

07-23376

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



DELEGATURA PROPIEDAD INDUSTRIAL

División de Nuevas Creaciones

PCT

SOLICITUD

PATENTE DE INVENCION

ENTRADA EN FASE NACIONAL

21. EXPEDIENTE No.

07-23376

54. TITULO

METODOS DE UTILIZAR ENLACDOS DE ACEPTOR DE FUENTE CELULAR Y
ANTICUERPOS CD20

51. CLASIFICACION INTERNACIONAL

71. SOLICITANTE

GENERATION, INC

DOMICILIO

SOUTH SAN FRANCISCO, CALIFORNIA, ESTADOS UNIDOS

74. APODERADO

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PETITORIO

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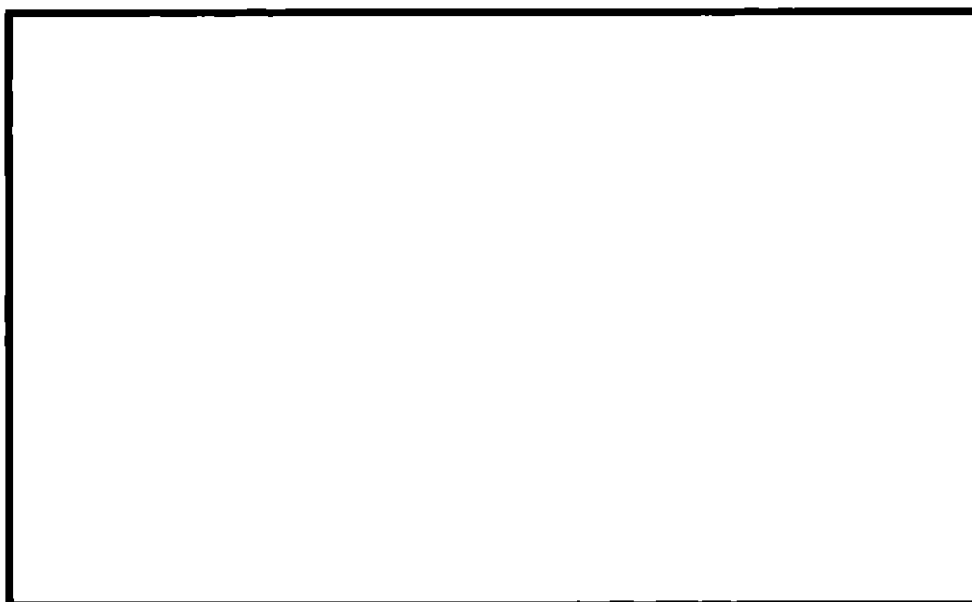


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Act 411 PRESENTACION Folios: 388

FORMULARIO ÚNICO DE SOLICITUD DE PATENTE
PCT
ENTRADA EN FASE NACIONAL

1 SOLICITUD DE:			
☒ Patente de Invención.		☐ Patente de Modelo de Utilidad.	
☒ Capítulo I.		☐ Capítulo II.	
2 SOLICITANTE (71)	Nombre: GENENTECH, INC. Dirección: 1 DNA Way, South San Francisco, CA 94080-4990, US Nacionalidad o Domicilio: Estados Unidos Lugar de Constitución: Estados Unidos Teléfono: Fax: E-mail		IDENTIFICACION C.C. ☐ NIT ☐ C.E. ☐ Otro ☐ Cual _____ Número _____
3 REPRESENTANTE O APODERADO	Nombre: JUAN PABLO CADENA SARMIENTO Dirección: Carrera 7 # 71 – 21 Torre B – Piso 4 Teléfono: 744-2200 Fax: 744-2700 E-mail:		IDENTIFICACION C.C. ☒ NIT ☐ C.E. ☐ Otro ☐ Cual _____ Número <u>80.410.337 TP 57492</u>
4 INVENTOR (ES) (72)	Nombre: Avi J. Ashkenazi Dirección: 1456 Tarrytown Street, San Mateo, California 94402, Estados Unidos de América Nacionalidad o Domicilio: Estados Unidos de América		
5 PCT (86) Solicitud Internacional No. <u>PCT/US2005/032015</u> Fecha <u>07-09-2005</u> Publicación Internacional No. <u>WO 2006/029275 A3</u> Fecha <u>16-03-2006</u>			
6 Título (54): <u>MÉTODOS DE UTILIZAR LIGANDOS DE RECEPTOR DE MUERTE CELULAR Y ANTICUERPOS CD20</u>			
7 Clasificación Internacional (51) _____			
8 Prioridad		(33) País de Origen	(32) Fecha
SI ☒ NO ☐		US	08-09-2004
		US	30-03-2005
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			60/607.834
			60/666.550
9 Comprobante de pago No. <u>07-7.214</u> Fecha <u>07-03-2007</u>			
Instrucciones para el diligenciamiento del presente formulario, ver reverso de esta página.			

- ☒ Comprobante de pago de la tasa de presentación de la solicitud. \$482.000
- ☒ Documento que acredita la existencia y representación legal cuando el solicitante sea persona jurídica.
- ☒ Poderes, si fuere el caso. Fotocopia Autenticada
- ☐ Documento de cesión del inventor al solicitante o a su causante.
- ☐ Declaraciones
- ☒ Copia de la solicitud internacional. (Resumen, descripción, reivindicaciones, dibujos)
- ☒ Traducción de la solicitud internacional al castellano. (Resumen, descripción, reivindicaciones, dibujos)
- ☐ Copia del examen preliminar efectuado por la IPEA.
- ☐ Traducción del examen preliminar efectuado por la IPEA.
- ☐ De ser el caso, copia del contrato de acceso.
- ☐ De ser el caso, documento que acredite la licencia o autorización de uso de conocimientos tradicionales de las comunidades indígenas.
- ☐ De ser el caso, certificado de depósito del material biológico.
- ☐ Arte final 12x12.
- ☐ De ser el caso, información sobre otras solicitudes de patente o títulos obtenidos en el extranjero por el mismo titular o su causante, relacionadas parcial o totalmente con la invención de esta solicitud.
- ☒ Otros: Listado de Secuencias
Tarjeta de Propietarios
Documentos PCT

11 FIGURA CARACTERÍSTICA:**12 Solicito la concesión de la patente,****NOMBRE: JUAN PABLO CADENA SARMIENTO****FIRMA: [Firma manuscrita]**
C.C. 80.410.337 de Usaquén TP 57492 del C.S.J.

Instrucciones para la presentación de los anexos, ver reverso de esta página.

3

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	Vr. PAGO
BRIGARD & CASTRO LTDA	CONSIGNACION	BANCO POPULAR	050-00110-6	52421261	24/01/2007	6,260,000.00

***** CONCEPTO *****

CANT. RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1 50005-01-01 SOLICITUDES	1 TRAMITES DE SOL. DE PATENTE DE IN	482,000.00
	TOTAL :	\$ 482,000.00

SON: CUATROCIENTOS OCHENTA Y DOS MIL PESOS

RESPONSABLE : _____

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4

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES

PATENTE ☒ X PCT/US2005/032015 MODELO DE UTILIDAD ☐ DISEÑO INDUSTRIAL ☐

SOLICITANTES. RAZON SOCIAL O APELLIDOS Y NOMBRES GENENTECH, INC.

DOMICILIO SOUTH SAN FRANCISCO, CALIFORNIA, ESTADOS UNIDOS DE AMERICA

APOGERADO JUAN PABLO CADENA SARMIENTO

TITULO METODOS DE UTILIZAR LIGANDOS DE RECEPTOR DE MUERTE CELULAR Y ANTICUERPOS
CD20

CLASIFICACION

EXPEDIENTE No. _____ FECHA DE SOLICITUD _____ ROLLO MICROFILM _____

CERTIFICADO No. _____ FECHA DE CONCESION _____ VIGENCIA DESDE _____ HASTA _____

PRORROGA _____ CAMBIO DE NOMBRE _____

TRASPASO A _____

INVENTOR (ES) AVI J. ASHKENAZI

OTROS _____

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60/666,550 30 March 2005 (30.03.2005) US

(71) Applicant (for all designated States except US): GENENTECH, INC. [US/US]; 1 DNA Way, South San Francisco, California 94080-4990 (US).

(72) Inventor; and

(75) Inventor/Applicant (for US only): ASHKENAZI, Avi J. [US/US]; 1456 Terrytown Street, San Mateo, California 94402 (US).

(74) Agents: MARSCHANG, Diane L. et al.; GENENTECH, INC., 1 DNA Way, South San Francisco, California 94080-4990 (US).

(81) Designated States (unless otherwise indicated, for every

kind of national protection available): AE, AG, AI, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

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(54) Title: METHODS OF USING DEATH RECEPTOR LIGANDS AND CD20 ANTIBODIES

(57) Abstract: Methods for using death receptor ligands, such as Apo-2 ligand/TRAIL polypeptides or death receptor antibodies, and CD20 antibodies to treat conditions such as cancer and immune related diseases are provided. Embodiments of the invention include methods of using Apo2L/TRAIL or death receptor antibodies such as DR5 antibodies and DR4 antibodies in combination with CD20 antibodies.



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(57) Abstract: Methods for using death receptor ligands, such as Apo-2 ligand/TRAIL polypeptides or death receptor antibodies, and CD20 antibodies to treat conditions such as cancer and immune related diseases are provided. Embodiments of the invention include methods of using Apo2L/TRAIL or death receptor antibodies such as DR5 antibodies and DR4 antibodies in combination with CD20 antibodies.

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METHODS OF USING DEATH RECEPTOR LIGANDS AND CD20 ANTIBODIES**RELATED APPLICATIONS**

5 This application claims priority under Section 119(e) to provisional application number 60/607,834 filed September 8, 2004 and to provisional application number 60/666,550 filed March 30, 2005, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

10 The present invention relates to methods of using death receptor ligands and CD20 antibodies. More particularly, the invention relates to methods of using Apo-2 ligand/TRAIL or death receptor antibodies in combination with CD20 antibodies to treat various pathological disorders,
15 such as cancer and immune related diseases.

BACKGROUND OF THE INVENTION

Various ligands and receptors belonging to the tumor necrosis factor (TNF) superfamily have been identified in the art. Included among such
20 ligands are tumor necrosis factor-alpha ("TNF-alpha"), tumor necrosis factor-beta ("TNF-beta" or "lymphotoxin-alpha"), lymphotoxin-beta ("LT-beta"), CD30 ligand, CD27 ligand, CD40 ligand, OX-40 ligand, 4-1BB ligand, LIGHT, Apo-1 ligand (also referred to as Fas ligand or CD95 ligand), Apo-2 ligand (also referred to as Apo2L or TRAIL), Apo-3 ligand (also referred to
25 as TWEAK), APRIL, OPG ligand (also referred to as RANK ligand, ODF, or TRANCE), and TALL-1 (also referred to as BlyS, BAFF or THANK) (See, e.g., Ashkenazi, Nature Review, 2:420-430 (2002); Ashkenazi and Dixit, Science, 281:1305-1308 (1998); Ashkenazi and Dixit, Curr. Opin. Cell Biol., 11:255-260 (2000); Golstein, Curr. Biol., 7:750-753 (1997) Wallach, Cytokine
30 Reference, Academic Press, 2000, pages 377-411; Locksley et al., Cell, 104:487-501 (2001); Gruss and Dower, Blood, 85:3378-3404 (1995); Schmid et al., Proc. Natl. Acad. Sci., 83:1881 (1986); Dealtry et al., Eur. J. Immunol., 17:689 (1987); Pitti et al., J. Biol. Chem., 271:12687-12690 (1996); Wiley et al., Immunity, 3:673-682 (1995); Browning et al., Cell,
35 72:847-856 (1993); Armitage et al. Nature, 357:80-82 (1992); WO 97/01633 published January 16, 1997; WO 97/25428 published July 17, 1997; Marsters et al., Curr. Biol., 8:525-528 (1998); Chicheportiche et al., Biol. Chem., 272:32401-32410 (1997); Hahne et al., J. Exp. Med., 188:1185-1190 (1998); WO98/28426 published July 2, 1998; WO98/46751 published October 22, 1998;
40 WO/98/18921 published May 7, 1998; Moore et al., Science, 285:260-263 (1999); Shu et al., J. Leukocyte Biol., 65:680 (1999); Schneider et al., J. Exp. Med., 189:1747-1756 (1999); Mukhopadhyay et al., J. Biol. Chem., 274:15978-15981 (1999)).

Induction of various cellular responses mediated by such TNF family ligands is typically initiated by their binding to specific cell receptors. Some, but not all, TNF family ligands bind to, and induce various biological activity through, cell surface "death receptors" to activate caspases, or enzymes that carry out the cell death or apoptosis pathway (Salvesen et al., Cell, 91:443-446 (1997)). Included among the members of the TNF receptor superfamily identified to date are TNFR1, TNFR2, TACI, GITR,, CD27, OX-40, CD30, CD40, HVEM, Fas (also referred to as Apo-1 or CD95), DR4 (also referred to as TRAIL-R1), DR5 (also referred to as Apo-2 or TRAIL-R2), DcR1, DcR2, osteoprotegerin (OPG), RANK and Apo-3 (also referred to as DR3 or TRAMP) (see, e.g., Ashkenazi, Nature Reviews, 2:420-430 (2002); Ashkenazi and Dixit, Science, 281:1305-1308 (1998); Ashkenazi and Dixit, Curr. Opin. Cell Biol., 11:255-260 (2000); Golstein, Curr. Biol., 7:750-753 (1997); Wallach, Cytokine Reference, Academic Press, 2000, pages 377-411; Locksley et al., Cell, 104:487-501 (2001); Gruss and Dower, Blood, 85:3378-3404 (1995); Hohman et al., J. Biol. Chem., 264:14927-14934 (1989); Brockhaus et al., Proc. Natl. Acad. Sci., 87:3127-3131 (1990); EP 417,563, published March 20, 1991; Loetscher et al., Cell, 61:351 (1990); Schall et al., Cell, 61:361 (1990); Smith et al., Science, 248:1019-1023 (1990); Lewis et al., Proc. Natl. Acad. Sci., 88:2830-2834 (1991); Goodwin et al., Mol. Cell. Biol., 11:3020-3026 (1991); Stamenkovic et al., EMBO J., 8:1403-1410 (1989); Mallett et al., EMBO J., 9:1063-1068 (1990); Anderson et al., Nature, 390:175-179 (1997); Chicheportiche et al., J. Biol. Chem., 272:32401-32410 (1997); Pan et al., Science, 276:111-113 (1997); Pan et al., Science, 277:815-818 (1997); Sheridan et al., Science, 277:818-821 (1997); Degli-Esposti et al., J. Exp. Med., 186:1165-1170 (1997); Marsters et al., Curr. Biol., 7:1003-1006 (1997); Tsuda et al., BBRC, 234:137-142 (1997); Nocentini et al., Proc. Natl. Acad. Sci., 94:6216-6221 (1997); vonBulow et al., Science, 278:138-141 (1997)).

Most of these TNF receptor family members share the typical structure of cell surface receptors including extracellular, transmembrane and intracellular regions, while others are found naturally as soluble proteins lacking a transmembrane and intracellular domain. The extracellular portion of typical TNFRs contains a repetitive amino acid sequence pattern of multiple cysteine-rich domains (CRDs), starting from the NH₂-terminus.

The ligand referred to as Apo-2L or TRAIL was identified several years ago as a member of the TNF family of cytokines. (see, e.g., Wiley et al., Immunity, 3:673-682 (1995); Pitti et al., J. Biol. Chem., 271:12697-12690 (1996); WO 97/01633; WO 97/25428; US Patent 5,763,223 issued June 9, 1998; US Patent 6,284,236 issued September 4, 2001). The full-length native sequence human Apo2L/TRAIL polypeptide is a 281 amino acid long, Type II transmembrane protein. Some cells can produce a natural soluble form of the polypeptide, through enzymatic cleavage of the polypeptide's extracellular region (Mariani et al., J. Cell. Biol., 137:221-229 (1997)).

Crystallographic studies of soluble forms of Apo2L/TRAIL reveal a homotrimeric structure similar to the structures of TNF and other related proteins (Hymowitz et al., *Molec. Cell*, 4:563-571 (1999); Cha et al., *Immunity*, 11:253-261 (1999); Mongkolsapaya et al., *Nature Structural Biology*, 6:1048 (1999); Hymowitz et al., *Biochemistry*, 39:633-644 (2000)). Apo2L/TRAIL, unlike other TNF family members however, was found to have a unique structural feature in that three cysteine residues (at position 230 of each subunit in the homotrimer) together coordinate a zinc atom, and that the zinc binding is important for trimer stability and biological activity. (Hymowitz et al., *supra*; Bodmer et al., *J. Biol. Chem.*, 275:20632-20637 (2000)).

It has been reported in the literature that Apo2L/TRAIL may play a role in immune system modulation, including autoimmune diseases such as rheumatoid arthritis [see, e.g., Thomas et al., *J. Immunol.*, 161:2195-2200 (1998); Johnsen et al., *Cytokine*, 11:664-672 (1999); Griffith et al., *J. Exp. Med.*, 189:1343-1353 (1999); Song et al., *J. Exp. Med.*, 191:1095-1103 (2000)].

Soluble forms of Apo2L/TRAIL have also been reported to induce apoptosis in a variety of cancer cells, including colon, lung, breast, prostate, bladder, kidney, ovarian and brain tumors, as well as melanoma, leukemia, and multiple myeloma (see, e.g., Wiley et al., *supra*; Pitti et al., *supra*; US Patent 6,030,945 issued February 29, 2000; US Patent 6,746,668 issued June 8, 2004; Rieger et al., *FEBS Letters*, 427:124-128 (1998); Ashkenazi et al., *J. Clin. Invest.*, 104:155-162 (1999); Walczak et al., *Nature Med.*, 5:157-163 (1999); Keane et al., *Cancer Research*, 59:734-741 (1999); Mixutani et al., *Clin. Cancer Res.*, 5:2605-2612 (1999); Gazitt, *Leukemia*, 13:1817-1824 (1999); Yu et al., *Cancer Res.*, 60:2384-2389 (2000); Chinnaiyan et al., *Proc. Natl. Acad. Sci.*, 97:1754-1759 (2000)). In vivo studies in murine tumor models further suggest that Apo2L/TRAIL, alone or in combination with chemotherapy or radiation therapy, can exert substantial anti-tumor effects (see, e.g., Ashkenazi et al., *supra*; Walczak et al., *supra*; Gliniak et al., *Cancer Res.*, 59:6153-6158 (1999); Chinnaiyan et al., *supra*; Roth et al., *Biochem. Biophys. Res. Comm.*, 265:1999 (1999); PCT Application US/00/15512; PCT Application US/01/23691). In contrast to many types of cancer cells, most normal human cell types appear to be resistant to apoptosis induction by certain recombinant forms of Apo2L/TRAIL (Ashkenazi et al., *supra*; Walczak et al., *supra*). Jo et al. has reported that a polyhistidine-tagged soluble form of Apo2L/TRAIL induced apoptosis in vitro in normal isolated human, but not non-human, hepatocytes (Jo et al., *Nature Med.*, 6:564-567 (2000); see also, Nagata, *Nature Med.*, 6:502-503 (2000)). It is believed that certain recombinant Apo2L/TRAIL preparations may vary in terms of biochemical properties and biological activities on diseased versus normal cells, depending, for example, on the presence or absence of a tag molecule, zinc content, and %

trimer content (See, Lawrence et al., *Nature Med.*, Letter to the Editor, 7:383-385 (2001); Qin et al., *Nature Med.*, Letter to the Editor, 7:385-386 (2001)).

Apo2L/TRAIL has been found to bind at least five different receptors. At least two of the receptors which bind Apo2L/TRAIL contain a functional, cytoplasmic death domain. One such receptor has been referred to as "DR4" (and alternatively as TR4 or TRAIL-R1) (Pan et al., *Science*, 276:111-113 (1997); see also WO98/32856 published July 30, 1998; WO99/37684 published July 29, 1999; WO 00/73349 published December 7, 2000; US 6,433,147 issued August 13, 2002; US 6,461,823 issued October 8, 2002, and US 6,342,383 issued January 29, 2002).

Another such receptor for Apo2L/TRAIL has been referred to as DR5 (it has also been alternatively referred to as Apo-2; TRAIL-R or TRAIL-R2, TR6, Tango-63, hAPO8, TRICK2 or KILLER) (see, e.g., Sheridan et al., *Science*, 277:818-821 (1997); Pan et al., *Science*, 277:815-818 (1997); WO98/51793 published November 19, 1998; WO98/41629 published September 24, 1998; Screaton et al., *Curr. Biol.*, 7:693-696 (1997); Walczak et al., *EMBO J.*, 16:5386-5387 (1997); Wu et al., *Nature Genetics*, 17:141-143 (1997); WO98/35986 published August 20, 1998; EP870,827 published October 14, 1998; WO98/46643 published October 22, 1998; WO99/02653 published January 21, 1999; WO99/09165 published February 25, 1999; WO99/11791 published March 11, 1999; US 2002/0072091 published August 13, 2002; US 2002/0098550 published December 7, 2001; US 6,313,269 issued December 6, 2001; US 2001/0010924 published August 2, 2001; US 2003/01255540 published July 3, 2003; US 2002/0160446 published October 31, 2002; US 2002/0048785 published April 25, 2002; US 6,342,369 issued February, 2002; US 6,569,642 issued May 27, 2003; US 6,072,047 issued June 6, 2000; US 6,642,358 issued November 4, 2003; US 6,743,625 issued June 1, 2004). Like DR4, DR5 is reported to contain a cytoplasmic death domain and be capable of signaling apoptosis upon ligand binding (or upon binding a molecule, such as an agonist antibody, which mimics the activity of the ligand). The crystal structure of the complex formed between Apo-2L/TRAIL and DR5 is described in Hymowitz et al., *Molecular Cell*, 4:563-571 (1999).

Upon ligand binding, both DR4 and DR5 can trigger apoptosis independently by recruiting and activating the apoptosis initiator, caspase-8, through the death-domain-containing adaptor molecule referred to as FADD/Mort1 (Kischkel et al., *Immunity*, 12:611-620 (2000); Sprick et al., *Immunity*, 12:599-609 (2000); Bodmer et al., *Nature Cell Biol.*, 2:241-243 (2000)).

Apo2L/TRAIL has been reported to also bind those receptors referred to as DcR1, DcR2 and OPG, which believed to function as inhibitors, rather than transducers of signaling (see., e.g., DCR1 (also referred to as TRID, LIT or TRAIL-R3) [Pan et al., *Science*, 276:111-113 (1997); Sheridan et al., *Science*, 277:818-821 (1997); McFarlane et al., *J. Biol. Chem.*, 272:25417-

25420 (1997); Schneider et al., FEBS Letters, 416:329-334 (1997); Degli-Esposti et al., J. Exp. Med., 186:1165-1170 (1997); and Mongkolsapaya et al., J. Immunol., 160:3-6 (1998); DCR2 (also called TRUMDD or TRAIL-R4) [Marsters et al., Curr. Biol., 7:1003-1006 (1997); Pan et al., FEBS Letters, 424:41-45 (1998); Degli-Esposti et al., Immunity, 7:813-820 (1997)], and OPG [Simonet et al., supra]. In contrast to DR4 and DR5, the DcR1 and DcR2 receptors do not signal apoptosis.

Certain antibodies which bind to the DR4 and/or DR5 receptors have been reported in the literature. For example, anti-DR4 antibodies directed to the DR4 receptor and having agonistic or apoptotic activity in certain mammalian cells are described in, e.g., WO 99/37684 published July 29, 1999; WO 00/73349 published July 12, 2000; WO 03/066661 published August 14, 2003. See, also, e.g., Griffith et al., J. Immunol., 162:2597-2605 (1999); Chuntharapai et al., J. Immunol., 166:4891-4898 (2001); WO 02/097033 published December 2, 2002; WO 03/042367 published May 22, 2003; WO 03/038043 published May 8, 2003; WO 03/037913 published May 8, 2003. Certain anti-DR5 antibodies have likewise been described, see, e.g., WO 98/51793 published November 8, 1998; Griffith et al., J. Immunol., 162:2597-2605 (1999); Ichikawa et al., Nature Med., 7:954-960 (2001); Hylander et al., "An Antibody to DR5 (TRAIL-Receptor 2) Suppresses the Growth of Patient Derived Gastrointestinal Tumors Grown in SCID mice", Abstract, 2d International Congress on Monoclonal Antibodies in Cancers, Aug. 29-Sept. 1, 2002, Banff, Alberta, Canada; WO 03/038043 published May 8, 2003; WO 03/037913 published May 8, 2003. In addition, certain antibodies having cross-reactivity to both DR4 and DR5 receptors have been described (see, e.g., US patent 6,252,050 issued June 26, 2001).

The CD20 antigen (also called human B-lymphocyte-restricted differentiation antigen, Bp35) is a hydrophobic transmembrane protein with a molecular weight of approximately 35 kD located on pre-B and mature B lymphocytes (Valentine et al. J. Biol. Chem. 264(19):11282-11287 (1989); and Einfeld et al. EMBO J. 7(3):711-717 (1988)). The antigen is also expressed on greater than 90% of B cell non-Hodgkin's lymphomas (NHL) (Anderson et al. Blood 63(6):1424-1433 (1984)), but is not found on hematopoietic stem cells, pro-B cells, normal plasma cells or other normal tissues (Tedder et al. J. Immunol. 135(2):973-979 (1985)). CD20 regulates an early step(s) in the activation process for cell cycle initiation and differentiation (Tedder et al., supra) and possibly functions as a calcium ion channel (Tedder et al. J. Cell. Biochem. 14D:195 (1990)). Given the expression of CD20 in B cell lymphomas, this antigen may serve as a candidate for "targeting" of such lymphomas.

The rituximab (RITUXAN®) antibody is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen. Rituximab is the antibody called "C2B8" in US Patent No. 5,736,137 issued April 7, 1998 (Anderson et al.). RITUXAN® is indicated for the

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treatment of patients with relapsed or refractory low-grade or follicular, CD20 positive, B cell non-Hodgkin's lymphoma. In vitro mechanism of action studies have demonstrated that RITUXAN® binds human complement and lyses lymphoid B cell lines through complement-dependent cytotoxicity (CDC) (Reff et al. Blood 83(2):435-445 (1994); Cragg and Marlin, Blood, 103: 2738-2743 (2004). Additionally, it has significant activity in assays for antibody-dependent cell-mediated cytotoxicity (ADCC). More recently, RITUXAN® has been shown to have anti-proliferative effects in tritiated thymidine incorporation assays and to induce apoptosis directly, while other anti-CD19 and CD20 antibodies do not (Maloney et al. Blood 88(10):637a (1996)). Synergy between RITUXAN® and certain chemotherapies and toxins has also been observed experimentally. In particular, RITUXAN® sensitizes drug-resistant human B cell lymphoma cell lines to the cytotoxic effects of doxorubicin, CDDP, VP-16, diphtheria toxin and ricin (Demidem et al. Cancer Chemotherapy & Radiopharmaceuticals 12(3):177-186 (1997)). In vivo preclinical studies have shown that RITUXAN® depletes B cells from the peripheral blood, lymph nodes, and bone marrow of cynomolgus monkeys, presumably through complement and cell-mediated processes (Reff et al. Blood 83(2):435-445 (1994)).

SUMMARY OF THE INVENTION

Methods for using death receptor ligands, such as Apo-2 ligand/TRAIL polypeptides or death receptor antibodies, and CD20 antibodies are provided herein. Embodiments of the invention include methods of treating cancer, comprising exposing cancer cells to an effective amount of Apo2L/TRAIL and CD20 antibody. Optionally, the cancer cells are exposed to an effective amount of death receptor antibody, such as an agonist DR4 antibody or agonist DR5 antibody, and CD20 antibody. Optionally, the amount of Apo2L/TRAIL or death receptor antibody and CD20 antibodies employed in the methods are effective to achieve synergy therapeutically, e.g., their combined anti-cancer effect is greater than the anti-cancer effect achieved when the Apo2L/TRAIL or antibodies are employed individually as a single therapeutic agent. The methods may entail in vitro use or in vivo use wherein the Apo2L/TRAIL or death receptor antibody and CD20 antibody are administered to a mammal (patient). Optionally, in the methods, the cancer cells treated with Apo2L/TRAIL or death receptor antibody and CD20 antibody are lymphoma cells.

Further embodiments of the invention include methods of treating an immune-related disease, comprising administering to a mammal an effective amount of Apo2L/TRAIL and CD20 antibody. Optionally, an effective amount of death receptor antibody, such as an agonist DR4 antibody or agonist DR5 antibody, and CD20 antibody is administered to the mammal. Optionally, the amount of Apo2L/TRAIL or death receptor antibody and CD20 antibodies

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employed in the methods are effective to achieve synergy therapeutically, e.g., their combined effect in treating the immune-related disease is greater than the effect achieved when the Apo2L/TRAIL or antibodies are employed individually as a single therapeutic agent. Optionally, in the methods, the immune-related disease is rheumatoid arthritis or multiple sclerosis.

Methods of the invention include methods of treating a disorder in a mammal, such as an immune-related disease or cancer, comprising steps of obtaining tissue or a cell sample from the mammal, examining the tissue or cells for expression of CD20, DR4, and/or DR5, and upon determining said tissue or cell sample expresses said one or more receptors, administering an effective amount of Apo2L/TRAIL or death receptor antibody and CD20 antibody to said mammal. The steps in the methods for examining expression of one or more of such receptors may be conducted in a variety of assay formats, including assays detecting mRNA expression and immunohistochemistry assays.

Optionally, the methods of the invention comprise, in addition to administering an effective amount of Apo2L/TRAIL and/or death receptor antibody and CD20 antibody, administering chemotherapeutic agent(s) or radiation therapy to said mammal.

More embodiments of the invention are illustrated by way of example in the following claims:

1. A method of treating cancer cells, comprising exposing mammalian cancer cells to a synergistic effective amount of agonist death receptor antibody and CD20 antibody.

2. The method of claim 1 wherein said agonist death receptor antibody is an anti-DR5 receptor monoclonal antibody.

3. The method of claim 1 wherein said agonist death receptor antibody is an anti-DR4 receptor monoclonal antibody.

4. The method of claim 1 wherein said cancer cells are exposed to said synergistic effective amount of agonist death receptor antibody and CD20 antibody *in vivo*.

5. The method of claim 2 or 3 wherein said agonist death receptor antibody is a chimeric antibody or a humanized antibody.

6. The method of claim 2 or 3 wherein said agonist death receptor antibody is a human antibody.

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7. The method of claim 1 wherein said agonist death receptor antibody is an antibody which cross-reacts with more than one Apo-2 ligand receptor.

5 8. The method of claim 1 wherein said cancer cells are lymphoma cells.

9. The method of claim 1 further comprising exposing the cancer cells to one or more growth inhibitory agents.

10 10. The method of claim 1 further comprising exposing the cells to radiation.

11. The method of claim 2 wherein said DR5 antibody has a DR5 receptor binding affinity of 10^8 M^{-1} to 10^{11} M^{-1} .

12. The method of claim 1 wherein said death receptor antibody and CD20 antibody is expressed in a recombinant host cell selected from the group consisting of a CHO cell, yeast cell and *E. coli*.

20 13. The method of claim 1 wherein said CD20 antibody is a monoclonal antibody.

14. The method of claim 13 wherein said CD20 antibody is the antibody Rituximab.

25 15. A method of treating an immune related disease, comprising administering to a mammal a synergistic effective amount of agonist death receptor antibody and CD20 antibody.

30 16. The method of claim 15 wherein said agonist death receptor antibody is an anti-DR5 receptor monoclonal antibody.

17. The method of claim 15 wherein said agonist death receptor antibody is an anti-DR4 receptor monoclonal antibody.

35 18. The method of claim 16 or 17 wherein said agonist death receptor antibody is a chimeric antibody or a humanized antibody.

40 19. The method of claim 16 or 17 wherein said agonist death receptor antibody is a human antibody.

20. The method of claim 15 wherein said agonist death receptor antibody is an antibody which cross-reacts with more than one Apo-2 ligand receptor.

21. The method of claim 15 wherein said immune related disease is rheumatoid arthritis or multiple sclerosis.

22. The method of claim 15 wherein said DR5 antibody has a DR5 receptor binding affinity of 10^8 M^{-1} to 10^{12} M^{-1} .

23. The method of claim 15 wherein said death receptor antibody and CD20 antibody is expressed in a recombinant host cell selected from the group consisting of a CHO cell, yeast cell and E. coli.

24. The method of claim 15 wherein said CD20 antibody is a monoclonal antibody.

25. The method of claim 24 wherein said CD20 antibody is the antibody Rituximab.

26. The method of claim 1 or 15 wherein said death receptor antibody and CD20 antibody are administered sequentially.

27. The method of claim 1 or 15 wherein said death receptor antibody and CD20 antibody are administered concurrently.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows the nucleotide sequence of human Apo-2 ligand cDNA (SEQ ID NO:2) and its derived amino acid sequence (SEQ ID NO:1). The "N" at nucleotide position 447 is used to indicate the nucleotide base may be a "T" or "G".

Figures 2A and 2B show the nucleotide sequence of a cDNA (SEQ ID NO:4) for full length human DR4 and its derived amino acid sequence (SEQ ID NO:3). The respective nucleotide and amino acid sequences for human DR4 are also reported in Pan et al., *Science*, 276:111 (1997).

Figure 3A shows the 411 amino acid sequence of human DR5 (SEQ ID NO:5) as published in WO 98/51793 on November 19, 1998. A transcriptional splice variant of human DR5 is known in the art. This DR5 splice variant encodes the 440 amino acid sequence of human DR5 (SEQ ID NO:6) shown in Figures 3B and 3C as published in WO 98/35986 on August 20, 1998.

Figure 4 illustrates the expression of Apo2L/TRAIL receptors in B lymphoma cell lines.

Figure 5 illustrates expression of CD20 in B lymphoma cell lines.

Figure 6 shows the effects of Apo2L/TRAIL, RITUXAN®, or combination treatment on the growth of pre-established subcutaneous BJAB lymphoma tumor xenografts in SCID mice.

Figure 7 shows further results on the effects of Apo2L/TRAIL, RITUXAN®, or combination treatment of Apo2L/TRAIL and RITUXAN® on the growth of pre-established subcutaneous BJAB lymphoma tumor xenografts in SCID mice.

Figure 8 shows the effects of Apo2L/TRAIL, RITUXAN®, or combination treatment of Apo2L/TRAIL and RITUXAN® on caspase processing in pre-established subcutaneous BJAB lymphoma tumor xenografts grown in SCID mice.

Figure 9 shows the effects of DR5 agonistic antibody, RITUXAN®, or combination treatment on the growth of pre-established subcutaneous BJAB lymphoma tumor xenografts in SCID mice.

Figure 10 shows the effects of DR5 agonistic antibody, RITUXAN®, or combination treatment on caspase processing in pre-established subcutaneous BJAB lymphoma tumor xenografts grown in SCID mice.

Figure 11 illustrates the expression of CD20 and Apo2L/TRAIL receptors in NHL cell lines.

Figure 12 shows the effects of Apo2L/TRAIL, Rituximab, or combination treatment on the growth of pre-established subcutaneous Ramos RA1 tumor xenografts in SCID mice.

Figure 13 shows the effects of Apo2L/TRAIL, Rituximab, or combination treatment on the growth of pre-established DOHH-2 follicular lymphoma xenografts in SCID mice.

Figure 14 illustrates the effects and mechanisms of cell killing by Apo2L/TRAIL and Rituximab or combination treatments on BJAB cells.

Figure 15 shows the effects of Apo2L/TRAIL, Rituximab, or combination treatment on the growth of Ramos T1 tumor xenografts in SCID mice.

Figure 16 shows the effects of Apo2L/TRAIL, Rituximab, or combination treatment on the BJAB-Luc tumor xenografts in SCID mice.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Unless otherwise defined, all terms of art, notations and other scientific terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this invention pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art. The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized molecular cloning methodologies described in Sambrook et al., Molecular Cloning: A Laboratory Manual 2nd. edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. As appropriate, procedures involving the use of commercially available kits and reagents are generally carried out in

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accordance with manufacturer defined protocols and/or parameters unless otherwise noted.

Before the present methods, kits and uses therefor are described, it is to be understood that this invention is not limited to the particular methodology, protocols, cell lines, animal species or genera, constructs, and reagents described as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

It must be noted that as used herein and in the appended claims, the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise.

All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. Publications cited herein are cited for their disclosure prior to the filing date of the present application. Nothing here is to be construed as an admission that the inventors are not entitled to antedate the publications by virtue of an earlier priority date or prior date of invention. Further the actual publication dates may be different from those shown and require independent verification.

Definitions

The terms "Apo-2 ligand", "Apo-2L", "Apo2L", "Apo2L/TRAIL", "Apo-2 ligand/TRAIL" and "TRAIL" are used herein interchangeably to refer to a polypeptide sequence which includes amino acid residues 114-281, inclusive, 95-281, inclusive, residues 92-281, inclusive, residues 91-281, inclusive, residues 41-281, inclusive, residues 39-281, inclusive, residues 15-281, inclusive, or residues 1-281, inclusive, of the amino acid sequence shown in Figure 1, as well as biologically active fragments, deletional, insertional, or substitutional variants of the above sequences. In one embodiment, the polypeptide sequence comprises residues 114-281 of Figure 1. Optionally, the polypeptide sequence comprises residues 92-281 or residues 91-281 of Figure 1. The Apo-2L polypeptides may be encoded by the native nucleotide sequence shown in Figure 1. Optionally, the codon which encodes residue Pro119 (Figure 1) may be "CCT" or "CCG". Optionally, the fragments or variants are biologically active and have at least about 80% amino acid sequence identity, more preferably at least about 90% sequence identity, and even more preferably, at least 95%, 96%, 97%, 98%, or 99% sequence identity with any one of the above sequences. The definition encompasses substitutional variants of Apo-2 ligand in which at least one of its native amino acids are substituted by another amino acid such as an alanine residue. Optional substitutional variants include one or more of

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the residue substitutions. Optional variants may comprise an amino acid sequence which differs from the native sequence Apo-2 ligand polypeptide sequence of Figure 1 and has one or more of the following amino acid substitutions at the residue position(s) in Figure 1: S96C; S101C; S111C; R170C; K179C. The definition also encompasses a native sequence Apo-2 ligand isolated from an Apo-2 ligand source or prepared by recombinant or synthetic methods. The Apo-2 ligand of the invention includes the polypeptides referred to as Apo-2 ligand or TRAIL disclosed in WO97/01633 published January 16, 1997, WO97/25428 published July 17, 1997, WO99/36535 published July 22, 1999, WO 01/00832 published January 4, 2001, WO02/09755 published February 7, 2002, and WO 00/75191 published December 14, 2000. The terms are used to refer generally to forms of the Apo-2 ligand which include monomer, dimer, trimer, hexamer or high oligomer forms of the polypeptide. All numbering of amino acid residues referred to in the Apo-2L sequence use the numbering according to Figure 1, unless specifically stated otherwise. For instance, "D203" or "Asp203" refers to the aspartic acid residue at position 203 in the sequence provided in Figure 1.

The term "Apo-2 ligand selective variant" as used herein refers to an Apo-2 ligand polypeptide which includes one or more amino acid mutations in a native Apo-2 ligand sequence and has selective binding affinity for either the DR4 receptor or the DR5 receptor. In one embodiment, the Apo-2 ligand variant has a selective binding affinity for the DR4 receptor and includes one or more amino acid substitutions in any one of positions 189, 191, 193, 199, 201 or 209 of a native Apo-2 ligand sequence. In another embodiment, the Apo-2 ligand variant has a selective binding affinity for the DR5 receptor and includes one or more amino acid substitutions in any one of positions 189, 191, 193, 264, 266, 267 or 269 of a native Apo-2 ligand sequence. Preferred Apo-2 ligand selective variants include one or more amino acid mutations and exhibit binding affinity to the DR4 receptor which is equal to or greater ($\geq$) than the binding affinity of native sequence Apo-2 ligand to the DR4 receptor, and even more preferably, the Apo-2 ligand variants exhibit less binding affinity ($<$) to the DR5 receptor than the binding affinity exhibited by native sequence Apo-2 ligand to DR5. When binding affinity of such Apo-2 ligand variant to the DR4 receptor is approximately equal (unchanged) or greater than (increased) as compared to native sequence Apo-2 ligand, and the binding affinity of the Apo-2 ligand variant to the DR5 receptor is less than or nearly eliminated as compared to native sequence Apo-2 ligand, the binding affinity of the Apo-2 ligand variant, for purposes herein, is considered "selective" for the DR4 receptor. Preferred DR4 selective Apo-2 ligand variants of the invention will have at least 10-fold less binding affinity to DR5 receptor (as compared to native sequence Apo-2 ligand), and even more preferably, will have at least 100-fold less binding affinity to DR5 receptor (as compared to native sequence Apo-2 ligand). The respective binding affinity of the

Apo-2 ligand variant may be determined and compared to the binding properties of native Apo-2L (such as the 114-281 form) by ELISA, RIA, and/or BIAcore assays, known in the art. Preferred DR4 selective Apo-2 ligand variants of the invention will induce apoptosis in at least one type of mammalian cell (preferably a cancer cell), and such apoptotic activity can be determined by known art methods such as the alamar blue or crystal violet assay. The DR4 selective Apo-2 ligand variants may or may not have altered binding affinities to any of the decoy receptors for Apo-2L, those decoy receptors being referred to in the art as DcR1, DcR2 and OPG.

Further preferred Apo-2 ligand selective variants include one or more amino acid mutations and exhibit binding affinity to the DR5 receptor which is equal to or greater ($\geq$) than the binding affinity of native sequence Apo-2 ligand to the DR5 receptor, and even more preferably, such Apo-2 ligand variants exhibit less binding affinity ($<$) to the DR4 receptor than the binding affinity exhibited by native sequence Apo-2 ligand to DR4. When binding affinity of such Apo-2 ligand variant to the DR5 receptor is approximately equal (unchanged) or greater than (increased) as compared to native sequence Apo-2 ligand, and the binding affinity of the Apo-2 ligand variant to the DR4 receptor is less than or nearly eliminated as compared to native sequence Apo-2 ligand, the binding affinity of the Apo-2 ligand variant, for purposes herein, is considered "selective" for the DR5 receptor. Preferred DR5 selective Apo-2 ligand variants of the invention will have at least 10-fold less binding affinity to DR4 receptor (as compared to native sequence Apo-2 ligand), and even more preferably, will have at least 100-fold less binding affinity to DR4 receptor (as compared to native sequence Apo-2 ligand). The respective binding affinity of the Apo-2 ligand variant may be determined and compared to the binding properties of native Apo-2L (such as the 114-281 form) by ELISA, RIA, and/or BIAcore assays, known in the art. Preferred DR5 selective Apo-2 ligand variants of the invention will induce apoptosis in at least one type of mammalian cell (preferably a cancer cell), and such apoptotic activity can be determined by known art methods such as the alamar blue or crystal violet assay. The DR5 selective Apo-2 ligand variants may or may not have altered binding affinities to any of the decoy receptors for Apo-2L, those decoy receptors being referred to in the art as DcR1, DcR2 and OPG.

Amino acid identification may use the single-letter alphabet or three-letter alphabet of amino acids, i.e.,

Asp	D	Aspartic acid	Ile	I	Isoleucine
Thr	T	Threonine	Leu	L	Leucine
Ser	S	Serine	Tyr	Y	Tyrosine
Glu	E	Glutamic acid	Phe	F	Phenylalanine
Pro	P	Proline	His	H	Histidine
Gly	G	Glycine	Lys	K	Lysine
Ala	A	Alanine	Arg	R	Arginine

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Cys	C	Cysteine	Trp	W	Tryptophan
Val	V	Valine	Gln	Q	Glutamine
Met	M	Methionine	Asn	N	Asparagine

5 The term "Apo2L/TRAIL extracellular domain" or "Apo2L/TRAIL ECD" refers to a form of Apo2L/TRAIL which is essentially free of transmembrane and cytoplasmic domains. Ordinarily, the ECD will have less than 1% of such transmembrane and cytoplasmic domains, and preferably, will have less than 0.5% of such domains. It will be understood that any transmembrane
10 domain(s) identified for the polypeptides of the present invention are identified pursuant to criteria routinely employed in the art for identifying that type of hydrophobic domain. The exact boundaries of a transmembrane domain may vary but most likely by no more than about 5 amino acids at either end of the domain as initially identified. In preferred
15 embodiments, the ECD will consist of a soluble, extracellular domain sequence of the polypeptide which is free of the transmembrane and cytoplasmic or intracellular domains (and is not membrane bound). Particular extracellular domain sequences of Apo-2L/TRAIL are described in PCT Publication Nos. WO97/01633 and WO97/25428.

20 The term "Apo2L/TRAIL monomer" or "Apo2L monomer" refers to a covalent chain of an extracellular domain sequence of Apo2L.

 The term "Apo2L/TRAIL dimer" or "Apo2L dimer" refers to two Apo-2L monomers joined in a covalent linkage via a disulfide bond. The term as used herein includes free standing Apo2L dimers and Apo2L dimers that are
25 within trimeric forms of Apo2L (i.e., associated with another, third Apo2L monomer).

 The term "Apo2L/TRAIL trimer" or "Apo2L trimer" refers to three Apo2L monomers that are non-covalently associated.

 The term "Apo2L/TRAIL aggregate" is used to refer to self-associated
30 higher oligomeric forms of Apo2L/TRAIL, such as Apo2L/TRAIL trimers, which form, for instance, hexameric and nanomeric forms of Apo2L/TRAIL. Determination of the presence and quantity of Apo2L/TRAIL monomer, dimer, or trimer (or other aggregates) may be made using methods and assays known in the art (and using commercially available materials), such as native
35 size exclusion HPLC ("SEC"), denaturing size exclusion using sodium dodecyl sulphate ("SDS-SEC"), reverse phase HPLC and capillary electrophoresis.

 "Apo-2 ligand receptor" includes the receptors referred to in the art as "DR4" and "DR5" whose polynucleotide and polypeptide sequences are shown in Figures 2 and 3 respectively. Pan et al. have described the TNF
40 receptor family member referred to as "DR4" (Pan et al., Science, 276:111-113 (1997); see also WO98/32856 published July 30, 1998; WO 99/37684 published July 29, 1999; WO 00/73349 published December 7, 2000; US 6,433,147 issued August 13, 2002; US 6,461,823 issued October 8, 2002, and US 6,342,383 issued January 29, 2002). Sheridan et al., Science, 277:818-

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821 (1997) and Pan et al., Science, 277:815-818 (1997) described another receptor for Apo2L/TRAIL (see also, WO98/51793 published November 19, 1998; WO98/41629 published September 24, 1998). This receptor is referred to as DR5 (the receptor has also been alternatively referred to as Apo-2; TRAIL-R, TR6, Tango-63, hAPO8, TRICK2 or KILLER; Screaton et al., Curr. Biol., 7:693-696 (1997); Walczak et al., EMBO J., 16:5386-5387 (1997); Wu et al., Nature Genetics, 17:141-143 (1997); WO98/35986 published August 20, 1998; EP870,827 published October 14, 1998; WO98/46643 published October 22, 1998; WO99/02653 published January 21, 1999; WO99/09165 published February 25, 1999; WO99/11791 published March 11, 1999; US 2002/0072091 published August 13, 2002; US 2002/0098550 published December 7, 2001; US 6,313,269 issued December 6, 2001; US 2001/0010924 published August 2, 2001; US 2003/01255540 published July 3, 2003; US 2002/0160446 published October 31, 2002; US 2002/0048785 published April 25, 2002; US 6,569,642 issued May 27, 2003; US 6,072,047 issued June 6, 2000; US 6,642,358 issued November 4, 2003). As described above, other receptors for Apo-2L include DcR1, DcR2, and OPG (see, Sheridan et al., supra; Marsters et al., supra; and Simonet et al., supra). The term "Apo-2L receptor" when used herein encompasses native sequence receptor and receptor variants. These terms encompass Apo-2L receptor expressed in a variety of mammals, including humans. Apo-2L receptor may be endogenously expressed as occurs naturally in a variety of human tissue lineages, or may be expressed by recombinant or synthetic methods. A "native sequence Apo-2L receptor" comprises a polypeptide having the same amino acid sequence as an Apo-2L receptor derived from nature. Thus, a native sequence Apo-2L receptor can have the amino acid sequence of naturally-occurring Apo-2L receptor from any mammal. Such native sequence Apo-2L receptor can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence Apo-2L receptor" specifically encompasses naturally-occurring truncated or secreted forms of the receptor (e.g., a soluble form containing, for instance, an extracellular domain sequence), naturally-occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants. Receptor variants may include fragments or deletion mutants of the native sequence Apo-2L receptor. Figure 3A shows the 411 amino acid sequence of human DR5 as published in WO 98/51793 on November 19, 1998. A transcriptional splice variant of human DR5 is known in the art. This DR5 splice variant encodes the 440 amino acid sequence of human DR5 shown in Figures 3B and 3C as published in WO 98/35986 on August 20, 1998.

"Death receptor antibody" is used herein to refer generally to antibody or antibodies directed to a receptor in the tumor necrosis factor receptor superfamily and containing a death domain capable of signalling apoptosis, and such antibodies include DR5 antibody and DR4 antibody.

"DR5 receptor antibody", "DR5 antibody", or "anti-DR5 antibody" is used in a broad sense to refer to antibodies that bind to at least one form

of a DR5 receptor or extracellular domain thereof. Optionally the DR5 antibody is fused or linked to a heterologous sequence or molecule. Preferably the heterologous sequence allows or assists the antibody to form higher order or oligomeric complexes. Optionally, the DR5 antibody binds to DR5 receptor but does not bind or cross-react with any additional Apo-2L receptor (e.g. DR4, DcR1, or DcR2). Optionally the antibody is an agonist of DR5 signalling activity.

Optionally, the DR5 antibody of the invention binds to a DR5 receptor at a concentration range of about 0.1 nM to about 20 nM as measured in a BIAcore binding assay. Optionally, the DR5 antibodies of the invention exhibit an I_{c50} value of about 0.6 nM to about 18 nM as measured in a BIAcore binding assay.

"DR4 receptor antibody", "DR4 antibody", or "anti-DR4 antibody" is used in a broad sense to refer to antibodies that bind to at least one form of a DR4 receptor or extracellular domain thereof. Optionally the DR4 antibody is fused or linked to a heterologous sequence or molecule. Preferably the heterologous sequence allows or assists the antibody to form higher order or oligomeric complexes. Optionally, the DR4 antibody binds to DR4 receptor but does not bind or cross-react with any additional Apo-2L receptor (e.g. DR5, DcR1, or DcR2). Optionally the antibody is an agonist of DR4 signalling activity.

Optionally, the DR4 antibody of the invention binds to a DR4 receptor at a concentration range of about 0.1 nM to about 20 nM as measured in a BIAcore binding assay. Optionally, the DR5 antibodies of the invention exhibit an I_{c50} value of about 0.6 nM to about 18 nM as measured in a BIAcore binding assay.

The term "agonist" is used in the broadest sense, and includes any molecule that partially or fully enhances, stimulates or activates one or more biological activities of Apo2L/TRAIL, DR4 or DR5, *in vitro*, *in situ*, or *in vivo*. Examples of such biological activities binding of Apo2L/TRAIL to DR4 or DR5, include apoptosis as well as those further reported in the literature. An agonist may function in a direct or indirect manner. For instance, the agonist may function to partially or fully enhance, stimulate or activate one or more biological activities of DR4 or DR5, *in vitro*, *in situ*, or *in vivo* as a result of its direct binding to DR4 or DR5, which causes receptor activation or signal transduction. The agonist may also function indirectly to partially or fully enhance, stimulate or activate one or more biological activities of DR4 or DR5, *in vitro*, *in situ*, or *in vivo* as a result of, e.g., stimulating another effector molecule which then causes DR4 or DR5 activation or signal transduction. It is contemplated that an agonist may act as an enhancer molecule which functions indirectly to enhance or increase DR4 or DR5 activation or activity. For instance, the agonist may enhance activity of endogenous Apo-2L in a mammal. This could be accomplished, for example, by pre-complexing DR4 or DR5 or by

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stabilizing complexes of the respective ligand with the DR4 or DR5 receptor (such as stabilizing native complex formed between Apo-2L and DR4 or DR5).

The term "DR4" and "DR4 receptor" as used herein refers to full length and soluble, extracellular domain forms of the receptor described in
5 Pan et al., Science, 276:111-113 (1997); WO98/32856 published July 30, 1998; US Patent 6,342,363 issued January 29, 2002; and WO99/37684 published July 29, 1999. The full length amino acid sequence of DR4 receptor is provided herein in Fig. 2.

The term "DR5" and "DR5 receptor" as used herein refers to the full
10 length and soluble, extracellular domain forms of the receptor described in Sheridan et al., Science, 277:818-821 (1997); Pan et al., Science, 277:815-818 (1997), US Patent 6,072,047 issued June 6, 2000; US Patent 6,342,369, WO98/51793 published November 19, 1998; WO98/41629 published September 24, 1998; Screaton et al., Curr. Biol., 7:693-696 (1997); Walczak et al., EMBO J., 16:5386-5387 (1997); Wu et al., Nature Genetics, 17:141-143 (1997);
15 WO98/35986 published August 20, 1998; EP870,827 published October 14, 1998; WO98/46643 published October 22, 1998; WO99/02653 published January 21, 1999; WO99/09165 published February 25, 1999; WO99/11791 published March 11, 1999. The DR5 receptor has also been referred to in the art as Apo-2;
20 TRAIL-R, TR6, Tango-63, hAPO8, TRICK2 or KILLER. The term DR5 receptor used herein includes the full length 411 amino acid polypeptide provided in Fig. 3A and the full length 440 amino acid polypeptide provided in Figs. 3B-C.

The term "polyol" when used herein refers broadly to polyhydric
25 alcohol compounds. Polyols can be any water-soluble poly(alkylene oxide) polymer for example, and can have a linear or branched chain. Preferred polyols include those substituted at one or more hydroxyl positions with a chemical group, such as an alkyl group having between one and four carbons. Typically, the polyol is a poly(alkylene glycol), preferably poly(ethylene glycol) (PEG). However, those skilled in the art recognize that other
30 polyols, such as, for example, poly(propylene glycol) and polyethylene-polypropylene glycol copolymers, can be employed using the techniques for conjugation described herein for PEG. The polyols of the invention include those well known in the art and those publicly available, such as from
35 commercially available sources.

The term "conjugate" is used herein according to its broadest definition to mean joined or linked together. Molecules are "conjugated" when they act or operate as if joined.

The term "extracellular domain" or "ECD" refers to a form of ligand
40 or receptor which is essentially free of transmembrane and cytoplasmic domains. Ordinarily, the soluble ECD will have less than 1% of such transmembrane and cytoplasmic domains, and preferably, will have less than 0.5% of such domains.

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The term "divalent metal ion" refers to a metal ion having two positive charges. Examples of divalent metal ions for use in the present invention include but are not limited to zinc, cobalt, nickel, cadmium, magnesium, and manganese. Particular forms of such metals that may be employed include salt forms (e.g., pharmaceutically acceptable salt forms), such as chloride, acetate, carbonate, citrate and sulfate forms of the above mentioned divalent metal ions. Divalent metal ions, as described herein, are preferably employed in concentrations or amounts (e.g., effective amounts) which are sufficient to, for example, (1) enhance storage stability of Apo-2L trimers over a desired period of time, (2) enhance production or yield of Apo-2L trimers in a recombinant cell culture or purification method, (3) enhance solubility (or reduce aggregation) of Apo-2L trimers, or (4) enhance Apo-2L trimer formation.

"Isolated," when used to describe the various proteins disclosed herein, means protein that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the protein, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the protein will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated protein includes protein in situ within recombinant cells, since at least one component of the protein's natural environment will not be present. Ordinarily, however, isolated protein will be prepared by at least one purification step.

An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the nucleic acid. An isolated Apo-2 ligand nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated Apo-2 ligand nucleic acid molecules therefore are distinguished from the Apo-2 ligand nucleic acid molecule as it exists in natural cells. However, an isolated Apo-2 ligand nucleic acid molecule includes Apo-2 ligand nucleic acid molecules contained in cells that ordinarily express Apo-2 ligand where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

"Percent (%) amino acid sequence identity" with respect to the sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the Apo-2 ligand sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence

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identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art can determine appropriate parameters for measuring alignment, including assigning algorithms needed to achieve maximal alignment over the full-length sequences being compared. For purposes herein, percent amino acid identity values can be obtained using the sequence comparison computer program, ALIGN-2, which was authored by Genentech, Inc. and the source code of which has been filed with user documentation in the US Copyright Office, Washington, DC, 20559, registered under the US Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available through Genentech, Inc., South San Francisco, CA. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

A "B cell" is a lymphocyte that matures within the bone marrow, and includes a naive B cell, memory B cell, or effector B cells (plasma cells). The B cell herein may be a normal or non-malignant B cell.

The "CD20" antigen is a ~35 kDa, non-glycosylated phosphoprotein found on the surface of greater than 90% of B cells from peripheral blood or lymphoid organs. CD20 is present on both normal B cells as well as malignant B cells, but is not expressed on stem cells. Other names for CD20 in the literature include "B-lymphocyte-restricted antigen" and "Bp35". The CD20 antigen is described in Clark et al. *PNAS (USA)* 82:1766 (1985), for example.

Examples of antibodies which bind the CD20 antigen include: "C2B8" which is now called "Rituximab" ("RITUXAN®") (US Patent No. 5,736,137); the yttrium-[90]-labeled 2B8 murine antibody designated "Y2B8" or "Ibritumomab Tiuxetan" ZEVALIN® commercially available from Idac Pharmaceuticals, Inc. (US Patent No. 5,736,137; 2B8 deposited with ATCC under accession no. HB11388 on June 22, 1993); murine IgG2a "B1," also called "Tositumomab," optionally labeled with ¹³¹I to generate the "¹³¹I-B1" antibody (Iodine I131 tositumomab, BEXXAR™) commercially available from Corixa (see, also, US Patent No. 5,595,721); murine monoclonal antibody "1F5" (Press et al. *Blood* 69(2):584-591 (1987)) and variants thereof including "framework patched" or humanized 1F5 (WO 2003/002607, Leung, ATCC Deposit HB-96450); murine 2H7 and chimeric 2H7 antibody (US Patent No. 5,677,180); humanized 2H7; HUMAX-CD20™ fully human, high-affinity antibody targeted at the CD20 molecule in the cell membrane of B-cells (Genmab, Denmark; see, for example, Glennie and van de Winkel, *Drug Discovery Today* 8: 503-510 (2003) and Cragg et al., *Blood* 101: 1045-1052 (2003)); the human monoclonal antibodies set forth in WO04/035607 (Teeling et al.); AME-133™ antibodies (Applied Molecular Evolution); A20 antibody or variants thereof such as chimeric or humanized A20 antibody (ca20, ha20, respectively) (US 2003/0219433, Immunomedics); and monoclonal antibodies L27, G28-2, 93-1B3, B-C1 or NU-B2 available from the International Leukocyte Typing Workshop (Valentine et al., In: *Leukocyte Typing III* (McMichael, Ed., p. 440, Oxford University Press (1987))). The preferred CD20 antibodies herein are chimeric, humanized, or human CD20 antibodies, more preferably rituximab, humanized 2H7, chimeric or humanized A20 antibody (Immunomedics), and HUMAX-CD20™ human CD20 antibody (Genmab).

The terms "rituximab" or "RITUXAN®" herein refer to the genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen and designated "C2B8" in US Patent No. 5,736,137, including fragments thereof which retain the ability to bind CD20.

Purely for the purposes herein and unless indicated otherwise, "humanized 2H7" refers to a humanized CD20 antibody, or an antigen-binding fragment thereof, wherein the antibody is effective to deplete primate B cells *in vivo*, the antibody comprising in the H chain variable region (V_H) thereof at least a CDR H3 sequence from an anti-human CD20 antibody and substantially the human consensus framework (FR) residues of the human heavy-chain subgroup III (V_HIII).

A preferred humanized 2H7 is an intact antibody or antibody fragment comprising the variable light chain sequence:

DIQMTQSPSSLSASVGRVTITCRASSSVSYMEHWYQQKPGKAPKPLIYAPENLASGVPSRFSGSGSGTDFTLTIS
SLQPEDFATYYCQQWSFNPPFTFGQGTKVEIKR (SEQ ID NO:7);

and the variable heavy chain sequence:

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMEWVRQAPGKLEWVGAIYPCNGDTSYNQKFKGRFTISVDKS
KNTLYLQMNSLRAEDTAVYYCARVYYFNSYWFYFDVWGQGTLTIVSS (SEQ ID NO:8).

Where the humanized 2H7 antibody is an intact antibody, preferably it comprises the light chain amino acid sequence:

DIQMTQSPBSLSASVGGDRVTITCRASSSVSYMHWYQQKPGKAPKPLIYAPENLASGVPSRFSGSGSGTDFTLTIS
SLQPEDFATYYCQQWSFNPPITFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVD
5 NALQSGNSQESVTEQDSKDSSTYSLSSTLTLSKADYKHKVYACEVTEQGLSPVTKBFRGEC (SEQ ID
NO:9);

and the heavy chain amino acid sequence:

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGAIYPGNGDTSYNQKFKGRFTISVDKS
KNTLYLQMNSLRAEDTAVYYCARVVYYSNYSYWFYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
10 VKDYFPEPVTVSWNSGALTSGVETFFPAVLQSSGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
DKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFWMYVDGVEVENAKTKPREEQ
YNSTYRVVSVLTIVLEQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRHEMTKNQVSLTCLVK
GFYPSTDAVEWESNGQPENNYKTTFFVLDSDGSFFLYSKLTVDKSRWQQGNVFSQSVMEALENHYTQKSLSLSP
GK (SEQ ID NO:10)

15 or the heavy chain amino acid sequence:

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGAIYPGNGDTSYNQKFKGRFTISVDKS
KNTLYLQMNSLRAEDTAVYYCARVVYYSNYSYWFYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVETFFPAVLQSSGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
DKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFWMYVDGVEVENAKTKPREEQ
20 YNATYRVVSVLTIVLEQDWLNGKEYKCKVSNKALPAPIAATISKAKGQPREPQVYTLPPSRHEMTKNQVSLTCLVK
GFYPSTDAVEWESNGQPENNYKTTFFVLDSDGSFFLYSKLTVDKSRWQQGNVFSQSVMEALENHYTQKSLSLSP
GK (SEQ ID NO:11).

"Antibody-dependent cell-mediated cytotoxicity" and "ADCC" refer to a
25 cell-mediated reaction in which nonspecific cytotoxic cells that express Fc
receptors (FcRs) (e.g. Natural Killer (NK) cells, neutrophils, and
macrophages) recognize bound antibody on a target cell and subsequently
cause lysis of the target cell. The primary cells for mediating ADCC, NK
cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and
30 FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on
page 464 of Ravetch and Kinet, Annu. Rev. Immunol 9:457-92 (1991). To
assess ADCC activity of a molecule of interest, an *in vitro* ADCC assay,
such as that described in US Patent No. 5,500,362 or 5,821,337 may be
performed. Useful effector cells for such assays include peripheral blood
35 mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or
additionally, ADCC activity of the molecule of interest may be assessed *in vivo*,
e.g., in a animal model such as that disclosed in Clynes et al. PNAS
(USA) 95:652-656 (1998).

"Human effector cells" are leukocytes which express one or more FcRs
40 and perform effector functions. Preferably, the cells express at least
FcγRIII and carry out ADCC effector function. Examples of human leukocytes
which mediate ADCC include peripheral blood mononuclear cells (PBMC),
natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils;
with PBMCs and NK cells being preferred.

"Native antibodies" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a β -sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β -sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cell-mediated cytotoxicity (ADCC).

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-binding sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and antigen-binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the V_H - V_L dimer. Collectively, the

six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear at least one free thiol group. F(ab')₂ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, antibodies can be assigned to different classes. There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA₁, and IgA₂. The heavy-chain constant domains that correspond to the different classes of antibodies are called α , δ , ϵ , γ , and μ , respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

"Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H - V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993).

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e.,

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the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature*, 352:624-628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)). Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (e.g. Old World Monkey, such as baboon, rhesus or cynomolgus monkey) and human constant region sequences (US Pat No. 5,693,780).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found

in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (e.g. residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a "hypervariable loop" (e.g. residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk J. *Mol. Biol.* 196:901-917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

An antibody "which binds" an antigen of interest, e.g. CD20 or DR4 or DR5, is one capable of binding that antigen with sufficient affinity and/or avidity such that the antibody is useful as a therapeutic agent for targeting a cell expressing the antigen.

For the purposes herein, "immunotherapy" will refer to a method of treating a mammal (preferably a human patient) with an antibody, wherein the antibody may be an unconjugated or "naked" antibody, or the antibody may be conjugated or fused with heterologous molecule(s) or agent(s), such as one or more cytotoxic agent(s), thereby generating an "immunoconjugate".

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antagonist or antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE

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under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody *in situ* within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated

5 antibody will be prepared by at least one purification step.

The expression "effective amount" refers to an amount of the Apo2L/TRAIL or death receptor antibody and CD20 antibody which is effective for preventing, ameliorating or treating the disease or condition in question.

10 The term "immunosuppressive agent" as used herein for adjunct therapy refers to substances that act to suppress or mask the immune system of the mammal being treated herein. This would include substances that suppress cytokine production, downregulate or suppress self-antigen expression, or mask the MHC antigens. Examples of such agents include 2-amino-6-aryl-5-

15 substituted pyrimidines (see U.S. Pat. No. 4,665,077, the disclosure of which is incorporated herein by reference); nonsteroidal antiinflammatory drugs (NSAIDs); azathioprine; cyclophosphamide; bromocryptine; danazol; dapsone; glutaraldehyde (which masks the MHC antigens, as described in U.S. Pat. No. 4,120,649); anti-idiotypic antibodies for MHC antigens and MHC

20 fragments; cyclosporin A; steroids such as glucocorticosteroids, e.g., prednisone, methylprednisolone, dexamethasone, and hydrocortisone; methotrexate (oral or subcutaneous); hydroxycycloquine; sulfasalazine; leflunomide; cytokine or cytokine receptor antagonists including anti-interferon- γ , - β , or - α antibodies, anti-tumor necrosis factor- α antibodies

25 (infliximab or adalimumab), anti-TNF α immunoabsorbent (etanercept), anti-tumor necrosis factor- β antibodies, anti-interleukin-2 antibodies and anti-IL-2 receptor antibodies; anti-LFA-1 antibodies, including anti-CD11a and anti-CD18 antibodies; anti-L3T4 antibodies; heterologous anti-lymphocyte globulin; pan-T antibodies, preferably anti-CD3 or anti-CD4/CD4a

30 antibodies; soluble peptide containing a LFA-3 binding domain (WO 90/08187 published 7/26/90); streptokinase; TGF- β ; streptodornase; RNA or DNA from the host; FK506; RS-61443; deoxyspergualin; rapamycin; T-cell receptor (Cohen et al., U.S. Pat. No. 5,114,721); T-cell receptor fragments (Offner et al., *Science*, 251: 430-432 (1991); WO 90/11294; Ianeway, *Nature*, 341: 482 (1989); and WO 91/01133); and T cell receptor antibodies (EP 340,109)

35 such as T10B9.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. At²¹¹, I¹³¹, I¹²⁵, Y⁹⁰, Re¹⁸⁶, Re¹⁸⁸, Sm¹⁵³, Bi²¹³, P³² and radioactive isotopes of Lu),

40 chemotherapeutic agents, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

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"Synergistic activity" or "synergy" or "synergistic effect" or "synergistic effective amount" for the purposes herein means that the effect observed when employing a combination of Apo2L/TRAIL or death receptor antibody and CD20 antibody is (1) greater than the effect achieved when that Apo2L/TRAIL, death receptor antibody or CD20 antibody is employed alone (or individually) and (2) greater than the sum added (additive) effect for that Apo2L/TRAIL or death receptor antibody and CD20 antibody. Such synergy or synergistic effect can be determined by way of a variety of means known to those in the art. For example, the synergistic effect of Apo2L/TRAIL or death receptor antibody and CD20 antibody can be observed in in vitro or in vivo assay formats examining reduction of tumor cell number or tumor mass.

The terms "apoptosis" and "apoptotic activity" are used in a broad sense and refer to the orderly or controlled form of cell death in mammals that is typically accompanied by one or more characteristic cell changes, including condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. This activity can be determined and measured using well known art methods, for instance, by cell viability assays, FACS analysis or DNA electrophoresis, binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). Assays which determine the ability of an antibody (e.g. Rituximab) to induce apoptosis have been described in Shan et al. *Cancer Immunol Immunother* 48:673-83 (2000); Pedersen et al. *Blood* 99:1314-9 (2002); Demidem et al. *Cancer Chemotherapy & Radiopharmaceuticals* 12(3):177-186 (1997), for example.

The terms "cancer", "cancerous", and "malignant" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma including adenocarcinoma, lymphoma, blastoma, melanoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, Hodgkin's and non-Hodgkin's lymphoma, pancreatic cancer, glioblastoma, glioma, cervical cancer, ovarian cancer, liver cancer such as hepatic carcinoma and hepatoma, bladder cancer, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, myeloma (such as multiple myeloma), salivary gland carcinoma, kidney cancer such as renal cell carcinoma and Wilms' tumors, basal cell carcinoma, melanoma, prostate cancer, vulval cancer, thyroid cancer, testicular cancer, esophageal cancer, and various types of head and neck cancer.

The term "immune related disease" means a disease in which a component of the immune system of a mammal causes, mediates or otherwise contributes to a morbidity in the mammal. Also included are diseases in

which stimulation or intervention of the immune response has an ameliorative effect on progression of the disease. Included within this term are autoimmune diseases, immune-mediated inflammatory diseases, non-immune-mediated inflammatory diseases, infectious diseases, and immunodeficiency diseases. Examples of immune-related and inflammatory diseases, some of which are immune or T cell mediated, which can be treated according to the invention include systemic lupus erythematosus, rheumatoid arthritis, juvenile chronic arthritis, spondyloarthropathies, systemic sclerosis (scleroderma), idiopathic inflammatory myopathies (dermatomyositis, polymyositis), Sjogren's syndrome, systemic vasculitis, sarcoidosis, autoimmune hemolytic anemia (immune pancytopenia, paroxysmal nocturnal hemoglobinuria), autoimmune thrombocytopenia (idiopathic thrombocytopenic purpura, immune-mediated thrombocytopenia), thyroiditis (Grave's disease, Hashimoto's thyroiditis, juvenile lymphocytic thyroiditis, atrophic thyroiditis), diabetes mellitus, immune-mediated renal disease (glomerulonephritis, tubulointerstitial nephritis), demyelinating diseases of the central and peripheral nervous systems such as multiple sclerosis, idiopathic demyelinating polyneuropathy or Guillain-Barré syndrome, and chronic inflammatory demyelinating polyneuropathy, hepatobiliary diseases such as infectious hepatitis (hepatitis A, B, C, D, E and other non-hepatotropic viruses), autoimmune chronic active hepatitis, primary biliary cirrhosis, granulomatous hepatitis, and sclerosing cholangitis, inflammatory and fibrotic lung diseases such as inflammatory bowel disease (ulcerative colitis: Crohn's disease), gluten-sensitive enteropathy, and Whipple's disease, autoimmune or immune-mediated skin diseases including bullous skin diseases, erythema multiforme and contact dermatitis, psoriasis, allergic diseases such as asthma, allergic rhinitis, atopic dermatitis, food hypersensitivity and urticaria, immunologic diseases of the lung such as eosinophilic pneumonias, idiopathic pulmonary fibrosis and hypersensitivity pneumonitis, transplantation associated diseases including graft rejection and graft-versus-host-disease. Infectious diseases include AIDS (HIV infection), hepatitis A, B, C, D, and E, bacterial infections, fungal infections, protozoal infections and parasitic infections.

A "B cell malignancy" is a malignancy involving B cells. Examples include Hodgkin's disease, including lymphocyte predominant Hodgkin's disease (LPHD); non-Hodgkin's lymphoma (NHL); follicular center cell (FCC) lymphoma; acute lymphocytic leukemia (ALL); chronic lymphocytic leukemia (CLL); hairy cell leukemia; plasmacytoid lymphocytic lymphoma; mantle cell lymphoma; AIDS or HIV-related lymphoma; multiple myeloma; central nervous system (CNS) lymphoma; post-transplant lymphoproliferative disorder (PTLD); Waldenstrom's macroglobulinemia (lymphoplasmacytic lymphoma); mucosa-associated lymphoid tissue (MALT) lymphoma; and marginal zone lymphoma/leukemia.

Non-Hodgkin's lymphoma (NHL) includes, but is not limited to, low grade/follicular NHL, relapsed or refractory NHL, front line low grade NHL, Stage III/IV NHL, chemotherapy resistant NHL, small lymphocytic (SL) NHL, intermediate grade/follicular NHL, intermediate grade diffuse NHL, diffuse large cell lymphoma, aggressive NHL (including aggressive front-line NHL and aggressive relapsed NHL), NHL relapsing after or refractory to autologous stem cell transplantation, high grade immunoblastic NHL, high grade lymphoblastic NHL, high grade small non-cleaved cell NHL, bulky disease NHL, etc.

10 An "autoimmune disease" herein is a disease or disorder arising from and directed against an individual's own tissues or a co-segregate or manifestation thereof or resulting condition therefrom. Examples of autoimmune diseases or disorders include, but are not limited to arthritis (rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, 15 psoriatic arthritis, and ankylosing spondylitis), psoriasis, dermatitis including atopic dermatitis; chronic idiopathic urticaria, including chronic autoimmune urticaria, polymyositis/dermatomyositis, toxic epidermal necrolysis, systemic scleroderma and sclerosis, responses associated with inflammatory bowel disease (IBD) (Crohn's disease, ulcerative colitis), and 20 IBD with co-segregate of pyoderma gangrenosum, erythema nodosum, primary sclerosing cholangitis, and/or episcleritis), respiratory distress syndrome, including adult respiratory distress syndrome (ARDS), meningitis, IgE-mediated diseases such as anaphylaxis and allergic rhinitis, encephalitis such as Rasmussen's encephalitis, uveitis, colitis such as 25 microscopic colitis and collagenous colitis, glomerulonephritis (GN) such as membranous GN, idiopathic membranous GN, membranous proliferative GN (MPGN), including Type I and Type II, and rapidly progressive GN, allergic conditions, eczema, asthma, conditions involving infiltration of T cells and chronic inflammatory responses, atherosclerosis, autoimmune 30 myocarditis, leukocyte adhesion deficiency, systemic lupus erythematosus (SLE) such as cutaneous SLE, lupus (including nephritis, cerebritis, pediatric, non-renal, discoid, alopecia), juvenile onset diabetes, multiple sclerosis (MS) such as spino-optical MS, allergic encephalomyelitis, immune responses associated with acute and delayed hypersensitivity mediated by 35 cytokines and T-lymphocytes, tuberculosis, sarcoidosis, granulomatosis including Wegener's granulomatosis, agranulocytosis, vasculitis (including Large Vessel vasculitis (including Polyarteritis Rheumatica and Giant Cell (Takayasu's) Arteritis), Medium Vessel vasculitis (including Kawasaki's Disease and Polyarteritis Nodosa), CNS vasculitis, and ANCA-associated 40 vasculitis, such as Churg-Strauss vasculitis or syndrome (CSS)), aplastic anemia, Coombs positive anemia, Diamond Blackfan anemia, immune hemolytic anemia including autoimmune hemolytic anemia (AIHA), pernicious anemia, pure red cell aplasia (PRCA), Factor VIII deficiency, hemophilia A, autoimmune neutropenia, pancytopenia, leukopenia, diseases involving

leukocyte diapedesis, CNS inflammatory disorders, multiple organ injury syndrome, myasthenia gravis, antigen-antibody complex mediated diseases, anti-glomerular basement membrane disease, anti-phospholipid antibody syndrome, allergic neuritis, Bechet disease, Castleman's syndrome, Goodpasture's Syndrome, Lambert-Eaton Myasthenic Syndrome, Reynaud's syndrome, Sjorgen's syndrome, Stevens-Johnson syndrome, solid organ transplant rejection (including pretreatment for high panel reactive antibody titers, IgA deposit in tissues, and rejection arising from renal transplantation, liver transplantation, intestinal transplantation, cardiac transplantation, etc.), graft versus host disease (GVHD), pemphigoid bullous, pemphigus (including vulgaris, foliaceus, and pemphigus mucosa-membrane pemphigoid), autoimmune polyendocrinopathies, Reiter's disease, stiff-man syndrome, immune complex nephritis, IgM polyneuropathies or IgM mediated neuropathy, idiopathic thrombocytopenic purpura (ITP), thrombotic thrombocytopenic purpura (TTP), thrombocytopenia (as developed by myocardial infarction patients, for example), including autoimmune thrombocytopenia, autoimmune disease of the testis and ovary including autoimmune orchitis and oophoritis, primary hypothyroidism; autoimmune endocrine diseases including autoimmune thyroiditis, chronic thyroiditis (Hashimoto's Thyroiditis), subacute thyroiditis, idiopathic hypothyroidism, Addison's disease, Grave's disease, autoimmune polyglandular syndromes (or polyglandular endocrinopathy syndromes), Type I diabetes also referred to as insulin-dependent diabetes mellitus (IDDM), including pediatric IDDM, and Sheehan's syndrome; autoimmune hepatitis, lymphoid interstitial pneumonitis (HIV), bronchiolitis obliterans (non-transplant) vs NSIP, Guillain-Barré Syndrome, Berger's Disease (IgA nephropathy), primary biliary cirrhosis, celiac sprue (gluten enteropathy), refractory sprue with co-segregate dermatitis herpetiformis, cryoglobulinemia, amyotrophic lateral sclerosis (ALS; Lou Gehrig's disease), coronary artery disease, autoimmune inner ear disease (AIED), autoimmune hearing loss, opsoclonus myoclonus syndrome (OMS), polychondritis such as refractory polychondritis, pulmonary alveolar proteinosis, amyloidosis, giant cell hepatitis, scleritis, monoclonal gammopathy of uncertain/unknown significance (MGUS), peripheral neuropathy, paraneoplastic syndrome, channelopathies such as epilepsy, migraine, arrhythmia, muscular disorders, deafness, blindness, periodic paralysis, and channelopathies of the CNS; autism, inflammatory myopathy, and focal segmental glomerulosclerosis (FSGS).

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to cancer cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stalla et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery,"

Directed Drug Delivery, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, beta-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described below.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. At²¹¹, I¹³¹, I¹²⁵, Y⁹⁰, Re¹⁸⁶, Re¹⁸⁸, Sm¹⁵³, Bi²¹³, P³² and radioactive isotopes of Lu), chemotherapeutic agents, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and CYTOXAN® cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CBI-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novambichin, phenasterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the enediyne antibiotics (e. g., calicheamicin, especially calicheamicin gammaII and calicheamicin omegaII (see, e.g., Agnew, Chem Intl. Ed. Engl., 33: 183-186 (1994)); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carubicin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin,

detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin (including
 morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-
 doxorubicin and decydoxorubicin), epirubicin, esorubicin, idarubicin,
 marcellomycin, mitomycins such as mitomycin C, mycophenolic acid,
 5 nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin,
 rodorubicin, streptonigrin, streptoxocin, tubercidin, ubenimex, zinostatin,
 zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU);
 folic acid analogues such as denopterin, methotrexate, pteropterin,
 trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine,
 10 thiamiprine, thioguanine; pyrimidine analogs such as ancitabine,
 azacitidine, 6-azauridine; carmofur, cytarabine, dideoxyuridine,
 doxifluridine, enocitabine, floxuridine; androgens such as calusterone,
 dromostanolone propionate, epitioostanol, mepitioostane, testolactone; anti-
 adrenals such as aminoglutethimide, mitotane, trilostane; folic acid
 15 replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside;
 aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene;
 edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium
 acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan;
 lonidainine; maytansinoids such as maytansine and ansamitocins;
 20 mitoguanzone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet;
 pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide;
 procarbazine; PSK® polysaccharide complex (JES Natural Products, Eugene,
 OR); razoxane; rhizoxin; sizofiran; spirogermanium; temuzonic acid;
 triaziquone; 2,2',2''-trichlorotriethylamine; trichothecenes (especially T-
 25 2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine;
 dacarbazine; mannometrine; mitobronitol; mitolactol; pipobroman;
 gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids,
 e.g., TAXOL® paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.),
 ABRAXANE™ Cremophor-free, albumin-engineered nanoparticle formulation of
 30 paclitaxel (American Pharmaceutical Partners, Schaumburg, Illinois), and
 TAXOTERE® docetaxel (Rhône-Poulenc Rorer, Antony, France); chlorambucil;
 GEMZAR® gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum
 analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide
 (VP-16); ifosfamide; mitoxantrone; vincristine; NAVELBINE® vinorelbine;
 35 novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda;
 ibandronate; CPT-11; topoisomerase inhibitor RFS 2000;
 difluoromethylornithine (DMFO); retinoids such as retinoic acid;
 capecitabine; and pharmaceutically acceptable salts, acids or derivatives
 of any of the above.

40 Also included in this definition are anti-hormonal agents that act to
 regulate or inhibit hormone action on tumors such as anti-estrogens and
 selective estrogen receptor modulators (SERMs), including, for example,
 tamoxifen (including NOLVADEX® tamoxifen), raloxifene, droloxifene, 4-
 hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and

FARESTON[®] toremifene; aromatase inhibitors that inhibit the enzyme
 aromatase, which regulates estrogen production in the adrenal glands, such
 as, for example, 4(5)-imidazoles, aminoglutethimide, MEKASE[®] megestrol
 acetate, AROMASIN[®] exemestane, formestane, fadrozole, RIVISOR[®] vorozole,
 5 FEMARA[®] letrozole, and ARIMIDEX[®] anastrozole; and anti-androgens such as
 flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; as well as
 troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense
 oligonucleotides, particularly those which inhibit expression of genes in
 signaling pathways implicated in adherent cell proliferation, such as, for
 10 example, PKC-alpha, Raf and H-Ras; ribozymes such as a VEGF expression
 inhibitor (e.g., ANGIOZYME[®] ribozyme) and a HER2 expression inhibitor;
 vaccines such as gene therapy vaccines, for example, ALLOVECTIN[®] vaccine,
 LEUVECTIN[®] vaccine, and VAXID[®] vaccine; PROLEUKIN[®] rIL-2, LURTOTECAN[®]
 topoisomerase 1 inhibitor; ABARELIX[®] rnrH; and pharmaceutically acceptable
 15 salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or
 composition which inhibits growth of a cell, either in vitro or in vivo.
 Thus, the growth inhibitory agent is one which significantly reduces the
 percentage of cells overexpressing such genes in S phase. Examples of
 20 growth inhibitory agents include agents that block cell cycle progression
 (at a place other than S phase), such as agents that induce G1 arrest and
 M-phase arrest. Classical M-phase blockers include the vincas (vincristine
 and vinblastine), taxol, and topo II inhibitors such as doxorubicin,
 epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that
 25 arrest G1 also spill over into S-phase arrest, for example, DNA alkylating
 agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine,
 cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information
 can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds.,
 Chapter 1, entitled "Cell cycle regulation, oncogens, and antineoplastic
 30 drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p.
 13.

The term "cytokine" is a generic term for proteins released by one
 cell population which act on another cell as intercellular mediators.
 Examples of such cytokines are lymphokines, monokines, and traditional
 35 polypeptide hormones. Included among the cytokines are growth hormones such
 as human growth hormone, N-methionyl human growth hormone, and bovine
 growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin;
 relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating
 hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone
 40 (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental
 lactogen; tumor necrosis factor- α and - β ; mullerian-inhibiting substance;
 mouse gonadotropin-associated peptide; inhibin; activin; vascular
 endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth
 factors; platelet-growth factor; transforming growth factors (TGFs) such as

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TGF- α and TGF- β ; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon- α , - β , and -gamma; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

10 A "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications, other therapeutic products to be combined with the packaged product, and/or warnings concerning the use of such therapeutic products, etc.

The terms "treating", "treatment" and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy.

The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.

II. Compositions and Methods of the Invention

A cytokine related to the TNF ligand family, the cytokine identified herein as "Apo-2 ligand" or "TRAIL" has been described. The predicted mature amino acid sequence of native human Apo-2 ligand contains 281 amino acids, and has a calculated molecular weight of approximately 32.5 kDa. The absence of a signal sequence and the presence of an internal hydrophobic region suggest that Apo-2 ligand is a type II transmembrane protein. Soluble extracellular domain Apo-2 ligand polypeptides have also been described. See, e.g., WO97/25428 published July 17, 1997. Apo-2L substitutional variants have further been described. Alanine scanning techniques have been utilized to identify various substitutional variant molecules having biological activity. Particular substitutional variants of the Apo-2 ligand include those in which at least one amino acid is substituted by another amino acids such as an alanine residue. These substitutional variants are identified, for example, as "D203A"; "D218A" and "D269A." This nomenclature is used to identify Apo-2 ligand variants wherein the aspartic acid residues at positions 203, 218, and/or 269 (using the numbering shown in Figure 1) are substituted by alanine residues. Optionally, the Apo-2L variants of the present invention may comprise one or more of the amino acid substitutions. Optionally, such Apo-2L variants will be DR4 or DR5 receptor selective variants.

The description below relates to methods of producing Apo-2 ligand, including Apo-2 ligand variants, by culturing host cells transformed or

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transfected with a vector containing Apo-2 ligand encoding nucleic acid and recovering the polypeptide from the cell culture.

The DNA encoding Apo-2 ligand may be obtained from any cDNA library prepared from tissue believed to possess the Apo-2 ligand mRNA and to express it at a detectable level. Accordingly, human Apo-2 ligand DNA can be conveniently obtained from a cDNA library prepared from human tissues, such as the bacteriophage library of human placental cDNA as described in WO97/25428. The Apo-2 ligand-encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

Libraries can be screened with probes (such as antibodies to the Apo-2 ligand or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook et al., *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding Apo-2 ligand is to use PCR methodology [Sambrook et al., *supra*; Dieffenbach et al., *PCR Primer: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, 1995)].

Amino acid sequence fragments or variants of Apo-2 ligand can be prepared by introducing appropriate nucleotide changes into the Apo-2 ligand DNA, or by synthesis of the desired Apo-2 ligand polypeptide. Such fragments or variants represent insertions, substitutions, and/or deletions of residues within or at one or both of the ends of the intracellular region, the transmembrane region, or the extracellular region, or of the amino acid sequence shown for the full-length Apo-2 ligand in Figure 1. Any combination of insertion, substitution, and/or deletion can be made to arrive at the final construct, provided that the final construct possesses, for instance, a desired biological activity, such as apoptotic activity, as defined herein. In a preferred embodiment, the fragments or variants have at least about 80% amino acid sequence identity, more preferably, at least about 90% sequence identity, and even more preferably, at least 95%, 96%, 97%, 98% or 99% sequence identity with the sequences identified herein for the intracellular, transmembrane, or extracellular domains of Apo-2 ligand, or the full-length sequence for Apo-2 ligand. The amino acid changes also may alter post-translational processes of the Apo-2 ligand, such as changing the number or position of glycosylation sites or altering the membrane anchoring characteristics.

Variations in the Apo-2 ligand sequence as described above can be made using any of the techniques and guidelines for conservative and non-conservative mutations set forth in U.S. Pat. No. 5,364,934. These include oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis.

Scanning amino acid analysis can be employed to identify one or more amino acids along a contiguous sequence. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine and cysteine. Alanine is typically a preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant. [Cunningham et al., Science, 244:1081 (1989)]. Alanine is also typically preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., NY); Chothia, J. Mol. Biol., 150:1 (1976)].

Amino acids may be grouped according to similarities in the properties of their side chains (in A. L. Lehninger, in Biochemistry, second ed., pp. 73-75, Worth Publishers, New York (1975)):

- (1) non-polar: Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M)
- (2) uncharged polar: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gln (Q)
- (3) acidic: Asp (D), Glu (E)
- (4) basic: Lys (K), Arg (R), His (H)

Alternatively, naturally occurring residues may be divided into groups based on common side-chain properties:

- (1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;
- (2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;
- (3) acidic: Asp, Glu;
- (4) basic: His, Lys, Arg;
- (5) residues that influence chain orientation: Gly, Pro;
- (6) aromatic: Trp, Tyr, Phe.

Table 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

5 Variations in the Apo-2 ligand sequence also included within the scope of the invention relate to amino-terminal derivatives or modified forms. Such Apo-2 ligand sequences include any of the Apo-2 ligand polypeptides described herein having a methionine or modified methionine (such as formyl methionyl or other blocked methionyl species) at the N-terminus of the polypeptide sequence.

10 The nucleic acid (e.g., cDNA or genomic DNA) encoding native or variant Apo-2 ligand may be inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is described below.

Optional signal sequences, origins of replication, marker genes, enhancer elements and transcription terminator sequences that may be employed are known in the art and described in further detail in WO97/25428.

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the Apo-2 ligand nucleic acid sequence. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, such as the Apo-2 ligand nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to Apo-2 ligand encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native Apo-2 ligand promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the Apo-2 ligand DNA.

Promoters suitable for use with prokaryotic and eukaryotic hosts are known in the art, and are described in further detail in WO97/25428.

A preferred method for the production of soluble Apo-2L in *E. coli* employs an inducible promoter for the regulation of product expression. The use of a controllable, inducible promoter allows for culture growth to the desirable cell density before induction of product expression and accumulation of significant amounts of product which may not be well tolerated by the host.

Several inducible promoter systems (T7 polymerase, trp and alkaline phosphatase (AP)) have been evaluated by Applicants for the expression of Apo-2L (form 114-281). The use of each of these three promoters resulted in significant amounts of soluble, biologically active Apo-2L trimer being recovered from the harvested cell paste. The AP promoter is preferred among these three inducible promoter systems tested because of tighter promoter control and the higher cell density and titers reached in harvested cell paste.

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline

resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced using standard techniques known in the art. [See, e.g., Messing et al., Nucleic Acids Res., 9:309 (1981); Maxam et al., Methods in Enzymology, 65:499 (1980)].

Expression vectors that provide for the transient expression in mammalian cells of DNA encoding Apo-2 ligand may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector [Sambrook et al., supra]. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of Apo-2 ligand that are biologically active Apo-2 ligand.

Other methods, vectors, and host cells suitable for adaptation to the synthesis of Apo-2 ligand in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantel et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

Suitable host cells for cloning or expressing the DNA in the vectors herein include prokaryote, yeast, or higher eukaryote cells. Suitable prokaryotes for this purpose include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, *Enterobacteriaceae* such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for Apo-2 ligand-encoding vectors. Suitable host cells for the expression of glycosylated Apo-2 ligand are derived from multicellular organisms. Examples of all such host cells, including CHO cells, are described further in WO97/25428.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors for Apo-2 ligand production and cultured in nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

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Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, CaPO_4 and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52:456-457 (1978) may be employed. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352 (1988).

Prokaryotic cells used to produce Apo-2 ligand may be cultured in suitable culture media as described generally in Sambrook et al., supra. Particular forms of culture media that may be employed for culturing *E. coli* are described further in the Examples below. Mammalian host cells used to produce Apo-2 ligand may be cultured in a variety of culture media.

Examples of commercially available culture media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an

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equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: A Practical Approach, M. Butler, ed. (IRL Press, 1991).

In accordance with one aspect of the present invention, one or more divalent metal ions will typically be added to or included in the culture media for culturing or fermenting the host cells. The divalent metal ions are preferably present in or added to the culture media at a concentration level sufficient to enhance storage stability, enhance solubility, or assist in forming stable Apo-2L trimers coordinated by one or more zinc ions. The amount of divalent metal ions which may be added will be dependent, in part, on the host cell density in the culture or potential host cell sensitivity to such divalent metal ions. At higher host cell densities in the culture, it may be beneficial to increase the concentration of divalent metal ions. If the divalent metal ions are added during or after product expression by the host cells, it may be desirable to adjust or increase the divalent metal ion concentration as product expression by the host cells increases. It is generally believed that trace levels of divalent metal ions which may be present in typical commonly available cell culture media may not be sufficient for stable trimer formation. Thus, addition of further quantities of divalent metal ions, as described herein, is preferred.

The divalent metal ions are preferably added to the culture media at a concentration which does not adversely or negatively affect host cell growth, if the divalent metal ions are being added during the growth phase of the host cells in the culture. In shake flask cultures, it was observed that ZnSO₄ added at concentrations of greater than 1 mM can result in lower host cell density. Those skilled in the art appreciate that bacterial cells can sequester metal ions effectively by forming metal ion complexes with cellular matrices. Thus, in the cell cultures, it is preferable to add the selected divalent metal ions to the culture media after the growth phase (after the desired host cell density is achieved) or just prior to product expression by the host cells. To ensure that sufficient amounts of divalent metal ions are present, additional divalent metal ions may be added or fed to the cell culture media during the product expression phase.

The divalent metal ion concentration in the culture media should not exceed the concentration which may be detrimental or toxic to the host cells. In the methods of the invention employing the host cell, *E. coli*, it is preferred that the concentration of the divalent metal ion

concentration in the culture media does not exceed about 1mM (preferably, $\leq$ 1mM). Even more preferably, the divalent metal ion concentration in the culture media is about 50 micromolar to about 250 micromolar. Most preferably, the divalent metal ion used in such methods is zinc sulfate.

5 It is desirable to add the divalent metal ions to the cell culture in an amount wherein the metal ions and Apo-2 ligand trimer can be present at a one to one molar ratio.

The divalent metal ions can be added to the cell culture in any acceptable form. For instance, a solution of the metal ion can be made using water, and the divalent metal ion solution can then be added or fed to the culture media.

Expression of the Apo-2L may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, and particularly ^{32}P . However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide.

15 The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorescers or enzymes. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected. Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like.

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native Apo-2 ligand polypeptide or against a synthetic peptide based on the DNA sequences provided herein or against exogenous sequence fused to Apo-2 ligand DNA and encoding a specific antibody epitope.

Apo-2 ligand preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates when directly produced without a secretory signal. If the Apo-2

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ligand is membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g. Triton-X 100) or its extracellular region may be released by enzymatic cleavage.

When Apo-2 ligand is produced in a recombinant cell other than one of human origin, the Apo-2 ligand is free of proteins or polypeptides of human origin. However, it is usually necessary to recover or purify Apo-2 ligand from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to Apo-2 ligand. As a first step, the culture medium or lysate may be centrifuged to remove particulate cell debris. Apo-2 ligand thereafter is purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE or CM; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; diafiltration and protein A Sepharose columns to remove contaminants such as IgG.

In a preferred embodiment, the Apo-2 ligand can be isolated by affinity chromatography. Apo-2 ligand fragments or variants in which residues have been deleted, inserted, or substituted are recovered in the same fashion as native Apo-2 ligand, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of an Apo-2 ligand fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing antibody to the antigen can be used to adsorb the fusion polypeptide.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native Apo-2 ligand may require modification to account for changes in the character of Apo-2 ligand or its variants upon expression in recombinant cell culture.

During any such purification steps, it may be desirable to expose the recovered Apo-2L to a divalent metal ion-containing solution or to purification material (such as a chromatography medium or support) containing one or more divalent metal ions. In a preferred embodiment, the divalent metal ions and/or reducing agent is used during recovery or purification of the Apo-2L. Optionally, both divalent metal ions and reducing agent, such as DTT or BME, may be used during recovery or purification of the Apo-2L. It is believed that use of divalent metal ions during recovery or purification will provide for stability of Apo-2L trimer or preserve Apo-2L trimer formed during the cell culturing step.

The description below also relates to methods of producing Apo-2 ligand covalently attached (hereinafter "conjugated") to one or more chemical groups. Chemical groups suitable for use in an Apo-2L conjugate of the present invention are preferably not significantly toxic or immunogenic. The chemical group is optionally selected to produce an Apo-2L conjugate that can be stored and used under conditions suitable for storage. A variety of exemplary chemical groups that can be conjugated to polypeptides are known in the art and include for example carbohydrates, such as those carbohydrates that occur naturally on glycoproteins, polyglutamate, and non-proteinaceous polymers, such as polyols (see, e.g., U.S. Patent No. 6,245,901).

A polyol, for example, can be conjugated to polypeptides such as an Apo-2L at one or more amino acid residues, including lysine residues, as is disclosed in WO 93/00109, *supra*. The polyol employed can be any water-soluble poly(alkylene oxide) polymer and can have a linear or branched chain. Suitable polyols include those substituted at one or more hydroxyl positions with a chemical group, such as an alkyl group having between one and four carbons. Typically, the polyol is a poly(alkylene glycol), such as poly(ethylene glycol) (PEG), and thus, for ease of description, the remainder of the discussion relates to an exemplary embodiment wherein the polyol employed is PEG and the process of conjugating the polyol to a polypeptide is termed "pegylation." However, those skilled in the art recognize that other polyols, such as, for example, poly(propylene glycol) and polyethylene-polypropylene glycol copolymers, can be employed using the techniques for conjugation described herein for PEG.

The average molecular weight of the PEG employed in the pegylation of the Apo-2L can vary, and typically may range from about 500 to about 30,000 daltons (D). Preferably, the average molecular weight of the PEG is from about 1,000 to about 25,000 D, and more preferably from about 1,000 to about 5,000 D. In one embodiment, pegylation is carried out with PEG having an average molecular weight of about 1,000 D. Optionally, the PEG homopolymer is unsubstituted, but it may also be substituted at one end with an alkyl group. Preferably, the alkyl group is a C1-C4 alkyl group, and most preferably a methyl group. PEG preparations are commercially available, and typically, those PEG preparations suitable for use in the present invention are nonhomogeneous preparations sold according to average molecular weight. For example, commercially available PEG(5000) preparations typically contain molecules that vary slightly in molecular weight, usually ± 500 D.

The Apo-2 ligand of the invention may be in various forms, such as in monomer form or trimer form (comprising three monomers). Optionally, an Apo-2L trimer will be pegylated in a manner such that a PEG molecule is linked or conjugated to one, two or each of the three monomers that make up the trimeric Apo-2L. In such an embodiment, it is preferred that the PEG

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employed have an average molecular weight of about 1,000 to about 5,000 D. It is also contemplated that the Apo-2L trimers may be "partially" pegylated, i.e., wherein only one or two of the three monomers that make up the trimer are linked or conjugated to PEG.

5 A variety of methods for pegylating proteins are known in the art. Specific methods of producing proteins conjugated to PEG include the methods described in U.S. Pat. No. 4,179,337, U.S. Pat. No. 4,935,465 and U.S. Patent No. 5,849,535. Typically the protein is covalently bonded via one or more of the amino acid residues of the protein to a terminal
10 reactive group on the polymer, depending mainly on the reaction conditions, the molecular weight of the polymer, etc. The polymer with the reactive group(s) is designated herein as activated polymer. The reactive group selectively reacts with free amino or other reactive groups on the protein. The PEG polymer can be coupled to the amino or other reactive group on the
15 protein in either a random or a site specific manner. It will be understood, however, that the type and amount of the reactive group chosen, as well as the type of polymer employed, to obtain optimum results, will depend on the particular protein or protein variant employed to avoid having the reactive group react with too many particularly active groups on
20 the protein. As this may not be possible to avoid completely, it is recommended that generally from about 0.1 to 1000 moles, preferably 2 to 200 moles, of activated polymer per mole of protein, depending on protein concentration, is employed. The final amount of activated polymer per mole of protein is a balance to maintain optimum activity, while at the same
25 time optimizing, if possible, the circulatory half-life of the protein.

It is further contemplated that the Apo2L described herein may be also be linked or fused to leucine zipper sequences using techniques known in the art.

30 Methods for generating death receptor antibodies and CD20 antibodies are also described herein. The antigen to be used for production of, or screening for, antibody may be, e.g., a soluble form of the antigen or a portion thereof, containing the desired epitope. Alternatively, or
35 additionally, cells expressing the antigen at their cell surface can be used to generate, or screen for, antibody. Other forms of the antigen useful for generating antibody will be apparent to those skilled in the art.

(i) Polyclonal antibodies

40 Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or

soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl_2 , or $\text{R}^1\text{N}=\text{C}=\text{NR}$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those
5 derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Manassas, Virginia USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal
10 antibodies (Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen.
15 Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be
20 determined by the Scatchard analysis of Munson et al., *Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding,
25 *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably
30 separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and
35 sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as E.
40 coli cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody

include Skerra et al., *Curr. Opinion in Immunol.*, 5:256-262 (1993) and Plückerthun, *Immunol. Revs.*, 130:151-188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature*, 348:552-554 (1990). Clackson et al., *Nature*, 352:624-628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.*, 21:2265-2266 (1993). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; Morrison, et al., *Proc. Natl Acad. Sci. USA*, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized antibodies

Methods for humanizing non-human antibodies have been described in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-327 (1988); Verhoeven et al., *Science*, 239:1534-1536 (1988)), by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.



The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993); Chothia et al., *J. Mol. Biol.*, 196:901 (1987)). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta et al., *J. Immunol.*, 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

(iv) Human antibodies

As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits et al., *Nature*, 362:255-

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258 (1993); Bruggermann et al., Year in Immuno., 7:33 (1993); and US Patent Nos. 5,591,669, 5,589,369 and 5,545,807.

Alternatively, phage display technology (McCafferty et al., Nature 348:552-553 (1990)) can be used to produce human antibodies and antibody fragments in vitro, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B cell. Phage display can be performed in a variety of formats; for their review see, e.g., Johnson, Kevin S. and Chiswell, David J., Current Opinion in Structural Biology 3:564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson et al., Nature, 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks et al., J. Mol. Biol. 222:581-597 (1991), or Griffith et al., EMBO J. 12:725-734 (1993). See, also, US Patent Nos. 5,565,332 and 5,573,905.

Human antibodies may also be generated by in vitro activated B cells (see US Patents 5,567,610 and 5,229,275).

(v) Antibody fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., Journal of Biochemical and Biophysical Methods 24:107-117 (1992) and Brennan et al., Science, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from E. coli and chemically coupled to form F(ab')₂ fragments (Carter et al., Bio/Technology 10:163-167 (1992)). According to another approach, F(ab')₂ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185; US Patent No. 5,571,894; and US Patent No. 5,587,458. The antibody fragment may also be a "linear

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antibody", e.g., as described in US Patent 5,641,870 for example. Such linear antibody fragments may be monospecific or bispecific.

(vi) Bispecific antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the CD20, DR4 or DR5 receptors. Bispecific antibodies may also be used to localize cytotoxic agents to a B cell. These antibodies possess a B cell marker-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon- α , vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. F(ab'), bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature*, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.*, 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was

found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation.

5 This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology*, 121:210 (1986). According to another approach described in US Patent No. 5,731,168, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers
10 which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar
15 size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

20 Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO
25 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody
30 fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science*, 229: 81 (1985); Shalaby et al., *J. Exp. Med.*, 175: 217-225 (1992).

Various techniques for making and isolating bispecific antibody
35 fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.*, 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody
40 homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making

bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., J. Immunol., 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. J. Immunol. 147: 60 (1991). Antibodies with three or more antigen binding sites are described in WO01/77342 (Miller and Presta), expressly incorporated herein by reference.

The antibody used in the methods or included in the articles of manufacture herein is optionally conjugated to a cytotoxic agent.

Chemotherapeutic agents useful in the generation of such antibody-cytotoxic agent conjugates have been described above.

Conjugates of an antibody and one or more small molecule toxins, such as a calicheamicin, a maytansine (US Patent No. 5,208,020), a trichothene, and CC1065 are also contemplated herein. In one embodiment of the invention, the antibody is conjugated to one or more maytansine molecules (e.g. about 1 to about 10 maytansine molecules per antibody molecule). Maytansine may, for example, be converted to May-SS-Me which may be reduced to May-SH3 and reacted with modified antibody (Chari et al. Cancer Research 52: 127-131 (1992)) to generate a maytansinoid-antibody conjugate.

Alternatively, the antibody is conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics is capable of producing double-stranded DNA breaks at sub-picomolar concentrations. Structural analogues of calicheamicin which may be used include, but are not limited to, γ_1^I , α_3^I , α_5^I , N-acetyl- γ_1^I , P8AG and θ^I , (Hinman et al. Cancer Research 53: 3336-3342 (1993) and Loda et al. Cancer Research 58: 2925-2928 (1998)).

Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolacca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, saponaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. See, for example, WO 93/21232 published October 28, 1993.

The present invention further contemplates antibody conjugated with a compound with nucleolytic activity (e.g. a ribonuclease or a DNA endonuclease such as a deoxyribonuclease, DNase).

A variety of radioactive isotopes are available for the production of radioconjugated antagonists or antibodies. Examples include At^{211} , I^{131} , I^{125} , Y^{90} , Re^{186} , Re^{188} , Sm^{153} , Bi^{213} , P^{32} and radioactive isotopes of Lu.

Conjugates of the antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. Science 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MK-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antagonist or antibody. See WO94/11026. The linker may be a "cleavable linker" facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, dimethyl linker or disulfide-containing linker (Chari et al. Cancer Research 52: 127-131 (1992)) may be used.

Alternatively, a fusion protein comprising the antibody and cytotoxic agent may be made, e.g. by recombinant techniques or peptide synthesis.

The antibodies of the present invention may also be conjugated with a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Patent No. 4,975,278.

The enzyme component of such conjugates includes any enzyme capable of acting on a prodrug in such a way so as to convert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β -galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β -lactamase useful for converting drugs derivatized with β -lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at

their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abszymes", can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, *Nature* 5 328: 457-458 (1987)). Antibody-abszyme conjugates can be prepared as described herein for delivery of the abszyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the antibody by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, 10 fusion proteins comprising at least the antigen binding region of an antibody linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g., Neuberger et al., *Nature*, 312: 604-608 (1984)).

15 Other modifications of the antibody are contemplated herein. For example, the antibody may be linked to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, polyoxyalkylenes, or copolymers of polyethylene glycol and polypropylene glycol.

20 To increase the serum half life of the antibody, one may incorporate a salvage receptor binding epitope into the antibody (especially an antibody fragment) as described in US Patent 5,739,277, for example. As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG₁, IgG₂, IgG₃, or IgG₄) 25 that is responsible for increasing the *in vivo* serum half-life of the IgG molecule. Alternatively, or additionally, one may increase, or decrease, serum half-life by altering the amino acid sequence of the Fc region of an antibody to generate variants with altered FcRn binding. Antibodies with altered FcRn binding and/or serum half life are described in WO00/42072 30 (Presta, L.).

Formulations comprising Apo2L/TRAIL, death receptor antibodies, and/or CD20 antibodies are also provided by the present invention. It is 35 believed that such formulations will be particularly suitable for storage as well as for therapeutic administration. The formulations may be prepared by known techniques. For instance, the formulations may be prepared by buffer exchange on a gel filtration column.

Typically, an appropriate amount of an acceptable salt or carrier is 40 used in the formulation to render the formulation isotonic. Examples of pharmaceutically-acceptable carriers include saline, Ringer's solution and dextrose solution. The pH of the formulation is preferably from about 6 to about 9, and more preferably from about 7 to about 7.5. It will be apparent to those persons skilled in the art that certain carriers may be

more preferable depending upon, for instance, the route of administration and concentrations of Apo-2 ligand, death receptor antibodies, and/or CD20 antibodies.

Therapeutic compositions can be prepared by mixing the desired molecules having the appropriate degree of purity with optional carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences, 16th edition, Osol, A. ed. (1980)), in the form of lyophilized formulations, aqueous solutions or aqueous suspensions. Acceptable carriers, excipients, or stabilizers are preferably nontoxic to recipients at the dosages and concentrations employed, and include buffers such as Tris, HEPES, PIPES, phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; and/or non-ionic surfactants such as TWEENSM, FLURONICSSM or polyethylene glycol (PEG).

Additional examples of such carriers include ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts, or electrolytes such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, and cellulose-based substances. Carriers for topical or gel-based forms include polysaccharides such as sodium carboxymethylcellulose or methylcellulose, polyvinylpyrrolidone, polyacrylates, polyoxyethylene-polyoxypropylene-block polymers, polyethylene glycol, and wood wax alcohols. For all administrations, conventional depot forms are suitably used. Such forms include, for example, microcapsules, nano-capsules, liposomes, plasters, inhalation forms, nose sprays, sublingual tablets, and sustained-release preparations.

Formulations to be used for in vivo administration should be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. The formulation may be stored in lyophilized form or in solution if administered systemically. If in lyophilized form, it is typically formulated in combination with other ingredients for reconstitution with an

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appropriate diluent at the time for use. An example of a liquid formulation is a sterile, clear, colorless unpreserved solution filled in a single-dose vial for subcutaneous injection.

Therapeutic formulations generally are placed into a container having
5 a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle. The formulations are preferably administered as repeated intravenous (i.v.), subcutaneous (s.c.), intramuscular (i.m.) injections or infusions, or as aerosol formulations suitable for intranasal or intrapulmonary delivery
10 (for intrapulmonary delivery see, e.g., EP 257,956).

Apo2L/TRAIL, death receptor antibodies, and CD20 antibodies can also be administered in the form of sustained-release preparations. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the protein, which matrices are in
15 the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (e.g., poly(2-hydroxyethyl-methacrylate) as described by Langer et al., J. Biomed. Mater. Res., 15: 167-277 (1981) and Langer, Chem. Tech., 12: 98-105 (1982) or poly(vinylalcohol)), polylactides (U.S. Patent No. 3,773,919, EP 58,481),
20 copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al., Biopolymers, 22: 547-556 (1983)), non-degradable ethylene-vinyl acetate (Langer et al., *supra*), degradable lactic acid-glycolic acid copolymers such as the Lupron Depot (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-
25 hydroxybutyric acid (EP 133,988).

The Apo2L/TRAIL, death receptor antibodies, and CD20 antibodies described herein can be employed in a variety of therapeutic applications. Among these applications are methods of treating various cancers and immune related diseases. Diagnosis in mammals of the various pathological
30 conditions described herein can be made by the skilled practitioner. Diagnostic techniques are available in the art which allow, e.g., for the diagnosis or detection of cancer or immune related disease in a mammal. For instance, cancers may be identified through techniques, including but not limited to, palpation, blood analysis, x-ray, NMR and the like. Immune
35 related diseases can also be readily identified. In systemic lupus erythematosus, the central mediator of disease is the production of auto-reactive antibodies to self proteins/tissues and the subsequent generation of immune-mediated inflammation. Multiple organs and systems are affected clinically including kidney, lung, musculoskeletal system, mucocutaneous,
40 eye, central nervous system, cardiovascular system, gastrointestinal tract, bone marrow and blood. Rheumatoid arthritis (RA) is a chronic systemic autoimmune inflammatory disease that mainly involves the synovial membranes of multiple joints with resultant injury to the articular cartilage. The pathogenesis is T lymphocyte dependent and is associated with the

production of rheumatoid factors, auto-antibodies directed against self IgG, with the resultant formation of immune complexes that attain high levels in joint fluid and blood. These complexes in the joint may induce the marked infiltrate of lymphocytes and monocytes into the synovium and subsequent marked synovial changes; the joint space/fluid is infiltrated by similar cells with the addition of numerous neutrophils. Tissues affected are primarily the joints, often in symmetrical pattern. However, extra-articular disease also occurs in two major forms. One form is the development of extra-articular lesions with ongoing progressive joint disease and typical lesions of pulmonary fibrosis, vasculitis, and cutaneous ulcers. The second form of extra-articular disease is the so called Felty's syndrome which occurs late in the RA disease course, sometimes after joint disease has become quiescent, and involves the presence of neutropenia, thrombocytopenia and splenomegaly. This can be accompanied by vasculitis in multiple organs with formations of infarcts, skin ulcers and gangrene. Patients often also develop rheumatoid nodules in the subcutis tissue overlying affected joints; the nodules late stage have necrotic centers surrounded by a mixed inflammatory cell infiltrate. Other manifestations which can occur in RA include: pericarditis, pleuritis, coronary arteritis, interstitial pneumonitis with pulmonary fibrosis, keratoconjunctivitis sicca, and rheumatoid nodules.

The Apo2L/TRAIL, death receptor antibodies, and CD20 antibodies can be administered in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Optionally, administration may be performed through mini-pump infusion using various commercially available devices.

Effective dosages and schedules for administering Apo2L/TRAIL, death receptor antibodies, and CD20 antibodies may be determined empirically, and making such determinations is within the skill in the art. Single or multiple dosages may be employed. It is presently believed that an effective dosage or amount of Apo2L/TRAIL used alone may range from about 1 µg/kg to about 100 mg/kg of body weight or more per day. Interspecies scaling of dosages can be performed in a manner known in the art, e.g., as disclosed in Mordenti et al., Pharmaceut. Res., 8:1351 (1991).

When in vivo administration of an Apo2L/TRAIL is employed, normal dosage amounts may vary from about 10 ng/kg to up to 100 mg/kg of mammal body weight or more per day, preferably about 1 µg/kg/day to 10 mg/kg/day, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature; see, for example, U.S. Pat. Nos. 4,657,760; 5,206,344; or 5,225,212. It is anticipated that different formulations will be effective for different treatment compounds and different disorders, that administration targeting

one organ or tissue, for example, may necessitate delivery in a manner different from that to another organ or tissue. Those skilled in the art will understand that the dosage of Apo2L/TRAIL that must be administered will vary depending on, for example, the mammal which will receive the Apo2L/TRAIL, the route of administration, and other drugs or therapies being administered to the mammal.

The CD20 antibody may be an antibody such as Rituximab or humanized 2H7, which is not conjugated to a cytotoxic agent. Suitable dosages for an unconjugated antibody are, for example, in the range from about 20 mg/m² to about 1000 mg/m². In one embodiment, the dosage of the antibody differs from that presently recommended for Rituximab. Exemplary dosage regimens for the CD20 antibody include 375 mg/m² weekly x 4 or 8; or 1000 mg x 2 (e.g. on days 1 and 15).

It is contemplated that yet additional therapies may be employed in the methods. The one or more other therapies may include but are not limited to, administration of radiation therapy, cytokine(s), growth inhibitory agent(s), chemotherapeutic agent(s), cytotoxic agent(s), tyrosine kinase inhibitors, ras farnesyl transferase inhibitors, angiogenesis inhibitors, and cyclin-dependent kinase inhibitors which are known in the art and defined further with particularity in Section I above.

Exemplary therapeutic antibodies include anti-HER2 antibodies including rhuMAb 4D5 (HERCEPTIN.) (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285-4289 (1992), U.S. Patent No. 5,725,856); anti-IL-8 (St John et al., *Chest*, 103:932 (1993), and International Publication No. WO 95/23865); anti-VEGF antibodies including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti-VEGF antibody huA4.6.1 AVASTIN. (Kim et al., *Growth Factors*, 7:53-64 (1992), International Publication No. WO 96/30046, and WO 98/45331, published October 15, 1998); anti-PSCA antibodies (WO01/40309); anti-CD40 antibodies, including S2C6 and humanized variants thereof (WO00/75348); anti-CD11a antibodies including Raptiva™ (US Patent No. 5,622,700, WO 98/23761, Steppe et al., *Transplant Intl.* 4:3-7 (1991), and Hourmant et al., *Transplantation* 58:377-380 (1994)); anti-IgE antibodies (Presta et al., *J. Immunol.* 151:2623-2632 (1993), and International Publication No. WO 95/19181; US Patent No. 5,714,338, issued February 3, 1998 or US Patent No. 5,091,313, issued February 25, 1992, WO 93/04173 published March 4, 1993, or International Application No. PCT/US98/13410 filed June 30, 1998, US Patent No. 5,714,338); anti-CD18 antibodies (US Patent No. 5,622,700, issued April 22, 1997, or as in WO 97/26912, published July 31, 1997); anti-Apo-2 receptor antibody antibodies (WO 98/51793 published November 19, 1998); anti-TNF-alpha antibodies including cA2 (REMICADE.), CDP571 and MAK-195 (See, US Patent No. 5,672,347 issued September 30, 1997, Lorenz et al. *J. Immunol.* 156(4):1646-1653 (1996), and Dhainaut et al. *Crit. Care Med.* 23(9):1461-1469 (1995)); anti-Tissue Factor (TF) antibodies (European Patent No. 0 420 937 B1 granted

November 9, 1994); anti-human $\alpha_4\beta_7$ integrin antibodies (WO 98/06248 published February 19, 1998); anti-EGFR antibodies (chimerized or humanized 225 antibody as in WO 96/40210 published December 19, 1996); anti-CD3 antibodies such as OKT3 (US Patent No. 4,515,893 issued May 7, 1985); anti-
5 CD25 or anti-Tac antibodies such as CHI-621 (SIMULECT.) and ZENAPAX. (See US Patent No. 5,693,762 issued December 2, 1997); anti-CD4 antibodies such as the cM-7412 antibody (Choy et al. *Arthritis Rheum* 39(1):52-56 (1996)); anti-CD52 antibodies such as CAMPATH-1H (Riechmann et al. *Nature* 332:323-337 (1988); anti-Fc receptor antibodies such as the M22 antibody directed
10 against Fc γ RI as in Graziano et al. *J. Immunol.* 155(10):4996-5002 (1995); anti-carcinoembryonic antigen (CEA) antibodies such as hMN-14 (Sharkey et al. *Cancer Res.* 55(23Suppl): 5935s-5945s (1995); antibodies directed against breast epithelial cells including huBrE-3, hu-Mc 3 and CHL6 (Ceriani et al. *Cancer Res.* 55(23): 5852s-5856s (1995); and Richman et al.
15 *Cancer Res.* 55(23 Suppl): 5916s-5920s (1995)); antibodies that bind to colon carcinoma cells such as C242 (Litton et al. *Eur J. Immunol.* 26(1):1-9 (1996)); anti-CD38 antibodies, e.g. AT 13/5 (Ellis et al. *J. Immunol.* 155(2):925-937 (1995)); anti-CD33 antibodies such as Hu M195 (Jurcic et al. *Cancer Res* 55(23 Suppl):5908s-5910s (1995) and CMA-676 or CDP771; anti-CD22
20 antibodies such as LL2 or LymphoCide (Juweid et al. *Cancer Res* 55(23 Suppl):5899s-5907s (1995); anti-EpCAM antibodies such as 17-1A (PANOREX.); anti-GpIIb/IIIa antibodies such as abciximab or c7E3 Fab (REOPRO.); anti-REV antibodies such as MEDI-493 (SYNAGIS.); anti-CMV antibodies such as PROTOVIR.; anti-HIV antibodies such as PRO542; anti-hepatitis antibodies
25 such as the anti-Hep B antibody OSTAVIR.; anti-CA 125 antibody OvaRax; anti-idiotypic GD3 epitope antibody BEC2; anti-.v.3 antibody VITAXIN.; anti-human renal cell carcinoma antibody such as ch-G250; ING-1; anti-human 17-1A antibody (3622W94); anti-human colorectal tumor antibody (A33); anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human
30 squamous-cell carcinoma (SF-25); and anti-human leukocyte antigen (HLA) antibodies such as Smart ID10 and the anti-HLA DR antibody Oncolym (Lym-1).

Preparation and dosing schedules for chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such
35 chemotherapy are also described in *Chemotherapy Service* Ed., M.C. Perry, Williams & Wilkins, Baltimore, MD (1992). The chemotherapeutic agent may precede, or follow administration of the Apo2L/TRAIL, death receptor antibody, and/or CD20 antibody, or may be given simultaneously therewith.

Sometimes, it may be beneficial to also administer one or more
40 cytokines or growth inhibitory agent.

The Apo2L/TRAIL, death receptor antibodies, and CD20 antibodies (and one or more other therapies) may be administered concurrently or sequentially. Following administration, treated cells in vitro can be analyzed. Where there has been in vivo treatment, a treated mammal can be

monitored in various ways well known to the skilled practitioner. For instance, cancer cells can be examined pathologically to assay for necrosis or serum can be analyzed for immune system responses.

For RA, and other autoimmune diseases, the Apo2L/TRAIL, death receptor antibody, and/or CD20 antibody may be combined with any one or more of the immunosuppressive agents, chemotherapeutic agents and/or cytokines listed in the definitions section above; any one or more disease-modifying antirheumatic drugs (DMARDs), such as hydroxychloroquine, sulfasalazine, methotrexate, leflunomide, azathioprine, D-penicillamine, Gold (oral), Gold (intramuscular), minocycline, cyclosporine, Staphylococcal protein A immunoadsorption; intravenous immunoglobulin (IVIG); nonsteroidal antiinflammatory drugs (NSAIDs); glucocorticoid (e.g. via joint injection); corticosteroid (e.g. methylprednisolone and/or prednisone); folate; an anti-tumor necrosis factor (TNF) antibody, e.g. etanercept/ENBREL[®], infliximab/REMICADE[®], D2E7 (Knoll) or CDP-870 (Celltech); IL-1R antagonist (e.g. Kineret); IL-10 antagonist (e.g. Ilodecakin); a blood clotting modulator (e.g. WinRho); an IL-6 antagonist/anti-TNF (CBP 1011); CD40 antagonist (e.g. IDEC 131); Ig-Fc receptor antagonist (MDX33); immunomodulator (e.g. thalidomide or ImmuDyn); anti-CD5 antibody (e.g. H5G1.1); macrophage inhibitor (e.g. MDX 33); costimulatory blocker (e.g. RMS 188667 or Tolerimab); complement inhibitor (e.g. h5G1.1, 3E10 or an anti-decay accelerating factor (DAF) antibody); or IL-2 antagonist (xxSMART).

For B cell malignancies, e.g., the Apo2L/TRAIL, death receptor antibody, and/or CD20 antibody may be combined with a chemotherapeutic agent; cytokine, e.g. a lymphokine such as IL-2, IL-12, or an interferon, such as interferon alpha-2a; other antibody, e.g., a radiolabeled antibody such as ibritumomab tiuxetan (ZEVALIN[®]), iodine I¹³¹ tositumomab (BEKKAR[®]), ¹³¹I Lym-1 (ONCOLYM[®]), ⁹⁰Y-LYMPHOICIDE[®]; anti-CD52 antibody, such as alemtuzumab (CAMPATH-1H[®]), anti-HLA-DR-β antibody, such as apolizumab, anti-CD80 antibody (e.g. IDEC-114), epratuzumab, Bu1D10 (SMART 1D10[®]), CD19 antibody, CD40 antibody or CD22 antibody; an immunomodulator (e.g. thalidomide or ImmuDyn); an inhibitor of angiogenesis (e.g. an anti-vascular endothelial growth factor (VEGF) antibody such as AVASTIN[®] or thalidomide); idiotype vaccine (EPOCH); ONCO-TCS[®]; HSPPC-96 (ONCOPHAGE[®]); liposomal therapy (e.g. daunorubicin citrate liposome), etc.

In another embodiment of the invention, articles of manufacture containing materials useful for the treatment of cancer or immune related disease, described above, are provided. In one aspect, the article of manufacture comprises (a) a container comprising CD20 antibody (preferably the container comprises the antibody and a pharmaceutically acceptable carrier or diluent within the container); (b) a container comprising Apo2L/TRAIL or death receptor antibody (preferably the container comprises the Apo2L/TRAIL or death receptor antibody and a pharmaceutically

acceptable carrier or diluent within the container); and (c) a package insert with instructions for treating cancer or immune related disease in a patient, wherein the instructions indicate that amounts of the CD20 antibody and the Apo2L/TRAIL or death receptor antibody are administered to the patient that are effective to provide synergistic activity in treating the disease.

In all of these aspects, the package insert is on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds or contains a composition that is effective for treating the cancer or immune related disease and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is the CD20 antibody, Apo2L/TRAIL or death receptor antibody. The label or package insert indicates that the composition is used for treating cancer or immune related disease in a patient or subject eligible for treatment with specific guidance regarding dosing amounts and intervals of antibody and any other medicament being provided. The article of manufacture may further comprise an additional container comprising a pharmaceutically acceptable diluent buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution, and/or dextrose solution. The article of manufacture may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLES

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by way of reference to the ATCC is the American Type Culture Collection, Manassas, Virginia.

Example 1

Analysis of Apo2L/TRAIL receptor expression in B lymphoma cell lines

To examine the cell-surface expression of Apo2L/TRAIL receptors (DR4, DR5, DcR1, and DcR2) in human lymphoma cell lines, the B lymphoma cell lines Ramos, Daudi, Raji, and BJAB (ATCC) were analyzed by FACS using monoclonal antibodies specific for DR4 (mAb 4B6.17.8; ATCC HB-12455), DR5

(mAb 3H3.14.5; HB-12534), DcR1 (mAb 6G9; Genentech, Inc.), or DcR2 (mAb 1G9, Genentech, Inc.) For Ramos cells, the analysis was carried out twice to ensure reproducibility (RAMOS A and B).

As illustrated in Figure 4, DR4 and DR5 were expressed at significant levels (mean fluorescence shift of approximately 0.5 - 1.7 units) in all four of the cell lines, while DcR1 and DcR2 were expressed at lower or minimal levels (mean fluorescence shift of approximately 0 - 0.3 units).

Example 2

Analysis of CD20 expression in B lymphoma cell lines

To examine the cell-surface expression of CD20 in human lymphoma cell lines, the B lymphoma cell lines Ramos, Daudi, Raji, and BJAB (ATCC) were analyzed by FACS using a monoclonal antibody specific for CD20 (RITUXAN®, Genentech, Inc.). For Ramos cells, the analysis was carried out twice to ensure reproducibility (RAMOS A and B).

As illustrated in Figure 5, all four cell lines expressed high levels of CD20, indicated by a mean fluorescence shift of approximately 5 - 15 units.

Example 3

Effect of Apo2L/TRAIL, RITUXAN®, or combination treatment on the growth of pre-established subcutaneous BJAB lymphoma tumor xenografts in SCID mice

SCID mice were injected subcutaneously with human B-cell non-Hodgkin's BJAB lymphoma cells (ATCC) (20 million cells per mouse) and tumors were allowed to grow to ~200 mm³. The mice were then divided into 4 study groups (8 mice per group) and treated with five intraperitoneal (IP) doses per week over 2 weeks (i.e., days 0-4 and 7-11) of vehicle (0.5M Arg-Succinate/20mM Tris/0.02% Tween 20 pH =7.2), Apo2L/TRAIL (amino acids 114-281 of Figure 1) (60 mg/kg), or with 1 IP dose per week over 2 weeks (i.e., days 0 and 7) of RITUXAN® (4 mg/kg, Genentech, Inc.), or the combination of these latter Apo2L/TRAIL and RITUXAN® regimens (Figure 6).

Tumors in vehicle-treated mice grew rapidly, while single-agent Apo2L/TRAIL or RITUXAN® treatment markedly delayed tumor growth. One mouse in the Apo2L/TRAIL group showed complete tumor ablation, leaving a tumor incidence (TI) of 7/8. RITUXAN® treatment did not ablate any tumors but showed a more prolonged effect. Importantly, combined treatment with Apo2L/TRAIL and RITUXAN® caused a dramatic reduction in tumor volume in all mice, with 5 out of 8 mice showing complete tumor ablation and 3/8 showing minimal tumor growth for at least 28 days. These results indicate that Apo2L/TRAIL and RITUXAN® can exert synergistic anti-tumor activity against lymphoma xenografts.

Example 4Effect of Apo2L/TRAIL, RITUXAN®, or combination treatment on the growth of pre-established subcutaneous BJAB lymphoma tumor xenografts grown in SCID mice

5 A similar study to the one described in Example 3 was conducted. SCID mice were injected subcutaneously with human B-cell non-Hodgkin's BJAB lymphoma cells (ATCC) (20 million cells per mouse) and tumors were allowed to grow to ~200 mm³. The mice were then divided into 4 study groups (8 mice per group) and treated with five intraperitoneal (IP) doses per week over
10 2 weeks (i.e., days 0-4 and 7-11) of vehicle (0.5M Arg-Succinate/20mM Tris/0.02% Tween 20 pH =7.2), Apo2L/TRAIL ("Apo2L.0"; amino acids 114-281 of Fig.1) (60 mg/kg), or with 1 IP dose per week over 2 weeks (i.e., days 0 and 7) of RITUXAN® (4 mg/kg), or the combination of these latter Apo2L/TRAIL and RITUXAN® regimens.

15 The results are shown in Fig. 7. Tumors in vehicle-treated mice grew rapidly, while single-agent Apo2L/TRAIL or RITUXAN® treatment markedly delayed tumor growth. Neither Apo2L/TRAIL nor RITUXAN® alone caused any complete regressions, while RITUXAN® showed a more prolonged effect. As in the study described in Example 3, combined treatment with Apo2L/TRAIL and
20 RITUXAN® caused a remarkable reduction in tumor volume in all mice, with 6 out of 7 mice showing complete tumor ablation. These results indicate that Apo2L/TRAIL and RITUXAN® can exert synergistic anti-tumor activity against lymphoma xenografts.

Example 5Effect of Apo2L/TRAIL, RITUXAN®, or combination treatment on caspase processing in pre-established subcutaneous BJAB lymphoma tumor xenografts grown in SCID mice

To examine the processing of apoptosis-mediating caspases in treated
30 tumors (indicated by proteolytic caspase processing), SCID mice were injected subcutaneously with human B-cell non Hodgkin's BJAB lymphoma cells (ATCC) (20 million cells per mouse) and tumors were allowed to grow to ~200 mm³. The mice were then treated with vehicle (0.5M Arg-Succinate/20mM Tris/0.02% Tween 20 pH =7.2) (n=1), 1 IP dose of Apo2L/TRAIL (60 mg/kg)
35 (n=1), or 1 IP dose of RITUXAN® (4 mg/kg, Genentech, Inc.) (n=2), or the combination of these latter Apo2L/TRAIL and RITUXAN® doses (n=2). Two days after treatment, the tumors were harvested, lysed in lysis buffer, and subjected to immunoblot with specific antibodies against Caspase 8, 3, 9, and 7 (with anti-beta actin antibody as a loading control) to visualize caspase processing (Figure 8).
40

Apo2L/TRAIL treatment (A) induced increased processing of caspase 8, 3, 9, and 7 as compared to the vehicle control (V), while RITUXAN® (R) did not induce caspase processing. Notably, combination treatment with Apo2L/TRAIL and RITUXAN® (AR) did not further increase caspase processing

as compared to Apo2L/TRAIL alone. These results suggest that the synergistic anti-tumor activity between Apo2L/TRAIL and RITUXAN® is not necessarily mediated by enhancement of apoptosis, suggesting that combination of apoptosis activation mediated by Apo2L/TRAIL and complement-dependent lysis together with ADCC mediated by RITUXAN® may underlie the observed anti-tumor synergy.

Example 6

10 Effect of agonistic DR5 antibody, RITUXAN®, or combination treatment on the growth of pre-established subcutaneous BJAB lymphoma tumor xenografts in SCID mice

SCID mice were injected subcutaneously with human B-cell non-Hodgkin's BJAB lymphoma cells (ATCC) (20 million cells per mouse) and tumors were allowed to grow to ~200 mm³. The mice were then divided into 4 study groups (7 mice per group) and treated with one intraperitoneal (IP) injection per week over 2 weeks (i.e., days 0 and 7) of vehicle (0.5M Arg-Succinate/20mM Tris/0.02% Tween 20 pH =7.2), agonist DR5 monoclonal antibody ("Apo2ab") (10 mg/kg), or RITUXAN® (4 mg/kg), or the combination of these latter DR5 antibody and RITUXAN® regimens (Figure 9). Tumors in vehicle-treated mice grew rapidly, while single-agent DR5 antibody or RITUXAN® treatment markedly delayed tumor growth. Importantly, combined treatment with DR5 antibody and RITUXAN® caused a dramatic reduction in tumor volume in all mice, with 5 out of 7 mice showing complete tumor ablation and 2/7 showing minimal tumor growth for at least 35 days. These results indicate that agonist DR5 antibody and RITUXAN® can exert synergistic anti-tumor activity against lymphoma xenografts.

Example 7

30 Effect of agonistic DR5 antibody, RITUXAN®, or combination treatment on caspase processing in pre-established subcutaneous BJAB lymphoma tumor xenografts grown in SCID mice

To examine the processing of apoptosis-mediating caspases in treated tumors (indicated by proteolytic caspase processing), SCID mice were injected subcutaneously with human B-cell non-Hodgkin's BJAB lymphoma cells (ATCC) (20 million cells per mouse) and tumors were allowed to grow to ~200 mm³. The mice were then treated with vehicle (0.5M Arg-Succinate/20mM Tris/0.02% Tween 20 pH =7.2) (n=1), or 1 IP dose of RITUXAN® (4 mg/kg) (n=2), or 1 IP dose of agonist DR5 antibody (10 mg/kg) (n=2), or the combination of these latter DR5 antibody and RITUXAN® doses (n=2). Two days after treatment, the tumors were harvested, lysed in lysis buffer, and subjected to immunoblot with specific antibodies against Caspase 8, 3, 9,

and 7 (with anti-beta actin antibody as a loading control) to visualize caspase processing (Figure 10).

Agonist DR5 antibody treatment (A) induced increased processing of caspase 8, 3, 9, and 7 as compared to the vehicle control (V), while RITUXAN® (R) did not induce caspase processing. Notably, combination treatment with DR5 antibody and RITUXAN® (AR) did not further increase caspase processing as compared to DR5 antibody alone. These results suggest that the synergistic anti-tumor activity between DR5 antibody and RITUXAN® is not necessarily mediated by enhancement of apoptosis, but rather that the combination of apoptosis activation mediated by agonist DR5 antibody and complement-dependent lysis together with ADCC mediated by RITUXAN® may underlie the observed anti-tumor synergy.

Further data illustrating expression of CD20 and Apo2L/TRAIL receptors in NHL cell lines and the effects of Rituximab, Apo2L/TRAIL and combinations thereof on cancer cells are provided in Figures 11-16.

WHAT IS CLAIMED IS:

1. A method of treating cancer cells, comprising exposing mammalian cancer cells to a synergistic effective amount of agonist death receptor antibody and CD20 antibody.

2. The method of claim 1 wherein said agonist death receptor antibody is an anti-DR5 receptor monoclonal antibody.

3. The method of claim 1 wherein said agonist death receptor antibody is an anti-DR4 receptor monoclonal antibody.

4. The method of claim 1 wherein said cancer cells are exposed to said synergistic effective amount of agonist death receptor antibody and CD20 antibody *in vivo*.

5. The method of claim 2 or 3 wherein said agonist death receptor antibody is a chimeric antibody or a humanized antibody.

6. The method of claim 2 or 3 wherein said agonist death receptor antibody is a human antibody.

7. The method of claim 1 wherein said agonist death receptor antibody is an antibody which cross-reacts with more than one Apo-2 ligand receptor.

8. The method of claim 1 wherein said cancer cells are lymphoma cells.

9. The method of claim 1 further comprising exposing the cancer cells to one or more growth inhibitory agents.

10. The method of claim 1 further comprising exposing the cells to radiation.

11. The method of claim 2 wherein said DR5 antibody has a DR5 receptor binding affinity of 10^8 M^{-1} to 10^{12} M^{-1} .

12. The method of claim 1 wherein said death receptor antibody and CD20 antibody is expressed in a recombinant host cell selected from the group consisting of a CHO cell, yeast cell and *E. coli*.

13. The method of claim 1 wherein said CD20 antibody is a monoclonal antibody.

14. The method of claim 13 wherein said CD20 antibody is the antibody Rituximab.
- 5 15. A method of treating an immune related disease, comprising administering to a mammal a synergistic effective amount of agonist death receptor antibody and CD20 antibody.
- 10 16. The method of claim 15 wherein said agonist death receptor antibody is an anti-DR5 receptor monoclonal antibody.
17. The method of claim 15 wherein said agonist death receptor antibody is an anti-DR4 receptor monoclonal antibody.
- 15 18. The method of claim 16 or 17 wherein said agonist death receptor antibody is a chimeric antibody or a humanized antibody.
19. The method of claim 16 or 17 wherein said agonist death receptor antibody is a human antibody.
- 20 20. The method of claim 15 wherein said agonist death receptor antibody is an antibody which cross-reacts with more than one Apo-2 ligand receptor.
- 25 21. The method of claim 15 wherein said immune related disease is rheumatoid arthritis or multiple sclerosis.
22. The method of claim 15 wherein said DR5 antibody has a DR5 receptor binding affinity of 10^8 M^{-1} to 10^{12} M^{-1} .
- 30 23. The method of claim 15 wherein said death receptor antibody and CD20 antibody is expressed in a recombinant host cell selected from the group consisting of a CHO cell, yeast cell and *E. coli*.
- 35 24. The method of claim 15 wherein said CD20 antibody is a monoclonal antibody.
25. The method of claim 24 wherein said CD20 antibody is the antibody Rituximab.
- 40 26. The method of claim 1 or 15 wherein said death receptor antibody and CD20 antibody are administered sequentially.
27. The method of claim 1 or 15 wherein said death receptor antibody and CD20 antibody are administered concurrently.

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1 ATGGCGCCAC CACCAGCTAG AGTACATCTA GGTGCGTTCC TGGCAGTGAC
 TACCGCGGTG GTGGTCGATC TCATGTAGAT CCACGCAAGG ACCGTCACTG
 1 MetAlaProP roProAlaAr gValHisLeu GlyAlaPheL euAlaValTh

51 TCGAATCCC GGGAGCGCAG CGAGTGGGAC AGAGGCAGCC GCGGCCACAC
 AGGCTTAGGG CCCTCGCGTC GCTCACCTG TCTCCGTGG CCGCGGTGTG
 rProAsnPro GlySerAlaA laSerGlyTh rGluAlaAla AlaAlaThrPro

101 CCAGCAAAGT GTGGGGCTCT TCCGCGGGGA GGATTGAACC ACGAGGCCGG
 GGTGCTTTCA CACCCCGAGA AGCGCGCCCT CCTAACTTGG TGCTCCGCC
 35 SerLysVa lTrpGlySer SerAlaGlyA rgIleGluPr oArgGlyGly

151 GGCCGAGGAG CGCTCCCTAC CTCCATGGGA CAGCACGGAC CCAGTGCCCG
 CCGGCTCCTC GCGAGGGATG GAGGTACCCT GTCGTGCCTG GGTACCGGSC
 GlyArgGlyA laLeuProTh rSerMetGly GlnHisGlyP roSerAlaArg

201 GGCCCGGGCA GGGCGCGCCC CAGGACCCAG GCCGCGCGCG GAAGCCAGCC
 CCGGGCCCGT CCGCGCGCGG GTCCTGGGTC CCGCCGCGCC CTTGGGTGCG
 68 AlaArgAla GlyArgAlaP roGlyProAr gProAlaArg GluAlaSerP

251 CTCGGCTCCG GGTCCACAAG ACCTTCAAGT TTGTGCTCGT CCGGGTCCTG
 GAGCCGAGGC CCAGGTGTTT TGGAAAGTTCA AACAGCAGCA GCCCCAGGAC
 roArgLeuAr gValHisLys ThrPheLysP heValValVa lGlyValLeu

301 CTGCAGGTCG TACCTAGCTC AGCTGCAACC ATGATCAATC AATTGGCACA
 GACGTCCAGC ATGGATCGAG TCGACGTTGG TAGTTTGAGG TACTAGTTAG
 101 LeuGlnValV alProSerSe rAlaAlaThr IleLysLeuH isAspGlnSe

351 AATTGGCACA CAGCAATGGG AACATAGCCC TTTGGGAGAG TTGTGTCCAC
 TTAACCGTGT GTCGTTACCC TTGTATCGGG AAACCTCTC AACACAGGTG
 rIleGlyThr GlnGlnTrpG luHisSerPr oLeuGlyGlu LeuCysProPro

401 CAGGATCTCA TAGATCAGAA CGTCCTGGAG CCTGTAACCG GTGCACAGAG
 GTCCTAGAGT ATCTAGTCTT GCAGGACCTC GGACATTGGC CAGGTGTCTC
 135 GlySerHi sArgSerGlu ArgProGlyA laCysAsnAr gCysThrGlu

451 GGTGTGGGTT ACACCAATGC TTCCAACAAT TTGTTTGCTT GCCTCCCATG
 CCACACCCAA TGTGGTTACG AAGGTTGTTA AACAAACGAA CGGAGGGTAC
 GlyValGlyT yrThrAsnAl aSerAsnAsn LeuPheAlaC ysLeuProCys

501 TACAGCTTGT AAATCAGATG AAGAAGAGAG AAGTCCCTGC ACCACGACCA
 ATGTCGAACA TTTAGTCTAC TTCCTCTCTC TTCAGGGACG TGGTGCTGGT
 168 ThrAlaCys LysSerAspG luGluGluAr gSerProCys ThrThrThra

551 GGAACACAGC ATGTCAGTGC AAACCAGGAA CTTTCCGGAA TGACAATTCT
 CCTTGTGTG TACAGTCACG TTGGTTCCTT GAAAGGCCTT ACTGTTAAGA
 rgAsnThrAl aCysGlnCys LysProGlyT hrPheArgAs nAspAsnSer

601 GCTGAGATGT GCCGGAAGTG CAGCACAGGG TGCCCCAGAG GGATGGTCAA
 CGACTCTACA CGGCCTTAC GTCGTGTCCC ACGGGGTCTC CCTACCAGTT
 201 AlaGluMetC ysArgLysCy sSerThrGly CysProArgG lyMetVally

651 GGTCAAGGAT TGTACGCCCT GGAGTGACAT CGAGTGTGTC CACAAAGAAT
 CCAGTTCCTA ACATGCGGGA CCTCACTGTA GCTCACACAG GTGTTTCTTA
 sVallyAsp CysThrProT rpSerAspIl eGluCysVal HisLysGluSer

FIG. 2A

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701 CAGGCAATGG ACATAATATA TGGGTGATTT TGGTTGTGAC TTGGTTGTT
 GTCCGTTACC TGTATTATAT ACCCACTAAA ACCAACACTG AAACCAACAA
 235 GlyAsnG1 yHisAsnIle TrpValIleL euValValTh rLeuValVal
 751 CCGTTGCTGT TGGTGGCTGT GCTGATTGTC TGTGTGTTCA TCGGCTCAGG
 GGCAACGACA ACCACCGACA CGACTAACAG ACAACAACGT AGCCCGAGTCC
 ProLeuLeuL euValAlaVa lLeuIleVal CysCysCysI leGlySerGly
 801 TTGTGGAGGG GACCCCAAGT GCATGGACAG GGTGTGTTTC TGCCGCTTGG
 AACACCTCCC CTGGGGTTCA CGTACCTGTC CCAGACAAAG ACCGCGAACC
 268 CysGlyGly AspProLysC ysMetAspAr gValCysPhe TrpArgLeuG
 851 GTCTCCTACG AGGGCCTGGG GCTGAGGACA ATGCTCACAA CGAGATTCTG
 CAGAGGATGC TCCCGGACCC CGACTCCTGT TACGAGTGTT GCTCTAAGAC
 lyLeuLeuAr gGlyProGly AlaGluAspA snAlaHisAs nGluIleLeu
 901 AGCAACGCAG ACTCGCTGTC CACTTTCGTC TCTGAGCAGC AAATGGAAAG
 TCGTTGCGTC TGAGCGACAG GTGAAAGCAG AGACTCGTCG TTACCTTTTC
 301 SerAsnAlaA spSerLeuSe rThrPheVal SerGluGlnG lnMetGluSe
 951 CCAGGAGCCC GCAGATTGA CAGGTGTCAC TGTACAGTCC CCAGGGGAGG
 GGTCTCTGGC CGTCTAACT GTCCACATGT ACATGTCAGG GGTCCCTCC
 rGlnGluPro AlaAspLeuT hrGlyValTh rValGlnSer ProGlyGluAla
 1001 CACAGTGCTT GCTGGGACCG GCAGAAGCTG AAGGGTCTCA GAGGAGGAGG
 GTGTCACAGA CGACCCTGGC CGTCTTCGAC TTCCAGAGT CTCTCTCTCC
 335 GlnCysLe uLeuGlyPro AlaGluAlaG luGlySerG1 nArgArgArg
 1051 CTGCTGGTTC CAGCAAATGG TGCTGACCCC ACTGAGACTC TGATGCTGTT
 GACGACCAAG GTCGTTTACC ACGACTGGGG TGA CTCTGAG ACTACGACAA
 LeuLeuValP roAlaAsnG1 yAlaAspPro ThrGluThrL euMetLeuPhe
 1101 CTTTGACAAG TTTGCAAACA TCCTGCCCTT TGA CTCTGG GACCAGCTCA
 GAAACTGTTT AAACGTTTGT AGCACGGGAA ACTGAGGACC CTGGTCAAGT
 368 PheAspLys PheAlaAsnI leValProPh eAspSerTrp AspGlnLeuM
 1151 TGAGGCAGCT GGACCTCACG AAAAATGAGA TCGATGTGGT CAGAGCTGGT
 ACTCCGTCGA CCTGGAGTGC TTCTTACTCT AGCTACACCA GTCTCGACCA
 etArgGlnLe uAspLeuThr LysAsnGluI leAspValVa lArgAlaGly
 1201 ACAGCAGGCC CAGGGGATGC CTGTATGCA ATGCTGATGA AATGGGTCAA
 TGTCTGTCGG GTCCCTTACG GAACATACGT TACGACTACT TTACCCAGTT
 401 ThrAlaGlyP roGlyAspAl aLeuTyrAla MetLeuMetL ysTrpValAs
 1251 CAAAACGGA CGGAACGCCT CGATCCACAC CCTGCTGGAT GCCTTGGAGA
 GTTTTGACCT GCCTTGCGGA GCTAGGTGTG GGACGACCTA CGGAACCTCT
 nLysThrGly ArgAsnAlaB erIleHisTh rLeuLeuAsp AlaLeuGluArg
 1301 GGATGGAAGA GAGACATGCA AAAGAGAAGA TTCAGGACCT CTTGGTGGAC
 CCTACCTTCT CTCTGTACGT TTCTCTTCTT AAGTCTTGA GAACCACTG
 435 MetGluG1 uArgHisAla LysGluLysI leGlnAspLe uLeuValAsp
 1351 TCTGGAAAGT TCATCTACTT AGAAGATGGC ACAGGCTCTG CCCTGTCTTT
 AGACCTTTCA AGTAGATGAA TCTTCTACCG TGTCCGAGAC GGCACAGGAA
 SerGlyLysP heIleTyrLe uGluAspGly ThrGlySerA leValSerLeu
 1401 GGAGTGA
 CCTCACT
 468 GluOP*

FIG. 2B

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1 MEQRCQNAPAASGAKCKRGPGPREANGARPGLVPKTLVLVAAVLLLVSAESALITQD
61 LAPQQRAAPQQKSSPSEGLCPPGHHISEDGRDCISCKYQDYSTHWNDLLFCLRCTRCD
121 SGEVELSPCTTIRNTVCQCKGTFREKDSPENCRKCRTGCPRGMVKVGDCTPNSDIECVH
181 KESGLIIGVTVAAVVLVAVFVCKLLNKVLFYLKGICBGGGDPERVDRSSQRPGAED
241 NVLNEIVSILQPTQVPEQENEVQEPAKPTGVNHLSPGESEHLLEPANAERSQRRRLVPA
301 NEGDPTETLRQCFDDFADLVPFDSNEPLMRKLGLMDNEIKVAKAEAAGHDTLYTMLIKN
361 VNKTGRDASVHTLLDALETLGERLAKOKIEDHLSSGKFMYLEGNADSALS

FIG. 3A

20

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Met	Glu	Gln	Arg	Gly	Gln	Asn	Ala	Pro	Ala	Ala	Ser	Gly	Ala	Arg	Lys
1				5					10					15	
Arg	His	Gly	Pro	Gly	Pro	Arg	Glu	Ala	Arg	Gly	Ala	Arg	Pro	Gly	Pro
		20						25					30		
Arg	Val	Pro	Lys	Thr	Leu	Val	Leu	Val	Val	Ala	Ala	Val	Leu	Leu	Leu
		35					40					45			
Val	Ser	Ala	Glu	Ser	Ala	Leu	Ile	Thr	Gln	Gln	Asp	Leu	Ala	Pro	Gln
	50					55					60				
Gln	Arg	Ala	Ala	Pro	Gln	Gln	Lys	Arg	Ser	Ser	Pro	Ser	Glu	Gly	Leu
65					70					75					80
Cys	Pro	Pro	Gly	His	His	Ile	Ser	Glu	Asp	Gly	Arg	Asp	Cys	Ile	Ser
				85					90					95	
Cys	Lys	Tyr	Gly	Gln	Asp	Tyr	Ser	Thr	His	Trp	Asn	Asp	Leu	Leu	Phe
			100					105					110		
Cys	Leu	Arg	Cys	Thr	Arg	Cys	Asp	Ser	Gly	Glu	Val	Glu	Leu	Ser	Pro
		115					120					125			
Cys	Thr	Thr	Thr	Arg	Asn	Thr	Val	Cys	Gln	Cys	Glu	Glu	Gly	Thr	Phe
	130					135					140				
Arg	Glu	Glu	Asp	Ser	Pro	Glu	Met	Cys	Arg	Lys	Cys	Arg	Thr	Gly	Cys
145					150					155					160
Pro	Arg	Gly	Met	Val	Lys	Val	Gly	Asp	Cys	Thr	Pro	Trp	Ser	Asp	Ile
				165					170					175	
Glu	Cys	Val	His	Lys	Glu	Ser	Gly	Thr	Lys	His	Ser	Gly	Glu	Ala	Pro
			180					185					190		
Ala	Val	Glu	Glu	Thr	Val	Thr	Ser	Ser	Pro	Gly	Thr	Pro	Ala	Ser	Pro
		195					200					205			
Cys	Ser	Leu	Ser	Gly	Ile	Ile	Ile	Gly	Val	Thr	Val	Ala	Ala	Val	Val
		210				215					220				
Leu	Ile	Val	Ala	Val	Phe	Val	Cys	Lys	Ser	Leu	Leu	Trp	Lys	Lys	Val
225					230					235					240
Leu	Pro	Tyr	Leu	Lys	Gly	Ile	Cys	Ser	Gly	Gly	Gly	Gly	Asp	Pro	Glu
				245					250					255	
Arg	Val	Asp	Arg	Ser	Ser	Gln	Arg	Pro	Gly	Ala	Glu	Asp	Asn	Val	Leu
		260						265					270		
Asn	Glu	Ile	Val	Ser	Ile	Leu	Gln	Pro	Thr	Gln	Val	Pro	Glu	Gln	Glu
		275					280					285			

FIG. 3B

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Met	Glu	Val	Gln	Glu	Pro	Ala	Glu	Pro	Thr	Gly	Val	Asn	Met	Leu	Ser
290						295					300				
Pro	Gly	Glu	Ser	Glu	His	Leu	Leu	Glu	Pro	Ala	Glu	Ala	Glu	Arg	Ser
305					310					315					320
Gln	Arg	Arg	Arg	Leu	Leu	Val	Pro	Ala	Asn	Glu	Gly	Asp	Pro	Thr	Glu
				325					330					335	
Thr	Leu	Arg	Gln	Cys	Phe	Asp	Asp	Phe	Ala	Asp	Leu	Val	Pro	Phe	Asp
			340					345					350		
Ser	Trp	Glu	Pro	Leu	Met	Arg	Lys	Leu	Gly	Leu	Met	Asp	Asn	Glu	Ile
		355					360					365			
Lys	Val	Ala	Lys	Ala	Glu	Ala	Ala	Gly	His	Arg	Asp	Thr	Leu	Tyr	Thr
		370					375				380				
Met	Leu	Ile	Lys	Trp	Val	Asn	Lys	Thr	Gly	Arg	Asp	Ala	Ser	Val	His
385					390					395					400
Thr	Leu	Leu	Asp	Ala	Leu	Glu	Thr	Leu	Gly	Glu	Arg	Leu	Ala	Lys	Gln
				405					410					415	
Lys	Ile	Glu	Asp	His	Leu	Leu	Ser	Ser	Gly	Lys	Phe	Met	Tyr	Leu	Glu
			420					425					430		
Gly	Asn	Ala	Asp	Ser	Ala	Met	Ser								*
		435					440								

FIG. 3C

Handwritten signature

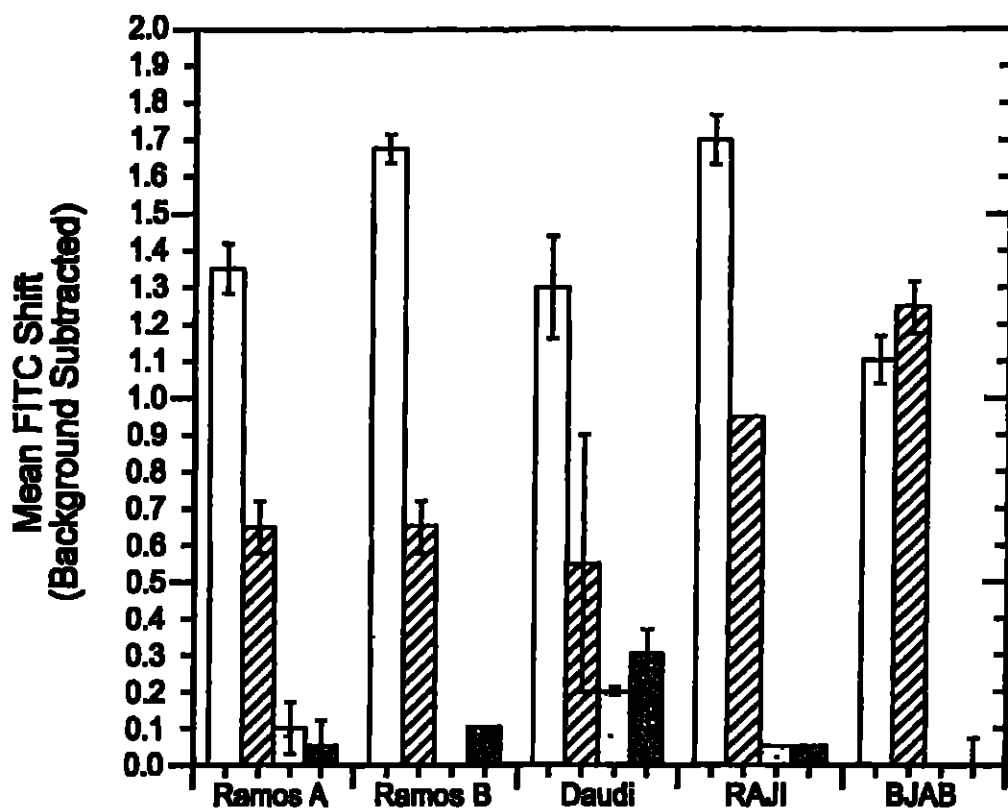


FIG. 4

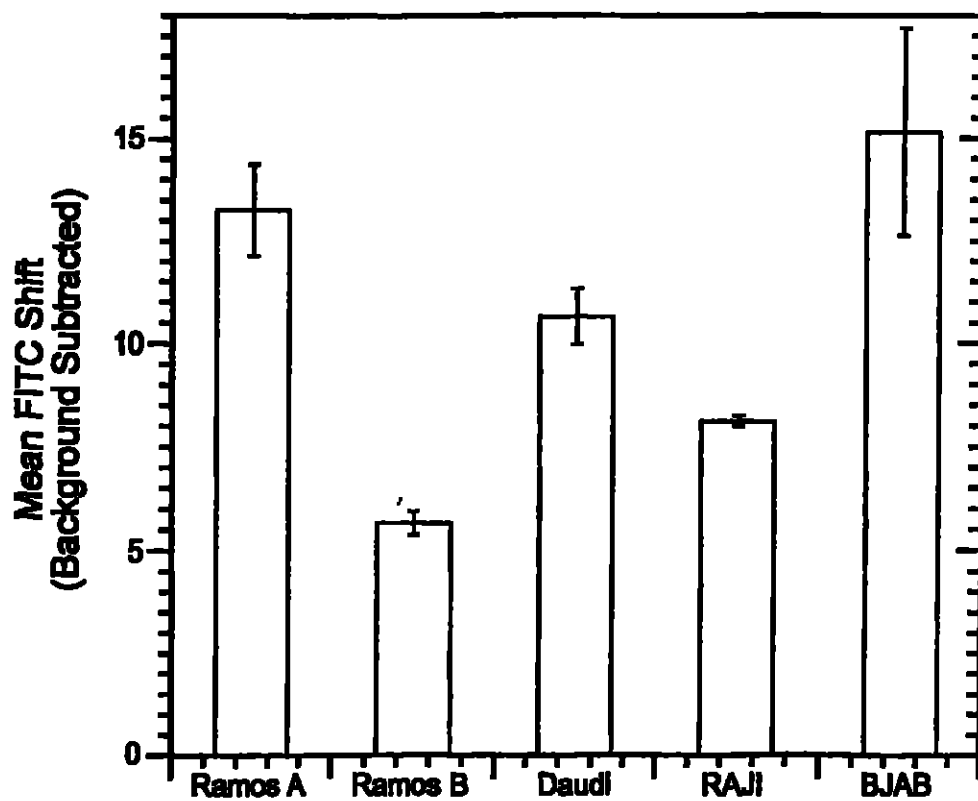


FIG. 5

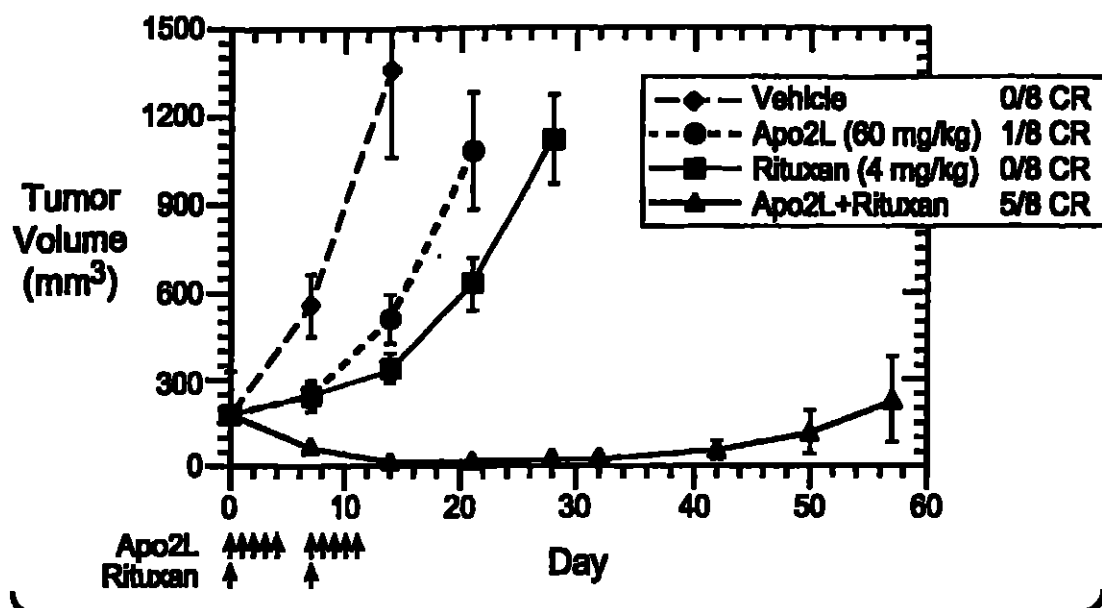


FIG. 6

Apo2L.0 (60 mg/kg/mouse; 5x/wk; IP) Alone and in Combination with Rituxan (4 mg/kg/mouse; 1x/wk; IP) vs. BJAB (SCID Mice) Xenograft

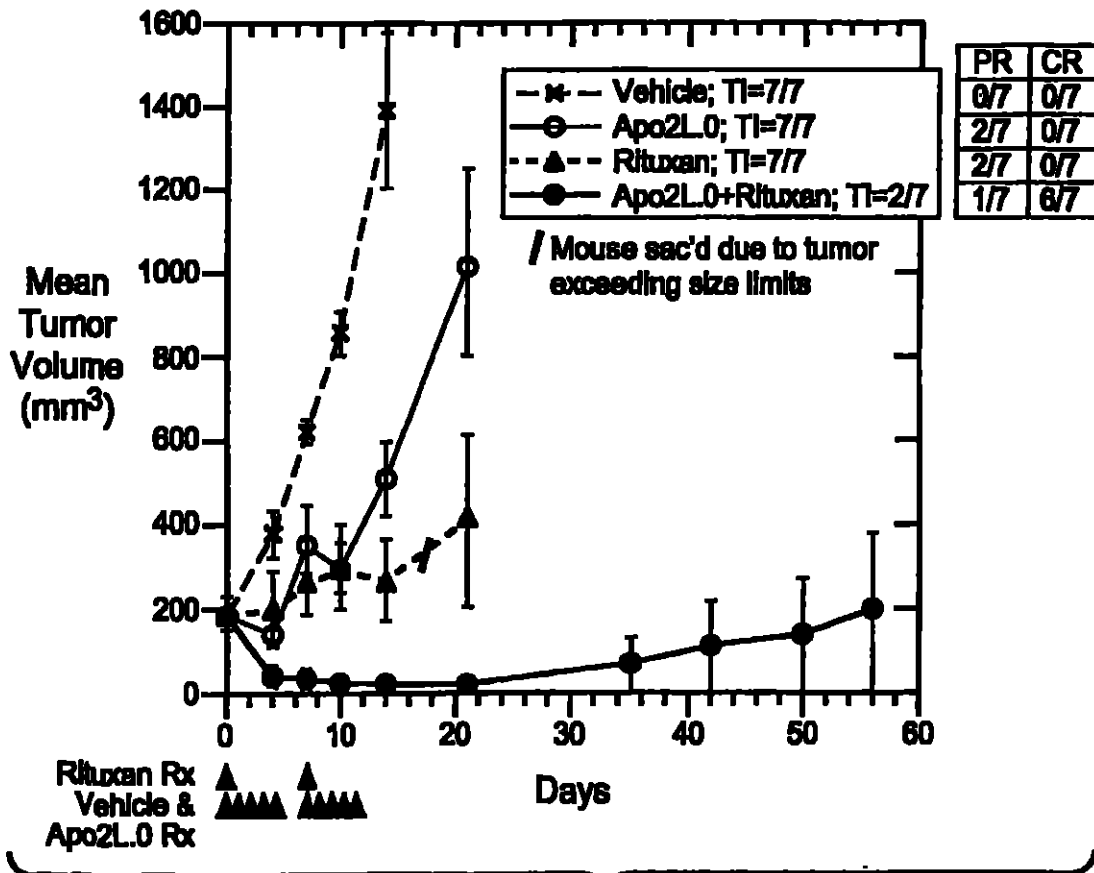


FIG. 7

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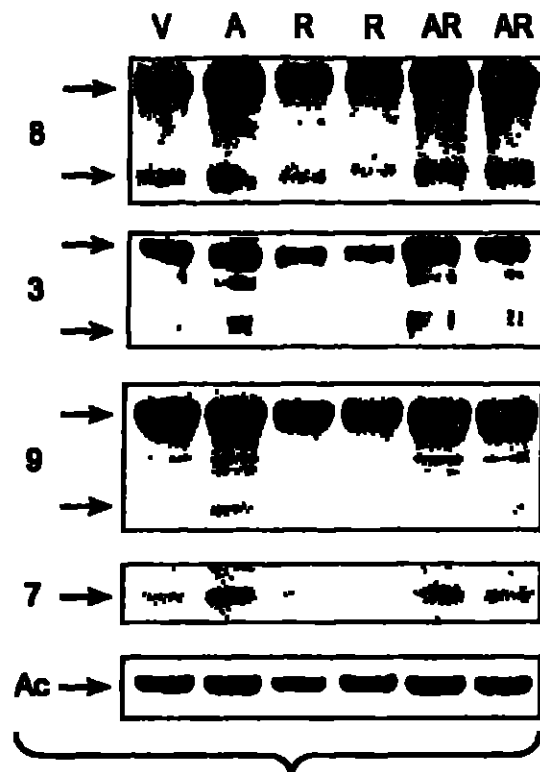


FIG. 8



FIG. 10

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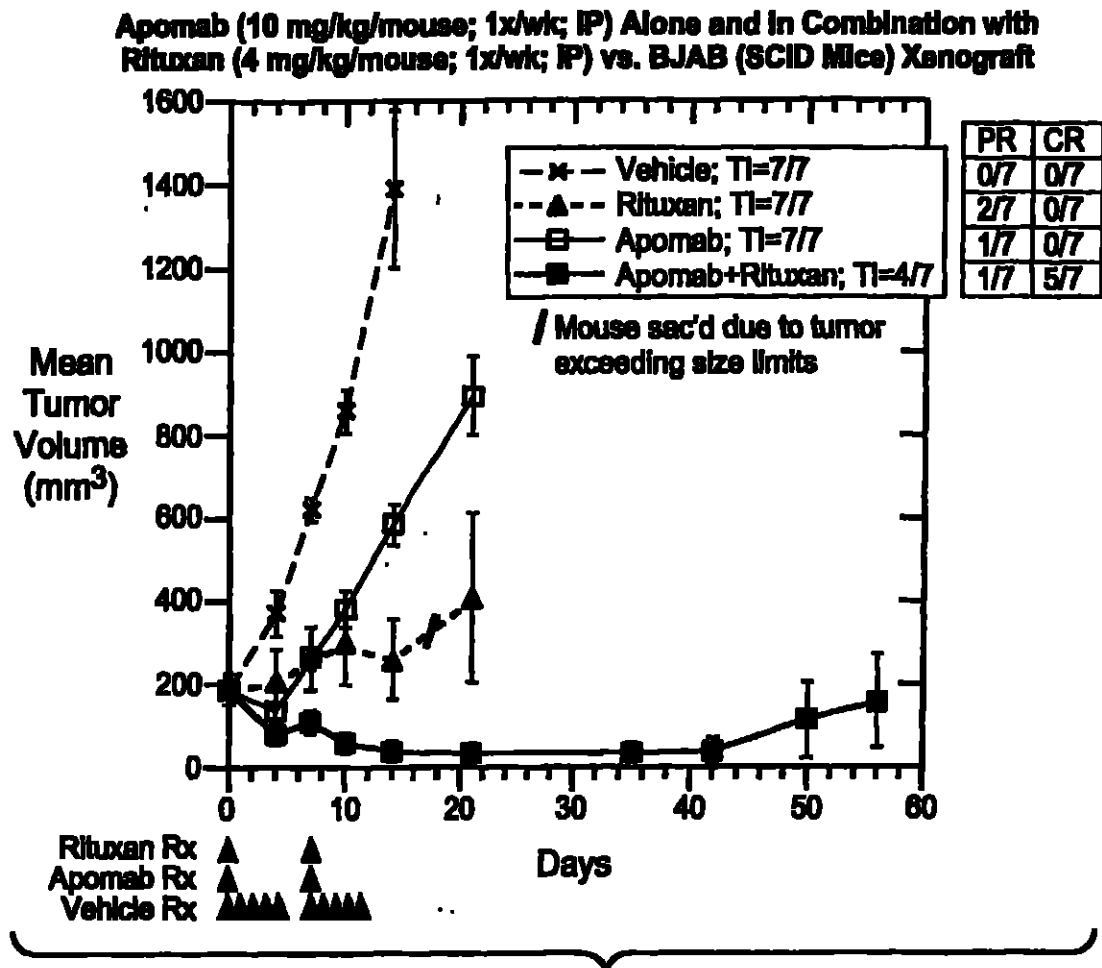


FIG. 9

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Expression of CD20 and Apo2L / TRAIL Receptors on NHL Cell Lines

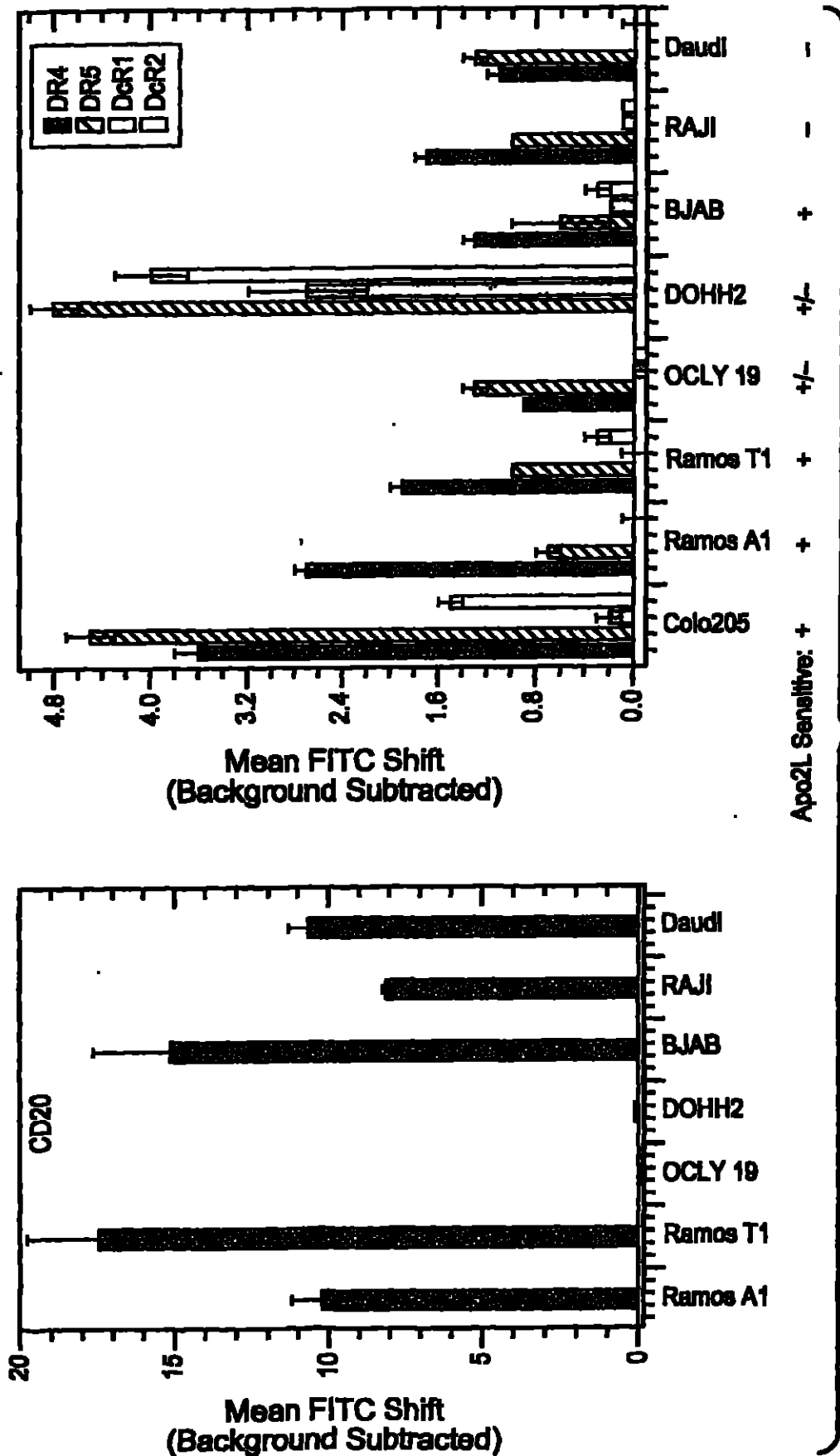


FIG. 11

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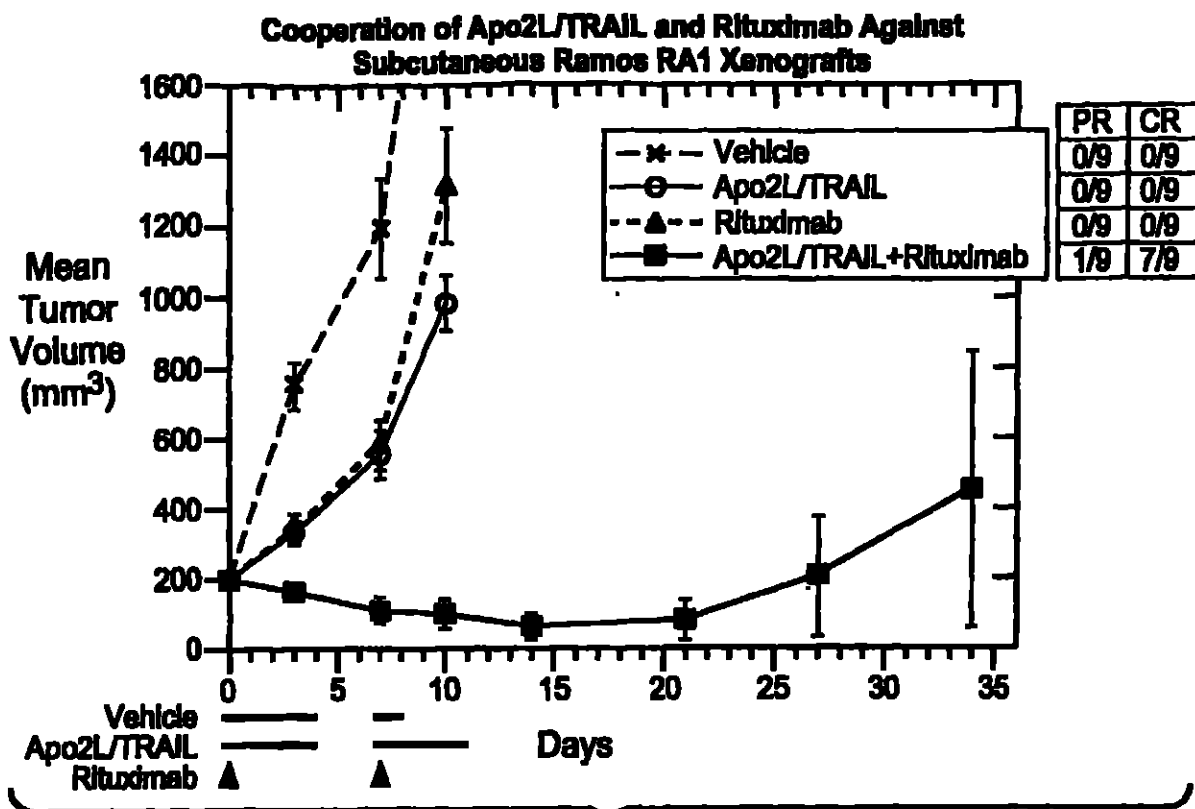


FIG. 12

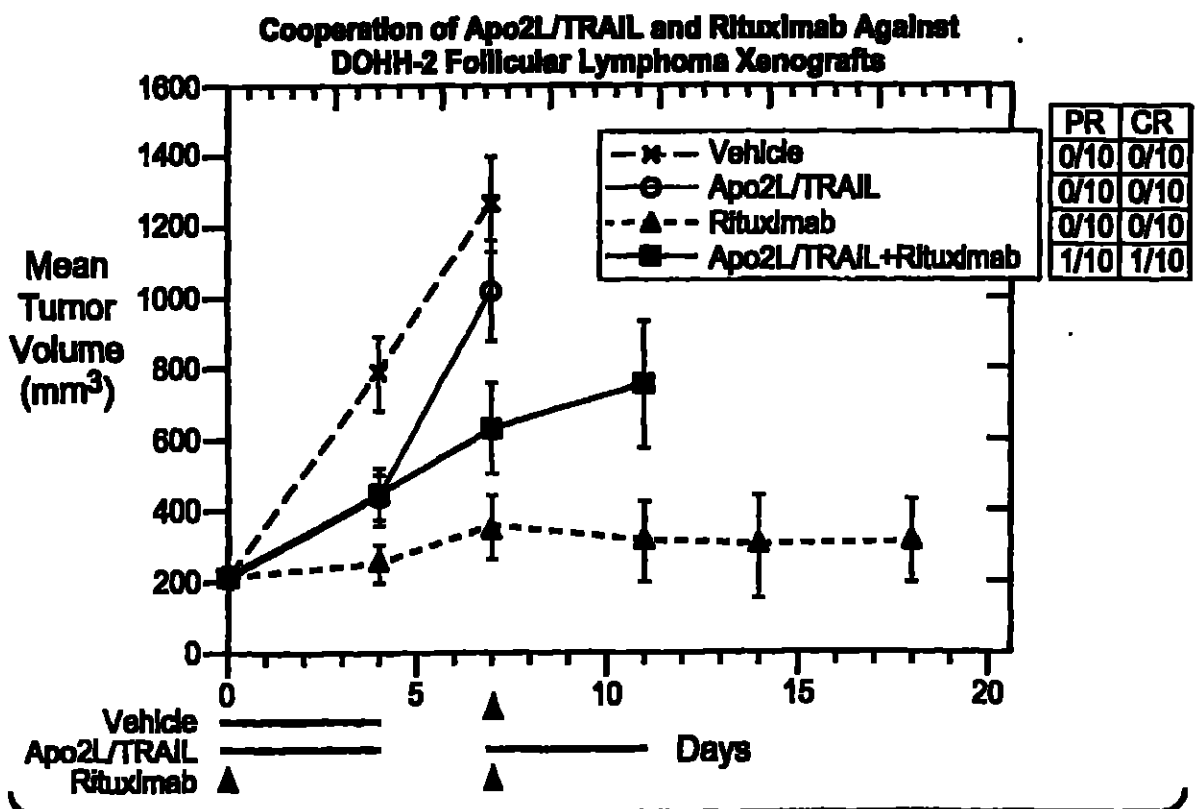


FIG. 13

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Rituximab and Apo2L/TRAIL Kill BJAB Cells by Independent Mechanisms

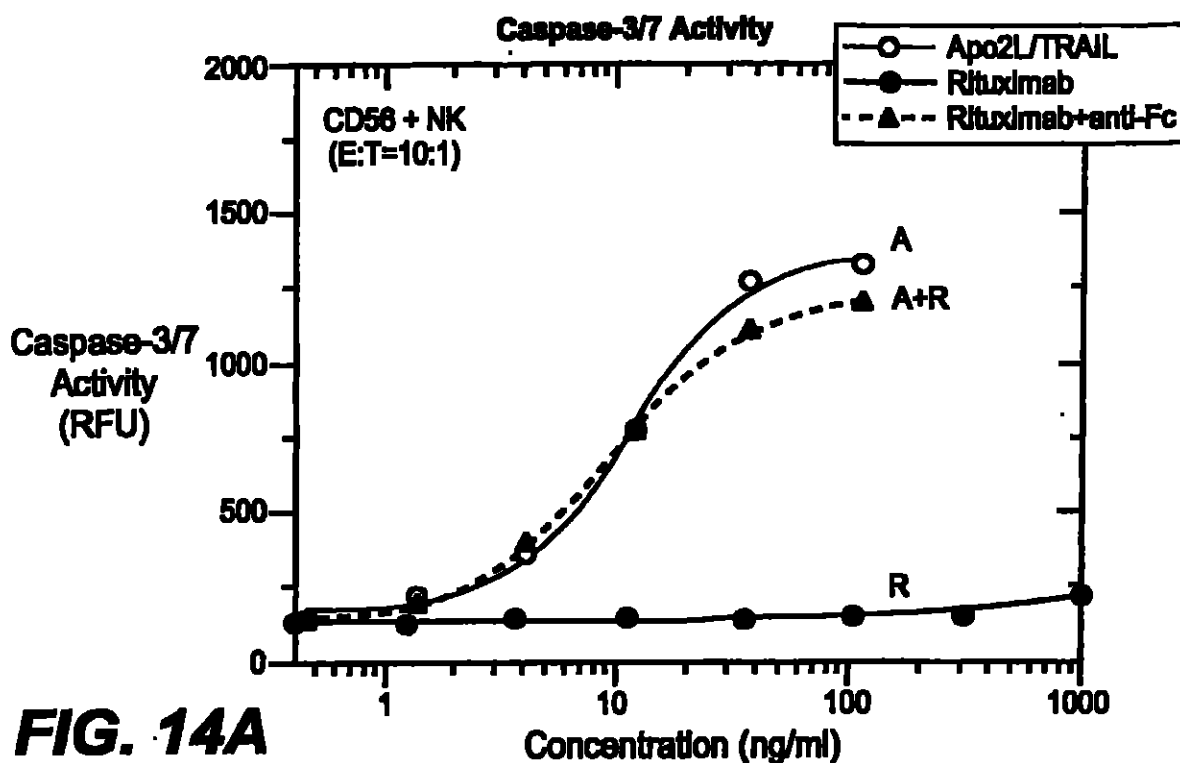


FIG. 14A

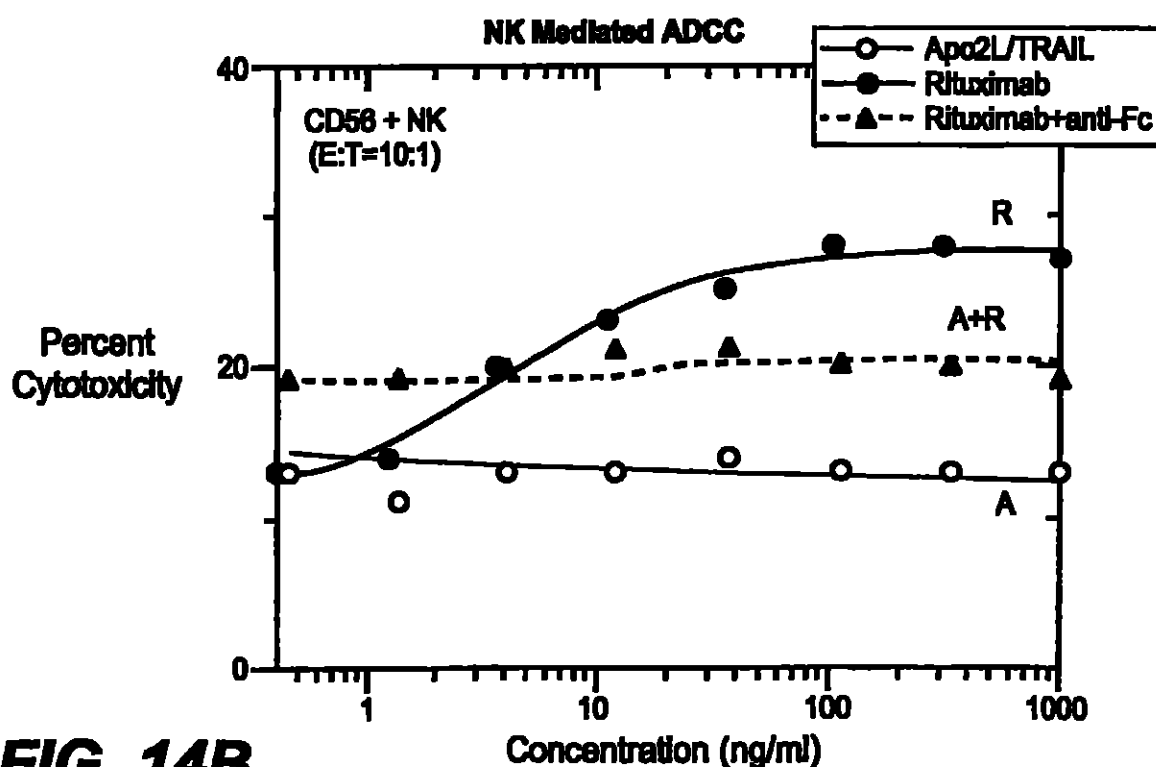


FIG. 14B

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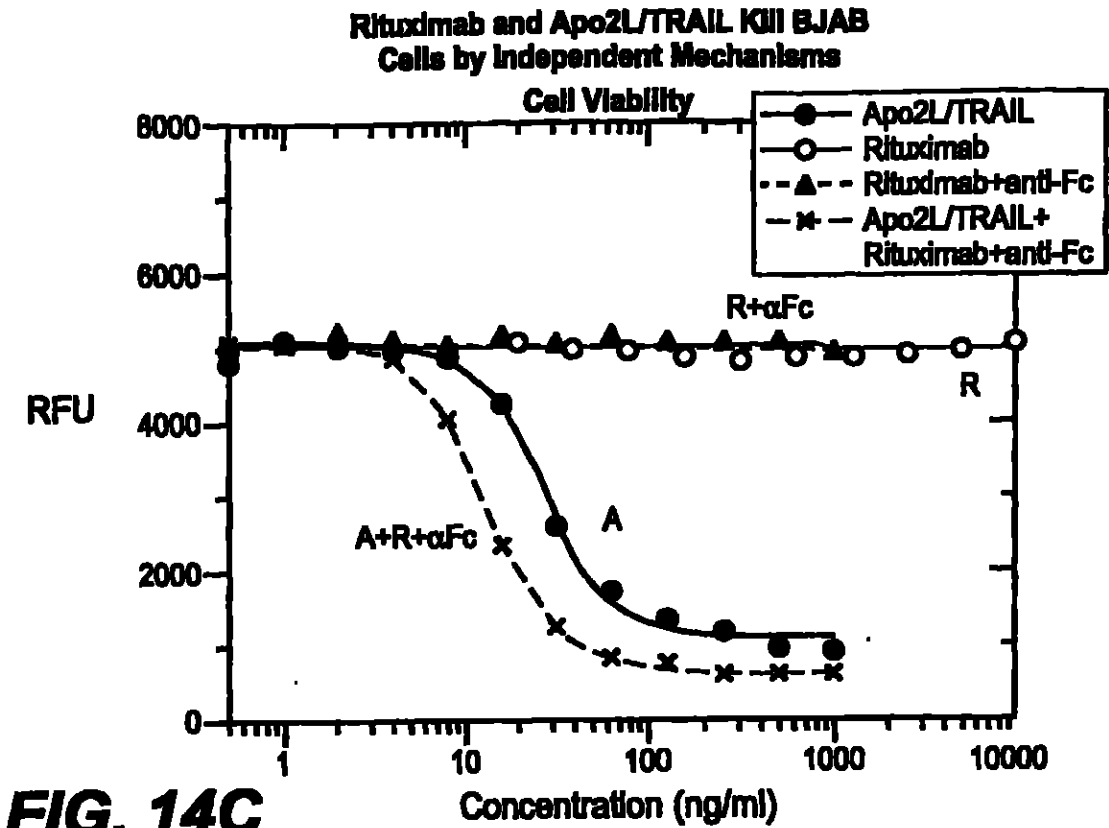


FIG. 14C

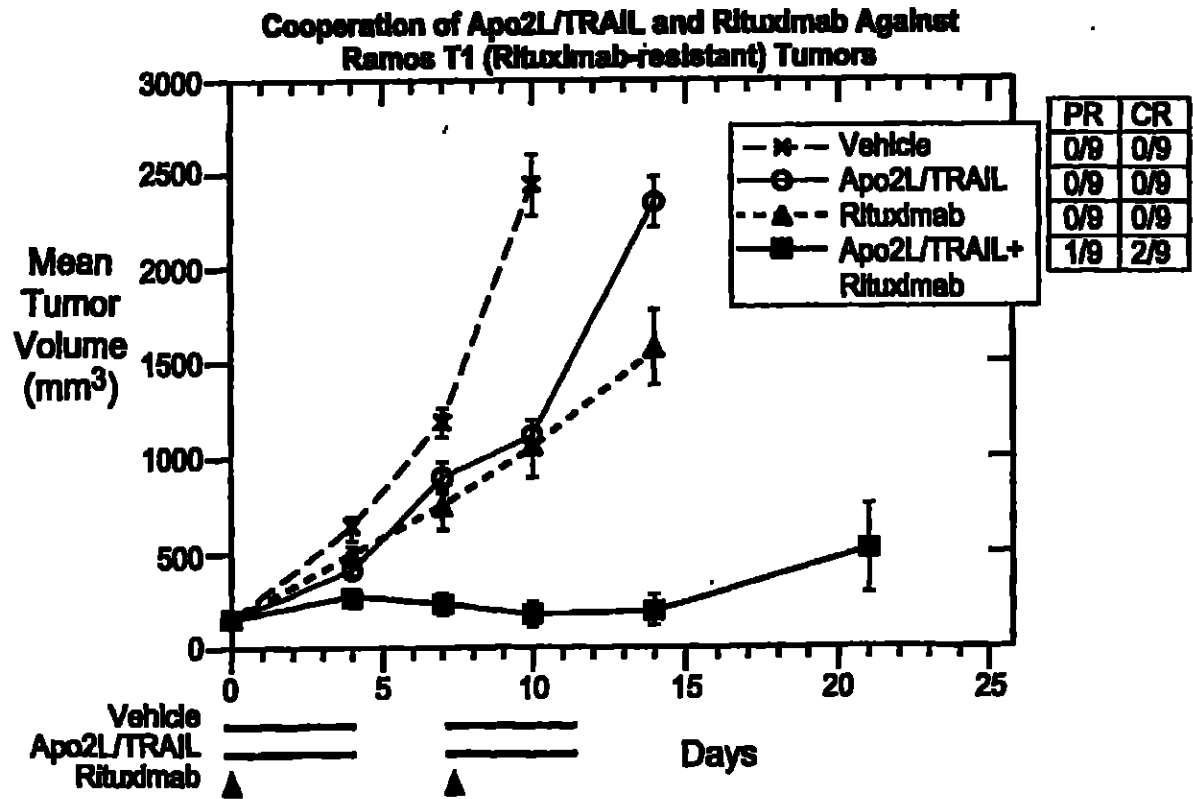


FIG. 15

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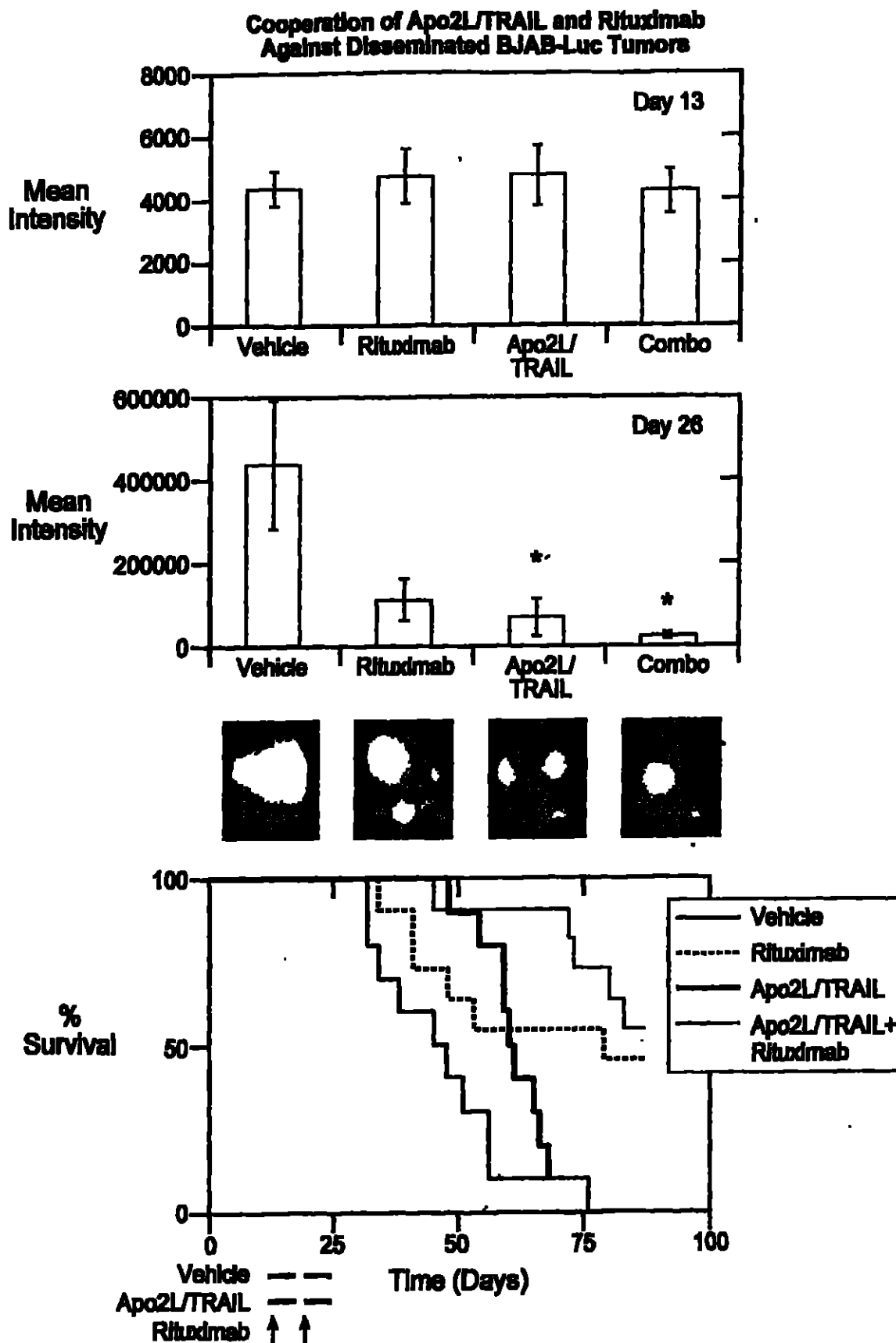


FIG. 16

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2005/032015

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K16/28 A61P35/02 A61P19/02 A61P25/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03/038043 A (UAB RESEARCH FOUNDATION; ZHOU, TONG; ICHIKAWA, KIMIHISA; KIMBERLY, ROB) 8 May 2003 (2003-05-08) page 6, line 1 - page 8, line 6 page 54, line 8 - line 14 claims 30, 66, 102 examples	1-27
A	WO 99/37684 A (GENENTECH, INC) 29 July 1999 (1999-07-29) abstract	1-27

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document relating to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *Z* document member of the same patent family

Date of the actual completion of the international search

24 May 2006

Date of mailing of the international search report

22/06/2006

Name and mailing address of the ISA/

European Patent Office, P.B. 6618 Patankaan 2
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Authorized officer

Rankin, R

9L

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2005/032015

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

- ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1-27 are directed to a method of treatment of the human body, the search has been carried out and based on the alleged effects of the composition.
- ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
- ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

- ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
- ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
- ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
- ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2005/032015

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 03038043	A	08-05-2003	CA 2465314 A1	08-05-2003
			CN 1630516 A	22-06-2005
			EP 1506285 A2	16-02-2005
			JP 2005519871 T	07-07-2005
			MX PA04004184 A	25-01-2005
WO 9937684	A	29-07-1999	AU 2338299 A	09-08-1999
			CA 2318405 A1	29-07-1999
			EP 1053256 A1	22-11-2000
			JP 2002500877 T	15-01-2002

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(12) SOLICITUD INTERNACIONAL PUBLICADA SEGÚN EL TRATADO DE COOPERACIÓN INTERNACIONAL
EN MATERIA DE PATENTES (PCT)

(19) Organización Mundial de Propiedad
Intelectual
Oficina Internacional



(43) Fecha de Publicación Internacional
16 de marzo de 2006 (16.03.2006)

PCT

(10) Número de Publicación Internacional
WO 2006/029275 A3

(51) Clasificación Internacional de Patentes⁷:

C07K 16/28 (2006.01) A61P 19/02 (2006.01)
A61K 35/02 (2006.01) A61K 25/00 (2006.01)

(81) Países designados (a menos que se indique lo contrario para cada clase de protección disponible): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(21) No. Solicitud Internacional: PCT/US2005/032015

(22) Fecha de Presentación Internacional:
7 de septiembre de 2005 (07.09.2005)

(25) Idioma de Presentación: Inglés

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(30) Datos relativos a la Prioridad:
60/607,634 8 de septiembre de 2004 (08.09.2004) US
60/666,550 30 de marzo de 2005 (30.03.2005) US

(84) Países designados (a menos que se indique lo contrario para cada clase de protección disponible): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasática (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), Europea (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(71) Solicitante (para todos los países destinatarios con excepción de US): GENENTECH, INC. [US/US]; 1 DNA Way, South San Francisco, California 94080-4990 (US).

Publicado:
— con informe internacional de búsqueda
— antes del vencimiento del tiempo límite para modificar las reivindicaciones y para volverse a publicar en el caso de recibo de modificaciones

(72) Inventores, e
(75) Inventores/Solicitantes (para US únicamente): ASHKENAZI, Avi J. [US/US]; 1456 Tarrytown Street, San Mateo, California 94402 (US).

(88) Fecha de publicación del informe de búsqueda internacional:

27 de julio de 2006

(74) Agentes: MARSCHANG, Diane L.; GENENTECH, INC., 1 DNA Way, South San Francisco, California 94080-4990 (US).

Para códigos de dos letras y otras abreviaturas, remítase a las "Notas Guía sobre Códigos y Abreviaturas" que aparece al comienzo de cada edición regular del Boletín Oficial PCT.

(54) Título: MÉTODOS DE UTILIZAR LIGANDOS DE RECEPTOR DE MUERTE CELULAR Y ANTICUERPOS CD20

(57) Extracto: Se proveen métodos para usar ligandos de receptor de muerte celular, tales como polipéptidos ligando Apo-2/TRAIL o anticuerpos de receptor de muerte celular, y anticuerpos CD20 para tratar afecciones tales como cáncer y enfermedades inmuno-relacionadas. Las incorporaciones de la invención incluyen métodos de usar Apo2L/TRAIL o anticuerpos de receptor de muerte celular tales como anticuerpos DR5 y anticuerpos DR4 en combinación con anticuerpos CD20.

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MÉTODOS DE UTILIZAR LIGANDOS DE RECEPTOR DE MUERTE CELULAR Y ANTICUERPOS CD20

SOLICITUDES RELACIONADAS

Esta solicitud reivindica prioridad bajo la Sección 119(e) a la solicitud provisional número 60/607,834 presentada el 8 de septiembre de 2004 y a la solicitud provisional número 60/666,550 presentada el 30 de marzo de 2005, cuyos contenidos se incorporan en esta solicitud por referencia.

CAMPO DE LA INVENCION

La presente invención se refiere a métodos de utilizar ligandos de receptor de muerte celular y anticuerpos CD20. Más particularmente, la invención concierne a métodos de emplear el ligando Apo 2/TRAIL o anticuerpos de receptor de muerte celular en combinación con anticuerpos CD20 para tratar varios trastornos patológicos, tales como cáncer y enfermedades inmunitarias relacionadas.

ANTECEDENTES DE LA INVENCION

En el arte se han identificado varios ligandos y receptores pertenecientes a la superfamilia del factor de necrosis tumoral (TNF). Incluidos entre tales ligandos se encuentran el factor alfa de necrosis tumoral ("TNF-alfa"), el factor beta de necrosis tumoral ("TNF-beta" o "linfotoxina-alfa"), linfotoxina-beta ("LT-beta"), ligando CD30, ligando CD27, ligando CD40, ligando OX-40, ligando 4-1BB, ligando Apo-1 (también denominado ligando Fas o ligando CD95), ligando Apo-2 (también denominado Apo2L ó TRAIL), ligando Apo-3 (también denominado TWEAK), APRIL, ligando OPG (también denominado ligando RANK, ODF, o TRANCE), y TALL-1 (también denominado BlyS, BAFF ó THANK) (Véase, por ejemplo, Ashkenazi, Nature Review, 2:420-430 (2002) ; Ashkenazi and Dixit, Science, 281:1305-1308 (1998); Ashkenazi and Dixit, Curr. Opin. Cell Biol., 11:255- 260 (2000); Golstein, Curr. Biol.,

7:750-753 (1997) Wallach, Cytokine Reference, Academic Press, 2000, pages 377-411; Locksley et al., Cell, 104:487-501 (2001); Gruss and Dower, Blood, 85:3378-3404 (1995); Schmid et al., Proc. Natl. Acad. Sci., 83:1881 (1986); Dealtry et al., Eur. J. Immunol., 17:689 (1987); Pitti et al., J. Biol. Chem., 271:12687-12690 (1996); Wiley et al., Immunity, 3:673-682 (1995); Browning et al., Cell, 72:847-856 (1993); Armitage et al. Nature, 357:80-82 (1992); WO 97/01633 publicada el 16 de enero de 1997; WO 97/25428 publicada el 17 de julio de 1997; Marsters et al., Curr. Biol., 8:525-528 (1998); Chicheportiche et al., Biol. Chem., 272:32401-32410 (1997); Hahne et al., J. Exp. Med., 188:1185-1190 (1998); WO98/28426 publicada el 2 de julio de 1998; WO98/46751 publicada el 22 de octubre de 1998; WO/98/18921 publicada el 7 de mayo de 1998; Moore et al., Science, 285:260-263 (1999); Shu et al., J. Leukocyte Biol., 65:680 (1999); Schneider et al., J. Exp. Med., 189:1747-1756 (1999); Mukhopadhyay et al., J. Biol. Chem., 274:15978-15981 (1999)).

La inducción de varias respuestas celulares, mediadas por tales ligandos de la familia TNF, se inicia típicamente por su unión a receptores celulares específicos. Algunos, pero no todos, los ligandos de la familia TNF se unen a, e inducen diferente actividad biológica a través de, "receptores de muerte celular" de superficie celular para activar caspasas, o enzimas que llevan a cabo la vía de muerte celular o apoptosis (Salvesen et al., Cell, 91:443-446 (1997). Incluidos entre los miembros de la superfamilia de receptores TNF, identificados hasta la fecha, se encuentran TNFR1, TNFR2, TACI, GITR, CD27, OX-40, CD30, CD40, HVEM, Fas (también denominado Apo-1 o CD95), DR4 (también denominado TRAIL-R1), DR5 (también denominado Apo-2 o TRAIL-R2), DcR1, DcR2, osteoprotegerina (OPG), RANK y Apo-3 (también denominado DR3 o TRAMP) (véase, por ejemplo, Ashkenazi, Nature Reviews, 2:420-430 (2002); Ashkenazi and Dixit, Science, 281:1305-1308 (1998); Ashkenazi and Dixit, Curr. Opin. Cell Biol., 11:255-260 (2000); Golstein, Curr. Biol., 7:750-753 (1997); Wallach, Cytokine Reference, Academic Press, 2000, pages 377-411; Locksley et al., Cell, 104:487-501 (2001); Gruss and

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Dower, Blood, 85:3378-3404 (1995); Hohman et al., J. Biol. Chem., 264:14927-14934 (1989); Brockhaus et al., Proc. Natl. Acad. Sci., 87:3127-3131 (1990); EP 417,563, publicada el 20 de marzo de 1991; Loetscher et al., Cell, 61:351 (1990); Schall et al., Cell, 61:361 (1990); Smith et al., Science, 248:1019-1023 (1990); Lewis et al., Proc. Natl. Acad. Sci., 88:2830-2834 (1991); Goodwin et al., Mol. Cell. Biol., 11:3020-3026 (1991); Stamenkovic et al., EMBO J., 8:1403-1410 (1989); Mallett et al., EMBO J., 9:1063-1068 (1990); Anderson et al., Nature, 390:175-179 (1997); Chicheportiche et al., J. Biol. Chem., 272:32401-32410 (1997); Pan et al., Science, 276:111-113 (1997); Pan et al., Science, 277:815-818 (1997); Sheridan et al., Science, 277:818-821 (1997); Degli-Esposti et al., J. Exp. Med., 186:1165-1170 (1997); Marsters et al., Curr. Biol., 7:1003-1006 (1997); Tsuda et al., BBRC, 234:137-142 (1997); Nocentini et al., Proc. Natl. Acad. Sci., 94:6216-6221 (1997); von Bulow et al., Science, 278:138-141 (1997)).

La mayoría de estos miembros de la familia de receptores TNF comparten la estructura típica de los receptores de superficie celular, incluidas las regiones extracelulares, de transmembrana e intracelulares, mientras que otros se encuentran naturalmente como proteínas solubles que carecen de un dominio transmembrana e intracelular. La porción extracelular de los TNFR típicos contiene un patrón repetitivo de secuencia de aminoácidos de múltiples dominios, ricos en cisteína (CRD – *cysteine-rich domains*), que se inician a partir del término NH₂.

El ligando designado como Apo-2L o TRAIL se identificó hace varios años como un miembros de la familia TNF de citocinas, (véase *por ejemplo*, Wiley et al., Immunity, 3:673-682 (1995); Pitti et al., J. Biol. Chem., 271:12697-12690 (1996); WO 97/01633; WO 97/25428; Patente de los Estados Unidos 5,763,223 expedida el 9 de junio de 1998; Patente de los Estados Unidos 6,284,236 expedida el 4 de septiembre de 2001). El polipéptido Apo2L/TRAIL humano de secuencia natural, de longitud total, es una proteína transmembrana tipo II, de 281 aminoácidos de larga. Algunas células pueden producir una forma soluble natural del polipéptido,

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mediante escisión enzimática de la región extracelular del polipéptido (Mariani et al., J. Cell. Biol., 137:221-229 (1997)). Estudios cristalográficos de formas solubles de Apo2L/TRAIL revelan una estructura homotrimérica similar a las estructuras de TNF y otras proteínas relacionadas (Hymowitz et al., Molec. Cell, 4:563-571 (1999); Cha et al., Immunity, 11:253-261 (1999); Mongkolsapaya et al., Nature Structural Biology, 6:1048 (1999); Hymowitz et al., Biochemistry, 39:633-644 (2000)). Sin embargo, se descubrió que Apo2L/TRAIL, a diferencia de otros miembros de la familia TNF, posee una característica estructural única, en la que tres residuos cisteína (en la posición 230 de cada subunidad en el homotrímero) coordinan conjuntamente un átomo de zinc, y que la unión de zinc es importante para la estabilidad y actividad biológica del trímero. (Hymowitz et al., supra; Bodmer et al., J. Biol. Chem., 275:20632-20637 (2000)).

Se ha informado en la literatura que Apo2L/TRAIL puede jugar un papel en la modulación del sistema inmunitario, incluidas enfermedades autoinmunitarias tales como artritis reumatoide [véase *por ejemplo*, Thomas et al., J. Immunol., 161:2195-2200 (1998); Johnsen et al., Cytokine, 11:664-672 (1999); Griffith et al., J. Exp. Med., 189:1343-1353 (1999); Song et al., J. Exp. Med., 191:1095-1103 (2000)].

Se ha divulgado asimismo que formas solubles de Apo2L/TRAIL inducen apoptosis en una variedad de células cancerosas, incluidos tumores de colon, pulmón, mama, próstata, vejiga, riñón, ovarios y cerebro, así como melanoma, leucemia, y mieloma múltiple (véase *por ejemplo*, Wiley et al., supra; Pitti et al., supra; Patente de los Estados Unidos 6,030,945 expedida el 29 de febrero de 2000; Patente de los Estados Unidos 6,746,668 expedida el 8 de junio de 2004; Rieger et al., FEBS Letters, 427:124-128 (1998); Ashkenazi et al., J. Clin. Invest., 104:155-162 (1999); Walczak et al., Nature Med., 5:157-163 (1999); Keane et al., Cancer Research, 59:734-741 (1999); Mizutani et al., Clin. Cancer Res., 5:2605-2612 (1999); Gazitt, Leukemia, 13:1817-1824 (1999); Yu et al., Cancer Res., 60:2384-2389 (2000); Chinnaiyan et al., Proc. Natl. Acad. Sci., 97:1754-1759 (2000)).

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Estudios *in vivo* en modelos de tumor murino sugieren además que Apo2L/TRAIL, solo o en combinación con quimioterapia o terapia de radiación, puede ejercer sustanciales efectos anti-neoplásicos (véase *por ejemplo*, Ashkenazi et al., *supra*; Walzcak et al., *supra*; Gliniak et al., *Cancer Res.*, 59:6153-6158 (1999); Chinnaiyan et al., *supra*; Roth et al., *Biochem. Biophys. Res. Comm.*, 265:1999 (1999); Solicitud PCT US/00/15512; Solicitud PCT US/01/23691). En contraste con muchos tipos de células cancerosas, la mayoría de los tipos de células humanas normales parecen ser resistentes a la inducción de apoptosis por ciertas formas recombinantes de Apo2L/TRAIL (Ashkenazi et al., *supra*; Walzcak et al., *supra*). Jo et al. ha divulgado que una forma soluble, marcada con polihistidina, de Apo2L/TRAIL indujo apoptosis *in vitro* en hepatocitos humanos aislados normales, más no en hepatocitos no humanos, (Jo et al., *Nature Med.*, 6:564-567 (2000); véase también, Nagata, *Nature Med.*, 6:502-503 (2000)). Se cree que ciertas preparaciones de Apo2L/TRAIL recombinantes pueden variar en términos de propiedades bioquímicas y actividades biológicas en células enfermas versus células normales, dependiendo, por ejemplo, de la presencia o ausencia de una molécula marcadora, el contenido de zinc, y el % de contenido de trímero (véase Lawrence et al., *Nature Med.*, Letter to the Editor, 7:383-385 (2001); Qin et al., *Nature Med.*, Letter to the Editor, 7:385-386 (2001)).

Se ha descubierto que Apo2L/TRAIL une por lo menos cinco receptores diferentes. Por lo menos dos de los receptores que unen Apo2L/TRAIL contienen un dominio de muerte celular citoplásmico funcional. Un receptor tal se ha designado "DR4" (y, alternativamente, TR4 o TRAIL-R1) (Pan et al., *Science*, 276:111-113 (1997); véase también WO98/32856 publicada el 30 de julio de 1998; WO99/37684 publicada el 29 de julio de 1999; WO 00/73349 publicada el 7 de diciembre de 2000; US 6,433,147 expedida el 13 de agosto de 2002; US 6,461,823 expedida el 8 de octubre de 2002, y US 6,342,383 expedida el 29 de enero de 2002).

Otro receptor similar para Apo2L/TRAIL se ha denominado DR5 (alternativamente, se ha designado también Apo-2; TRAIL-R, TR6, Tango-

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63, hAPO8, TRICK2 ó KILLER (véase, por ejemplo, Sheridan et al., Science, 277:818-821 (1997); Pan et al., Science, 277:815-818 (1997); WO98/51793 publicada el 19 de noviembre de 1998; WO98/41629 publicada el 24 de septiembre de 1998; Scream et al., Curr. Biol., 7:693-696 (1997); Walczak et al., EMBO J., 16:5386-5387 (1997); Wu et al., Nature Genetics, 17:141-143 (1997); WO98/35986 publicada el 20 de agosto de 1998; EP870,827 publicada el 14 de octubre de 1998, WO98/46643 publicada el 22 de octubre de 1998; WO99/02653 publicada el 21 de enero de 1999; WO99/09165 publicada el 25 de febrero de 1999, WO99/11791 publicada el 11 de marzo de 1999); US 2002/0072091 publicada el 13 de agosto de 2002; US 2002/0098550 publicada el 7 de diciembre de 2001; US 6,313,269 expedida el 6 de diciembre de 2001; US 2001/0010924 publicada el 2 de agosto de 2001; US 2003/01255540 publicada el 3 de julio de 2003; US 2002/0160446 publicada el 31 de octubre de 2002; US 2002/0048785 publicada el 25 de abril 2002; US 6,342,369 expedida en febrero de 2002; US 6,569,642 expedida el 27 de mayo de 2003; US 6,072,047 expedida el 6 de junio de 2000; US 6,642,358 expedida el 4 de noviembre de 2003; US 6,743,625 expedida el 1 de junio de 2004). Al igual que DR4, se divulga que DR5 contiene un dominio de muerte celular citoplásmico y es capaz de señalar apoptosis tras la unión del ligando (o tras de unir una molécula, tal como un anticuerpo agonista, que imita la actividad del ligando. La estructura cristalina del complejo formado entre Apo-2L/TRAIL y DR5 se describe en Hymowitz et al., Molecular Cell, 4:563-571 (1999).

Luego de la unión del ligando, los dos DR4 y DR5 pueden desencadenar apoptosis independientemente, reclutando y activando el iniciador de apoptosis, caspasa-8, a través de la molécula de adaptador que contiene el dominio de muerte celular, denominada FADD/Mort1 [Kischkel et al., Immunity, 12:611-620 (2000); Sprick et al., Immunity, 12:599-609 (2000); Bodmer et al., Nature Cell Biol., 2:24:1-24:3 (2000)].

Se ha divulgado que Apo2L/TRAIL une también aquellos receptores designados DcR1, DcR2 y OPG, los cuales se cree que funcionan como

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inhibidores, más bien que como transductores de señalización (véase, por ejemplo, DCR1 (también denominado TRID, LIT o TRAIL-R3) [Pan et al., Science, 276:111-113 (1997); Sheridan et al., Science, 277:818-821 (1997); McFarlane et al., J. Biol. Chem., 272:25417-25420 (1997); Schneider et al., FEBS Letters, 416:329-334 (1997); Degli-Esposti et al., J. Exp. Med., 186:1165-1170 (1997); y Mongkolsapaya et al., J. Immunol., 160:3-6 (1998)] y DCR2 (llamado igualmente TRUNDD ó TRAIL-R4) [Marsters et al., Curr. Biol., 7:1003-1006 (1997); Pan et al., FEBS Letters, 424:41-45 (1998); Degli-Esposti et al., Immunity, 7:813-820 (1997)], y OPG [Simonet et al., supra]. En contraste con DR4 y DR5, los receptores DcR1 y DcR2 no señalizan apoptosis.

Algunos anticuerpos que se unen a los receptores DR4 y/o DR5 se han divulgado en la literatura. Por ejemplo, los anticuerpos anti-DR4 dirigidos al receptor DR4 y que poseen actividad agonística o apoptótica en ciertas células mamíferas se describen en, por ejemplo, WO 99/37684 publicada el 29 de julio de 1999, WO 00/73349 publicada el 12 de julio de 2000; WO 03/066661 publicada el 14 de agosto de 2003. Véase, además, por ejemplo, Griffith et al., J. Immunol., 162:2597-2605 (1999); Chuntharapai et al., J. Immunol., 166:4891-4898 (2001); WO 02/097033 publicada el 2 de diciembre de 2002; WO 03/042367 publicada el 22 de mayo de 2003; WO 03/038043 publicada el 8 de mayo de 2003; WO 03/037913 publicada el 8 de mayo de 2003. Igualmente, se han descrito ciertos anticuerpos anti-DR5, véase, por ejemplo, WO 98/51793 publicada el 8 de noviembre de 1998; Griffith et al., J. Immunol., 162:2597-2605 (1999); Ichikawa et al., Nature Med., 7:954-960 (2001); Hylander et al., "An Antibody to DR5 (TRAIL-Receptor 2) Suppresses the Growth of Patient Derived Gastrointestinal Tumors Grown in SCID mice", Abstract, 2d International Congress on Monoclonal Antibodies in Cancers, Aug. 29-Sept. 1, 2002, Banff, Alberta, Canada; WO 03/038043 publicada el 8 de mayo de 2003; WO 03/037913 publicada el 8 de mayo de 2003. Además, se han descrito ciertos anticuerpos con reactividad cruzada frente a los dos

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receptores, DR4 y DR5 (véase, por ejemplo, Patente de los Estados Unidos 6,252,050 expedida el 26 de junio de 2001).

El antígeno CD20 (también llamado antígeno de diferenciación restringida a linfocito B humano, Bp35) es una proteína transmembrana hidrófoba con un peso molecular de aproximadamente 35 kD localizada en linfocitos pre-B y linfocitos B maduros (Valentine et al. *J. Biol. Chem.* 264 (19):11282-11287 (1989); and Einfeld et al. *EMBO J.* 7(3):711-717 (1988)). El antígeno se expresa también en más del 90% de linfomas no hodgkinianos de linfocitos B (NHL) (Anderson et al. *Blood* 63 (6):1424-1433 (1984)), pero no se encuentra en células madre hematopoyéticas, linfocitos pre-B, células plasmáticas normales u otros tejidos normales (Tedder et al. *J. Immunol.* 135 (2):973-979 (1985)). CD20 regula un paso(s) previo en el proceso de activación para la iniciación y diferenciación del ciclo celular (Tedder et al., *supra*) y, posiblemente, funciona como un canal de iones de calcio (Tedder et al. *J. Cell. Biochem.* 14D:195 (1990)). Dada la expresión de CD20 en linfomas de linfocitos B, este antígeno puede servir como un candidato para el "direccionamiento" ("*targeting*") de tales linfomas.

El anticuerpo rituximab (RITUXAN®) es un anticuerpo monoclonal murino/humano modificado genéticamente, dirigido contra el antígeno CD20. Rituximab es el anticuerpo denominado "C2B8" en la Patente de los Estados Unidos No. 5,736,137 expedida el 7 de abril de 1998 (Anderson et al.). RITUXAN® es indicado para el tratamiento de pacientes con linfoma no hodgkiniano de linfocitos B, CD20 positivo, de bajo grado o folicular, con recaída o resistente al tratamiento. Estudios del mecanismo de acción *in vitro* han demostrado que RITUXAN® une el complemento humano y disuelve líneas de linfocitos B linfoides mediante citotoxicidad dependiente de complemento (CDC) (Reff et al. *Blood* 83 (2):435-445 (1994); Cragg and Marlin, *Blood*, 103: 2738-2743 (2004). Adicionalmente, posee una actividad significativa en análisis de citotoxicidad celular mediada por anticuerpo (ADCC). Más recientemente, se ha demostrado que el RITUXAN posee efectos anti-proliferativos en los análisis de

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incorporación de timidina tritiada y que induce apoptosis directamente, mientras que otros anticuerpos anti-CD19 y CD20 no (Maloney et al. Blood 88(10):637a (1996)). De igual manera, se ha observado experimentalmente la sinergia entre el RITUXAN® y ciertas quimioterapias y toxinas. En particular, el RITUXAN® sensibiliza líneas de células de linfocitos B humanos, resistentes a los medicamentos, a los efectos citotóxicos de la doxorubicina, CDDP, VP-16, la toxina de la difteria y la ricina (Demidem et al. Cancer Chemotherapy & Radiopharmaceuticals 12 (3):177-186 (1997)). Estudios preclínicos *in vivo* han demostrado que el RITUXAN® agota los linfocitos B de la sangre periférica, nódulos linfáticos, y médula ósea de monos macacos cynomolgus, presumiblemente a través de procesos mediados por célula y complemento (Reff et al. Blood 83 (2) :435-445 (1994)).

SUMARIO DE LA INVENCION

Métodos para usar ligando de receptor de muerte celular, tales como polipéptidos ligando Apo-2/TRAIL o anticuerpos de receptor de muerte celular, y anticuerpos CD20 se proveen en esta solicitud. Incorporaciones de la invención incluyen métodos de tratar cáncer, que comprenden exponer células cancerosas a una cantidad efectiva de Apo2L/TRAIL y anticuerpo CD20. Opcionalmente, las células cancerosas se exponen a una cantidad efectiva de anticuerpo de receptor de muerte celular, tal como un anticuerpo DR4 agonista o anticuerpo DR5 agonista, y anticuerpo CD20. Opcionalmente, la cantidad de Apo2L/TRAIL o anticuerpo de receptor de muerte celular y anticuerpos CD20 empleados en los métodos son efectivos para obtener sinergia en forma terapéutica, por ejemplo, su efecto anti-cáncer combinado es mayor que el efecto anti-cáncer conseguido cuando el Apo2L/TRAIL o anticuerpos se emplean individualmente como un agente terapéutico único. Los métodos pueden incluir uso *in vitro* o uso *in vivo*, donde el Apo2L/TRAIL o anticuerpo de receptor de muerte celular y el anticuerpo CD20 se administran a un mamífero (paciente). Opcionalmente,

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en los métodos, las células cancerosas tratadas con Apo2L/TRAIL o anticuerpo de receptor de muerte celular y anticuerpo CD20 son células de linfoma.

Otras incorporaciones de la invención incluyen métodos de tratar una enfermedad inmuno-relacionada, que comprende administrar a un mamífero una cantidad efectiva de Apo2L/TRAIL y anticuerpo CD20. Opcionalmente, una cantidad efectiva de anticuerpo de receptor de muerte celular, tal como un anticuerpo DR4 agonista o anticuerpo DR5 agonista, y anticuerpo CD20 se administra al mamífero. Opcionalmente, la cantidad de Apo2L/TRAIL o anticuerpo de receptor de muerte celular y anticuerpos CD20 empleados en los métodos son efectivos para lograr sinergia en forma terapéutica, por ejemplo, su efecto combinado en tratar la enfermedad inmuno-relacionada es mayor que el efecto alcanzado cuando el Apo2L/TRAIL o anticuerpos se emplean individualmente como un agente terapéutico único. Opcionalmente, en los métodos, la enfermedad inmuno-relacionada es artritis reumatoide o esclerosis múltiple.

Métodos de la invención incluyen métodos de tratar un trastorno en un mamífero, tal como una enfermedad inmuno-relacionada o cáncer, que comprenden los pasos de obtener una muestra de tejido o células del mamífero, examinar el tejido o células para expresión de CD20, DR4, y/o DR5, y, tras determinar que dicha muestra de tejido o células expresan dichos uno o más receptores, administrar una cantidad efectiva de Apo2L/TRAIL o anticuerpo de receptor de muerte celular y anticuerpo CD20 a dicho mamífero. Los pasos en los métodos para examinar la expresión de uno o más de tales receptores pueden conducirse en una variedad de formatos de análisis, incluidos análisis para detectar la expresión de ARNm y análisis de inmunohistoquímica.

Opcionalmente, los métodos de la invención comprenden, además de administrar una cantidad efectiva de Apo2L/TRAIL y/o anticuerpo de receptor de muerte celular y anticuerpo CD20, administrar agente(s) quimioterapéutico o terapia de radiación a dicho mamífero. Más

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incorporaciones de la invención se ilustran a manera de ejemplo en las siguientes reivindicaciones:

1. Un método de tratar células cancerosas, que comprende exponer células cancerosas de mamífero a una cantidad efectiva sinérgica de anticuerpo de receptor de muerte celular agonista y anticuerpo CD20.
2. El método de la reivindicación 1 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo monoclonal de receptor anti-DR5.
3. El método de la reivindicación 1 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo monoclonal de receptor anti-DR4.
4. El método de la reivindicación 1 en donde dichas células cancerosas se exponen a dicha cantidad efectiva sinérgica de anticuerpo de receptor de muerte celular agonista y anticuerpo CD20 *in vivo*.
5. El método de la reivindicación 2 ó 3 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo quimérico o anticuerpo humanizado.
6. El método de la reivindicación 2 o 3 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo humano.
7. El método de la reivindicación 1 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo que reacciona cruzadamente con más de un receptor de ligando Apo-2.

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8. El método de la reivindicación 1 en donde dichas células cancerosas son células de linfoma.

9. El método de la reivindicación 1 que comprende además exponer las células cancerosas a uno o más agentes inhibidores de crecimiento.

10. El método de la reivindicación 1 que comprende además exponer las células a radiación.

11. El método de la reivindicación 2 en donde dicho anticuerpo DR5 tiene una afinidad de unión de receptor de 10^8 M^{-1} a 10^{12} M^{-1} .

12. El método de la reivindicación 1 en donde dicho anticuerpo de receptor de muerte celular y anticuerpo CD20 se expresa en una célula hospedera recombinante seleccionada del grupo conformado por una célula CHO, célula de levadura y *E. coli*.

13. El método de la reivindicación 1 en donde dicho anticuerpo CD20 es un anticuerpo monoclonal.

14. El método de la reivindicación 13 en donde dicho anticuerpo CD20 es el anticuerpo Rituximab.

15. Un método de tratar una enfermedad inmuno-relacionada, que comprende administrar a un mamífero una cantidad efectiva sinérgica de anticuerpo de receptor de muerte celular agonista y anticuerpo CD20.

16. El método de la reivindicación 15 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo monoclonal de receptor anti-DR5.

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17. El método de la reivindicación 15 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo monoclonal de receptor anti-DR4.

18. El método de la reivindicación 16 ó 17 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo quimérico o un anticuerpo humanizado.

19. El método de la reivindicación 16 ó 17 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo humano.

20. El método de la reivindicación 15 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo que reacciona cruzadamente con más de un receptor de ligando Apo-2.

21. El método de la reivindicación 15 en donde dicha enfermedad inmuno-relacionada es artritis reumatoide o esclerosis múltiple.

22. El método de la reivindicación 15 en donde dicho anticuerpo DR5 tiene una afinidad de unión de receptor DR5 de 10^8 M^{-1} a 10^{12} M^{-1} .

23. El método de la reivindicación 15 en donde dicho anticuerpo de receptor de muerte celular y anticuerpo CD20 se expresa en una célula hospedera recombinante seleccionada del grupo conformado por una célula CHO, una célula de levadura y *E. coli*.

24. El método de la reivindicación 15 en donde dicho anticuerpo CD20 es un anticuerpo monoclonal.

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25. El método de la reivindicación 24 en donde dicho anticuerpo CD20 es el anticuerpo Rituximab.

26. El método de la reivindicación 1 ó 15 en donde dicho anticuerpo de receptor de muerte celular y anticuerpo CD20 se administran en forma secuencial.

27. El método de la reivindicación 1 ó 15 en donde dicho anticuerpo de receptor de muerte celular y anticuerpo CD20 se administran en forma concurrente.

BREVE DESCRIPCIÓN DE LOS DIBUJOS

Figura 1A muestra la secuencia de nucleótidos del ADNc de ligando Apo-2 humano (SEQ ID NO:2) y su secuencia derivada de aminoácidos (SEQ ID NO:1). La "N" en la posición de nucleótido 447 es usa para indicar que la base del nucleótido puede ser una "T" o "G".

Figuras 2A y 2B muestran la secuencia de nucleótidos de un ADNc (SEQ ID NO:4) para DR4 humano de longitud total y su secuencia derivada de aminoácidos (SEQ ID NO:3). Las respectivas secuencias de nucleótidos y aminoácidos para DR4 humano se divulgan también en Pan et al., Science, 276:111 (1997).

Figura 3A muestra la secuencia de 411 aminoácidos de DR5 humano (SEQ ID NO:5) como se publica en WO 98/51793 el 19 de noviembre de 1998. Una variante de empalme de transcripción de DR5 humano es conocida en el arte. Esta variante de empalme de DR5 codifica la secuencia de 440 aminoácidos de DR5 humano (SEQ ID NO:6) mostrada en Figuras 3B y 3C como publica en WO 98/35986 el 20 de agosto de 1998.

Figura 4 ilustra la expresión de receptores Apo2L/TRAIL en líneas de células de linfoma B.

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Figura 5 ilustra la expresión de CD20 en líneas de células de linfoma B.

Figura 6 muestra los efectos de Apo2L/TRAIL, RITUXAN®, o tratamiento de combinación sobre el crecimiento xenoinjertos de tumor de linfoma BJAB subcutáneos pre-establecidos en ratones con inmunodeficiencia combinada grave (SCID - *Severe Combined Immunodeficiency*).

Figura 7 muestra otros resultados sobre los efectos de Apo2L/TRAIL, RITUXAN®, o tratamiento de combinación de Apo2L/TRAIL y RITUXAN® sobre el crecimiento de xenoinjertos de tumor de linfoma BJAB subcutáneos pre-establecidos en ratones SCID.

Figura 8 muestra los efectos de Apo2L/TRAIL, RITUXAN®, o tratamiento de combinación de Apo2L/TRAIL y RITUXAN® sobre el procesamiento de caspasa en xenoinjertos de tumor de linfoma BJAB subcutáneos pre-establecidos, desarrollados en ratones SCID.

Figura 9 muestra los efectos del anticuerpo DR5 agonístico, RITUXAN®, o tratamiento de combinación sobre el crecimiento de sobre el crecimiento de xenoinjertos de tumor de linfoma BJAB subcutáneos pre-establecidos en ratones SCID.

Figure 10 muestra los efectos del anticuerpo DR5 agonístico, RITUXAN®, o tratamiento de combinación en el procesamiento de caspasa en xenoinjertos de tumor de linfoma BJAB subcutáneos pre-establecidos, desarrollados en ratones SCID.

Figura 11 ilustra la expresión de receptores de CD20 y Apo2L/TRAIL en líneas de células de linfoma no hodgkiniano (NHL - *Non-Hodgkin's lymphoma*).

Figura 12 muestra los efectos de Apo2L/TRAIL, Rituximab, o tratamiento de combinación sobre el crecimiento de xenoinjertos de tumor Ramos RA1 subcutáneos pre-establecidos en ratones SCID.

Figura 13 muestra los efectos de Apo2L/TRAIL, Rituximab, o tratamiento de combinación sobre el crecimiento de xenoinjertos de linfoma folicular DOHH-2 pre-establecidos en ratones SCID.

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Figura 14 ilustra los efectos y mecanismos de eliminación celular por Apo2L/TRAIL y Rituximab o tratamientos de combinación sobre células BJAB.

Figura 15 muestra los efectos de Apo2L/TRAIL, Rituximab, o tratamiento de combinación sobre el crecimiento de xenoinjertos de tumor Ramos T1 en ratones SCID.

Figure 16 muestra los efectos de Apo2L/TRAIL, Rituximab, o tratamiento de combinación sobre los xenoinjertos de tumor BJAB-Luc en ratones SCID.

DESCRIPCIÓN DETALLADA DE LAS INCOPORACIONES PREFERIDAS

A menos que se defina lo contrario, todos los términos del arte, notaciones y demás terminología científica, utilizados en esta solicitud, se proponen tener los significados, entendidos comúnmente por aquellas personas expertas en el arte, a los cuales pertenece esta invención. En algunos casos, términos con significados comúnmente conocidos se definen en esta solicitud para claridad y/o facilidad de referencia, y la inclusión de tales definiciones en esta solicitud no debe interpretarse necesariamente que representan una diferencia sustancial sobre lo que se entiende por lo general en el arte. Las técnicas y procedimientos descritos o referenciados en esta solicitud se entienden generalmente bien y se emplean comúnmente usando metodología convencional por las personas expertas en el arte, tal como, por ejemplo, las metodologías de clonación molecular ampliamente utilizadas, descritas en Sambrook et al., Molecular Cloning: A Laboratory Manual 2nd. edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. Según se considere apropiado, los procedimientos que involucran el uso de kits y reactivos disponibles en el comercio se llevan generalmente a cabo de acuerdo con protocolos definidos por el fabricante y/o parámetros, a menos que se anote lo contrario.

Antes de describir los presentes métodos, kits y usos de los mismos, hay que entender que esta invención no se limita a la metodología, protocolos, líneas de células, especies animales o géneros, constructos particulares y que, naturalmente, los reactivos descritos, como tales, pueden variar. Hay que entender asimismo que la terminología utilizada en esta solicitud tiene como finalidad describir incorporaciones particulares únicamente, y no pretende limitar el ámbito de la presente invención, la cual no se limitará únicamente por las reivindicaciones anexas.

Hay que anotar que, tal como se emplea en esta solicitud y en las reivindicaciones anexas, las formas del singular "un", "uno", y "el" incluyen referentes del plural, a menos que el contexto establezca claramente lo contrario.

Todas las publicaciones mencionadas en esta solicitud se incorporan aquí por referencia para divulgar y describir los métodos y/o materiales en conexión con los cuales se citan las publicaciones. Las publicaciones citadas aquí se citan para su divulgación antes de la fecha de presentación de la presente solicitud. Nada de lo aquí contenido tiene que interpretarse como una aceptación de que los inventores no están autorizados a poner una fecha anterior a las publicaciones en virtud de una fecha de prioridad anterior o fecha previa de la invención. Además, las fechas actuales de publicación pueden ser diferentes de las indicadas y requieren verificación independiente.

Definiciones

Los términos "ligando Apo-2", "Apo-2L", "Apo2L", "Apo2L/TRAIL", "ligando Apo-2/TRAIL" y "TRAIL" se usan indistintamente en esta solicitud para referirse a una secuencia de polipéptidos que incluye residuos de aminoácidos 114-281, inclusive, 95-281, inclusive, residuos 92-281, inclusive, residuos 91-281, inclusive, residuos 41-281, inclusive, residuos 39-281, inclusive, residuos 15-281, inclusive, o residuos 1-281, inclusive, de la secuencia de aminoácidos mostrada en Figura 1, así como fragmentos biológicamente activos,

variantes de eliminación, inserción, o sustitución de las secuencias anteriores. En una incorporación, la secuencia de polipéptidos comprende residuos 114-281 de Fig. 1. Opcionalmente, la secuencia de polipéptido comprende residuos 92-281 o residuos 91-281 o Figura 1. Los polipéptidos Apo-2L pueden codificarse por la secuencia de nucleótidos naturales mostrada en Figura 1. Opcionalmente, el codón que codifica el residuo Pro119 (Figura 1) puede ser "CCT" o "CCG". Opcionalmente, los fragmentos o variantes son biológicamente activos y tienen por lo menos alrededor de 80% de identidad de secuencia de aminoácidos, más preferiblemente por lo menos alrededor de 90% de identidad de secuencia y, aún más preferiblemente, por lo menos 95%, 96%, 97%, 98%, o 99% de identidad de secuencia con una cualquiera de las secuencias anteriores. La definición abarca variantes de sustitución del ligando Apo-2 en el cual por lo menos uno de sus aminoácidos naturales se sustituyen por otro aminoácido tal como un residuo de alanina. Variantes de sustitución opcionales incluyen una o más de las sustituciones de residuo. Variantes opcionales de sustitución pueden comprender una secuencia de aminoácidos que difiere de la secuencia de polipéptidos de ligando Apo-2 de secuencia natural de Figura 1 y tiene una o más de las siguientes sustituciones de aminoácidos en la posición (posiciones) de residuos en Figura 1: S96C; S101C; S111C; R170C; K179C. La definición abarca asimismo un ligando Apo-2 de secuencia natural, aislado de una fuente de ligando Apo-2, o preparado por métodos recombinantes o sintéticos. El ligando Apo-2 de la invención incluye los polipéptidos designados como ligando Apo-2 o TRAIL divulgados en WO97/01633 publicada el 16 de enero de 1997, WO97/25428 publicada el 17 de julio de 1997, WO99/36535 publicada el 22 de julio de 1999, WO 01/00832 publicada el 4 de enero de 2001, WO02/09755 publicada el 7 de febrero de 2002, y WO 00/75191 publicada el 14 de diciembre de 2000. Los términos se utilizan para referirse generalmente a formas del ligando Apo-2, que incluyen formas monoméricas, diméricas, triméricas, hexaméricas o formas oligoméricas altas del polipéptido. Toda la numeración de residuos de aminoácidos



mencionados en la secuencia Apo-2L usa la numeración de acuerdo con la Figura 1, a menos que se indique específicamente lo contrario. Por ejemplo, "D203" o "Asp203" se refiere al residuo de ácido aspártico en la posición 203 en la secuencia provista en Figura 1.

El término "variante selectiva de ligando Apo-2", tal como se emplea en esta solicitud, se refiere a un polipéptido de ligando Apo-2 que incluye una o más mutaciones de aminoácidos en una secuencia de ligando Apo-2 natural y posee una afinidad de unión selectiva para cualquiera de los dos receptores, el DR4 ó DR5. En una incorporación, la variante del ligando Apo-2 posee una afinidad de unión selectiva para el receptor DR4 e incluye una o más sustituciones de aminoácidos en una cualquiera de las posiciones 189, 191, 193, 199, 201 ó 209 de una secuencia de ligando Apo-2 natural. En otra incorporación, la variante de ligando Apo-2 tiene una afinidad de unión selectiva para el receptor DR5 e incluye una o más sustituciones de aminoácidos en una cualquiera de las posiciones 189, 191, 193, 264, 266, 267 ó 269 de una secuencia de ligando Apo-2 natural. Variantes preferidas selectivas del ligando Apo-2 incluyen una o más mutaciones de aminoácidos y exhiben una afinidad de unión al receptor DR4, que es mayor o igual ($\geq$) que la afinidad de unión del ligando Apo-2 de secuencia natural al receptor DR4 y, aún más preferiblemente, las variantes del ligando Apo-2 exhiben menos afinidad de unión ($<$) al receptor DR5 que la afinidad de unión exhibida por el ligando Apo-2 de secuencia natural a DR5. Cuando la afinidad de unión de tal variante de ligando Apo-2 al receptor DR4 es aproximadamente igual (sin cambio) o mayor que (incrementada), comparada con el ligando Apo-2 de secuencia natural, y la afinidad de unión de la variante del ligando Apo-2 al receptor DR5 es menor que, o casi eliminada, comparada con el ligando Apo-2 de secuencia natural, la afinidad de unión de la variante del ligando Apo-2, para los propósitos indicados aquí, se considera "selectiva" para el receptor DR4. Variantes preferidas del ligando Apo-2, selectivas para DR4 de la invención, tendrán como mínimo 10 veces menos afinidad de unión al receptor DR5 (comparada con el ligando Apo-2 de secuencia natural) y, aún

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más preferiblemente, tendrá como mínimo 100 veces menos afinidad de unión al receptor DR5 (comparada con el ligando Apo-2 de secuencia natural). La respectiva afinidad de unión de la variante del ligando Apo-2 puede determinarse y compararse con las propiedades de unión del Apo-2L natural (tal como la forma 114-281) mediante análisis ELISA, RIA, y/o BIAcore, conocidos en el arte. Variantes preferidas del ligando Apo-2 selectivas de DR4 de la invención inducirán apoptosis en por lo menos un tipo de célula mamífera (preferiblemente una célula cancerosa), y tal actividad apoptótica puede determinarse por métodos conocidos, tales como el análisis alamar blue o cloruro de metil rosanilina ("*crystal violet*"). Las variantes de ligando Apo-2 selectivas de DR4 pueden tener o no tener afinidades de unión alteradas a cualquiera de los receptores señuelos para Apo-2L, designándose aquellos receptores señuelos en el arte como DcR1, DcR2 y OPG.

Otras variantes selectivas preferidas del ligando Apo-2 incluyen una o más mutaciones de aminoácidos y exhiben afinidad de unión al receptor DR5, que es igual o mayor ($\geq$) que la afinidad de unión del ligando Apo-2 de secuencia natural al receptor DR5 y, aún más preferiblemente, tales variantes de ligando Apo-2 exhiben menos afinidad de unión ($<$) al receptor DR4 que la afinidad de unión exhibida por el ligando Apo-2 de secuencia natural a DR4. Cuando la afinidad de unión de tal variante del ligando Apo-2 al receptor DR5 es aproximadamente igual (sin cambio) o mayor que (incrementada), comparada con el ligando Apo-2 de secuencia natural, y la afinidad de unión de la variante del ligando Apo-2 al receptor DR4 es menor que, o casi se elimina, comparada con el ligando Apo-2 de secuencia natural, la afinidad de unión de la variante del ligando Apo-2, para lo propósitos contemplados en esta solicitud, se considera "selectiva" para el receptor DR5. Variantes preferidas del ligando Apo-2 selectivas de DR5 de la invención tendrán como mínimo 10 veces menos afinidad de unión al receptor DR4 (comparada con el ligando Apo-2 de secuencia natural) y, aún más preferiblemente, tendrá como mínimo 100 menos afinidad de unión al receptor DR4 (comparada con el ligando Apo-2 de secuencia natural). La

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afinida de unión respectiva de la variante del ligando Apo-2 puede determinarse y compararse con las propiedades de unión del ligando Apo-2 natural Apo2L (tal como la forma 114-281) mediante análisis ELISA, RIA, y/o BIAcore, conocidos en el arte. Variantes preferidas del ligando Apo-2 selectivas de DR5 de la invención inducirán apoptosis en por lo menos un tipo de célula mamífera (preferiblemente una célula cancerosa), y tal actividad apoptótica puede determinarse por métodos conocidos, tales como el análisis alamar blue o cloruro de metil rosanilina ("*crystal violet*"). Las variantes de ligando Apo-2 selectivas de DR5 pueden tener o no tener afinidades de unión alteradas a cualquiera de los receptores señuelos para Apo-2L, designándose aquellos receptores señuelos en el arte como DcR1, DcR2 y OPG.

La identificación de aminoácidos puede utilizar el alfabeto de letra única o el alfabeto de tres letras de aminoácidos, es decir,

Asp	D	Ácido aspártico	Ile	I	Isoleucina
Thr	T	Treonina	Leu	L	Leucina
Ser	S	Serina	Tyr	Y	Tirosina
Glu	E	Ácido glutámico	Phe	F	Fenilalanina
Pro	P	Prolina	His	H	Histidina
Gly	G	Glicina	Lys	K	Lisina
Ala	A	Alanina	Arg	R	Arginina
Cys	C	Cisteína	Trp	W	Triptófano
Val	V	Valina	Gln	Q	Glutamina
Met	M	Metionina	Asn	N	Asparagina

El término dominio extracelular "Apo2L/TRAIL" o "ECD Apo2L/TRAIL" se refiere a una forma de Apo2L/TRAIL, que está sustancialmente libre de dominios transmembrana y citoplásmicos. Comúnmente, el ECD tendrá menos de 1% de tales dominios transmembrana y citoplásmicos y, preferiblemente, tendrán menos de 0.5% de tales dominios. Se entenderá que cualquier dominio(s) transmembrana identificado para los polipéptidos de la presente invención se identifican

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según los criterios empleados rutinariamente en el arte para identificar ese tipo de dominio hidrófobo. Las fronteras exactas de un dominio transmembrana pueden variar, pero muy probablemente, en no más de alrededor de 5 aminoácidos en cualquiera de los dos extremos del dominio, como identificado inicialmente. En incorporaciones preferidas, el ECD consistirá de una secuencia de dominio extracelular, soluble, del polipéptido, que está libre de los dominios transmembrana y citoplásmicos o intracelulares (y no está unido a la membrana). Secuencias de dominios extracelulares particulares de Apo-2L/TRAIL se describen en la Publicación PCT No. WO97/01633 y WO97/25428.

El término "monómero Apo2L/TRAIL" o "monómero Apo2L" se refiere a una cadena covalente de una secuencia de dominio extracelular de Apo2L.

El término "dímero Apo2L/TRAIL" o "dímero Apo2L" se refiere a dos monómeros Apo-2L unidos en un enlace covalente mediante un enlace disulfuro. Los términos utilizados en esta solicitud incluyen dímeros Apo2L independientes y dímeros Apo2L que son formas triméricas de Apo2L (es decir, asociadas con otro tercer monómero Apo2L).

El término "trímero Apo2L/TRAIL" o "trímero Apo2L" se refiere a tres monómeros Apo2L que no se asocian covalentemente.

El término "agregado Apo2L/TRAIL" se utiliza para referirse a formas oligoméricas más altas, auto-asociadas, de Apo2L/TRAIL, tales como trímeros Apo2L/TRAIL, los cuales forman, por ejemplo, formas hexaméricas y nanoméricas de Apo2L/TRAIL. La determinación de la presencia y cantidad de monómero Apo2L/TRAIL, dímero, o trímero (u otros agregado) puede hacerse usando métodos y análisis conocidos en el arte (y usando materiales obtenibles en el comercio), tales como HPLC de exclusión de tamaño natural ("SEC"), exclusión de tamaño desnaturizante usando dodecilsulfato de sodio ("SDS-SEC"), HPLC de fase inversa y electroforesis capilar.

"Receptor de ligando Apo-2" incluye los receptores mencionados en el arte como "DR4" y "DR5", cuyas secuencias de polinucleótidos y



polipéptidos se muestran en Figuras 2 y 3 respectivamente. Pan et al. han descrito el miembro de la familia de receptor TNF designado como "DR4" (Pan et al., Science, 276:111- 113 (1997); véase también WO98/32856 publicada el 30 de julio de 1998; WO 99/37684 publicada el 29 de julio de 1999; WO 00/73349 publicada el 7 de diciembre de 2000; US 6,433,147 expedida el 13 de agosto de 2002; US 6,461,823 expedida el 8 de octubre de 2002, y US 6,342,383 expedida el 29 de enero de 2002). Sheridan et al., Science, 277:818- 821 (1997) y Pan et al., Science, 277:815-818 (1997) describieron otro receptor para Apo2L/TRAIL (véase asimismo, WO98/51793 publicada el 19 de noviembre de 1998; WO98/41629 publicada el 24 de septiembre de 1998). Este receptor se designa como DR5 (el receptor se ha designado alternativamente como Apo-2; TRAIL-R, TR6, Tango-63, hAPO8, TRICK2 o KILLER; Screatton et al., Curr. Biol., 7:693-696 (1997); Walczak et al., EMBO J., 16:5386-5387 (1997); Wu et al., Nature Genetics, 17:141-143 (1997); WO98/35986 publicada el 20 de agosto de 1998; EP870,827 publicada el 14 de octubre de 1998; WO98/46643 publicada el 22 de octubre de 1998; WO99/02653 publicada el 21 de enero de 1999; WO99/09165 publicada el 25 de febrero de 1999; WO99/11791 publicada el 11 de marzo de 1999; US 2002/0072091 publicada el 13 de agosto de 2002; US 2002/0098550 publicada el 7 2001; US 6,313,269 expedida el 6 de diciembre de 2001; US 2001/0010924 publicada el 2 de agosto de 2001; US 2003/01255540 publicada el 3 de julio de 2003; US 2002/0160446 publicada el 31 de octubre de 2002, US 2002/0048785 publicada el 25 de abril de 2002; US 6,569,642 expedida el 27 de mayo de 2003, US 6,072,047 expedida el 6 de junio de 2000, US 6,642,358 expedida el 4 de noviembre de 2003). Como se describe anteriormente, otros receptores para Apo-2L incluyen DcR1, DcR2, y OPG (véase Sheridan et al., supra; Marsters et al., supra, y Simonet et al., supra). El término "receptor Apo-2L", cuando se utiliza en esta solicitud, abarca receptor de secuencia natural y variantes de receptor. Estos términos comprenden el receptor Apo-2L expresado en una variedad de mamíferos, incluidos humanos. El receptor Apo-2L puede expresarse endógenamente,

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ya que ocurre naturalmente en una variedad de linajes de tejido humano, o puede expresarse por métodos recombinantes o sintéticos. Un "receptor Apo-2L de secuencia natural" comprende un polipéptido que posee la misma secuencia de aminoácidos como un receptor Apo-2L, derivado de la naturaleza. Por consiguiente, un receptor Apo-2L de secuencia natural puede tener la secuencia de aminoácidos del receptor Apo-2L de ocurrencia natural de cualquier mamífero. Tal receptor Apo-2L de secuencia natural puede aislarse de la naturaleza o puede producirse por medios recombinantes o sintéticos. El término "receptor Apo-2L de secuencia natural" abarca específicamente formas truncadas o secretadas, de ocurrencia natural, del receptor (*por ejemplo*, una forma soluble que contiene, *por ejemplo*, una secuencia de dominio extracelular), formas de variantes de ocurrencia natural (*por ejemplo*, formas alternativamente empalmadas) y variantes alélicas de ocurrencia natural. Variantes de receptor pueden incluir fragmentos o mutantes de eliminación del receptor Apo-2L de secuencia natural. Figura 3A muestra la secuencia de 411 aminoácidos de DR5 humano, publicada en WO 98/51793 el 19 de noviembre de 1998. Una variante de empalme de transcripción de DR5 humano es conocida en el arte. Esta variante de empalme DR5 codifica la secuencia de 440 aminoácidos de DR5 humano, mostrada en Figuras 3B y 3C como se publica en WO 98/35986 el 20 de agosto de 1998.

"Anticuerpo de receptor de muerte celular" se usa en esta solicitud para referirse generalmente a un anticuerpo o anticuerpos dirigidos a un receptor en la superfamilia de receptores de factor de necrosis tumoral y que contienen un dominio de muerte celular capaz de señalar apoptosis, y tales anticuerpos incluyen el anticuerpo DR5 y el anticuerpo DR4.

"Anticuerpo de receptor DR5", "anticuerpo DR5", o "anticuerpo anti-DR5" se usan en un sentido amplio para referirse a anticuerpos que unen por lo menos una forma de un receptor DR5 o su dominio extracelular. Opcionalmente el anticuerpo DR5 se fusiona o enlaza a una secuencia heteróloga o molécula. Preferiblemente la secuencia heteróloga permite o ayuda al anticuerpo a formar complejos oligoméricos o de orden más

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elavado. Opcionalmente, el anticuerpo DR5 se un al receptor DR5 pero no se une o reacciona cruzadamente con cualquier receptor Apo-2L adicional (por ejemplo, DR4, DcR1, o DcR2). Opcionalmente, el anticuerpo es un agonista de actividad señalizadora de DR5.

Opcionalmente, el anticuerpo DR5 de la invención se une a un receptor DR5 en un rango de concentración de alrededor de 0.1 nM a alrededor de 20 mM, medido en un análisis de unión BIAcore. Opcionalmente, los anticuerpos DR5 de la invención exhiben un valor IC₅₀ de alrededor de 0.6 nM a alrededor de 18 mM, medido en un análisis de unión BIAcore.

"Anticuerpo de receptor DR4", "anticuerpo DR4", o "anticuerpo anti-DR4" se usan en un sentido amplio para referirse a anticuerpos que unen por lo menos una forma de un receptor DR4 o su dominio extracelular. Opcionalmente el anticuerpo DR4 se fusiona o enlaza a una secuencia heteróloga o molécula. Preferiblemente la secuencia heteróloga permite o ayuda al anticuerpo a formar complejos oligoméricos o de orden más elavado. Opcionalmente, el anticuerpo DR4 se un al receptor DR4 pero no se une o reacciona cruzadamente con cualquier receptor Apo-2L adicional (por ejemplo, DR5, DcR1, o DcR2). Opcionalmente, el anticuerpo es un agonista de actividad señalizadora de DR4.

Opcionalmente, el anticuerpo DR4 de la invención se une a un receptor DR4 en un rango de concentración de alrededor de 0.1 nM a alrededor de 20 mM, medido en un análisis de unión BIAcore. Opcionalmente, los anticuerpos DR4 de la invención exhiben un valor IC₅₀ de alrededor de 0.6 nM a alrededor de 18 mM, medido en un análisis de unión BIAcore.

El término "agonista" se utiliza en el sentido más amplio, e incluye cualquier molécula, que mejore, estimule o active parcial o completamente una o más actividades biológicas de Apo2L/TRAIL, DR4 o DR5, *in vitro*, *in situ*, o *in vivo*. Ejemplos de tales actividades biológicas de unión de Apo2L/TRAIL a DR4 o DR5, incluyen apoptosis, así como aquellas que se describen en la literatura. Un agonista puede funcionar en una manera

directa o indirecta. Por ejemplo, el agonista puede funcionar para mejorar, estimular o activar parcial o completamente una o más actividades biológicas de DR4 o DR5, *in vitro*, *in situ*, o *in vivo* como resultado de su unión directa a DR4 o DR5, que produce la activación del receptor o la transducción de señales. El agonista puede funcionar asimismo indirectamente para mejorar, estimular o activar parcial o completamente una o más actividades biológicas de DR4 o DR5, *in vitro*, *in situ*, o *in vivo* como resultado de, por ejemplo, estimular otra molécula efectora, que causa entonces activación de DR4 o DR5 o transducción de señales. Se contempla que un agonista puede actuar como una molécula potenciadora, que funciona indirectamente para mejorar o incrementar la activación o actividad de DR4 o DR5. Por ejemplo, el agonista puede mejorar la actividad de Apo-2L endógeno en un mamífero. Esto podría realizarse, por ejemplo, pre-acomplejando DR4 o DR5 o estabilizando complejos del ligando respectivo con el receptor DR4 o DR5 (tal como estabilizar el complejo natural formado entre Apo-2L y DR4 o DR5).

El término "receptor DR4" y "DR4", tal como se emplea en esta solicitud, se refiere a formas de dominio extracelular, soluble y de cadena total, del receptor descrito en Pan et al., Science, 276:111-113 (1997); WO98/32856 publicada el 30 de julio de 1998; Patente de los Estados Unidos 6,342,363 expedida el 29 de enero de 2002; y WO99/37684 publicada el 29 de julio de 1999. La secuencia de aminoácidos de longitud total del receptor DR4 se provee aquí en Fig. 2.

El término "DR5" y "receptor DR5", tal como se emplea en esta solicitud, se refiere a formas de dominio extracelular, soluble y de cadena total, del receptor descrito en Sheridan et al., Science, 277:818-821 (1997); Pan et al., Science, 277:815-818 (1997), Patente de los Estados Unidos 6,072,047 expedida el 6 de junio de 2000; Patente de los Estados Unidos 6,342,369, WO98/51793 publicada el 19 de noviembre de 1998; WO98/41629 publicada el 24 de septiembre de 1998, Screatton et al., Curr. Biol., 7:693-696 (1997); Walczak et al., EMBO J., 16:5386-5387 (1997); Wu et al., Nature Genetics, 17:141-143 (1997); WO98/35986 publicada el

20 de agosto de 1998, EP870,827 publicada el 14 de octubre de 1998; WO98/46643 publicada el 22 de octubre de 1998; WO99/02653 publicada el 21 de enero de 1999; WO99/09165 publicada el 25 de febrero de 1999; WO99/11791 publicada el 11 de marzo de 1999. El receptor DR5 se ha designado también el arte como Apo-2; TRAIL-R, TR6, Tango-63, hAPO8, TRICK2 o KILLER. El término receptor DR5, empelado en esta solicitud, incluye el polipéptido de 411 aminoácidos de longitud total provisto en Fig. 3A y el polipéptido de 440 aminoácidos de longitud total provisto en Figs. 3B-C.

El término "poliol", cuando se utiliza en esta solicitud, se refiere ampliamente a compuestos de alcohol polihídrico. Polioles pueden ser cualquier polímero óxido de poli(alquilen) soluble en agua, por ejemplo, y pueden tener una cadena lineal o ramificada. Polioles preferidos incluyen aquellos sustituidos en una o más posiciones hidroxilo con un grupo químico, tal como un grupo alquilo que tiene entre uno y cuatro carbonos. Típicamente, el poliol es un poli(alquilen glicol), preferiblemente poli(etilen glicol) (PEG). No obstante, aquellas personas expertas en el arte reconocen que pueden emplearse otros polioles, tales como, por ejemplo, poli(propilen glicol) y copolímeros polietilen-polipropilen glicol, usando las técnicas de conjugación descritas en esta solicitud para PEG. Los polioles de la invención incluyen aquellos bien conocidos en el arte y aquellos de disposición pública, tales como de formas disponibles en el comercio.

El término "conjugado" se emplea aquí, de acuerdo con su definición más amplia, para significar unido o enlazado conjuntamente. Moléculas son "conjugadas" cuando actúan u operan como si estuvieran unidas.

El término "dominio extracelular" o "ECD" se refiere a una forma de ligando o receptor, que está esencialmente libre de dominios transmembrana y citoplásmicos. Comúnmente, el ECD soluble tendrá menos de 1% de tales dominios transmembrana y citoplásmicos y, preferiblemente, tendrá menos de 0.5% de tales dominios.

El término "ión de metal divalente" se refiere a un ión de metal que tiene dos cargas positivas. Ejemplos de iones de metal divalentes para uso

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en la presente invención incluyen, pero no se limitan a zinc, cobalto, níquel, cadmio, magnesio, y manganeso. Formas particulares de tales metales, que pueden emplearse, incluyen formas de sal (por ejemplo, formas de sal farmacéuticamente aceptable), tales como formas cloruro, acetato, carbonato, citrato y sulfato de los iones ya mencionados de metal divalentes. Iones de metal divalentes, como se describe en esta solicitud, se emplean preferiblemente en concentraciones o cantidades (por ejemplo, cantidades efectivas) que son suficientes, por ejemplo, para (1) mejorar la estabilidad de almacenamiento de trímeros Apo-2L durante un período deseado de tiempo, (2) aumentar la producción o rendimiento de trímeros Apo-2L en un método de purificación o cultivo celular recombinante, (3) potenciar la solubilidad (o reducir la agregación) de trímeros Apo-2L, o (4) reforzar la formación de trímeros Apo-2L.

"Aislado," cuando se usa para describir las diferentes proteínas divulgadas aquí, significa una proteína que se ha identificado y separado y/o recuperado de un componente de su ambiente natural. Componentes contaminantes de su entorno natural son materiales que, típicamente, interferirían los usos diagnósticos o terapéuticos de la proteína, y pueden incluir enzimas, hormonas, y otros solutos proteináceos o no proteináceos. En incorporaciones preferidas, la proteína se purificará (1) a un grado suficiente para obtener por lo menos 15 residuos de la secuencia de aminoácidos N-terminal o interna mediante el uso de un secuenciador de vaso giratorio, o (2) a homogeneidad por SDS-PAGE bajo condiciones reductoras o no reductoras usando azul Coomassie o, preferiblemente, tinción de plata. Una proteína aislada incluye proteína *in situ* en células recombinantes, puesto que por lo menos un componente del ambiente natural de la proteína no estará presente. Ordinariamente, sin embargo, la proteína aislada se preparará en por lo menos un paso de purificación.

Una molécula de ácido nucleico "aislada" es una molécula de ácido nucleico que se identifica y separa de, por lo menos, una molécula de ácido nucleico contaminante, con la cual se asocia comúnmente en la fuente natural del ácido nucleico. Una molécula de ácido nucleico de ligando

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Apo-2, aislada, es distinta de la forma o escenario en el cual se encuentra en la naturaleza. Por consiguiente, moléculas de ácido nucleico del ligando Apo-2, aisladas, se distinguen de la molécula de ácido nucleico del ligando Apo-2 tal como existe en células naturales. Sin embargo, una molécula de ácido nucleico del ligando Apo-2 incluye moléculas de ácido nucleico del ligando Apo-2, contenidas en células que ordinariamente expresan el ligando Apo-2 donde, por ejemplo, la molécula de ácido nucleico está en una localización cromosómica diferente de la de las células naturales.

"Identidad porcentual (%) de secuencia de aminoácidos" con respecto a secuencias identificadas en esta solicitud se define como el porcentaje de residuos de aminoácidos en una secuencia candidata, que son idénticos con los residuos de aminoácidos en la secuencia de ligando Apo-2L, luego de alinear las secuencias e introducir vacíos ("*gaps*"), si es necesario, para lograr la máxima identidad porcentual de secuencia, y sin considerar sustituciones conservadoras de ninguna clase como parte de la identidad de secuencia. La alineación para efectos de determinar la identidad porcentual de secuencia de aminoácidos puede lograrse en varias formas que se encuentran al alcance de la habilidad en el arte... (*sic*) pueden determinar parámetros apropiados para medir la alineación, incluidos todos los algoritmos necesarios para lograr la alineación máxima sobre la longitud completa de las secuencias que se están comparando. Para los propósitos de esta solicitud pueden obtenerse valores de identidad de secuencia de aminoácidos usando el programa de computador de comparación de secuencia ALIGN-2, el cual se creó por Genentech, Inc., y cuyo código fuente se ha depositado con documentación de usuario ante la Oficina de Derechos de Autor, Washington, D.C, 20559, registrado bajo el Registro de Derechos de Autor de los Estados Unidos No. TXU510087. El programa ALIGN-2 se encuentra a disposición pública a través de Genentech, Inc., South San Francisco, California. Todos los parámetros de comparación de secuencias se fijan por el programa ALIGN-2 y no varían.

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El término "secuencias de control" se refiere a secuencias de ADN necesarias para la expresión de una secuencia codificante, operacionalmente enlazada, en un organismo hospedero particular. Las secuencias de control que son apropiadas para procariotas, por ejemplo, incluyen un promotor, opcionalmente una secuencia operadora, y a un sitio de unión a ribosoma. Las células eucariotas son conocidas por utilizar promotores, señales de poliadenilación y potenciadores.

El ácido nucleico está "operacionalmente enlazado" cuando se pone en una relación funcional con otra secuencia de ácidos nucleicos. Por ejemplo, ADN para una pre-secuencia o líder secretor está operacionalmente enlazado a ADN para un polipéptido, si se expresa como una pre-proteína que participa en la secreción de un polipéptido; un promotor o potenciador está operacionalmente enlazado a una secuencia codificante si afecta la transcripción de la secuencia; o un sitio de unión a ribosoma está operacionalmente enlazado a una secuencia codificante si se posiciona de manera que facilite la traducción. Generalmente, "operacionalmente enlazado" significa que las secuencias de ADN que se están enlazando son contiguas, y, en el caso de un líder secretor, contiguas en la fase de lectura. No obstante, los potenciadores no necesitan ser contiguos. El enlazamiento se lleva a cabo mediante ligamiento en sitios de restricción convenientes. Si tales sitios no existen, los adaptadores o conectores de oligonucleótidos sintéticos se usan de acuerdo con la práctica convencional.

Un "linfocito B" es un linfocito que madura en la médula ósea, e incluye un linfocito B virgen, un linfocito B de memoria, o linfocitos B efectoros (células plasmáticas). El linfocito B en esta solicitud puede ser un linfocito B normal o no maligna.

El antígeno "CD20" es una fosfoproteína no glucosilado de ~35 kDa, hallado en la superficie de más del 90% de los linfocitos B de sangre periférica u órganos linfoides. CD20 está presente tanto en linfocitos B normales como en linfocitos B malignos, pero no se expresa en las células madre. Otros nombres para CD20 en la literatura incluyen "antígeno

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restringido de linfocito B" y "Bp35". El antígeno CD20 se describe en Clark et al. *PNAS (USA)* 82:1766 (1985), por ejemplo.

Ejemplos de anticuerpos que unen el antígeno CD20 incluyen: "C2B8" que actualmente se denomina "Rituximab" ("RITUXAN®") (Patente de los Estados Unidos No. 5,736,137); el anticuerpo murino 2B8 marcado con itrio-[90], designado "Y2B8" o "Ibritumotnab Tiuxetan" ZEVALIN® obtenible comercialmente de Idex Pharmaceuticals, Inc. (Patente de los Estados Unidos No. 5,736,137; 2B8 depositado con la ATCC bajo el No. de Acceso HB11388 el 22 de junio de 1993); IgG2a "B1" murino, también llamado "Tositumomab," opcionalmente marcado con ^{131}I para generar el anticuerpo " ^{131}I -B1" (tositumomab yodo ^{131}I , BEXXAR™) disponible en el comercio de Corixa (véase también, Patente de los Estados Unidos No. 5,595,721); anticuerpo monoclonal murino "1F5" (*Press et al. Blood* 69(2):584-591 (1987)) y sus variantes, incluidos 1F5 "framework patched" o humanizado (WO 2003/002607, Leung, ATCC Depósito HB-96450); anticuerpo 2H7 murino y 2H7 quimérico (Patente de los Estados Unidos No. 5,677,180); 2H7 humanizado; HUMAX-CD20™ totalmente humano, anticuerpo de alta afinidad dirigido en la molécula CD20 en la membrana celular de linfocitos B (Genmab, Denmark; véase, por ejemplo, Glennie and van de Winkel, *Drug Discovery Today* 8: 503-510 (2003) y Cragg et al., *Blood* 101: 1045-1052 (2003)); los anticuerpos monoclonales humanos divulgados en WO04/035607 (Teeling et al.); anticuerpos AME-133™ (Applied Molecular Evolution); anticuerpo A20 o sus variantes tales como el anticuerpo A20 quimérico o humanizado (cA20, hA20, respectivamente) (US 2003/0219433, Immunomedics); y anticuerpos monoclonales L27, G28-2, 93-1B3, B-C1 o NU-B2 disponibles de the International Leukocyte Typing Workshop (Valentine et al., In: *Leukocyte Typing III* (McMichael, Ed., p. 440, Oxford University Press (1987)). Los anticuerpos CD20 preferidos en esta solicitud son anticuerpos CD20 quiméricos, humanizados, o humanos, más preferiblemente rituximab, 2H7 humanizado, anticuerpo A20 quimérico o humanizado (Immunomedics), y HUMAX-CD20™ anticuerpo CD20 humano (Genmab).

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Los términos "rituximab" o "RITUXAN®" en esta solicitud se refieren a un anticuerpo monoclonal murino/humano quimérico, genéticamente modificado, dirigido contra el antígeno CD20 y designado "C2B8" en la Patente de los Estados Unidos No. 5,736,137, incluidos sus fragmentos que conservan la capacidad de unir CD20.

Puramente para los propósitos de esta solicitud y a menos que se indique lo contrario, "2H7 humanizado" se refiere a un anticuerpo CD20 humanizado, o su fragmento de unión a antígeno, en donde el anticuerpo es efectivo para agotar linfocitos B de primate *in vivo*, comprendiendo el anticuerpo en su región variable de cadena H (V_H) por lo menos una secuencia H3 CDR de un anticuerpo CD20 anti-humano y sustancialmente los residuos del marco (FR) de consenso humano del subgrupo III de cadena pesada humana (V_{HIII}).

Un 2H7 humanizado preferido es un anticuerpo intacto o fragmento de anticuerpo que comprende la secuencia de cadena ligera:

DIQMTQSPSSLSASVGRVTITCRASSSVSYMEHWYQQKPGKAPKPLIYAPENLASGVPSRFSGSGSGTDFTLTIS
SLQPEDFATYYCOOWSFNPPTFGQGTKVEIKR (SEQ ID NO:7);

y la secuencia de cadena pesada variable:

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMEHWVRQAPGKGLNWGAITYPGNGDTSYNQKFKGRFTISVDKS
KNTLYLQMNSLRAEDTAVYYCARVVYYMSYNYFDVWGQGTLVTVSS (SEQ ID NO:8).

Donde el anticuerpo 2H7 humanizado es un anticuerpo intacto, preferiblemente comprende la secuencia de aminoácidos de cadena ligera:

DIQMTQSPSSLSASVGRVTITCRASSSVSYMEHWYQQKPGKAPKPLIYAPENLASGVPSRFSGSGSGTDFTLTIS
SLQPEDFATYYCOOWSFNPPTFGQGTKVEIKRTVAAPSVFIFPPPSDEQLKSGTASVVCILNNFYPREAKVQWKVD
NALQSGNSQESVTEQDSKIDSTYLSSTLTLSKADYEKHKVYACEVTEQGLSSPVTKSFNRGEC (SEQ ID
NO:9);

y la secuencia de aminoácidos de cadena pesada:

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMEHWVRQAPGKGLNWGAITYPGNGDTSYNQKFKGRFTISVDKS
KNTLYLQMNSLRAEDTAVYYCARVVYYMSYNYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVETFPFAVLQSSGLYSLSSVTVTPSSSLGTQTYICHVNEHKPSNTKVDKKVEPKSC
DKTHTCPPCPAPKLLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVENAKTKPREEQ
YMSYRVSIVLTIVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRREMTKNQVSLTCLVK
GFYPGSDIAVENESFNGQPENNYKTPFPVLDSDGSFFLYSKLTVDKSRNQQGNVFSCSVMEALEHNYTQKSLSLSP
GK (SEQ ID NO:10)

o la secuencia de aminoácidos de cadena pesada:

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EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMEHWVRQAPGKLEWVGAIYPGSGDTSYNQKFKGRFTISVDKS
 KNTLYLQMNSLRAEDTAVYYCARVVYYSMBYWYFDVWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
 VKDYFPEPVTVSWNSGALTSGVETFPFAVLQSSGLYSLSSVTVPSSSLGTQTYICNVMHKPSNTKVDKKVEPKSC
 DKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
 YNATYRVVEVLTVLHQDWLNGKEYKCKVSNKALPAPIAAATISKARGQPREPQVYTLPPSRREMTKNQVSLTCLVK
 GFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSGFFLYSKLTVDKSRWQQGNVFSQSVMHAELENEYTQKSLSLSP
 GK (SEQ ID NO:11).

"Citotoxicidad mediada por célula dependiente de anticuerpo" o "ADCC" se refiere a una reacción mediada por células en la cual células citotóxicas no específicas que expresan receptores Fc (FcRs) (*por ejemplo*, linfocitos citolíticos naturales (NK), neutrófilos y macrófagos) reconocen anticuerpo unido en una célula diana y, posteriormente, ocasionan lisis de la célula diana. Las células primarias para mediar ADCC, linfocitos citolíticos naturales, expresan FcγRIII únicamente, mientras que los monocitos expresan FcγRI, FcγRII y FcγRIII. La expresión de FcR en las células hematopoyéticas se resume en la Tabla 3, página 464 de Ravetch y Kinet, *Annu. Rev. Immunol.* 9:457-92 (1991). Para evaluar la actividad ADCC de una molécula de interés, puede llevarse a cabo un análisis ADCC *in vitro*, tal como el descrito en la Patente de los Estados Unidos No. 5,500,362 ó 5,821,337. Células efectoras útiles para tales análisis incluyen células mononucleares de sangre periférica (PBMC) y linfocitos citolíticos naturales (NK). Alternativa, o adicionalmente, la actividad ADCC de la molécula de interés puede evaluarse *in vivo*, *por ejemplo*, en un modelo animal tal como el divulgado en Clynes *et. al.*, *PNAS USA* 95:652-656 (1998).

"Células efectoras humanas" son leucocitos que expresan uno o más FcR y realizan funciones efectoras. Preferiblemente, las células expresan por lo menos FcγRIII y llevan a cabo la función efectora ADCC. Ejemplos de leucocitos humanos, que median ADCC, incluyen células mononucleares de sangre periférica (PBMC), linfocitos citolíticos naturales (NK), monocitos, linfocitos T citotóxicos y neutrófilos, prefiriéndose PBMC y linfocitos citolíticos naturales (NK).

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Los términos "receptor Fc" o "FcR" se usan para describir un receptor que se une a la región Fc de un anticuerpo. El FcR preferido es un FcR humano de secuencia natural. Más aún, un FcR preferido es uno que une un anticuerpo IgG (un receptor gamma) e incluye receptores de las subclases FcγRI, FcγRII, y FcγRIII, incluidas variantes alélicas y, alternativamente, formas empalmadas de estos receptores. Los receptores FcγRII incluyen FcγRIIA (un "receptor activador") y FcγRIIB (un "receptor inhibidor"), los cuales poseen secuencias de aminoácidos similares que difieren principalmente en sus dominios citoplásmicos. El receptor activador FcγRIIA contiene un motivo de activación a base de tirosina de inmunorreceptor (ITAM) en su dominio citoplásmico. El receptor inhibidor FcγRIIB contiene un motivo de inhibición a base de tirosina de inmunorreceptor (ITIM) en su dominio citoplásmico, (véase Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997). Los FcR se reseñan en Ravetch y Kinet, *Annu. Rev. Immunol.* 9: 457-92 (1991); Capel *et. al.*, *Immunomethods* 4: 25-34 (1994); y de Haas *et. al.*, *J. Lab. Clin. Med.* 126: 330-41 (1995). Otros FcR, incluidos aquellos por identificar en el futuro, se abarcan por el término "FcR" contenido aquí. El término comprende asimismo el receptor neonatal, FcRn, que es responsable de la transferencia de IgG maternas al feto. Guyer *et. al.*, *J. Immunol.* 117: 587 (1976) y Kim *et. al.*, *J. Immunol.* 24: 249 (1994). Los FcR en esta solicitud incluyen polimorfismos tales como dimorfismo genético en el gen que codifica FcγRIIIa resultando en una fenilalanina (F) o una valina (V) en la posición de aminoácido 158, localizada en la en la región del receptor que se une a IgG1. Se ha demostrado que el FcγRIIIa de valina homocigótica (FcγRIIIa-158V) posee una mayor afinidad para la IgG1 humana y media la ADCC incrementada *in vitro* en relación con el FcγRIIIa de fenilalanina homocigótica (FcγRIIIa-158F) o receptores heterocigóticos (FcγRIIIa-158F/V).

"Citotoxicidad dependiente de complemento" o "CDC" se refiere a la capacidad de una molécula de descomponer por lisis una diana, en la presencia de complemento. La ruta de activación del complemento se inicia por la unión del primer componente del sistema complemento (C1q) a una

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molécula (*por ejemplo*, un anticuerpo) acomplejado con un antígeno afín. Para evaluar la activación de complemento, puede realizarse un análisis CDC, *por ejemplo*, como se describe en Gazzano-Santoro *et. al.*, *J. Immunol Methods* 202: 163 (1996).

El término "anticuerpo" en esta solicitud se usa en el sentido más amplio y, específicamente, cubre anticuerpos monoclonales intactos, anticuerpos policlonales, anticuerpos multiespecíficos (*por ejemplo*, anticuerpos biespecíficos) formados de por lo menos dos anticuerpos intactos, y fragmentos de anticuerpo, siempre que exhiban la actividad biológica deseada.

"Fragmentos de anticuerpo" comprenden una porción de un anticuerpo intacto, que comprende la región variable o de unión a antígeno del mismo. Ejemplos de fragmentos de anticuerpo incluyen fragmentos Fab, Fab', F(ab')₂ y Fv; diacuerpos; anticuerpos lineales; moléculas de anticuerpo de cadena única y anticuerpos multiespecíficos formados de fragmentos de anticuerpo.

"Anticuerpos naturales" son usualmente glucoproteínas heterotetraméricas de alrededor de 150.000 daltons, compuestas de dos cadenas ligeras (L) idénticas y dos cadenas pesadas (H) idénticas. Cada cadena ligera se enlaza a una cadena pesada por un enlace disulfuro covalente, mientras que el número de enlazamientos disulfuro varía entre las cadenas pesadas de diferentes isotipos de inmunoglobulina. Cada cadena ligera y pesada posee también puentes disulfuro intracadenas regularmente espaciados. Cada cadena pesada tiene en un extremo un dominio variable (V_H) seguido por un número de dominios constantes. Cada cadena ligera tiene un dominio variable en un extremo (V_L) y un dominio constante en su otro extremo; el dominio constante de la cadena ligera se alinea con el primer dominio constante de la cadena pesada, y el dominio variable de la cadena ligera se alinea con el dominio variable de la cadena pesada. Se cree que residuos de aminoácidos particulares forman una interfase entre los dominios variables de cadena ligera y cadena pesada.

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El término "variable" se refiere al hecho de que ciertas porciones de los dominios variables que difieren extensivamente en secuencia entre anticuerpos y se utilizan en la unión y especificidad de cada anticuerpo particular para su antígeno particular. No obstante, la variabilidad no está uniformemente distribuida a través de los dominios variables de los anticuerpos. Ésta se concentra en tres segmentos denominados regiones hipervariables, tanto en los dominios variables de cadena pesada como en los de cadena ligera. Las porciones más altamente conservadas de los dominios variables se llaman las regiones marco (FR). Los dominios variables de cadena naturales pesada y ligera comprenden cada una cuatro FR que adoptan en gran medida una configuración de hoja β (" *β -sheet configuration*"), conectada por tres regiones hipervariables, que forman lazos que conectan y, en algunos casos, forman parte de la estructura de la hoja β . Las regiones hipervariables en cada cadena se mantienen juntas en estrecha proximidad por las FR y, con las regiones hipervariables de la otra cadena, contribuyen a la formación del sitio de unión de antígeno de anticuerpos (véase Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). Los dominios constantes no están implicados directamente en unir un anticuerpo a un antígeno, pero exhiben varias funciones efectoras, tales como participación del anticuerpo en citotoxicidad mediada por célula, dependiente de anticuerpo (ADCC).

La digestión con papaína de anticuerpos produce dos fragmentos de unión de antígeno idénticos, llamados fragmentos "Fab", cada uno con un solo sitio de unión de antígeno, y un fragmento "Fc" residual, cuyo nombre refleja su capacidad de cristalizarse rápidamente. El tratamiento con pepsina proporciona un fragmento $F(ab')_2$ que posee dos sitios de unión de antígeno y es aún capaz de entrecruzar antígeno.

"Fv" es el fragmento de anticuerpo mínimo, que contiene un sitio completo de unión de antígeno y reconocimiento de antígeno. Esta región consta de un dímero de un dominio de región variable de una cadena pesada y una cadena ligera en asociación estrecha, no covalente. Es en esta

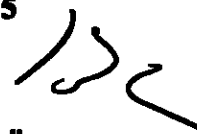
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configuración que las tres regiones hipervariables de cada dominio variable interactúan para definir un sitio de fijación de antígeno en la superficie del dímero V_H-V_L . Colectivamente, las seis regiones hipervariables confieren especificidad de unión de antígeno al anticuerpo. No obstante, incluso un dominio variable único (o la mitad de un Fv que comprende únicamente tres regiones hipervariables específicas para un antígeno) tiene la capacidad de reconocer y fijar antígeno, si bien con una afinidad más baja que todo el sitio de fijación.

Los fragmentos Fab contienen igualmente los dominios constantes de la cadena ligera y el primer dominio constante (CH1) de la cadena pesada. Los fragmentos Fab' difieren de los fragmentos Fab por la adición de unos pocos residuos en el término carboxi del dominio CH1 de cadena pesada, incluyendo una o más cisteínas de la región de unión flexible del anticuerpo. Fab'-SH es la designación en esta solicitud para Fab', en el cual el residuo(s) de cisteína de los dominios constantes lleva por lo menos un grupo tiol libre. Fragmentos $F(ab')_2$ de anticuerpo se produjeron originalmente como pares de fragmentos Fab', que tienen cisteínas de unión flexible entre ellos. Otros acoplamientos químicos de fragmentos de anticuerpo son también conocidos.

Las "cadenas livianas" de anticuerpos (inmunoglobulinas) de cualquier especie vertebrada pueden asignarse a uno de dos tipos claramente distintos, llamados kappa (κ) y lambda (λ), basados en las secuencias de aminoácidos de sus dominios constantes.

Dependiendo de la secuencia de aminoácidos del dominio constante de sus cadenas pesadas, los anticuerpos pueden asignarse a diferentes clases. Existen cinco clases principales de anticuerpos intactos: IgA, IgD, IgE, IgG, e IgM, y varias de éstas pueden subdividirse en subclases (isotipos), *por ejemplo*, IgG1, IgG2, IgG3, IgG4, IgA, e IgA2. Los dominios constantes de cadena pesada, que corresponden a las diferentes clases de anticuerpos, se denominan α , δ , ϵ , γ y μ , respectivamente. Las estructuras subunitarias y configuraciones tridimensionales de diferentes clases de inmunoglobulinas son bien conocidas.



Fragmentos de anticuerpo "Fv de cadena única" o "scFv" comprenden los dominios V_H y V_L de anticuerpo, en donde estos dominios están presentes en una sola cadena polipeptídica. Preferiblemente, el polipéptido Fv comprende además un conector de polipéptido entre los dominios V_H y V_L , que permite que el scFv forme la estructura deseada para unión de antígeno. Para una reseña del scFv, véase Plückthun en *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, páginas 269-315(1994).

El término "diacuerpos" se refiere a pequeños fragmentos de anticuerpo con dos sitios de unión de antígeno, los cuales fragmentos comprenden un dominio variable de cadena pesada (V_H) conectado a un dominio variable de cadena ligera (V_L) en la misma cadena polipeptídica ($V_H - V_L$). Usando un conector que es demasiado corto para permitir el emparejamiento entre los dos dominios en la misma cadena, los dominios son forzados a emparejarse con los dominios complementarios de otra cadena y a crear dos sitios de unión de antígeno. Los diacuerpos se describen más completamente en, por ejemplo, EP 404,097; WO 93/11161; y Hollinger *et. al.*, *Proc. Natl. Acad. Sci. USA*, 90: 6444-6448 (1993).

El término "anticuerpo monoclonal", como se emplea en esta solicitud, se refiere a un anticuerpo obtenido de una población de anticuerpos sustancialmente homogéneos, *es decir*, los anticuerpos individuales que componen la población son idénticos, excepto por posibles mutaciones de ocurrencia natural, que puedan estar presentes en cantidades menores. Los anticuerpos monoclonales son altamente específicos, dirigiéndose contra un solo sitio antigénico. Más aún, en contraste con preparaciones convencionales de anticuerpo policlonal, que incluyen diferentes anticuerpos dirigidos contra diferentes determinantes (epítomos), cada anticuerpo monoclonal se dirige contra un solo determinante en el antígeno. Además de su especificidad, los anticuerpos monoclonales son ventajosos en el sentido de que pueden sintetizarse por el cultivo de hibridoma, incontaminados por otras inmunoglobulinas. El calificativo "monoclonal" indica el carácter del anticuerpo como obtenido de una

población sustancialmente homogénea de anticuerpos, y no tiene que interpretarse como que requiere la producción del anticuerpo por algún método particular. Por ejemplo, los anticuerpos monoclonales a utilizarse de acuerdo con la presente invención pueden elaborarse por el método del hibridoma, descrito por primera vez por Kohler et al., *Nature*, 256:495 (1975), o pueden elaborarse por métodos de ADN recombinante (véase, *por ejemplo*, Patente de los Estados Unidos 4,816,567). Los "anticuerpos monoclonales" pueden aislarse también de bibliotecas de anticuerpos de fagos usando las técnicas descritas en Clackson et al., *Nature*, 352:624-628 (1991); Marks et al., *J. Mol. Biol.*, 222:581-597 (1991), por ejemplo.

Los anticuerpos monoclonales en esta solicitud incluyen específicamente anticuerpos "quiméricos" (inmunoglobulinas), en los cuales una porción de la cadena pesada y/o ligera es idéntica con, u homóloga a, secuencias correspondientes en anticuerpos derivados de una especie particular, o pertenecientes a una clase o subclase de anticuerpos, mientras que el resto de la cadena(s) es idéntico con, u homólogo a, secuencias correspondientes en anticuerpos derivados de otra especie, o pertenecientes a otra clase o subclase de anticuerpos, así como fragmentos de tales anticuerpos, siempre que exhiban la actividad biológica deseada (Patente de los Estados Unidos 4,816,567; y Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)). Los anticuerpos quiméricos de interés indicados aquí incluyen anticuerpos "primatizados" que comprenden secuencias de unión de antígeno de dominio variable, derivadas de un primate no humano (*por ejemplo*, simio del viejo mundo, tales como babuino, mono rhesus o cynomolgus) y secuencias de región constante humana (Patente de los Estados Unidos No. 5,693,780).

Formas "humanizadas" de anticuerpos no humanos (*por ejemplo*, murinos) son anticuerpos quiméricos que contienen una secuencia mínima derivada de inmunoglobulina no humana. En su mayor parte, los anticuerpos humanizados son inmunoglobulinas humanas (anticuerpo destinatario), en las cuales residuos de una región hipervariable del destinatario se reemplazan por residuos de una región hipervariable de una

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especie no humana (anticuerpo donante) tal como ratón, rata, conejo o primate no humano, que tienen la especificidad, afinidad y capacidad deseadas. En algunos casos, residuos de la región marco (FR) de la inmunoglobulina humana se reemplazan por residuos no humanos correspondientes. Más aún, los anticuerpos humanizados pueden comprender residuos, que no se encuentran en el anticuerpo destinatario ni en anticuerpo donante. Estas modificaciones se hacen para refinar aún más el desempeño del anticuerpo. En general, el anticuerpo humanizado comprenderá sustancialmente la totalidad de por lo menos uno y, típicamente dos, dominios variables, en los cuales la totalidad o sustancialmente todos los lazos hipervariable corresponden a los de una inmunoglobulina no humana, y todas o sustancialmente todas las FR son las de una secuencia de inmunoglobulina humana. Opcionalmente, el anticuerpo humanizado comprenderá también por lo menos una porción de una región constante de inmunoglobulina (Fc), típicamente la de una inmunoglobulina humana. Para mayores detalles, véase Jones *et al.*, *Nature*, 321:522-525 (1986); Riechmann *et al.*, *Nature*, 332:323-329 (1988); y Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992).

El término "región hipervariable", cuando se emplea aquí, se refiere a los residuos de aminoácidos de un anticuerpo que son responsables de la unión de antígeno. La región hipervariable comprende residuos de aminoácidos de una "región determinante de complementariedad" o "CDR" (*por ejemplo*, residuos 24-34 (L1), 50-56 (L2) y 89-97 (L3) en el dominio variable de cadena ligera y 31-35 (H1), 50-65 (H2) y 95-102 (H3) en el dominio variable de cadena pesada; Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) y/o aquellos residuos de un "lazo hipervariable" (*por ejemplo*, residuos 26-32 (L1), 50-52 (L2) y 91-96 (L3) en el dominio variable de cadena ligera y 26-32 (H1), 53-55 (H2) y 96-101 (H3) en el dominio variable de cadena pesada; Chothia y Lesk *J. Mol. Biol.* 196:901-917 (1987)). Residuos "marco" o "FR" son aquellos residuos de



dominio variable distintos de los residuos de región hipervariable, como se definen aquí.

Un anticuerpo "que une" un antígeno de interés, *por ejemplo*, CD20 o DR4 o DR5, es uno capaz de unir ese antígeno con suficiente afinidad y/o avidez, de modo que el anticuerpo es útil como agente terapéutico para direccionar ("*for targeting*") una célula que expresa el antígeno.

Para los propósitos de esta solicitud, "inmunoterapia" se referirá a un método de tratar un mamífero (preferiblemente un paciente humano) con un anticuerpo, en donde el anticuerpo puede ser un anticuerpo no conjugado o "desnudo", o el anticuerpo puede conjugarse o fusionarse con molécula(s) o agente(s) heterólogo, tal como uno o más agente(s) citotóxicos, generando en esa forma un "inmunoconjugado".

Un anticuerpo "aislado" es uno que se ha identificado y separado y/o recuperado de un componente de su entorno natural. Componentes contaminantes de su entorno natural son materiales que interferirían los usos diagnósticos o terapéuticos para el antagonista o anticuerpo, y pueden incluir enzimas, hormonas, y otros solutos proteináceos o no proteináceos. En incorporaciones preferidas, el anticuerpo se purificará (1) a más del 95% en peso del anticuerpo según se determine por el método de Lowry y, muy preferiblemente, más del 99% en peso, (2) a un grado suficiente para obtener por lo menos 15 residuos de secuencia de aminoácidos interna o N-terminal mediante el uso de un secuenciador de vaso, o (3) a homogeneidad por SDS-PAGE bajo condiciones reductoras o no reductoras usando azul Coomassie o, preferiblemente, tintura de plata. El anticuerpo aislado incluye el anticuerpo *in situ* en células recombinantes, puesto que por lo menos un componente del entorno natural del anticuerpo no estará presente. Por lo regular, sin embargo, el antagonista aislado se preparará por un paso de purificación, como mínimo.

La expresión "cantidad efectiva" se refiere a una cantidad del Apo2L/TRAIL o anticuerpo de receptor de muerte celular y anticuerpo CD20, que es efectiva para prevenir, mejorar o tratar la enfermedad o afección en cuestión.

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El término "agente inmunodepresor", tal como se emplea aquí para terapia complementaria, se refiere a sustancias que actúan para suprimir o enmascarar el sistema inmunitario del mamífero que está siendo tratado aquí. Esto incluiría sustancias que suprimen la producción de citocinas, regulan por disminución o suprimen la expresión de auto-antígeno, o enmascaran los antígenos MHC. Ejemplos de tales agentes incluyen pirimidinas 2-amino-6-aril-5-sustituídas (véase Patente de los Estados Unidos No. 4,665,077, cuya divulgación se incorpora aquí por referencia); medicamentos anti-inflamatorios no esteroideos (AINE); azatioprina; ciclofosfamida; bromocriptina; danazol; dapsona; glutaraldehído (que enmascara los antígenos MHC, como se describe en la Patente de los Estados Unidos No. 4,120,649); anticuerpos anti-idiotípicos para antígenos MHC y fragmentos MHC; ciclosporina A; esteroides tales como glucocorticoesteroides, *por ejemplo*, prednisona, metilprednisolona y dexametasona; metotrexato (oral o subcutáneo); hidroxiclороquina; sulfasalazina; leflunomida; antagonistas de citocina o receptor de citocina incluidos anti-interferón- γ , anticuerpos α o β , o, anticuerpos de factor α de necrosis anti-tumoral (infiximab o adalimumab), inmunoadhesina de anti-TNF α (etanercept), anticuerpos de factor β de necrosis anti-tumoral, anticuerpos de anti-interleucina-2 y anticuerpos de receptor de anti-IL-2; anticuerpos de anti-LFA-1, incluidos anticuerpos de anti-CD11a y anti-CD18; anticuerpos de anti-L3T4; globulina anti-linfocito heterólogo; anticuerpos pan-T, preferiblemente anticuerpos anti-CD3 o anti-CD4/CD4a; péptido soluble que contiene un dominio de unión de LFA-3 (WO 90/08187 publicada el 7/26/90); estreptocinasa; TGF- β ; estreptodornasa; ARN o ADN del hospedero; FK506; RS-61443; deoxiespergualina; rapamicina; receptor de linfocito T (Cohen et al., Patente de los Estados Unidos 5,114,721); fragmentos de receptor de linfocitos T (Offner *et al.*, *Science*, 251: 430-432 (1991); WO 90/11294; Ianeway, *Nature*, 341: 482 (1989); y WO 91/01133); y anticuerpos de receptor de linfocitos T (EP 340,109) tales como T10B9.

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El término "agente citotóxico", tal como se usa en esta solicitud, se refiere a una sustancia que inhibe o previene la función de las células y/o causa destrucción de las células. El término tiene como finalidad incluir isótopos radioactivos (*por ejemplo*, At^{211} , I^{131} , I^{125} , Y^{90} , Re^{186} , Re^{188} , Sm^{153} , Bi^{212} , P^{32} e isótopos radioactivos de Lu), agentes quimioterapéuticos, y toxinas tales como toxinas de molécula pequeña o toxinas enzimáticamente activas de origen bacteriano, fúngico, vegetal o animal, incluidos sus fragmentos y/o variantes.

"Actividad sinérgica" o "sinergia" o "efecto sinérgico" o "cantidad sinérgicamente efectiva", para los propósitos contemplados en esta solicitud, significa que el efecto observado, cuando se emplea una combinación de Apo2L/TRAIL o anticuerpo de receptor de muerte celular y anticuerpo CD20 es (1) mayor que el efecto logrado cuando ese Apo2L/TRAIL, anticuerpo de receptor de muerte celular o anticuerpo CD20 se emplea solo (o individualmente) y (2) mayor que el efecto de la suma agregada (aditivo) para el Apo2L/TRAIL o anticuerpo de receptor de muerte celular y el anticuerpo CD20. Tal sinergia o efecto sinérgico puede determinarse mediante una variedad de medios conocidos por los expertos en el arte. Por ejemplo, el efecto sinérgico de Apo2L/TRAIL o anticuerpo de receptor de muerte celular y anticuerpo CD20 puede observarse en formatos de análisis *in vitro* o *in vivo* examinando la reducción del número de células tumorales o masa tumoral.

Los términos "apoptosis" y "actividad apoptótica" se usan en un sentido amplio y se refieren a la forma ordenada o controlada de muerte celular en mamíferos, que típicamente se acompaña por uno o más cambios celulares característicos, incluidos condensación de citoplasma, pérdida de microvellosidades de la membrana plasmática, segmentación del núcleo, degradación de ADN cromosómico o pérdida de función mitocondrial. Esta actividad puede determinarse y medirse usando métodos bien conocidos en el arte, por ejemplo, mediante análisis de viabilidad celular, análisis FACS o electroforesis de ADN, unión de anexina V, fragmentación de ADN, encogimiento celular, dilatación del retículo endoplásmico, fragmentación



celular, y/o formación de vesículas de membrana (llamados cuerpos apoptóticos). Análisis que determinan la capacidad de un anticuerpo (por ejemplo, Rituximab) para inducir apoptosis se han descrito en Shan et al. *Cancer Immunol Immunother* 48:673-83 (2000); Pedersen et al. *Blood* 99:1314-9 (2002); Demidem et al. *Cancer Chemotherapy & Radiopharmaceuticals* 12 (3):177-186 (1997), por ejemplo.

Los términos "cáncer" y "canceroso" o "maligno" se refieren a, o describen, la condición fisiológica en mamíferos, que se caracteriza típicamente por un crecimiento celular descontrolado. Ejemplos de cáncer incluyen, pero no se limitan a, carcinoma incluidos adenocarcinoma, linfoma, blastoma, melanoma, sarcoma y leucemia. Ejemplos más particulares de tales neoplasias malignas incluyen carcinoma epidermoide ("*squamous cell cancer*"), cáncer de pulmón microcítico, cáncer de pulmón amicrocítico, cáncer gastrointestinal, linfoma hodgkiniano y no hodgkiniano, cáncer pancreático, glioblastoma, glioma, cáncer cervical, cáncer de ovarios, cáncer del hígado tal como carcinoma hepático y hepatoma, cáncer de la vejiga, cáncer de próstata, cáncer de colon, cáncer colorrectal, carcinoma del endometrio, mieloma (tal como mieloma múltiple), carcinoma de glándula salival, cáncer del riñón tal como carcinoma de célula renal y tumores de Wilms, carcinoma de célula basal, melanoma, cáncer de próstata, cáncer de la vulva, cáncer de tiroides, cáncer testicular, cáncer esofágico, y varios tipos de cáncer de cabeza y cuello.

El término "enfermedad inmuno-relacionada" significa una enfermedad en la cual un componente del sistema inmunitario de un mamífero causa, media o contribuye de otro modo a la morbilidad en el mamífero. Se incluyen además enfermedades, en las cuales la estimulación o intervención de la respuesta inmunitaria tiene un efecto aliviador sobre la evolución de la enfermedad. Incluidas en este término están las enfermedades autoinmunitarias, enfermedades inflamatorias mediadas inmunitariamente, enfermedades inflamatorias no mediadas inmunitariamente, enfermedades infecciosas, y enfermedades de inmunodeficiencia. Ejemplos de enfermedades inflamatorias e inmuno-

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relacionadas, algunas de las cuales son mediadas inmunitariamente o por linfocitos T, que pueden tratarse de acuerdo con la invención incluyen lupus sistémico eritematoso, artritis reumatoide, artritis crónica juvenil, espondiloartropatías, esclerosis sistémica (escleroderma), miopatías inflamatorias idiopáticas (dermatomiositis, polimiositis), síndrome de Sjögren, vasculitis sistémica, sarcoidosis, anemia hemolítica autoinmunitaria (pancitopenia inmunitaria, hemoglobinuria nocturna paroxística), trombocitopenia autoinmunitaria (púrpura trombocitopénica idiopática, trombocitopenia mediada inmunitariamente), tiroiditis (enfermedad de Grave, tiroiditis de Hashimoto, tiroiditis linfocítica juvenil, tiroiditis atrófica), diabetes sacarina, enfermedad renal mediada inmunitariamente (glomerulonefritis, nefritis tubulointersticial), enfermedades desmielinizantes de los sistemas nervioso central y periférico, tales como esclerosis múltiple, polineuropatía desmielinizante idiopática o síndrome de Guillain-Barré, y polineuropatía desmielinizante inflamatoria crónica, enfermedades hepato biliares tales como hepatitis infecciosa (hepatitis A, B, C, D, E y otros virus no hepatotrópicos), hepatitis activa crónica autoinmunitaria, cirrosis biliar primaria, hepatitis granulomatosa, y colangitis esclerosante, enfermedades pulmonares fibróticas e inflamatorias, tal como síndrome del intestino irritable (colitis ulcerosa: enfermedad de Crohn), enteropatía de sensibilidad al gluten, y enfermedad de Whipple; enfermedades cutáneas autoinmunitarias o mediadas inmunitariamente incluidas enfermedades cutáneas vesiculares, eritema multiforme y dermatitis por contacto, psoriasis; enfermedades alérgicas tales como asma, rinitis alérgica, dermatitis atópica, hipersensibilidad a los alimentos y urticaria; enfermedades inmunológicas del pulmón tales como neumonías eosinófilas, fibrosis pulmonar idiopática y neumonitis por hipersensibilidad, enfermedades asociada con transplantes incluido rechazo de injertos y enfermedad del injerto contra el hospedero. Las enfermedades infecciosas incluyen SIDA (infección por VIH), hepatitis A, B, C, D, y E, infecciones bacterianas, infecciones fúngicas, infecciones protozoarias e infecciones parasitarias.

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Una "neoplasia maligna de linfocitos B" es una neoplasia maligna que involucra linfocitos B. Los ejemplos comprenden enfermedad de Hodgkin, incluidas la enfermedad de Hodgkin de predominio linfocítico (LPHD); linfoma no hodgkiniano (NHL); linfoma de células centrofoliculares (FCC); leucemia linfocítica aguda (ALL); leucemia linfocítica crónica (CLL); tricoleucemia; linfoma linfocítico plasmacitoide; linfoma de células del manto; SIDA o linfoma relacionada con VIH; mieloma múltiple; linfoma del sistema nervioso central (SNC); trastorno linfoproliferativo pos-transplante (PTLD); macroglobulinemia de Waldenstrom (linfoma linfoplasmacítico); linfoma del tejido linfoide asociado con la mucosa (MALT); y linfoma/leucemia de la zona marginal.

El linfoma no hodgkiniano (NHL) incluye, pero no se limita a, NHL de grado bajo/folicular, NHL con recaída o resistente a tratamiento, NHL de grado bajo de primera línea, NHL de fase III/IV, NHL resistente a la quimioterapia, NHL linfocítico pequeño (SL), NHL de grado intermedio/folicular, NHL difuso de grado intermedio, linfoma difuso de células grandes, NHL agresivo (incluido NHL de primera línea, agresivo y NHL con recaída, agresivo), NHL reincidente luego de o resistente al transplante de células madre autólogas, NHL inmunoblástico de grado elevado, NHL linfoblástico de grado elevado, NHL de microcitosis no segmentados, de grado elevado, NHL de enfermedad de tumor sólido ("*bulky disease NHL*"), etc.

Una "enfermedad autoinmunitaria" contemplada aquí es una enfermedad o trastorno que se origina y dirige contra los propios tejidos de un individuo o un co-segregado o manifestación o afección resultante del mismo. Ejemplos de enfermedades o trastornos autoinmunitarios incluyen, pero no se limitan a artritis (artritis reumatoide, artritis reumatoide en jóvenes, osteoartritis, artritis psoriásica y espondilitis anquilosante), psoriasis, dermatitis incluida dermatitis atópica; urticaria idiopática crónica, incluida urticaria autoinmunitaria crónica, polimiositis/dermatomiositis, necrólisis epidérmica tóxica, escleroderma sistémico y esclerosis, respuestas asociadas con síndrome de intestino irritable (IBD) (enfermedad

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de Crohn, colitis ulcerosa), e IBD con co-segregado de pyoderma gangrenosum, erythema nodosum, colangitis esclerosante primaria, y/o episcleritis), síndrome disneico, incluidos síndrome disneico en adultos (ARDS), meningitis, enfermedades mediadas por IgE tales como anafilaxis y rinitis alérgica, encefalitis tal como encefalitis de Rasmussen, uveítis, colitis tales como colitis microscópica y colitis colágena, glomerulonefritis (GN) tales como GN, membranosa, GN idiopática, GN proliferativa membranosa (MPGN), incluidas GN Tipo I y Tipo II, y GN de rápida evolución, afecciones alérgicas, eczema, asma, estados que involucran la infiltración de linfocitos T y respuestas inflamatorias crónicas, arterioesclerosis, miocarditis autoinmunitaria, deficiencia de adhesión de leucocitos, lupus eritematoso sistémico (SLE) tal como SLE cutáneo, lupus (incluidas nefritis, cerebritis, pediátrico, no renal, discoide, alopecia), diabetes de aparición en jóvenes, esclerosis múltiple (MS) tal como MS espino-óptica, encefalomiелitis alérgica, respuestas inmunitarias asociadas con hipersensibilidad aguda y diferida mediada por citocinas y linfocitos T, tuberculosis, sarcoidosis, granulomatosis incluidas granulomatosis de Wegener, agranulocitosis, vasculitis (incluidas vasculitis de vasos grandes (incluidas *polymyalgia rheumatica* y arteritis de células gigantes (de Takayasu)), vasculitis de vasos medianos (incluidas la enfermedad de Kawasaki y *polyarteritis nodosa*), Vasculitis SNC, y vasculitis asociada con anticuerpos anticitoplasma de los neutrófilos (ANCA), tal como vasculitis o síndrome de Churg-Strauss (CSS)), anemia aplásica, anemia positiva de Coombs, anemia de Diamond Blackfan, anemia hemolítica inmunitaria incluida anemia hemolítica autoinmunitaria (AIHA), anemia perniciosa, aplasia de glóbulos rojos puros (PRCA), deficiencia de factor VIII, hemofilia A, neutropenia autoinmunitaria, pancitopenia, leucopenia, enfermedades que involucran diapedesis de leucocitos, sistemas inflamatorios CNS, síndrome de lesión de órganos múltiples, *myasthenia gravis*, enfermedades mediadas por complejo antígeno-anticuerpo, enfermedad de membrana basal anti-glomerular, síndrome de anticuerpo anti-fosfolípido, neuritis alérgica, enfermedad de Behçet, síndrome de

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Castleman, síndrome de Goodpasture, síndrome miasténico de Lambert-Eaton, síndrome de Reynaud, síndrome de Sjögren, síndrome de Stevens-Johnson, rechazo de trasplante de órganos sólidos (incluidos pre-tratamiento para concentraciones elevadas de anticuerpos reactivos ("*high panel reactive antibody titers*"), depósito de IgA en tejidos, y rechazo derivado de trasplante renal, trasplante de hígado, trasplante intestinal, trasplante cardíaco, etc.), enfermedad injerto versus huésped (GVHD), penfigoide vesicular, pénfigo (incluidos pemphigus vulgaris, foliaceus, y penfigoide pemphigus mucus-membrana), poliendocrinopatías autoinmunitarias, enfermedad de Reiter, síndrome de Stiffman, nefritis de complejo inmunitario, polineuropatías IgM o neuropatía mediada por IgM, púrpura trombocitopénica idiopática (ITP), púrpura trombocitopénica trombótica (TTP), trombocitopenia (desarrollada por pacientes con infarto del miocardio, por ejemplo), incluidas trombocitopenia autoinmunitaria, enfermedad autoinmunitaria de los testículos y ovario incluidas orquitis autoinmunitarias y ooforitis, hipotiroidismo primario; enfermedades endocrinas autoinmunitarias incluida tiroiditis autoinmunitaria, tiroiditis crónica (tiroiditis de Hashimoto), tiroiditis subaguda, hipotiroidismo idiopático, enfermedad de Addison, enfermedad de Grave, síndromes poliglandulares autoinmunitarios (o síndromes de endocrinopatía poliglandular), diabetes tipo I también conocida como diabetes sacarina insulín dependiente (IDDM), incluida IDDM pediátrica, y síndrome de Sheehan; hepatitis autoinmunitaria, neumonitis intersticial linfocítica (VIH), bronchiolitis obliterans (no trasplante) vs NSIP (neumonitis intersticial no específica - *non-specific interstitial pneumonitis*), síndrome de Guillain-Barré, enfermedad de Berger (nefropatía IgA), cirrosis biliar primaria, esprúe celíaco (enteropatía del gluten), esprúe resistente al tratamiento con co-segregado dermatitis herpetiformis, crioglobulinemia, esclerosis lateral amiotrófica (ALS; enfermedad de Lou Gehrig), arteriopatía coronaria, enfermedad autoinmunitaria del oído interno (AIED), pérdida de la audición autoinmunitaria, síndrome opsoclonus mioclonus (OMS), policondritis tal como policondritis resistente al tratamiento, proteinosis alveolar pulmonar,

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amiloidosis, hepatitis de células gigantes, escleritis, gamopatía monoclonal de significación incierta/desconocida (MGUS), neuropatía periférica, síndrome paraneoplásico, conductopatías tales como epilepsia, migraña, arritmia, trastornos musculares, sordera, ceguera, parálisis periódica, y conductopatías del SNC; autismo, miopatía inflamatoria, y glomerulosclerosis segmental focal (FSGS).

El término "profármaco", tal como se emplea en esta solicitud, se refiere a un precursor o forma derivada de una sustancia farmacéuticamente activa, que es menos citotóxica a las células cancerosas, comparada con el medicamento original, y que es capaz de activarse enzimáticamente o convertirse en la forma original más activa. Véase, *por ejemplo*, Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) y Stella *et al.*, "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt *et al.*, (ed.), pp. 247-267, Humana Press (1985). Los profármacos de esta invención incluyen, pero no se limitan a, profármacos con contenido de fosfato, profármacos con contenido de tiofosfato, profármacos con contenido de sulfato, profármacos con contenido de péptido, profármacos modificados con D-aminoácidos, profármacos glucosilados, profármacos con contenido de β -lactama, profármacos con contenido de fenoxiacetamida opcionalmente sustituida, o profármacos con contenido de fenilacetamida opcionalmente sustituida, profármacos de 5-fluorocitosina y otros profármacos de 5-fluorouridina, que pueden convertirse en el medicamento libre citotóxico más activo. Ejemplos de medicamentos citotóxicos, que pueden derivarse en una forma profármaco para uso en esta invención, incluyen, pero no se limitan a, aquellos agentes quimioterapéuticos descritos más adelante.

El término "agente citotóxico", tal como se usa en esta solicitud, se refiere a una sustancia que inhibe o previene la función de las células y/o causa destrucción de las células. El término tiene como finalidad incluir isótopos radioactivos (*por ejemplo*, At^{211} , I^{131} , I^{125} , Y^{90} , Re^{186} , Re^{188} , Sm^{153} , Bi^{212} , P^{32} e isótopos radioactivos de Lu), agentes quimioterapéuticos, y

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toxinas tales como toxinas de molécula pequeña o toxinas enzimáticamente activas de origen bacteriano, fúngico, vegetal o animal, incluidos sus fragmentos y/o variantes.

Un "agente quimioterapéutico" es un compuesto químico útil en el tratamiento del cáncer. Ejemplos de agentes quimioterapéuticos incluyen agentes alquilantes tales como tiotepa y ciclosfosfamida CYTOXAN®; alquilosulfonatos tales como busulfán, improsulfán y piposulfán; aziridinas tales como benzodopa, carbocadona, meturedopa y uredopa; etileniminas y metilamelaminas incluidas altretamina, trietilenmelamina, trietilenfosforamida, trietilentionfosforamida y trimetilomelamina; acetogeninas (especialmente bulatacina y bulatacinona); una camptotecina (incluido el análogo sintético topotecán); briostatina; calistatina; CC-1065 (incluidos sus análogos sintéticos adozelesina, carzelesina y bizelesina); criptoficinas (particularmente criptoficina 1 y criptoficina 8); dolastatina; duocarmicina (incluidos los análogos sintéticos, KW-2189 y CB1-TM1); eleuterobina; pancratistatina; una sarcodictina; espongistatina; mostazas nitrogenadas tales como clorambucil, clornafazina, colofosfamida, estramustina, ifosfamida, mecloretamina, clorhidrato de óxido de mecloretamina, melfalán, novembichin, phenesterine, prednimustina, trofosfamida, mostaza de uracilo; nitrosureas tales como carmustina, clorozotocina, fotemustina, lomustina, nimustina, y ranimustina; antibióticos tales como los antibióticos enediyne (*por ejemplo*, caliqueamicina, especialmente caliqueamicina gamma11 y caliqueamicina omega11 (véase, *por ejemplo*, Agnew, Chem Intl. Ed. Engl., 33: 183-186 (1994))); dinemicina, incluida dinemicina A; bisfosfonatos, tales como clodronato; una esperamicina; así como cromóforo de neocarzinostatina y cromóforos antibióticos relacionados de cromoproteína enedifino), aclacinomisinas, actinomicina, autramicina, azaserina, bleomicinas, cactinomicina, carabicina, carminomicina, carzinofilina, cromomicinas, dactinomicina, daunorubicina, detorubicina, 6-diazo-5-oxo-L-norleucina, ADRLAMYCIN® doxorubicina (incluidas morfolino-doxorubicina, cianomorfolino-doxorubicina, 2-pirrolino-doxorubicina y

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deoxidoxorubicina), epirubicina, esorubicina, idarubicina, marcelomicina, mitomicinas tales como mitomicina C, ácido micofenólico, nogalamicina, olivomicinas, peplomicina, potfiromicina, puromicina, quelamicina, rodorubicina, estreptonigrina, estreptozocina, tubercidina, ubenimex, zinostatina, zorubicina; anti-metabolitos tales como metotrexato y 5-fluorouracilo (5-FU); análogos de ácido fólico tales como denopterina, metotrexato, pteropterina, trimetrexato; análogos de purina tales como fludarabina, 6-mercaptopurina, tiamiprina, tioguanina; análogos de pirimidina tales como ancitabina, azacitidina, 6-azauridina, carmofur, citarabina, dideoxiuridina, doxifluridina, enocitabina, floxuridina; andrógenos tales como calusterona, dromostanolona propionato, epitioestanol, mepitioestano, testolactona; anti-adrenales tales como aminoglutetimida, mitotano, trilostano; reabastecedor de ácido fólico tal como ácido frolínico; aceglatona; glucósido de aldofosfamida; ácido aminolevulínico; eniluracil; amsacrina; bestrabucil; bisantreno; edatraxato; defofamine; demecolcine; diaziquone; elfornithine; acetato de eliptinio; una epotilona; etoglucid; nitrato de galio; hidroxiiurea; lentinan; lonidamine; maitansinoides tales como maitansina y ansamitocinas; mitoguazona; mitoxantrona; mopidamol; nitracrine; pentostatina; phenamet; pirarubicina; losoxantrona, ácido podofilínico; 2-etilhidrazida; procarbazona; complejo polisacárido PSK® (JHS Natural Products, Eugene, OR); razoxano; rizoxina; sizofirano; espirogermanio; ácido tenuazónico; triazicuona; 2,2',2"- triclortrietilamina; tricotecenos (especialmente T-2 toxina, verracurin A, roridin A y anguidine); uretano; vindesina; dacarbazina; mannomustina; mitobronitol; mitolactol; pipobroman; gacytosine; arabinósido ("Ara-C"); ciclofosfamida; tiotepa; taxoides, *por ejemplo*, TAXOL® paclitaxel (Bristol-Myers Squibb Oncology, Princeton, NJ), ABRAAXANE™ libre de cremóforo, formulación de nanopartículas modificadas con albúmina de paclitaxel (American Pharmaceutical Partners, Schaumburg, Illinois), y TAXOTERE® doxetaxel (Rhône-Poulenc Rorer, Antony, Francia); clorambucil; GEMZAR® gemcitabina; 6-tioguanina; mercaptopurina; metotrexato; análogos de platino tales como

cisplatino y carboplatino; vinblastina; platino; etopósido (VP-16); ifosfamida; mitoxantrona; vincristina; NAVELBINE® vinorelbina; novantrona; tenipósido, edatrexato; daunomicina; aminopterina; xeloda; ibandronato; CPT-11; inhibidor de topoisomerasa RFS 2000; difluorometilornitina (DMFO); retinoides tales como ácido retinoico; capecitabina; y sales, ácidos o derivados farmacéuticamente aceptables de cualquiera de los anteriores.

También incluidos en esta definición se encuentran agentes anti-hormonales que actúan para regular o inhibir la acción hormonal en tumores, tales como anti-estrógenos y moduladores selectivos de receptor de estrógeno (SERMs), incluidos por ejemplo tamoxifeno (incluido NOLVADEX® tamoxifeno), raloxifeno, droloxifeno, 4-hidroxitamoxifeno, trioxifeno, keoxifeno, LY117018, onapristona, y FARESTON® toremifeno; inhibidores de aromatasa que inhiben la enzima aromatasa, la cual regula la producción de estrógenos en las glándulas suprarrenales, tales como, por ejemplo, 4 (5)-imidazoles, aminoglutetimida, MEGASE® acetato de megestrol, AROMASIN® exemestano, formestanie, fadrozol, RIVISOR® vorozol, FEMARA® letrozol, y ARIMIDEX® anastrozol; y anti-andrógenos tales como flutamida, nilutamida, bicalutamida, leuprolida, y goserelina; así como troxacitabina (un análogo de citosina 1,3-dioxolano nucleósido); oligonucleótidos complementarios, particularmente aquellos que inhiben la expresión de genes en vías de señalización implicadas en la proliferación celular aberrante, tal como, por ejemplo, PKC-alfa, Ralf y H-Ras; ribozimas tales como un inhibidor de expresión de VEGF (por ejemplo, ribozima ANGIOZYME®) y un inhibidor de la expresión de HER2, vacunas tales como vacunas de terapia génica, por ejemplo, la vacuna ALLOVECTIN®, la vacuna LEUVECTIN®, y la vacuna VAXID®; PROLEUKIN® rIL-2; LURTOTECAN® inhibidor de topoisomerasa 1, ABARELIX® rmRH, y sus sales farmacéuticamente aceptables, ácidos o derivados de cualquiera de los anteriores.

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Un "agente inhibidor de crecimiento" se refiere a un compuesto o composición que inhibe el crecimiento de una célula, *in vitro* o *in vivo*. Por consiguiente, el agente inhibidor de crecimiento puede ser uno que reduzca significativamente el porcentaje de células que sobreexpresan tales genes en la fase S. Ejemplos de agentes inhibidores de crecimiento incluyen agentes que bloquean la progresión del ciclo celular (en un sitio distinto de la fase S), tales como agentes que inducen la detención G1 y la detención de fase M. Bloqueadores clásicos de fase M incluyen las vincas (vincristina y vinblastina), TAXOL®, e inhibidores topo II tales como doxorubicina, epirubicina, daunorubicina, etopósido, y bleomicina. Aquellos agentes que detienen G1 pasan también a la detención de la fase S, por ejemplo, agentes alquilantes de ADN tales como tamoxifeno, prednisona, dacarbazina, mecloretamina, cisplatino, metotrexato, 5-fluorouracil, y ara-C. Más información puede encontrarse en *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especialmente página 13).

El término "citocina" es un término genérico para proteínas liberadas por una población de células que actúan sobre otra célula como mediadores intercelulares. Ejemplos de tales citocinas son linfocinas, monocinas, y hormonas polipeptídicas tradicionales. Incluidas entre las citocinas se encuentran somatotropina tal como somatotropina humana, somatotropina humana N-metionilo, y somatotropina bobina; hormona paratiroide; tiroxina; insulina; proinsulina; relaxina; prorrelaxina; hormonas de glucoproteína tales como folitropina (FSH), tirotropina (TSH), y lutropina (LH); somatotropina hepática; factor de crecimiento de fibroblastos; prolactina; lactógeno placentario; factor de necrosis tumoral α y β ; sustancia inhibidora de Müller; péptido asociado con gonadotropina de ratón; inhibina; activina; factor de crecimiento endotelial vascular; integrina; trombopoyetina (TPO); factores de crecimiento neurales; factor de crecimiento plaquetario; factores de crecimiento transformantes (TGF) tales como TGF- α y TGF- β ; factor de crecimiento insulinoide tipo I y II;

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eritropoyetina (EPO); factores osteoinductores; interferones tales como interferón α , β , y γ ; factores estimulantes de colonias (CSF) tales como CSF de macrófago (M-CSF); CSF de granulocito-macrófago (GM-CSF); y CSF de granulocito (G-CSF); interleucinas (IL) tales como IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; y otros factores polipeptídicos incluido LIF y ligando de kit (KL). Tal como se emplea en esta solicitud, el término citocina incluye proteínas de fuentes naturales o de cultivo de células recombinantes y equivalentes biológicamente activos de las citocinas de secuencia natural.

El término "prospecto del envase" se emplea para referirse a instrucciones usualmente incluidas en los envases comerciales de productos terapéuticos, que contienen información acerca de las indicaciones, uso, posología, administración, contraindicaciones y/o advertencias concernientes al empleo de tales productos terapéuticos, etc.

Los términos "tratar", "tratamiento" y "terapia", tal como se emplean en esta solicitud, se refieren a terapia curativa, terapia profiláctica, y terapia preventiva.

El término "mamífero", tal como se emplea en esta solicitud, se refiere a cualquier animal clasificado como mamífero, incluidos humanos, vacas, caballos, perros y gatos. En una incorporación preferida la invención, el mamífero es humano.

II. Composiciones y Métodos de la Invención

Se han descrito una citocina relacionada con la familia de ligandos TNF, la citocina identificada en esta solicitud como "ligando Apo-2" o "TRAIL". La secuencia de aminoácidos madura predicha del ligando Apo-2 humano natural contiene 281 aminoácidos, y tiene un peso molecular calculado de aproximadamente 32.5 kDa. La ausencia de una señal de secuencia y la presencia de una región hidrófoba interna sugieren que el ligando Apo-2 es una proteína transmembrana tipo II. Asimismo se han descrito polipéptidos de ligando Apo-2 de dominio extracelular soluble. Véase, por ejemplo, WO97/25428 publicada el 17 de julio de 1997. De

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igual modo, se han descrito variantes de sustitución de Apo-2L. Se han utilizado técnicas de exploración de alanina para identificar varias moléculas variantes de sustitución con actividad biológica. Variantes particulares de sustitución del ligando Apo-2 incluyen aquellas en las cuales por lo menos un aminoácido se sustituye por otros aminoácidos, tal como un residuo de alanina. Estas variantes de sustitución se identifican, por ejemplo, como "D203A"; "D218A" y "D269A." Esta nomenclatura se usa para identificar variantes de ligando Apo-2, en donde los residuos de ácido aspártico en las posiciones 203, 218, y/o 269 (empleando la numeración mostrada en Figura 1) se sustituyen por residuos de alanina. Opcionalmente, las variantes Apo-2L de la presente invención pueden comprender una o más de las sustituciones de aminoácido. Opcionalmente, tales variantes Apo-2L serán variantes selectivas de receptor DR4 o DR5.

La descripción siguiente se refiere a métodos de producir el ligando Apo-2, incluidas las variantes de ligando Apo-2, cultivando células hospederas transformadas o transfectadas con un vector que contiene el ligando Apo-2, codificando el ácido nucleico y recuperando el polipéptido del cultivo celular.

El ADN que codifica el ligando Apo-2 puede obtenerse de cualquier biblioteta de ADNc, preparada de tejido del que se supone posee el ARNm del ligando Apo-2 y lo expresa en un nivel detectable. Por consiguiente, ADN del ligando Apo-2 humano puede obtenerse convenientemente de una biblioteca de ADNc preparada de tejidos humanos, tales como la biblioteca de bacteriófagos de ADNc placentario humano, como se describe en WO97/25428. El gen que codifica el ligando Apo-2 puede obtenerse asimismo de una biblioteca genómica o mediante síntesis de oligonucleótidos.

Las bibliotecas pueden cribarse con sondas (tales como anticuerpos contra el ligando Apo-2 u oligonucleótidos de por lo menos alrededor de 20-80 bases) diseñadas para identificar el gen de interés o la proteína codifica por él. El cribado del ADNc o biblioteca genómica con la sonda seleccionada puede conducirse usando procedimientos estándar, tales como

los descritos en Sambrook et al., *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1989). Un medio alternativo para aislar el gen que codifica el ligando Apo-2 es utilizar la metodología PCR [Sambrook et al., supra; Dieffenbach et al., *PCR Primer: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, 1995)].

Fragmentos de segmentos de aminoácidos o variantes del ligando Apo-2 pueden prepararse introduciendo cambios de nucleótidos apropiados en el ADN del ligando Apo-2, o por síntesis del polipéptido del ligando Apo-2 deseado. Tales fragmentos o variantes representan inserciones, sustituciones, y/o eliminaciones de residuos dentro o en uno o dos de los extremos de la región intracelular, la región transmembrana, o la región extracelular, o de la secuencia de aminoácidos mostrada para el ligando Apo-2 de longitud total en Figura 1. Puede elaborarse cualquier combinación de inserción, sustitución, y/o eliminación para llegar al constructo final, siempre que el constructo final posea, por ejemplo, una actividad biológica deseada, tal como actividad apoptótica, como se define en esta solicitud. En una incorporación preferida, los fragmentos o variantes tienen por lo menos alrededor de 80% de identidad de secuencia de aminoácidos, más preferiblemente por lo menos alrededor de 90% de identidad de secuencia, y, aún más preferiblemente, por lo menos 95%, 96%, 97%, 98% o 99% de identidad de secuencia con las secuencias identificadas en esta solicitud para los dominios intracelulares, transmembrana, o extracelulares del ligando Apo-2, o la secuencia de longitud total para el ligando Apo-2. Los cambios de aminoácidos pueden alterar también procesos pos-traduccion del ligando Apo-2, tales como cambiar el número o posición de sitios de glucosilación o alterando las características de anclaje de la membrana.

Variaciones en la secuencia del ligando Apo-2, como se describe anteriormente, pueden introducirse usando cualquiera de las técnicas y pautas para mutaciones conservadoras y no conservadoras divulgadas en la Patente de los Estados Unidos No. 5,364,934. Éstas incluyen la

mutagénesis mediada por oligonucleótidos (dirigida), rastreo de alanina ("*alanine scanning*"), y mutagénesis PCR.

El análisis de rastreo de aminoácidos puede emplearse para identificar uno o más aminoácidos a lo largo de una secuencia contigua. Entre los aminoácidos de rastreo preferidos se encuentran los aminoácidos neutros, relativamente pequeños. Dichos aminoácidos incluyen alanina, glicina, serina y cisteína. Típicamente, la alanina es un aminoácido de barrido preferido entre este grupo, porque elimina la cadena lateral más allá del carbono beta y tiene menos tendencia a alterar la conformación de cadena principal de la variante. [Cunningham et al., *Science*, 244:1081 (1989)]. Además, la alanina es generalmente preferida por ser el aminoácido más común. De igual modo, se encuentra frecuentemente tanto en las posiciones ocultas como en las expuestas [Creighton, *The Proteins*, (W.H. Freeman & Co., NY); Chothia, *J. Mol. Biol.*, 150:1 (1976)].

Los aminoácidos pueden agruparse de acuerdo con similitudes en las propiedades de sus cadenas laterales (in A. L. Lehninger, in *Biochemistry*, second ed., pp. 73-75, Worth Publishers, New York (1975)):

- (1) no polares: Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M)
- (2) polares no cargados: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gln (Q)
- (3) ácidos: Asp (D), Glu (E)
- (4) básicos: Lys (K), Arg (R), His (H)

Alternativamente, residuos de ocurrencia natural pueden dividirse en grupos, basados en propiedades comunes de cadena lateral:

- (1) hidrófobos: Norleucina, Met, Ala, Val, Leu, Ile;
- (2) hidrófilos neutros: Cys, Ser, Thr, Asn, Gln;
- (3) ácidos: Asp, Glu;
- (4) básicos: His, Lys, Arg;
- (5) residuos que influyen en la orientación de la cadena: Gly, Pro;
- (6) aromáticos: Trp, Tyr, Phe.

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Tabla 1

Residuo Original	Sustituciones Ejemplares	Sustituciones Preferidas
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn, Glu	Asn
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucina	Leu
Leu (L)	Norleucina; He; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu, Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; He; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	He; Leu; Met; Phe; Ala; Norleucina	Leu

Variaciones en la secuencia del ligando Apo-2, incluidas asimismo en el ámbito de la invención, se refieren a derivados de terminal amino o formas modificadas. Tales secuencias de ligando Apo-2 incluyen cualquiera de los polipéptidos de ligando Apo-2, descritos en esta solicitud, que tienen una metionina o metionina modificada (tal como formil metionilo u otra especie metionilo bloqueado) en el término N de la secuencia de polipéptidos.

El ácido nucleico (por ejemplo, ADNc o ADN genómico) que codifica el ligando Apo-2 natural o variante puede insertarse en un vector replicable para clonación adicional (amplificación del ADN) o para

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expresión. Varios vectores se encuentran disponibles al público. Los componentes del vector incluyen generalmente, pero no se limitan a, uno o más de los siguientes: una secuencia de señal, un origen de replicación, uno o más genes marcadores, un elemento potenciador, un promotor, y una secuencia de terminación de transcripción, cada uno de los cuales se describe más adelante. Secuencias de señal opcionales, orígenes de replicación, genes marcadores, elementos potenciadores y secuencias terminadoras de transcripción que pueden emplearse son conocidas en el arte y se describen con mayor detalle en WO97/25428.

Los vectores de expresión y clonación contienen usualmente un promotor que es reconocido por el organismo hospedero y se enlaza operacionalmente a la secuencia de ácido nucleico del ligando Apo-2. Los promotores son secuencias no traducidas localizadas corriente arriba (5') del codón de iniciación de un gen estructural (generalmente con alrededor de 100-1.000 bp), que controlan la transcripción y traducción de una secuencia particular de ácido nucleico, tal como la secuencia de ácido nucleico del ligando Apo-2, a la cual se enlazan operacionalmente. Típicamente, tales promotores caen dentro de dos clases, inducibles y constitutivos. Los promotores inducibles son promotores que inician niveles incrementados de transcripción de ADN bajo su control, en respuesta a algún cambio en las condiciones de cultivo, por ejemplo, la presencia o ausencia de un nutriente o un cambio en temperatura. Actualmente es bien conocido un gran número de promotores, reconocidos por una variedad de células hospederas potenciales. Estos promotores se enlazan operacionalmente al ligando Apo-2 que codifica el ADN retirando el promotor de la fuente de ADN por digestión de enzimas de restricción e insertando la secuencia promotora aislada en el vector. Tanto la secuencia promotora del ligando Apo-2 natural como muchos promotores heterólogos pueden utilizarse para dirigir la amplificación y/o expresión del ADN del ligando Apo-2.

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Promotores apropiados para uso con hospederos procariotas y eucariotas son conocidos en el arte, y se describen con mayor detalle en WO97/25428.

Un método preferido para la producción de Apo-2L soluble en *E. coli* emplea un promotor inducible para la regulación de la expresión del producto. El uso de un promotor inducible, controlable, permite el crecimiento del cultivo a la densidad celular deseable antes de la inducción de la expresión del producto y la acumulación de cantidades significativas de producto que pueden no ser bien toleradas por el hospedero.

Varios sistemas promotores inducibles (T7 polimerasa, trp y alcalina fosfatasa (AP)) se han evaluado por los Solicitantes para la expresión de Apo-2L (forma 114-281). El uso de cada uno de estos tres promotores resultó en cantidades significativas de trímero de Apo-2L biológicamente activo, soluble, que se recuperó de la pasta celular recogida. El promotor AP se prefiere entre estos tres sistemas promotores inducibles ensayados debido al control de promotor más estricto y a la mayor densidad celular y a las concentraciones volumétricas alcanzadas en la pasta celular recogida.

La construcción de vectores apropiados, que contienen uno o más de los componentes ya enumerados, emplea técnicas estándar de ligamiento. Plásmidos aislados o fragmentos de ADN se segmentan, se ajustan a la medida, y se re-ligan en la forma deseada para generar los plásmidos requeridos.

En el análisis para confirmar las secuencias correctas en los plásmidos contruidos, las mezclas de ligamiento pueden usarse para transformar la cepa K12 294 de *E. coli* (ATCC 31,446) y transformantes exitosos seleccionados por resistencia a la ampicilina o tetraciclina, donde sea apropiado. Plásmidos de los transformantes se preparan, se analizan por digestión de endonucleasa de restricción, y/o se secuencian usando técnicas estándar conocidas en el arte. [Véase, por ejemplo, Messing et al., Nucleic Acids Res., 9:309 (1981); Maxam et al., Methods in Enzymology, 65:499 (1980)].

Pueden emplearse vectores de expresión que permiten la expresión transitoria en células mamíferas de ADN que codifica el ligando Apo-2. En general, la expresión transitoria involucra el uso de un vector de expresión que es capaz de replicarse eficientemente en una célula hospedera, de modo que la célula hospedera acumula muchas copias del vector de expresión y, a su vez, sintetiza altos niveles de un polipéptido deseado codificado por el vector de expresión [Sambrook et al., supra]. Sistemas de expresión transitoria, que comprenden un vector de expresión apropiado y una célula hospedera, permiten la identificación positiva conveniente de polipéptidos codificados por ADN clonados, así como el cribado rápido de tales polipéptidos respecto a propiedades biológicas o fisiológicas deseadas. Por consiguiente, sistemas de expresión transitoria son particularmente útiles en la invención para propósitos de identificar análogos y variantes del ligando Apo-2, que son el ligando Apo-2 biológicamente activo.

Otros métodos, vectores, y células hospederas apropiadas para adaptación a la síntesis del ligando Apo-2 en cultivo de células de vertebrado recombinantes se describen en Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; y EP 117,058.

Células hospederas apropiadas para clonar o expresar el ADN en los vectores en esta solicitud son las células de procariota, levadura, o eucariotas superiores. Procariotas apropiados para este propósito incluyen, pero no se limitan a, eubacterias, tales como organismos gram-negativos o gram-positivos, por ejemplo, *Enterobacteriaceae* tales como *Escherichia*, por ejemplo, *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, por ejemplo, *Salmonella typhimurium*, *Serratia*, por ejemplo, *Serratia marcescans*, y *Shigella*, así como *Bacilli* tales como *B. subtilis* y *B. licheniformis* (por ejemplo, *B. licheniformis* 41P divulgado en DD 266,710 publicada el 12 de abril de 1989), *Pseudomonas* tales como *P. aeruginosa*, y *Streptomyces*. Preferiblemente, la célula hospedera debe segregar cantidades mínimas de enzimas proteolíticas.

Aparte de los procariotas, microbios eucarióticos tales como hongos filamentosos o levadura son hospederos de clonación o expresión apropiados para vectores que codifican el ligando Apo-2. Células hospederas apropiadas para la expresión del ligando Apo-2 glucosilado se derivan de organismos multicelulares. Ejemplos de tales células hospederas, incluidas las células CHO, se describe también en WO97/25428.

Células hospederas se transfectan y preferiblemente se transforman con los vectores de expresión y clonación ya descritos para producción del ligando Apo-2 y se cultivan en medio modificado, según sea apropiado, para inducir promotores, seleccionar transformantes, o amplificar los genes que codifican las secuencias deseadas.

La transfección se refiere a la absorción de un vector de expresión por una célula hospedera, sin importar si, efectivamente, se expresan o no secuencias codificantes cualesquiera. Numerosos métodos de transfección son conocidos al experto de habilidad ordinaria, por ejemplo, CaPO_4 y electroporación. La transfección exitosa se reconoce generalmente cuando ocurre cualquier indicación de la operación de este vector en las células hospedera.

Transformación significa introducir ADN en un organismo de modo que el ADN es replicable, o bien como elemento extracromosómico o bien por un integrante cromosómico. Dependiendo de la célula huésped utilizada, la transformación se realiza usando técnicas estándar apropiadas a tales células. El tratamiento con calcio empleando cloruro de calcio, como se describe en Sambrook *et al.*, supra, o electroporación se utiliza generalmente para procariotas u otras células que contienen barreras sustanciales de pared celular. La infección con *Agrobacterium tumefaciens* se usa para la transformación de ciertas células vegetales, como se describe por Shaw *et al.*, Gene, 23:315 (1983) y WO 89/05859 publicada el 29 de junio de 1989. Además, pueden transfectarse plantas usando tratamiento con ultrasonido como se describe en WO 91/00358 publicada el 10 de enero de 1991.

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Para células mamíferas sin tales paredes celulares, puede emplearse el método de precipitación de fosfato de calcio de Graham y van der Eb, Virology, **52**:456-457 (1978). Aspectos generales de transfecciones del sistema huésped de células mamíferas se han descrito en la Patente de los Estados Unidos No. 4,399,216. Las transformaciones en levadura se llevan típicamente a cabo de acuerdo con el método de Van Solingen et al., J. Biol. Chem., **252**:946 (1977) y Hsiao et al., Proc. Natl Acad. Sci. (USA), **76**:3829 (1979). No obstante, también pueden usarse otros métodos para introducir ADN en las células, tales como por microinyección nuclear, electroporación, fusión de protoplasto bacteriano con células intactas, o policondensationes, por ejemplo, polibreno, poliornitina. Para varias técnicas de transformar células mamíferas, véase Keown et al., Methods in Enzymology, **185**:527-537 (1990) y Mansour et al., Nature, **336**:348-352 (1988).

Células procariotas usadas para producir el ligando Apo-2 pueden cultivarse en un medio de cultivo apropiado, como se describe generalmente en Sambrook et al., supra. Formas particulares de medios de cultivo, que pueden emplearse para cultivar *E. coli*, se describen además en los Ejemplos que siguen. Células hospederas mamíferas usadas para producir el ligando Apo-2 pueden cultivarse en una variedad de medios de cultivo.

Ejemplos de medios de cultivo disponibles en el comercio incluyen Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), y Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). Cualquiera de estos medios pueden suplementarse, según sea necesario, con hormonas y/u otros factores de crecimiento (tales como insulina, transferrina, o factor de crecimiento epidérmico), sales (tales como cloruro de sodio, calcio, magnesio, y fosfato), tampones (tales como HEPES), nucleótidos (tales como adenosina y timidina), antibióticos (tales como el medicamento Gentamycin™), oligoelementos (definidos como compuestos inorgánicos usualmente presentes en concentraciones finales en el rango micromolar), y glucosa o una fuente de energía equivalente. Cualesquiera

otros suplementos necesarios pueden incluirse asimismo en concentraciones apropiadas serían conocidos a los expertos en el arte. Las condiciones de cultivo, tales como temperatura, pH, y similares, son las empleadas previamente con la célula hospedera seleccionada para expresión, y serán evidentes para el profesional de experiencia ordinaria.

En general, principios, protocolos, y técnicas prácticas para maximizar la productividad de cultivos de células mamíferas pueden encontrarse en Mammalian Cell Biotechnology: A Practical Approach, M. Butler, ed. (IRL Press, 1991).

De acuerdo con un aspecto de la presente invención, uno o más iones de metal divalente se agregarán típicamente a o se incluirán en el medio de cultivo para cultivar o fermentar las células anfitrionas. Los iones de metal divalente están preferiblemente presentes en o se agregan al medio de cultivo en un nivel de concentración suficiente para mejorar la capacidad de almacenamiento, incrementar la solubilidad, o contribuir a la formación de trímeros estables de Apo-2L, coordinados por uno o más iones de zinc. La cantidad de iones de metal divalente que pueden agregarse dependerá, en parte, de la densidad de las células hospederas en el medio de cultivo o la sensibilidad potencial de las células hospederas a dichos iones de metal divalente. Con densidades mayores de células hospederas en el cultivo, puede ser ventajoso incrementar la concentración de iones de metal divalentes. Si los iones de metal divalente se agregan durante o después de la expresión del producto por las células hospederas, puede ser deseable ajustar o aumentar la concentración de iones de metal divalente a medida que se incrementa la expresión de producto por las células hospederas. Generalmente se cree que niveles trazadores de iones de metal divalente presentes en medios típicos de cultivo celular, comúnmente obtenibles, pueden no ser suficientes para una formación de trímeros estable. Por consiguiente, se prefiere la adición de cantidades adicionales de iones de metal divalente, como se describe en esta solicitud.

Preferiblemente, los iones de metal divalente se agregan al medio de cultivo en una concentración que no afecte adversa o negativamente el

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crecimiento de las células hospederas, si los iones de metal divalente se están agregando durante la fase de crecimiento de las células hospederas en el cultivo. En cultivos en matraces de agitación, se observó que el ZnSO_4 agregado en concentraciones de más de 1 mM pueden resultar en una densidad menor de las células hospederas. Los expertos en el arte observarán que las células bacterianas pueden secuestrar iones de metal en forma efectiva, formando complejos de iones de metal con matrices celulares. En consecuencia, en los cultivos celulares, es preferible agregar los iones seleccionados de metal divalente al medio de cultivo después de la fase de crecimiento (una vez alcanzada la densidad deseada de células hospederas) o justo antes de la expresión de producto por las células hospederas. Para garantizar que estén presentes cantidades suficientes de iones de metal divalente, pueden agregarse iones adicionales de metal divalente o alimentarse al medio de cultivo durante la fase de expresión de producto.

La concentración de iones de metal divalente en el medio de cultivo no debe exceder la concentración que pueda ser perjudicial o tóxica a las células hospederas. En los métodos de la invención que emplean la célula hospedera, *E. coli*, se prefiere que la concentración de los iones de metal divalente en el medio de cultivo no exceda de alrededor de 1mM (preferiblemente, $\leq 1\text{mM}$). Aún más preferiblemente, la concentración de iones de metal divalente en el medio de cultivo es alrededor de 50 micromolar a alrededor de 250 micromolar. Muy preferiblemente, el ión de metal divalente usado en tales métodos es sulfato de zinc. Es deseable agregar los iones de metal divalente al cultivo celular en una cantidad en donde los iones de metal y el trímero de ligando Apo-2 puedan estar presentes en una relación molar 1:1.

Los iones de metal divalente pueden agregarse al medio de cultivo en cualquier forma aceptable. Por ejemplo, puede prepararse una solución del ión de metal utilizando agua, y la solución del ión de metal divalente puede agregarse o alimentarse al medio de cultivo.

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La expresión del Apo-2L puede medirse en una muestra directamente, por ejemplo, mediante transferencia convencional de Southern ("*Southern blotting*"), transferencia de Northern para cuantificar la transcripción de mRNA [Thomas, *Proc. Natl. Acad. Sci. USA*, **77**:5201-5205 (1980)], *dot blotting* (análisis de ADN), o transferencia *in situ*, usando una sonda apropiadamente marcada, basada en las secuencias suministradas aquí. Pueden emplearse varias etiquetas, muy comúnmente radioisótopos y, particularmente, ^{32}P . No obstante, pueden emplearse también otras técnicas, tales como utilizar nucleótidos modificados con biotina para introducción en un polinucleótido. La biotina sirve entonces como el sitio de unión a avidina o anticuerpos, que pueden marcarse con una extensa variedad de etiquetas, tales como radionucleótidos, fluorescentes o enzimas. Alternativamente, pueden emplearse anticuerpos que puedan reconocer bicatenarios específicos, incluidos bicatenarios de ADN, bicatenarios de ARN, y bicatenarios híbridos ADN-ARN o bicatenarios de proteína-ADN. Los anticuerpos, a su vez, pueden marcarse y el análisis puede llevarse a cabo donde el bicatenario se une a la superficie de modo que, después de la formación del bicatenario en la superficie, pueda detectarse la presencia de anticuerpo unido al bicatenario. Alternativamente, la expresión de los genes puede medirse por métodos inmunológicos, tales como tinción inmunohistoquímica de células o secciones de tejido y análisis de cultivo celular o fluidos corporales, para cuantificar directamente la expresión del producto génico. Con técnicas de tinción inmunohistoquímica, se prepara una muestra de células, típicamente por deshidratación y fijación, seguida por la reacción con anticuerpos marcados, específicos para el producto génico acoplado, donde las etiquetas se utilizan por lo general en forma detectable visualmente, tal como etiquetas enzimáticas, etiquetas fluorescentes, etiquetas luminescentes, y similares.

Anticuerpos útiles para tinción inmunohistoquímica y/o análisis de fluidos de muestra pueden ser monoclonales o policlonales, y pueden prepararse en cualquier mamífero. Convenientemente, los anticuerpos pueden prepararse contra un polipéptido de ligando Apo-2 natural o contra

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un péptido sintético, basados en las secuencias de ADN, suministradas en esta solicitud, o contra una secuencia exógena fusionada al ADN del ligando Apo-2 y que codifique un epítipo de anticuerpo específico.

El ligando Apo-2 se recupera preferiblemente del medio de cultivo como un polipéptido segregado, si bien puede recuperarse también de los lisados de células hospederas cuando se producen directamente sin una señal secretora. Si el ligando Apo-2 es unido a la membrana, puede desprenderse de la membrana usando una solución detergente apropiada (*por ejemplo*, Triton-X 100) o su región extracelular puede liberarse por segmentación enzimática.

Cuando el ligando Apo-2 se produce en una célula recombinante distinta de una de origen humano, el ligando Apo-2 está libre de proteínas o polipéptidos de origen humano. No obstante, usualmente es necesario recuperar o purificar el ligando Apo-2 de proteínas de células recombinantes o polipéptidos para obtener preparaciones que sean sustancialmente homogéneas en cuanto al ligando Apo-2. Como un primer paso, el medio de cultivo o lisado puede centrifugarse para retirar desechos celulares particulados. A continuación el ligando Apo-2 se purifica de proteínas solubles contaminantes y polipéptidos, siendo ejemplares los siguientes procedimientos de procedimientos apropiados de purificación: por fraccionamiento en columna de intercambio iónico, precipitación en etanol, HPLC de fase inversa; cromatografía en sílice o en una resina de intercambio catiónico tal como DEAE o CM; cromatofocado; SDS-PAGE; precipitación en sulfato de amonio; filtración en gel usando, por ejemplo, Sephadex G-75; columnas de diafiltración y proteína A Sepharose para eliminar contaminantes tales como IgG.

En una incorporación preferida, el ligando Apo-2 puede aislarse por cromatografía de afinidad. Fragmentos o variantes del ligando Apo-2, en los cuales se han eliminado, insertado o substituido residuos se recuperan en la misma forma que el ligando Apo-2 natural, tomando en consideración cualesquiera cambios sustanciales en las propiedades, ocasionados por la variación. Por ejemplo, la preparación de una fusión de ligando Apo-2 con

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otra proteína o polipéptido, verbigracia, un antígeno bacteriano o vírico, facilita la purificación; una columna de inmunoadfinidad, que contenga un anticuerpo para el antígeno, puede usarse para absorber el polipéptido de fusión.

Igualmente puede ser útil un inhibidor de proteasa tal como fenil metil sulfonil fluoruro (PMSF) para inhibir la degradación proteolítica durante la purificación, y pueden incluirse antibióticos para prevenir el crecimiento de contaminantes adventicios. Un experto en el arte observará que métodos de purificación apropiados para el ligando Apo-2 natural pueden requerir modificación para explicar los cambios en el carácter del ligando Apo-2 o sus variantes luego de la expresión en un cultivo de células recombinantes.

Durante cualquiera de tales pasos de purificación, puede ser deseable exponer el Apo-2L recuperado a una solución que contenga iones de metal divalente o a un material de purificación (tal como un medio de cromatografía o soporte) que contenga uno o más iones de metal divalente. En una incorporación preferida, los iones de metal divalente y/o agente reductor se usa durante la recuperación o purificación del Apo-2L. Opcionalmente, los dos, los iones de metal divalente y el agente reductor, tal como DTT o BME, pueden utilizarse durante la recuperación o purificación del Apo-2L. Se cree que el uso de iones de metal divalente durante la recuperación o purificación permitirá la estabilidad del trímero Apo-2L o preservará el trímero Apo-2L formado durante el paso de cultivar las células.

La siguiente descripción se refiere también a métodos de producir el ligando Apo-2 unido covalentemente (en lo sucesivo, "conjugado") a uno o más grupos químicos. Grupos químicos apropiados para uso en un conjugado de Apo-2L de la presente invención son preferiblemente no significativamente tóxicos o inmunógenos. Opcionalmente, el grupo químico se selecciona para producir un conjugado Apo-2L que pueda guardarse y usarse bajo condiciones apropiadas de almacenamiento. Una variedad de grupos químicos ejemplares que pueden conjugarse a

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polipéptidos son bien conocidos en el arte e incluyen, por ejemplo, carbohidratos, tales como aquellos carbohidratos que ocurren naturalmente en glucoproteínas, poliglutamato, y polímeros no proteináceos, tales como polioles (véase, por ejemplo, Patente de los Estados Unidos No. 6,245,901).

Un poliol, por ejemplo, puede conjugarse a polipéptidos tales como un Apo-2L en uno o más residuos de aminoácidos, incluidos residuos de lisina, como se divulga en WO 93/00109, supra. El poliol empleado puede ser cualquier polímero óxido de poli(alquilen) y puede tener una cadena lineal o ramificada. Polioles apropiados incluyen los sustituidos en una o más posiciones hidroxilo con un grupo químico, tal como un grupo alquilo que tiene entre uno y cuatro carbonos. Típicamente, el poliol es un poli(alquilen glicol), tal como poli(etilen glicol) (PEG) y, en consecuencia, para facilidad de la descripción, el resto de la discusión se refiere a una incorporación ejemplar en donde el poliol empleado es PEG y el proceso de conjugar el poliol a un polipéptido se designa "PEGilación". No obstante, los expertos en el arte reconocerán que pueden utilizarse otros polioles, tales como, por ejemplo, copolímeros poli(propilen glicol) y polietilen-polipropilen glicol, usando las técnicas de conjugación descritas en esta solicitud para PEG.

El peso molecular promedio del PEG empleado en la pegilación del Apo-2L puede variar y, típicamente, está en el rango desde alrededor de 500 a alrededor de 30.000 daltons (D). Preferiblemente, el peso molecular promedio del PEG va desde alrededor de 1.000 a alrededor de 25.000 D y, más preferiblemente, desde alrededor de 1.000 a alrededor de 5.000 D. En una incorporación, la pegilación se lleva a cabo con un PEG que tiene un peso molecular promedio de alrededor de 1.000 D. Opcionalmente, el homopolímero PEG es no sustituido, pero puede sustituirse también en un extremo con un grupo alquilo. Preferiblemente, el grupo alquilo es un grupo C1-C4 alquilo y, muy preferiblemente, un grupo metilo. Las preparaciones PEG se encuentran disponibles en el comercio y, típicamente, aquellas preparaciones PEG apropiadas para uso en la presente invención son preparaciones no homogéneas comercializadas de acuerdo con el peso

molecular promedio. Por ejemplo, preparaciones PEG(5000) disponibles en el comercio contienen, por lo general, moléculas que varían ligeramente en peso molecular, usualmente ± 500 D.

El ligando Apo-2 de la invención puede estar en varias formas, tal como en forma de monómero o forma de trímero (que comprende tres monómeros). Opcionalmente, un trímero Apo-2L se pegilará en una manera tal que una molécula PEG se enlace o conjugue a uno, dos o cada uno de los tres monómeros que componen el Apo-2L trimérico. En dicha incorporación, se prefiere que el PEG empleado tenga un peso molecular promedio de alrededor de 1.000 a alrededor de 5.000 D. Se contempla además que los trímeros Apo-2L puedan pegilarse "parcialmente", es decir, en donde sólo uno o dos de los tres monómeros que componen el trímero se enlazan o conjugan a PEG.

En el arte se conoce una variedad de métodos para pegilar proteínas. Métodos específicos de producir proteínas conjugadas a PEG incluyen los métodos descritos en la Patente de los Estados Unidos No. 4,179,337, Patente de los Estados Unidos No. 4,935,465 y Patente de los Estados Unidos No. 5,849,535. Típicamente la proteína se enlaza covalentemente mediante uno o más de los residuos de aminoácidos de la proteína a un grupo reactivo terminal en el polímero, dependiendo principalmente de las condiciones de reacción, el peso molecular del polímero, etc. El polímero con el grupo(s) reactivo se designa en esta solicitud como polímero activado. El grupo reactivo reacciona selectivamente con los grupos amino libres u otros grupos reactivos en la proteína. El polímero PEG puede acoplarse al grupo amino u otro grupo reactivo en la proteína en una manera aleatoria o específica del sitio. Se entenderá, sin embargo, que el tipo y cantidad del grupo reactivo seleccionado, así como el tipo de polímero empleado, para obtener resultados óptimos, dependerá de la proteína particular o variante de proteína empleada para impedir que el grupo reactivo tenga que reaccionar con demasiados grupos particularmente activos en la proteína. Puesto que esto no es completamente posible de evitar, se recomienda que generalmente se emplee desde alrededor de 0.1 a

1.000 moles, preferiblemente 2 a 200 moles, de polímero activado por mol de proteína, dependiendo de la concentración de proteína. La cantidad final de polímero activado por mol de proteína es un saldo para mantener la actividad óptima, optimizando al mismo tiempo, si es posible, la semivida circulatoria de la proteína.

Se contempla asimismo que el Apo2L descrito en esta solicitud puede enlazarse o fusionarse además a secuencias de cremallera de leucina usando técnicas conocidas en el arte.

Métodos para generar anticuerpos de receptor de muerte celular y anticuerpos CD20 se describen también en esta solicitud. El antígeno a usarse para la producción, o cribado, de anticuerpo puede ser, por ejemplo, una forma soluble del antígeno o una porción del mismo, que contenga el epítipo deseado. Alternativa o, adicionalmente, pueden usarse células que expresen el antígeno en su superficie celular para generar, o cribar, el anticuerpo. Otras formas del antígeno, útiles para generar anticuerpo, serán evidentes a los expertos en el arte.

(i) *Anticuerpos policlonales*

Los anticuerpos policlonales se producen preferiblemente en animales por inyecciones múltiples subcutáneas (sc) o intraperitoneales (ip) del antígeno relevante y un adyuvante. Puede ser útil conjugar el antígeno relevante a una proteína que sea inmunógena en la especie a inmunizar, *por ejemplo*, hemocianina de lapa, seroalbúmina, tiroglobulina bovina, o inhibidor de tripsina de soya, usando un agente bi-funcional o derivatizante, *por ejemplo*, éster de maleimidobenzoyl sulfosuccinimida (conjugación mediante residuos de cisteína), N-hidroxisuccinimida (mediante residuos de lisina), glutaraldehído, anhídrido succínico, SOCl_2 , o $\text{R}^1\text{N}=\text{C}=\text{NR}$, donde R y R^1 son diferentes grupos alquilo.

Los animales se inmunizan contra el antígeno, conjugados inmunógenos, o derivados combinando, *por ejemplo*, 100 μg ó 5 μg de la proteína o conjugado (para conejos o ratones, respectivamente) con 3

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volúmenes de adyuvante completo de Freund e inyectando la solución intradérmicamente en múltiples sitios. Un mes después, los animales se revacunaron/reforzaron con 1/5 a 1/10 la cantidad original de péptido o conjugado en adyuvante completo de Freund por inyección subcutánea en múltiples sitios. Siete a catorce días después, los animales se sangran y el suero se analiza para establecer la concentración de anticuerpos. Los animales se revacunan hasta que se nivela la concentración. Preferiblemente, el animal se revacuna con el conjugado del mismo antígeno, pero se conjuga a una proteína diferente y/o a través de un reactivo diferente de enlazamiento cruzado. Asimismo pueden elaborarse conjugados en cultivo de células recombinantes como fusiones de proteína. Además, agentes agregantes tales como sulfato de aluminio son apropiados para intensificar la respuesta inmunitaria.

(ii) *Anticuerpos monoclonales*

Anticuerpos monoclonales se obtienen de una población de anticuerpos sustancialmente homogéneos, *es decir*, los anticuerpos individuales que comprenden la población son idénticos, excepto por posibles mutaciones que ocurran naturalmente, que puedan estar presentes en cantidades menores. Por consiguiente, el calificativo "monoclonal" indica el carácter del anticuerpo como no siendo una mezcla de anticuerpos discretos.

Por ejemplo, los anticuerpos monoclonales pueden elaborarse usando el método del hibridoma, descrito por primera vez por Kohler et al., *Nature*, 256:495 (1975), o puede elaborarse por métodos de ADN recombinante (Patente de los Estados Unidos No. 4,816,567).

En el método del hibridoma, un ratón u otro animal huésped apropiado, tal como un hámster, se inmuniza, tal como se describe más arriba en el texto, para provocar linfocitos que produzcan o sean capaces de producir anticuerpos, que se unirán específicamente a la proteína usada para inmunización. Alternativamente, los linfocitos pueden inmunizarse *in vitro*.

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En seguida, los linfocitos se fusionan con células de mieloma usando un agente fusionante apropiado, tal como polietilen glicol, para formar una célula de hibridoma (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986))

Las células de hibridoma así preparadas se siembran y cultivan en un medio de cultivo adecuado, que preferiblemente contiene una o más sustancias que inhiben el crecimiento o supervivencia de las células de mieloma progenitoras no fusionadas. Por ejemplo, si las células de mieloma progenitoras carecen de la enzima hipoxantina guanina fosforibosil transferasa (HGPRT o HPRT), el medio de cultivo para los hibridomas incluirá típicamente hipoxantina, aminopterina, y timidina (medio HAT), sustancias que impiden el crecimiento de células deficientes en HGPRT.

Células preferidas de mieloma, son aquellas que se fusionan eficientemente, favorecen una producción estable de alto nivel de anticuerpo por las células seleccionadas productoras de anticuerpos, y son sensibles a un medio tal como HAT. Entre éstas, líneas preferidas de células de mieloma son líneas de mieloma murino, tales como las derivadas de tumores de ratón MOPC-21 y MPC-11 disponibles del Salk Institute Cell Distribution Center, San Diego, California USA, y células SP-2 o células X63-Ag8-653 disponibles de la American Type Culture Collection, Manassas, Virginia USA. También se han descrito líneas de células de mieloma humano y heteromieloma ratón-humano para la producción de anticuerpos monoclonales humanos (Kozbor, *J. Immunol*, 133:3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, páginas 51-63 (Marcel Dekker, Inc., New York, 1987)).

El medio de cultivo en el cual están creciendo las células del hibridoma se analiza para determinar la producción de anticuerpos monoclonales dirigidos contra el antígeno. Preferiblemente, la especificidad de unión de anticuerpos monoclonales producidos por células del hibridoma se determina por inmunoprecipitación o por un análisis de

unión *in vitro*, tal como radioinmunoanálisis (RIA) o análisis de inmunoabsorción ligado a enzima (ELISA).

Por ejemplo, la afinidad de unión del anticuerpo monoclonal puede determinarse mediante el análisis Scatchard de Munson *et al.*, *Anal. Biochem.*, 107:220 (1980).

Una vez identificadas las células del hibridoma que producen anticuerpos de la especificidad, afinidad y/o actividad deseada, los clones pueden subclonarse limitando los procesos de dilución y cultivarse por métodos estándar (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Medios apropiados de cultivo para este propósito incluyen, por ejemplo, medio D-MEM o RPMI-1640. Adicionalmente, las células del hibridoma pueden cultivarse *in vivo* como tumores ascíticos en un animal.

Los anticuerpos monoclonales segregados por los subclones se separan convenientemente del medio de cultivo, fluido ascítico, o suero mediante procedimientos convencionales de purificación de inmunoglobulina tales como, por ejemplo, cromatografía de hidroxilapatita proteína A-sefarosa, electroforesis en gel, diálisis, o cromatografía de afinidad.

El ADN que codifica los anticuerpos monoclonales se aísla fácilmente y se secuencia usando procedimientos convencionales (*por ejemplo*, usando sondas de oligonucleótidos que son capaces de unirse específicamente a genes que codifican las cadenas pesada y ligera de anticuerpos murinos). Las células del hibridoma sirven como fuente preferida de tal ADN. Una vez aislado, el ADN puede ponerse en vectores de expresión, los cuales se transfectan luego en células hospederas tales como células de *E. coli*, células COS de simio, células derivadas de ovario de hámster chino (CHO), o células de mieloma, que no producen de otro modo proteína de inmunoglobulina, con el fin de sintetizar anticuerpos monoclonales en tales células hospederas. Artículos de reseña sobre la expresión recombinante en bacterias de ADN que codifica el anticuerpo

incluyen Skerra *et al.*, *Curr. Opinion in Immunol*, 5:256-262 (1993) y Plückthun, *Immunol. Revs.* 130:151-188 (1992).

En una incorporación adicional, los anticuerpos pueden aislarse de bibliotecas de fagos de anticuerpos, generadas usando las técnicas descritas en McCafferty *et al.*, *Nature*, 348:552-554 (1990). Clackson *et al.*, *Nature*, 352:624-628 (1991) y Marks *et al.*, *J. Mol Biol*, 222:581-597 (1991) describen el aislamiento de anticuerpos humanos y murinos, respectivamente, usando bibliotecas de fagos. Publicaciones subsiguientes describen la producción de anticuerpos humanos de alta afinidad (rango nM) mediante entremezcla de cadenas (Marks *et al.*, *Bio/Technology*, 10:779-783 (1992)), así como infección combinatoria y recombinación *in vivo* como una estrategia para construir bibliotecas muy grandes de fagos (Waterhouse *et al.*, *Nucl Acids Res.*, 21:2265-2266 (1993)). Por consiguiente, estas técnicas son alternativas viables a las técnicas tradicionales de hibridoma de anticuerpo monoclonal para el aislamiento de anticuerpos monoclonales.

El ADN puede modificarse además, por ejemplo, sustituyendo la secuencia codificante para dominios constantes de cadena ligera y pesada en vez de las secuencias murinas homólogas (Patente de los Estados Unidos No. 4,816,567; Morrison, *et al.*, *Proc. Natl Acad. Sci. USA*, 81:6851 (1984)), o uniendo a la secuencia codificante de inmunoglobulina toda, o parte, de la secuencia codificante para un polipéptido no inmunoglobulina.

Típicamente tales polipéptidos no-inmunoglobulina se sustituyen por los dominios constantes de un anticuerpo, o se sustituyen por los dominios variables de un sitio combinador de antígeno de un anticuerpo para crear un anticuerpo bivalente, quimérico, que comprende un sitio combinador de antígeno, con especificidad para un antígeno, y otro sitio combinador de antígeno con especificidad para un antígeno diferente..

(iii) Anticuerpos humanizados

Métodos para humanizar anticuerpos no humanos son bien conocidos en el arte. Preferiblemente, un anticuerpo humanizado tiene uno o más residuos de aminoácidos introducidos en él de una fuente que es no

humana. Estos residuos de aminoácidos no humanos se denominan a menudo residuos de "importación", los cuales se toman típicamente de un dominio variable de "importación". La humanización puede realizarse esencialmente siguiendo el método de Winter *et al.*, Jones *et al.*, *Nature* 321:522-525 (1986); Riechmann *et al.*, *Nature* 332:323-327 (1988); Verhoeven *et al.*, *Science* 239:1534-1536 (1988), o mediante sustitución de secuencias de región hipervariable para las secuencias correspondientes de un anticuerpo humano. En consecuencia, tales anticuerpos "humanizados" son anticuerpos quiméricos (Patente de los Estados Unidos No. 4,816,567), en donde sustancialmente menos de un dominio variable humano intacto se ha sustituido por la secuencia correspondiente de una especie no humana. En la práctica, anticuerpos humanizados son típicamente anticuerpos humanos en los cuales algunos residuos de la región hipervariable y, posiblemente, algunos residuos FR se sustituyen con residuos de sitios análogos en anticuerpos de roedor.

La elección de dominios variables humanos, livianos y pesados, para usarlos en elaborar los anticuerpos humanizados es muy importante para reducir la antigenicidad. De acuerdo con el denominado método de "ajuste óptimo", se criba la secuencia del dominio variable de un anticuerpo de roedor contra toda la biblioteca de secuencias de dominio variable conocidas. La secuencia humana que es más próxima a la del roedor se acepta entonces como la región marco (FR) humano para el anticuerpo humanizado. Sims *et al.*, *J. Immunol.*, 151:2296 (1993); Chothia *et al.*, *J. Mol. Biol.*, 196:901 (1987)). Otro método usa una región marco particular derivado de la secuencia de consenso de todos los anticuerpos humanos de un subgrupo particular de cadenas ligeras y pesadas. El mismo marco puede utilizarse para varios anticuerpos humanizados diferentes. Carter *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta *et al.*, *J. Immunol.*, 151:2623 (1993)).

Es además importante que los anticuerpos se humanicen conservando una alta afinidad para el antígeno y otras propiedades biológicas favorables. Para lograr este objetivo, de acuerdo con un método preferido, anticuerpos

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humanizados se preparan por un proceso de análisis de las secuencias progenitoras y varios productos humanizados conceptuales usando modelos tri-dimensionales de las secuencias progenitoras y humanizadas. Comúnmente se dispone de modelos tri-dimensionales de inmunoglobulina y son familiares a los expertos en el arte. Se dispone de programas de computador, los cuales ilustran y visualizan estructuras probables de conformación tri-dimensional de secuencias seleccionadas de inmunoglobulina candidata. La inspección de estas visualizaciones permite el análisis del papel probable de los residuos en el funcionamiento de la secuencia de inmunoglobulina candidata, *es decir*, el análisis de residuos que influyen en la capacidad de la inmunoglobulina candidata de unir su antígeno. De esta forma, pueden seleccionarse residuos FR y combinarse de las secuencias receptoras y de importación, de modo que se logre la característica del anticuerpo deseado, tal como creciente afinidad para el antígeno(s) diana. En general, los residuos de región hipervariable se hallan directa y, la mayoría de las veces, sustancialmente implicados en influir en la unión del antígeno.

(iv) Anticuerpos humanos

Como una alternativa a la humanización, pueden generarse anticuerpos humanos. Por ejemplo, actualmente es posible producir animales transgénicos (*verbigracia*, ratones) que son capaces, luego de la inmunización, de producir un completo repertorio de anticuerpos humanos en la ausencia de producción de inmunoglobulina endógena. Por ejemplo, se ha descrito que la eliminación homocigótica del gen de la región de unión a la cadena pesada (J_H) del anticuerpo en ratones mutantes quiméricos y de línea germinatural resulta en completa inhibición de la producción de anticuerpos endógenos. La transferencia del arreglo de genes de inmunoglobulina de línea germinatural humana en tales ratones mutantes de línea germinatural resultará en la producción de anticuerpos humanos luego de la exposición al antígeno. Véase, *por ejemplo*, Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al.*, *Nature*, 362:255-

258 (1993); Bruggermann *et al.*, *Year in Immuno.*, 7:33 (1993); Patentes de los Estados Unidos No. 5,591,669, 5,589,369 y 5,545,807.

Alternativamente, tecnología de muestrario de fagos puede usarse para producir anticuerpos humanos y fragmentos de anticuerpo *in vitro*, a partir de repertorios de genes de dominio variable (V) de inmunoglobulina de donantes no inmunizados. De acuerdo con esta técnica, genes de dominio V de anticuerpo se clonan sin cambio en la fase de lectura ("*are cloned in-frame*") en un gen de cubierta proteínica (cápside) mayor o menor de un bacteriófago filamentoso, tal como M13 ó fd, y se visualizan como fragmentos funcionales de anticuerpo en la superficie de la partícula del fago. Debido a que la partícula filamentosa contiene una copia de ADN de una sola hebra del genoma del fago, las selecciones basadas en las propiedades funcionales del anticuerpo resultan igualmente en la selección del gen codificante del anticuerpo que exhibe estas propiedades. Por consiguiente, el fago imita algunas de las propiedades del linfocito B. El muestrario de fagos puede realizarse en una variedad de formatos, reseñados en, *por ejemplo*, Johnson, Kevin S. y Chiswell, David J., *Current Opinion in Structural Biology* 3:564-571 (1993). Para el muestrario de fagos pueden emplearse varias fuentes de segmentos de gen V. Clackson *et al.*, *Nature* 352:624-628 (1991) aislaron un arreglo diverso de anticuerpos de anti-oxazolona de una pequeña biblioteca combinatoria aleatoria de genes V derivados de los bazo de ratones inmunizados. Puede construirse un repertorio de genes V de donantes no inmunizados humanos, y anticuerpos a un arreglo diverso de antígenos (incluidos auto-antígenos) pueden aislarse siguiendo esencialmente las técnicas descritas por Marks *et al.*, *J. Mol. Biol.* 222:581-597 (1991), o Griffith *et al.*, *EMBO J.* 12:725-734 (1993). Véase también, Patentes de los Estados Unidos No. 5,565,332 y 5,573,905.

Anticuerpos humanos pueden generarse igualmente *in vitro* por linfocitos B activados (véase Patentes de los Estados Unidos No. 5,567,610 y 5,229,275).

(v) *Fragmentos de anticuerpo*

Se han desarrollado varias técnicas para la producción de fragmentos de anticuerpo. Tradicionalmente, estos fragmentos se derivaron por digestión proteolítica de anticuerpos intactos (véase, *por ejemplo*, Morimoto *et al.*, *Journal Biochemical and Biophysical Methods* 24:107-117 (1992); y Brennan *et al.*, *Science* 229:81 (1985)). Sin embargo, estos fragmentos pueden producirse ahora directamente por células hospederas recombinantes. Por ejemplo, los fragmentos de anticuerpo de anticuerpo pueden aislarse de bibliotecas de fagos de anticuerpos discutidas anteriormente. Alternativamente, fragmentos Fab'-SH pueden recuperarse directamente de *E. coli* y acoplarse químicamente para formar fragmentos F(ab')₂ (Carter *et al.*, *Bio/Technology* 10:163-167 (1992)). De acuerdo con otra metodología, fragmentos F(ab')₂ pueden aislarse directamente de cultivo de células hospederas recombinantes. Otras técnicas para la producción de fragmentos anticuerpo serán evidentes al profesional experto. En otras incorporaciones, el anticuerpo de elección es un fragmento Fv de cadena única (scFv). Véase WO 93/16185; Patente de los Estados Unidos No. 5,571,894 y Patente de los Estados Unidos No. 5,587,458. El fragmento de anticuerpo puede ser igualmente un "anticuerpo lineal", por ejemplo, como se describe en la Patente de los Estados Unidos 5,641,870. Tales fragmentos de anticuerpo lineal pueden ser monoespecíficos o bi-específicos.

(vi) Anticuerpos biespecíficos

Anticuerpos biespecíficos son anticuerpos que poseen afinidades de unión para dos epítopos diferentes, como mínimo. Anticuerpos biespecíficos ejemplares pueden unirse a dos epítopos diferentes de los receptores CD20, DR4 o DR5. Los anticuerpos biespecíficos pueden usarse igualmente para localizar agentes citotóxicos a un linfocito B. Estos anticuerpos poseen un brazo de unión a marcador de linfocito B y un brazo que une el agente citotóxico (por ejemplo, saporina anti-interferon- α , vinca alcaloide, cadena de ricina A, metotrexato o hapteno de isótopo radioactivo). Los anticuerpos biespecíficos pueden prepararse como



anticuerpos de longitud total o fragmentos de anticuerpo (por ejemplo, anticuerpos biespecíficos $F(ab')_2$).

Métodos para elaborar anticuerpos biespecíficos son conocidos en el arte. La producción tradicional de anticuerpos biespecíficos de longitud total se basa en la co-expresión de dos parejas de cadena ligera-cadena pesada de inmunoglobulina, donde las dos cadenas tienen especificidades diferentes. Millstein *et al.*, *Nature*, 305:537-539 (1983). Debido a la clasificación aleatoria de cadenas ligeras y pesadas de inmunoglobulina, estos hibridomas (cuadromas) producen una mezcla potencial de 10 diferentes moléculas de anticuerpo, de las cuales sólo una posee la estructura biespecífica correcta. La purificación de la molécula correcta, lo cual se hace usualmente por los pasos de cromatografía de afinidad, es más bien difícil, y los rendimientos del producto son bajos. Procedimientos similares se divulgan en WO 93/08829 y en Traunecker *et al.*, *EMBO J.*, 10:3655-3659 (1991).

De acuerdo con un enfoque diferente, dominios variables de anticuerpo con las especificidades de unión deseadas (sitios combinadores de anticuerpo-antígeno) se fusionan a secuencias de dominio constante de inmunoglobulina. La fusión es preferiblemente con un dominio constante de cadena pesada de inmunoglobulina, que comprende por lo menos parte de las regiones de unión flexible, CH2 y CH3. Se prefiere tener la primera región constante de cadena pesada (CH1) conteniendo el sitio necesario para unión de la cadena ligera, presente en por lo menos una de las fusiones. ADN que codifican las fusiones de cadena pesada de inmunoglobulina y, si se desea, la cadena ligera de inmunoglobulina, se insertan en factores de expresión separados, y se co-transfectan en un organismo hospedero apropiado. Esto facilita una gran flexibilidad en ajustar las proporciones mutuas de los tres fragmentos del polipéptido en incorporaciones cuando proporciones desiguales de las tres cadenas de polipéptido usadas en la construcción proporcionan los rendimientos óptimos. No obstante, es posible insertar las secuencias codificantes para las dos o todas las tres cadenas de polipéptido en un vector de expresión cuando la expresión de

por lo menos dos cadenas de polipéptido en proporciones iguales resulta en altos rendimientos, o cuando las proporciones no son de significancia particular.

En una incorporación preferida de este enfoque, los anticuerpos biespecíficos se componen de una cadena pesada de inmunoglobulina híbrida con una primera especificidad de unión en un brazo, y una pareja cadena ligera-cadena pesada de inmunoglobulina híbrida (que proporciona una segunda especificidad de unión) en el otro brazo. Se encontró que esta estructura asimétrica facilita la separación del compuesto biespecífico deseado de combinaciones no deseadas de cadena de inmunoglobulina, en la medida en que la presencia de una cadena ligera de inmunoglobulina en sólo una mitad de las moléculas biespecíficas ofrece un modo fácil de separación. Esta metodología se divulga en WO 94/04690. Para mayores detalles de generar anticuerpos biespecíficos, véase, por ejemplo, Suresh et al., *Methods in Enzymology* 121: 210 (1986). De acuerdo con otro enfoque descrito en WO 96/27011 o Patente de los Estados Unidos 5,731,168, la interfase entre una pareja de moléculas de anticuerpo puede diseñarse para maximizar el porcentaje de heterodímeros que se recuperan del cultivo de células recombinantes. La interfase preferida comprende por lo menos una parte de la región C_H3 de un dominio constante de anticuerpo. En este método, una o más cadenas laterales pequeñas de aminoácidos de la interfase de la primera molécula de anticuerpo se reemplazan con cadenas laterales más grandes (*por ejemplo*, tirosina o triptófano). "Cavidades" compensatorias de tamaño idéntico o similar a la cadena(s) lateral grande se crean en la interfase de la segunda molécula de anticuerpo reemplazando cadenas laterales de aminoácidos grandes con más pequeñas (*por ejemplo*, alanina o treonina). Esto proporciona un mecanismo para aumentar el rendimiento del heterodímero sobre otros productos finales indeseados tales como homodímeros.

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Anticuerpos biespecíficos incluyen anticuerpos enlazados cruzadamente o de "heteroconjugado". Por ejemplo, uno de los anticuerpos en el heteroconjugado puede acoplarse a avidina, el otro a biotina. Tales anticuerpos, por ejemplo, han sido propuestos para apuntar células del sistema inmunitario a células indeseadas (Patente de los Estados Unidos No. 4,676,980), y para tratamiento de infección de VIH (WO 91/00360, WO 92/200373, y EP 03089). Los anticuerpos de heteroconjugado pueden elaborarse usando cualesquiera métodos convenientes de enlazamiento cruzado. Agentes adecuados de enlazamiento cruzado son bien conocidos en el arte, y se divulgan en la Patente de los Estados Unidos No. 4,676,980, junto con un número de técnicas de enlazamiento cruzado.

Asimismo, se han descrito en la literatura técnicas para generar anticuerpos biespecíficos a partir de fragmentos de anticuerpo. Por ejemplo, anticuerpos biespecíficos pueden prepararse usando ligamiento químico. Brennan et al., Science, 229: 81 (1985); Shalaby et al., J. Exp. Med., 175: 217-225 (1992).

Se han descrito asimismo varias técnicas para elaborar y aislar fragmentos de anticuerpo bivalentes, directamente de cultivo de células recombinantes. Por ejemplo, se han producido anticuerpos biespecíficos usando cremalleras de leucina. Kostelny et al., J. Immunol., 148(5): 1547-1553 (1992). Los péptidos de cremallera de leucina de las proteínas Fos y Jun se enlazaron a las porciones Fab' de dos anticuerpos diferentes mediante fusión de genes. Los homodímeros de anticuerpo se redujeron en la región de unión flexible para formar monómeros y luego se re-oxidaron para formar los heterodímeros del anticuerpo. La tecnología "diacuerpo" descrita por Hollinger et al., Proc. Natl. Acad. Sci. USA, 90: 6444-6448 (1993) ha proporcionado un mecanismo alternativo para elaborar fragmentos de anticuerpos biespecíficos. Los fragmentos comprenden un dominio variable de cadena pesada (V_H) conectado a un dominio variable de cadena ligera (V_L) por un conector que es demasiado corto para permitir el emparejamiento entre los dos dominios en la misma cadena. Por consiguiente, los dominios V_H y V_L de un fragmento son forzados a

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emparejarse con los dominios V_L y V_H complementarios del otro fragmento, formando así dos sitios de unión de antígeno. En la misma forma se ha informado de otra estrategia para elaborar fragmentos de anticuerpos biespecíficos mediante el uso de dímeros Fv de cadena única (sFv). Véase Gruber *et al.*, *J. Immunol.*, 152:5368 (1994).

Se contemplan anticuerpos con más de dos valencias. Por ejemplo, pueden prepararse anticuerpos triespecíficos. Tutt *et al.* *J. Immunol.* 147: 60 (1991). Anticuerpos con tres o más sitios de unión a antígeno se describen en WO01/77342 (Miller and Presta), incorporada expresamente en esta solicitud por referencia.

El anticuerpo usado en los métodos o incluido en los artículos de fabricación en esta solicitud se conjuga opcionalmente a un agente citotóxico.

Agentes quimioterapéuticos útiles en la generación de tales conjugados anticuerpo-agente citotóxico se han descrito anteriormente.

También se contemplan en esta solicitud conjugados de un anticuerpo y una o más toxinas de molécula pequeña tales como caliqueamicina, maitansina (Patente de los Estados Unidos 5,208,020), tricoteno y CC1065. En una incorporación de la invención, el anticuerpo se conjuga a una o más moléculas de maitansina (por ejemplo, alrededor de 1 a alrededor de 10 moléculas de maitansina por molécula de anticuerpo). La maitansina puede, por ejemplo, convertirse en May-SS-Me, la cual puede reducirse a May-SH3 y hacerse reaccionar con anticuerpo modificado (Chari *et al.* *Cancer Research* 52: 127-131 (1992)) para generar un inmunoconjugado maitansinoide-anticuerpo.

Alternativamente, el anticuerpo se conjuga a una o más moléculas de caliqueamicina. La familia de caliqueamicina de antibióticos es capaz de producir cortes de ADN de doble hebra en concentraciones sub-picomolares. Análogos estructurales de caliqueamicina que pueden usarse incluyen, pero no se limitan a, γ_1^I , α_2^I , α_3^I , N-acetil- γ_1^I , PSAG y θ^I (Hinman *et al.*, *Cancer Res.* 53:3336-3342 (1993) y Lode *et al.*, *Cancer Res.* 58:2925-2928 (1998)).

TOXINAS ENZIMÁTICAMENTE ACTIVAS Y SUS FRAGMENTOS

Toxinas enzimáticamente activas y sus fragmentos que pueden usarse incluyen cadena A de la difteria, fragmentos activos no unión de toxina de la difteria, cadena A de exotoxina (de *Pseudomonas aeruginosa*), cadena A de ricina, cadena A de abrina, cadena A de modeccina, alfa-sarcina, proteínas de *Aleurites fordii*, proteínas de diantina, proteínas de *Phytolaca americana* (PAPI, PAPII, y PAP-S), inhibidor de momordica charantia, curcina, crotina, inhibidor de saponaria officinalis, gelonina, mitogelina, restrictocina, fenomicina, fenomicina y los tricotecenos. Véase, por ejemplo, WO 93/21232 publicada el 28 de octubre de 1993.

La presente invención contempla además un anticuerpo conjugado con un compuesto con actividad nucleolífica (por ejemplo, una ribonucleasa o ADNendonucleasa tal como deoxiribonucleasa, ADNasa).

Se dispone de una variedad de isótopos radioactivos para la producción de antagonistas radioconjugados o anticuerpos. Los ejemplos incluyen At^{211} , I^{131} , I^{125} , Y^{90} , Re^{186} , Re^{188} , Sm^{153} , Bi^{212} y P^{32} e isótopos radioactivos de Lu.

Conjugados del anticuerpo y agente citotóxico pueden elaborarse usando una variedad de agentes de acoplamiento de proteínas bifuncionales tales como N-succinimidil-3-(2-piridilditiol) propionato (SPDP), succinimidil-4-(N-maleimidometil)ciclohexano-1-carboxilato, iminotiolano (IT), derivados bifuncionales de imidoésteres (tales como HCl de dimetil adipimidato), ésteres activos (tales como disuccinimidil suberato), aldehídos (tales como glutaraldehído), compuestos bis-azido (tales como bis (p-azidobenzoil)hexanodiamina), derivados de bis-diazonio (tales como bis-(p-diazoniobenzoil)-etilendiamina), diisocianatos (tales como tolien 2,6-diisocianato), y compuestos de flúor bis-activo (tales como 1,5-difluoro-2,4-dinitrobenceo). Por ejemplo, puede prepararse una inmunotoxina de ricina como se describe en Vitetta *et al.*, *Science*, 238: 1098 (1987). Ácido 1-isotiocianatobencil-3-metildietilen triaminapentaacético marcado con carbono 14 (MX-DTPA) es un agente quelante ejemplar para conjugación de radionucleótido al antagonista o anticuerpo. Véase WO94/11026. El conector puede ser un "conector segmentable" que facilite la liberación del

medicamento citotóxico en la célula. Por ejemplo, pueden usarse un conector lábil al ácido, un conector sensible a peptidasa, un conector dimetilico o conector que contenga disulfuro (Chari *et al.*, *Cancer Res.* 52:127-131 (1992)).

Alternativamente, una proteína de fusión que comprenda un anticuerpo y el agente citotóxico puede elaborarse, por ejemplo, por técnicas recombinantes o síntesis de péptidos.

Los anticuerpos de la presente invención pueden conjugarse también con una enzima activadora de profármaco, que convierte un profármaco (*por ejemplo*, un agente quimioterapéutico peptídico, véase WO 81/01145) en un medicamento antineoplásico activo. Véase, por ejemplo, WO 88/07378 y Patente de los Estados Unidos No. 4,975,278.

El componente enzimático de tales conjugados incluye cualquier enzima capaz de actuar sobre un profármaco, de tal manera que lo convierta en su forma citotóxica, más activa.

Las enzimas que son útiles en el método de esta invención incluyen, pero no se limitan a, alcalina fosfatasa útil para convertir profármacos que contienen fosfatasa en medicamentos libres; arilsulfatasa útil para convertir profármacos que contienen sulfato en medicamentos libres; citosina deaminasa útil para convertir 5-fluorocitosina no tóxica en el medicamento antineoplásico 5-fluorouracilo; proteasas, tales como serratia proteasa, termolisina, subtilisina, carboxipeptidasas y catepsinas (tales como catepsinas B y L), que son útiles para convertir profármacos que contienen péptidos en medicamentos libres; D-alanilcarboxipeptidasas, útiles para convertir profármacos que contienen sustituyentes D-aminoácidos; enzimas de segmentación de carbohidratos tales como β -galactosidasa y neuraminidasa útiles para convertir profármacos glucosilados en medicamentos libres; β -lactamasa útil para convertir medicamentos derivados con β -lactamas en medicamentos libres; y penicilina amidasas, tales como penicilina V amidasa o penicilina G amidasa, útiles para convertir medicamentos derivados en sus nitrógenos de amina con grupos fenoxiacetilo o fenilacetilo, respectivamente en medicamentos libres.

Alternativamente, anticuerpos con actividad enzimática, también conocidos en el arte como "abzimas" pueden emplearse para convertir los profármacos de la invención en medicamentos libres activos (véase, *por ejemplo*, Massey, *Nature* 328: 457-458 (1987)). Conjugados anticuerpo-abzima pueden prepararse como se describe aquí para entrega de la abzima a una población de células neoplásicas.

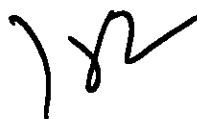
Las enzimas de esta invención pueden unirse covalentemente al anticuerpo mediante técnicas bien conocidas en el arte, tales como el uso de agentes de entrecruzamiento heterobifuncional, ya discutidos. Alternativamente, proteínas de fusión que comprenden por lo menos la región de unión a antígeno de un anticuerpo, enlazada a por lo menos una porción funcionalmente activa de una enzima de la invención, pueden construirse utilizando técnicas de ADN recombinante bien conocidas en el arte (véase, *por ejemplo*, Neuberger et al., *Nature* 312: 604-608 (1984)).

Otras modificaciones del anticuerpo se contemplan en esta solicitud. Por ejemplo, el anticuerpo puede enlazarse a uno de una variedad de polímeros no proteináceos, por ejemplo, polietilen glicol, polipropilen glicol, polioxialquilenos, o copolímeros de polietilen glicol y polipropilen glicol. Para aumentar la semivida sérica del anticuerpo, se puede incorporar un epítipo de unión a receptor de salvamento en el anticuerpo (especialmente un fragmento de anticuerpo) como se describe en la Patente de los Estados Unidos 5,739,277, por ejemplo. Tal como se emplea en esta solicitud, el término "epítipo de unión a receptor de salvamento" se refiere a un epítipo de la región Fc de una molécula de IgG (por ejemplo, IgG₁, IgG₂, IgG₃, o IgG₄) que es responsable de aumentar la semivida sérica *in vivo* de la molécula de IgG. Alternativa, o adicionalmente, se puede incrementar, o disminuir, la semivida sérica alterando la secuencia de aminoácidos de la región Fc de un anticuerpo para generar variantes con unión FcRn alterada. Anticuerpos con unión FcRn alterada y/o semivida sérica se describen en WO00/42072 (Presta, L.).

Formulaciones que comprenden Apo2L/TRAIL, anticuerpos de receptor de muerte celular, y/o anticuerpos CD20 se proveen también por la presente invención. Se cree que tales formulaciones serán particularmente apropiadas para almacenamiento, así como para administración terapéutica. Las formulaciones pueden prepararse por técnicas conocidas. Por ejemplo, las formulaciones pueden prepararse por intercambio de tampón en una columna de filtración en gel.

Típicamente, una cantidad apropiada de una sal aceptable o vehículo se usa en la formulación para volver la formulación isotónica. Ejemplos de vehículos farmacéuticamente aceptables incluyen solución salina, solución de Ringer y solución de dextrosa. El pH de la formulación va preferiblemente desde alrededor de 6 a alrededor de 9 y, más preferiblemente, desde alrededor de 7 a alrededor de 7.5. Será evidente a los expertos en el arte que ciertos vehículos pueden ser más preferibles dependiendo de, por ejemplo, la vía de administración y las concentraciones del ligando Apo-2, anticuerpos de receptor de muerte celular, y/o anticuerpos CD20.

Composiciones terapéuticas pueden prepararse mezclando las moléculas deseadas, que tienen el grado apropiado de pureza, con vehículos, excipientes, o estabilizantes opcionales (Remington's Pharmaceutical Sciences, 16th edition, Osol, A. ed. (1980)), en la forma de formulaciones liofilizadas, soluciones acuosas o suspensiones acuosas. Vehículos, diluyentes, excipientes y estabilizantes aceptables son preferiblemente no tóxicos a los destinatarios en las dosis y concentraciones empleadas, e incluyen tampones tales como Tris, HEPES, PIPES, fosfato, citrato, y otros ácidos orgánicos; antioxidantes incluidos ácido ascórbico y metionina; preservantes (tales como cloruro de octadecildimetilbencilamonio; cloruro de hexametonio; cloruro de benzalconio, cloruro de benzetonio; fenol, alcohol butílico o bencílico; alquil parabenos tales como metil o propil parabeno; catecol; resorcinol; ciclohexanol; 3-pentanol; y m-cresol); polipéptidos de bajo peso molecular (menos de alrededor de 10 residuos); proteínas, tales como albúmina sérica, gelatina, o



inmunoglobulinas; polímeros hidrófilos tales como polivinilpirrolidona; aminoácidos tales como glicina, glutamina, asparagina, histidina, arginina, o lisina; monosacáridos, disacáridos, y otros carbohidratos incluidos glucosa, mannososa, o dextrinas; azúcares tales como sacarosa, manitol, trehalosa o sorbitol; iones contrarios formadores de sales tales como sodio; y/o surfactantes no iónicos tales como TWEEN™, PLURONIC™ o polietilen glicol (PEG).

Ejemplos adicionales de tales vehículos incluyen intercambiadores iónicos, alúmina, estearato de aluminio, lecitina, proteínas séricas, tales como albúmina sérica humana, sustancias tamponantes tales como glicina, ácido sórbico, sorbato de potasio, mezclas parciales de glicérido de ácidos grasos vegetales saturados, agua, sales, o electrolitos tales como sulfato de protamina, hidrogenofosfato disódico, hidrogenofosfato potásico, cloruro de sodio, sílice coloidal, trisilicato de magnesio, polivinilpirrolidona, y sustancias a base de celulosa. Vehículos para formas tópicas o a base de gel incluyen polisacáridos tales como carboximetilcelulosa o metilcelulosa sódica, polivinilpirrolidona, poliacrilatos, polímeros de bloques polioxietilen-polioxipropilen, polietilen glicol, y alcoholes de madera. Para todas las administraciones, se usan convenientemente formas de efecto prolongado. Tales formas incluyen, por ejemplo, microcápsulas, nanocápsulas, liposomas, emplastos, formas para inhalación, aerosoles nasales, tabletas sub-linguales, y preparaciones de liberación sostenida.

Las formulaciones a usarse para administración *in vivo* tienen que ser estériles. Esto se logra fácilmente por filtración a través de membranas de filtración estériles, antes de, o luego de, la liofilización y reconstitución. La formulación puede almacenarse en forma liofilizada o en solución, si se administra sistemáticamente. Si en forma liofilizada, se formula típicamente en combinación con otros ingredientes para reconstitución con un diluyente apropiado en el momento del uso. Un ejemplo de una formulación líquida es una solución no preservada, incolora, transparente y estéril, envasada en un frasquito de dosis única para inyección subcutánea.

Las formulaciones terapéuticas se ponen por lo general en un envase que contiene un puerto de acceso estéril, por ejemplo, una bolsa para solución intravenosa o frasco que tiene un tapón perforable por una aguja de inyección hipodérmica. Las formulaciones se administran preferiblemente como inyecciones intravenosas (i.v.), subcutáneas (s.c), intramusculares (i.m.) repetidas o infusiones, o como formulaciones en aerosol, apropiadas para administración intranasal o intrapulmonar (para administración intrapulmonar véase, por ejemplo, EP 257,956).

Apo2L/TRAIL, los anticuerpos de receptor de muerte celular, y anticuerpos CD20 pueden administrarse también en la forma de preparaciones de liberación sostenida. Ejemplos apropiados de preparaciones de liberación sostenida incluyen matrices semi-permeables de polímeros hidrófobos sólidos que contienen la proteína, matrices que están en la forma de artículos moldeados, *por ejemplo*, películas, o microcápsulas. Ejemplos de matrices de liberación sostenida incluyen poliésteres, hidrogeles (por ejemplo, poli(2-hidroxietil-metacrilato) como se describe por Langer et al., J. Biomed. Mater. Res., **15**: 167-277 (1981) y Langer, Chem. Tech., **12**: 98-105 (1982), o poli(vinilalcohol)), poliláctidos (Patente de los Estados Unidos 3,773,919, EP 58,481), copolímeros de ácido L-glutámico y γ etil-L-glutamato (Sidman *et al.*, Biopolymers, **22**: 547-556 (1983)), etilen-vinil acetato no degradable (Langer *et al.*, *supra*), copolímeros degradables ácido láctico-ácido glicólico tales como el Lupron Depot (microesferas inyectables compuestas de copolímero ácido láctico-ácido glicólico y acetato de leuprolida), y ácido poli-D-(-)-3-hidroxibutírico (EP 133,988).

Apo2L/TRAIL, los anticuerpos de receptor de muerte celular, y anticuerpos CD20 descritos en esta solicitud pueden emplearse en una variedad de aplicaciones terapéuticas. Entre estas aplicaciones se encuentran métodos de tratar varias neoplasias malignas y enfermedades inmuno-relacionadas. El diagnóstico en mamíferos de los diferentes estados patológicos descritos en esta solicitud puede realizarse por el profesional experto. En el arte se dispone de técnicas diagnósticas que

permiten, por ejemplo, el diagnóstico o detección de cáncer o enfermedad inmuno-relacionada en un mamífero. Por ejemplo, pueden identificarse neoplasias malignas mediante técnicas, incluidas pero no limitadas a, palpación, examen de sangre, rayos X, NMR y similares. En igual forma pueden identificarse fácilmente enfermedades inmuno-relacionadas. En lupus eritematoso sistémico, el mediador central de la enfermedad es la producción de anticuerpos auto-reactivos a autoproteínas/tejidos y la generación subsiguiente de inflamación mediada inmunitariamente. Clínicamente se afectan órganos múltiples y sistemas incluidos el riñón, pulmón, sistema músculo-esquelético, mucocutáneo, ojo, sistema nervioso central, sistema cardiovascular, tubo digestivo, médula ósea y sangre. La artritis reumatoide (RA) es una enfermedad inflamatoria autoinmunitaria sistémica, que involucra principalmente la membrana sinovial de articulaciones múltiples, con lesión resultante al cartílago articular. La patogénesis es dependiente de los linfocitos T y se asocia con la producción de factores reumatoides, auto-anticuerpos dirigidos contra auto IgG, con la resultante formación de complejos inmunitarios que alcanzan niveles elevados en líquido sinovial y la sangre. Estos complejos en la articulación pueden inducir el infiltrado marcado de linfocitos y monocitos en la sinovia y cambios sinoviales marcados subsiguientes; el espacio articular/líquido sinovial es infiltrado por células similares con la adición de numerosos neutrófilos. Los tejidos afectados son principalmente las articulaciones, a menudo en un patrón simétrico. No obstante, ocurre también una afección, extra-articular en dos formas principales. Una forma es el desarrollo de lesiones extra-articulares con enfermedad articular progresiva continuada y lesiones típicas de fibrosis pulmonar, vasculitis, y úlceras cutáneas. La segunda forma de enfermedad extra-articular es el denominado síndrome de Felty, que ocurre tardíamente en el curso de la enfermedad RA, a veces después de que la enfermedad articular se ha vuelto latente, e implica la presencia de neutropenia, trombocitopenia y esplenomegalia. Esto puede acompañarse por vasculitis en órganos múltiples con formaciones de infartos, úlceras cutáneas y gangrena. A menudo los pacientes también

desarrollan nódulos reumatoides en el tejido subcutáneo que recubre las articulaciones afectadas; los nódulos de fase tardía exhiben centros necróticos rodeados por un infiltrado celular inflamatorio mixto. Otras manifestaciones que pueden ocurrir en la RA incluyen: pericarditis, pleuritis, arteritis coronaria, pneumonitis intersticial con fibrosis pulmonar, queratoconjuntivitis seca, y nódulos reumatoides.

Apo2L/TRAIL, los anticuerpos de receptor de muerte celular, y anticuerpos CD20 pueden administrarse de acuerdo con métodos conocidos, tales como administración intravenosa como una inyección intravenosa rápida o por infusión continua durante un período de tiempo, o por vías intramuscular, intraperitoneal, intracerebrospinal, subcutánea, intra-articular, intrasinovial, intratecal, oral, tópica, o mediante inhalación. Opcionalmente, la administración puede llevarse a cabo mediante infusión de mini-bomba usando varios dispositivos disponibles en el comercio.

Posologías efectivas y pautas de administración de Apo2L/TRAIL, los anticuerpos de receptor de muerte celular, y anticuerpos CD20 pueden determinarse empíricamente, y realización de tales determinaciones se encuentra dentro de las habilidades corrientes en el arte. Se pueden emplear posologías únicas o múltiples. En la actualidad se cree que una posología efectiva o cantidad de Apo2L/TRAIL usado solo puede variar desde alrededor de 1 µg/kg a alrededor de 100 mg/kg de peso corporal o más al día. El aumento de la escala interespecies de las posologías puede llevarse a cabo en una manera conocida en el arte, por ejemplo, como se divulga en Mordenti et al., Pharmaceut. Res., 8:1351 (1991). Cuando se emplea la administración *in vivo* de un Apo2L/TRAIL, cantidades de posología normales pueden variar desde alrededor de 10 ng/kg hasta 100 mg/kg de peso corporal del mamífero o más por día, preferiblemente alrededor de 1 µg/kg/día a 10 mg/kg/día, dependiendo de la ruta de administración. En la literatura se ofrece orientación con respecto a posologías y métodos particulares de aplicación; véase, por ejemplo, Patente de los Estados Unidos Nos. 4,657,760; 5,206,344; o 5,225,212. Se anticipa que diferentes formulaciones serán efectivas para diferentes compuestos de tratamiento y



diferentes trastornos, que la administración que se dirige a un órgano o tejido, por ejemplo, puede requerir la aplicación en una manera diferente de la administración a otro órgano o tejido. Los expertos en el arte entenderán que la posología de Apo2L/TRAIL, que tiene que administrarse, variará dependiendo de, por ejemplo, el mamífero que recibirá el Apo2L/TRAIL, la vía de administración, y otros medicamentos o terapias que se estén administrando al mamífero.

El anticuerpo CD20 puede ser un anticuerpo tal como Rituximab o 2H7 humanizado, que no se conjuga a un agente citotóxico. Posologías apropiadas para un anticuerpo no conjugado están, por ejemplo, en el rango desde alrededor de 20 mg/m² a alrededor de 1.000 mg/m². En una incorporación, la posología del anticuerpo difiere de la actualmente recomendada para Rituximab. Regímenes posológicos ejemplares para el anticuerpo CD20 incluyen 375 mg/m² semanales x 4 o 8; ó 1.000 mg x 2 (por ejemplo, los días 1 y 15). Se contempla todavía el posible empleo de terapias adicionales en los métodos. La una o más terapias pueden incluir, pero no se limitan a, administración de terapia de radiación, citocina(s), agente(s) inhibidores de crecimiento, agentes(s) quimioterapéutico, agentes(s) citotóxico, inhibidores de tirosina cinasa, inhibidores de ras farnesil transferasa, inhibidores de angiogénesis, e inhibidores de cinasa dependiente de ciclina, que son conocidos en el arte y se definen con particularidad en la anterior Sección I.

Anticuerpos terapéuticos ejemplares incluyen anticuerpos anti-HER2 incluidos rhuMAb 4D5 (HERCEPTIN.) (Carter *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:4285-4289 (1992), Patente de los Estados Unidos No. 5,725,856); anti-IL-8 (St John *et al.*, *Chest*, 103:932 (1993), y Publicación Internacional No. WO 95/23865); anticuerpos anti-VEGF incluidos anticuerpos anti-VEGF humanizados y/o madurados por afinidad tales como el anticuerpo anti-VEGF humanizado huA4.6.1 AVASTIN. (Kim *et al.*, *Growth Factors*, 7:53-64 (1992), Publicación Internacional No. WO 96/30046, y WO 98/45331, publicada el 15 de octubre de 1998); anticuerpos anti-PSCA (WO01/40309); anticuerpos anti-CD40, incluidos



S2C6 y sus variantes humanizadas (WO00/75348); anticuerpos anti-CD11a incluidos Raptiva™ (Patente de los Estados Unidos No. 5,622,700, WO 98/23761, Steppe *et al.*, *Transplant Intl.* 4:3-7 (1991), y Hourmant *et al.*, *Transplantation* 58:377-380 (1994)); anticuerpos anti-IgE (Presta *et al.*, *J. Immunol.* 151:2623-2632 (1993), y Publicación Internacional No. WO 95/19181; Patente de los Estados Unidos No. 5,714,338, expedida el 3 de febrero de 1998 o Patente de los Estados Unidos No. 5,091,313, expedida el 25 de febrero de 1992, WO 93/04173 publicada el 4 de marzo de 1993, o Publicación Internacional No. PCT/US98/13410 presentada el 30 de junio de 1998, Patente de los Estados Unidos No. 5,714,338); anticuerpos anti-CD18 (Patente de los Estados Unidos No. 5,622,700, expedida el 22 de abril de 1997, o como en WO 97/26912, publicada el 31 de julio de 1997); anticuerpos de anticuerpo de receptor anti-Apo-2 (WO 98/51793 publicada el 19 de noviembre de 1998); anticuerpos anti-TNF-alfa incluidos cA2 (REMICADE), CDP571 y MAK-195 (véase, Patente de los Estados Unidos No. 5,672,347 expedida el 30 de septiembre de 1997, Lorenz *et al.* *J. Immunol.* 156 (4):1646-1653 (1996), y Dhainaut *et al.* *Crit. Care Med.* 23 (9):1461-1469 (1995)); anticuerpo de Factor anti-Tisular (TF) (Patente Europea No. 0 420 937 B1 otorgada el 9 de noviembre de 1994); anticuerpos de integrina $\alpha 4\text{-}\beta 7$ anti-humana (WO 98/06248 publicada el 19 de febrero de 1998); anticuerpos anti-EGFR (anticuerpo 225 quimérico o humanizado como en WO 96/40210 publicada el 19 de diciembre de 1996); anticuerpos anti-CD3 tales como OKT3 (Patente de los Estados Unidos No. 4,515,893 expedida el 7 de mayo de 1985); anticuerpos anti-CD25 o anti-Tac tales como CHI-621 (SIMULECT) y ZENAPAX. (Véase Patente de los Estados Unidos No. 5,693,762 expedida el 2 de diciembre de 1997); anticuerpos anti-CD4 tales como el anticuerpo cM-7412 (Choy *et al.* *Arthritis Rheum* 39(1):52-56 (1996)); anticuerpos anti-CD52 tales como CAMPATH-IH (Riechmann *et al.* *Nature* 332:323-337 (1988); anticuerpos de receptor anti-Fc tales como el anticuerpo M22 dirigido contra Fc.RI como en Graziano *et al.* *J. Immunol.* 155 (10):4996-5002 (1995); anticuerpos de antígeno anti-carcinoembrionario (CEA) tales como hMN-

14 (Sharkey *et al. Cancer Res.* 55 (23 Suppl):5935s-5945s (1995); anticuerpos dirigidos contra células epiteliales de mama incluidas huBrE-3, hu-Mc 3 y CHL6 (Ceriani *et al. Cancer Res.* 55(23):5852s-5856s (1995); y Richman *et al. Cancer Res.* 55(23 Suppl):5916s-5920s (1995)); anticuerpos que se unen a células de carcinoma de colon tales como C242 (Litton *et al. Eur J. Immunol.* 26(1):1-9 (1996)); anticuerpos anti-CD38, por ejemplo AT 13/5 (Ellis *et al. J. Immunol.* 155(2):925-937 (1995)); anticuerpos anti-CD33 tales como Hu M195 (Jurcic *et al. Cancer Res* 55(23 Suppl):5908s-5910s (1995) y CMA-676 o CDP771; anticuerpos anti-CD22 tales como LL2 o LymphoCide (Juweid *et al. Cancer Res* 55(23 Suppl):5899s-5907s (1995); anticuerpos anti-EpCAM tales como 17-1A (PANOREX); anticuerpos anti-GpIIb/IIIa tales como abciximab o c7E3 Fab (REOPRO); anticuerpos anti-RSV tales como MEDI-493 (SYNAGIS), anticuerpos anti-CMV tales como PROTOVIR; anticuerpos anti-VIH tales como PRO542; anticuerpos anti-hepatitis tales como el anticuerpo anti-Hep B OSTAVIR; anticuerpo anti-CA 125 OvaRex; anticuerpo de epítipo anti-idiotípico GD3 BEC2; anticuerpo anti-v.3 VITAXIN; anticuerpo de carcinoma de células renales anti-humano tal como ch-G250; ING-1; anticuerpo anti-human 17-1A (3622W94); anticuerpos de tumor colorrectal anti-humano (A33); anticuerpo de melanoma anti-humano R24 dirigido contra el gangliósido GD3; anticuerpos de carcinoma escamoso anti-humano (SF-25); y anticuerpos de antígeno de leucocito anti-humano (HLA) tales como Smart ID1O y el anticuerpo anti-HLA DR Oncolym (Lym-1).

La preparación y pautas posológicas para los agentes quimioterapéuticos pueden usarse de acuerdo con las instrucciones del fabricante o como se determine empíricamente por el profesional experto. La preparación y pautas posológicas para tal quimioterapia se describen igualmente en *Chemotherapy Service* Ed., M.C. Perry, Williams & Wilkins, Baltimore, MD (1992). El agente quimioterapéutico puede preceder o seguir a la administración del Apo2L/TRAIL, el anticuerpo de receptor de muerte celular, y/o el anticuerpo CD20, o puede administrarse simultáneamente con aquél. A veces, puede ser beneficioso administrar

también una o más citocinas o agente inhibidor del crecimiento. El Apo2L/TRAIL, los anticuerpos de receptor de muerte celular, y los anticuerpos CD20 (y una o más de otras terapias) pueden administrarse concurrente o secuencialmente. Luego de la administración, pueden analizarse células tratadas *in vitro*. Donde ha habido tratamiento *in vivo*, un mamífero tratado puede monitorearse en varias formas bien conocidas por el profesional experto. Por ejemplo, pueden examinarse patológicamente células cancerosas para analizarlas en cuanto a necrosis o puede analizarse el suero para detectar respuestas del sistema inmunitario. Para la RA, y otras enfermedades autoinmunitarias, el Apo2L/TRAIL, el anticuerpo de receptor de muerte celular, y/o el anticuerpo CD20 pueden combinarse con una cualquiera o más de los agentes inmunosupresores, agentes quimioterapéuticos y/o citocinas enumerados en la anterior Sección Definiciones; uno cualquiera o más de los fármacos antirreumáticos modificadores de la enfermedad (DMARDs), tales como hidroxicloroquina, sulfasalazina, metotrexato, leflunomida, azatioprina, D-penicilamina, Gold (oral), Gold (intramuscular), minociclina, ciclosporina, inmunoadsorción de proteína A estafilocócica; inmunoglobulina intravenosa (IVIG); fármacos anti-inflamatorios no esteroides (AINE); glucocorticoide (por ejemplo, mediante inyección en las articulaciones); corticosteroide (por ejemplo, metilprednisolona y/o prednisona); folato; un anticuerpo de factor de necrosis anti-tumoral (TNF), por ejemplo, etanercept/ENBREL™, infliximab/REMICADE™, D2E7 (Knoll) o CDP-870 (Celltech); antagonista IL-1R (por ejemplo, Kineret); antagonista IL-10 (por ejemplo, Ilodecakin); un modulador de coagulación sanguínea (por ejemplo, WinRho); un antagonista IL-6/anti-TNF (CBP 1011); antagonista CD40 (por ejemplo, IDEC 131); antagonista de receptor Ig-Fc (MDX33); inmunomodulador (por ejemplo, talidomida o ImmuDyn); anticuerpo anti-CD5 (por ejemplo, H5g1.1); inhibidor de macrófagos (por ejemplo, MDX 33); bloqueador co-estimulador (por ejemplo, BMS 188667 o Tolerimab); inhibidor de complemento (por ejemplo, h5G1.1, 3E10 un anticuerpo de factor acelerador anti-deterioro (DAF)); o antagonista IL-2 (zxSMART).

Para tumores malignos de linfocitos B, por ejemplo, el Apo2L/TRAIL, anticuerpo de receptor de muerte celular, y/o anticuerpo CD20 pueden combinarse con un agente quimioterapéutico; citocina, por ejemplo, una linfocina tal como IL-2, IL-12, o un interferón, tal como interferón alfa-2a; otro anticuerpo, por ejemplo, un anticuerpo radiomarcado tal como ibritumomab tiuxetan (ZEVALIN®), yodo I¹³¹ tositumomab (BEXXAR™), ¹³¹I Lym-1 (ONCOLYM™), 90Y-LYMPHOCIDE11™; anticuerpo anti-CD52, tal como alemtuzumab (CAMPATH-1H™), anticuerpo anti-HLA-DR-β, tal como apolizumab, anticuerpo anti-CD80 (por ejemplo, IDEC-114), epratuzumab, Hu1D10 (SMART 1D10™), anticuerpo CD19, anticuerpo CD40 o anticuerpo CD22; un inmunomodulador (por ejemplo, talidomida o ImmuDyn); un inhibidor de angiogénesis (por ejemplo, un anticuerpo de factor de crecimiento endotelial anti-vascular (VEGF) tal como AVASTIN™ o talidomida); vacuna de idiotipo (EPOCH); ONCO-TCS™; HSPPC-96 (ONCOPHAGE™); terapia liposómica (por ejemplo, liposoma de citrato de daunorubicina), etc.

En otra incorporación de la invención, se proveen artículos de fabricación que contienen materiales útiles para el tratamiento de cáncer o enfermedad inmuno-relacionada, descritos anteriormente. En un aspecto, el artículo de fabricación comprende (a) un envase que contiene el anticuerpo CD20 (preferiblemente, el envase contiene el anticuerpo y un vehículo o diluyente farmacéuticamente aceptable dentro del envase); (b) un envase que contiene Apo2L/TRAIL o anticuerpo de receptor de muerte celular (preferiblemente, el envase contiene el Apo2L/TRAIL o anticuerpo de receptor de muerte celular y un vehículo o diluyente farmacéuticamente aceptable dentro del envase); y (c) un prospecto de envase con instrucciones para tratar cáncer o una enfermedad inmuno-relacionada en un paciente, en donde las instrucciones indican que las cantidades del anticuerpo CD20 y el Apo2L/TRAIL o anticuerpo de receptor de muerte celular, que se administran al paciente, son efectivas para proporcionar una actividad sinérgica en tratar la enfermedad. En todos estos aspectos, el prospecto de

envase se encuentra sobre o asociado con el envase. Envases apropiados incluyen, por ejemplo, frascos, ampolletas, jeringas, etc. Los envases pueden formarse de una variedad de materiales tales como vidrio o plástico. El envase contiene una composición que es efectiva para tratar el cáncer o enfermedad inmuno-relacionada y puede contar con un puerto de acceso estéril (por ejemplo el envase puede ser una bolsa de solución intravenosa o una ampolla con un tapón perforable por una aguja de inyección hipodérmica). Por lo menos un agente activo en la composición es el anticuerpo CD20, Apo2L/TRAIL o anticuerpo de receptor de muerte celular. La etiqueta o prospecto de envase indica que la composición se utiliza para tratar cáncer o una enfermedad inmuno-relacionada en un paciente o sujeto elegible para tratamiento, incluyendo orientación específica en relación con las cantidades posológicas e intervalos de anticuerpo y cualquier otro medicamento que se esté suministrando. El artículo de manufactura puede comprender además un envase adicional que contenga un tampón diluyente farmacéuticamente aceptable, tal como agua bacteriostática para inyección (BWFI), solución salina tamponada con fosfato, solución salina de Ringer, y/o solución de dextrosa. El artículo de fabricación puede incluir además otros materiales deseables desde un punto de vista comercial y del usuario, incluidos otros tampones, diluyentes, filtros, agujas, y jeringas. Los siguientes ejemplos se ofrecen con fines ilustrativos únicamente, y no pretenden limitar el ámbito de la presente invención en ninguna forma. Todas las referencias de patentes y la literatura citadas en la presente especificación se incorporan por la presente solicitud por referencia en su integridad.

EJEMPLOS

Los reactivos disponibles en el comercio, mencionados en los ejemplos, se utilizaron según las instrucciones del fabricante, salvo que se indique lo contrario. La fuente de aquellas células identificadas en los siguientes ejemplos, y a todo lo largo de la especificación, a modo de referencia a la ATCC es la American Type Culture Collection, Manassas, Virginia.

Ejemplo 1**Análisis de la expresión de receptor Apo2L/TRAIL en líneas de células de linfoma B**

Para examinar la expresión en la superficie celular de los receptores Apo2L/TRAIL (DR4, DR5, DcR1, y DcR2) en líneas de células de linfoma humano, las líneas de células de linfoma B Ramos, Daudi, Raji, y BJAB (ATCC) se analizaron mediante FACS usando anticuerpos monoclonales específicos para DR4 (mAb 4H6.17.8; ATCC HB-12455), DR5 (mAb 3H3.14.5; HB-12534), DcR1 (mAb 6G9; Genentech, Inc.), o DcR2 (mAb 1G9, Genentech, Inc.) Para las células Ramos, el análisis se llevó a cabo dos veces para garantizar la reproducibilidad (RAMOS A y B). Como se ilustra en la Figura 4, DR4 y DR5 se expresaron en niveles significativos (cambio de la fluorescencia media de aproximadamente 0.5 - 1.7 unidades) en todas las cuatro líneas de células, mientras que DcR1 y DcR2 se expresaron en niveles más bajos o mínimos (cambio de la fluorescencia media de aproximadamente 0 - 0.3 unidades).

Ejemplo 2**Análisis de la expresión de CD20 en líneas de células de linfoma B**

Para examinar la expresión en la superficie celular de CD20 en líneas de células de linfoma humano, las líneas de células de linfoma B Ramos, Daudi, Raji, y BJAB (ATCC) se analizaron mediante FACS usando un anticuerpo monoclonal específico para CD20 (RITUXAN®, Genentech, Inc.). Para células Ramos, el análisis se llevó a cabo dos veces para garantizar la reproducibilidad (RAMOS A y B). Como se ilustra en Figura 5, todas las cuatro líneas de células expresaron niveles elevados de CD20, indicados por un cambio de fluorescencia media de aproximadamente 5 - 15 unidades.

Ejemplo 3**Efecto de Apo2L/TRAIL, RITUXAN®, o tratamiento de combinación sobre el crecimiento de xenoinjertos de tumor linfoma BJAB subcutáneos, pre-establecidos en ratones SCID**

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Ratones SCID se inyectaron subcutáneamente con células de linfoma BJAB no hodgkiniano de linfocitos B humanos (ATCC) (20 millones de células por ratón), y los tumores se dejaron crecer a $\sim 200 \text{ mm}^3$. A continuación los ratones se dividieron en 4 grupos de estudio (8 ratones por grupo) y se trataron con cinco dosis intraperitoneales (IP) por semana durante 2 semanas (es decir, los días 0-4 y 7-11) de vehículo (0.5M Arg-Succinato/20mM Tris/O.02% Tween 20 pH =7.2), Apo2L/TRAIL (aminoácidos 114- 281 de Figura 1) (60 mg/kg), o con 1 dosis IP por semana durante 2 semanas (es decir, los días 0 y 7) de RITUXAN® (4 mg/kg, Genentech, Inc.), o la combinación de estos últimos regímenes Apo2L/TRAIL y RITUXAN® (Figura 6). Los tumores en ratones tratados con vehículo crecieron rápidamente, mientras que el tratamiento con un solo agente Apo2L/TRAIL o RITUXAN® retrasó notoriamente el crecimiento del tumor. Un ratón en el grupo Apo2L/TRAIL mostró la completa extirpación completa del tumor, dejando una incidencia tumoral (TI) de 7/8. El tratamiento con RITUXAN® no extirpó tumores pero mostró un efecto más prolongado. En forma digna de consideración, el tratamiento combinado con Apo2L/TRAIL y RITUXAN® produjo una reducción dramática en el volumen del tumor en todos los ratones, con 5 de 8 ratones mostrando la extirpación completa del tumor y 3/8 mostrando un crecimiento mínimo del tumor durante un mínimo de 28 días. Estos resultados indican que Apo2L/TRAIL y RITUXAN® pueden ejercer una actividad anti-tumoral sinérgica contra xenoinjertos de linfoma.

Ejemplo 4

Efecto de Apo2L/TRAIL, RITUXAN®, o tratamiento de combinación sobre el crecimiento de xenoinjertos de tumor linfoma BJAB subcutáneos, pre-establecidos, desarrollados en ratones SCID

Se condujo un estudio similar al descrito en Ejemplo 3. Ratones SCID se inyectaron subcutáneamente con células de linfoma BJAB no hodgkiniano de linfocitos B humanos (ATCC) (20 millones de células por ratón), y los tumores se dejaron crecer a $\sim 200 \text{ mm}^3$. A continuación los ratones se dividieron en 4 grupos de estudio (8 ratones por grupo) y se

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trataron con cinco dosis intraperitoneales (IP) por semanas durante 2 semanas (es decir, los días 0-4 y 7-11) de vehículo (0.5M Arg-Succinato/20mM Tris/0.02% Tween 20 pH =7,2), Apo2L/TRAIL ("Apo2L.0"; aminoácidos 114-281 de Fig. 1) (60 mg/kg), o con 1 dosis IP por semana durante 2 semanas (es decir, los días 0 y 7) de RITUXAN® (4 mg/kg), o la combinación de estos últimos regímenes Apo2L/TRAIL y RITUXAN®. Los resultados se muestran en Fig. 7. Los tumores en los ratones tratados con vehículo crecieron rápidamente, mientras que el tratamiento con un solo agente Apo2L/TRAIL o RITUXAN® retrasó notablemente el crecimiento del tumor. Ni Apo2L/TRAIL ni RITUXAN® produjeron regresiones completas, mientras que RITUXAN® mostró un efecto más prolongado. Al igual que en el estudio descrito en Ejemplo 3, el tratamiento combinado con Apo2L/TRAIL y RITUXAN® produjo una reacción notoria en el volumen del tumor en todos los ratones, con 6 de 7 ratones mostrando la completa extirpación del tumor. Estos resultados indican que Apo2L/TRAIL y RITUXAN® pueden ejercer una actividad anti-tumoral sinérgica contra xenoinjertos de linfoma.

Ejemplo 5

Efecto de Apo2L/TRAIL, RITUXAN®, o tratamiento de combinación sobre el procesamiento de caspasa en xenoinjertos de tumor linfoma BJAB subcutáneos, pre-establecidos, desarrollados en ratones SCID

Para examinar el procesamiento de caspasas mediadoras de apoptosis en tumores tratados (indicado por procesamiento de caspasa proteolítica), ratones SCID se inyectaron subcutáneamente con células de linfoma BJAB no hodgkiniano de linfocitos B humanos (ATCC) (20 millones de células por ratón), y los tumores se dejaron crecer a ~200 mm³. Los ratones se trataron entonces con vehículo (0.5M Arg-Succinato/20mM Tris/0.02% Tween 20 pH =7.2) (n=1), 1 dosis IP de Apo2L/TRAIL (60 mg/kg) (n=1), o 1 dosis IP de RITUXAN® (4 mg/kg, Genentech, Inc.) (n=2), o la combinación de estas dos últimas dosis de Apo2L/TRAIL y RITUXAN® (n=2). Dos días después del tratamiento, los tumores se recogieron, se desintegraron en tampón de lisis, y se sometieron a inmunotransferencia

con anticuerpos específicos contra Caspasa 8, 3, 9, y 7 (con anticuerpo actina anti-beta como control de carga) para visualizar el procesamiento de caspasa (Figura 8). El tratamiento con Apo2L/TRAIL (A) indujo un procesamiento incrementado de caspasa 8, 3, 9, y 7, comparado con el control de vehículo (V), mientras que el RITUXAN® (R) no indujo procesamiento de caspasa. En particular, el tratamiento de combinación con Apo2L/TRAIL y RITUXAN® (AR) no incrementó más el procesamiento de caspasa, comparada con Apo2L/TRAIL solo. Estos resultados sugieren que la actividad anti-tumoral sinérgica entre Apo2L/TRAIL y RITUXAN® no está necesariamente mediada por la intensificación de apoptosis, insinuando que la combinación de la activación de apoptosis, mediada por Apo2L/TRAIL y lisis dependiente de complemento, junto con ADCC mediada por RITUXAN®, puede estar en la base de la sinergia anti-tumoral observada.

Ejemplo 6

Efecto del anticuerpo DR5 agonístico, RITUXAN®, o tratamiento de combinación sobre el crecimiento de xenoinjertos de tumor linfoma BJAB subcutáneos, pre-establecidos en ratones SCID

Ratones SCID se inyectaron subcutáneamente con células de linfoma BJAB no hodgkiniano de linfocitos B humanos (ATCC) (20 millones de células por ratón), y los tumores se dejaron crecer a ~200 mm³. Los ratones se dividieron entonces en 4 grupos de estudio (7 ratones por grupo) y se trataron con una inyección intraperitoneal (IP) por semana durante 2 semanas (es decir, los días 0 y 7) de vehículo (0.5M Arg- Succinato/20mM Tris/0.02% Tween 20 pH =7.2), anticuerpo monoclonal DR5 agonista ("Apomab") (10 mg/kg), o RITUXAN® (4 mg/kg), o la combinación de estos últimos regímenes de anticuerpo DR5 y RITUXAN® (Figura 9). Los tumores en los ratones tratados con vehículo crecieron rápidamente, mientras que el tratamiento con agente único de anticuerpo DR5 o RITUXAN® retrasó notoriamente el crecimiento del tumor. Hay que resaltar que el tratamiento combinado con anticuerpo DR5 y RITUXAN® produjo una reducción dramática en el volumen del tumor en todos los

ratones, con 5 de 7 ratones mostrando la completa extirpación del tumor y 2/7 mostrando un crecimiento mínimo del tumor durante 35 días como mínimo. Estos resultados indican que el anticuerpo DR5 agonista y RITUXAN® pueden ejercer actividad anti-tumoral sinérgica contra xenoinjertos de linfoma.

Ejemplo 7

Efecto del anticuerpo DR5 agonístico, RITUXAN®, o tratamiento de combinación sobre el procesamiento de caspasa en xenoinjertos de tumor linfoma BJAB subcutáneos, pre-establecidos, desarrollados en ratones SCID

Para examinar el procesamiento de caspasas mediadoras de apoptosis en tumores tratados (indicado por procesamiento de caspasa proteolítica), ratones SCID se inyectaron subcutáneamente con células de linfoma BJAB no hodgkiniano de linfocitos B humanos (ATCC) (20 millones de células por ratón), y los tumores se dejaron crecer a $\sim 200 \text{ mm}^3$. A continuación los ratones se trataron con vehículo (0.5M. Arg-Succinato/20mM Tris/0.02% Tween 20 pH = 7.2) (n=1), o 1 dosis IP de RITUXAN® (4 mg/kg) (n=2), o 1 dosis IP de anticuerpo DR5 agonista (10 mg/kg) (n=2), o la combinación de estas últimas dosis de anticuerpo DR5 y RITUXAN® (n=2). Dos días después del tratamiento, los tumores se recogieron, se desintegraron en tampón de lisis, y se sometieron a inmunotransferencia con anticuerpos específicos contra Caspasa 8, 3, 9, y 7 (con anticuerpo actina anti-beta como control de carga) para visualizar el procesamiento de caspasa (Figura 10). El tratamiento con anticuerpo DR5 agonista (A) indujo el procesamiento incrementado de caspasa 8, 3, 9, y 7, comparado con el control de vehículo (V), mientras que el RITUXAN® (R) no indujo el procesamiento de caspasa. Notoriamente, el tratamiento de combinación con anticuerpo DR5 y RITUXAN® (AR) no aumentó más el procesamiento de caspasa, comparado con el anticuerpo DR5 solo. Estos resultados sugieren que la actividad anti-tumoral sinérgica entre el anticuerpo DR5 y RITUXAN® no está necesariamente mediada por la intensificación de

apoptosis, sino más bien que la combinación de la activación de apoptosis mediada por el anticuerpo DR5 agonista y lisis dependiente de complemento, junto con ADCC mediada por RITUXAN®, pueden estar en la base de la sinergia anti-tumoral observada.

Mayores datos que ilustran la expresión de los receptores de CD20 y Apo2L/TRAIL en líneas de células NHL y los efectos de Rituximab, Apo2L/TRAIL y sus combinaciones sobre las células cancerosas se presentan en las Figuras 11-16.

LO QUE SE REIVINDICA ES:

1. Un método de tratar células cancerosas, que comprende exponer células cancerosas mamíferas a una cantidad efectiva sinérgica de anticuerpo de receptor de muerte celular y anticuerpo CD20.
2. El método de la reivindicación 1 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo monoclonal de receptor anti-DR5.
3. El método de la reivindicación 1 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo monoclonal de receptor anti-DR4.
4. El método de la reivindicación 1 en donde dichas células cancerosas se exponen a dicha cantidad efectiva sinérgica de anticuerpo de receptor de muerte celular agonista y anticuerpo CD20 *in vivo*.
5. El método de la reivindicación 2 ó 3 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo quimérico o un anticuerpo humanizado.
6. El método de la reivindicación 2 ó 3 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo humano.
7. El método de la reivindicación 1 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo que reacciona cruzadamente con más de un receptor de ligando Apo-2.
8. El método de la reivindicación 1 en donde dichas células cancerosas son células de linfoma.

9. El método de la reivindicación 1 que comprende además exponer las células cancerosas a uno o más agentes inhibidores de crecimiento.
10. El método de la reivindicación 1 que comprende además exponer las células a radiación.
11. El método de la reivindicación 2 en donde dicho anticuerpo DR5 tiene una afinidad de unión a receptor DR5 de 10^8 M^{-1} a 10^{12} M^{-1} .
12. El método de la reivindicación 1 en donde dicho anticuerpo de receptor de muerte celular y anticuerpo CD20 se expresa en una célula hospedera recombinante seleccionada del grupo conformado por una célula CHO, una célula de levadura y *E. coli*.
13. El método de la reivindicación 1 en donde dicho anticuerpo CD20 es un anticuerpo monoclonal.
14. El método de la reivindicación 13 en donde dicho anticuerpo CD20 es el anticuerpo Rituximab.
15. Un método de tratar una enfermedad inmuno-relacionada, que comprende administrar a un mamífero una cantidad efectiva sinérgica de anticuerpo de receptor de muerte celular agonista y anticuerpo CD20.
16. El método de la reivindicación 15 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo monoclonal de receptor anti-DR5.
17. El método de la reivindicación 15 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo monoclonal de receptor anti-DR4.

18. El método de la reivindicación 16 ó 17 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo quimérico o un anticuerpo humanizado.

19. El método de la reivindicación 16 o 17 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo humano.

20. El método de la reivindicación 15 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo que reacciona cruzadamente con más de un receptor de ligando Apo-2.

21. El método de la reivindicación 15 en donde dicha enfermedad inmuno-relacionada es artritis reumatoide o esclerosis múltiple.

22. El método de la reivindicación 15 en donde dicho anticuerpo DR5 tiene una afinidad de unión a receptor DR5 de 10^8 M^{-1} a 10^{12} M^{-1} .

23. El método de la reivindicación 15 en donde dicho anticuerpo de receptor de muerte celular y el anticuerpo CD20 se expresa en una célula hospedera recombinante seleccionada del grupo conformado por una célula CHO, una célula de levadura y *E. coli*.

24. El método de la reivindicación 15 en donde dicho anticuerpo CD20 es un anticuerpo monoclonal.

25. El método de la reivindicación 24 en donde dicho anticuerpo CD20 es el anticuerpo Rituximab.

26. El método de la reivindicación 1 ó 15 en donde dicho anticuerpo de receptor de muerte celular y el anticuerpo CD20 se administran secuencialmente.

27. El método de la reivindicación 1 ó 15 en donde dicho anticuerpo de receptor de muerte celular y el anticuerpo CD20 se administran concurrentemente.

1 TTTCCCTCACTGACTATATAAGAAATAGAGAAAGGAGGCTTCAGTGACCGGCTGCCTGGCTGACTTACAGCAGTCAAGCTCTGACAGGATC
 1 ATGGCTATGATGGAGGTCCAGGGGGACCCAGCCTGGGACAGACCTGCTGCTGATCCTGATCTTTCACAGTGCCTCCATGCAATCTCTCTGT
 1 MetAlaMetMetGluValGlnGlyGlyProSerLeuGlyGlnThrCysValLeuIleValIlePheThrValLeuLeuGlnSerLeuCys
 181 GTGGCTGTAACCTACGTGTACTTTACCAACGAGCTGAAGCAGATGCAGGACAAAGTACTCCAAAGGTGGCATTTGCTTTCTTAAAGAA
 31 ValAlaValThrTyValTyPheThrAsnGluLeuLysGlnMetGlnAspLysTyrSerLysSerGlyIleAlaCysPheLeuLysGlu
 171 GATGACAGTTATTGGGACCCCAATGACGAAGAGAGATATGAACAGCCCTGCTGGCAAGTCAAGTGGCAACTCCGTCAAGCTCGTTAGAAAG
 61 AspAspSerTyrTrpAspProAsnAspGluGluSerMetAsnSerProCysTrpGlnValLysTrpGlnLeuArgGlnLeuValArgLys
 161 ATGATTTTGAGAACCTCTGAGGAAACCATTTCTACAGTTCAAGAAAGCAACAAATATTTCTCCCTTAGTGAGAGAGAAAGGTCCTCNCAG
 91 MetIleLeuArgThrSerGluGluThrIleSerThrValGlnGluLysGlnAsnIleSerProLeuValArgGluArgGlyProGln
 151 AGAGTAGCAGCTCACATACTGGGACCAGAGGAAGAAAGCAACACATTTCTCCAAACTCCAAAGAAATGAAGAGGCTCTGGGCCGCAAA
 121 ArgValAlaAlaHisIleThrGlyThrArgGlyArgSerAsnThrLeuSerSerProAsnSerLysAsnGluLysAlaLeuGlyArgLys
 141 ATAAACTCCTGGGAATCATCAAGGAGTGGGCATTTCTCTGAGCAACTTGCACTTGAGGAATGGTGAAGTGGTCACTCCATGAAGAAAGGG
 151 IleAsnSerTrpGluSerSerArgSerGlyHisSerPheLeuSerAsnLeuHisLeuArgAsnGlyGluLeuValIleHisGluLysGly
 131 TTTTACTACATCTATTCCCAACATACATCTTTCGATTTTCAGGAGGAATAAAGAAACACAAAGACAAACAAATGGTCCAAATATATT
 181 PheTyrTyrIleTyrSerGlnThrTyrPheArgPheGlnGluIleLysGluAsnThrLysAsnAspLysGlnMetValGlnTyrIle
 121 TACAAATACACAAAGTTATCCTGACCCCTATATTGTTGATGAAGAGTGCTAGAAATAGTTGTTGCTTAAAGATGCAGAAATATGGACTCTAT
 211 TyrLysTyrThrSerTyrProAspProIleLeuLeuMetLysSerAlaArgAsnSerCysTrpSerLysAspAlaGluTyrGlyLeuTyr
 111 TCCATCTATCAAGGGGAAATATTTGAGCTTAAGGAAATATGACAGAAATTTTGTGTTCTGTAAACAAATGAGCAGCTTGATAGACATGGACCAT
 241 SerIleTyrGlnGlyGlyIlePheGluLeuLysGluAsnAspArgIlePheValSerValThrAsnGluHisLeuIleAspMetAspHis
 101 GAAGCCAGTTTTCGGGGCCTTTTAGTTGGCTAACTGACCTGGAAAGAAAGCAATAACCTCAAGTGACTATTTCAGTTTTCAGGAT
 271 GluAlaSerPhePheGlyAlaPheLeuValGlyGly
 991 GATACACTATGAAGATGTTTCAAAAAAATCTGACCAAAACAAACACAGAAA

FIG. 1

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1 ATGCGCCAC CACCAGCTAG AGTACATCTA GGTGCGTTCC TGGCAGTGAC
 TACCGCGGTG GTGGTCGATC TCATGTAGAT CCACGCAAGG ACCGTCACTG
 1 MetAlaProP roProAlaAr gValHisLeu GlyAlaPheL euAlaValTh

 51 TCCGAATCCC GGGAGCGCAG CGAGTGGGAC AGAGGCAGCC GCGGCCACAC
 AGGCTTAGGG CCCTCGCGTC GCTCACCTG TCTCCGTCGG CCGCGGTGTG
 rProAsnPro GlySerAlaA laSerGlyTh rGluAlaAla AlaAlaThrPro

 101 CCAGCAAAGT GTGGGGCTCT TCCGCGGGGA GGATTGAACC ACCAGGCGGG
 GGTGCTTTCA CACCCGAGA AGGCGCCCTT CCTAACTTGG TGCTCCGCCC
 35 SerLysVa lTrpGlySer SerAlaGlyA rgileGluPr oArgGlyGly

 151 GCGCGAGGAG CGCTCCCTAC CTCATGGGA CAGCACGGAC CCAGTGCCCG
 CCGGCTCCTC GCGAGGGATG GAGGTACCCT GTCGTGCCTG GGTACGGGC
 GlyArgGlyA laLeuProTh rSerMetGly GlnHisGlyP roSerAlaArg

 201 GCGCCGGGCA GGGCGCGCCC CAGGACCCAG GCCGGCGCGG GAAGCCAGCC
 CCGGCCCCGT CCGCGCGCGG GTCCTGGGTC CCGCGCGCGC CTCGGTCCGG
 68 AlaArgAla GlyArgAlaP roGlyProAr gProAlaArg GluAlaSerP

 251 CTCGGCTCCG GGTCCACAAG ACCTTCAAGT TTGTGTCGT CCGGGTCTTG
 GAGCCGAGGC CCAGGTGTTT TGAAGTTCA AACAGCAGCA GCCCCAGGAC
 roArgLeuAr gValHisLys ThrPheLysP heValValVa lGlyValLeu

 301 CTGCAGGTCG TACCTAGCTC AGCTGCAACC ATGATCAATC AATTGGCACA
 GACGTCCAGC ATGGATCGAG TCGACGTTGG TAGTTTGAAG TACTAGTTAG
 101 LeuGlnValV alProSerSe rAlaAlaThr ileLysLeuE isAspGlnSe

 351 AATTGGCACA CAGCAATGGG AACATAGCCC TTTGGGAGAG TTGTGTCCAC
 TTAACCGTGT GTCGTTACCC TTGTATCGGG AAACCTCTC AACACAGGTG
 rIleGlyThr GlnGlnTrpG luHisSerPr oLeuGlyGlu LeuCysProPro

 401 CAGGATCTCA TAGATCAGAA CGTCCTGGAG CCTGTAACCG GTGCACAGAG
 GTCCTAGAGT ATCTAGTCTT GCAGGACCTC GGACATTGGC CACGTGTCTC
 135 GlySerHi sArgSerGlu ArgProGlyA laCysAsnAr gCysThrGlu

 451 GGTGTGGGTT ACACCAATGC TTCCAACAAT TTGTTTGCTT GCCTCCCATG
 CCACACCCAA TGTGGTTACG AAGGTTGTTA AACAAACGAA CGGAGGGTAC
 GlyValGlyT yrThrAsnAl aSerAsnAsn LeuPheAlaC ysLeuProCys

 501 TACAGCTTGT AAATCAGATG AAGAAGAGAG AAGTCCCTGC ACCACGACCA
 ATGTCGAACA TTTAGTCTAC TTCTTCTCTC TTCAGGGACG TGGTGCTGGT
 168 ThrAlaCys LysSerAspG luGluGluAr gSerProCys ThrThrThrA

 551 GGAACACAGC ATGTCAGTGC AAACCAGGAA CTTTCCGGAA TGACAATTCT
 CCTGTGTGTC TACAGTCACG TTTGGTCCTT GAAAGGCCTT ACTGTTAAGA
 rgAsnThrAl aCysGlnCys LysProGlyT hrPheArgAs nAspAsnSer

 601 GCTGAGATGT GCCGGAAGTG CAGCACAGGG TGCCCCAGAG GGATGGTCAA
 CGACTCTACA CGGCTTCAC GTCGTGTCCC ACGGGGTCTC CCTACCAGTT
 201 AlaGluMetC ysArgLysCy sSerThrGly CysProArgG lyMetVally

 651 GGTCAGGAT TGTACGCCCT GGAGTGACAT CGAGTGTGTC CACAAAGAAT
 CCAGTTCTTA ACATGCGGGA CCTCACTGTA GCTCACACAG GTGTTTCTTA
 sValLysAsp CysThrProT rpSerAspIl eGluCysVal HisLysGluSer

FIG. 2A

HOJA DE REEMPLAZO (REGLA 26)

701 CAGGCAATGG ACATAATATA TGGGTGATTT TGGTTGTGAC TTTGGTTGTT
 GTCCGTTACC TGTATTATAT ACCCACTAAA ACCAACACTG AAACCAACAA
 235 GlyAsnGly yHisAsnIle TrpValIleL euValValTh rLeuValVal
 751 CCGTTGCTGT TGGTGGCTGT GCTGATTGTC TGTGTGTCGA TCGGCTCAGG
 GGCAACGACA ACCACCGACA CGACTAACAG ACAACAACGT AGCCGAGTCC
 ProLeuLeuL euValAlaVa lLeuIleVal CysCysCysI leGlySerGly
 801 TTGTGGAGGG GACCCCAAGT GCATGGACAG GGTGTGTTTC TGGCGCTTGG
 AACACCTCCC CTGGGGTTCA CGTACCTGTC CCACACAAAG ACCCGGAACC
 268 CysGlyGly AspProLysC ysMetAspAr gValCysPhe TrpArgLeuG
 851 GTCTCCTACG AGGGCCTGGG GCTGAGGACA ATGCTCACAA CGAGATTCTG
 CAGAGGATGC TCCCGGACCC CGACTCCTGT TACGAGTGTT GCTCTAAGAC
 lyLeuLeuAr gGlyProGly AlaGluAspA snAlaHisAs nGluIleLeu
 901 AGCAACGCAG ACTCGCTGTC CACTTTCGTC TCTGAGCAGC AAATGGAAAG
 TCGTTGCGTC TGAGCGACAG GTGAAAGCAG AGACTCGTCG TTTACCTTTC
 301 SerAsnAlaA spSerLeuSe rThrPheVal SerGluGlnG lNmetGluSe
 951 CCAGGAGCCG GCAGATTGTA CAGGTGTCAC TGTACAGTCC CCAGGGGAGG
 GGTCTCTGCG CGTCTAAACT GTCCACATGT ACATGTCAGG GGTCCCCTCC
 rGlnGluPro AlaAspLeuT hrGlyValTh rValGlnSer ProGlyGluAla
 1001 CACAGTGTCT GCTGGGACCG GCAGAAGCTG AAGGGTCTCA GAGGAGGAGG
 GTGTACAGA CGACCCTGGC CGTCTTCGAC TTCCGAGAGT CTCCTCCTCC
 335 GlnCysLe uLeuGlyPro AlaGluAlaG luGlySerGl nArgArgArg
 1051 CTGCTGGTTC CAGCAAATGG TGCTGACCCC ACTGAGACTC TGATGCTGTT
 GACGACCAAG GTCGTTTACC ACGACTGGGG TGA CTCTGAG ACTACGACAA
 LeuLeuValP roAlaAsnGly yAlaAspPro ThrGluThrL euMetLeuPhe
 1101 CTTTGACAAG TTGCAAACA TCGTGCCCTT TGA CTCTGAG GACCAGCTCA
 GAACTGTTC AAACGTTTGT AGCACGGGAA ACTGAGGACC CTGGTCGAGT
 368 PheAspLys PheAlaAsnI leValProPh eAspSerTrp AspGlnLeuN
 1151 TGAGGCAGCT GGACCTCACG AAAAATGAGA TCGATGTGGT CAGAGCTGGT
 ACTCCGTCGA CCTGGAGTGC TTTTACTCT AGCTACACCA GTCTCGACCA
 etArgGlnLe uAspLeuThr LysAsnGluI leAspValVa lArgAlaGly
 1201 ACAGCAGGCC CAGGGGATGC CTTGTATGCA ATGCTGATGA AATGGGTCAA
 TGTGCTCCGG GTCCCCTACG GAACATACGT TACGACTACT TTACCCAGTT
 401 ThrAlaGlyP roGlyAspAl eLeuTyrAla MetLeuMetL ysTrpValAs
 1251 CAAAACCTGA CGGAACGCCT CGATCCACAC CCTGCTGGAT GCCTTGGAGA
 GTTTTGACCT GCCTTGCGGA GCTAGGTGTG GGACGACCTA CGGAACCTCT
 nLysThrGly ArgAsnAlaS erIleHisTh rLeuLeuAsp AlaLeuGluArg
 1301 GGATGGAAGA GAGACATGCA AAAGAGAAGA TTCAGGACCT CTTGGTGGAC
 CCTACCTTCT CTCTGTACGT TTTCTTCTT AAGTCCTGGA GAACCACCTG
 435 MetGluGl uArgHisAla LysGluLysI leGlnAspLe uLeuValAsp
 1351 TCTGGAAAGT TCATCTACTT AGAAGATGGC ACAGGCTCTG CCGTGTCTTT
 AGACCTTTCA AGTAGATGAA TCTTCTACCG TGTCCGAGAC GGCACAGGAA
 SerGlyLysP heIleTyrLe uGluAspGly ThrGlySerA laValSerLeu
 1401 GGAGTGA
 CCTCACT
 468 GluOP*

FIG. 2B

205

1 NEORGQNDPAASGAKKSHGPGFREARGARPGLEVPKFLVLVVAAVLLLVSAESALITQQD
 61 LAPQQRAPQQRSSPSEGLCPPGHRISEDGRDCISCKYGGDYSTHNDLLFCLRCTRCQ
 121 SGEVELSPCTTTRATVCCKEGTFREEDSPENCRCRTGCPFGMVKVGDCFPWSDIEQVE
 181 KEGGITIGVTVAAVLLVAVPVCKSLNKKVLFYIKGICSGGGGQDPERVDRSSQRFGAED
 241 NVLNRIVSTLQPTQVPEQNEVQEPAPKPTGVNMLSPGESKELLLEPASAERSQRRRLVPA
 301 NEGDPTFTLRQCTFNADLVPTDSWEPINRKLQISDDEIKVAKAKAAGHRDTLYTMLDQW
 361 VNKTGRDASVHTLLDALSTLGERLAKQCKDEHLLSSGKFMYLEGNADSALS

FIG. 3A

Met Glu Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg Lys
1 5 10 15
Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala Arg Pro Gly Pro
20 25 30
Arg Val Pro Lys Thr Leu Val Leu Val Val Ala Ala Val Leu Leu Leu
35 40 45
Val Ser Ala Glu Ser Ala Leu Ile Thr Gln Gln Asp Leu Ala Pro Gln
50 55 60
Gln Arg Ala Ala Pro Gln Gln Lys Arg Ser Ser Pro Ser Glu Gly Leu
65 70 75 80
Cys Pro Pro Gly His His Ile Ser Glu Asp Gly Arg Asp Cys Ile Ser
85 90 95
Cys Lys Tyr Gly Gln Asp Tyr Ser Thr His Trp Asn Asp Leu Leu Phe
100 105 110
Cys Leu Arg Cys Thr Arg Cys Asp Ser Gly Glu Val Glu Leu Ser Pro
115 120 125
Cys Thr Thr Thr Arg Asn Thr Val Cys Gln Cys Glu Glu Gly Thr Phe
130 135 140
Arg Glu Glu Asp Ser Pro Glu Met Cys Arg Lys Cys Arg Thr Gly Cys
145 150 155 160
Pro Arg Gly Met Val Lys Val Gly Asp Cys Thr Pro Trp Ser Asp Ile
165 170 175
Glu Cys Val His Lys Glu Ser Gly Thr Lys His Ser Gly Glu Ala Pro
180 185 190
Ala Val Glu Glu Thr Val Thr Ser Ser Pro Gly Thr Pro Ala Ser Pro
195 200 205
Cys Ser Leu Ser Gly Ile Ile Ile Gly Val Thr Val Ala Ala Val Val
210 215 220
Leu Ile Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys Val
225 230 235 240
Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly Gly Asp Pro Glu
245 250 255
Arg Val Asp Arg Ser Ser Gln Arg Pro Gly Ala Glu Asp Asn Val Leu
260 265 270
Asn Glu Ile Val Ser Ile Leu Gln Pro Thr Gln Val Pro Glu Gln Glu
275 280 285

FIG. 3B

Met	Glu	Val	Gln	Glu	Pro	Ala	Glu	Pro	Thr	Gly	Val	Asn	Met	Leu	Ser	290	295	300
Pro	Gly	Glu	Ser	Glu	His	Leu	Leu	Glu	Pro	Ala	Glu	Ala	Glu	Arg	Ser	305	310	315
Gln	Arg	Arg	Arg	Leu	Leu	Val	Pro	Ala	Asn	Glu	Gly	Asp	Pro	Thr	Glu	325	330	335
Thr	Leu	Arg	Gln	Cys	Phe	Asp	Asp	Phe	Ala	Asp	Leu	Val	Pro	Phe	Asp	340	345	350
Ser	Trp	Gln	Pro	Leu	Met	Arg	Lys	Leu	Gly	Leu	Met	Asp	Asn	Glu	Ile	355	360	365
Lys	Val	Ala	Lys	Ala	Glu	Ala	Ala	Gly	His	Arg	Asp	Thr	Leu	Tyr	Thr	370	375	380
Met	Leu	Ile	Lys	Trp	Val	Asn	Lys	Thr	Gly	Arg	Asp	Ala	Ser	Val	His	385	390	395
Thr	Leu	Leu	Asp	Ala	Leu	Glu	Thr	Leu	Gly	Glu	Arg	Leu	Ala	Lys	Gln	405	410	415
Lys	Ile	Glu	Asp	His	Leu	Leu	Ser	Ser	Gly	Lys	Phe	Met	Tyr	Leu	Glu	420	425	430
Gly	Asn	Ala	Asp	Ser	Ala	Met	Ser									435	440	*

FIG. 3C

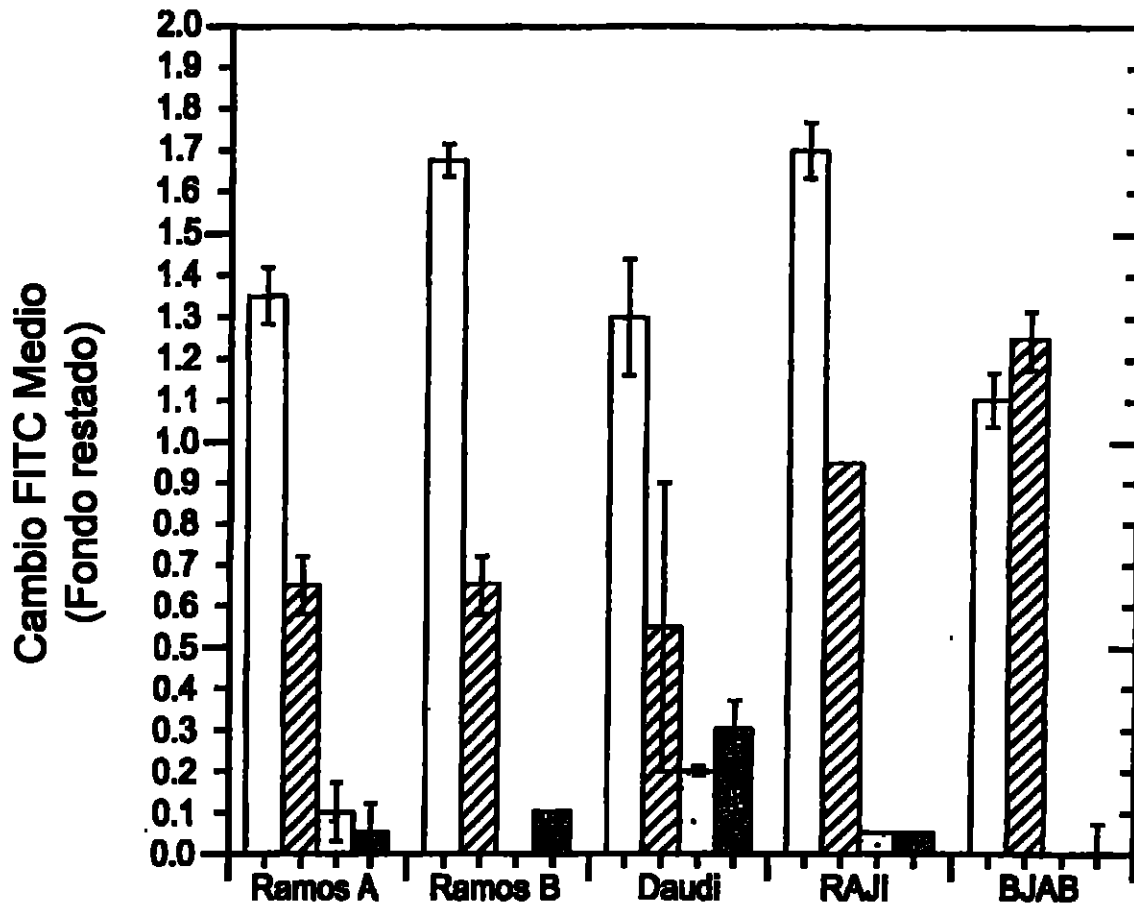


FIG. 4

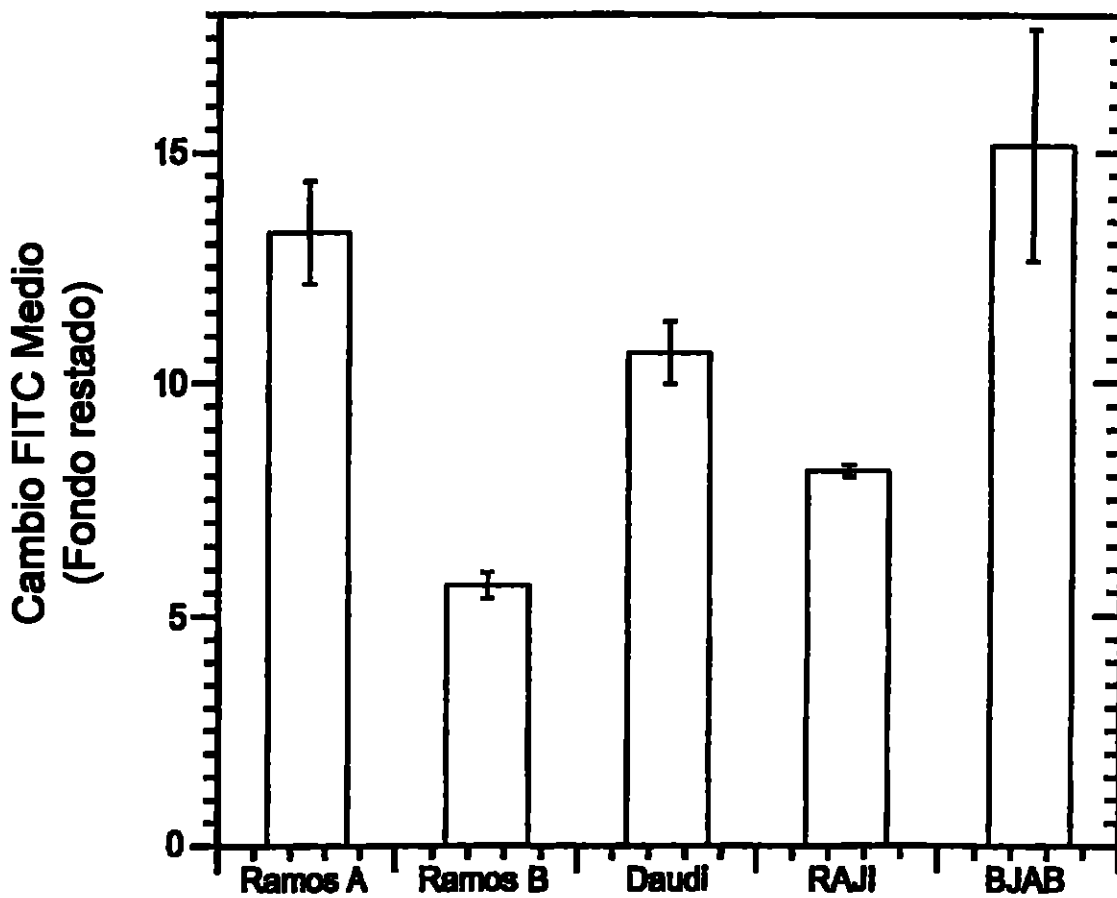


FIG. 5

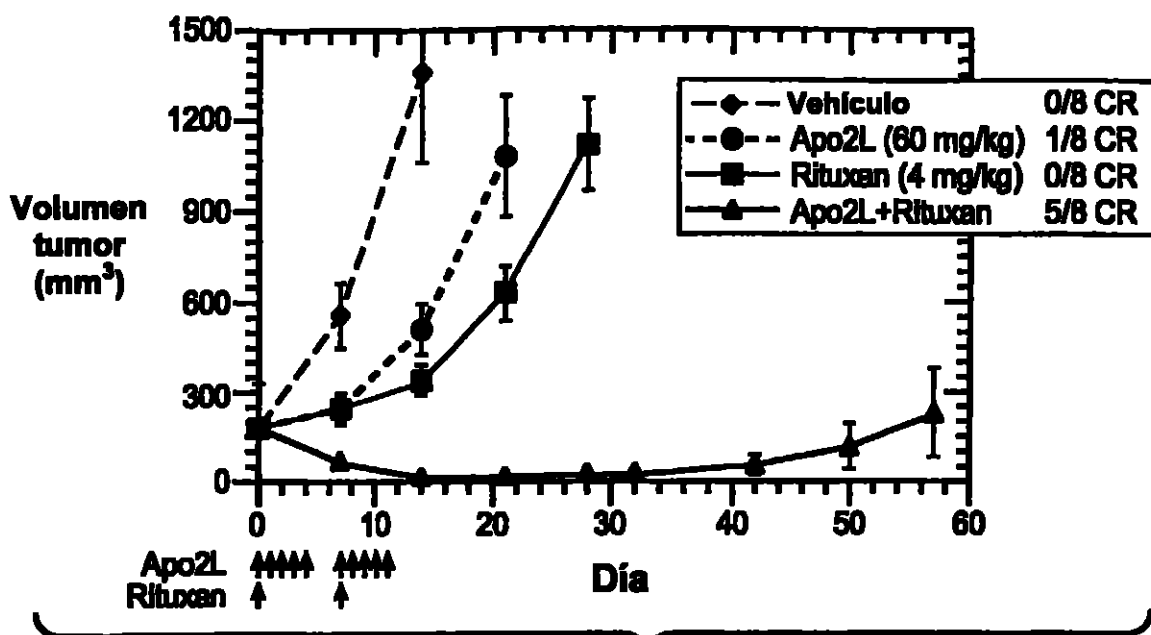


FIG. 6

Apo2L.0 (60 mg/kg/ratón; 5x/semana; IP) solo o en combinación con Rituxan (4 mg/kg/ratón; 1x/semana; IP) vs. xenoinjerto BJAB (ratones SCID)

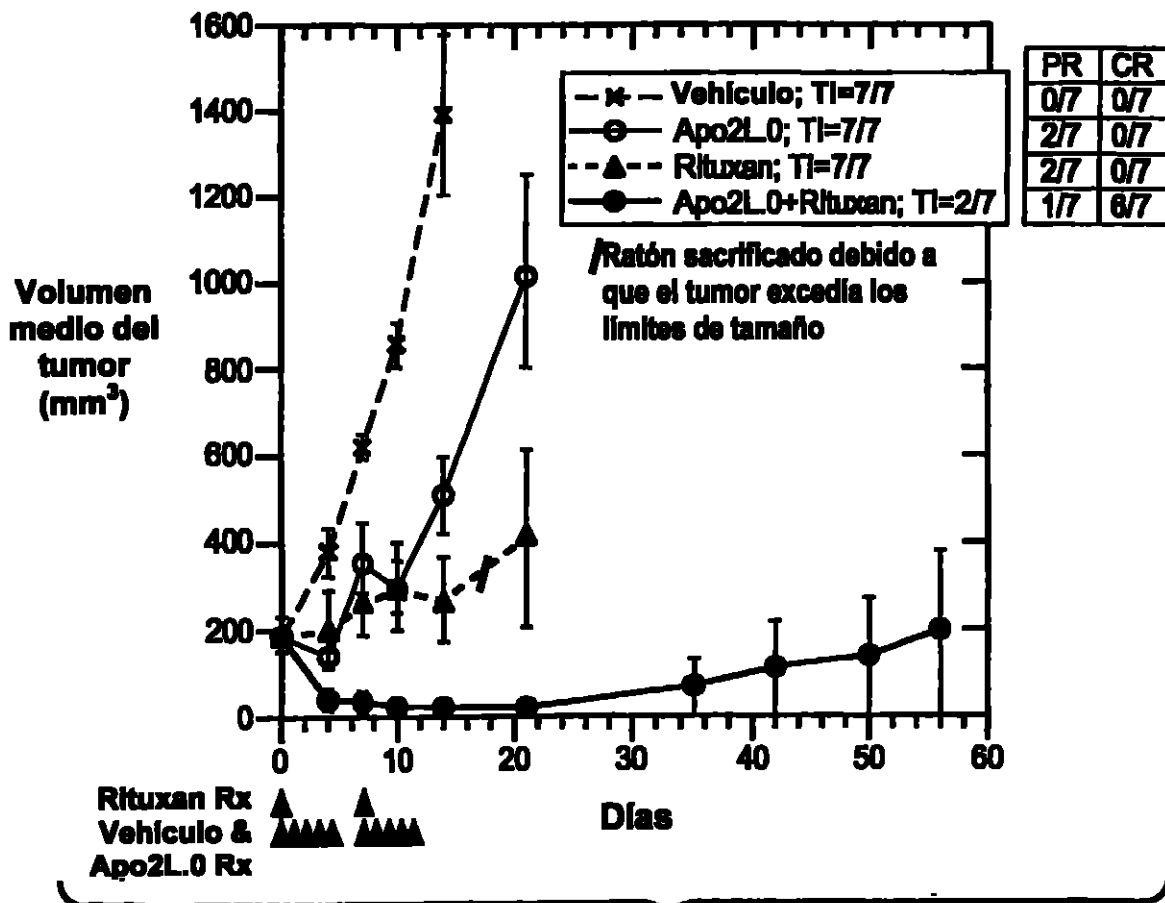


FIG. 7

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FIG. 8

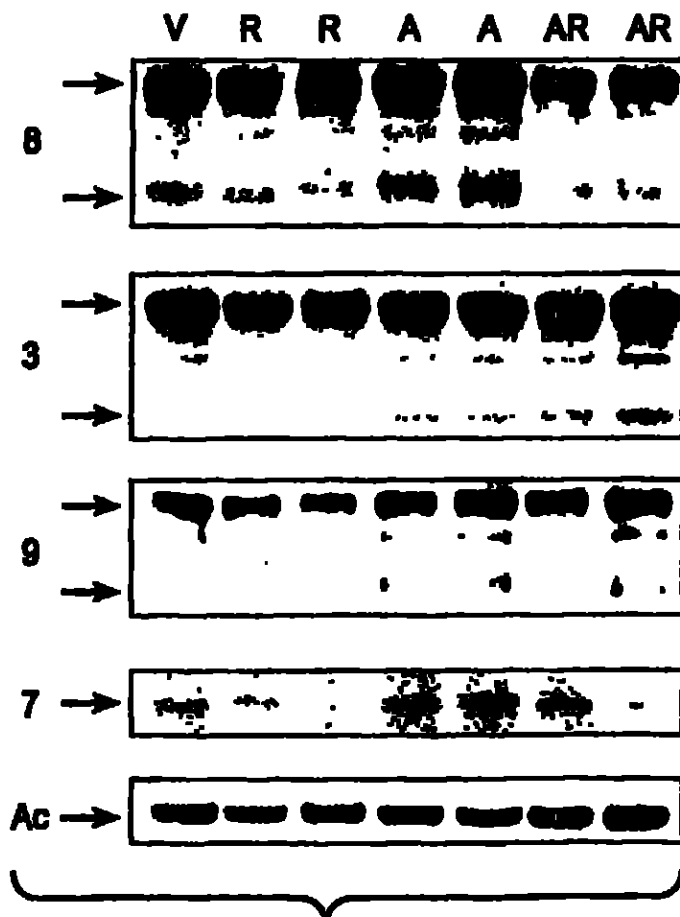


FIG. 10

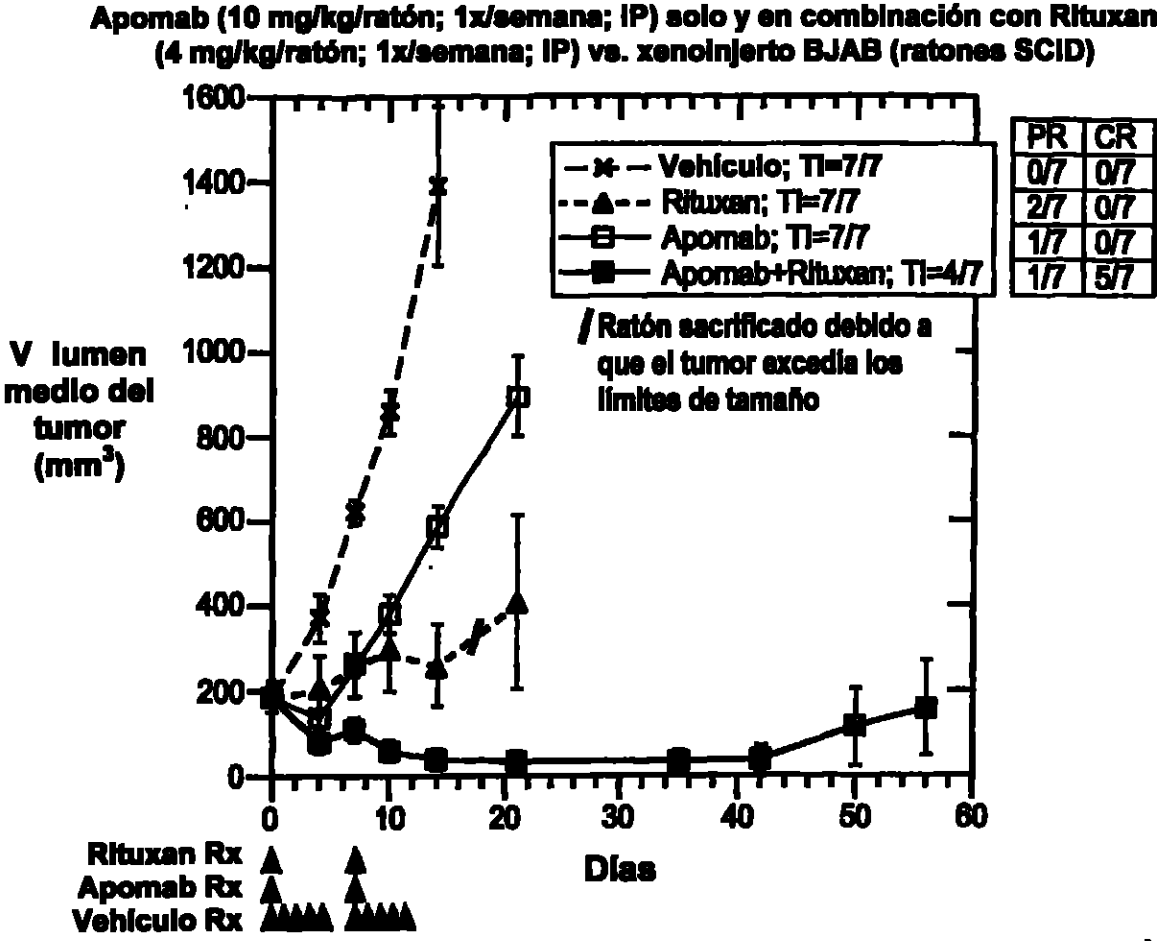


FIG. 9

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Expresión de Receptores CD20 y Ap 2L/TRAIL s bre Líneas Celulares de NHL (Linfoma N H dgkinian)

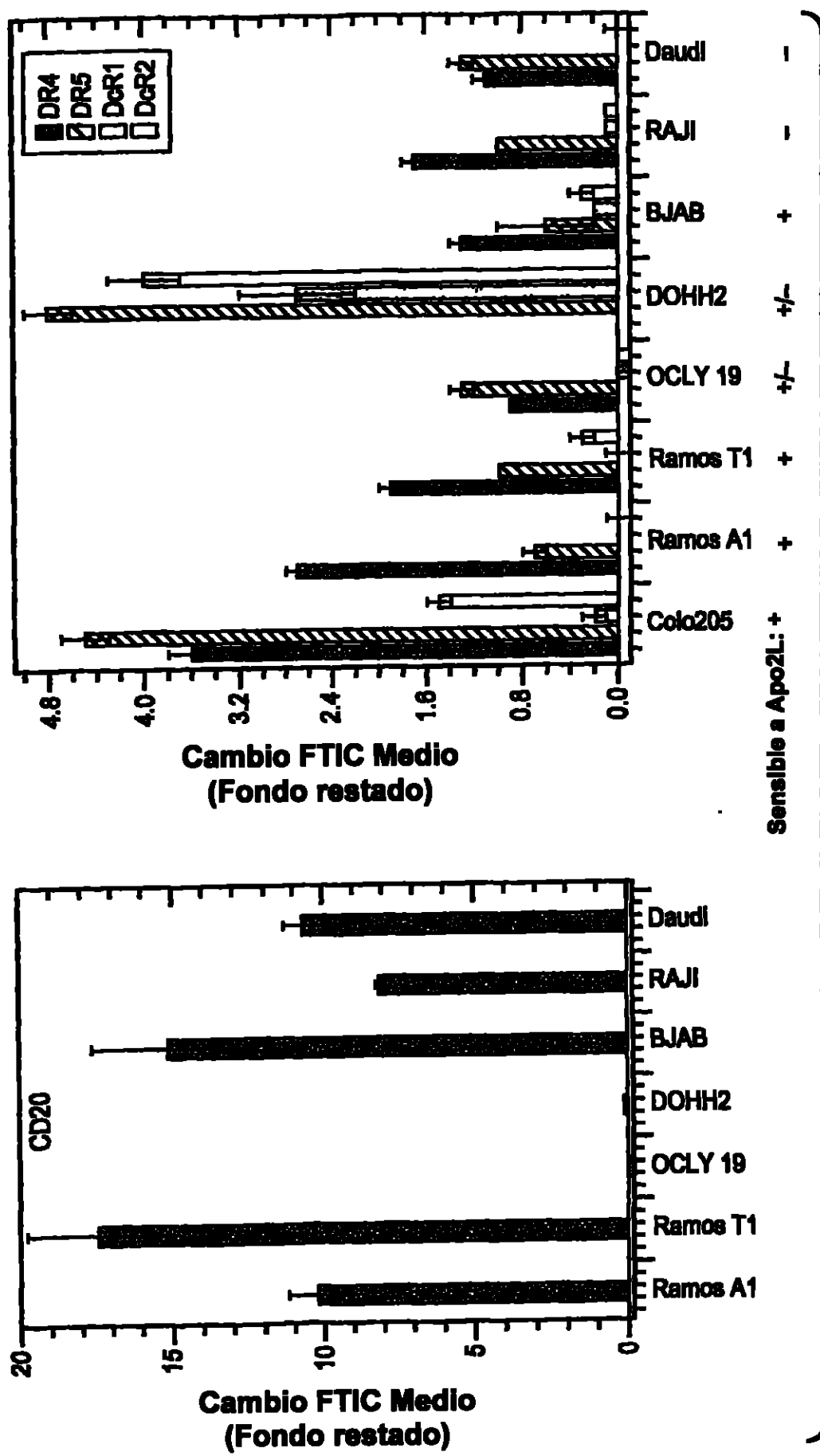


FIG. 11

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[Handwritten signature]

Cooperación de Apo2L/TRAIL y Rituximab contra Xenoinjertos
Ramos RA1 Subcutáneos

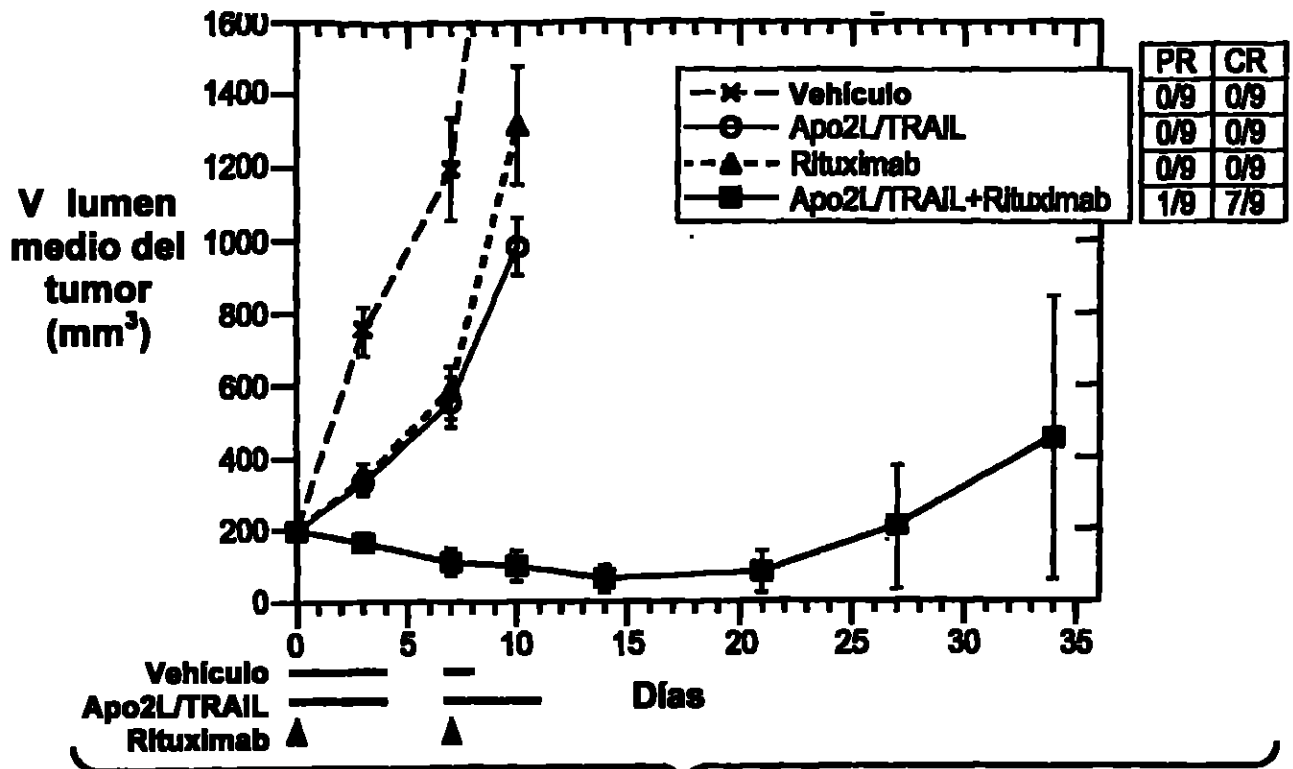


FIG. 12

Cooperación de Apo2L/TRAIL y Rituximab contra
Xenoinjertos de Linfoma Follicular DOHH-2

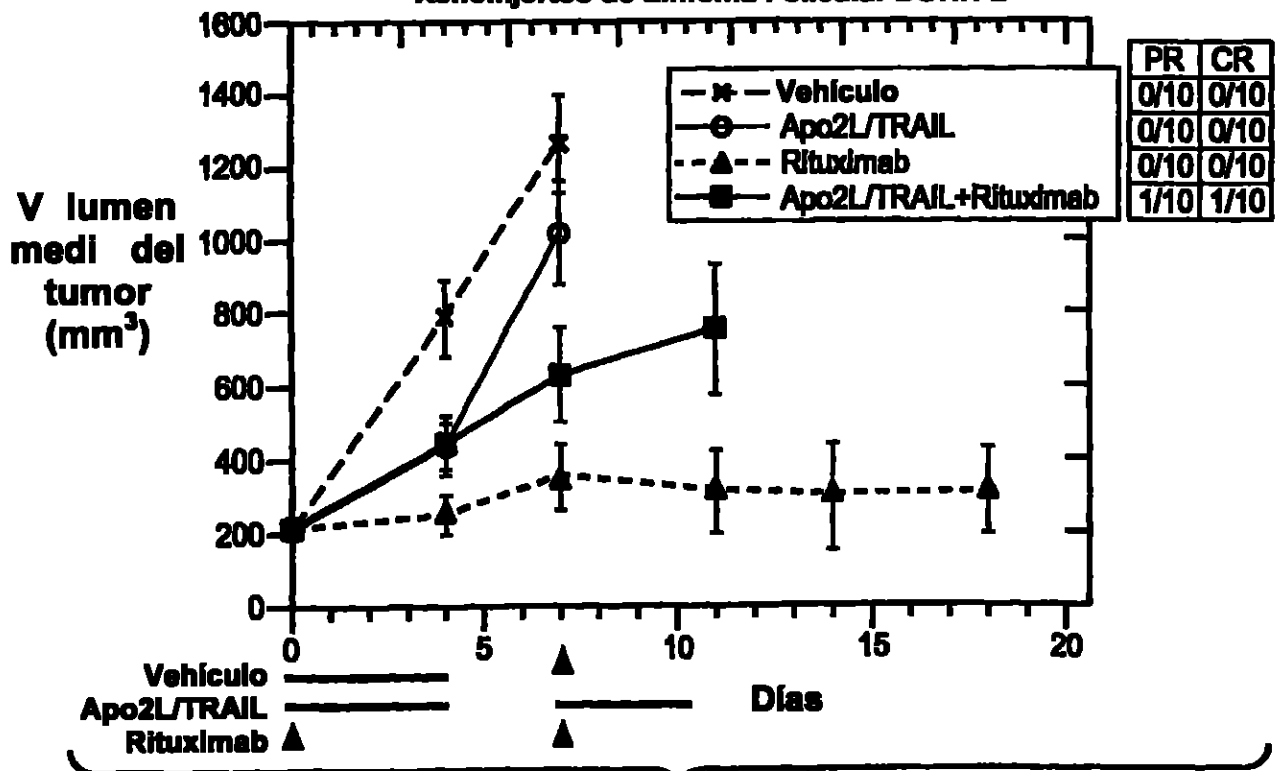


FIG. 13

Rituximab y Apo2L/TRAIL eliminan Células BJAB
Por Mecanismo Independiente

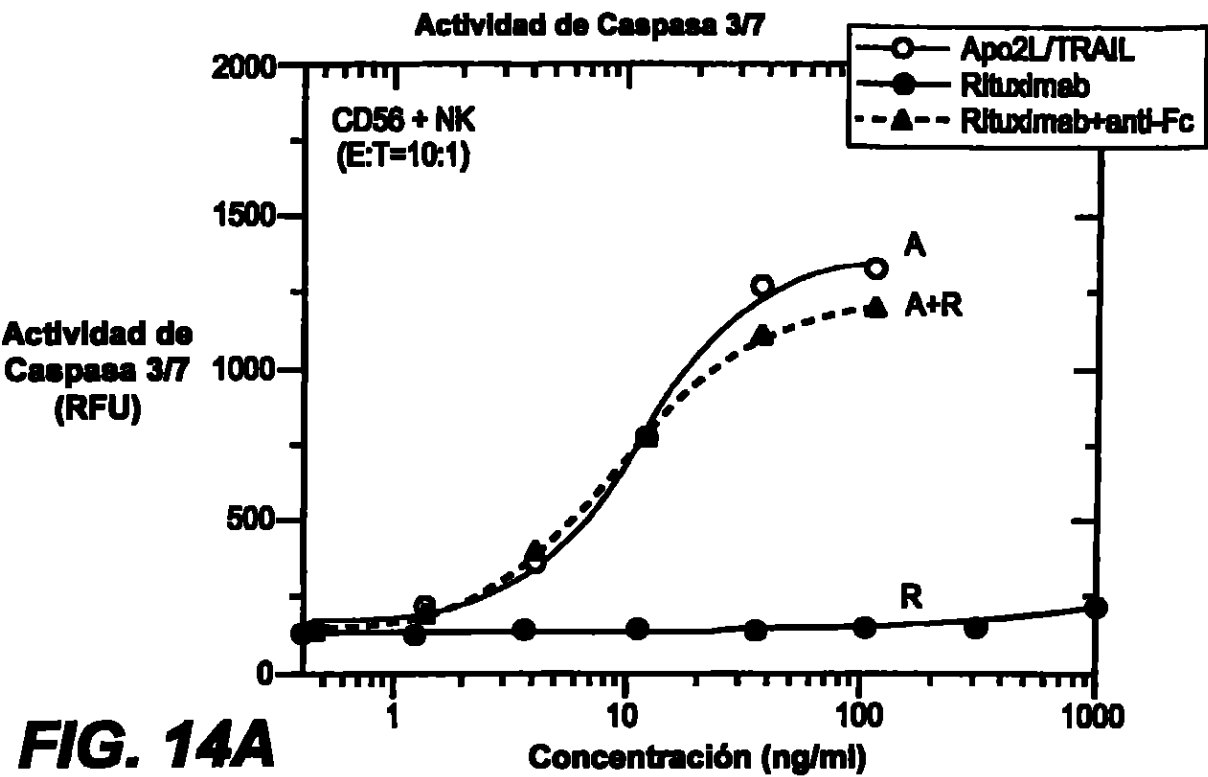


FIG. 14A

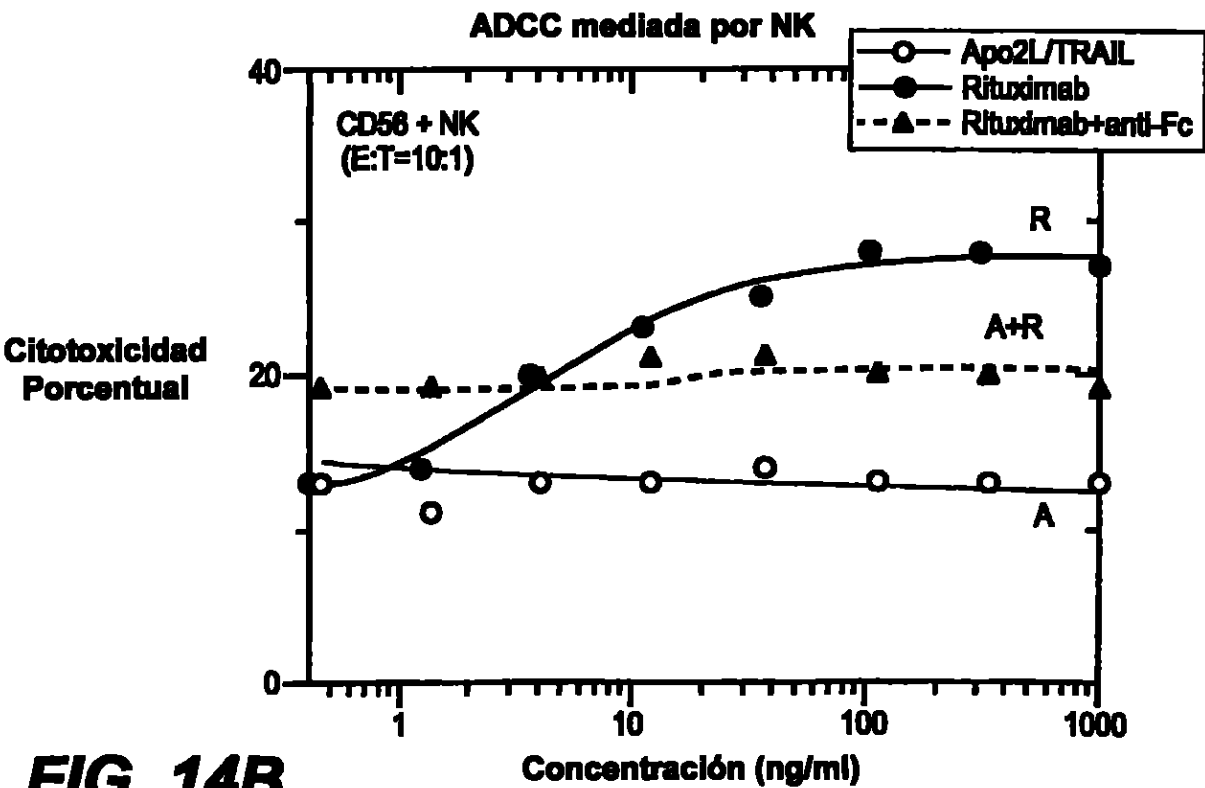
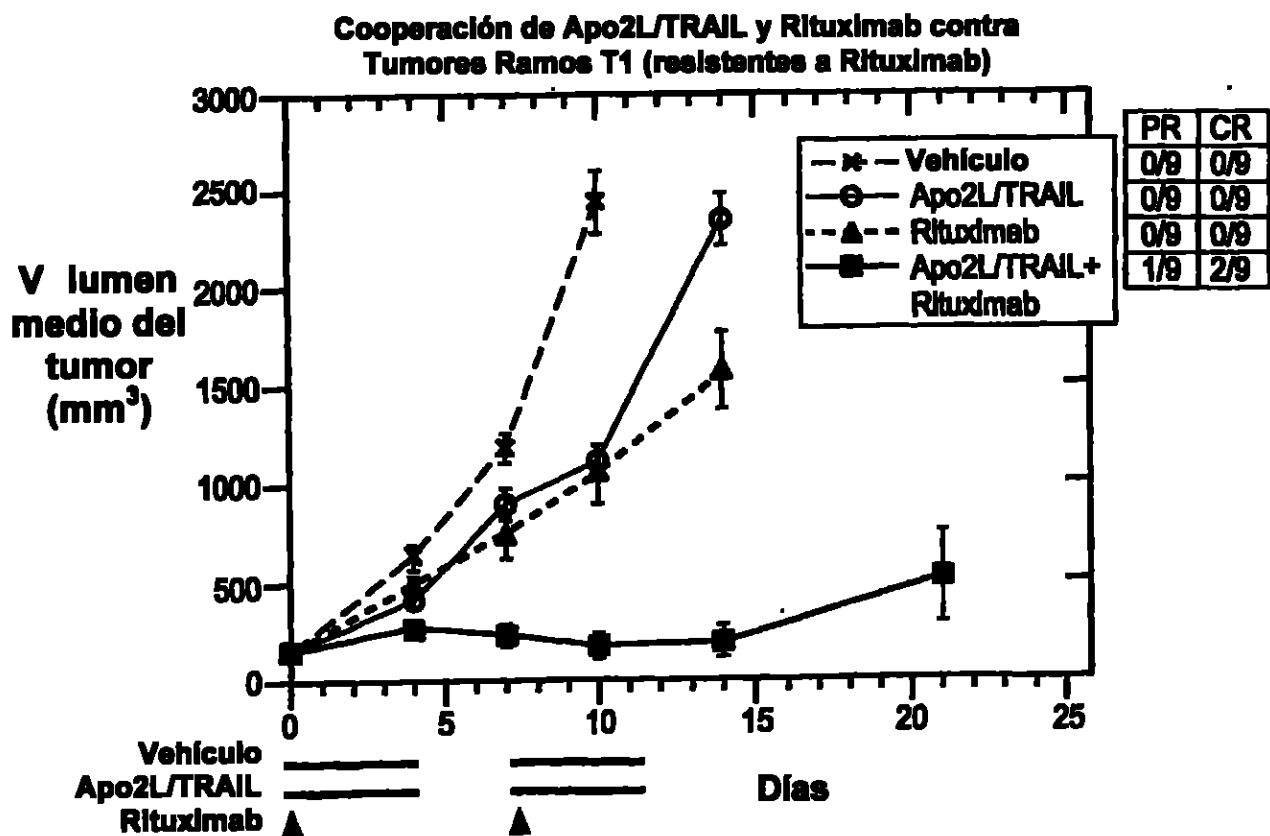
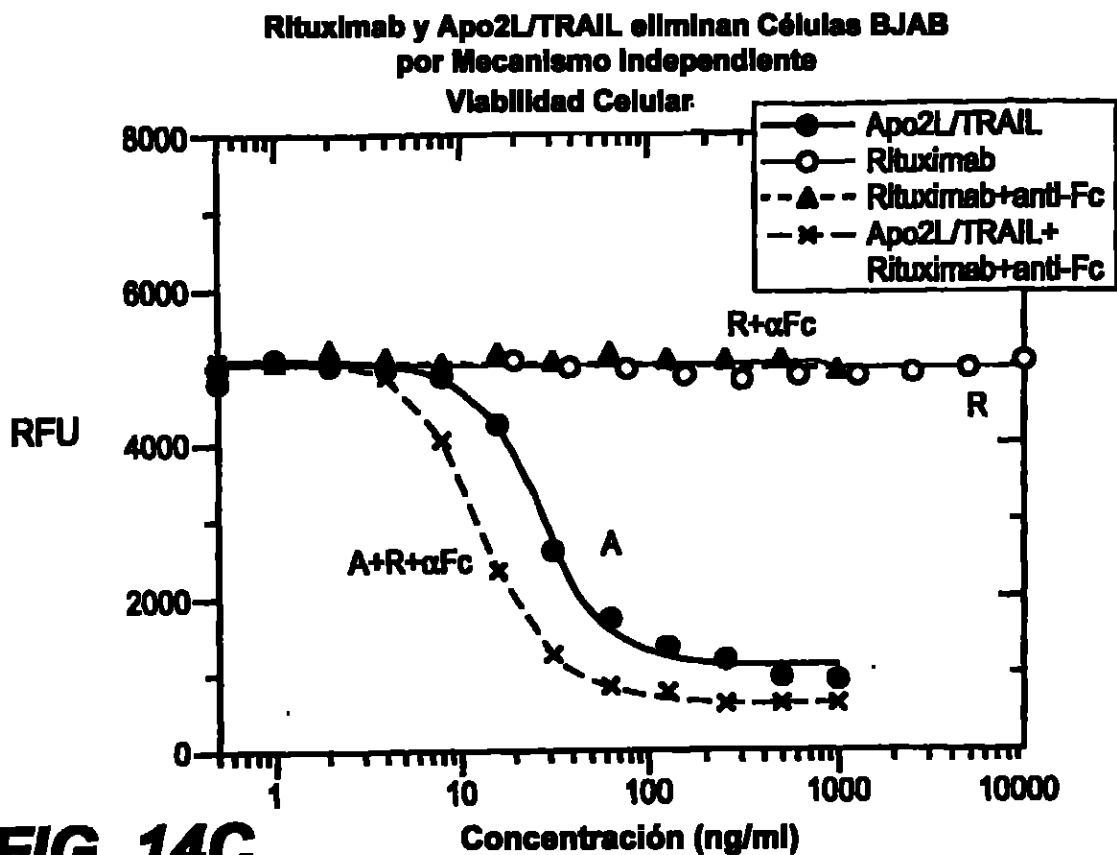


FIG. 14B



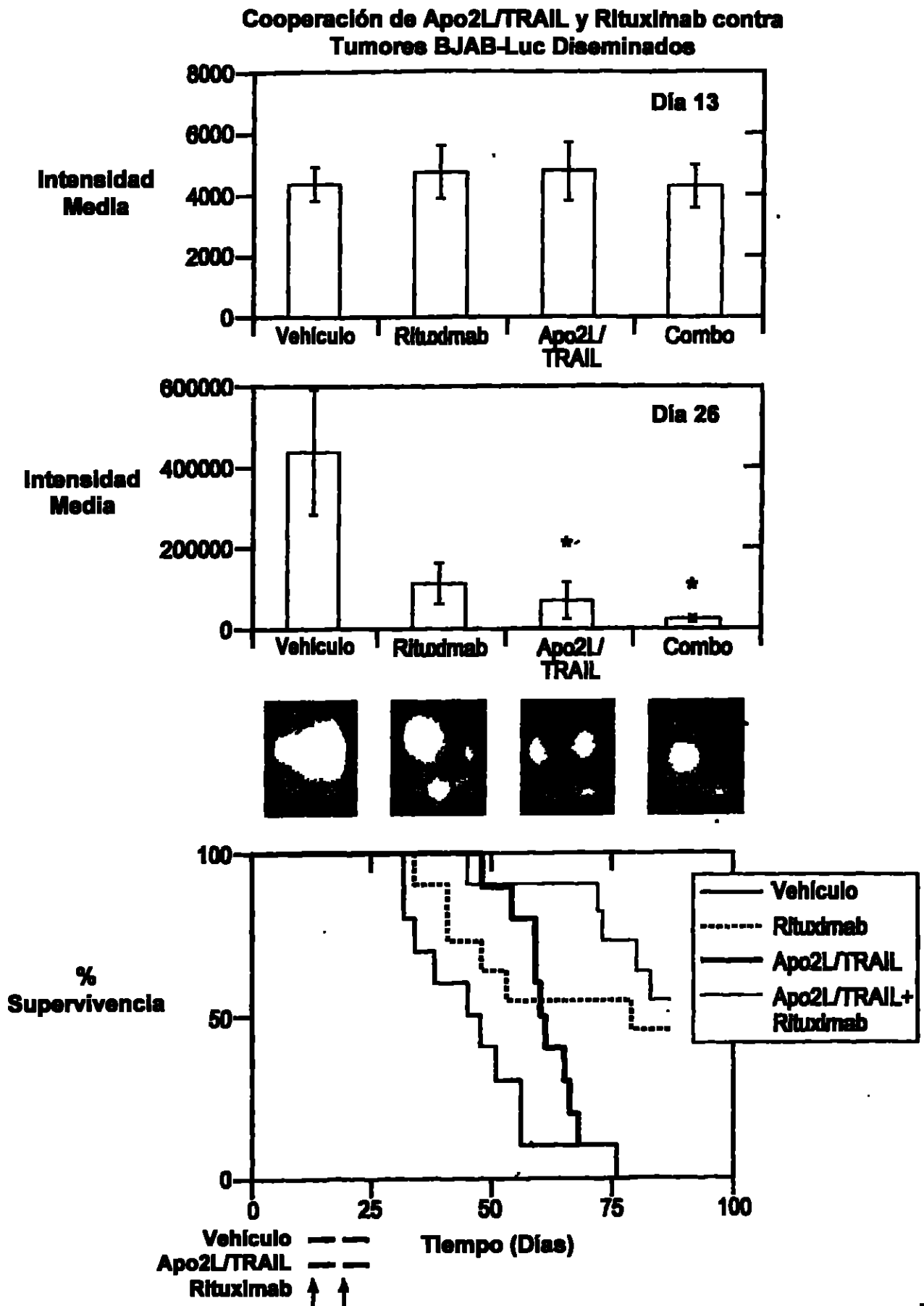


FIG. 16

P2031R1 PCT Sequence Listing
Sequence Listing

212

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<120> METHODS OF USING DEATH RECEPTOR LIGANDS AND CD20 ANTIBODIES

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<140> PCT/US2005/032015

<141> 2005-09-07

<150> US 60/607,834

<151> 2004-09-08

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Gln Asp Lys Tyr Ser Lys Ser Gly Ile Ala Cys Phe Leu Lys Glu
          50          55          60
Asp Asp Ser Tyr Trp Asp Pro Asn Asp Glu Glu Ser Met Asn Ser
          65          70          75
Pro Cys Trp Gln Val Lys Trp Gln Leu Arg Gln Leu Val Arg Lys
          80          85          90
Met Ile Leu Arg Thr Ser Glu Glu Thr Ile Ser Thr Val Gln Glu
          95          100          105
Lys Gln Gln Asn Ile Ser Pro Leu Val Arg Glu Arg Gly Pro Gln
          110          115          120
Arg Val Ala Ala His Ile Thr Gly Thr Arg Gly Arg Ser Asn Thr
          125          130          135
Leu Ser Ser Pro Asn Ser Lys Asn Glu Lys Ala Leu Gly Arg Lys
          140          145          150
Ile Asn Ser Trp Glu Ser Ser Arg Ser Gly His Ser Phe Leu Ser
          155          160          165
Asn Leu His Leu Arg Asn Gly Glu Leu Val Ile His Glu Lys Gly
          170          175          180
Phe Tyr Tyr Ile Tyr Ser Gln Thr Tyr Phe Arg Phe Gln Glu Glu
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Ile Lys Glu Asn Thr Lys Asn Asp Lys Gln Met Val Gln Tyr Ile
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P2031R1 PCT Sequence Listing

226

215 220 225
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Ser Ile Tyr Gln Gly Gly Ile Phe Glu Leu Lys Glu Asn Asp Arg
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gaagccagtt ttttcggggc ctttttagtt ggctaactga cctggaaaga 950
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P2031R1 PCT Sequence Listing

<211> 468
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Ala	Ala	Thr	Pro	Ser	Lys	Val	Trp	Gly	Ser	Ser	Ala	Gly	Arg	Ile	35	40	45	
Glu	Pro	Arg	Gly	Gly	Gly	Arg	Gly	Ala	Leu	Pro	Thr	Ser	Met	Gly	50	55	60	
Gln	His	Gly	Pro	Ser	Ala	Arg	Ala	Arg	Ala	Gly	Arg	Ala	Pro	Gly	65	70	75	
Pro	Arg	Pro	Ala	Arg	Glu	Ala	Ser	Pro	Arg	Leu	Arg	Val	His	Lys	80	85	90	
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Gly	Val	Gly	Tyr	Thr	Asn	Ala	Ser	Asn	Asn	Leu	Phe	Ala	Cys	Leu	155	160	165	
Pro	Cys	Thr	Ala	Cys	Lys	Ser	Asp	Glu	Glu	Glu	Arg	Ser	Pro	Cys	170	175	180	
Thr	Thr	Thr	Arg	Asn	Thr	Ala	Cys	Gln	Cys	Lys	Pro	Gly	Thr	Phe	185	190	195	
Arg	Asn	Asp	Asn	Ser	Ala	Glu	Met	Cys	Arg	Lys	Cys	Ser	Thr	Gly	200	205	210	
Cys	Pro	Arg	Gly	Met	Val	Lys	Val	Lys	Asp	Cys	Thr	Pro	Trp	Ser	215	220	225	
Asp	Ile	Glu	Cys	Val	His	Lys	Gly	Ser	Gly	Asn	Gly	His	Asn	Ile	230	235	240	
Trp	Val	Ile	Leu	Val	Val	Thr	Leu	Val	Val	Pro	Leu	Leu	Leu	Val	245	250	255	
Ala	Val	Leu	Ile	Val	Cys	Cys	Cys	Ile	Gly	Ser	Gly	Cys	Gly	Gly	260	265	270	
Asp	Pro	Lys	Cys	Met	Asp	Arg	Val	Cys	Phe	Trp	Arg	Leu	Gly	Leu	275	280	285	
Leu	Arg	Gly	Pro	Gly	Ala	Glu	Asp	Asn	Ala	His	Asn	Glu	Ile	Leu	290	295	300	
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P2031R1 PCT Sequence Listing

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				350					355					360
Thr	Glu	Thr	Leu	Met	Leu	Phe	Phe	Asp	Lys	Phe	Ala	Asn	Ile	Val
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Pro	Phe	Asp	Ser	Trp	Asp	Gln	Leu	Met	Arg	Gln	Leu	Asp	Leu	Thr
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Lys	Asn	Glu	Ile	Asp	Val	Val	Arg	Ala	Gly	Thr	Ala	Gly	Pro	Gly
				395					400					405
Asp	Ala	Leu	Tyr	Ala	Met	Leu	Met	Lys	Trp	Val	Asn	Lys	Thr	Gly
				410					415					420
Arg	Asn	Ala	Ser	Ile	His	Thr	Leu	Leu	Asp	Ala	Leu	Glu	Arg	Met
				425					430					435
Glu	Glu	Arg	His	Ala	Lys	Glu	Lys	Ile	Gln	Asp	Leu	Leu	Val	Asp
				440					445					450
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ggcccgggca gggcgcgccc caggaccag gccggcgcgg gaagccagcc 250
ctcggctccg ggtccacaag accttcaagt ttgtcgtcgt cggggtcctg 300
ctgcaggtcg tacctagctc agctgcaacc atgatcaatc aattggcaca 350
aattggcaca cagcaatggg aacatagccc ttgggagag ttgtgtccac 400
caggatctca tagatcagaa cgtcctggag cctgtaaccg gtgcacagag 450
ggtgtgggtt acaccaatgc ttccaacaat ttgtttgctt gcctcccatg 500
tacagcttgt aaatcagatg aagaagagag aagtcctgc accacgacca 550
ggaacacagc atgtcagtc aaaccaggaa ctttccggaa tgacaattct 600
gctgagatgt gccggaagt cagcacagg tgccccagag ggatgggtcaa 650
ggtcaaggat tgtacgccct ggagtgacat cgagtgtgtc cacaagaat 700
caggcaatgg acataatata tgggtgattt tggttgtgac ttggttgtt 750

P2031R1 PCT Sequence Listing

ccgttgctgt tggtagctgt gctgattgtc tgttggtgca tcggctcagg 800
 ttgtggaggg gaccccaagt gcatggacag ggtgtgtttc tggcgcttgg 850
 gtctcctacg agggcctggg gctgaggaca atgctcaca cgagattctg 900
 agcaacgcag actcgtgtc cactttcgtc tctgagcagc aaatggaaaag 950
 ccaggagccg gcagatttga caggtgtcac tgtacagtcc ccaggggagg 1000
 cacagtgtct gctgggaccg gcagaagctg aaggggtctca gaggaggagg 1050
 ctgctggttc cagcaaattg tgctgacccc actgagactc tgatgctgtt 1100
 ctttgacaag ttgcaaaca tcgtgccctt tgactcctgg gaccagctca 1150
 tgaggcagct ggacctcacg aaaaatgaga tcgatgtggt cagagctggt 1200
 acagcaggcc caggggatgc cttgtatgca atgctgatga aatgggtcaa 1250
 caaaactgga cggaacgcct cgatccacac cctgctggat gccttgagaga 1300
 ggatggaaga gagacatgca aaagagaaga ttcaggacct cttggtggac 1350
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<210> 5
 <211> 411
 <212> PRT
 <213> Homo sapiens

<400> 5
 Met Glu Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg
 1 5 10
 Lys Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala Arg Pro
 20 25 30
 Gly Leu Arg Val Pro Lys Thr Leu Val Leu Val Val Ala Ala Val
 35 40 45
 Leu Leu Leu Val Ser Ala Glu Ser Ala Leu Ile Thr Gln Gln Asp
 50 55 60
 Leu Ala Pro Gln Gln Arg Ala Ala Pro Gln Gln Lys Arg Ser Ser
 65 70 75
 Pro Ser Glu Gly Leu Cys Pro Pro Gly His His Ile Ser Glu Asp
 80 85 90
 Gly Arg Asp Cys Ile Ser Cys Lys Tyr Gly Gln Asp Tyr Ser Thr
 95 100 105
 His Trp Asn Asp Leu Leu Phe Cys Leu Arg Cys Thr Arg Cys Asp
 110 115 120
 Ser Gly Glu Val Glu Leu Ser Pro Cys Thr Thr Thr Arg Asn Thr
 125 130 135
 Val Cys Gln Cys Glu Glu Gly Thr Phe Arg Glu Glu Asp Ser Pro
 140 145 150
 Glu Met Cys Arg Lys Cys Arg Thr Gly Cys Pro Arg Gly Met Val
 155 160 165

257 222

P2031R1 PCT Sequence Listing

Lys Val Gly Asp Cys Thr Pro Trp Ser Asp Ile Glu Cys Val His	170	175	180
Lys Glu Ser Gly Ile Ile Ile Gly Val Thr Val Ala Ala Val Val	185	190	195
Leu Ile Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys	200	205	210
Val Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly Gly Asp	215	220	225
Pro Glu Arg Val Asp Arg Ser Ser Gln Arg Pro Gly Ala Glu Asp	230	235	240
Asn Val Leu Asn Glu Ile Val Ser Ile Leu Gln Pro Thr Gln Val	245	250	255
Pro Glu Gln Glu Met Glu Val Gln Glu Pro Ala Glu Pro Thr Gly	260	265	270
Val Asn Met Leu Ser Pro Gly Glu Ser Glu His Leu Leu Glu Pro	275	280	285
Ala Glu Ala Glu Arg Ser Gln Arg Arg Arg Leu Leu Val Pro Ala	290	295	300
Asn Glu Gly Asp Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp Asp	305	310	315
Phe Ala Asp Leu Val Pro Phe Asp Ser Trp Glu Pro Leu Met Arg	320	325	330
Lys Leu Gly Leu Met Asp Asn Glu Ile Lys Val Ala Lys Ala Glu	335	340	345
Ala Ala Gly His Arg Asp Thr Leu Tyr Thr Met Leu Ile Lys Trp	350	355	360
Val Asn Lys Thr Gly Arg Asp Ala Ser Val His Thr Leu Leu Asp	365	370	375
Ala Leu Glu Thr Leu Gly Glu Arg Leu Ala Lys Gln Lys Ile Glu	380	385	390
Asp His Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu Gly Asn	395	400	405
Ala Asp Ser Ala Leu Ser	410		

<210> 6
 <211> 440
 <212> PRT
 <213> Homo sapiens

<400> 6
 Met Glu Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg
 1 5 10 15
 Lys Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala Arg Pro
 20 25 30
 Gly Pro Arg Val Pro Lys Thr Leu Val Leu Val Val Ala Ala Val
 35 40 45
 Leu Leu Leu Val Ser Ala Glu Ser Ala Leu Ile Thr Gln Gln Asp
 50 55 60

P2031R1 PCT Sequence Listing

237 223

Leu	Ala	Pro	Gln	Gln	Arg	Ala	Ala	Pro	Gln	Gln	Lys	Arg	Ser	Ser	
			65						70					75	
Pro	Ser	Glu	Gly	Leu	Cys	Pro	Pro	Gly	His	His	Ile	Ser	Glu	Asp	
			80						85					90	
Gly	Arg	Asp	Cys	Ile	Ser	Cys	Lys	Tyr	Gly	Gln	Asp	Tyr	Ser	Thr	
			95						100					105	
His	Trp	Asn	Asp	Leu	Leu	Phe	Cys	Leu	Arg	Cys	Thr	Arg	Cys	Asp	
			110						115					120	
Ser	Gly	Glu	Val	Glu	Leu	Ser	Pro	Cys	Thr	Thr	Thr	Arg	Asn	Thr	
			125						130					135	
Val	Cys	Gln	Cys	Glu	Glu	Gly	Thr	Phe	Arg	Glu	Glu	Asp	Ser	Pro	
			140						145					150	
Glu	Met	Cys	Arg	Lys	Cys	Arg	Thr	Gly	Cys	Pro	Arg	Gly	Met	Val	
			155						160					165	
Lys	Val	Gly	Asp	Cys	Thr	Pro	Trp	Ser	Asp	Ile	Glu	Cys	Val	His	
			170						175					180	
Lys	Glu	Ser	Gly	Thr	Lys	His	Ser	Gly	Glu	Ala	Pro	Ala	Val	Glu	
			185						190					195	
Glu	Thr	Val	Thr	Ser	Ser	Pro	Gly	Thr	Pro	Ala	Ser	Pro	Cys	Ser	
			200						205					210	
Leu	Ser	Gly	Ile	Ile	Ile	Gly	Val	Thr	Val	Ala	Ala	Val	Val	Leu	
			215						220					225	
Ile	Val	Ala	Val	Phe	Val	Cys	Lys	Ser	Leu	Leu	Trp	Lys	Lys	Val	
			230						235					240	
Leu	Pro	Tyr	Leu	Lys	Gly	Ile	Cys	Ser	Gly	Gly	Gly	Gly	Asp	Pro	
			245						250					255	
Glu	Arg	Val	Asp	Arg	Ser	Ser	Gln	Arg	Pro	Gly	Ala	Glu	Asp	Asn	
			260						265					270	
Val	Leu	Asn	Glu	Ile	Val	Ser	Ile	Leu	Gln	Pro	Thr	Gln	Val	Pro	
			275						280					285	
Glu	Gln	Glu	Met	Glu	Val	Gln	Glu	Pro	Ala	Glu	Pro	Thr	Gly	Val	
			290						295					300	
Asn	Met	Leu	Ser	Pro	Gly	Glu	Ser	Glu	His	Leu	Leu	Glu	Pro	Ala	
			305						310					315	
Glu	Ala	Glu	Arg	Ser	Gln	Arg	Arg	Arg	Leu	Leu	Val	Pro	Ala	Asn	
			320						325					330	
Glu	Gly	Asp	Pro	Thr	Glu	Thr	Leu	Arg	Gln	Cys	Phe	Asp	Asp	Phe	
			335						340					345	
Ala	Asp	Leu	Val	Pro	Phe	Asp	Ser	Trp	Glu	Pro	Leu	Met	Arg	Lys	
			350						355					360	
Leu	Gly	Leu	Met	Asp	Asn	Glu	Ile	Lys	Val	Ala	Lys	Ala	Glu	Ala	
			365						370					375	
Ala	Gly	His	Arg	Asp	Thr	Leu	Tyr	Thr	Met	Leu	Ile	Lys	Trp	Val	
			380						385					390	
Asn	Lys	Thr	Gly	Arg	Asp	Ala	Ser	Val	His	Thr	Leu	Leu	Asp	Ala	

234 224

P2031R1 PCT Sequence Listing

395

400

405

Leu Glu Thr Leu Gly Glu Arg Leu Ala Lys Gln Lys Ile Glu Asp
410 415 420

His Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu Gly Asn Ala
425 430 435

Asp Ser Ala Met Ser
440

<210> 7
<211> 107
<212> PRT
<213> Artificial sequence

<220>
<223> synthetic

<400> 7
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15
Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Val Ser
20 25 30
Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Pro
35 40 45
Leu Ile Tyr Ala Pro Ser Asn Leu Ala Ser Gly Val Pro Ser Arg
50 55 60
Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
65 70 75
Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Trp
80 85 90
Ser Phe Asn Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile
95 100 105

Lys Arg

<210> 8
<211> 122
<212> PRT
<213> Artificial sequence

<220>
<223> synthetic

<400> 8
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15
Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr
20 25 30
Ser Tyr Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45
Glu Trp Val Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr
50 55 60
Asn Gln Lys Phe Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser
65 70 75

25 / 225

P2031R1 PCT Sequence Listing

Lys	Asn	Thr	Leu	Tyr	Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp
				80					85					90
Thr	Ala	Val	Tyr	Tyr	Cys	Ala	Arg	Val	Val	Tyr	Tyr	Ser	Asn	Ser
			95						100					105
Tyr	Trp	Tyr	Phe	Asp	Val	Trp	Gly	Gln	Gly	Thr	Leu	Val	Thr	Val
			110						115					120

Ser Ser

<210> 9
 <211> 213
 <212> PRT
 <213> Artificial sequence

<220>
 <223> synthetic

<400> 9

Asp	Ile	Gln	Met	Thr	Gln	Ser	Pro	Ser	Ser	Leu	Ser	Ala	Ser	Val
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Gly	Asp	Arg	Val	Thr	Ile	Thr	Cys	Arg	Ala	Ser	Ser	Ser	Val	Ser
			20						25					30
Tyr	Met	His	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Lys	Ala	Pro	Lys	Pro
			35						40					45
Leu	Ile	Tyr	Ala	Pro	Ser	Asn	Leu	Ala	Ser	Gly	Val	Pro	Ser	Arg
			50						55					60
Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr	Leu	Thr	Ile	Ser
			65						70					75
Ser	Leu	Gln	Pro	Glu	Asp	Phe	Ala	Thr	Tyr	Tyr	Cys	Gln	Gln	Trp
			80						85					90
Ser	Phe	Asn	Pro	Pro	Thr	Phe	Gly	Gln	Gly	Thr	Lys	Val	Glu	Ile
			95						100					105
Lys	Arg	Thr	Val	Ala	Ala	Pro	Ser	Val	Phe	Ile	Phe	Pro	Pro	Ser
			110						115					120
Asp	Glu	Gln	Leu	Lys	Ser	Gly	Thr	Ala	Ser	Val	Val	Cys	Leu	Leu
			125						130					135
Asn	Asn	Phe	Tyr	Pro	Arg	Glu	Ala	Lys	Val	Gln	Trp	Lys	Val	Asp
			140						145					150
Asn	Ala	Leu	Gln	Ser	Gly	Asn	Ser	Gln	Glu	Ser	Val	Thr	Glu	Gln
			155						160					165
Asp	Ser	Lys	Asp	Ser	Thr	Tyr	Ser	Leu	Ser	Ser	Thr	Leu	Thr	Leu
			170						175					180
Ser	Lys	Ala	Asp	Tyr	Glu	Lys	His	Lys	Val	Tyr	Ala	Cys	Glu	Val
			185						190					195
Thr	His	Gln	Gly	Leu	Ser	Ser	Pro	Val	Thr	Lys	Ser	Phe	Asn	Arg
			200						205					210

Gly Glu Cys

<210> 10
 <211> 452

P2031R1 PCT Sequence Listing

<212> PRT

<213> Artificial sequence

<220>

<223> synthetic

<400> 10

Glu	Val	Gln	Leu	Val	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln	Pro	Gly
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Gly	Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Tyr	Thr	Phe	Thr
				20					25					30
Ser	Tyr	Asn	Met	His	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu
				35					40					45
Glu	Trp	Val	Gly	Ala	Ile	Tyr	Pro	Gly	Asn	Gly	Asp	Thr	Ser	Tyr
				50					55					60
Asn	Gln	Lys	Phe	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Val	Asp	Lys	Ser
				65					70					75
Lys	Asn	Thr	Leu	Tyr	Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp
				80					85					90
Thr	Ala	Val	Tyr	Tyr	Cys	Ala	Arg	Val	Val	Tyr	Tyr	Ser	Asn	Ser
				95					100					105
Tyr	Trp	Tyr	Phe	Asp	Val	Trp	Gly	Gln	Gly	Thr	Leu	Val	Thr	Val
				110					115					120
Ser	Ser	Ala	Ser	Thr	Lys	Gly	Pro	Ser	Val	Phe	Pro	Leu	Ala	Pro
				125					130					135
Ser	Ser	Lys	Ser	Thr	Ser	Gly	Gly	Thr	Ala	Ala	Leu	Gly	Cys	Leu
				140					145					150
Val	Lys	Asp	Tyr	Phe	Pro	Glu	Pro	Val	Thr	Val	Ser	Trp	Asn	Ser
				155					160					165
Gly	Ala	Leu	Thr	Ser	Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu	Gln
				170					175					180
Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser
				185					190					195
Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	Asn	Val	Asn	His	Lys
				200					205					210
Pro	Ser	Asn	Thr	Lys	Val	Asp	Lys	Lys	Val	Glu	Pro	Lys	Ser	Cys
				215					220					225
Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu
				230					235					240
Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr
				245					250					255
Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp
				260					265					270
Val	Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp
				275					280					285
Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln
				290					295					300
Tyr	Asn	Ser	Thr	Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His

237 227

P2031R1 PCT Sequence Listing

305		310		315
Gln Asp Trp Leu	Asn Gly Lys Glu Tyr	Lys Cys Lys Val Ser	Asn	
	320	325	330	
Lys Ala Leu Pro	Ala Pro Ile Glu Lys	Thr Ile Ser Lys Ala	Lys	
	335	340	345	
Gly Gln Pro Arg	Glu Pro Gln Val Tyr	Thr Leu Pro Pro Ser	Arg	
	350	355	360	
Glu Glu Met Thr	Lys Asn Gln Val Ser	Leu Thr Cys Leu Val	Lys	
	365	370	375	
Gly Phe Tyr Pro	Ser Asp Ile Ala Val	Glu Trp Glu Ser Asn	Gly	
	380	385	390	
Gln Pro Glu Asn	Asn Tyr Lys Thr Thr	Pro Pro Val Leu Asp	Ser	
	395	400	405	
Asp Gly Ser Phe	Phe Leu Tyr Ser Lys	Leu Thr Val Asp Lys	Ser	
	410	415	420	
Arg Trp Gln Gln	Gly Asn Val Phe Ser	Cys Ser Val Met His	Glu	
	425	430	435	
Ala Leu His Asn	His Tyr Thr Gln Lys	Ser Leu Ser Leu Ser	Pro	
	440	445	450	

Gly Lys

- <210> 11
- <211> 452
- <212> PRT
- <213> Artificial sequence

- <220>
- <223> synthetic

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Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr	
20 25 30	
Ser Tyr Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu	
35 40 45	
Glu Trp Val Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr	
50 55 60	
Asn Gln Lys Phe Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser	
65 70 75	
Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp	
80 85 90	
Thr Ala Val Tyr Tyr Cys Ala Arg Val Val Tyr Tyr Ser Asn Ser	
95 100 105	
Tyr Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val	
110 115 120	
Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro	
125 130 135	

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P2031R1 PCT Sequence Listing

Ser	Ser	Lys	Ser	Thr	Ser	Gly	Gly	Thr	Ala	Ala	Leu	Gly	Cys	Leu
				140					145					150
Val	Lys	Asp	Tyr	Phe	Pro	Glu	Pro	Val	Thr	Val	Ser	Trp	Asn	Ser
				155					160					165
Gly	Ala	Leu	Thr	Ser	Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu	Gln
				170					175					180
Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser
				185					190					195
Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	Asn	Val	Asn	His	Lys
				200					205					210
Pro	Ser	Asn	Thr	Lys	Val	Asp	Lys	Lys	Val	Glu	Pro	Lys	Ser	Cys
				215					220					225
Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu
				230					235					240
Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr
				245					250					255
Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp
				260					265					270
Val	Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp
				275					280					285
Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln
				290					295					300
Tyr	Asn	Ala	Thr	Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His
				305					310					315
Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn
				320					325					330
Lys	Ala	Leu	Pro	Ala	Pro	Ile	Ala	Ala	Thr	Ile	Ser	Lys	Ala	Lys
				335					340					345
Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg
				350					355					360
Glu	Glu	Met	Thr	Lys	Asn	Gln	Val	Ser	Leu	Thr	Cys	Leu	Val	Lys
				365					370					375
Gly	Phe	Tyr	Pro	Ser	Asp	Ile	Ala	Val	Glu	Trp	Glu	Ser	Asn	Gly
				380					385					390
Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr	Thr	Pro	Pro	Val	Leu	Asp	Ser
				395					400					405
Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	Lys	Leu	Thr	Val	Asp	Lys	Ser
				410					415					420
Arg	Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	Cys	Ser	Val	Met	His	Glu
				425					430					435
Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	Leu	Ser	Leu	Ser	Pro
				440					445					450

Gly Lys

P2031R1 PCT Listado de Secuencias
Listado de Secuencias

229

<110> Genentech, Inc.

<120> MÉTODOS DE UTILIZAR LIGANDOS DE RECEPTOR DE MUERTE CELULAR Y ANTICUERPOS CD20

<130> P2031R1 PCT

<140> PCT/US2005/032015

<141> 2005-09-07

<150> US 60/607,834

<151> 2004-09-08

<150> US 60/666,550

<151> 2005-03-30

<160> 11

<210> 1

<211> 281

<212> PRT

<213> Homo sapiens

<400> 1

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Met Ala Met Met Glu Val Gln Gly Gly Pro Ser Leu Gly Gln Thr
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Cys Val Leu Ile Val Ile Phe Thr Val Leu Leu Gln Ser Leu Cys
          20          25          30

Val Ala Val Thr Tyr Val Tyr Phe Thr Asn Glu Leu Lys Gln Met
          35          40          45

Gln Asp Lys Tyr Ser Lys Ser Gly Ile Ala Cys Phe Leu Lys Glu
          50          55          60

Asp Asp Ser Tyr Trp Asp Pro Asn Asp Glu Glu Ser Met Asn Ser
          65          70          75

Pro Cys Trp Gln Val Lys Trp Gln Leu Arg Gln Leu Val Arg Lys
          80          85          90

Met Ile Leu Arg Thr Ser Glu Glu Thr Ile Ser Thr Val Gln Glu
          95          100          105

Lys Gln Gln Asn Ile Ser Pro Leu Val Arg Glu Arg Gly Pro Gln
          110          115          120

Arg Val Ala Ala His Ile Thr Gly Thr Arg Gly Arg Ser Asn Thr
          125          130          135

Leu Ser Ser Pro Asn Ser Lys Asn Glu Lys Ala Leu Gly Arg Lys
          140          145          150

Ile Asn Ser Trp Glu Ser Ser Arg Ser Gly His Ser Phe Leu Ser
          155          160          165

Asn Leu His Leu Arg Asn Gly Glu Leu Val Ile His Glu Lys Gly
          170          175          180

Phe Tyr Tyr Ile Tyr Ser Gln Thr Tyr Phe Arg Phe Gln Glu Glu
          185          190          195

Ile Lys Glu Asn Thr Lys Asn Asp Lys Gln Met Val Gln Tyr Ile
          200          205          210

Tyr Lys Tyr Thr Ser Tyr Pro Asp Pro Ile Leu Leu Met Lys Ser

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Handwritten signature

230

Ala	Arg	Asn	Ser	Cys	Trp	Ser	Lys	Asp	Ala	Glu	Tyr	Gly	Leu	Tyr
				230					235					240
Ser	Ile	Tyr	Gln	Gly	Gly	Ile	Phe	Glu	Leu	Lys	Glu	Asn	Asp	Arg
				245					250					255
Ile	Phe	Val	Ser	Val	Thr	Asn	Glu	His	Leu	Ile	Asp	Met	Asp	His
				260					265					270
Glu	Ala	Ser	Phe	Phe	Gly	Ala	Phe	Leu	Val	Gly				
				275					280					

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 <212> DNA
 <213> Homo sapiens

<220>
 <221> N
 <222> 447
 <223> Puede ser T o G

<400> 2
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 atcttcacag tgctcctgca gtctctctgt gtggctgtaa cttacgtgta 200
 cttaccaac gagctgaagc agatgcagga caagtactcc aaaagtggca 250
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 gagagtatga acagcccctg ctggcaagtc aagtggcaac tccgtcagct 350
 cgrtagaaaag atgattttga gaacctctga ggaaccatt tctacagttc 400
 aagaaaagca acaaaatatt tctcccctag tgagagaaaag aggtccncag 450
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 aacaaatggt ccaatatatt tacaataca caagttatcc tgaccctata 750
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<400> 3

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 35 40 45
 Glu Pro Arg Gly Gly Gly Arg Gly Ala Leu Pro Thr Ser Met Gly
 50 55 60
 Gln His Gly Pro Ser Ala Arg Ala Arg Ala Gly Arg Ala Pro Gly
 65 70 75
 Pro Arg Pro Ala Arg Glu Ala Ser Pro Arg Leu Arg Val His Lys
 80 85 90
 Thr Phe Lys Phe Val Val Val Gly Val Leu Leu Gln Val Val Pro
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 110 115 120
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 125 130 135
 Ser His Arg Ser Glu Arg Pro Gly Ala Cys Asn Arg Cys Thr Glu
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 Gly Val Gly Tyr Thr Asn Ala Ser Asn Asn Leu Phe Ala Cys Leu
 155 160 165
 Pro Cys Thr Ala Cys Lys Ser Asp Glu Glu Glu Arg Ser Pro Cys
 170 175 180
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 185 190 195
 Arg Asn Asp Asn Ser Ala Glu Met Cys Arg Lys Cys Ser Thr Gly
 200 205 210
 Cys Pro Arg Gly Met Val Lys Val Lys Asp Cys Thr Pro Trp Ser
 215 220 225
 Asp Ile Glu Cys Val His Lys Gly Ser Gly Asn Gly His Asn Ile
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 Trp Val Ile Leu Val Val Thr Leu Val Val Pro Leu Leu Leu Val
 245 250 255
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 Asp Pro Lys Cys Met Asp Arg Val Cys Phe Trp Arg Leu Gly Leu
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 Ser Asn Ala Asp Ser Leu Ser Thr Phe Val Ser Glu Gln Gln Met
 305 310 315

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Glu Ser Gln Glu Pro Ala Asp Leu Thr Gly Val Thr Val Gln Ser
320 325 330

Pro Gly Glu Ala Gln Cys Leu Leu Gly Pro Ala Glu Ala Glu Gly
335 340 345

Ser Gln Arg Arg Arg Leu Leu Val Pro Ala Asn Gly Ala Asp Pro
350 355 360

Thr Glu Thr Leu Met Leu Phe Phe Asp Lys Phe Ala Asn Ile Val
365 370 375

Pro Phe Asp Ser Trp Asp Gln Leu Met Arg Gln Leu Asp Leu Thr
380 385 390

Lys Asn Glu Ile Asp Val Val Arg Ala Gly Thr Ala Gly Pro Gly
395 400 405

Asp Ala Leu Tyr Ala Met Leu Met Lys Trp Val Asn Lys Thr Gly
410 415 420

Arg Asn Ala Ser Ile His Thr Leu Leu Asp Ala Leu Glu Arg Met
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237

233

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 Leu Leu Leu Val Ser Ala Glu Ser Ala Leu Ile Thr Gln Gln Asp
 50 55 60
 Leu Ala Pro Gln Gln Arg Ala Ala Pro Gln Gln Lys Arg Ser Ser
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 Pro Ser Glu Gly Leu Cys Pro Pro Gly His His Ile Ser Glu Asp
 80 85 90
 Gly Arg Asp Cys Ile Ser Cys Lys Tyr Gly Gln Asp Tyr Ser Thr
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 His Trp Asn Asp Leu Leu Phe Cys Leu Arg Cys Thr Arg Cys Asp
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 Ser Gly Glu Val Glu Leu Ser Pro Cys Thr Thr Thr Arg Asn Thr
 125 130 135
 Val Cys Gln Cys Glu Glu Gly Thr Phe Arg Glu Glu Asp Ser Pro
 140 145 150
 Glu Met Cys Arg Lys Cys Arg Thr Gly Cys Pro Arg Gly Met Val
 155 160 165

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Lys Val Gly Asp Cys Thr Pro Trp Ser Asp Ile Glu Cys Val His
170 175 180

Lys Glu Ser Gly Ile Ile Ile Gly Val Thr Val Ala Ala Val Val
185 190 195

Leu Ile Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys
200 205 210

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215 220 225

Pro Glu Arg Val Asp Arg Ser Ser Gln Arg Pro Gly Ala Glu Asp
230 235 240

Asn Val Leu Asn Glu Ile Val Ser Ile Leu Gln Pro Thr Gln Val
245 250 255

Pro Glu Gln Glu Met Glu Val Gln Glu Pro Ala Glu Pro Thr Gly
260 265 270

Val Asn Met Leu Ser Pro Gly Glu Ser Glu His Leu Leu Glu Pro
275 280 285

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290 295 300

Asn Glu Gly Asp Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp Asp
305 310 315

Phe Ala Asp Leu Val Pro Phe Asp Ser Trp Glu Pro Leu Met Arg
320 325 330

Lys Leu Gly Leu Met Asp Asn Glu Ile Lys Val Ala Lys Ala Glu
335 340 345

Ala Ala Gly His Arg Asp Thr Leu Tyr Thr Met Leu Ile Lys Trp
350 355 360

Val Asn Lys Thr Gly Arg Asp Ala Ser Val His Thr Leu Leu Asp
365 370 375

Ala Leu Glu Thr Leu Gly Glu Arg Leu Ala Lys Gln Lys Ile Glu
380 385 390

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395 400 405

Ala Asp Ser Ala Leu Ser
410

<210> 6
<211> 440
<212> PRT
<213> Homo sapiens

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Gly Pro Arg Val Pro Lys Thr Leu Val Leu Val Val Ala Ala Val
35 40 45

Leu Leu Leu Val Ser Ala Glu Ser Ala Leu Ile Thr Gln Gln Asp
50 55 60

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24 (235

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80 85 90

Gly Arg Asp Cys Ile Ser Cys Lys Tyr Gly Gln Asp Tyr Ser Thr
95 100 105

His Trp Asn Asp Leu Leu Phe Cys Leu Arg Cys Thr Arg Cys Asp
110 115 120

Ser Gly Glu Val Glu Leu Ser Pro Cys Thr Thr Thr Arg Asn Thr
125 130 135

Val Cys Gln Cys Glu Glu Gly Thr Phe Arg Glu Glu Asp Ser Pro
140 145 150

Glu Met Cys Arg Lys Cys Arg Thr Gly Cys Pro Arg Gly Met Val
155 160 165

Lys Val Gly Asp Cys Thr Pro Trp Ser Asp Ile Glu Cys Val His
170 175 180

Lys Glu Ser Gly Thr Lys His Ser Gly Glu Ala Pro Ala Val Glu
185 190 195

Glu Thr Val Thr Ser Ser Pro Gly Thr Pro Ala Ser Pro Cys Ser
200 205 210

Leu Ser Gly Ile Ile Ile Gly Val Thr Val Ala Ala Val Val Leu
215 220 225

Ile Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys Val
230 235 240

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245 250 255

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260 265 270

Val Leu Asn Glu Ile Val Ser Ile Leu Gln Pro Thr Gln Val Pro
275 280 285

Glu Gln Glu Met Glu Val Gln Glu Pro Ala Glu Pro Thr Gly Val
290 295 300

Asn Met Leu Ser Pro Gly Glu Ser Glu His Leu Leu Glu Pro Ala
305 310 315

Glu Ala Glu Arg Ser Gln Arg Arg Arg Leu Leu Val Pro Ala Asn
320 325 330

Glu Gly Asp Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp Asp Phe
335 340 345

Ala Asp Leu Val Pro Phe Asp Ser Trp Glu Pro Leu Met Arg Lys
350 355 360

Leu Gly Leu Met Asp Asn Glu Ile Lys Val Ala Lys Ala Glu Ala
365 370 375

Ala Gly His Arg Asp Thr Leu Tyr Thr Met Leu Ile Lys Trp Val
380 385 390

Asn Lys Thr Gly Arg Asp Ala Ser Val His Thr Leu Leu Asp Ala

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395

400

405

Leu Glu Thr Leu Gly Glu Arg Leu Ala Lys Gln Lys Ile Glu Asp
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<213> Secuencia artificial

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35 40 45

Leu Ile Tyr Ala Pro Ser Asn Leu Ala Ser Gly Val Pro Ser Arg
50 55 60

Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
65 70 75

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<213> Secuencia artificial

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Ser Tyr Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr
50 55 60

Asn Gln Lys Phe Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser
65 70 75

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237

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
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<223> Sintética

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35 40 45

Leu Ile Tyr Ala Pro Ser Asn Leu Ala Ser Gly Val Pro Ser Arg
50 55 60

Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
65 70 75

Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Trp
80 85 90

Ser Phe Asn Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile
95 100 105

Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser
110 115 120

Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu
125 130 135

Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp
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Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln
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 Glu Trp Val Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr
 50 55 60
 Asn Gln Lys Phe Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser
 65 70 75
 Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
 80 85 90
 Thr Ala Val Tyr Tyr Cys Ala Arg Val Val Tyr Tyr Ser Asn Ser
 95 100 105
 Tyr Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val
 110 115 120
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 155 160 165
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 185 190 195
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 200 205 210
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 215 220 225
 Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
 230 235 240
 Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr
 245 250 255
 Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp
 260 265 270
 Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp
 275 280 285
 Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln
 290 295 300
 Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His

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239

305	310	315
Gln Asp Trp Leu Asn Gly Lys Glu Tyr	Lys Cys Lys Val Ser Asn	
320	325	330
Lys Ala Leu Pro Ala Pro Ile Glu Lys	Thr Ile Ser Lys Ala Lys	
335	340	345
Gly Gln Pro Arg Glu Pro Gln Val Tyr	Thr Leu Pro Pro Ser Arg	
350	355	360
Glu Glu Met Thr Lys Asn Gln Val Ser	Leu Thr Cys Leu Val Lys	
365	370	375
Gly Phe Tyr Pro Ser Asp Ile Ala Val	Glu Trp Glu Ser Asn Gly	
380	385	390
Gln Pro Glu Asn Asn Tyr Lys Thr Thr	Pro Pro Val Leu Asp Ser	
395	400	405
Asp Gly Ser Phe Phe Leu Tyr Ser Lys	Leu Thr Val Asp Lys Ser	
410	415	420
Arg Trp Gln Gln Gly Asn Val Phe Ser	Cys Ser Val Met His Glu	
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 Ser Tyr Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
 35 40 45
 Glu Trp Val Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr
 50 55 60
 Asn Gln Lys Phe Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser
 65 70 75
 Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
 80 85 90
 Thr Ala Val Tyr Tyr Cys Ala Arg Val Val Tyr Tyr Ser Asn Ser
 95 100 105
 Tyr Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val
 110 115 120
 Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro
 125 130 135

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Ser Ser Lys Ser	Thr Ser Gly Gly Thr	Ala Ala Leu Gly Cys Leu	140	145	150
Val Lys Asp Tyr	Phe Pro Glu Pro Val	Thr Val Ser Trp Asn Ser	155	160	165
Gly Ala Leu Thr	Ser Gl Val His Thr	Phe Pro Ala Val Leu Gln	170	175	180
Ser Ser Gly Leu	Tyr Ser Leu Ser Ser	Val Val Thr Val Pro Ser	185	190	195
Ser Ser Leu Gly	Thr Gln Thr Tyr Ile	Cys Asn Val Asn His Lys	200	205	210
Pro Ser Asn Thr	Lys Val Asp Lys Lys	Val Glu Pro Lys Ser Cys	215	220	225
Asp Lys Thr His	Thr Cys Pro Pro Cys	Pro Ala Pro Glu Leu Leu	230	235	240
Gly Gly Pro Ser	Val Phe Leu Phe Pro	Pro Lys Pro Lys Asp Thr	245	250	255
Leu Met Ile Ser	Arg Thr Pro Glu Val	Thr Cys Val Val Val Asp	260	265	270
Val Ser His Glu	Asp Pro Glu Val Lys	Phe Asn Trp Tyr Val Asp	275	280	285
Gly Val Glu Val	His Asn Ala Lys Thr	Lys Pro Arg Glu Glu Gln	290	295	300
Tyr Asn Ala Thr	Tyr Arg Val Val Ser	Val Leu Thr Val Leu His	305	310	315
Gln Asp Trp Leu	Asn Gly Lys Glu Tyr	Lys Cys Lys Val Ser Asn	320	325	330
Lys Ala Leu Pro	Ala Pro Ile Ala Ala	Thr Ile Ser Lys Ala Lys	335	340	345
Gly Gln Pro Arg	Glu Pro Gln Val Tyr	Thr Leu Pro Pro Ser Arg	350	355	360
Glu Glu Met Thr	Lys Asn Gln Val Ser	Leu Thr Cys Leu Val Lys	365	370	375
Gly Phe Tyr Pro	Ser Asp Ile Ala Val	Glu Trp Glu Ser Asn Gly	380	385	390
Gln Pro Glu Asn	Asn Tyr Lys Thr Thr	Pro Pro Val Leu Asp Ser	395	400	405
Asp Gly Ser Phe	Phe Leu Tyr Ser Lys	Leu Thr Val Asp Lys Ser	410	415	420
Arg Trp Gln Gln	Gly Asn Val Phe Ser	Cys Ser Val Met His Glu	425	430	435
Ala Leu His Asn	His Tyr Thr Gln Lys	Ser Leu Ser Leu Ser Pro	440	445	450
Gly Lys					

PATENT COOPERATION TREATY

PCT

From the INTERNATIONAL SEARCHING AUTHORITY

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT AND
THE WRITTEN OPINION OF THE INTERNATIONAL
SEARCHING AUTHORITY, OR THE DECLARATION

To:
GENENTECH, INC.
Attn. Marschang, Diane L.
1 DNA Way
South San Francisco, CA 94080-1390
UNITED STATES OF AMERICA

Calendar/CLK
Resp to D&R/Conrad. Ch II - 22 Aug 06
Chap. II Dem/Resp to WO - 22 Sep 06
Due Date

Date of mailing
(day/month/year)

22/06/2006

(PCT Rule 44.1)

Applicant's or agent's file reference

P2031R1

FOR FURTHER ACTION

See paragraphs 1 and 4 below

International application No.

PCT/US2005/032015

International filing date
(day/month/year)

07/09/2005

Applicant

GENENTECH, INC.

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

Filing of amendments and statement under Article 19:

The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

Where? Directly to the International Bureau of WIPO, 34 chemin des Colombettes
1211 Geneva 20, Switzerland, Facsimile No.: (41-22) 338.82.70

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.

3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

- ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.
- ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. Reminders

Shortly after the expiration of 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90bis.1 and 90bis.3, respectively, before the completion of the technical preparations for international publication.

The applicant may submit comments on an informal basis on the written opinion of the International Searching Authority to the International Bureau. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established. These comments would also be made available to the public but not before the expiration of 30 months from the priority date.

Within 18 months from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later); otherwise, the applicant must, within 20 months from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

In respect of other designated Offices, the time limit of 30 months (or later) will apply even if no demand is filed within 18 months.

See the Annex to Form PCT/IB/301 and, for details about the applicable time limits, Office by Office, see the PCT Applicant's Guide, Volume II, National Chapters and the WIPO Internet site.

Name and mailing address of the International Searching Authority

 European Patent Office, P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer

Catriona Cleere

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NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and these requirements, the latter are applicable. For more detailed information, see also the *PCT Applicant's Guide*, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report and the written opinion of the International Searching Authority, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purpose of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only (see *PCT Applicant's Guide*, Annexes B1 and B2).

The attention of the applicant is drawn to the fact that amendments to the claims under Article 19 are not allowed where the International Searching Authority has declared, under Article 17(2), that no international search report would be established (see *PCT Applicant's Guide*, Volume IA, paragraph 286).

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 18 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference P2031R1	FOR FURTHER ACTION see Form PCT/ISA/220 as well as, where applicable, Item 5 below.	
International application No. PCT/US2005/032015	International filing date (day/month/year) 07/09/2005	(Earliest) Priority Date (day/month/year) 08/09/2004
Applicant GENENTECH, INC.		

This International search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International search report consists of a total of 5 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the language, the international search was carried out on the basis of:



the international application in the language in which it was filed



a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))

b. ☒ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, see Box No. I.

2. ☒ Certain claims were found unsearchable (See Box No. II)

3. ☐ Unity of invention is lacking (see Box No. III)

4. With regard to the title,



the text is approved as submitted by the applicant



the text has been established by this Authority to read as follows:

5. With regard to the abstract,



the text is approved as submitted by the applicant



the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the drawings,

a. the figure of the drawings to be published with the abstract is Figure No. _____



as suggested by the applicant



as selected by this Authority, because the applicant failed to suggest a figure



as selected by this Authority, because this figure better characterizes the invention

b. ☒ none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2005/032015

Box No. 1 Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
 - a. type of material
 - ☒ a sequence listing
 - ☐ table(s) related to the sequence listing
 - b. format of material
 - ☒ on paper
 - ☒ in electronic form
 - c. time of filing/furnishing
 - ☐ contained in the international application as filed
 - ☐ filed together with the international application in electronic form
 - ☒ furnished subsequently to this Authority for the purpose of search
2. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K16/28 A61P35/02 A61P19/02 A61P25/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data bases consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03/038043 A (UAB RESEARCH FOUNDATION; ZHOU, TONG; ICHIKAWA, KIMIHISA; KIMBERLY, ROB) 8 May 2003 (2003-05-08) page 6, line 1 - page 8, line 6 page 54, line 8 - line 14 claims 30,66,102 examples	1-27
A	WO 99/37684 A (GENENTECH, INC) 29 July 1999 (1999-07-29) abstract	1-27

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

- 'A' document defining the general state of the art which is not considered to be of particular relevance
- 'E' earlier document but published on or after the international filing date
- 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- 'O' document referring to an oral disclosure, use, exhibition or other means
- 'P' document published prior to the international filing date but later than the priority date claimed

- 'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- 'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- 'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- 'A' document member of the same patent family

Date of the actual completion of the international search

24 May 2006

Date of mailing of the international search report

22/06/2006

Name and mailing address of the ISA/

European Patent Office, P.O. Box 18 Patentstr. 2
NL - 2280 HV Rijswijk
Tel: (+31-70) 340-2340, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3015

Authorized officer

Rankin, R

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US2005/032015

Box II Observations where certain claims were found unsearchable (Continuation of Item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1-27 are directed to a method of treatment of the human body, the search has been carried out and based on the alleged effects of the composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 5.4(a).

Box III Observations where unity of invention is lacking (Continuation of Item 3 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

II INTERNATIONAL SEARCH REPORT
Information on patent family members

7/27 242
International application No
PCT/US2005/032015

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 03038043	A	08-05-2003	CA 2465314 A1	08-05-2003
			CN 1630516 A	22-06-2005
			EP 1506285 A2	16-02-2005
			JP 2005519871 T	07-07-2005
			MX PA04004184 A	25-01-2005
WO 9937684	A	29-07-1999	AU 2338299 A	09-08-1999
			CA 2318405 A1	29-07-1999
			EP 1053256 A1	22-11-2000
			JP 2002500877 T	15-01-2002

PATENT COOPERATION TREATY

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From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY (PCT Rule 43bis.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.
PCT/US2005/032015

International filing date (day/month/year)
07.09.2005

Priority date (day/month/year)
08.09.2004

International Patent Classification (IPC) or both national classification and IPC
INV. C07K16/28 A61P35/02 A61P19/02 A61P25/00

Applicant
GENENTECH, INC.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 68.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA:



European Patent Office
D-80298 Munich
Tel. +49 89 2339 - 0 Tlx 523656 epnu d
Fax: +49 89 2339 - 4465

Date of completion of
this opinion

see form
PCT/ISA/210

Authorized Officer

Rankin, R
Telephone No. +49 89 2339-2559



**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/US2005/032015

257 249

Box No. I Basis of the opinion

1. With regard to the language, this opinion has been established on the basis of:
 - ☒ the international application in the language in which it was filed
 - ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).
2. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:
 - a. type of material:
 - ☒ a sequence listing
 - ☐ table(s) related to the sequence listing
 - b. format of material:
 - ☒ on paper
 - ☒ in electronic form
 - c. time of filing/furnishing:
 - ☐ contained in the international application as filed.
 - ☐ filed together with the international application in electronic form.
 - ☒ furnished subsequently to this Authority for the purposes of search.
3. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
4. Additional comments:

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/US2005/032015

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of

- ☐ the entire international application
- ☒ claims Nos. 1-27 (For reasons of industrial applicability)

because:

- ☒ the said international application, or the said claims Nos. 1-27 (for reasons of industrial applicability) relate to the following subject matter which does not require an international search (*specify*):

see separate sheet

- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):
- ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed (*specify*):
- ☐ no international search report has been established for the whole application or for said claims Nos.
- ☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:
- ☐ furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.
- ☐ furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.
- ☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rules 13ter.1(a) or (b).
- ☐ a meaningful opinion could not be formed without the tables related to the sequence listings; the applicant did not, within the prescribed time limit, furnish such tables in electronic form complying with the technical requirements provided for in Annex C-bis of the Administrative Instructions, and such tables were not available to the International Searching Authority in a form and manner acceptable to it.
- ☐ the tables related to the nucleotide and/or amino acid sequence listing, if in electronic form only, do not comply with the technical requirements provided for in Annex C-bis of the Administrative Instructions.
- ☐ See Supplemental Box for further details

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/US2005/032015

**Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or
industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Yes: Claims	11, 22
	No: Claims	1-10, 12-21, 23-27
Inventive step (IS)	Yes: Claims	
	No: Claims	1-27
Industrial applicability (IA)	Yes: Claims	
	No: Claims	

2. Citations and explanations

see separate sheet

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING
AUTHORITY (SEPARATE SHEET)**

262 252
International application No.

PCT/US2005/032015

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claims 1-27 relate to subject matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject matter of these claims (Article 34(4)(a)(i) PCT)

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

For the assessment of the present claims 1-27 on the question whether they are industrially applicable, no unified criteria exist within the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognise as industrially applicable the subject matter of claims to the use of a compound in medical treatment, but may allow, however, the use of such a compound for the manufacture of a medicament for a new medical treatment.

Cited Documents

Reference is made to the following document:

D1: WO 03/038043 A (UAB RESEARCH FOUNDATION; ZHOU, TONG; ICHIKAWA, KIMIHISA; KIMBERLY, ROB) 8 May 2003 (2003-05-08)

Article 33(2) PCT

D1 discloses a therapeutic method of treating cancers and other ailments by a combination of antibody against CD20 and antibody against DR4 or DR5 (page 6, line 1 to page 8, line 6; page 54, lines 8-14; claims). The methods of D1 may also be employed treat rheumatoid arthritis or multiple sclerosis. A preferred anti-CD20 MAb is Rituximab and the antibody against the DR4 or DR5 molecule may be humanized, chimeric or of human origin. The subject matter of claims 1-10, 12-21 and 23-27 is therefore not new in light of D1 (Article 33(2) PCT).

Article 33(3) PCT

The subject matter of claims 11 and 22 are not considered inventive as the skilled person would seek to utilize antibodies with the binding characteristics outline in said claims in

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING
AUTHORITY (SEPARATE SHEET)**

253
International application No.

PCT/US2005/032015

order to be able to use them in a therapeutic setting (Article 33(3) PCT).

Re Item VIII

Certain observations on the international application

Claims 1, 3-10, 12-15, 17-21, 23-27 do not meet the requirements of Article 6 PCT since there is no support within the application as filed for any synergistic action of anti-DR4 antibodies in combination with an anti-CD20 antibody. Since there are no experimental data supporting such features of the above claims they do not fulfill the provisions of Article 6 PCT.

From the INTERNATIONAL BUREAU

PCT

FIRST NOTICE INFORMING THE APPLICANT OF
THE COMMUNICATION OF THE INTERNATIONAL
APPLICATION (TO DESIGNATED OFFICES WHICH
DO NOT APPLY THE 30 MONTH TIME LIMIT
UNDER ARTICLE 22(1))

(PCT Rule 47.1(c))

To:

MARSCHANG, Diane L.
GENENTECH, INC.
1 DNA Way
South San Francisco, California 94080-4980
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 13 April 2006 (13.04.2006)		IMPORTANT NOTICE	
Applicant's or agent's file reference P2031R1			
International application No. PCT/US2005/032015	International filing date (day/month/year) 07 September 2005 (07.09.2005)	Priority date (day/month/year) 08 September 2004 (08.09.2004)	
Applicant GENENTECH, INC. et al			

1. ATTENTION: For any designated Office(s), for which the time limit under Article 22(1), as in force from 1 April 2002 (30 months from the priority date), does apply, please see Form PCT/IB/308 (Second and Supplementary Notice) (to be issued promptly after the expiration of 28 months from the priority date).

2. Notice is hereby given that the following designated Office(s), for which the time limit under Article 22(1), as in force from 1 April 2002, does not apply, has/have requested that the communication of the international application, as provided for in Article 20, be effected under Rule 93bis.1. The International Bureau has effected that communication on the date indicated below:
16 March 2006 (16.03.2006)

CH

In accordance with Rule 47.1(c-bis)(i), those Offices will accept the present notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

3. The following designated Offices, for which the time limit under Article 22(1), as in force from 1 April 2002, does not apply, have not requested, as at the time of mailing of the present notice, that the communication of the international application be effected under Rule 93bis.1:

LU, SE, TZ, UG, ZM

In accordance with Rule 47.1(c-bis)(ii), those Offices accept the present notice as conclusive evidence that the Contracting State for which that Office acts as a designated Office does not require the furnishing, under Article 22, by the applicant of a copy of the international application.

4. TIME LIMITS for entry into the national phase

For the designated Office(s) listed above, and unless a demand for international preliminary examination has been filed before the expiration of 19 months from the priority date (see Article 39(1)), the applicable time limit for entering the national phase will, subject to what is said in the following paragraph, be 20 MONTHS from the priority date.

In practice, time limits other than the 20-month time limit will continue to apply, for various periods of time, in respect of certain of the designated Offices listed above. For regular updates on the applicable time limits (20 or 21 months, or other time limit), Office by Office, refer to the *PCT Gazette*, the *PCT Newsletter* and the *PCT Applicant's Guide*, Volume II, National Chapters, all available from WIPO's Internet site, at <http://www.wipo.int/pct/en/index.html>.

It is the applicant's sole responsibility to monitor all these time limits.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Authorized officer

Athina Nickitas-Etienne

Facsimile No. +41 22 740 14 35

Facsimile No. +41 22 338 89 95

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10/20/05 12:07

PAGE 001/004

Fax Server

Facsimile Transmission

*This company uses RightFAX® fax
server software from RightFAX, Inc.*

From: **Name:** USPTO ASSIGNMENT DIVISION
 Fax Number:
 Voice Phone: 703-308-9723

To: **Name:** GENENTECH, INC.
 Company: DIANE L. MARSCHANG
 Fax Number: 16509529881
 Voice Phone:

Fax Notes:

Pg# **Description**

1 Cover Page

2 387.TXT

4 Document 1, Batch 517236

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Date and time of transmission: Thursday, October 20, 2005 12:06:56 PM
Number of pages including this cover sheet: 04

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UNITED STATES PATENT AND TRADEMARK OFFICE

UNDER SECRETARY OF COMMERCE FOR INTELLECTUAL PROPERTY AND
DIRECTOR OF THE UNITED STATES PATENT AND TRADEMARK OFFICE

OCTOBER 20, 2005

PTAS

700205562A

700205562A

GENENTECH, INC.
DIANE L. MARSCHANG
1 DNA WAY
SOUTH SAN FRANCISCO, CA 94080

**UNITED STATES PATENT AND TRADEMARK OFFICE
NOTICE OF RECORDATION OF ASSIGNMENT DOCUMENT**

THE ENCLOSED DOCUMENT HAS BEEN RECORDED BY THE ASSIGNMENT DIVISION OF THE U.S. PATENT AND TRADEMARK OFFICE. A COMPLETE MICROFILM COPY IS AVAILABLE AT THE ASSIGNMENT SEARCH ROOM ON THE REEL AND FRAME NUMBER REFERENCED BELOW.

PLEASE REVIEW ALL INFORMATION CONTAINED ON THIS NOTICE. THE INFORMATION CONTAINED ON THIS RECORDATION NOTICE REFLECTS THE DATA PRESENT IN THE PATENT AND TRADEMARK ASSIGNMENT SYSTEM. IF YOU SHOULD FIND ANY ERRORS OR HAVE QUESTIONS CONCERNING THIS NOTICE, YOU MAY CONTACT THE EMPLOYEE WHOSE NAME APPEARS ON THIS NOTICE AT 571-272-3350. PLEASE SEND REQUEST FOR CORRECTION TO: U.S. PATENT AND TRADEMARK OFFICE, MAIL STOP: ASSIGNMENT SERVICES DIVISION, P.O. BOX 1450, ALEXANDRIA, VA 22313.

RECORDATION DATE: 08/24/2005

REEL/FRAME: 016663/0168
NUMBER OF PAGES: 2

BRIEF: ASSIGNMENT OF ASSIGNOR'S INTEREST (SEE DOCUMENT FOR DETAILS).

ASSIGNOR:

ASHKENAZI, AVI J.

DOC DATE: 08/15/2005

ASSIGNEE:

GENENTECH, INC.

1 DNA WAY

SOUTH SAN FRANCISCO, CALIFORNIA

94080-4990

SERIAL NUMBER: 60666550

FILING DATE: 03/30/2005

PATENT NUMBER:

ISSUE DATE:

TITLE: METHODS OF USING DEATH RECEPTOR LIGANDS AND CD20 ANTIBODIES

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PAGE 003/004

Fax Server

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[Handwritten signature]

016663/0168 PAGE 2

KIMBERLY WHITE, EXAMINER
ASSIGNMENT DIVISION
OFFICE OF PUBLIC RECORDS

UB/24/2005
700205562

RECOMMENDATION FORM COVER SHEET PATENTS ONLY Consolidated cover for Patents. Please record the attached original documents or copy thereof.		U.S. DEPARTMENT OF COMMERCE Patent and Trademark Office Any Dis No. FPM301A
1. Name of conveying party(ies): Avi J. Ashkenazi Additional name(s) of conveying party(ies) attached? ☐ Yes ☒ No	2. Name and address of receiving party(ies): Name: GENENTECH, INC. Internal Address: Street Address: 1 DNA Way City: South San Francisco State: California ZIP: 94080-4990 Additional name(s) & address(es) attached? ☐ Yes ☒ No	
3. Nature of conveyance: ☒ Assignment ☐ Merger ☐ Security Agreement ☐ Change of Name ☐ Other: Execution Date: 8/15/05	4. Application number(s) or patent number(s): If this document is being filed together with a new application, the execution date of the application is: A. Patent Application No.(s) B. Patent No.(s) 60/666,550 Additional numbers attached? ☐ Yes ☒ No	
5. Name and address of party to whom correspondence concerning document should be mailed: Diane L. Marchant GENENTECH, INC. 1 DNA Way South San Francisco, CA 94080	6. Total number of applications and patents involved: 1 7. Total fee (37 CFR 3.41): \$ 40.00 ☐ Enclosed ☒ Authorized to be charged to deposit account 8. Deposit account number: 07-0630	
DO NOT USE THIS SPACE		
9. Statement and signature. To the best of my knowledge and belief, the foregoing information is true and correct and my attached copy is a true copy of the original document. Diane L. Marchant Name of Person Signing Registration No. 33,600 <i>Diane L. Marchant</i> Signature August 24, 2005 Date Total number of pages comprising cover sheet, attachments and document: 3		

✓✓✓ 259

Genentech, Inc.

G n ntech, Inc.

Genentech, Inc.

FACSIMILE TRANSMITTAL

✓✓

1 DNA WAY
South San Francisco, CA 94080
(415) 225-1000
Facsimile: (650) 952-9881

CERTIFICATION OF FACSIMILE TRANSMISSION UNDER 37 CFR § 1.8	
I hereby certify that this paper is being facsimile transmitted to the Patent and Trademark Office on the date shown below.	
<u>Diane L. Marschang</u>	
Type or print name of person signing certification	
Signature <u>Diane L. Marschang</u>	Date: <u>8/24/05</u>

DATE: August 24, 2005

Please deliver the following page(s) to:

NAME: Mail Stop: Assignments
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Fax No.: 1-703-306-5995

FROM: Diane L. Marschang
Registration No.: 35,600

RE: U.S. Serial No.: 80/666,550
Our Docket No.: PR2031A

Number of Pages including this cover sheet - 3

CONFIDENTIAL NOTE

The documents accompanying this facsimile transmission contain information from GENENTECH, INC. which is confidential or privileged. The information is intended only for the individual or entity named on this transmission sheet. If you are not the intended recipient, be aware that any disclosure, copying, distribution, or use of the contents of this faxed information is strictly prohibited. If you have received this facsimile in error, please notify us by telephone immediately so that we can arrange for the return of the original documents to us and the retransmission of them to the intended recipient.

If you do not receive all pages, please notify Mona Beltran at (650) 225-6383.

✓ 260

RECORDATION FORM COVER SHEET
PATENTS ONLY

U.S. DEPARTMENT OF COMMERCE
Patent and Trademark Office

Commissioner for Patents: Please record the attached original documents or copy thereof.

Atty Dist No: PR2031A

1. Name of conveying party(ies):

Avi J. Ashkenazi

Additional name(s) of conveying party(ies) attached? _ Yes ☒ No

2. Name and address of receiving party(ies):

Name: GENENTECH, INC.

Internal Address:

Street Address: 1 DNA Way

City: South San Francisco State: California

ZIP: 94080-4990

Additional name(s) & address(es) attached? _ Yes ☒ No

3. Nature of conveyance:

☒ Assignment

Merger

Security Agreement

Change of Name

Other:

Execution Date: 8/15/05

4. Application number(s) or patent number(s):

If this document is being filed together with a new application, the execution date of the application is:

A. Patent Application No.(s)

B. Patent No.(s)

60/666,550

Additional numbers attached? _ Yes ☒ No

5. Name and address of party to whom correspondence concerning document should be mailed:

Diane L. Marschang
GENENTECH, INC.

1 DNA Way

South San Francisco, CA 94080

6. Total number of applications and patents involved: 1

7. Total fee (37 CFR 3.41): \$ 40.00

_ Enclosed

☒ Authorized to be charged to deposit account

8. Deposit account number:

07-0630

DO NOT USE THIS SPACE

9. Statement and signature.

To the best of my knowledge and belief, the foregoing information is true and correct and any attached copy is a true copy of the original document.

Diane L. Marschang
Name of Person Signing
Registration No. 35,600

Diane L. Marschang
Signature

August 24, 2005
Date

Total number of pages comprising cover sheet, attachments and document: 3

Revised (10/31/98)

221 261

Docket No. PR2031A

ASSIGNMENT

WHEREAS, Avi J. Ashkenazi, a citizen of US, residing at 1458 Tarrytown Street, San Mateo, CA 94402 (hereinafter "ASSIGNOR"), has invented a new and useful invention in

Methods of using death receptor ligands and CD20 antibodies

for which a provisional application Serial No. 60/866,550, if received (Docket No. PR2031A) has been filed by him on March 30, 2005; and

WHEREAS, GENENTECH, INC., a corporation organized and existing under and by virtue of the laws of the State of Delaware, having a place of business at 1 DNA Way, South San Francisco, California 94080-4990, is desirous of acquiring an interest in and to said invention, and in and to the Letters Patents to be obtained therefor;

NOW, THEREFORE, be it known by all whom it may concern;

That for good and valuable consideration the receipt of which is hereby acknowledged, the said ASSIGNOR have and do hereby sell, assign, transfer and set over unto the said GENENTECH, INC., its successors and assigns, the full and exclusive right, title and interest including all rights under the Paris Convention for the Protection of Industrial Property, in and to said invention, and in and to any and all Letters Patents to be granted and issued therefor or any continuation, division, renewal, or substitute thereof, and as to Letters Patents any reissue or re-examination thereof, not only for, to, and in the United States of America, its territories and possessions, but for, to and in all other countries; and it has been and is hereby authorized and requested that the appropriate government agencies issue said Letters Patents to said GENENTECH, INC., in accordance with this Assignment.

Said ASSIGNOR covenants and agrees to cooperate with GENENTECH, INC., to enable said GENENTECH, INC. to enjoy to the fullest extent the right, title and interest herein conveyed in the United States and foreign countries. Such cooperation by said ASSIGNOR includes prompt production of pertinent facts and documents, giving of testimony, execution of petitions, oaths, specifications, declarations or other papers, and other assistance all to the extent deemed necessary or desirable by said GENENTECH, INC., (a) for perfecting the right, title and interest herein conveyed; (b) for prosecuting any of said applications; (c) for filing and prosecuting applications for reissuance of any of said patents; (d) for interference or other priority proceedings involving said invention; and (e) for legal proceedings involving said invention and any applications therefor and any patents granted thereon, including without limitation opposition proceedings, cancellation proceedings, priority contests, public use proceedings, infringement actions and court actions; provided, however, that the expense incurred by said ASSIGNOR in providing such cooperation shall be paid for by said GENENTECH, INC.

The terms and covenants of this assignment shall inure to the benefit of said GENENTECH, INC., its successors, assigns and other legal representatives, and shall be binding upon said ASSIGNOR, their respective heirs, legal representatives and assigns.

Said ASSIGNOR hereby warrants and represents that he has not entered and will not enter into any assignment, contract, or understanding in conflict herewith.

IN WITNESS WHEREOF I undersign as follows;

South San Francisco

Avi J. Ashkenazi

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3/21/05 5:04

PAGE 001/004

Fax Server

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PR2031

Facsimile Transmission

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From: **Name:** USPTO ASSIGNMENT DIVISION
 Fax Number:
 Voice Phone: 703-308-9723

To: **Name:** GENENTECH, INC.
 Company: DIANE L. MARSCHANG
 Fax Number: 16509529881
 Voice Phone:

Fax Notes:

Pg# **Description**

1 Cover Page

2 814.TXT

4 Document 1, Batch 427683

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MARCH 21, 2005

PTAS

7001575244

GENENTECH, INC.
DIANE L. MARSCHANG
1 DNA WAY
SOUTH SAN FRANCISCO, CA 94080

UNITED STATES PATENT AND TRADEMARK OFFICE
NOTICE OF RECORDATION OF ASSIGNMENT DOCUMENT

THE ENCLOSED DOCUMENT HAS BEEN RECORDED BY THE ASSIGNMENT DIVISION OF THE U.S. PATENT AND TRADEMARK OFFICE. A COMPLETE MICROFILM COPY IS AVAILABLE AT THE ASSIGNMENT SEARCH ROOM ON THE REEL AND FRAME NUMBER REFERENCED BELOW.

PLEASE REVIEW ALL INFORMATION CONTAINED ON THIS NOTICE. THE INFORMATION CONTAINED ON THIS RECORDATION NOTICE REFLECTS THE DATA PRESENT IN THE PATENT AND TRADEMARK ASSIGNMENT SYSTEM. IF YOU SHOULD FIND ANY ERRORS OR HAVE QUESTIONS CONCERNING THIS NOTICE, YOU MAY CONTACT THE EMPLOYEE WHOSE NAME APPEARS ON THIS NOTICE AT 703-308-9723. PLEASE SEND REQUEST FOR CORRECTION TO: U.S. PATENT AND TRADEMARK OFFICE, ASSIGNMENT DIVISION, BOX ASSIGNMENTS, CG-4, 1213 JEFFERSON DAVIS HWY, SUITE 320, WASHINGTON, D.C. 20231.

RECORDATION DATE: 02/28/2005

REEL/FRAME: 015798/0787
NUMBER OF PAGES: 2

BRIEF: ASSIGNMENT OF ASSIGNOR'S INTEREST (SEE DOCUMENT FOR DETAILS).

ASSIGNOR:

ASHKENAZI, AVI J.

DOC DATE: 02/17/2005

ASSIGNEE:

GENENTECH, INC.
1 DNA WAY
SOUTH SAN FRANCISCO, CALIFORNIA

94080-4990

SERIAL NUMBER: 60607834

FILING DATE: 09/08/2004

PATENT NUMBER:

ISSUE DATE:

TITLE: METHODS OF USING DEATH RECEPTOR LIGANDS AND CD20 ANTIBODIES

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277

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PAGE 003/004

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015798/0787 PAGE 2

TARA WASHINGTON, EXAMINER
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Asst. Commissioner of Patents: Please record the attached original document or copy thereof.		Any Dkt No: PR2005
1. Name of conveying party(ies): Avi J. Antkowiak Additional name(s) of conveying party(ies) attached? ☐ Yes ☒ No	2. Name and address of receiving party(ies): Name: GENENTECH, INC. Internal Address: Street Address: 1 DNA Way City: South San Francisco State: California ZIP: 94080-4990 Additional name(s) & address(es) attached? ☐ Yes ☒ No	
3. Nature of conveyance: ☒ Assignment Security Agreement Change of Name Other: Execution Date: 2/17/2005	Marger	
4. Application number(s) or patent number(s): If this document is being filed together with a new application, the execution date of the application is: A. Patent Application No.(s) 60/607,834 B. Patent No.(s) Additional numbers attached? ☐ Yes ☒ No		
5. Name and address of party to whom correspondence concerning document should be mailed: Diane L. Marchant GENENTECH, INC. 1 DNA Way South San Francisco, CA 94080	6. Total number of applications and patents involved: 1 7. Total fee (37 CFR 3.41): \$ 40.00 Enclosed ☒ Authorized to be charged to deposit account 8. Deposit account number: 07-0630	
DO NOT USE THIS SPACE		
9. Statement and signature: To the best of my knowledge and belief, the foregoing information is true and correct and any attached copy is a true copy of the original document. Diane L. Marchant Name of Person Signing Registration No. 35,600 Signature: <u>Diane L. Marchant</u> Date: <u>2/28/2005</u>		
Total number of pages comprising cover sheet, attachments and document: 3 (total pages)		

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Genentech, Inc.

Genentech, Inc.

Genentech, Inc.

FACSIMILE TRANSMITTAL

1 DNA WAY

South San Francisco, CA 94080

(415) 225-1000

Facsimile: (650) 952-9881

CERTIFICATION OF FACSIMILE TRANSMISSION UNDER 37 CFR 1.18

I hereby certify that this paper is being facsimile transmitted to the Patent and Trademark Office on the date shown below.

Diane L. Marschang

Type or print name of person signing certification

Signature

Diane L. Marschang

Date:

2/28/2005

DATE: February 28, 2005

Please deliver the following page(s) to:

NAME: Mail Stop: Assignments
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P.O. Box 1450
Alexandria, VA 22313-1450

Fax No.: 1-703-306-6995

FROM: Diane L. Marschang
Registration No.: 35,600

RE: U.S. Serial No.: 60/607,834
Our Docket No.: PR2031

Number of Pages including this cover sheet - 3

CONFIDENTIAL NOTE

The documents accompanying this facsimile transmission contain information from GENENTECH, INC. which is confidential or privileged. The information is intended only for the individual or entity named on this transmission sheet. If you are not the intended recipient, be aware that any disclosure, copying, distribution, or use of the contents of this faxed information is strictly prohibited. If you have received this facsimile in error, please notify us by telephone immediately so that we can arrange for the return of the original documents to us and the retransmission of them to the intended recipient.

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Atty Dkt No: PR2038

Assistant Commissioner of Patents: Please record the attached original documents or copy thereof.

1. Name of conveying party(ies):

Avi J. Ashkenazi

Additional name(s) of conveying party(ies) attached? _ Yes ☒ No

2. Name and address of receiving party(ies):

Name: GENENTECH, INC.

Internal Address:

Street Address: 1 DNA Way

City: South San Francisco State: California

ZIP: 94080-4990

Additional name(s) & address(es) attached? _ Yes ☒ No

3. Nature of conveyance:

☒ Assignment

Merger

Security Agreement

Change of Name

Other:

Execution Date: 2/17/2005

4. Application number(s) or patent number(s):

If this document is being filed together with a new application, the execution date of the application is:

A. Patent Application No.(s)

B. Patent No.(s)

60/607,834

Additional numbers attached? _ Yes ☒ No

5. Name and address of party to whom correspondence concerning document should be mailed:

Diane L. Marschang
GENENTECH, INC.

1 DNA Way
South San Francisco, CA 94080

6. Total number of applications and patents involved: 1

7. Total fee (37 CFR 3.41): \$ 40.00

_ Enclosed

☒ Authorized to be charged to deposit account

8. Deposit account number:

07-0630

DO NOT USE THIS SPACE

9. Statement and signature.

To the best of my knowledge and belief, the foregoing information is true and correct and any attached copy is a true copy of the original document.

Diane L. Marschang
Name of Person Signing
Registration No. 35,600

Diane L. Marschang
Signature

2/28/2005
Date

Total number of pages comprising cover sheet, attachments and document: 3

Revised (10/11/03)

27/8 260

Docket No. PR2031

ASSIGNMENT

WHEREAS, Avi J. Ashkenazi, a citizen of US, residing at 1456 Terrytown Street, San Mateo, CA 94402 (hereinafter "ASSIGNOR"), has invented a new and useful invention in

METHODS OF USING DEATH RECEPTOR LIGANDS AND CD20 ANTIBODIES

for which a provisional application Serial No. 60/607,834, if received (Docket No. PR2031) has been filed by him on September 8, 2004; and

WHEREAS, GENENTECH, INC., a corporation organized and existing under and by virtue of the laws of the State of Delaware, having a place of business at 1 DNA Way, South San Francisco, California 94080-4980, is desirous of acquiring an interest in and to said invention, and in and to the Letters Patents to be obtained therefor;

NOW, THEREFORE, be it known by all whom it may concern;

That for good and valuable consideration the receipt of which is hereby acknowledged, the said ASSIGNOR have and do hereby sell, assign, transfer and set over unto the said GENENTECH, INC., its successors and assigns, the full and exclusive right, title and interest including all rights under the Paris Convention for the Protection of Industrial Property, in and to said invention, and in and to any and all Letters Patents to be granted and issued therefor or any continuation, division, renewal, or substitute thereof, and as to Letters Patents any reissue or re-examination thereof, not only for, to, and in the United States of America, its territories and possessions, but for, to and in all other countries; and it has been and is hereby authorized and requested that the appropriate government agencies issue said Letters Patents to said GENENTECH, INC., in accordance with this Assignment.

Said ASSIGNOR covenants and agrees to cooperate with GENENTECH, INC., to enable said GENENTECH, INC. to enjoy to the fullest extent the right, title and interest herein conveyed in the United States and foreign countries. Such cooperation by said ASSIGNOR includes prompt production of pertinent facts and documents, giving of testimony, execution of petitions, oaths, specifications, declarations or other papers, and other assistance all to the extent deemed necessary or desirable by said GENENTECH, INC., (a) for perfecting the right, title and interest herein conveyed; (b) for prosecuting any of said applications; (c) for filing and prosecuting applications for reissuance of any of said patents; (d) for interference or other priority proceedings involving said invention; and (e) for legal proceedings involving said invention and any applications therefor and any patents granted thereon, including without limitation opposition proceedings, cancellation proceedings, priority contests, public use proceedings, infringement actions and court actions; provided, however, that the expense incurred by said ASSIGNOR in providing such cooperation shall be paid for by said GENENTECH, INC.

The terms and covenants of this assignment shall inure to the benefit of said GENENTECH, INC., its successors, assigns and other legal representatives, and shall be binding upon said ASSIGNOR, their respective heirs, legal representatives and assigns.

Said ASSIGNOR hereby warrants and represents that he has not entered and will not enter into any assignment, contract, or understanding in conflict herewith.

IN WITNESS WHEREOF I undersign as follows;

A - A A I

P2031R1

1/4

PCT REQUEST

Original (for SUBMISSION)

0	For receiving Office use only	
0-1	International Application No.	
0-2	International Filing Date	
0-3	Name of receiving Office and "PCT International Application"	
0-4	Form PCT/RO/101 PCT Request	
0-4-1	Prepared Using	PCT-SAFE [EASY mode] Version 3.50 (Build 0002.173)
0-5	Petition The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	Receiving Office (specified by the applicant)	United States Patent and Trademark Office (USPTO) (RO/US)
0-7	Applicant's or agent's file reference	P2031R1
I	Title of invention	METHODS OF USING DEATH RECEPTOR LIGANDS AND CD20 ANTIBODIES
II	Applicant	
II-1	This person is	applicant only
II-2	Applicant for	all designated States except US
II-4	Name	GENENTECH, INC.
II-5	Address	1 DNA Way South San Francisco, California 94080-4990 United States of America
II-6	State of nationality	US
II-7	State of residence	US
II-8	Telephone No.	650-225-1000
II-9	Facsimile No.	650-952-9881
III-1	Applicant and/or inventor	
III-1-1	This person is	applicant and inventor
III-1-2	Applicant for	US only
III-1-4	Name (LAST, First)	ASHKENAZI, Avi J.
III-1-5	Address	1456 Tarrytown Street San Mateo, California 94402 United States of America
III-1-6	State of nationality	US
III-1-7	State of residence	US

PCT REQUEST

Original (for SUBMISSION)

IV-1	Agent or common representative or address for correspondence The person identified below is hereby/ has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	agent
IV-1-1	Name (LAST, First)	MARSCHANG, Diane L.
IV-1-2	Address	GENENTECH, INC. 1 DNA Way South San Francisco, California 94080-4990 United States of America
IV-1-3	Telephone No.	650-225-5416
IV-1-4	Facsimile No.	650-952-9881
IV-1-5	Agent's registration No.	35,600
IV-2	Additional agent(s)	additional agent(s) with same address as first named agent
IV-2-1	Name(s)	AGARWAL, Atulya R.; ANDRUS, Alex; BARNES, Elizabeth M.; CARPENTER, David A.; COBURN, Cara M.; CONLEY, Deirdre L.; CUI, Steven X.; ELLIOTT, Jennifer L.; EVANS, David W.; HASAK, Janet E.; JOHNSTON, Sean A.; KALINOWSKI, Grant; KRESNAK, Mark T.; KUBINEC, Jeffrey S.; LEE, Wendy M.; NAIK, Paul; PLEASURE, Irene; SCHWARTZ, Timothy R.; SHIN, Elinor K.; SVOBODA, Craig G.; TAN, Lee K.; YEUNG, Bonny G.
V	DESIGNATIONS	
V-1	The filing of this request constitutes under Rule 4.9(a), the designation of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.	
VI-1	Priority claim of earlier national application	
VI-1-1	Filing date	08 September 2004 (08.09.2004)
VI-1-2	Number	60/607,834
VI-1-3	Country	US
VI-2	Priority claim of earlier national application	
VI-2-1	Filing date	30 March 2005 (30.03.2005)
VI-2-2	Number	60/666,550
VI-2-3	Country	US

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PCT REQUEST

Original (for SUBMISSION)

VI-3	Priority document request The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s):	VI-1, VI-2	
VII-1	International Searching Authority Chosen	European Patent Office (EPO) (ISA/EP)	
VIII	Declarations	Number of declarations	
VIII-1	Declaration as to the identity of the inventor	-	
VIII-2	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	-	
VIII-3	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	-	
VIII-4	Declaration of inventorship (only for the purposes of the designation of the United States of America)	-	
VIII-5	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	-	
IX	Check list	number of sheets	electronic file(s) attached
IX-1	Request (including declaration sheets)	4	✓
IX-2	Description	67	-
IX-3	Claims	2	-
IX-4	Abstract	1	✓
IX-5	Drawings	19	-
IX-7	TOTAL	93	
IX-8	Accompanying items	paper document(s) attached	electronic file(s) attached
IX-8	Fee calculation sheet	✓	-
IX-11	Copy of general power of attorney	reference no. none	-
IX-17	PCT-SAFE physical media	-	✓
IX-19	Figure of the drawings which should accompany the abstract	none	
IX-20	Language of filing of the international application	English	
X-1	Signature of applicant, agent or common representative		
X-1-1	Name (LAST, First)	MARSCHANG, Diane L.	
X-1-2	Name of signatory		
X-1-3	Capacity		

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PCT REQUEST

Original (for SUBMISSION)

FOR RECEIVING OFFICE USE ONLY

10-1	Date of actual receipt of the purported international application	
10-2	Drawings:	
10-2-1	Received	
10-2-2	Not received	
10-3	Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application	
10-4	Date of timely receipt of the required corrections under PCT Article 11(2)	
10-5	International Searching Authority	ISA/EP
10-6	Transmittal of search copy delayed until search fee is paid	

FOR INTERNATIONAL BUREAU USE ONLY

11-1	Date of receipt of the record copy by the International Bureau	
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PCT

GENERAL POWER OF ATTORNEY

(for several international applications filed under the Patent Cooperation Treaty)

(PCT Rule 90.5)

The undersigned person(s):

(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

JOHNSTON, Sean A.
Vice President
GENENTECH, INC.
1 DNA Way
South San Francisco, California
94080-4990 US

hereby appoints (appoint) the following person as:



agent



common representative

Name and address

(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

ANDRUS, Alex
AGARWAL, Atulya R.
BARNES, Elizabeth M.
EVANS, David E.
CARPENTER, David A.
COBURN, Cara M.
CONLEY, Dolores L.
CUI, Steven X.
ELLIOTT, Jennifer E.

HASAK, Janet E.
JOHNSTON, Sean A.
KALINOWSKI, Grant
KREBNAK, Mark T.
KUBINEC, Jeffrey S.
LEE, Wendy M.
MARSHANG, Diane L.
NAK, Paul

PLEASURE, Irene T.
SCHWARTZ, Timothy R.
SHERMAN, Daniel E.
SHIN, Einar K.
SVOBODA, Craig G.
TAN, Lee K.
YEUNG, Bonny G.

All of: GENENTECH, INC
1 DNA Way
South San Francisco, California 94080-4990

to represent the undersigned before



all the competent International Authorities



the International Searching Authority only



the International Preliminary Examining Authority only

In connection with any and all international applications filed by the undersigned with the following Office

United States Patent and Trademark Office

as receiving Office

and to make or receive payments on behalf of the undersigned.

Signatures of the applicant(s) (where there are several persons, each of them must sign; next to each signature, indicate the name of the person signing and the capacity in which the person signs. If such capacity is not obvious from reading this power):

Sean Johnston
JOHNSTON, Sean A.
Vice President
GENENTECH, INC.
1 DNA Way
South San Francisco, California
94080-4990 US

METHODS OF USING DEATH RECEPTOR LIGANDS AND CD20 ANTIBODIES

RELATED APPLICATIONS

5 This application claims priority under Section 119(e) to provisional application number 60/607,834 filed September 8, 2004 and to provisional application number 60/666,550 filed March 30, 2005, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

10 The present invention relates to methods of using death receptor ligands and CD20 antibodies. More particularly, the invention relates to methods of using Apo-2 ligand/TRAIL or death receptor antibodies in combination with CD20 antibodies to treat various pathological disorders,
15 such as cancer and immune related diseases.

BACKGROUND OF THE INVENTION

Various ligands and receptors belonging to the tumor necrosis factor (TNF) superfamily have been identified in the art. Included among such
20 ligands are tumor necrosis factor-alpha ("TNF-alpha"), tumor necrosis factor-beta ("TNF-beta" or "lymphotoxin-alpha"), lymphotoxin-beta ("LT-beta"), CD30 ligand, CD27 ligand, CD40 ligand, OX-40 ligand, 4-1BB ligand, LIGHT, Apo-1 ligand (also referred to as Fas ligand or CD95 ligand), Apo-2 ligand (also referred to as Apo2L or TRAIL), Apo-3 ligand (also referred to
25 as TWEAK), APRIL, OPG ligand (also referred to as RANK ligand, ODF, or TRANCE), and TALL-1 (also referred to as BlyS, BAFF or THANK) (See, e.g., Ashkenazi, Nature Review, 2:420-430 (2002); Ashkenazi and Dixit, Science, 281:1305-1308 (1998); Ashkenazi and Dixit, Curr. Opin. Cell Biol., 11:255-260 (2000); Golstein, Curr. Biol., 7:750-753 (1997) Wallach, Cytokine Reference, Academic Press, 2000, pages 377-411; Locksley et al., Cell,
30 104:487-501 (2001); Gruss and Dower, Blood, 85:3378-3404 (1995); Schmid et al., Proc. Natl. Acad. Sci., 83:1881 (1986); Dealtry et al., Eur. J. Immunol., 17:689 (1987); Pitti et al., J. Biol. Chem., 271:12687-12690 (1996); Wiley et al., Immunity, 3:673-682 (1995); Browning et al., Cell,
35 72:847-856 (1993); Armitage et al. Nature, 357:80-82 (1992); WO 97/01633 published January 16, 1997; WO 97/25428 published July 17, 1997; Marsters et al., Curr. Biol., 8:525-528 (1998); Chicheportiche et al., Biol. Chem., 272:32401-32410 (1997); Hahne et al., J. Exp. Med., 188:1185-1190 (1998); WO98/28426 published July 2, 1998; WO98/46751 published October 22, 1998;
40 WO/98/18921 published May 7, 1998; Moore et al., Science, 285:260-263 (1999); Shu et al., J. Leukocyte Biol., 65:680 (1999); Schneider et al., J. Exp. Med., 189:1747-1756 (1999); Mukhopadhyay et al., J. Biol. Chem., 274:15978-15981 (1999)).

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Induction of various cellular responses mediated by such TNF family ligands is typically initiated by their binding to specific cell receptors. Some, but not all, TNF family ligands bind to, and induce various biological activity through, cell surface "death receptors" to activate caspases, or enzymes that carry out the cell death or apoptosis pathway (Salvesen et al., Cell, 91:443-446 (1997)). Included among the members of the TNF receptor superfamily identified to date are TNFR1, TNFR2, TACI, GITR,, CD27, OX-40, CD30, CD40, HVEM, Fas (also referred to as Apo-1 or CD95), DR4 (also referred to as TRAIL-R1), DR5 (also referred to as Apo-2 or TRAIL-R2), DcR1, DcR2, osteoprotegerin (OPG), RANK and Apo-3 (also referred to as DR3 or TRAMP) (see, e.g., Ashkenazi, Nature Reviews, 2:420-430 (2002); Ashkenazi and Dixit, Science, 281:1305-1308 (1998); Ashkenazi and Dixit, Curr. Opin. Cell Biol., 11:255-260 (2000); Golstein, Curr. Biol., 7:750-753 (1997); Wallach, Cytokine Reference, Academic Press, 2000, pages 377-411; Locksley et al., Cell, 104:487-501 (2001); Gruss and Dower, Blood, 85:3378-3404 (1995); Hohman et al., J. Biol. Chem., 264:14927-14934 (1989); Brockhaus et al., Proc. Natl. Acad. Sci., 87:3127-3131 (1990); EP 417,563, published March 20, 1991; Loetscher et al., Cell, 61:351 (1990); Schall et al., Cell, 61:361 (1990); Smith et al., Science, 248:1019-1023 (1990); Lewis et al., Proc. Natl. Acad. Sci., 88:2830-2834 (1991); Goodwin et al., Mol. Cell. Biol., 11:3020-3026 (1991); Stamenkovic et al., EMBO J., 8:1403-1410 (1989); Mallett et al., EMBO J., 9:1063-1068 (1990); Anderson et al., Nature, 390:175-179 (1997); Chicheportiche et al., J. Biol. Chem., 272:32401-32410 (1997); Pan et al., Science, 276:111-113 (1997); Pan et al., Science, 277:815-818 (1997); Sheridan et al., Science, 277:818-821 (1997); Degli-Esposti et al., J. Exp. Med., 186:1165-1170 (1997); Marsters et al., Curr. Biol., 7:1003-1006 (1997); Tsuda et al., BBRC, 234:137-142 (1997); Nocentini et al., Proc. Natl. Acad. Sci., 94:6216-6221 (1997); vonBulow et al., Science, 278:138-141 (1997)).

Most of these TNF receptor family members share the typical structure of cell surface receptors including extracellular, transmembrane and intracellular regions, while others are found naturally as soluble proteins lacking a transmembrane and intracellular domain. The extracellular portion of typical TNFRs contains a repetitive amino acid sequence pattern of multiple cysteine-rich domains (CRDs), starting from the NH₂-terminus.

The ligand referred to as Apo-2L or TRAIL was identified several years ago as a member of the TNF family of cytokines. (see, e.g., Wiley et al., Immunity, 3:673-682 (1995); Pitti et al., J. Biol. Chem., 271:12697-12690 (1996); WO 97/01633; WO 97/25428; US Patent 5,763,223 issued June 9, 1998; US Patent 6,284,236 issued September 4, 2001). The full-length native sequence human Apo2L/TRAIL polypeptide is a 281 amino acid long, Type II transmembrane protein. Some cells can produce a natural soluble form of the polypeptide, through enzymatic cleavage of the polypeptide's extracellular region (Mariani et al., J. Cell. Biol., 137:221-229 (1997)).

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Crystallographic studies of soluble forms of Apo2L/TRAIL reveal a homotrimeric structure similar to the structures of TNF and other related proteins (Hymowitz et al., *Molec. Cell*, 4:563-571 (1999); Cha et al., *Immunity*, 11:253-261 (1999); Mongkolsapaya et al., *Nature Structural Biology*, 6:1048 (1999); Hymowitz et al., *Biochemistry*, 39:633-644 (2000)). Apo2L/TRAIL, unlike other TNF family members however, was found to have a unique structural feature in that three cysteine residues (at position 230 of each subunit in the homotrimer) together coordinate a zinc atom, and that the zinc binding is important for trimer stability and biological activity. (Hymowitz et al., *supra*; Bodmer et al., *J. Biol. Chem.*, 275:20632-20637 (2000)).

It has been reported in the literature that Apo2L/TRAIL may play a role in immune system modulation, including autoimmune diseases such as rheumatoid arthritis [see, e.g., Thomas et al., *J. Immunol.*, 161:2195-2200 (1998); Johnsen et al., *Cytokine*, 11:664-672 (1999); Griffith et al., *J. Exp. Med.*, 189:1343-1353 (1999); Song et al., *J. Exp. Med.*, 191:1095-1103 (2000)].

Soluble forms of Apo2L/TRAIL have also been reported to induce apoptosis in a variety of cancer cells, including colon, lung, breast, prostate, bladder, kidney, ovarian and brain tumors, as well as melanoma, leukemia, and multiple myeloma (see, e.g., Wiley et al., *supra*; Pitti et al., *supra*; US Patent 6,030,945 issued February 29, 2000; US Patent 6,746,668 issued June 8, 2004; Rieger et al., *FEBS Letters*, 427:124-128 (1998); Ashkenazi et al., *J. Clin. Invest.*, 104:155-162 (1999); Walczak et al., *Nature Med.*, 5:157-163 (1999); Keane et al., *Cancer Research*, 59:734-741 (1999); Mizutani et al., *Clin. Cancer Res.*, 5:2605-2612 (1999); Gazitt, *Leukemia*, 13:1817-1824 (1999); Yu et al., *Cancer Res.*, 60:2384-2389 (2000); Chinnaiyan et al., *Proc. Natl. Acad. Sci.*, 97:1754-1759 (2000)). In vivo studies in murine tumor models further suggest that Apo2L/TRAIL, alone or in combination with chemotherapy or radiation therapy, can exert substantial anti-tumor effects (see, e.g., Ashkenazi et al., *supra*; Walczak et al., *supra*; Gliniak et al., *Cancer Res.*, 59:6153-6158 (1999); Chinnaiyan et al., *supra*; Roth et al., *Biochem. Biophys. Res. Comm.*, 265:1999 (1999); PCT Application US/00/15512; PCT Application US/01/23691). In contrast to many types of cancer cells, most normal human cell types appear to be resistant to apoptosis induction by certain recombinant forms of Apo2L/TRAIL (Ashkenazi et al., *supra*; Walczak et al., *supra*). Jo et al. has reported that a polyhistidine-tagged soluble form of Apo2L/TRAIL induced apoptosis in vitro in normal isolated human, but not non-human, hepatocytes (Jo et al., *Nature Med.*, 6:564-567 (2000); see also, Nagata, *Nature Med.*, 6:502-503 (2000)). It is believed that certain recombinant Apo2L/TRAIL preparations may vary in terms of biochemical properties and biological activities on diseased versus normal cells, depending, for example, on the presence or absence of a tag molecule, zinc content, and %

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trimer content (See, Lawrence et al., *Nature Med.*, Letter to the Editor, 7:383-385 (2001); Qin et al., *Nature Med.*, Letter to the Editor, 7:385-386 (2001)).

Apo2L/TRAIL has been found to bind at least five different receptors.

At least two of the receptors which bind Apo2L/TRAIL contain a functional, cytoplasmic death domain. One such receptor has been referred to as "DR4" (and alternatively as TR4 or TRAIL-R1) (Pan et al., *Science*, 276:111-113 (1997); see also WO98/32856 published July 30, 1998; WO99/37684 published July 29, 1999; WO 00/73349 published December 7, 2000; US 6,433,147 issued August 13, 2002; US 6,461,823 issued October 8, 2002, and US 6,342,383 issued January 29, 2002).

Another such receptor for Apo2L/TRAIL has been referred to as DR5 (it has also been alternatively referred to as Apo-2; TRAIL-R or TRAIL-R2, TR6, Tango-63, hAPO8, TRICK2 or KILLER) (see, e.g., Sheridan et al., *Science*, 277:818-821 (1997); Pan et al., *Science*, 277:815-818 (1997); WO98/51793 published November 19, 1998; WO98/41629 published September 24, 1998; Screaton et al., *Curr. Biol.*, 7:693-696 (1997); Walczak et al., *EMBO J.*, 16:5386-5387 (1997); Wu et al., *Nature Genetics*, 17:141-143 (1997); WO98/35986 published August 20, 1998; EP870,827 published October 14, 1998; WO98/46643 published October 22, 1998; WO99/02653 published January 21, 1999; WO99/09165 published February 25, 1999; WO99/11791 published March 11, 1999; US 2002/0072091 published August 13, 2002; US 2002/0098550 published December 7, 2001; US 6,313,269 issued December 6, 2001; US 2001/0010924 published August 2, 2001; US 2003/01255540 published July 3, 2003; US 2002/0160446 published October 31, 2002; US 2002/0048785 published April 25, 2002; US 6,342,369 issued February, 2002; US 6,569,642 issued May 27, 2003; US 6,072,047 issued June 6, 2000; US 6,642,358 issued November 4, 2003; US 6,743,625 issued June 1, 2004). Like DR4, DR5 is reported to contain a cytoplasmic death domain and be capable of signaling apoptosis upon ligand binding (or upon binding a molecule, such as an agonist antibody, which mimics the activity of the ligand). The crystal structure of the complex formed between Apo-2L/TRAIL and DR5 is described in Hymowitz et al., *Molecular Cell*, 4:563-571 (1999).

Upon ligand binding, both DR4 and DR5 can trigger apoptosis independently by recruiting and activating the apoptosis initiator, caspase-8, through the death-domain-containing adaptor molecule referred to as FADD/Mort1 [Kischkel et al., *Immunity*, 12:611-620 (2000); Sprick et al., *Immunity*, 12:599-609 (2000); Bodmer et al., *Nature Cell Biol.*, 2:241-243 (2000)].

Apo2L/TRAIL has been reported to also bind those receptors referred to as DcR1, DcR2 and OPG, which believed to function as inhibitors, rather than transducers of signaling (see., e.g., DCR1 (also referred to as TRID, LIT or TRAIL-R3) [Pan et al., *Science*, 276:111-113 (1997); Sheridan et al., *Science*, 277:818-821 (1997); McFarlane et al., *J. Biol. Chem.*, 272:25417-

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25420 (1997); Schneider et al., FEBS Letters, 416:329-334 (1997); Degli-Esposti et al., J. Exp. Med., 186:1165-1170 (1997); and Mongkolsapaya et al., J. Immunol., 160:3-6 (1998); DCR2 (also called TRUNDD or TRAIL-R4) [Marsters et al., Curr. Biol., 7:1003-1006 (1997); Pan et al., FEBS Letters, 424:41-45 (1998); Degli-Esposti et al., Immunity, 7:813-820 (1997)], and OPG [Simonet et al., supra]. In contrast to DR4 and DR5, the DcR1 and DcR2 receptors do not signal apoptosis.

Certain antibodies which bind to the DR4 and/or DR5 receptors have been reported in the literature. For example, anti-DR4 antibodies directed to the DR4 receptor and having agonistic or apoptotic activity in certain mammalian cells are described in, e.g., WO 99/37684 published July 29, 1999; WO 00/73349 published July 12, 2000; WO 03/066661 published August 14, 2003. See, also, e.g., Griffith et al., J. Immunol., 162:2597-2605 (1999); Chuntharapai et al., J. Immunol., 166:4891-4898 (2001); WO 02/097033 published December 2, 2002; WO 03/042367 published May 22, 2003; WO 03/038043 published May 8, 2003; WO 03/037913 published May 8, 2003. Certain anti-DR5 antibodies have likewise been described, see, e.g., WO 98/51793 published November 8, 1998; Griffith et al., J. Immunol., 162:2597-2605 (1999); Ichikawa et al., Nature Med., 7:954-960 (2001); Hylander et al., "An Antibody to DR5 (TRAIL-Receptor 2) Suppresses the Growth of Patient Derived Gastrointestinal Tumors Grown in SCID mice", Abstract, 2d International Congress on Monoclonal Antibodies in Cancers, Aug. 29-Sept. 1, 2002, Banff, Alberta, Canada; WO 03/038043 published May 8, 2003; WO 03/037913 published May 8, 2003. In addition, certain antibodies having cross-reactivity to both DR4 and DR5 receptors have been described (see, e.g., US patent 6,252,050 issued June 26, 2001).

The CD20 antigen (also called human B-lymphocyte-restricted differentiation antigen, Bp35) is a hydrophobic transmembrane protein with a molecular weight of approximately 35 kD located on pre-B and mature B lymphocytes (Valentine et al. J. Biol. Chem. 264(19):11282-11287 (1989); and Einfeld et al. EMBO J. 7(3):711-717 (1988)). The antigen is also expressed on greater than 90% of B cell non-Hodgkin's lymphomas (NHL) (Anderson et al. Blood 63(6):1424-1433 (1984)), but is not found on hematopoietic stem cells, pro-B cells, normal plasma cells or other normal tissues (Tedder et al. J. Immunol. 135(2):973-979 (1985)). CD20 regulates an early step(s) in the activation process for cell cycle initiation and differentiation (Tedder et al., supra) and possibly functions as a calcium ion channel (Tedder et al. J. Cell. Biochem. 14D:195 (1990)). Given the expression of CD20 in B cell lymphomas, this antigen may serve as a candidate for "targeting" of such lymphomas.

The rituximab (RITUXAN®) antibody is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen. Rituximab is the antibody called "C2B8" in US Patent No. 5,736,137 issued April 7, 1998 (Anderson et al.). RITUXAN® is indicated for the

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treatment of patients with relapsed or refractory low-grade or follicular, CD20 positive, B cell non-Hodgkin's lymphoma. In vitro mechanism of action studies have demonstrated that RITUXAN® binds human complement, and lyses lymphoid B cell lines through complement-dependent cytotoxicity (CDC) (Reff et al. *Blood* 83(2):435-445 (1994); Cragg and Marlin, *Blood*, 103: 2738-2743 (2004). Additionally, it has significant activity in assays for antibody-dependent cell-mediated cytotoxicity (ADCC). More recently, RITUXAN® has been shown to have anti-proliferative effects in tritiated thymidine incorporation assays and to induce apoptosis directly, while other anti-CD19 and CD20 antibodies do not (Maloney et al. *Blood* 88(10):637a (1996)). Synergy between RITUXAN® and certain chemotherapies and toxins has also been observed experimentally. In particular, RITUXAN® sensitizes drug-resistant human B cell lymphoma cell lines to the cytotoxic effects of doxorubicin, CDDP, VP-16, diphtheria toxin and ricin (Demidem et al. *Cancer Chemotherapy & Radiopharmaceuticals* 12(3):177-186 (1997)). In vivo preclinical studies have shown that RITUXAN® depletes B cells from the peripheral blood, lymph nodes, and bone marrow of cynomolgus monkeys, presumably through complement and cell-mediated processes (Reff et al. *Blood* 83(2):435-445 (1994)).

SUMMARY OF THE INVENTION

Methods for using death receptor ligands, such as Apo-2 ligand/TRAIL polypeptides or death receptor antibodies, and CD20 antibodies are provided herein. Embodiments of the invention include methods of treating cancer, comprising exposing cancer cells to an effective amount of Apo2L/TRAIL and CD20 antibody. Optionally, the cancer cells are exposed to an effective amount of death receptor antibody, such as an agonist DR4 antibody or agonist DR5 antibody, and CD20 antibody. Optionally, the amount of Apo2L/TRAIL or death receptor antibody and CD20 antibodies employed in the methods are effective to achieve synergy therapeutically, e.g., their combined anti-cancer effect is greater than the anti-cancer effect achieved when the Apo2L/TRAIL or antibodies are employed individually as a single therapeutic agent. The methods may entail in vitro use or in vivo use wherein the Apo2L/TRAIL or death receptor antibody and CD20 antibody are administered to a mammal (patient). Optionally, in the methods, the cancer cells treated with Apo2L/TRAIL or death receptor antibody and CD20 antibody are lymphoma cells.

Further embodiments of the invention include methods of treating an immune-related disease, comprising administering to a mammal an effective amount of Apo2L/TRAIL and CD20 antibody. Optionally, an effective amount of death receptor antibody, such as an agonist DR4 antibody or agonist DR5 antibody, and CD20 antibody is administered to the mammal. Optionally, the amount of Apo2L/TRAIL or death receptor antibody and CD20 antibodies

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employed in the methods are effective to achieve synergy therapeutically, e.g., their combined effect in treating the immune-related disease is greater than the effect achieved when the Apo2L/TRAIL or antibodies are employed individually as a single therapeutic agent. Optionally, in the methods, the immune-related disease is rheumatoid arthritis or multiple sclerosis.

Methods of the invention include methods of treating a disorder in a mammal, such as an immune-related disease or cancer, comprising steps of obtaining tissue or a cell sample from the mammal, examining the tissue or cells for expression of CD20, DR4, and/or DR5, and upon determining said tissue or cell sample expresses said one or more receptors, administering an effective amount of Apo2L/TRAIL or death receptor antibody and CD20 antibody to said mammal. The steps in the methods for examining expression of one or more of such receptors may be conducted in a variety of assay formats, including assays detecting mRNA expression and immunohistochemistry assays.

Optionally, the methods of the invention comprise, in addition to administering an effective amount of Apo2L/TRAIL and/or death receptor antibody and CD20 antibody, administering chemotherapeutic agent(s) or radiation therapy to said mammal.

More embodiments of the invention are illustrated by way of example in the following claims:

1. A method of treating cancer cells, comprising exposing mammalian cancer cells to a synergistic effective amount of agonist death receptor antibody and CD20 antibody.
2. The method of claim 1 wherein said agonist death receptor antibody is an anti-DR5 receptor monoclonal antibody.
3. The method of claim 1 wherein said agonist death receptor antibody is an anti-DR4 receptor monoclonal antibody.
4. The method of claim 1 wherein said cancer cells are exposed to said synergistic effective amount of agonist death receptor antibody and CD20 antibody *in vivo*.
5. The method of claim 2 or 3 wherein said agonist death receptor antibody is a chimeric antibody or a humanized antibody.
6. The method of claim 2 or 3 wherein said agonist death receptor antibody is a human antibody.

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7. The method of claim 1 wherein said agonist death receptor antibody is an antibody which cross-reacts with more than one Apo-2 ligand receptor.

5 8. The method of claim 1 wherein said cancer cells are lymphoma cells.

9. The method of claim 1 further comprising exposing the cancer cells to one or more growth inhibitory agents.

10 10. The method of claim 1 further comprising exposing the cells to radiation.

15 11. The method of claim 2 wherein said DR5 antibody has a DR5 receptor binding affinity of 10^8 M^{-1} to 10^{12} M^{-1} .

12. The method of claim 1 wherein said death receptor antibody and CD20 antibody is expressed in a recombinant host cell selected from the group consisting of a CHO cell, yeast cell and *E. coli*.

20 13. The method of claim 1 wherein said CD20 antibody is a monoclonal antibody.

14. The method of claim 13 wherein said CD20 antibody is the antibody Rituximab.

25 15. A method of treating an immune related disease, comprising administering to a mammal a synergistic effective amount of agonist death receptor antibody and CD20 antibody.

30 16. The method of claim 15 wherein said agonist death receptor antibody is an anti-DR5 receptor monoclonal antibody.

17. The method of claim 15 wherein said agonist death receptor antibody is an anti-DR4 receptor monoclonal antibody.

35 18. The method of claim 16 or 17 wherein said agonist death receptor antibody is a chimeric antibody or a humanized antibody.

40 19. The method of claim 16 or 17 wherein said agonist death receptor antibody is a human antibody.

20. The method of claim 15 wherein said agonist death receptor antibody is an antibody which cross-reacts with more than one Apo-2 ligand receptor.

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21. The method of claim 15 wherein said immune related disease is rheumatoid arthritis or multiple sclerosis.

5 22. The method of claim 15 wherein said DR5 antibody has a DR5 receptor binding affinity of 10^8 M^{-1} to 10^{13} M^{-1} .

10 23. The method of claim 15 wherein said death receptor antibody and CD20 antibody is expressed in a recombinant host cell selected from the group consisting of a CHO cell, yeast cell and *E. coli*.

24. The method of claim 15 wherein said CD20 antibody is a monoclonal antibody.

15 25. The method of claim 24 wherein said CD20 antibody is the antibody Rituximab.

20 26. The method of claim 1 or 15 wherein said death receptor antibody and CD20 antibody are administered sequentially.

27. The method of claim 1 or 15 wherein said death receptor antibody and CD20 antibody are administered concurrently.

BRIEF DESCRIPTION OF THE DRAWINGS

25 Figure 1A shows the nucleotide sequence of human Apo-2 ligand cDNA (SEQ ID NO:2) and its derived amino acid sequence (SEQ ID NO:1). The "N" at nucleotide position 447 is used to indicate the nucleotide base may be a "T" or "G".

30 Figures 2A and 2B show the nucleotide sequence of a cDNA (SEQ ID NO:4) for full length human DR4 and its derived amino acid sequence (SEQ ID NO:3). The respective nucleotide and amino acid sequences for human DR4 are also reported in Pan et al., Science, 276:1111 (1997).

35 Figure 3A shows the 411 amino acid sequence of human DR5 (SEQ ID NO:5) as published in WO 98/51793 on November 19, 1998. A transcriptional splice variant of human DR5 is known in the art. This DR5 splice variant encodes the 440 amino acid sequence of human DR5 (SEQ ID NO:6) shown in Figures 3B and 3C as published in WO 98/35986 on August 20, 1998.

Figure 4 illustrates the expression of Apo2L/TRAIL receptors in B lymphoma cell lines.

40 Figure 5 illustrates expression of CD20 in B lymphoma cell lines.

Figure 6 shows the effects of Apo2L/TRAIL, RITUXAN®, or combination treatment on the growth of pre-established subcutaneous BJAB lymphoma tumor xenografts in SCID mice.

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Figure 7 shows further results on the effects of Apo2L/TRAIL, RITUXAN®, or combination treatment of Apo2L/TRAIL and RITUXAN® on the growth of pre-established subcutaneous BJAB lymphoma tumor xenografts in SCID mice.

Figure 8 shows the effects of Apo2L/TRAIL, RITUXAN®, or combination treatment of Apo2L/TRAIL and RITUXAN® on caspase processing in pre-established subcutaneous BJAB lymphoma tumor xenografts grown in SCID mice.

Figure 9 shows the effects of DR5 agonistic antibody, RITUXAN®, or combination treatment on the growth of pre-established subcutaneous BJAB lymphoma tumor xenografts in SCID mice.

Figure 10 shows the effects of DR5 agonistic antibody, RITUXAN®, or combination treatment on caspase processing in pre-established subcutaneous BJAB lymphoma tumor xenografts grown in SCID mice.

Figure 11 illustrates the expression of CD20 and Apo2L/TRAIL receptors in NHL cell lines.

Figure 12 shows the effects of Apo2L/TRAIL, Rituximab, or combination treatment on the growth of pre-established subcutaneous Ramos RAI tumor xenografts in SCID mice.

Figure 13 shows the effects of Apo2L/TRAIL, Rituximab, or combination treatment on the growth of pre-established DOHH-2 follicular lymphoma xenografts in SCID mice.

Figure 14 illustrates the effects and mechanisms of cell killing by Apo2L/TRAIL and Rituximab or combination treatments on BJAB cells.

Figure 15 shows the effects of Apo2L/TRAIL, Rituximab, or combination treatment on the growth of Ramos T1 tumor xenografts in SCID mice.

Figure 16 shows the effects of Apo2L/TRAIL, Rituximab, or combination treatment on the BJAB-Luc tumor xenografts in SCID mice.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Unless otherwise defined, all terms of art, notations and other scientific terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this invention pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art. The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized molecular cloning methodologies described in Sambrook et al., Molecular Cloning: A Laboratory Manual 2nd. edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. As appropriate, procedures involving the use of commercially available kits and reagents are generally carried out in

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accordance with manufacturer defined protocols and/or parameters unless otherwise noted.

Before the present methods, kits and uses therefor are described, it is to be understood that this invention is not limited to the particular methodology, protocols, cell lines, animal species or genera, constructs, and reagents described as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

It must be noted that as used herein and in the appended claims, the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise.

All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. Publications cited herein are cited for their disclosure prior to the filing date of the present application. Nothing here is to be construed as an admission that the inventors are not entitled to antedate the publications by virtue of an earlier priority date or prior date of invention. Further the actual publication dates may be different from those shown and require independent verification.

Definitions

The terms "Apo-2 ligand", "Apo-2L", "Apo2L", "Apo2L/TRAIL", "Apo-2 ligand/TRAIL" and "TRAIL" are used herein interchangeably to refer to a polypeptide sequence which includes amino acid residues 114-281, inclusive, 95-281, inclusive, residues 92-281, inclusive, residues 91-281, inclusive, residues 41-281, inclusive, residues 39-281, inclusive, residues 15-281, inclusive, or residues 1-281, inclusive, of the amino acid sequence shown in Figure 1, as well as biologically active fragments, deletional, insertional, or substitutional variants of the above sequences. In one embodiment, the polypeptide sequence comprises residues 114-281 of Figure 1. Optionally, the polypeptide sequence comprises residues 92-281 or residues 91-281 of Figure 1. The Apo-2L polypeptides may be encoded by the native nucleotide sequence shown in Figure 1. Optionally, the codon which encodes residue Pro119 (Figure 1) may be "CCT" or "CCG". Optionally, the fragments or variants are biologically active and have at least about 80% amino acid sequence identity, more preferably at least about 90% sequence identity, and even more preferably, at least 95%, 96%, 97%, 98%, or 99% sequence identity with any one of the above sequences. The definition encompasses substitutional variants of Apo-2 ligand in which at least one of its native amino acids are substituted by another amino acid such as an alanine residue. Optional substitutional variants include one or more of

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the residue substitutions. Optional variants may comprise an amino acid sequence which differs from the native sequence Apo-2 ligand polypeptide sequence of Figure 1 and has one or more of the following amino acid substitutions at the residue position(s) in Figure 1: S96C; S101C; S111C; R170C; K179C. The definition also encompasses a native sequence Apo-2 ligand isolated from an Apo-2 ligand source or prepared by recombinant or synthetic methods. The Apo-2 ligand of the invention includes the polypeptides referred to as Apo-2 ligand or TRAIL disclosed in WO97/01633 published January 16, 1997, WO97/25428 published July 17, 1997, WO99/36535 published July 22, 1999, WO 01/00832 published January 4, 2001, WO02/09755 published February 7, 2002, and WO 00/75191 published December 14, 2000. The terms are used to refer generally to forms of the Apo-2 ligand which include monomer, dimer, trimer, hexamer or hight oligomer forms of the polypeptide. All numbering of amino acid residues referred to in the Apo-2L sequence use the numbering according to Figure 1, unless specifically stated otherwise. For instance, "D203" or "Asp203" refers to the aspartic acid residue at position 203 in the sequence provided in Figure 1.

The term "Apo-2 ligand selective variant" as used herein refers to an Apo-2 ligand polypeptide which includes one or more amino acid mutations in a native Apo-2 ligand sequence and has selective binding affinity for either the DR4 receptor or the DR5 receptor. In one embodiment, the Apo-2 ligand variant has a selective binding affinity for the DR4 receptor and includes one or more amino acid substitutions in any one of positions 189, 191, 193, 199, 201 or 209 of a native Apo-2 ligand sequence. In another embodiment, the Apo-2 ligand variant has a selective binding affinity for the DR5 receptor and includes one or more amino acid substitutions in any one of positions 189, 191, 193, 264, 266, 267 or 269 of a native Apo-2 ligand sequence. Preferred Apo-2 ligand selective variants include one or more amino acid mutations and exhibit binding affinity to the DR4 receptor which is equal to or greater ($\geq$) than the binding affinity of native sequence Apo-2 ligand to the DR4 receptor, and even more preferably, the Apo-2 ligand variants exhibit less binding affinity ($<$) to the DR5 receptor than the binding affinity exhibited by native sequence Apo-2 ligand to DR5. When binding affinity of such Apo-2 ligand variant to the DR4 receptor is approximately equal (unchanged) or greater than (increased) as compared to native sequence Apo-2 ligand, and the binding affinity of the Apo-2 ligand variant to the DR5 receptor is less than or nearly eliminated as compared to native sequence Apo-2 ligand, the binding affinity of the Apo-2 ligand variant, for purposes herein, is considered "selective" for the DR4 receptor. Preferred DR4 selective Apo-2 ligand variants of the invention will have at least 10-fold less binding affinity to DR5 receptor (as compared to native sequence Apo-2 ligand), and even more preferably, will have at least 100-fold less binding affinity to DR5 receptor (as compared to native sequence Apo-2 ligand). The respective binding affinity of the

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Apo-2 ligand variant may be determined and compared to the binding properties of native Apo-2L₂ (such as the 114-281 form) by ELISA, RIA, and/or BIAcore assays, known in the art. Preferred DR4 selective Apo-2 ligand variants of the invention will induce apoptosis in at least one type of mammalian cell (preferably a cancer cell), and such apoptotic activity can be determined by known art methods such as the alamar blue or crystal violet assay. The DR4 selective Apo-2 ligand variants may or may not have altered binding affinities to any of the decoy receptors for Apo-2L, those decoy receptors being referred to in the art as DcR1, DcR2 and OPG.

Further preferred Apo-2 ligand selective variants include one or more amino acid mutations and exhibit binding affinity to the DR5 receptor which is equal to or greater ($\geq$) than the binding affinity of native sequence Apo-2 ligand to the DR5 receptor, and even more preferably, such Apo-2 ligand variants exhibit less binding affinity ($<$) to the DR4 receptor than the binding affinity exhibited by native sequence Apo-2 ligand to DR4. When binding affinity of such Apo-2 ligand variant to the DR5 receptor is approximately equal (unchanged) or greater than (increased) as compared to native sequence Apo-2 ligand, and the binding affinity of the Apo-2 ligand variant to the DR4 receptor is less than or nearly eliminated as compared to native sequence Apo-2 ligand, the binding affinity of the Apo-2 ligand variant, for purposes herein, is considered "selective" for the DR5 receptor. Preferred DR5 selective Apo-2 ligand variants of the invention will have at least 10-fold less binding affinity to DR4 receptor (as compared to native sequence Apo-2 ligand), and even more preferably, will have at least 100-fold less binding affinity to DR4 receptor (as compared to native sequence Apo-2 ligand). The respective binding affinity of the Apo-2 ligand variant may be determined and compared to the binding properties of native Apo2L (such as the 114-281 form) by ELISA, RIA, and/or BIAcore assays, known in the art. Preferred DR5 selective Apo-2 ligand variants of the invention will induce apoptosis in at least one type of mammalian cell (preferably a cancer cell), and such apoptotic activity can be determined by known art methods such as the alamar blue or crystal violet assay. The DR5 selective Apo-2 ligand variants may or may not have altered binding affinities to any of the decoy receptors for Apo-2L, those decoy receptors being referred to in the art as DcR1, DcR2 and OPG.

Amino acid identification may use the single-letter alphabet or three-letter alphabet of amino acids, i.e.,

Asp	D	Aspartic acid	Ile	I	Isoleucine
Thr	T	Threonine	Leu	L	Leucine
Ser	S	Serine	Tyr	Y	Tyrosine
Glu	E	Glutamic acid	Phe	F	Phenylalanine
Pro	P	Proline	His	H	Histidine
Gly	G	Glycine	Lys	K	Lysine
Ala	A	Alanine	Arg	R	Arginine

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Cys	C	Cysteine	Trp	W	Tryptophan
Val	V	Valine	Gln	Q	Glutamine
Met	M	Methionine	Asn	N	Asparagine

5 The term "Apo2L/TRAIL extracellular domain" or "Apo2L/TRAIL ECD" refers to a form of Apo2L/TRAIL which is essentially free of transmembrane and cytoplasmic domains. Ordinarily, the ECD will have less than 1% of such transmembrane and cytoplasmic domains, and preferably, will have less than 0.5% of such domains. It will be understood that any transmembrane domain(s) identified for the polypeptides of the present invention are identified pursuant to criteria routinely employed in the art for identifying that type of hydrophobic domain. The exact boundaries of a transmembrane domain may vary but most likely by no more than about 5 amino acids at either end of the domain as initially identified. In preferred embodiments, the ECD will consist of a soluble, extracellular domain sequence of the polypeptide which is free of the transmembrane and cytoplasmic or intracellular domains (and is not membrane bound). Particular extracellular domain sequences of Apo-2L/TRAIL are described in PCT Publication Nos. WO97/01633 and WO97/25428.

20 The term "Apo2L/TRAIL monomer" or "Apo2L monomer" refers to a covalent chain of an extracellular domain sequence of Apo2L.

The term "Apo2L/TRAIL dimer" or "Apo2L dimer" refers to two Apo-2L monomers joined in a covalent linkage via a disulfide bond. The term as used herein includes free standing Apo2L dimers and Apo2L dimers that are within trimeric forms of Apo2L (i.e., associated with another, third Apo2L monomer).

The term "Apo2L/TRAIL trimer" or "Apo2L trimer" refers to three Apo2L monomers that are non-covalently associated.

30 The term "Apo2L/TRAIL aggregate" is used to refer to self-associated higher oligomeric forms of Apo2L/TRAIL, such as Apo2L/TRAIL trimers, which form, for instance, hexameric and nanomeric forms of Apo2L/TRAIL. Determination of the presence and quantity of Apo2L/TRAIL monomer, dimer, or trimer (or other aggregates) may be made using methods and assays known in the art (and using commercially available materials), such as native size exclusion HPLC ("SEC"), denaturing size exclusion using sodium dodecyl sulphate ("SDS-SEC"), reverse phase HPLC and capillary electrophoresis.

40 "Apo-2 ligand receptor" includes the receptors referred to in the art as "DR4" and "DR5" whose polynucleotide and polypeptide sequences are shown in Figures 2 and 3 respectively. Pan et al. have described the TNF receptor family member referred to as "DR4" (Pan et al., Science, 276:111-113 (1997); see also WO98/32856 published July 30, 1998; WO 99/37684 published July 29, 1999; WO 00/73349 published December 7, 2000; US 6,433,147 issued August 13, 2002; US 6,461,823 issued October 8, 2002, and US 6,342,383 issued January 29, 2002). Sheridan et al., Science, 277:818-

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821 (1997) and Pan et al., Science, 277:815-818 (1997)* described another receptor for Apo2L/TRAIL (see also, WO98/51793 published November 19, 1998; WO98/41629 published September 24, 1998). This receptor is referred to as DR5 (the receptor has also been alternatively referred to as Apo-2; TRAIL-R, TR6, Tango-63, hAPO8, TRICK2 or KILLER; Screaton et al., Curr. Biol., 7:693-696 (1997); Walczak et al., EMBO J., 16:5386-5387 (1997); Wu et al., Nature Genetics, 17:141-143 (1997); WO98/35986 published August 20, 1998; EP870,827 published October 14, 1998; WO98/46643 published October 22, 1998; WO99/02653 published January 21, 1999; WO99/09165 published February 25, 1999; WO99/11791 published March 11, 1999; US 2002/0072091 published August 13, 2002; US 2002/0098550 published December 7, 2001; US 6,313,269 issued December 6, 2001; US 2001/0010924 published August 2, 2001; US 2003/01255540 published July 3, 2003; US 2002/0160446 published October 31, 2002; US 2002/0048785 published April 25, 2002; US 6,569,642 issued May 27, 2003; US 6,072,047 issued June 6, 2000; US 6,642,358 issued November 4, 2003). As described above, other receptors for Apo-2L include DcR1, DcR2, and OPG (see, Sheridan et al., supra; Marsters et al., supra; and Simonet et al., supra). The term "Apo-2L receptor" when used herein encompasses native sequence receptor and receptor variants. These terms encompass Apo-2L receptor expressed in a variety of mammals, including humans. Apo-2L receptor may be endogenously expressed as occurs naturally in a variety of human tissue lineages, or may be expressed by recombinant or synthetic methods. A "native sequence Apo-2L receptor" comprises a polypeptide having the same amino acid sequence as an Apo-2L receptor derived from nature. Thus, a native sequence Apo-2L receptor can have the amino acid sequence of naturally-occurring Apo-2L receptor from any mammal. Such native sequence Apo-2L receptor can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence Apo-2L receptor" specifically encompasses naturally-occurring truncated or secreted forms of the receptor (e.g., a soluble form containing, for instance, an extracellular domain sequence), naturally-occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants. Receptor variants may include fragments or deletion mutants of the native sequence Apo-2L receptor. Figure 3A shows the 411 amino acid sequence of human DR5 as published in WO 98/51793 on November 19, 1998. A transcriptional splice variant of human DR5 is known in the art. This DR5 splice variant encodes the 440 amino acid sequence of human DR5 shown in Figures 3B and 3C as published in WO 98/35986 on August 20, 1998.

"Death receptor antibody" is used herein to refer generally to antibody or antibodies directed to a receptor in the tumor necrosis factor receptor superfamily and containing a death domain capable of signalling apoptosis, and such antibodies include DR5 antibody and DR4 antibody.

"DR5 receptor antibody", "DR5 antibody", or "anti-DR5 antibody" is used in a broad sense to refer to antibodies that bind to at least one form

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of a DR5 receptor or extracellular domain thereof. Optionally the DR5 antibody is fused or linked to a heterologous sequence or molecule. Preferably the heterologous sequence allows or assists the antibody to form higher order or oligomeric complexes. Optionally, the DR5 antibody binds to DR5 receptor but does not bind or cross-react with any additional Apo-2L receptor (e.g. DR4, DcR1, or DcR2). Optionally the antibody is an agonist of DR5 signalling activity.

Optionally, the DR5 antibody of the invention binds to a DR5 receptor at a concentration range of about 0.1 nM to about 20 mM as measured in a BIAcore binding assay. Optionally, the DR5 antibodies of the invention exhibit an I_c 50 value of about 0.6 nM to about 18 mM as measured in a BIAcore binding assay.

"DR4 receptor antibody", "DR4 antibody", or "anti-DR4 antibody" is used in a broad sense to refer to antibodies that bind to at least one form of a DR4 receptor or extracellular domain thereof. Optionally the DR4 antibody is fused or linked to a heterologous sequence or molecule. Preferably the heterologous sequence allows or assists the antibody to form higher order or oligomeric complexes. Optionally, the DR4 antibody binds to DR4 receptor but does not bind or cross-react with any additional Apo-2L receptor (e.g. DR5, DcR1, or DcR2). Optionally the antibody is an agonist of DR4 signalling activity.

Optionally, the DR4 antibody of the invention binds to a DR4 receptor at a concentration range of about 0.1 nM to about 20 mM as measured in a BIAcore binding assay. Optionally, the DR5 antibodies of the invention exhibit an I_c 50 value of about 0.6 nM to about 18 mM as measured in a BIAcore binding assay.

The term "agonist" is used in the broadest sense, and includes any molecule that partially or fully enhances, stimulates or activates one or more biological activities of Apo2L/TRAIL, DR4 or DR5, *in vitro*, *in situ*, or *in vivo*. Examples of such biological activities binding of Apo2L/TRAIL to DR4 or DR5, include apoptosis as well as those further reported in the literature. An agonist may function in a direct or indirect manner. For instance, the agonist may function to partially or fully enhance, stimulate or activate one or more biological activities of DR4 or DR5, *in vitro*, *in situ*, or *in vivo* as a result of its direct binding to DR4 or DR5, which causes receptor activation or signal transduction. The agonist may also function indirectly to partially or fully enhance, stimulate or activate one or more biological activities of DR4 or DR5, *in vitro*, *in situ*, or *in vivo* as a result of, e.g., stimulating another effector molecule which then causes DR4 or DR5 activation or signal transduction. It is contemplated that an agonist may act as an enhancer molecule which functions indirectly to enhance or increase DR4 or DR5 activation or activity. For instance, the agonist may enhance activity of endogenous Apo-2L in a mammal. This could be accomplished, for example, by pre-complexing DR4 or DR5 or by

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stabilizing complexes of the respective ligand with the DR4 or DR5 receptor (such as stabilizing native complex formed between Apo-2L and DR4 or DR5).

The term "DR4" and "DR4 receptor" as used herein refers to full length and soluble, extracellular domain forms of the receptor described in Pan et al., Science, 276:111-113 (1997); WO98/32856 published July 30, 1998; US Patent 6,342,363 issued January 29, 2002; and WO99/37684 published July 29, 1999. The full length amino acid sequence of DR4 receptor is provided herein in Fig. 2.

The term "DR5" and "DR5 receptor" as used herein refers to the full length and soluble, extracellular domain forms of the receptor described in Sheridan et al., Science, 277:818-821 (1997); Pan et al., Science, 277:815-818 (1997), US Patent 6,072,047 issued June 6, 2000; US Patent 6,342,369, WO98/51793 published November 19, 1998; WO98/41629 published September 24, 1998; Screaton et al., Curr. Biol., 7:693-696 (1997); Walczak et al., EMBO J., 16:5386-5387 (1997); Wu et al., Nature Genetics, 17:141-143 (1997); WO98/35986 published August 20, 1998; EP870,827 published October 14, 1998; WO98/46643 published October 22, 1998; WO99/02653 published January 21, 1999; WO99/09165 published February 25, 1999; WO99/11791 published March 11, 1999. The DR5 receptor has also been referred to in the art as Apo-2; TRAIL-R, TR6, Tango-63, hAPO8, TRICK2 or KILLER. The term DR5 receptor used herein includes the full length 411 amino acid polypeptide provided in Fig. 3A and the full length 440 amino acid polypeptide provided in Figs. 3B-C.

The term "polyol" when used herein refers broadly to polyhydric alcohol compounds. Polyols can be any water-soluble poly(alkylene oxide) polymer for example, and can have a linear or branched chain. Preferred polyols include those substituted at one or more hydroxyl positions with a chemical group, such as an alkyl group having between one and four carbons. Typically, the polyol is a poly(alkylene glycol), preferably poly(ethylene glycol) (PEG). However, those skilled in the art recognize that other polyols, such as, for example, poly(propylene glycol) and polyethylene-polypropylene glycol copolymers, can be employed using the techniques for conjugation described herein for PEG. The polyols of the invention include those well known in the art and those publicly available, such as from commercially available sources.

The term "conjugate" is used herein according to its broadest definition to mean joined or linked together. Molecules are "conjugated" when they act or operate as if joined.

The term "extracellular domain" or "ECD" refers to a form of ligand or receptor which is essentially free of transmembrane and cytoplasmic domains. Ordinarily, the soluble ECD will have less than 1% of such transmembrane and cytoplasmic domains, and preferably, will have less than 0.5% of such domains.

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The term "divalent metal ion" refers to a metal ion having two positive charges. Examples of divalent metal ions for use in the present invention include but are not limited to zinc, cobalt, nickel, cadmium, magnesium, and manganese. Particular forms of such metals that may be employed include salt forms (e.g., pharmaceutically acceptable salt forms), such as chloride, acetate, carbonate, citrate and sulfate forms of the above mentioned divalent metal ions. Divalent metal ions, as described herein, are preferably employed in concentrations or amounts (e.g., effective amounts) which are sufficient to, for example, (1) enhance storage stability of Apo-2L trimers over a desired period of time, (2) enhance production or yield of Apo-2L trimers in a recombinant cell culture or purification method, (3) enhance solubility (or reduce aggregation) of Apo-2L trimers, or (4) enhance Apo-2L trimer formation.

"Isolated," when used to describe the various proteins disclosed herein, means protein that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the protein, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the protein will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated protein includes protein in situ within recombinant cells, since at least one component of the protein's natural environment will not be present. Ordinarily, however, isolated protein will be prepared by at least one purification step.

An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the nucleic acid. An isolated Apo-2 ligand nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated Apo-2 ligand nucleic acid molecules therefore are distinguished from the Apo-2 ligand nucleic acid molecule as it exists in natural cells. However, an isolated Apo-2 ligand nucleic acid molecule includes Apo-2 ligand nucleic acid molecules contained in cells that ordinarily express Apo-2 ligand where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

"Percent (%) amino acid sequence identity" with respect to the sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the Apo-2 ligand sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence

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identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art can determine appropriate parameters for measuring alignment, including assigning algorithms needed to achieve maximal alignment over the full-length sequences being compared. For purposes herein, percent amino acid identity values can be obtained using the sequence comparison computer program, ALIGN-2, which was authored by Genentech, Inc. and the source code of which has been filed with user documentation in the US Copyright Office, Washington, DC, 20559, registered under the US Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available through Genentech, Inc., South San Francisco, CA. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

A "B cell" is a lymphocyte that matures within the bone marrow, and includes a naive B cell, memory B cell, or effector B cells (plasma cells). The B cell herein may be a normal or non-malignant B cell.

The "CD20" antigen is a 35 kDa, non-glycosylated phosphoprotein found on the surface of greater than 90% of B cells from peripheral blood or lymphoid organs. CD20 is present on both normal B cells as well as malignant B cells, but is not expressed on stem cells. Other names for CD20 in the literature include "B-lymphocyte-restricted antigen" and "Bp35". The CD20 antigen is described in Clark et al. PNAS (USA) 82:1766 (1985), for example.

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Examples of antibodies which bind the CD20 antigen include: "C2B8" which is now called "Rituximab" ("RITUXAN®") (US Patent No. 5,736,137); the yttrium-[90]-labeled 2B8 murine antibody designated "Y2B8" or "Ibritumomab Tiuxetan" ZEVALIN® commercially available from Idec Pharmaceuticals, Inc. (US Patent No. 5,736,137; 2B8 deposited with ATCC under accession no. HB11388 on June 22, 1993); murine IgG2a "B1," also called "Tositumomab," optionally labeled with ¹³¹I to generate the "¹³¹I-B1" antibody (iodine I131 tositumomab, BEXXAR™) commercially available from Corixa (see, also, US Patent No. 5,595,721); murine monoclonal antibody "1F5" (Press et al. *Blood* 69(2):584-591 (1987)) and variants thereof including "framework patched" or humanized 1F5 (WO 2003/002607, Leung, ATCC Deposit HB-96450); murine 2H7 and chimeric 2H7 antibody (US Patent No. 5,677,180); humanized 2H7; HUMAX-CD20™ fully human, high-affinity antibody targeted at the CD20 molecule in the cell membrane of B-cells (Genmab, Denmark; see, for example, Glennie and van de Winkel, *Drug Discovery Today* 8: 503-510 (2003) and Cragg et al., *Blood* 101: 1045-1052 (2003)); the human monoclonal antibodies set forth in WO04/035607 (Teeling et al.); AME-133™ antibodies (Applied Molecular Evolution); A20 antibody or variants thereof such as chimeric or humanized A20 antibody (ca20, ha20; respectively) (US 2003/0219433, Immunomedics); and monoclonal antibodies L27, G28-2, 93-1B3, B-C1 or NU-B2 available from the International Leukocyte Typing Workshop (Valentine et al., In: *Leukocyte Typing III* (McMichael, Ed., p. 440, Oxford University Press (1987))). The preferred CD20 antibodies herein are chimeric, humanized, or human CD20 antibodies, more preferably rituximab, humanized 2H7, chimeric or humanized A20 antibody (Immunomedics), and HUMAX-CD20™ human CD20 antibody (Genmab).

The terms "rituximab" or "RITUXAN®" herein refer to the genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen and designated "C2B8" in US Patent No. 5,736,137, including fragments thereof which retain the ability to bind CD20.

Purely for the purposes herein and unless indicated otherwise, "humanized 2H7" refers to a humanized CD20 antibody, or an antigen-binding fragment thereof, wherein the antibody is effective to deplete primate B cells *in vivo*, the antibody comprising in the H chain variable region (V_H) thereof at least a CDR H3 sequence from an anti-human CD20 antibody and substantially the human consensus framework (FR) residues of the human heavy-chain subgroup III (V_HIII).

A preferred humanized 2H7 is an intact antibody or antibody fragment comprising the variable light chain sequence:

DIQMTQSPSSLSASVGRVTITCRASSSVSYMHWYQQKPGKAPKPLIYAPSNLASGVPSRFSGSGSGTDFLTIS
SLOPEDFATYYCQWSFNPPTFGQGTKVEIKR (SEQ ID NO:7);
and the variable heavy chain sequence:
EVQLVESGGGLVQPGGSLRLSCAASGYTFSTSYNMHWYRQAPGKGLEWVGAIYPNGDTSYNQKFKGRFTISVDKS
KNTLYLQMNSLRAEDTAVYYCARVVYYSNSYWFYFDVWGQGTLVTVSS (SEQ ID NO:8).

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Where the humanized 2H7 antibody is an intact antibody, preferably it comprises the light chain amino acid sequence:

DIQMTQSPSSLSASVGDRVTTITCRASSSVSYMHYQQKPGKAPKPLIYAPSNLASGVPSRFSGSGSGTDFTLTIS
SLQPEDFATYYCQOWSFNPPTFGQGTKVEIKRTVAAPSVFIFPPPSDEQLKSGTASVVCLLNFFYPREAKVQWKVD
5 NALQSGNSQESVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID
NO:9);

and the heavy chain amino acid sequence:

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGAIYPGNGDTSYNQKFKGRFTISVDKS
KNTLYLQMNSLRAEDTAVYYCARVVYYSNSYWFYFDVWGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
10 VKDYFPEPFTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
DKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNSTYRVVSVLTVTLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVK
GFYPDSIAVEWESNGQPENNYKTTPFVLDSGDSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSP
GK (SEQ ID NO:10)

or the heavy chain amino acid sequence:

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGAIYPGNGDTSYNQKFKGRFTISVDKS
KNTLYLQMNSLRAEDTAVYYCARVVYYSNSYWFYFDVWGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
20 VKDYFPEPFTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
DKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNATYRVVSVLTVTLHQDWLNGKEYKCKVSNKALPAPIAATISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVK
GFYPDSIAVEWESNGQPENNYKTTPFVLDSGDSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSP
GK (SEQ ID NO:11).

"Antibody-dependent cell-mediated cytotoxicity" and "ADCC" refer to a
25 cell-mediated reaction in which nonspecific cytotoxic cells that express Fc
receptors (FcRs) (e.g. Natural Killer (NK) cells, neutrophils, and
macrophages) recognize bound antibody on a target cell and subsequently
cause lysis of the target cell. The primary cells for mediating ADCC, NK
cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and
30 FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on
page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To
assess ADCC activity of a molecule of interest, an *in vitro* ADCC assay,
such as that described in US Patent No. 5,500,362 or 5,821,337 may be
performed. Useful effector cells for such assays include peripheral blood
35 mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or
additionally, ADCC activity of the molecule of interest may be assessed *in*
vivo, e.g., in a animal model such as that disclosed in Clynes et al. *PNAS*
(USA) 95:652-656 (1998).

"Human effector cells" are leukocytes which express one or more FcRs
40 and perform effector functions. Preferably, the cells express at least
FcγRIII and carry out ADCC effector function. Examples of human leukocytes
which mediate ADCC include peripheral blood mononuclear cells (PBMC),
natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils;
with PBMCs and NK cells being preferred.

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The terms "Fc receptor" or "FcR" are used to describe a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-92 (1991); Capel et al., *Immunomethods* 4:25-34 (1994); and de Haas et al., *J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., *J. Immunol.* 117:587 (1976) and Kim et al., *J. Immunol.* 24:249 (1994)). FcRs herein include polymorphisms such as the genetic dimorphism in the gene that encodes FcγRIIIa resulting in either a phenylalanine (F) or a valine (V) at amino acid position 158, located in the region of the receptor that binds to IgG1. The homozygous valine FcγRIIIa (FcγRIIIa-158V) has been shown to have a higher affinity for human IgG1 and mediate increased ADCC in vitro relative to homozygous phenylalanine FcγRIIIa (FcγRIIIa-158F) or heterozygous (FcγRIIIa-158F/V) receptors.

"Complement dependent cytotoxicity" or "CDC" refer to the ability of a molecule to lyse a target in the presence of complement. The complement activation pathway is initiated by the binding of the first component of the complement system (C1q) to a molecule (e.g. an antibody) complexed with a cognate antigen. To assess complement activation, a CDC assay, e.g. as described in Gazzano-Santoro et al., *J. Immunol. Methods* 202:163 (1996), may be performed.

The term "antibody" herein is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of an intact antibody, preferably comprising the antigen-binding or variable region thereof. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

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"Native antibodies" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a β -sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β -sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cell-mediated cytotoxicity (ADCC).

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')$ fragment that has two antigen-binding sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and antigen-binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the V_H - V_L dimer. Collectively, the

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six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear at least one free thiol group. F(ab')₂ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, antibodies can be assigned to different classes. There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of antibodies are called α , δ , ϵ , γ , and μ , respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

"Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H - V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993).

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e.,

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the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature*, 352:624-628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)). Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (e.g. Old World Monkey, such as baboon, rhesus or cynomolgus monkey) and human constant region sequences (US Pat No. 5,693,780).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found

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in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (e.g. residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a "hypervariable loop" (e.g. residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

An antibody "which binds" an antigen of interest, e.g. CD20 or DR4 or DR5, is one capable of binding that antigen with sufficient affinity and/or avidity such that the antibody is useful as a therapeutic agent for targeting a cell expressing the antigen.

For the purposes herein, "immunotherapy" will refer to a method of treating a mammal (preferably a human patient) with an antibody, wherein the antibody may be an unconjugated or "naked" antibody, or the antibody may be conjugated or fused with heterologous molecule(s) or agent(s), such as one or more cytotoxic agent(s), thereby generating an "immunoconjugate".

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antagonist or antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE

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under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody *in situ* within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The expression "effective amount" refers to an amount of the Apo2L/TRAIL or death receptor antibody and CD20 antibody which is effective for preventing, ameliorating or treating the disease or condition in question.

The term "immunosuppressive agent" as used herein for adjunct therapy refers to substances that act to suppress or mask the immune system of the mammal being treated herein. This would include substances that suppress cytokine production, downregulate or suppress self-antigen expression, or mask the MHC antigens. Examples of such agents include 2-amino-6-aryl-5-substituted pyrimidines (see U.S. Pat. No. 4,665,077, the disclosure of which is incorporated herein by reference); nonsteroidal antiinflammatory drugs (NSAIDs); azathioprine; cyclophosphamide; bromocryptine; danazol; dapsone; glutaraldehyde (which masks the MHC antigens, as described in U.S. Pat. No. 4,120,649); anti-idiotypic antibodies for MHC antigens and MHC fragments; cyclosporin A; steroids such as glucocorticosteroids, e.g., prednisone, methylprednisolone, dexamethasone, and hydrocortisone; methotrexate (oral or subcutaneous); hydroxycloquine; sulfasalazine; leflunomide; cytokine or cytokine receptor antagonists including anti-interferon- γ , - β , or - α antibodies, anti-tumor necrosis factor- α antibodies (infliximab or adalimumab); anti-TNF α immunoahesin (etanercept); anti-tumor necrosis factor- β antibodies, anti-interleukin-2 antibodies and anti-IL-2 receptor antibodies; anti-LFA-1 antibodies, including anti-CD11a and anti-CD18 antibodies; anti-L3T4 antibodies; heterologous anti-lymphocyte globulin; pan-T antibodies, preferably anti-CD3 or anti-CD4/CD4a antibodies; soluble peptide containing a LFA-3 binding domain (WO'90/08187 published 7/26/90); streptokinase; TGF- β ; streptodornase; RNA or DNA from the host; FK506; RS-61443; deoxyspergualin; rapamycin; T-cell receptor (Cohen et al., U.S. Pat. No. 5,114,721); T-cell receptor fragments (Offner et al., *Science*, 251: 430-432 (1991); WO 90/11294; Ianeway, *Nature*, 341: 482 (1989); and WO 91/01133); and T cell receptor antibodies (EP'340,109) such as T10B9.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. At²¹¹, I¹³¹, I¹²⁵, Y⁹⁰, Re¹⁸⁶, Re¹⁸⁸, Sm¹⁵³, Bi²¹³, P³² and radioactive isotopes of Lu), chemotherapeutic agents, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

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"Synergistic activity" or "synergy" or "synergistic effect" or "synergistic effective amount" for the purposes herein means that the effect observed when employing a combination of Apo2L/TRAIL or death receptor antibody and CD20 antibody is (1) greater than the effect achieved when that Apo2L/TRAIL, death receptor antibody or CD20 antibody is employed alone (or individually) and (2) greater than the sum added (additive) effect for that Apo2L/TRAIL or death receptor antibody and CD20 antibody. Such synergy or synergistic effect can be determined by way of a variety of means known to those in the art. For example, the synergistic effect of Apo2L/TRAIL or death receptor antibody and CD20 antibody can be observed in in vitro or in vivo assay formats examining reduction of tumor cell number or tumor mass.

The terms "apoptosis" and "apoptotic activity" are used in a broad sense and refer to the orderly or controlled form of cell death in mammals that is typically accompanied by one or more characteristic cell changes, including condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. This activity can be determined and measured using well known art methods, for instance, by cell viability assays, FACS analysis or DNA electrophoresis, binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). Assays which determine the ability of an antibody (e.g. Rituximab) to induce apoptosis have been described in Shan et al. *Cancer Immunol. Immunother* 48:673-83 (2000); Pedersen et al. *Blood* 99:1314-9 (2002); Demidem et al. *Cancer Chemotherapy & Radiopharmaceuticals* 12(3):177-186 (1997), for example.

The terms "cancer", "cancerous", and "malignant" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma including adenocarcinoma, lymphoma, blastoma, melanoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, Hodgkin's and non-Hodgkin's lymphoma, pancreatic cancer, glioblastoma, glioma, cervical cancer, ovarian cancer, liver cancer such as hepatic carcinoma and hepatoma, bladder cancer, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, myeloma (such as multiple myeloma), salivary gland carcinoma, kidney cancer such as renal cell carcinoma and Wilms' tumors, basal cell carcinoma, melanoma, prostate cancer, vulval cancer, thyroid cancer, testicular cancer, esophageal cancer, and various types of head and neck cancer.

The term "immune related disease" means a disease in which a component of the immune system of a mammal causes, mediates or otherwise contributes to a morbidity in the mammal. Also included are diseases in

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which stimulation or intervention of the immune response has an ameliorative effect on progression of the disease. Included within this term are autoimmune diseases, immune-mediated inflammatory diseases, non-immune-mediated inflammatory diseases, infectious diseases, and immunodeficiency diseases. Examples of immune-related and inflammatory diseases, some of which are immune or T cell mediated, which can be treated according to the invention include systemic lupus erythematosus, rheumatoid arthritis, juvenile chronic arthritis, spondyloarthropathies, systemic sclerosis (scleroderma), idiopathic inflammatory myopathies (dermatomyositis, polymyositis), Sjogren's syndrome, systemic vasculitis, sarcoidosis, autoimmune hemolytic anemia (immune pancytopenia, paroxysmal nocturnal hemoglobinuria), autoimmune thrombocytopenia (idiopathic thrombocytopenic purpura, immune-mediated thrombocytopenia), thyroiditis (Grave's disease, Hashimoto's thyroiditis, juvenile lymphocytic thyroiditis, atrophic thyroiditis), diabetes mellitus, immune-mediated renal disease (glomerulonephritis, tubulointerstitial nephritis), demyelinating diseases of the central and peripheral nervous systems such as multiple sclerosis, idiopathic demyelinating polyneuropathy or Guillain-Barré syndrome, and chronic inflammatory demyelinating polyneuropathy, hepatobiliary diseases such as infectious hepatitis (hepatitis A, B, C, D, E and other non-hepatotropic viruses), autoimmune chronic active hepatitis, primary biliary cirrhosis, granulomatous hepatitis, and sclerosing cholangitis, inflammatory and fibrotic lung diseases such as inflammatory bowel disease (ulcerative colitis: Crohn's disease), gluten-sensitive enteropathy, and Whipple's disease, autoimmune or immune-mediated skin diseases including bullous skin diseases, erythema multiforme and contact dermatitis, psoriasis, allergic diseases such as asthma, allergic rhinitis, atopic dermatitis, food hypersensitivity and urticaria, immunologic diseases of the lung such as eosinophilic pneumonias, idiopathic pulmonary fibrosis and hypersensitivity pneumonitis, transplantation associated diseases including graft rejection and graft-versus-host-disease. Infectious diseases include AIDS (HIV infection), hepatitis A, B, C, D, and E, bacterial infections, fungal infections, protozoal infections and parasitic infections.

A "B cell malignancy" is a malignancy involving B cells. Examples include Hodgkin's disease, including lymphocyte predominant Hodgkin's disease (LPHD); non-Hodgkin's lymphoma (NHL); follicular center cell (FCC) lymphoma; acute lymphocytic leukemia (ALL); chronic lymphocytic leukemia (CLL); hairy cell leukemia; plasmacytoid lymphocytic lymphoma; mantle cell lymphoma; AIDS or HIV-related lymphoma; multiple myeloma; central nervous system (CNS) lymphoma; post-transplant lymphoproliferative disorder (PTLD); Waldenstrom's macroglobulinemia (lymphoplasmacytic lymphoma); mucosa-associated lymphoid tissue (MALT) lymphoma; and marginal zone lymphoma/leukemia.

Non-Hodgkin's lymphoma (NHL) includes, but is not limited to, low grade/follicular NHL, relapsed or refractory NHL, front line low grade NHL, Stage III/IV NHL, chemotherapy resistant NHL, small lymphocytic (SL) NHL, intermediate grade/follicular NHL, intermediate grade diffuse NHL, diffuse large cell lymphoma, aggressive NHL (including aggressive front-line NHL and aggressive relapsed NHL), NHL relapsing after or refractory to autologous stem cell transplantation, high grade immunoblastic NHL, high grade lymphoblastic NHL, high grade small non-cleaved cell NHL, bulky disease NHL, etc.

An "autoimmune disease" herein is a disease or disorder arising from and directed against an individual's own tissues or a co-segregate or manifestation thereof or resulting condition therefrom. Examples of autoimmune diseases or disorders include, but are not limited to arthritis (rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis, and ankylosing spondylitis), psoriasis, dermatitis including atopic dermatitis; chronic idiopathic urticaria, including chronic autoimmune urticaria, polymyositis/dermatomyositis, toxic epidermal necrolysis, systemic scleroderma and sclerosis, responses associated with inflammatory bowel disease (IBD) (Crohn's disease, ulcerative colitis), and IBD with co-segregate of pyoderma gangrenosum, erythema nodosum, primary sclerosing cholangitis, and/or episcleritis), respiratory distress syndrome, including adult respiratory distress syndrome (ARDS), meningitis, IgE-mediated diseases such as anaphylaxis and allergic rhinitis, encephalitis such as Rasmussen's encephalitis, uveitis, colitis such as microscopic colitis and collagenous colitis, glomerulonephritis (GN) such as membranous GN, idiopathic membranous GN, membranous proliferative GN (MPGN), including Type I and Type II, and rapidly progressive GN, allergic conditions, eczema, asthma, conditions involving infiltration of T cells and chronic inflammatory responses, atherosclerosis, autoimmune myocarditis, leukocyte adhesion deficiency, systemic lupus erythematosus (SLE) such as cutaneous SLE, lupus (including nephritis, cerebritis, pediatric, non-renal, discoid, alopecia), juvenile onset diabetes, multiple sclerosis (MS) such as spino-optical MS, allergic encephalomyelitis, immune responses associated with acute and delayed hypersensitivity mediated by cytokines and T-lymphocytes, tuberculosis, sarcoidosis, granulomatosis including Wegener's granulomatosis, agranulocytosis, vasculitis (including Large Vessel vasculitis (including Polymyalgia Rheumatica and Giant Cell (Takayasu's) Arteritis), Medium Vessel vasculitis (including Kawasaki's Disease and Polyarteritis Nodosa), CNS vasculitis, and ANCA-associated vasculitis, such as Churg-Strauss vasculitis or syndrome (CSS)), aplastic anemia, Coombs positive anemia, Diamond Blackfan anemia, immune hemolytic anemia including autoimmune hemolytic anemia (AIHA), pernicious anemia, pure red cell aplasia (PRCA), Factor VIII deficiency, hemophilia A, autoimmune neutropenia, pancytopenia, leukopenia, diseases involving

leukocyte diapedesis, CNS inflammatory disorders, multiple organ injury syndrome, myasthenia gravis, antigen-antibody complex mediated diseases, anti-glomerular basement membrane disease, anti-phospholipid antibody syndrome, allergic neuritis, Bechet disease, Castleman's syndrome, 5 Goodpasture's Syndrome, Lambert-Eaton Myasthenic Syndrome, Reynaud's syndrome, Sjorgen's syndrome, Stevens-Johnson syndrome, solid organ transplant rejection (including pretreatment for high panel reactive antibody titers, IgA deposit in tissues, and rejection arising from renal transplantation, liver transplantation, intestinal transplantation, cardiac 10 transplantation, etc.), graft versus host disease (GVHD), pemphigoid bullous, pemphigus (including vulgaris, foliaceus, and pemphigus mucus-membrane pemphigoid), autoimmune polyendocrinopathies, Reiter's disease, stiff-man syndrome, immune complex nephritis, IgM polyneuropathies or IgM mediated neuropathy, idiopathic thrombocytopenic purpura (ITP), thrombotic 15 thrombocytopenic purpura (TTP), thrombocytopenia (as developed by myocardial infarction patients, for example), including autoimmune thrombocytopenia, autoimmune disease of the testis and ovary including autoimmune orchitis and oophoritis, primary hypothyroidism; autoimmune endocrine diseases including autoimmune thyroiditis, chronic thyroiditis (Hashimoto's Thyroiditis), 20 subacute thyroiditis, idiopathic hypothyroidism, Addison's disease, Grave's disease, autoimmune polyglandular syndromes (or polyglandular endocrinopathy syndromes), Type I diabetes also referred to as insulin-dependent diabetes mellitus (IDDM), including pediatric IDDM, and Sheehan's syndrome; autoimmune hepatitis, Lymphoid interstitial pneumonitis (HIV), 25 bronchiolitis obliterans (non-transplant) vs NSIP, Guillain-Barré Syndrome, Berger's Disease (IgA nephropathy), primary biliary cirrhosis, celiac sprue (gluten enteropathy), refractory sprue with co-segregate dermatitis herpetiformis, cryoglobulinemia, amyotrophic lateral sclerosis (ALS; Lou Gehrig's disease), coronary artery disease, autoimmune inner ear disease 30 (AIED), autoimmune hearing loss, opsoclonus myoclonus syndrome (OMS), polychondritis such as refractory polychondritis, pulmonary alveolar proteinosis, amyloidosis, giant cell hepatitis, scleritis, monoclonal gammopathy of uncertain/unknown significance (MGUS), peripheral neuropathy, paraneoplastic syndrome, channelopathies such as epilepsy, migraine, 35 arrhythmia, muscular disorders, deafness, blindness, periodic paralysis, and channelopathies of the CNS; autism, inflammatory myopathy, and focal segmental glomerulosclerosis (FSGS).

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less 40 cytotoxic to cancer cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery,"

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Directed Drug Delivery, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, beta-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described below.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. At²¹¹, I¹³¹, I¹²⁵, Y⁹⁰, Re¹⁸⁶, Re¹⁸⁸, Sm¹⁵³, Bi²¹³, P³² and radioactive isotopes of Lu), chemotherapeutic agents, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and CYTOXAN® cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the enediyne antibiotics (e. g., calicheamicin, especially calicheamicin gammaII and calicheamicin omegaII (see, e.g., Agnew, Chem Intl. Ed. Engl., 33: 183-186 (1994)); dynemicin, including dynemicin-A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabycin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin,

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- deterubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid,
- 5 nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine,
- 10 thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiothane, testolactone; anti-adrenals such as aminogluthethimide, mitotane, trilostane; folic acid
- 15 replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; ansacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins;
- 20 mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-
- 25 2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g., TAXOL® paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE™ Cremophor-free, albumin-engineered nanoparticle formulation of
- 30 paclitaxel (American Pharmaceutical Partners, Schaumburg, Illinois), and TAXOTERE® doxetaxel (Rhône-Poulenc Rorer, Antony, France); chloranbucil; GEMZAR® gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; NAVELBINE® vinorelbine;
- 35 novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluorometilhyloornithine (DMFO); retinoids such as retinoic acid; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above.
- 40 Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEX® tamoxifen), raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and

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FARESTON[®] toremifene; aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, MEGASE[®] megestrol acetate, AROMASIN[®] exemestane, formestane, fadrozole, RIVISOR[®] vorozole, 5 FEMARA[®] letrozole, and ARIMIDEX[®] anastrozole; and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; as well as troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those which inhibit expression of genes in signaling pathways implicated in aberrant cell proliferation, such as, for 10 example, PKC-alpha, Raf and H-Ras; ribozymes such as a VEGF expression inhibitor (e.g., ANGIOZYME[®] ribozyme) and a HER2 expression inhibitor; vaccines such as gene therapy vaccines, for example, ALLOVECTIN[®] vaccine, LEUVECTIN[®] vaccine, and VAXID[®] vaccine; PROLEUKIN[®] rIL-2; LURTOTECAN[®] topoisomerase 1 inhibitor; ABARELIX[®] rmrh; and pharmaceutically acceptable 15 salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, either *in vitro* or *in vivo*. Thus, the growth inhibitory agent is one which significantly reduces the percentage of cells overexpressing such genes in S phase. Examples of 20 growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), taxol, and topo. II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that 25 arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogens, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 30 13.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional 35 polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone 40 (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor- α and - β ; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors; platelet-growth factor; transforming growth factors (TGFs) such as

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TGF- α and TGF- β ; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon- α , - β , and -gamma; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

A "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications, other therapeutic products to be combined with the packaged product, and/or warnings concerning the use of such therapeutic products, etc.

The terms "treating", "treatment" and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy.

The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.

II. Compositions and Methods of the Invention

A cytokine related to the TNF ligand family, the cytokine identified herein as "Apo-2 ligand" or "TRAIL" has been described. The predicted mature amino acid sequence of native human Apo-2 ligand contains 281 amino acids, and has a calculated molecular weight of approximately 32.5 kDa. The absence of a signal sequence and the presence of an internal hydrophobic region suggest that Apo-2 ligand is a type II transmembrane protein. Soluble extracellular domain Apo-2 ligand polypeptides have also been described. See, e.g., WO97/25428 published July 17, 1997. Apo-2L substitutional variants have further been described. Alanine scanning techniques have been utilized to identify various substitutional variant molecules having biological activity. Particular substitutional variants of the Apo-2 ligand include those in which at least one amino acid is substituted by another amino acids such as an alanine residue. These substitutional variants are identified, for example, as "D203A"; "D218A" and "D269A." This nomenclature is used to identify Apo-2 ligand variants wherein the aspartic acid residues at positions 203, 218, and/or 269 (using the numbering shown in Figure 1) are substituted by alanine residues. Optionally, the Apo-2L variants of the present invention may comprise one or more of the amino acid substitutions. Optionally, such Apo-2L variants will be DR4 or DR5 receptor selective variants.

The description below relates to methods of producing Apo-2 ligand, including Apo-2 ligand variants, by culturing host cells transformed or

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transfected with a vector containing Apo-2 ligand encoding nucleic acid and recovering the polypeptide from the cell culture.

The DNA encoding Apo-2 ligand may be obtained from any cDNA library prepared from tissue believed to possess the Apo-2 ligand mRNA and to express it at a detectable level. Accordingly, human Apo-2 ligand DNA can be conveniently obtained from a cDNA library prepared from human tissues, such as the bacteriophage library of human placental cDNA as described in WO97/25428. The Apo-2 ligand-encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

Libraries can be screened with probes (such as antibodies to the Apo-2 ligand or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding Apo-2 ligand is to use PCR methodology [Sambrook et al., supra; Dieffenbach et al., PCR Primer: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)].

Amino acid sequence fragments or variants of Apo-2 ligand can be prepared by introducing appropriate nucleotide changes into the Apo-2 ligand DNA, or by synthesis of the desired Apo-2 ligand polypeptide. Such fragments or variants represent insertions, substitutions, and/or deletions of residues within or at one or both of the ends of the intracellular region, the transmembrane region, or the extracellular region, or of the amino acid sequence shown for the full-length Apo-2 ligand in Figure 1. Any combination of insertion, substitution, and/or deletion can be made to arrive at the final construct, provided that the final construct possesses, for instance, a desired biological activity, such as apoptotic activity, as defined herein. In a preferred embodiment, the fragments or variants have at least about 80% amino acid sequence identity, more preferably, at least about 90% sequence identity, and even more preferably, at least 95%, 96%, 97%, 98% or 99% sequence identity with the sequences identified herein for the intracellular, transmembrane, or extracellular domains of Apo-2 ligand, or the full-length sequence for Apo-2 ligand. The amino acid changes also may alter post-translational processes of the Apo-2 ligand, such as changing the number or position of glycosylation sites or altering the membrane anchoring characteristics.

Variations in the Apo-2 ligand sequence as described above can be made using any of the techniques and guidelines for conservative and non-conservative mutations set forth in U.S. Pat. No. 5,364,934. These include oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis.

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Scanning amino acid analysis can be employed to identify one or more amino acids along a contiguous sequence. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine and cysteine. Alanine is typically a preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant. [Cunningham et al., Science, 244:1081 (1989)]. Alanine is also typically preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., NY); Chothia, J. Mol. Biol., 150:1 (1976)].

Amino acids may be grouped according to similarities in the properties of their side chains (in A. L. Lehninger, in Biochemistry, second ed., pp. 73-75, Worth Publishers, New York (1975)):

- (1) non-polar: Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M)
- (2) uncharged polar: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gln (Q)
- (3) acidic: Asp (D), Glu (E)
- (4) basic: Lys (K), Arg (R), His (H)

Alternatively, naturally occurring residues may be divided into groups based on common side-chain properties:

- (1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;
- (2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;
- (3) acidic: Asp, Glu;
- (4) basic: His, Lys, Arg;
- (5) residues that influence chain orientation: Gly, Pro;
- (6) aromatic: Trp, Tyr, Phe.

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Table 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

5 Variations in the Apo-2 ligand sequence also included within the scope of the invention relate to amino-terminal derivatives or modified forms. Such Apo-2 ligand sequences include any of the Apo-2 ligand polypeptides described herein having a methionine or modified methionine (such as formyl methionyl or other blocked methionyl species) at the N-terminus of the polypeptide sequence.

10 The nucleic acid (e.g., cDNA or genomic DNA) encoding native or variant Apo-2 ligand may be inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is described below.

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Optional signal sequences, origins of replication, marker genes, enhancer elements and transcription terminator sequences that may be employed are known in the art and described in further detail in WO97/25428.

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the Apo-2 ligand nucleic acid sequence. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, such as the Apo-2 ligand nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to Apo-2 ligand encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native Apo-2 ligand promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the Apo-2 ligand DNA.

Promoters suitable for use with prokaryotic and eukaryotic hosts are known in the art, and are described in further detail in WO97/25428.

A preferred method for the production of soluble Apo-2L in *E. coli* employs an inducible promoter for the regulation of product expression. The use of a controllable, inducible promoter allows for culture growth to the desirable cell density before induction of product expression and accumulation of significant amounts of product which may not be well tolerated by the host.

Several inducible promoter systems (T7 polymerase, trp and alkaline phosphatase (AP)) have been evaluated by Applicants for the expression of Apo-2L (form 114-281). The use of each of these three promoters resulted in significant amounts of soluble, biologically active Apo-2L trimer being recovered from the harvested cell paste. The AP promoter is preferred among these three inducible promoter systems tested because of tighter promoter control and the higher cell density and titers reached in harvested cell paste.

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline

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resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced using standard techniques known in the art. [See, e.g., Messing et al., Nucleic Acids Res., 9:309 (1981); Maxam et al., Methods in Enzymology, 65:499 (1980)].

Expression vectors that provide for the transient expression in mammalian cells of DNA encoding Apo-2 ligand may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector [Sambrook et al., supra]. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of Apo-2 ligand that are biologically active Apo-2 ligand.

Other methods, vectors, and host cells suitable for adaptation to the synthesis of Apo-2 ligand in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantel et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

Suitable host cells for cloning or expressing the DNA in the vectors herein include prokaryote, yeast, or higher eukaryote cells. Suitable prokaryotes for this purpose include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, *Enterobacteriaceae* such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for Apo-2 ligand-encoding vectors. Suitable host cells for the expression of glycosylated Apo-2 ligand are derived from multicellular organisms. Examples of all such host cells, including CHO cells, are described further in WO97/25428.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors for Apo-2 ligand production and cultured in nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

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Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, CaPO₄ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52:456-457 (1978) may be employed. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352 (1988).

Prokaryotic cells used to produce Apo-2 ligand may be cultured in suitable culture media as described generally in Sambrook et al., supra. Particular forms of culture media that may be employed for culturing *E. coli* are described further in the Examples below. Mammalian host cells used to produce Apo-2 ligand may be cultured in a variety of culture media.

Examples of commercially available culture media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as GentamycinTM drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an

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equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: A Practical Approach, M. Butler, ed. (IRL Press, 1991).

In accordance with one aspect of the present invention, one or more divalent metal ions will typically be added to or included in the culture media for culturing or fermenting the host cells. The divalent metal ions are preferably present in or added to the culture media at a concentration level sufficient to enhance storage stability, enhance solubility, or assist in forming stable Apo-2L trimers coordinated by one or more zinc ions. The amount of divalent metal ions which may be added will be dependent, in part, on the host cell density in the culture or potential host cell sensitivity to such divalent metal ions. At higher host cell densities in the culture, it may be beneficial to increase the concentration of divalent metal ions. If the divalent metal ions are added during or after product expression by the host cells, it may be desirable to adjust or increase the divalent metal ion concentration as product expression by the host cells increases. It is generally believed that trace levels of divalent metal ions which may be present in typical commonly available cell culture media may not be sufficient for stable trimer formation. Thus, addition of further quantities of divalent metal ions, as described herein, is preferred.

The divalent metal ions are preferably added to the culture media at a concentration which does not adversely or negatively affect host cell growth, if the divalent metal ions are being added during the growth phase of the host cells in the culture. In shake flask cultures, it was observed that ZnSO₄ added at concentrations of greater than 1 mM can result in lower host cell density. Those skilled in the art appreciate that bacterial cells can sequester metal ions effectively by forming metal ion complexes with cellular matrices. Thus, in the cell cultures, it is preferable to add the selected divalent metal ions to the culture media after the growth phase (after the desired host cell density is achieved) or just prior to product expression by the host cells. To ensure that sufficient amounts of divalent metal ions are present, additional divalent metal ions may be added or fed to the cell culture media during the product expression phase.

The divalent metal ion concentration in the culture media should not exceed the concentration which may be detrimental or toxic to the host cells. In the methods of the invention employing the host cell, *E. coli*, it is preferred that the concentration of the divalent metal ion

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concentration in the culture media does not exceed about 1mM (preferably, $\leq$ 1mM). Even more preferably, the divalent metal ion concentration in the culture media is about 50 micromolar to about 250 micromolar. Most preferably, the divalent metal ion used in such methods is zinc sulfate.

5 It is desirable to add the divalent metal ions to the cell culture in an amount wherein the metal ions and Apo-2 ligand trimer can be present at a one to one molar ratio.

The divalent metal ions can be added to the cell culture in any acceptable form. For instance, a solution of the metal ion can be made

10 using water, and the divalent metal ion solution can then be added or fed to the culture media.

Expression of the Apo-2L may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205

15 (1980)], dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, and particularly ^{32}P . However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide.

20 The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorescers or enzymes. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The

25 antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected. Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay

30 of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels,

35 fluorescent labels, luminescent labels, and the like.

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native Apo-2 ligand polypeptide or against a synthetic peptide based on the

40 DNA sequences provided herein or against exogenous sequence fused to Apo-2 ligand DNA and encoding a specific antibody epitope.

Apo-2 ligand preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates when directly produced without a secretory signal. If the Apo-2

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ligand is membrane-bound, it can be released from the membrane; using a suitable detergent solution (e.g. Triton-X 100) or its extracellular region may be released by enzymatic cleavage.

When Apo-2 ligand is produced in a recombinant cell other than one of human origin, the Apo-2 ligand is free of proteins or polypeptides of human origin. However, it is usually necessary to recover or purify Apo-2 ligand from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to Apo-2 ligand. As a first step, the culture medium or lysate may be centrifuged to remove particulate cell debris. Apo-2 ligand thereafter is purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE or CM; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; diafiltration and protein A Sepharose columns to remove contaminants such as IgG.

In a preferred embodiment, the Apo-2 ligand can be isolated by affinity chromatography. Apo-2 ligand fragments or variants in which residues have been deleted, inserted, or substituted are recovered in the same fashion as native Apo-2 ligand, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of an Apo-2 ligand fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing antibody to the antigen can be used to adsorb the fusion polypeptide.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native Apo-2 ligand may require modification to account for changes in the character of Apo-2 ligand or its variants upon expression in recombinant cell culture.

During any such purification steps, it may be desirable to expose the recovered Apo-2L to a divalent metal ion-containing solution or to purification material (such as a chromatography medium or support) containing one or more divalent metal ions. In a preferred embodiment, the divalent metal ions and/or reducing agent is used during recovery or purification of the Apo-2L. Optionally, both divalent metal ions and reducing agent, such as DTT or BME, may be used during recovery or purification of the Apo-2L. It is believed that use of divalent metal ions during recovery or purification will provide for stability of Apo-2L trimer or preserve Apo-2L trimer formed during the cell culturing step.

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The description below also relates to methods of producing Apo-2 ligand covalently attached (hereinafter "conjugated") to one or more chemical groups. Chemical groups suitable for use in an Apo-2L conjugate of the present invention are preferably not significantly toxic or immunogenic. The chemical group is optionally selected to produce an Apo-2L conjugate that can be stored and used under conditions suitable for storage. A variety of exemplary chemical groups that can be conjugated to polypeptides are known in the art and include for example carbohydrates, such as those carbohydrates that occur naturally on glycoproteins, polyglutamate, and non-proteinaceous polymers, such as polyols (see, e.g., U.S. Patent No. 6,245,901).

A polyol, for example, can be conjugated to polypeptides such as an Apo-2L at one or more amino acid residues, including lysine residues, as is disclosed in WO 93/00109, supra. The polyol employed can be any water-soluble poly(alkylene oxide) polymer and can have a linear or branched chain. Suitable polyols include those substituted at one or more hydroxyl positions with a chemical group, such as an alkyl group having between one and four carbons. Typically, the polyol is a poly(alkylene glycol), such as poly(ethylene glycol) (PEG), and thus, for ease of description, the remainder of the discussion relates to an exemplary embodiment wherein the polyol employed is PEG and the process of conjugating the polyol to a polypeptide is termed "pegylation." However, those skilled in the art recognize that other polyols, such as, for example, poly(propylene glycol) and polyethylene-polypropylene glycol copolymers, can be employed using the techniques for conjugation described herein for PEG.

The average molecular weight of the PEG employed in the pegylation of the Apo-2L can vary, and typically may range from about 500 to about 30,000 daltons (D). Preferably, the average molecular weight of the PEG is from about 1,000 to about 25,000 D, and more preferably from about 1,000 to about 5,000 D. In one embodiment, pegylation is carried out with PEG having an average molecular weight of about 1,000 D. Optionally, the PEG homopolymer is unsubstituted, but it may also be substituted at one end with an alkyl group. Preferably, the alkyl group is a C1-C4 alkyl group, and most preferably a methyl group. PEG preparations are commercially available, and typically, those PEG preparations suitable for use in the present invention are nonhomogeneous preparations sold according to average molecular weight. For example, commercially available PEG(5000) preparations typically contain molecules that vary slightly in molecular weight, usually ± 500 D.

The Apo-2 ligand of the invention may be in various forms, such as in monomer form or trimer form (comprising three monomers). Optionally, an Apo-2L trimer will be pegylated in a manner such that a PEG molecule is linked or conjugated to one, two or each of the three monomers that make up the trimeric Apo-2L. In such an embodiment, it is preferred that the PEG

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employed have an average molecular weight of about 1,000 to about 5,000 D. It is also contemplated that the Apo-2L trimers may be "partially" pegylated, i.e., wherein only one or two of the three monomers that make up the trimer are linked or conjugated to PEG.

5 A variety of methods for pegylating proteins are known in the art. Specific methods of producing proteins conjugated to PEG include the methods described in U.S. Pat. No. 4,179,337, U.S. Pat. No. 4,935,465 and U.S. Patent No. 5,849,535. Typically the protein is covalently bonded via one or more of the amino acid residues of the protein to a terminal
10 reactive group on the polymer, depending mainly on the reaction conditions, the molecular weight of the polymer, etc. The polymer with the reactive group(s) is designated herein as activated polymer. The reactive group selectively reacts with free amino or other reactive groups on the protein. The PEG polymer can be coupled to the amino or other reactive group on the
15 protein in either a random or a site specific manner. It will be understood, however, that the type and amount of the reactive group chosen, as well as the type of polymer employed, to obtain optimum results, will depend on the particular protein or protein variant employed to avoid having the reactive group react with too many particularly active groups on
20 the protein. As this may not be possible to avoid completely, it is recommended that generally from about 0.1 to 1000 moles, preferably 2 to 200 moles, of activated polymer per mole of protein, depending on protein concentration, is employed. The final amount of activated polymer per mole of protein is a balance to maintain optimum activity, while at the same
25 time optimizing, if possible, the circulatory half-life of the protein.

It is further contemplated that the Apo2L described herein may be also be linked or fused to leucine zipper sequences using techniques known in the art.

30 Methods for generating death receptor antibodies and CD20 antibodies are also described herein. The antigen to be used for production of, or screening for, antibody may be, e.g., a soluble form of the antigen or a portion thereof, containing the desired epitope. Alternatively, or
35 additionally, cells expressing the antigen at their cell surface can be used to generate, or screen for, antibody. Other forms of the antigen useful for generating antibody will be apparent to those skilled in the art.

(i) Polyclonal antibodies

40 Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or

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soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl_2 , or $\text{R}^1\text{N}=\text{C}=\text{NR}$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

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Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those
5 derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Manassas, Virginia USA. Human myeloma and mouse-human heteromyeloma cell
10 lines also have been described for the production of human monoclonal antibodies (Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen.
15 Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be
20 determined by the Scatchard analysis of Munson et al., *Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding,
25 *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably
30 separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and
35 sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E.*
40 *coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody

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include Skerra et al., *Curr. Opinion in Immunol.*, 5:256-262 (1993) and Plückerthun, *Immunol. Revs.*, 130:151-188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature*, 348:552-554 (1990). Clackson et al., *Nature*, 352:624-628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.*, 21:2265-2266 (1993). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; Morrison, et al., *Proc. Natl Acad. Sci. USA*, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized antibodies

Methods for humanizing non-human antibodies have been described in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-327 (1988); Verhoeven et al., *Science*, 239:1534-1536 (1988)), by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

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The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993); Chothia et al., *J. Mol. Biol.*, 196:901 (1987)). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta et al., *J. Immunol.*, 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

(iv) Human antibodies

As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits et al., *Nature*, 362:255-

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258 (1993); Bruggermann et al., Year in Immuno., 7:33 (1993); and US Patent Nos. 5,591,669, 5,589,369 and 5,545,807.

Alternatively, phage display technology (McCafferty et al., Nature 348:552-553 (1990)) can be used to produce human antibodies and antibody fragments in vitro, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B cell. Phage display can be performed in a variety of formats; for their review see, e.g., Johnson, Kevin S. and Chiswell, David J., Current Opinion in Structural Biology 3:564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson et al., Nature, 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks et al., J. Mol. Biol. 222:581-597 (1991), or Griffith et al., EMBO J. 12:725-734 (1993). See, also, US Patent Nos. 5,565,332 and 5,573,905.

Human antibodies may also be generated by in vitro activated B cells (see US Patents 5,567,610 and 5,229,275).

(v) Antibody fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., Journal of Biochemical and Biophysical Methods 24:107-117 (1992) and Brennan et al., Science, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from E. coli and chemically coupled to form F(ab')₂ fragments (Carter et al., Bio/Technology 10:163-167 (1992)). According to another approach, F(ab')₂ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185; US Patent No. 5,571,894; and US Patent No. 5,587,458. The antibody fragment may also be a "linear

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antibody", e.g., as described in US Patent 5,641,870 for example. Such linear antibody fragments may be monospecific or bispecific.

(vi) Bispecific antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the CD20, DR4 or DR5 receptors. Bispecific antibodies may also be used to localize cytotoxic agents to a B cell. These antibodies possess a B cell marker-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon- α , vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. F(ab'), bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., Nature, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was

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found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation.

5 This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986). According to another approach described in US Patent No. 5,731,168, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

20 Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent, No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 25 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

30 Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., Science, 229: 81 (1985); Shalaby et al., J. Exp. Med., 175: 217-225 (1992).

35 Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., J. Immunol., 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993) has provided an alternative mechanism for making

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bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., J. Immunol., 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. J. Immunol. 147: 60 (1991). Antibodies with three or more antigen binding sites are described in WO01/77342 (Miller and Presta), expressly incorporated herein by reference.

The antibody used in the methods or included in the articles of manufacture herein is optionally conjugated to a cytotoxic agent.

Chemotherapeutic agents useful in the generation of such antibody-cytotoxic agent conjugates have been described above.

Conjugates of an antibody and one or more small molecule toxins, such as a calicheamicin, a maytansine (US Patent No. 5,208,020), a trichothene, and CC1065 are also contemplated herein. In one embodiment of the invention, the antibody is conjugated to one or more maytansine molecules (e.g. about 1 to about 10 maytansine molecules per antibody molecule). Maytansine may, for example, be converted to May-SS-Me which may be reduced to May-SH3 and reacted with modified antibody (Chari et al. Cancer Research 52: 127-131 (1992)) to generate a maytansinoid-antibody conjugate.

Alternatively, the antibody is conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics is capable of producing double-stranded DNA breaks at sub-picomolar concentrations. Structural analogues of calicheamicin which may be used include, but are not limited to, γ_1^I , α_1^I , α_3^I , N-acetyl- γ_1^I , PSAG and θ_1^I (Hinman et al. Cancer Research 53: 3336-3342 (1993) and Lode et al. Cancer Research 58: 2925-2928 (1998)).

Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. See, for example, WO 93/21232 published October 28, 1993.

The present invention further contemplates antibody conjugated with a compound with nucleolytic activity (e.g. a ribonuclease or a DNA endonuclease such as a deoxyribonuclease; DNase).

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A variety of radioactive isotopes are available for the production of radioconjugated antagonists or antibodies. Examples include At^{211} , I^{131} , I^{125} , Y^{90} , Re^{186} , Re^{188} , Sm^{153} , Bi^{212} , P^{32} and radioactive isotopes of Lu.

Conjugates of the antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. Science 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antagonist or antibody. See WO94/11026. The linker may be a "cleavable linker" facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, dimethyl linker or disulfide-containing linker (Chari et al. Cancer Research 52: 127-131 (1992)) may be used.

Alternatively, a fusion protein comprising the antibody and cytotoxic agent may be made, e.g. by recombinant techniques or peptide synthesis.

The antibodies of the present invention may also be conjugated with a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Patent No. 4,975,278.

The enzyme component of such conjugates includes any enzyme capable of acting on a prodrug in such a way so as to convert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β -galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β -lactamase useful for converting drugs derivatized with β -lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at

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their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, Nature 328: 457-458 (1987)). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the antibody by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g., Neuberger et al., Nature, 312: 604-608 (1984)).

Other modifications of the antibody are contemplated herein. For example, the antibody may be linked to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, polyoxyalkylenes, or copolymers of polyethylene glycol and polypropylene glycol.

To increase the serum half life of the antibody, one may incorporate a salvage receptor binding epitope into the antibody (especially an antibody fragment) as described in US Patent 5,739,277, for example. As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG₁, IgG₂, IgG₃, or IgG₄) that is responsible for increasing the in vivo serum half-life of the IgG molecule. Alternatively, or additionally, one may increase, or decrease, serum half-life by altering the amino acid sequence of the Fc region of an antibody to generate variants with altered FcRn binding. Antibodies with altered FcRn binding and/or serum half life are described in WO00/42072 (Presta, L.).

Formulations comprising Apo2L/TRAIL, death receptor antibodies, and/or CD20 antibodies are also provided by the present invention. It is believed that such formulations will be particularly suitable for storage as well as for therapeutic administration. The formulations may be prepared by known techniques. For instance, the formulations may be prepared by buffer exchange on a gel filtration column.

Typically, an appropriate amount of an acceptable salt or carrier is used in the formulation to render the formulation isotonic. Examples of pharmaceutically-acceptable carriers include saline, Ringer's solution and dextrose solution. The pH of the formulation is preferably from about 6 to about 9, and more preferably from about 7 to about 7.5. It will be apparent to those persons skilled in the art that certain carriers may be

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more preferable depending upon, for instance, the route of administration and concentrations of Apo-2 ligand, death receptor antibodies, and/or CD20 antibodies.

Therapeutic compositions can be prepared by mixing the desired molecules having the appropriate degree of purity with optional carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences, 16th edition, Osol, A. ed. (1980)), in the form of lyophilized formulations, aqueous solutions or aqueous suspensions. Acceptable carriers, excipients, or stabilizers are preferably nontoxic to recipients at the dosages and concentrations employed, and include buffers such as Tris, HEPES, PIPES, phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

Additional examples of such carriers include ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts, or electrolytes such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, and cellulose-based substances. Carriers for topical or gel-based forms include polysaccharides, such as sodium carboxymethylcellulose or methylcellulose, polyvinylpyrrolidone, polyacrylates, polyoxyethylene-polyoxypropylene-block polymers, polyethylene glycol, and wood wax alcohols. For all administrations, conventional depot forms are suitably used. Such forms include, for example, microcapsules, nano-capsules, liposomes, plasters, inhalation forms, nose sprays, sublingual tablets, and sustained-release preparations.

Formulations to be used for in vivo administration should be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. The formulation may be stored in lyophilized form or in solution if administered systemically. If in lyophilized form, it is typically formulated in combination with other ingredients for reconstitution with an.

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appropriate diluent at the time for use. An example of a liquid formulation is a sterile, clear, colorless unpreserved solution filled in a single-dose vial for subcutaneous injection.

Therapeutic formulations generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle. The formulations are preferably administered as repeated intravenous (i.v.), subcutaneous (s.c.), intramuscular (i.m.) injections or infusions, or as aerosol formulations suitable for intranasal or intrapulmonary delivery (for intrapulmonary delivery see, e.g., EP 257,956).

Apo2L/TRAIL, death receptor antibodies, and CD20 antibodies can also be administered in the form of sustained-release preparations. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the protein, which matrices are in the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (e.g., poly(2-hydroxyethyl-methacrylate) as described by Langer et al., J. Biomed. Mater. Res., 15: 167-277 (1981) and Langer, Chem. Tech., 12: 98-105 (1982) or poly(vinylalcohol)), polylactides (U.S. Patent No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al., Biopolymers, 22: 547-556 (1983)), non-degradable ethylene-vinyl acetate (Langer et al., *supra*), degradable lactic acid-glycolic acid copolymers such as the Lupron Depot (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133,988).

The Apo2L/TRAIL, death receptor antibodies, and CD20 antibodies described herein can be employed in a variety of therapeutic applications. Among these applications are methods of treating various cancers and immune related diseases. Diagnosis in mammals of the various pathological conditions described herein can be made by the skilled practitioner. Diagnostic techniques are available in the art which allow, e.g., for the diagnosis or detection of cancer or immune related disease in a mammal. For instance, cancers may be identified through techniques, including but not limited to, palpation, blood analysis, x-ray, NMR and the like. Immune related diseases can also be readily identified. In systemic lupus erythematosus, the central mediator of disease is the production of auto-reactive antibodies to self proteins/tissues and the subsequent generation of immune-mediated inflammation. Multiple organs and systems are affected clinically including kidney, lung, musculoskeletal system, mucocutaneous, eye, central nervous system, cardiovascular system, gastrointestinal tract, bone marrow and blood. Rheumatoid arthritis (RA) is a chronic systemic autoimmune inflammatory disease that mainly involves the synovial membrane of multiple joints with resultant injury to the articular cartilage. The pathogenesis is T lymphocyte dependent and is associated with the

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production of rheumatoid factors, auto-antibodies directed against self IgG, with the resultant formation of immune complexes that attain high levels in joint fluid and blood. These complexes in the joint may induce the marked infiltrate of lymphocytes and monocytes into the synovium and subsequent marked synovial changes; the joint space/fluid is infiltrated by similar cells with the addition of numerous neutrophils. Tissues affected are primarily the joints, often in symmetrical pattern. However, extra-articular disease also occurs in two major forms. One form is the development of extra-articular lesions with ongoing progressive joint disease and typical lesions of pulmonary fibrosis, vasculitis, and cutaneous ulcers. The second form of extra-articular disease is the so called Felty's syndrome which occurs late in the RA disease course, sometimes after joint disease has become quiescent, and involves the presence of neutropenia, thrombocytopenia and splenomegaly. This can be accompanied by vasculitis in multiple organs with formations of infarcts, skin ulcers and gangrene. Patients often also develop rheumatoid nodules in the subcutis tissue overlying affected joints; the nodules late stage have necrotic centers surrounded by a mixed inflammatory cell infiltrate. Other manifestations which can occur in RA include: pericarditis, pleuritis, coronary arteritis, interstitial pneumonitis with pulmonary fibrosis, keratoconjunctivitis sicca, and rheumatoid nodules.

The Apo2L/TRAIL, death receptor antibodies, and CD20 antibodies can be administered in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Optionally, administration may be performed through mini-pump infusion using various commercially available devices.

Effective dosages and schedules for administering Apo2L/TRAIL, death receptor antibodies, and CD20 antibodies may be determined empirically, and making such determinations is within the skill in the art. Single or multiple dosages may be employed. It is presently believed that an effective dosage or amount of Apo2L/TRAIL used alone may range from about 1 :g/kg to about 100 mg/kg of body weight or more per day. Interspecies scaling of dosages can be performed in a manner known in the art, e.g., as disclosed in Mordenti et al., Pharmaceut. Res., 8:1351 (1991).

When *in vivo* administration of an Apo2L/TRAIL is employed, normal dosage amounts may vary from about 10 ng/kg to up to 100 mg/kg of mammal body weight or more per day, preferably about 1 µg/kg/day to 10 mg/kg/day, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature; see, for example, U.S. Pat. Nos. 4,657,760; 5,206,344; or 5,225,212. It is anticipated that different formulations will be effective for different treatment compounds and different disorders, that administration targeting

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one organ or tissue, for example, may necessitate delivery in a manner different from that to another organ or tissue. Those skilled in the art will understand that the dosage of Apo2L/TRAIL that must be administered will vary depending on, for example, the mammal which will receive the Apo2L/TRAIL, the route of administration, and other drugs or therapies being administered to the mammal.

The CD20 antibody may be an antibody such as Rituximab or humanized 2H7, which is not conjugated to a cytotoxic agent. Suitable dosages for an unconjugated antibody are, for example, in the range from about 20 mg/m² to about 1000 mg/m². In one embodiment, the dosage of the antibody differs from that presently recommended for Rituximab. Exemplary dosage regimens for the CD20 antibody include 375 mg/m² weekly x 4 or 8; or 1000 mg x 2 (e.g. on days 1 and 15).

It is contemplated that yet additional therapies may be employed in the methods. The one or more other therapies may include but are not limited to, administration of radiation therapy, cytokine(s), growth inhibitory agent(s), chemotherapeutic agent(s), cytotoxic agent(s), tyrosine kinase inhibitors, ras farnesyl transferase inhibitors, angiogenesis inhibitors, and cyclin-dependent kinase inhibitors which are known in the art and defined further with particularity in Section I above.

Exemplary therapeutic antibodies include anti-HER2 antibodies including rhuMab 4D5 (HERCEPTIN) (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285-4289 (1992), U.S. Patent No. 5,725,856); anti-IL-8 (St John et al., *Chest*, 103:932 (1993), and International Publication No. WO 95/23865); anti-VEGF antibodies including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti-VEGF antibody huA4.6.1 AVASTIN (Kim et al., *Growth Factors*, 7:53-64 (1992), International Publication No. WO 96/30046, and WO 98/45331, published October 15, 1998); anti-PSCA antibodies (WO01/40309); anti-CD40 antibodies, including S2C6 and humanized variants thereof (WO00/75348); anti-CD11a antibodies including Raptiva[®] (US Patent No. 5,622,700, WO 98/23761, Steppe et al., *Transplant Intl.* 4:3-7 (1991), and Hourmant et al., *Transplantation* 58:377-380 (1994)); anti-IgE antibodies (Presta et al., *J. Immunol.* 151:2623-2632 (1993), and International Publication No. WO 95/19181; US Patent No. 5,714,338, issued February 3, 1998 or US Patent No. 5,091,313, issued February 25, 1992, WO 93/04173 published March 4, 1993, or International Application No. PCT/US98/13410 filed June 30, 1998, US Patent No. 5,714,338); anti-CD18 antibodies (US Patent No. 5,622,700, issued April 22, 1997, or as in WO 97/26912, published July 31, 1997); anti-Apo-2 receptor antibody antibodies (WO 98/51793 published November 19, 1998); anti-TNF-alpha antibodies including cA2 (REMICADE), CDP571 and MAK-195 (See, US Patent No. 5,672,347 issued September 30, 1997, Lorenz et al. *J. Immunol.* 156(4):1646-1653 (1996), and Dhainaut et al. *Crit. Care Med.* 23(9):1461-1469 (1995)); anti-Tissue Factor (TF) antibodies (European Patent No. 0 420 937 B1, granted

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November 9, 1994); anti-human $\alpha_4\beta_1$ integrin antibodies (WO 98/06248 published February 19, 1998); anti-EGFR antibodies (chimerized or humanized 225 antibody as in WO 96/40210 published December 19, 1996); anti-CD3 antibodies such as OKT3 (US Patent No. 4,515,893 issued May 7, 1985); anti-
 5 CD25 or anti-Tac antibodies such as CHI-621 (SIMULACT.) and ZENAPAX. (See US Patent No. 5,693,762 issued December 2, 1997); anti-CD4 antibodies such as the CM-7412 antibody (Choy et al. *Arthritis Rheum* 39(1):52-56 (1996)); anti-CD52 antibodies such as CAMPATH-1H (Riechmann et al. *Nature* 332:323-337 (1988); anti-Fc receptor antibodies such as the M22 antibody directed
 10 against Fc γ RI as in Graziano et al. *J. Immunol.* 155(10):4996-5002 (1995); anti-carcinoembryonic antigen (CEA) antibodies such as hMN-14 (Sharkey et al. *Cancer Res.* 55(23Suppl): 5935s-5945s (1995); antibodies directed against breast epithelial cells including huBrE-3, hu-Mc 3 and CHL6 (Ceriani et al. *Cancer Res.* 55(23): 5852s-5856s (1995); and Richman et al.
 15 *Cancer Res.* 55(23 Suppl): 5916s-5920s (1995)); antibodies that bind to colon carcinoma cells such as C242 (Litton et al. *Eur J. Immunol.* 26(1):1-9 (1996)); anti-CD38 antibodies, e.g. AT 13/5 (Ellis et al. *J. Immunol.* 155(2):925-937 (1995)); anti-CD33 antibodies such as Hu M195 (Jurcic et al. *Cancer Res* 55(23 Suppl):5908s-5910s (1995) and CMA-676 or CDP771; anti-CD22
 20 antibodies such as LL2 or LymphoCide (Juweid et al. *Cancer Res* 55(23 Suppl):5899s-5907s (1995); anti-EpCAM antibodies such as 17-1A (PANOREX.); anti-GpIIb/IIIa antibodies such as abciximab or c7E3 Fab (REOPRO.); anti-RSV antibodies such as MEDI-493 (SYNAGIS.); anti-CMV antibodies such as PROTOVIR.; anti-HIV antibodies such as PRO542; anti-hepatitis antibodies
 25 such as the anti-Hep B antibody OSTAVIR.; anti-CA 125 antibody OvaRex; anti-idiotypic GD3 epitope antibody BEC2; anti-v.3 antibody VITAXIN.; anti-human renal cell carcinoma antibody such as ch-G250; ING-1; anti-human 17-1A antibody (3622W94); anti-human colorectal tumor antibody (A33); anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human
 30 squamous-cell carcinoma (SF-25); and anti-human leukocyte antigen (HLA) antibodies such as Smart ID10 and the anti-HLA DR antibody Oncolym (Lym-1).

Preparation and dosing schedules for chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such
 35 chemotherapy are also described in *Chemotherapy Service Ed.*, M.C. Perry, Williams & Wilkins, Baltimore, MD (1992). The chemotherapeutic agent may precede, or follow administration of the Apo2L/TRAIL, death receptor antibody, and/or CD20 antibody, or may be given simultaneously therewith.

Sometimes, it may be beneficial to also administer one or more
 40 cytokines or growth inhibitory agent.

The Apo2L/TRAIL, death receptor antibodies, and CD20 antibodies (and one or more other therapies) may be administered concurrently or sequentially. Following administration, treated cells *in vitro* can be analyzed. Where there has been *in vivo* treatment, a treated mammal can be

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monitored in various ways well known to the skilled practitioner. For instance, cancer cells can be examined pathologically to assay for necrosis or serum can be analyzed for immune system responses.

For RA, and other autoimmune diseases, the Apo2L/TRAIL, death receptor antibody, and/or CD20 antibody may be combined with any one or more of the immunosuppressive agents, chemotherapeutic agents and/or cytokines listed in the definitions section above; any one or more disease-modifying antirheumatic drugs (DMARDs), such as hydroxychloroquine, sulfasalazine, methotrexate, leflunomide, azathioprine, D-penicillamine, Gold (oral), Gold (intramuscular), minocycline, cyclosporine, Staphylococcal protein A immunoadsorption; intravenous immunoglobulin (IVIG); nonsteroidal antiinflammatory drugs (NSAIDs); glucocorticoid (e.g. via joint injection); corticosteroid (e.g. methylprednisolone and/or prednisone); folate; an anti-tumor necrosis factor (TNF) antibody, e.g. etanercept/ENBREL[™], infliximab/REMICADE[™], D2E7 (Knoll) or CDP-870 (Celltech); IL-1R antagonist (e.g. Kineret); IL-10 antagonist (e.g. Ilodecakin); a blood clotting modulator (e.g. WinRho); an IL-6 antagonist/anti-TNF (CBP 1011); CD40 antagonist (e.g. IDEC 131); Ig-Fc receptor antagonist (MDX33); immunomodulator (e.g. thalidomide or ImmuDyn); anti-CD5 antibody (e.g. H5g1.1); macrophage inhibitor (e.g. MDX 33); costimulatory blocker (e.g. BMS 188667 or Tolerimab); complement inhibitor (e.g. h5G1.1, 3E10 or an anti-decay accelerating factor (DAF) antibody); or IL-2 antagonist (zxSMART).

For B cell malignancies, e.g., the Apo2L/TRAIL, death receptor antibody, and/or CD20 antibody may be combined with a chemotherapeutic agent; cytokine, e.g. a lymphokine such as IL-2, IL-12, or an interferon, such as interferon alpha-2a; other antibody, e.g., a radiolabeled antibody such as ibritumomab tiuxetan (ZEVALIN[®]), iodine I¹³¹ tositumomab (BEXXAR[™]), ¹³¹I Lym-1 (ONCOLYM[™]), ⁹⁰Y-LYMPHOCIDE[™]; anti-CD52 antibody, such as alemtuzumab (CAMPATH-1H[™]), anti-HLA-DR-β antibody, such as apolizumab, anti-CD80 antibody (e.g. IDEC-114), epratuzumab, Hu1D10 (SMART 1D10[™]), CD19 antibody, CD40 antibody or CD22 antibody; an immunomodulator (e.g. thalidomide or ImmuDyn); an inhibitor of angiogenesis (e.g. an anti-vascular endothelial growth factor (VEGF) antibody such as AVASTIN[™] or thalidomide); idiotype vaccine (EPOCH); ONCO-TCS[™]; HSPPC-96 (ONCOPHAGE[™]); liposomal therapy (e.g. daunorubicin citrate liposome), etc.

In another embodiment of the invention, articles of manufacture containing materials useful for the treatment of cancer or immune-related disease, described above, are provided. In one aspect, the article of manufacture comprises (a) a container comprising CD20 antibody (preferably the container comprises the antibody and a pharmaceutically acceptable carrier or diluent within the container); (b) a container comprising Apo2L/TRAIL or death receptor antibody (preferably the container comprises the Apo2L/TRAIL or death receptor antibody and a pharmaceutically

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acceptable carrier or diluent within the container); and (c) a package insert with instructions for treating cancer or immune related disease in a patient, wherein the instructions indicate that amounts of the CD20 antibody and the Apo2L/TRAIL or death receptor antibody are administered to the patient that are effective to provide synergistic activity in treating the disease.

In all of these aspects, the package insert is on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds or contains a composition that is effective for treating the cancer or immune related disease and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is the CD20 antibody, Apo2L/TRAIL or death receptor antibody. The label or package insert indicates that the composition is used for treating cancer or immune related disease in a patient or subject eligible for treatment with specific guidance regarding dosing amounts and intervals of antibody and any other medicament being provided. The article of manufacture may further comprise an additional container comprising a pharmaceutically acceptable diluent buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution, and/or dextrose solution. The article of manufacture may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLES

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by way of reference to the ATCC is the American Type Culture Collection, Manassas, Virginia.

Example 1

Analysis of Apo2L/TRAIL receptor expression in B lymphoma cell lines

To examine the cell-surface expression of Apo2L/TRAIL receptors (DR4, DR5, DcR1, and DcR2) in human lymphoma cell lines, the B lymphoma cell lines Ramos, Daudi, Raji, and BJAB (ATCC) were analyzed by FACS using monoclonal antibodies specific for DR4 (mAb 4H6.17.8; ATCC HB-12455), DR5

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(mAb 3H3.14.5; HB-12534), DcR1 (mAb 6G9; Genentech, Inc.), or DcR2 (mAb 1G9, Genentech, Inc.) For Ramos cells, the analysis was carried out twice to ensure reproducibility (RAMOS A and B).

As illustrated in Figure 4, DR4 and DR5 were expressed at significant levels (mean fluorescence shift of approximately 0.5 - 1.7 units) in all four of the cell lines, while DcR1 and DcR2 were expressed at lower or minimal levels (mean fluorescence shift of approximately 0 - 0.3 units).

Example 2

Analysis of CD20 expression in B lymphoma cell lines

To examine the cell-surface expression of CD20 in human lymphoma cell lines, the B lymphoma cell lines Ramos, Daudi, Raji, and BJAB (ATCC) were analyzed by FACS using a monoclonal antibody specific for CD20 (RITUXAN®, Genentech, Inc.). For Ramos cells, the analysis was carried out twice to ensure reproducibility (RAMOS A and B).

As illustrated in Figure 5, all four cell lines expressed high levels of CD20, indicated by a mean fluorescence shift of approximately 5 - 15 units.

Example 3

Effect of Apo2L/TRAIL, RITUXAN®, or combination treatment on the growth of pre-established subcutaneous BJAB lymphoma tumor xenografts in SCID mice

SCID mice were injected subcutaneously with human B-cell non-Hodgkin's BJAB lymphoma cells (ATCC) (20 million cells per mouse) and tumors were allowed to grow to ~200 mm³. The mice were then divided into 4 study groups (8 mice per group) and treated with five intraperitoneal (IP) doses per week over 2 weeks (i.e., days 0-4 and 7-11) of vehicle (0.5M Arg-Succinate/20mM Tris/0.02% Tween 20 pH =7.2), Apo2L/TRAIL (amino acids 114-281 of Figure 1) (60 mg/kg), or with 1 IP dose per week over 2 weeks (i.e., days 0 and 7) of RITUXAN® (4 mg/kg, Genentech, Inc.), or the combination of these latter Apo2L/TRAIL and RITUXAN® regimens (Figure 6).

Tumors in vehicle-treated mice grew rapidly, while single-agent Apo2L/TRAIL or RITUXAN® treatment markedly delayed tumor growth. One mouse in the Apo2L/TRAIL group showed complete tumor ablation, leaving a tumor incidence (TI) of 7/8. RITUXAN® treatment did not ablate any tumors but showed a more prolonged effect. Importantly, combined treatment with Apo2L/TRAIL and RITUXAN® caused a dramatic reduction in tumor volume in all mice, with 5 out of 8 mice showing complete tumor ablation and 3/8 showing minimal tumor growth for at least 28 days. These results indicate that Apo2L/TRAIL and RITUXAN® can exert synergistic anti-tumor activity against lymphoma xenografts.

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Example 4Effect of Apo2L/TRAIL, RITUXAN®, or combination treatment on the growth of pre-established subcutaneous BJAB lymphoma tumor xenografts grown in SCID mice

5 A similar study to the one described in Example 3 was conducted. SCID mice were injected subcutaneously with human B-cell non-Hodgkin's BJAB lymphoma cells (ATCC) (20 million cells per mouse) and tumors were allowed to grow to ~200 mm³. The mice were then divided into 4 study groups (8 mice per group) and treated with five intraperitoneal (IP) doses per week over 10 2 weeks (i.e., days 0-4 and 7-11) of vehicle (0.5M Arg-Succinate/20mM Tris/0.02% Tween 20 pH =7.2), Apo2L/TRAIL ("Apo2L.0"; amino acids 114-281 of Fig.1) (60 mg/kg), or with 1 IP dose per week over 2 weeks (i.e., days 0 and 7) of RITUXAN® (4 mg/kg), or the combination of these latter Apo2L/TRAIL and RITUXAN® regimens.

15 The results are shown in Fig. 7. Tumors in vehicle-treated mice grew rapidly, while single-agent Apo2L/TRAIL or RITUXAN® treatment markedly delayed tumor growth. Neither Apo2L/TRAIL nor RITUXAN® alone caused any complete regressions, while RITUXAN® showed a more prolonged effect. As in the study described in Example 3, combined treatment with Apo2L/TRAIL and 20 RITUXAN® caused a remarkable reduction in tumor volume in all mice, with 6 out of 7 mice showing complete tumor ablation. These results indicate that Apo2L/TRAIL and RITUXAN® can exert synergistic anti-tumor activity against lymphoma xenografts.

Example 5Effect of Apo2L/TRAIL, RITUXAN®, or combination treatment on caspase processing in pre-established subcutaneous BJAB lymphoma tumor xenografts grown in SCID mice

25 To examine the processing of apoptosis-mediating caspases in treated 30 tumors (indicated by proteolytic caspase processing), SCID mice were injected subcutaneously with human B-cell non Hodgkin's BJAB lymphoma cells (ATCC) (20 million cells per mouse) and tumors were allowed to grow to ~200 mm³. The mice were then treated with vehicle (0.5M Arg-Succinate/20mM Tris/0.02% Tween 20 pH =7.2) (n=1), 1 IP dose of Apo2L/TRAIL (60 mg/kg) 35 (n=1), or 1 IP dose of RITUXAN® (4 mg/kg, Genentech, Inc.) (n=2), or the combination of these latter Apo2L/TRAIL and RITUXAN® doses (n=2). Two days after treatment, the tumors were harvested, lysed in lysis buffer, and subjected to immunoblot with specific antibodies against Caspase 8, 3, 9, and 7 (with anti-beta actin antibody as a loading control) to visualize 40 caspase processing (Figure 8).

Apo2L/TRAIL treatment (A) induced increased processing of caspase 8, 3, 9, and 7 as compared to the vehicle control (V), while RITUXAN® (R) did not induce caspase processing. Notably, combination treatment with Apo2L/TRAIL and RITUXAN® (AR) did not further increase caspase processing

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as compared to Apo2L/TRAIL alone. These results suggest that the synergistic anti-tumor activity between Apo2L/TRAIL and RITUXAN® is not necessarily mediated by enhancement of apoptosis, suggesting that combination of apoptosis activation mediated by Apo2L/TRAIL and complement-dependent lysis together with ADCC mediated by RITUXAN® may underlie the observed anti-tumor synergy.

Example 6

Effect of agonistic DR5 antibody, RITUXAN®, or combination treatment on the growth of pre-established subcutaneous BJAB lymphoma tumor xenografts in SCID mice

SCID mice were injected subcutaneously with human B-cell non-Hodgkin's BJAB lymphoma cells (ATCC) (20 million cells per mouse) and tumors were allowed to grow to ~200 mm³. The mice were then divided into 4 study groups (7 mice per group) and treated with one intraperitoneal (IP) injection per week over 2 weeks (i.e., days 0 and 7) of vehicle (0.5M Arg-Succinate/20mM Tris/0.02% Tween 20 pH =7.2), agonist DR5 monoclonal antibody ("Apomab") (10 mg/kg), or RITUXAN® (4 mg/kg), or the combination of these latter DR5 antibody and RITUXAN® regimens (Figure 9). Tumors in vehicle-treated mice grew rapidly, while single-agent DR5 antibody or RITUXAN® treatment markedly delayed tumor growth. Importantly, combined treatment with DR5 antibody and RITUXAN® caused a dramatic reduction in tumor volume in all mice, with 5 out of 7 mice showing complete tumor ablation and 2/7 showing minimal tumor growth for at least 35 days. These results indicate that agonist DR5 antibody and RITUXAN® can exert synergistic anti-tumor activity against lymphoma xenografts.

Example 7

Effect of agonistic DR5 antibody, RITUXAN®, or combination treatment on caspase processing in pre-established subcutaneous BJAB lymphoma tumor xenografts grown in SCID mice

To examine the processing of apoptosis-mediating caspases in treated tumors (indicated by proteolytic caspase processing), SCID mice were injected subcutaneously with human B-cell non-Hodgkin's BJAB lymphoma cells (ATCC) (20 million cells per mouse) and tumors were allowed to grow to ~200 mm³. The mice were then treated with vehicle (0.5M Arg-Succinate/20mM Tris/0.02% Tween 20 pH =7.2) (n=1), or 1 IP dose of RITUXAN® (4 mg/kg) (n=2), or 1 IP dose of agonist DR5 antibody (10 mg/kg) (n=2), or the combination of these latter DR5 antibody and RITUXAN® doses (n=2). Two days after treatment, the tumors were harvested, lysed in lysis buffer, and subjected to immunoblot with specific antibodies against Caspase 8, 3, 9,

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and 7 (with anti-beta actin antibody as a loading control) to visualize caspase processing (Figure 10).

Agonist DR5 antibody treatment (A) induced increased processing of caspase 8, 3, 9, and 7 as compared to the vehicle control (V), while RITUXAN® (R) did not induce caspase processing. Notably, combination treatment with DR5 antibody and RITUXAN® (AR) did not further increase caspase processing as compared to DR5 antibody alone. These results suggest that the synergistic anti-tumor activity between DR5 antibody and RITUXAN® is not necessarily mediated by enhancement of apoptosis, but rather that the combination of apoptosis activation mediated by agonist DR5 antibody and complement-dependent lysis together with ADCC mediated by RITUXAN® may underlie the observed anti-tumor synergy.

Further data illustrating expression of CD20 and Apo2L/TRAIL receptors in NHL cell lines and the effects of Rituximab, Apo2L/TRAIL and combinations thereof on cancer cells are provided in Figures 11-16.

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WHAT IS CLAIMED IS:

1. A method of treating cancer cells, comprising exposing mammalian cancer cells to a synergistic effective amount of agonist death receptor antibody and CD20 antibody.

2. The method of claim 1 wherein said agonist death receptor antibody is an anti-DR5 receptor monoclonal antibody.

3. The method of claim 1 wherein said agonist death receptor antibody is an anti-DR4 receptor monoclonal antibody.

4. The method of claim 1 wherein said cancer cells are exposed to said synergistic effective amount of agonist death receptor antibody and CD20 antibody *in vivo*.

5. The method of claim 2 or 3 wherein said agonist death receptor antibody is a chimeric antibody or a humanized antibody.

6. The method of claim 2 or 3 wherein said agonist death receptor antibody is a human antibody.

7. The method of claim 1 wherein said agonist death receptor antibody is an antibody which cross-reacts with more than one Apo-2 ligand receptor.

8. The method of claim 1 wherein said cancer cells are lymphoma cells.

9. The method of claim 1 further comprising exposing the cancer cells to one or more growth inhibitory agents.

10. The method of claim 1 further comprising exposing the cells to radiation.

11. The method of claim 2 wherein said DR5 antibody has a DR5 receptor binding affinity of 10^8 M^{-1} to 10^{12} M^{-1} .

12. The method of claim 1 wherein said death receptor antibody and CD20 antibody is expressed in a recombinant host cell selected from the group consisting of a CHO cell, yeast cell and *E. coli*.

13. The method of claim 1 wherein said CD20 antibody is a monoclonal antibody.

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14. The method of claim 13 wherein said CD20 antibody is the antibody Rituximab.

5 15. A method of treating an immune related disease, comprising administering to a mammal a synergistic effective amount of agonist death receptor antibody and CD20 antibody.

10 16. The method of claim 15 wherein said agonist death receptor antibody is an anti-DR5 receptor monoclonal antibody.

17. The method of claim 15 wherein said agonist death receptor antibody is an anti-DR4 receptor monoclonal antibody.

15 18. The method of claim 16 or 17 wherein said agonist death receptor antibody is a chimeric antibody or a humanized antibody.

19. The method of claim 16 or 17 wherein said agonist death receptor antibody is a human antibody.

20 20. The method of claim 15 wherein said agonist death receptor antibody is an antibody which cross-reacts with more than one Apo-2 ligand receptor.

25 21. The method of claim 15 wherein said immune related disease is rheumatoid arthritis or multiple sclerosis.

22. The method of claim 15 wherein said DR5 antibody has a DR5 receptor binding affinity of 10^8 M^{-1} to 10^{12} M^{-1} .

30 23. The method of claim 15 wherein said death receptor antibody and CD20 antibody is expressed in a recombinant host cell selected from the group consisting of a CHO cell, yeast cell and *E. coli*.

35 24. The method of claim 15 wherein said CD20 antibody is a monoclonal antibody.

25. The method of claim 24 wherein said CD20 antibody is the antibody Rituximab.

40 26. The method of claim 1 or 15 wherein said death receptor antibody and CD20 antibody are administered sequentially.

27. The method of claim 1 or 15 wherein said death receptor antibody and CD20 antibody are administered concurrently.

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ABSTRACT OF THE DISCLOSURE

Methods for using death receptor ligands, such as Apo-2 ligand/TRAIL polypeptides or death receptor antibodies, and CD20 antibodies to treat conditions such as cancer and immune related diseases are provided.

- 5 Embodiments of the invention include methods of using Apo2L/TRAIL or death receptor antibodies such as DR5 antibodies and DR4 antibodies in combination with CD20 antibodies.

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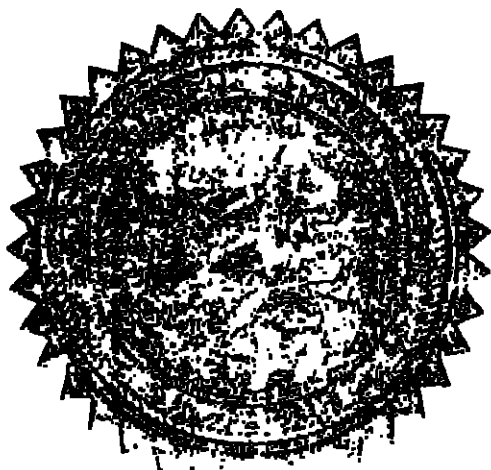
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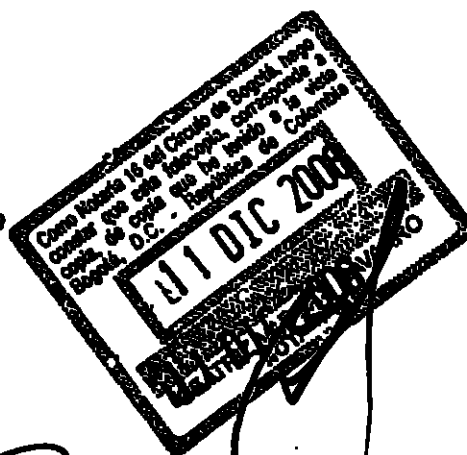
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Secretary of State



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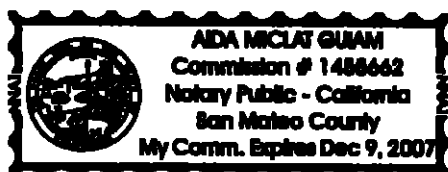
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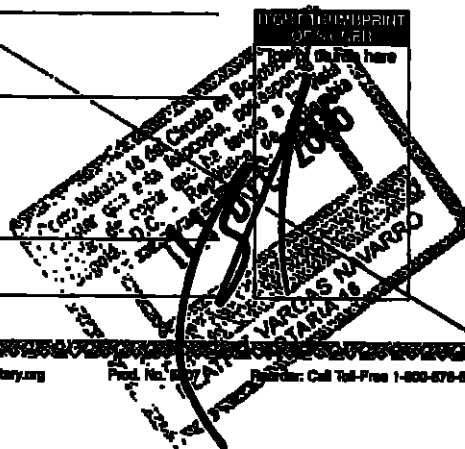
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Nombro(amos) a: JUAN PABLO CADENA SARMIENTO y/o BEATRIZ JARAMILLO y/o CARLOS H. PALACIO EASTMAN y/o HECTOR GOMEZ MEJIA, de Bogotá, Colombia, apoderados verdaderos y legales con poder especial, amplio y suficiente, para recabar de las oficinas y autoridades colombianas administrativas, judiciales y de policía, en cualquier instancia o recurso, la obtención de patentes de invención, registros de marcas, diseños industriales, modelos de utilidad, depósitos de nombres comerciales y enseññas, registros de derechos de autor y contratos sobre los mismos, obtención de certificados de obtentor de variedades vegetales, así como sus prórrogas, renovaciones, anotaciones de traspaso, cambios de nombre y/o domicilio; elaborar, tramitar y registrar contratos de licencia y licencias de cualesquiera clases; intervenir como demandante o como demandado en cualquier instancia y recurso ante cualquier juez, corporación, funcionario público o autoridad en acciones judiciales, administrativas y de policía tales como: oposiciones al registro de marcas; acciones de caducidad, cancelación y nulidad; acciones reivindicatorias, acciones derivadas de la obtención o explotación de licencias; acciones de competencia desleal; demandas penales; acciones de indemnización de perjuicios, reclamos y observaciones a solicitudes de patentes, diseños industriales, modelos de utilidad, en el ejercicio de medidas cautelares y en otras acciones o medidas similares.

A este efecto, lo(s) faculto(amos) para elevar solicitudes, formular descripciones, enmiendas, protestas, declaraciones, oposiciones, cancelaciones, nulidades, apelaciones y reclamos; pagar impuestos, cuotas y gravámenes prescritos por la ley; renunciar o desistir a derechos concedidos o en curso de concesión; recibir toda clase de documentos y valores, dando el descargo respectivo, y, en general, para dar ante dichas autoridades todos los pasos necesarios al efecto indicado y tomar todas las medidas que creyeran conducentes al resguardo de mis (nuestros intereses), con todas las demás facultades legales necesarias.

Este poder comprende la facultad de ratificar o confirmar en mi (nuestro) nombre lo actuado así como la facultad de desistir, transigir, comprometer, y la de sustituirlo en todo o en parte y revocar sustituciones. Asimismo nombro(amos) a: JUAN PABLO CADENA SARMIENTO y/o BEATRIZ JARAMILLO y/o CARLOS H. PALACIO EASTMAN y/o HECTOR GOMEZ MEJIA, domiciliado(s) en la Carrera 7 No. 16-56 Piso 9, mis (nuestros) apoderados en Colombia, con facultades para recibir notificaciones y designar apoderados judiciales o extrajudiciales.

Otorgado y firmado en

hoy . de de 200_

Firma

Nombre
Cargo

POWER OF ATTORNEY

I (We)

Genentech, Inc.
1 DNA Way

domiciled in South San Francisco, California 94080-4990
USA

Hereby appoints JUAN PABLO CADENA SARMIENTO and/or BEATRIZ JARAMILLO and/or CARLOS H. PALACIO EASTMAN and/or HECTOR GOMEZ MEJIA, of Bogotá, Colombia, my (our) true and lawful attorneys with special, ample and sufficient power to obtain from the Colombian administrative, judicial and police offices and authorities, in any instance, privileges of patents of invention, registration of trademarks, industrial designs and utility models; registration of commercial names and emblems, registration of copyright and contracts related thereto, certificates of obtainers of vegetables varieties, as well as extensions, renewals and recordal of assignments and changes of name and domicile related to the above; to prepare, prosecute and register licenses of any kind; to act as plaintiff or defendant in any instance or recourse, and before any judge, corporation, public official or authority in judicial, administrative and police actions, such as: oppositions to the registration of trademarks; caducity, cancellation and nullity actions; claim actions derived from the obtaining or exploitation of licenses, action of unfair competition; criminal proceedings, actions for payment of damages; claims or observations on patent applications, models, industrial designs and utility models; in the exercise of preventive measures and in other actions or similar proceedings.

To that effect they hereby empowered to file applications, formulate descriptions, amendments, protests, declarations, oppositions, cancellations, nullities, appeals and claims; to pay taxes, quotas and assessments prescribed by law, renounce or waive the rights granted or in the process of granting; to receive all kinds of documents and valuables, giving the respective release of receipt, and, in general, to take before said authorities the necessary steps to such effect, and any measures that they may deem pertinent to the safeguard of my (our) interests, with all the other necessary legal powers.

This power includes the faculty to ratify or confirm, desist, settle and compromise on my (our) behalf and to substitute this power in whole or in part and to revoke substitutions. At the same time I (We) hereby appoints JUAN PABLO CADENA SARMIENTO and/or BEATRIZ JARAMILLO and/or CARLOS H. PALACIO EASTMAN and/or HECTOR GOMEZ MEJIA, domiciled in Carrera 7 No. 16-56, Piso 9, my (our) attorney(s) in fact, with powers to receive notifications and to designate judicial and extrajudicial attorneys.

Granted and signed in South San Francisco, California
USA

this 6th day of December 2005

Signature

Name Timothy R. Schwartz, Ph.D.
Title Section Patent Counsel
Authorized Corporate Signatory
Genentech, Inc.

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AUTHORIZATION

Genentech, Inc. hereby authorizes any of Janet E. Hasak, Mark T. Kresnak, Jeffrey S. Kubinec and Timothy R. Schwartz, for so long as they remain employees of Genentech, Inc., to execute, on its behalf, any submissions associated with the filing, prosecution, granting, and extension and maintenance of grant of patent and trademark applications in the U.S. and foreign countries, contracts relating to biological deposits with the American Type Culture Collection, and assignments to publishers of copyrights covering works authored by Genentech employees.

August 27, 2002

Dated

Sean Johnston

Sean A. Johnston
Vice President, Intellectual Property
Genentech, Inc.



370 348

TRADUCCION AL ESPAÑOL del Documento de Poder otorgado por Genentech, Inc. domiciliada en 1 DNA Way, South San Francisco, California 94080-4990, Estados Unidos de América.

**ESTADO DE CALIFORNIA
SECRETARIA DE ESTADO**

APOSTILLA

(Convención de La Haya del 5 de Octubre de 1961)

1. País: Estados Unidos de América
Esta documento público
2. ha sido firmado por AIDA MICLAT GUIAM
3. actuando en capacidad de Notario Público, Estado de California
4. lleva el sello/timbre de AIDA MICLAT GUIAM, Notario Público
Estado de California

CERTIFICADO

5. en Sacramento, California
6. el 15 de diciembre de 2005
7. por el Secretario de Estado Delegado, Estado de California
8. No. 267083
9. Sello/Timbre

10. Firma

(Firma)

Secretario de Estado



~~257~~ 349

RECONOCIMIENTO TODO-PROPOSITO DE CALIFORNIA

Estado de California)
) ss.
Condado de San Mateo)

El 6 de diciembre de 2005, ante mi, AIDA MICLAT GUIAM, apareció personalmente Thimoty R. Schwartz

☐ a quien conozco personalmente

☒ quien demostró ante mi en base a evidencia satisfactoria ser la persona cuyo nombre esta suscrito en el instrumento y quien reconoció ante mi que ejecutó el mismo en su capacidad autorizada, y que por su firma en el instrumento la persona o la entidad a nombre de la cual la persona actuó, ejecutó el instrumento.

**Hay un sello que dice:
AIDA MICLAT GUIAM
COMISIÓN # 14556812
Notario Público-California
Condado de San Mateo
Mi comisión expira Dic. 9 de 2007**

Atestigua mi firma y sello oficial,

AIDA MICLAT GUIAM

OPCIONAL

Aunque la información en esta sección no es requerida por la ley, puede probar ser valiosa para las personas que se basan en el documento y puede prevenir la remoción y readhesión fraudulenta de esta forma a otro documento

Descripción adicional de cualquier documento anexo

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Nombre del firmante: _____

- ☐ Individuo
☐ Funcionario Corporativo – Título(s): _____
☐ Socio - ☐ Limitado ☐ General
☐ Abogado de hecho
☐ Fideicomisario
☐ Guardán o conservador
☐ Otro: _____

El firmante representa: _____



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AUTORIZACION

Genentech, Inc. por la presente autoriza a cualquiera de Janet E. Hasak, Mark T. Kresnak, Jeffrey S. Kubinec y Thomity R. Schwartz, mientras permanezcan como empleados de Genentech, Inc., a ejecutar, a su nombre, cualquier solicitud asociada con presentación, procesamiento, consecución, y extensión y mantenimiento de concesión de solicitudes de patente y marca registrada en los Estados Unidos y países extranjeros, contratos relacionados con depósitos biológicos con la Colección Americana de Tipo de Cultivo, y cesiones a publicistas de derechos de autor que cubran los trabajos de autoría de los empleados de Genentech.

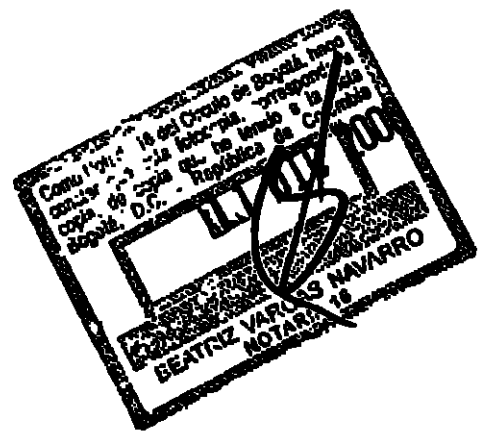
27 de Agosto de 2002

Fechado

(Firma)

Sean A. Johnston

Vicepresidente, Propiedad Intelectual
Genentech, Inc.



Apostille

(Convention de La Haye du 5 Octobre 1961)

1. Country: United States of America

This public document:

2. has been signed by Harriet Smith Windsor

3. acting in the capacity of Secretary of State of Delaware

4. bears the seal/stamp of Office of Secretary of State

Certified

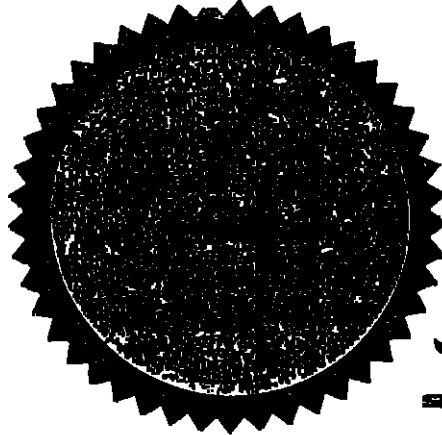
5. at Dover, Delaware

6. the twenty-fourth day of May, A.D. 2005

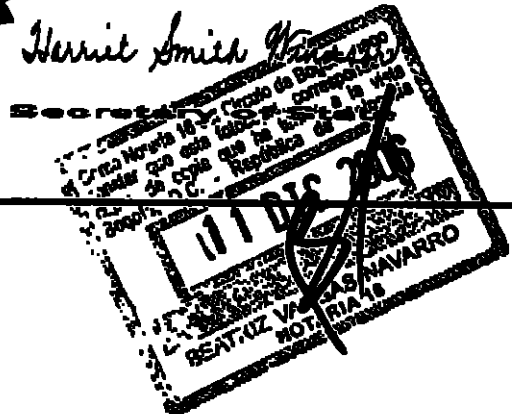
7. by Secretary of State, Delaware Department of State

8. No. 0254030

9. Seal/Stamp:



10. Signature:

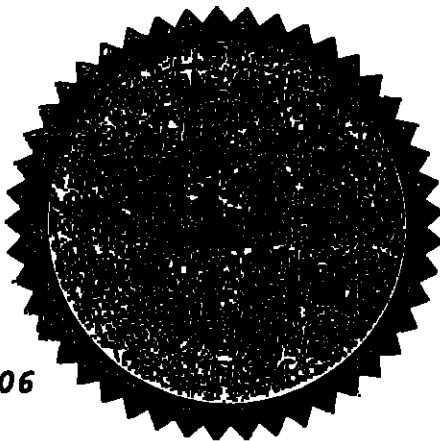


Delaware

PAGE 1

The First State

I, HARRIET SMITH WINDSOR, SECRETARY OF STATE OF THE STATE OF DELAWARE, DO HEREBY CERTIFY THE ATTACHED IS A TRUE AND CORRECT COPY OF THE RESTATED CERTIFICATE OF "GENENTECH, INC.", FILED IN THIS OFFICE ON THE TWENTY-SECOND DAY OF JULY, A.D. 1999, AT 9 O'CLOCK A.M.

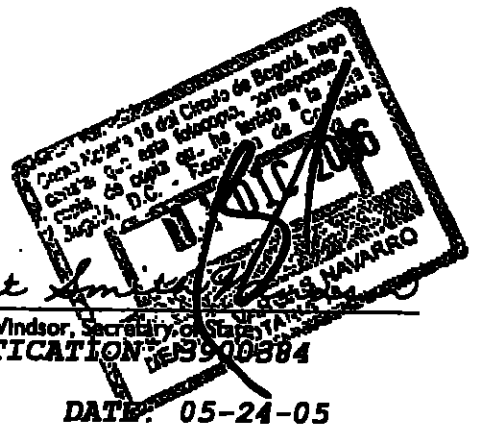


2112549

050426506

Harriet Smith Windsor
Harriet Smith Windsor, Secretary of State
AUTHENTICATION

DATE: 05-24-05



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AMENDED AND RESTATED
CERTIFICATE OF INCORPORATION

GENENTECH, INC.

Genentech, Inc. (the "Corporation"), a corporation organized and existing under and by virtue of the General Corporation Law of the State of Delaware, does hereby amend the Certificate of Incorporation of the Corporation, which was originally filed on December 23, 1986 under the name "Genentech Delaware, Inc.", and subsequently amended.

The Certificate of Incorporation of the Corporation is hereby restated and further amended to read in its entirety as follows:

ARTICLE 1

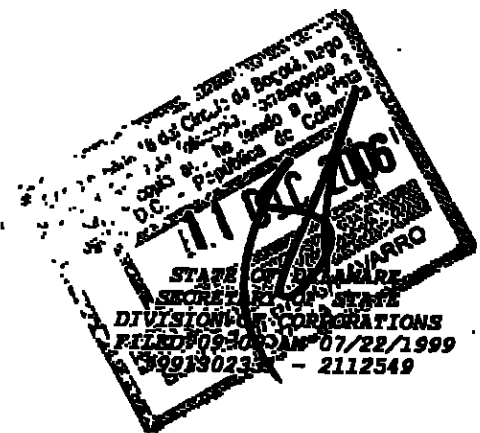
The name of the Corporation is: Genentech, Inc.

ARTICLE 2

The address of the registered office of the Corporation in the State of Delaware is Corporation Service Company, 1013 Centre Road, City of Wilmington, County of New Castle, Delaware 19805. The name of its registered agent at such address is Corporation Service Company.

ARTICLE 3

The purpose of the Corporation is to engage in any lawful act or activity for which corporations may be organized under the General Corporation Law of the State of Delaware as the same exists or may hereafter be amended ("Delaware Law").



ARTICLE 4

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SECTION 4.01. *Capital Stock.* (a) The Corporation is authorized to issue two classes of stock to be designated, respectively, preferred stock and common stock. The total number of shares which the Corporation is authorized to issue is four hundred million (400,000,000) shares. One hundred million (100,000,000) shares shall be designated preferred stock, par value \$0.02 per share ("Preferred Stock"). Three hundred million (300,000,000) shares shall be designated common stock, par value \$0.02 per share ("Common Stock") The Common Stock of the Corporation shall be all of one class.

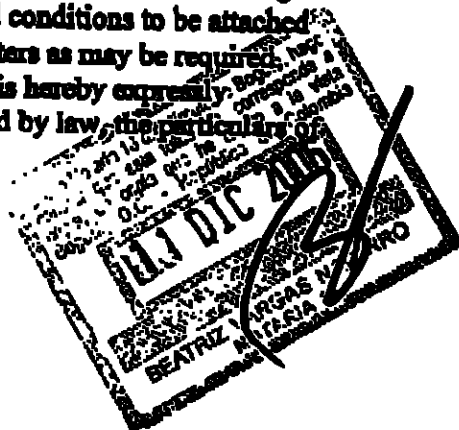
(b) The number of authorized shares of any class or classes of stock may be increased or decreased (but not below the number of shares thereof then outstanding) by the affirmative vote of a majority in voting power of the outstanding stock of the Corporation entitled to vote thereon irrespective of the provisions of Section 242(b)(2) of Delaware Law.

SECTION 4.02. *Common Stock.* (a) *Issuance and Consideration.* Any unissued or treasury shares of the Common Stock may be issued for such consideration as may be fixed in accordance with applicable law from time to time by the Board of Directors.

(b) *Dividends.* Subject to the rights of holders of the Preferred Stock, the holders of the Common Stock shall be entitled to receive, when and as declared by the Board of Directors, out of the assets of the Corporation which are by law available therefor, dividends payable either in cash, in property, or in shares of stock and the holders of the Preferred Stock shall not be entitled to participate in any such dividends (unless otherwise provided by the Board of Directors in any resolution providing for the issue of a series of Preferred Stock).

SECTION 4.03. *Preferred Stock.*

(a) *Series and Limits of Variations between Series.* Any unissued or treasury shares of the Preferred Stock may be issued from time to time in one or more series for such consideration as may be fixed from time to time by the Board of Directors. Before any shares of Preferred Stock of any particular series shall be issued, a certificate shall be filed with the Secretary of State of Delaware setting forth the designation, rights, privileges, restrictions, and conditions to be attached to the Preferred Stock of such series and such other matters as may be required and the Board of Directors shall fix and determine, and is hereby expressly empowered to fix and determine, in the manner provided by law, the particulars of



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the shares of such series (so far as not inconsistent with the provisions of this Article 4 applicable to all series of Preferred Stock), including, but not limited to, the following:

- (i) the distinctive designation of such series and the number of shares which shall constitute such series, which number may be increased (except where otherwise provided by the Board of Directors in creating such series) or decreased (but not below the number of shares thereof then outstanding) from time to time by like action of the Board of Directors;
- (ii) the annual rate of dividends payable on shares of such series, the conditions upon which such dividends shall be payable and the date from which dividends shall be cumulative in the event the Board of Directors determines that dividends shall be cumulative;
- (iii) whether such series shall have voting rights, in addition to the voting rights provided by law and, if so, the terms of such voting rights;
- (iv) whether such series shall have conversion privileges and, if so, the terms and conditions of such conversion, including, but not limited to, provision for adjustment of the conversion rate upon such events and in such manner as the Board of Directors shall determine;
- (v) whether or not the shares of such series shall be redeemable and, if so, the terms and conditions of such redemption, including the date or dates upon or after which they shall be redeemable, and the amount per share payable in case of redemption, which amount may vary under different conditions and at different redemption dates;
- (vi) whether such series shall have a sinking fund for the redemption or purchase of shares of that series and, if so, the terms and amount of such sinking fund;
- (vii) the rights of the shares of such series in the event of voluntary or involuntary liquidation, dissolution or winding up of the Corporation, and the relative rights of priority, if any, of payment of shares of that series; and
- (viii) any other relative rights, preferences and limitations of such series.

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ARTICLE 5

SECTION 5.01. *Amendment of Bylaws by Directors.* (a) In furtherance and not in limitation of the powers conferred by statute, the Board of Directors is expressly authorized to make, alter, amend, repeal and rescind the bylaws of the Corporation, subject to Sections 5.01(b) and 5.01(c) hereof.

(b) Sections 2.02, 3.02, 3.03(a), 3.03(b), 3.04, 3.05, 3.14, and 5.06 of the bylaws may only be altered, amended, repealed, or rescinded by the affirmative vote of holders of capital stock entitled to vote thereon representing more than 60% of the shares entitled to be voted thereon.

(c) Sections 3.03(c) and 3.15 of the bylaws may only be altered, amended, repealed, or rescinded by the affirmative vote of holders of capital stock entitled to vote thereon representing more than 90% of the shares entitled to be voted thereon.

SECTION 5.02. *Amendment of Certificate of Incorporation.* Section 5.01(b) of this Certificate of Incorporation may only be altered, amended, repealed, or rescinded by the affirmative vote of holders of capital stock entitled to vote thereon representing more than 60% of the shares entitled to be voted thereon. Section 5.01(c) and Articles 8 and 9 of this Certificate of Incorporation may only be altered, amended, repealed, or rescinded by the affirmative vote of holders of capital stock entitled to vote thereon representing more than 90% of the shares entitled to be voted thereon.

ARTICLE 6

SECTION 6.01. *Election of Directors by Holders of Preferred Stock.* During any period when the holders of any Preferred Stock or any one or more series thereof, voting as a class, shall be entitled to elect a specified number of directors, by reason of dividend arrearages or other provisions giving them the right to do so, then and during such time as such right continues (i) the then otherwise authorized number of directors shall be increased by such specified number of directors, and the holders of such Preferred Stock or such series thereof, voting as a class, shall be entitled to elect the additional directors so provided for, pursuant to the provisions of such Preferred Stock or series; (ii) each such additional director shall serve for such term, and have such voting powers, as shall be stated in the provisions pertaining to such Preferred Stock or series; and (iii) whenever the holders of any such Preferred Stock or series thereof are

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divested of such rights to elect a specified number of directors, voting as a class, pursuant to the provisions of such Preferred Stock or series, the terms of office of all directors elected by the holders of such Preferred Stock or series, voting as a class pursuant to such provisions or elected to fill any vacancies resulting from the death, resignation or removal of directors so elected by the holders of such Preferred Stock or series, shall forthwith terminate and the authorized number of directors shall be reduced accordingly.

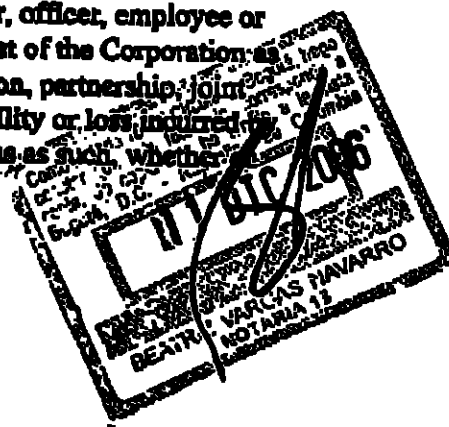
SECTION 6.02. Ballots. Elections of directors at an annual or special meeting of stockholders need not be by written ballot unless the bylaws of the Corporation shall provide otherwise.

SECTION 6.03. Elimination of Certain Personal Liability of Directors. (a) A director of the Corporation shall not be liable to the Corporation or its stockholders for monetary damages for breach of fiduciary duty as a director to the fullest extent permitted by Delaware Law.

(b) Each person (and the heirs, executors or administrators of such person) who was or is a party or is threatened to be made a party to, or is involved in any threatened, pending or completed action, suit or proceeding, whether civil, criminal, administrative or investigative, by reason of the fact that such person is or was a director of the Corporation or is or was serving at the request of the Corporation as a director of another corporation, partnership, joint venture, trust or other enterprise, shall be indemnified and held harmless by the Corporation to the fullest extent permitted by Delaware Law. The right to indemnification conferred in this Section 6.03 shall also include the right to be paid by the Corporation the expenses incurred in connection with any such proceeding in advance of its final disposition to the fullest extent authorized by Delaware Law. The right to indemnification conferred in this Section 6.03 shall be a contract right.

(c) The Corporation may, by action of its Board of Directors, provide indemnification to such of the officers, employees and agents of the Corporation to such extent and to such effect as the Board of Directors shall determine to be appropriate and authorized by Delaware Law.

(d) The Corporation shall have power to purchase and maintain insurance on behalf of any person who is or was a director, officer, employee or agent of the Corporation, or is or was serving at the request of the Corporation as a director, officer, employee or agent of another corporation, partnership, joint venture, trust or other enterprise against any expense, liability or loss incurred by such person in any such capacity or arising out of his status as such, whether



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not the Corporation would have the power to indemnify him against such liability under Delaware Law.

(e) The rights and authority conferred in this Section 6.03 shall not be exclusive of any other right which any person may otherwise have or hereafter acquire.

(f) Neither the alteration, amendment, repeal or rescission of this Section 6.03, nor the adoption of any provision of this Certificate of Incorporation or the bylaws of the Corporation, nor, to the fullest extent permitted by Delaware Law, any modification of law, shall eliminate or reduce the effect of this Section 6.03 in respect of any acts or omissions occurring prior to such alteration, amendment, repeal, rescission, adoption or modification.

ARTICLE 7

SECTION 7.01. *Section 203 of Delaware Law.* The Corporation hereby elects not to be governed by Section 203 of Delaware Law.

ARTICLE 8

SECTION 8.01. *Relationship With Roche.* In anticipation that the Corporation will cease to be a wholly owned subsidiary of Roche Holdings, Inc., but that Roche (as defined in Section 8.05) will remain a stockholder of the Corporation, and in anticipation that the Corporation (as defined in Section 8.05) and Roche may engage in the same or similar activities or lines of business and have an interest in the same areas of corporate opportunities, and in recognition of (i) the benefits to be derived by the Corporation through its continued contractual, corporate and business relations with Roche (including service of officers and directors of Roche as directors of the Corporation) and (ii) the difficulties attendant to any director, who desires and endeavors fully to satisfy such director's fiduciary duties, in determining the full scope of such duties in any particular situation, the provisions of this Article 8 are set forth to regulate, define and guide the conduct of certain affairs of the Corporation as they may involve Roche and its officers and directors, and the powers, rights, duties and liabilities

of the Corporation and its officers, directors and stockholders in connection therewith.

SECTION 8.02. *Similar Business Activities.* Except as Roche Holdings, Inc. may otherwise agree in writing,

(a) Roche shall not have a duty to refrain from engaging directly or indirectly in the same or similar business activities or lines of business as the Corporation, and

(b) neither Roche nor any officer or director thereof shall be liable to the Corporation or its stockholders for breach of any fiduciary duty by reason of any such activities of Roche or of such person's participation therein.

In the event that Roche acquires knowledge of a potential transaction or matter that may be a corporate opportunity for both Roche and the Corporation, Roche shall have no duty to communicate or offer such corporate opportunity to the Corporation and shall not be liable to the Corporation or its stockholders for breach of any fiduciary duty as a stockholder of the Corporation or controlling person of a stockholder by reason of the fact that Roche pursues or acquires such corporate opportunity for itself, directs such corporate opportunity to another person or entity, or does not communicate information regarding, or offer, such corporate opportunity to the Corporation.

SECTION 8.03. *Corporate Opportunities.* In the event that a director, officer or employee of the Corporation who is also a director, officer or employee of Roche acquires knowledge of a potential transaction or matter that may be a corporate opportunity for the Corporation and Roche (whether such potential transaction or matter is proposed by a third-party or is conceived of by such director, officer or employee of the Corporation), such director, officer or employee shall be entitled to offer such corporate opportunity to the Corporation or Roche as such director, officer or employee deems appropriate under the circumstances in his sole discretion, and no such director, officer or employee shall be liable to the Corporation or its stockholders for breach of any fiduciary duty or duty of loyalty or failure to act in (or not opposed to) the best interests of the Corporation or the derivation of any improper personal benefit by reason of the fact that (i) such director, officer or employee offered such corporate opportunity to Roche (rather than the Corporation) or did not communicate information regarding such corporate opportunity to the Corporation or (ii) Roche pursues or acquires such corporate opportunity for itself or directs such corporate opportunity to another person or does not communicate information regarding such corporate opportunity to the Corporation.



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SECTION 8.04. Deemed Consent of Stockholders. Any person or entity purchasing or otherwise acquiring any interest in any shares of capital stock of the Corporation shall be deemed to have notice of and to have consented to the provisions of this Article 8.

SECTION 8.05. Definitions. For purposes of this Article 8 and Article 9 only, (i) the term "Corporation" shall mean the Corporation and all corporations, partnerships, joint ventures, associations and other entities in which the Corporation beneficially owns (directly or indirectly) fifty percent or more of the outstanding voting stock, voting power or similar voting interests, and (ii) the term "Roche" shall mean Roche Holdings, Inc. and all corporations, partnerships, joint ventures, associations and other entities (other than the Corporation, defined in accordance with clause (i) of this Section 8.05) that are "affiliates" (as defined in Rule 12b-2 under the Securities Exchange Act of 1934, as amended) of Roche Holdings, Inc.

SECTION 8.06. Termination: Binding Effect. Notwithstanding anything in this Certificate of Incorporation to the contrary, the foregoing provisions of this Article 8 shall expire on the date that Roche ceases to own beneficially Common Stock representing at least 5% of the number of outstanding shares of Common Stock of the Corporation and no person who is a director or officer of the Corporation is also a director or officer of Roche. Neither such expiration, nor the alteration, amendment, change or repeal of any provision of this Article 8 nor the adoption of any provision of this Amended and Restated Certificate of Incorporation inconsistent with any provision of this Article 8 shall eliminate or reduce the effect of this Article 8 in respect of any matter occurring, or any cause of action, suit or claim that, but for this Article 8, would accrue or arise, prior to such expiration, alteration, amendment, repeal or adoption.

SECTION 8.07. Article 9. The provisions of this Article 8 are in addition to the provisions of Article 9.

ARTICLE 9

SECTION 9.01. Contracts Not Void. No contract, agreement, arrangement or transaction (or any amendment, modification or termination thereof) between the Corporation and Roche shall be void or voidable solely for the reason that Roche is a party thereto, or solely because any directors or officers of the Corporation who are affiliated with Roche are present at or participate in the



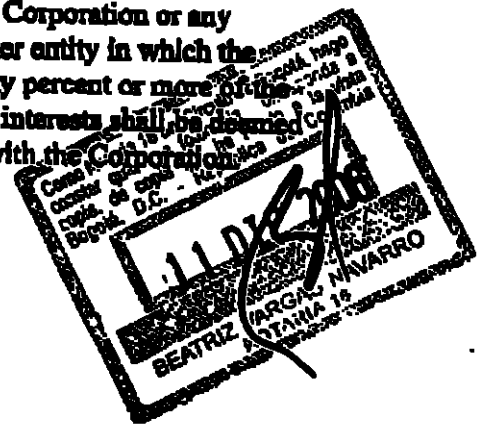
meeting of the Board of Directors or committee thereof which authorizes the contract, agreement, arrangement, transaction, amendment, modification or termination or solely because his or their votes are counted for such purpose, but any such contract, agreement, arrangement or transaction (or any amendment, modification or termination thereof) shall be governed by the provisions of this Amended and Restated Certificate of Incorporation, the Corporation's Bylaws, Delaware Law and other applicable law. For purposes of this Article 9, the terms the "Corporation" and "Roche" have the meanings set forth in Section 8.05.

SECTION 9.02. *Quorum.* Directors of the Corporation who are also directors or officers of Roche may be counted in determining the presence of a quorum at a meeting of the Board of Directors or of a committee that authorizes or approves any such contract, agreement, arrangement or transaction (or amendment, modification or termination thereof). Outstanding shares of Common Stock owned by Roche may be counted in determining the presence of a quorum at a meeting of stockholders that authorizes or approves any such contract, agreement, arrangement or transaction (or amendment, modification or termination thereof).

SECTION 9.03. *No Liability For Good Faith Actions.* Neither Roche nor any officer or director thereof shall be liable to the Corporation or its stockholders for breach of any fiduciary duty or duty of loyalty or failure to act in (or not opposed to) the best interests of the Corporation or the derivation of any improper personal benefit by reason of the fact that Roche or an officer or director thereof in good faith takes any action or exercises any rights or gives or withholds any consent in connection with any agreement or contract between Roche and the Corporation. No vote cast or other action taken by any person who is an officer, director or other representative of Roche, which vote is cast or action is taken by such person in his capacity as a director of this Corporation, shall constitute an action of or the exercise of a right by or a consent of Roche for the purpose of any such agreement or contract.

SECTION 9.04. *Deemed Consent by Stockholders.* Any person or entity purchasing or otherwise acquiring any interest in any shares of capital stock of the Corporation shall be deemed to have notice of and to have consented to the provisions of this Article 9.

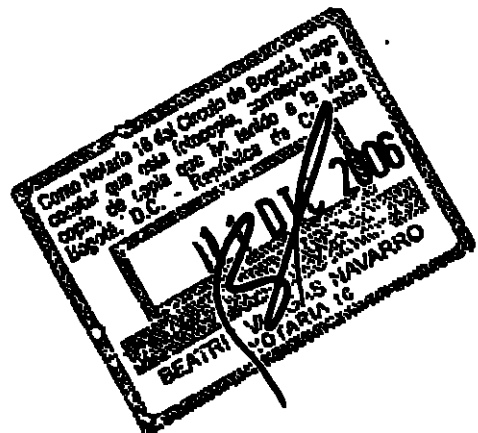
SECTION 9.05. *Contracts Covered.* For purposes of this Article 9, any contract, agreement, arrangement or transaction with the Corporation or any corporation, partnership, joint venture, association or other entity in which the Corporation beneficially owns (directly or indirectly) fifty percent or more of the outstanding voting stock, voting power or similar voting interests shall be deemed to be a contract, agreement, arrangement or transaction with the Corporation.



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SECTION 9.06. *Binding Effect.* Neither the alteration, amendment, change or repeal of any provision of this Article 9 nor the adoption of any provision inconsistent with any provision of this Article 9 shall eliminate or reduce the effect of this Article 9 in respect of any matter occurring, or any cause of action, suit or claim that, but for this Article 9, would accrue or arise, prior to such alteration, amendment, change, repeal or adoption.

SECTION 9.07. *Article 8.* The provisions of this Article 9 are in addition to the provisions of Article 8.

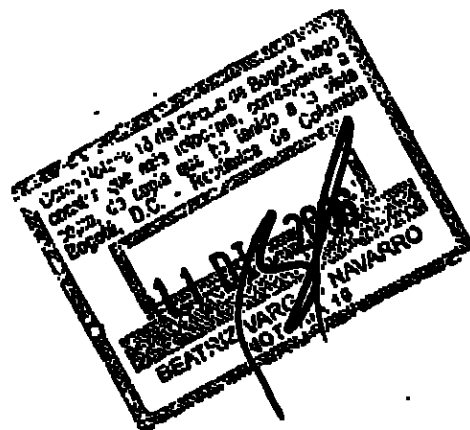


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IN WITNESS WHEREOF, this Amended and Restated Certificate of Incorporation, having been duly adopted in accordance with Sections 242 and 245 of the General Corporation Law of the State of Delaware and by the written consent of the sole stockholder of the Corporation in accordance with Section 228 of the General Corporation Law of the State of Delaware, has been executed this 22nd day of July, 1999.

GENENTECH, INC.

By: /s/ Stephen G. Juelsgaard
Name: Stephen G. Juelsgaard
Title: Senior Vice President,
General Counsel and Secretary



APOSTILLA

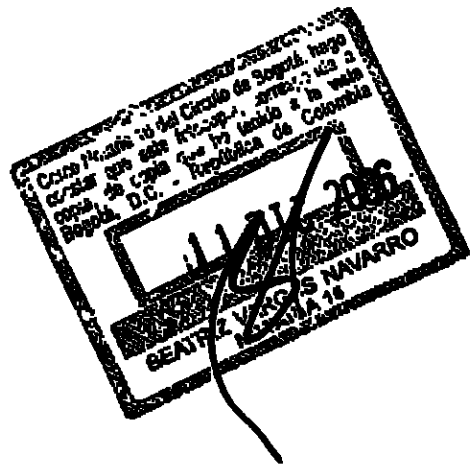
(Convención de La Haya del 5 de Octubre de 1961)

1. País: Estados Unidos de América
Este documento público
2. ha sido firmado por Harriet Smith Windsor
3. actuando en capacidad de Secretaria de Estado de Delaware
4. lleva el sello/timbre de la Oficina del Secretario de Estado

Certificado

5. en Dover, Delaware
6. el veinticuatro de mayo, A. D. 2005
7. por el Secretario de Estado, Departamento de Estado de Delaware
8. No. 0254030
9. Sello/timbre
10. Firma

Harriet Smith Windsor (Firma)
Secretaria de Estado



277 365
preferenciales, por un valor de \$0.02 por acción ("Acción Preferencial"). Trecientos millones (300,000,000) de acciones serán designadas acciones comunes, por un valor de \$0.02 por acción ("Acción Común"). La acción común de la Corporación será toda de una misma clase.

(b) El número de acciones autorizadas de cualquier clase o clases de acciones puede incrementarse o disminuirse (pero no por debajo del número del número de acciones de las mismas que en su momento están vigentes) por el voto afirmativo de una mayoría con poder de votación de las acciones vigentes de la Corporación con derecho a votar por la misma independiente de las consideraciones de la Sección 242(b)(2) de la Ley de Delaware.

SECCION 4.02. Acción Común. (a) *Emisión y Consideración.* Cualquier acción sin emitir o del tesoro del las Acciones Comunes puede emitirse para tal consideración como pueda fijarse de acuerdo con la ley aplicable cada cierto tiempo por la Junta Directiva.

(b) *Dividendos.* Sujeto a los derechos de los accionistas de las Acciones Preferenciales, los accionistas de Acciones Comunes deberán tener el derecho a recibir, cuando y como se declare por la Junta Directiva, de los activos de la Corporación los cuales están disponibles para ello por ley, dividendos pagables ya sea en dinero, propiedad, o acciones y los accionistas de la Acción Preferencial no tendrán derecho a participar en tales dividendos (a menos que la Junta Directiva determine lo contrario en cualquier resolución que condiciona la emisión de una serie de Acciones Preferenciales).

SECCIÓN 4.03. Acción Preferencial

(a) *Serie y Límites de Variaciones entre Series.* Cualquier acción no emitida o del tesoro de las Acciones Preferenciales puede emitirse cada cierto tiempo en una o más series para tal consideración como pueda ser fijada cada cierto tiempo por la Junta Directiva. Antes de que se emita cualquier acción de las Acciones Preferenciales de cualquier serie particular, se deberá presentar un certificado ante el Secretario de Estado de Delaware estableciendo las designaciones, derechos, privilegios, restricciones, y condiciones que irán anexas a las Acciones Preferenciales de tal serie y tales otras cuestiones como puedan requerirse, y la Junta Directiva deberá fijar y determinar, y por la presente está expresamente autorizada para fijar y determinar, en la forma provista por la ley, los



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CORREGIDO Y VUELTO A REDACTAR
CERTIFICADO DE INCORPORACIÓN
GENENTECH, INC.

Genentech, Inc (la "Corporación"), una corporación organizada y existente bajo y por virtud de la Ley General de Corporación del Estado de Delaware, por la presente corrige el Certificado de Incorporación, el fue presentado originalmente el 23 de diciembre de 1986, bajo el nombre "Genentech Delaware, Inc.", y corregido subsiguientemente.

Por la presente se vuelve a redactar el certificado de incorporación de la Corporación y se corrige aun más para que diga en su totalidad lo que sigue:

ARTICULO 1

El nombre de la Corporación es: Genentech, Inc.

ARTICULO 2

La dirección de la oficina registrada de la Corporación en el Estado de Delaware es Corporation Service Company, 1013 Centre Road, Ciudad de Wilmington, Condado de New castle, Delaware 19805. El nombre de su agente registrado en tal dirección es Corporation Service Company.

ARTICULO 3

El propósito de a Corporación es el de participar en cualquier acto o actividad legal para las cuales las corporaciones pueden ser organizadas bajo la Ley General de Corporación del Estado de Delaware como entidad o puede ser modificada la misma en adelante ("Delaware Law").

ARTICULO 4

SECCION 4.01. *Acciones de Capital.* (a) la Corporación está autorizada a emitir dos clases de acciones que deben designarse, respectivamente, acciones preferenciales y acciones comunes. El número total de acciones que la Corporación esta autorizada a emitir es de cuatrocientos millones (400,000,000) de acciones. Cien millones (100,000,000) de acciones serán designadas acciones



particulares de las acciones de tal serie (siempre y cuando no sean inconsistentes con las condiciones de este Artículo 4 aplicables a todas las series de Acciones Preferenciales), incluyendo, pero sin limitarse a, las siguientes:

(i) la designación distintiva de tal serie y el número de las acciones que han de constituir tal serie, numero que puede incrementarse (excepto cuando lo determine de otro modo la Junta Directiva al crear tal serie) o reducida (pero no por debajo del número de acciones de las mismas que están vigentes) cada cierto tiempo por una acción semejante de la Junta Directiva;

(ii) la tasa anual de dividendos a pagar sobre las acciones de tal serie, las condiciones sobre las cuales se pagarán tales dividendos y le fecha a partir de la cual los dividendos serán acumulativos en el evento que la Junta Directiva determine que los dividendos serán acumulativos;

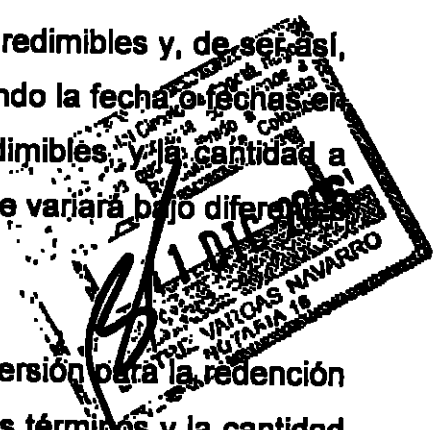
(iii) si la serie tendrá o no derechos de voto, además de los derechos de voto provistos por la ley, y de ser así, los términos de tales derechos de voto;

(iv) si tal serie tendrá o no privilegios de conversión y, de ser así, los términos y condiciones de tal conversión, incluyendo, pero sin estar limitado a, la condición para ajuste de la tasa de conversión en tales eventos y de tal forma como la Junta Directiva lo determine;

(v) si las acciones de tal serie serán o no redimibles y, de ser así, los términos y condiciones de tal redención, incluyendo la fecha o fechas en las cuales o después de las cuales pueden ser redimibles, y la cantidad a pagar por acción en caso de redención, cantidad que variará bajo diferentes condiciones y en diferentes fechas de redención;

(vi) si tal serie tendrá o no un fondo de inversión para la redención o compra de acciones de esa serie y, de ser así, los términos y la cantidad de tal fondo de inversión;

(vii) los derechos de las acciones de tal serie en caso de una liquidación, disolución o cierre voluntaria o involuntaria de la Corporación, y



los derechos relativos de prioridad, de haber, de pago de acciones de esa serie; y

(viii) cualquier otro derecho relativo, preferencia y limitación de tal serie.

ARTICULO 5

SECCION 5.01. Enmiendas a los estatutos por los Directores. (a) En adición y no limitando los poderes conferidos por estatutos, la Junta Directiva esta expresamente autorizada para hacer, alterar, enmendar, rechazar y rescindir los estatutos de la Corporación, sujetos a las secciones 5.01(b) y 5.01(c) a continuación.

(b) Las secciones 2.02, 3.02, 3.03(a), 3.04, 3.05, 3.14 y 5.06 de los estatutos solo pueden alterarse, enmendarse, repelerse, o rescindirse por el voto afirmativo de los accionistas de capital con derecho a votar las mismas que representen más del 60% de las acciones con derecho a votar las mismas.

(c) Las secciones 3.03(c) y 3.15 de los estatutos solo pueden ser alteradas, enmendadas, repelidas, o rescindidas por le voto afirmativo de los accionistas de capital con derecho a votar las mismas que representen más del 90% de las acciones con derecho a votar las mismas.

SECCION 5.02. Enmienda del Certificado de Incorporación. La sección 5.01 (b) de este Certificado de Incorporación solo puede ser alterada, enmendada, repelida, o rescindida por el voto afirmativo de los accionistas de capital con derecho a voto sobre la misma que representen a más del 60% de las acciones con derecho a votar la misma. La sección 5.01(c) y los Artículos 8 y 9 de este Certificado de Incorporación solo pueden alterarse, enmendarse, repelerse o rescindirse por el voto afirmativo de los accionistas de capital con derecho a votar la misma que representen más del 90% de las acciones con derecho a votar la misma.

ARTICULO 6

SECCION 6.01. Elección del Directorio por los Accionistas de Acción Preferencial. Durante cualquier periodo cuando los accionistas de cualquier

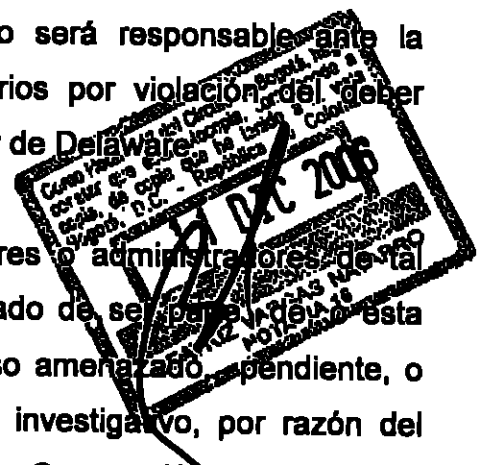
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acción preferencial cualquiera de una o más series de las mismas, que voten como una clase, tengan derecho a elegir un número específico de directores, por razón a atrasos en los dividendos u otras condiciones que les dan el derecho a hacerlo, entonces y durante el tiempo que continúe el derecho (i) el número de directores autorizado de otro modo será incrementado por tal número de directores especificado, y los accionistas de tales Acciones Preferenciales o tales series de las mismas, que votan como una clase, tendrán derecho a elegir los directores adicionales para lo que se provee, de acuerdo con las condiciones de tales Acciones Preferenciales o series; (ii) cada uno de tales directores adicionales servirá durante tal termino, y tendrá tales poderes de voto, como se han de otorgar en las provisiones pertinentes a tales Acciones Preferenciales o series; y (iii) siempre que los accionistas de cualquiera de tales Acciones Preferenciales o series de las mismas están desprovistos de tales derechos a elegir un número específico de directores, votando como clase, de acuerdo con las condiciones de tales Acciones preferenciales o series, los términos de oficio de todos los directores elegidos por los accionistas de tales Acciones preferenciales o series, que votan como clase, de acuerdo con las condiciones o elegidos para llenar cualquier vacante resultante de la muerte, renuncia o remoción de directores así elegidos por los accionistas de tales Acciones Preferenciales o series, terminarán en adelante y el número autorizado de directores será reducido en concordancia.

SECCION 6.02. *Votaciones.* Las elecciones de directores en una reunión anual o especial de accionistas necesaria no serán por votos escritos a menos que los estatutos de la Corporación digan lo contrario.

SECCION 6.03. *Eliminación de cierta responsabilidad personal de los directores.* (a) Un director de la Corporación no será responsable ante la Corporación o sus accionistas por daños monetarios por violación del deber fiduciario como director hasta donde lo permita la Ley de Delaware.

(b) Cada persona (y sus herederos, ejecutores o administradores de tal persona) quien fue o es una parte o esta amenazado de ser parte en esta involucrado en cualquier acción, demanda o proceso amenazado, pendiente, o completado, ya sea civil, criminal, administrativo o investigativo, por razón del hecho de que esa persona es o fue un director de la Corporación o es o estaba sirviendo por solicitud de la Corporación como un director de otra corporación, sociedad, sociedad de riesgo compartido, fideicomiso u otra empresa, será indemnizado y completamente protegido por la Corporación hasta donde lo



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permita la Ley de Delaware. El derecho a la indemnización concedido en esta sección 6.03 también debe incluir el derecho a que la Corporación le pague los gastos incurridos en conexión con cualquiera de tales procesos por adelantado de su decisión final hasta donde los autoriza la Ley de Delaware. El derecho a indemnización conferido en esta sección 6.03 será un derecho contractual.

(c) La Corporación puede, por acción de su Junta Directiva, proporcionar indemnización a tales funcionarios, empleados y agentes de la Corporación hasta tal punto y a tal efecto como lo determine apropiado la Junta Directiva y lo autorice la Ley de Delaware.

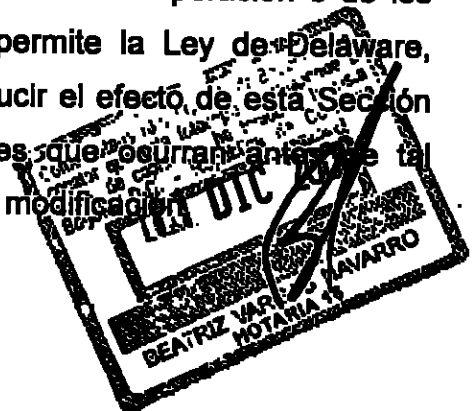
(d) La Corporación tendrá el poder de comprar y mantener un seguro a nombre de cualquier persona quien es o fue director, funcionario, empleado o agente de la corporación, o esta o estuvo en servicio por pedido de la Corporación como director, funcionario, empleado o agente de otra corporación, sociedad, sociedad de riesgo compartido, fideicomiso u otra empresa contra cualquier gasto, responsabilidad o pérdida incurrida por tal persona en cualquier capacidad o que surge de su estado como tal, ya sea si la Corporación tiene o no el poder de indemnizarlo contra tal responsabilidad bajo la ley de Delaware.

(e) Los derechos y autoridad conferidos en esta Sección 6.03 no serán exclusivos de ningún otro derecho el cual cualquier otra persona pueda tener o adquirir de aquí en adelante.

(f) Ni la alteración, enmienda, rechazo o rescisión de esta Sección 6.03, ni la adopción de ninguna condición de este Certificado de Incorporación o de los Estatutos de la Corporación, ni, hasta donde lo permite la Ley de Delaware, ninguna modificación de la ley, debe eliminar o reducir el efecto de esta Sección 6.03 respecto a ninguno de los actos u omisiones que ocurran antes de tal alteración, enmienda, rechazo, rescisión, adopción o modificación.

ARTICULO 7

SECCION 7.01, Sección 203 de la Ley de Delaware. Por la presente la Corporación elige no ser gobernada por la Sección 203 de la Ley de Delaware.



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ARTICULO 8

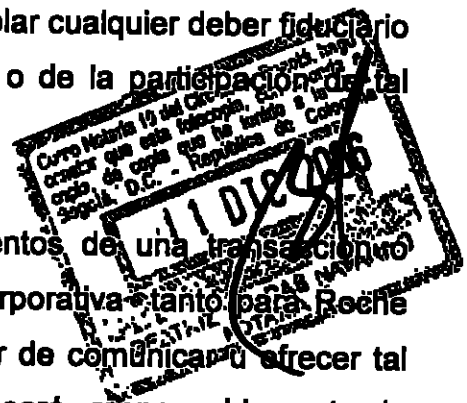
SECCION 8.01. *Relación con Roche.* Anticipándose a que la Corporación cese de ser un subsidiaria completamente de propiedad de Roche Holdings, Inc., pero que Roche (como se define en la sección 8.05) permanezca siendo un accionista de la Corporación, y anticipándose a que la Corporación (como se define en la Sección 8.05) y Roche puede entrar en actividades o líneas de negocios semejantes o similares y tener un interés en las mismas áreas de oportunidades corporativas, y en reconocimiento de (i) los beneficios a ser derivados por la Corporación a través de sus relaciones contractuales, corporativas y de negocios con Roche (incluyendo el servicio de funcionarios y directores de Roche como directores de la Corporación) y (ii) las dificultades concomitantes a cualquier director, quien desea y se propone satisfacer completamente tales deberes fiduciarios del director, al determinar el alcance completo de tales deberes en cualquier situación particular, las condiciones de este artículos 8 se establecen para regular, definir y guiar la conducta de ciertas cuestiones de la Corporación ya que pueden involucrar a Roche y sus funcionarios y directores, y los poderes, derechos, deberes y responsabilidades de la Corporación y sus funcionarios, directores y accionistas en conexión con éste.

SECCION 8.02. *Actividades en Negocios Similares.* Excepto que Roche Holdings, Inc. pueda de otra forma acordarlo por escrito,

(a) Roche no tendrá el deber de abstenerse de entrar directamente o indirectamente en actividades de negocios o líneas de negocios semejantes o similares a las de la Corporación, y

(b) ni Roche ni ninguno de sus funcionarios o director de la misma será responsable a la Corporación o sus accionistas por violar cualquier deber fiduciario por razón de alguna de tales actividades de Roche o de la participación de tal persona en las mismas.

En el evento que Roche adquiera conocimientos de una transacción o materia potencial que pueda ser una oportunidad corporativa tanto para Roche como para la Corporación, Roche no tendrá el deber de comunicar o ofrecer tal oportunidad corporativa a la Corporación y no será responsable ante la Corporación o sus accionistas por violar ningún deber fiduciario como accionista de la Corporación o la persona que controla a un accionista por razón del hecho



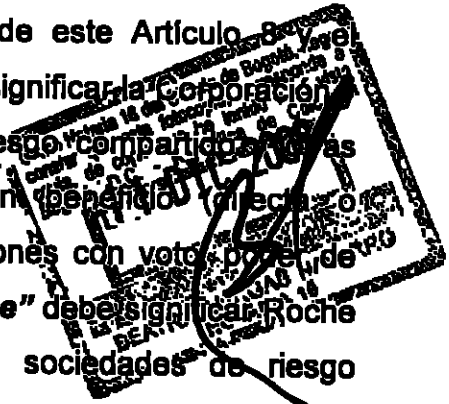
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que Roche prosigue o adquiere tal oportunidad corporativa para si misma, dirige tal oportunidad corporativa a otra persona o entidad, o no comunica información respecto, u ofrece, tal oportunidad corporativa a la Corporación.

SECCION 8.03. Oportunidades Corporativas. En el evento que un director, funcionario o empleado de la Corporación quien también es director, funcionario o empleado de Roche adquiera conocimiento de una transacción o materia potencial que pueda ser una oportunidad corporativa para la Corporación y Roche (ya sea si esta transacción o materia potencial es propuesta por un tercero o es concebida por tal director, funcionario o empleado de la Corporación o no), tal director, funcionario o empleado debe tener el derecho a ofrecer tal oportunidad corporativa a la Corporación o Roche como tal director, funcionario o empleado los considere apropiado bajo las circunstancias de su discreción, y tal director, funcionario o empleado no será responsable a la Corporación o sus accionistas por violar ningún deber fiduciario o deber de lealtad o faltar en actuar en (o no oponerse a) los mejores intereses de la Corporación o la derivación de ningún beneficio personal inapropiado por razón del hecho que (i) tal director, funcionario o empleado ofreció tal oportunidad corporativa a Roche (en lugar de a la Corporación) o no comunicó información respecto a tal oportunidad corporativa a la Corporación o (ii) Roche busca o adquiere tal oportunidad corporativa para si misma o dirige tal oportunidad corporativa a otra persona o no comunica información respecto a tal oportunidad corporativa a la Corporación.

SECCION 8.04. Consentimiento estimado de los accionistas. Se considerará que cualquier persona o entidad que compre o de otro modo adquiera algún interés en cualquiera de las acciones de capital de la Corporación ha tenido noticia o ha estado de acuerdo con las condiciones de este Artículo 8.

SECCION 8.05, Definiciones. Para propósitos de este Artículo 8 y el Artículo 9 solamente, (i) el término "**Corporación**" debe significar la Corporación, todas las corporaciones, sociedades, sociedades de riesgo compartido, y otras entidades en las cuales la Corporación posee con beneficio (directo o indirectamente) cincuenta por ciento o más de las acciones con voto, poder de voto o intereses de voto similares, y (ii) el término "**Roche**" debe significar Roche Holdings, Inc y todas las corporaciones, sociedades, sociedades de riesgo compartido, asociaciones y otras entidades (diferentes a la Corporación, definida de acuerdo con la cláusula (i) de esta sección 8.05) que son "**afiliados**" (como se



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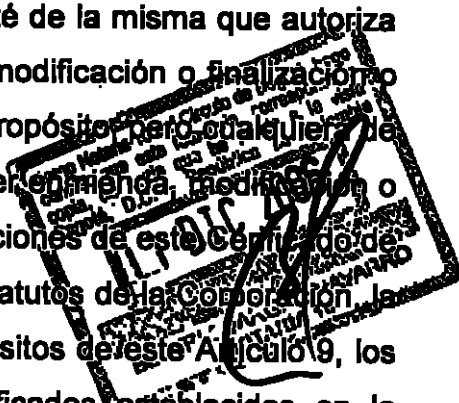
define en la Regla 12b-2 bajo el Acta de Intercambio de Valores de 1934, como se enmendó) de Roche Holdings, Inc.

SECCION 8.06, Finalización: Efecto Obligante. Son consideración con nada en este Certificado de Incorporación que lo contradiga, las condiciones anteriores de este Artículo 8 deberán expirar en la fecha que Roche cese de poseer con beneficios Acciones Comunes que representen al menos 5% del número de acciones vigentes de las Acciones Comunes de la Corporación y nadie que se director o funcionario de la Corporación sea también director o funcionario de Roche. Ni tal vencimiento, ni la alteración, enmienda, cambio o rechazo de alguna de las condiciones de este Artículo 8 así como tampoco ninguna condición de este Certificado de Incorporación Enmendado y Vuelto a Redactar inconsistente con alguna de las condiciones de este Artículo 8 eliminará o reducirá el efecto de este Artículo 8 respecto a cualquier materia, o cualquier causa de acción, demanda o reclamo que, excepto por este Artículo 8, pueda acaecer o surgir, antes de tal vencimiento, alteración, enmienda o adopción.

SECCION 8.07. Artículo 9. Las condiciones de este Artículo son adicionales a las condiciones del Artículo 9.

ARTICULO 9

SECCION 9.01. Contratos no nulos. Ningún contrato, acuerdo, arreglo o transacción (o cualquier enmienda, modificación o finalización de los mismos) entre la Corporación y Roche será nulo u anulable solamente por razón de que Roche es parte del mismo, o solamente porque cualquiera de los directores o funcionarios de la Corporación quienes están afiliados a Roche están presentes en o participan en la reunión de la Junta Directiva o comité de la misma que autoriza el contrato, acuerdo, arreglo, transacción, enmienda, modificación o finalización o solamente porque su o sus votos se cuentan para tal propósito, pero cualquiera de tal contrato, acuerdo, arreglo o transacción (o cualquier enmienda, modificación o finalización del mismo) estará gobernado por las condiciones de este Certificado de Incorporación Enmendado y vuelto a Redactar, los Estatutos de la Corporación, la Ley de Delaware y otras leyes aplicables. Para propósitos de este Artículo 9, los términos "**Corporación**" y "**Roche**" tienen los significados establecidos en la Sección 8.05.



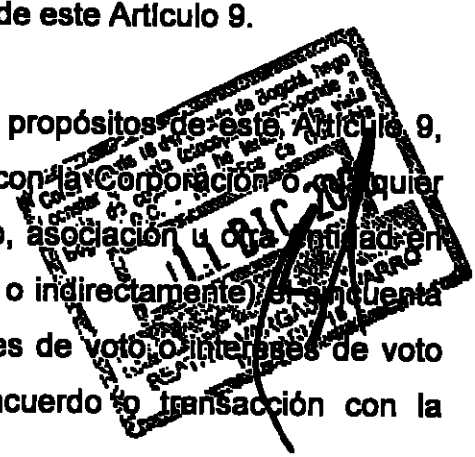
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SECCION 9.02. *Quórum.* Los Directores de la Corporación que son también directores o funcionarios de Roche pueden contarse al determinar la presencia de un quórum en una reunión de la Junta Directiva o de un comité que autoriza o aprueba cualquiera de tal contrato, acuerdo, arreglo o transacción (o cualquier enmienda, modificación o finalización del mismo). Las acciones vigentes de las Acciones Comunes de propiedad de Roche pueden contarse al determinar la presencia de un quórum en una reunión de accionistas que autorice o apruebe tal contrato, acuerdo, arreglo o transacción (o cualquier enmienda, modificación o finalización del mismo).

SECCION 9.03. *No responsabilidad por acciones de buena fe.* Ni Roche ni ningún funcionario o director de la misma será responsable ante la Corporación o sus accionistas por violar cualquiera de los deberes fiduciarios o deberes de lealtad o por fallar en actuar en (o no oponerse a) los mejores intereses de la Corporación o por derivación de cualquier beneficio personal por razón del hecho que Roche o un funcionario o director de la misma de buena fe tome cualquier acción o ejerza cualquier derecho o de o retenga cualquier consentimiento en conexión con cualquier acuerdo o contrato entre Roche y la Corporación. Ningún voto dado u otra acción tomada por cualquier persona que es un funcionario, director o representante de Roche, donde el voto o acción es tomado por tal persona en su capacidad como director de esta Corporación, constituirá una acción de o el ejercicio de un derecho o un consentimiento de Roche para los propósitos de cualquiera de tal acuerdo o contrato.

SECCION 9.04. *Consentimiento estimado de los accionistas.* Se considerará que cualquier persona o entidad que compre o de otro modo adquiera algún interés en cualquiera de las acciones de capital de la Corporación ha tenido noticia o ha estado de acuerdo con las condiciones de este Artículo 9.

SECCION 9.05. *Contratos cubiertos.* Para propósitos de este Artículo 9, cualquier contrato, acuerdo, arreglo o transacción con la Corporación o cualquier corporación, sociedad, sociedad de riesgo conjunto, asociación u otra entidad en la cual la Corporación posee con beneficio (directa o indirectamente) el cincuenta por ciento o más de las acciones con voto, poderes de voto o intereses de voto similares vigentes se considerará un contrato, acuerdo o transacción con la Corporación.



SECCION 9.06. *Efecto obligante.* Ni la alteración, enmienda, cambio o rechazo de alguna de las condiciones de este Artículo 9 ni la adopción de alguna condición inconsistente con cualquier condición de este Artículo 9 eliminará o reducirá el efecto de este Artículo 9 respecto a cualquier materia que ocurra, o cualquier causa de acción, demanda o reclamo que, a no ser por este Artículo 9, pueda acaecer o surgir, antes de tal alteración, enmienda, cambio, rechazo o adopción.

SECCION 9.07. *Artículo 8.* Las condiciones de este Artículo 9 son adicionales a las condiciones del Artículo 8.

EN TESTIMONIO DE LO CUAL, este Certificado de Incorporación Enmendado y vuelto a Redactar, el cual ha sido adoptado de acuerdo con las secciones 242 y 245 de la Ley General de Corporación del Estado de Delaware y por consentimiento escrito de el único accionista de la Corporación de acuerdo con la sección 228 de Ley General de Corporación del Estado de Delaware, ha sido ejecutado este 22 de Julio de 1999.

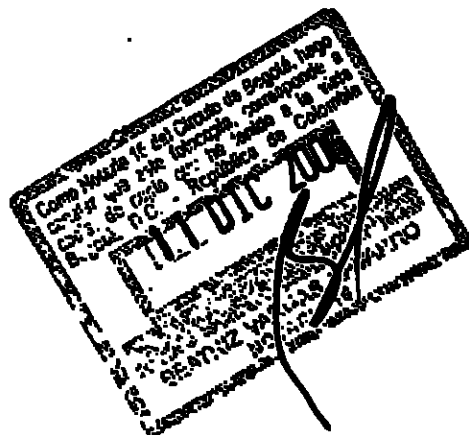
GENENTECH, INC.

Por: /s/ Stephen G. Juelsgaard

Nombre: Stephen G. Juelsgaard

Título: Vicepresidente

Consejo General y Secretaría



Apostille

(Convention de La Haye du 5 Octobre 1961)

1. Country: United States of America

This public document:

2. has been signed by Harriet Smith Windsor

3. acting in the capacity of Secretary of State of Delaware

4. bears the seal/stamp of Office of Secretary of State

Certified

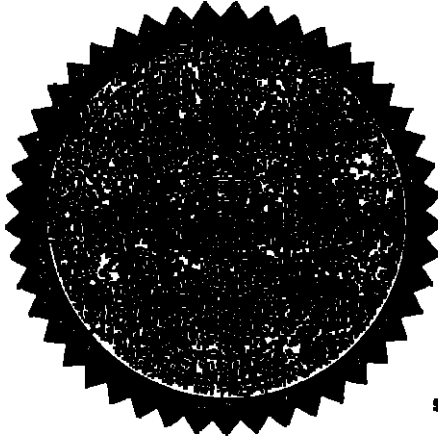
5. at Dover, Delaware

6. the twenty-fourth day of May, A.D. 2005

7. by Secretary of State, Delaware Department of State

8. No. 0254031

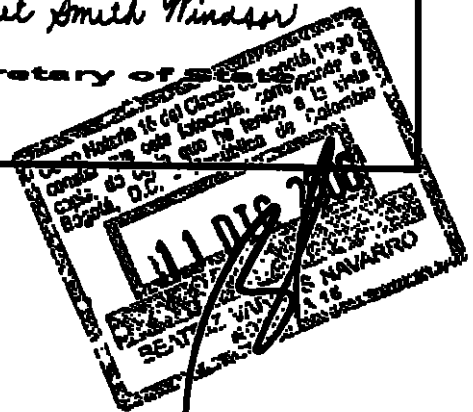
9. Seal/Stamp:



10. Signature:

Harriet Smith Windsor

Secretary of State

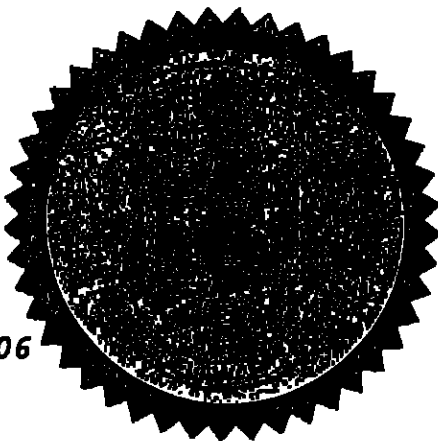


Delaware

PAGE 1

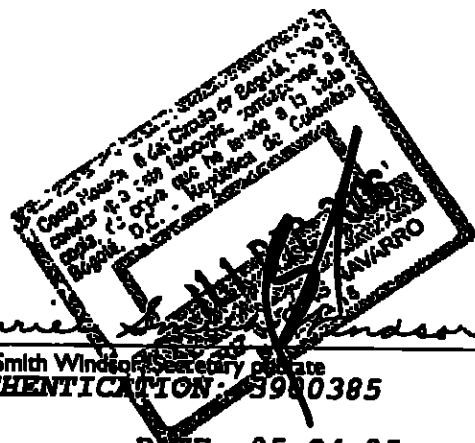
The First State

I, HARRIET SMITH WINDSOR, SECRETARY OF STATE OF THE STATE OF DELAWARE, DO HEREBY CERTIFY THE ATTACHED IS A TRUE AND CORRECT COPY OF THE CERTIFICATE OF AMENDMENT OF "GENENTECH, INC.", FILED IN THIS OFFICE ON THE FIFTEENTH DAY OF MAY, A.D. 2000, AT 5 O'CLOCK P.M.



2112549

050426506



Harriet Smith Windsor, Secretary of State
AUTHENTICATION: 5980385

DATE: 05-24-05

CERTIFICATE OF AMENDMENT
OF AMENDED AND RESTATED
CERTIFICATE OF INCORPORATION

Genentech, Inc. a corporation organized and existing under and by virtue of the General Corporation Law of the State of Delaware.

DOES HEREBY CERTIFY:

FIRST: That at a meeting of the Board of Directors of Genentech, Inc. resolutions were duly adopted setting forth a proposed amendment of the Amended and Restated Certificate of Incorporation of said corporation, declaring said amendment to be advisable and calling a meeting of the stockholders of said corporation for consideration thereof. The resolution setting forth the proposed amendment is as follows:

RESOLVED, that the Amended and Restated Certificate of Incorporation of this corporation be amended by changing "Section 4.01(a)" of the Article thereof numbered "4" so that, as amended, said Section of said Article shall be and read as follows:

"Section 4.01. *Capital Stock.* (a) The Corporation is authorized to issue two classes of stock to be designated, respectively, preferred stock and common stock. The total number of shares which the Corporation is authorized to issue is seven hundred million (700,000,000) shares. One hundred million (100,000,000) shares shall be designated preferred stock, par value \$0.02 per share ("Preferred Stock"). Six hundred million (600,000,000) shares shall be designated common stock, par value \$0.02 per share ("Common Stock"). The Common Stock of the Corporation shall be all of one class."

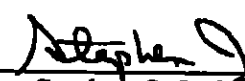
SECOND: That thereafter, pursuant to resolution of its Board of Directors, the regular meeting of the stockholders of said corporation was duly called and held upon notice in accordance with Section 222 of the General Corporation Law of the State of Delaware at which meeting the necessary number of shares as required by statute were voted in favor of the amendment.

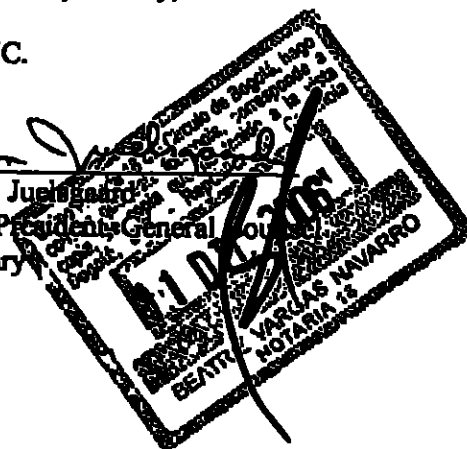
THIRD: That said amendment was duly adopted in accordance with the provisions of Section 242 of the General Corporation Law of the State of Delaware.

FOURTH: That the capital of said corporation shall not be reduced under or by reason of said amendment.

IN WITNESS WHEREOF, said Genentech, Inc. has caused this Certificate of Amendment to be signed by Stephen G. Juelsgaard, an Authorized Officer, this 15th day of May, 2000.

GENENTECH, INC.

By: 
Name: Stephen G. Juelsgaard
Title: Senior Vice President, General
and Secretary



APOSTILLA

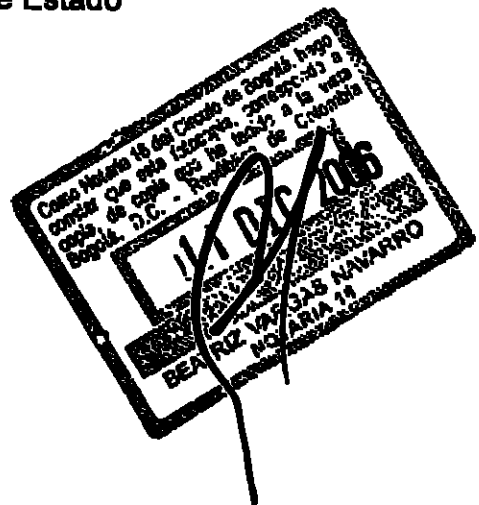
(Convención de La Haya del 5 de Octubre de 1961)

1. País: Estados Unidos de América
Este documento público
2. ha sido firmado por Harriet Smith Windsor
3. actuando en capacidad de Secretaria de Estado de Delaware
4. lleva el sello/timbre de la Oficina del Secretario de Estado

Certificado

5. en Dover, Delaware
6. el veinticuatro de mayo, A. D. 2005
7. por el Secretario de Estado, Departamento de Estado de Delaware
8. No. 0254031
9. Sello/timbre
10. Firma

Harriet Smith Windsor (Firma)
Secretaria de Estado



DELAWARE
EL PRIMER ESTADO

380
THT

YO, HARRIET SMITH WINDSOR, SECRETARIA DE ESTADO DEL ESTADO DE DELAWARE, CERTIFICO POR LA PRESENTE QUE EL ANEXO ES UNA COPIA AUTÉNTICA Y CORRECTA DEL CERTIFICADO VUELTO A REDACTAR DE "GENENTECH, INC", PRESENTADO EN ESTA OFICINA EL DECIMOQUINTO DIA DE MAYO, A. D. 2000, A LAS 5 EN PUNTO DE LA TARDE.



Harriet Smith Windsor (Firma)
Harriet Smith Windsor, Secretaria de Estado
AUTENTICACIÓN: 3900385

FECHA: 05 - 24 - 05

DELAWARE
EL PRIMER ESTADO

381
JH

YO, HARRIET SMITH WINDSOR, SECRETARIA DE ESTADO DEL ESTADO DE DELAWARE, CERTIFICO POR LA PRESENTE QUE EL ANEXO ES UNA COPIA AUTÉNTICA Y CORRECTA DEL CERTIFICADO VUELTO A REDACTAR DE "GENENTECH, INC", PRESENTADO EN ESTA OFICINA EL VIGÉSIMO SEGUNDO DÍA DE JULIO, A. D. 1999, A LAS 9 EN PUNTO DE LA MAÑANA.



Harriet Smith Windsor (Firma)
Harriet Smith Windsor, Secretaria de Estado
AUTENTICACIÓN: 3900384

FECHA: 05 - 24 - 05

CERTIFICADO DE ENMIENDA DEL
CERTIFICADO DE INCORPORACIÓN
CORREGIDO Y VUELTO A REDACTAR

382
TAD

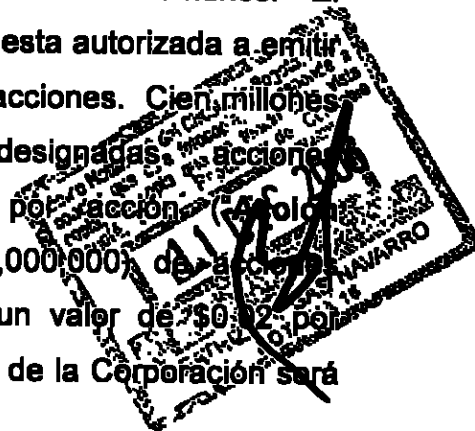
Genentech, Inc. una corporación organizada y existente bajo y por virtud de la Ley General de Corporación del Estado de Delaware.

CERTIFICA POR LA PRESENTE:

PRIMERO: Que en una reunión de la Junta Directiva de Genentech, Inc. se adoptaron debidamente resoluciones que establecen una enmienda propuesta del certificado de Incorporación Corregido y vuelto a Redactar de dicha corporación, declarando dicha enmienda como aconsejable y llamando a una reunión de accionistas de dicha corporación para consideración de la misma. La resolución establece la enmienda propuesta de la siguiente manera:

RESUELVE, que el Certificado de Incorporación Corregido y vuelto a Redactar de esta corporación se enmienda cambiando "la Sección 4.01(a)" del Artículo allí numerado "4" tal que, como se enmienda, dicha Sección de dicho Artículo se lea de la siguiente manera:

SECCION 4.01. *Acciones de Capital.* (a) la Corporación está autorizada a emitir dos clases de acciones que deben designarse, respectivamente, acciones preferenciales y acciones comunes. El número total de acciones que la Corporación esta autorizada a emitir es de setecientos millones (700,000,000) de acciones. Cien millones (100,000,000) de acciones serán designadas acciones preferenciales, por un valor de \$0.02 por acción ("Acción Preferencial"). Seiscientos millones (600,000,000) de acciones serán designadas acciones comunes, por un valor de \$0.02 por acción ("Acción Común"). La acción común de la Corporación será toda de una misma clase.



SEGUNDO: Que por lo tanto, de acuerdo con la resolución de su Junta Directiva, la reunión ordinaria de accionistas de dicha corporación se convocó debidamente y se sostuvo al ser anunciada de acuerdo con la sección 222 de la Ley General de Corporación del Estado de Delaware, en donde el número necesario de acciones requerido por los estatutos votaron a favor de la enmienda.

383
T
TERCERO: Que dicha enmienda se adoptó debidamente de acuerdo con lo
previsto en la Sección 242 de la Ley General de Corporación del Estado de
Delaware.

CUARTO: Que el capital de dicha corporación no será reducido bajo o por razón
de dicha enmienda

EN TESTIMONIO DE LO CUAL, dicha Genentech, Inc. ha causado este
Certificado de Enmienda para que sea firmado por Stephen G. Juelsgaard, un
Funcionario Autorizado, este 15 de Mayo de 2000.

GENENTECH, INC.

Por: (Firma)

Nombre: Stephen G. Juelsgaard

Título: Vicepresidente, Consejo
y Secretaría General.



384

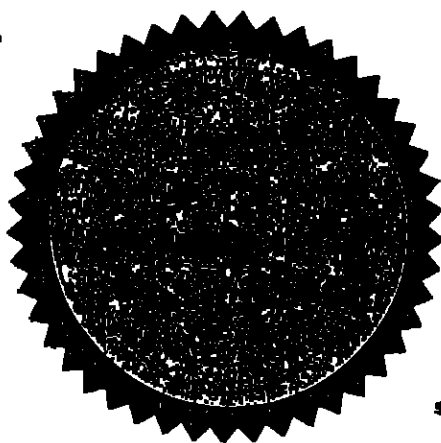
Apostille

(Convention de La Haye du 5 Octobre 1961)

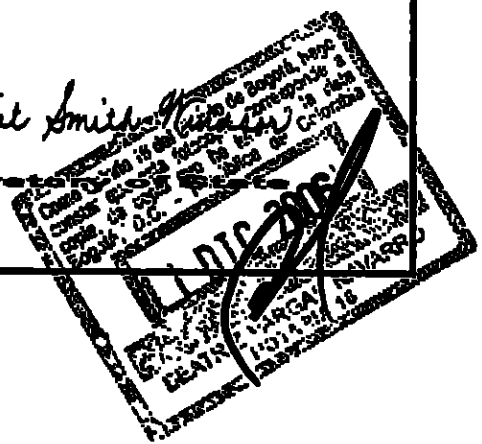
1. Country: United States of America
2. This public document:
has been signed by Harriet Smith Windsor
3. acting in the capacity of Secretary of State of Delaware
4. bears the seal/stamp of Office of Secretary of State

Certified

5. at Dover, Delaware
6. the twenty-fourth day of May, A.D. 2005
7. by Secretary of State, Delaware Department of State
8. No. 0254032
9. Seal/Stamp:
10. Signature:



Harriet Smith Windsor
Secretary of State



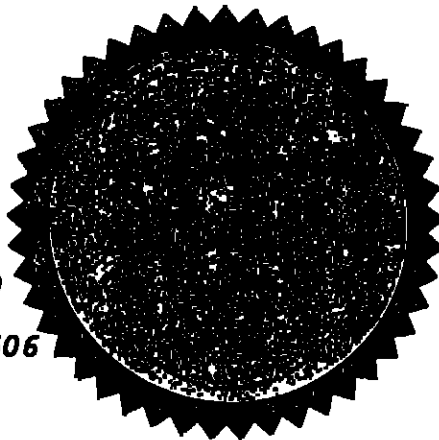
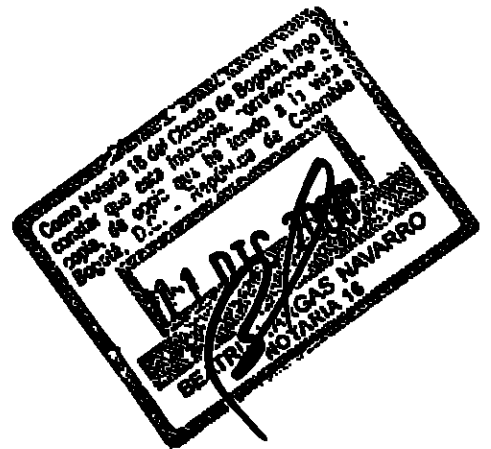
Delaware

PAGE 1

385
7/7

The First State

I, HARRIET SMITH WINDSOR, SECRETARY OF STATE OF THE STATE OF DELAWARE, DO HEREBY CERTIFY THE ATTACHED IS A TRUE AND CORRECT COPY OF THE CERTIFICATE OF AMENDMENT OF "GENENTECH, INC.", FILED IN THIS OFFICE ON THE TENTH DAY OF MAY, A.D. 2001, AT 2:05 O'CLOCK P.M.



2112549

050426506

Harriet Smith Windsor

Harriet Smith Windsor, Secretary of State
AUTHENTICATION: 3900386

DATE: 05-24-05

244 386

CERTIFICATE OF AMENDMENT
OF AMENDED AND RESTATED
CERTIFICATE OF INCORPORATION

Genentech, Inc. a corporation organized and existing under and by virtue of the General Corporation Law of the State of Delaware.

DOES HEREBY CERTIFY:

FIRST: That at a meeting of the Board of Directors of Genentech, Inc. resolutions were duly adopted setting forth a proposed amendment of the Amended and Restated Certificate of Incorporation of said corporation, declaring said amendment to be advisable and calling a meeting of the stockholders of said corporation for consideration thereof. The resolution setting forth the proposed amendment is as follows:

RESOLVED, that the Amended and Restated Certificate of Incorporation of this corporation be amended by changing "Section 4.01(a)" of the Article thereof numbered "4" so that, as amended, said Section of said Article shall be and read as follows:

"Section 4.01. *Capital Stock.* (a) The Corporation is authorized to issue two classes of stock to be designated, respectively, preferred stock and common stock. The total number of shares which the Corporation is authorized to issue is one billion three hundred million (1,300,000,000) shares. One hundred million (100,000,000) shares shall be designated preferred stock, par value \$0.02 per share ("Preferred Stock"). One billion two hundred million (1,200,000,000) shares shall be designated common stock, par value \$0.02 per share ("Common Stock"). The Common Stock of the Corporation shall be all of one class."

SECOND: That thereafter, pursuant to resolution of its Board of Directors, the regular meeting of the stockholders of said corporation was duly called and held upon notice in accordance with Section 222 of the General Corporation Law of the State of Delaware at which meeting the necessary number of shares as required by statute were voted in favor of the amendment.

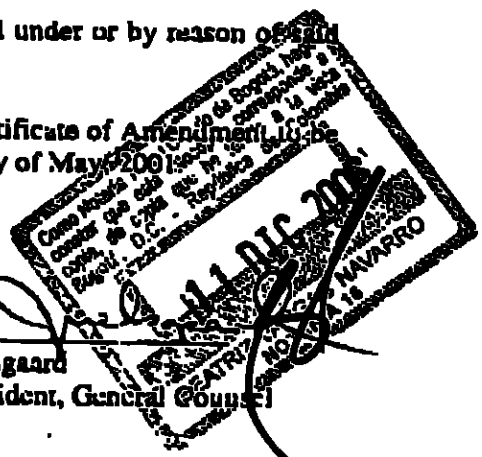
THIRD: That said amendment was duly adopted in accordance with the provisions of Section 242 of the General Corporation Law of the State of Delaware.

FOURTH: That the capital of said corporation shall not be reduced under or by reason of said amendment.

IN WITNESS WHEREOF, said Genentech, Inc. has caused this Certificate of Amendment to be signed by Stephen G. Juelsgaard, an Authorized Officer, this 10th day of May 2001.

GENENTECH, INC.

By: Stephen G. Juelsgaard
Name: Stephen G. Juelsgaard
Title: Senior Vice President, General Counsel
and Secretary



APOSTILLA

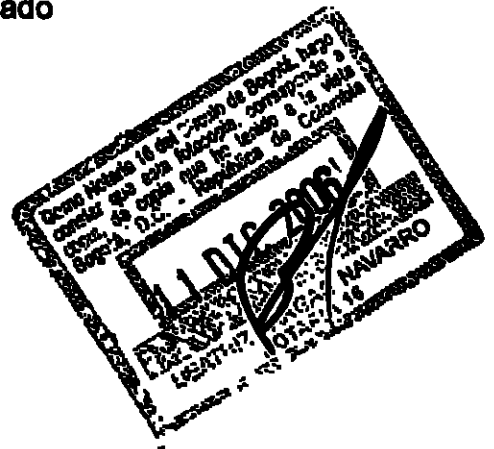
(Convención de La Haya del 5 de Octubre de 1961)

1. País: Estados Unidos de América
Este documento público
2. ha sido firmado por Harriet Smith Windsor
3. actuando en capacidad de Secretaria de Estado de Delaware
4. lleva el sello/timbre de la Oficina del Secretario de Estado

Certificado

5. en Dover, Delaware
6. el veinticuatro de mayo, A. D. 2005
7. por el Secretario de Estado, Departamento de Estado de Delaware
8. No. 0254032
9. Sello/timbre
10. Firma

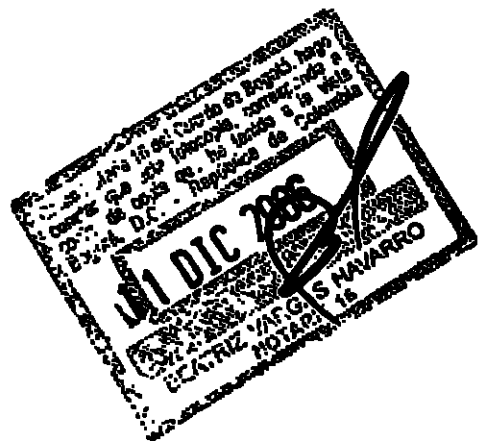
Harriet Smith Windsor (Firma)
Secretaria de Estado



DELAWARE
EL PRIMER ESTADO

388
HP

YO, HARRIET SMITH WINDSOR, SECRETARIA DE ESTADO DEL ESTADO DE DELAWARE, CERTIFICO POR LA PRESENTE QUE EL ANEXO ES UNA COPIA AUTÉNTICA Y CORRECTA DEL CERTIFICADO DE ENMIENDA DE "GENENTECH, INC", PRESENTADO EN ESTA OFICINA EL DECIMO DIA DE MAYO, A. D. 2001, A LAS 2:05 EN PUNTO DE LA TARDE.



Harriet Smith Windsor (Firma)
Harriet Smith Windsor, Secretaria de Estado
AUTENTICACIÓN: 3900386

FECHA: 05 - 24 - 05

CERTIFICADO DE TERCERA ENMIENDA DEL
CERTIFICADO DE INCORPORACIÓN
CORREGIDO Y VUELTO A REDACTAR

389
3/14

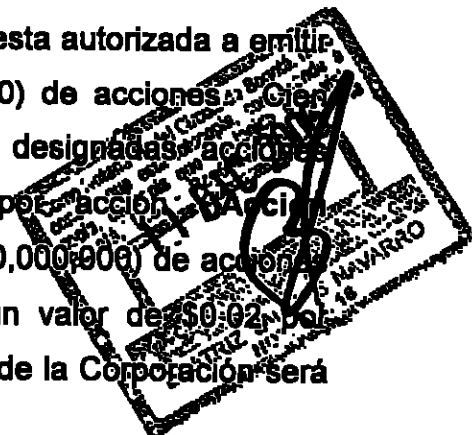
Genentech, Inc. una corporación organizada y existente bajo y por virtud de la Ley General de Corporación del Estado de Delaware.

CERTIFICA POR LA PRESENTE:

PRIMERO: Que en una reunión de la Junta Directiva de Genentech, Inc. se adoptaron debidamente resoluciones que establecen una enmienda propuesta del certificado de Incorporación Corregido y vuelto a Redactar de dicha corporación, declarando dicha enmienda como aconsejable y llamando a una reunión de accionistas de dicha corporación para consideración de la misma. La resolución establece la enmienda propuesta de la siguiente manera:

RESUELVE, que el Certificado de Incorporación Corregido y vuelto a Redactar de esta corporación se enmiende cambiando "la Sección 4.01(a)" del Artículo allí numerado "4" tal que, como se enmienda, dicha Sección de dicho Artículo se lea de la siguiente manera:

SECCION 4.01. *Acciones de Capital.* (a) la Corporación está autorizada a emitir dos clases de acciones que deben designarse, respectivamente, acciones preferenciales y acciones comunes. El número total de acciones que la Corporación esta autorizada a emitir es de mil trescientos millones (1,300,000,000) de acciones. Cien millones (100,000,000) de acciones serán designadas acciones preferenciales, por un valor de \$0.02 por acción ("Acción Preferencial"). Mil doscientos millones (1,200,000,000) de acciones serán designadas acciones comunes, por un valor de \$0.02 por acción ("Acción Común"). La acción común de la Corporación será toda de una misma clase.



SEGUNDO: Que por lo tanto, de acuerdo con la resolución de su Junta Directiva, la reunión ordinaria de accionistas de dicha corporación se convocó debidamente y se sostuvo al ser anunciada de acuerdo con la sección 222 de la Ley General de Corporación del Estado de Delaware, en donde el número necesario de acciones requerido por los estatutos votaron a favor de la enmienda.

TERCERO: Que dicha enmienda se adoptó debidamente de acuerdo con lo previsto en la Sección 242 de la Ley General de Corporación del Estado de Delaware.

CUARTO: Que el capital de dicha corporación no será reducido bajo o por razón de dicha enmienda

EN TESTIMONIO DE LO CUAL, dicha Genentech, Inc. ha causado este Certificado de Enmienda para que sea firmado por Stephen G. Juelsgaard, un Funcionario Autorizado, este 10 de Mayo de 2001.

GENENTECH, INC.

Por: (Firma)

Nombre: Stephen G. Juelsgaard

Título: Vicepresidente, Consejo
y Secretaría General.



391

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
Rad: 07-023376- -00000-0000
Fee: 2007-03-08 16:06:40 Dep. 2020 NUECREACIONES
Trib. 11 PATENTEYI
Eve: 378 FASENACIONALI
Act 411 PRESENTACION Folios: 398



Industria y Comercio
SUPERINTENDENCIA

**REQUISITOS MINIMOS PARA ADMISION A TRAMITE
PCT**

PATENTE DE INVENCION

☒

MODELO DE UTILIDAD ☐

- ◆ Indicación de que se solicita la concesión de una patente ☒
- ◆ Datos de identificación del solicitante o de la persona que presenta la solicitud ☒
- ◆ Descripción de la invención ☒
- ◆ Dibujos de ser estos pertinentes ☒
- ◆ Comprobante de pago de las tasas establecidas ☒

COMPLETA ☐

INCOMPLETA ☐

DISEÑO INDUSTRIAL ☐

- ◆ Indicación de que se solicita el registro de Diseño Industrial ☐
- ◆ Datos de identificación del solicitante o de la persona que presenta la solicitud ☐
- ◆ Representación gráfica y fotográfica del Diseño Industrial o muestra del material que incorpora el diseño ☐
- ◆ Comprobante de pago de las tasas establecidas ☐

COMPLETA ☐

INCOMPLETA ☐

ESQUEMA DE TRAZADO ☐

- ◆ Indicación de que se solicita el registro de un Esquema de Trazado ☐
- ◆ Datos de identificación del solicitante o de la persona que presenta la solicitud ☐
- ◆ Representación gráfica del esquema trazado ☐
- ◆ Comprobante de pago de las tasas establecidas ☐

COMPLETA ☐

INCOMPLETA ☐

Claudia M Neiva
Firma Responsable

Fecha _____

Carrera 13 No. 27-00 Piso 5, 7 y 10
Conmutador: 3 82 08 40
Fax: 350 52 20
E-mail: info@slc.gov.co
www.slc.gov.co
Bogotá, D.C., Colombia

392

AS: 103.282



REGISTRAR GENERAL DE MARCAS Y DISEÑOS
Rad: 07-023376- -00000-0000
Fec: 2007-03-08 18:08:40 Dep. 2820 NUBCREACIONES
Tra. 11 PATENTENYI
Ere: 378 FASENACIONAL
Aut. 411 PRESENTACION Folios: 398

FORMULARIO ÚNICO DE SOLICITUD DE PATENTE
PCT
ENTRADA EN FASE NACIONAL

1 SOLICITUD DE:			
☒ Patente de Invención.		☐ Patente de Modelo de Utilidad.	
☒ Capítulo I.		☐ Capítulo II.	
2 SOLICITANTE (71)	Nombre: GENENTECH, INC. Dirección: 1 DNA Way, South San Francisco, CA 94080-4990, US Nacionalidad o Domicilio: Estados Unidos Lugar de Constitución: Estados Unidos Teléfono: Fax: E-mail:		IDENTIFICACION C.C. ☐ NIT ☐ C.E. ☐ Otro ☐ Cual _____ Número _____
3 REPRESENTANTE O APODERADO	Nombre: JUAN PABLO CADENA SARMIENTO Dirección: Carrera 7 # 71 - 21 Torre B - Piso 4 Teléfono: 744-2200 Fax: 744-2700 E-mail:		IDENTIFICACION C.C. ☒ NIT ☐ C.E. ☐ Otro ☐ Cual _____ Número <u>80.410.337 TP 57492</u>
4 INVENTOR (ES) (72)	Nombre: Avi J. Ashkenazi Dirección: 1458 Terrytown Street, San Mateo, California 94402, Estados Unidos de América Nacionalidad o Domicilio: Estados Unidos de América		
5 PCT (86) Solicitud Internacional No. <u>PCT/US2005/032015</u> Fecha <u>07-09-2005</u> Publicación Internacional No. <u>WO 2006/028275 A3</u> Fecha <u>16-03-2006</u>			
6 Título (54): <u>MÉTODOS DE UTILIZAR LIGANDOS DE RECEPTOR DE MUERTE CELULAR Y ANTICUERPOS CD20</u>			
7 Clasificación Internacional (51) _____			
8 Prioridad	(33) País de Origen	(32) Fecha	(31) Número de Solicitud
SI ☒ NO ☐	US	08-09-2004	60/607.834
	US	30-03-2005	60/666.550
9 Comprobante de pago No. <u>07-03-2007</u> Fecha <u>07-03-2007</u>			

QUE SE REIVINDICA ES:

En método de tratar células cancerosas, que comprende exponer células cancerosas mamíferas a una cantidad efectiva sinérgica de anticuerpo de receptor de muerte celular y anticuerpo CD20.

393

El método de la reivindicación 1 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo monoclonal de receptor anti-5.

El método de la reivindicación 1 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo monoclonal de receptor anti-14.

El método de la reivindicación 1 en donde dichas células cancerosas se ponen a dicha cantidad efectiva sinérgica de anticuerpo de receptor de muerte celular agonista y anticuerpo CD20 *in vivo*.

El método de la reivindicación 2 ó 3 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo quimérico o un anticuerpo humanizado.

El método de la reivindicación 2 ó 3 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo humano.

El método de la reivindicación 1 en donde dicho anticuerpo de receptor de muerte celular agonista es un anticuerpo que reacciona cruzadamente con más de un receptor de ligando Apo-2.

El método de la reivindicación 1 en donde dichas células cancerosas son células de linfoma.

394

REPUBLICA DE COLOMBIA
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

Bogotá,
2020

Doctor(a)
CADENA SARMIENTO JUAN PABLO
Apoderado(a) y/o Representante de
GENENTECH, INC.

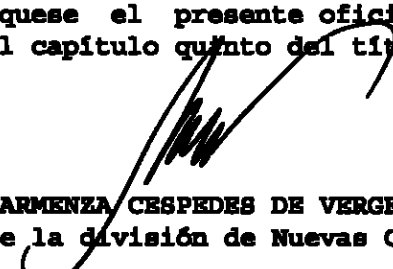
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	Solicitud internacional	PCT/US2005/032015
	Fecha inicio fase nacional	08/04/2007
	Trámite	11
	Evento	378
	Actuación	430
	Oficio No.	6556

Como resultado del examen de forma, realizado con fundamento en el artículo 38 de la Decisión 486 de la Comisión de la Comunidad Andina, artículo 27 del Tratado de Cooperación en Materia de Patentes (PCT), regla 51 bis del Reglamento y Circular Única de la Superintendencia de Industria y Comercio, se le comunica que:

1. La solicitud no contiene la copia del documento en que consta la cesión del derecho a la patente del inventor al solicitante o a su causante. (Art. 26-k) Decisión 486.

En cumplimiento de lo dispuesto por el artículo 39 de la Decisión 486, se le requiere para que dentro del término de dos meses siguientes a la fecha de notificación del presente oficio, complete los requisitos anteriormente enunciados. En caso de no dar cumplimiento al presente requerimiento, la solicitud se considerará abandonada y perderá su prelación.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No.10 de 2001.


ALIX CARMENZA CESPEDES DE VERGEL
Jefe de la división de Nuevas Creaciones

El anterior requerimiento fue notificado por fijación en lista de fecha 07 JUN 2007
adpall