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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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División de Nuevas Creaciones

PCT
SOLICITUD

PATENTE DE INVENCION

ENTRADA EN FASE NACIONAL

21 EXPEDIENTE No

07-22892

54 TITULO

METODOS PARA UTILIZAR LIGANDOS DE RECEPTOR MUERTO Y ANTICUERPOS

CD20

51 CLASIFICACION INTERNACIONAL

71 SOLICITANTE

GENENTECH INC

DOMICILIO

SOUTH SAN FRANCISCO, CALIFORNIA, ESTADOS UNIDOS

74 APODERADO

JUAN PABLO CADENA SARMIENTO

22 SANTAFE DE BOGOTA, D C

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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.

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Lugar de Constitución: Estados Unidos

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IDENTIFICACION

C.C. ☐ NIT ☐C.E. ☐ Otro ☐

Cual _____

Número _____

3

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Número 80.410.337 TP 57492

4

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60/807,909

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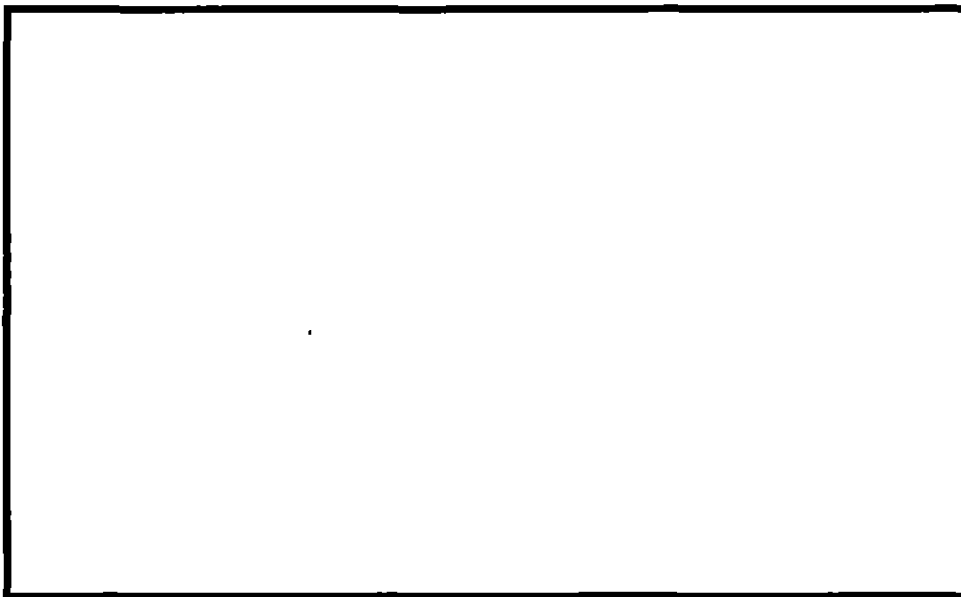
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Instrucciones para el diligenciamiento del presente formulario, ver reverso de esta página.

2020-F06 (01-11-15)

- ☒ 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.
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- ☐ 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:

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3

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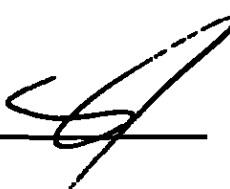
CONSIGNACION

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
BRIGARD & CASTRO LTDA	CONSIGNACION	BANCO POPULAR	050-00110-6	52423538	14/02/2007	17,370,000.00

CONCEPTO

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

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4

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES

PATENTE ☒ PCT/US2005/031907

MODELO DE UTILIDAD ☐

DESENÑO INDUSTRIAL ☐

SOLICITANTE/S. RAZON SOCIAL O APELLIDOS Y NOMBRES GENENTECH, INC.

DOMICILIO SOUTH SAN FRANCISCO, CALIFORNIA, ESTADOS UNIDOS DE AMERICA

APODERADO JUAN PABLO CADENA SARMIENTO

TITULO METODOS PARA UTILIZAR LIGANDOS DE RECEPTOR MUERTO Y ANTICUERPOS CD20

CLASIFICACION _____

EXPEDIENTE No. _____

FECHA DE SOLICITUD _____

ROLLO MICROFILM _____

CERTIFICADO No. _____

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VIGENCIA DESDE _____

HASTA _____

PRORROGA _____

CAMBIO DE NOMBRE _____

TRASPASO A _____

INVENTOR (ES) AVI J. ASHKENAZI

OTROS _____

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60/607,909 8 September 2004 (08.09.2004) US
60/666,553 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 Tarrytown Street, San Mateo, California 94402 (US).

(74) Agents: MARSHANG, 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, 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.

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

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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 60/607,909 filed September 8, 2004 and to provisional application number 60/666,553 filed March 30, 2005.

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, such as cancer and immune related diseases.

BACKGROUND OF THE INVENTION

15 Various ligands and receptors belonging to the tumor necrosis factor (TNF) superfamily have been identified in the art. Included among such 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 as TWEAK), APRIL, OPG ligand (also referred to as RANK ligand, ODF, or 20 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, 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 25 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; 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); 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 % 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)).

D

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 TRUND 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 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)

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(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-dependant 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 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 Apo2L/TRAIL polypeptide and CD20 antibody.
2. The method of claim 1 wherein said Apo2L/TRAIL polypeptide comprises amino acids 1-281 of Fig. 1 (SEQ ID NO:1) or a fragment or variant thereof.
3. The method of claim 1 wherein said Apo2L/TRAIL polypeptide comprises amino acids 114-281 of Fig. 1 (SEQ ID NO:1).
4. The method of claim 1 wherein said cancer cells are exposed to said synergistic effective amount of Apo2L/TRAIL polypeptide and CD20 antibody in vivo.
5. The method of claim 1 wherein said cancer cells are lymphoma cells.
6. The method of claim 1 further comprising exposing the cancer cells to one or more growth inhibitory agents.
7. The method of claim 1 further comprising exposing the cells to radiation.

8. The method of claim 1 wherein said Apo2L/TRAIL polypeptide is expressed in a recombinant host cell selected from the group consisting of a CHO cell, yeast cell and *E. coli*.

5 9. The method of claim 1 wherein said Apo2L/TRAIL polypeptide is linked to a polyethylene glycol molecule.

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

11. The method of claim 10 wherein said CD20 antibody is the antibody Rituximab.

15 12. A method of treating an immune related disease, comprising administering to a mammal a synergistic effective amount of Apo2L/TRAIL polypeptide and CD20 antibody.

20 13. The method of claim 12 wherein said Apo2L/TRAIL polypeptide comprises amino acids 1-281 of Fig. 1 (SEQ ID NO:1) or a fragment or variant thereof.

14. The method of claim 12 wherein said Apo2L/TRAIL polypeptide comprises amino acids 114-281 of Fig. 1 (SEQ ID NO:1).

25 15. The method of claim 12 wherein said Apo2L/TRAIL polypeptide is expressed in a recombinant host cell selected from the group consisting of a CHO cell, yeast cell and *E. coli*.

30 16. The method of claim 12 wherein said Apo2L/TRAIL polypeptide is linked to a polyethylene glycol molecule.

17. The method of claim 12 wherein said immune related disease is rheumatoid arthritis or multiple sclerosis.

35 18. The method of claim 12 wherein said CD20 antibody is a monoclonal antibody.

40 19. The method of claim 18 wherein said CD20 antibody is the antibody Rituximab.

20. The method of claim 1 or 12 wherein said Apo2L/TRAIL polypeptide and CD20 antibody are administered sequentially.

21. The method of claim 1 or 12 wherein said Apo2L/TRAIL polypeptide

and CD20 antibody are administered concurrently.

BRIEF DESCRIPTION OF THE DRAWINGS

5 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".

10 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).

15 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.

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

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

30 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.

35 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.

40 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.

45 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.

50 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.

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

60 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.

65 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 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 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

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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

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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.,

15	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
20	Gly	G	Glycine	Lys	K	Lysine
	Ala	A	Alanine	Arg	R	Arginine
	Cys	C	Cysteine	Trp	W	Tryptophan
	Val	V	Valine	Gln	Q	Glutamine
	Met	M	Methionine	Asn	N	Asparagine

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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.

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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

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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.

5 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
10 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.

"Apo-2 ligand receptor" includes the receptors referred to in the art as "DR4" and "DR5" whose polynucleotide and polypeptide sequences are shown
15 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
20 US 6,342,383 issued January 29, 2002). Sheridan et al., Science, 277:818-821 (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;
25 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
30 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
35 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
40 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
45 from nature. Thus, a native sequence Apo-2L receptor can have the amino acid

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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

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exhibit an I_c 50 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 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, hAPO2, 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.

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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.

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 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

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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 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, BEKKAR™) 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:

DIQMTQSPSSLSASVGDRVTITCRASSSVSYMHYQKPGKAPKPLIYAPSNLASGVPSRFSGSGSGTDFTLTIS
SLQPEDFATYYCQQWSFNPPFTFGQGTKVEIKR (SEQ ID NO:7);

and the variable heavy chain sequence:

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGAIYPGNGDTSYNQKFGRFTISVDKS
KNTLYLQMNSLRAEDTAVYYCARVVYYSNBYWYFDVWGQGTLLVTVSS (SEQ ID NO:8).

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

DIQMTQSPSSLSASVGDRVTITCRASSSVSYMHYQKPGKAPKPLIYAPSNLASGVPSRFSGSGSGTDFTLTIS
SLQPEDFATYYCQQWSFNPPFTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCCLNNFYPREAKVQWKVD
NALQSGNSQESVTEQDSKDSYSLSSITLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID
NO:9);

and the heavy chain amino acid sequence:

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGAIYPGNGDTSYNQKFGRFTISVDKS
KNTLYLQMNSLRAEDTAVYYCARVVYYSNBYWYFDVWGQGTLLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
DKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNSTYRVVSVLTVQLHQLDNLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVK
GFYPSDIAVEWESNGQPENNYKITTPFVLDSDGSFFLYSKLTVDKSRWQQGNVFSQSVMHREALHNYTQKSLSLSP
GK (SEQ ID NO:10)

or the heavy chain amino acid sequence:

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGAIYPGNGDTSYNQKFGRFTISVDKS
KNTLYLQMNSLRAEDTAVYYCARVVYYSNBYWYFDVWGQGTLLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
DKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNATYRVVSVLTVQLHQLDNLNGKEYKCKVSNKALPAPIAATISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVK
GFYPSDIAVEWESNGQPENNYKITTPFVLDSDGSFFLYSKLTVDKSRWQQGNVFSQSVMHREALHNYTQKSLSLSP
GK (SEQ ID NO:11).

"Antibody-dependent cell-mediated cytotoxicity" and "ADCC" refer to a 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 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 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 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.

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.

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"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.

"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, 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.

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"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., 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

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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.

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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.

5 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".

10 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
20 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.

25 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
30 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
35 fragments; cyclosporin A; steroids such as glucocorticosteroids, e.g., prednisone, methylprednisolone, dexamethasone, and hydrocortisone; methotrexate (oral or subcutaneous); hydroxychloroquine; 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 immunoashe~~in~~ (etanercept), anti-tumor
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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, Bi²¹², 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.

"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.

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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

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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

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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, 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

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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, migraines, 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 Stella 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. At^{211} , I^{131} , I^{125} , Y^{90} , Re^{186} , Re^{188} , Sr^{90} , Bi^{213} , P^{32} 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 "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 methylenamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylololomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatins; calystatin; CC-1065 (including its adozelesin,

carbazepine and bixlesin synthetic analogues; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KM-2189 and CBI-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongiostatins; nitrogen mustards such as chlorambucil, chlornaphazine, chlophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard, nitrosoureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antidiotics such as the enediyne antidiotics (e.g., calicheamicin, especially calicheamicin gamma1 and calicheamicin omega1 (see, e.g., Agnew, Chem. Int'l. Ed., Engl., 33: 183-186 (1994)); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an espartanin; as well as neocarzinostatin chromophore and related chromoproteins enediyne antidiotics chromophores), acalcinomyasins, actinomycin, anthracycline, bleomycin, cactinomycin, carabidin, carthomycin, carzinophyllin, chromomycins, dactinomycin, daunorubicin, decarubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin (including morbolino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and desoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycin such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peryomycin, pottiomycin, puromycin, quealamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zibosatin, sorubicin; 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, thiamiprine, thioguanine; pyrimidine analogs such as encitabine, azacitidine, 6-azauridine, carmustine, cytarabine, diideoxuridine, doxifluridine, encitabine, flouxuridine; androgens such as calusterone, dromostanolone propionate, epitestosterone, mepitosterone, testolactone; anti-adrenals such as aminoglutethimide, mifepristone, trilostane; folic acid replenisher such as trolic acid; acetylcholine; aldophosphamide glycosides; aminoglutethimide acid; eniluracil; amastatine; bestabucil; bisantrene; edatraxate; defotamine; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucic acid; gallium nitrate; hydroxyurea; leflunomide; lomidastine; maytansinoids such as maytansine and ansamitocins; mitoguanzone; mitoxantrone; mofidamole; nitazepine; pentostatin; phenacet; pitarubicin; josoxantrone; podophyllinic acid; 2-ethylhydrazide; procabazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); taxotane; thixotin; lisofyllin; epirgesterminium; tenaxonic acid; triaziquone; 2',2"-trichloro-2'-trichloroethylamine; trichothecenes (especially T-2 toxin; verrucin A, roxidin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside (Ara-C); cyclophosphamide; chloroquine; taxoids; e.g., TAXOL® paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABIRAXANE™ cremophor-free, albumin-engineered nanoparticle formulation of

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paclitaxel (American Pharmaceutical Partners, Schaumburg, Illinois), and TAXOTERE[®] doxorubicin (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; novantrone; teniposide; adatrexate; 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.

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, MEGASE[®] megestrol acetate, AROMASIN[®] exemestane, formestane, fadrozole, RIVISOR[®] vorozole, 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 example, PKC- α , 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; LORTOTECAN[®] topoisomerase I inhibitor; ABARELIX[®] rnrR; and pharmaceutically acceptable 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 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 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. 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 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 (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 TGF- α and TGF- β ; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon- α , - β , and - γ ; 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 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)].

5 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
10 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
15 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
20 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,
25 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
30 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-
35 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
40 cultured in nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed.

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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.

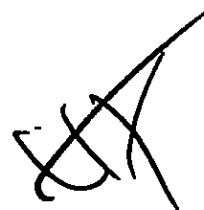
5 Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment
10 employing calcium 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
15 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.
20 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
25 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
30 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
35 (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),
40 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 equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled

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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.

5 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).

10 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
15 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
20 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
25 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
30 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
35 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
40 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
45 culture media is about 50 micromolar to about 250 micromolar. Most

preferably, the divalent metal ion used in such methods is zinc sulfate. 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.

5 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.

10 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
15 particularly ³²P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. 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
20 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.
25 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
30 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
35 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
40 secreted polypeptide, although it also may be recovered from host cell lysates when directly produced without a secretory signal. If the Apo-2 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.

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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 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.

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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 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 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 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 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.

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 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

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.

5 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

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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
5 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
10 production of monoclonal antibodies directed against the antigen. 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).

15 The binding affinity of the monoclonal antibody can, for example, be 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
20 by limiting dilution procedures and grown by standard methods (Goding, *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.

25 The monoclonal antibodies secreted by the subclones are suitably 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.

30 DNA encoding the monoclonal antibodies is readily isolated and 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
35 expression vectors, which are then transfected into host cells such as *E. 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
40 include Skerra et al., *Curr. Opinion in Immunol.*, 5:256-262 (1993) and Plückthun, *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

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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
5 (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
10 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
15 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
20 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
25 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
30 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
35 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.

40 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*, 362:255-258 (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

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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 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

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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')₂ bispecific antibodies).

5 (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
10 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
15 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
20 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-
25 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
30 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
35 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
40 half of the bispecific molecule provides for a facile way of separation. 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
45 molecules can be engineered to maximize the percentage of heterodimers

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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.

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 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 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 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 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-S8-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_2^I , α_3^I , N-acetyl- γ_1^I , PSAG and θ^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, sapaomaria 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} , Ra^{226} , Re^{186} , 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

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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
5 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.

10 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.
15 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
20 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,
25 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
30 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
35 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
40 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

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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
5 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
10 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,
15 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.).

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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
25 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
30 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
35 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
40 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
5 (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
10 sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; and/or non-ionic surfactants such as TWEENTM, PLURONICSTM or polyethylene glycol (PEG).

Additional examples of such carriers include ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin,
15 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.
20 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
25 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
30 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 appropriate diluent at the time for use. An example of a liquid formulation is a sterile, clear, colorless unpreserved solution filled in a
35 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.),
40 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 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,

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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 $\mu\text{g/kg}$ 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 $\mu\text{g/kg/day}$ 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/m^2 to about 1000 mg/m^2 . 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 82C6 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-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 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 (Cariani et al. *Cancer Res.* 55(23): 5852s-5856s (1995); and Richman et al. *Cancer Res.* 55(23 Supp): 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 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 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 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 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 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

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(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/ENBRELV[®], 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 (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 (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); idiotypic 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

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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 (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 4

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

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 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.

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 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 5

Effect 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 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) (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).

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

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

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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, 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 Apo2L/TRAIL polypeptide and CD20 antibody.

2. The method of claim 1 wherein said Apo2L/TRAIL polypeptide comprises amino acids 1-281 of Fig. 1 (SEQ ID NO:1) or a fragment or variant thereof.

3. The method of claim 1 wherein said Apo2L/TRAIL polypeptide comprises amino acids 114-281 of Fig. 1 (SEQ ID NO:1).

4. The method of claim 1 wherein said cancer cells are exposed to said synergistic effective amount of Apo2L/TRAIL polypeptide and CD20 antibody in vivo.

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

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

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

8. The method of claim 1 wherein said Apo2L/TRAIL polypeptide is expressed in a recombinant host cell selected from the group consisting of a CHO cell, yeast cell and *E. coli*.

9. The method of claim 1 wherein said Apo2L/TRAIL polypeptide is linked to a polyethylene glycol molecule.

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

11. The method of claim 10 wherein said CD20 antibody is the antibody Rituximab.

12. A method of treating an immune related disease, comprising administering to a mammal a synergistic effective amount of Apo2L/TRAIL polypeptide and CD20 antibody.

Fig 23
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13. The method of claim 12 wherein said Apo2L/TRAIL polypeptide comprises amino acids 1-281 of Fig. 1 (SEQ ID NO:1) or a fragment or variant thereof.
- 5 14. The method of claim 12 wherein said Apo2L/TRAIL polypeptide comprises amino acids 114-281 of Fig. 1 (SEQ ID NO:1).
15. The method of claim 12 wherein said Apo2L/TRAIL polypeptide is expressed in a recombinant host cell selected from the group
- 10 consisting of a CHO cell, yeast cell and *E. coli*.
16. The method of claim 12 wherein said Apo2L/TRAIL polypeptide is linked to a polyethylene glycol molecule.
- 15 17. The method of claim 12 wherein said immune related disease is rheumatoid arthritis or multiple sclerosis.
18. The method of claim 12 wherein said CD20 antibody is a monoclonal antibody.
- 20 19. The method of claim 18 wherein said CD20 antibody is the antibody Rituximab.
- 25 20. The method of claim 1 or 12 wherein said Apo2L/TRAIL polypeptide and CD20 antibody are administered sequentially.
21. The method of claim 1 or 12 wherein said Apo2L/TRAIL polypeptide and CD20 antibody are administered concurrently.

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FIG. 1

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1 ATGGCGCCAC CACCAGCTAG AGTACATCTA GGTCCGTTCC TGGCAATGAC
TACCGCGGTG GTGGTCGATC TCATGTAGAT CCACGCAAGG ACCGTCACTG
1 MetAlaProP roProAlaAr gValHisLeu GlyAlaPheL euAlaValTh

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

101 CCAGCAAGT GTGGGGCTCT TCCGCGGGGA GGATTGAACC ACGAGGCGGG
GGTCGTTTCA CACCCCGAGA AGGCGCCCTT CCTAAGTTGG TGCTCCGCCC
35 SerLysVa lTrpGlySer SerAlaGlyA rgIleGluPr oArgGlyGly

151 GCGCGAGGAG CGCTCCCTAC CTCATGGGA CAGCACGAC CCAATGCCCC
CCGGCTCCTC GCGAGGGATG GAGGTACCTT GTCGTGCTG GGTACGGGC
GlyArgGlyA laLeuProTh rSerMetGly GlnHisGlyP roSerAlaArg

201 GCGCCGGGCA GGGCGCGCCC CAGGACCCAG GCGCGCGCGG GAAGCCAGCC
CGGGGCGCGT CCGCGCGCGG GTCTCGGTC CCGCGCGCGC CTTCGCTCGG
68 AlaArgAla GlyArgAlaP roGlyProAr gProAlaArg GluAlaSerP

251 CTGGCTCCG GGTCCACAAG ACCTTCAAGT TTGTCGCTGT CCGGGTCTTG
GAGCCGAGGC CCAGGTGTTT TGGAAATTCA AACAGCAGCA GCGCCAGGAC
roArgLeuAr gValHisLys ThrPheLysP heValValVa lGlyValLeu

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

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

401 CAGGATCTCA TAGATCAGAA CGTCCTGGAG CCTGTAAACC GTGCACAGAG
GTCTTAGAGT ATCTAGTCTT GCAGGACCTC GGACATTGGC CACGTGTCTC
135 GlySerHi sArgSerGlu ArgProGlyA laCysAsnAr gCysThrGlu

451 GGTGTGGGTT ACACCAATGC TTCCAACAAT TTGTTGCTT GCTTCCCATG
CCACACCCAA TGTGGTTACG AAGGTTGTTA AACAAACGAA CCGAGGGTAC
GlyValGlyT yrThrAsnAl aSerAsnAsn LeuPheAlaC ysLeuProCys

501 TACAGCTTGT AAATCAGATG AAGAAGAGAG AAGTCCCTGC ACCACGACCA
ATGTGGAACA TTTAGTCTAC TTCTTCTCTC TTCAGGAGCG TGGTGTCTGT
168 ThrAlaCys LysSerAspG luGluGluAr gSerProCys ThrThrThra

551 GGAACACAGC ATGTCACTGC AAACCAGGAA CTTTCCGGAA TGACAATTCT
CCTGTGTCTG TACAGTCACG TTGTGTCCTT GAAAGGCCTT ACTGTTAAGA
rgAsnThrAl aCysGlnCys LysProGlyT hrPheArgAs nAspAsnSer

601 GCTGAGATGT GCGGGAAGTG CAGCACAGGG TCGCCGAGAG GGATGGTCAA
CGACTCTACA CGGCCTTCAC GTCGTGTCCC ACGGGGTCTC CCTACCAATT
201 AlaGluMetC ysArgLysCy sSerThrGly CysProArgG lyMetVally

651 GGTCAAGGAT TGTACGCCCT GGAGTGACAT CGAGTGTGTC CACAAAGAAT
CCAGTTCTTA ACATGCGGGA CCTCACTGTA GCTCACACAG GTGTTTCTTA
sValLysAsp CysThrProT xpSerAspIl eGluCysVal HisLysGluSer

FIG. 2A
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701 CAGGCAATGG ACATAATATA TGGGTGATTT TGGTTGTGAC TTTGGTTGTT
 GTCCGTTACC TGTATTATAT ACCCACTAAA ACCAACACTG AAACCAACAA
 235 GlyAsnGly yHisAsnIle TrpValIleL euValValTh rLeuValVal

751 CCGTTGCTGT TGGTGGCTGT GCTGATTGTC TGTGTGTGCA TCGGCTCAGG
 GGCAACGACA ACCACCGACA CGACTAACAG ACAACAACGT AGCCGAGTCC
 ProLeuLeuL euValAlaVa lLeuIleVal CysCysCysI leGlySerGly

801 TTGTGGAGGG GACCCCAAGT GCATGGACAG GGTGTGTTTC TGGCGCTTGG
 AACACCTCCC CTGGGGTTCA CGTACCTGTC CCAGACAAAG 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 GCAGATTGA CAGGTGTCAC TGTACAGTCC CCAGGGGAGG
 GGTCTCTGGC CGTCTAAACT GTCCACATGT ACATGTCAGG GGTCCCCTCC
 rGlnGluPro AlaAspLeuT hrGlyValTh rValGlnSer ProGlyGluAla

1001 CACAGTGCTT GCTGGGACCG GCAGAGGCTG AAGGGTCTCA GAGGAGGAGG
 GTGTCACAGA CGACCTGCGC CGTCTTCGAC TTCCAGAGT CTCTCTCTCC
 335 GlnCysLe uLeuGlyPro AlaGluAlaG luGlySerGl nArgArgArg

1051 CTGCTGGTTC CAGCAATGG TGCTGACCCC ACTGAGACTC TGATGCTGTT
 GACGACCAAG GTCGTTTACC ACCACTGGGG TGACTCTGAG ACTACGACAA
 LeuLeuValP roAlaAsnGly yAlaAspPro ThrGluThrL euMetLeuPhe

1101 CTTTGACAAG TTTGCAACA TCGTGCCCTT TGACTCCTGG GACCAGCTCA
 GAACTGTTTC AAACGTTTGT AGCACGGGAA ACTGAGGACC CTGGTGGAGT
 368 PheAspLys PheAlaAsnI leValProPh eAspSerTrp AspGlnLeuM

1151 TGAGGCAGCT GCACCTCAGC AAAAATGAGA TCGATGTGGT CAGAGCTGGT
 ACTCCGTCGA CCTGGAGTGC TTTTACTCT AGCTACACCA GTCTCGACCA
 etArgGlnLe uAspLeuThr LysAsnGluI leAspValVa lArgAlaGly

1201 ACAGCAGGCC CAGGGGATGC CTTGTATGCA ATGCTGATGA AATGGGTCAA
 TGTGCTCCGG GTCCCTTACG GAACATACGT TACGACTACT TTACCCAGTT
 401 ThrAlaGlyP roGlyAspAl aLeuTyrAla MetLeuMetL ysTrpValAs

1251 CAAAACCTGA CGGAACGCTT CGATCCACAC CCTGCTGGAT GCCTTGGAGA
 GTTTTGAACCT GCCTTGCAGA GCTAGGTGTG GGACGACCTA CGGAACCTCT
 nLysThrGly ArgAsnAlaS exIleHisTh rLeuLeuAsp AlaLeuGluArg

1301 GGATGGAAGA GAGACATGCA AAAGAGAAGA TTCAGGACCT CTTGGTGGAC
 CCTACCTTCT CTCTGTACGT TTTCTCTTCT AAGTCTCTGA GAACCACCTG
 435 MetGluGly 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

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1 MEQRGCHAPAAAGAKRHGPGPREARGHAPGLKVPKTLVLVVAAVLLLVHAESALITQGD
61 LAPQORAAAPQOKRSPSEGLCPFGHHSDEGRDCISCKYQGDYSTHNDLLFCLECTRCD
121 SGVELSPCTTTANTFVQCCEGTFRKEDSPESCRRCTGCFRGMVKVGDCTPNSDIECVH
181 KESGIIIGVTVAAVVLIVAVFVCKELLKQVLPLYLGICSGGGGDPKRVDRSSQRPQARD
241 NVLNEIVSILQPTQVFPQEMEVQEPAPPTGVNELSPGESKELLAPANAERSQRRLVPA
301 NEGDPISTLRQCFDDFADLVFPDSWEPLMKLGLMDNEIKVAKAAACHROFLYTNLIKW
361 VNRFGRDASVHTLLDALFTLGRRLAKQKEDALLSGGKFNYLEGNADSAIS

FIG. 3A

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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 Gln 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

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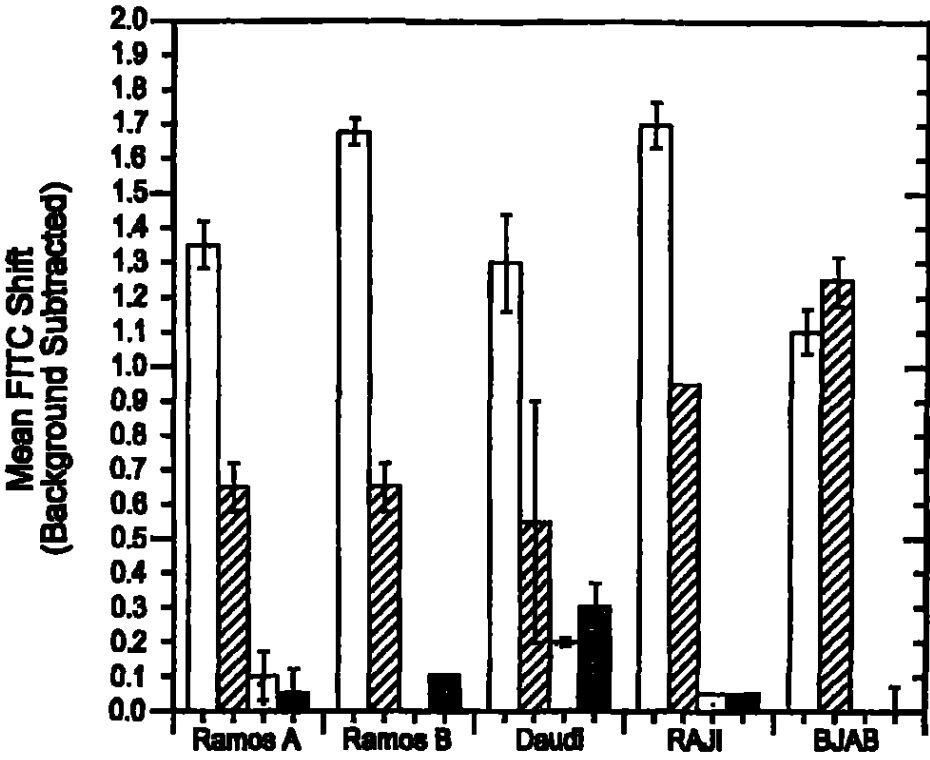


FIG. 4

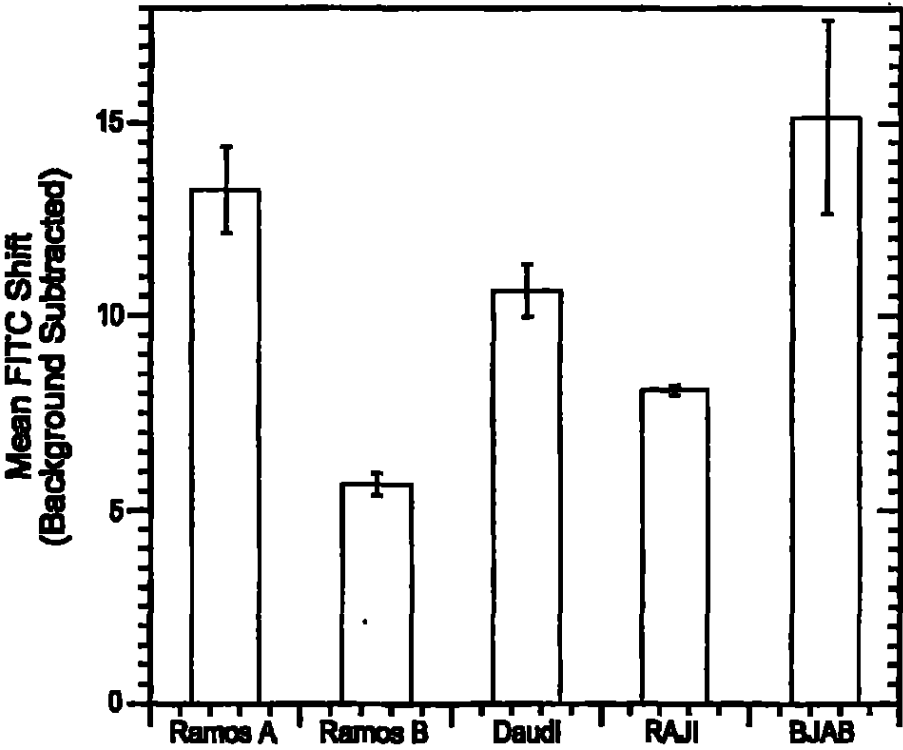


FIG. 5

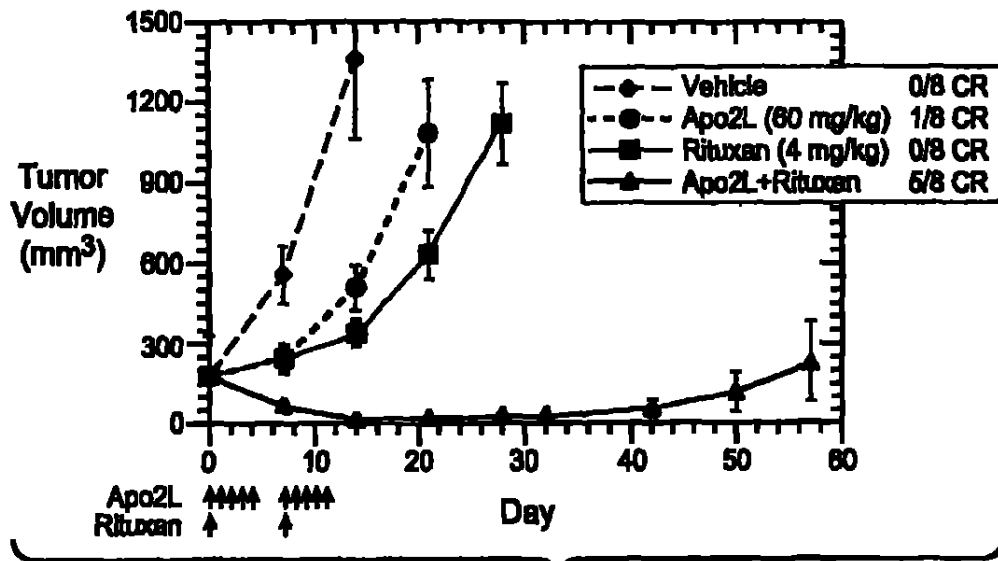


FIG. 6

Apo2L.0 (80 mg/kg/mouse; 5x/wk; IP) Alone and in Combination with Rituxan (4 mg/kg/mouse; 1x/wk; IP) vs. BJAB (SCID Mice) Xenograft

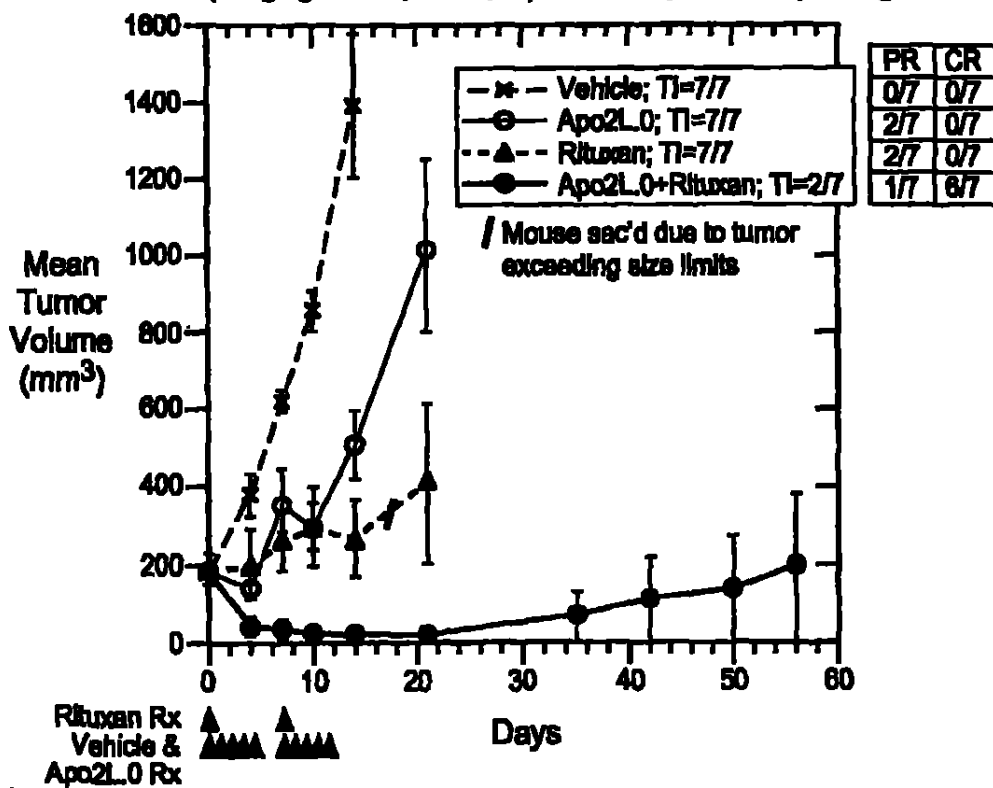


FIG. 7

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**FIG. 8****FIG. 10**

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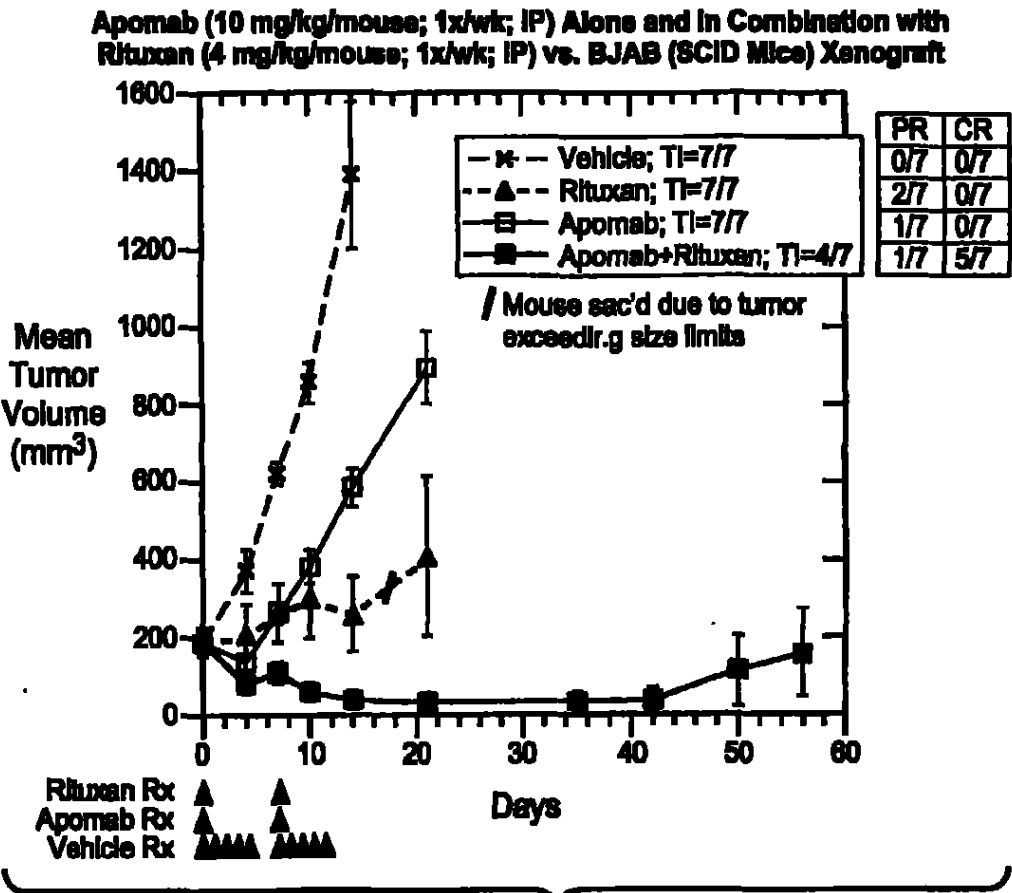


FIG. 9

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[Signature]

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

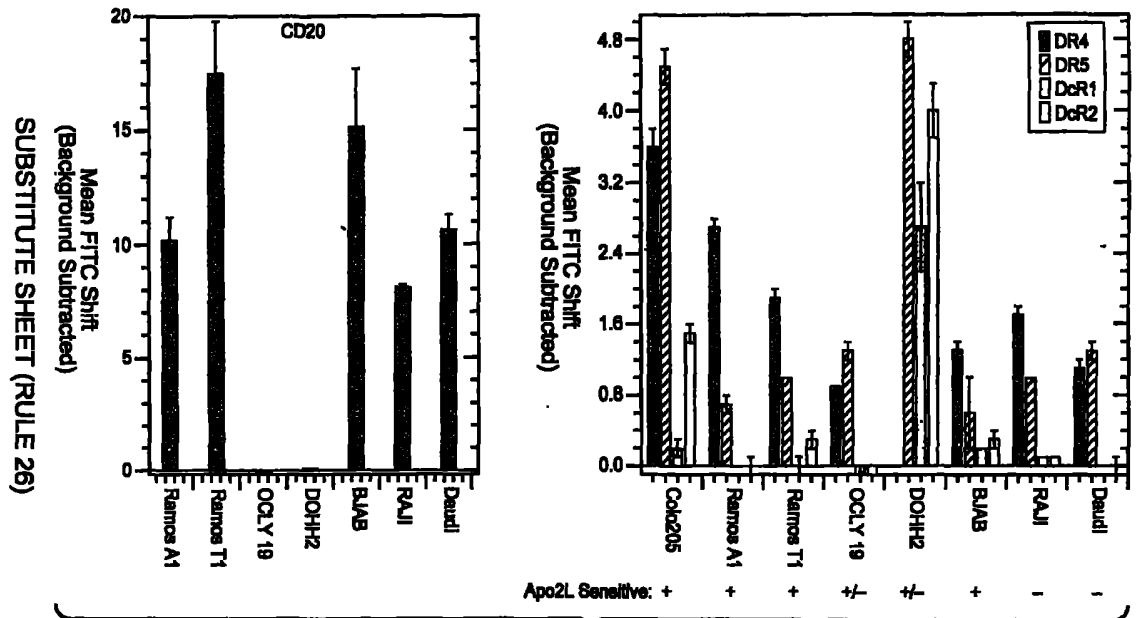


FIG. 11

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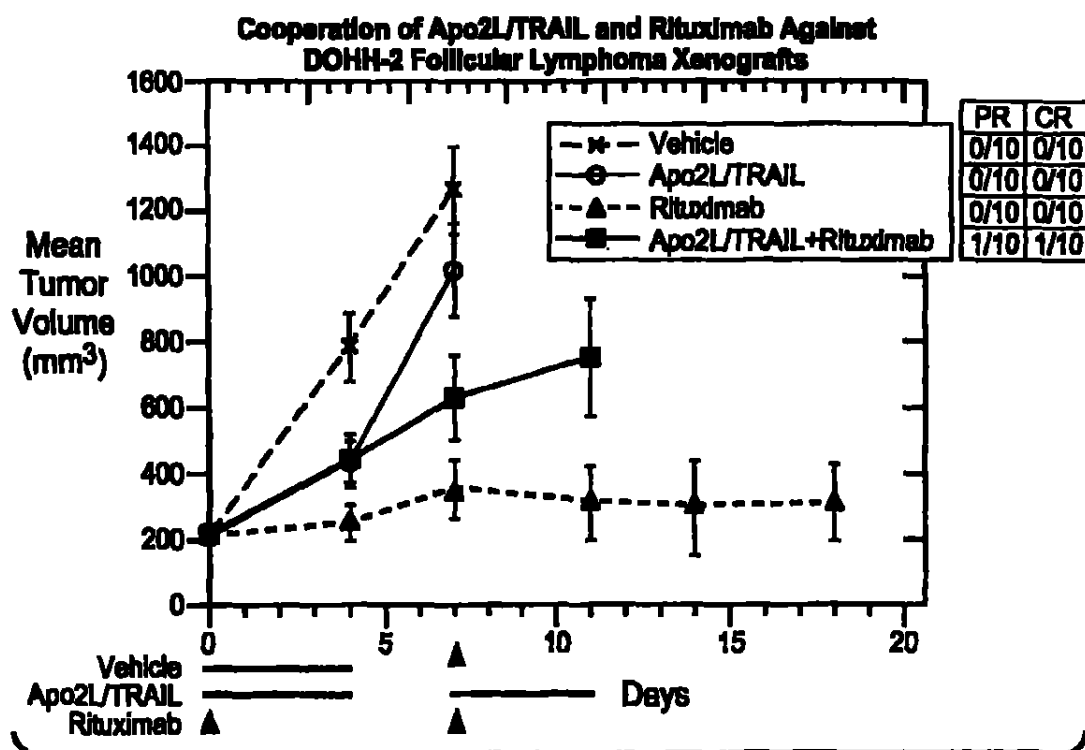
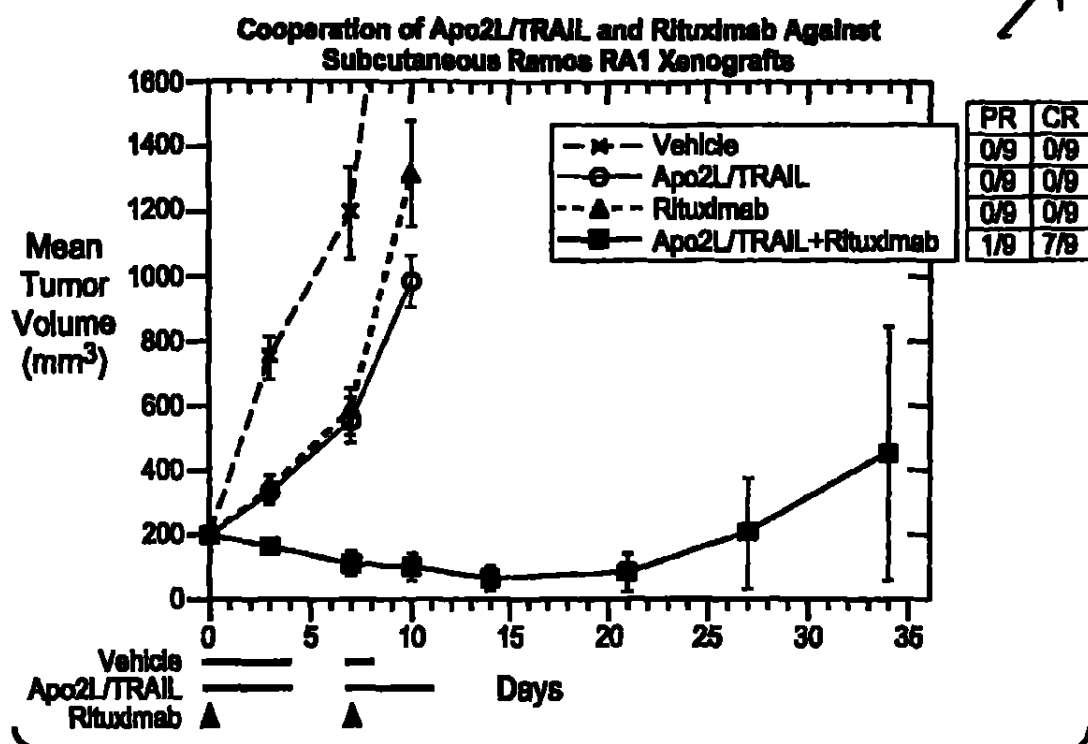


FIG. 13
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Rituximab and Apo2L/TRAIL Kill BJAB Cells by Independent Mechanisms

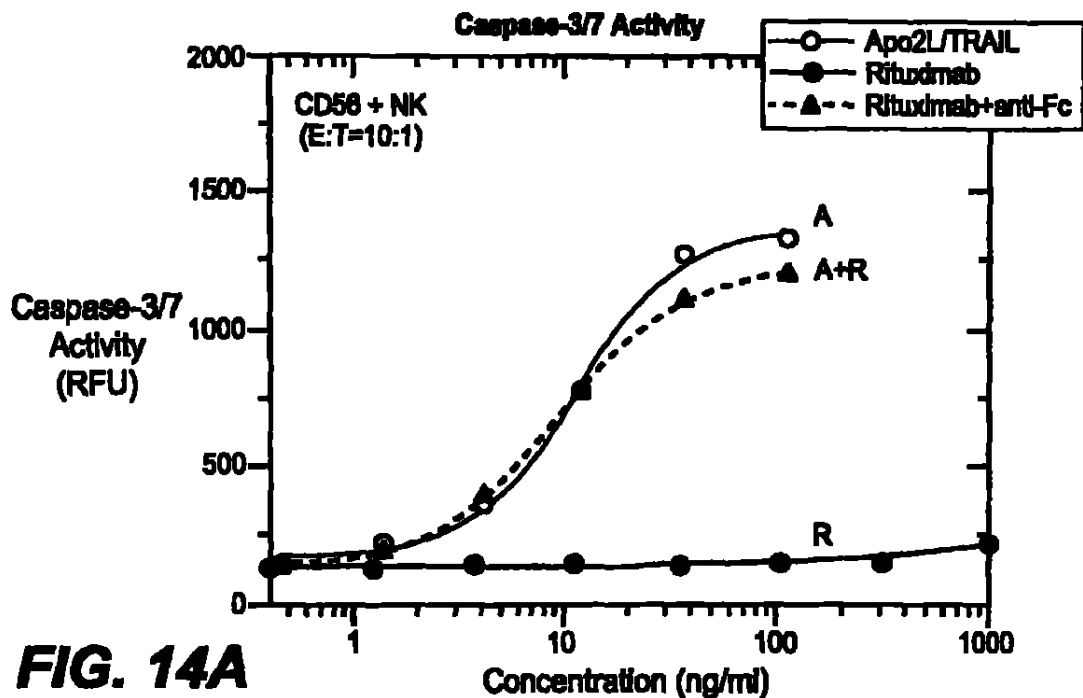


FIG. 14A

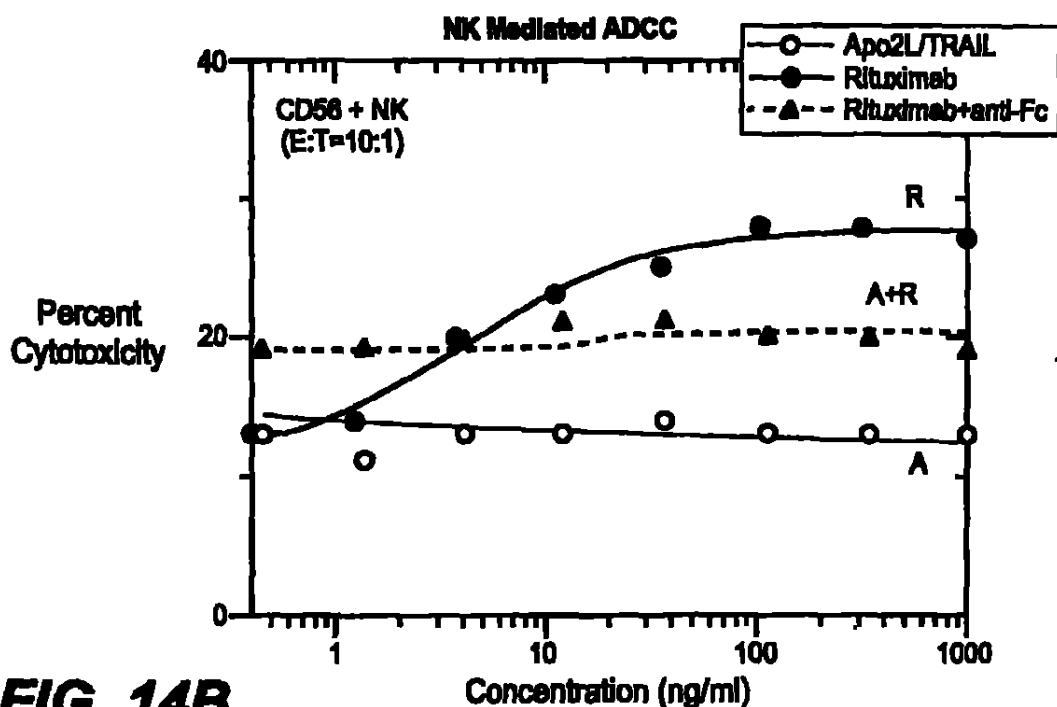


FIG. 14B

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Rituximab and Apo2L/TRAIL Kill BJAB Cells by Independent Mechanisms

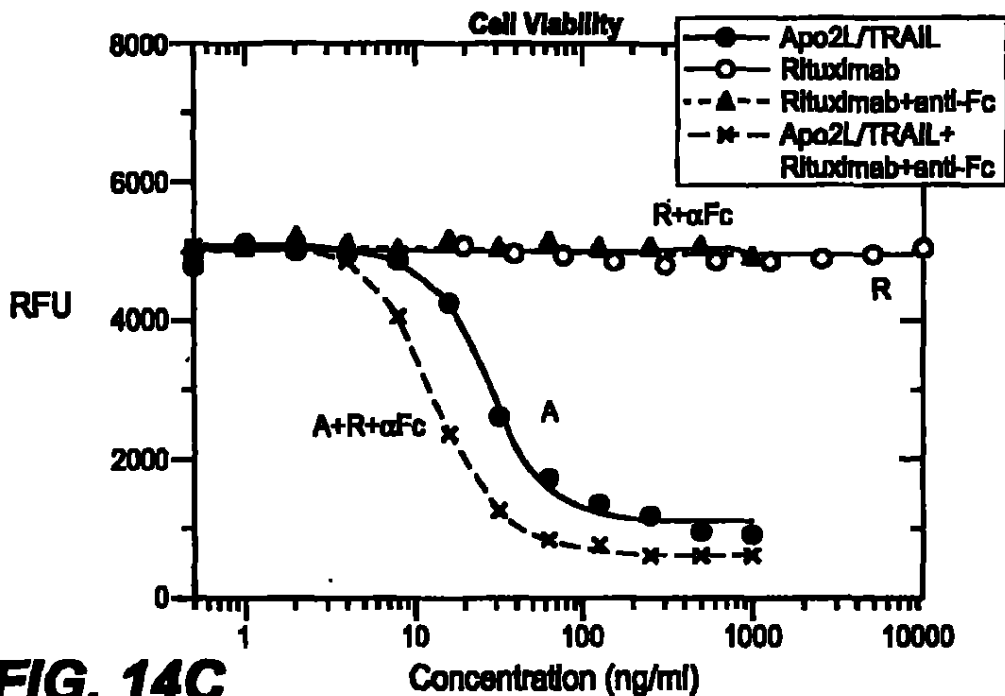


FIG. 14C

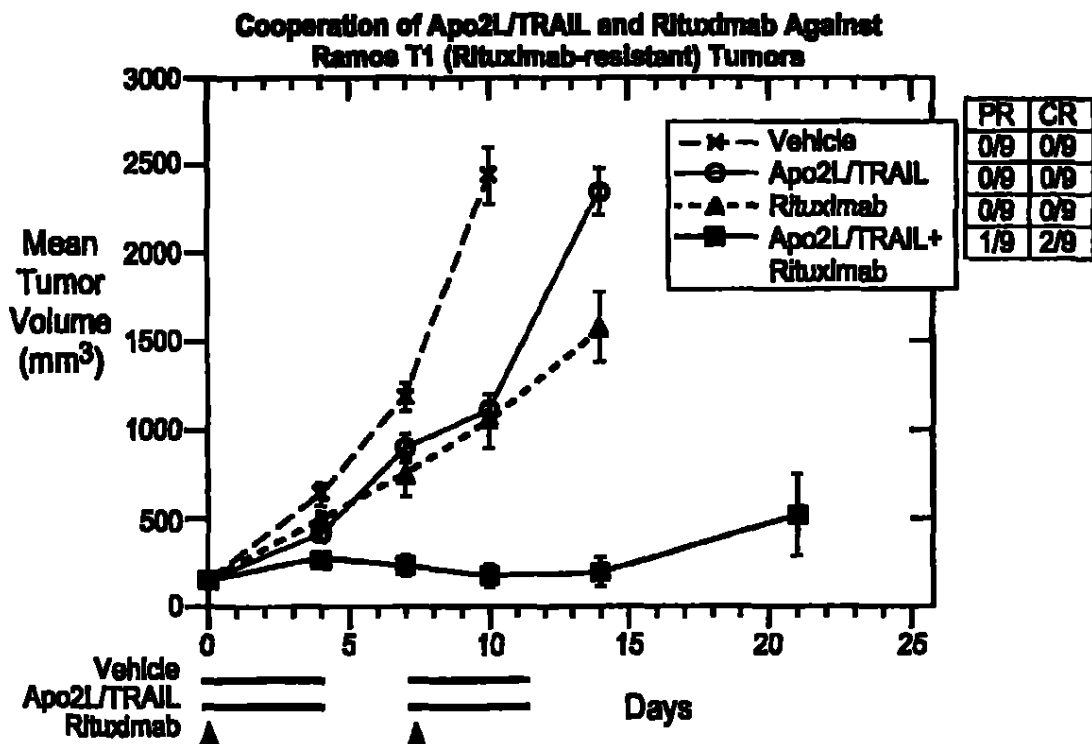


FIG. 15

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X

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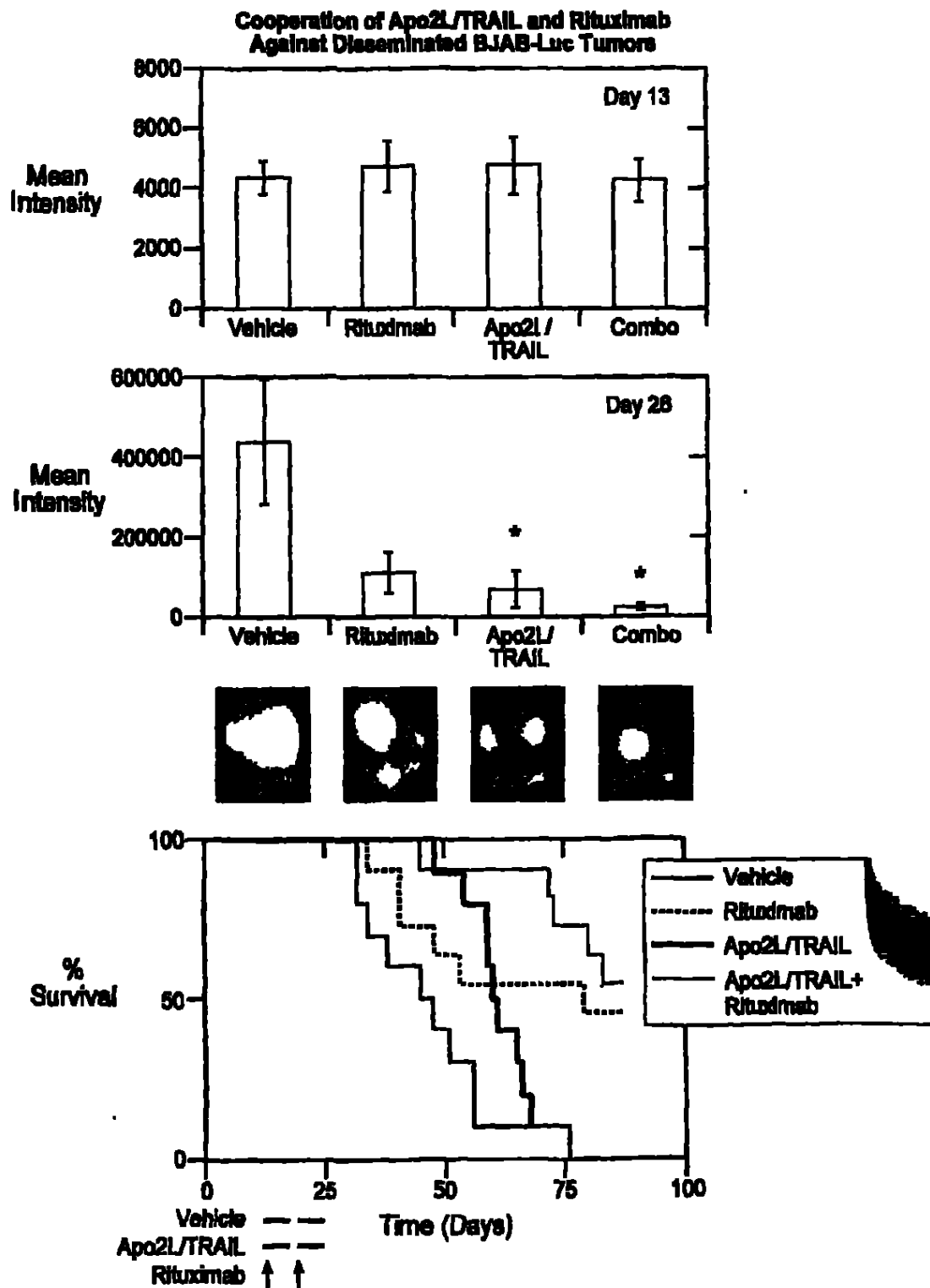


FIG. 16
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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2005/031907

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K14/705 C07K16/28 A61P35/02 A61K39/395

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.
A	WO 03/039486 A (IDEC PHARMACEUTICALS CORPORATION; HARIHARAN, KANDASAMY; HANNA, NABIL) 15 May 2003 (2003-05-15) paragraph [0006]	1-21
A	WO 03/038043 A (UAB RESEARCH FOUNDATION; ZHOU, TONG; ICHIKAWA, KIMIHISA; KIMBERLY, ROB) 8 May 2003 (2003-05-08) page 54, line 8 - line 14 claims 30,66,102	1-21
A	LEBLANC H N ET AL: "Apo2L/TRAIL and its death and decoy receptors." CELL DEATH AND DIFFERENTIATION. JAN 2003, vol. 10, no. 1, January 2003 (2003-01), pages 66-75, XP002382355 ISSN: 1350-9047 abstract	1-21

☒ 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
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"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

23 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 2911 Patentamt
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Fax: (+31-70) 340-8018

Authorized officer

Rankin, R

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2005/031907

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LUDWIG DALE L ET AL: "Monoclonal antibody therapeutics and apoptosis." ONCOGENE. 8 DEC 2003, vol. 22, no. 56, 8 December 2003 (2003-12-08), pages 9097-9106, XP008050059 ISSN: 0950-9232 abstract	1-21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2005/031907

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 1-21 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 compound/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 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:

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.

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2005/031907

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 03039486	A	15-05-2003	NONE
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

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(12) SOLICITUD INTERNACIONAL PUBLICADA DE ACUERDO CON EL TRATADO DE COOPERACIÓN DE PATENTES (PCT)

(43) Fecha de Publicación Internacional **(10) Número de Publicación Internacional**
 16 Marzo 2006 (16.03.2006) PCT WO 2006/029224 A3

(51) Clasificación Internacional de Patente:
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 C07K 16/28 (2006.01)
 A61P 35/02 (2006.01)
 A61K 39/395 (2006.01)

(21) Número de Solicitud Internacional:
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(25) Idioma de Presentación: Inglés

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 60/607,909 08 Septiembre 2004 EU
 (08.09.2004)
 60/666,553 30 Marzo 2005 EU
 (30.03.2005)

(71) Solicitante (para todos los estados designados excepto EU): GENENTECH, INC. [US/US]; 1 DNA Way, South San Francisco, California 94080-4990 (EU).

(72) Inventor; y

(75) Inventor/Solicitante (solo para EU): ASHKENAZI, Avi, J. [US/US]; 1456 Tarrytown Street, San Mateo, California 94402 (US).

(74) Agente: MARSCHANG, Diane, L. et al.; Genentech, Inc., 1 DNA Way, South San Francisco, California 94080-4990 (US).

(81) Estados Designados (nacional): 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.

(84) Estados Designados (regional): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasia (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), Europa (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)

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Publicado

— Con reporte de búsqueda internacional:

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Para códigos de dos letras y otras abreviaturas, refiérase "Notas Gula sobre Códigos y Abreviaturas" que aparecen al inicio de cada publicación regular de la Gaceta PCT.

(54) Título: MÉTODOS PARA UTILIZAR LIGANDOS DE RECEPTOR MUERTO Y ANTICUERPOS CD20

(57) Resumen: Se suministran métodos para utilizar ligandos de receptor muerto, tales como los polipéptidos de ligando/TRAIL APo-2 o los anticuerpos receptores muertos, y los anticuerpos CD20 para tratar condiciones tales como cáncer y enfermedades relacionadas con el sistema inmune. Las modalidades de la invención incluyen métodos para utilizar el Apo2L/trail o los anticuerpos de receptor muerto tales como los anticuerpos DR5 y los anticuerpos DR4 en combinación con los anticuerpos CD20.

MÉTODOS PARA UTILIZAR LIGANDOS DE RECEPTOR MUERTO Y ANTICUERPOS**CD20****SOLICITUDES RELACIONADAS**

Esta solicitud reivindica prioridad bajo la sección 119 (e) solicitud provisional No 60/607,909 presentada en septiembre 8, 2004 y la solicitud provisional No 60/666,553 presentada en marzo 30, 2005.

CAMPO DE LA INVENCION

La presente invención se relaciona con métodos para utilizar ligandos de receptor muerto y anticuerpo CD20. Más particularmente, la invención se relaciona con métodos para utilizar el ligando/TRAIL Apo-2 o los anticuerpos de receptor muerto en combinación con los anticuerpos CD20 para tratar varios desórdenes patológicos, tales como el cáncer y las enfermedades relacionadas con el sistema inmune.

Antecedentes de la invención

Varios ligandos y receptores pertenecen a la súper familia del factor de necrosis tumoral (TNF) que han sido identificados en la técnica. Incluidos entre tales ligandos está el factor α de necrosis tumoral ("TNF- α "), el factor β de necrosis tumoral ("TNF- β ") el factor β de necrosis tumoral ("TNF- β " o linfotoxina- α "), o la linfotoxicidad- β ("LT- β "), ligando CD30,

ligando CD27, ligando CD40, ligando OX-40, ligando 4-1 de B, LIGHT, ligando Apo-1 (también denominado como ligando FAS o el ligando CD95), ligando Apo-2 (también denominado como el Apo2L o TRAIL), ligando Apo-3 (también denominado como TWEAK), APRIL, ligando OPG (también denominado como ligando RANK, ODF, o TRANCE), y el TALL-I (también denominado como BlyS, BAFF o THANK) (ver 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, páginas 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 en enero 16, 1997; WO 97/25428 publicada en julio 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 publicada en julio 2, 1998; WO98/46751 publicada en octubre 22, 1998; WO/98/18921 publicada en mayo 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)).

La inducción de varias respuestas celulares mediadas por tales ligandos de la familia TNF es típicamente iniciada por su unión a los receptores de célula específicos.

Algunos, pero no todos, los ligandos de la familia TNF se unen a, e inducen varias actividades biológicas a través, de la superficie celular "receptores muertos" para activar caspazas, o enzimas que llevan acabo la muerte celular o la senda de apoptosis ((Salvesen et al., Cell, 91:443-446 (1997). incluidos entre los miembros de la súper familia del sector TNF hasta la fecha están el TNFR1, TNFR2, TACI, GITR,, CD27, OX-40, CD30, CD40, HVEM, Fas (también denominado como Apo-1 o CD95), DR4 (también denominado como TRAIL-R1), DR5 (también denominado como Apo-2 o TRAIL-R2), DcR1, DcR2, oste oprotogerina (OPG), RANK y Apo-3 (también denominado como DR3 o TRAMP) (ver, por ejemplo., Ashkenazi, Nature Reviews, 2:420- 430 (2002); Ashkenazi y 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, páginas 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, publicada en Marzo 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))

La mayoría de estos miembros de la familia del receptor TNF comparten la estructura típica de los receptores de superficie celular que incluyen las regiones extra celulares, de transmembrana e intracelular, mientras que otros se encuentran naturalmente como proteínas solubles a los que les falta el dominio de transmembrana e intracelular. La porción extra celular de los TNFR típicos contiene un patrón de secuencia de aminoácido repetitiva de los dominios múltiples ricos en cisteína (CDR), partiendo del terminal NH₂.

El ligando denominado como el Apo-2L o TRAIL se identificó hace varios años como un miembro de la familia TNF α de las citoquinas, (ver, 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 estadounidense No. 5,763,223 publicada en Junio 9, 1998; Patente estadounidense 6,284,236 publicada en septiembre 4, 2001).

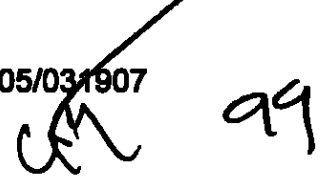
El polipéptido Apo2L/TRAIL humano de la secuencia nativa de longitud completa es una proteína de transmembrana tipo II de 281

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aminoácidos de largo, algunas células pueden producir una forma natural soluble de polipéptido, a través del clivaje enzimático de los estudios cristalográficos de las formas solubles del Apo2L/TRAIL de polipéptido revelan una estructura homo trimérica similar a la estructura del TLF y otras proteínas relacionadas (Mariani et al., J. Cell. Biol., 137:221-229 (1997)). El Apo2L/TRAIL, a diferencia de otros miembros de la familia TNF α sin embargo, se encontró que tiene una característica estructural única porque tres residuos de cisteína (en la posición 230 de cada sub unidad en el homo trímero) coordinan juntos un átomo de zinc, y que la unión zinc que es importante para la estabilidad del trímero y la actividad biológica. (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)).

Se ha reportado en la literatura que el Apo2L/TRAIL, puede jugar un papel en la modulación del sistema inmune, que incluye enfermedades autoinmunes tales como la artritis reumatoide (Hymowitz et al., supra; Bodmer et al., J. Biol. Chem., 275:20632-20637 (2000)). [ver, 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)]

Las formas solubles del Apo2L/TRAIL se han reportado por inducir apoptosis en una variedad de células de cáncer, que incluyen tumores de colón, pulmón, cerebro, próstata, vejiga, riñón, ovario y cerebro, así como también melanoma, leucemia y mieloma



múltiple (ver, por ejemplo, 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)). Los estudios in vivo en modelos de tumor de murino sugieren además que el Apo2L/TRAIL, sólo o en combinación con quimio terapia o terapia de radiación, pueden ejercer efectos anti tumorales sustanciales (ver, por ejemplo, Ashkenazi et al., supra; Walczak et al., supra; Gliniak et al., Cáncer 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). En contraste a muchos tipos de células cancerígenas, los mayores tipos de células humanas normales parecen ser resistentes a la inducción de apoptosis mediante ciertas formas recombinantes de Apo2L/TRAIL (Ashkenazi et al., supra; Walczak et al., supra). Jo et al. Ha reportado que la forma soluble de poli histidina-etiquetada de Apo2L/TRAIL indujo apoptosis in Vitro en hepatocitos humanos normales aislados, pero no humanos (Jo et al., Nature Med., 6:564-567 (2000); ver también, Nagata, Nature Med., 6:502-503 (2000)). se cree que ciertas preparaciones de Apo2L/TRAIL recombinantes pueden variar en términos de las propiedades bioquímicas y las actividades biológicas o las células enfermas de las normales, dependiendo, por ejemplo, de la presencia o

ausencia de una molécula etiquetada, el contenido de 5 y/o el contenido de trímero (ver, por ejemplo, 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)).

El Apo2L/TRAIL se ha encontrado que se une a cinco diferentes receptores. Al menos dos de los receptores que se unen al Apo2L/TRAIL contienen un dominio muerto cito plasmático funcional. Uno de tales receptores se ha denominado como "DR4" (y alternativamente TR4 o TRAIL-R1) (Pan et al., *Science*, 276:111-113 (1997); ver también WO98/32856 publicada en julio 30, 1998; WO99/37684 publicada en julio 29, 1999; WO 00/73349 publicada en diciembre 7, 2000; US 6,433,147 otorgado en diciembre 13, 2002; US 6,461,823 otorgada en octubre 8, 2002, y US 6,342,383 otorgada en enero 29, 2002).

Otro de tales receptores para el Apo2L/TRAIL se ha denominado como DR5 (este se ha denominado alternativamente como Apo2L/TRAIL; TRAIL-R o TRAIL-R2, TR6, Tango-63, hAPO8, TRICK2 o KILLER) (ver, por ejemplo, Sheridan et al., *Science*, 277:818-821 (1997); Pan et al., *Science*, 277:815-818 (1997); WO98/51793 publicado en noviembre 19, 1998; WO98/41629 publicado en septiembre 24, 1998; Screatton et al., *Curr. Biol.*, 7:693-69e (1997); Walczak et al., *EMBO J.*, 16:5386-5387 (1997); Wu et al., *Nature Genetics*, 17:141-143 (1997); WO98/35986 publicado en agosto 20, 1998; EP870,827 publicado en octubre 14, 1998; WO98/46643 publicado en octubre 22, 1998; WO99/02653 publicado en enero 21, 1999; WO99/09165 publicado en febrero 25, 1999; WO99/11791 publicado en marzo 11, 1999; US 2002/0072091 publicado

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en agosto 13, 2002; US 2002/0098550 publicado en diciembre 7, 2001; US 6,313,269 otorgado en diciembre 6, 2001; US 2001/0010924 publicado en agosto 2, 2001; US 2003/01255540 publicado en julio 3, 2003; US 2002/0160446 publicado en octubre 31, 2002; US 2002/0048785 publicado en abril 25, 2002; US 6,342,369 otorgado en febrero, 2002; US 6,569,642 publicado en mayo 27, 2003; US 6,072,047 publicado en junio 6, 2000; US 6,642,358 publicado en noviembre 4, 2003; US 6,743,625 publicado en junio 1, 2004). Similar al DR4, el DR5 se reportó por contener un dominio citoplasmático muerto y es capaz de señalar la apoptosis luego de la unión de ligando (luego de unir una molécula tal como un anticuerpo antagonista que semeja la actividad del ligando). La estructura de cristal del complejo formado entre el Apo-2L/TRAIL y el DR5 se describe en Hymowitz et al., Molecular Cell, 4:563-571 (1999).

Luego de la unión de ligando, tanto el DR4 como el DR5 pueden disparar apoptosis independientemente al reclutar y activar el iniciador de apoptosis, las caspasa -8, a través de la molécula adaptadora que contiene el dominio de muerte denominado como 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)].

El Apo2L/TRAIL se ha reportado también por unirse a aquellos receptores denominados como DSR1, DcR2 y OPG, que se cree que funcionan como inhibidores, en lugar de traductores de señalización (ver, por ejemplo, DCR1 (también denominado como TRID, LIT o TRAIL-R3) [Pan et al., Science, 276:111-113 (1997);

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Sheridan et al., Science, 277:818-821 (1997); McFarlane et al., J. Biol. Chem., 272:25417- 25420 (1997); Schneider et al., FBBS 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); DCR2 (también llamado 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, 2:813-820 (1997)], and OPG [Simonet et al., supra]. Como contraste al DR4 y DR, los receptores de DcR1 y DcR2 no señalan apoptosis.

Ciertos anticuerpos que se unen a los receptores DR4 y/o DR5 se han reportado en la literatura. Por ejemplo, los anticuerpos anti-DR4 dirigidos al receptor DR4 y que tienen actividad agonística y apoptósica en ciertas células de mamífero se describen en, por ejemplo, la WO 99/37684 publicada en julio 29, 1999; WO 00/73349 publicada en junio 12, 2000; WO 03/066661 publicada en agosto 14, 2003. Ver, también, por ejemplo, Griffith et al., J. Immunol., 162:2597-2605 (1999); Chuntharapai et al., J. Immunol., 166:4891-4898 (2001); WO 02/097033 publicada en diciembre 2, 2002; WO 03/042367 publicada en mayo 22, 2003; WO 03/038043 publicado en mayo 8, 2003; WO 03/037913 publicado en mayo 8, 2003. Ciertos anticuerpos anti-DR5 que tienen forma similar se describen, ver, por ejemplo, WO 98/51793 Publicada en noviembre 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,

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Canadá; WO 03/038043 publicada en mayo 8, 2003; WO 03/037913 publicada en mayo 8, 2003.

Además, se han descrito ciertos anticuerpos que tienen reactividad cruzada tanto los receptores DR4 como DR5 (ver, por ejemplo, patente US 6,252,050 otorgada en junio 26, 2001).

El antígeno CD20 (también denominado antígeno de diferenciación restringido de linfocito B humano, Bp35) es una proteína de transmembrana hidrófoba con peso molecular de aproximadamente 35 kD localizado sobre linfocitos pre-B y B maduros (Valentine et al. J. Biol. Chem. 264 (19):11282-11287 (1989); y Einfeld et al. EMBO J. 7(3):711-717 (1988)). El antígeno también se expresa sobre más del 90% de los linfomas Hodgkin de célula B (NHL) (Anderson et al. Blood 63 (6):1424-1433 (1984)), pero se encuentra sobre células madre ematopoyéticas, células pro-B, células de plasma normales u otros tejidos normales (Tedder et al. J. Immunol. 135 (2):973-979 (1985)). El CD20 regula una o unas etapas tempranas en el proceso de activación para la iniciación de un ciclo de la célula y la diferenciación (Tedder et al., supra) y posiblemente funciona como un canal de ión de calcio (Tedder et al. J. Cell. Biochem. 14D:195 (1990)). Dada la expresión de CD20 en los linfomas de célula B, este antígeno puede servir como un candidato para "apuntamiento" de tales linfomas.

El anticuerpo rituximab (RITUXAN®) es un anticuerpo monoclonal de murino/humano quimérico trabajado por ingeniería genética dirigido contra el antígeno CD20. Rituximab es el anticuerpo

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denominado "C2B8" en la patente US No. 5, 736,137 otorgada en abril 7, 1998 (Anderson et al.). RITUXAN® se indica para el tratamiento de pacientes con linfoma no Hodgkin relapsado o refractario de bajo grado o folicular positivo para CD20. En el mecanismo de los estudios de acción in Vitro se ha demostrado que el RITUXAN® se une al complemento humano y liza las líneas de célula B linfoide a través de la citotoxicidad dependiente de complemento (CDC). (Reff et al. Blood 83 (2):435-445 (1994); Cragg and Marlin, Blood, 103: 2738-2743 (2004)).

Adicionalmente, este tiene actividad significativa en los ensayos para citotoxicidad mediada por célula dependiente de anticuerpo (ADCC). Más recientemente, el, RITUXAN® ha mostrado tener efectos anti proliferativos en ensayos de incorporación de timidina tritiada y para inducir apoptosis directamente, aunque otros anticuerpos anti-CD19 y CD20 no (Maloney et al. Blood 88(10):637a (1996)). La sinergia entre el RITUXAN® y ciertas quimio terapias y toxinas también se han observado experimentalmente. En particular, el RITUXAN® sensibiliza las líneas células de linfoma de célula B humana resistente a droga a los efectos cito tóxicos de la dextrorubicina, CDDP, VP-16, toxina de difteria y ricina (Demidem et al. Cancer Chemotherapy & Radiopharmaceuticals 12 (3):177-186 (1997)). En estudios pre clínicos in Vivo se han mostrado que el RITUXAN® vacía las células B de la sangre periférica, los nodos linfáticos, y la médula ósea de los monos cinomólogos, presumiblemente a través de los procesos de complemento y mediados por células (Reff et al. Blood 83 (2):435-445 (1994)).

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RESUMEN DE LA INVENCION

Los métodos para utilizar ligandos de receptor de muerte, tales como los polipéptidos de ligando Apo-2 /TRAIL o los anticuerpos de receptor de muertos y los anticuerpos CD20 se suministran aquí. Las modalidades de la invención incluyen métodos para tratar cáncer, que comprenden células cancerosas expuestas a una cantidad efectiva de anticuerpo Apo2L/TRAIL y CD20. Opcionalmente, las células cancerígenas están expuestas a una cantidad efectiva del anticuerpo receptor de muerte, tal como un anticuerpo DR4 agonista o un anticuerpo DR5 agonista, y un anticuerpo CD20. Opcionalmente, la cantidad de Apo2L/TRAIL o de anticuerpo receptor de muerte y anticuerpo CD20 empleado en los métodos son efectivos para lograr sinergia terapéuticamente, por ejemplo, su efecto anti cancerígeno combinado es mayor que el efecto anticancerígeno logrado con el Apo2L/TRAIL o los anticuerpos son empleados individualmente como un agente terapéutico simple. Los métodos que implican uso in Vitro o uso in vivo en donde el Apo2L/TRAIL o el anticuerpo receptor de muerte y el anticuerpo CD20 son administrados a un mamífero (paciente). Opcionalmente, en los métodos, las células de cáncer tratadas con Apo2L/TRAIL o el anticuerpo receptor de muerte y el anticuerpo CD20 son células de de linfoma.

Las modalidades adicionales de la invención incluyen métodos para tratar una enfermedad relacionada con la inmunidad, que comprende administrar a un mamífero una cantidad efectiva de Apo2L/TRAIL o anticuerpo CD20. Opcionalmente, una cantidad efectiva de anticuerpo receptor de muerte, tal como el anticuerpo DR4

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agonista o el anticuerpo DR5 agonista, y el anticuerpo CD20 se administran al mamífero. Opcionalmente, la cantidad del Apo2L/TRAIL o el anticuerpo receptor de muerte y los anticuerpos CD20 empleados en los métodos son efectivos para lograr sinergia terapéuticamente, por ejemplo, su efecto combinado para tratar enfermedades relacionadas con la inmunidad es, mayor que el efecto logrado cuando el Apo2L/TRAIL o los anticuerpos son empleados individualmente como un agente terapéutico simple. Opcionalmente, en los métodos, la enfermedad relacionada con la inmunidad es artritis remautoide o esclerosis múltiple.

Los métodos de la invención incluyen métodos para tratar un trastorno en un mamífero, tal como las enfermedades relacionadas con la inmunidad o el cáncer, que comprenden las etapas tejido o una muestra de célula del mamífero, examinar el tejido o las células para expresión de CD20, DR4 y/o DR5, y luego determinar dicho tejido o muestra de célula que expresa dicho uno o más receptores, que administran una cantidad efectiva de Apo2L/TRAIL o anticuerpo receptor de muerte y anticuerpo CD20 a dicho mamífero. Las etapas en los métodos para examinar la expresión de uno o más de tales receptores se puede conducir en una variedad de formatos de ensayo, que incluyen los ensayos que detectan la expresión de mRNA y los ensayos de inmuno histoquímica.

Opcionalmente, los métodos de la invención comprende, además de administrar una cantidad efectiva de Apo2L/TRAIL y/o anticuerpo receptor de muerte y anticuerpo CD20, administrar el agente o agentes quimioterapéuticos o de terapia de radiación a dicho mamífero.

[b]

Más modalidades de la invención se ilustran por vía de ejemplo en las siguientes reivindicaciones:

1. Un método para tratar células cancerígenas, que comprende exponer las células cancerígenas de mamífero a una cantidad sinérgicamente efectiva de polipéptido Apo2L/TRAIL y anticuerpo CD20.
2. El método de la reivindicación 1 en donde dicho polipéptido Apo2L/TRAIL comprende los aminoácidos 1-281 de la Fig. 1 (SEQ ID NO:1) o usualmente variante de este.
3. El método de la reivindicación 1 en donde dicho polipéptido Apo2L/TRAIL comprende los aminoácidos 114-281 de la Fig. 1 (SEQ ID NO: 1).
4. El método de la reivindicación 1 en donde dichas células cancerígenas están expuestas a una cantidad efectiva sinérgica del polipéptido Apo2L/TRAIL y el anticuerpo CD20.
5. El método de la reivindicación 1 en donde dichas células cancerígenas son células de linfoma.
6. El método de la reivindicación 1 que comprende además exponer las células cancerígenas a uno o más agentes inhibitorios del crecimiento.

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7. El método de la reivindicación 1 que comprende además exponer las células a radiación.
8. El método de la reivindicación 1 en donde dicho polipéptido Apo2L/TRAIL se expresa en una célula huésped recombinante de el grupo que consiste de una célula CHO, célula de levadura I. e Coli.
9. El método de la reivindicación 1 en donde dicho polipéptido Apo2L/TRAIL está ligado a una molécula de polietilenglicol.
10. El método de la reivindicación 1 en donde dicho anticuerpo CD20 es un anticuerpo monoclonal.
11. El método de la reivindicación 10 en donde dicho anticuerpo CD20 es el anticuerpo Rituximab.
12. Un método para tratar una enfermedad relacionada con la inmunidad, que comprende administrar a un mamífero una cautividad efectiva sinérgica del polipéptido Apo2L/TRAIL y el anticuerpo CD20.
13. El método de la reivindicación 12 en donde dicho polipéptido Apo2L/TRAIL comprende los aminoácidos 1-281 de la Fig. 1 (SEQ ID N0:1) o un fragmento o variantes de estos.

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14. El método de la reivindicación 12 en donde dicho polipéptido Apo2L/TRAIL comprende los aminoácidos 114-281 of Fig. 1 (SEQ ID NO:1).
15. El método de la reivindicación 12 en donde dicho polipéptido Apo2L/TRAIL se expresa en una célula huésped recombinante seleccionada del grupo que consiste de una célula CHO, célula de levadura I. e Coli.
16. El método de la reivindicación 12 en donde dicho polipéptido Apo2L/TRAIL está ligado a una molécula de polietilenglicol.
17. El método de la reivindicación 12 en donde dicha enfermedad relacionada con la inmunidad es la artritis reumatoide o la esclerosis múltiple.
18. El método de la reivindicación 12 en donde dicho anticuerpo CD20 es un anticuerpo monoclonal.
19. El método de la reivindicación 12 en donde dicho anticuerpo CD20 es el anticuerpo Rituximab.
20. El método de la reivindicación 1 o 12 en donde dicho polipéptido Apo2L/TRAIL y el anticuerpo CD20 se administran secuencialmente.

21. El método de la reivindicación 1 o 12 en donde dicho polipéptido Apo2L/TRAIL y el anticuerpo CD20 se administran concurrentemente.

BREVE DESCRIPCIÓN DE LOS DIBUJOS

La figura 1A muestra la secuencia de nucleótido del cADN de ligando Apo-2 humano (SEQ ID NO:2) y sus secuencia de aminoácido derivada (SEQ ID NO:1). El "N" en la posición de nucleótido 447 se utilizan para indicar que el nucleótido base puede ser un "T" o "G".

Las figuras 2A y 2B muestran la secuencia de nucleótido de cADN (SEQ ID NO:4) para DR4 humano de longitud completa y su secuencia de aminoácido derivada (SEQ ID NO:3). Las respectivas secuencias de nucleótido y aminoácido para el DR4 humano son reportadas en Pan et al., Science, 276:111 (1997).

La Figura 3A muestra las secuencias del aminoácido 411 del DR5 humano (SEQ ID NO:5) como se publicó en la WO 98/51793 en Noviembre 19, 1998. Una variante de empalme trascripcional del DR5 humano es conocida en la técnica. Esta variante de empalme DR5 codifica la secuencia de aminoácido 440 del DR5 humano (SEQ ID NO:6) mostrada en las figuras 3B y 3C como se publicó en la WO 98/35986 en Agosto 20, 1998.

La Figura 4 ilustra la expresión de los receptores Apo2L/TRAIL en las líneas celulares de linfoma B.

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La figura 5 ilustra la expresión de CD20 de las líneas células de linfoma b.

La figura 6 muestra los efectos del Apo2L/TRAIL, RITUXAN®, o tratamiento de combinación sobre el crecimiento de los seno injertos tumorales de linfoma BJAB sub cutáneo preestablecidos en ratones SCID.

La figura 7 muestra los resultados adicionales sobre los efectos del Apo2L/TRAIL, RITUXAN®, o tratamiento de combinación del Apo2L/TRAIL y RITUXAN® sobre el crecimiento de seno injertos tumorales de BJAB sub cutáneo preestablecidos en ratones SCID.

La figura 8 muestra los efectos del Apo2L/TRAIL, RITUXAN®, o tratamiento de combinación del Apo2L/TRAIL y RITUXAN® sobre el procesamiento de caspaza en seno injertos tumorales de linfomas BJAB sub cutáneo preestablecidos en ratones SCID.

La figura 9 muestra los efectos del anticuerpo agonista DR5 como RITUXAN®, o tratamiento de combinación sobre el crecimiento de seno injertos tumorales de linfoma BJAB sub cutáneo preestablecidos en ratones SCID.

La figura 10 muestra los efectos del anticuerpo agonista DR5 como RITUXAN®, o tratamiento de combinación sobre el procesamiento de caspaza en seno injertos tumorales de linfoma BJAB sub cutáneo preestablecidos en ratones SCID.

La figura 11 ilustra la expresión del CD20 y los receptores Apo2L/TRAIL en líneas celulares NHL.

La figura 12 muestra los efectos del Apo2L/TRAIL, RITUXAN®, o tratamiento de combinación sobre el crecimiento de los seno injertos tumorales RA1 ramos sub cutáneos pre establecidos en ratones SCID.

La figura 13 muestra los efectos del Apo2L/TRAIL, RITUXAN®, o tratamiento de combinación sobre el crecimiento de los seno injertos de linfoma folicular de ADOHH-2 pre establecidos en ratones SCID.

La figura 14 ilustra el efecto y los mecanismos de muerte celular mediante Apo2L/TRAIL, y RITUXIMAB o tratamientos de combinación sobre células BJAB.

La figura 15 muestra los efectos del Apo2L/TRAIL, y RITUXIMAB, o tratamiento de combinación sobre el crecimiento de los seno injertos tumorales T1 ramos en ratones SCDI.

La figura 16 muestra los efectos del Apo2L/TRAIL, y RITUXIMAB, o tratamiento de combinación sobre los seno injertos tumorales DJAB-luc en ratones SCDI.

DESCRIPCIÓN DETALLADA DE LAS MODALIDADES PREFERIDAS

A menos que se defina otra cosa, todos los términos de la técnica, anotaciones y otra terminología científica utilizada

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aquí pretenden tener los significados que se entienden comúnmente por aquellos expertos en la técnica a los cuales pertenece la invención. En Algunos casos, los términos con los significados comúnmente entendidos se definen aquí para claridad y/o para fácil referencia, y la inclusión de tales definiciones aquí no debe ser necesariamente considerada por representar una diferencia sustancial sobre lo que se entiende generalmente en la técnica. Las técnicas y procedimientos descritos o referenciados aquí son generalmente bien entendidos y comúnmente empleados utilizando la metodología convencional de aquellos expertos, tales, 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 ha apropiado, los procedimientos que involucran el uso de kits comercialmente disponibles y reactivos son generalmente llevados a cabo de acuerdo con los protocolos definidos por el fabricante y/o los parámetros a menos que se establezca otra cosa.

Antes de que los métodos presentes, los kits y los usos para estos sean descritos, se debe entender que esta invención está limitada a la metodología, protocolos, líneas celulares, especies de animales o géneros particulares, las construcciones y reactivos descritos como tales, pueden, por supuesto, variar. También se debe entender que la terminología utilizada aquí es para propósitos de describir solamente las modalidades particulares, y no está destinada a limitar el alcance de la

presente invención que estará limitada solamente a las reivindicaciones finales.

Se debe notar que como se utiliza aquí y las reivindicaciones finales, las formas singulares "A" y "I" y "El", incluyen las referencias plurales a menos que el contexto diste claramente otra cosa.

Todas las publicaciones mencionadas aquí se incorporan como referencia para mostrar y describir los métodos y/o materiales en relación con las cuales son citadas las publicaciones. Las publicaciones citadas aquí son citadas por su descripción anterior a la fecha de presentación de la presente solicitud. Nada aquí debe ser considerado como una admisión de que los inventores no tienen derecho a anticipar las publicaciones en virtud de una fecha de prioridad anterior o a una fecha anterior de invención. Además las fechas de publicaciones actuales pueden ser diferentes de aquellas mostradas y requieren verificación independiente.

DEFINICIONES

Los términos "ligando Apo-2", "Apo-2L", "Apo2L", "Apo2L/TRAIL", "ligando Apo-2 /TRAIL" y "TRAIL" se utilizan aquí intercambiabilmente para referirse a una secuencia de polipéptido que incluye los residuos de aminoácido 114-281, inclusive, 95-281, inclusive, los residuos 92-281, inclusive, los residuos 91-281, inclusive, los residuos 41-281, inclusive, los residuos 39-281, inclusive, los residuos 15-281, inclusive, los residuos 1-281,

inclusive, de la secuencia de aminoácido mostrada en la figura 1, así como también los fragmentos biológicamente activos, supresionales, insercionales, o variantes sustitucionales de las secuencias anteriores. En una modalidad, la secuencia de polipéptido comprende los residuos 114-281 de la figura 1. Opcionalmente, la secuencia del polipéptido comprende los residuos 92-281 o los residuos 91-281 de la figura 1. Los polipéptidos Apo-2L se pueden codificar mediante la secuencia de nucleótido mostrada en la figura 1. Opcionalmente, el codón que codifica el residuo pro 119 (figura 1) puede ser "CCT" o "CCG". Opcionalmente, los fragmentos o variantes son biológicamente activos y tienen al menos aproximadamente 80% de identidad de secuencia de aminoácido, más preferiblemente números aproximadamente 90 de identidad de secuencia, y aún más preferiblemente, al menos 95%, 96%, 97%, 98%, o 99% de identidad de secuencia con una cualquiera de las secuencias anteriores. La definición comprende las variantes sustitucionales del ligando Apo-2 en la cual al menos uno de sus aminoácidos nativo son sustituidos por otro aminoácido tales como un residuo de alanina. Las variantes sustitucionales opcionales incluyen una o más de las sustituciones de residuo. Las variantes opcionales pueden comprender una secuencia de aminoácido que difiere de la secuencia nativa de la secuencia del polipéptido ligando Apo-2 de la figura 1 y tiene uno o más de las siguientes sustituciones de aminoácido en la posición o posiciones de residuo en la figura 1: S96C; S101C; S111C; R170C,- K179C. La definición también comprende ligando Apo-2 de secuencia nativa de una fuente de ligando Apo-2 o preparada mediante métodos recombinantes o sintéticos. El ligando Apo-2 de la invención incluye los

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polipéptidos denominados como ligando Apo-2 o TRAIL descritos en la WO97/01633 publicada en enero 16, 1997, WO97/25428 publicada en julio 17, 1997, WO99/36535 publicada en julio 22, 1999, WO 01/00832 publicada en enero 4, 2001, WO02/09755 publicada en febrero 7, 2002, y WO 00/75191 publicada en diciembre 14, 2000.

Los términos son utilizados para referirse de manera general a las formas de ligando Apo-2 que incluyen las formas de monómero, dímero, trímero, exámero, u oligómero alto del polipéptido. Toda la numeración de los residuos de aminoácido a que se hace referencia en la secuencia Apo-2L usa la numeración de acuerdo a la figura 1, a menos que se establezca específicamente otra cosa. Por ejemplo, el "D203" o "AST203" se refiere al residuo de ácido aspártico de la posición 203 de la secuencia suministrada en la figura 1.

El término "variante selectiva de ligando Apo-2" como se utiliza aquí se refiere a un polipéptido de ligando Apo-2 el cual incluye una o más mutaciones de aminoácido en la secuencia de ligando Apo-2 nativa y tiene una afinidad de unión selectiva para el receptor DR4 o el receptor DR5. En una modalidad, la variante del ligando Apo-2 tiene una a afinidad de unión selectiva para el receptor DR4 e incluye una o más sustituciones de aminoácido en una cualquiera de las posiciones 189, 191, 193, 199, 201 o 209 de la secuencia de ligando Apo-2 nativa. En otra modalidad, la variante de ligando Apo-2 tiene una afinidad de unión selectiva para el receptor DR5 e incluye una o más sustituciones de aminoácido en cualquiera de las posiciones 189, 191, 193, 264, 266, 267 o 269 de la secuencia de ligando Apo-2 nativa.- Las

variantes selectivas de ligando Apo-2 preferidas incluyen una o más mutaciones de aminoácido y exhiben afinidad de unión al receptor DR4 el cual es igual o mayor ($\geq$) que la afinidad de unión del ligando Apo-2 de la secuencia nativa al receptor DR4, y aún más preferiblemente, las variables de ligando Apo-2 exhiben menos afinidad de unión ($<$) a su afinidad de unión al receptor DR5 que la afinidad de unión exhibida por la secuencia nativa del ligando Apo-2 al DR5. cuando la afinidad de unión et al variante de ligando APO-2 al receptor DR4 es aproximadamente igual (sin cambio) o mayor que (incrementada) comparada con la secuencia nativa del ligando Apo-2 y la afinidad de unión de la variante de ligando Apo-2 al sector DR5 es menos o aproximadamente eliminada comparada con el ligando Apo-2 de secuencia nativa, la afinidad de unión de la variante del ligando Apo-2 para los propósitos de esta, se considera "selectiva" para el receptor DR4. Las variantes del ligando Apo-2 selectivas Dr4 preferidas de la invención tendrán al menos 10 veces menos afinidad de unión al receptor DR5 (comparadas con el ligando Apo-2 de secuencia nativa), y aún más preferiblemente. Tendrán al menos 100 veces menos de afinidad de unión al receptor DR5 (comparadas con el ligando Apo-2 de secuencia nativa).

La afinidad de unión respectiva de la variante de ligando Apo-2 se puede determinar y comparar con las propiedades de unión del Apo-2 nativo (tal como las formas 114-281) mediante ELISA, RIA, y/o ensayos BIAcore, conocidos en la técnica. Las variantes de ligando Apo-2 selectivas DR4 preferidas de la invención inducirán apoptosis en al menos un tipo de célula del mamífero

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(preferiblemente de célula de cáncer), y tal actividad apoptósica se puede determinar mediante métodos conocidos en la técnica tales como el alamar blue o ensayo de cristal violeta. Las variantes de ligando Apo-2 selectivas DR4 pueden o no tener afinidades de unión alteradas con cualquiera de los receptores de señuelo para el Apo-2L, aquellos receptores de señuelo que son denominados en la técnica como DcR1, DcR2 y OPG.

Las variantes selectivas del ligando Apo-2 preferidas adicionales incluyen una o más mutaciones de aminoácido 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 nativa 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 nativa al DR4. cuando la afinidad de unión de tal variante de ligando Apo-2 al receptor DR5 es aproximadamente igual (sin cambios) o mayor que (incrementada) comparada con la secuencia nativa del ligando Apo-2 y la afinidad de unión de la variante de ligando Apo-2 al receptor DR4 es menor o casi eliminada comparada con el ligando Apo-2 de secuencia nativa, la afinidad de unión de la variante del ligando Apo-2 para los propósitos de esta, se considera "selectiva" para el receptor DR5. Las variantes del ligando Apo-2 selectivas Dr5 preferidas de la invención tendrán al menos 10 veces menos de afinidad de unión al receptor DR4 (comparadas con el ligando Apo-2 de secuencia nativa), y aún más preferiblemente. Tendrán al menos 100 veces menos de afinidad de unión al receptor DR4 (comparadas con el ligando Apo-2 de secuencia nativa).

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La afinidad de unión respectiva de la variante de ligando Apo-2 se puede determinar y comparar con las propiedades de unión del Apo-2L nativo (tal como las formas 114-281) mediante ensayos ELISA, RIA, y/o ensayos BIAcore, conocidos en la técnica. Las variantes de ligando Apo-2 selectivas DR5 preferidas de la invención inducirán apoptosis en al menos un tipo de célula del mamífero (preferiblemente de célula de cáncer), y tal actividad apoptósica se puede determinar mediante métodos conocidos en la técnica tales como el alamar blue o ensayo de cristal violeta. Las variantes de ligando Apo-2 selectivas DR5 pueden o no tener afinidades de unión alteradas con cualquiera de los receptores de señuelo para el Apo-2L, aquellos receptores de señuelo que son denominados en la técnica como DcR1, DcR2 y OPG.

La identificación del aminoácido puede utilizar el alfabeto de letra simple o el alfabeto de tres letras de los aminoácidos, es decir,

Asp D ácido Aspártico

Thr T Treonina

Ser S Serina

Glu E ácido Glutámico

Pro P Prolina

Gly G Glicina

Ala A Alanita

Cys C Cisteina

Val V Valina

Met M Metionina

Ile I Isoleucina

Leu L Leucina

Tyr Y Tirosina

Phe F fenilalanina

His H Histidina

Lys K Lisina

Arg R Arginina

Trp W Tripofan

Gln Q Glutamina

Asn N Asparagina.

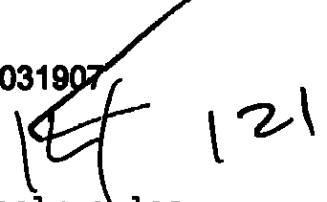
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El término dominio extracelular "Apo2L/TRAIL" o "Apo2L/TRAIL ECD" se refiere a una forma de Apo2L/TRAIL la cual está esencialmente libre de dominio citoplasmático y de transmembrana. Ordinariamente, el SD tendrá menos de 1% de tales dominios citoplasmático y de transmembrana y preferiblemente, tendrá menos de 0.5 de tales dominios. Se entenderá que cualquiera de los dominios de transmembrana identificados por los polipéptidos de la presente invención son identificados de acuerdo a los criterios rutinariamente empleados en la técnica para identificar el tipo de dominio hidrófobo. Los límites exactos de un dominio de transmembrana pueden variar pero más probablemente por no más de aproximadamente 5 aminoácidos en cualquier extremo del dominio como se identificó inicialmente. En las modalidades preferidas, el SD consistirá de una secuencia de dominio soluble, extracelular del polipéptido que es calibre de transmembrana y de los dominios citoplasmático o intracelular (y no está unido a membrana).

Las secuencias de dominio extra celular particular del Apo-2L/TRAIL se describen en la publicación PCT Nos. WO97/01633 y WO97/25428.

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

El término "dímero Apo2L/TRAIL" o "dímero Apo2L" se refiere a los dos monómeros Apo2L unidos en un enlace covalente por vía de



una unión disulfuro. El término como se utiliza aquí incluye los dímeros Apo2L aislados y los dímeros Apo2L que están dentro de las formas triméricas del Apo2L (es decir, asociadas con otro, el tercer monómero Apo2L).

El término "dímero Apo2L/TRAIL " o "dímero Apo2L" se refiere a los tres monómeros Apo2L que son asociados no covalentemente.

El término "agregado Apo2L/TRAIL " se utiliza para referirse a las formas oligoméricas mayores auto asociadas del Apo2L/TRAIL tal como los dímeros Apo2L/TRAIL, cuya forma, por ejemplo, las formas hexaméricas y nanoméricas del Apo2L/TRAIL. La determinación de la presencia y la cantidad del monómero, dímero o trímero Apo2L/TRAIL, (u otros agregados) se puede hacer utilizando métodos y ensayos conocidos en la técnica (que utilizan materiales comercialmente disponibles), tales como el HPLC de exclusión y tamaño nativo ("SEC"), la exclusión del tamaño desnaturalizante utilizando dodecil sulfato de sodio ("SDS-SEC"), HPLC de fase inversa y electroforesis de capilar.

El "receptor ligando Apo-2" incluye los receptores denominados en la técnica como "DR4" y "DR5" cuyas secuencias de poli nucleótido y polipéptido se muestran en las figuras 2 y 3 respectivamente. Pan et al. Han descrito la familia del receptor TNF α denominada como "DR4"(Pan et al., Science, 276:111- 113 (1997); ver también WO98/32856 publicada en julio 30, 1998; WO 99/37684 publicada en julio 29, 1999; WO 00/73349 publicada en diciembre 7, 2000; US 6,433,147 otorgada en agosto 13, 2002; US 6,461,823 otorgada en octubre 8, 2002, y US 6,342,383 otorgada en enero 29, 2002).

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Sheridan et al., Science, 277:818- 821 (1997) y Pan et al., Science, 277:815-818 (1997) descrita en otro receptor para Apo2L/TRAIL (ver también, WO98/51793 publicada en noviembre 19, 1998; WO98/41629 publicada en septiembre 24, 1998).

Este receptor se denomina como DR5 (receptor también se ha denominado alternativamente como Apo-2; TRAIL-R, TR6, Tango-63, hAPO8, TRICK2 o KILLER; Screaton et al., Curr. Biol., 2:693-696 (1997); Walczak et al., BMBO J., 1(5:5386-5387 (1997); Wu et al., Nature Genetics, 3/7:141-143 (1997); WO98/35986 publicada en agosto 20, 1998; EP870,827 publicada en octubre 14, 1998; WO98/46643 publicada en octubre 22, 1998; WO99/02653 publicada en enero 21, 1999; WO99/09165 publicada en febrero 25, 1999; WO99/11791 publicada en marzo 11, 1999; US 2002/0072091 publicada en agosto 13, 2002; US 2002/0098550 publicada en diciembre 7, 2001; US 6,313,269 otorgada en diciembre 6, 2001; US 2001/0010924 publicada en agosto 2, 2001; US 2003/01255540 publicada en julio 3, 2003; US 2002/0160446 publicada en octubre 31, 2002, US 2002/0048785 publicada en abril 25, 2002; US 6,569,642 otorgado en mayo 27, 2003, US 6,072,047 otorgado en 6, 2000, US 6,642,358 otorgada en noviembre 4, 2003). Como se describió anteriormente, otros receptores para el Apo-2L incluyen el DcR1, DcR2, y OPG (ver, Sheridan et al., supra; Marsters et al., supra; and Simonet et al., supra). El término "receptor Apo-2L "cuando se utiliza aquí comprende el receptor de secuencia nativa y las variantes del receptor. Estos términos comprenden el receptor Apo-2L expresado en una variedad de mamíferos, que incluyen losa humanos. El receptor Apo-2L puede ser endógenamente expresado como ocurre naturalmente en una variedad de linajes de tejido

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humano, o se puede expresar mediante métodos recombinantes o sintéticos. Un "receptor Apo-2L de secuencia nativa" comprende un polipéptido que tiene la misma secuencia de aminoácido que el receptor Apo-2L derivado de la naturaleza. Así, el receptor Apo-2L de secuencia nativa puede tener la secuencia de aminoácido del receptor Apo-2L de ocurrencia natural proveniente de cualquier mamífero. Tal receptor Apo-2L de secuencia nativa se puede aislar de la naturaleza o se puede producir mediante medios recombinantes o sintéticos. El término "receptor Apo-2L de secuencia nativa" comprende 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 variantes de ocurrencia natural, (por ejemplo formas alternativamente empalmadas) y variantes de ocurrencia natural. Las variantes del receptor pueden incluir fragmentos o mutantes de supresión del receptor Apo-2L de secuencia nativa. La figura 3A muestra la secuencia de aminoácido 411 del DR5 humano como se publicó en la WO 98/51793 en Noviembre 19, 1998. Una variante de empalme transcripcional del DR5 humano es conocida en la técnica. Esta variante de empalme DR5 codifica la secuencia del aminoácido 440 del DR5 humano mostrado en las figuras 3B y 3C como se publicó en la 98/35986 en Agosto 20, 1998.

"Anticuerpo del receptor de muerte" se utiliza aquí para referirse de manera general ala anticuerpo o anticuerpos dirigidos a un receptor en la súper familia del factor de necrosis tumoral y que contiene el dominio de muerte capaz de

señalizar apoptosis, y tales anticuerpos incluyen el anticuerpo DR5 y el anticuerpo DR4.

"El anticuerpo receptor DR5", "anticuerpo DR5" o "anticuerpo anti-DR5" se utiliza en un amplio sentido para referirse a los anticuerpos que se unen al menos una forma de un receptor DR5 o un dominio extracelular de este. Opcionalmente el anticuerpo DR5 se liga a una secuencia heteróloga o molécula. Preferiblemente la secuencia heteróloga permite o ayuda al anticuerpo a formar complejos de orden superior u oligomérico. Opcionalmente, el anticuerpo DR5 se une al receptor DR5 pero no reacciona de manera cruzada con cualquier receptor Apo-2L adicional (por ejemplo, DR4, DcR1, o DcR2). Opcionalmente, el anticuerpo es un agonista de la actividad de señalización DR5.

Opcionalmente, el anticuerpo DR5 de la invención se une a un receptor DR5 en un rango de concentración de aproximadamente 0.nM a aproximadamente 20nM medido en un ensayo de unión Biacore. Opcionalmente, los anticuerpos DR5 de la invención exhiben un valor I_{c50} de aproximadamente 0.6 nM a aproximadamente 18 nM medido en un ensayo de unión Biacore.

"Anticuerpo receptor DR4" anticuerpo DR4" o "anticuerpo anti-DR4" se utiliza en un amplio sentido para referirse a los anticuerpos que se unen al menos una forma de un receptor DR4 o un dominio extracelular de este. Opcionalmente el anticuerpo DR4 se liga a una secuencia heteróloga o molécula. Preferiblemente la secuencia heteróloga permite o ayuda al anticuerpo a formar complejos de orden superior u oligomérico. Opcionalmente, el anticuerpo DR4 se

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

Opcionalmente, el anticuerpo DR4 de la invención se une a un receptor DR4 en un rango de concentración de aproximadamente 0.1 n a aproximadamente 20mn medido en un ensayo de unión Biacore. Opcionalmente, los anticuerpos DR5 de la invención exhiben un valor Ic_{50} de aproximadamente 0.6 nm a aproximadamente 18 nm medido en un ensayo de unión Biacore.

El término "agonista" se utiliza en el sentido más amplio, e incluye cualquier molécula que mejora completamente, estimula, o activa una o más actividades biológicas del Apo2L/TRAIL, DR4 o DR5, in Vitro, in situ, o in vivo. Ejemplos de tales actividades biológicas que unen al Apo2L/TRAIL al DR4 o DR5 incluyen la apoptosis así como también aquellas reportadas adicionalmente en la literatura. Un agonista puede funcionar de una manera directa o indirecta. Por ejemplo, el agonista puede funcionar para mejorar parcial o completamente, estimular o activar una o más actividades biológicas del DR4 o DR5, in Vitro, in situ, in vivo como resultado de su unión directa al DR4 o DR5, que origina la activación del receptor o la transducción de señal. El agonista también puede funcionar indirectamente para mejorar parcial o completamente, estimular o activar una o más actividades biológicas del DR4 o DR5, in Vitro, in situ, o in vivo como resultado del, por ejemplo, estimular otra molécula efectuada

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que luego que entonces origina la activación del DR4 o DR5 o la transducción de señal.

Se contempla que un agonista puede actuar como una molécula mejoradora que funciona indirectamente para mejorar o incrementar la activación o actividad DR4 o DR5. Por ejemplo, el agonista puede mejorar la actividad del Apo2L endógeno en un mamífero. Esto se podría lograr, por ejemplo, al precompletar DR4 o DR5 o al estabilizar complejos del ligando respectivo con el receptor DR4 o DR5 (tal como estabilizar complejos negativos formados en el Apo2L y el DR4 o DR5).

El término "DR4" y "receptor DR4 como se utiliza aquí se refiere a formas de dominio extracelular de longitud completa y solubles del receptor descrito en Pan et al., Science, 276:111-113 (1997); WO98/32856 publicada en julio 30, 1998; patente US 5,342,363 otorgado en enero 29, 2002; y WO99/37684 publicado en julio 29, 1999. La secuencia de aminoácido de longitud completa del receptor DR4 se suministra aquí en la Fig. 2.

El término "DR5" y "receptor DR5" como se utiliza aquí se refiere a las formas de dominio extracelular de longitud completa y soluble del receptor descrito en Sheridan et al., Science, 277:818-821 (1997); Pan et al., Science, 277:815-818 (1997), US Patent 6,072,047 otorgada en junio 6, 2000; US Patent 6,342,369, WO98/51793 publicada en noviembre 19, 1998; WO98/41629 publicada en septiembre 24, 1998; Screatton et al., Curr. Biol., 2:693-696 (1997); Walczak et al., BMBO J[^], 1(5:5386-5387 (1997); Wu et al., Mature Genetics, 1/7:141-143 (1997); WO98/35986 publicada en

agosto 20, 1998; EP870,827 publicada en octubre 14, 1998; WO98/46643 publicada en octubre 22, 1998; WO99/02653 publicada en enero 21, 1999; WO99/09165 publicada en febrero 25, 1999; WO99/11791 publicada en marzo 11, 1999.

El receptor DR5 también se ha denominado en la técnica como Apo-2; TRAIL-R, TR6, Tango-63, hAPO8, TRICK2 o KILLER. El término receptor DR5 utilizado aquí incluye el polipéptido del aminoácido 411 de longitud completa suministrado en la Fig. 3A y el polipéptido del aminoácido 440 suministrado en las Figs. 3B-C.

El término "poliol" cuando se utiliza aquí se refiere ampliamente a compuestos de alcohol poli hidrico. Los polioles pueden ser cualquier polímero poli (óxido de alquilen) soluble en agua, y puede tener una cadena lineal o ramificada. Los 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 (etilenglicol) (PEG).

Sin embargo, aquellos expertos en la técnica reconocen que otros polioles, tales como por ejemplo, poli (propilenglicol) y copolímeros de polietileno-polipropilenglicol se pueden emplear utilizando las técnicas para la conjugación descritas aquí para PEG. Los polioles de la invención incluyen aquellos bien conocidos en la técnica y aquellos públicamente disponibles, tales como de las fuentes comercialmente disponibles.

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El término "conjugado" se utiliza aquí de acuerdo a su definición más amplia para significar unido o ligado junto. Las moléculas son "conjugadas" cuando ellas 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 transmembrana y dominio citoplasmáticos. Ordinariamente, el ECD soluble tendrá menos de 1% de tales dominios de transmembrana cito plasmáticos, y preferiblemente tendrán 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 divalente para uso en la presente invención incluyen pero no están limitados a zinc, cobalto, níquel, caseína, magnesio, y manganeso. Las formas particulares de tales metales que se pueden emplear incluyen formas de sales (por ejemplo formas de sales farmacéuticamente aceptables), tales como cloruro, acetato, carbonato, citrato, y sulfato de los iones de metal divalente anteriormente mencionados. Los iones de metal divalente, como se describieron aquí, son preferiblemente empleados en concentraciones o cantidades (por ejemplo, cantidades efectivas) que son suficientes para, por ejemplo, (1) mejorar la estabilidad de almacenamiento de los dímeros Apo-2L durante un periodo deseado de tiempo, (2) mejorar la producción o rendimiento de los dímeros Apo-2L en un cultivo de célula recombinante o método de purificación, (3) mejorar la solubilidad (o reducir la

agregación) de los dímeros Apo-2L, o (4) mejorar la formación del trímero Apo-2L.

"Aislado" cuando se utiliza para describir las varias proteínas descritas aquí, significan proteína que se ha identificado y separado y/o recubierto de un componente de su ambiente natural. Los componentes con contaminantes de su ambiente natural son materiales que interferirían típicamente con los usos diagnósticos o terapéuticos para la proteína, y pueden incluir enzimas, hormonas, y otros solutos proteínica o no proteínica. En modalidades preferidas, la proteína será purificada (1) a un grado suficiente para obtener al menos 15 residuos de secuencia N-terminal o de aminoácido interno mediante el uso de un secuenciadora de copa de giro, o (2) para homogeneidad mediante un SDS-page bajo condiciones no reductoras o reductoras utilizando Coomassie blue o, preferiblemente, teñido con plata. Las proteínas celulares incluyen la proteína in situ dentro de las células recombinantes, en razón a que al menos un componente del ambiente natural de la proteína no estará presente. De manera ordinaria, sin embargo, la proteína aislada se preparará mediante al menos una etapa de purificación.

Una molécula de ácido nucleico "aislada" es una molécula de ácido nucleico que se identifica y se separa de al menos una molécula de ácido nucleico contaminante con la cual este está ordinariamente asociada en la fuente natural del ácido nucleico. La molécula de ácido nucleico del ligando Apo-2 aislada es diferente en la forma o establecimiento de la cual esto se encuentre en la naturaleza.

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Las moléculas de ácido nucleico de ligando Apo-2 aisladas por lo tanto se distinguen de la molécula de ácido nucleico de ligando Apo-2 en la medida en que esta exista en células naturales. Sin embargo, una molécula de ácido nucleico de ligando Apo-2 aislada incluye las moléculas de ácido nucleico de ligando Apo-2 contenidas en las células que expresan ordinariamente el ligando Apo-2 donde, por ejemplo, la molécula de ácido nucleico está en un sitio cromosomal diferente de aquellas de las células naturales.

"identidad de secuencia de aminoácido %" con respecto a las secuencias identificadas aquí se define como el porcentaje de las resinas de aminoácido en una secuencia candidato que son idénticos con los residuos de aminoácido en la secuencia de ligando Apo-2, después de alinear la secuencia e introducir espacios, si es necesario, para lograr la identidad de secuencia porcentual máxima, y no considerar ninguna sustituciones conservadoras, a parte de la identidad de secuencia. El alineamiento para los propósitos de determinar la identidad de secuencia de aminoácido porcentual se puede lograr de varias maneras que son del conocimiento del experto y pueden determinar parámetros apropiados para medir el alineamiento, incluyendo asignar algoritmos necesarios para lograr el alineamiento máximo sobre las secuencias de longitud completa que son comparadas. Para los presentes propósitos, los valores de identidad de aminoácido porcentual se pueden obtener utilizando el programa de computadora de comparación de secuencia ALING-2, que fue elaborado por Genentech, Inc. y el código fuente que ha sido

presentado con la documentación de usuario con la oficina de derechos de autor de los Estados Unidos US Copyright Office, Washington, DC, 20559, registrado bajo el registro de derecho de autor US No. TXU510087. El programa ALIGN-2 está públicamente disponible a través de Genentech, Inc., South San Francisco, CA. Todos los parámetros de comparación de secuencia se establecen mediante el programa ALIGN-2 y no varían.

El término "secuencias de control" se refieren a unas secuencias de ADN necesarias para la expresión de una secuencia codificante operablemente ligada a un organismo huésped particular. Las secuencias de control que son adecuadas para los procariotes, por ejemplo, incluyen un promotor, opcionalmente una secuencia operadora, y un sitio de unión de ribosoma. Las células eucarióticas son conocidas por utilizar los promotores, señales de poliadenilación, y mejoradores.

El ácido nucleico está, "operablemente ligado" cuando este se coloca en una relación funcional con otra secuencia de ácido nucleico. Por ejemplo, el ADN para pre secuencia o el líder secretorio está operablemente ligado al ADN para un polipéptido si este se expresa como un AP proteína que participa en la secreción del polipéptido; un promotor o mejorador está operablemente ligado a una secuencia codificante si este afecta la transcripción de las secuencias; o un sitio de grande ribosoma está operablemente ligado a una secuencia codificante si este se ubica de tal forma que facilite la traducción. De manera general, "operablemente ligado" significa que las secuencias de ADN a las que están ligadas son contiguas, y, en el caso del líder

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secretorio, contiguo y en fase de lectura. Sin embargo, los mejoradotes no tienen que ser contiguos. El enlace se logra mediante la ligación en sitios de restricción convenientes. Si tales sitios no existen, los adaptare de oligo nucleótido sintéticos o enlazadores no son utilizados de acuerdo con la práctica convencional.

Una "célula B" es un linfocito que madura dentro de la médula ósea, e incluyen una célula B naive, célula B de memoria, o células B efectuatoras (células de plata). La célula B aquí puede ser una célula B normal o no maligna.

El antígeno "CD20" es una fosfoproteína no glicosilada de 35 KDA encontrada en la superficie de más del 90% de las células B provenientes de sangre periférica u órganos linfoides. El CD20 está presente tanto en células B normales, así como también en células B malignas, pero no se expresa sobre las células madre. Otros nombres para el CD20 en la literatura incluyen el "antígeno restringido linfocito B" y "BP35". El antígeno CD20 se describe en Clark et al. PNAS (USA) 82:1766 (1985), por ejemplo.

Ejemplos de los anticuerpos que se unen al antígeno CD20 incluyen: "C2B8" el cual es ahora denominado "Rituximab" ("RITUXAN®") (US Patent No. 5,736,137); el anticuerpo de murino 2B8 marcada con ittrio- [90] designada "Y2B8" o el "Ibritumomab Tiuxetan" ZEVALIN® comercialmente disponible de Idee Pharmaceuticals, Inc. (patente US No. 5,736,137; 2B8 depositado con el número depositado con la ATCC bajo número de acceso no. HB11388 en Junio 22, 1993); el IgG2a de murino "B1," también

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denominado "Tositumomab," opcionalmente marcado con ^{131}I para generar el anticuerpo " ^{131}I -B1" (yodo y ^{131}I tositumomab, BEXXAR[™]) comercialmente disponible de Corixa. (ver, también, patente US No. 5,595,721); el anticuerpo monoclonal de murino "1F5" (Press et al. Blood 69 (2):584-591 (1987)) y las variantes de estos que incluyen "la estructura parcheada" o humanizada 1F5 (WO 2003/002607, Leung, ATCC Deposit HB-96450); en anticuerpo 2H7 de murino y 2H7 quimérico (patente US No. 5,677,180); en 2H7 humanizado; HUMAX- CD20[™] completamente humano, el anticuerpo de alta afinidad apuntado en la molécula CD20 en la membrana celular de las células B (Genmab, Denmark; see, for example, 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 establecidos en la WO04/035607 (Teeling et al.); los anticuerpos AME-133[™] (Applied Molecular Evolution); el anticuerpo A20 o las variantes de estos tales como el anticuerpo A20 quimérico humanizado (cA20, hA20, respectivamente) (US 2003/0219433, Immunomedics); y los anticuerpos monoclonales L27, G28-2, 93-1B3, B-C1 o NU-B2 disponibles del . International Leukocyte Typing Workshop (Valentine et al., In: Leukocyte Typing III (McMichael, Ed., p. 440, Oxford University Press (1987))). El anticuerpo CD20 preferido aquí son anticuerpos CD20 quiméricos, humanizados, más preferiblemente el anticuerpo rituximab, 2H7 humanizado, y A20 quimérico o humanizado (Immunomedics), y el anticuerpo CD20 humano HUMAX-CD20[™] (Genmab).

Los términos "rituximab" o "RITUXAN®" se refieren aquí a anticuerpos de murino/humanos quiméricos trabajados por ingeniería genética dirigidos contra el antígeno CD20 y

designados "C2B8" en la patente US No. 5,736,137, que incluyen fragmentos de estos que retienen la capacidad al CD20. 134

Puramente para los propósitos de esto al menos que se indique otra cosa, el "2H7 humanizado" se refiere al anticuerpo CD20 humanizado, o a un fragmento de unión de antígeno de este, en donde el anticuerpo es efectivo para vaciar las células B de primate in vivo, el anticuerpo comprende la región variable de cadena H (B_h) de esta a al menos una secuencia CRH3 proveniente del anticuerpo CD20 anti humano y sustancialmente los residuos de la estructura de consenso humano (FR) del sub grupo III de la cadena pesada humana (V_{HIII}).

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

DIQMTQSPSSLSASVGDRVTITCRASSSVSYMHWYQQKPGKAPKPLIYAPSNLASGVPSRFSGSG
SGTDFTLTIS SLQPEDFATYYCQQWSFNPPTEFGQGTKVEIKR (SEQ ID NO: 7);

Y la secuencia de cadena pesada variable:

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGAIYPGNGDTSYNQKFK
GRFTISVDKS KNTLYLQMNSLRAEDTAVYYCARWYYSNSYWFYFDVWGQGTLLTVSS (SEQ ID
NO: 8)

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

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DIQMTQEPSSLSASVGDRVTITCRASSSVSYMHWYQQKPGKAPKPLIYAPSNLASGVPSRFSGSG
SGTDFTLTIS
SLQPEDFATYYCQWSFNPPTEGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASWCLLNNFYPR
EAKVQWKVD
NALQSGNSQESVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
(SEQ ID NO:9);

Y la secuencia de aminoácido de cadena pesada:

EVQLVESGGGLVQPGGSLRLSCAASGYTFSTSYNMHWVRQAPGKGLEWVGAIYPGNGDTSYNQKFK
GRFTISVDKS
KNTLYLQMNSLRAEDTAVYYCARWYYSNSYWFVDVWGQGTLLTVSSASTKGPSVFPLAPSSKSTS
GGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSWTVPSSSLGTQTYICNVNHKPSNTKV
DKKVEPKSC
DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVWDVSHEDPEVKFNWYVDGVEVHN
AKTKPREEQ
YNSTYRWSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKN
QVSLTCLVK
GFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH
YTQKSLSLSP GK (SEQ ID NO:10)

O la secuencia de aminoácido de cadena pesada:

EVQLVESGGGLVQPGGSLRLSCAASGYTFSTSYNMHWVRQAPGKGLEWVGAIYPGNGDTSYNQKFK
GRFTISVDKS
KNTLYLQMNSLRAEDTAVYYCARWYYSNSYWFVDVWGQGTLLTVSSASTKGPSVFPLAPSSKSTS
GGTAALGCL

VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSWTVPSSSLGTQTYICNHNKPSNTKV
DKKVEPKSC
DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVWDVSHEDPEVKFNWYVDGVEVHN
AKTKPREEQ
YNATYRWSVLTVLHQDWLNGKEYKCKVSNKALPAPIAATISKAKGQPREPQVYTLPPSREEMTKN
QVSLTCLVK
GFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNNH
YTQKSLSLSP GK (SEQ ID NO: 11).

"La citotoxicidad mediada por célula dependiente de anticuerpo" y "ADCC" se refieren a una reacción mediada por célula en la cual las células citotóxicas que expresan los receptores FC (FCRS) (por ejemplo células asesinas naturales (NK) neutrógenos, y macrófagos).

Reconoce el anticuerpo unido sobre una célula blanco y subsecuentemente causa la lisis de la célula blanco. Las células primarias para mediar ADCC, células NK, expresan solamente FC α RIII, mientras que los monocitos expresan FC α RI, FC α RII, y FC α RIII. La expresión de FCR sobre células hematopoyéticas se resume en la tabla 3 en la página 464 de Ravetch y Kinet, Annu. Rev. Immunol 9:457-92 (1991). Para evaluar la actividad ADCC de una molécula de interés, un ensayo ADCC in Vitro, tal como aquel descrito en la patente US No. 5,500,362 o 5,821,337 se puede desarrollar. Las células efectuatoras útiles para tales ensayos incluyen células mononucleares de sangre periférica (PBMC) y células asesinas naturales (NK). Alternativamente, o adicionalmente, la actividad ADCC de la molécula de interés se

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puede evaluar in vivo, por ejemplo, en un modelo de animal tal como el descrito en en Clynes et al. PNAS (USA) 95:652-656 (1998).

"células efectuatoras humanas "son leucocitos que expresan uno o más FcR y desarrollan funciones efectuatoras. Preferiblemente, las células que expresan al menos FC α RIII, y llevan a cabo la función efectuatora ADCC. Los ejemplos de leucocito humanos que median ADCC incluyen células mono nucleares de sangre periférica (PBMC), células asesino naturales (NK), monocitos, células T citotóxicas y neutrófilos; siendo las células PBMC y NK las preferidas.

Los términos "receptor Fc " o "FcR" son utilizados para describir un receptor que se une a la región FcR de un anticuerpo. El FcR preferido es un FCR humano de secuencia nativa. Más aún el FcR preferido es aquel que se une a un anticuerpo IgG (un receptor gama) e incluyen los receptores de las sub clases FC α RI, FC α RII, y FC α RIII que incluyen las variantes alélicas y alternativamente las formas y empalmadas de estos receptores. Los receptores FC α RII incluyen el FC α RIIA (un "receptor activante") y el FC α RIIB (un "receptor de inhibición "), que tiene secuencias de aminoácido similares que difieren principalmente en los dominios citoplasmáticos de esta. El receptor activante FC α RIIA contiene un motivo de activación basado en tiroxina inmunoreceptora (ITAM) en su dominio citoplasmático. El receptor de inhibición FC α RIIB contiene un motivo de inhibición basado en tiroxina inmunoreceptora (ITIM) en su dominio citoplasmático. (Ver Daeron,

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Annu..Rev. Immunol. 15:203-234 (1997)). Los FcR son o se pueden
revisar 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, que incluyen aquellos por ser identificados en el futuro, están comprendidos por el término "FcR" aquí. El término también incluye el receptor neonatal, FcRn, que es responsable por la transferencia de los IgGs maternos al feto (Guyer et al., J. Immunol. 117:587 (1976) and Kim et al., J. Immunol. 24:249 (1994)). Los FcR incluyen aquí polinucleótidos tales como el dimorfismo genético en el gen que codifica el FcαRIIIa en una fenilalanina (F) o una valina (V) en la posición de aminoácido 158, localizada en la región del receptor que se une al IgG1. La valina homocigoto FcαRIIIa (FcαRIIIa- 158V) se ha mostrado por tener una afinidad superior por el IgG1 humano y media el ADCC incrementado in Vitro en relación a la fenilalanina homocigota FcαRIIIa (FcαRIIIa-158F) o receptores heterocigotos (FcαRIIIa-158F/v).

"citotoxicidad dependiente de Complemento" o "CDC" se refiere a la capacidad de una molécula a lisar un blanco en la presencia de complemento. La celda de activación de complemento se inicia como la unión del primer componente del sistema de complemento (C1q) a una molécula (por ejemplo un anticuerpo) complejoado con un antígeno innato. Para evaluar la activación de complemento, un ensayo CDC, por ejemplo, como se describió en Gazzano-Santoro et al., J. Immunol. Methods 202:163 (1996), se desarrolló.

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El término "anticuerpo" se utiliza aquí en el sentido más amplio y cubre específicamente los anticuerpos monoclonales intactos, los anticuerpos poli clonales, los anticuerpos multi específicos (por ejemplo anticuerpos bi específicos) formados de al menos dos anticuerpos intactos, y los fragmentos de anticuerpo en tanto ellos exhiban la actividad biológica deseada.

"fragmentos de anticuerpo" comprende una porción de un anticuerpo intacto, preferiblemente que comprende el antígeno de unión o la región variable de este. Ejemplos de fragmentos de unión incluyen Fab, Fab1, F(ab')₂, y los fragmentos Fv; diacuerpos; anticuerpos lineales, moléculas de anticuerpo de cadena simple; y anticuerpos multi específicos formados de fragmentos de anticuerpo.

"anticuerpos nativos" son usualmente lico proteínas heterotetraméricas de aproximadamente 150000 Daltons, compuestos de dos cadenas ligeras (L) idénticas y dos cadenas pesadas (H) idénticas. Cada cadena ligera está ligada a una cadena pesada mediante una unión disulfuro covalente, aunque el número de enlaces disulfuro varía entre las cadenas pesadas de diferentes isotipos de inmunoglobulina. Cada cadena pesada y ligera también tiene puente disulfuro intra cadenas regularmente espaciados. Cada pesada tiene en un extremo un dominio variable (VH) por un número de dominios constantes. Cada cadena ligera tiene un dominio variable en un extremo (VN) 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

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variable de cadena ligera se línea con el dominio variable de la cadena pesada.

Los residuos de aminoácido particulares se cree que forman una interfase entre los dominios variables de cadena ligera y de cadena pesada.

El término "variable" se refiere al hecho de que ciertas porciones de los dominios variables difieren extensivamente en las secuencias de los anticuerpos y son utilizados en la unión y especificidad de cada anticuerpo particular para su antígeno particular. Sin embargo la variabilidad no está homogéneamente distribuida a través de los dominios variables de los anticuerpos. Se concentran en tres dominios variables de cadena. Las porciones más altamente conservadas de los dominios variables son denominadas las regiones de estructura (FR),. Los dominios variables de las cadenas pesadas y livianas nativas comprenden cada uno 4FR, adaptando grandemente una configuración de lámina beta, concentrada a tres regiones hipervariables, que forman conexiones de arco, y en algunos casos forman parte de, la estructura de lámina beta. En las regiones hipervariables en cada cadena son mantenidas juntas en proximidad cercana mediante los FÓRMULA y, con las regiones hipervariables de la otro cadena, contribuyen en la formación de un sitio de unión de antígeno de los anticuerpos (ver, 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 directamente involucrados en unir un anticuerpo o un antígeno, sino que exhiben varias funciones

efectuadoras, tales, como la participación del anticuerpo en citotoxicidad mediada por célula dependiente de anticuerpo (ASC).

La digestión de papaína de los anticuerpos produce dos fragmentos de unión de antígeno idénticos, denominados fragmentos "FAB", cada uno con un sitio de unión de antígeno simple, y un fragmento "FC" residual, cuyo nombre refleja su capacidad para cristalizar fácilmente. El tratamiento de toxina produce un fragmento F (ab')² que tiene dos sitios de unión de antígeno y es aún capaz de reticular antígeno.

"FV" es el fragmento de anticuerpo mínimo que contiene un reconocimiento de antígeno completo y un sitio de unión de antígeno. Esta región consiste de un dímero de una cadena pesada y un dominio variable de cadena ligera en asociación estrecha, no covalente. Es en esta configuración que las tres regiones hipervariables de cada dominio variable interactúan para definir un sitio de unión de antígeno sobre la superficie del dímero VH-VL. Colectivamente, las seis regiones hipervariables confieren especificidad de unión de antígeno al anticuerpo. Sin embargo, aún un dominio variable simple (o la mitad de un FV que comprende solamente tres regiones hipervariables específicas para un antígeno) tiene la capacidad de reconocer y unirse al antígeno, aunque a una afinidad inferior de la del sitio de unión completo.

El fragmento FAB también contiene el dominio constante de la cadena ligera y el primer dominio constante (CH1) de la cadena pesada. Los fragmentos FAB' difieren de los fragmentos FAB mediante la adhesión de unos pocos residuos en el terminal

carboxi del dominio CH1 de cadena pesada que incluye una o más cisteínas provenientes de la región pivote de anticuerpo. El FAb'-SH es la designación aquí para el fAB' en el cual el o los residuos de cisteína de los dominios constantes llevan al menos un grupo tiol libre. Los fragmentos de anticuerpo (FAB')² originalmente fueron producidos como pares de los fragmentos FAb' que tienen cisteínas pivote entre ellas. Otros acoplamientos químicos de fragmento de uní cuerpo también son conocidos.

Las "cadenas ligeras" de anticuerpos (inmunoglobulina) provenientes de las especies de vertebrados se pueden asignar a uno o dos tipos claramente distintos, denominados capa (κ) y landa (λ), con base en las secuencias de aminoácido de sus dominios constantes.

Dependiendo de la secuencia de aminoácido del dominio constante de sus cadenas pesadas, los anticuerpos pueden ser asignados a diferentes clases. Existen cinco clases principales de anticuerpos intactos: IgA, IgD, IgE, IgG, y IgM, y varios de estos pueden ser adicionalmente divididos en sub clases (isotipos), por ejemplo, IgG-1, IgG2, IgG3, IgG4, IgA, y IgA2. Los dominios constantes de cadena pesada que corresponden a las clases diferentes de los anticuerpos son denominada α , δ , ϵ , γ , y μ , respectivamente.

Las estructuras sub unitarias de las configuraciones tridimensionales de clases diferentes de inmunoglobulinas son bien conocidas.

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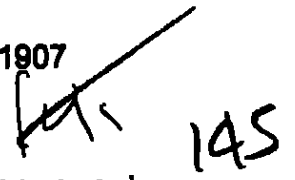
Los fragmentos de anticuerpo "Fv de cadena simple" o "SCFV" comprenden los dominios Vh y VL del anticuerpo, en donde estos dominios están presentes en una cadena de polipéptido simple. Preferiblemente, el polipéptido FV comprende además un ligador de polipéptido entre los dominios VH y VL que le posibilitan al scFv formar la estructura deseada para la unión de antígeno. Para una revisión del ScFv ver Plückerthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

El término "diacuerpo" se refiere a fragmentos de anticuerpo pequeños con dos sitios de unión de antígeno, cuyos fragmentos comprenden un dominio variable de cadena pesada (VH) conectado a un dominio variable de cadena ligera (VL) en la misma cadena de polipéptido (VH-VL). Al utilizar un ligador que es demasiado corto para permitir el pareado entre los dos dominios sobre la misma cadena, los dominios son forzados a aparearse con los dominios complementarios de otra cadena y crear dos sitios de unión de antígeno. Los diacuerpos se describen más completamente en, por ejemplo, EP 404,097; WO 93/11161; and Hollinger et al., Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993).

El término "anticuerpo monoclonal" como se utiliza aquí se refiere a un anticuerpo obtenido de una población de anticuerpos sustancialmente homogéneos, es decir, los anticuerpos individuales que comprenden la población son idénticos excepto por las mutaciones posibles de ocurrencia natural que puede estar presentes en cantidades menores. Los anticuerpos monoclonales son altamente específicos, los cuales están dirigidos contra el sitio

anti higiénico simple. Adicionalmente, como contraste a las preparaciones de anti cuerpo convencional (policlonal) que incluyen típicamente diferentes anticuerpos dirigidos contra diferentes determinantes (epítopos), cada anticuerpo monoclonal está dirigido contra un determinante simple sobre el antígeno. Además de su especificidad, los anticuerpos monoclonales son ventajosos porque ellos son sintetizados en el cultivo de hibridoma, no contaminado por otras inmunoglobulinas. El modificador "monoclonal" indica el carácter del anticuerpo a ser obtenido de una población de anticuerpos sustancialmente homogénea, y no se debe considerar como que requiera producción del anticuerpo por ningún método particular. Por ejemplo, los anticuerpos monoclonales a ser utilizados de acuerdo con la presente invención se pueden hacer mediante el método de hibridoma primero descrito por Kohler et al., Nature, 256:495 (1975), o se pueden hacer mediante métodos de ADN recombinante (ver, por ejemplo., patente estadounidense No. 4,816,567) Los "anticuerpos monoclonales" también se pueden aislar de librerías de anticuerpo de fago utilizando las técnicas descritas en Clackson et al., Nature, 352:624-628 (1991) y Marks et al., J. Mol. Biol., 222:581-597 (1991), por ejemplo.

Los anticuerpos monoclonales específicamente aquí incluyen anticuerpos "quiméricos" (inmunoglobulinas) en la cual una porción de la cadena pesada y/o ligera es idéntica o homóloga a las secuencias correspondientes en anticuerpos derivados de especies particulares o que pertenecen a una clase o sub clase de anticuerpo particular, aunque el resto de las cadenas es idéntica u homóloga a las secuencias correspondientes en anticuerpos



derivados de otras especies o que pertenecen a otras clases o subclases de anticuerpo, así, como también fragmentos de tales anticuerpos, en tanto ellas exhiban la actividad biológica deseada (patente.S. No. 4,816,567; Morrison et al., Proc. Natl. Acad. Sci. USA, 81:6851-6855 (1984)).

Los anticuerpos quiméricos de interés incluyen anticuerpos "privatizados" que comprenden las secuencias de unión de antígeno de dominio variable derivadas de un primate no humano (por ejemplo mono del viejo mundo, tal como el baboon, rhesus o el mono cinomolgus) y las secuencias de región constante humana (patente US No. 5,693,780).

Las formas "humanizadas" de los anticuerpos no humanos (por ejemplo murino) son anticuerpos quiméricos que contienen secuencias mínimas derivadas de inmunoglobulina no humana. Para la mayor parte, los anticuerpos humanizados son inmunoglobulinas humanas (anticuerpo receptor) en los cuales el residuo proveniente de la región hipervariable del receptor son reemplazados por residuos de la región hipervariable de las especies no humanas, (anticuerpo donador) tal como ratón, rata, conejo o primate no humano que tiene la especificidad deseada, afinidad, y capacidad. En algunos casos, los residuos de estructura (FR) de la inmunoglobulina no humana son reemplazados por los residuos no humanos correspondientes. Adicionalmente, los anticuerpos humanizados pueden comprender residuo que nos son encontrados en el anticuerpo receptor o en el anticuerpo donador. Estas modificaciones son hechas para refinar adicionalmente el desempeño del anticuerpo. En general, el anticuerpo humanizado

comprenderá esencialmente todas o al menos una, y químicamente dos, dominios variable, en los cuales o sustancialmente todos los aros hipervariables corresponden a aquellos de la inmunoglobulina no humana, y a todas o a sustancialmente todos los FR son aquellos de una secuencia de inmunoglobulina humana. El anticuerpo humanizado también comprenderá al menos una porción de una región constante de inmunoglobulina (FC), típicamente que de una inmunoglobulina humana. Para detalles adicionales, ver, 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 términos "región hipervariable" como se utiliza aquí se refiere a los residuos de aminoácido de un anticuerpo los cuales son responsables por la unión de antígeno. La región hipervariable comprende los residuos de aminoácido de una región de determinante complementariedad o "CR" por ejemplo los residuos 24-34 (L1) 50-56 (L2) y 89-97 (L3) en el dominio de variable de cadena ligera y 31-35 (H1), 50-56 (L2) y 89-97 (L3) en el dominio variable de cadena pesada; 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 "aro 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 and Lesk J. Mol. Biol. 196:901-917 (1987)). Los residuos de "estructuras" o "FR" son

aquellos residuos de dominio variables diferentes de los residuos de región hipervariable como se definieron aquí.

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Un anticuerpo "que une" un antígeno de interés, por ejemplo CD20 o DR4 o DR5, es uno capaz de unir aquel antígeno con suficiente afinidad y/o avidéz de tal forma que el anticuerpo es útil como un agente terapéutico para apuntar a una célula que exprese el antígeno.

Para los propósitos de esto "inmuno terapia" se referirá a un método para 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 se puede conjugar o fusionar con R las moléculas heterólogas o el o los agentes, tal como uno o más agente o agentes cito tóxicos, generando de esta manera un "inmunoconjugado".

Un anticuerpo "aislado" es aquel que ha sido identificado y separado y/o recuperado de un componente de su ambiente natural. Los componentes contaminantes de su ambiente natural son materiales que interferirían con los usos diagnósticos o terapéuticos del agonista o anticuerpo, y pueden incluir enzimas, hormonas, y otros solutos, proteináceos o no proteináceos. En modalidades preferidas, el anticuerpo será purificado (1) a más del 95% en peso del anticuerpo como se determinó mediante el método Lowry, y más preferiblemente más del 99% en peso, (2) a un grado suficiente para obtener al menos 15 residuos de la secuencia de aminoácido N-terminal o interna (mediante el uso de un secuenciador de copa de giro) o (3) hasta homogeneidad

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mediante SDS - PAGE bajo condiciones reductoras y no reductoras utilizando azul Coomassie o Coomassie blue, o, preferiblemente, tinte de plata. El anticuerpo aislado incluye el anticuerpo in situ dentro de las células recombinantes en razón a que al menos un componente del ambiente natural del anticuerpo no estará presente. De manera habitual, sin embargo, el anticuerpo aislado se preparará mediante al menos una etapa de purificación.

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

El término "agente inmuno supresor" como se utiliza aquí para terapia adjunta se refiere a sustancias que actúan para suprimir o enmascarar el sistema inmune del mamífero que está siendo tratado. Este incluirá sustancias que suprimen la producción de citoquina, sub regulan o suprimen la expresión de auto antígeno, o enmascaran los antígenos NHC. Ejemplos de tales agentes incluyen 2-amino-6-arilo-5- pirimidinas sustituidas (ver patente estadounidense No. 4,665,077, cuya descripción se incorpora aquí como referencia); drogas anti inflamatorias no esteroides (NSAIDs); azatioprina; ciclofosfamida; bromocriptina; danazol; dapsona; glutaraldehído (que enmascara los antígenos MHC como se describieron en la patente estadounidense No. 4,120,649); anticuerpos anti-idiotípicos para los antígenos MHC y los fragmentos MHC; la ciclosporina A; los esteroides tales como glucocorticosteroides, por ejemplo prednisona, metilprednisolona, dexametasona, y hidrocortisona; metotrexato (oral o sub cutáneo);

hidroxicloroquina; sulfasalazina; leflunomida; agonistas
receptores de citoquina o citoquina que incluyen los anticuerpos
anti- interferón- γ , - β , o - α , anticuerpos del factor- α de
necrosis antitumoral (infliximab o adalimumab), la inmunoahesina
anti-TNF α (etanercept), los anticuerpos de factor- β de necrosis
anti-tumoral, los anticuerpos anti-interleukina-2 y los
anticuerpos del receptor anti-IL-2; los anticuerpos anti-LFA-I,
que incluyen anti-CD11a y los anticuerpos anti- CD18; los
anticuerpos anti-L3T4; las globulinas anti-linfocito heterólogas;
los anticuerpos pan-T, preferiblemente anti-CD3 o anticuerpos
anti-CD4/CD4a; el péptido soluble que contiene el dominio de
unión LFA-3 (WO 90/08187 publicada 7/26/90); estreptoquinasa;
TGF- β ; estreptodornasa; ARN o ADN proveniente del hésped; FK506;
RS-61443; deoxispergualina; rapamicina; receptor de célula T
(Cohen et al., U.S. Pat. No. 5,114,721); fragmentos receptores de
célula T (Offner et al., Science, 251: 430-432 (1991); WO
90/11294; Ianeway, Nature, 341: 482 (1989); y WO 91/01133); y los
anticuerpos receptores de célula T (EP 340,109) tal como T10B9.

El término "agente cito tóxico " como se utiliza aquí, se refiere
a una sustancia que inhibe o evita las funciones de las células
y/o origina la destrucción de las células. El término está
destinado a incluir los isótopos radiactivos (por ejemplo, At²¹¹,
I¹³¹, I¹²⁵, Y⁹⁰, Re¹⁸⁶, Re¹⁸⁸, Sm¹⁵³, Bi²¹², P³² y los isótopos
radioactivo de Lu), los agentes quimioterapéuticos, y las toxinas
tales como las toxinas de molécula pequeña o las toxinas
enzimáticamente activa de origen bacterial, fungoso, vegetal o
animal, o fragmentos de estos.

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"Actividad sinérgica" o "sinergia" o "efecto sinérgico" o "cantidad sinérgicamente efectiva" para los propósitos de estas significan que el efecto observado cuando emplea una combinación de Apo2L/TRAIL o anticuerpo receptor de muerte y el anticuerpo CD20 es (1) mayor que el efecto logrado cuando aquel Apo2L/TRAIL, anticuerpo receptor de muerte o anticuerpo CD20 se emplea sólo (o individualmente) y (2) mayor que la suma agregada (aditiva) efecto para aquel Apo2L/TRAIL o anticuerpo receptor de muerte y anticuerpo. Tal efecto de sinergia o sinérgico se puede determinar por vía de una variedad de medios conocidos por aquellos expertos. Por ejemplo, el efecto sinérgico del Apo2L/TRAIL o anticuerpo receptor de muerte y anticuerpo CD20 se puede observar en formatos de ensayo in Vitro o in vivo examinando la reducción del número de células tumorales o de masa de tumor.

Los términos "apoptosis" y "actividad apoptósica" son utilizados en un amplio sentido para referirse a una forma ordenadamente o controlada de muerte celular en mamíferos que es típicamente acompañada por uno o más cambios celulares característicos, que incluyen la condensación del citoplasma, la pérdida de la membrana de plasma microvilli, la segmentación de los núcleos, la degradación del ADN cromosomal o la pérdida de la función mitocondrial. Esta actividad se puede determinar al medir utilizando métodos bien conocidos en la técnica, por ejemplo, mediante ensayos de viabilidad celular, los análisis FAC o la electroforesis de ADN, la unión de anexina V, la fragmentación de ADN, el encogimiento celular, la dilatación del retículo endoplasmático, la fragmentación celular y/o la formación de

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vesícula de membrana (denominadas cuerpos apoptóticos). Los ensayos que determinan la capacidad de un anticuerpo (por ejemplo Rituximab) a inducir la apoptosis se han descrito en Shan et al. Cáncer Immunol Immunther 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", "canceroso", y "maligno" se refieren o describen las condiciones fisiológicas en mamíferos que están típicamente caracterizados por el crecimiento celular no regulado. Ejemplos de cáncer incluyen pero no están limitados, a, carcinoma que incluyen adenocarcinoma, linfoma, melastroma, melanoma, sarcoma y leucemia. Ejemplos más particulares de tales cánceres incluyen cáncer de célula escamosa, cáncer pulmonar de célula pequeña, cáncer pulmonar de célula no pequeña, cáncer gastrointestinal, linfoma de Hodgkin' y no Hodgkin', cáncer pancreático, glioblastoma, glicoma, cáncer cervical, cáncer de ovario, cáncer de hígado tal como el carcinoma hepático y la hepatoma, cáncer de vejiga, cáncer de mama, cáncer de colon, cáncer colo rectal, carcinoma endometrial, mieloma (tal como mieloma múltiple), carcinoma de glándula salivar, cáncer de riñón tal como carcinoma de célula de próstata y tumores Wilms, carcinoma de célula basal, melanoma cáncer de próstata, cáncer de vulva, cáncer de tiroides, cáncer testicular, cáncer esofágico y varios tipos de cáncer de cabeza y cuello.

El término "enfermedad relacionada con sistema inmune" significa una enfermedad en la cual un componente del sistema inmune de un mamífero origina, media o contribuye de otra manera a la

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morbilidad en el mamífero. También están incluidas enfermedades en las cuales la estimulación o intervención de la respuesta inmune tiene un efecto mejorado o de prohibición de la enfermedad. Incluida en este término están las enfermedades autoinmunes, las enfermedades inflamatorias no mediadas por inmunidad, las enfermedades infecciosas, y las enfermedades de inmuno deficiencia. Ejemplos de enfermedades relacionadas con inmunidad e inflamatorias, algunas de las cuales son inmunes o mediadas por célula T, que se pueden tratar de acuerdo con la invención, incluyen lupus heritematoso, artritis remautoide, a artritis crónica juvenil, espóndil artropatías (esclerosis múltiple (esclerodermia)), niopatías inflamatorias y diopáticas (dermatositis poliomiocitis), síndrome de Sjogren, vasculitis sistémica, sarcoidosis, anemia hemolítica autoinmune (pancitopenia inmune, hemoglobinuria nocturna paroxismal), trombositopenia autoinmune (púrpura trombocitopénica idiopática, trombositotenia mediada por inmunidad), tiroiditis (enfermedad de Grave, tiroiditis Hashimoto, tiroiditis linfocítica juvenil, tiroiditis atropica), diabetes mellitus, enfermedad renal mediada por sistema inmune (glomerulonefritis, tubulointerstitial nefritis), enfermedades desminilizantes del sistema nervioso central y periférico tal como esclerosis múltiple, poli neuropatía desmienilizante a síndrome de - Barre, y poli neuropatía desmienilizante inflamatoria crónica, enfermedad heptobiliar tal como hepatitis infecciosa (hepatitis A, B, C, D, E y otros virus no hepatotrópicos), hepatitis activa crónica autoinmune, cirrosis filial primaria, hepatitis granulomatosa, y colangitis esclerosante, enfermedad del pulmón inflamatoria y fibrótica tal como enfermedad inflamatoria del intestino (colitis

ulcerativa: enfermedad de Crohn's), enteropatía sensible gluten, y enfermedad de Whipple, enfermedades de la piel autoinmunes y mediadas por inmunidad que incluyen enfermedades de piel bulosa, eritema multiforme y dermatitis de contacto, soriasis, enfermedades alérgicas tales como asma, rinitis alérgica, dermatitis atópica, hipersensibilidad a los alimentos y urticaria, enfermedades inmunológicas del pulmón tales como neumonías eosinofílicas, fibrosis pulmonar e idiopática y neumotes de hipersensibilidad enfermedades asociadas a trasplante que incluyen rechazo de injerto y enfermedad de injerto vs. huésped. Las enfermedades infecciosas incluyen SIDA (infección por HIV), hepatitis A, B, C, D, y E, e infecciones bacteriales, infecciones fúngicas, infecciones por protozoarios e infecciones parasíticas.

Una "malignidad de célula B" es una malignidad que involucra la célula B. ejemplos incluyen enfermedad de Hodgkin (LPHD); linfoma de no Hodgkin (NHL); linfoma de célula de centro folicular (PCC); leucemia linfocítica aguda (ALL); leucemia linfocítica crónica (CLL); leucemia de célula mielosa, linfoma linfocítico plasmocitoide; linfoma de célula de manto, linfoma relacionado con SIDA o HIV; mieloma múltiple; linfoma del sistema nervioso central (CNS); trastorno linfoproliferativo post trasplante (PTLD); macroglobulina de Waldenstrom (linfoma linfoplasmacítico); tejido de linfoide asociado a mucosa (MALT); y linfoma/leucemia de célula marginal.

El linfoma no Hodgkin (NHL) incluye, pero no se limita a, NHL grado/folicular, NHL relapsado o refractario, NHL de grado bajo

de liga frontal, NHL etapa III/IV, NHL resistente a quimio
terapia, NHL linfocítico pequeño (SL), NHL de grado intermedio/
folicular, NHL difuso de grado intermedio, linfoma de célula
grande difusa, NHL agresivo (que incluye NHL de línea frontal
agresiva y NHL relapsado agresivo), NHL relapsante después o
refractario a trasplante de célula madre autóloga, NHL inmuno
blástico de alto grado, NHL linfoblástico de grado alto, línea
celular no clivada pequeña de alto grado, NHL de enfermedad de
masa,, etc.

Una "enfermedad autoinmune" aquí es una enfermedad o trastorno
que surge de y está dirigida contra unos tejidos propios de
individuos o co segregado o manifestación de este o condición
resultante de estos. Ejemplos de enfermedades autoinmunes o
desórdenes incluyen, pero no están limitados a artritis (artritis
reumatoides, artritis remautoide juvenil, osteoartritis, artritis
soriática, y espodilitis anquilosante), soriasis, dermatitis que
incluye dermatitis atópica; urticaria idiopática crónica,
urticaria autoinmune crónica, poliomyositis/dermatomiositis,
necrolisis epidérmica tóxica, escleroderma sistémica y
esclerosis, respuestas asociadas con enfermedad inflamatoria del
intestino (IBD) (enfermedad de Crohn, colitis ulcerativa), e IBD
con co-segregado de pyoderma gangrenosum, Eritema nodoso,
colangitis esclerosante primaria y/o episcleritis), síndrome de
enfermedad respiratoria, que incluye síndrome de enfermedad
respiratoria de adulto (ARDS), meningitis, enfermedades mediadas
por IgE tales como anafilaxis y rinitis alérgica, encefalitis
tales como encefalitis de Rasmussen, uveitis, colitis tales como
colitis microscópica y colitis colagenosa, glomerulonefritis (GN)

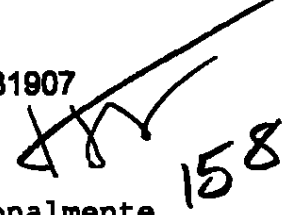
tal como GN membranosa, GN membranoso idiopático, GN Proliferativo membranoso (MPGN), GN tipo I y tipo II, y rápidamente progresiva, condiciones alérgicas, eczema, asma, condiciones que involucran la infiltración de células T respuestas inflamatorias crónicas, aterosclerosis, miocarditis autoinmune, eficiencia de adhesión de leucocito, lupus sistémico heritematoso(SLE) tal como SLE cutáneo, lupus (que incluye nefritis, cerebritis, pediátricos, alopecia discordia no renal pediátrica), diabetes de incidencia juvenil, esclerosis múltiple (MS) tal como MS espino óptica, encefalomiелitis alérgica, respuesta inmune asociada con hipersensibilidad aguda y retrasada mediada por citoquinas y linfocitos T, tuberculosis, sarcoidosis, granulomatosis que incluye granulomatosis Wegener, agranulocitosis, vasculitis (que incluye vasculitis de bazo grande (que incluyen reumapolimialgia reumática y artritis de célula grande (Takayasu)), vasculitis de bazo medio (que incluyen enfermedad de kawasaki y Poliarteritis Nodosa), vasculitis del CNS, y vasculitis asociada a ANCA, tal como vasculitis o síndrome de Churg-Strauss (CSS)), anemia aplástica, anemia positiva Coombs, anemia Diamond Blackfan, anemia hemolítica inmune, anemia hemolítica autoinmune que incluye (AIHA), anemia perniciosa, aplasia de célula roja pura (PRCA), deficiencia del Factor VIII, hemofilia A, neutropenia autoinmune, pancitopenia, leucopenia, enfermedad que involucra diapedesis leucocítica, trastornos inflamatorios del CNS, síndrome del daño de órgano múltiple, miastenia gravis, enfermedades mediadas por el complejo de anticuerpo de antígeno, enfermedad de membrana con base anti-glomerular, síndrome de anticuerpo anti-fosfolípido neuritis alérgico, síndrome de Castleman, síndrome de Goodpasture,

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síndrome misaténica Lambert-Eaton, síndrome Reynaud, síndrome Sjorgen, síndrome de Stevens-Johnson, rechazo de trasplante de órgano sólido (que incluyen pre tratamiento para títulos de anti cuerpo reactivos de panel alto, depósito IgA en tejidos, y rechazo que surge de trasplante renal, trasplante de hígado, trasplante intestinal, trasplante cardiaco, etc.) enfermedad de injerto Vs huésped (GVHD), bocio pemfigoides, fenfigus (que incluyen pemfigoide de membrana de mucosa vulgaris, foliaceus, y fencigus), poliendocrinopatías autoinmunes, enfermedad de Reiter, síndrome de hombre rígido, nefritis de complejo inmune, polineuropatías IgM o neuropatía mediada por IgM, púrpura trombocitopénica y hepática (ITP), púrpura trombocitopénica trombótica (TTP), trombocitopenia (desarrollada mediante pacientes de infarto del miocardio de, por ejemplo), que incluye trombocitopenia autoinmune, enfermedad autoinmune de testículos y ovarios que incluyen orquitis autoinmune y ooforitis, hipotiroidismo primario, enfermedades endocrinas autoinmunes que incluyen tiroiditis autoinmune, tiroiditis crónica (tiroiditis de Hashimoto), tiroiditis sub aguda, hipotiroidismo idiopático, enfermedad de Addison, enfermedad de Grave, síndromes poliglandulares autoinmunes (o síndromes endocrinopatía poliglandular), diabetes tipo I también denominado como diabetes mellitus dependiente de insulina (IDDM), que incluye IDDM pediátrica, y síndrome de Sheehan; hepatitis autoinmune, neumonitis intersticial de linfocitos (HIV), bronquiolitis obliterans (no-trasplantes) vs. NSIP, síndrome de Guillain-Barre, enfermedad de Berger's (neuropatía IgA), cirrosis biliar primaria, celiaquía (enteropatía de gluten), celiaquía refractaria con dermatitis co-segregada queratofolliculiformis,

crioglobulinemia, esclerosis lateral aminotrófica (ALS; enfermedad de Lou Gehrig), enfermedad de arteria coronaria, enfermedad de oído interior autoinmune (AIED), pérdida autoinmune de escucha, síndrome de mioclonus opsoclonus (OMS), policondritis tal como policondritis refractaria, proteinosis alveolar pulmonar, amiloidosis, hepatitis de célula gigante, escleritis, gamopatía monoclonal de significancia incierta/ desconocida (MGUS), neuropatía periférica, síndrome paraneoplásico, canelopatías tales como epilepsia, migraña, arritmia, desórdenes musculares, sordera, ceguera, parálisis periódica, y canelopatías tales como CNS; autismo, miopatía inflamatoria, y glomeruloesclerosis focal segmental (FSGS).

El término "prodrogas" como se utiliza en esta solicitud se refiere a una forma precursora o derivada de una sustancia farmacéuticamente activa que es menos citotóxica a las células cancerígenas comparadas con la droga padre que es capaz de ser enzimáticamente activada o convertida en la forma parenteral más activa. Ver, 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). Las prodrogas de esta invención incluyen, pero no están limitadas a, prodrogas que contienen fosfato, prodrogas que contienen tiofosfato, prodrogas que contienen sulfato, prodrogas que contienen péptido, prodrogas modificadas por D-aminoácido, prodrogas glicosiladas, prodrogas que contienen beta-lactam-, opcionalmente prodrogas que contienen fenoxiacetamida sustituida



o prodrogas que contienen fenilacetamida opcionalmente sustituida, 5-fluorocitoquina y otras prodrogas de 5-fluorouridina que se pueden convertir en las drogas libres citotóxicas más activas. Ejemplos de drogas citotóxicas que se pueden derivar en la forma de prodroga para uso en esta invención incluyen, pero no están limitadas a, aquellos agentes quimioterapéuticos descritos adelante.

El término "agentes cito tóxicos" como se utiliza aquí se refiere a una sustancia que inhibe o evita la función de las células y/o origina la destrucción de las células. El término está destinado a incluir isótopos radio activos (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 o bacteriales, fungosas, o de origen animal, que incluyen fragmentos y/o variantes de estos.

Un "agente quimioterapéutico" es un "agente quimioterapéutico" es un compuesto químico útil en el tratamiento de cáncer. Ejemplos de agentes quimioterapéuticos incluyen agentes alquilantes tales como tiotepa, y CYTOXAN® ciclosfosfamida,- alquil sulfonatos tales como busulfan, improsulfan y piposulfan,- aziridinas tales como benzodopa, carboguone, meturedopa, y uredopa; eteleniminas y metilamelaminas que incluyen altretamina, trietilenemelamina, trietilenefosforamida, trietilenetiofosforamida y trimetilolomelamina; acetogeninas (especialmente bulatacina y bulatacinona); y camptotecina (que incluyen el análogo sintético de cantotecan); briostatina;

calistatina; CC-1065 (que incluyen su adozelesina, carzelesina y análogos sintéticos de bizelesina); criptoficinas (particularmente criptoficina 1 y criptoficina 8); dolastatina; duocarmicina (que incluyen los análogos sintéticos, KW-2189 y CBI-TM1); eleuterobina; pancratistatina; una sarcodictina; espongistatina, mustardasas de nitrógeno tales como clorambucil, clornafazina, colofosfamida, estramustina, ifosfamida, mecloretamina, clorhidrato de óxido de mecloretamina, melfalan, novembichin, fenesterina, prednimustina, trofosfamida, mustaza de uracilo; nitrosureas tales como carmustina, clorozotocina, fotemustina, lomustina, nimustina, y ranimustina; antibióticos tales como los antibióticos enedina (por ejemplo, caliqueamicina, especialmente caliqueamicina gama II y caliqueamicina omega 11 (ver,. Por ejemplo, Agnew, Chem Intl. Ed. Engl., 33: 183-186 (1994)); dinemicina, que incluye dinemicina A,- bisfosfonatos, tales como clodronato; una esperamicina, así como también neocarcinostatina cromóforo y cromóforos anti éticos de cromoproteína enedina), aclacinomisinas, actinomicina, autramicina, azaserina, bleomicinas, cactinomicina, carabicina, carminomicina, carzinofilina, cromomicinas, dactinomicina, daunorubicina, detorubicina, 6-díazo-5-oxo-L-norleucina, ADRIAMYCIN® doxorubicina (que incluye morfolino-doxorubicina, cianomorfolino-doxorubicina, 2-pirrolino- doxorubicina y deoxidoxorubicina), epirubicina, esorubicina, idarubicina, marcellomicina, 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

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y 5-fluorouracilo (5-FU); antólogos de ácido fólico tales como denopterina, metotrexato, pteropterina, trimetrexato; análogos de purina tales como fludarabina, 6-mercaptapurina, 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- adrenérgicos tales como aminoglutetimida, mitotano, trilostano; relleno de ácido fólico tal como ácido frolínico; aceglatona; aldofosfámidico glicosido; ácido aminolevulínico,- eniluracilo; amsacrina; bestrabucilo; bisantreno; edatraxato; defofamina; demecolcina; diaziadona; elfornitina; acetato de eliptínico; y epotilona; etoglucido; nitrato de galio; hidroxiaurea; lentinano,- lonidainina; maitansinoides tales como maitansina y ansamitocinas; mitoguazona; mitoxantrona; mopidanmol; nitraerina; pentostatina; fenamet, -pirarubicina; losoxantrona; ácido podofilínico; 2-etilhidrazida; procarbazona; complejo de polisacárido PSK® (JHS Natural Products, Eugene, OR); razoxenol; rizoxina; sizofirano; espirogermanio; ácido tenuazonico; triaziadona; 2,2 ',2"-triclorotrietilamina; tricotecenos (especialmente toxina T- 2, verracurina A, roridina A y anguidina); uretano; vindesina; dacarbazina; mannomustina; mitobronitol; mitolactol; pipobromano; gacitoquina; arabinosido ("Ara-C"); ciclofosfámidico; tiotepa; taxoides, por ejemplo, TAXOL® paclitaxel (Bristol- Myers Squibb Oncology, Princeton, N.J.), ABRAXANE™ libre de cremóforo, formulación de nanopartícula trabajada por ingeniería de albúmina de paclitaxel (American Pharmaceutical Partners, Schaumburg, Illinois), y TAXOTERE®

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doxetaxel (Rhone- Poulenc Rorer, Antony, France); clorambucil; GEMZAR® gemcitabina; 6- tioguanina; mercaptopurina; metotrexato; análogos de platino tales como cisplatina y carboplatina,- vinblastina; platino; etoposida (VP-16); ifosfamida; mitoxantrona; vincristina; NAVELBINE® vinorelbina; novantrona; teniposida; edatrexato; daunomycina,- aminopterina; xeloda; ibandronato; CPT-11; inhibidor de topoisomerasa RFS 2000; difluorometilornitina (DMFO); retinoides tales como ácido retinóico; capecitabina; y sales farmacéuticamente aceptables, ácidos o derivados de cualquiera de los anteriores.

También se ha incluido en ésta definición los agentes anti-hormonales que actúan para regular o inhibir la acción de la hormona en tumores tales como anti-estrógenos y moduladores del receptor de estrógeno selectivo (SERMs), incluyendo, por ejemplo, tamoxifen (incluyendo NOLVADEX® tamoxifen), raloxifeno, droloxifeno, 4-hidroxitamoxifen, trioxifeno, keoxifenp, LY117018, onapristona, y FARESTON- toremifeno; inhibidores de aromatasa que inhiben la enzima aromatasa, la cual regula la producción de estrógeno en glándulas adrenales, tales como, por ejemplo, 4 (5) -imidazoles, aminoglutetimida, MEGASE® acetato de megestrol, AROMASIN® exemestano, formestania, fadrozol, RIVISOR® vorozol, FEMARA® letrozol, y ARIMIDEX® anastrozol; y anti-androgenos tales como flutamida, nilutamida, bicalutamida, leuprolida, y goserelina; lo mismo que troxacitabina (un análogo del nucleocido de citoquina 1,3-dioxolano); oligonucleótidos antisentido, particularmente aquellas que inhiben la expresión de genes en trayectos de señalización implicados en la proliferación aberrante de células, tales como, por ejemplo, PKC-alfa, Ralf y

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H-Ras; ribozimas tales como un inhibidor de la expresión VEGF (por ejemplo, ANGIOZYMA® ribozima) y inhibidor de la expresión HER2; vacunas tales como vacunas para terapia de genes, por ejemplo, la vacuna ALLOVECTIN®, la vacuna LEUVECTIN®, y la vacuna VAXID®; PROLEUKIN® rIL-2; LURTOTECAN® inhibidor de la topoisomerasa 1; ABARELIX® rMRH; y sales farmacéuticamente aceptables, ácidos o derivados de cualquiera de los anteriores.

Un "agente inhibitorio de l crecimiento" cuando se usa aquí se refiere a un compuesto o composición que inhibe el compuesto de una célula, sea in vitro o in vivo. Así, el agente inhibitorio de crecimiento es uno que reduce significativamente el porcentaje de células que sobro expresan tales genes en la fase S. Ejemplos de agentes inhibidores de crecimiento, incluyen gentes que bloquean la progresión del ciclo celular (en un lugar diferente a la fase S), tales como agentes que inducen la detención del G1 y la detención de la fase M. Los bloqueadores clásicos de la fase M incluyen las vincas (vincristina y vinblastina), taxol, e inhibidores de topo II tales como doxorubicina, epirubicina, daunorubicina, etoposida, y bleomycina. Aquellos agentes que detienen detienen G1 también derraman doble la detención de la fase S, por ejemplo, agentes alquilantes de ADN tales como tamoxifen, prednisona, dacarbazina, mecloretamina, cisplatina, methotrexato, 5-fluorouracilo, y ara-C. Mayor información puede encontrarse en The Molecular Basis of Cancer, Mendelsohn and Israel, eds., Capítulo 1, titulado "Cell cycle regulation, oncogens, and antineoplastic drugs" por Murakami et al. (WB Saunders: Philadelphia, 1995), especialmente en la página 13.

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El término "citoquina" es un término genérico para proteínas liberadas por una población celular que actúan sobre otra célula como mediadores intercelulares. Ejemplos de tales citoquinas, son las lymfocinas, monocinas, y las hormonas de polipéptidos tradicionales. Incluidos entre las citoquinas están las hormonas de crecimiento tales como la hormona de crecimiento humano, la hormona de crecimiento humano N-metionilo, y la hormona de crecimiento ovino; hormonas paratiroides; tiroxina; insulina; proinsulina,- relaxina; prorelaxina,- hormona de glicoproteína tales como las hormonas estimulantes del folículo (FSH), la hormona estimuladora de la tiroides (TSH), y la hormona luteinizante (LH); el factor de crecimiento hepático; el factor de crecimiento del fibroblasto; la prolactina; el lactógeno placentar; el factor- α y - β de la necrosis del tumor; sustancia inhibidora mulleriana; péptido asociado con la gonadotropin de ratón; inhibina; activina; factor de crecimiento endotelial vascular; integrina; trombopoyetina (TPO); factores de crecimiento de los nervios; factor de crecimiento de plaquetas; factores de crecimiento transformantes (TGFs) tales como TGF- α y TGF- β ; factor I y II de crecimiento similar a insulina; eritropoyetina (EPO); factores osteoinductivos; interferonas tales como el interferón- α , - β , y -gamma; factores estimulantes de colonia (CSFs) tales como macrófago-CSF (M-CSF); granulocito-macrófago-CSF (GM-CSF); y granulocito-CSF (G-CSF); interleucinas (ILs) 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 de polipéptido que incluyen el LIF y el kit ligando (KL). Como se usa aquí, el término citoquina, incluye proteínas de fuentes naturales o de cultivos

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celulares recombinantes y equivalentes biológicamente activos de las secuencias de las citoquinas nativas.

Un "inserto en paquete" es usado para referirse a instrucciones usualmente incluidas en empaques comerciales de productos terapéuticos, que contienen información acerca de las indicaciones, uso, dosificación, administración, contraindicaciones, otros productos terapéuticos para ser combinados con el producto empacado y/o advertencias concernientes al uso de tales productos terapéuticos, etc.

El término "tratar", "tratamiento", "terapia", como se usa aquí se refiere a terapia curativa, terapia profiláctica, terapia preventiva.

El término "mamífero" como se usa aquí se refiere a cualquier mamífero clasificado como mamífero, incluyendo humanos, vacas, caballos, perros y gatos. En una modalidad preferida de la invención, el mamífero es un humano.

II. Composiciones y métodos de la invención

Una citoquina relacionada con la familia del ligando TNF, la citoquina identificada aquí como "ligando Apo-2" o "TRAIL" ha sido descrita. La secuencia de aminoácidos madura predicha del ligando Apo-2 humano nativo contiene 281 aminoácidos, y tiene un peso molecular calculado de aproximadamente 32,5 kDa. La ausencia de una secuencia de señal y la presencia de una región hidrófoba interna sugiere que el ligando Apo-2 es una proteína

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transmembrana tipo II. Los polipéptidos del ligando Apo-2 del dominio extracelular solubles, han sido también descritos. Ver, cpor ejemplo, EO97/25428 publicado en Julio 17, 1997. Las variantes sustitucionales de Apo-2L también han sido descritas adicionalmente. Las técnicas de barrido con alamina han sido utilizadas para identificar diversas moléculas variantes sustitucionales que tienen actividad biológica. Las variantes sustitucionales del ligando Apo-2 incluyen aquellas en las cuales por lo menos un aminoácido es sustituido por otros aminoácidos tales como un residuo de alamina. Estas variantes sustitucionales son identificadas, por ejemplo, de "D203A"; "D218A" y "D269". Esta nomenclatura es utiliza para identificar las variantes del ligando Apo-2, en donde los residuos de ácido aspártico en las posiciones 203, 218 y/o 269 (usando la numeración mostrada en la figura 1) son sustituidas por residuos de alamina. Opcionalmente las variantes de Apo-2L de la presente invención pueden comprender una o más de las sustituciones de aminoácidos Opcionalmente, tales variantes de Apo-2L serán variantes selectivas de receptor DR4 o DR5.

La descripción más adelante se relaciona con métodos para producir el ligando Apo-2, incluyendo las variantes del ligando Apo-2, mediante cultivar células anfitrionas transformadas o transfectadas con un vector que contiene el ácido nucleico que codifica al ligando Apo-2 y recuperar el polipéptido del cultivo celular. El ADN que codifica el ligando Apo-2 puede ser obtenido de cualquier librería de cADN prepara de tejido que se cree que posee mRNA del ligando Apo-2 y expresarlo a un nivel detectable. De acuerdo el ADN del ligando Apo-2 humano, puede ser obtenido

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convenientemente de una librería de cADN preparad a partir de tejidos humanos, tales como la librería de bacteriófagos de cADN placentar humano como se describió en la WO97/25428. El gen que codifica el ligando Apo-2 también puede ser obtenido de una librería genómica o mediante síntesis de oligonucleótido.

Las librerías pueden ser seleccionadas con probetas, (tales como anticuerpos para el ligando Apo-2 u oligonucleótidos de por lo menos cerca de 20 a 80 cases) diseñadas para identificar el gen de interés o la proteína codificada por éste. La selección del cADN o de la librería genómica con la probeta seleccionada, puede ser conducida usando procedimientos estándar, tales como los descritos en Sambrook et al., Molecular Cloning: A Laboratory Manual (Nueva York: Cold Spring Harbor Laboratory Press, 1989). Una medio alternativo para aislar el gen que codifica el ligando Apo-2 es usar la metodología PCR [Sambrook et al., supra; Dieffenbach et al., PCR Primer: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)].

Los fragmentos de secuencia del aminoácido o variantes de ligando Apo-2 puede ser preparados mediante introducir cambios de nucleótidos apropiados en el ADN del ligando Apo-2 o mediante síntesis del polipéptido del ligando Apo-2 deseado. Tales fragmentos o variantes representan inserciones, sustituciones, y/o eliminaciones de residuos dentro de, o en uno o ambos extremos de la región intracelular, la región transmembrana, o la región extracelular, o de la secuencia de aminoácido mostrada para el ligando Apo-2 de longitud completa en la figura 1. Cualquier combinación de inserción, sustitución y/o eliminación,

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puede ser hecha para llegar a la construcción final, siempre que la construcción final, posea, por ejemplo, una actividad biológica deseada, tal como la actividad apoptótica, como se definió aquí. En modalidades preferidas, los fragmentos o variantes, tiene por lo menos cerca de 80% de identidad de la secuencia de aminoácido, más preferiblemente, por lo menos cerca 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 aquí para los dominios intracelular, de transmembrana o extracelular del ligando Apo-2, o la secuencia de longitud completa del ligando Apo-2. Los cambios de aminoácidos también pueden alterar los procesos post-translacionales del ligando Apo-2, tal como cambiar el número o posición de los sitios de glicosilación o alterar las características de anclaje de la membrana.

Las variaciones en la secuencia del ligando Apo-2 como se describieron anteriormente, puede hacerse usando cualquiera de las técnicas e instrucciones para mutaciones conservativas y no conservativas establecidas en la patente estadounidense No. 5,364,934. Éstas incluyen mutagénesis mediada por oligonucleótido (dirigida a sitios), barrido por alamina, y mutagénesis PCR.

El análisis de barrido de aminoácido puede ser empleado para identificar uno o más aminoácidos a lo largo de una secuencia contigua. Entre los aminoácidos de barrido preferidos están los aminoácidos relativamente pequeños, y neutrales. Tales aminoácidos incluyen la alanina, glicina, serina y cisteína. La alanina es típicamente un aminoácido de barrido preferido entre

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éste grupo porque éste elimina la cadena lateral, más allá del carbón beta, y es menos probable que altere la conformación de la cadena principal de la variante. [Cunningham et al., Science, 244:1081 (1989)]. La alanina también es preferida típicamente porque es el aminoácido más común. Adicionalmente, es frecuentemente encontrado tanto en posiciones enterradas como expuestas [Creighton, The Proteins, (W.H. Freeman & Co., NY); Chothia, J. Mol. Biol., 150:1 (1976)].

Los aminoácidos pueden ser agrupados de acuerdo con las similitudes en las propiedades de sus cadenas laterales (en A. L. Lehninger, in Biochemistry, second ed., pp. 73-75, Worth Publishers, Nueva 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) Acídicos: Asp (D), Glu (E)
- (4) Básicos: Lys (K), Arg (R), His (H)

Alternativamente, los residuos de ocurrencia natural pueden ser divididos en grupos con base en las propiedades de cadena lateral comunes:

- (1) Hidrófobos: Norleucina, Met, Ala, Val, Leu, Ile;
- (2) Hidrofílicos neutrales: Cys, Ser, Thr, Asn, Gln;
- (3) Acídicos: Asp, Glu;
- (4) Básicos: His, Lys, Arg;

(5) Residuos que tienen influencia en la orientación de la

cadena: Gly, Pro;

(6) Aromáticos: Trp, Tyr, Phe.

Tabla 1.

Residuo Original	Substituciones Ilustrativas	Substituciones 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; 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

Las variaciones en la secuencia del ligando Apo-2 también incluidas dentro del alcance de la invención se relacionan con derivados o formas modificadas de terminales aminos. Tales secuencias del ligando Apo-2 incluyen cualquiera de los

polipéptidos del ligando Apo-2 descritos aquí, que tiene una metionina o una metionina modificada (tal como formal metionilo u otras especies de metionilo bloqueadas), en los terminales N de la secuencia del polipéptido.

El ácido nucleico (por ejemplo, cADN o ADN genómico) que codifica el ligando Apo-2 nativo o variante puede ser insertado en un vector replicable para clonación adicional (amplificación del ADN) o para su expresión. Varios vectores están disponibles públicamente. Los componentes de vector, generalmente incluyen, pero no se limitan, a uno o más de los siguientes: Una secuencia de señal un origen de replicación, u no o más genes marcadores, un elemento mejorador, un promotor y una secuencia de terminación de transcripción, cada uno de los cuales se describe más adelante. Las secuencias de señal opcionales, orígenes de replicación, genes marcadores, elementos mejoradores, y secuencias terminadas de transcripción, que pueden ser empleadas, son conocidas en la técnica y descritas en mayor detalle en WO97/25428.

Los vectores de expresión y de clonación usualmente contienen un promotor que es reconocido por el organismo anfitrión y está ligado operativamente a la secuencia del ácido nucleico del ligando Apo-2. Los promotores son secuencias no traducidas, localizadas aguas arriba (5') del codón de partida de un gen estructural (generalmente dentro de 100 a 1000 bp) que controla la transcripción y la traducción de una secuencia de ácido nucleico particular, tal como la secuencia de ácido nucleico del ligando Apo-2, al cual ellos éstas ligados operativamente. Tales

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promotores típicamente caen en dos clases, inducible y constitutiva. Los promotores inducibles son promotores que inician niveles incrementados de transcripción del ADN bajo su control en respuesta a algunos cambios en las condiciones del cultivo, por ejemplo, la presencia o ausencia de un nutriente o un cambio en la temperatura. En éste tiempo, un número grande de promotores reconocidos por una variedad de células huésped o anfitrionas potenciales, son bien conocidos. Éstos promotores están ligados operativamente al ADN que codifica el ligando Apo-2 mediante la remoción del promotor de la fuente de ADN por una digestión de enzima por restricción y la inserción de la secuencia promotora aislada en el vector. Tanto en la secuencia del promotor del ligando Apo-2 nativo, y muchos promotores heterólogos, pueden ser usados para dirigir la amplificación y/o la expresión del ADN ligando Apo-2.

Los promotores adecuados para uso con anfitriones eucarióticos y procarióticos son conocidos en la técnica, y son descritos con mayor detalle en la WO97/25428.

Un método preferido para la producción de Apo-2 soluble en E. coli, emplea un promotor inducible para la regulación de la expresión del producto. El uso de un promotor controlable, inducible, permite el crecimiento de cultivos a densidades celulares deseables, antes de la inducción de la expresión del producto y la acumulación de cantidades significativas del producto que pueden no ser toleradas bien por el anfitrión.

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Diversos sistemas promotores e inducibles (polimerasa T7, fosfata trp y alcalina (AP)) han sido evaluadas por los solicitantes para la expresión del Apo-2L (forma 114-281). El uso de cada uno de éstos tres promotores resulto en cantidades significativas del trímero Apo-2L activo biológicamente y soluble siendo recuperado de la pasta celular cosechada. El promotor AP es el preferido entre estos tres sistemas promotores inducibles ensayados, por el control más fuerte del promotor y por la densidad mayor y los títulos alcanzados en la pasta celular cosechada.

Las construcciones de vectores adecuados que contiene uno o más de los anteriores componentes, emplea técnicas de ligación estándar. Los plásmidos aislados, o fragmentos de ADN, son divididos, cortados y religados en la forma deseada para generar los plásmidos requeridos.

Para análisis para confirmar las secuencias correctas en los plásmidos contruidos, las mezclas de ligación pueden ser usadas para transformar la sepa 294 K12 del E. coli (ATCC 31,446) y los transformantes exitosos seleccionados por la resistencia a la ampicilina o a la tetraciclina en donde se ha apropiado. Los plásmidos de los transformantes son preparados, analizados por digestión de endonucleasa por restricción, y/o secuenciados usando técnicas estándar conocidas en la técnica. [Ver, por ejemplo, Messing et al., Nucleic Acids Res., 9:309 (1981); Maxam et al., Methods in Enzymology, 65:499 (1980)].

Los vectores de expresión se suministran para la expresión transitorio en células de mamíferos que codifica el ligando Apo-2

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pueden ser empleadas. En general, las expresiones transitorias, involucran el uso de vector de expresión que es capaz de replicarse eficientemente en una célula anfitriona, de modo que la célula anfitriona 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]. Los sistemas de expresión transitorias, comprende un vector de expresión adecuado y una célula anfitriona, permiten la identificación positiva conveniente de polipéptidos codificados por ADN clonados, lo mismo que para un tamizado rápido de tales polipéptidos en cuanto a las propiedades biológicas o fisiológicas deseadas. Así los sistemas de expresión transitorias son particularmente útiles en la invención para los propósitos de identificar los análogos y variantes del ligando Apo-2 que son ligando Apo-2 biológicamente activos.

Otros métodos, vectores y células anfitrionas adecuadas para la adopción a la síntesis del ligando Apo-2 en cultivos celulares de vertebrados recombinantes son descritos en Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; y EP 117,058.

Las células anfitrionas adecuadas para la clonación y expresión del DN en los sectores, incluyen aquí células procariotas, de levadura o eucariota mayores. Las procariotas adecuadas para éste propósito incluyen pero no se limitan a eubacteria, tal como los organismos Gram-negativo o Gram-positivo, por ejemplo, enterobacteriaceae tales como Esquiriquia, por ejemplo, la E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, por

ejemplo, la *Salmonella typhimurium*, *Serratia*, por ejemplo, la *Serratia marcescans*, y *Shigella*, lo mismo que Bacilos tales como *B. subtilis* y el *B. liqueniformes* (por ejemplo, el *B. liqueniformes* 41P descrito en DD 266,710 publicado el 12 de Abril de 1989), *Pseudomonas* tales como la *P. aeruginosa*, y *Streptomyces*. Preferiblemente, las células anfitrionas deben secretar cantidades mínimas de enzimas proteolíticas.

En adición a las procariotas, los microbios eucarióticos tales como los hongos filamentosos o la levadura son adecuados para clonar o expresar en anfitriones los vectores que codifican el ligando Apo-2. Las células anfitrionas adecuadas para la expresión del ligando Apo-2 glicosilado, son derivadas de organismos multicelulares. Ejemplos de todas aquellas células anfitrionas, incluyen las células CHO, como se describieron adicionalmente en la WO97/97258. Las células anfitrionas son transfectadas y preferiblemente transformadas con los vectores de expresión o de clonación, descritos anteriormente para la producción del ligando Apo-2 y cultivados en un medio nutriente modificado según sea apropiado para inducir promotores, seleccionar transformantes, o amplificar los genes que codifican las secuencias deseadas.

La transacción se refiere a la toma de un vector de expresión por una célula anfitriona, haya o no, ningunas secuencias de codificación de hecho expresada.

Numerosos métodos de transfección son conocidos por aquellos basados en la técnica, por ejemplo, CaPO_4 y la electroporación.

La transfección exitosa es generalmente reconocida cuando cualquier indicación de la operación de éste vector ocurre dentro de la célula anfitriona.

Los medios de transformación que introducen el ADN en un organismo de modo que el ADN sea replicable, sea como un elemento extracromosomal, o por integrante cromosomal. Dependiendo de la célula anfitriona usada, la transformación se hace usando técnicas estándar apropiadas a tales células. El tratamiento por calcio que emplea cloruro de calcio, como se Sambrook et al., supra, o por electroporación es generalmente usado para procariotas u otras células que contienen barreras de paredes celulares sustanciales. La infección con *Agrobacterium tumefaciens* es usada para transformación de ciertas células plantas, como se describió por Shaw et al., Gene, 23:315 (1983) y la WO 89/05859 publicadas el 29 de Junio de 1989. En adición, las plantas pueden ser transfectadas usando tratamiento por ultrasonido como se describió en la WO 91/00358 publicada el 10 de Enero de 1991.

Para células de mamíferos sin tales paredes celulares, el método de precipitación por sustrato de calcio de Graham y van der Eb, Virology, 52:456-457 (1978) puede ser empleado. Aspectos generales de transformaciones del sistema anfitrión de células d mamíferos, han sido descritos en la patente estadounidense No. 4,399,216. Las transformaciones en levadura son llevadas a cabo típicamente de acuerdo con el método Van Solingen et al., J. Bact., 130:946 (1977) y Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76: 3829 (1979). Sin embargo, otros métodos para

introducir ADN en la célula en las células, tales como mediante microinyección nuclear, electroporación, fusión de protoplasto bacterial con células intactas, o policondicionamiento, por ejemplo, polibromo, poliornitina, también pueden ser usados. Para diversas técnicas para transformar células de mamífero, ver Keown et al., Methods in Enzymology, 185:527-537 (1990) y Mansour et al., Nature, 336:348-352 (1988).

Las células procarióticas usadas para producir el ligando de Apo-2 pueden ser cultivadas en un medio de cultivo adecuado, según se describió en general en Sambrook et al., supra. Las formas particulares de medios de cultivo que pueden ser empleados para cultivar el E. coli se describen adicionalmente en los ejemplos adelante. Las células anfitrionas de mamífero, usadas para producir el ligando Apo-2, pueden ser cultivadas en una variedad de medios de cultivo.

Ejemplos de medios de cultivo disponibles comercialmente incluyen el Ham's F10 (Sigma), Medio esencial mínimo ("MEM", Sigma), RPMI-1640 (Sigma), y el Medio Eagle's modificado por Dulbecco ("DMEM", Sigma). Cualquiera de tales medios puede ser suplementado como sea necesario con hormonas y/o otros factores de crecimiento (tal como insulina, transferrina, o factor de crecimiento epidérmico) sales (tales como cloruro de sodio, calcio, magnesio, y fosfato), amortiguadores (tales como HEPES), nucleósidos (tales como adenosina y timidina), antibióticos (tales como la droga Gentamicin^{GM}), trazos de elementos (definidos como compuestos inorgánicos usualmente presentes en concentraciones finales en el rango de micromolar), y glucosa o una fuente de energía

equivalente. Cuales quiera otros suplementos necesarios pueden ser también incluidos en concentraciones apropiadas lo que seria conocido por aquellos versados en la técnica. Las condiciones del cultivo, tales como temperatura, pH, y similares, son aquellas usadas previamente con la célula anfitriona seleccionada para la expresión, y serán evidentes para aquellos versados en la técnica.

En general, los principios, protocolos, y técnicas prácticas, para maximizar la productividad de cultivos celulares de mamíferos 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 serán agregados típicamente a, o incluidos en el medio de cultivo, para cultivar o fermentar las células anfitrionas. Los iones de metal divalente, están presentes preferiblemente en, o agregados al medio de cultivo en un nivel de concentración suficiente para promover o mejorar la estabilidad de almacenamiento, mejorar la solubilidad, o ayudar en la formación de los trímeros de Apo-2L estables, coordinados mediante uno o más iones de Zinc. La cantidad de iones de metal divalente que pueden ser agregados, dependerá, en parte, de la densidad celular anfitriona en el cultivo, o la sensibilidad de las células anfitrionas potenciales a tales iones metálicos divalentes. A densidades de células anfitrionas mayores, puede ser benéfico incrementar la concentración de los iones de metal divalente. Si los iones de metal divalente son agregados durante o después de la expresión del producto por las células

anfitrionas, puede ser deseable ajustar o incrementar la concentración de ión de metal divalente a medida que la expresión del producto por las células anfitrionas se incrementa. Se cree en general que niveles de trazas de iones de metal divalente que puedan estar presentes, en medios de cultivo de células comúnmente disponibles no sean suficientes para una formación de trímero estable. Así, la adición de cantidades adicionales de iones de metal divalente, como se describió aquí, es preferido.

Los iones de metal divalente son agregados preferiblemente al medio de cultivo en una concentración que no afecte adversamente o negativamente, el crecimiento de las células anfitrionas, si en los iones de metal divalente son agregados durante la fase de crecimiento de las células anfitrionas en el cultivo. En cultivos en frascos con agitación, se observó que se agregó ZnSO_4 a concentraciones mayores a 1 mM puede resultar en una densidad de células anfitrionas inferior. Aquellos versados en la técnica podrán apreciar que las células bacterianas pueden secuestrar las iones de metal efectivamente mediante formar complejos de ión metálico con matrices celulares. Así, en los cultivos de células, es preferible agregar los iones de metal divalente seleccionados al medio de cultivo después de la fase de crecimiento (después de que la densidad de células anfitrionas deseadas se ha logrado) o justo antes de la expresión del producto por medio de las células anfitrionas. Para asegurar que suficientes cantidades de iones de metal divalente están presentes, iones de metal divalentes adicionales pueden ser agregados o alimentados al medio de cultivo de células durante la fase de expresión del producto.

La concentración de ión de metal divalente en el medio de cultivo, no debería exceder la concentración que puede ser dañina o tóxica a las células anfitrionas. En los métodos de la invención, que emplean las células anfitrionas, E.coli, se prefiere que la concentración del ión de metal divalente, en el medio de cultivo no exceda alrededor de 1mM (preferiblemente $\leq$ 1mM). Aún más preferiblemente, la concentración de ión de metal divalente en el medio de cultivo, es de cerca de 50 micromolar a cerca de 250 micromolar. Más preferiblemente, el ión de metal divalente usado en tales métodos es el sulfato de Zinc. Es deseable agregar los iones de metal divalente al cultivo de células, en una cantidad en donde los metales de metal y el trómero del ligando Apo-2 pueda estar presente en una proporción uno a uno molar.

Los iones de metal divalentes, pueden ser agregados al cultivo de células en cualquier forma aceptable. Por ejemplo, una solución del ión de metal puede ser hecha usando agua, y la solución de ión de metal divalente puede ser entonces ser agregada o alimentada el medio de cultivo.

La expresión del Apo-2 puede ser medida en una muestra directamente, por ejemplo, por secado Southern convencional, secado Northern para cuantificar la transcripción del mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], secado de punto (análisis de ADN), o hibridización in situ, usando una probeta marcada apropiadamente, con base en las secuencias suministradas aquí. Diversas marcas pueden ser empleadas, las más comunes son los radio isótopos, y particularmente ^{32}P . Sin

embargo, otras técnicas también pueden ser empleadas, tales como usar nucleótidos modificados por biotina, para la introducción en un polinucleótido. La biotina, entonces sirve como el sitio para ligar a la avidina o los anticuerpos, que pueden ser marcados con una amplia variedad de marcas, tales como radio nucleótidos, fluorescencias o enzimas. Alternativamente, los anticuerpos pueden ser empleados en cuanto a que reconozcan duplexos específicos, incluyendo duplexos de ADN, duplexos de ARN, y duplexos híbridos de ADN-ARN o duplexos de ADN-proteína. Los anticuerpos a su vez pueden estar marcados y el ensayo se puede llevar a cabo, en donde el duplex es ligado a una superficie, de modo que luego de la formación del duplex sobre la superficie, la presencia del anticuerpo ligado al duplex puede ser detectada. La expresión de genes, alternativamente, puede ser medida por métodos inmunológicos, tales como, manchado inmunohistoquímico de células o secciones de tejido, y ensayo de cultivos de células o fluido corporal, para cuantificar directamente la expresión del producto de genes. Con las técnicas de manchado inmunohistoquímico, una muestra de célula es preparada, típicamente por deshidratación y fijación, seguido por reacción con anticuerpos marcados específicos para el producto de gen acoplado, en donde las marcas son usualmente detectables visualmente, tal como marcas enzimáticas, marcas fluorescentes, marcas luminiscentes, y similares.

Los anticuerpos útiles para el manchado inmunohistoquímico y/o ensayo de fluidos de muestra, puede ser monoclonales y policlonales, y pueden ser preparados en cualquier mamífero. Convenientemente, los anticuerpos pueden ser preparados contra un

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polipéptido de ligando Apo-2 nativo o contra un péptico sintético con base en las secuencias de ADN provistas aquí, o contra una secuencia exógena fundida al ADN del ligando Apo-2, y que codifica un epítipo de anticuerpo específico.

El ligando de Apo-2 preferiblemente es recuperado del medio de cultivo como un polipéptido secretado, aunque también puede ser recuperadote los lisatos de la célula anfitriona cuando son producidos directamente sin una señal secretoria. Si el ligando Apo-2 es ligado a membrana, éste puede ser liberado de la membrana usando una solución detergente adecuada (por ejemplo, Triton-X 100) o su región extracelular puede ser liberada mediante división enzimática.

Cuando el ligando Apo-2 es producido en una célula recombinante diferente a aquellas de origen humano, el ligando Apo-2 está libre de proteínas o polipéptidos de origen humano. Sin embargo, es usualmente necesario recuperar o purificar el ligando Apo-2 de proteínas de células recombinantes o polipéptidos recombinantes, para obtener preparaciones que sean substancialmente homogéneas en cuanto al ligando Apo-2. Como una primera etapa, el medio de cultivo o lisato puede ser centrifugado para remover los desechos de células particulares. El ligando Apo-2 de ahí en adelante es purificado de las proteínas y polipéptidos solubles contaminantes, con los siguientes procedimientos ilustrativos de los procedimientos de purificación adecuados: por fraccionación sobre una columna de intercambio iónico; precipitación con etanol; HPLC de fase inversa; cromatografía sobre sílice o una resina de intercambio catiónico tal como el DEAE o CM;

cromatofocamiento; SDS-PAGE; precipitación por sulfato de amonio; filtración con gel usando, por ejemplo, Sephadex G-75; diafiltración y columnas de Sefarosa de proteína A, para remover los contaminantes como el IgG. 182

En una modalidad preferidas, el ligando Apo-2 puede ser aislado mediante cromatografía de afinidad. Los fragmentos del ligando Apo-2 o las variantes en las cuales los residuos han sido eliminados, insertados o sustituidos, son recuperados de la misma manera que el ligando Apo-2 nativo, tomando en cuenta cualesquier cambio substanciales en las propiedades ocasionadas por la variación. Por ejemplo, la preparación de una fusión del ligando Apo-2 con otra proteína o polipéptido, por ejemplo, un antígeno bacteriano o viral, facilita la purificación; una columna de inmutafinidad que contiene el anticuerpo al antígeno puede ser usada para absorber al polipéptido de fusión.

Un inhibidor de proteasa tal como fenil metil sulfonil floruro (PMSF), puede también ser útil para inhibir la degradación proteolítica durante la purificación, y los antibióticos pueden ser incluidos para impedir el crecimiento de los contaminantes ventajosos. Una persona versada en la técnica podrá apreciar que los métodos de purificación adecuados para el ligando Apo-2 nativo, puede requerir modificación, o tener en cuenta cambios en el carácter del ligando Apo-2 o sus variantes luego de la expresión en cultivos de células recombinantes.

Durante cualquiera de las etapas de purificación, puede ser deseable exponer el Apo-2L recuperado a una solución que contiene

ión de metal divalente o a material de purificación (tal como un medio de cromatografía o soporte de cromatografía), que contiene uno o más iones de metal divalente. En una modalidad preferida, en los iones de metal divalente y/o los agentes reductores se usan durante la recuperación o purificación del Apo-2L. Opcionalmente, tanto los iones de metal divalente, como los agentes de reducción, tales como DTT o BME, pueden ser usados durante la recuperación o purificación del Apo-2L. Se cree, que el uso de iones de metal divalente, durante la recuperación o purificación, suministrarán la estabilidad del trímero Apo-2L o preservan el trímero Apo-2L formado durante la etapa del cultivo celular.

La descripción que sigue, también se relacionaron métodos para producir el ligando Apo-2 covalentemente sujeto (de aquí en adelante "conjugado") a uno o más grupos químicos. Los grupos químicos adecuados para uso en un conjugado de Apo-2L de la presente invención, son preferiblemente no significativamente tóxicos o inmunogénicos. El grupo químico es seleccionado opcionalmente para producir un conjugado de Apo-2L que pueda ser almacenado y usado bajo condiciones adecuadas para almacenamiento. Una variedad de grupos químicos ilustrativos que pueden ser conjugados a polipéptidos son conocidos en la técnica e incluyen, por ejemplo carbohidratos, tales como aquellos carbohidratos que ocurren naturalmente en glicoproteínas, poliglutamato, y polímeros no proteináceos, tales como folioles (ver, por ejemplo, la patente estadounidense No. 6,245,901).

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Un poliol, por ejemplo, puede ser conjugado con polipéptidos tal como un Apo-2L en uno o más residuos de aminoácidos, incluyendo residuos de lisina, como se describe en la WO 93/00109, supra. El poliol empleado puede ser cualquier polímero poli (óxido de alquilen) soluble, y puede tener una cadena lineal o ramificada. Los polioles adecuados incluyen aquellos sustituidos en uno o más posiciones hidroxilo con un grupo químico, tal como un grupo alquilo que tiene entre uno a cuatro carbonos. Típicamente, el poliol es un poli (alquilen glicol), tal como el poli (etilen glicol) (PEG) y por lo tanto para facilidad de descripción, el resto de la discusión se relaciona con una modalidad ilustrativa, en donde el poliol empleado es el PEG, y el proceso de conjugar el poliol a un polipéptido se llama "pegilación". Sin embargo aquellos versados en la técnica reconocen que otros polioles, tal como, por ejemplo, poli (propilenglicol) y copolímeros de polietileno-poli propilenglicol, pueden ser empleados usando las técnicas para conjugación descritas aquí para el PEG.

El peso molecular promedio del PEG empleado en la pegilación, del Apo-2L, puede variar y típicamente puede estar en el rango entre cerca de 500 a cerca de 30.000 Daltons (D). Preferiblemente, el peso molecular promedio del PEG está entre cerca de 1.000 a cerca de 25.000 D, y más preferiblemente entre cerca de 1.000 a cerca de 5.000 D. En una modalidad la pegilación, se lleva a cabo con el PEG que tiene un peso molecular promedio de cerca de 1.000 D. Opcionalmente, el homopolímero de PEG es no sustituido, y éste puede también ser sustituido en un extremo con un grupo alquilo. Preferiblemente, el grupo alquilo, es un grupo alquilo con uno a cuatro carbonos, y más preferiblemente un grupo metilo. Las

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preparaciones PEG son disponibles comercialmente y típicamente, aquellas preparaciones PEG adecuadas para uso en la presente invención, con preparaciones no homogéneas vendidas de acuerdo con el peso molecular promedio. Por ejemplo, PEG comercialmente disponible (5.000) típicamente contienen moléculas que varían ligeramente en un peso molecular, usualmente más o menos 500 D.

El ligando Apo-2 de la invención puede estar en diversas formas tal como en forma de monómero o en forma de trímero (que comprende tres monómeros). Opcionalmente, un trímero Apo-2L puede ser pegilado de una manera tal que una molécula PEG es enlazada o conjugada con una, dos o cada una de los tres monómeros que forman el Apo-2L trimérico. En tal modalidad, es preferido que el PEG empleado tenga un peso molecular promedio de cerca de 1.000 a cerca de 5.000 D. También se ha contemplado que en los trimeros Apo-2L pueden ser pegilados "parcialmente", es decir, en donde sólo uno o dos de los tres monómeros que forman el trímero son enlazados o conjugados con el PEG. Una variedad de métodos para pegilar proteínas son conocidos en la técnica. Los métodos específicos para producir proteínas conjugadas al PEG, incluyen los métodos descritos en la patente estadounidense No. 4,179,337, la patente estadounidense No. 4,935,465, Y la patente estadounidense No. 5,849,535. Típicamente la proteínas es ligada covalentemente mediante uno o más de los residuos de a aminoácido 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 los grupos reactivos es designado aquí como polímero activado. El grupo reactivo selectivamente reacciona con amino libre, y otros grupos

reactivos en la proteína. El polímero PEG puede ser acoplado al amino o a otro grupo reactivo en la proteína sea de una manera aleatoria o de una manera específica a sitio. Se deberá entender, sin embargo, que el tipo y la cantidad de grupo reactivo escogido, lo mismo que el tipo de polímero empleado, para obtener resultados óptimos, dependerá de la proteína particular, o la variante de la proteína empleada para evitar que tenga la reacción del grupo reactivo con muchos grupos activos particularmente en la proteína. Como esto no es posible o puede no ser posible de evitar completamente, se recomienda que generalmente de cerca de 0.1 a 1.000 moles, preferiblemente 2 a 200 moles de polímero activado por mol de proteína, dependiendo la concentración de proteína sea lo empleado. La cantidad final de polímero activado por mol de proteína, es un balance para mantener la actividad óptima, mientras que al mismo tiempo, se optimiza, si es posible, la vida promedio circulatoria de la proteína.

Se ha contemplado adicionalmente, que el Apo-2L descrito aquí, puede ser también ligado o fusionado a secuencias de cremallera de leusina usando técnicas conocidas en el arte.

Los métodos para generar anticuerpos receptores de muerte y anticuerpo CD20 también son descritos aquí. En antígeno a ser usado para la producción de, o el tamizado para, anticuerpo, puede ser, por ejemplo, una forma soluble de antígeno o una porción del mismo, que contiene el epítipo deseado.

Alternativamente, o adicionalmente, las células que expresan el antígeno en su superficie celular puede ser usadas para generar o tamizar, anticuerpos. Otras formas de antígeno útiles para generar anticuerpo, ser serán evidentes para aquellos versados en la técnica.

(i) *Anticuerpos policlonales.*

Los anticuerpos policlonales, son preferiblemente cultivados en animales mediante múltiples inyecciones subcutáneas (sc), o intraperitoneales (ip) del antígeno relevante y un adyuvante. Puede ser útil conjugar el antígeno relevante a una proteína que es inmunogénica es las especies a ser inmunizadas, por ejemplo, hemocianina de agujero de llave, suero de albúmina, triroglobulina bovina, o inhibidor de tripsina de soya, usando un agente bifuncional o derivatizante, por ejemplo, éster de maleimidobenzoyl sulfosuccinimida (conjugación mediante residuos de sisteína), N-hidroxisuccinimida (mediante residuos de lisina), glutaraldehído, anhídrido succinico, SOCl_2 , o $\text{R}^1\text{N}=\text{C}=\text{NR}$, donde R y R^1 don grupos alquilos diferentes.

Los animales son inmunizados contra el antígeno, conjugados inmunogénicos o derivados mediante combinar, por ejemplo, 100 μg o 5 μg de proteína o conjugado (para conejos o ratones, respectivamente) con tres volúmenes de adyuvante completo de Freund, e inyectar la solución intradérmicamente en múltiples sitios. Un mes después los animales son reforzados con 1/5 a 1/10 de la cantidad original del péptido o conjugado en adyuvante

completo de Freund, mediante inyección subcutánea en múltiples sitios. De 7 a 14 días después, a los animales se les toma sangre, y el suero es ensayado en cuanto al título de anticuerpo. Los animales son reforzados hasta el título plateaus. Preferiblemente, el animal es reforzado con el conjugado del mismo antígeno, pero conjugado a una proteína diferente y/o mediante un reactivo de reticulación diferente, Los conjugados también pueden hacerse en cultivos de células recombinantes como proteínas de fusión. También, los agentes agregantes tales como alud son adecuadamente usados para mejorar la respuesta inmune.

(ii) *Anticuerpos monoclonales*

Los anticuerpos monoclonales, son obtenidos de una población de anticuerpos substancialmente homogéneos, es decir, los anticuerpos individuales que comprende la población son idénticos excepto por posibles mutaciones de ocurrencia natural que puedan estar presentes en cantidades menores. Así, el modificador "monoconal" indica el carácter del anticuerpo de que no es una mezcla de anticuerpos discretos.

Por ejemplo, los anticuerpos monoclonales pueden ser hechos usando el método de hibridoma primero descrito por Kohler et al., Nature, 256:495 (1975), o puede ser hecho mediante métodos de ADN recombinante (Patente estadounidense No. 4,816,567).

En el método de hibridoma, un ratón u otro animal anfitrión apropiado, tal como un hámster es inmunizado, como se describió anteriormente para producir linfocitos que produzcan o sean

capaces de producir anticuerpos que se ligarán específicamente a la proteína usada para la inmunización. Alternativamente, los linfocitos pueden ser inmunizados in vitro. Los linfocitos son entonces fusionados con células de mieloma, usando un agente de fusión adecuado, tal como el polietilenglicol, para formar una células hibridoma, (Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986)).

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

Las células de mieloma preferidas son aquellas que se funden eficientemente, soportan una producción estable a alto nivel de anticuerpo, mediante células productoras de anticuerpo seleccionadas y son sensibles a un medio tal como le medio HAT. Entre éstas, las líneas de células del mieloma preferidas, son las líneas del mieloma de murino, tales como aquellas derivadas de los tumores de ratón MOPC-21 y mpc-11, disponibles de SaIk Institute Cell Distribution Center, San Diego, California USA, y células SP-2 o X63-Ag8-653 disponibles del American Type Culture Collection, Manassas, Virginia USA. Las células de mieloma humano

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y heteromieloma de ratón-huano también han sido descritas para la producción de anticuerpos monoclonales humanos (Kozbor, J. *Immunol.*, 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., Nueva York, 1987)).

El medio de cultivo en el cuál las células hibridoma se hacen crecer es ensayado en cuánto a la producción de anticuerpos monoclonales dirigida contra el antígeno. Preferiblemente, la especificidad de ligado de los anticuerpos monoclonales producidos por las células hibridoma se determina por inmunoprecipitación o por un ensayo de ligado in vitro, tal como radio inmuno ensayo (RIA) o ensayo inmuno absorbente ligado a enzima (ELISA).

La afinidad del ligado del anticuerpo monoclonal, puede, por ejemplo, ser determinada por el análisis Scatchard de Munson et al., *Anal. Biochem.*, 107:220 (1980).

Después las células de hibridoma son identificadas las que producen anticuerpos de especificidad deseada, afinidad, y/o actividad, los clones pueden ser subclonados por procedimientos de dilución limitativa, y se hacen crecer por métodos estándar (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). El medio de cultivo adecuado para éste propósito incluye, por ejemplo, al medio D-MEM o RPMI-1640. En adición, las células de hibridoma pueden hacerse crecer in vivo como tumores de ascitas en un animal.

Los anticuerpos monoclonales secretados por los clones son separados adecuadamente del medio de cultivo, los fluidos ascitas, o el suero por procedimientos de purificación de inmunoglobulina convencional, tal Composición, por ejemplo, la proteína A-Sefarosa, cromatografía por hidroxilapatita, electrofóresis de gel, diálisis, o cromatografía de afinidad.

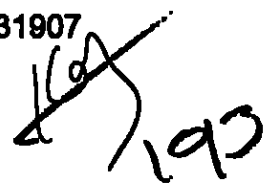
El ADN que codifica los anticuerpos monoclonales es fácilmente aislado y secuenciado, usando procedimientos convencionales (por ejemplo, mediante usar probetas de oligonucleótidos que son capaces de ligarse específicamente a los genes que codifican las cadenas pesadas y livianas de los anticuerpos de murino). Las células de hibridoma, sirven como una fuente preferida de tal ADN. Una vez aislado, el ADN puede ser colocado en vectores de expresión, que entonces son transfectados en células anfitrionas tales como células de E. coli, células COS de simio, células de ovario de hámster chino (CHO), o células de mieloma, que de otra manera no producen proteína inmunoglobulina, para obtener la síntesis de los anticuerpos monoclonales en las células anfitrionas recombinantes. Artículos de revistas 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 modalidad adicional, los anticuerpos o fragmentos de anticuerpo, pueden ser aislados de librerías de anticuerpos fago, generados usando las técnicas descritas 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),

describe el aislamiento de los anticuerpos de murino y humanos respectivamente usando librerías fago. Publicaciones subsiguientes describen la producción de anticuerpos humanos de alta afinidad (rango nM) mediante mezclado de cadenas (Marks et al., Bio/Technology, 10:779-783 (1992)), lo mismo que la infección combinatoria y la recombinación in vivo como una estrategia para la construcción de librerías fago muy grandes (Waterhouse et al., Nuc. Acids. Res., 21:2265-2266 (1993)). Así, éstas técnicas son alternativas viables a técnicas tradicionales de hibridoma de anticuerpo monoclonal para el aislamiento de anticuerpos monoclonales.

El ADN también puede ser modificado, por ejemplo, mediante la sustitución de de la secuencia de codificación para los dominios constantes de cadena pesada y liviana humanos en lugar de las secuencias de murino homólogas (Patente estadounidense No. 4,816,567; Morrison, et al., Proc. Natl Acad. Sci. USA, 81:6851 (1984)), o mediante unir covalentemente a la secuencia de codificación de inmunoglobulina todo o parte de la secuencia de codificación para un polipéptido no inmunoglobulina.

Típicamente, tales polipéptidos no inmunoglobulina son sustituidos por dominios constantes de un anticuerpo, o ellos son sustituidos por los dominios variables de un sitio de combinación con antígeno de un anticuerpo para crear un anticuerpo divalente quimérico, que comprende un sitio para combinar antígeno que tiene especificidad por un antígeno y otro sitio para combinar antígeno que tiene especificidad para un antígeno diferente.



(iii) *Anticuerpos humanizados*

Los métodos para humanizar anticuerpos no humanos, han sido descritos en la técnica. Preferiblemente, un anticuerpo humanizado tiene uno o más residuos de aminoácido introducidos en éste de una fuente que no es humano. Éstos residuos de aminoácidos no humanos, se llaman frecuentemente como "residuos importados", que son típicamente tomados de un dominio variable "importado". La humanización, puede llevarse a cabo esencialmente siguiendo el método de Winter y los co-trabajadores (Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)), mediante sustituir las secuencias de región hipervariable para las secuencias correspondientes de un anticuerpo humano. De acuerdo a lo anterior, tales anticuerpos "humanizados" son anticuerpos quiméricos (Patente estadounidense No. 4,816,567) en donde substancialmente menos de un dominio variable humano intacto ha sido sustituido por la secuencia correspondiente desde una especie no humana. En la práctica, los anticuerpos humanizados, son típicamente anticuerpos humanos en los cuales algunos residuos de región hipervariable, y posiblemente algunos residuos FR son sustituidos por residuos de sitios análogos en anticuerpos de roedores.

La escogencia de los dominios variables humanos, tanto livianos y pesados, para ser usados para fabricar los anticuerpos humanizados es muy importante para reducir la antigenicidad. De acuerdo con lo que se ha llamado el método del "mejor ajuste" la secuencia del dominio variable de un anticuerpo de roedor, es

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tamizada contra la librería completa de las secuencias de dominio variable humanas conocidas. La secuencia humana que es más cercana a aquella del roedor, es entonces aceptada como la región de estructura de trabajo humano (FR) 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 de estructura de trabajo o marco de trabajo particular, derivado de la secuencia de consenso de todos los anticuerpos humanos de un subgrupo particular de cadenas livianas o pesadas. El mismo marco de trabajo puede ser usado para diversos anticuerpos humanizados diferentes (Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta et al., J. Immunol., 151:2623 (1993)).

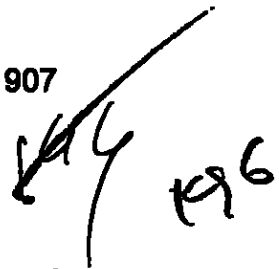
Es adicionalmente importante, que los anticuerpos sena humanizados con retención de la alta afinidad para el antígeno, y otras propiedades biológicas favorables. Para lograr éste objetivo, de acuerdo con un método preferido, los anticuerpos humanizados, son preparados por un proceso de análisis de las secuencia parientes y diversos productos humanizados conceptuales que usan modelos tridimensionales de las secuencias parentales y humanizadas. Modelos tridimensionales de inmunoglobulina son comúnmente disponibles y son familiares para aquellos versados en la técnica. Los programas de computadora están disponibles los cuales ilustran y muestran estructuras conformacionales tridimensionales probables de secuencias de candidato de inmunoglobulina seleccionado. La inspección de éstas visualizaciones permite el análisis se papel probable de los residuos en el funcionamiento de la secuencia candidato de

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inmunoglobulina, es decir, el análisis de los residuos que influyen en la habilidad del candidato de inmunoglobulina para aligarse a su antígeno. De ésta manera, los residuos FR pueden ser seleccionados y combinados desde el recipiente y las secuencias de importación de modo que las características del anticuerpo deseadas, tales como la afinidad incrementada para el antígeno objetivo, es logrado. En general, los residuos de región hipervariable, están directamente, y más substancialmente involucrados en influenciar el ligado del antígeno.

(iv) *Anticuerpos humanos*

Como una alternativa a la humanización, anticuerpos humanos pueden ser generados. Por ejemplo, es ahora posible producir animales transgénicos (por ejemplo ratones) que son capaces luego de inmunización, de producir un repertorio completo de anticuerpos humanos en la ausencia de producción de inmunoglobulina endovenosa. Por ejemplo, se ha descrito que la eliminación homocigota de la región de unión de cadena pesada del anticuerpo en el gen (J_H) en ratos quiméricos y mutantes de líneas de gérmenes, resulta en la inhibición completa de la producción de cuerpo endogenoso. La transferencia de la disposición o del arreglo de genes de inmunoglobulina línea de gérmenes humanos en tales ratones mutantes de línea, resultarán en la producción de anticuerpos humanos luego del reto o de la infección con el antígeno. Ver, 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); y las Patentes estadounidenses Nos. 5,591,669, 5,589,369 y 5,545,807.



Alternativamente, la tecnología de visualización de fagos (McCafferty et al., Nature 348:552-553 (1990)), puede ser usada para producir anticuerpos humanos, y fragmentos de anticuerpos in vitro, a partir de repertorios de dominio variable (V) de inmunoglobulina de donantes no inmunizados. De acuerdo con ésta técnica, los genes de dominio de anticuerpo V son clonados en el marco dentro de un gen de proteína recubierta mayor o menor de un bacteriófago filamentoso, tal como el M13 o el fd, y mostrado como fragmentos de anticuerpo funcionales sobre la superficie de las partículas fago. Debido a que la partícula filamentosa contiene una copia de ADN de cadena única del genoma del fago, las selecciones con base en las propiedades funcionales del anticuerpo también resultan en la selección del gen que codifica el anticuerpo que exhibe esas propiedades. Así, el fago minimiza algo de las propiedades de las células B. La muestra de fago puede ser llevada acabo en una variedad de formatos; para su revisión, ver, por ejemplo, Johnson, Kevin S. y Chiswell, David J., Current Opinion in Structural Biology 3:564-571 (1993). Diversas fuentes de segmentos de gen V pueden ser usados para mostrar fagos. Clackson et al., Nature, 352:624- 628 (1991), aisló un arreglo diverso de anticuerpos anti-oxazolonas de una librería de genes combinatorios aleatorios pequeños, derivados de riñones de ratones inmunizados. Un repertorio de genes V, de donantes humanos no inmunizados pueden ser construidos, y los anticuerpos a un arreglo diverso de antígenos (incluyendo auto-antígenos), pueden ser aislados esencialmente siguiendo las técnicas descritas por Marks et al., J. Mol. Biol. 222:581-597

(1991), o Griffith et al., EMBO J. 12:725-734 (1993). Ver, también, las Patentes estadounidenses Nos. 5,565,332 y 5,573,905.

Los anticuerpos humanos pueden también ser generados por células B activadas in vitro (ver las patentes estadounidenses 5,567,610 y 5,229,275).

(v) *Fragmentos de anticuerpo*

Diversas técnicas han sido desarrolladas para la producción de fragmentos de anticuerpos. Tradicionalmente, estos fragmentos fueron derivados vía la digestión proteolítica de anticuerpos intactos (ver, por ejemplo, Morimoto et al., Journal of Biochemical and Biophysical Methods 24:107-117 (1992) y Brennan et al., Science, 229:81 (1985)). Sin embargo, estos fragmentos pueden ahora ser producidos directamente mediante células anfitrionas recombinantes. Por ejemplo, los fragmentos de anticuerpos pueden ser aislados de las librerías de fagos de anticuerpos discutidos anteriormente. Alternativamente, fragmentos Fab'-SH pueden ser directamente recuperados del E. coli y químicamente acoplados para formar fragmentos F(ab')₂ ((Carter et al., Bio/Technology 10:163-167 (1992)). De acuerdo con otro modelo, fragmentos F(ab')₂ pueden ser aislados directamente de cultivos de células anfitrionas recombinantes. Otras técnicas para la producción de fragmentos de anticuerpos serán evidentes para aquellos practicantes versados. En otras modalidades, el anticuerpo de escogencia es un fragmento Fv de cadena única (scFv). Ver WO 93/16185; la Patente estadounidense No. 5,571,894; y Patente estadounidense No.

5,587,458. El fragmento de anticuerpo, puede también ser un "anticuerpo lineal", por ejemplo, como se describió en la patente estadounidense 5,641,870 por ejemplo. Tales fragmentos de anticuerpo lineal pueden ser monoespecíficos o biespecíficos.

(vi) *Anticuerpos biespecíficos*

Los anticuerpos biespecíficos, son anticuerpos que tienen especificidades de ligados para por lo menos dos epítopos diferentes. Anticuerpos biespecíficos ilustrativos pueden ligarse a dos epítopos diferentes de los receptores CD20, DR4 o DR5. Los anticuerpos biespecíficos, también pueden ser usados para localizar agentes citotóxicos en una célula B. éstos anticuerpos poseen un brazo de ligado marcador de célula B, y una brazo que liga al agente citotóxico (por ejemplo saponina, anti-interferón- α , alcaloide vinca, cadena de ricino A, metotrexato o isótopo radiactivo haptén). Los anticuerpos biespecíficos, pueden ser preparados como anticuerpos de longitud completa, o fragmentos de anticuerpos (por ejemplo, anticuerpos biespecíficos $F(ab')_2$).

Los métodos para hacer anticuerpos biespecíficos son conocidos en la técnica. La producción tradicional de anticuerpos biespecíficos de longitud completa se basa en la co expresión de dos pares de cadenas livianas o pesadas de inmunoglobulina, en donde las dos cadenas tienen diferentes especificidades (Millstein et al., Nature, 305:537- 539 (1983)). Debido a la clasificación aleatoria de las cadenas pesada y liviana de la inmunoglobulina, estos hibridomas (cuadromas) producen una mezcla potencial de 10 diferentes moléculas de anticuerpo, de las cuales

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solo una tiene la estructura di específica correcta. La purificación de la molécula correcta, que se hace usualmente por etapas de cromatografía de afinidad, es algo problemática, y las producciones de producto son bajas. Segmentos similares se describen en la WO 93/08829, y en Traunecker et al., EMBO J., 10:3655-3659 (1991).

De acuerdo con un modelo diferente, los dominios variables de anticuerpo con las especificidades de ligado deseadas (sitios de combinación anticuerpo-antígenos) son fusionados a las secuencias de dominio constante de la inmunoglobulina. La fusión preferiblemente es con un dominio constante de cadena pesada de inmunoglobulina, que comprende por lo menos parte de la bisagra, y las regiones CH₂, y CH₃. Es preferido tener en la primera región constante de cadena pesada (CH1) que contiene el sitio necesario para el ligado con la cadena liviana, presente en por lo menos una de las funciones. Los ADN que codifican las fusiones de cadena pesada de la inmunoglobulina, y se desea, la cadena liviana de inmunoglobulina, son insertados en vectores de expresión separados, y son co transfectados en un organismo anfitrión adecuado. Esto suministra una gran flexibilidad al ajustar las proporciones mutuas de los tres fragmentos de polipéptido en modalidades con proporciones desiguales de las tres cadenas de polipéptido usadas en la construcción lo que suministra producciones óptimas. Es, sin embargo, posible insertar las secuencias de codificación para dos o para 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 producciones mayores o cuando las proporciones no son de significancia particular.

En una modalidad preferida de este modelo, los anticuerpos di-específicos están compuestos de una cadena pesada de inmunoglobulina híbrida con una primera especificidad de ligado en un brazo, y un par de cadena pesada-liviana de inmunoglobulina híbrida (siempre que suministra una segunda especificidad de ligado) en el otro brazo. Se encontró que esta estructura asimétrica facilita la separación del compuesto bi-específico deseado de las combinaciones de cadena de inmunoglobulina no deseadas, ya que la presencia de una cadena liviana de inmunoglobulina en solamente la mitad de la molécula di-específica suministra una vía más fácil de separación. Este modelo está descrito en la WO 94/04690. Para mayores detalles de generara anticuerpos bi-específicos ver, por ejemplo, Suresh et al., *Methods in Enzymology*, 121:210 (1986). De acuerdo a otro modelo descrito en la patente estadounidense No 5, 731,168, la interfase entre un par de moléculas de anticuerpo puede ser construida por ingeniería para maximizar el porcentaje de heterodímeros que son recubiertos del cultivo de células recombinantes. La interfase preferida comprende por lo menos una parte del dominio C_H3 de un dominio constante de anticuerpo. En este método, uno o más cadenas laterales pequeñas de aminoácidos de la interfase de la primera molécula de anticuerpos son reemplazados con cadenas laterales más grandes (por ejemplo tirozina o triptofan). Las "cavidades" compensatorias de tamaño idéntico o similar a la cadena lateral grande son creadas sobre la interfase de la segunda molécula de anticuerpo mediante



reemplazar las cadenas laterales de aminoácidos grandes con unas pequeñas, por ejemplo adanina o trionina. Esto suministra un mecanismo para incrementar la producción del heterodímero sobre otros productos finales no deseados tales como los homodímeros.

Los anticuerpos diespecíficos incluyen anticuerpos reticulados o "heteroconjugados". Por ejemplo, uno de los anticuerpos en el heteroconjugado puede estar acoplado a la avidina, el otro a la biotina. Tales anticuerpos han sido, por ejemplo, propuestos para células de sistema inmune objetivo a células no deseadas (patente estadounidense No. 4,676,980), y para el tratamiento de infecciones de HIV (WO 91/00360, WO 92/200373, y EP 03089). Los anticuerpos heteroconjugados pueden estar hechos usando cualquiera de los métodos de reticulación convenientes. Los agentes de reticulación adecuados son bien conocidos en la técnica, y se describen en la patente estadounidense No. 4,676,980,, junto con un número de técnicas de reticulación.

Las técnicas para generar anticuerpos biespecíficos de fragmentos de anticuerpo también han sido descritas en la literatura. Por ejemplo, los anticuerpos bi específicos pueden ser preparados enlaces químicos. Brennan et al., Science, 229: 81 (1985); Shalaby et al., J. Exp. Med., 175: 217-225 (1992).

Diversas técnicas para hacer y aislar fragmentos de anticuerpos bi específicos directamente de cultivos de células recombinantes también han sido descritas. Por ejemplo, los anticuerpos di específicos han sido producidos usando cremalleras de leucina. Kostelny et al., J. Immunol., 148 (5):1547-1553 (1992). Los

péptidos de cremallera de leucina de las proteínas SOS y JUM fueron enlazados a las porciones Fab' de dos anticuerpos diferentes por fusión de genes. Los homo dímeros del anticuerpo fueron reducidos en la región de bisagra para formar monómeros y entonces reoxidados para formar heterodímeros del anticuerpo. Este método también puede ser utilizado para la producción de homodímeros de anticuerpo. La tecnología de "diacuerpos" descrita por Hollinger et al., Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993) ha provisto un mecanismo alternativo para hacer fragmentos de anticuerpo bi específicos. Los fragmentos comprenden un dominio variable de cadena pesada (VH) conectado a un dominio variable de cadena liviana (VL) mediante un enlazador que es demasiado corto para permitir el apareamiento o el emparejamiento entre los dos dominios en la misma cadena. De acuerdo a lo anterior, los dominios VH y VL de un fragmento son forzados para emparejarse con los dominios VL y VH complementarios de otro fragmento, formando así dos sitios de ligado por antígeno. Otra estrategia para hacer fragmentos de anticuerpo di específicos mediante el uso de dímeros Fb de cadena única (sFv) también han sido reportados. Ver Gruber et al., J. Immunol., 152:5368 (1994).

Los anticuerpos con más de dos valencias son contemplados también. Por ejemplo, anticuerpos tri específicos pueden ser preparados. Tutt et al. J. Immunol. 147: 60 (1991). Los anticuerpos con tres o más sitios de ligado de antígeno son descritos en la WO01/77342 (Miller y Presta), incorporados expresamente aquí como referencia.

Los anticuerpos usados en los métodos o incluidos en los artículos de fabricación aquí son conjugados opcionalmente con un agente cito tóxico.

Los agentes quimioterapéuticos útiles en la generación de tales conjugados de anticuerpo- agentes cito tóxicos han sido descritos anteriormente.

Los conjugados de un anticuerpo y uno o más toxinas de molécula pequeña, tales como caliqueamicina, una maitansina (patente estadounidense No. 5, 208,020), un tricoteno, y CC1065 también son contemplados aquí.

En otra modalidad de la invención, el anticuerpo es conjugado a uno o más moléculas de maitansina (por ejemplo cerca de 1 a cerca de 10 moléculas de maitansina por molécula de anticuerpo). La maitansina puede por ejemplo, ser convertida en May-SS-Me que puede ser reducida a May-SH3 y se hace reaccionar con el anticuerpo modificado (Chari et al. Cancer Research 52: 127-131 (1992)) para generar un conjugado maitansinoide - anticuerpo.

Alternativamente, el anticuerpo es conjugado con uno o más moléculas de caliqueamicina. La familia de las caliqueamicinas de antibióticos es capaz de producir rupturas de ADN de doble cadena en concentraciones submicromolares. Los análogos estructurales de la caliqueamicina que pueden ser usados incluyen, pero no se limitan a, δ_1^1 , α_2^1 , α_3^1 , N-acetil- δ_1^1 , PSAG y θ_1^1 ; (Hinman et al. Cancer Research 53: 3336-3342 (1993) y Lode et al. Cancer Research 58: 2925-2928 (1998)).

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Las toxinas y fragmentos enzimáticamente activos de los mismos que pueden ser usados incluyen la cadena A de la difteria, fragmentos activos que no se ligan de la toxina de la difteria, cadena de la exotoxina A (de *Sedumonas aeruginosa*), cadena de ricina A, la cadena de afina A, la cadena de modicina A, la alfa-sarcina, proteínas de aleuritas forddie, proteínas de diantina, proteínas de fitolaca americana PAPI, PAPII, y PAP-S), inhibidor de momordica carantia, curcina, crotina, sapaonaria officinalis como inhibidor, gelonina, mitogelina, restrictocina, fenomicina, enomicina y los tricotecenos. Ver, por ejemplo, WO 93/21232 publicado en octubre 28, 1993.

La presente invención contempla adicionalmente anticuerpo conjugado con un compuesto con actividad nucleolítica (por ejemplo una ribonucleasa o una endonucleasa de ADN tal como desoxiribonucleasa;- DNasa).

Una variedad de isótopos radioactivos están disponibles para la producción de antagonistas radio conjugados o anticuerpos radio conjugados. Ejemplo incluyen At^{211} , I^{131} , I^{125} , Y^{90} , Re^{106} , Re^{188} , Sm^{153} , Bi^{212} , P^{32} e isótopos reactivos de Lu.

Los conjugados de los anticuerpos y del agente citotóxico pueden ser hechos usando una variedad de agentes de acoplamiento de proteína bifuncional tal como propionato de N-succinimidil-3- (2-piridilditiol) SPDP), succinimidil-4-(N-maleimidometil) cyclohexano-1-carboxilato, iminotiolano (IT), derivados bifuncionales de imidoésteres (tales como adipimidato de dimitilo HCL) ésteres

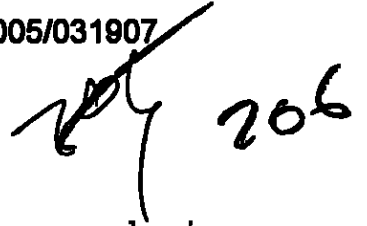
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activos (tales como suberato de disuccinimidilo), aldehidos (tales como glutarealdehido), compuestos bis-azido (tales como bis (p-azidobenzoyl) hexanodiamina), derivados bis-diazonio (tales como bis- (p-diazoniobenzoyl) -etilenediamina), diisocianatos (tales como 2, 6-diisocianato de tolieno), y compuestos de flúor bis-activo tales como 1,5-difluoro-2,4-dinitrobenzeno). Por ejemplo, una inmunotoxina de ricino que puede ser preparada según se describió en Vitetta et al. Science 238: 1098 (1987). El ácido -isotiocianatobenzil-3~metildietileno triaminepentaacético marcado con Carbón-14- (MX-DTPA) es un agente quelante ilustrativo para la conjugación del radio nucleótido al antagonista o al anticuerpo. Ver WO94/11026.

El enlazador puede ser un enlazador de "división" que facilita la liberación de la droga citotóxica en la célula. Por ejemplo, un enlazador azido-labil, un enlazador sensible a la peptidasa, un enlazador de dimetilo o un enlazador que contiene disulfuro (Chari et al. Cancer Research 52: 127-131 (1992)). Pueden ser usados.

Alternativamente, una proteína de fusión que comprende el anticuerpo y el agente cito tóxico puede hacerse, por ejemplo mediante técnicas recombinantes o síntesis de péptido.

Los anticuerpos de la presente invención también pueden ser conjugados con un enzima activadora de prodroga que convierte una prodroga (por ejemplo un agente quimioterapéutico de peptidilo, ver WO81/01145) en una droga anti cáncer activa. Ver, por ejemplo WO 88/07378 y la patente estadounidense No. 4,975,278.



El componente de enzima de tales conjugados incluye cualquier enzima capaz de actuar sobre una prodroga de tal manera que la convierta en su forma citotóxica más activa.

Las enzimas que son útiles en el método de esta invención incluyen, pero no se limitan a, fosfatasa alcalina útil para convertir las prodrogas que contienen fosfato en drogas libres; aril sulfatasa útil para convertir prodrogas que contienen sulfato en drogas libres; de aminasa de citoquina útil para convertir 5-fluorocitoxina no tóxica en la droga anti cáncer, 5-fluorouracilo; proteasas, tales como la proteasa serratia, termolisina, subtilisina, carboxipeptidasas y catepsinas (tal como catepsinas B y L), que son útiles para convertir prodrogas que contiene péptido en drogas libres; D-alanilcarboxipeptidasas, útiles para convertir prodrogas que contienen sustituyentes D-aminoácido; enzimas divisoras de carbohidrato tales como la β -galactosidasa y neuraminidasa útiles para convertir prodrogas glicosiladas en drogas libres; β -lactamasa útil para convertir drogas derivadas con β -lactamasa en drogas libres; amidasas de penicilina, tales como la amidasa de penicilina V o la amidasa de penicilina G, útiles para convertir drogas derivadas en sus nitrógenos de amina con fenoxiacetilo o grupos fenil acetilo, respectivamente, en drogas libres. Alternativamente, los anticuerpos con actividad enzimática, también conocidos en la técnica como "abzimas", pueden ser usados para convertir las prodrogas de la invención en drogas libres activas (ver, por ejemplo, Massey, Nature 328: 457-458 (1987)).

Los conjugados anticuerpo-abzima pueden ser preparados como se describe aquí para el suministro de la abzima a una población celular de tumor.

Las enzimas de esta invención pueden ser ligadas covalentemente al anticuerpo mediante técnicas bien conocidas en el arte tales como el uso de reactivos de reticulación heterobifuncionales discutidos anteriormente. Alternativamente, las proteínas de fusión que comprenden por lo menos la región de ligado de antígeno de un anticuerpo ligado a por lo menos una porción funcionalmente activa de una enzima de la invención pueden ser contruidos usando técnicas de ADN recombinante bien conocidas en la técnica (ver, por ejemplo, Neuberger et al., Nature, 312: 604-608 (1984)).

Otras modificaciones del anticuerpo se contemplan aquí. Por ejemplo, el anticuerpo puede ser enlazado de una variedad de polímeros no proteináceos, por ejemplo, politilenglicol, polipropilenglicol, polioxiaquilenos, o copolímeros de polietilenglicol y polipropilenglicol.

Para incrementar la vida promedio del suero del anticuerpo, uno puede incorporar un receptor salvaje que se liga al epítipo en el anticuerpo (especialmente un fragmento de anticuerpo) como se describió en la patente estadounidense No 5,739,277, por ejemplo. Como se usa aquí, el término "epítipo que se liga al receptor salvaje" se refiere a un epítipo de la región FC de una molécula IgG (por ejemplo, IgG₁, IgG₂, IgG₃, o IgG₄) que es responsable de incrementar la vida promedio del suero in vivo de la molécula

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IgG. Alternativamente, o adicionalmente, uno puede incrementar, o disminuir, la vida promedio del suero mediante alterar la secuencia de aminoácido de la región FC de un anticuerpo para generar variantes con ligado F CRN alterado. Los anticuerpos con el ligado FCRN alterado y/o con barra promedio del suero alterada son descritos en la WO 00/42072 (Presta, L.).

Las formulaciones que comprenden el Apo2L/TRAIL, anticuerpos receptores de muerte y/o anticuerpos CD20 también son suministradas por la presente invención. Se cree que tales formulaciones pueden ser adecuadas particularmente para almacenamiento lo mismo que para administración terapéutica. Las formulaciones pueden ser preparadas por técnicas conocidas. Por ejemplo, las formulaciones pueden ser preparadas mediante intercambio de amortiguador sobre una columna de filtración de gel.

Típicamente, una cantidad apropiada de una sal o vehículo aceptable es usada en la formulación para convertir la formulación en isotónica. Ejemplos de vehículos farmacéuticamente aceptables incluyen salina, solución de Ringer y la solución de dextrosa. El ph de la formulación preferiblemente está entre cerca de 6 a cerca de 9, y más preferiblemente entre cerca de 7 a cerca de 7.5.

Será evidente para aquellas personas versadas en la técnica que ciertos vehículos pueden ser más preferibles dependiendo de, por

ejemplo, la vía de administración y las concentraciones del ligando Apo-2, los anticuerpos receptores de muerte, y/o los anticuerpos CD20.

Las composiciones terapéuticas pueden ser preparadas mediante mezclar las moléculas deseadas que tienen el grado apropiado de pureza con vehículos opcionales, excipientes, o estabilizadores (Remington's Pharmaceutical Sciences, 16th edition, Osol, A. ed. (1980)), en la forma de formulaciones liofilizadas, soluciones acuosas o suspensiones acuosas. Los vehículos, excipientes o estabilizadores aceptables son preferiblemente no tóxicos a los recipientes en dosificaciones y concentraciones empleadas, e incluyen amortiguadores tales como tris, HEPES, PIPES, fosfato, citrato, y otros ácidos orgánicos; los anti oxidantes incluyen ácido ascórbico y metionina; preservativos (tales como cloruro de octadecildimetilbenzilo amonio; cloruro de hexametonio; cloruro de benzalconio, cloruro de benzetonio e; fenol, butol o benzil alcohol; parabenos de alquilo tales como parabeno de metilo o de propilo; catecol; resorcinol; ciclo hexanol; 3-pentanol; y m-cresol); polipéptidos de bajo peso molecular (menos de cerca de 10 residuos); proteínas, tales como suero de albúmina, gelatina, o inmunoglobulinas; polímeros hidrofílicos tales como polivinil pirrolidona; aminoácidos tales como glicina, glutamina, asparagina, histidina, arginina o lisina; mono sacáridos, disacáridos, y otros carbohidratos que incluyen glucosa, manosa, o dextrinas; azúcares tales como sacarosa, manitol, trealosa, o sorbitol; contra guiones formadores de sal tales como sodio; y/o tenso activos no iónicos tales como TWEEN™, PLURONICS™ o polietilenglicol (PEG).

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Ejemplos adicionales de tales vehículos incluyen los intercambiadores iónicos, alúmina, estearato de aluminio, lecitina, proteínas de suero, tales como albúmina de suero, sustancias amortiguadoras tales como glicina, ácido sórbico, sorbato de potasio, mezclas parciales de glicérido de ácidos grasos de vegetales saturados, agua, sales o electrolitos tales como sulfato de protamina, hidrogenfosfato de disodio, hidrogen fosfato de potasio, cloruro de sodio, sílice coloidal, trisilicato de magnesio, polivinilpirrolidona y sustancias con base en celulosa. Vehículos para formas tópicas o con base en gel que incluyen poli sacáridos tales como carboximetilcelulosa de sodio o metilcelulosa, de sodio, polivinilpirrolidona, poliacrilatos, polímeros de bloque de polioxietileno-polioxipropileno s, polietilenglicol, y alcoholes de cera de madera. Para todas las administraciones, las formas de depósito convencionales son usadas adecuadamente. Tales formas incluyen, por ejemplo, micro cápsulas, nano cápsulas, liposomas, plastos, formas de inhalación, rociados de nariz, tabletas sublinguales, y preparaciones de liberación sostenida.

Las formulaciones que son usadas para la administración in vivo deben ser estériles. Esto es fácilmente logrado mediante filtración a través de membranas estériles de filtración, antes de o luego de la liofilización y la reconstitución. La formulación puede ser almacenada en forma liofilizada o en solución si es administrada sistémicamente.

Si está en la forma liofilizada, es típicamente formulada en combinación con otros ingredientes para la reconstitución con un diluyente apropiado en el momento del uso. Un ejemplo de una formulación líquida es una solución no preservada sin color clara y estéril llena en un frasco de dosis única para inyección sub cutánea.

Las formulaciones terapéuticas generalmente están colocadas en un contenedor que tiene una puerta de acceso estéril, por ejemplo, una bolsa o frasco de solución intravenosa que tiene un tapón perforable mediante una aguja de inyección hipodérmica. Las formulaciones son administradas preferiblemente como intra venosa repetida (I.V.), sub cutánea (S.C.), intra muscular (I.M) o infusiones, o como formulaciones de aerosol adecuadas para suministro intra nasal o intra pulmonar (para suministro intra pulmonar ver, por ejemplo, EP 257,956).

La Apo2L/TRAIL, los anticuerpos de receptor de muerte, y los anticuerpos CD20 pueden también ser administrados en la forma de preparaciones de liberación sostenida. Ejemplos adecuados de preparaciones de liberación sostenida incluyen matrices semipermeables de polímeros hidrófobos sólidos que contienen la proteína, dichas matrices están en la forma de artículos formados, por ejemplo, películas o micro cápsulas. Ejemplos de matrices de liberación sostenida incluyen los poli ésteres, hidro geles, (por ejemplo poli(2- hidroxietil-metacrilato) como se describió por Langer et al., J. Biomed. Mater. Res., 15: 167-277 (1981) y Langer, Chem. Tech., 12: 98-105 (1982) o poli(vinil alcohol)), poliláctidos (patente estadounidense No. 3,773,919,

EP 58,481), copolímeros de ácido L-glutamico y gamma etil-L-glutamato (Sidman et al., Biopolymers, 22: 547-556 (1983)), etileno-acetato de vinilo no degradable (Langer et al., supra), copolímeros de ácido láctico degradable- ácido glicólico tales como Lupron Depot (micro esferas inyectables compuestas de ácido láctico- ácido glicólico en copolímero y acetato leuprolida), y ácido poli-D- (-) -3- hidroxibutírico (EP 133,988).

El Apo2L/TRAIL, los anticuerpos de receptor de muerte, y los anticuerpos CD20 descritos aquí pueden ser empleados en una variedad de aplicaciones terapéuticas. Entre estas aplicaciones están los métodos para tratar diversos cánceres y enfermedades relacionadas con inmunidad. El diagnóstico en mamíferos de diversas condiciones patológicas descritas aquí puede hacerse por el practicante versado. Las técnicas de diagnóstico están disponibles en la técnica lo que permite, por ejemplo, para el diagnóstico o detección de cáncer o la enfermedad relacionada con inmunología en un mamífero. Por ejemplo, los cánceres pueden ser identificados mediante técnicas, que incluyen pero no se limitan a, palpación, análisis sanguíneo, rayos X, NMR y similares. Las enfermedades relacionadas con inmunidad también son fácilmente identificadas. El lupus heritematoso sistémico, el mediador central de la enfermedad es la producción de anticuerpos auto reactivos para auto proteínas/tejidos y la subsecuente generación de inflamación mediada por la inmunización. Los órganos y sistemas múltiples son afectados clínicamente incluyendo el riñón, el pulmón, el sistema músculo esquelético, mucocutáneo, el ojo, el sistema nervioso central, el sistema cardiovascular, el tracto gastrointestinal, la médula ósea y la sangre. La artritis

reumatoide (RA) es una enfermedad inflamatoria autoinmune sistémica crónica que principalmente involucra la membrana sinovial de las múltiples uniones con daño resultante en el cartílago articular. La patogénesis es dependiente del linfocito T y está asociada con la producción de factores reumatoides, auto anticuerpos dirigidos contra el auto IgG con la formación resultante de complejos inmunes que logran altos niveles en el fluido de las uniones y la sangre.

Estos complejos en las coyunturas puede inducir en el infiltrado de marcado de linfocitos y monolitos en la cinofia y subsecuentemente cambios marcados sinoviales; el espacio de las coyunturas / fluido si es infiltrado por células similares con la adición de numerosos neutrófilos. Los tejidos afectados son principalmente las coyunturas, frecuentemente en patrón simétrico. Sin embargo, la enfermedad extra articular también ocurre en dos formas principales. Una forma es el desarrollo de lesiones extra articulares con el establecimiento progresivo de la enfermedad en la articulación y lesiones típicas de fibrosis pulmonar, vasculitis, y úlceras cutáneas. La segunda forma de enfermedad extra articular es la llamada síndrome de felti que ocurre tardíamente en el curso de la enfermedad RA, algunas veces después de que la enfermedad de la articulación se ha vuelto grave, e involucra la presencia de neutropenia, trombositopenia y esplenomegalia.

Esto puede estar acompañado por vasculitis en múltiples órganos con la formación de infartos, úlceras de piel y gangrenas. Los pacientes también frecuentemente desarrollan nódulos reumatoides

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en el tejido sub cutis que se sobrepone a las articulaciones afectadas; la etapa tardía de los nódulos tiene centros necróticos rodeados por un infiltrado celular inflamatorio mezclado. Otras manifestaciones que pueden ocurrir en el NA incluyen: pericarditis, pleuritis, arteritis coronaria, neumonitis intersticial con fibrosis pulmonar, keratoconjunctivitis sicca, y nódulos reumatoides.

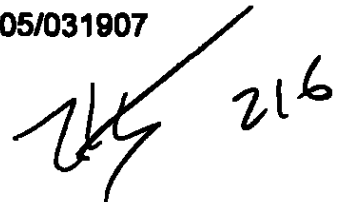
El Apo2L/TRAIL, los anticuerpos receptores de muerte, y los anticuerpos CD 20 pueden ser administrados de acuerdo con métodos conocidos tales como administración intravenosa como un bolo o por infusión continua durante un periodo de tiempo, por vías intra muscular, intra peritoneal, intra cerebro espinal, sub cutánea, intra articular, intra sinovial, intratecal, oral, tópica o por inhalación. Opcionalmente, la administración puede llevarse a cabo mediante infusión con mini bomba usando diversos dispositivos disponibles comercialmente.

Las dosificaciones y programaciones efectivas para administrar el Apo2L/TRAIL, los anticuerpos receptores de muerte, y los anticuerpos CD20 puede ser determinado empíricamente, y hacer tales determinaciones está dentro del experticio de la técnica. Dosis únicas o múltiples pueden ser empleadas. Actualmente se cree que una dosis efectiva o cantidad efectiva de Apo2L/TRAIL, usada sola puede variar entre cerca de 1 µg/ kg cerca de 100ng/kg con respecto al peso del cuerpo o más por día. La escala de las inter especies de dosis puede llevarse a cabo de una manera conocida en la técnica, por ejemplo; como se describe en Mordenti et al., Pharmaceut. Res., 8:1351 (1991).

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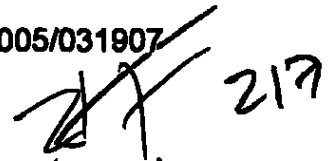
Cuando se emplea la administración in vivo de un Apo2L/TRAIL, las cantidades de dosificación normales pueden variar entre cerca de 10 ng/kg hasta cerca de 100 mg/kg del peso del cuerpo del mamífero o más por día, preferiblemente cerca de 1µg/ kg/ día a 10 mg/kg/ día, dependiendo de la vía de administración. La guía en cuanto a dosificaciones y métodos particulares de suministro se da en la literatura; ver, por ejemplo, las patentes estadounidenses No 657,760; 5,206,344; o 5,225,212. Se anticipado que diferentes formulaciones pueden ser efectivas para diferentes compuestos de tratamiento y diferentes desórdenes que el objetivo de la administración en un órgano o tejido, por ejemplo, puede requerir el suministro de una manera diferente a aquella para otro órgano o tejido. Aquellos versados en la técnica podrán entender que la dosificación de Apo2L/TRAIL que puede ser administrada dependerá variadamente en, o de, por ejemplo, el mamífero que va a recibir el Apo2L/TRAIL la vía de administración, y otras drogas o terapias que estén siendo administradas al mamífero.

El anticuerpo CD20 puede ser un anticuerpo tal como Rituximab o el 2H7, humanizado, el cual no está conjugado con un agente citotóxico. Las dosificaciones adecuadas para un anti cuerpo no conjugado son, por ejemplo, en el rango de entre cerca 20 mg/m² hasta cerca de 1000 mg/m². En una modalidad, la dosificación del anticuerpo difiere de aquella actualmente recomendada para el Rituximab. Regímenes de dosificación ilustrativos para el anticuerpo CD20 375 mg/m² semanalmente por 4 o 8; o 1000 mg/m² por 2 (por ejemplo en los días 1 y 15).

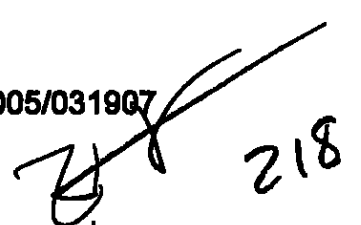


Se ha contemplado que todavía otras terapias adicionales pueden ser empleadas en los métodos. La una o más de otras terapias que pueden incluirse pero no se limitan a, la administración de terapia de radiación, citoquinas, agentes inhibitorios del crecimiento, agentes quimioterapéuticos, agentes cito tóxicos, inhibidores de tirocina quinasa, inhibidoras de ras farnesil transferasa, inhibidores de angiogénesis, e inhibidores de quinasa dependiente de ciclina que son conocidos en la técnica y definidos adicionalmente con particularidad en la sección 1 anterior.

Anticuerpos terapéuticos ilustrativos incluyen los anticuerpos anti-HER2 que incluyen el rhuMAb 4D5 (HERCEPTIN.) (Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285-4289 (1992), la patente estadounidense No. 5,725,856); anti-IL-8 (St John et al., Chest, 103:932 (1993), y la publicaciónj internacional No. WO 95/23865); anticuerpos anti-VEGF que incluyen anticuerpos anti-DEGF madurados humanizados y/o por afinidad tales comop 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 la WO 98/45331, publicada en octubre 15, 1998); anticuerpos anti-PSCA (WO01/40309); anticuerpos anti-CD40, incluyendo el S2C6 y las variantes humanizadas de el (WO00/75348); anticuerpos anti-CD11a incluyendo Raptiva™ (patente estadounidense 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 la publicación internacional No.



WO 95/19181; la patente estadounidense No. 5,714,338, otorgada en febrero 3, 1998 o la patente estadounidense No. 5,091,313, otorgada en febrero 25, 1992, WO 93/04173 publicada en marzo 4, 1993, o la solicitud internacional No. PCT/US98/13410 presentada en junio 30, 1998, la patente estadounidense No. 5,714,338); anticuerpos anti-CD18 (patente estadounidense No. 5,622,700, otorgada en abril 22, 1997, o como en la WO 97/26912, publicada en julio 31, 1997); anticuerpos de anticuerpo receptor anti-Apo-2 (WO 98/51793 publicado en noviembre 19, 1998); anticuerpos anti-TNF-alpha incluyendo cA2 (REMICADE.), CDP571 y MAK-195 (ver, patente estadounidense No. 5,672,347 otorgada en septiembre 30, 1997, Lorenz et al. J. Immunol. 156 (4):1646-1653 (1996), y Dhainaut et al. Crit. Care Med. 23 (9):1461-1469 (1995)); anticuerpos del factor anti- tejido (TF) (patente europea No. 0 420 937 B1 otorgada en noviembre 9, 1994),- anticuerpos $\alpha 4$ - $\beta 7$ de integrina antihumana (WO 98/06248 published February 19, 1998); anticuerpos anti-EGFR (anticuerpo 225 quimerizado o humanizado como en la WO 96/40210 publicada en diciembre 19, 1996); anticuerpos anti-CD3 tales como el OKT3 (patente estadounidense No. 4,515,893 otorgada en mayo 7, 1985); anticuerpos anti- CD25 o anti-Tac tales como el CHI-621 (SIMULECT.) y ZENAPAX. (ver patente estadounidense No. 5,693,762 otorgada en diciembre 2, 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-1H (Riechmann et al. Nature 332:323-337 (1988); anticuerpos receptores anti-Fc tales como el anticuerpo M22 dirigido contra el Fc.RI como en Graziano et al. J. Immunol. 155 (10):4996-5002 (1995); anticuerpos de antígeno anti-carcinoembrionario (CEA) tales como el hMN-14 (Sharkey et al.



Cancer Res. 55 (23Suppl): 5935s-5945s (1995); anticuerpos dirigidos contra las células epiteliales de pecho incluyendo 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 ligan a las células del carcinoma del colón tales como la 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 LimpoCide (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 or c7E3 Fab (REOPRO.); anticuerpos anti- RSV tales como MEDI-493 (SYNAGIS.); anticuerpos anti-CMV tales como PROTOVIR.; anticuerpos anti-HIV tales como PRO542; anticuerpos anti-hepatitis tales como el anticuerpo anti-Hep B OSTAVIR.; anticuerpos anti-CA 125 OvaRex; anticuerpo del epítipo anti-idiotípico GD3 BEC2; anticuerpo anti-.v.3 VITAXIN.; anticuerpo del carcinoma de célula renal antihumana tal como el ch-G250; ING-I; anticuerpo anti-human 17-1A (3622W94); anticuerpo anti tumor colo rectal (A33); anticuerpo del melano anti humanoR24 dirigido contra la gangliosida GD3; carcinoma anti-humano de la célula escamosa (SF-25); antígeno del leucocito (HLA) anticuerpos tales como Smart ID10 y el anticuerpo anti-HLA DR Oncolym (Lym-1).

La preparación y los programas de dosificación para agentes quimioterapéuticos pueden ser usados de acuerdo con las

instrucciones de los fabricantes o como se determine empíricamente por el practicante versado. La preparación en los programas de dosificación para tal quimioterapia también están descritos 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, anticuerpo de receptor de muerte, y/o anticuerpo CD20. o puede ser dada simultáneamente con ellos.

Algunas veces puede ser beneficioso también administrar una o más citoquinas o agentes inhibidores del crecimiento.

El Apo2L/TRAIL, los anticuerpos de receptor de muerte, y los anticuerpos de CD20 (y uno o más de otras terapias) pueden ser administradas concurrentemente o secuencialmente. Siguiendo la administración, las células tratadas in vitro pueden ser analizadas. En donde A ha sido tratamiento in vivo, un mamífero tratado puede ser monitoreado de diversas maneras bien conocidas por aquellos practicantes versados. Por ejemplo, las células de cáncer pueden ser examinadas patológicamente para ensayar en cuanto a necrosis o el suero puede ser analizado para las respuestas del sistema inmune.

Para el RA, y otras enfermedades autoinmunes el Apo2L/TRAIL, el anticuerpo de receptor de muerte, y/o el anticuerpo CD20 pueden estar combinados con cualquiera uno o más de agentes inmuno supresores, agentes quimioterapéuticos y/o citoquinas listadas en las definiciones de la sección anterior; cualquiera una o más de las drogas anti reumáticas modificantes de enfermedad (DMARD),

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tal como hidroxyciclorotina, sulfasalacina, metrotexato, lefrunomida, asatiopina, D-penicilinamina, gold (oral), Gold (intramuscular), minociclina, ciclosporina, proteína eStafilococcal A de inmuno absorción; inmunoglobulina intravenosa (IVIG); drogas antiinflamatorias no esteróides (NSAIDs); glucocorticoides (por ejemplo inyección en articulaciones); corticosteroide (por ejemplo. metilprednisolona y/o prednisona); folato; un anticuerpo del factor de necrosis anti-tumor (TNF) por ejemplo etanercept/ENBREL™, infliximab/REMICADE™, D2E7 (Knoll) o CDP-870 (Celltech); antagonista de IL-1R (por ejemplo. Kineret); antagonista de IL-10 (por ejemplo Ilodecakin); un modulador de coagulación de sangre (por ejemplo. WinRho); un antagonista IL-6 /anti-TNF (CBP 1011); antagonista CD40 (por ejemplo. IDEC 131); antagonista del receptor de Ig-Fc (MDX33); inmuno modulador (por ejemplo talidomida o ImmuDyn); anticuerpo anti-CD5 (por ejemplo. H5gl.1); inhibidor de macrófago (por ejemplo. MDX 33); bloqueador costimulatorio (por ejemplo BMS 188667 o Tolerimab); inhibidor complemento (por ejemplo. h5Gl.1, 3E10 o un anticuerpo de factor acelerante anti decaimiento DAF) antibody); o el antagonista de IL-2 (zxSMART).

Para las malignas células B, por ejemplo, el Apo2L/TRAIL, el anticuerpo receptor de muerte, y/o el anticuerpo CD20 pueden ser combinados con un agente quimioterapéutico, citoquina, por ejemplo, una linfoquina tal como la IL-2, IL-12, o un interferón, tal como el interferón alfa-2a; otros anticuerpos, por ejemplo un anticuerpo radio marcado tal como ibritumomab tiuxetan (ZEVALIN™), yodo I131 tositumomab (BEXXAR™), ¹³¹I Lym-1 (ONCOLYM™), ⁹⁰Y-LYMPHOCIDE™; anticuerpo anti-CD52, tal como

alemtuzumab (CAMPATH-IH[™]), anticuerpo anti-HLA-DR- β tal como apolizumab, anticuerpo anti-CD80 (por ejemplo IDEC-114), epratuzumab, HuID10 (SMART 1D10[™]), anticuerpo CD19, anticuerpo CD40 o anticuerpo CD22; un inmuno modulador (por ejemplo. talidomida o ImmuDyn); un inhibidor de angiogenesis (por ejemplo un anticuerpo de factor de crecimiento endotelial anti- vascular (VEGF) tal como AVASTIN[™] o talidomida); una vacuna idiотipo (EPOCH); ONCO-TCS[™]; HSPPC-96 (ONCOPHAGE[™]); terapia liposomal (por ejemplo liposoma de citrato de daunorubicina, etc.

En otra modalidad de la invención, los artículos de fabricación que contienen materiales útiles para el tratamiento del cáncer o enfermedades relacionadas con la inmunidad, descritos anteriormente, se suministra. En un aspecto, el artículo de fabricación comprende (a) un contenedor que comprende el anticuerpo CD20 (preferiblemente el contenedor comprende el anticuerpo y un vehículo farmacéuticamente aceptable o un diluyente dentro del contenedor),- (b) un contenedor que comprende Apo2L/TRAIL o el anticuerpo receptor de muerte (preferiblemente el contenedor comprende el Apo2L/TRAIL o el anticuerpo receptor de muerte y un vehículo o diluyente farmacéuticamente aceptable dentro del contenedor; y (c) un injerto de empaque con instrucciones para tratar el cáncer o enfermedad relacionada con la inmunidad en un paciente, en donde las instrucciones indican las cantidades del anticuerpo CD20 y el Apo2L/TRAIL o del anticuerpo receptor de muerte que son administradas al paciente que son efectivas para suministrar actividad sinérgica en el tratamiento de la enfermedad.

En todos estos aspectos, el inserto del empaque esta sobre o asociado con el contenedor. Los contenedores adecuados incluyen, por ejemplo, botellas frascos, jeringas, etc. Los contenedores pueden estar formados de una variedad de materiales tales como plástico o vidrio. El contenedor contiene una composición que es efectiva para tratar el cáncer o la enfermedad relacionada con la inmunidad y puede tener una puerta de acceso estéril (por ejemplo el contenedor puede ser una bolsa de solución intravenosa o un frasco que tenga un tapón perforable mediante una aguja de inyección hipodérmica). Por lo menos un agente activo en la combinación es el anticuerpo CD20 el Apo2L/TRAIL o el anticuerpo de receptor de muerte. La etiqueta o el inserto del empaque indica que la composición es usada para tratar cáncer o una enfermedad relacionada con la inmunidad en un paciente o sujeto elegible para el tratamiento con guía específica con respecto a las cantidades de dosificación e intervalos de dosificación del anticuerpo y de cualquier otro medicamento que este siendo suministrado.

El artículo de fabricación puede comprender adicionalmente un contenedor adicional que comprende un amortiguador diluyente farmacéuticamente aceptable, tal como agua bacterioestática para inyección (VWFI), una salina amortiguada con fosfato, solución de Ringer y/o solución de dextrosa. El artículo de fabricación puede incluir adicionalmente otros materiales deseables desde un punto de vista comercial y de usuario, incluyendo otros amortiguadores, diluyentes, filtros, agujas y jeringas.

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Los siguientes ejemplos se ofrecen para propósitos ilustrativos solamente, y no tienen el propósito de limitar el alcance de la presente invención de ninguna manera. Todas las patentes y referencia de literatura citada en la presente especificación se incorporan aquí como referencia en su totalidad.

Ejemplos

Los reactivos, comercialmente disponibles en referidos en los ejemplos fueron usados de acuerdo con las instrucciones del fabricante a menos que se indique otra cosa. La fuente de aquellas células identificadas en los siguientes ejemplos, y en toda la especificación, como vía de referencia al ATCC es el American Type Culture Collection, Manassas, Virginia.

Ejemplo 1

Análisis de la expresión del receptor Apo2L/TRAIL en líneas de células de linfoma B

Para examinar la expresión de 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) fueron analizadas por 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 ramo, el análisis se llevó na cabo dos veces para asegurar la reproductibilidad (ramos A y B).

Como se ilustró en la figura 4, DR4 y DR5 fueron expresados en niveles significativos (cambios de fluorescencia media de aproximadamente 0.05-1,7 unidades) en todas las 4 líneas de células, mientras que DCR1 y DCR2 fueron expresados a niveles más bajos o mínimos (cambio de fluorescencia media de aproximadamente 0 a 0,3 unidades)

Ejemplo 2

Análisis de la expresión de CD20 en líneas de células de linfoma

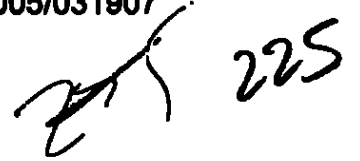
B

Para examinar la expresión de superficie celular del CD20 en las líneas de células de linfoma humano, líneas de células de linfoma B ramos, Daudi, Raji, y BJAB (ATCC) fueron analizadas por FACS usando anticuerpo monoclonal específico para el CD20 (RITUXAN, Genentech, Inc.). Para las células ramos, el análisis se llevó a cabo doble vez para asegurar la reproducibilidad (RAMOS A y B).

Como se ilustró en la figura 5, todas las 4 líneas de células expresaron altos niveles de CD20, se indicó por un cambio de fluorescencia medio de aproximadamente 5 a 15 unidades.

Ejemplo 3

Efecto del Apo2L/TRAIL, RITUXAN®, o tratamiento de combinación sobre el crecimiento de el xeno injertos de tumor de linfoma BJAB subcutáneos preestablecidos en ratones Sc y D



Los ratones Sc fueron inyectados sub cutáneamente con células B humanas no Hodgkin de las células de linfoma BJAB (ATCC) (20 millones de células por ratón) y se permitió que los tumores crecieran hasta aproximadamente 200 mm³. Los ratones fueron entonces divididos en 4 grupos de estudio (8 ratones por grupo) y fueron tratados con cinco dosis intra peritoneales (IP) por semana durante dos semanas (es decir, días 0 a 4 y 7 a 11) del vehículo (0,5 M de Arg- Succinato/20mM de Tris/0.02% Tween 20 pH =7.2), el Apo2L/TRAIL (aminoácidos 114- 281 de la Figura 1) (60 mg/kg), o con 1 dosis IP por semana durante dos semanas 2 (es decir, días 0 a 7) de RITUXAN® (4 mg/kg, Genentech, Inc.), o la combinación de estos dos últimos regímenes de Apo2L/TRAIL y RITUXAN® (Figure 6).

Los tumores en los ratones tratados con el vehículo crecieron rápidamente, mientras que el tratamiento con el agente único Apo2L/TRAIL o y RITUXAN® retardó marcadamente el crecimiento del tumor. Un ratón en el grupo Apo2L/TRAIL mostró una ablación completa del tumor, dejando una incidencia de tumor (TI) de 7/8. El tratamiento con RITUXAN® no dañó ninguno de los tumores pero mostró un efecto más prolongado. De manera muy importante, el tratamiento combinado con Apo2L/TRAIL o y RITUXAN® causó una reducción dramática en el volumen del tumor en todos los ratones, con cinco de ocho ratones que mostraron ablación completa del tumor y tres de ocho mostraron un crecimiento del tumor mínimo durante por lo menos 28 días. Estos resultados indican que el Apo2L/TRAIL y RITUXAN® pueden ejercer una actividad anti tumor sinérgica contra los seno injertos de linfoma.

Ejemplo 4

Efecto del Apo2L/TRAIL y RITUXAN® o tratamiento en combinación sobre el crecimiento de seno injertos de tumor de linfoma DJAB sub cutáneo preestablecido en ratones CSID

Un estudio similar al descrito en el ejemplo 3 se condujo. Los ratones CS y D fueron inyectados subcutáneamente con células B humanas no Hodgkin's de las células de linfoma BJAB (ATCC) (20 millones de células por ratón) y se permitió que los tumores crecieran hasta aproximadamente 200 mm³. Los ratones fueron entonces divididos en 4 grupos de estudio (8 ratones por grupo) y fueron tratados con cinco dosis intra peritoneales (IP) por semana durante dos semanas (es decir, días 0 a 4 y 7 a 11) del vehículo (0,5 M de Arg- Succinato/20mM de Tris/0.02% Tween 20 pH =7.2), el Apo2L/TRAIL (aminoácidos 114- 281 de la Figura 1) (60 mg/kg), o con 1 dosis IP por semana durante dos semanas (es decir, días 0 a 7) de RITUXAN® (4 mg/kg, Genentech, Inc.), o la combinación de estos dos últimos regímenes de Apo2L/TRAIL y RITUXAN®.

Los resultados se muestran en la figura 7. Los tumores en los ratones tratados con el vehículo, crecieron rápidamente, mientras que el tratamiento con el agente solo Apo2L/TRAIL o RITUXAN retardó marcadamente el crecimiento del tumor. Ninguno entra el Apo2L/TRAIL o el RITUXAN® solos, causaron ningunas regresiones completas, mientras que el RITUXAN® mostró un efecto más prolongado. Como en el estudio descrito en el ejemplo 3, el

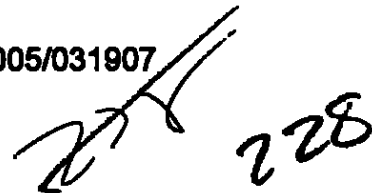
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tratamiento combinado con Apo2L/TRAIL y RITUXAN, causó una reducción muy notable en el volumen del tumor en todos los ratones, con 6 de 7 ratones que mostraron una ablación del tumor completa. Estos resultados indican que el Apo2L/TRAIL y el RITUXAN® pueden ejercer una actividad anti-tumor sinérgica contra los xenoinjertos de linfoma.

Ejemplo 5

Efecto del Apo2L/TRAIL o RITUXAN®, o tratamiento en combinación sobre el procesamiento de caspasa en xenoinjerto de tumor de linfoma BJAD subcutáneo pre-establecido en ratones SCID

Para examinar el procesamiento de caspasas que median la apoptosis en tumores tratados (indicado por procesamiento de traspasas proteolíticas) los ratones SCID fueron inyectados subcutáneamente con células B humanas no Hodgkin de células del linfoma BJAB (ATCC) (20 millones de células por ratón) y se permitió que los tumores crecieran hasta aproximadamente 200mm³. Los ratones fueron tratados entonces con el vehículo (0.05M de Arg-Succinato/20mM de Tris/0.02% de Tween 20pH = 7.2) (n=1), una dosis de IP Apo2L/TRAIL (60 mg/kg) (n=1), o una dosis de IP de o RITUXAN® (4 mg/kg, Genentech, Inc.) (n=2), o la combinación de estas últimas dosis de Apo2L/TRAIL y RITUXAN® (n=2). Dos días después del tratamiento, los tumores fueron cosechados, lisados en amortiguador de lisis, y sometidos a inmunomanchado con anticuerpos específicos contra caspasas 8, 3, 9, 7 (con anticuerpo de actina anti-beta como un control de carga), para visualizar el procesamiento de la caspasa (Fig 8).



El tratamiento con Apo2L/TRAIL (A), indujo un procesamiento incrementado de la caspasa 8, 3,9 y 7 en comparación con el vehículo de control (V), mientras que el RITUXAN® (R), no indujo procesamiento de caspasa. Notablemente, el tratamiento en combinación Apo2L/TRAIL y RITUXAN® (AR) no incremento adicionalmente el procesamiento de caspasa en comparación con el Apo2L/TRAIL solo. Estos resultados sugieren que en la actividad anti-tumor sinérgica entre el Apo2L/TRAIL y el RITUXAN®, no es mediada necesariamente mediante mejorar la apoptosis, sugiriendo que la combinación de la activación de apoptosis mediada por Apo2L/TRAIL y la lisis dependiente del complemento juntos con el ADCC mediado por RITUXAN® puede subyacer la sinergia anti-tumor observada.

Ejemplo 6

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

Los ratones SCID fueron inyectados subcutáneamente con células de linfoma BJAB no Hodgkin de células B humanas (ATCC) (20 millones de células por ratón), y se permitió que los tumores crecieran hasta aproximadamente 200mm³. Los ratones fueron entonces divididos en 4 grupos de estudio (7 ratones por grupo) y se trataron con una inyección intraperitoneal (IP) por semana, durante 2 semanas (es decir, días 0 y 7) del vehículo (0.5M de Arg-Succinato/20mM de Tris/0.02% de Tween 20 pH = 7.2), el .

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® (Fig 9). Los tumores en los ratones tratados por el vehículo crecieron rápidamente, mientras que el tratamiento con anticuerpos DR5 o RITUXAN® solos marcadamente retardaron el crecimiento del tumor. De manera muy importante, el tratamiento combinado con anticuerpo DR5 y RITUXAN®, causó una reducción dramática en el volumen del tumor en todos los ratones, con 5 de 7 ratones que mostraron que mostraron una ablación completa del tumor y 2 de 7 mostraron un crecimiento mínimo del tumor durante por lo menos 35 días. Estos resultados indican que el agonista del anticuerpo DR5 y RITUXAN® pueden ejercer una actividad anti-tumor sinérgica contra xenoinjertos de linfoma.

Ejemplo 7

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

Al examinar el procesamiento de las caspasas que median la apoptosis en tumores tratados (indicado por el procesamiento de caspasa proteolítica), los ratones SCID fueron inyectados subcutáneamente con células de linfoma BJAB no Hodgkin de células B humanas (ATCC) (20 millones de células por ratón) y se permitió que los tumores crecieran hasta aproximadamente 200mm³. Los ratones fueron entonces tratados con el vehículo (0.5M de Arg-Succinato/20mM de Tris/0.02% de Tween 20 pH = 7.2) (n=1), o una sosis IP de RITUXAN (4 mg/kg) (n=2), o una dosis IP del

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anticuerpo DR5 agonista (10mg/kg) (n=2), o la combinación de éstas dosis últimas de anticuerpo DR5 y RITUXAN® (n=2). Dos días después del tratamiento, los tumores fueron cosechados, lisados en amortiguador de lisis, y sometidos a inmunomanchado con anticuerpos específicos contra la caspasa 8, 3, 9, y 7 (con el anticuerpo anti-beta actina como un control de carga), para visualizar el procesamiento de caspasa (Fig 10).

El tratamiento con el anticuerpo agonista DR5 (A), indujo un procesamiento incrementado de la caspasa 8, 3, 9 y 7 en comparación con el vehículo de control (V), mientras que el RITUXAN® (R), no indujo procesamiento de caspasa. Notablemente, el tratamiento en combinación con anticuerpo DR5 y RITUXAN® (AR), no incremento adicionalmente el procesamiento de caspasa en comparación con el anticuerpo DR5 solo. Estos resultados sugieren que en la actividad anti-tumor sinérgica entre el anticuerpo DR5 y el RITUXAN®, no es mediada necesariamente mediante mejorar la apoptosis, sugiriendo que la combinación de la activación de apoptosis mediada por anticuerpo agonista DR5 y la lisis dependiente del complemento juntos con el ADCC mediado por RITUXAN® puede subyacer la sinergia anti-tumor observada.

Datos adicionales que ilustran la expresión DC20 y de los receptores Apo2L/TRAIL en líneas de células NHL, y los efectos de RITUXAN®, Apo2L/TRAIL y las combinaciones de ellos sobre células de cáncer se suministran en las Figuras 11 a 16.

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REIVINDICACIONES

Lo que se reivindica es:

1. Un método para tratar células cancerosas, que comprende exponer células de cáncer de mamíferos, a una cantidad sinérgicamente efectiva del polipéptido Apo2L/TRAIL y el anticuerpo CD20.
2. El método de la reivindicación 1, en donde dicho polipéptido Apo2L/TRAIL comprende los aminoácidos 1 a 281 de la Fig.1 (ID de (SEQ. No.: ID NO: 1) o un fragmento o variante de el.
3. El método de la reivindicación 1, en donde dicho polipéptido Apo2L/TRAIL comprende los aminoácidos 114 a 281 de la Fig.1 (SEQ. No.: ID NO: 1).
4. El método de la reivindicación 1, en donde dichas células cancerosas son expuestas a dicha cantidad sinérgicamente efectiva del polipéptido Apo2L/TRAIL y el anticuerpo CD20 in vivo.
5. El método de la reivindicación 1, en donde dichas células cancerosas son células de linfoma.
6. El método de la reivindicación 1, que comprende adicionalmente exponer las células cancerosas a uno o más agentes inhibidores del crecimiento.

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7. El método de la reivindicación 1, que comprende adicionalmente exponer las células a radiación.
8. El método de la reivindicación 1, en donde dicho polipéptido Apo2L/TRAIL es expresado en una célula anfitriona recombinante seleccionada del grupo que consiste de una célula CHO, una célula de levadura y *E. coli*.
9. El método de la reivindicación 1, en donde dicho polipéptido Apo2L/TRAIL está ligado a una molécula de polietilenglicol.
10. El método de la reivindicación 1, en donde dicho anticuerpo CD20 es un anticuerpo monoclonal.
11. El método de la reivindicación 10, en donde dicho anticuerpo CD20 es el anticuerpo Rituximab.
12. Un método para tratar una enfermedad relacionada con inmunidad, que comprende administrar a un mamífero una cantidad sinérgica efectiva de Apo2L/TRAIL y el anticuerpo CD20.
13. El método de la reivindicación 12, en donde dicho polipéptido Apo2L/TRAIL comprende los aminoácidos 1 a 281 de la Fig.1 (SEQ ID NO:1) o un fragmento o variante de el.
14. El método de la reivindicación 12, en donde dicho polipéptido Apo2L/TRAIL comprende los aminoácidos 114 a 281 de la Fig.1 (SEQ ID NO:1).

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15. El método de la reivindicación 12, en donde dicho polipéptido Apo2L/TRAIL es expresado en una célula anfitriona recombinante seleccionada entre el grupo que consiste de una célula CHO, una células de levadura y *E. coli*.
16. El método de la reivindicación 12, en donde dicho polipéptido Apo2L/TRAIL está ligado a una molécula de polietilenglicol.
17. El método de la reivindicación 12, en donde dicha enfermedad relacionada con inmunidad es artritis reumatoide o esclerosis múltiple.
18. El método de la reivindicación 12, en donde dicho anticuerpo CD20 es un anticuerpo monoclonal.
19. El método de la reivindicación 18, en donde dicho anticuerpo CD20 es el anticuerpo Rituximab.
20. El método de la reivindicación 1 o 12, en donde dicho polipéptido Apo2L/TRAIL y el anticuerpo CD20 son administrados secuencialmente.
21. El método de la reivindicación 1 o 12, en donde dicho polipéptido Apo2L/TRAIL y el anticuerpo CD20 son administrados concurrentemente.

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**MÉTODOS PARA UTILIZAR LIGANDOS DE RECEPTOR MUERTO Y ANTICUERPOS
CD20**

Resumen

Se suministran métodos para utilizar ligandos de receptor muerto, tales como los polipéptidos de ligando/trail APO-2 o los anticuerpos receptores muertos, y los anticuerpos CD20 para tratar condiciones tales como cáncer y enfermedades relacionadas con el sistema inmune. Las modalidades de la invención incluyen métodos para utilizar el Apo2L/trail o los anticuerpos de receptor muerto tales como los anticuerpos DR5 y los anticuerpos DR4 en combinación con los anticuerpos CD20.

1 TTTCTCTACTGACTATATAAAGAAATAGAGAAAGGAGGCTTCAGTGTACCGGGCTGCTCTGGCTGTGACTTACAGACAGTCAGACTCTGTGACAGGATC
1 ATGGCTATGATGGAGGTCACGGGGGACCCAGCCTGGGACAGACCTTCGCTGCTGTGATCTTCACAGTGTCTCTCTGCTCTCTCTCTGT
1 MetAlaMetMetGluValGlnGlyGlyProSerLeuGlyGlnThrCysValLeuIleValIlePheThrValLeuLeuGlnSerLeuCys
1181 GTGGCTGTAACTTACGTGTACTTTACCAACGAGCTGAAGCAGTACGAGGACAAAGTACTCCAAAGTGGCATTCCTTGTCTTCTTAAAGAA
31 ValAlaValThrTyValTyValTyPheThrAsnGluLeuLysGlnMetGlnAspLysTySerLysSerGlyIleAlaCysPheLeuLysGlu
271 GATGACAGTTATTGGACGCCCAATGACGAAAGAGAGATATGAACAGCCCTGCTGGCAAGTCAAGTGGCAGACTCCGTCAGCTCGTTAGAAAG
61 AspAspSerTyTrpAspProAsnAspGluGluSerMetAsnSerProCysTrpGlnValLysTrpGlnLeuArgGlnLeuValAspLys
361 ATGATTTTGTAGAACCTCTGGAGGAACCATTTCTACAGTTCAAGAAAGAACCAAAATATTTCTCCCTAGTGTGAGAGAAAGAGGTCCTCAG
91 MetIleLeuArgThrSerGluGluThrIleSerThrValGlnGlnLysGlnGlnAsnIleSerProLeuValArgGluArgGlyProGln
451 AGAGTAGCAGCTCACATAACTGGGACCCAGAGGAGAAAGCAACACATTCCTTCTCCAAACTCCAAAGATGAAAGAGGCTCTGGGCCCGCAAA
121 ArgValAlaAlaHisIleIleThrGlyThrArgGlyArgSerAsnThrLeuSerSerProAsnSerLysAsnGluLysAlaLeuGlyIleAspLys
541 ATAACTCTCGGAATCATCAAGGAGTGGGCATTCATTCTCTGAGCAACTTGCACTTGAGGAAATGGTGAACTGGTCACTCCATCGAAAGAGG
151 IleAsnSerTrpGluSerSerArgSerGlyHisSerPheLeuSerAsnLeuHisLeuArgAsnGlyGluLeuValIleHisGluLysGly
631 TTTTACTACATCTATTCTCCAAACATCTTTCGATTTTCAGGAGGAAATAAAGAAACACAAAGACGACAAACAAATGGTCCCAATATATT
181 PheTyTyIleTySerGlnThrTyPheArgPheGlnGluIleLysGluAsnThrLysAsnAspLysGlnMetValGlnTyIle
771 TACAAATACACAAAGTTATCTCTGACCTATATTGTTGATGAAAGTGCTAGAAATATGTTTGGTCTTAAAGATGCAGAAATATGGACTCTAT
211 TyLeuTyThrSerTyProAspProIleLeuLeuMetLysSerAlaArgAsnSerCysTrpSerLysAspAlaGlnGluTyGlyLeuTy
811 TCATCTATCAAGGGGGAATATTGAGCTTAAGGAAATATGACAGAAATTTTGTCTGTAAACAANTAGGACCATTTGATAGACATGGACCAT
241 SerIleTyGlnGlyIlePheGluLeuLysGluAsnAspArgIlePheValSerValThrAsnGlnHisLeuIleAspMetAspHis
901 GAGCCAGTTTTCGGGGCTTTTATAGTTGGCTAACTGACCTGGAAAGAAAGCAATTAACCTCAAAGTGAATATTTCAGTTTTCAGGAT
271 GluIleSerPhePheGlyAlaPheLeuValGlyStp
991 GATACACTATGAAGATGTTTCAAAAAAATCTGACCAAAACAAACAAACAGAA

HOJA SUSTITUTA (REGLA 26)

1 ATGGCGCCAC CACCACTAG AGTACATCTA GGTGCTTCC TGGCAGTGC
TACCGCGGTG GTGGTCGATC TCAITGATAT CCACGCAAGG ACCGTCACTG
1 MetAlaProP roProAlaAr gValHisLeu GlyAlaPheL euAlaValTh

51 TCCGATCCC GGGAGCCGAG CGAGTGGGAC AGAGGCAGCC UCGGCCACAC
AGGCTTAGGG CCGTCGCGTC GCTCACCCTG TCTCCGTCGG CGCCGGTGTG
rProAsnPro GlySerAlaA laSerGlyTh xGluAlaAla AlaAlaThrPro

101 CCAGCAAGT GTGGGCTCT TCCGCGGGA GGATTGAACC ACGAGCGGG
GGTCTTTCA CACCCGAGA AGCGGCCCT CTAACCTTG TGTCCGCCC
35 SerLysVa lTrpGlySer SerAlaGlyA rgIleGluPr oArgGlyGly

151 GCGCGAGGAG CGCTCCCTAC CTCATGGGA CAGCACGGAC CCAGTCCCCG
CGGCTCCTC GCGAGGGATG GAGGTACCT GTCTGCTCG GTTCACGGGC
GlyArgGlyA laLeuProTh xSerMetGly GlnHisGlyP roSerAlaArg

201 GCGCGGGCA GGGCGGCCC CAGGACCCAG GCGCGCGCG GAAGCCAGCC
CGGCGCCGT CCGCGCGCG GTCCTGGTC CGCGCGCGC CTTCGGTCGG
68 AlaArgAla GlyArgAlaP roGlyProAr gProAlaArg GluAlaSerP

251 CTCGCTCCG GGTCCCAAG ACCTTCAGT TTGCTGCTT CGGGGTCCTG
GAGCGAGGC CCAGGTGTTT TGAAGTTCA AACAGCAGCA GCGCCAGGAC
roArgLeuAr gValHisLys ThrPheLysP heValValVa lGlyValLeu

301 CTCAGGTCG TACCTAGCTC AGCTGCAACC ATGATCAATC AATTGGCACA
GAGCTCCAGC ATGGATCGAG TCGACCTTG TAGTTTGAAG TACTAGTTAG
101 LeuGlnValV alProSerSe rAlaAlaThr IleLysLeuH isAspGlnSe

351 AATTGGCACA CAGCAATGG AACATAGCC TTTGGAGAG TTGTGTCCAC
TTACCGTGT GTGCTTACC TTGTATCGG AAACCTCTC AACACAGGTG
rIleGlyThr GlnGlnTrpG luHisSerPr oLeuGlyGlu LeuCysProPro

401 CAGGATCTCA TAGATCAGAA GTTCTGGAG CTTGTAACCG GTGCACAGAG
GTCTAGAGT ATCTAGCTT GCGGACCTC GAGCATTTGC CACGTGTCTC
135 GlySerHi sArgSerGlu ArgProGlyA laCysAsnAr gCysThrGlu

451 GGTGTGGTT ACACCAATG TTCCAACAAT TTGTTGCTT GCTTCCCATG
CCACACCCAA TGTGTTTACG AAGTTGTTA AACAAACGAA CGGAGGGTAC
GlyValGlyT yxThrAsnAl aSerAsnAsn LeuPheAlaC ysLeuProCys

501 TACAGCTTGT AAATCAGATG AAGAAGAGAG AAGTCCCTGC ACCACGACCA
ATGTGGAACA TTAGTCTAC TTCTTCTCTC TTGAGGAGCG TGTGCTGTT
168 ThrAlaCys LysSerAspG luGluGluAr gSerProCys ThrThrThra

551 GGAACACAGC ATGTCACTG AAACCAAGAA CTTTCCGGA TGACAACTCT
CCTTGTGTCG TACAGTCAGC TTGCTCCTT GAAAGGCTT ACTGTTAGA
rgAsnThrAl aCysGlnCys LysProGlyT hrPheArgAs nAspAsnSer

601 GCTGAGATGT GCCGGAAGTG CAGCACAGGG TGCCCCAGAG GGATGCTCAA
CGACTCTACA CGGCTTACG GTGTTGTCCTC ACGGGTCTC CTTACCAATT
201 AlaGluMetC ysArgLysCy sSerThrGly CysProArgG lyMetVally

651 GGTCAAGGAT TGTACGCCCT GAGTGACAT CGAGTGTGTC CACAAAGAT
CCAGTTCTTA ACATGCGGGA CCTCACTGTA GCTCACACAG GTGTTCTTA
sValLysAsp CysThrProT rpSerAsp11 eGluCysVal HisLysGluSer

FIG. 2A
HOJA SUSTITUTA (REGLA 26)

701 CAGGCAATGG ACATAATATA TGGGTGATTT TGGTTGTGAC TTGGTTGTTT
 GTCCGTTACC TGTATTATAT ACCCACTAAA ACCAACACTG AAACCAACAA
 235 GlyAsnGly yHisAsnIle TrpValIleL euValValTh rLeuValVal
 751 CCGTTGCTGT TGGTGGCTGT GCTGATGTGTC TGTGTGTGCA TCGGCTCAGG
 GGCACGACA ACCACCGACA CCACTAACAG ACAACACAGT AGCCGAGTCC
 ProLeuLeuL euValAlaVa lLeuIleVal CysCysCysI leGlySerGly
 801 TTGTGGAGGG GACCCCAAGT GCATGGACAG GGTGTGTTTC TGGCGCTTGG
 AACACCTCCC CTGGGTTTCA GGTACCTGTC CCACACAAAG ACCCGGAACC
 268 CysGlyGly AspProLysC ysMetAspAr gValCysPhe TrpArgLeuG
 851 GTCTCCTACG AGGCGCTTGG GCTGAGGACA ATGCTCACAA CGAGATTCTG
 CAGAGGATGC TCCCGGACCC GCACTGCTGT TACGAGTGTG GCTCTAAGAC
 lylLeuLeuAr gGlyProGly AlaGluAspA snAlaHisAs nGluIleLeu
 901 AGCAACGAG ACTGCTGTGC CACTTTCGTC TCTGAGCAGC AAATGGAAAG
 TCCTTCCGTC TGAGCGACAG GTGAAAGCAG AGACTGCTCG TTACCTTTTC
 301 SerAsnAlaA spSerLeuSe rThrPheVal SerGluGlnG lnMetGluSe
 951 CCAAGAGCCG GCAGATTGGA CAGGTGTGAC TGTACAGTCC CCAGGGGAGG
 GGTCTCTGCG COTCTAACT GTCCACATGT ACATGTCAGG GGTCCCTTCC
 rGlnGluPro AlaAspLeuT hrGlyValTh rValGlnSer ProGlyGluAla
 1001 CACAOTGTCT GCTGGGACCG GCAGAAGCTG AAGGGTCTCA GAGGAGGAGG
 GTGTACAGA CGACCTTGGC COTCTTCGAC TTCCCAAGAT CTCTCTCTCC
 335 GluCysLe uLeuGlyPro AlaGluAlaG luGlySerG l nArgArgArg
 1051 CTGCTGCTTC CAGCAATGG TGGTGACCCC ACTGAGACTC TGATGCTGTT
 GACGACCAAG GTGCTTTACC ACGACTGGGG TGACTCTGAG ACTACGACAA
 LeuLeuValP roAlaAsnG l yAlaAspPro ThrGluThrL euMetLeuPhe
 1101 CTTTGACAAG TTTCGAAACA TCGTGCCCTT TGACTCCTGG GACCACTCA
 GAACTGTTC AAACGTTTGT AGCAGGGGAA ACTGAGGACC CTGGTCGAGT
 368 PheAspLys PheAlaAsnI leValProPh eAspSerTrp AspGlnLeuH
 1151 TGAGGCAGCT GGACCTCACG AAAAATGAGA TCGATGTGCT CAGAGCTGTT
 ACTCCGTGCA CCTGGAGTGC TTTTACTCT AGCTACACCA GTCTCGACCA
 etArgGlnLe uAspLeuThr LysAsnGluI leAspValVa lArgAlaGly
 1201 ACAGCAAGCC CAGGGGATGC CTTGTATGCA ATGCTGATGA AATGGGTCAA
 TGTGTTCCCG GTCCCTTACG GACATACGT TACGACTACT TTACCCAGTT
 401 ThrAlaGlyP roglyAspAl aLeuTyrAla MetLeuMetL ysTrpValAs
 1251 CAAACTGGA CGGAACGCTT CATTCCACAC CTTGCTGGAT GCCTTGGAGA
 GTTTTGACCT GCCTTGGAGA GCTAGGTGTG GGACGACCTA CGGAACCTCT
 nLysThrGly ArgAsnAlaS erIleHisTh rLeuLeuAsp AlaLeuGluArg
 1301 GGATGGAGA GAGACATGCA AAAGAGAAGA TTCAGGACCT CTTGGTGGAC
 CCTACCTTCT CTCTGTACGT TTCTCTTCT AAGTCTTGA GAACCACTG
 435 MetGluG l uArgHisAla LysGluLysI leGlnAspLe uLeuValAsp
 1351 TCTGGAAAGT TCATCTACTT AGAAGATGGC ACAGGCTCTG CCGTGTCTTT
 AGACCTTTCA AGTAGATGAA TCTTCTACCG TGTCCGAGAC GGCACAGGAA
 SerGlyLysP heIleTyrLe uGluAspGly ThrGlySerA leValSerLeu
 1401 GGAGTGA
 CCTCACT
 468 GluOP*

FIG. 2B
HOJA SUSTITUTA (REGLA 26)

1 NEQRGCHAPAAAGAKCHGGPGPREARGAKPSLRVFKTLVLVVAVLLLVSAESALITQGD
 61 LAPQQRALPQQKRSSPSEGLCPFGHETSDGGRDCISCKYQDDYSTENQDLFCIRCTACD
 121 SGEVELSPCTTFRNTVCQCEGTFREDSFEMCKKCTGCPFMHVKVGDCFPWSDLECVH
 181 KHSGITIGVTVAKVYLIVAVFVCKHLLAKKVLFFYLKQICSGGGGDPFVRVSSQRPGAKD
 241 NVLNIVELLQPTQVFGQMEVOEPAPPTGVNMLSPGHSNHLLEPAHAERSQRKRLVFA
 301 NEGDPTEFLNQCFDDFADLVFFDSNEPTLKKLGLNDSNLIKVAKARAASHROTLYTMLIKN
 361 VNLTQPDASVHTLLDALFTLGRLANQKIDHLLSGGKPHYLSGNADGALS

FIG. 3A

239

Met Glu Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg Lys
 1 3 10 15
 Arg His Gly Pro Gly Pro Arg Gln 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 Gln Ile Val Ser Ile Leu Gln Pro Thr Gln Val Pro Glu Gln Glu
 275 280 285

FIG. 3B
HOJA SUSTITUTA (REGLA 26)

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246

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

HOJA SUSTITUTA (REGLA 26)

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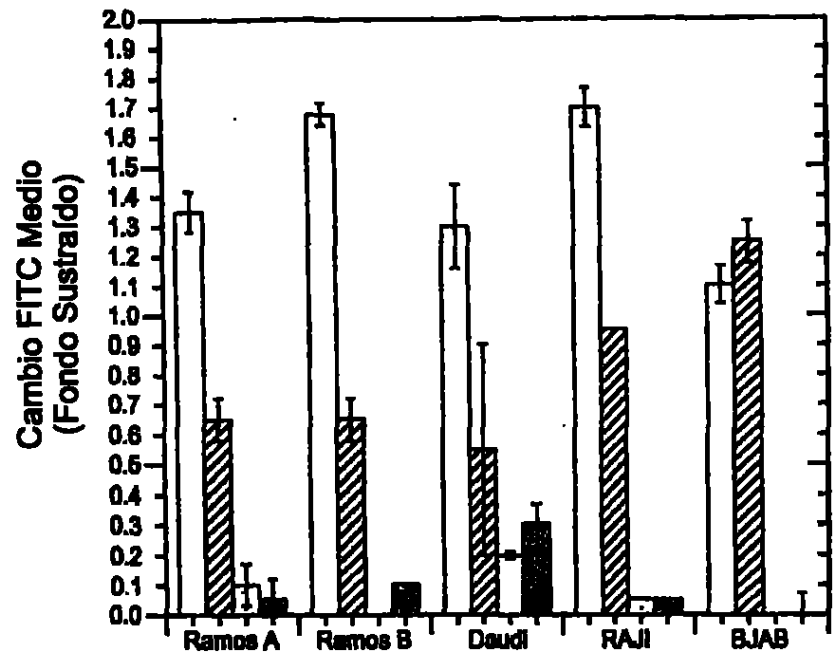


FIG. 4

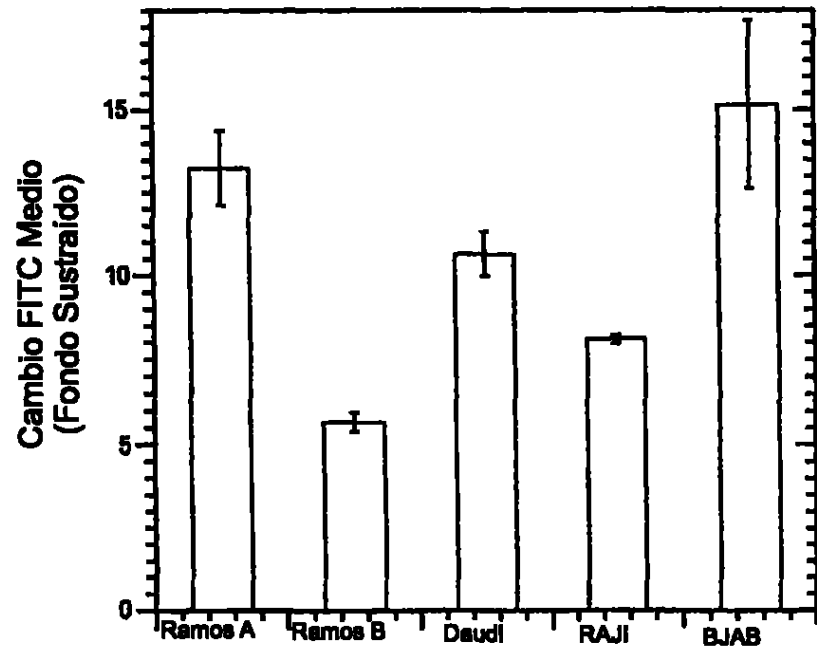


FIG 5

HOJA SUSTITUTA (REGLA 26)

242

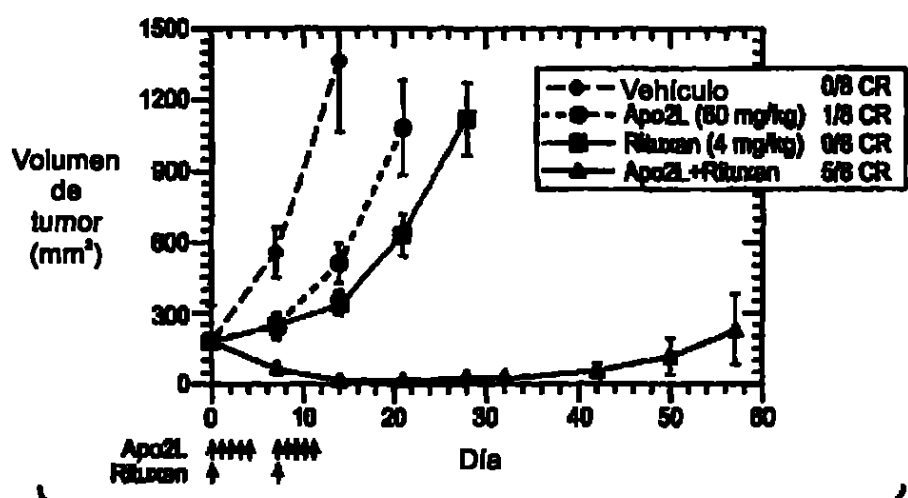


FIG. 6

Apo2L.0 (60mg/kg/ratón; 5x/wk; IP) solo y en combinación con Rituxan (4 mg/kg/ratón; 1x/wk; IP) vs. Xenoinjertos de (SCIDraton) BJAB

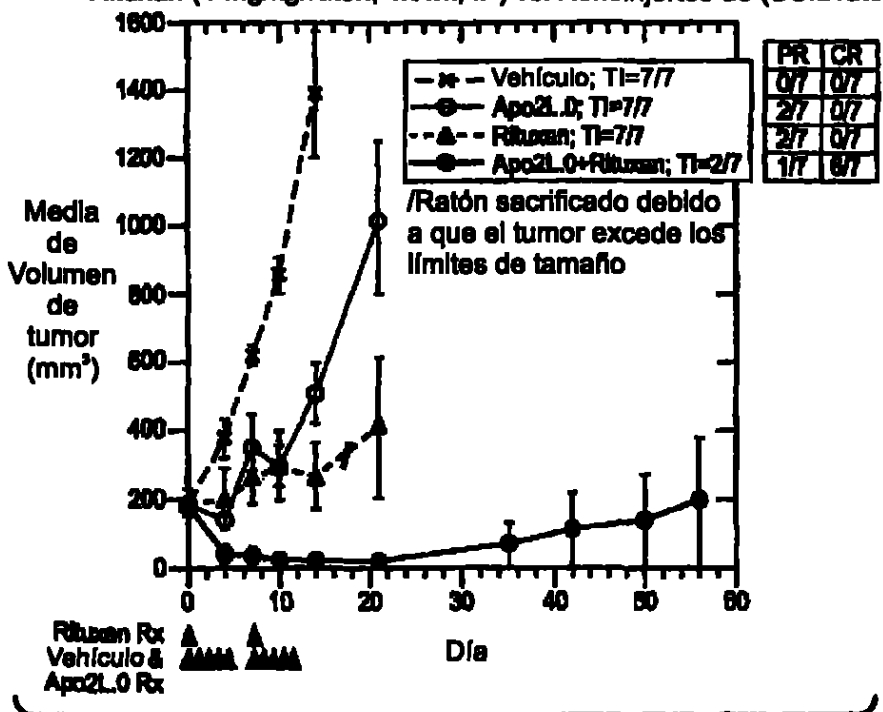


FIG. 7

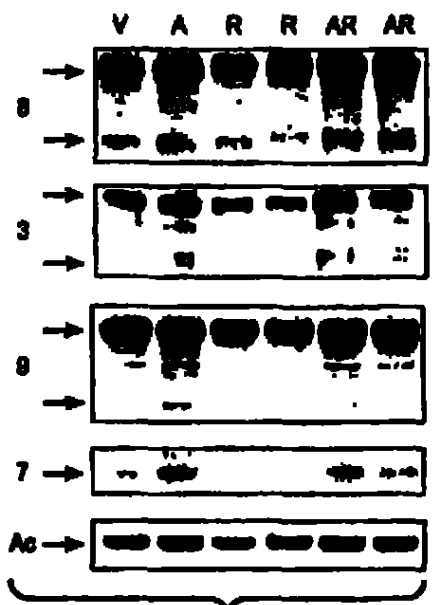


FIG. 8

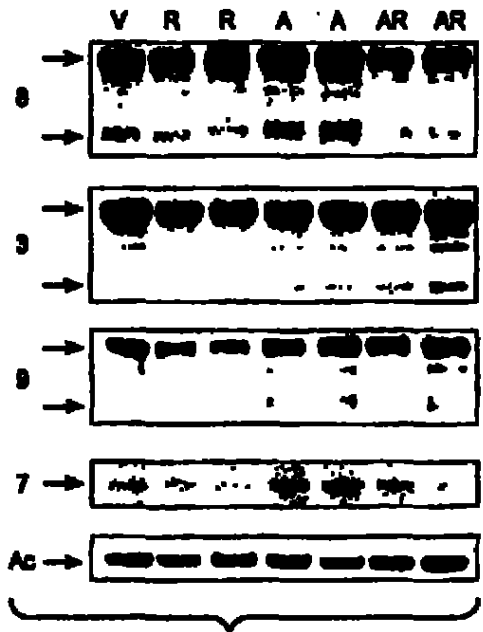


FIG. 10

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Apomab (10mg/kg/ratón; 1x/wk; IP) solo y en combinación con Rituxan (4 mg/kg/ratón; 1x/wk; IP) vs. Xenoinjertos de (SCID ratón) BJAB

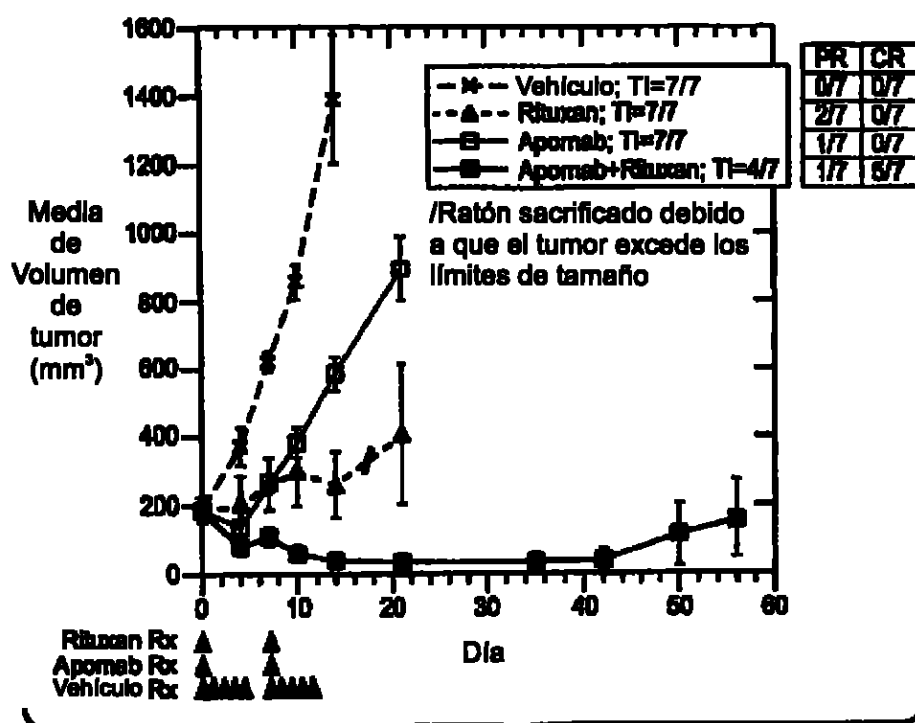


FIG. 9

295

WFO 2006/029224

11/15

PCT/US2005/031907

2011.2

Expresión de Receptores CD20 y Apo2L/TRAIL en líneas celulares NHL

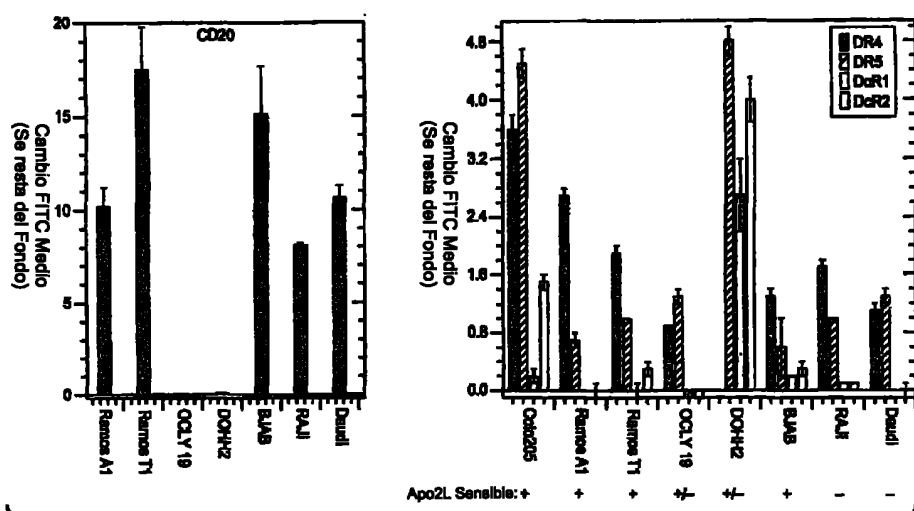


FIG. 11

HOJA SUSTITUTA (REGLA 26)

12/15

246

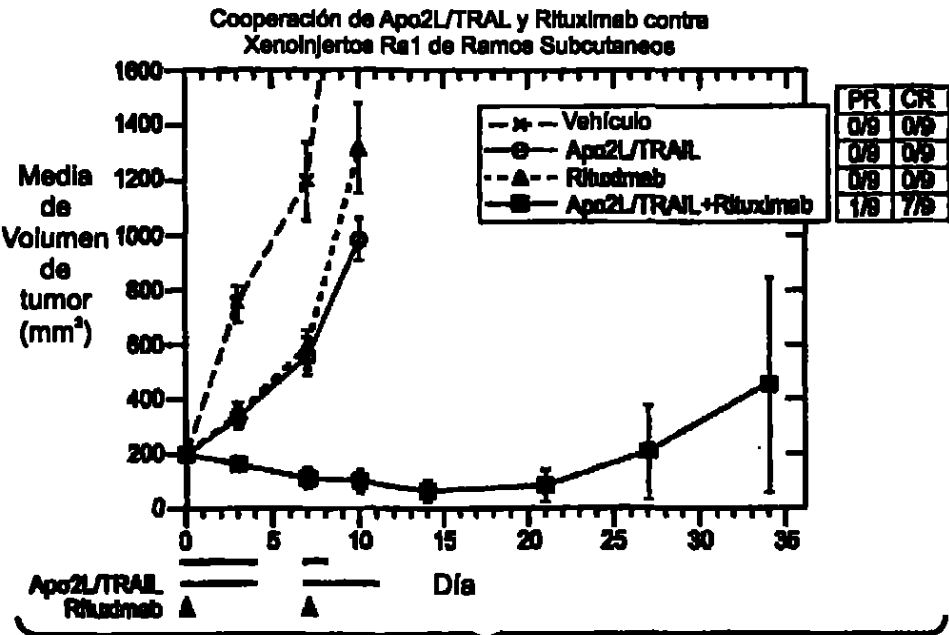


FIG. 12

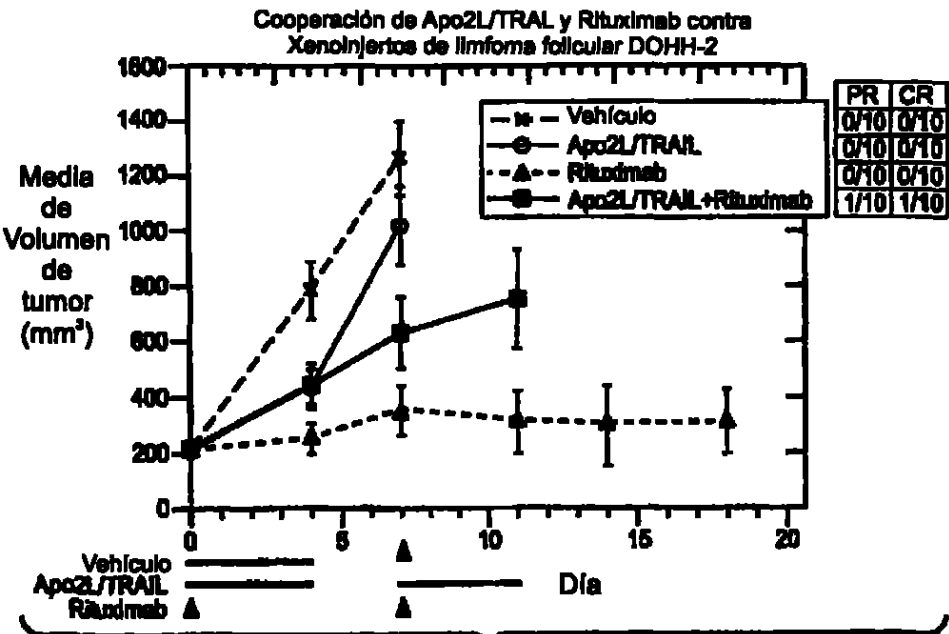
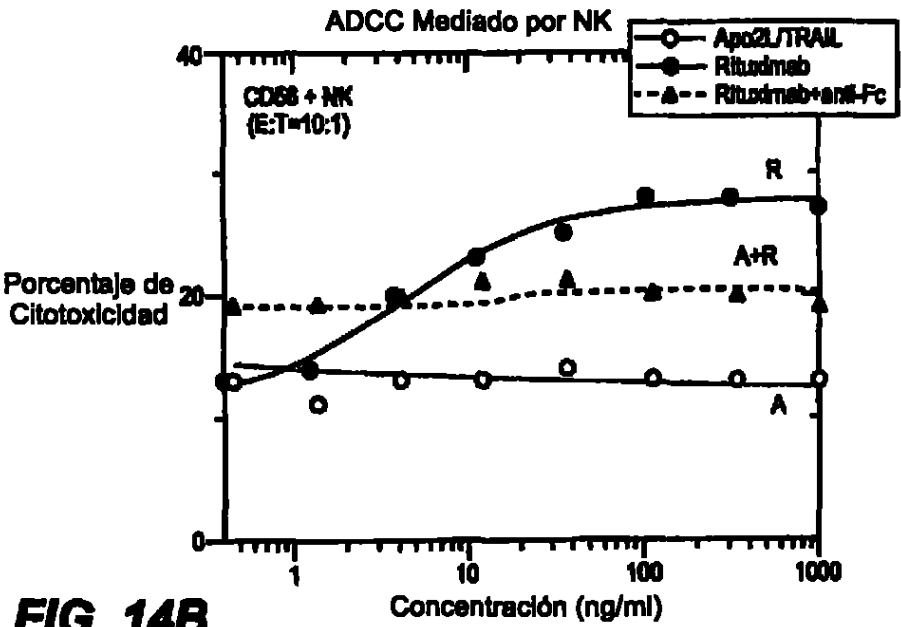
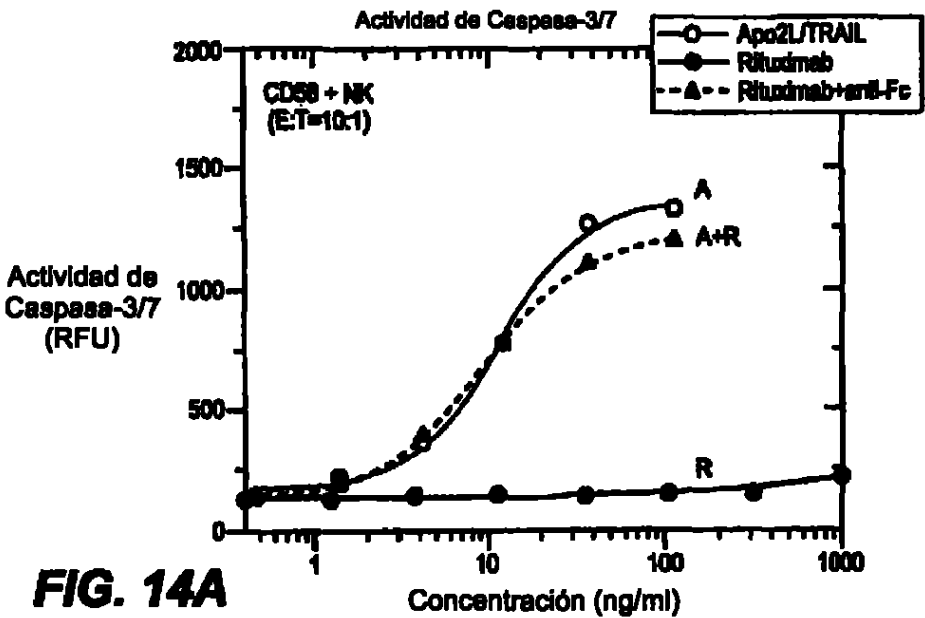


FIG. 13

Rituximab y Apo2L/TRAIL muerte a células BJAB por mecanismos independientes



248

Rituximab y Apo2L/TRAIL muerte a células BJAB por mecanismos independientes

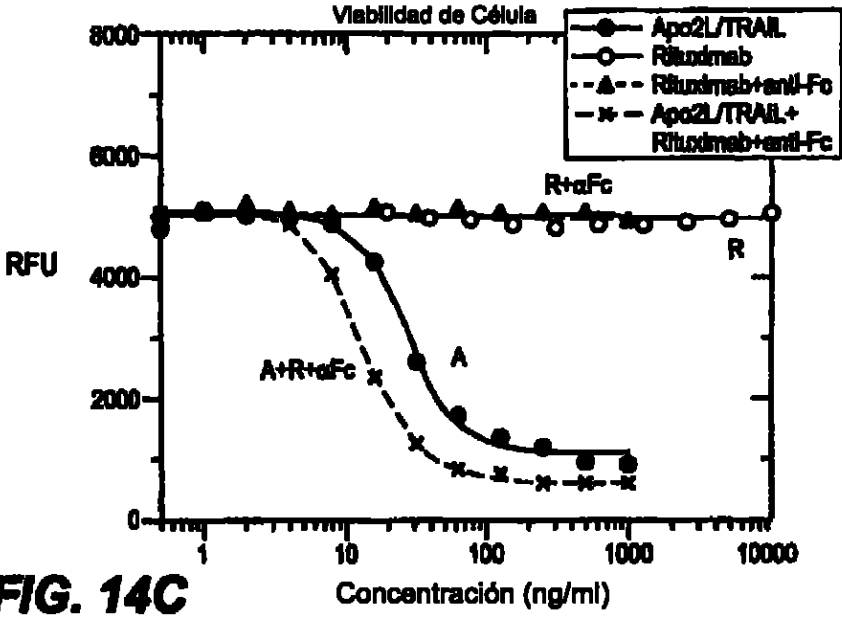


FIG. 14C

Cooperación de Apo2L/TRAIL y Rituximab contra Tumores (resistentes a Rituximab) Ramos T1

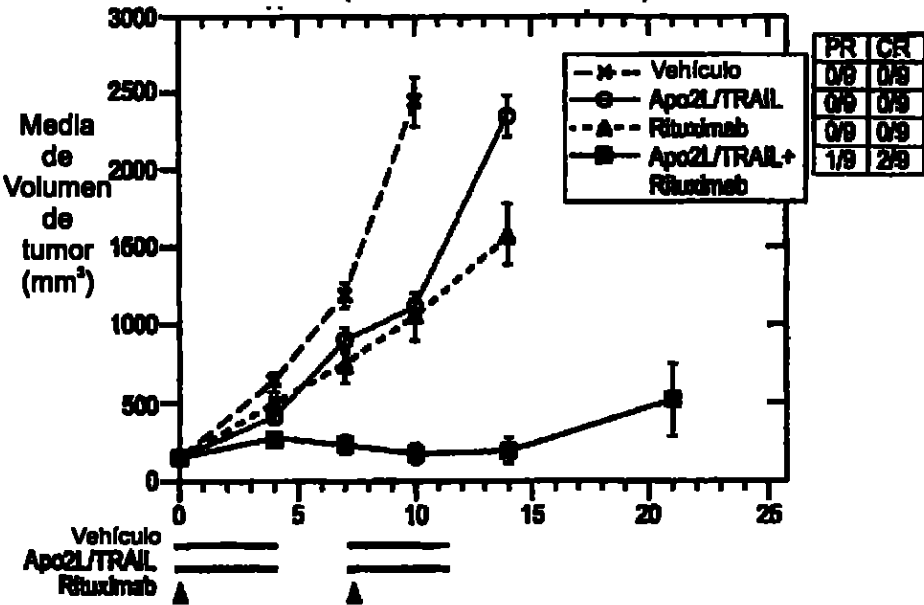
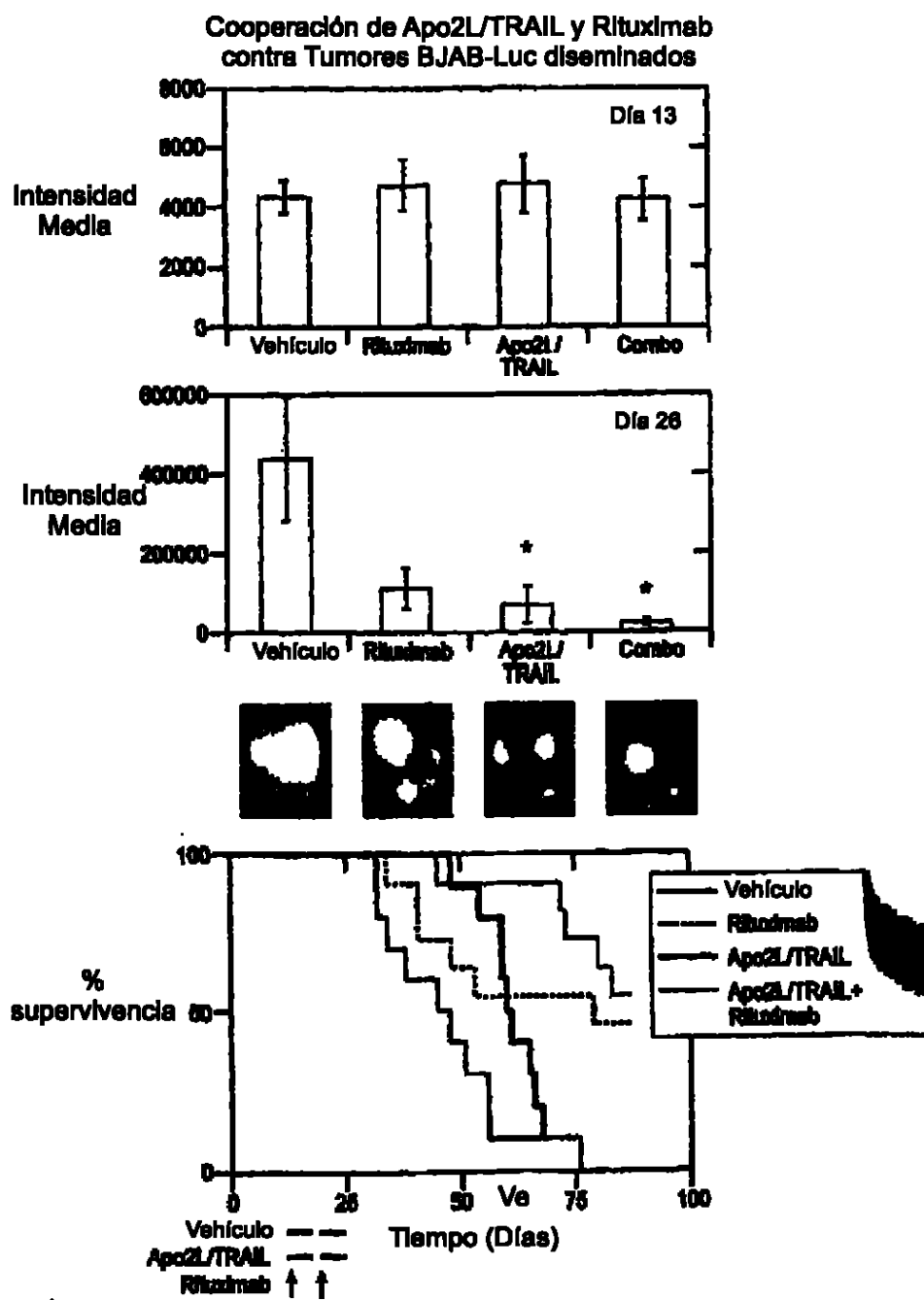


FIG. 15



P2031R1A PCT Sequence Listing
Sequence Listing

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<130> P2031R1A PCT

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<151> 2004-09-08

<150> US 60/666,553

<151> 2005-03-30

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<400> 1

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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
     215     220     225
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
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Ile Phe Val Ser Val Thr Asn Glu His Leu Ile Asp Met Asp His
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251

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acagaatttt tgtttctgta acaaatgagc acttgataga catggaccat 900
gaagccagtt ttttcggggc ctttttagtt ggctaactga cctggaaaga 950
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<212> PRT
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35 40 45
Glu Pro Arg Gly Gly Gly Arg Gly Ala Leu Pro Thr Ser Met Gly 60
50 55 60
Gln His Gly Pro Ser Ala Arg Ala Arg Gly Arg Ala Pro Gly 75
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95 100 105

252
252

P2031R1A PCT Sequence Listing

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				140					145					150
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
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Asp	Ile	Glu	Cys	Val	His	Lys	Glu	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
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Ala	Val	Leu	Ile	Val	Cys	Cys	Cys	Ile	Gly	Ser	Gly	Cys	Gly	Gly
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Ser	Asn	Ala	Asp	Ser	Leu	Ser	Thr	Phe	Val	Ser	Glu	Gln	Gln	Met
				305					310					315
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
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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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ggccgaggag cgctccctac ctccatggga cagcacggac ccagtgcctg 200
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ctcggctccg ggtccacaag accttcaagt ttgtcgtcgt cggggctctg 300
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aattggcaca cagcaatggg aacatagccc tttgggagag ttgtgtccac 400
caggatctca tagatcagaa cgtcctggag cctgtaaccg gtgcacagag 450
ggtgtggggt acaccaatgc ttccaacaat ttgtttgctt gcctcccatg 500
tacagcttgt aaatcagatg aagaagagag aagtccttgc accacgacca 550
ggaacacagc atgtcagtg c aaaccaggaa ctttccggaa tgacaattct 600
gctgagatgt gccggaagtg cagcacaggg tgccccagag ggatggtcaa 650
ggtcaaggat tgtacgccct ggagtgacat cgagtgtgtc cacaagaat 700
caggcaatgg acataatata tgggtgattt tgggtgtgac tttggttgtt 750
ccgttgctgt tgggtggctgt gctgattgtc tgttgttgca tcggctcagg 800
ttgtggaggg gaccccaagt gcatggacag ggtgtgtttc tggcgcttgg 850
gtctcctacg agggcctggg gctgaggaca atgctcaca cgagattctg 900
agcaacgcag actcgtgtc cactttcgtc tctgagcagc aaatggaaag 950
ccaggagccg gcagatttga cagggtgtcac tgtacagtc ccaggggagg 1000
cacagtgtct gctgggaccg gcagaagctg aagggtctca gaggaggagg 1050
ctgctggttc cagcaaattg tgctgacccc actgagactc tgatgctgtt 1100
ctttgacaag tttgcaaaca tcgtgccctt tgactcctgg gaccagctca 1150
tgaggcagct ggacctcacg aaaaatgaga tcgatgtggt cagagctggg 1200
acagcaggcc caggggatgc cttgtatgca atgctgatga aatgggtcaa 1250
caaaactgga cggaacgcct cgatccacac cctgctggat gccttgga 1300
ggatggaaga gagacatgca aaagagaaga ttcaggacct cttggtggac 1350
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ggagtga 1407

<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 15
1 5 10
Lys Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala Arg Pro 30
20 25 30
Gly Leu Arg Val Pro Lys Thr Leu Val Leu Val Val Ala Ala Val 45
35 40 45
Leu Leu Leu Val Ser Ala Glu Ser Ala Leu Ile Thr Gln Gln Asp 60
50 55 60
Leu Ala Pro Gln Gln Arg Ala Ala Pro Gln Gln Lys Arg Ser Ser 75
65 70 75
Pro Ser Glu Gly Leu Cys Pro Pro Gly His His Ile Ser Glu Asp 90
80 85 90

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254

P2031R1A PCT Sequence Listing

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	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

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255

P2031R1A PCT Sequence Listing

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
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	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		

P2031R1A PCT Sequence Listing

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256

<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
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
1 5 10 15

P2031R1A PCT Sequence Listing

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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
<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
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
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

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P2031R1A PCT Sequence Listing

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
				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

<400> 11
 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

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P2031R1A PCT Sequence Listing

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 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

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P2031R1A PCT Sequence Listing

Gly Lys

Lista de Secuencia PCT p2031R1A
 Listado de Secuencia

261

<110> Genentech, Inc.
 <120> MÉTODOS PARA UTILIZAR LIGANDOS DE RECEPTOR MUERTO Y ANTICUERPOS CD20
 <130> P2031R1A PCT
 <140> PCT/US2005/031907
 <141> 2005-09-07
 <150> US 60/607,909
 <151> 2004-09-08
 <150> US 60/666,553
 <151> 2005-03-30
 <160> 11
 <210> 1
 <211> 281
 <212> PRT
 <213> Homo sapiens
 <400> 1
 Met Ala Met Met Glu Val Gln Gly Gly Pro Ser Leu Gly Gln Thr
 1 5 10 15
 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
 215 220 225
 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

Lista de Secuencia PCT p2031R1A

262

<210> 2
<211> 1042
<212> DNA
<213> Homo sapiens

<220>
<221> N
<222> 447
<223>

<400> 2
tttctctact gactataaaa gaatagagaa ggaagggctt cagtgcaccg 50
ctgcctggct gacttacagc agtcagactc tgacaggatc atggctatga 100
tggaggtcca ggggggaccc agcctgggac agacctgcgt gctgattcgt 150
atcttcacag tgctcctgca gtctctctgt gtggctgtaa cttacgtgta 200
ctttaccanc gagctgaagc agatgcagga caagtactcc aaaagtggca 250
ttgcttgttt cttaaaagaa gatgacagtt attgggaccc caatgacgaa 300
gagagtatga acagccctcg ctggcaagtc aagtggcaac tccgtcagct 350
cgttagaag atgattttga gaaacctctg ggaaccatt tctacagttc 400
aagaaaagca acaaaatatt tctcccctag tgagagaaag aggtccncag 450
agagtagcag ctacataac tgggaccaga ggaagaagca acacattgtc 500
ttctccaac tccaagaatg aaaaggctct gggccgcaa ataaactcct 550
gggaatcctc aaggagtggg cattcattcc tgagcaactt gcacttgagg 600
aatggtgaac tggcatcca tgaanaagg tttactaca tctattccca 650
aacatacttt cgatttcagg aggaataaa agaaacaca aagaacgaca 700
aacaantggt ccaatatatt tacaatata caagttatcc tgaccctata 750
ttgttgatga aaagtgctag aantagttgt tggcttaag atgcagaata 800
tggactctat tccatctatc aagggggaat atttgagctt aaggaaatg 850
acagaatttt tgtttctgta acaaatgagc acttgataga catggaccat 900
gaagccagtt ttttcggggc ctttttagtt ggctaactga cctggaaaga 950
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gaagatgttt caaaaantct gaccnaaca acaaacaga aa 1042

<210> 3
<211> 468
<212> PRT
<213> Homo sapiens

<400> 3
Met Ala Pro Pro Pro Ala Arg Val His Leu Gly Ala Phe Leu Ala
1 5 10 15
Val Thr Pro Asn Pro Gly Ser Ala Ala Ser Gly Thr Glu Ala Ala
20 25 30
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
Thr Phe Lys Phe Val Val Val Gly Val Leu Leu Gln Val Val Pro
95 100 105

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Lista de Secuencia PCT p2031R1A

Ser Ser Ala Ala Thr Ile Lys Leu His Asp Gln Ser Ile Gly Thr
110 115 120
Gln Gln Trp Glu His Ser Pro Leu Gly Glu Leu Cys Pro Pro Gly
125 130 135
Ser His Arg Ser Glu Arg Pro Gly Ala Cys Asn Arg Cys Thr Glu
140 145 150
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 Glu 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
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Asp Pro Lys Cys Met Asp Arg Val Cys Phe Trp Arg Leu Gly Leu
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Leu Arg Gly Pro Gly Ala Glu Asp Asn Ala His Asn Glu Ile Leu
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Ser Asn Ala Asp Ser Leu Ser Thr Phe Val Ser Glu Gln Gln Met
305 310 315
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
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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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Ser Leu Glu

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264

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ctcggctccg ggtccacaag accttcaagt ttgtcgtcgt cggggctcctg 300
ctgcaggtcg tacctagctc agctgcaacc atgatcaatc aattggcaca 350
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caggatctca tagatcagaa cgtcctggag cctgtaaccg gtgcacagag 450
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acagcaggcc caggggatgc cttgtatgca atgctgatga aatgggtcaa 1250
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ggaatggaag gagacatgca aaagagaaga ttcaggacct cttgggtggc 1350
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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

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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 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
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395 400 405
Ala Asp Ser Ala Leu Ser
410

<210> 6
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<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

Handwritten signature

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Leu	Leu	Leu	Val	Ser	Ala	Glu	Ser	Ala	Leu	Ile	Thr	Gln	Gln	Asp
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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
				395					400					405
Leu	Glu	Thr	Leu	Gly	Glu	Arg	Leu	Ala	Lys	Gln	Lys	Ile	Glu	Asp
				410					415					420
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				440										

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26X

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35 40 45
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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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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
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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
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155 160 165
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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
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Val	Lys	Asp	Tyr	Phe	Pro	Glu	Pro	Val	Thr	Val	Ser	Trp	Asn	Ser
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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
				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
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 Ser Tyr Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
 35 40 45

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Glu Trp Val Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr
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 Asn Gln Lys Phe Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser
 65 70 75
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 80 85 90
 Thr Ala Val Tyr Tyr Cys Ala Arg Val Val Tyr Tyr Ser Asn Ser
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 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 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

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Gly Lys

JUL 05 2006

PCT
PCT DEPT.

From the INTERNATIONAL SEARCHING AUTHORITY

To:
GENENTECH, INC.
Attn: Marschang, Diane L.
1 DNA Way
South San Francisco, CA 94080-4990
UNITED STATES OF AMERICA

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT AND
THE WRITTEN OPINION OF THE INTERNATIONAL
SEARCHING AUTHORITY, OR THE DECLARATION

Calendared/CLK

Resp. to ISR/Corr'd Cl II - 22 Aug 06

Ch II Deadline/Resp. to WO - 22 Sep 06

(PCT Rule 44.1)

Date of mailing (day/month/year)	22/06/2006
Applicant's or agent's file reference P2031RIA	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/US2005/031907	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 18:

The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 48):

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) 338.92.70

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. 5518 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 those 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 letter to be published for the purposes 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 296).

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 48.1).

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 48.2).

Where a demand for international preliminary examination has been/is 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.

From the INTERNATIONAL BUREAU

PCT**NOTIFICATION CONCERNING
AVAILABILITY OF THE PUBLICATION
OF THE INTERNATIONAL APPLICATION**

To:

MARSCHANG, Diane, L.
Genentech, Inc.
1 DNA Way
South San Francisco, California 94080-4990
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year)
08 September 2006 (08.09.2006)

Applicant's or agent's file reference
P2031R1A

IMPORTANT NOTICE

International application No.
PCT/US2005/031907

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

RECEIVED

The applicant is hereby notified that the International Bureau:

SEP 21 2006

GENENTECH, INC.
LEGAL DEPT.

☐ has published the above-indicated international application on under
No. WO

☒ has republished the above-indicated international application on 08 September 2006 (08.09.2006) under
No. WO 2006/029324

For an explanation as to the reason for this republication of the international application, reference is made to INID codes (15), (48)
or (88) (as the case may be) on the front page of the published international application.

A copy of the international application is available for viewing and downloading on WIPO's website at the following address:
<http://www.wipo.int/pctdb> (under "Query" enter the PCT or WO number).

The applicant may also request a paper copy of the published international application from the International Bureau in writing from
pcleproducts@wipo.int or the contact details provided below.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. +41 22 338 91 70

Authorized officer

Beate Giffo-Schmitt

e-mail: pt03@wipo.int

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference P2031R1A	FOR FURTHER ACTION see Form PCT/ISA/220 as well as, where applicable, item 5 below.	
International application No. PCT/US2005/031907	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 6 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/US2005/031907

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Box No. I 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. C07K14/705

C07K16/28

A61P35/02

A61K39/395

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	Classification of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 03/039486 A (IDEC PHARMACEUTICALS CORPORATION; HARIHARAN, KANDASAMY; HANNA, NABIL) 15 May 2003 (2003-05-15) paragraph [0006]	1-21
A	WO 03/038043 A (UAB RESEARCH FOUNDATION; ZHOU, TONG; ICHIKAWA, KIMIHISA; KIMBERLY, ROB) 8 May 2003 (2003-05-08) page 54, line 8 - line 14 claims 30,66,102	1-21
A	LEBLANC H N ET AL: "Apo2L/TRAIL and its death and decoy receptors." CELL DEATH AND DIFFERENTIATION. JAN 2003, vol. 10, no. 1, January 2003 (2003-01), pages 66-75, XP002382355 ISSN: 1350-9047 abstract	1-21



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
- "Z" document member of the same patent family

Date of the actual completion of the international search

23 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. 5818 Patenlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3018

Authorized officer

Rankin, R

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LUDWIG DALE L ET AL: "Monoclonal antibody therapeutics and apoptosis." ONCOGENE. 8 DEC 2003, vol. 22, no. 56, 8 December 2003 (2003-12-08), pages 9097-9106, XP008050059 ISSN: 0950-9232 abstract	1-21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2005/031907

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Box I 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 1-21 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 compound/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 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:

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.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 03039486	A	15-05-2003	NONE
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

28.0

From the
INTERNATIONAL SEARCHING AUTHORITY

PCT

To:

see form PCT/ISA/220

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/220FOR FURTHER ACTION
See paragraph 2 belowInternational application No.
PCT/US2005/031907International filing date (day/month/year)
07.09.2005Priority date (day/month/year)
08.09.2004International Patent Classification (IPC) or both national classification and IPC
INV. C07K14/705 C07K16/28 A81P35/02 A81K39/395Applicant
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-80296 Munich
Tel. +49 89 2399-0 Tx: 523656 sprmu d
Fax: +49 89 2399-4465

Date of completion of
this opinionsee form
PCT/ISA/210

Authorized Officer

Rankin, R

Telephone No. +49 89 2399-2659



**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/US2005/031907

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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/US2005/031907

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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. 1-21 (for reasons of industrial applicability)

because:

- ☒ the said international application, or the said claims Nos. 1-21 (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/US2005/031907

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**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	1-21
	No: Claims	
Inventive step (IS)	Yes: Claims	1-21
	No: Claims	
Industrial applicability (IA)	Yes: Claims	
	No: Claims	

2. Citations and explanations

see separate sheet

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claims 1-21 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 1-21 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

The subject matter of claims 1-21 is novel. No prior art document discloses a combination therapy consisting of administering APO2L/TRAIL and a CD20 antibody to a patient.

Article 33(3) PCT

The closest prior art is D1 which 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 difference between the subject matter of claim 1 and D1 is that in claim 1 the combination is that of CD20 antibody and the APO2L/TRAIL protein.

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING
AUTHORITY (SEPARATE SHEET)**

286
International application No.

PCT/US2005/031907

The problem to be solved may therefore be considered as being to provide a therapy against cancer which may lead to tumour ablation.

The subject matter of claim 1 solves this problem and is considered inventive since the skilled person would not have been prompted by the prior art to arrive at the combination of claim 1. Nor would the skilled person have been prompted to attempt the solution of claim 1 as there would have been no indication that success was likely.

Though it is known that Apo2/TRAIL is the antigen corresponding in activity to the anti-DR5 antibodies of the prior art and that this isolated antigen may have the same effect as the antibody of D1, the skilled person would not have expected that the anti-tumour effect would be as prominent as shown in the examples and that the said technical problem could be solved by the claimed combination. The subject matter of claim 1 is therefore inventive as is that of independent claim 12, for the same reasoning (Article 33(3) PCT).



722031-1

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Facsimile Transmission

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From: **Name:** USPTO ASSIGNMENT DIVISION
 Fax Number:
 Voice Phone: 703-308-9723

To: **Name:** GENETECH, INC.
 Company: DIANE L. MARSCHANG
 Fax Number: 16509529881
 Voice Phone:

Fax Notes:

Pg# **Description**

1 Cover Page

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MARCH 21, 2005

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GENETECH, INC.
DIANE L. MARSCHANG
1 NDA 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: 015798/0778

NUMBER OF PAGES: 3

BRIEF: ASSIGNMENT OF ASSIGNOR'S INTEREST (SEE DOCUMENT FOR DETAILS).

ASSIGNOR:

ASHKENAZI, AVI J.

DOC DATE: 02/17/2005

ASSIGNEE:

GENENTECH, INC.

1 NDA WAY

SOUTH SAN FRANCISCO, CALIFORNIA

94080-4990

SERIAL NUMBER: 60607909

FILING DATE: 09/08/2004

PATENT NUMBER:

ISSUE DATE:

TITLE: METHODS OF USING DEATH RECEPTOR LIGANDS AND CD20 ANTIBODIES

fy

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015798/0778 PAGE 2

STEVEN POST, EXAMINER
ASSIGNMENT DIVISION
OFFICE OF PUBLIC RECORDS

U2/28/2005
700157518U.S. DEPARTMENT OF COMMERCE
Patent and Trademark Office
Asst Dir. No. PR2031-1RECORDATION FORM COVER SHEET
PATENTS ONLY

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)

60/607,909

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. Marshchang
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:

07-0690

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. Marshchang
Name of Person Signing
Registration No. 35,600Diane L. Marshchang 2/28/2005
Signature Date

Total number of pages comprising cover sheet, attachments and document: 3

20050306 09:40:30 00007506

Genentech, Inc.

Genentech, Inc.

Genentech, Inc.

291
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.

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:

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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.: 60/607,909
Our Docket No.: PR2031-1

Number of Pages including this cover sheet - 3

CONFIDENTIAL NOTE

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PATENTS ONLY

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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 WayCity: South San Francisco State: CaliforniaZIP: 94080-4990Additional 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)

60/607,909

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: 17. 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. 35,600Diane L. Marschang
Signature2/28/2005
DateTotal number of pages comprising cover sheet, attachments and document: 3

Revised (10/7/04)

A S S I G N M E N T

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. 60/807,909, if received (Docket No. PR2031-1) 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 94080-4990, 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 expenses 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

Dated: 2/17/05

Avi J. Ashkenazi
Avi J. Ashkenazi

Facsimile Transmission

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From: Name: USPTO ASSIGNMENT DIVISION
Fax Number:
Voice Phone: 703-308-9723

To: Name: DIANE L. MARSCHANG
Company: GENENTECH, INC.
Fax Number: 15509529881
Voice Phone:

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Pg# Description

1 Cover Page

2 382.TXT

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OCTOBER 20, 2005

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DIANE L. MARSCHANG
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1 DNA WAY
SOUTH SAN FRANCISCO, CA 94080UNITED STATES PATENT AND TRADEMARK OFFICE
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REEL/FRAME: 016663/0176
NUMBER OF PAGES: 2BRIEF: ASSIGNMENT OF ASSIGNOR'S INTEREST (SEE DOCUMENT FOR DETAILS).
DOCKET NUMBER: PR2031-1A

ASSIGNOR:

ASHKENAZI, AVI J.

DOC DATE: 08/15/2005

ASSIGNEE:

GENENTECH, INC.
1 DNA WAY
SOUTH SAN FRANCISCO, CALIFORNIA

94080-4990

SERIAL NUMBER: 60666553

FILING DATE: 03/30/2005

PATENT NUMBER:

ISSUE DATE:

TITLE: METHODS OF USING DEATH RECEPTOR LIGANDS AND CD20 ANTIBODIES

~~296~~ 296

016663/0176 PAGE 2

KIMBERLY WHITE, EXAMINER
ASSIGNMENT DIVISION
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Comprehension for Patent: 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

Mortgage

Security Agreement

Change of Name

Other:

Execution Date: 8/15/05

4. Application number(s) or patent number(s):

With 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)

60/666,553

Additional numbers attached? Yes ☒ No

5. Name and address of party to whom correspondence concerning document should be mailed:

Diane L. Marchand
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

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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. Marchand
Name of Person Signing
Registration No. 35,600Diane L. Marchand
SignatureAugust 24, 2005
Date

Total number of pages comprising cover sheet, attachments and document: 3

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16
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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.

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Please deliver the following page(s) to:

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P.O. Box 1450
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Fax No.: 1-703-306-5995

FROM: Diane L. Marschang
Registration No.: 35,600

RE: U.S. Serial No.: 60/666,553
Our Docket No.: PR2031-1A

Number of Pages Including this cover sheet - 3

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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: <u>GENENTECH, INC.</u> Internal Address: Street Address: <u>1 DNA Way</u> City: <u>South San Francisco</u> State: <u>California</u> ZIP: <u>94080-4990</u> Additional name(s) & address(es) attached? ☐ Yes ☒ No	
3. Nature of conveyance: ☒ Assignment Security Agreement Change of Name Other: Execution Date: <u>8/15/05</u>	Merger	
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) <u>60/666,553</u> 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. Marschang GENENTECH, INC. 1 DNA Way South San Francisco, CA 94080	6. Total number of applications and patents involved: <u>1</u> 7. Total fee (37 CFR 3.41): <u>\$ 40.00</u> ☐ Enclosed ☒ Authorized to be charged to deposit account 8. Deposit account number: <u>07-0630</u>	
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 <u>Diane L. Marschang</u> Signature August 24, 2005 Date Total number of pages comprising cover sheet, attachments and document: <u>3</u> Revised (10/1/03)		

ASSIGNMENT

WHEREAS, Avi J. Ashkenazi, a citizen of US, residing at 1456 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. 60/556,553, if received (Docket No. PR2031-1A) 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 expenses 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

Dated: 8/15/05

Avi J. Ashkenazi
Avi J. Ashkenazi

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 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	P2031R1A
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)	AGARNAL, 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	60/607,909
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	60/666,553
VI-2-3	Country	US

PCT REQUEST

Original (for SUBMISSION)

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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	65	-
IX-3	Claims	2	-
IX-4	Abstract	1	✓
IX-5	Drawings	19	-
IX-7	TOTAL	91	
	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	<i>Diane L. Marschang</i>	
X-1-1	Name (LAST, First)	MARSCHANG, Diane L.	
X-1-2	Name of signatory		
X-1-3	Capacity		

PCT REQUEST

Original (for SUBMISSION)

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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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METHODS OF USING DEATH RECEPTOR LIGANDS AND CD20 ANTIBODIES

RELATED APPLICATIONS

5 This application claims priority under Section 119(e) to provisional application number 60/607,909 filed September 8, 2004 and to provisional application number 60/666,553 filed March 30, 2005.

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, such as cancer and immune related diseases.

15

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 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 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, 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 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; 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.

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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.,

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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 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)).

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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 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 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)

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(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 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 Apo2L/TRAIL polypeptide and CD20 antibody.
2. The method of claim 1 wherein said Apo2L/TRAIL polypeptide comprises amino acids 1-281 of Fig. 1 (SEQ ID NO:1) or a fragment or variant thereof.
3. The method of claim 1 wherein said Apo2L/TRAIL polypeptide comprises amino acids 114-281 of Fig. 1 (SEQ ID NO:1).
4. The method of claim 1 wherein said cancer cells are exposed to said synergistic effective amount of Apo2L/TRAIL polypeptide and CD20 antibody *in vivo*.
5. The method of claim 1 wherein said cancer cells are lymphoma cells.
6. The method of claim 1 further comprising exposing the cancer cells to one or more growth inhibitory agents.
7. The method of claim 1 further comprising exposing the cells to radiation.

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8. The method of claim 1 wherein said Apo2L/TRAIL polypeptide is expressed in a recombinant host cell selected from the group consisting of a CHO cell, yeast cell and *E. coli*.

9. The method of claim 1 wherein said Apo2L/TRAIL polypeptide is linked to a polyethylene glycol molecule.

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

11. The method of claim 10 wherein said CD20 antibody is the antibody Rituximab.

12. A method of treating an immune related disease, comprising administering to a mammal a synergistic effective amount of Apo2L/TRAIL polypeptide and CD20 antibody.

13. The method of claim 12 wherein said Apo2L/TRAIL polypeptide comprises amino acids 1-281 of Fig. 1 (SEQ ID NO:1) or a fragment or variant thereof.

14. The method of claim 12 wherein said Apo2L/TRAIL polypeptide comprises amino acids 114-281 of Fig. 1 (SEQ ID NO:1).

15. The method of claim 12 wherein said Apo2L/TRAIL polypeptide is expressed in a recombinant host cell selected from the group consisting of a CHO cell, yeast cell and *E. coli*.

16. The method of claim 12 wherein said Apo2L/TRAIL polypeptide is linked to a polyethylene glycol molecule.

17. The method of claim 12 wherein said immune related disease is rheumatoid arthritis or multiple sclerosis.

18. The method of claim 12 wherein said CD20 antibody is a monoclonal antibody.

19. The method of claim 18 wherein said CD20 antibody is the antibody Rituximab.

20. The method of claim 1 or 12 wherein said Apo2L/TRAIL polypeptide and CD20 antibody are administered sequentially.

21. The method of claim 1 or 12 wherein said Apo2L/TRAIL polypeptide

and CD20 antibody are administered concurrently.

BRIEF DESCRIPTION OF THE DRAWINGS

5 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".

10 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).

15 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.

20 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.

25 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.

30 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.

35 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.

40 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.

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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.

5 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

10 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
15 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
20 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 accordance with manufacturer defined protocols and/or parameters unless
25 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
30 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
35 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
40 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
45 verification.

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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 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

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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 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.,

15	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
20	Gly	G	Glycine	Lys	K	Lysine
	Ala	A	Alanine	Arg	R	Arginine
	Cys	C	Cysteine	Trp	W	Tryptophan
	Val	V	Valine	Gln	Q	Glutamine
	Met	M	Methionine	Asn	N	Asparagine

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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.

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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

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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 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.

"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, 277:818-821 (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_c 50 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_c 50 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 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.

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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.

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

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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 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

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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 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).

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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.

5 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

10 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:

DIQMTQSPSSLSASVGDRVTITCRASSSVSYMHYQQKPGKAPKPLIYAPSNLASGVPSRFSGSGSGTDFTLTIS
15 SLQPEDFATYYCQQWSFNPPTFGQGTKVEIKR (SEQ ID NO:7);

and the variable heavy chain sequence:

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGAIYPGNGDTSYNQKFKGRFTISVDKS
KNTLYLQMNSLRAEDTAVYYCARVYYNSYWFVDVWGQGLTVTVSS (SEQ ID NO:8).

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

DIQMTQSPSSLSASVGDRVTITCRASSSVSYMHYQQKPGKAPKPLIYAPSNLASGVPSRFSGSGSGTDFTLTIS
20 SLQPEDFATYYCQQWSFNPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYFREAKVQWKVD
NALQSGNSQESVTEQDSKDYSLSSITLTLKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID
NO:9);

25 and the heavy chain amino acid sequence:

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGAIYPGNGDTSYNQKFKGRFTISVDKS
KNTLYLQMNSLRAEDTAVYYCARVYYNSYWFVDVWGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVFSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
DKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
30 YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVK
GFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSP
GK (SEQ ID NO:10)

or the heavy chain amino acid sequence:

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGAIYPGNGDTSYNQKFKGRFTISVDKS
35 KNTLYLQMNSLRAEDTAVYYCARVYYNSYWFVDVWGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVFSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNATYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIAATISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVK
GFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSP
40 GK (SEQ ID NO:11).

"Antibody-dependent cell-mediated cytotoxicity" and "ADCC" refer to a cell-mediated reaction in which nonspecific cytotoxic cells that express Fc receptors (FcRs) (e.g. Natural Killer (NK) cells, neutrophils, and

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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 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 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 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.

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.

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"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.

"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

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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, 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.

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"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., 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.

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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.

5 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".

10 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
15 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
20 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.

25 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
30 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
35 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.,
40 prednisone, methylprednisolone, dexamethasone, and hydrocortisone; methotrexate (oral or subcutaneous); hydroxychloroquine; 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. ^{211}At , ^{131}I , ^{125}I , ^{90}Y , ^{186}Re , ^{188}Re , ^{153}Sm , ^{212}Bi , ^{32}P 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.

"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.

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, 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 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 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

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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 Stella 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, Bi²¹², 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, 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 antibiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabycin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 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, 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, 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 aminoglutethimide, mitotane, trilostane; folic acid 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; 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-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

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paclitaxel (American Pharmaceutical Partners, Schaumburg, Illinois), and TAXOTERE® doxorubicin (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; 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.

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, MEGASE® megestrol acetate, AROMASIN® exemestane, formestane, fadrozole, RIVISOR® vorozole, 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 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® rrmh; and pharmaceutically acceptable 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 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 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. 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 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 (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 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

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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 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.

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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.

Optional signal sequences, origins of replication, marker genes, enhancer

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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)].

5 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
10 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
15 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
20 described in Gething et al., Nature, 293:620-625 (1981); Mantei 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,
25 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
30 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-
35 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
40 cultured in nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed.

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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.

5 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
10 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
15 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.
20 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
25 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
30 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
35 (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),
40 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 equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled

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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 $ZnSO_4$ 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

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preferably, the divalent metal ion used in such methods is zinc sulfate. 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.

5 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.

10 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
15 particularly ^{32}P . However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. 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
20 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.
25 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
30 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
35 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.

40 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 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 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.

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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 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 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 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 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.

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 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*

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

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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 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. 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 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, *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 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 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. 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 Plückthun, *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

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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*, 362:255-258 (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

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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 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

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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).

5 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
10 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
15 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
20 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-
25 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
30 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
35 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
40 half of the bispecific molecule provides for a facile way of separation. 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
45 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.

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 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 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 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 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_2^I , α_3^I , N-acetyl- γ_1^I , PSAG and θ^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, 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 (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 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 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

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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 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 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 if 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,

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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 μ 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 μ g/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). 363

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 $\alpha_4\beta_1$ 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-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 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. *Cancer Res.* 55(23 Suppl): 5916s-5920s (1995)); antibodies that bind to colon carcinoma cells such as C242 (Littón 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 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 such as the anti-Hep B antibody OSTAVIR.; anti-CA 125 antibody OvaRex; anti-idiotypic GD3 epitope antibody B8C2; 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 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 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 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

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(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[™]), ¹²⁵I 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); idiotypic 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

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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 (mAb 3H3.17.5; 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

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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 4

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

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 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.

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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
5 the study described in Example 3, combined treatment with Apo2L/TRAIL and 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.

10 Example 5

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

15 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
20 Tris/0.02% Tween 20 pH =7.2) (n=1), 1 IP dose of Apo2L/TRAIL (60 mg/kg) (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,
25 and 7 (with anti-beta actin antibody as a loading control) to visualize 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
30 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-
35 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

40 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

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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, 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 Apo2L/TRAIL polypeptide and CD20 antibody.

2. The method of claim 1 wherein said Apo2L/TRAIL polypeptide comprises amino acids 1-281 of Fig. 1 (SEQ ID NO:1) or a fragment or variant thereof.

3. The method of claim 1 wherein said Apo2L/TRAIL polypeptide comprises amino acids 114-281 of Fig. 1 (SEQ ID NO:1).

4. The method of claim 1 wherein said cancer cells are exposed to said synergistic effective amount of Apo2L/TRAIL polypeptide and CD20 antibody in vivo.

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

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

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

8. The method of claim 1 wherein said Apo2L/TRAIL polypeptide is expressed in a recombinant host cell selected from the group consisting of a CHO cell, yeast cell and *E. coli*.

9. The method of claim 1 wherein said Apo2L/TRAIL polypeptide is linked to a polyethylene glycol molecule.

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

11. The method of claim 10 wherein said CD20 antibody is the antibody Rituximab.

12. A method of treating an immune related disease, comprising administering to a mammal a synergistic effective amount of Apo2L/TRAIL polypeptide and CD20 antibody.

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13. The method of claim 12 wherein said Apo2L/TRAIL polypeptide comprises amino acids 1-281 of Fig. 1 (SEQ ID NO:1) or a fragment or variant thereof.

5 14. The method of claim 12 wherein said Apo2L/TRAIL polypeptide comprises amino acids 114-281 of Fig. 1 (SEQ ID NO:1).

10 15. The method of claim 12 wherein said Apo2L/TRAIL polypeptide is expressed in a recombinant host cell selected from the group consisting of a CHO cell, yeast cell and E. coli.

16. The method of claim 12 wherein said Apo2L/TRAIL polypeptide is linked to a polyethylene glycol molecule.

15 17. The method of claim 12 wherein said immune related disease is rheumatoid arthritis or multiple sclerosis.

18. The method of claim 12 wherein said CD20 antibody is a monoclonal antibody.

20 19. The method of claim 18 wherein said CD20 antibody is the antibody Rituximab.

25 20. The method of claim 1 or 12 wherein said Apo2L/TRAIL polypeptide and CD20 antibody are administered sequentially.

30 21. The method of claim 1 or 12 wherein said Apo2L/TRAIL polypeptide 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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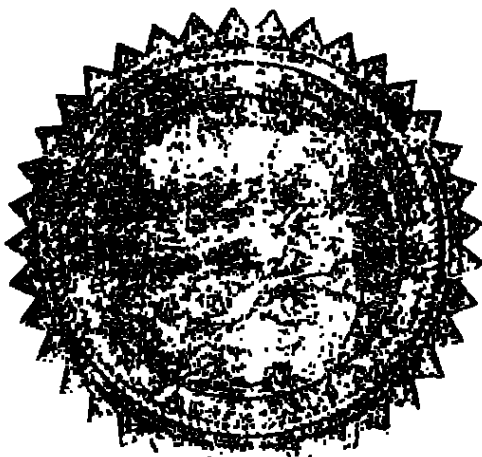
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(Convention de La Haye du 5 octobre 1961)

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State of California

CERTIFIED

5. At Sacramento, California
6. the 15th day of December 2005
7. by Deputy Secretary of State, State of California
8. No. 267083
9. Seal/Stamp:



10. Signature



[Signature]

Secretary of State

[Signature]

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CALIFORNIA ALL-PURPOSE ACKNOWLEDGMENT

State of California

County of

San Mateo

} ss.

On December 6, 2005 before me,

Aida Micala Guian

Name and Title of Officer (e.g., "Jurat Notary Public")

personally appeared

Timothy R. Schwartz

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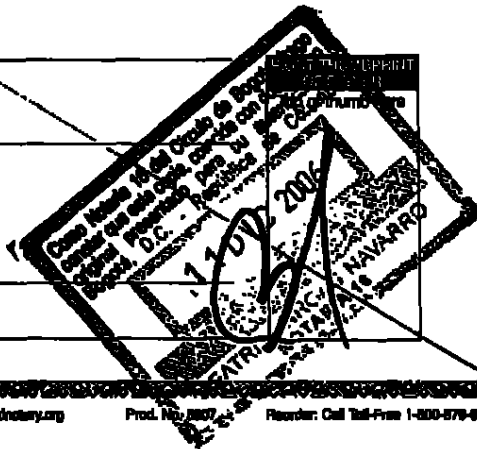
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USA

Hereby appoints JUAN PABLO CADENA SARMIENTO and/or BEATRIZ JARAMILLO and/or CARL S 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) interest(s) 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.

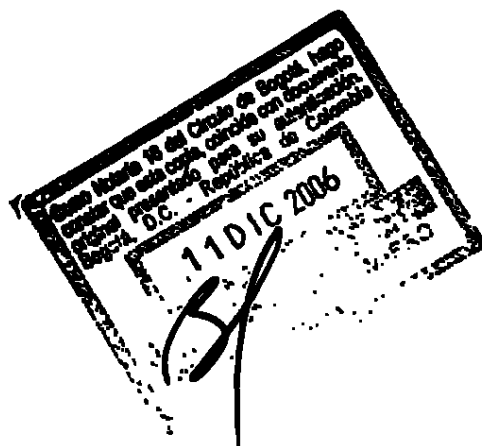
August 27, 2002

Dated

Sean Johnston

Sean A. Johnston

Vice President, Intellectual Property
Genentech, Inc.



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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

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 # 14558612
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

Título o tipo de documento: _____

Fecha del Documento: _____ Número de páginas: _____

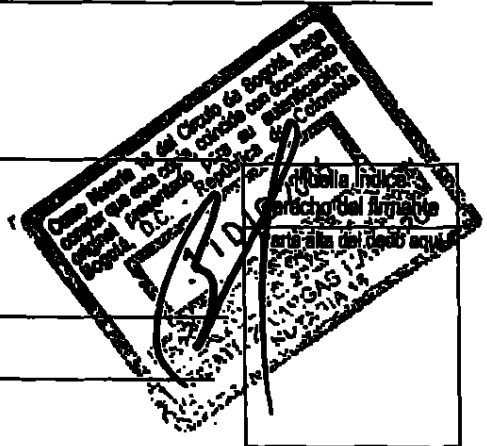
Firmante(s) diferente(s) a (los) nombrado(s) arriba: _____

Capacidad(es) Relvindicada(s) por el (los) firmante(s)

Nombre del firmante: _____

- ☐ Individuo
☐ Funcionario Corporativo – Título(s): _____
☐ Socio - ☐ Limitado ☐ General
☐ Abogado de hecho
☐ Fideicomisario
☐ Guardián 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

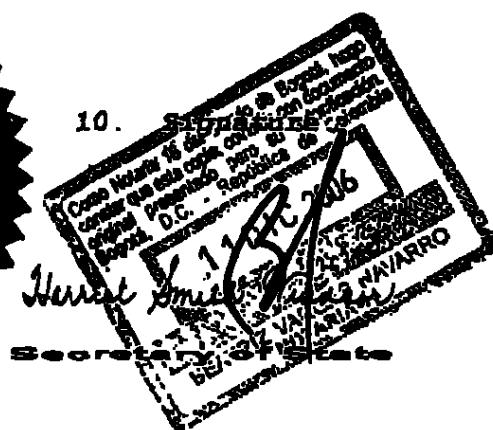
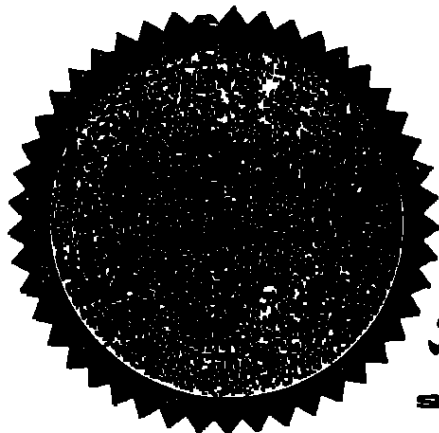
5. at Dover, Delaware

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:



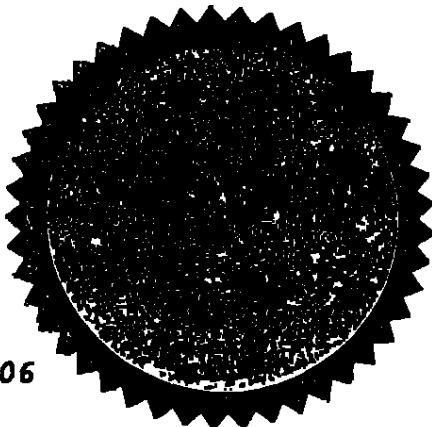
Delaware

PAGE 1

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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, Secretary of State
AUTHENTICATION: 3900384

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

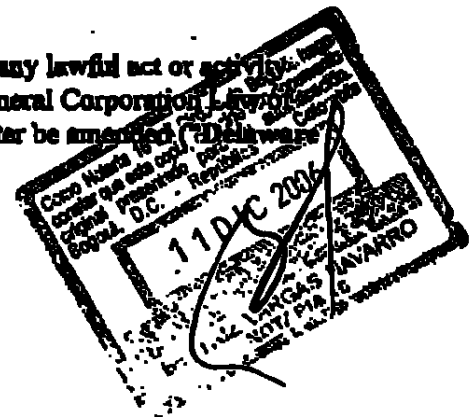
The name of the Corporation is: Genentech, Inc.

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").



STATE OF DELAWARE
SECRETARY OF STATE
DIVISION OF CORPORATIONS
FILED 09:00 AM 07/22/1999
991302331 - 2112549

ARTICLE 4

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.

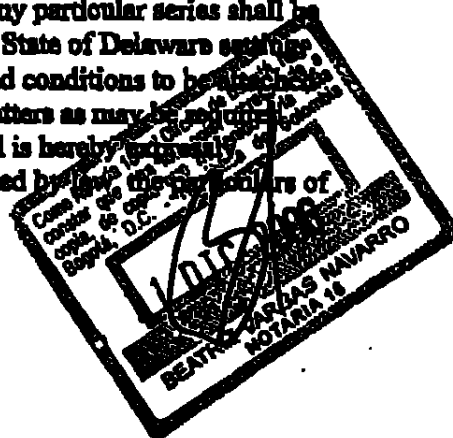
(b) The number of authorized shares of any class or classes of stock may be increased or decreased (but not below the number of shares thereof then outstanding) by the affirmative vote of a majority in voting power of the outstanding stock of the Corporation entitled to vote thereon irrespective of the 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 by law and the Board of Directors shall fix and determine, and is hereby authorized and empowered to fix and determine, in the manner provided by law the rights 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;

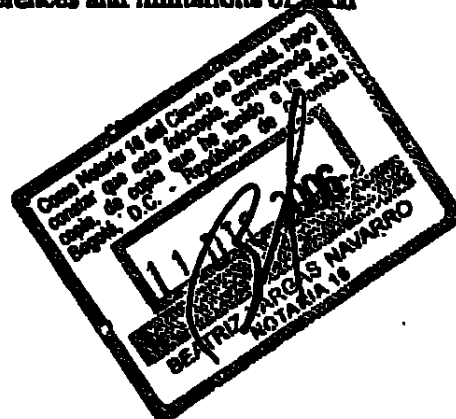
(iv) whether such series shall have conversion privileges and, if so, the terms and conditions of such conversion, including, but not limited to, provision for adjustment of the conversion rate upon such events and in such manner as the Board of Directors shall determine;

(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.



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.

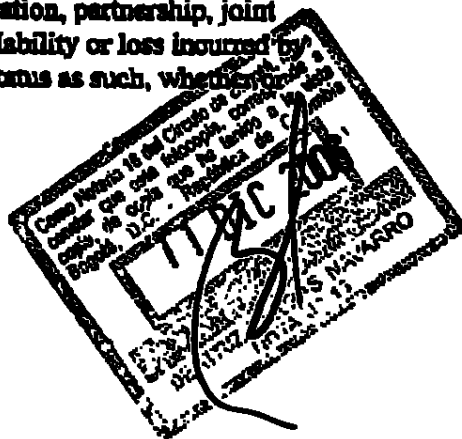
SECTION 6.02. Ballots. Elections of directors at an annual or special meeting of stockholders need not be by written ballot unless the bylaws of the Corporation shall provide otherwise.

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 or not



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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.

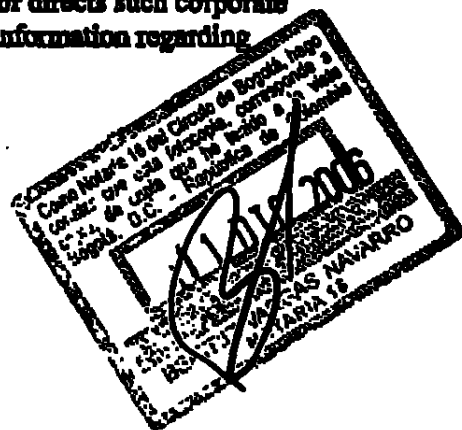
SECTION 8.02. *Similar Business Activities.* Except as Roche Holdings, Inc. may otherwise agree in writing.

(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.

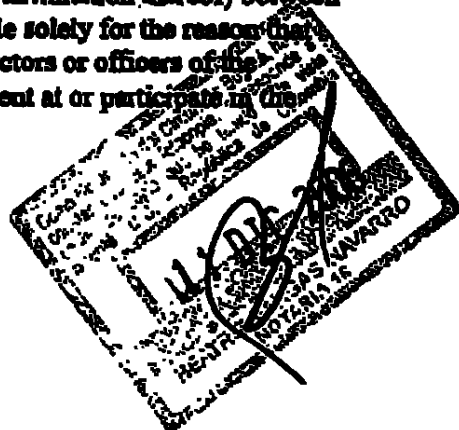
SECTION 8.05. *Definitions.* For purposes of this Article 8 and Article 9 only, (i) the term "Corporation" shall mean the Corporation and all corporations, partnerships, joint ventures, associations and other entities in which the Corporation beneficially owns (directly or indirectly) fifty percent or 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



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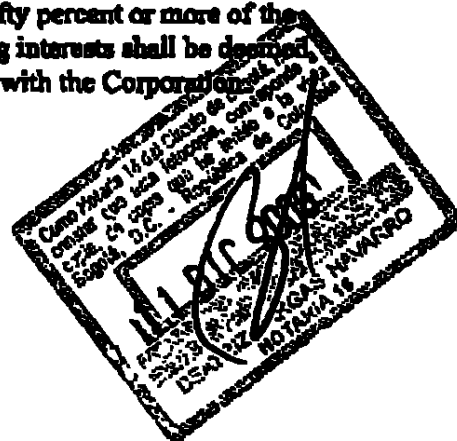
meeting of the Board of Directors or committee thereof which authorizes the contract, agreement, arrangement, transaction, amendment, modification or termination or solely because his or their votes are counted for such purpose, but any such contract, agreement, arrangement or transaction (or any amendment, 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.

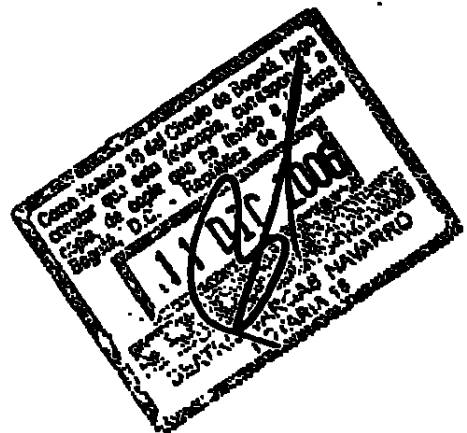
SECTION 9.04. Deemed Consent by Stockholders. Any person or entity purchasing or otherwise acquiring any interest in any shares of capital stock of the Corporation shall be deemed to have notice of and to have consented to the provisions of this Article 9.

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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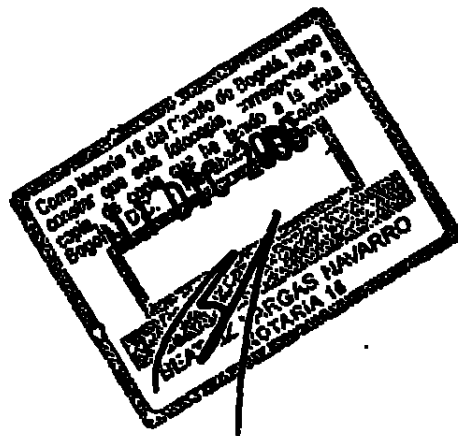
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IN WITNESS WHEREOF, this Amended and Restated Certificate of Incorporation, having been duly adopted in accordance with Sections 242 and 245 of the General Corporation Law of the State of Delaware and by the written consent of the sole stockholder 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



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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) *Series y Limites 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 la 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 existe 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



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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;

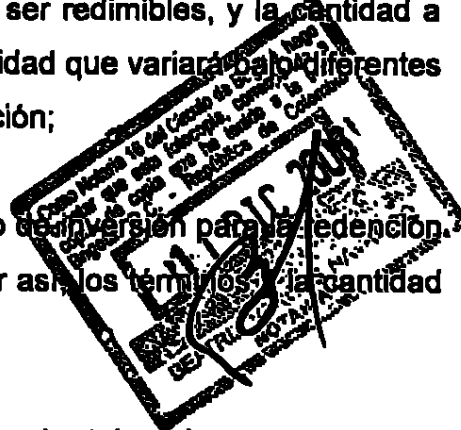
(iii) si la serie tendrá o no derechos de voto, además de los derechos de voto provistos por la ley, y de ser así, los términos de tales derechos de voto;

(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



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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 el 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.

o

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 de 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 artículo 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 adquiriera 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 u 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

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 se 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 y todas las corporaciones, sociedades, sociedades de riesgo compartido y otras entidades en las cuales la Corporación posee con beneficio (directa 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

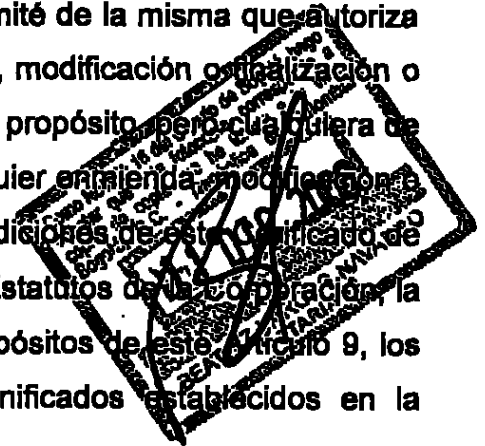
define en la Regla 12b-2 bajo el Acta de Intercambio de Valores de 1934, como se enmendó) de Roche Holdings, Inc.

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.



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.

SECCION 9.05. Contratos cubiertos. Para propósitos de este artículo, 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 indirecta) 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.

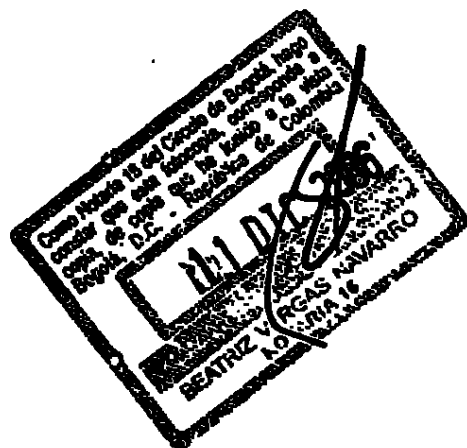
GENENTECH, INC.

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

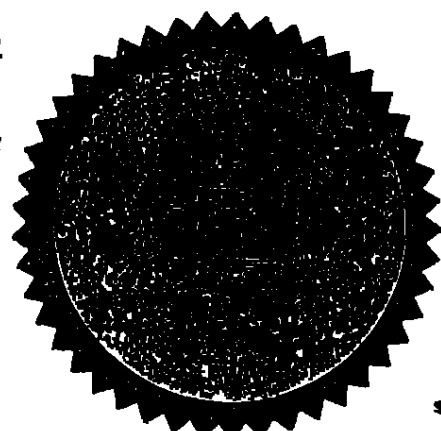
5. at Dover, Delaware

6. the twenty-fourth day of May, A.D. 2005

7. by Secretary of State, Delaware Department of State

8. No. 0254031

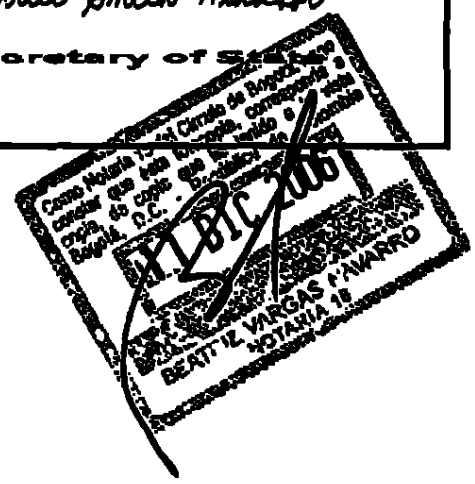
9. Seal/Stamp:



10. Signature:

Harriet Smith Windsor

Secretary of State



Delaware

PAGE 1

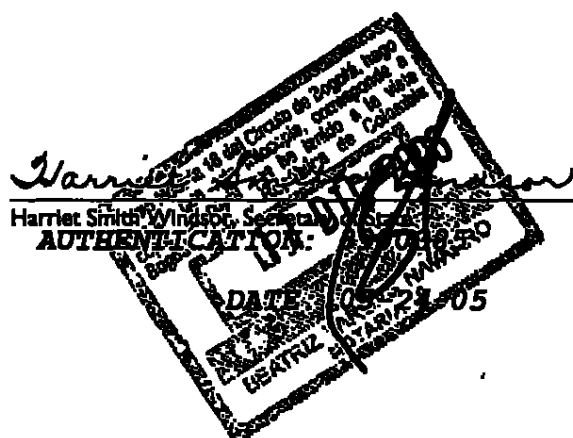
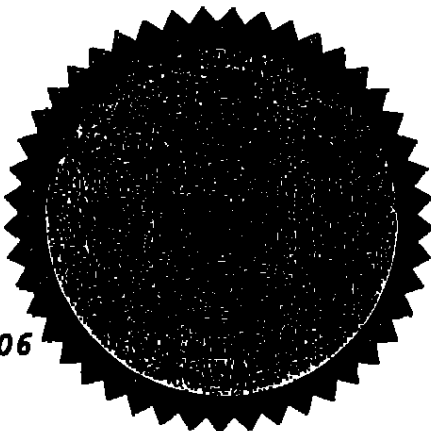
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The First State

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



CERTIFICATE OF AMENDMENT
OF AMENDED AND RESTATED
CERTIFICATE OF INCORPORATION

40X

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.

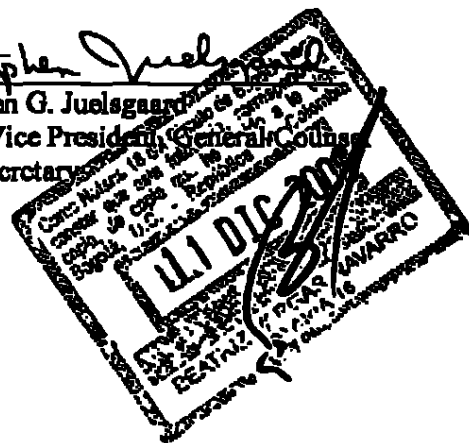
FOURTH: That the capital of said corporation shall not be reduced under or by reason of said amendment.

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 Counsel
and Secretary



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TRADUCCION AL ESPAÑOL del Certificado de Incorporación de Genentech, Inc.

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



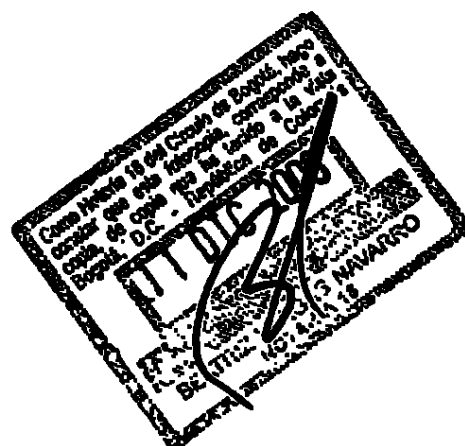
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FECHA: 05 - 24 - 05

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DELAWARE
EL PRIMER ESTADO

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

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**CERTIFICADO DE ENMIENDA DEL
CERTIFICADO DE INCORPORACIÓN
CORREGIDO Y VUELTO A REDACTAR**

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 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.

4/2 412

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

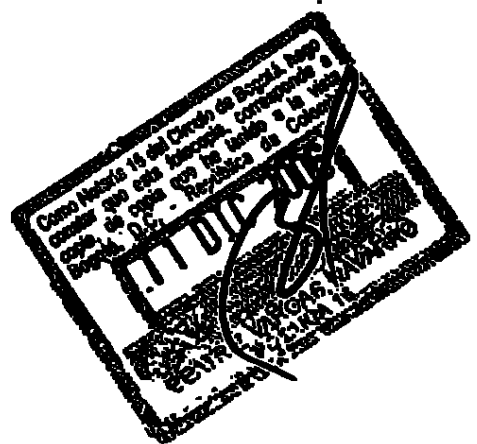
EN TESTIMONIO DE LO CUAL, dicha Genentech, Inc. ha causado este Certificado de Enmienda para que sea firmado por Stephen G. Juelsgaard, un Funcionario Autorizado, este 15 de Mayo de 2000.

GENENTECH, INC.

Por: (Firma)

Nombre: Stephen G. Juelsgaard

**Título: Vicepresidente, Consejo
y Secretaría General.**



43

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

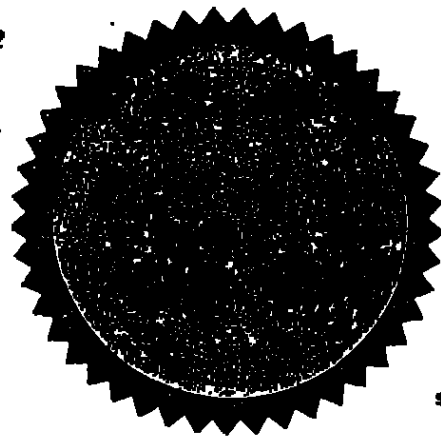
5. at Dover, Delaware

6. the twenty-fourth day of May, A.D. 2005

7. by Secretary of State, Delaware Department of State

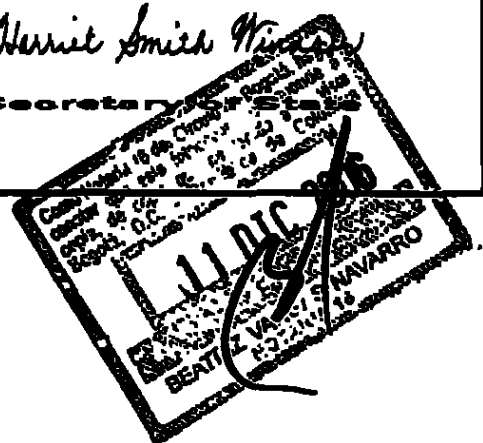
8. No. 0254032

9. Seal/Stamp:



10. Signature:

Harriet Smith Windsor
Secretary of State



Delaware

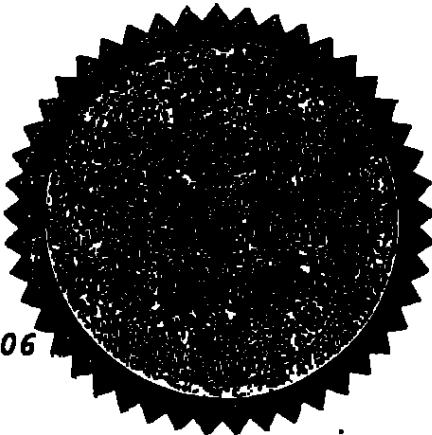
414
PAGE 1

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 TENTH DAY OF MAY, A.D. 2001, AT 2:05 O'CLOCK P.M.

2112549

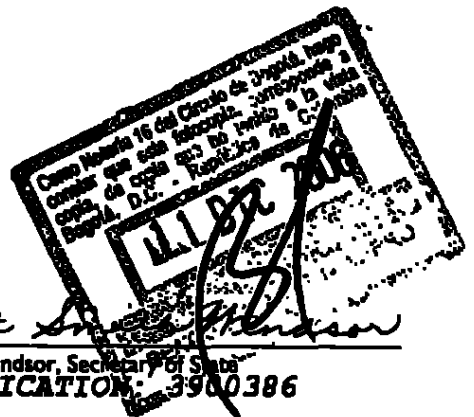
050426506



Harriet Smith Windsor

Harriet Smith Windsor, Secretary of State
AUTHENTICATION: 3900386

DATE: 05-24-05



CERTIFICATE OF AMENDMENT
OF AMENDED AND RESTATED
CERTIFICATE OF INCORPORATION

411 415

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.

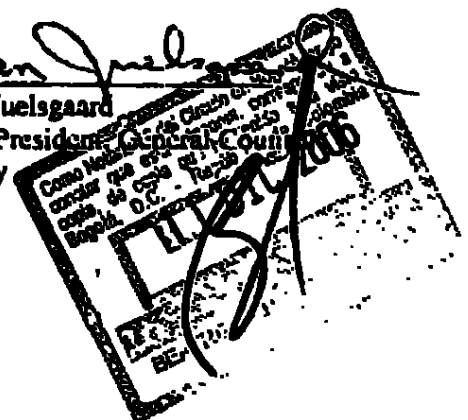
THIRD: That said amendment was duly adopted in accordance with the provisions of Section 242 of the General Corporation Law of the State of Delaware.

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
and Secretary



APOSTILLA

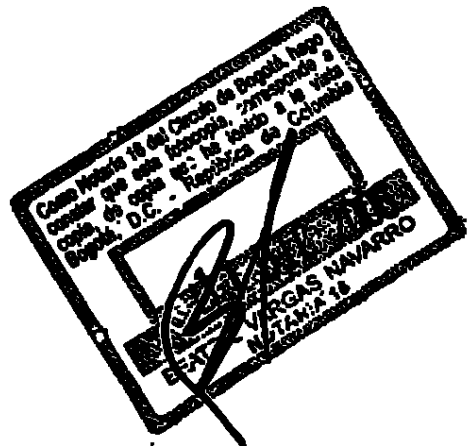
(Convención de La Haya del 5 de Octubre de 1961)

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



41X

DELAWARE
EL PRIMER ESTADO

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

418

**CERTIFICADO DE TERCERA ENMIENDA DEL
CERTIFICADO DE INCORPORACIÓN
CORREGIDO Y VUELTO A REDACTAR**

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 está 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.

419

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

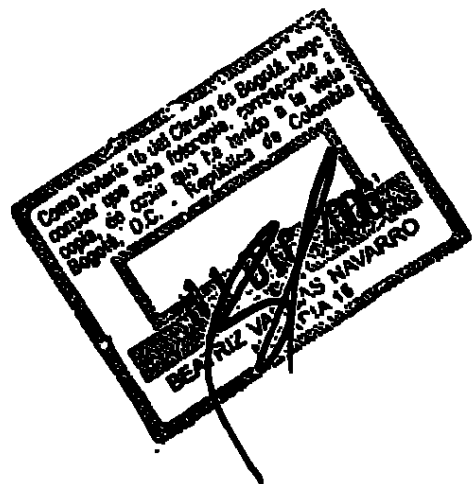
EN TESTIMONIO DE LO CUAL, dicha Genentech, Inc. ha causado este Certificado de Enmienda para que sea firmado por Stephen G. Juelsgaard, un Funcionario Autorizado, este 10 de Mayo de 2001.

GENENTECH, INC.

Por: (Firma)

Nombre: Stephen G. Juelsgaard

Título: Vicepresidente, Consejo
y Secretaría General.



SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
Rad: 07-022892- -00000-0000
Fee: 2007-03-07 18:01:51 Dep. 2020 NUECREACIONES
Tm. 11 PATENTE/II
Eve: 378 FASENACIONAL
Act 411 PRESENTACION Folios: 418



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. Neria
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

AS: 103.274



SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
 Rext: 07-022882- -00000-0000
 Fax: 2807-03-87 1801:31 Dep. 2800 NUECREACIONES
 Tlx. 11 PATENTEYH
 Ene: 378 FASENACIONALI
 Ant: 611 PRESENTACION Folio: 410

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 94080-4980, 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</u> TP <u>57492</u>
4 INVENTOR (ES) (72)	Nombre: Avi J. Ashkanazi 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/US2005/031907</u> Fecha <u>07-09-2005</u> Publicación Internacional No. <u>WO 2006/029224 A3</u> Fecha <u>18-03-2006</u>			
6 Título (54): <u>MÉTODOS PARA UTILIZAR LIGANDOS DE RECEPTOR MUERTO Y ANTICUERPOS CD20</u>			
7 Clasificación Internacional (51) <u>título (mala traducción)</u>			
8 Prioridad	(33) País de Origen	(32) Fecha	(31) Número de Solicitud
SI ☒ NO ☐	US	08-09-2004	60/607.909
	US	30-03-2005	60/666.553

Instrucciones para el diligenciamiento del presente formulario, ver reverso de esta página.

se reivindica es:

422

método para tratar células cancerosas, que comprende exponer células de cáncer de mamíferos, a una cantidad sinérgicamente efectiva del polipéptido Apo2L/TRAIL y el anticuerpo CD20.

método de la reivindicación 1, en donde dicho polipéptido Apo2L/TRAIL comprende los aminoácidos 1 a 281 de la Fig.1 ID de (SEQ. No.: ID NO: 1) o un fragmento o variante de él.

método de la reivindicación 1, en donde dicho polipéptido Apo2L/TRAIL comprende los aminoácidos 114 a 281 de la Fig.1 SEQ. No.: ID NO: 1).

método de la reivindicación 1, en donde dichas células cancerosas son expuestas a dicha cantidad sinérgicamente efectiva del polipéptido Apo2L/TRAIL y el anticuerpo CD20 in vivo.

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

método de la reivindicación 1, que comprende adicionalmente exponer las células cancerosas a uno o más agentes inhibidores del crecimiento.

423

REPUBLICA DE COLOMBIA
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

Bogotá,
2020

Doctor(a)
CADENA SARMIENTO JUAN PABLO
Apoderado(a) y/o Representante de
GENENTECH, INC.

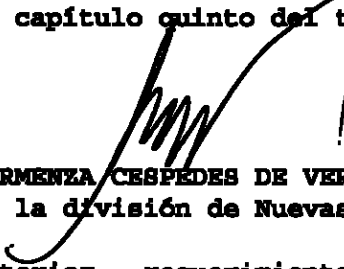
REFERENCIA	Radicación No.	7 22892
	Solicitud internacional	PCT/US2005/031907
	Fecha inicio fase nacional	08/04/2007
	Trámite	11
	Evento	378
	Actuación	430
	Oficio No.	6555

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 CARMONA CESPEDES DE VERGEL
Jefe de la división de Nuevas Creaciones

El anterior requerimiento fue notificado por fijación en lista de
fecha _____
adpall _____ 07 JUN 2007