

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES**

2020
Bogotá, D.C.,

Abogado
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ASUNTO	Radicación	09-22898
	Trámite	011
	Evento	378
	Actuación	519
	Oficio	
	Folios	016

15058

Patente de Invención	☒	Patente de Modelo de Utilidad	☐
Vía Nacional	☐		
Vía PCT (fase nacional)	☐	Cap. I	☐
		Cap. II	☐

No. Solicitud Internacional PCT/US2007/017546
Fecha de Presentación Internacional 07 agosto 2007

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 44 a 61	05 marzo 2009
Reivindicaciones:	Folio 62	05 marzo 2009
	Folios 100 a 101	20 octubre 2010
Dibujos:	Folios 64 a 72	05 marzo 2009
Secuencias:	Folios 73 a 80	05 marzo 2009
Prioridad:	US 60/836.003	07 agosto 2006

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486), y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Métodos terapéuticos para el tratamiento de trastornos oculares vasculares con antagonistas de DLL4"

El problema planteado en esta solicitud consiste en la necesidad de suministrar tratamiento para trastornos vasculares e isquémicos mediante la administración de composiciones que inhiban la interacción de DLL4 con los receptores Notch.

Para resolver este problema técnico la solicitud en estudio proporciona un anticuerpo o fragmento de anticuerpo que se une específicamente a DLL4 y bloquea la unión del DLL4 a un receptor Notch y métodos de tratamiento para trastornos

Primer Artículo 45- Expediente 09-22898

vasculares e isquémicos

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 39/039

4. No es una invención (Art 15 D 486 literal (b))

El objeto de la reivindicación 3 no se considera una invención, de acuerdo con el Art citado puesto que corresponde a un fragmento de anticuerpo completamente humano.

5. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 6 y 7 no es patentable de acuerdo con el Art citado pues hace referencia de manera implícita a un método de tratamiento para lesión isquémica o insuficiencia vascular.

6. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

El capítulo reivindicatorio no caracteriza adecuadamente el o los anticuerpos reclamados ya que deben caracterizarlo a partir de su estructura completa, mencionando las secuencias que lo conforman en la cadena ligera y cadena pesada, o las secuencias variables de las cadenas ligera y pesada o las secuencias de los 6 CDR's que conforman ambas cadenas. La referencia a estructuras parciales del anticuerpo no son válidas puesto que no es una estructura activa, probada ni definida. De igual manera la caracterización por su función no es adecuada puesto que corresponde a un resultado a alcanzar y dicha reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado, es así, que se sugiere reemplazarlo por las características esenciales, estructurales o funcionales, que contribuyen a la obtención de ese efecto.

La reivindicación 1 contiene la expresión "ligadura del tipo delta 4(DLL4) antagonista en la fabricación de un medicamento" la cual no es clara puesto que no se sabe si se reclama el anticuerpo o el medicamento que lo contiene, asimismo, las expresiones "a un receptor de muesca" y "conectada a un componente multimerizante" no definen a que estructura hace referencia. Se sugiere hacer las aclaraciones correspondientes y establecer términos concretos.

La reivindicación 1 contiene las expresiones "para prevenir o reducir la pérdida de vasos sanguíneos o promover angiogénesis productiva, o ambos en un sujeto que tenga una lesión isquémica o insuficiencia vascular", "anticuerpo que específicamente enlace la DLL4 y bloquee el enlace de la DLL4 a un receptor de muesca" las cuales hacen referencia a la función o efecto que ejerce el anticuerpo reclamado. Lo anterior no es válido puesto que estas no corresponden a características esenciales, estructurales o funcionales, que contribuyen a la obtención de ese efecto. se sugiere establecer estas expresiones en el preámbulo de la reivindicación o eliminarla de esta.

Las reivindicaciones 6 y 7 hacen referencia a las enfermedades que son tratadas con los anticuerpos reclamados, lo anterior hace referencia implícita a un método de tratamiento por lo cual se sugiere al solicitante eliminar dichas reivindicaciones.

Descripción (Art 28 D 486)

La Descripción no es clara porque la información que contiene no permite que el la persona del oficio normalmente versada en la materia pueda comprender el problema técnico y la solución aportada por la invención ya que no se evidencia la obtención de anticuerpos específicos que sean caracterizados por sus secuencias, ni composiciones que los contengan.

Se requiere al Solicitante hacer las aclaraciones necesarias.

7. Determinación del Estado de la Técnica

El Estado de la Técnica se determinó con base en la fecha de presentación de la solicitud de prioridad (07 agosto 2006) y en la fecha de presentación de la solicitud nacional (05 marzo 2009) y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	CO 08-129885*	Anticuerpos anti- DLL4 y métodos que los usan	Fecha presentación (05 diciembre 2008) Fecha prioridad (06 junio 2006)
D2	Leukemia (2004) vol. 18, no. 4, pag 788-797	Membrane-bound Delta-4 Notch ligand reduces the proliferative activity of primitive human hematopoietic CD34+CD38 low cells while maintaining their LTC-IC potential	01 abril 2004
D3	<u>US2006134121</u>	Dll4 antagonists, assays, and therapeutic methods thereof	22 junio 2006

D1 (correspondiente a la solicitud WO2008/060705) divulga anticuerpos monoclonales quiméricos y humanizados anti-DLL4 y composiciones que los contienen. (folios 113 a 118)

D2 divulga anticuerpos monoclonales anti DLL4 y dominio extracelular de la Dll4 unido a un Fc de tipo IgG1 como antagonistas de Notch que intervienen en los mecanismos de proliferación celular. (folios 119 a 123)

D3 divulga anticuerpos monoclonales quiméricos y humanizados anti-DLL4, composiciones que los contienen, métodos de tratamiento para enfermedades de proliferación celular y métodos de diagnóstico.

URL:http://worldwide.espacenet.com/publicationDetails/biblio?CC=US&NR=2006030529&KC=&FT=E&locale=en_EP

8. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La **reivindicación 1** consiste en una ligadura del tipo delta 4(DLL4) antagonista en la fabricación de un medicamento para prevenir o reducir la perdida de vasos sanguíneos o promover angiogénesis productiva, o ambos en un sujeto que tenga una lesión isquémica o insuficiencia vascular, en donde el Dll4 antagonista comprende un anticuerpo o fragmento de anticuerpo que específicamente enlace la Dll4 y bloquee el enlace de la Dll4 a un receptor de muesca o comprende el dominio extracelular de la Dll4, opcionalmente conectada a un componente multimerizante.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2	D3
Medicamento	Composición farmacéutica	Formulación	Composición farmacéutica
Dll4 antagonista	Antagonista anti- Dll4	Antagonista Dll4	Antagonista anti-Dll4
Anticuerpo o fragmento de anticuerpo que específicamente enlace la Dll4, o dominio extracelular de la Dll4, opcionalmente conectada a un componente multimerizante	Anticuerpo monoclonal quimérico o humanizado	Anticuerpo monoclonal ó dominio extracelular de la Dll4 unido a un Fc de tipo IgG1	Anticuerpo monoclonalo proteínas de fusión que comprenden dominio extracelular de la Dll4 unido a un Fc de tipo IgG1

Novedad frente a D1

Las características **esenciales** del anticuerpo reclamado en la reivindicación 1 habían sido divulgadas previamente en D1, el cual da a conocer composiciones farmacéuticas (folio 118, reivindicación 31, folio 115, línea 20 a 26) para el tratamiento de un trastorno de proliferación celular, que comprende un anticuerpo monoclonal quimérico o humanizado anti -Dll4 (folio 115, línea 29 a 34; folio 116, reivindicación 1 a 6) o fragmentos de anticuerpos como los fragmentos Fab, Fab', fab'-SH y F(ab')2.(folio 115, línea 29 a 34).

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 2 a 4 no mencionan alguna característica que haga nueva a la composición de la reivindicación 1, por lo cual se considera que no son nuevas.

Novedad frente a D2

Las características **esenciales** del anticuerpo reclamado en la reivindicación 1 habían sido divulgadas previamente en D2, el cual da a conocer formulaciones (folio) para el tratamiento y evaluación de un trastorno de proliferación celular especialmente en el endotelio vascular (folio 119, pág. 788). D3 hace referencia a la generación de proteínas Delta-4-Fc de secuencias específicas las cuales corresponden a un dominio extracelular de la Dll4 unido a un Fc de tipo IgG1 (folio 119, pág. 789; folio 122, pág. 795). De igual, manera enseña un anticuerpo policlonal anti-Dll4 (folio 119, pág. 789) denominado mbDelta-4 (folio 122, pág. 794) el cual es específico y tiene la acción de bloquear e inactivar las funciones propias de DLL4 y sobre los ligandos tipo Notch (folio 123, pág. 795) como son la reducción de la proliferación celular.

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en el documento D2.

Las reivindicaciones dependientes 2 y 5 no mencionan alguna característica que haga nueva a la composición de la reivindicación 1, por lo cual se considera que no son nuevas.

Novedad frente a D3

Las características **esenciales** de anticuerpo reclamado en la reivindicación 1 habían sido divulgadas previamente en D3, el cual da a conocer proteínas DLL4, fragmentos de proteína (párrafo 009), anticuerpos monoclonales quiméricos y/o humanizados (párrafo 0028, 0037, reivindicaciones 10 y 11) o proteínas de fusión que comprenden una proteína específica (dominio extracelular de la Dll4) (párrafo 0052) junto con un componente multimerizante como es una Fc de tipo IgG (párrafo 0012, 0013, 0038, 0052, ejemplo 2) los cuales son capaces de bloquear la actividad de unión de DLL4 para el tratamiento de un trastorno de proliferación celular (párrafo 0024). De igual manera se reclaman composiciones o medicamentos que contienen dichos antagonistas (párrafo 0017). También se divulgan fragmentos de anticuerpos como los fragmentos Fab, Fab', fab'-SH y F(ab')2 (párrafo 0040)

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 2 a 7 no mencionan alguna característica que haga nueva a la composición de la reivindicación 1, por lo cual se considera que no son nuevas.

9. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	CO 08-129885*	1 a 4	Novedad
D2	Leukemia (2004) vol. 18, no. 4, pag 788-797	1, 2, 5	Novedad
D3	US2006134121	1 a 7	Novedad

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante y presentarla por escrito

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.


JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha 28 AGO. 2012

Elaborado por: Ana Delia Pinzón García
Revisado Por: Consuelo Leguizamón Leguizamón 
Aprobado por: José Luis Salazar López



Organización y
Digitalización CSA Ltda.

DELEGATURA PROPIEDAD INDUSTRIAL
División de Nuevas Creaciones

SOLICITUD
PATENTE DE INVENCION

ENTRADA EN FASE NACIONAL

08-129885

21. EXPEDIENTE No.

54. TITULO

ANTICUERPOS ANTI-DLL4 Y MÉTODOS QUE LOS USAN

51. CLASIFICACION INTERNACIONAL

71. SOLICITANTE

GENENTECH. INC.

DOMICILIO

SAN FRANCISCO, CA, ESTADOS UNIDOS DE AMÉRICA

74. APODERADO

JUAN PABLO CADENA SARMIENTO

22. SANTAFE DE BOGOTÁ, D.C.

PETITORIO			
AS: 108.822  Industria y Comercio SUPERINTENDENCIA		SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO  No. 08-128885-00000-0000 Fecha: 2008-12-05 18:51:42 Dep. 2020 NUECREACION Tra 11 PATENTEYII Evg. 378 FASENACIONALI Act. 411 PRESENTACION Folios. 433	
FORMULARIO ÚNICO DE SOLICITUD DE PATENTE PCT ENTRADA EN FASE NACIONAL			
06 ene 2009			
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, California 94080, Estados Unidos de América Nacionalidad o Domicilio: Estados Unidos de América Lugar de Constitución: Estados Unidos de América Teléfono: Fax: E-mail:		IDENTIFICACIÓN C.C. ☐ NIT ☐ C.E. ☐ Otro ☐ Cual _____ Número _____
3 REPRESENTANTE O APODERADO	Nombre: JUAN PABLO CADENA SARMIENTO Dirección: Cra. 7 No. 71-21 Torre B - Piso 4, Bogotá, Colombia. Teléfono: 744-2200 Fax: 742-2200 E-mail:		IDENTIFICACIÓN C.C. ☒ NIT ☐ C.E. ☐ Otro ☐ Cual _____ Número 80.410.337 TP 57492
4 INVENTOR (ES) (72)	Nombre: Minhong YAN, Yan WU. Dirección: 411 Nantucket Street, Foster City, California 94404, US; 1160 Blythe Street, Foster City, California 94404, US (Respectivamente). Nacionalidad o Domicilio: Estados Unidos de América		
5 PCT (86) Solicitud Internacional No. <u>PCT/US2007/070513</u> Fecha <u>06-06-2007</u> Publicación Internacional No. <u>WO 2008/060705 A2</u> Fecha <u>22-05-2008</u> <i>Folios</i> <u>WO 2008/060705</u>			
6 Título (54): <u>ANTICUERPOS ANTI-DLL4 Y MÉTODOS QUE LOS USAN</u>			
7 Clasificación Internacional (51): _____			
8 Prioridad SI ☒ NO ☐	(33) País de Origen	(32) Fecha	(31) Número de Solicitud
	US	06-06-2006	60/811,349
	US	06-06-2006	60/811,357
	US	21-11-2006	60/866,772
	US	21-11-2006	60/866,767
9 Comprobante de pago No. <u>08 - 85.493</u> Fecha <u>05-12-2008</u>			
Instrucciones para el diligenciamiento del presente formulario, ver reverso de esta página.			

transformarse en el fármaco más activo no citotóxico. Ejemplos de fármacos citotóxicos que pueden ser derivatizados para obtener un profármaco para su uso en esta invención son los agentes quimioterapéuticos descritos anteriormente.

Un "agente antiangiogénico" o "inhibidor angiogénico" es una sustancia de bajo peso molecular, un polinucleótido (incluyendo, por ejemplo, un ARN inhibidor (ARNi o siARN)), un polipéptido, una proteína aislada, una proteína recombinante, un anticuerpo, o conjugados o proteínas de fusión de los mismos, que inhiben la angiogénesis, la vasculogénesis o la permeabilidad vascular no deseada, bien directa bien indirectamente. Por ejemplo, un agente antiangiogénico es un anticuerpo u otro antagonista a un agente angiogénico tal como se define anteriormente, por ejemplo, anticuerpos anti-VEGF, anticuerpos contra receptores del VEGF, fragmentos solubles del receptor del VEGF o pequeñas moléculas que bloquean la señalización de los receptores del VEGF (por ejemplo, PTK787/ZK2284, SU6668, SUTENT®/SU11248 (sunitinib malato), AMG706, o las descritas en, por ejemplo, la solicitud de patente internacional WO 2004/113304). Los agentes antiangiogénicos también incluyen inhibidores nativos de la angiogénesis, por ejemplo, angiostatina, endostatina, etc. Véase, por ejemplo, Klagsbrun y D'Amore, *Annu. Rev. Physiol.*, 53:217-39 (1991); Streit y Detmar, *Oncogene*, 22:3172-3179 (2003) (por ejemplo, la Tabla 3, que enumera los tratamientos antiangiogénicos en el melanoma maligno); Ferrara y Allitalo, *Nature Medicine* 5(12):1359-1364 (1999); Tonini et al., *Oncogene*, 22:6549-6556 (2003) (por ejemplo, la Tabla 2, que enumera factores antiangiogénicos); y Sato *Int. J. Clin. Oncol.*, 8:200-206 (2003) (por ejemplo, la Tabla 1, que enumera antiangiogénicos usados en ensayos clínicos).

20 Composiciones de la invención y métodos para su elaboración

Esta invención engloba composiciones, incluidas composiciones farmacéuticas, que comprenden un anticuerpo anti-DLL4, y polinucleótidos, que incluyen secuencias que codifican un anticuerpo anti-DLL4. Como se usa en la presente memoria descriptiva, las composiciones comprenden uno o más anticuerpos que se unen a DLL4 y/o uno o más polinucleótidos que comprenden secuencias que codifican uno o más anticuerpos que se unen a DLL4. Estas composiciones pueden contener, además, vehículos aptos, como excipientes farmacéuticamente aceptables que incluyan soluciones tamponadas, que son bien conocidas en la materia.

La invención también engloba formas de realización de polinucleótidos y anticuerpos aislados. La invención también engloba formas de realización de polinucleótidos y anticuerpos sustancialmente puros.

Los anticuerpos anti-DLL4 de la invención son preferiblemente monoclonales. También se incluyen dentro del ámbito de la invención fragmentos de anticuerpos como los fragmentos Fab, Fab', Fab'-SH y F(ab')₂, de los anticuerpos anti-DLL4 proporcionados en la presente memoria descriptiva. Estos fragmentos de anticuerpos se pueden crear por medios tradicionales, como digestión enzimática, o se pueden generar mediante técnicas recombinantes. Estos fragmentos de anticuerpos pueden ser quiméricos o humanizados. Estos fragmentos son útiles para los fines diagnósticos y terapéuticos que se exponen a continuación.

Los anticuerpos monoclonales se obtienen a partir de una población de anticuerpos sustancialmente homogéneos, es decir, los anticuerpos individuales que forman la población son idénticos, excepto en posibles mutaciones de formación natural que pueden estar presentes en cantidades insignificantes. Por lo tanto, el modificador "monoclonal" indica el carácter del anticuerpo, que no es una mezcla de anticuerpos discretos.

LAS REIVINDICACIONES SON:

1. Un anticuerpo anti-DLL4 aislado que comprende:

(a) al menos uno, dos, tres, cuatro o cinco secuencias de la región hipervariable (HVR) seleccionadas en el grupo formado por:

- 5 (i) HVR-L1 que comprende la secuencia A1-A11, donde A1-A11 es RASQDVSTAVA (identificador de secuencia n.º: 10)
- (ii) HVR-L2 que comprende la secuencia B1-B7, donde B1-B7 es SASFLYS (identificador de secuencia n.º: 11)
- (iii) HVR-L3 que comprende la secuencia C1-C9, donde C1-C9 es QQSYTGTVT (identificador de
- 10 secuencia n.º: 18)
- (iv) HVR-H1 que comprende la secuencia D1-D10, donde D1-D10 es GFTFTDNWIS (identificador de secuencia n.º: 1);
- (v) HVR-H2 que comprende la secuencia E1-E18, donde E1-E18 es GYISPNSGFTYYADSVKG (identificador de secuencia n.º: 8) y
- 15 (vi) HVR-H3 que comprende la secuencia F1-F15, donde F1-F15 es VYYCARDNFGGYFDY (identificador de secuencia n.º: 9); y
- (b) al menos una variante de la HVR, donde la secuencia de la variante de la HVR incluye la modificación de al menos un residuo de la secuencia descrita en los identificadores de secuencia n.º: 1-18.

20 2. Un anticuerpo anti-DLL4 aislado que comprende:

(a) al menos uno, dos, tres, cuatro o cinco secuencias de la región hipervariable (HVR) seleccionadas en el grupo formado por:

- (i) HVR-L1 que comprende la secuencia A1-A11, donde A1-A11 es RASQDVSTAVA (identificador de secuencia n.º: 10)
- 25 (ii) HVR-L2 que comprende la secuencia B1-B7, donde B1-B7 es SASFLYS (identificador de secuencia n.º: 11)
- (iii) HVR-L3 que comprende la secuencia C1-C9, donde C1-C9 es QQSYNGPST (identificador de secuencia n.º: 15)
- (iv) HVR-H1 que comprende la secuencia D1-D10, donde D1-D10 es GFTFTDNWIS (identificador de
- 30 secuencia n.º: 1)
- (v) HVR-H2 que comprende la secuencia E1-E18, donde E1-E18 es GVINPNSGATÉYADSVKG (identificador de secuencia n.º: 5) y
- (vi) HVR-H3 que comprende la secuencia F1-F15, donde F1-F15 es VYYCARDNFGGYFDY (identificador de secuencia n.º: 9); y
- 35 (b) al menos una variante de la HVR, donde la secuencia de la variante de la HVR incluye la modificación de al menos un residuo de la secuencia descrita en los identificadores de secuencia n.º: 1-18.

3. El anticuerpo de la reivindicación 1 ó 2, donde una variante de la HVR-L3 comprende de 1 a 6 (1, 2, 3, 4, 5 ó 6) sustituciones en cualquier combinación de las posiciones siguientes: 91 (S o W), 92 (Y o F), 93 (T, N o S), 94 (T o G), 95 (P, Q, A o T), y/o 96 (P, S, A o V).
- 5 4. El anticuerpo de la reivindicación 1 ó 2, donde una variante de la HVR-H2 comprende de 1 a 4 (1, 2, 3 ó 4) sustituciones en cualquier combinación de las posiciones siguientes: 50 (V, L o Y), 52 (N o S), 52a (P o S) o 53 (N, Q, T o I).
- 10 5. Un anticuerpo anti-DLL4 aislado que comprende uno, dos, tres, cuatro, cinco o seis HVR, donde cada HVR comprende, consiste o consiste esencialmente en una secuencia seleccionada entre el grupo formado por los identificadores de secuencia núms: 1-18, y donde el identificador de secuencia n.º 10 se corresponde con una HVR-L1; el identificador de secuencia n.º 11 se corresponde con una HVR-L2; los identificadores de secuencia n.º 12, 13, 14, 15, 16, 17 o 18 se corresponden con una HVR-L3; los identificadores de secuencia n.º 1 ó 2 se corresponden con una HVR-H1; los identificadores de secuencia n.º 3, 4, 5, 6, 7 u 8 se corresponden con una HVR-H2; y el
- 15 identificador de secuencia n.º 9 se corresponde con una HVR-H3.
- 20 6. El anticuerpo de la reivindicación 5, donde el anticuerpo de la invención comprende HVR-L1, HVR-L2, HVR-L3, HVR-H1, HVR-H2 y HVR-H3, donde cada una de ellas, por orden, comprende los identificadores de secuencia n.º: 10, 11, 14, 2, 3, 9.
- 25 7. El anticuerpo de la reivindicación 5, donde el anticuerpo de la invención comprende HVR-L1, HVR-L2, HVR-L3, HVR-H1, HVR-H2 y HVR-H3, donde cada una de ellas, por orden, comprende los identificadores de secuencia n.º: 10, 11, 15, 1, 5, 9.
- 30 8. El anticuerpo de la reivindicación 5, donde el anticuerpo de la invención comprende HVR-L1, HVR-L2, HVR-L3, HVR-H1, HVR-H2 y HVR-H3, donde cada una de ellas, por orden, comprende los identificadores de secuencia n.º: 10, 11, 16, 1, 6, 9.
- 35 9. El anticuerpo de la reivindicación 5, donde el anticuerpo de la invención comprende HVR-L1, HVR-L2, HVR-L3, HVR-H1, HVR-H2 y HVR-H3, donde cada una de ellas, por orden, comprende los identificadores de secuencia n.º: 10, 11, 17, 1, 7, 9.
- 40 10. El anticuerpo de la reivindicación 5, donde el anticuerpo de la invención comprende HVR-L1, HVR-L2, HVR-L3, HVR-H1, HVR-H2 y HVR-H3, donde cada una de ellas, por orden, comprende los identificadores de secuencia n.º: 10, 11, 18, 1, 8 y 9.
- 45 11. El anticuerpo de cualquiera de las reivindicaciones 1-10, donde al menos una porción de la secuencia de estructura es una secuencia de estructura de consenso humana.

28. Un método para la detección de DLL4, que comprenda la detección del complejo DLL4- anticuerpo anti-DLL4 en una muestra biológica.

29. Un método para diagnosticar un trastorno asociado a la expresión de DLL4, que incluya la detección del complejo formado por DLL4 y anticuerpos anti-DLL4 en una muestra biológica procedente de un paciente que tenga o posiblemente tenga el trastorno.

30. El método de cualquiera de las reivindicaciones 28-29, donde el anticuerpo anti-DLL4 se detecta mediante marcado.

31. Una composición que comprende un anticuerpo anti-DLL4 de cualquiera de las reivindicaciones 1-16.

32. Una composición que comprende un polinucleótido de cualquiera de las reivindicaciones 17-19.

33. La composición de la reivindicación 31 ó 32, donde la composición comprende además una molécula vehículo.

34. Un método para el tratamiento de un tumor, un cáncer o un trastorno de proliferación celular que comprenda la administración de una cantidad eficaz de un anticuerpo de cualquiera de las reivindicaciones 1 a 16 a un sujeto que necesite dicho tratamiento, mediante el cual se trata el tumor, el cáncer o el trastorno de proliferación celular.

35. El método de la reivindicación 34, donde el tumor, cáncer o trastorno de proliferación celular es cáncer de colon, cáncer de pulmón o cáncer de mama.

36. El método de la reivindicación 34 ó 35, que comprende, además, la administración de una cantidad eficaz de un agente antiangiogénico.

37. El método de la reivindicación 36, donde el agente antiangiogénico es un antagonista del factor de crecimiento endotelial vascular (VEGF).

38. El método de la reivindicación 37, donde el antagonista del VEGF es un anticuerpo anti-VEGF.

39. El método de la reivindicación 38, donde el anticuerpo anti-VEGF es bevacizumab.

40. El método de cualquiera de las reivindicaciones 34-39, que supone administrar también una cantidad eficaz de un agente quimioterapéutico.

Membrane-bound Delta-4 Notch ligand reduces the proliferative activity of primitive human hematopoietic CD34⁺ CD38^{low} cells while maintaining their LTC-IC potential

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To examine the role of the Notch ligand Delta-4 on hematopoietic stem cells, human CD34⁺ CD38^{low} cord blood cells were cocultured on S17 cells transduced with transmembrane Delta-4 (mbD4/S17) or an empty vector (C/S17). By the end of a 3-week culture, mbD4/S17 induced a 25-fold reduction in nucleated cell production, as compared to C/S17, by maintaining a higher proportion of cells in G0/G1 phase. A specific retention of a high proportion of CD34⁺ cells throughout the culture was observed with mbD4/S17, contrary to C/S17. Although mbD4/S17 promoted expansion of cells with the phenotype of committed lymphoid precursors (CD34⁺ CD7⁺ CD45RA⁺), these cells still retained their myeloid differentiation potential. mbD4/S17 maintained a higher LTC-IC frequency in output CD34⁺ cells, compared to C/S17, as in the subsets of cells having completed the same number of divisions on mbD4/S17. A Delta4-Fc protein (extracellular part of human Delta4 fused to Fc human IgG1 portion), immobilized on plastic, also reduced cell production and retained the LTC-IC potential. Transplantation of cells grown on mbD4/S17 into NOD/SCID mice showed no significant enhancement of the long-term repopulating ability. Thus, Delta4 appears to inhibit hematopoietic stem cell proliferation, in association with the maintenance of short-term lymphoid and myeloid repopulation capacity.

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Introduction

Hematopoiesis is a continuous developmental process in which hematopoietic stem cells (HSC) and their progeny make sequential cell fate decisions, producing mature blood cells of the various lineages. The constant generation of appropriate numbers and types of mature cells, as well as the maintenance of HSC, requires a complex regulatory network of exogenous and endogenous signalling which are incompletely understood.¹ In invertebrate and vertebrate developmental systems, molecules belonging to the highly conserved Notch family are known to modulate cell fate.^{2–4} Accumulated evidence indicates that Notch signalling modulates the ability of nonterminally differentiated cells to respond to differentiation and proliferation signals in order to progress to the next developmental stage. Notch receptors are transmembrane glycoproteins that bind to ligands expressed on adjoining cells. They represent a single-pass transmembrane protein composed of a large extracellular domain containing between 29 and 36 epidermal growth factor (EGF)-like repeats in tandem and three cysteine-rich

Notch/LIN-12 repeats and an intracellular domain consisting of six tandem cdc10/ankyrin repeats, a glutamine-rich domain (OPA), and a PEST sequence critical to downstream signalling.⁵ The Notch receptor interacts with a family of transmembrane proteins that serve as ligands, containing EGF repeats and a highly conserved Delta-Serrate-Lag-2 (DSL) domain in the extracellular region. Activation of Notch signalling is mediated through interactions between the DSL domain and specific EGF repeats located in the extracellular portion of the Notch receptor. In the prevailing model of Notch activation, once the ligand is bound, signal transduction takes place, resulting in the appearance of transcription factors such as Hes-1, capable to suppress the transcription of lineage-specific genes. The cell becomes refractory to differentiation signals, an effect transient and reversible; once Notch activation ceases the cell is able to differentiate.

If a role for Notch-1 has been clearly demonstrated in promoting T-cell development while inhibiting B-cell differentiation,⁶ a role for Notch receptors and their ligands in the regulation of HSC has recently emerged. Four different Notch genes have been identified in mice and humans, two of which, Notch-1 and Notch-2, are known to be expressed in the hematopoietic system, in primitive and mature cells within the different cell compartment^{7,8} as well as in adult medullar stroma cells. Two Notch ligand families, Delta and Serrate/Jagged ligands, have been identified in mammals, including Delta-1, 3 for the Delta family and Jagged-1, 2 for the Serrate/Jagged family. Human Delta-1 as well as human Jagged-1 are expressed in human CD34⁺ cells and in adult bone marrow stroma cells.^{7,8} Similarly, Jagged-2 is expressed on murine Lin[−]c-kit⁺ cells.⁹ A number of recent studies have provided evidence that ligand-induced Notch signalling favored HSC self-renewal,^{10–13} increased the numbers of progenitors,^{7,8,14–17} and promoted HSC survival.¹⁸

A new member of the Delta family has been cloned by us and others, from either endothelial cell or placenta libraries, named Delta-4.^{19–21} Like other Delta ligands, Delta-4 possesses an extracellular region containing several EGF-like domains and a DSL domain required for receptor binding. Human Delta-4 was found to be highly expressed within the vascular endothelium,²⁰ but its expression was also found in a wide range of adult and fetal tissues, including sites of hematopoiesis.⁸ We investigated if Delta-4 was able to influence *in vitro* the fate of human cord blood CD34⁺ CD38^{low} cells stimulated by early acting cytokines. Since Notch ligands were considered to exert their effects through membrane-bound expression from stroma cells, we ascertained the effects of Delta-4 on HSC by using a coculture between HSC and S17 cells engineered to express Delta-4 as membrane-bound protein (mbDelta4). Our results reveal that the major activity of mbDelta-4 consists in reducing HSC proliferation, thus leading to the maintenance of the LTC-IC potential.

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Methods

Construction, and production of retroviruses

Retroviral vectors coding for full-length ¹⁹ human Delta-4 cDNA were derived from the MP5Vpac vector carrying the puromycin N-acetyl transferase (pac) gene driven by an internal murine phosphoglycerase kinase (pgk) promoter and a murine embryonic stem cell virus (MESV) LTR.²² The unmodified vector served as control. Full-length Delta-4 cDNA (2058 kb) (mbDelta-4) was PCR-amplified to introduce unique 5'-XhoI and 3'-EcoRI restriction sites and a Kozak sequence (ACCGCC) in the original cDNA (Advantage-HF kit, Clontech Lab. Inc, Palo Alto CA, USA). The PCR products were ligated into the MSCVpac retroviral vector, sequenced, and one clone was selected for base perfect match with the original cDNA.

Viral supernatants were generated using 293-EBNA cells (Invitrogen, Carlsbad, CA, USA) by transfecting the Delta-4 constructs using the Exgen 500 reagent (Euromedex, France) as previously described.²³

Generation of Delta-4-Fc protein

This construct was engineered to encode the extracellular domain of human Delta-4 fused in frame to a sequence of human IgG1-Fc fragment. The human Delta-4 cDNA was PCR-amplified using a sense primer (5'-CGTACTCGAGATGGCGG-CAGCGTCCCGGAGC-3') and an antisense primer (5'-CTGAG-GATCCGAGGGGAAGCTGGCGGCAAGCCAC-3'). The product of amplification was digested by XhoI and BamHI, and cloned into the XhoI and BamHI sites of pHT7555-hlgG1Fc expression vector. The resulting construct was introduced into HEK293 cells using the Exgen 500 reagent to generate a stable cell population for protein production. The presence of soluble Delta-4Fc in the conditioned medium of G418-resistant cells (Invitrogen) was revealed by slot-dot using Protein A horse-radish peroxidase linked (Amersham). Conditioned medium was first 10-fold concentrated into dialysis tubing by contact with a layer of polyethylene glycol compound (Sigma), and dialyzed against 2 M NaCl 50 mM borate pH 8.5. The dialyzed protein concentrate was pumped over a Protein A column (HiTrap Protein A, Amersham), and the column was extensively washed with 2 M NaCl 50 mM borate pH 8.5. Bound protein was eluted with 100 mM glycine, pH 3, and neutralized with 2 M Tris pH 11. Purity assessed by separating proteins using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining (SilverQuest kit, Invitrogen) was always superior to 80%.

Generation of S17 and NIH3T3 stromal cell populations

S17 cells²⁴ were grown in minimal essential medium (α -MEM) supplemented with 10% fetal calf serum (FCS, Invitrogen, France). They were infected with 0.1 ml of the viral supernatants (containing the control vector, or the mbDelta-4-expressing vector) in the presence of 4 μ M polybrene (Sigma, France). Two days after infection, puromycin selection (4 μ g/ml, Sigma, France) was performed to generate stably transduced cell populations. Each of the two S17 populations (C/S17, or mbD4/S17) was the result of a mixture of at least 10 different clones. NIH3T3 cells were transfected with the same procedure.

Polyclonal antibodies against a peptide in the extracellular domain of mouse Delta-4 (TPQDDLRPETSPGNS) were raised in

rabbits and subsequently peptide affinity purified (Research Genetics, Huntsville, AL, USA). C/S17 and mbD4/S17 cells were labelled according to standard procedures using a biotinylated anti-rabbit antibody (Immunotech, Marseille, France) and streptavidin conjugated to R-Phycoerythrin (Caltag, Burlingame, CA, USA). Fluorescence analysis was performed on a FACScan (Becton Dickinson, San Diego, CA, USA).

Separation of CD34⁺CD38^{low} cells

Cord blood samples were obtained with informed consent according to approved institutional guidelines, and were subjected to standard CD34 immunomagnetic bead separation (Miltenyi Biotec, Auburn, CA, USA). Sorting of the CD34⁺CD38^{low} fraction was performed using a FACSVantage (Becton-Dickinson) as previously described.²⁵ In some experiments, CD34⁺CD38^{low} cells were labelled with the carboxy fluorescein diacetate succinimidyl ester (CFSE) to track the successive cell division during culture, as previously described.²⁶

Cell culture

Cultures were initiated by plating 5000 CD34⁺CD38^{low} cells in 24-well plates precoated with C/S17 or mbD4/S17 in α -MEM containing 10% FCS (Stem Cell Technology, Vancouver, Canada). The following cytokines were added: PEG-rhu-MGDF (50 ng/ml; Kirin, Brewery, Japan), rhu-IL3 (100 U/ml, Novartis), rhu-Flt-3 ligand (100 ng/ml, Immunex), and rhu-SCF (50 ng/ml, Amgen). At day 7, to avoid any deleterious effect of 7-day aged stroma, the cultures were disrupted by vigorous pipetting and scraping. Nucleated cells were counted, and 5000 output nucleated cells were replated into a new 24-well plate precoated with C/S17 and mbD4/S17 stroma lines. This procedure was repeated for two consecutive weeks. At days 7, 14, 21, and 28, output CD34⁺ cells were sorted to assess their functional potential.

Some cultures were initiated at single CD34⁺CD38^{low} cell level in the same conditions. At day 10, the resulting clones were examined, and their size was evaluated (10–100 cells; 101–1000 cells; >1000 cells).

In another series of experiments, CFSE-labelled CD34⁺CD38^{low} cells were cultured on the different S17 stroma lines as described above. Cells having divided once during 48 h were selected, and plated onto fresh stromas. Two days later, cells having performed four more divisions (a total of five from the onset of the culture) were sorted again, and their LTC-IC potentials were analyzed. All cultures were kept at 37°C in an air atmosphere supplemented with 5% CO₂ and saturated with humidity.

The activity of the Delta4-Fc protein was assessed by precoating for 1 h at 37°C wells of nontissue culture-treated plates with a solution of 10 μ g/ml of goat F(ab')₂ anti-human IgG1 Fc specific (Caltag, Tebu), and of 25 ng/ml Retronectin (Takara, Biowhittaker, UK) in 1 $\times$ PBS. Wells were washed with 1 $\times$ PBS, blocked with 1 $\times$ PBS containing 10% FCS, incubated with a solution of Delta4-Fc (10 μ g/ml) in 1 $\times$ PBS for 2 h at 37°C, and washed extensively with 1 $\times$ PBS. Irrelevant human IgG1 (10 μ g/ml) serves as negative control. Culture experiments of CD34⁺CD38^{low} cells with Delta4-Fc were performed in the same conditions as above, with the exception of the culture medium (STEMSPAN SFEM medium, Stem Cell Technologies, Vancouver, Canada + cytokines).

Assessment for cell apoptosis and cell cycle by propidium iodide analysis

Seven days after the onset of the culture, nucleated cells were resuspended at a concentration of 5×10^6 cells per ml in 0.15 mol/l NaCl, 0.1 mol/l Tris-HCl pH 7.6, then diluted with 20 vol of 0.1% (wt/vol) Na citrate, pH 7.6, 10 mmol/l NaCl, 50 mg/ml RNase A, 0.1% NP40, and 10 mg/ml propidium iodide. The cells were then incubated at 4°C for 24 h before flow cytometric analysis (Becton Dickinson, FACSort, France). At least 10 000 cells per sample were analyzed for cell cycle progression.

Apoptosis was analyzed as follows. Purified CD34⁺ cells were pelleted, and cells were washed once with $1 \times$ PBS, and once in Annexin V binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). Cells were transferred to a tube, and 1 μ l of Annexin V-FITC and 2.5 μ l dissolved propidium iodide were added per tube and allowed to incubate on ice for 15 min in the dark. Cells were analyzed for fluorescence on a FACScan using the CellQuest software.

Phenotype of output CD34⁺ cells

The phenotype of output CD34⁺ cells was assessed by flow cytometry after labelling with the following mAbs: CD7-PE (Becton Dickinson), CD38-PE (Becton Dickinson), CD90-FITC (Immunotech), CD45RA (Pharmingen) and CD10PE (Becton Dickinson). Analysis was performed on a FACScanTM using CellQuest software.

Functional assays

Quantification of CFC in CD34⁺ cells was performed using standard methylcellulose colony assay using previously described criteria.²⁷

LTC-IC potential was performed as previously described.²⁷ The LTC-IC frequency was established by culturing them in limiting dilution. The absolute number of LTC-IC was then derived from the calculate frequency.²⁷

Myeloid (G/M) and/or lymphoid (B, NK) potential of CD34⁺ cells was assessed in a permissive culture as previously described.²⁸ Wells with significant cell proliferation were selected, cells were collected and counted, and their phenotype assessed by flow cytometry after labelling with the following mAbs: CD19-PE (Becton Dickinson), CD15-FITC (Dako Corp.), and CD56-PE-Cy5 (Immunotech).

Transplantation of output CD34⁺ cells into NOD-SCID mice was performed using mice bred and maintained at the Gustave Roussy Institute. NOD/SCID mice at 8–10 weeks of age were γ -irradiated at 250 cGy the day of transplantation, and human cells were injected via the retro-orbital sinus. When fresh CD34⁺CD38^{low} cells were transplanted, 10^5 irradiated (20 Gy) CD34^{neg} cord blood cells were coinjected as carrier cells. At 10 weeks, mice were killed, cells were harvested from both femurs and tibias, and analyzed for engraftment. After blocking with anti-mouse CD16/32 Fc γ III/II receptor (Pharmingen, Becton Dickinson) antibody, cells were stained with FITC-conjugated human CD45 (Immunotech).

Real-time RT-PCR studies of Hes-1 expression

Total RNA was extracted from output CD34⁺ cells using the SV total RNA isolation kit, and DNase-treated (Promega) before synthesizing cDNA using the SuperScriptII kit (Invitrogen Life

Technologies, Inc.). Primers and probes of Hes-1 were as follows: Hes-1 forward primer, 5'TGGAAATGACAGTGAA GCACCT3', Hes-1 reverse primer, 5'GTTTCATGCACTCGCT GAAGC3', Hes-1 probe 5'GCCAGATGACGGCTGCGCTG3'. Probes were labelled at the 5' end with the reporter dye molecule FAM and at the 3' end with the quencher dye molecule TAMRA (Applied Biosystems, Foster City, CA, USA). The β 2-microglobulin gene was used as an endogenous control (Applied Biosystems). PCR amplification was carried out for 10 min at 95°C followed by 50 cycles of 1 min at 60°C and 15 s at 95°C in an ABI PRISM 7700 Sequence Detector 5 (Applied Biosystems). Hes-1 RNA expression was normalized using β 2-microglobulin as the endogenous standard and JY cells (American Type Culture Collection, Manassas, VA, USA) as a positive standard (normalized target value).

Statistical analysis

Statistical significance was determined by Student's *t*-test (paired data analysis).

Results

Ectopic expression of Delta-4 in S17 cells and coculture condition

Since Notch ligands have been described to exert their activity as membrane-bound molecule, we first assessed the role of Delta-4 in modulating HSC fate via activation of their Notch receptors using a feeder engineered to express the human membrane-bound Delta-4 (mbDelta-4). The choice of the appropriate feeder was conditioned by the amount of expression of the various Notch ligands evaluated by RT-PCR. S17 and NIH3T3 cells were chosen, since they did not express Delta-4 and were already used to evaluate the role of several Notch ligands on HSC potential.^{14–17} Of note, S17 cells slightly expressed Jagged-1 transcripts but not the others Notch ligands (data not shown).

The effects of mbDelta-4 were first analyzed by using S17 cells. Two cell feeders have been established: one line containing the control vector (C/S17), and the other one expressing the mbDelta-4 protein (mbD4/S17). Expression of mbDelta-4 protein at the surface of mbD4/S17 cells was detected by flow cytometric analysis (Figure 1), without inducing the expression of the other Notch ligands (data not shown). To evaluate the effects of Delta-4-expressing stromas on the progeny of HSC during 4 weeks of culture, human primitive CD34⁺CD38^{low} cord blood cells were plated onto the different transfected stromas in the presence of early acting cytokines (rhuIL-3, rhuSCF, rhuPEG-MGDF, and rhuFlt3-L). At day 7, nucleated cells were enumerated, and output CD34⁺ cells were sorted to analyze their potential (CFCs, lympho/myeloid differentiative potential, LTC-IC). The study was pursued another week by replating 5000 output nucleated cells onto fresh stromas. The same procedure was repeated at day 14 and 21 for two more weeks, thus, enabling the evaluation from day 7 to 28 of culture, of the impact of Delta-4 on the number of nucleated cells, CD34⁺ cells, CFCs, and LTC-ICs.

mbD4/S17 reduces nucleated cell production by modifying the cell cycle status

The fold increase in total cell number obtained during the 4-week coculture on S17 cells is depicted in Figure 2. During the

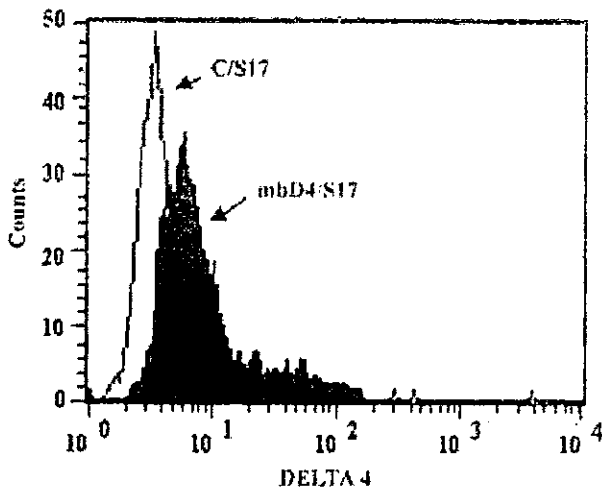


Figure 1 Delta-4 expression in S17 cells. Fluorescent histograms of S17 cells stained with antibodies, an affinity-purified rabbit antiserum raised to the extracellular domain of murine Delta-4. The y-axis represents cell number, and the x-axis represents log fluorescence intensity. The gray histogram represents mbD4/S17 stroma and the white C/S17 stroma. Mean fluorescence intensity: C/S17 = 4.77; mbD4/S17 = 11.90.

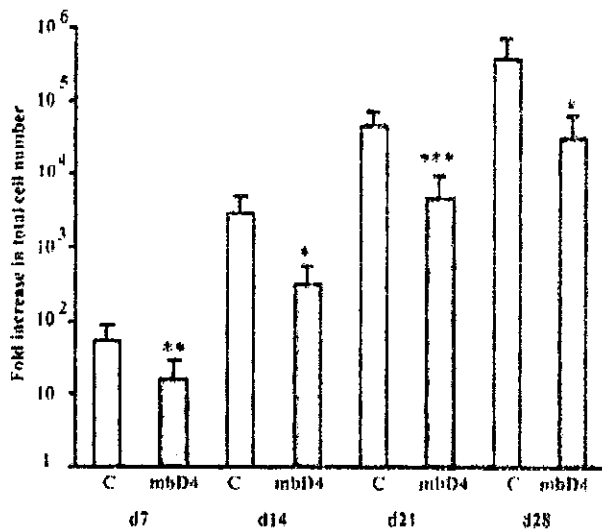


Figure 2 Effect of mbD4/S17 on nucleated cell production. 5000 CD34⁺CD38^{low} cells were added to wells coated with C/S17, or mbD4/S17 stromas in the presence of rhuSCF, rhuIL-3, rhuPGE-MGDF, and rhuFlt-3L. Cells were harvested at the indicated times, and 5000 output nucleated cells were replated onto the two different S17 stromas for another week of culture. This procedure was repeated for two additional weeks (results from five experiments). Significant difference, * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$.

first week, the fold increase in the total cell number was lower for cells growing on mbD4/S17 cells (16 ± 13 , mean $\pm$ s.d.; $P < 0.05$), as compared to cells growing on C/S17 stromas (54 ± 24). Despite the wide inter-cord blood variability between the samples, the cumulative nucleated cell production in the population grown on mbD4/S17 was always lower in all four timepoints, as compared to that grown on C/S17. Thus, on day 28, culture of CD34⁺CD38^{low} cells on mbD4/S17 led to a 12-fold reduced cell production ($P < 0.05$). Nucleated cell production between populations grown on C/S17 and parental S17 was not different (data not shown).

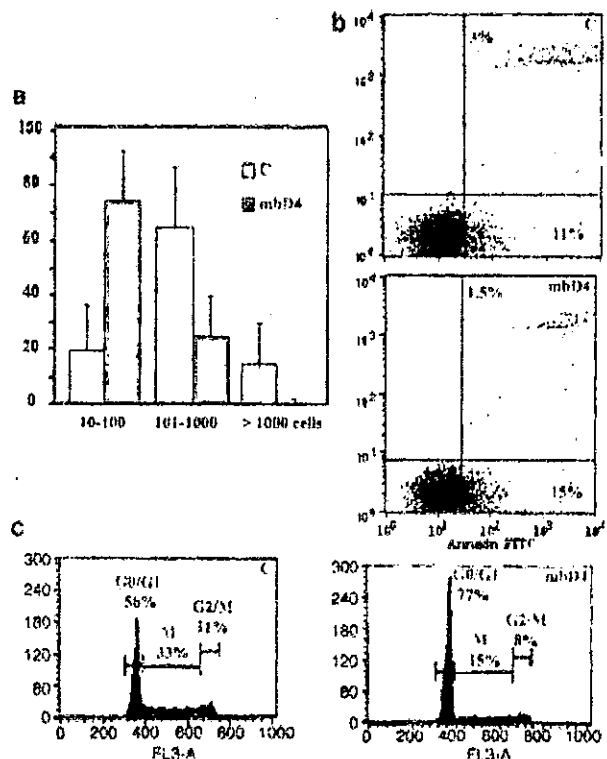


Figure 3 Effect of mbD4/S17 on viability and cell cycle status of nucleated cells. (a) Single cell cultures were established from CD34⁺CD38^{low} in the presence of cytokines on the C/S17, and mbD4/S17 stromas. The numbers of nucleated cells were estimated in each well at day 10 and the clones were classified according to their size (cell number per well) as indicated. At least 25 clones per experimental point have been analyzed. Each histogram represents the proportion of the proliferating wells containing the indicated number of nucleated cells (mean of four experiments). (b) Apoptosis analysis of one representative experiment. Output nucleated cells were harvested after 7 days of culture on both S17 stromas stained with propidium iodide and Annexin V, and analyzed by flow cytometry. (c) Cell cycle status of cells. Output nucleated cells were harvested after 7 days of culture on the S17 stromas, stained with propidium iodide and analyzed by flow cytometry. FACS analysis of one representative experiment.

To explore the mechanisms involved in the decrease of nucleated cell production by mbD4/S17, we analyzed the cloning efficiency and the proliferative potential of single CD34⁺CD38^{low} cells cultured on the S17 stromas. The number and the size of the proliferative clones were evaluated at day 10 (Figure 3a). The cloning efficiency, that is, the number of wells containing active cell proliferation (more than 10 cells/well) was identical whatever the stroma ($31 \pm 2\%$ C/S17, $31 \pm 2\%$ mbD4/S17), suggesting that mbD4/S17 did not act on input CD34⁺CD38^{low} cell survival. However, mbD4/S17 gave rise to mainly small-size clones (10–100 cells), while C/S17 gave rise to intermediate-size clones (101–1000 cells). These observations indicated that mbD4/S17 modulated either apoptosis or cell proliferation rate of daughter cells. Involvement of apoptosis in mbD4/S17 activity was excluded since Annexin V staining of output cells after 7 days of culture revealed the similarity in the mean viability of the two populations (Figure 3b; 11 ± 4 for C/S17 and 15 ± 4 for mbD4/S17). On the other hand, in agreement with the decreased cell production mediated by mbD4/S17, we observed in Figure 3c a lower percentage of cells in S phase, as compared to the cells grown on C/S17 (13 ± 4 vs $23 \pm 7\%$; $P = 0.003$), with a higher proportion of cells in G0/G1

phase (81 ± 4 vs $69 \pm 10\%$; $n=4$; $P=0.005$). All these data clearly indicated that mbD4/S17 counteracted cell proliferation induced by cytokines.

mbD4/S17 maintains a high proportion of CD34^{bright} cells

We next evaluated the influence of mbD4/S17 on the CD34⁺ cell production with high mean fluorescence intensity. At day 7, a high percentage of output CD34^{bright} cells (68 ± 5 – $74 \pm 7\%$) was maintained with no difference between the two different cultures (Figure 4a). Thereafter, in contrast to C/S17, mbD4/S17 was able to maintain a high proportion of CD34^{bright} cells (65 ± 8 , day 14; 40 ± 10 , day 21, 47 ± 3 , day 28 for mbD4/S17 vs 29 ± 16 , day 14; 9 ± 9 , day 21, 11 ± 2 , day 28, for C/S17). However, the total number of CD34^{bright} cells was similar to both conditions of culture due to reduced expansion of

nucleated cells with mbD4/S17 (data not shown). These data indicated that mbD4/S17 reduced cell production with a long-term maintenance of high percentage of CD34^{bright} cells.

mbD4/S17 enhances BFU-E production

We analyzed whether the ectopic expression of mbDelta-4 in S17 cells would alter the output CFC progenitors from input CD34⁺CD38^{low} cells. The clonogenicity in semisolid medium of output CD34⁺ cells cultured on mbD4/S17 and C/S17, sequentially generated at day 7, 14, and 21, was progressively lowered (data not shown). Examination of the type of colony did not reveal any difference in the number of CFU-GM per 1000 output CD34⁺ cells between the different cultures (data not shown). However, a constant and significant 2–3-fold increase in the number of BFU-E among the CD34⁺ cells exposed to mbD4/S17 was observed throughout the culture (Figure 4b), responsible for a slight but not significant increase in total CFC number from CD34⁺ cells stimulated by mbD4/S17.

mbDelta4 maintains the lymphoid and myeloid potential of output CD34^{bright} cells

To assess the early commitment of output CD34⁺ cells grown on mbD4/S17 and C/S17, we analyzed the expression of antigens such as CD7 and CD45RA representing committed lymphoid progenitors and CD38, Thy-1, and CD10 representing immature progenitors. At 7 days of culture, 60% of CD34^{bright} cells derived from mbD4/S17 culture coexpressed high levels of the CD7 antigen vs only 24% for CD34^{bright} cells generated on C/S17 (Figure 5a). CD45RA as well as the other markers studied did not display any modification of expression in the two CD34⁺ populations (Figure 5 and data not shown). Coexpression of CD7 and CD45RA on CD34⁺ cells represents the phenotype for the committed lymphoid precursor,²⁹ suggesting that activation by mbD4/S17 stroma promotes lymphoid differentiation of input CD34⁺CD38^{low} cells. To test this hypothesis, fresh CD34⁺CD38^{low} cells and output CD34⁺ cells from the 7-day culture on S17 stromas were seeded at one cell per well on MS-5 cells, maintained for 4 weeks in culture in the presence of six cytokines. The phenotype of each clone was determined (CD15 = G/M; CD19 = B; CD56 = NK) (Figure 5b). The cloning efficiency, that is, the number of wells containing active cell proliferation (more than 10 cells/well) was identical independent of the stroma ($52 \pm 5\%$ C/S17, $54 \pm 6\%$ mbD4/S17), slightly lower than the one of fresh CD34⁺CD38^{low} cells ($64 \pm 7\%$). Output CD34⁺ cells cultured on C/S17 stroma were characterized by a diminished differentiative potential, compared to the one of fresh CD34⁺CD38^{low} cells, with a decreased proportion of two-/three-lineage clones (31 ± 7 for CD34⁺ cells cultured on C/S17 vs 74 ± 11 for fresh CD34⁺CD38^{low} cells), and an increased proportion of one-lineage clones (69 ± 7 for CD34⁺ cells cultured on C/S17 stroma vs 26 ± 10 for fresh CD34⁺CD38^{low} cells). Inversely, mbD4/S17 significantly prevented the loss of the differentiative potential of output CD34⁺ cells. CD34⁺ cells derived from cultures on mbD4/S17 stroma generated higher proportion of two-/three-lineage clones (56 ± 13 , $n=6$, $P<0.01$), and a lower proportion of one-lineage clones (44 ± 13 , $n=6$, $P<0.01$). These data indicate that mbD4/S17 limited the loss of the differentiative potential in culture. Furthermore, examination of the differentiation potential of CD34⁺CD7⁺ cells (putative committed lymphoid progenitors) vs CD34⁺CD7[−] cells at the end of

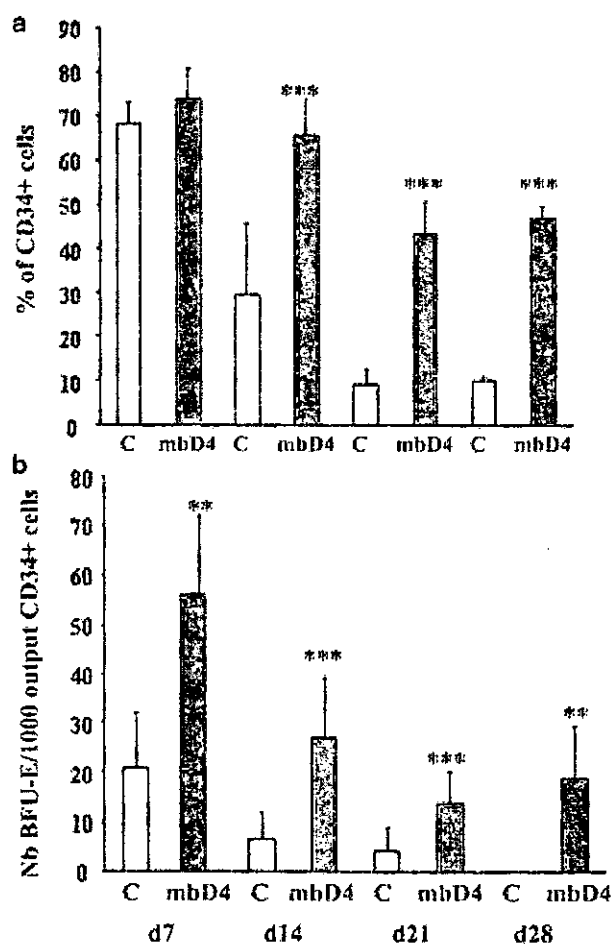


Figure 4 Effect of mbD4/S17 on CD34⁺ cells and BFU-E. 5000 CD34⁺CD38^{low} cells were added to wells coated with C/S17, or mbD4/S17 stromas in the presence of rhuSCF, rhuIL-3, rhuPEG-MGDF, and rhuFlt-3L. Cells were harvested at the indicated times, and 5000 output nucleated cells were replated onto the two different S17 stromas for another week of culture. This procedure was repeated for two additional weeks (results from five experiments). At days 7, 14, 21, and 28, output CD34⁺ cells were sorted and analyzed. (a) Changes in the frequency of CD34⁺ cells in the populations. (b) Number of BFU-E per 1000 output CD34⁺ cells. Significant difference, ** $P<0.02$; *** $P<0.01$.

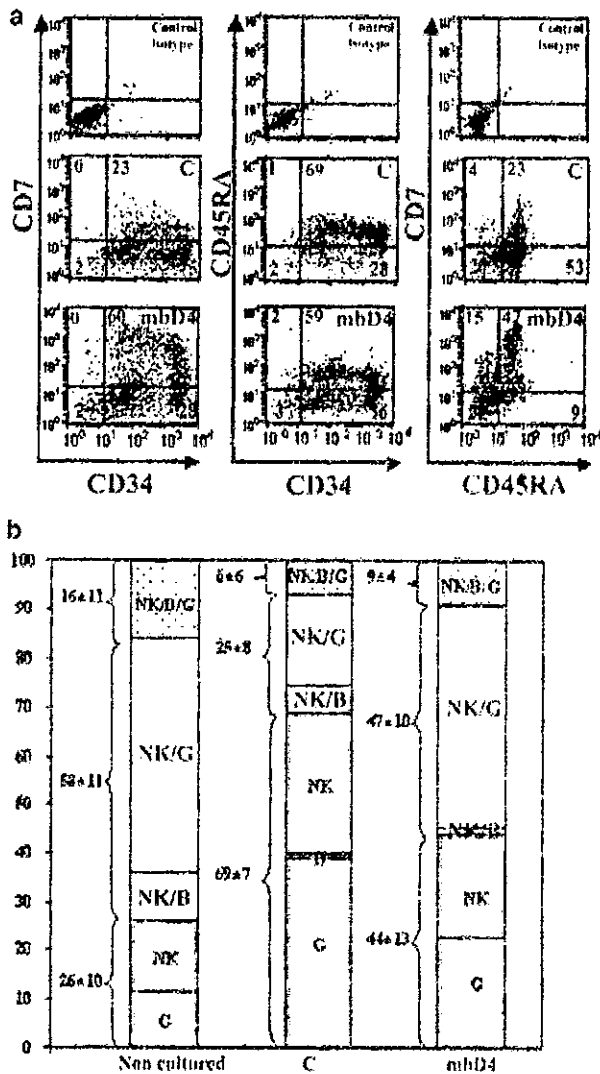


Figure 5 (a) FACS analysis for CD34, CD7, and CD45RA expression. 5000 CD34⁺CD38^{low} cells were added to wells coated with C/S17 or mbD4/S17 stromas as previously described. Seven days later nucleated cells were sorted and analyzed for CD34, CD7, and CD45RA expression (FACS analysis of one out of three independent experiments). Numbers in the graph are the percentages of gate events within the quadrant. (b) Phenotype of clones derived from input CD34⁺CD38^{low} cells and output CD34⁺ cells grown on C/S17 and mbD4/S17 stroma. 5000 CD34⁺CD38^{low} cells were added to wells coated with C/S17 or mbD4/S17 stromas as previously described. CD34⁺ cells were sorted 7 days later and examined for their myeloid (C/M) and lymphoid potential (B/NK) in a permissive culture. Proportions of the different types of clones were calculated as the ratio of clones containing one, two, or three lineages per total number of clones analyzed (mean of six experiments).

the 7-day culture on mbD4/S17 stroma cells did not reveal any difference (data not shown), indicating that CD34⁺CD7⁺ cells have the same capacity as CD34⁺CD7⁻ cells to differentiate towards myelopoiesis.

mbD4/S17 maintains the LTC-IC potential of input CD34⁺CD38^{low} cells

We next investigated whether mbDelta-4 would preserve in culture the LTC-IC potential of input CD34⁺CD38^{low} cells. In a

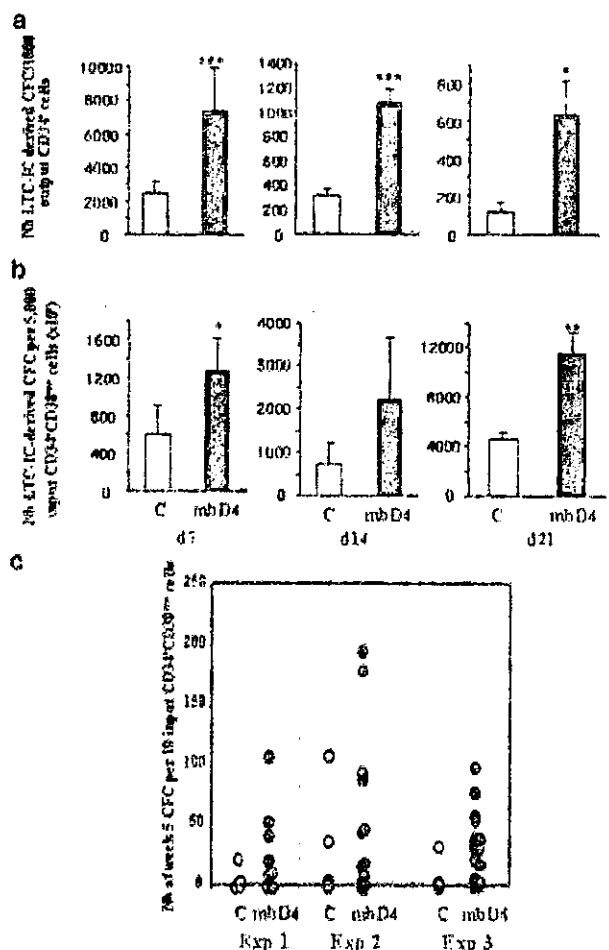


Figure 6 (a) Effect of mbD4/S17 on LTC-IC-derived CFC during the culture. 5000 CD34⁺CD38^{low} cells were added to wells coated with C/S17 or mbD4/S17 stromas in the presence of rhuSCF, rhuIL-3, rhuPEG-MGDF, and rhuFlt-3L. Cells were harvested at the indicated times and 5000 output nucleated cells were replated onto the two different S17 stromas for another week of culture. This procedure was repeated for an additional week (results from five experiments). At days 7, 14, and 21, output CD34⁺ cells were sorted and 1000 output CD34⁺ cells from each were then assayed for LTC-IC activity. (b) Number of LTC-IC-derived CFC per 5000 input CD34⁺CD38^{low} cells at day 0, day 7 ($n=5$), day 14 ($n=3$), day 21 ($n=3$). Significant difference, * $P<0.05$; ** $P<0.02$; *** $P<0.01$. (c) Comparison of the LTC-IC activity of cells having completed five divisions. CFSE-labelled CD34⁺CD38^{low} cells were cultured with cytokines in the presence of C/S17 (white circle) or mbD4/S17 (gray circle). On day 2, cells from each culture that had completed 1 division were sorted and replated on fresh stromas. On day 4, cells having accomplished five divisions were sorted and assayed for LTC-IC activity. Each point represents the number of CFC produced after 5 weeks on MS-5 cells by 10 CD34⁺CD38^{low} cells that have accomplished five divisions.

first series of experiments, we assessed the LTC-IC potential by plating output CD34⁺ cells harvested at day 7 until day 21 in long-term culture. As shown in Figure 6a, the total number of LTC-IC-derived CFC from output CD34⁺ cells decreased throughout the 3-week coculture whatever the stroma. The striking observation was the significantly higher production of CFC-derived LTC-IC from CD34⁺ cells exposed to mbD4/S17 at day 7, 14 ($P<0.01$), and day 21 ($P<0.05$). The total number of LTC-IC-derived CFC produced from 5000 input CD34⁺CD38^{low} cells was also significantly higher in the presence of mbD4/S17 by day 7 ($P<0.05$) and day 21

($P < 0.02$), as compared to C/S17 (Figure 6b). A higher but not significant number of LTC-IC-derived CFC was also observed in $CD34^+CD38^{low}$ cells stimulated by mbD4/S17 at day 14.

In a second series of experiments, we quantified the LTC-IC in $CD34^+CD38^{low}$ cells at day 0 and in output $CD34^+$ cells at day 7, by plating cells at limiting dilution. LTC-IC frequency in output $CD34^+$ cells grown on mbD4/S17 was not significantly different from LTC-IC frequency in input $CD34^+CD38^{low}$ cells (0.14 ± 0.08 vs 0.18 ± 0.08). Conversely, LTC-IC frequencies of output $CD34^+$ cells grown on C/S17 were significantly diminished (0.02 ± 0.02 , $P < 0.01$; $n = 5$), compared to that in input cells. Interestingly, mbD4/S17 maintained LTC-IC with high proliferative capacity at day 7, since the average number of CFC per LTC-IC was identical to that from input $CD34^+CD38^{low}$ (respectively, 23 ± 11 vs 26 ± 5). In contrast, the average number of CFC per LTC-IC was reduced in LTC-IC produced on C/S17 (13 ± 8). On day 7, the absolute number of LTC-IC was significantly higher ($P < 0.05$) in cell population grown on mbD4/S17. From 5000 input $CD34^+CD38^{low}$ cells containing initially an average of 915 (± 916) LTC-ICs, the mean production was 4765 (± 2125) LTC-ICs at day 7 on mbD4/S17, compared to 1660 (± 990) LTC-IC for C/S17. Thus mbD4/S17 induced a true expansion of LTC-IC.

Since an inverse correlation has been observed between LTC-IC potential and the number of cell division,^{25,26,30} the maintenance of the LTC-IC potential by mbDelta-4 could be only due to the reduced number of cell divisions. To test this hypothesis, we compared the LTC-IC potential of $CD34^+CD38^{low}$ cells having undergone the same number of divisions. CFSE-labelled $CD34^+CD38^{low}$ cells were cultured on the different S17 stromas, and at day 2, cells having accomplished one division in each culture were purified, and replated on fresh stromas for two additional days. LTC-IC activity was assessed in subset of cells having undergone five divisions from day 0. The results of three experiments are depicted in Figure 6c. The retention of LTC-IC activity mediated by mbD4/S17 was observed in all experiments: the geometric mean of LTC-IC-derived CFCs per 10 input $CD34^+CD38^{low}$ cells was significantly higher in the population grown on mbD4/S17 (14 ± 27 in Exp1; 40 ± 62 in Exp2; 27 ± 29 in Exp3, $P = 0.03$ for all experiments), as compared to the control population (2 ± 5 in Exp1; 9 ± 27 in Exp2; 4 ± 8 in Exp3). Thus, mbDelta-4 still maintained a high LTC-IC potential in cells having performed a similar number of doubling, indicating that LTC-IC retention by mbDelta4 is also mediated by mechanisms independent of the cell cycle.

mbD4/S17 does not enhance the in vivo marrow-repopulating ability in NOD-SCID mice

Since mbD4/S17 maintained the LTC-IC potential of output $CD34^+$ cells, we next tested the activity of mbDelta4 on the *in vivo* marrow-repopulating ability in NOD-SCID mice. For this purpose, we transplanted 2500 fresh $CD34^+CD38^{low}$ cells along with 10^5 irradiated (20 Gy) $CD34^{neg}$ cord blood cells, or all the progeny of 2500 or 10000 $CD34^+CD38^{low}$ cells cultured for 10 days onto C/S17 and mbD4/S17, into sublethally irradiated NOD/SCID mice. To cast off the difference of proliferation, we injected all the progeny of $CD34^+CD38^{low}$ cells grown on C/S17 and mbD4/S17 stromas whatever the number of cells. As expected, total cell production was higher in the populations grown on C/S17 (585 ± 15 and 600 ± 80 fold for 2500 or 10000 $CD34^+CD38^{low}$ cells, respectively) than in the populations grown on mbD4/S17

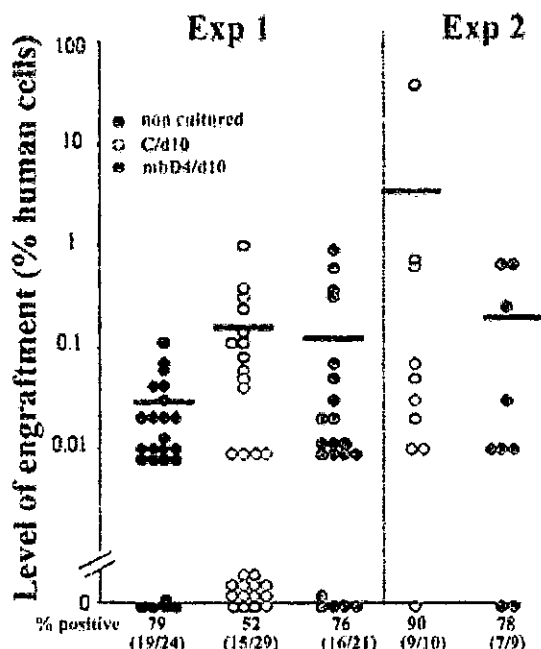


Figure 7 Effect of mbD4/S17 on engraftment of human cells in marrow of NOD/SCID mice. In all, 2500 fresh $CD34^+CD38^{low}$ cells along with 10^5 irradiated (20 Gy) $CD34^{neg}$ cord blood cells, or all the progeny of 2500 or 10000 $CD34^+CD38^{low}$ cells cultured for 10 days onto C/S17 or mbD4/S17, were transplanted into sublethally irradiated NOD/SCID mice. Analysis of the presence of human $CD45^+$ cells in the bone marrow of engrafted NOD-SCID mice was performed 10 weeks later. The percent of human $CD45^+$ cells was established from FACS analysis of at least 10^5 events. Each symbol represents the levels of a single chimeric mouse.

(110 ± 12 and 88 ± 16 for 2500 and 10000 $CD34^+CD38^{low}$ cells, respectively). Analysis of the presence of human $CD45^+$ cells in the bone marrow of engrafted NOD-SCID mice 10 weeks later showed a low chimerism in all conditions. We did not observe any significant difference in the percent of positive mice (Figure 7) in both experiments. Moreover, the level of engraftment between populations grown on C/S17 and mbD4/S17, whatever the number of expanded $CD34^+CD38^{low}$ cells was not different (0.17 ± 0.25 in Exp1; 4.4 ± 13 in Exp2 for C/S17 stroma vs 0.15 ± 0.26 in Exp1; 0.2 ± 0.3 in Exp2 for mbD4/S17 stroma). These data indicated that although mbDelta4 was able to enhance the production of progenitors such as LTC-IC, it did not enhance the production of primitive stem cells such as the long-term SCID repopulating cells.

Assessment of mbDelta-4 specificity

We next raised the question of whether the reduction of cell production and the retention of the primitive potential were specific for mbDelta4. To rule out any role of Jagged-1 (since S17 cells also slightly coexpressed the Jagged-1 ligand), we derived NIH3T3 lines expressing mbDelta-4 to assess Delta-4 activity on $CD34^+CD38^{low}$ cells in the same protocol as described above (NIH3T3 cells do not express Jagged-1 and neither other Notch ligands). Expression of mbDelta4 was confirmed on mbD4/3T3 cells (data not shown). The results from two experiments revealed that mbD4/3T3, like mbD4/S17, reduced nucleated cell production from input $CD34^+CD38^{low}$

cells. In contrast to C/3T3, it maintained a high proportion of CD34^{high} cells characterized by a high LTC-IC potential (data not shown). To assess the specific role of mbDelta-4 on cell production with regard to other Notch ligands, we have examined the activity of another Notch ligand, mbDelta-1, by culturing CD34⁺CD38^{low} cells onto a S17 stroma expressing mbDelta-1 using the protocol described above. In our culture conditions, mbDelta-1 did not modify nucleated cell expansion as compared to control stromas (data not shown). Finally, to assess the potential role of the cytokines, we examined the activity of mbD4/S17 on the progeny of CD34⁺CD38^{low} using other combinations of cytokines (PEG-MGDF/IL3/Flt-3L/SCF/IL-7 or PEG-MGDF/IL3/Flt-3L/SCF/G-CSF or PEG-MGDF/IL3/Flt-3L/SCF/GM-CSF). Whatever the combinations used, we still observed the reduced cell expansion in cell populations grown onto mbD4/S17 stroma, indicating that the reduced cell production was not relative to the cytokines used. Taken together, all these data strongly suggested the specific involvement of mbDelta-4 in the observed effects.

To evaluate whether the culture of CD34⁺ cells on mbDelta4-expressing stromas induces Notch signalling, we assessed the expression of Hes-1, a well-known target gene of Notch signalling, using quantitative RT-PCR in cells generated by the 7-day coculture of CD34⁺CD38^{low} cells on both NIH3T3 stromas. Indeed, output CD34⁺ cells generated in culture with mbD4/3T3 showed a significant increase in Hes-1 mRNA expression, ranging from 1.4- to 28-fold according to the sample, leading to a mean value of 11-fold ($n=5$, $P=0.04$), as compared to control cells.

Having established the activity of Delta4 on CD34⁺CD38^{low} cells as membrane-bound molecule, we next wondered whether the purified protein would produce the same effects. We therefore performed cultures with a chimeric Delta-4 protein, consisting of the extracellular domain of human Delta4 fused to the Fc portion of human IgG1. Since previous work has demonstrated that the Notch ligands must be immobilized on a plastic surface for Notch activation,^{12,13,31,32} we therefore assessed the role of Delta4-Fc on CD34⁺CD38^{low} cells according to this way of presentation. To strengthen the interactions between Delta4-Fc and CD34⁺CD38^{low} cells, we coated the plastic with retronectin,¹² and cultured CD34⁺CD38^{low} cells for 7 days. By the end of the culture, we examined the number of nucleated cells, the percent of CD34⁺ cells, and their content of CFC and LTC-IC. Like mbDelta4, the presence of immobilized Delta4-Fc and retronectin led to a reduced production of nucleated cell (30 ± 17 fold, $n=6$, $P<0.001$), compared to the culture with irrelevant human IgG and retronectin (137 ± 58 fold) (Figure 8a). The same proportion of CD34⁺ cells was observed at this time of culture between Delta4-Fc-activated population (33% of CD34⁺ cells), and control culture (36% of CD34⁺ cells) (Figure 8b). Furthermore, Delta4-Fc induced a three-fold increase in the number of BFU-E among CD34⁺ cells ($n=4$, $P<0.01$). The LTC-IC frequency among these CD34⁺ cells was higher in the population stimulated with Delta4-Fc (0.38 ± 0.17), compared to the frequency determined in the control population (0.07 ± 0.05 , $n=6$, $P<0.01$) (Figure 8c). However, the total number of LTC-IC was similar in both conditions of culture due to reduced expansion of nucleated cells with Delta4-Fc (Figure 8d). These data indicate that Delta4-Fc shared most of the activities of the membrane-bound protein such as reduced nucleated cell production, enhanced BFU-E production, maintaining of a high LTC-IC potential but does not enhance LTC-IC expansion.

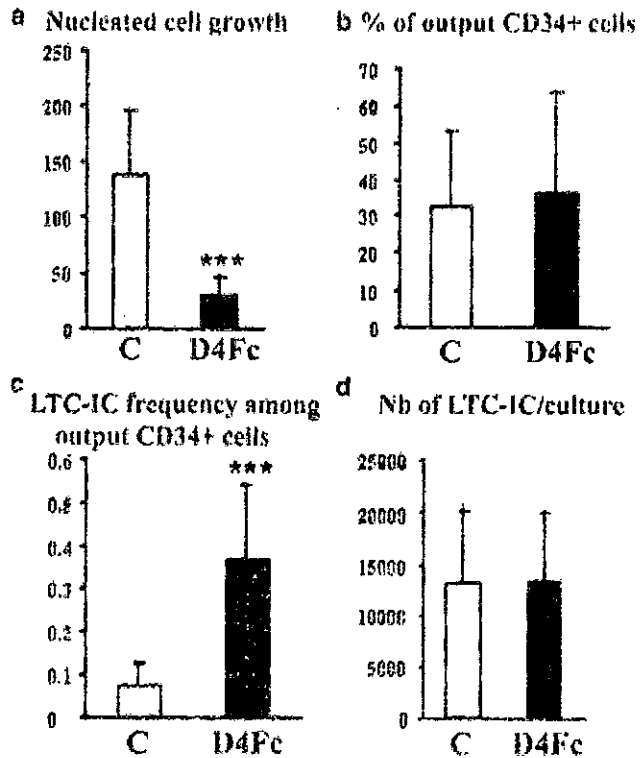


Figure 8 Effect of Delta4-Fc on the progeny of CD34⁺CD38^{low} cells. 5000 CD34⁺CD38^{low} cells were added to wells coated with Delta4-Fc as previously described. Seven days later, nucleated cells were counted, CD34⁺ cells were sorted, and their LTC-IC content was examined (mean of four experiments). (a) Nucleated cell growth. (b) % of CD34⁺ cells. (c) Frequency of LTC-IC in CD34⁺ cells. (d) Number of LTC-IC/culture. *** $P<0.01$.

Discussion

We report here a novel activity of a Notch ligand, the ligand Delta4, in which the main activity consists in counteracting cell proliferation of hematopoietic stem cells induced by cytokines. mbDelta4 maintained a high LTC-IC activity in output CD34⁺ cells, without retaining a long-term *in vivo* marrow-repopulating ability in NOD-SCID mice. mbDelta-4 induced the generation of cells of the phenotype of committed lymphoid precursors (CD34⁺CD45RA⁺CD7⁺), but these cells still retained their capacity to differentiate towards myelopoiesis. All these biological effects were associated with an overexpression of Hes-1, one of the well-known Notch target genes. These results are in agreement with a report indicating that overexpression of mbDelta-4 in murine HSC prevents their reconstitutive ability by blocking their differentiation into preCFU-S and day12CFU-S.³³

The first striking observation was the reduction in the total number of nucleated cells generated in the presence of mbDelta-4, compared to control culture. mbDelta-4 decreases the production of these cells, with neither impact on cell survival nor the recruitment of input CD34⁺CD38^{low} cells to proliferate, as indicated by the similar frequency of proliferating clones in single cell culture experiments between the different feeders. Instead, mbDelta-4 alters the cell cycle progression of daughter cells, by reducing cell proliferation, as indicated by the higher proportion of cells in G0/G1 phase, and the high proportion of small-size clones. Notch activation in other cellular contexts mediated growth arrest by upregulation of p21^{Cip} and p27^{Kip34} or cell proliferation by upregulation of

cyclin D1 and activation of CDK2.³⁵ Molecular mechanisms specific to the G0/G1 phase involved in the alteration of the cell cycle progression in CD34⁺ cells by mbDelta-4 remain to be identified.

The second major result is the enhancement of LTC-IC expansion by mbDelta4, contrary to control cultures. During the first week of culture, mbDelta-4 promotes a five-fold expansion over input LTC-IC, while the LTC-IC quality at day 7 was similar to that of parental CD34⁺CD38^{low} cells (ie same number of CFC per LTC-IC). These results correlated with the activity of mbDelta4 on the cell cycle and suggest that mbDelta-4 is a regulator of the LTC-IC potential by principally altering their cell cycle progression. However, when we compared the LTC-IC potential in a subset of cells having performed the same number of divisions, mbDelta-4 still maintained a higher LTC-IC potential. This suggests, besides the direct consequence of the reduced number of cell division, the existence of an alternative mechanism unrelated to cell proliferation implicated in the maintenance of LTC-IC potential by mbDelta4. Such maintenance of the LTC-IC potential independent of the mitotic history has been previously demonstrated with the MS-5 cell line, known to express Jagged2 and Delta1 Notch ligands.^{25,26}

Although mbDelta4 enhances LTC-IC expansion as compared to control cultures, culture of CD34⁺CD38^{low} cells on mbD4/S17 stroma did not enhance the long-term repopulation ability of output CD34⁺ cells in NOD-SCID mice. These data are in agreement with previous work showing that contrary to soluble Delta1, soluble Delta4 did not increase the long-term repopulation ability in NOD-SCID mice.⁸ Although the NOD/SCID model is not sufficient to observe a two-fold expansion of SRC, which needs recently developed models such as NOD/SCID $\beta 2m^{-/-}$ ³⁶ or NOD/SCID $\gamma c^{-/-}$,³⁷ we can conclude that mbDelta4 has no major effect on SRC expansion.

CD34⁺CD38^{low} cells stimulated by mbDelta4 mostly generated cells of the seemingly committed lymphoid precursor phenotype (CD34⁺CD45RA⁺CD7⁺).²⁹ This observation is in agreement with the work of Bernstein's group,¹² which described the generation of such cells after stimulation of CD34⁺CD38⁺ cells by Delta-1 immobilized on plastic. However, by comparing the lympho/myeloid differentiative potential of single output CD34⁺/CD7⁺ vs CD34⁺/CD7⁻ cells generated in culture with mbDelta4 (NK, G/M, B), we did not observe any difference between both populations. mbDelta4 rather maintains a high rate of multipotent clones, thus limiting the loss of the primitive potential. This suggests that in our hands activation of CD34⁺CD38^{low} cells by mbDelta4 maintains the level of pluripotent precursors, and does not promote the generation of cells exclusively committed towards the lymphoid lineage. Our results question the CD34⁺CD45RA⁺CD7⁺ phenotype as phenotype of cells possessing only the lymphoid potential.²⁹

The results with the chimeric Delta4Fc protein, presented as immobilized molecule, indicate that it shares most of the activities of mbDelta4. Delta4Fc reduces cell proliferation, and maintains a higher LTC-IC frequency, as compared to control population. However, Delta4Fc, contrary to mbDelta4, does not increase the total number of LTC-IC, due to the reduced expansion of nucleated cells. This discrepancy suggests that Delta4, presented as membrane-bound molecule, provides additional signals, enabling access of enzymes or other accessory molecules necessary for the complete response.

mbDelta4, as Notch ligand, exerts a particular activity by keeping HSC out of the cell cycle, preventing them to lose their primitive potential. These results are in keeping with the general conundrum surrounding the role of Notch receptors in the

regulation of HSC. The literature reveals that Notch signalling is able to enhance HSC self-renewal, to enhance HSC proliferation, or to promote cell survival. The role of Notch in HSC self-renewal has been clearly demonstrated in the case of constitutive activation of the intracellular domain of Notch1 in murine HSC^{10,11} and of activation of the Notch receptors by membrane-bound Jagged1 or Jagged2 or immobilized Delta1.¹²⁻¹⁶ An enhancement of cell proliferation with the retention of the primitive potential has been ascribed to soluble forms of Jagged1 and Delta1.^{2,8,18} In another study, membrane-bound Jagged2 was enhancing HSC survival.⁹ All these studies describing pleiotropic activities of Notch signalling on HSC suggest the existence of different ways of altering the conformation of Notch. Notch signalling occurs through several pathways that may be equally important. Some imply the Notch $\rightarrow$ CSL $\rightarrow$ Hes $\rightarrow$ bHLH pathways, other pathways are Hes independent.⁴ Nevertheless, even if Notch signalling enhances Hes-1 expression, this can lead to opposite activities in the same cellular context, for example, mbDelta-4 inhibits cell proliferation while immobilized Delta1 rather favors HSC self-renewal.¹² The different Notch ligands as well as their different modes of presentation could not act through the same Notch receptor. Alternatively, they could interact with the same receptor but may recruit different intracellular partners depending on signalling intensity, as hypothesized in the signalling threshold model on stem cell fate decision.³⁸

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