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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 13-296752-00006-0000

Fecha: 2015-03-10 16:36:51 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 523 RESPUESTA Folios: 25

Señor

SUPERINTENDENTE DE INDUSTRIA Y COMERCIO

DIVISIÓN DE NUEVAS CREACIONES

E. _____ S. _____ D. _____

Trámite: 11
Evento: 378
Actuación: 523

Re: PI.-INCYTE CORPORATION.-Solicitud de Patente de Invención en Fase Nacional PCT para "DERIVADOS DE AZETIDINIL FENIL, PIRIDIL O PIRAZINIL CARBOXAMIDA COMO INHIBIDORES DE JAK".-Clase C07D 471/04.-Expediente número 13-296.752.-

1. Me refiero al auto dictado por ese Despacho notificado por fijación en lista de 04 de Noviembre de 2014, así como a mi solicitud de plazo del 21 de Enero de 2015 para dar respuesta al auto arriba mencionado.

2. Con el fin de responder a las objeciones expuestas por el Señor Examinador en su Concepto Técnico correspondiente al Oficio No. **13618**, en relación con Excepción a la Patentabilidad, Claridad del Capítulo Reivindicatorio y Nivel Inventivo, me permito presentar los siguientes argumentos técnicos y las siguientes modificaciones en el capítulo reivindicatorio de la solicitud de patente en cuestión, por medio de lo cual se superan completamente dichas objeciones.

3. **EXCEPCIÓN A LA PATENTABILIDAD**

El Señor Examinador objeta las reivindicaciones 34 a 49 aduciendo que éstas no se consideran materia patentable en vista del literal (d) del artículo 20 de la Decisión 486 de 2000.

En respuesta a lo anterior, me permito señalar que el solicitante ha decidido **eliminar las reivindicaciones 34 a 39 presentadas originalmente,** con lo cual se supera la objeción realizada por el Señor Examinador a éste respecto.

4. **CLARIDAD A LAS REIVINDICACIONES**

De conformidad con los argumentos expuestos por el Señor examinador en su Concepto Técnico, el capítulo reivindicatorio originalmente presentado carece de claridad porque:

- Las reivindicaciones 1, 17 y 21 emplean el término "*opcionalmente*", el cual no permite distinguir la invención del estado de la técnica.

Adicionalmente a lo anterior, la reivindicación 1 no es concisa, ya que las definiciones de W,X,R1,R2 y R5 son generales, por lo que el Señor Examinador sugiere mencionar únicamente los sustituyentes debidamente sustentados mediante ejemplos concretos.

Con el fin de superar las observaciones anteriormente señaladas, me permito dirigir la atención del señor Examinador al hecho de que el solicitante ha decidido eliminar las reivindicaciones 17 y 21, y modificar la reivindicación 1 originalmente presentada, especificando cada uno de los sustituyentes mencionados y eliminando el término "*opcionalmente*".

- Por otro lado, la reivindicación 33 define una composición que incluye los compuestos reclamados incorporados en excipientes farmacéuticamente aceptables. Sin embargo, una composición debe definirse mediante la mención de los ingredientes junto con el nombre común de los excipientes y las proporciones de cada uno de estos.

Ahora bien, con el fin de superar la observación anteriormente mencionada, el solicitante ha decidido eliminar la reivindicación 33 originalmente presentada.

- Igualmente, las reivindicaciones dependientes 22, 25 y 26, 23, 27 y 28, 24, 29 y 30 repiten materia reclamada de forma innecesaria. Por lo cual el Señor Examinador sugiere dejar una de cada grupo de las reivindicaciones mencionadas.

Con el fin de superar las observaciones anteriormente señaladas, me permito dirigir la atención del señor Examinador al hecho de que el solicitante ha decidido eliminar las reivindicaciones 22, 23, 24, 26, 27 y 28, y modificar las reivindicaciones 25, 29 y 30 originalmente presentadas, con su respectivo sustento.

5. MODIFICACIONES AL CAPÍTULO REIVINDICATORIO

Ahora bien, me permito manifestar que de acuerdo a las objeciones mencionadas en el numeral 4, es deseo del solicitante presentar un **capítulo reivindicatorio modificado**, que reemplaza al ya presentado.

En conexión con el mencionado **capítulo reivindicatorio modificado** me permito resaltar que este no constituye de ninguna manera una ampliación al alcance de la invención, tal como fue originalmente presentada, dichas modificaciones se exponen a continuación:

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Nueva reivindicación 1: Ha sido modificada, eliminando la palabra "opcionalmente", además de especificar claramente que:

W es N o CR⁶.

X es N o CR⁴.

R¹ es isopropil, etil, 1-metilpropil, 2,2,2-trifluoro-1-metiletil, 1-ciclopropiletil, 1-cicloexiletil, ciclopropil, 1-trifluorometilciclopropil, 3,3-difluorociclobutil, 1-(1-metilpiperidin-4-il)etil, 1-ciclopropil-2,2,2-trifluoroetil, 2,2,2-trifluoroetil, o 2,2-difluoroetil.

R² es H o metilo.

R³ es H, F, o Cl.

R⁵ es H o F.

Donde dichos sustituyentes se encuentran debidamente soportados por las reivindicaciones 18 a 20 originalmente presentadas.

Nuevas reivindicaciones 2 y 3: Corresponden a las reivindicaciones 2 y 3 originalmente presentadas.

Nuevas reivindicaciones 4 y 5: Corresponden a las reivindicaciones 5 y 6 originalmente presentadas, donde ha sido modificada la dependencia a las reivindicaciones 1 a 3.

Nuevas reivindicaciones 6 y 7: Corresponden a las reivindicaciones 8 y 9 originalmente presentada, donde ha sido modificada la dependencia a las reivindicaciones 1 a 5.

Nueva reivindicación 8: Corresponde a la reivindicación 15 originalmente presentada, donde ha sido modificada la dependencia a las reivindicaciones 1 a 7.

Nueva reivindicaciones 9, 10 y 11: Corresponden a las reivindicaciones 25, 29 y 30 originalmente presentada, donde ha sido modificada la dependencia a la reivindicación 1.

Nueva reivindicación 12: Corresponde a una nueva reivindicación, la cual se encuentra debidamente soportada por el ejemplo 28 de la solicitud presentada.

Nueva reivindicación 13: Corresponde a una nueva reivindicación, la cual se encuentra debidamente soportada por el ejemplo 29 de la solicitud presentada.

Nueva reivindicación 14: Corresponde a una nueva reivindicación, la cual se encuentra debidamente soportada por el ejemplo 18 de la solicitud presentada.

Nueva reivindicación 15: Corresponde a una nueva reivindicación, la cual se encuentra debidamente soportada por el ejemplo 34 de la solicitud presentada.

Nueva reivindicación 16: Corresponde a las reivindicaciones 31 y 32, donde se ha eliminado la dependencia.

6. **ANÁLISIS DEL NIVEL INVENTIVO DE LA SOLICITUD**

De otro lado, el Señor Examinador ha objetado el nivel inventivo de la solicitud en cuestión, basándose en las enseñanzas de los siguientes documentos:

D1 → WO2010039939

D2 → US20090233903

En este sentido, el señor Examinador argumenta que la reivindicación 1 y las reivindicaciones dependientes carecen de nivel inventivo, exponiendo tres modalidades diferentes, **Modalidad donde W y X son C, Modalidad donde W es N o C o X es N o C, y Modalidad donde W es N y X es N**, en donde, el Señor Examinador señala que el documento D1 y D2 se consideran el estado de la técnica cercano a la invención, ya que D1 divulga compuestos derivados de pirazol-cianociclobutano como inhibidores de la actividad de los receptores JAK quinasa, los cuales difieren en el anillo ciclobutano, a diferencia de los compuestos reclamados que presentan en su estructura un anillo azetidino, y D2 divulga derivados de pirazol-azetidina, que incluyen un anillo azetidino, como moduladores de la actividad JAK útiles en el tratamiento de enfermedades inflamatorias, desordenes autoinmunes y cáncer.

Sin embargo es importante resaltar que los compuestos reclamados contienen diferencias estructurales significativas con respecto a los divulgados en las referencias D1 y D2.

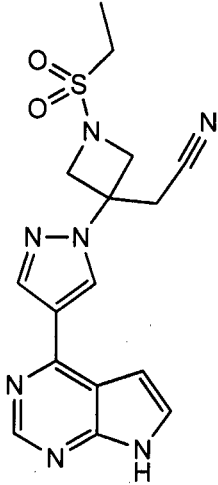
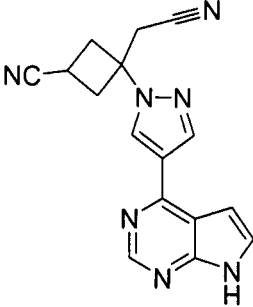
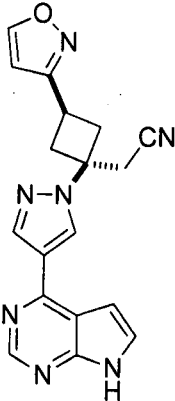
En particular, los compuestos reclamados presentan una amida sustituida por fenil, piridil o pirazinil, la cual se encuentra unida al anillo de azetidina, a diferencia de los compuestos divulgados en D1 o D2, que no presentan esta particularidad. En consecuencia, una persona normalmente versada en la materia no se sentiría motivada en realizar modificaciones estructurales necesarias para llegar a los compuestos reclamados.

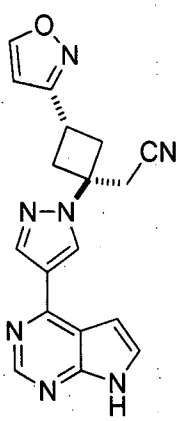
Por otra parte, el Señor Examinador establece que el único efecto de la materia reclamada consiste en la actividad inhibitoria JAK. Frente a esta afirmación, me permito dirigir la atención del Señor Examinador, a que los compuestos reivindicados son inhibidores selectivos de JAK1, y cada uno de los compuestos descritos en los ejemplos muestran una selectividad 10 veces mayor para JAK1 sobre JAK2, cuando estos se

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ensayaron a 1 mM ATP (Descripción de la presente solicitud, página 122, línea 18 hasta la página 124, línea 3). Por el contrario, ninguno de los compuestos de los ejemplos de D1 y D2 son selectivos para JAK1 sobre JAK2. Por ejemplo, el Señor Examinador identifica varios compuestos en D1 y D2 que se muestran en la siguiente tabla, donde cada uno de los ejemplos señalados no muestra ninguna selectividad para JAK1 sobre JAK2.

Estructura de los compuestos de D1 o D2	JAK1 IC ₅₀ (nM)	JAK2 IC ₅₀ (nM)
 D1 y D2	4.3	3.7
 D1 y D2	3.0	1.8
 D1 y D2	3.5	< 1.1

 D1 y D2	2.2	1.9
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Como se detalla en la descripción de la presente solicitud en la página 32, línea 15 a página 33, línea 4, los inhibidores selectivos de JAK1, en relación con otras quinasas JAK, puede tener varias ventajas terapéuticas sobre aquellos inhibidores menos selectivos.

Teniendo en cuenta lo anterior, es importante resaltar que la inhibición selectiva de JAK1 sobre JAK2 puede reducir los efectos secundarios indeseables resultantes de la inhibición de JAK2, ya que esta inhibición, puede presentar efectos secundarios, tales como la supresión inmune, anemia y trombocitopenia, mediante la reducción de la señalización de Epo y Tpo.

Epo es un factor clave de crecimiento para la producción de glóbulos rojos; por lo tanto, una escasez en la señal dependiente de EPO puede resultar en un número reducido de glóbulos rojos y anemia (Anexo 1. *Kaushansky K, NEJM 354: 2034-45, 2006*).

Tpo, es otro ejemplo de un factor de crecimiento dependiente de JAK2, el cual desempeña un papel central en el control de la proliferación y maduración de los megacariocitos - las células de las que se producen las plaquetas (Anexo 1. *Kaushansky K, NEJM 354: 2034-45, 2006*). Como tal, una disminución en la señal Tpo, reduciría la cantidad de megacariocitos y un menor recuento de plaquetas (trombocitopenia), lo que puede resultar en hemorragia indeseable y/o incontrolable.

En adición a esto, la reducción de la inhibición de otras JAKs, tales como JAK3 y Tyk2, también puede ser deseable, como han demostrado los seres humanos que carecen de la versión funcional de estas quinasas. Estas personas, sufren de numerosas enfermedades tales como la inmunodeficiencia severa combinada o síndrome de hiper IgE E (Anexo 2. *Minegishi, Y, y col. Inmunidad 25:745-55, 2006*; Anexo 3. *Macchi P, y*

col. Naturaleza 377: 65-8, 1995). Por lo tanto un inhibidor de JAK1 con afinidad reducida para otros JAKs tendría ventajas significativas sobre un inhibidor menos selectivo con respecto a la reducción de los efectos secundarios relacionados con la supresión inmune, anemia y trombocitopenia.

Con base en lo anteriormente mencionado, la selectividad inesperada de los compuestos reclamados para JAK1 sobre JAK2 está ligada directamente a un perfil terapéutico no visto desde los inhibidores no selectivos.

Por lo tanto, atentamente me permito solicitar al Señor Examinador la reconsideración de las objeciones realizadas en el Concepto Técnico N° 13618 a éste respecto.

7. **CONCLUSIÓN**

Para finalizar me permito reiterar que el capítulo reivindicatorio modificado presentado junto con este memorial es totalmente claro y conciso al reclamar los compuestos de la invención, y está plenamente soportado dentro del texto de la descripción, cumpliendo así con lo exigido por la mencionada Decisión Andina 486 de 2000.

Por otro lado, de conformidad con el análisis referente al Nivel Inventivo de la Solicitud, es claro que la solicitud en estudio cumple con los requisitos de patentabilidad y por lo tanto es merecedora del privilegio de patente solicitado.

8. Anexo al presente memorial me permito presentar:

- a) Copia de las páginas **131 a 137** que corresponden al capítulo reivindicatorio modificado, el cual está compuesto por **16** reivindicaciones y reemplaza al documento actualmente presentado.
- b) Copia del artículo: *Kaushansky K, NEJM 354: 2034-45, 2006*.
- c) Copia del artículo: *Minegishi, Y, y col. Inmunidad 25:745-55, 2006*; y
- d) Copia del artículo: *Macchi P, y col. Naturaleza 377: 65-8, 1995*.

los cuales sustentan el Análisis Inventivo de la Solicitud.

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9. En vista de lo anterior, ruego a usted proceder a estudiar nuevamente la solicitud a la luz de los argumentos presentados y conceder el privilegio de patente solicitado.

Señor Superintendente,

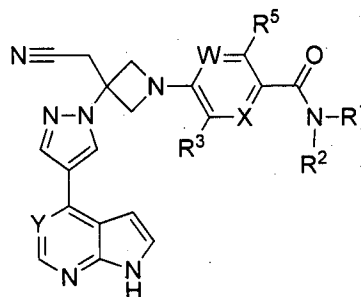


ALICIA LLOREDA RICAURTE

T.P. 53.215

REIVINDICACIONES

1. Un compuesto de Fórmula I:



(I)

o una sal farmacéuticamente aceptable del mismo, donde:

X es N o CR⁴;

W es N o CR⁶;

Y es N o CR⁷;

R¹ es isopropil, etil, 1-metilpropil, 2,2,2-trifluoro-1-metiletil, 1-ciclopropiletil, 1-cicloexiletil, ciclopropil, 1-trifluorometilciclopropil, 3,3-difluorociclobutil, 1-(1-metilpiperidin-4-il)etil, 1-ciclopropil-2,2,2-trifluoroetil, 2,2,2-trifluoroetil, o 2,2-difluoroetil;

R² es H o metilo;

R³ es H, F, o Cl;

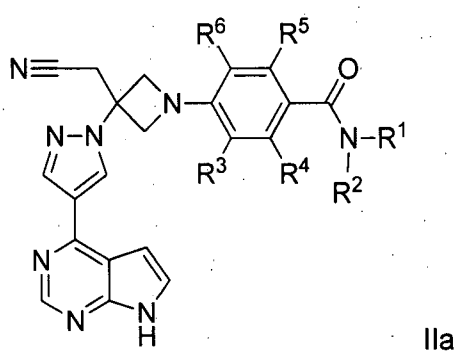
R⁴ es H o F;

R⁵ es H o F;

R⁶ es H; y

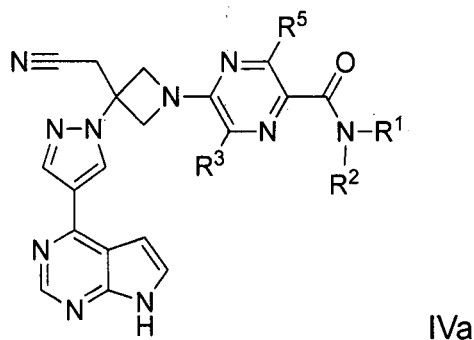
R⁷ es H.

2. El compuesto de acuerdo con la reivindicación 1, o una sal farmacéuticamente aceptable de este, donde Y es N.
3. El compuesto de acuerdo con la reivindicación 1, o una sal farmacéuticamente aceptable de este, donde Y es CR⁷.
4. El compuesto de acuerdo con cualquiera de las reivindicaciones 1 a 3, o una sal farmacéuticamente aceptable de este, donde X es N.
5. El compuesto de acuerdo con cualquiera de las reivindicaciones 1 a 3, o una sal farmacéuticamente aceptable de este, donde X es CR⁴.
6. El compuesto de acuerdo con cualquiera de las reivindicaciones 1 a 5, o una sal farmacéuticamente aceptable de este, donde W es N.
7. El compuesto de acuerdo con cualquiera de las reivindicaciones 1 a 5, o una sal farmacéuticamente aceptable de este, donde W es CR⁶.
8. El compuesto de acuerdo con cualquiera de las reivindicaciones 1 a 7, o una sal farmacéuticamente aceptable de este, donde R² es H.
9. El compuesto de acuerdo con la reivindicación 1 que tiene la Fórmula IIa:



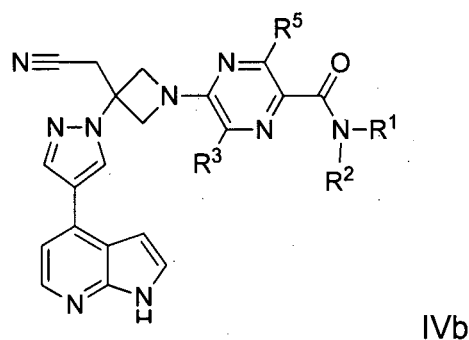
o una sal farmacéuticamente aceptable de este.

10. El compuesto de acuerdo con la reivindicación 1 que tiene la Fórmula IVa:



o una sal farmacéuticamente aceptable de este.

11. El compuesto de acuerdo con la reivindicación 1 que tiene la Fórmula IVb:



o una sal farmacéuticamente aceptable de este.

12. El compuesto, el cual es 4-{3-(cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-2,5-difluoro-N-[(1S)-2,2,2-trifluoro-1-metiletil]benzamida, o una sal farmacéuticamente aceptable de este.
13. El compuesto, el cual es 4-{3-(cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-2,5-difluoro-N-[(1R)-2,2,2-trifluoro-1-metiletil]benzamida, o una sal farmacéuticamente aceptable de este.
14. El compuesto, el cual es 5-{3-(cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-isopropilpirazine-2-carboxamida.

15. El compuesto, el cual es 5-{3-(cianometil)-3-[4-(1H-pirrolo[2,3-b]piridin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-isopropilpirazina-2-carboxamida, o una sal farmacéuticamente aceptable de este.

16. Un compuesto que se selecciona de:

4-{3-(Cianometil)-3-[4-(1H-pirrolo[2,3-b]piridin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-isopropilbenzamida;

5-{3-(Cianometil)-3-[4-(1H-pirrolo[2,3-b]piridin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-[(1S)-1-ciclopropiletil]piridina-2-carboxamida;

4-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-3-fluoro-N-isopropilbenzamida;

4-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-[(1R)-1-ciclopropiletil]-3-fluorobenzamida;

4-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-[(1S)-1-ciclopropiletil]-3-fluorobenzamida;

4-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-2,5-difluoro-N-isopropilbenzamida;

4-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-ciclopropil-3-fluoro-N-metilbenzamida;

5-Cloro-4-{3-(cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-2-fluoro-N-isopropilbenzamida;

5-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-isopropilpiridina-2-carboxamida;

4-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-3-fluoro-N-[(1S)-2,2,2-trifluoro-1-metiletil]benzamida;

5-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-[(1S)-1-ciclopropiletil]piridina-2-carboxamida;

5-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-(3,3-difluorociclobutil)piridina-2-carboxamida;

4-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-isopropilbenzamida;

4-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-2-fluoro-N-isopropilbenzamida;

4-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-[(1S)-1-ciclohexiletil]-2-fluorobenzamida;

4-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-3-fluoro-N-[(1R)-2,2,2-trifluoro-1-metiletil]benzamida;

5-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-[1-(trifluorometil)ciclopropil]piridina-2-carboxamida;

5-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-isopropilpirazina-2-carboxamida;

4-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-[1-(1-metilpiperidin-4-il)etil]benzamida;

4-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-[(1R)-1-ciclopropiletil]-2,5-difluorobenzamida;

5-Cloro-4-{3-(cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-[(1R)-1-ciclopropiletil]-2-fluorobenzamida;

4-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-2-fluoro-N-[(1S)-2,2,2-trifluoro-1-metiletil]benzamida;

4-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-[(1R)-2,2,2-trifluoro-1-metiletil]benzamida;

5-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-etilpiridina-2-carboxamida;

4-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-[(1R)-1-metilpropil]benzamida; y

4-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-(2,2,2-trifluoro-1-metiletil)benzamida;

4-{3-(Cianometil)-3-[4-(1H-pirrolo[2,3-b]piridin-4-il)-1H-pirazol-1-il]azetidin-1-il}-3-fluoro-N-isopropilbenzamida;

4-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-2,5-difluoro-N-[(1S)-2,2,2-trifluoro-1-metiletil]benzamida;

4-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-2,5-difluoro-N-[(1R)-2,2,2-trifluoro-1-metiletil]benzamida;

5-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-[(1R)-2,2,2-trifluoro-1-metiletil]pirazina-2-carboxamida;

5-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-[(1S)-2,2,2-trifluoro-1-metiletil]pirazina-2-carboxamida;

5-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-[(1S)-1-ciclopropil-2,2,2-trifluoroetil]pirazina-2-carboxamida;

5-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-[1-(trifluorometil)ciclopropil]pirazina-2-carboxamida;

5-{3-(Cianometil)-3-[4-(1H-pirrolo[2,3-b]piridin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-isopropilpirazina-2-carboxamida;

5-{3-(Cianometil)-3-[4-(1H-pirrolo[2,3-b]piridin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-[(1S)-2,2,2-trifluoro-1-metiletil]pirazina-2-carboxamida;

5-{3-(Cianometil)-3-[4-(1H-pirrolo[2,3-b]piridin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-(2,2,2-trifluoroetil)pirazina-2-carboxamida;

4-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-(2,2-difluoroetil)-2,5-difluorobenzamida;

4-{3-(Cinaometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-[(1S)-1-ciclopropil-2,2,2-trifluoroetil]benzamida;

4-{3-(Cianometil)-3-[4-(7H-pirrolo[2,3-d]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-[(1R)-1-ciclopropiletil]-2-fluorobenzamida;

5-{3-(Cianometil)-3-[4-(1H-pirrolo[2,3-b]pirimidin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-[1-(trifluorometil)ciclopropil]piridina-2-carboxamida;

5-{3-(Cianometil)-3-[4-(1H-pirrolo[2,3-b]piridin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-[(1S)-1-ciclopropiletil]pirazina-2-carboxamida; y

5-{3-(cianometil)-3-[4-(1H-pirrolo[2,3-b]piridin-4-il)-1H-pirazol-1-il]azetidin-1-il}-N-[(1S)-1-ciclopropil-2,2,2-trifluoroetil]pirazina-2-carboxamida;

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MECHANISMS OF DISEASE

Lineage-Specific Hematopoietic Growth Factors

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N Engl J Med 2006;354:2034-45.

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HEMATOPOIESIS IS THE PROCESS THAT GENERATES BLOOD CELLS OF ALL lineages. Calculations based on the blood volume and the level and half-life of each type of blood cell in the circulation indicate that each day an adult produces approximately 200 billion erythrocytes, 100 billion leukocytes, and 100 billion platelets. Moreover, these rates can increase by a factor of 10 or more when the demand for blood cells increases.

In 1906, Carnot¹ found that injecting healthy rabbits with serum from anemic animals prompted a rapid increase of erythrocytes in the recipients. The responsible humoral substance was first termed "hemopoietine" and was later named "erythropoietin." In 1957, Jacobson and colleagues² identified the kidney as the source of erythropoietin; in 1985, the factor was purified and its gene was cloned, thereby making it available for physiological study and therapeutic use.³ In a similar fashion, humoral substances that support the production of leukocytes⁴ and platelets⁵ were defined, cloned, and studied. Simultaneously with these advances, a model of blood-cell production was constructed in which hematopoiesis was envisioned as a hierarchical progression of multipotential hematopoietic stem cells that gradually lose one or more developmental options, becoming progenitor cells committed to a single lineage; these progenitors then mature into the corresponding types of peripheral-blood cells (Fig. 1).

LINEAGE-SPECIFIC HEMATOPOIETIC GROWTH FACTORS

Growth factors are required for the survival and proliferation of hematopoietic cells at all stages of development (Fig. 1). Of the factors that affect multipotential cells, steel factor, Fms-like tyrosine kinase 3 (FLT3) ligand, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-2, interleukin-3, and interleukin-7 are the best characterized. Each of these proteins supports the survival and proliferation of a number of distinct target cells, and with the exception of interleukin-7 and steel factor, the elimination of any one of them does little harm because of the redundancy in the functions of these early-acting growth factors.

ERYTHROPOIETIN

Blood levels of erythropoietin (normal, 20 mU per milliliter) are inversely related to tissue oxygenation — the level can increase up to 20,000 mU per milliliter in response to anemia or arterial hypoxemia.⁶ The juxtatubular interstitial cells of the renal cortex, which produce approximately 90 percent of the erythropoietin in blood (Fig. 2), sense oxygen levels through a newly identified, oxygen-dependent prolyl hydroxylase that regulates the stability of the primary transcription factor for erythropoietin, hypoxia-inducible factor 1 α (HIF-1 α). Once hydroxylated, HIF-1 α binds the von Hippel-Lindau (VHL) protein, thereby targeting it for ubiquitin-mediated de-

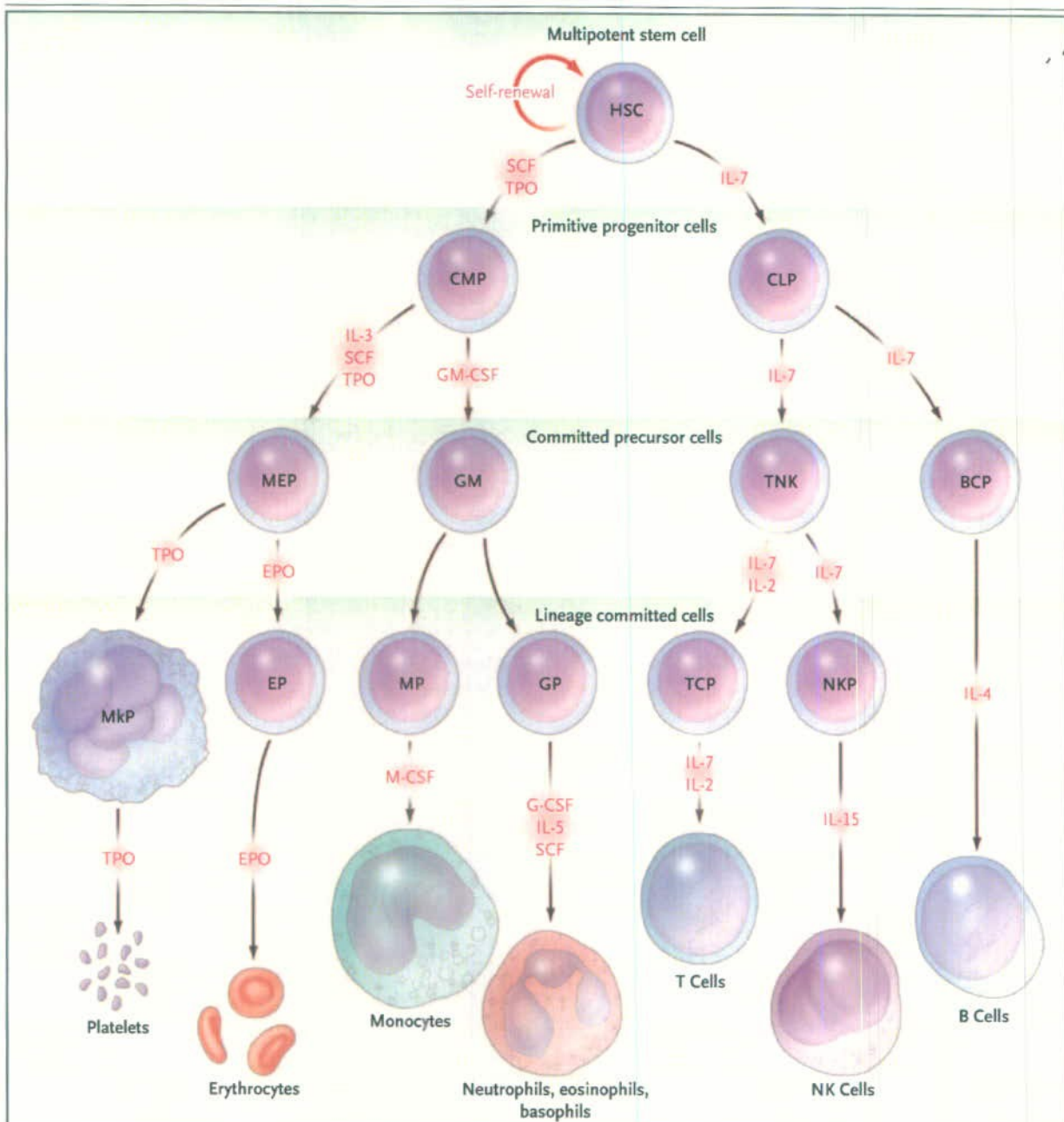


Figure 1. A General Model of Hematopoiesis.

Blood-cell development progresses from a hematopoietic stem cell (HSC), which can undergo either self-renewal or differentiation into a multilineage committed progenitor cell: a common lymphoid progenitor (CLP) or a common myeloid progenitor (CMP). These cells then give rise to more-differentiated progenitors, comprising those committed to two lineages that include T cells and natural killer cells (TNKs), granulocytes and macrophages (GMs), and megakaryocytes and erythroid cells (MEPs). Ultimately, these cells give rise to uni-lineage committed progenitors for B cells (BCPs), NK cells (NKPs), T cells (TCPs), granulocytes (GPs), monocytes (MPs), erythrocytes (EPs), and megakaryocytes (MkPs). Cytokines and growth factors that support the survival, proliferation, or differentiation of each type of cell are shown in red. For simplicity, the three types of granulocyte progenitor cells are not shown; in reality, distinct progenitors of neutrophils, eosinophils, and basophils or mast cells exist and are supported by distinct transcription factors and cytokines (e.g., interleukin-5 in the case of eosinophils, stem-cell factor [SCF] in the case of basophils or mast cells, and G-CSF in the case of neutrophils). IL denotes interleukin, TPO thrombopoietin, M-CSF macrophage colony-stimulating factor, GM-CSF granulocyte-macrophage CSF, and EPO erythropoietin.

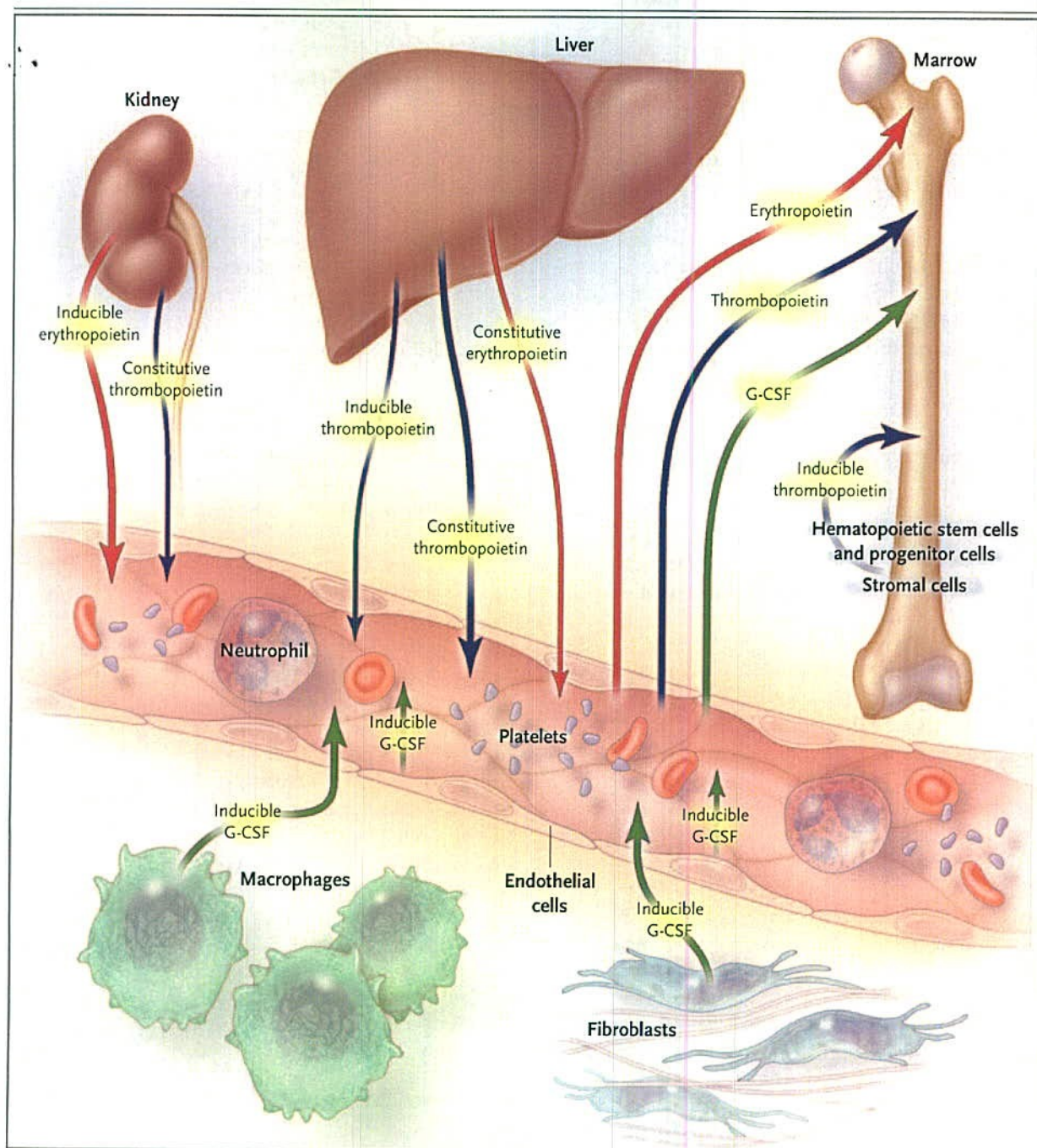


Figure 2. Regulation of the Production of Hematopoietic Growth Factors.

The production of growth factors by various tissues and cells (arrows) is both constitutive and inducible and subject to adsorption by receptor-bearing cells. Production of erythropoietin (red arrows) is inducible by hypoxia in the kidney and constitutive in the liver (10 percent of total body erythropoietin), and it is excreted in the urine. G-CSF (green arrows) is mostly inducible in tissues from fibroblasts and macrophages and from endothelial cells by inflammatory mediators (interleukin-1, interleukin-6, and tumor necrosis factor α). Thrombopoietin (blue arrows) is produced constitutively by the liver (accounting for approximately 50 percent of baseline thrombopoietin levels) and kidney (accounting for a minority of the total amount) and is inducible in the liver by inflammatory mediators (especially interleukin-6) and from bone marrow stromal cells by thrombocytopenia. In addition, both G-CSF and thrombopoietin are removed from the circulation by receptor-mediated uptake and destruction by the mature cells that bear the corresponding receptors on neutrophils and platelets. Arrows indicate the production and secretion of hematopoietic growth factors.

struction.⁷ Under conditions of low oxygen tension, the hydroxylase is inactive; consequently, HIF-1 α is not hydroxylated and cannot bind VHL, thereby maintaining its stability and capacity to drive the production of erythropoietin. This mechanism accounts for the erythrocytosis associated with high-altitude, heart or lung disease leading to hypoxemia and hemoglobin variants with a low affinity for oxygen. The discovery of a familial form of erythrocytosis in the Chuvashian population of the capital of the Chuvash Republic, Shupashkar (in Russian, Cheboksary), is of considerable interest because affected members of these families have a missense mutation in the *VHL* gene and high erythropoietin levels.⁸

Erythropoietin promotes the proliferation of erythroid progenitor cells by reducing the level of cell-cycle inhibitors and augmenting transcription of cyclins and supports their survival by increasing the antiapoptosis protein BCLX_L.⁶ Elimination of the erythropoietin gene or its receptor in mice causes severe anemia and death.⁹ The administration of erythropoietin to animals or humans increases the number of erythroid progenitor cells, which differentiate into normoblasts, enucleate, and leave the bone marrow.⁶ Within one or two weeks after the administration of the hormone, the reticulocyte count rises because of a large increase in red-cell production and the premature exit of erythrocytes from the bone marrow.

Erythropoietin was initially assumed to be an erythroid-specific hormone, but messenger RNA for erythropoietin and its receptor is readily detectable in neuronal and glial cells of the central nervous system and in the retina.^{10,11} Moreover, the hormone protects neurons from noxious stimuli¹² and induces the proliferation of neurons after neuronal injury.¹³ Preclinical trials have shown that exogenously administered erythropoietin crosses the blood-brain barrier and exerts a protective effect on ischemic neurons. As compared with control treatment, erythropoietin was associated with an improvement in outcome scales and infarct size in experimental stroke^{14,15} and in a single clinical trial.¹⁶ Should additional clinical studies confirm these results, an important new application for an "erythroid-specific" hormone will become available.

GRANULOCYTE COLONY-STIMULATING FACTOR

Granulocyte colony-stimulating factor (G-CSF) has been identified as a factor that stimulates the

growth of neutrophil colonies when added to cultures of murine or human marrow cells in a semi-solid medium. In contrast, GM-CSF stimulates the production of neutrophils, eosinophils, basophils, monocytes, and dendritic cells in culture and activates most of these types of mature cells. The gene for G-CSF was cloned in 1987, and the recombinant protein was assessed in clinical trials soon thereafter.⁴ Genetic studies in knockout mice demonstrated that G-CSF is essential for normal neutrophil production and for the leukocytosis that occurs in inflammation,¹⁷ whereas elimination of GM-CSF affected only the function and number of alveolar macrophages.

G-CSF supports the survival and stimulates the proliferation of neutrophil progenitors and promotes their differentiation into mature neutrophils.^{4,18} In addition, the cytokine causes premature release of neutrophils from the bone marrow and enhances the phagocytic capacity, generation of superoxide anions, and bacterial killing by these cells. The administration of G-CSF causes toxic granulation of neutrophils in the peripheral blood, which is a morphologic correlate of their heightened functional state, and a leftward shift (immaturity) in the leukocyte differential count. Activation of neutrophils in the bone marrow by G-CSF causes them to release matrix metalloproteases. This effect helps to explain the mobilization of hematopoietic stem cells out of the bone marrow in response to the administration of G-CSF,¹⁹ a finding that has greatly facilitated the collection of hematopoietic stem cells for transplantation.

Endothelial cells, fibroblasts, and macrophages in virtually all organs produce G-CSF (Fig. 2). Inflammatory cytokines such as tumor necrosis factor α , interleukin-1, and interleukin-6, derived from activated monocytes, stimulate G-CSF production, helping to explain the leukocytosis in patients with infection or inflammation.²⁰

THROMBOPOIETIN

Thrombopoietin is the primary regulator of platelet production.⁵ It supports the survival and proliferation of megakaryocyte progenitors by initiating many of the mechanisms that are triggered by erythropoietin and G-CSF, including increased expression of the cell-cycle regulator cyclin D and the antiapoptosis molecule BCLX_L and suppression of the cell-cycle inhibitor p27^{Kip}. In vitro, thrombopoietin induces the differentiation of progenitor cells into large megakaryocytes, each one capable

of producing thousands of platelets. Thrombopoietin, a potent stimulator of platelet production, causes thrombocytosis when administered to animals or humans. It also primes platelets to aggregate in response to otherwise subthreshold levels of thrombin, collagen, or adenosine diphosphate by activating the phosphoinositol 3-kinase signaling pathway in platelets.²¹ In mice, elimination of the thrombopoietin gene or its receptor reduces the level of production of megakaryocytes and platelets to approximately 10 percent of normal.²²

In addition to stimulating megakaryocytopoiesis, thrombopoietin has an important and non-redundant role in the survival and expansion of hematopoietic stem cells. When tested alone or in combination with other cytokines *in vitro*, thrombopoietin supports the survival and proliferation of hematopoietic stem cells.²³ Moreover, genetic elimination of thrombopoietin or its receptor in mice reduces by a factor of 7 to 8 the number of stem cells with the capacity to repopulate the bone marrow and decreases by a factor of 17 the expansion in the number of hematopoietic stem cells that occurs after transplantation.^{24,25} The effects of thrombopoietin on stem cells are mediated by enhanced expression or nuclear localization of several transcription factors — signal transducers and activators of transcription, homeobox B4, and homeobox A9 — and by autocrine production of vascular endothelial growth factor.²⁶ As a result of these studies, virtually every current attempt in the laboratory to expand hematopoietic stem cells includes thrombopoietin or a thrombopoietin mimetic in the culture medium, and current clinical trials of thrombopoietic agents are designed to detect the panhematopoietic effects that were seen in the preclinical trials of the hormone.

The cause of congenital amegakaryocytic thrombocytopenia has been traced to a deficiency of the thrombopoietin receptor.²⁷ In children with this disorder, aplastic anemia develops within one to five years after birth because of stem-cell exhaustion, consistent with the effects of thrombopoietin on hematopoietic stem cells. Thrombopoietin levels in blood are inversely related to the number of platelets in the blood and megakaryocytes in the bone marrow⁵; unlike erythropoietin production, however, the regulation of thrombopoietin levels is multifaceted (Fig. 2). At least half the production of thrombopoietin occurs in the liver,²⁸ but the kidney and skeletal muscles also produce

the hormone. Mature platelets and megakaryocytes display thrombopoietin receptors and are capable of removing the hormone from the circulation.²⁹ Thus, one mechanism of the inverse relationship between the platelet count and the thrombopoietin level is adsorption of the hormone from the circulation, a finding that can explain how platelet transfusions delay the rate of recovery from chemotherapy-related thrombocytopenia.³⁰ In addition to these steady-state mechanisms of thrombopoietin production and catabolism, stromal cells in the bone marrow increase production of the hormone substantially in states of severe thrombocytopenia.^{31,32} Furthermore, hepatic thrombopoietin production can be enhanced by inflammatory mediators such as interleukin-6,^{33,34} providing a molecular explanation for the common finding of reactive thrombocytosis in rheumatoid arthritis, Crohn's disease, and several other inflammatory and infectious conditions.³⁵

HEMATOPOIETIC GROWTH FACTOR RECEPTORS

The cytokine-receptor superfamily consists of the cell-surface receptors for erythropoietin, thrombopoietin, several colony-stimulating factors, most interleukins, and several structurally related cytokines and hormones (e.g., growth hormone).³⁶ Each of these transmembrane proteins includes one or two extracellular cytokine-binding domains containing approximately 200 amino acids, a transmembrane domain of 20 to 25 residues, and an intracellular domain of approximately 100 to 500 amino acids with the box 1 and box 2 motifs that recruit kinases of the Janus kinase (JAK) family (Fig. 3). The functional unit of the erythropoietin, G-CSF, and thrombopoietin receptors is a homodimer³⁷; the engagement of a single molecule of hematopoietic growth factor by a homodimeric receptor induces a major conformational shift in the receptor, bringing the two tethered cytoplasmic JAKs into close juxtaposition, thereby triggering activation of the kinases by mutual cross-phosphorylation. Mutations of the thrombopoietin receptor that affect its transmembrane region have been identified in patients with congenital thrombocytosis.³⁸ These mutations probably cause conformational shifts in receptor geometry similar to those triggered by thrombopoietin binding.

Once the JAKs are activated by the lineage-specific hematopoietic growth factors, a number of secondary signaling molecules are phosphorylated, and these initiate numerous events that

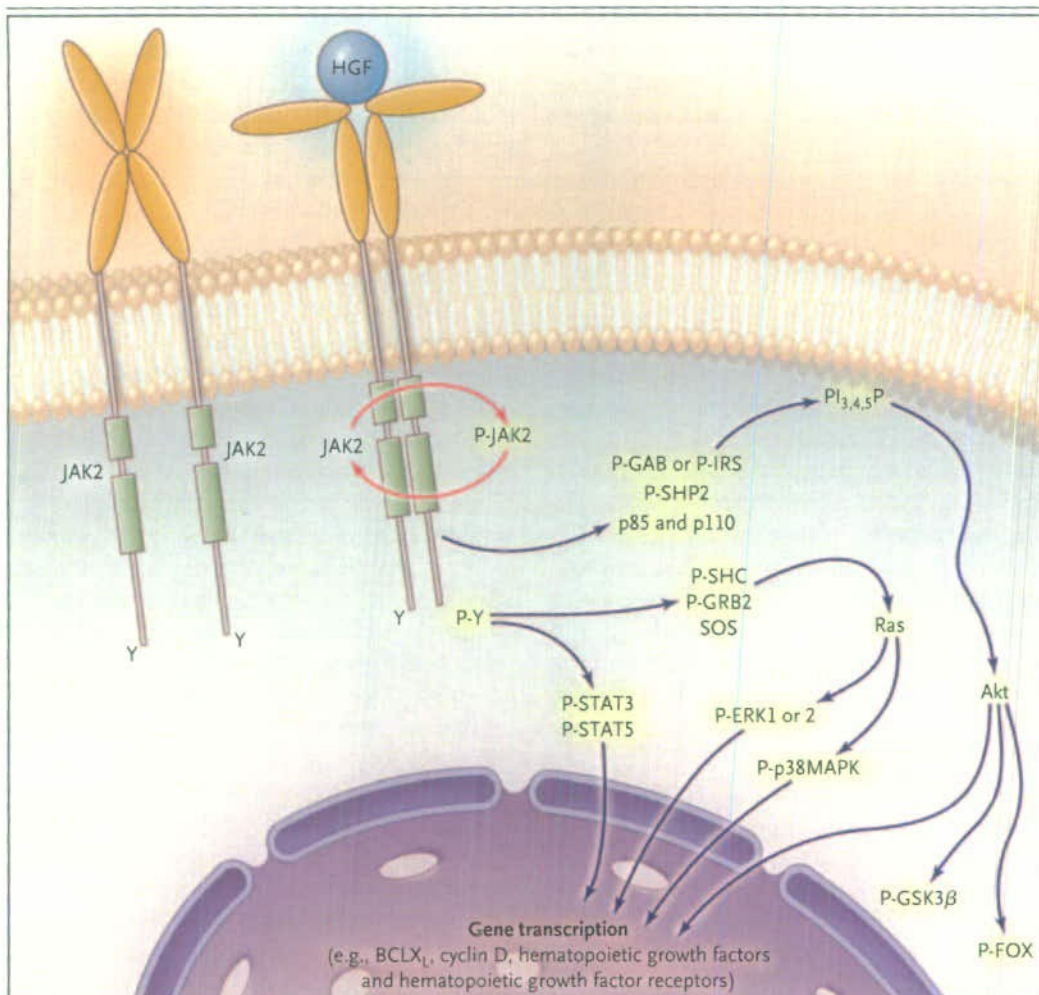


Figure 3. Hematopoietic Growth Factor Signaling.

Each hematopoietic growth factor–receptor dimer is composed of two subunits consisting of one or two 200-amino-acid motifs (each oval represents 100 amino acids), which bind two molecules of Janus kinase 2 (JAK2) but assume an open conformation (left) in the absence of the cognate growth factor. Ligand binding substantially changes the conformation of the receptor, bringing the cytoplasmic domains into close juxtaposition and allowing the tethered kinases to cross-phosphorylate (activate) each other. One of the first substrates for activated JAK2 is receptor tyrosine (Y) residues; when these are phosphorylated (P), they serve as docking sites for adaptors (SHC [Src homology containing], GRB2 [growth factor receptor–bound protein 2], GAB [Grb binding], and IRS [insulin–receptor substrate]), phosphatases (SHP2 [Shc homology domain containing phosphatase]), G-protein activators (SOS [son of seven-less]), and lipid kinases (p85 and p110 and phosphoinositol 3-kinase). Additional effectors of growth factor signaling include signal transducer and activator of transcription 3 and 5 (STAT3 and STAT5, respectively), membrane phospholipids (phosphoinositol 3,4,5-phosphate [$PI_{3,4,5}P$]), the small guanine nucleotide–binding protein Ras, and the kinases AKT, p38 mitogen-activated protein kinase (MAPK), extracellular response–stimulated kinase (ERK) 1 and 2, phosphatase forkhead (P-FOX), and phosphatase glycogen synthase kinase 3 β (P-GSK3 β). Eventually, most such signals affect nuclear transcription of antiapoptosis molecules (BCLX₁), cell-cycle regulators (cyclins), and additional growth factors and their receptors. Arrows indicate signaling pathways.

promote cell survival, proliferation, and differentiation. The cytokine-signaling pathways we know most about entail activation of the signal transducer and activator of transcription factors,³⁹ phosphoinositol-3 kinase,⁴⁰ and mitogen-activated

protein kinase,⁴¹ each of which activates an overlapping subgroup of tertiary signaling molecules (Fig. 3). Of these, transcription factors (homeobox, Ets, and forkhead box O^{42–44}), cell-cycle activators (cyclins⁴⁵) and inhibitors (p27^{Kip}),⁴⁶ anti-

apoptosis molecules (BCLX_L and inhibitor of apoptosis⁴⁷), and other growth factors (transforming growth factor β^{48} and vascular endothelial growth factor⁴⁹) are ultimately responsible for the effects induced by the binding of a hematopoietic growth factor to its receptor. In addition to these mediators of cell proliferation, the activation of hematopoietic growth factor receptors instigates three events that terminate the growth signal: internalization of receptors, activation of phosphatases, and production of suppressors of cytokine signaling.⁵⁰ An interesting finding is that a form of familial erythrocytosis that differs from Chuvash erythrocytosis is due to truncation of the erythropoietin receptor, which eliminates an important, phosphatase-binding negative signaling region.⁵¹

Numerous signaling kinases in hematopoietic cells have been implicated in pathologic processes. The ABL kinase is persistently activated in patients with chronic myelogenous leukemia⁵²; continual activation is also a feature of the FLT3 receptor tyrosine kinase or the JAK2 in patients with acute myelogenous leukemia, the platelet-derived growth factor receptor α tyrosine kinase in those with chronic eosinophilic leukemia, and the platelet-derived growth factor receptor β kinase in patients with chronic myelomonocytic leukemia.⁵³ In most cases, the aberrantly activated kinase mimics a kinase that is activated by a hematopoietic growth factor; many of the signaling pathways triggered by erythropoietin, G-CSF, and thrombopoietin are also constitutively active in the leukemic cells.

Recently, a change in a single amino acid in the regulatory domain of JAK2 (in which phenylalanine replaces valine at position 617 [V617F]) was found in hematopoietic cells from most patients with polycythemia vera and from approximately half the patients with essential thrombocythemia or idiopathic myelofibrosis.⁵⁴ Molecular modeling predicts that the mutation destabilizes the inactive form of enzyme, thereby causing constitutive activity and signaling. Consistent with this model are the observations that several signaling molecules that mediate the actions of hematopoietic growth factors are constitutively active in hematopoietic cells from patients with polycythemia vera and essential thrombocythemia and that bone marrow cells from such patients are hypersensitive to growth factors in vitro. This work on the JAK2 mutation has led to improved diag-

nostic tests for these myeloproliferative disorders. But there is much more work to do, including identifying the cell-surface receptors with which the mutant kinase associates, determining the mechanism by which the same point mutation leads to three phenotypically distinct diseases, deciding whether patients with polycythemia vera or essential thrombocythemia and the mutant kinase differ from those who do not, and developing a specific inhibitor of the mutant JAK2.

FORMS OF AND CLINICAL USES FOR HEMATOPOIETIC GROWTH FACTORS

In principle, there are four general indications for the administration of a hematopoietic growth factor: correction of cytopenia owing to the deficiency of a growth factor, stimulation of the recovery of hematopoietic stem and progenitor cells in iatrogenic and naturally occurring deficiency states, augmentation of hematopoiesis to compensate for pathologically rapid blood-cell destruction, and activation of mature hematopoietic cells. Although the use of these agents in states of growth-factor deficiency (e.g., erythropoietin in the anemia of renal insufficiency) is widely accepted, the efficacy of hematopoietic growth factors in the other three conditions was, a priori, uncertain. However, it is now clear from the results of controlled clinical trials that this group of agents is effective in a variety of clinical states.

ERYTHROPOIETIN

Erythropoietin was approved for use by the Food and Drug Administration in 1989. The initial clinical trial leading to its approval was for treatment of anemia due to chronic renal insufficiency.⁵⁵ Thereafter, its use quickly spread to the treatment of anemia owing to many other causes, making it one of the most frequently prescribed products of recombinant DNA technology. Erythropoietin or its derivatives have been administered to treat anemia in over 3 million patients worldwide, including those in intensive care units, those about to undergo surgery, and those undergoing therapy for infection with the human immunodeficiency virus or hepatitis C virus.

Anemia is often multifactorial in patients with cancer. Its causes include suppression of hematopoiesis by the tumor, therapy-induced myelosuppression, and the anemia of chronic inflammation. For these reasons, the effectiveness of epoetin

therapy in patients with cancer and the degree of anemia at which the hormone could be of benefit were uncertain. These questions have been addressed in several controlled trials encompassing nearly 2000 patients with cancer-related anemia. The trials have established that patients with a hemoglobin level of 10 g per deciliter or less benefit from epoetin therapy; they require fewer blood transfusions and have an enhanced sense of well-being. Evidence-based guidelines on the use of epoetin in patients with cancer have been published by the American Society of Hematology and the American Society of Clinical Oncology⁵⁶ and by the European Organisation for the Research and Treatment of Cancer,⁵⁷ and recent studies have reinforced the validity of a treatment threshold of 10 g of hemoglobin per deciliter. Treatment with epoetin is not risk-free, however. Intensive therapy in patients with head and neck or breast cancer has been associated with hypertension, bleeding, and an increased risk of thrombotic complications.^{58,59}

The response to exogenous epoetin is often determined by the patient's ability to produce erythropoietin in response to anemia; one reason that patients with anemia associated with cancer have a response to exogenous epoetin is that they are producing an insufficient amount of endogenous erythropoietin.⁶⁰ Conversely, administration of the hormone does not ameliorate anemia in patients with hematologic cancer in whom the blood level of erythropoietin exceeds 500 mIU per milliliter.⁶¹ Administration of epoetin is effective in patients undergoing myelosuppressive chemotherapy because chemotherapy induces apoptosis in both cancer cells and erythroid progenitors and epoetin blocks apoptosis of the erythroid progenitors.⁶

Eliciting a clinical response to a recombinant protein depends on the body's ability to avoid inducing an immune response to the protein. There had been almost no reports of adverse immunologic responses to epoetin for more than a decade, but between 1998 and 2003, pure red-cell aplasia due to antibodies against erythropoietin developed in over 200 patients with end-stage renal disease who were treated with one preparation of recombinant erythropoietin.⁶² Immunosuppressive therapy restored responsiveness to the recombinant hormone in about two thirds of the patients. Although the cause of the antierythropoietin antibodies in this condition continues to be explored,

partial denaturation of the erythropoietin probably provoked the immune response.

G-CSF

Neutropenia does not in itself cause symptoms, but it predisposes patients to infection, especially if the neutrophil count falls below 500 per cubic millimeter and persists longer than 10 to 14 days. In patients receiving chemotherapy, the administration of G-CSF can lessen the incidence and severity of neutropenia, and in some settings, it can reduce the mortality rate. Moreover, with the recognition that the cytokine mobilizes hematopoietic stem cells from the bone marrow into the peripheral blood,⁶³ the use of G-CSF to aid in the collection of stem cells for transplantation has now made harvesting of bone marrow for this indication nearly obsolete.

The use of G-CSF in patients receiving myelosuppressive chemotherapy has been evaluated by several expert panels. It is recommended for primary prophylaxis against neutropenia only if the risk of febrile neutropenia historically exceeds 40 percent for the planned chemotherapeutic protocol.⁶⁴ Of the 200 most commonly used chemotherapeutic protocols, only 12 are associated with a risk of febrile neutropenia of at least 20 percent.⁶⁵ In populations in which the risk is at least 40 percent, such as patients 65 years of age or older who are undergoing aggressive chemotherapy for lymphoma, patients with small-cell carcinoma of the lung or uroepithelial tumors, and patients of any age who have received chemotherapy for acute myelogenous leukemia, G-CSF therapy has proved effective in avoiding the risk of severe neutropenia.^{66,67} It is also recommended as secondary prophylaxis in patients with solid tumors who have had chemotherapy-induced febrile neutropenia and who continue to receive intensive chemotherapy, if a dose reduction will impair the antitumor response.

The use of G-CSF in patients undergoing chemotherapy has been linked to an increased risk of treatment-related myeloid leukemia. Initially, the increased risk was seen only after therapy with topoisomerase II inhibitors,⁶⁸ but more recent studies indicate that patients treated for breast cancer with cyclophosphamide and doxorubicin may have an increased risk of myelodysplasia or acute myelogenous leukemia if they have also received G-CSF.⁶⁹ What might explain this association? Chemotherapy given for a specific cancer

may induce otherwise lethal mutations in a myeloid stem cell or progenitor cell, but the antiapoptotic effect of G-CSF saves the mutant cell from destruction, thereby allowing it to develop into a myeloid cancer. Clearly, additional study is necessary to clarify the risk of acute myelogenous leukemia in patients who receive G-CSF chemotherapy and to determine the mechanism of the association.

The ability to use G-CSF to mobilize hematopoietic stem cells from the bone marrow into the peripheral blood has represented an important advance in hematopoietic stem-cell transplantation.⁶³ As compared with transplantation of bone marrow cells, the use of G-CSF-mobilized stem cells that originate in the peripheral blood reduces the duration of neutropenia after the administration of the myeloablative preparatory regimen and the need for platelet and red-cell transfusions, reducing both the need for hospitalization and costs. However, the value of the use of the cytokine in recipients of stem-cell transplants to further shorten the duration of neutropenia has not been established.⁷⁰

The administration of G-CSF to patients with congenital neutropenia or cyclic neutropenia has revolutionized the care of children with these disorders, since these conditions were usually fatal before the advent of recombinant G-CSF. The number of neutrophilic progenitor cells is severely reduced in both conditions as a result of mutations of the neutrophil elastase gene.⁷¹ Administration of G-CSF increases neutrophil levels substantially, allowing these children to remain free of life-threatening infections.

Despite this progress, G-CSF has been found to increase the risk of acute myelogenous leukemia in patients with congenital neutropenia. Before recombinant G-CSF was available for clinical use, there were occasional reports of acute myelogenous leukemia or myelodysplasia in patients with congenital neutropenia who survived infancy. Since the introduction of G-CSF, patients with congenital neutropenia live longer, but a malignant myeloid disorder develops in approximately 10 percent.⁷² The gene for the G-CSF receptor in the malignant bone marrow cells from these patients usually displays a somatic mutation that eliminates one or more growth inhibitory signals,⁷³ akin to the truncation in the erythropoietin receptor in patients with familial erythrocytosis.⁵¹ Therefore, it is likely that the administration

of G-CSF to patients with congenital neutropenia selects for cells with a mutation in the G-CSF receptor that enhances the proliferation of myeloid cells.

THROMBOPOIETIN

The gene for thrombopoietin was cloned 12 years ago, but the protein has not yet been approved for clinical use. Nevertheless, results of a number of clinical trials point to its eventual clinical utility. It is likely that a thrombopoietic agent will be useful to accelerate platelet recovery after moderately intensive chemotherapy,⁷⁴ but not after the intensive chemotherapy necessary for treating acute myelogenous leukemia, or at least with the use of the dosing strategies tested thus far.⁷⁵ It can also augment the number of hematopoietic stem cells mobilized by G-CSF⁷⁶ and increase the number of platelets available for apheresis from platelet donors.

The potential for the clinical use of recombinant thrombopoietin was irreversibly altered by a study to determine whether the hormone could improve platelet yields from platelet donors. The thrombopoietin used in most of the clinical trials that were conducted between 1995 and 1998 was a truncated form of the protein produced in *Escherichia coli* and modified with polyethylene glycol. Termed "megakaryocyte growth and differentiation factor" (MGDF), the recombinant protein and native thrombopoietin have important differences that probably explain an important adverse effect. Native thrombopoietin contains three sites of serine glycosylation, which are not present in the truncated recombinant protein produced in bacteria. Moreover, because MGDF is a truncated version of thrombopoietin, the C-terminal amino acid of MGDF is not normally a terminal residue. Hence, several nonnative structures (potential neoepitopes) were created in MGDF. When administered subcutaneously to platelet donors, some of the donors produced antibodies against MGDF that cross-reacted with endogenous thrombopoietin, thereby causing severe thrombocytopenia.⁷⁷ This adverse event — an important lesson in the engineering of recombinant proteins meant for clinical use — led to the abandonment of the use of MGDF and full-length forms of thrombopoietin as therapeutic proteins.

The experience with MGDF instigated a search for molecules that bind to and stimulate the thrombopoietin receptor on hematopoietic cells. Sophisticated screening techniques have led to the dis-

covery of several small-molecule thrombopoietin mimics that bind and stimulate the thrombopoietin receptor. These agents are undergoing clinical testing. The results of a recent trial of a molecule carrying four copies of a thrombopoietin receptor-binding peptide on an immunoglobulin scaffold point to the potential clinical utility of a thrombopoietin-receptor agonist.⁷⁸

The bone marrow of patients with immune thrombocytopenic purpura usually contains numerous megakaryocytes, reflecting an attempt to compensate for the thrombocytopenia. The abundance of megakaryocytes in these patients argued against an approach involving stimulation of the bone marrow to raise platelet levels. However, thrombopoietin levels are not elevated in patients with immune thrombocytopenic purpura, which raises the possibility that thrombopoietin therapy could be advantageous in such patients. In a recent phase 2 trial,⁷⁸ nearly 80 percent of patients with refractory immune thrombocytopenic purpura had a response to the thrombopoietin agonist AMG531, a peptide mimic of the hormone. This observation is evidence that pharmacologic levels of growth factors can increase hematopoi-

esis above the levels attained in a maximal physiologic response.

CONCLUSIONS

The clinical use of hematopoietic growth factors has improved the care of patients with inadequate blood-cell production, but these agents are costly, require parenteral administration, and can occasionally induce untoward effects. Recent work has focused on understanding and exploiting unexpected biologic activities of hematopoietic growth factors, identifying small-molecule mimics of these proteins, reducing their costs, and eliminating adverse effects. In the clinical setting, we need to be careful to restrict their use to indications that emerge from controlled clinical trials. On the basis of current knowledge, we may soon enter an era in which oral agents that stimulate hematopoiesis become part of routine care in patients with anemia, neutropenia, thrombocytopenia, or all these conditions as a result of natural or iatrogenic causes.

No potential conflict of interest relevant to this article was reported.

REFERENCES

1. Carnot P, Deflandre C. Sur l'activité hématopoïétique du sérum au cours de la régénération du sang. *C R Acad Sci Med* 1906;3:384-6.
2. Jacobson LO, Goldwasser E, Fried W, Plzak L. Role of the kidney in erythropoiesis. *Nature* 1957;179:633-4.
3. Jacobs K, Shoemaker C, Rudersdorf R, et al. Isolation and characterization of genomic and cDNA clones of human erythropoietin. *Nature* 1985;313:806-10.
4. Welte K, Gabrilove J, Bronchud MH, Platzer E, Morstyn G. Filgrastim (r-metHuG-CSF): the first 10 years. *Blood* 1996;88:1907-29.
5. Kaushansky K. Thrombopoietin. *N Engl J Med* 1998;339:746-54.
6. Krantz SB. Erythropoietin. *Blood* 1991;77:419-34.
7. Ratcliffe PJ, O'Rourke JF, Maxwell PH, Pugh CW. Oxygen sensing, hypoxia-inducible factor-1 and the regulation of mammalian gene expression. *J Exp Biol* 1998;201:1153-62.
8. Pastore YD, Jelinek J, Ang S, et al. Mutations in the VHL gene in sporadic apparently congenital polycythemia. *Blood* 2003;101:1591-5.
9. Wu H, Liu X, Jaenisch R, Lodish HF. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell* 1995;83:59-67.
10. Digicaylioglu M, Bichet S, Marti HH, et al. Localization of specific erythropoietin binding sites in defined areas of the mouse brain. *Proc Natl Acad Sci U S A* 1995;92:3717-20.
11. Juul SE, Yachnis AT, Christensen RD. Tissue distribution of erythropoietin and erythropoietin receptor in the developing human fetus. *Early Hum Dev* 1998;52:235-49.
12. Brines ML, Ghezzi P, Keenan S, et al. Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. *Proc Natl Acad Sci U S A* 2000;97:10526-31.
13. Kretz A, Hapold CJ, Marticke JK, Isenmann S. Erythropoietin promotes regeneration of adult CNS neurons via Jak2/Stat3 and PI3K/AKT pathway activation. *Mol Cell Neurosci* 2005;29:569-79.
14. Lu D, Mahmood A, Qu C, Goussev A, Schallert T, Chopp M. Erythropoietin enhances neurogenesis and restores spatial memory in rats after traumatic brain injury. *J Neurotrauma* 2005;22:1011-7.
15. Chang YS, Mu D, Wendland M, et al. Erythropoietin improves functional and histological outcome in neonatal stroke. *Pediatr Res* 2005;58:106-11.
16. Ehrenreich H, Hasselblatt M, Dembowski C, et al. Erythropoietin therapy for acute stroke is both safe and beneficial. *Mol Med* 2002;8:495-505.
17. Lieschke GJ, Grail D, Hodgson G, et al. Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. *Blood* 1994;84:1737-46.
18. Spiekermann K, Roesler J, Emmendorffer A, Elsner J, Welte K. Functional features of neutrophils induced by G-CSF and GM-CSF treatment: differential effects and clinical implications. *Leukemia* 1997;11:466-78.
19. van Os R, van Schie ML, Willemze R, Fibbe WE. Proteolytic enzyme levels are increased during granulocyte colony-stimulating factor-induced hematopoietic stem cell mobilization in human donors but do not predict the number of mobilized stem cells. *J Hematother Stem Cell Res* 2002;11:513-21.
20. Hareng L, Hartung T. Induction and regulation of endogenous granulocyte colony-stimulating factor formation. *Biol Chem* 2002;383:1501-17.
21. Kojima H, Shinagawa A, Shimizu S, et al. Role of phosphatidylinositol-3 kinase and its association with Gab1 in throm-

- bopoietin-mediated up-regulation of platelet function. *Exp Hematol* 2001;29:616-22.
22. de Sauvage FJ, Carver-Moore K, Luoh S-M, et al. Physiological regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. *J Exp Med* 1996;183:651-6.
 23. Sitnicka E, Lin N, Priestley GV, et al. The effect of thrombopoietin on the proliferation and differentiation of murine hematopoietic stem cells. *Blood* 1996;87:4998-5005.
 24. Solar GP, Kerr WG, Zeigler FC, et al. Role of c-mpl in early hematopoiesis. *Blood* 1998;92:4-10.
 25. Fox N, Priestley GV, Papayannopoulou T, Kaushansky K. Thrombopoietin expands hematopoietic stem cells after transplantation. *J Clin Invest* 2002;110:389-94.
 26. Kaushansky K. Thrombopoietin and the hematopoietic stem cell. *Ann N Y Acad Sci* 2005;1044:139-41.
 27. Ballmaier M, Germeshausen M, Schulze H, et al. c-mpl Mutations are the cause of congenital amegakaryocytic thrombocytopenia. *Blood* 2001;97:139-46.
 28. Qian S, Fu F, Li W, Chen Q, deSautage FJ. Primary role of the liver in thrombopoietin production shown by tissue-specific knockout. *Blood* 1998;92:2189-91.
 29. Yang C, Li YC, Kuter DJ. The physiological response of thrombopoietin (c-Mpl ligand) to thrombocytopenia in the rat. *Br J Haematol* 1999;105:478-85.
 30. Slichter SJ. Relationship between platelet count and bleeding risk in thrombocytopenic patients. *Transfus Med Rev* 2004;18:153-67.
 31. McCarty JM, Sprugel KH, Fox NE, Sabath DE, Kaushansky K. Murine thrombopoietin mRNA levels are modulated by platelet count. *Blood* 1995;86:3668-75.
 32. Sungaran R, Markovic B, Chong BH. Localization and regulation of thrombopoietin mRNA expression in human kidney, liver, bone marrow, and spleen using in situ hybridization. *Blood* 1997;89:101-7.
 33. Wolber EM, Jelkmann W. Interleukin-6 increases thrombopoietin production in human hepatoma cells HepG2 and Hep3B. *J Interferon Cytokine Res* 2000;20:499-506.
 34. Kaser A, Brandacher G, Steurer W, et al. Interleukin-6 stimulates thrombopoiesis through thrombopoietin: role in inflammatory thrombocytosis. *Blood* 2001;98:2720-5.
 35. Griesshammer M, Bangerter M, Sauer T, Wennauer R, Bergmann L, Heimpel H. Aetiology and clinical significance of thrombocytosis: analysis of 732 patients with an elevated platelet count. *J Intern Med* 1999;245:295-300.
 36. Cosman D. The hematopoietin receptor superfamily. *Cytokine* 1993;5:95-106.
 37. Livnah O, Stura EA, Middleton SA, Johnson DL, Jolliffe LK, Wilson IA. Crystallographic evidence for preformed dimers of erythropoietin receptor before ligand activation. *Science* 1999;283:987-90.
 38. Ding J, Komatsu H, Wakita A, et al. Familial essential thrombocythemia associated with a dominant-positive activating mutation of the c-MPL gene, which encodes for the receptor for thrombopoietin. *Blood* 2004;103:4198-200.
 39. Ihle JN, Kerr IM. JAKs and STATs in signaling by the cytokine receptor superfamily. *Trends Genet* 1995;11:69-74.
 40. Miyakawa Y, Rojnuckarin P, Habib T, Kaushansky K. Thrombopoietin induces phosphoinositol 3-kinase activation through SHP2, Gab, and insulin receptor substrate proteins in BAF3 cells and primary murine megakaryocytes. *J Biol Chem* 2001;276:2494-502.
 41. Miura Y, Miura O, Ihle JN, Aoki N. Activation of the mitogen-activated protein kinase pathway by the erythropoietin receptor. *J Biol Chem* 1994;269:29962-9.
 42. Kirito K, Fox NE, Kaushansky K. Thrombopoietin stimulates Hoxb4 expression: an explanation for the favorable effects of TPO on hematopoietic stem cells. *Blood* 2003;102:3172-8.
 43. Mora-Garcia P, Sakamoto KM. Granulocyte colony-stimulating factor induces Egr-1 up-regulation through interaction of serum response element-binding proteins. *J Biol Chem* 2000;275:22418-26.
 44. Dijkers PF, Medema RH, Pals C, et al. Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). *Mol Cell Biol* 2000;20:9138-48.
 45. Henry MK, Nimbalkar D, Hohl RJ, Quelle FW. Cytokine-induced phosphoinositide 3-kinase activity promotes Cdk2 activation in factor-dependent hematopoietic cells. *Exp Cell Res* 2004;299:257-66.
 46. Bouscary D, Pene F, Claessens YE, et al. Critical role for PI 3-kinase in the control of erythropoietin-induced erythroid progenitor proliferation. *Blood* 2003;101:3436-43.
 47. Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF. Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. *Cell* 1999;98:181-91.
 48. Yanagida M, Ide Y, Imai A, et al. The role of transforming growth factor-beta in PEG-rHuMGDF-induced reversible myelofibrosis in rats. *Br J Haematol* 1997;99:739-45.
 49. Kirito K, Fox NE, Komatsu N, Kaushansky K. Thrombopoietin enhances expression of vascular endothelial cell growth factor (VEGF) in primitive hematopoietic cells through induction of HIF-1 α . *Blood* 2005;105:4258-63.
 50. Starr R, Willson TA, Viney EM, et al. A family of cytokine-inducible inhibitors of signalling. *Nature* 1997;387:917-21.
 51. de la Chapelle A, Traskelin AL, Juvenon E. Truncated erythropoietin receptor causes dominantly inherited benign human erythrocytosis. *Proc Natl Acad Sci U S A* 1993;90:4495-9.
 52. Mauro MJ, Druker BJ. Chronic myelogenous leukemia. *Curr Opin Oncol* 2001;13:3-7.
 53. Chan IT, Gilliland DG. Oncogenic K-ras in mouse models of myeloproliferative disease and acute myeloid leukemia. *Cell Cycle* 2004;3:536-7.
 54. Kaushansky K. On the molecular origins of the chronic myeloproliferative disorders: it all makes sense. *Blood* 2005;105:4187-90.
 55. Eschbach JW, Egrie JC, Downing MR, Browne JK, Adamson JW. Correction of the anemia of end-stage renal disease with recombinant human erythropoietin: results of a combined phase I and II clinical trial. *N Engl J Med* 1987;316:73-8.
 56. Rizzo JD, Lichtin AE, Woolf SH, et al. Use of epoetin in patients with cancer: evidence-based clinical practice guidelines of the American Society of Clinical Oncology and the American Society of Hematology. *Blood* 2002;100:2303-20.
 57. Bokemeyer C, Aapro MS, Courdi A, et al. EORTC guidelines for the use of erythropoietic proteins in anaemic patients with cancer. *Eur J Cancer* 2004;40:2201-16.
 58. Henke M, Laszig R, Rube C, et al. Erythropoietin to treat head and neck cancer patients with anaemia undergoing radiotherapy: randomised, double-blind, placebo-controlled trial. *Lancet* 2003;362:1255-60.
 59. Leyland-Jones B. Breast cancer trial with erythropoietin terminated unexpectedly. *Lancet Oncol* 2003;4:459-60.
 60. Miller CB, Jones RJ, Piantadosi S, Abloff MD, Spivak JL. Decreased erythropoietin response in patients with the anemia of cancer. *N Engl J Med* 1990;322:1689-92.
 61. Cazzola M, Messinger D, Battistel V, et al. Recombinant human erythropoietin in the anemia associated with multiple myeloma or non-Hodgkin's lymphoma: dose finding and identification of predictors of response. *Blood* 1995;86:4446-53.
 62. Bennett CL, Cournoyer D, Carson KR, et al. Long-term outcome of individuals with pure red cell aplasia and antierythropoietin antibodies in patients treated with recombinant epoetin: a follow-up report from the Research on Adverse Drug Events and Reports (RADAR) Project. *Blood* 2005;106:3343-7.
 63. Sheridan WP, Begley CG, Juttner CA, et al. Effect of peripheral-blood progenitor cells mobilised by filgrastim (G-CSF) on platelet recovery after high-dose chemotherapy. *Lancet* 1992;339:640-4.
 64. Calhoun EA, Schumock GT, McKay JM, et al. Granulocyte colony-stimulating factor for chemotherapy-induced neutropenia in patients with small cell lung cancer: the 40% rule revisited. *Pharmacoeconomics* 2005;23:767-75.
 65. Heuser M, Ganser A. Colony-stimulat-

ing factors in the management of neutropenia and its complications. *Ann Hematol* 2005;84:697-708.

66. Repetto L, Biganzoli L, Koehne CH, et al. EORTC Cancer in the Elderly Task Force guidelines for the use of colony-stimulating factors in elderly patients with cancer. *Eur J Cancer* 2003;39:2264-72.

67. Ohno R, Tomonaga M, Kobayashi T, et al. Effect of granulocyte colony-stimulating factor after intensive induction therapy in relapsed or refractory acute leukemia. *N Engl J Med* 1990;323:871-7.

68. Relling MV, Boyett JM, Blanco JG, et al. Granulocyte colony-stimulating factor and the risk of secondary myeloid malignancy after etoposide treatment. *Blood* 2003;101:3862-7.

69. Smith RE, Bryant J, DeCillis A, Anderson S. Acute myeloid leukemia and myelodysplastic syndrome after doxorubicin-cyclophosphamide adjuvant therapy for operable breast cancer: the National Surgical Adjuvant Breast and Bowel Project Experience. *J Clin Oncol* 2003;21:1195-204.

70. Ringden O, Labopin M, Gorin NC, et al. Treatment with granulocyte colony-stimulating factor after allogeneic bone marrow transplantation for acute leuke-

mia increases the risk of graft-versus-host disease and death: a study from the Acute Leukemia Working Party of the European Group for Blood and Marrow Transplantation. *J Clin Oncol* 2004;22:416-23.

71. Dale DC, Cottle TE, Fier CJ, et al. Severe chronic neutropenia: treatment and follow-up of patients in the Severe Chronic Neutropenia International Registry. *Am J Hematol* 2003;72:82-93.

72. Dong F, Brynes RK, Tidow N, Welte K, Löwenberg B, Touw IP. Mutations in the gene for the granulocyte colony-stimulating-factor receptor in patients with acute myeloid leukemia preceded by severe congenital neutropenia. *N Engl J Med* 1995;333:487-93.

73. Hunter MG, Jacob A, O'Donnell LC, et al. Loss of SHIP and CIS recruitment to the granulocyte colony-stimulating factor receptor contribute to hyperproliferative responses in severe congenital neutropenia/acute myelogenous leukemia. *J Immunol* 2004;173:5036-45.

74. Vadhan-Raj S, Verschraegen CF, Bueso-Ramos C, et al. Recombinant human thrombopoietin attenuates carboplatin-induced severe thrombocytopenia and the need for platelet transfusions in patients

with gynecologic cancer. *Ann Intern Med* 2000;132:364-8.

75. Archimbaud E, Ottmann OG, Yin JA, et al. A randomized, double-blind, placebo-controlled study with pegylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF) as an adjunct to chemotherapy for adults with de novo acute myeloid leukemia. *Blood* 1999;94:3694-701.

76. Somlo G, Sniecinski I, ter Veer A, et al. Recombinant human thrombopoietin in combination with granulocyte colony-stimulating factor enhances mobilization of peripheral blood progenitor cells, increases peripheral blood platelet concentration, and accelerates hematopoietic recovery following high-dose chemotherapy. *Blood* 1999;93:2798-806.

77. Li J, Yang C, Xia Y, et al. Thrombocytopenia caused by the development of antibodies to thrombopoietin. *Blood* 2001;98:3241-8.

78. Bussel JB, Kuter DJ, George JN, et al. Long-term dosing of AMG 531 is effective and well tolerated in thrombocytopenic patients with immune thrombocytopenic purpura. *Blood* 2005;106:68a. abstract.

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Human Tyrosine Kinase 2 Deficiency Reveals Its Requisite Roles in Multiple Cytokine Signals Involved in Innate and Acquired Immunity

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Summary

Tyrosine kinase 2 (Tyk2) is a nonreceptor tyrosine kinase that belongs to the Janus kinase (Jak) family. Here we identified a homozygous Tyk2 mutation in a patient who had been clinically diagnosed with hyper-IgE syndrome. This patient showed unusual susceptibility to various microorganisms including virus, fungi, and mycobacteria and suffered from atopic dermatitis with elevated serum IgE. The patient's cells displayed defects in multiple cytokine signaling pathways including those for type I interferon (IFN), interleukin (IL)-6, IL-10, IL-12, and IL-23. The cytokine signals were successfully restored by transducing the intact Tyk2 gene. Thus, the Tyk2 deficiency is likely to account for the patient's complex clinical manifestations, including the phenotype of impaired T helper 1 (Th1) differentiation and accelerated Th2 differentiation. This study identifies human Tyk2 deficiency and demonstrates that Tyk2 plays obligatory roles in multiple cytokine signals involved in innate and acquired immunity of humans, which differs substantially from Tyk2 function in mice.

Introduction

The majority of helical bundled cytokines transduce their signals via the Janus kinase (Jak) family kinases that are constitutively associated with cytokine receptors. Four mammalian Jak proteins have been described to date: Jak1, Jak2, Jak3, and Tyk2 (Ihle, 1995; Liu et al., 1998; O'Shea et al., 2002). When cytokines bind to their corresponding receptors expressed on the cell surface, receptor-associated Jaks are activated, and they in turn phosphorylate both cytokine receptors and neighboring Jaks. Phosphorylation of specific tyrosine motifs in the cytokine receptors provides docking sites for signal transducers and activators of transcription (STAT), and STATs recruited to the receptor are phosphorylated by the Jaks. Upon phosphorylation, STATs dimerize via their Src homology domain-2 (SH2) domains and translocate to the nucleus, where they activate different genes. Recent studies have demonstrated that several inhibitory mechanisms are involved in the regulation of the Jak-STAT pathway to prevent it from overactivation, including SOCS (suppressor of cytokine signaling) proteins that are induced through the Jak-STAT pathway to make a negative feedback loop (Alexander and Hilton, 2004).

The functional importance of the Jaks was first investigated in studies that used cell lines defective in cytokine signaling and was subsequently confirmed by the establishment of Jak-deficient mice (Velazquez et al., 1992; Muller et al., 1993; Neubauer et al., 1998; Nosaka et al., 1995; Parganas et al., 1998; Park et al., 1995; Rodig et al., 1998). *Jak1*^{-/-} mice die perinatally and exhibit profound defects in lymphoid development (Rodig et al., 1998). Analyses of cells from *Jak1*^{-/-} mice demonstrated that Jak1 is essential for signaling by type I and II IFN, in agreement with prior studies that used human mutant cell lines. *Jak2*^{-/-} mice show embryonic lethality due to failure of erythropoiesis, while *Jak3*^{-/-} mice suffer from severe combined immunodeficiency in accord

with observations for human Jak3 deficiency (Macchi et al., 1995; Russell et al., 1995). These findings clearly demonstrated the nonredundant roles of Jak1, Jak2, and Jak3 in signaling through the corresponding cytokine receptors.

Tyk2 is the first member of the Jak kinase family to be isolated and was originally described as essential for type I IFN signaling in a human fibroblast cell line (Firmbach-Kraft et al., 1990; Velazquez et al., 1992). However, the establishment of Tyk2-deficient mice revealed that Tyk2 was not absolutely essential for type I IFN signaling in mice (Karaghiosoff et al., 2000; Shimoda et al., 2000). *Tyk2*^{-/-} mice displayed a lack of responsiveness to a small amount of IFN α , but a high concentration of IFN α could fully transduce its signals even in the absence of Tyk2. Although Tyk2 is a component of several other cytokine signaling pathways, including those of IL-6 and IL-12 (Bacon et al., 1995; Ihle, 1995; Stahl et al., 1994), in *Tyk2*^{-/-} mice the IL-6-mediated signals are essentially normal and IL-12 signaling is partially impaired. Thus, the function of Tyk2 in mice appears to be compensated for in vivo, partly by other kinase(s), most likely other Jak(s). Whether the discrepancy in the stringency of Tyk2 requirement in cytokine signaling between the human cell lines and the mutant mice can be attributed to the difference between established cell lines and primary cells or the species difference as observed in several primary immunodeficiencies such as the Btk, BLNK, or $\lambda 5$ deficiency has been unclear (Conley et al., 2000; Minegishi et al., 1998, 1999b).

In the present study, we investigated immunological abnormalities in a patient who had been clinically diagnosed with hyper-IgE syndrome (HIES). HIES is a unique primary immunodeficiency, characterized by recurrent skin abscesses, pneumonia, and highly elevated serum IgE (Grimbacher et al., 2005). Its etiology is totally unknown even though the autosomal dominant or recessive inheritance has been reported (Grimbacher et al., 1999a; Renner et al., 2004). The patient investigated in this study showed susceptibility to various microorganisms including virus, fungi, and mycobacteria and suffered from atopic dermatitis with elevated serum IgE. Peripheral blood cells from the patient showed almost complete defects in both IL-12 and IFN α/β signaling, prompting us to identify a homozygous mutation of Tyk2 in this patient, a signaling component shared by both cytokines. The patient's cells also displayed severe defects in IL-6 and IL-10 signaling, in contrast to previous observations in Tyk2-deficient mice. Transduction of the intact Tyk2 gene rescued the patient's cells from the cytokine signaling defects. Thus, the present study identified human Tyk2 deficiency as a type of primary immunodeficiency displaying the phenotype of the autosomal recessive HIES accompanied by susceptibility to intracellular bacterial infection and highlighted a unique and indispensable role played by Tyk2 in the innate and acquired immune responses in humans.

Results

Both IL-12 and IFN α Signaling Pathways Are Defective in T Cells from a Patient with HIES

We investigated, from an immunological point of view, a 22-year-old Japanese male who had been clinically

diagnosed as HIES. According to the HIES scoring system developed by the NIH group (Grimbacher et al., 1999b), the patient scored 48 points, similar to those with typical HIES (see Experimental Procedures). He showed complex clinical manifestations, including susceptibility to various types of microorganisms (virus, intracellular and extracellular bacteria, and fungi), atopic dermatitis-like skin inflammation starting from 1 month of age, and a high amount of serum IgE (2100 IU/ml). He has suffered recurrently from otitis media, sinusitis, pneumonias, skin abscesses, oral candidiasis, molluscum contagiosum, and herpes simplex infection in skin and mucosa. He also experienced an episode of Bacille Calmette-Guerin (BCG) infection at 22 months and nontyphi salmonella gastroenteritis at 15 years of age. The numbers of T, B, and NK cells in the peripheral blood were all within the normal range, and neutrophils showed normal functions in laboratory tests (data not shown). The patient's parents are consanguineous, indicating a possible recessive hereditary disorder. Because the patient experienced an infection with BCG, we first analyzed the genes encoding IL-12 p40, IL-12R β 1, IFNGR1, IFNGR2, and STAT1, which have been identified as responsible genes for primary immunodeficiency with susceptibility to mycobacterial infection (Casanova and Abel, 2004). No mutation was detected in any of the corresponding cDNAs isolated from the patient (data not shown).

We next examined whether the patient had any defect in IL-12 signaling. When peripheral blood mononuclear cells (PBMCs) from the patient were stimulated with mitogens, IL-12 production was normal (data not shown). On the other hand, CD4⁺ T cells isolated from the patient's PBMCs failed to produce detectable amounts of IFN γ when stimulated with IL-12 plus IL-18, a potent IFN γ inducer for T cells (Figure 1A). The failure could not be attributed to a total defect in the machinery for IFN γ production, because the patient's T cells produced considerable amounts of IFN γ in response to a combination of phorbol 12-myristate 13-acetate (PMA) and ionomycin, although the amount was a bit lower than normal (Figure 1A). Furthermore, IL-18 signaling appeared to be intact in the patient's T cells, as assessed by I κ B α degradation in response to IL-18 stimulation (Figure 1B), implying a defect in the IL-12 signal. Indeed, the patient's T cells showed almost no response to IL-12 in contrast to those from a normal subject, as judged by the tyrosine phosphorylation of STAT4, a component of the IL-12 signaling pathway (Figure 1C). Interestingly, the patient's T cells also failed to respond to IFN α (Figure 1C). This finding was in sharp contrast to human IL-12R β 1 deficiency, the only known human defect in IL-12 signaling, in which STAT4 phosphorylation is defective in response to IL-12, but its phosphorylation is intact in response to IFN α (Fieschi et al., 2004).

A Homozygous Mutation in the Patient's Tyk2 Gene

The defect in both IL-12 and IFN α signaling strongly suggested an abnormality in one or more molecules involved in a signaling pathway shared by both cytokines. STAT4 was a good candidate; however, STAT4 was expressed at a normal amount in the patient's T cells (Figure 1C), and no sequence alteration was found in the coding region of the STAT4 gene (data not shown).

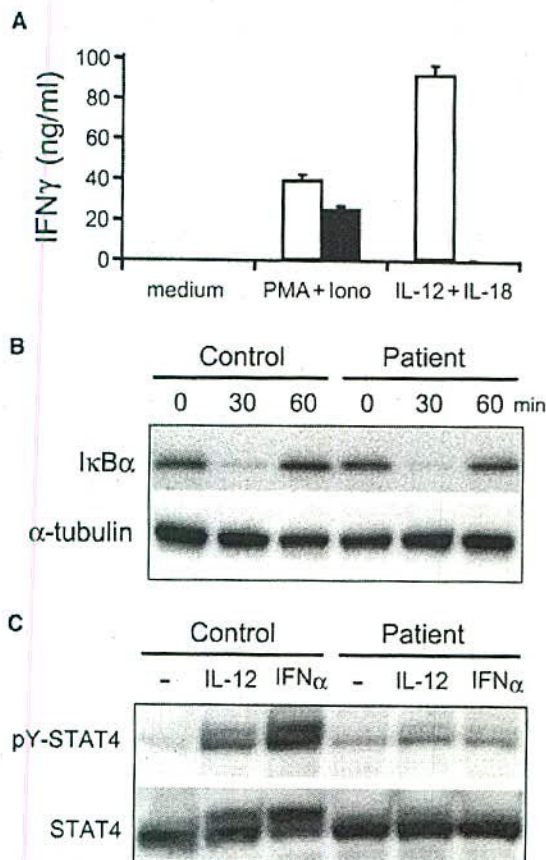


Figure 1. Impaired Responses to IL-12 and IFN α in a Patient with HIES

(A) The patient's T cells were defective in IFN γ production in response to IL-12 and IL-18. The concentration of IFN γ in culture supernatants was measured after CD4⁺ T cells from a healthy control and the patient were cultured for 24 hr with medium alone, a combination of PMA and ionomycin, or a combination of IL-12 and IL-18. White bars indicate a control, and black bars indicate the patient. Error bars are standard deviations.

(B) The patient's T cells had intact IL-18 signaling. I κ B α and α -tubulin proteins were detected by immunoblotting at the indicated time points after the T cells from a control subject and the patient were stimulated with IL-18.

(C) The patient's T cells had defects in both IL-12 and IFN α signaling. Tyrosine 694-phosphorylated and total STAT4 proteins were detected by immunoblotting after the T cells from a control subject and the patient were stimulated with IL-12 or IFN α for 15 min. Results are representative of at least three independent experiments.

The second candidate we investigated was Tyk2, because a mutant human cell line unresponsive to IFN α turned out to be deficient in Tyk2 (Velazquez et al., 1992), even though Tyk2 appeared to be not absolutely essential for IL-12 and IFN α signals in mice (Karaghiosoff et al., 2000; Shimoda et al., 2000). Sequencing of the patient's cDNA and genomic DNA coding for Tyk2 revealed a homozygous deletion of four nucleotides, GCTT, at nt 550–553, that was not found in 50 unrelated healthy individuals (Figures 2A and 2B). This deletion resulted in a frame-shift mutation from codon 70 to 89 and generated a premature stop codon at aa 90 in the 1187 amino acid-long Tyk2 protein (Figure 2B). The predicted truncation of Tyk2 deletes most of the protein, including

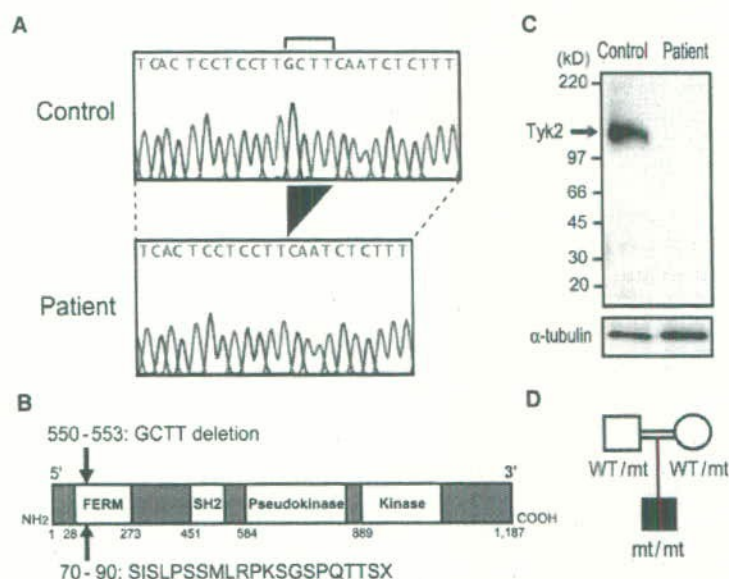


Figure 2. A Homozygous Mutation of the Tyk2 Gene in the Patient

(A) Electropherograms show the part of the Tyk2 cDNA sequences from a control subject and the patient, from which a deletion of four nucleotides, GCTT, was found in the patient. (B) The structure of Tyk2 is shown schematically, and the mutation is indicated: the 4-nucleotide deletion (above) and the frame-shift and truncation of the protein (below). (C) The Tyk2 protein was undetectable in the patient's T cells. Tyk2 and α -tubulin proteins in the T cells from a healthy control subject and the patient were detected by immunoblotting. (D) The patient was homozygous for the mutation, and both parents were heterozygous for it. WT, wild-type Tyk2; mt, mutated Tyk2.

the FERM domain, the SH2 domain, and the pseudo-kinase and kinase domains. Indeed, no Tyk2 protein was detected by immunoblotting with two specific antibodies that recognize different regions of Tyk2 (Figure 2C). Both parents of the patient were apparently healthy and showed normal amounts of serum IgE, but the analysis of genomic DNAs from the parents revealed that both were heterozygous for the same deletion (Figure 2D), establishing that the patient had an autosomal recessive Tyk2 deficiency.

Tyk2 Is Indispensable for Type I IFN Signaling in Humans

Stimulation of the patient's T cells with various concentrations of IFN α or IFN β up to 50 ng/ml (equivalent to 10,000 IU/ml) failed to induce tyrosine phosphorylation of not only STAT4 (Figure 1C) but also Jak1, STAT1, STAT2, and STAT3 (Figure 3A and see Figure S1 in the Supplemental Data available online), in contrast to the observation in Tyk2 $^{-/-}$ mice (Karaghiosoff et al., 2000; Shimoda et al., 2000). Furthermore, no tyrosine phosphorylation of STAT1 and STAT2 was detected at any time points (5, 15, 30, and 60 min) after the treatment with IFNs (Figure S2). Interestingly, the amount of STAT1 protein was reduced in the patient's T cells in accord with the observations in Tyk2-deficient mice (Karaghiosoff et al., 2000), whereas those of STAT2, STAT3, and STAT4 proteins appeared unaffected (Figures 1C and 3A, and Figure S3). The patient's T cells displayed a reduced amount of surface IFNAR1 expression (Figure S4). This is consistent with previous observations in a Tyk2-deficient human cell line but in contrast with that in the Tyk2-deficient mice (Gauzzi et al., 1997; Karaghiosoff et al., 2000; Shimoda et al., 2000). Tyk2 has been shown to stabilize IFNAR1 at the cell surface in human cells (Gauzzi et al., 1997; Ragimbeau et al., 2003). Thus, the defect in the type I IFN signaling in the patient's cells could be explained by the absence of Tyk2 accompanied with reduced amounts of STAT1 and IFNAR1.

Consistent with the signaling defects, the transcriptional upregulation of a panel of IFN-inducible genes

including NMI and MxA, in response to IFN α or IFN β , was completely abrogated in PBMCs from the patient (Figure 3B and Figure S5). We further investigated biological consequences of the compromised IFN signaling. The upregulation of HLA class I expression in response to IFN α was not observed in patient PBMCs (Figure 3C). Unexpectedly, the patient's cells displayed a higher basal amount of HLA class I expression than the control cells, suggesting a possible compensatory mechanism to regulate expression in a type I IFN-independent manner under conditions of the Tyk2 deficiency. Even at high concentrations, neither IFN α nor IFN β produced any antiviral protection against herpes simplex virus (HSV) within the patient B cells (Figures 3D and 3E). Thus, human Tyk2 deficiency showed a complete defect in type I IFN responses under our experimental conditions, in contrast to observations in Tyk2 $^{-/-}$ mice (Karaghiosoff et al., 2000; Shimoda et al., 2000).

We next examined the response to IFN γ in the patient (Figure 3F). Stimulation with IFN γ upregulated the transcription of two IFN γ -responsive genes, IRF1 and RING4, in PBMCs from the patient and from a healthy control. However, the extent of the induction was much smaller in the patient's PBMC sample, in accord with the impairment of IFN γ signaling reported in Tyk2 $^{-/-}$ mice (Karaghiosoff et al., 2000).

Tyk2 Is Critical for IL-23, IL-6, and IL-10 Signaling in Humans

We next examined the response to IL-23 in the patient's cells, because IL-23 shares signaling pathways with IL-12 (Watford et al., 2004). The patient's T cells showed no detectable tyrosine phosphorylation of STAT4 in response to IL-23 even at high concentrations (500 ng/ml) in contrast to T cells from a control subject (Figure 4A). Control T cells secreted IFN γ in response to IL-23 in a dose-dependent manner (Figure 4B) as reported previously (Oppmann et al., 2000). In contrast, the patient's T cells showed no response to IL-23 in IFN γ secretion even though the background amount of

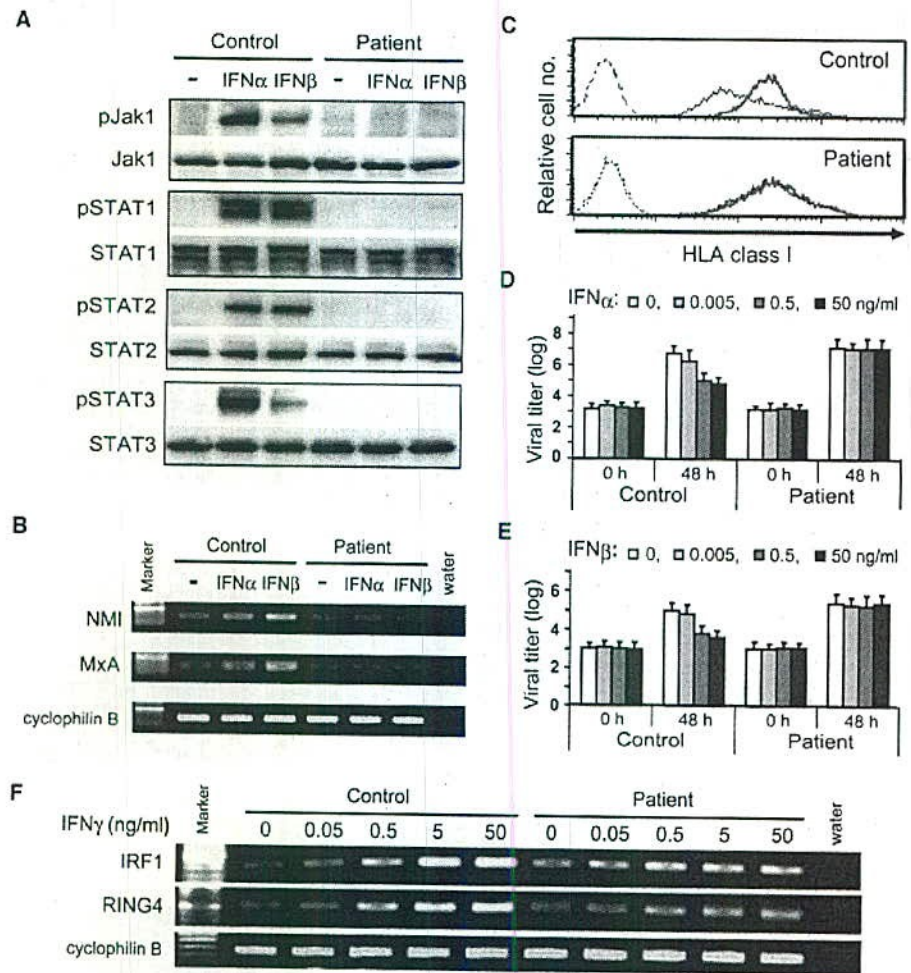


Figure 3. Defects in the Type I and II IFN Signals in the Patient's T Cells
(A) No detectable phosphorylation of the Jak and STAT molecules in the patient's T cells was seen upon stimulation with type I IFN. The indicated proteins (p, phosphorylated form) were detected by immunoblotting after the T cells from a control subject and the patient were stimulated with or without 50 ng/ml (equivalent to 10,000 IU/ml) of IFN α or IFN β for 15 min.
(B) There was no detectable upregulation of the IFN-inducible gene expression in the patient's cells upon stimulation with type I IFN. The amounts of NMI, MxA, and cyclophilin B transcripts were evaluated by semiquantitative RT-PCR after PBMCs from a control subject and the patient were stimulated or not with IFN α or IFN β for 2 hr.
(C) No detectable upregulation of HLA class I in the patient's cells upon stimulation with type I IFN. The level of HLA class I on PBMCs from a control subject and the patient was determined with flow cytometry after the cells were cultured with (histograms with thick gray lines) or without (histograms with thin black lines) IFN α for 48 hr. Histograms with dotted lines show the control staining with an isotype-matched mAb.
(D and E) No antiviral activity was detected in the patient's B cells upon stimulation with type I IFN. EBV-transformed B cells from a control subject and the patient were treated for 24 hr with indicated concentrations of IFN α (D) or IFN β (E) prior to the infection with HSV. The viral titers in culture supernatant of 0 and 48 hr culture were measured by plaque assay with Vero cells. Data are representative of two independent experiments. Error bars are standard deviations.
(F) Impaired response to IFN γ in the patient's cells. The amounts of IRF1, RING4, and cyclophilin B transcripts were evaluated by semiquantitative RT-PCR, after PBMCs from a control subject and the patient were stimulated with the indicated concentration of IFN γ for 2 hr.

IFN γ was slightly higher in the patient's T cells than in the control T cells (Figure 4B). These results indicated that human Tyk2 played an essential role in IL-23 signaling, in accord with observations in the *Tyk2*-deficient mice (Shaw et al., 2003).
It has been demonstrated that Tyk2 is also activated upon stimulation with IL-6 or IL-10 (Ihle, 1995; Stahl et al., 1994). PBMCs from the patient failed to upregulate SOCS3 transcripts in response to IL-6 stimulation (Figure 4C). They secreted IgM when stimulated by infection with Epstein-Barr Virus (EBV), as did those

from a healthy control subject. However, further stimulation with IL-6 induced only a little increase in IgM secretion from the patient's PBMCs, although IgM secretion did increase 3 times in the control PBMCs (Figure 4D). The patient's PBMCs had a very poor response to IL-10 stimulation in their induction of SOCS3 (Figure 4E). Moreover, the inhibition of LPS-induced TNF α production by IL-10 was severely impaired in macrophages from the patient (Figure 4F). These results indicated that Tyk2 plays an obligatory role in signaling of IL-6 and IL-10 in humans, in contrast to *Tyk2*^{-/-} mice, which

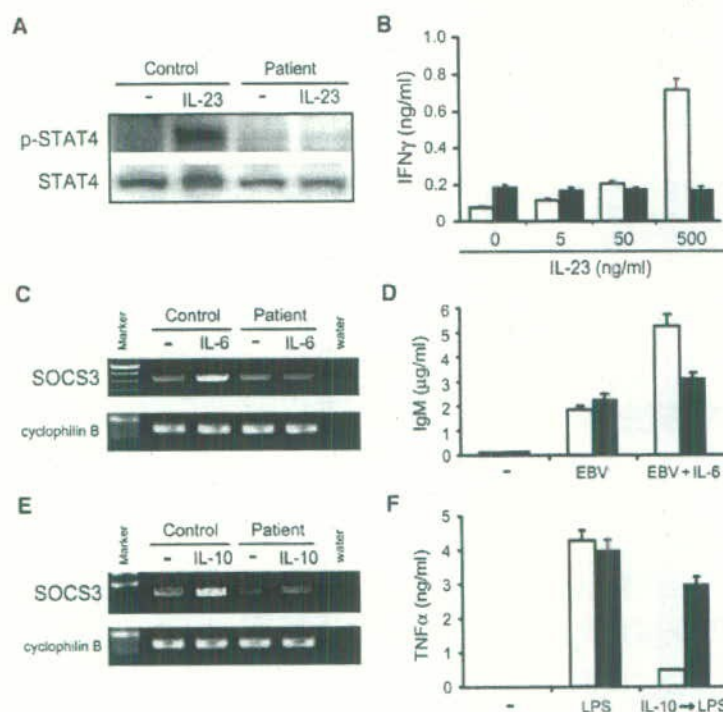


Figure 4. Defects in IL-23, IL-6, and IL-10 signals in the patient's cells

(A) The patient's T cells showed impaired IL-23 signaling. Tyrosine phosphorylated and total STAT4 proteins were detected by immunoblotting after the T cells from a control subject and the patient were stimulated with IL-23 (500 ng/ml) for 15 min.

(B) The patient's T cells showed no substantial response to IL-23 in the IFN γ production. The concentration of IFN γ in culture supernatants was determined by ELISA after the T cells from a control subject and the patient were cultured with the indicated concentrations of IL-23 for 48 hr. White bars indicate a control, and black bars indicate the patient. Error bars are standard deviations.

(C) No detectable upregulation of the SOCS3 gene expression in the patient's cells upon stimulation with IL-6. The amounts of SOCS3 and cyclophilin B transcripts were evaluated by semiquantitative RT-PCR after PBMCs from a control subject and from the patient were stimulated with or without IL-6 for 4 hr. (D) Little enhancement of IgM secretion in the patient's B cells upon stimulation with IL-6. The concentration of IgM in the culture supernatants was determined by ELISA after PBMCs from a control subject and the patient were cultured for 10 days with or without Epstein-Barr virus (EBV), alone or in combination with IL-6. White bars indicate a control, and black bars indicate the patient. Error bars are standard deviations.

(E) Poor upregulation of the SOCS3 gene expression in the patient's cells upon stimulation with IL-10. The amounts of SOCS3 and cyclophilin B transcripts were evaluated by semiquantitative RT-PCR after PBMCs from a control and the patient were cultured with or without IL-10 for 4 hr. (F) Poor inhibition of LPS-induced TNF α production by IL-10 in the patient's macrophages. The concentration of TNF α in the culture supernatants was determined by ELISA after macrophages derived from a control subject's and the patient's PBMCs were cultured with or without IL-10 for 24 hr, and then stimulated or not with LPS for 48 hr. White bars indicate a control, and black bars indicate the patient. Error bars are standard deviations. Results are representative of at least three independent experiments.

respond normally to IL-6 (Karaghiosoff et al., 2000; Shimoda et al., 2000). *Tyk2*^{-/-} mice were originally reported to respond normally to IL-10 (Karaghiosoff et al., 2000; Shimoda et al., 2000), while a recent study demonstrates partial impairment in IL-10 signaling (Shaw et al., 2006).

Imbalance in Th1 and Th2 Differentiation in the Patient

We next examined *in vitro* the Th1 and Th2 differentiation of T cells from the patient (Figure 5), because the patient displayed Th2-dominant phenotypes such as atopic dermatitis and highly elevated serum IgE. Even under Th1-biased culture conditions, the frequency of IFN γ -producing Th1 cells remained low in the patient's T cells (Figure 5A, middle). On the other hand, their differentiation into IL-4-producing Th2 cells under Th2-biased culture conditions appeared to be accelerated (Figure 5A, right). When cultured for 14 days in the presence of anti-CD3 and IL-2, the patient's T cells showed helper T cell differentiation that was extremely skewed toward Th2, compared with the control T cells (Figure 5B). Naive CD4⁺ T cells isolated from the patient's PBMCs showed reduced secretion of IFN γ and enhanced secretion of IL-5 and IL-13 as compared to the control T cells when stimulated with anti-CD3 and anti-CD28 (Figure 5C). These results clearly indicated that *Tyk2* is critical for the IL-12-induced Th1 differentiation and represses the development of Th2 cells, in

accord with the observations in mice (Seto et al., 2003; Shaw et al., 2003).

Expression of the Intact *Tyk2* Gene Rescues the Patient's T Cells from Defects in IL-12 and Type I IFN Signaling

The wild-type *Tyk2* gene in a retroviral vector was introduced into activated primary T cells from the patient. The infected T cells acquired the expression of *Tyk2* proteins at approximately 70% of the normal amount (Figure 6A). Upon combined stimulation with the IL-12 and IL-18, the infected *Tyk2*-expressing cells produced IFN γ , as did T cells from a normal control subject (Figure 6B). In contrast, the patient's T cells infected with virus carrying the empty vector did not produce IFN γ in response to this stimulation, nor did uninfected cells. Furthermore, *Tyk2*-infected T cells from the patient acquired the ability to upregulate the transcription of *MxA* in response to IFN α (Figure 6C). A complementary experiment was performed in which human HeLa cells were subjected to the knockdown of *Tyk2* gene expression with siRNAs. In cells treated with *Tyk2*-specific siRNAs, the tyrosine phosphorylation of STAT1 and STAT2 in response to IFN α was almost completely abrogated, concomitantly with the suppression of *Tyk2* expression, as observed in the patient's cells (Figure S6). These results indicated that the defects in the IL-12 and type I IFN signaling observed in the patient's T cells were caused primarily by the absence of functional *Tyk2*.

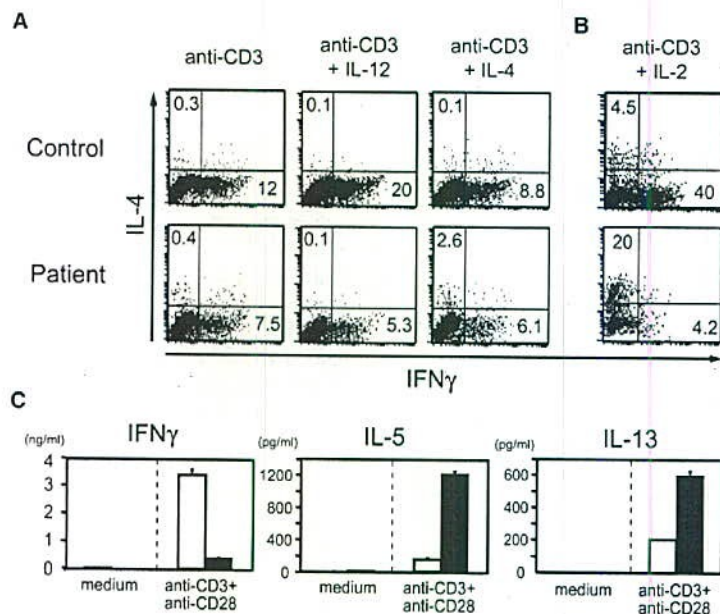


Figure 5. Impaired Th1 and Accelerated Th2 Differentiation in the Patient's T Cells

(A) Intracellular staining with anti-IFN γ and anti-IL-4 was performed after PBMCs from a control subject and the patient were stimulated with immobilized anti-CD3 alone, in combination with IL-12 and anti-IL-4 (Th1-promoting condition) or in combination with IL-4 (Th2-promoting condition) for 5 days. The percentage of IFN γ - or IL-4-producing cells is shown in each panel.

(B) PBMCs from a control subject and the patient were stimulated with immobilized anti-CD3 and IL-2 for 14 days, and intracellular staining was performed as in (A).

(C) Naive CD4 $^{+}$ T cells from a control subject and the patient were stimulated with anti-CD3 and anti-CD28 for 3 days, and the concentration of IFN γ , IL-5, and IL-13 in culture supernatants was measured by ELISA. White bars indicate a control, and black bars indicate the patient. Error bars are standard deviations. Results are representative of at least three independent experiments.

Discussion

We have identified a human deficiency in Tyk2. Among the four members of the Jak family, Jak3 deficiency was previously reported in human, demonstrating the crucial role of Jak3 in lymphocyte development, in accord with findings in Jak3-deficient mice (Macchi et al., 1995; Nosaka et al., 1995; Park et al., 1995; Russell et al., 1995). The identification of a human Tyk2 deficiency highlighted the obligatory roles of human Tyk2 in multiple cytokine signals involved in innate and acquired immune responses, including type I IFN, IL-6, IL-10, IL-12, and IL-23 signals. Although Tyk2 is ubiquitously expressed (unlike Jak3), the Tyk2 deficiency displayed apparently normal embryogenesis, postnatal growth, and hematopoiesis, while it resulted in a primary immunodeficiency with clinical manifestations characteristic to HIES, indicating a unique and indispensable role played by Tyk2 in the immune system.

The present study contrasted differences in the stringency of Tyk2 requirement between human and mice. The nonredundant roles of human Tyk2 in multiple cytokine signals in vivo could not be assumed from the studies on mouse Tyk2. The Tyk2 $^{-/-}$ mice responded normally to IL-6, even though IL-6 activated Tyk2 in vitro. The mice also showed a leaky phenotype in type I IFN responses: a high concentration of IFN α showed antiviral effects and upregulated MHC class I expression in cells from the Tyk2 $^{-/-}$ mice to an extent comparable to that observed in wild-type mice (Karaghiosoff et al., 2000; Shimoda et al., 2000). This was a sharp contrast to the observation in human Tyk2-deficient fibroblast cell lines. Our results indicated that the discrepancy between these two observations was due to a species difference rather than the difference between primary cells and particular cultured cell lines. We observed no detectable tyrosine phosphorylation of Jak1 in response to type I IFN in the human Tyk2 deficiency, supporting the idea that Tyk2 and Jak1 transphosphorylate each

other, and hence both are independently required for the initial activation of IFN signaling. In contrast, in the mouse Tyk2 deficiency, the Jak1 phosphorylation was observed when stimulated with type I IFN, albeit to a lesser extent than in wild-type mice. Thus, Tyk2 is the obligatory requirement for the initiation of type I IFN signaling in human whereas it is redundant in the initiation and might function as an amplifier of signaling in mice. This also appears to be the case in the IL-12 signaling. Tyk2 $^{-/-}$ mice showed compromised signaling and biological effects in response to IL-12 (Karaghiosoff et al., 2000; Seto et al., 2003; Shaw et al., 2003; Shimoda et al., 2000), but the impairment was less drastic than that observed in IL-12 receptor-deficient mice (Wu et al., 1997).

The in vitro findings of defects in multiple cytokine signals due to the Tyk2 deficiency appeared to account for the complex clinical manifestations of the patient investigated in the present study. First, the susceptibility to viral infection could be attributed to the defect in IFN signaling. Indeed, even a high titer of type I IFNs did not show any protective effect against herpes simplex virus in the patient's cells, unlike in Tyk2 $^{-/-}$ mice (Karaghiosoff et al., 2000; Shimoda et al., 2000). Interestingly, the response to type II IFN (IFN γ) stimulation was also impaired in the patient's T cells, albeit to a lesser extent, even though Tyk2 has not been assigned to the type II IFN signaling pathway. The partial impairment of IFN γ signaling could be explained, at least in part, by the reduced amounts of STAT1 protein or by the observation that IFN γ signaling depends on constitutive subthreshold type I IFN signaling (Takaoka et al., 2000). Thus, the type II IFN response is reduced but not absent in the Tyk2 deficiency in contrast to its complete lack in the STAT1 deficiency (Dupuis et al., 2003), even though the type I IFN response is absent in both deficiencies. This could account for the apparently milder phenotype of the Tyk2 deficiency in viral infections, compared to the lethal phenotype of the STAT1 deficiency. It remains

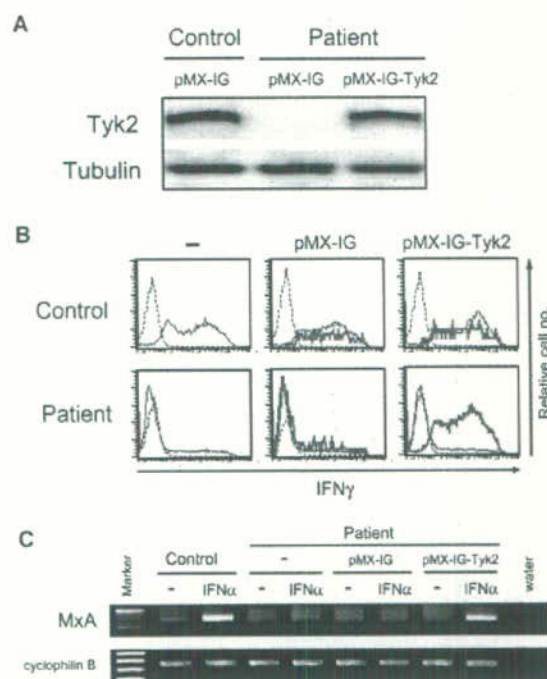


Figure 6. Transduction of the Intact Tyk2 Gene Rescued the Patient's T Cells from Defects in the IL-12 and Type I IFN Signals
(A) Reconstitution of Tyk2 in the patient's T cells. T cells from the patient and a control subject were infected with either the empty retroviral vector (pMX-IG) or the Tyk2 cDNA-containing vector (pMX-IG-Tyk2). The protein expression of Tyk2 and tubulin in GFP⁺-infected cells were detected by immunoblotting.
(B) Retrovirus-mediated Tyk2 transfer restored the IFN γ production in response to IL-12 and IL-18 in the patient's T cells. The production of IFN γ was detected by intracellular staining with an IFN γ mAb in uninfected and infected T cells after stimulation with IL-12 and IL-18 for 48 hr. Histograms with thick gray lines show the staining profile of GFP⁺-infected cells, and those with thin black lines show the staining profile of GFP⁻ uninfected cells. Histograms with dotted lines show the control staining with an isotype-matched mAb.
(C) Retrovirus-mediated Tyk2 transfer restored the upregulation of IFN-inducible gene expression in response to IFN α in the patient's T cells. The amounts of MxA and cyclophilin B transcripts were evaluated by semiquantitative RT-PCR after stimulation of the indicated T cells with IFN α for 2 hr. Results are representative of at least three independent experiments.

to be determined whether the type III IFN (IL-28 and IL-29) signaling is defective in the Tyk2 deficiency.

Second, the susceptibility of the patient to infection with intracellular bacteria, such as mycobacteria and salmonella, could be explained by the defect in IL-12 signaling, as seen in IL-12R β 1 deficiency (Altare et al., 1998; de Jong et al., 1998). The differentiation into Th1 cells, which are important for the elimination of these bacteria, was severely impaired in the patient's T cells, even under culture conditions that strongly promote the differentiation of Th1 cells. Given that type I IFNs promote Th1 differentiation in humans and not in mice, the defect in the IFN signal might also account in part for the failure of Th1 differentiation in this patient. The susceptibility of Tyk2 knockout mice to infection of intracellular bacteria has not been reported, but a natural mutant strain B10.Q/J, which is susceptible to toxoplasma infection and resistant to induction of autoimmune arthritis, was reported to carry Tyk2 mutation

(Shaw et al., 2003). Mice deficient for IL-23 showed the susceptibility to *Klebsiella pneumoniae* owing to their impairment in IL-17 production by T cells (Happel et al., 2005). Therefore, the patient's susceptibility to extracellular bacteria might be explained at least in part by the defect in IL-23 signaling.

Third, the high amount of serum IgE and the atopic dermatitis-like skin inflammation in the patient could be due to the accelerated Th2 differentiation. Indeed, the Tyk2 deficiency was shown to accelerate Th2-mediated allergic airway inflammation in mice (Seto et al., 2003). In a previous report, enhanced Th2 differentiation was not observed in an IL-12R β 1 deficiency, although the Th1 differentiation was severely impaired (Wood et al., 2005). Thus, the defect in IL-12 signaling does not account for the abnormally accelerated Th2 differentiation in the Tyk2-deficient patient. PBMCs and macrophages from the patient showed a severe signaling defect in the IL-10 pathway. IL-10 has been reported to suppress the differentiation of both Th1 and Th2 cells, so the defect in the IL-10 pathway might explain, at least in part, the accelerated Th2 differentiation under conditions in which Th1 differentiation was defective as observed in mice deficient for both IL-10 and IL-12 (Hoffmann et al., 2000).

The patient investigated in the present study had been clinically diagnosed as HIES, fulfilling the criteria for the diagnosis according to the NIH scoring system (Grimbacher et al., 1999b). HIES is a primary immunodeficiency with unknown etiology, characterized typically by high serum IgE amounts, recurrent staphylococcal skin abscesses, and pneumonia with pneumatocele formation (Grimbacher et al., 2005). While most cases of HIES are sporadic, families displaying autosomal dominant (AD) or autosomal recessive (AR) inheritance have been reported (Grimbacher et al., 1999a; Renner et al., 2004). In most sporadic and AD cases, these clinical manifestations are part of a multisystem disorder including abnormalities of the soft tissue, skeletal, and dental systems (Grimbacher et al., 1999a). In contrast, patients with AR-HIES lack skeletal and dental involvement and lung cyst formation and suffer from fungal and viral infections such as herpes simplex and molluscum contagiosum (Renner et al., 2004). According to this classification, the present case could be categorized into the AR-HIES. It is notable that the infection with intracellular bacteria experienced by the present case has not been frequently observed in HIES patients, even though three cases with mycobacterial infection were reported in the literatures (Grimbacher et al., 1999a; Metin et al., 2004; Pasic et al., 1998). Therefore, we would like to propose that the present case represents a subset of the AR-HIES. The whole picture of HIES including its etiology has not yet drawn clearly, so further genetic analyses of HIES is needed to evaluate this proposal and define the Tyk2 deficiency as either a variant of HIES or a distinct disease entity. In either case, the present study highlighted a requisite and nonredundant role played by Tyk2 in the innate and acquired immune responses in human.

Experimental Procedures

Patient

This study was approved by the institutional review board at the Tokyo Medical and Dental University. Written informed consent

was obtained from all the subjects studied. The patient investigated in this study is a 22-year-old Japanese male with a history of infantile atopic dermatitis starting from 1 month of age and recurrent infections, such as otitis media, sinusitis, pneumonias, and skin abscesses, starting from 12 months of age. At 22 months, he developed BCG lymphadenitis. Histological examination revealed paucibacillary, well-circumscribed granulomas with epithelioid and giant multinucleated cells, as observed in the human IL-12R β 1 deficiency (Altare et al., 1998; de Jong et al., 1998). The immunological work-up performed at this occasion revealed mild eosinophilia (700–800/mm³) and high serum IgE (2100 IU/ml) with normal amounts of other Ig classes and subclasses. All other laboratory data examined were within the normal range, including complement components, the oxidative burst of granulocytes, the number and the size of platelets, lymphocyte subpopulations, and their proliferative responses to mitogens. The patient's illness was tentatively diagnosed as HIES though he did not show any apparent skeletal abnormality. Thereafter, in addition to infections with bacteria including *Staphylococcus aureus*, he recurrently suffered from viral and fungal infections, such as molluscum contagiosum, herpes simplex infection of skin and mucosa, and oral candidiasis. At 15 years of age, the patient developed severe non-typhi salmonella gastroenteritis leading to sepsis. According to the HIES scoring system (Grimbacher et al., 1999b), the patient gained 48 points; 10 in IgE titers, 8 in skin abscess, 8 in pneumonia, 3 in eosinophil count, 4 in newborn rash, 4 in eczema, 2 in upper respiratory infection, 1 in oral candidiasis, 4 in other serious infections, and 4 in fatal infection.

Antibodies and Cytokines

The mAbs for the N-terminal region (aa 46–258) of human Tyk2, IL-12R β 1, IL-12R β 2, IL-4, and IFN γ were purchased from BD Pharmingen, and the CD3 mAb (OKT3) was from Janssen Pharmaceutical. The Ab for the C-terminal region of Tyk2 (C-8) was from Santa Cruz. Rabbit antibodies for Jak1, STAT1, STAT2, STAT3, and STAT4 and their phosphorylated forms were purchased from Cell Signaling. Anti-IFNAR1 was from R&D Systems. HRP-conjugated rabbit anti-mouse and goat anti-rabbit antibodies were from Calbiochem, and mouse anti- α -tubulin was from Sigma-Aldrich. Recombinant human IL-4, IL-12, IFN α , IFN β , IFN γ , and GM-CSF were purchased from Peprotech, recombinant human IL-2 was from Shionogi, and IL-18 was from MBL.

Isolation and Culture of T Cells and Macrophages from PBMCs

Heparinized blood samples were obtained from the patient and from healthy age-matched control subjects. PBMCs were isolated from blood samples through density gradient centrifugation via Histopaque-1077 (Sigma-Aldrich). For T cell activation, PBMCs were cultured with 5 μ g/ml plate-bound CD3 mAb and 300 U/ml IL-2 in RPMI 1640 (Sigma-Aldrich) containing 10% fetal bovine serum (FBS), 50 μ M 2-mercaptoethanol (Life Technologies), 2% L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 1% penicillin-streptomycin. CD4⁺ T cells were enriched with immunomagnetic beads (BD Pharmingen) with more than 98% purity. After 14 days of culture, more than 98% of the cells from both the patient and control subjects were effector memory T cells (CD45RO⁺CCR7⁻). Naive CD4⁺ T cells were purified by removing CD45RO⁺ cells from CD4⁺ T cells by immunomagnetic beads (BD Pharmingen) with more than 90% purity. To obtain macrophages, PBMCs were cultured for 2 hr, and the adherent cells were further cultured with 10 ng/ml GM-CSF for 7 days as described (Hart et al., 1995). All the experiments were performed at least three times with three different controls.

Construction of Retroviral Vectors and Infection of Cells

The retroviral vector pMX-IRES-GFP-Tyk2 was constructed by inserting the Tyk2 cDNA into the BamHI and NotI sites of the pMX-IRES-GFP vector (a gift from T. Kitamura) (Nosaka et al., 1999). Infectious retroviral particles were generated with the Plat-GP cell line (a gift from T. Kitamura and H. Miyoshi). Retroviral supernatant stocks were produced by culturing the producer cells at 37°C for 72 hr. For the infection, PBMCs were stimulated in culture with 5 μ g/ml plate-bound CD3 mAb and 300 IU/ml IL-2. Cells (1 $\times$ 10⁶/ml) were then spin-infected with the retroviral supernatant at 32°C for 30 min. The transduction procedure was repeated on days 2 and 3. The

efficiency of infection was approximately 3% as judged by the frequency of GFP⁺ cells.

Tyk2 Knockdown

RNA interference for Tyk2 was designed with the aid of the online software from Invitrogen. The sequences of siRNA sense strands (5' $\rightarrow$ 3') were as follows: Tyk2 siRNA1, CCCAGAGCAAGCCU GAUGCUAU, and Tyk2 siRNA2, CCAUUCUGAAGACAGUCCAUGA GAA. HeLa cells were grown in DMEM supplemented with 10% FBS. Transfection of the cells with siRNAs was performed with Lipofectamine-RNAiMAX reagent (Invitrogen). 48 hr after transfection, the cells were treated with IFN α (50 ng/ml) for 15 min before lysis. Lysates were analyzed by immunoblotting with the antibodies indicated.

RT-PCR and Direct Sequence Analysis

Extraction of total RNA, cDNA synthesis, PCR, and semiquantitative RT-PCR were performed as previously described (Minegishi et al., 1999a). PCR primers were designed as follows: Tyk2, sense, 5'-TTGCTTGAGTTGACACAGGGAGCT-3', antisense, 5'-TCTCTAGAC AGGAGTAAGGCACAC-3'; STAT4, sense, 5'-CTGGGACCTGTGCTG AGAGAGC-3', antisense, 5'-ACTTTTTCATTGCTTCCTT-3'; NMI, sense, 5'-TTAAGGAGCATTGCCAGAT-3', antisense, 5'-TTCGAGC TCATTGAAACGA-3'; MxA, sense, 5'-GAGGTGCAGGAGAACAG CTC-3', antisense 5'-CTCCTCATACTGGCTGCACA-3'; cyclophilin B, sense, 5'-GCCCAAAGTCACCGTCAAGG-3', antisense, 5'-GGAA GCGCTCACCCTAGATG-3'; IRF1, sense, 5'-GCTGGGACATCAACA AGGAT-3', antisense, 5'-GTGGAAGCATCCGGTACACT-3'; RING4, sense, 5'-AGGGCTGGCTGGCTGCTTGA-3', antisense, 5'-ACGTG GCCCATGGTGTGTTAT-3'; and SOCS3, sense, 5'-TTCAGCTCCA AGAGCGAGTA-3', antisense, 5'-CGGAGTAGATGTAATATGGCTC-3'. Sequencing was performed with the ABI Prism dRhodamine Terminator kit and analyzed with an ABI Prism 310 DNA Sequencer (Perkin-Elmer Applied Biosystems).

Stimulation of Cells with Cytokines and the Measurement of Cytokine and Ig Production

Cells were stimulated for the indicated time in culture with PMA (40 nM), ionomycin (0.5 μ g/ml), IL-12 (10 ng/ml), IL-18 (50 ng/ml), IFN α (50 ng/ml), IFN β (50 ng/ml), IL-6 (10 ng/ml), or IL-10 (100 ng/ml). The concentration of IFN γ in the culture supernatants was determined by ELISA (BD-Pharmingen) according to the manufacturer's instructions, after the cells (1 $\times$ 10⁶/ml) were cultured in 96-well plates at 37°C for 24 hr with or without stimulation. The concentration of TNF α in the culture supernatants was determined by ELISA (BD-Pharmingen), after the cells (1 $\times$ 10⁵/well) were cultured in 96-well plates at 37°C for 48 hr with or without stimulation. The measurement of IgM secretion from Epstein-Barr virus-infected B cells was determined by ELISA as previously described (Minegishi and Conley, 2001). To evaluate helper T cell differentiation in vitro, PBMCs were cultured for 5 days with immobilized anti-CD3 alone or in combination with IL-12 (10 ng/ml) and anti-IL-4 (10 μ g/ml) to promote Th1 differentiation, or in combination with IL-4 (20 ng/ml) to promote Th2 differentiation, or the PBMCs were cultured for 14 days with immobilized anti-CD3 and IL-2 (300 IU/ml). Alternatively, naive CD4⁺ T cells were cultured with immobilized anti-CD3 and soluble anti-CD28 for 3 days. The concentration of IFN γ , IL-5, and IL-13 in the culture supernatants was determined by ELISA (BD Pharmingen).

Flow Cytometric Analysis

The surface immunophenotype was analyzed as described (Minegishi et al., 1999a). For cytokine intracellular staining, cells were restimulated with PMA and ionomycin in the presence of 2 μ M of monensin for 5 hr, followed by fixation and cytoplasmic staining with FITC- or PE-conjugated mAbs specific for the indicated cytokines. An irrelevant FITC- or PE-conjugated IgG1 mAb was used as the negative control.

Immunoblotting and Immunoprecipitation

Cells were washed and cultured for 18 hr in starvation medium consisting of RPMI 1640 and 1% FBS. Cells were then stimulated at 37°C for 15 min with medium alone, 10 ng/ml IL-12, 50 ng/ml IL-18, 50 ng/ml IFN α , or 50 ng/ml IFN β . After the stimulation, the cells

were lysed on ice for 30 min in lysis buffer containing 1% Triton X-100, 50 mM Tris (pH 8.0), 150 mM NaCl, 2 mM EDTA, 2 μ g/ml aprotinin, 100 μ g/ml PMSF, 1 mM sodium orthovanadate, and 1 mM NaF. The cell lysates were subjected to SDS-PAGE, followed by electrotransfer to PVDF membranes and immunoblotting with the indicated antibodies.

Antiviral Activity Assay

EBV-transformed B cells were pretreated with or without 0.005, 0.5, 50 ng/ml IFN α or IFN β for 24 hr, and then infected with HSV (10^3 plaque-forming units/ml). The viral titers in culture supernatants were determined at the indicated time points by plaque assay with Vero cells.

Supplemental Data

Six Supplemental Figures can be found with this article online at <http://www.immunity.com/cgi/content/full/25/5/745/DC1/>.

Acknowledgments

We appreciate the willingness of the patient and the parents to participate in this research study. This work is supported by Grants-in-Aid 16616004, 17047013, and 18659299 from the Japanese Ministry of Education, Culture, Sports, Science, and Technology and Grants-in-Aid 2212131 and 2211932 from the Japanese Ministry of Health, Labor, and Welfare.

Received: May 3, 2006

Revised: June 27, 2006

Accepted: September 19, 2006

Published online: November 9, 2006

References

- Alexander, W.S., and Hilton, D.J. (2004). The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response. *Annu. Rev. Immunol.* 22, 503–529.
- Altare, F., Durandy, A., Lammas, D., Emile, J.F., Lamhamedi, S., Le Deist, F., Drysdale, P., Jouanguy, E., Doffinger, R., Bernaudin, F., et al. (1998). Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. *Science* 280, 1432–1435.
- Bacon, C.M., McVicar, D.W., Ortaldo, J.R., Rees, R.C., O'Shea, J.J., and Johnston, J.A. (1995). Interleukin 12 (IL-12) induces tyrosine phosphorylation of JAK2 and TYK2: differential use of Janus family tyrosine kinases by IL-2 and IL-12. *J. Exp. Med.* 181, 399–404.
- Casanova, J.L., and Abel, L. (2004). The human model: a genetic dissection of immunity to infection in natural conditions. *Nat. Rev. Immunol.* 4, 55–66.
- Conley, M.E., Rohrer, J., Rapalus, L., Boylin, E.C., and Minegishi, Y. (2000). Defects in early B-cell development: comparing the consequences of abnormalities in pre-BCR signaling in the human and the mouse. *Immunol. Rev.* 178, 75–90.
- de Jong, R., Altare, F., Haagen, I.A., Elferink, D.G., Boer, T., van Breda Vriesman, P.J., Kabel, P.J., Draaisma, J.M., van Dissel, J.T., Kroon, F.P., et al. (1998). Severe mycobacterial and *Salmonella* infections in interleukin-12 receptor-deficient patients. *Science* 280, 1435–1438.
- Dupuis, S., Jouanguy, E., Al-Hajjar, S., Fieschi, C., Al-Mohsen, I.Z., Al-Jumaah, S., Yang, K., Chappier, A., Eidenschenk, C., Eid, P., et al. (2003). Impaired response to interferon- α/β and lethal viral disease in human STAT1 deficiency. *Nat. Genet.* 33, 388–391.
- Fieschi, C., Bosticardo, M., de Beaucoudrey, L., Boisson-Dupuis, S., Feinberg, J., Santos, O.F., Bustamante, J., Levy, J., Candotti, F., and Casanova, J.L. (2004). A novel form of complete IL-12/IL-23 receptor β 1 deficiency with cell surface-expressed nonfunctional receptors. *Blood* 104, 2095–2101.
- Firmbach-Kraft, I., Byers, M., Shows, T., Dalla-Favera, R., and Krolewski, J.J. (1990). tyk2, prototype of a novel class of non-receptor tyrosine kinase genes. *Oncogene* 5, 1329–1336.
- Gauzzi, M.C., Barbieri, G., Richter, M.F., Uze, G., Ling, L., Fellous, M., and Pellegrini, S. (1997). The amino-terminal region of Tyk2 sustains

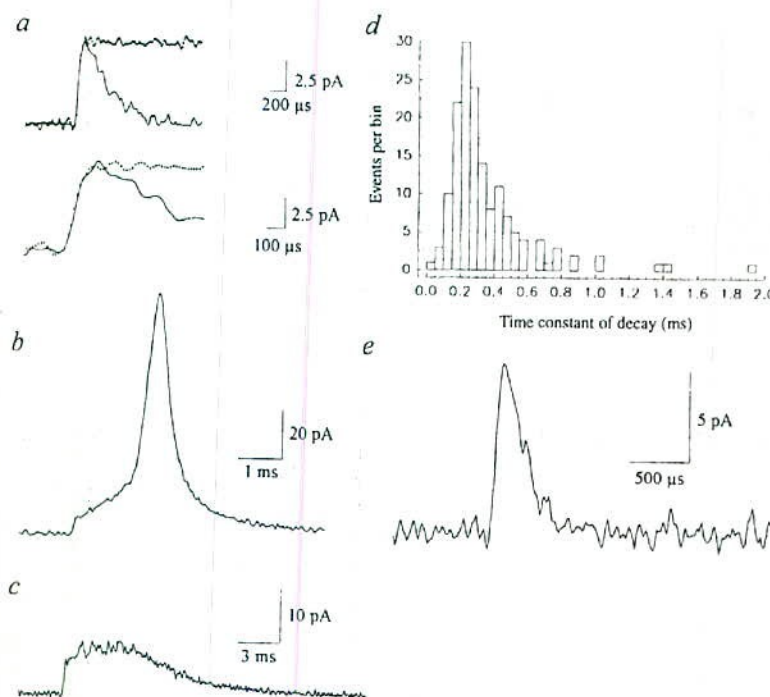
the level of interferon alpha receptor 1, a component of the interferon α/β receptor. *Proc. Natl. Acad. Sci. USA* 94, 11839–11844.

- Grimbacher, B., Holland, S.M., Gallin, J.I., Greenberg, F., Hill, S.C., Malech, H.L., Miller, J.A., O'Connell, A.C., and Puck, J.M. (1999a). Hyper-IgE syndrome with recurrent infections—an autosomal dominant multisystem disorder. *N. Engl. J. Med.* 340, 692–702.
- Grimbacher, B., Schaffer, A.A., Holland, S.M., Davis, J., Gallin, J.I., Malech, H.L., Atkinson, T.P., Belohradsky, B.H., Buckley, R.H., Cossu, F., et al. (1999b). Genetic linkage of hyper-IgE syndrome to chromosome 4. *Am. J. Hum. Genet.* 65, 735–744.
- Grimbacher, B., Holland, S.M., and Puck, J.M. (2005). Hyper-IgE syndromes. *Immunol. Rev.* 203, 244–250.
- Happel, K.L., Dubin, P.J., Zheng, M., Ghilardi, N., Lockhart, C., Quinton, L.J., Odden, A.R., Shellito, J.E., Bagby, G.J., Nelson, S., and Kolls, J.K. (2005). Divergent roles of IL-23 and IL-12 in host defense against *Klebsiella pneumoniae*. *J. Exp. Med.* 202, 761–769.
- Hart, P.H., Jones, C.A., and Finlay-Jones, J.J. (1995). Monocytes cultured in cytokine-defined environments differ from freshly isolated monocytes in their responses to IL-4 and IL-10. *J. Leukoc. Biol.* 57, 909–918.
- Hoffmann, K.F., Cheever, A.W., and Wynn, T.A. (2000). IL-10 and the dangers of immune polarization: excessive type 1 and type 2 cytokine responses induce distinct forms of lethal immunopathology in murine schistosomiasis. *J. Immunol.* 164, 6406–6416.
- Ihle, J.N. (1995). Cytokine receptor signalling. *Nature* 377, 591–594.
- Karaghiannis, M., Neubauer, H., Lassnig, C., Kovarik, P., Schindler, H., Pircher, H., McCoy, B., Bogdan, C., Decker, T., Brem, G., et al. (2000). Partial impairment of cytokine responses in Tyk2-deficient mice. *Immunity* 13, 549–560.
- Liu, K.D., Gaffen, S.L., and Goldsmith, M.A. (1998). JAK/STAT signaling by cytokine receptors. *Curr. Opin. Immunol.* 10, 271–278.
- Macchi, P., Villa, A., Giliani, S., Sacco, M.G., Frattini, A., Porta, F., Ugazio, A.G., Johnston, J.A., Candotti, F., O'Shea, J.J., et al. (1995). Mutations of Jak-3 gene in patients with autosomal severe combined immune deficiency (SCID). *Nature* 377, 65–68.
- Metin, A., Uysal, G., Guven, A., Unlu, A., and Ozturk, M.H. (2004). Tuberculous brain abscess in a patient with hyper IgE syndrome. *Pediatr. Int.* 46, 97–100.
- Minegishi, Y., and Conley, M.E. (2001). Negative selection at the pre-BCR checkpoint elicited by human mu heavy chains with unusual CDR3 regions. *Immunity* 14, 631–641.
- Minegishi, Y., Coustan-Smith, E., Wang, Y.H., Cooper, M.D., Campana, D., and Conley, M.E. (1998). Mutations in the human λ 5/14.1 gene result in B cell deficiency and agammaglobulinemia. *J. Exp. Med.* 187, 71–77.
- Minegishi, Y., Coustan-Smith, E., Rapalus, L., Ersoy, F., Campana, D., and Conley, M.E. (1999a). Mutations in Ig α (CD79a) result in a complete block in B-cell development. *J. Clin. Invest.* 104, 1115–1121.
- Minegishi, Y., Rohrer, J., Coustan-Smith, E., Lederman, H.M., Pappu, R., Campana, D., Chan, A.C., and Conley, M.E. (1999b). An essential role for BLNK in human B cell development. *Science* 286, 1954–1957.
- Muller, M., Briscoe, J., Laxton, C., Guschin, D., Ziemiecki, A., Silvennoinen, O., Harpur, A.G., Barbieri, G., Witthuhn, B.A., Schindler, C., et al. (1993). The protein tyrosine kinase JAK1 complements defects in interferon- α/β and γ signal transduction. *Nature* 366, 129–135.
- Neubauer, H., Cumano, A., Muller, M., Wu, H., Hufstadt, U., and Pfeffer, K. (1998). Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis. *Cell* 93, 397–409.
- Nosaka, T., van Deursen, J.M., Tripp, R.A., Thierfelder, W.E., Witthuhn, B.A., McMickle, A.P., Doherty, P.C., Grosveld, G.C., and Ihle, J.N. (1995). Defective lymphoid development in mice lacking Jak3. *Science* 270, 800–802.
- Nosaka, T., Kawashima, T., Misawa, K., Ikuta, K., Mui, A.L., and Kitamura, T. (1999). STAT5 as a molecular regulator of proliferation, differentiation and apoptosis in hematopoietic cells. *EMBO J.* 18, 4754–4765.
- Oppmann, B., Lesley, R., Blom, B., Timans, J.C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., et al. (2000). Novel p19 protein

- engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 13, 715–725.
- O'Shea, J.J., Gadina, M., and Schreiber, R.D. (2002). Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. *Cell* 109 (Suppl), S121–S131.
- Parganas, E., Wang, D., Stravopodis, D., Topham, D.J., Marine, J.C., Teglund, S., Vanin, E.F., Bodner, S., Colamonici, O.R., van Deursen, J.M., et al. (1998). Jak2 is essential for signaling through a variety of cytokine receptors. *Cell* 93, 385–395.
- Park, S.Y., Saijo, K., Takahashi, T., Osawa, M., Arase, H., Hirayama, N., Miyake, K., Nakauchi, H., Shirasawa, T., and Saito, T. (1995). Developmental defects of lymphoid cells in Jak3 kinase-deficient mice. *Immunity* 3, 771–782.
- Pasic, S., Lilic, D., Pejnovic, N., Vojvodic, D., Simic, R., and Abinun, M. (1998). Disseminated *Bacillus Calmette-Guerin* infection in a girl with hyperimmunoglobulin E syndrome. *Acta Paediatr.* 87, 702–704.
- Ragimbeau, J., Dondi, E., Alcover, A., Eid, P., Uze, G., and Pellegrini, S. (2003). The tyrosine kinase Tyk2 controls IFNAR1 cell surface expression. *EMBO J.* 22, 537–547.
- Renner, E.D., Puck, J.M., Holland, S.M., Schmitt, M., Weiss, M., Frosch, M., Bergmann, M., Davis, J., Belohradsky, B.H., and Grimbacher, B. (2004). Autosomal recessive hyperimmunoglobulin E syndrome: a distinct disease entity. *J. Pediatr.* 144, 93–99.
- Rodig, S.J., Meraz, M.A., White, J.M., Lampe, P.A., Riley, J.K., Arthur, C.D., King, K.L., Sheehan, K.C., Yin, L., Pennica, D., et al. (1998). Disruption of the Jak1 gene demonstrates obligatory and nonredundant roles of the Jak3 in cytokine-induced biologic responses. *Cell* 93, 373–383.
- Russell, S.M., Tayebi, N., Nakajima, H., Riedy, M.C., Roberts, J.L., Aman, M.J., Migone, T.S., Noguchi, M., Markert, M.L., Buckley, R.H., et al. (1995). Mutation of Jak3 in a patient with SCID: essential role of Jak3 in lymphoid development. *Science* 270, 797–800.
- Seto, Y., Nakajima, H., Suto, A., Shimoda, K., Saito, Y., Nakayama, K.I., and Iwamoto, I. (2003). Enhanced Th2 cell-mediated allergic inflammation in Tyk2-deficient mice. *J. Immunol.* 170, 1077–1083.
- Shaw, M.H., Boyartchuk, V., Wong, S., Karaghiosoff, M., Ragimbeau, J., Pellegrini, S., Muller, M., Dietrich, W.F., and Yap, G.S. (2003). A natural mutation in the Tyk2 pseudokinase domain underlies altered susceptibility of B10.Q/J mice to infection and autoimmunity. *Proc. Natl. Acad. Sci. USA* 100, 11594–11599.
- Shaw, M.H., Freeman, G.J., Scott, M.F., Fox, B.A., Bzik, D.J., Belkaid, Y., and Yap, G.S. (2006). Tyk2 negatively regulates adaptive Th1 immunity by mediating IL-10 signaling and promoting IFN- γ -dependent IL-10 reactivation. *J. Immunol.* 176, 7263–7271.
- Shimoda, K., Kato, K., Aoki, K., Matsuda, T., Miyamoto, A., Shibamori, M., Yamashita, M., Numata, A., Takase, K., Kobayashi, S., et al. (2000). Tyk2 plays a restricted role in IFN α signaling, although it is required for IL-12-mediated T cell function. *Immunity* 13, 561–571.
- Stahl, N., Boulton, T.G., Farruggella, T., Ip, N.Y., Davis, S., Witthuhn, B.A., Quelle, F.W., Silvennoinen, O., Barbieri, G., Pellegrini, S., et al. (1994). Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 β receptor components. *Science* 263, 92–95.
- Takaoka, A., Mitani, Y., Suemori, H., Sato, M., Yokochi, T., Noguchi, S., Tanaka, N., and Taniguchi, T. (2000). Cross talk between interferon- γ and - α/β signaling components in caveolar membrane domains. *Science* 288, 2357–2360.
- Velazquez, L., Fellous, M., Stark, G.R., and Pellegrini, S. (1992). A protein tyrosine kinase in the interferon α/β signaling pathway. *Cell* 70, 313–322.
- Watford, W.T., Hissong, B.D., Bream, J.H., Kanno, Y., Muul, L., and O'Shea, J.J. (2004). Signaling by IL-12 and IL-23 and the immunoregulatory roles of STAT4. *Immunol. Rev.* 202, 139–156.
- Wood, P.M., Fieschi, C., Picard, C., Ottenhoff, T.H., Casanova, J.L., and Kumararatne, D.S. (2005). Inherited defects in the interferon- γ receptor or interleukin-12 signalling pathways are not sufficient to cause allergic disease in children. *Eur. J. Pediatr.* 164, 741–747.
- Wu, C., Ferrante, J., Gately, M.K., and Magram, J. (1997). Characterization of IL-12 receptor β 1 chain (IL-12R β 1)-deficient mice: IL-12R β 1 is an essential component of the functional mouse IL-12 receptor. *J. Immunol.* 159, 1658–1665.

LETTERS TO NATURE

FIG. 3 Kinetic characteristics of transmitter discharge from SSV and LDCV. **a**, Initiation of transmitter discharge from SSV. Ten individual small events were averaged after alignment with respect to the midpoint of their rising phase. The same procedure was followed to obtain the instrument's step response, using records from capacitively injected current steps (amplitude 8 pA). The step response (dotted line) is superimposed on the rising phase of the small event (continuous line) after amplitude scaling. The 10–90% rise time of the step response was 60–70 μ s. The time course of the rising phase of the small event is indistinguishable from the step response of the measuring system (difference less than 10 μ s, maximum digitizing rate). **b**, 'Foot' signals often precede the main phase of transmitter discharge from LDCV. The foot had a duration of 540 ± 40 μ s and a maximal amplitude of 10 ± 1 pA. The integral of the foot signal reflects on average $4 \pm 0.5\%$ of the event's total integral ($n = 230$). Half of the foot signals were initiated by a stepwise increase of the current signal with an amplitude of 7.5 ± 0.7 pA (median, 4.5 pA) and a 50–90% rise time of 110 ± 10 μ s (median, 60 μ s; $n = 110$). Owing to the low rate of spontaneously occurring events (0.05 Hz) it is extremely unlikely that the initial step results from an accidental superimposition of small and large events. Large events initiated by such a step presumably arise from vesicles opening closest to the detector, in agreement with their short half-width (1.1 ± 0.8 ms, one third of the average half-width of large events; Table 1). **c**, Recording of transmitter release from an LDCV (charge 75 fC), which is initiated by a rapid stepwise increase of the transmitter signal but is not followed by a large amperometric spike. These events had an average half-width of 8 ± 11 ms ($n = 45$, 5% of all large events). Note the fast fluctuations of the current trace, which clearly exceed the baseline noise and might represent flickering of the vesicle fusion pore. Our experiments do not exclude the possibility that other mechanisms, like the dissolution of a storage matrix (as suggested by experiments on mast cells granules¹²), may also influence the kinetics and the magnitude of transmitter discharge from SSV and LDCV. **d**, Frequency distribution of the time constant of decay of spontaneously occurring small events. Decays of small events were fitted by single exponentials. The



median was 260 μ s, the mean was 360 ± 30 μ s ($n = 150$). LDCV release their content with a time constant of 2.3 ± 0.2 ms (median, 1.3 ms; $n = 140$). It should be noted that our measurements are limited by the time of diffusion of transmitter to the surface of the carbon fibre, hence reflecting an upper limit for the speed of transmitter discharge. **e**, Exemplary recording of transmitter discharge from a SSV at high resolution. The vesicle released 4,200 transmitter molecules with a time constant of 160 μ s.

through an undilated fusion pore. Although the fate of a SSV after transmitter discharge remains unclear, it is possible that SSV are recycled without ever undergoing full fusion, which would be advantageous for rapid and selective retrieval of vesicle membrane¹⁸.

Received 26 April; accepted 5 July 1995.

- Augustine, G. J., Charlton, M. P. & Smith, S. J. *A. Rev. Neurosci.* **10**, 633–693 (1987).
- Chow, R. H., von Rüden, L. & Neher, E. *Nature* **356**, 60–63 (1992).
- Wightman, R. M. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 10754–10758 (1991).
- Henderson, L. J. *Physiol.* **399**, 309–324 (1983).
- Bruno, D., Engert, F. & Lux, H. D. *Neuron* **10**, 559–572 (1993).
- Bartfai, T., Iverfeldt, K., Fisiore, G. & Serfozo, P. A. *Rev. Pharmac. Toxicol.* **28**, 285–310 (1988).
- Fuchs, P. A., Henderson, L. P. & Nicholls, J. G. *J. Physiol.* **323**, 195–210 (1982).
- Schiavo, G. et al. *Nature* **359**, 832–835 (1992).
- Link, E. et al. *Biochem. biophys. Res. Commun.* **189**, 1017–1023 (1992).
- Kuffler, S. W. & Yosikami, D. *J. Physiol.* **251**, 465–482 (1975).
- Henderson, L. P., Kuffler, D. P., Nicholls, J. G. & Ren-Ji-Zhang *J. Physiol.* **340**, 347–358 (1983).
- Alvarez de Toledo, G., Fernandez-Chacon, R. & Fernandez, J. M. *Nature* **363**, 554–558 (1993).
- Almers, W., Breckenridge, L. J. & Spruce, A. E. *Secretion and Its Control* (eds Oxford, G. & Armstrong, C.) 269–282 (Rockefeller Univ. Press, New York, 1989).
- Adler, E. M., Augustine, G. J., Duffy, M. P. & Charlton, M. P. *J. Neurosci.* **11**, 1496–1507 (1991).
- Linás, R., Sugimori, M. & Silver, R. B. *Science* **256**, 677–679 (1992).
- Heidelberger, R., Heinemann, C., Neher, E. & Matthews, G. *Nature* **371**, 513–515 (1994).
- Spruce, A. E., Breckenridge, L. J., Lee, A. K. & Almers, W. *Neuron* **4**, 643–654 (1990).
- Ceccarelli, B. & Hurlbut, W. P. *Physiol. Rev.* **60**, 396–441 (1980).
- Wrona, M. Z. & Dryhurst, G. *Bioorg. Chem.* **18**, 291–317 (1990).
- Kuffler, D. P., Nicholls, J. G. & Drapeau, P. J. *comp. Neurol.* **256**, 516–526 (1987).
- Beaudet, A. & Descarries, L. *J. Physiol., Paris* **77**, 193–203 (1981).

ACKNOWLEDGEMENTS. We thank F. Sigworth, P. Hanson, H. Otto, E. Chapman and R. Ossig for their suggestions regarding the manuscript; S. Engers for cultured neurons and for technical assistance; R. Chow for providing information on the carbon fibre technique; L. Daniell and P. Steen for help with the EM part of this study; and H. Niemann for tetanus-toxin light chain. This work was supported by a Max-Planck-Society fellowship to D.B.

Mutations of Jak-3 gene in patients with autosomal severe combined immune deficiency (SCID)

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SEVERE combined immune deficiency (SCID) represents a heterogeneous group of hereditary diseases. Mutations in the common γ -chain (γ^c), which is part of several cytokine receptors including those for interleukin (IL)-2, IL-4, IL-7, IL-9 and IL-15, are responsible for X-linked SCID^{1,2}, which is usually associated with a lack of circulating T cells and the presence of B lymphocytes

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LETTERS TO NATURE

(T⁻B⁺ SCID). The gene(s) responsible for autosomal recessive T⁻B⁺ SCID is still unknown. The Jak-3 protein kinase^{3,4} has been found to associate with the γ^c -chain-containing cytokine receptors⁴⁻⁹. Therefore Jak-3 or other STAT proteins with which it interacts^{10,11} are candidate genes for autosomal recessive T⁻B⁺ SCID⁷. Here we investigate two unrelated T⁻B⁺ SCID patients (both from consanguineous parents) who have homozygous mutations in the gene for Jak-3. One patient carries a mutation (Tyr100→Cys) in a conserved tyrosine residue in the JH7 domain of Jak-3 which is absent in more than 150 investigated chromosomes. The other patient carries a homozygous 151-base-pair deletion in the kinase-like domain, leading to a frameshift and premature termination. Both mutations resulted in markedly reduced levels of Jak-3. These findings show that abnormalities in the Jak/STAT signalling pathway can account for SCID in humans.

To facilitate the detection of putative Jak-3 mutations, we selected two unrelated male T⁻B⁺ SCID patients, both with consanguineous parents, in which homozygous abnormalities could be expected. Despite an increased proportion of B lymphocytes in peripheral blood cells (the CD19⁺ lymphocytes being 94% of circulating mononuclear cells in subject G.M. and 90% of circulating mononuclear cells in subject C.M.), both patients showed clear signs of impairment of B-cell function *in vivo*. At 3 months of age, before treatment with intravenous immunoglobulins, both patients were severely hypogammaglobulinaemic (patient G.M., IgG 1,000 mg l⁻¹, IgA < 66 mg l⁻¹, IgM 300 mg l⁻¹; patient C.M., IgG 1,300 mg l⁻¹, IgA < 66 mg l⁻¹, IgM 150 mg l⁻¹), and both lacked isoagglutinins.

Analysis of the entire γ_c gene sequence in patient G.M. showed that it was normal, ruling out an X-linked origin of the disorder. To investigate whether a deficiency in Jak-3 might explain the immunological defect of this patient, we sequenced reverse transcription-polymerase chain reaction (RT-PCR) amplification products obtained from Jak-3 messenger RNA extracted from an Epstein-Barr virus (EBV)-transformed B-cell line, which appeared normal in size (data not shown).

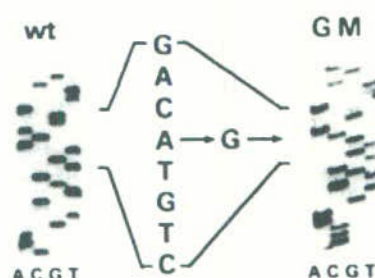


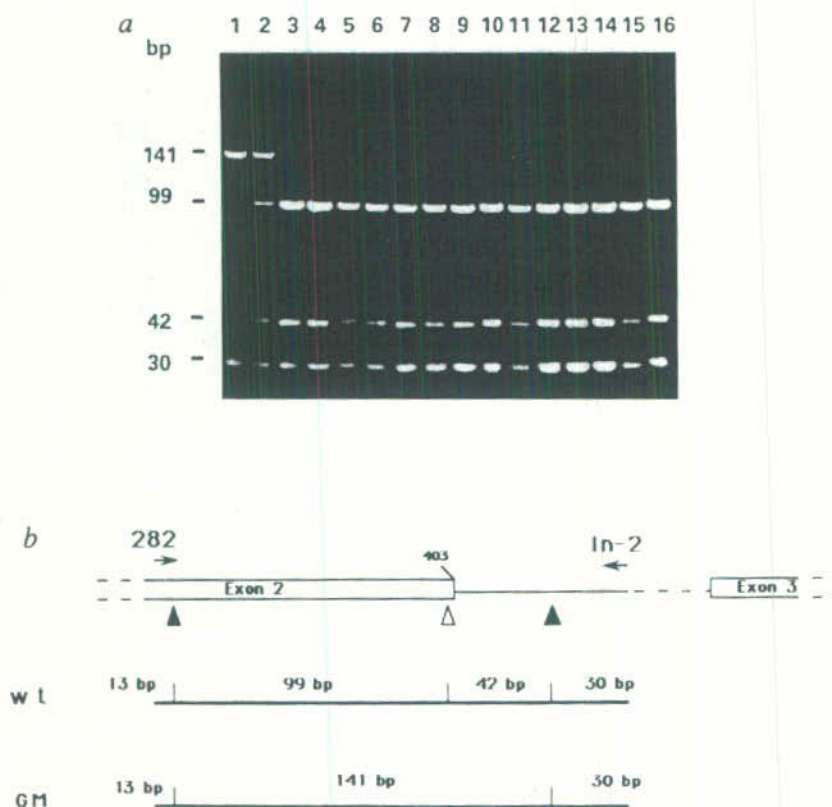
FIG. 1 Tyr100→Cys mutation in the Jak-3 gene of patient G.M. with autosomal SCID. The sequence of the region around nucleotide 394 of the Jak-3 sequence (Accession Number U09607), demonstrating an A→G transition (arrow) at this nucleotide, is shown.

METHODS. RNA was extracted from an EBV-transformed lymphoblastoid cell line obtained from peripheral blood cells of patient GM, according to standard techniques¹⁸. RT-PCR was performed with primer pair 96F/600R (96F: ATGGCACCTCCAAGTGAAG; 600R: CCAACACGCCAGGCTGAGA). The numbers refer to the nucleotide position in the complementary DNA Jak-3 sequence, and F means forward and R reverse. The amplification conditions were as follows: 30 cycles of denaturation at 94 °C, annealing at 59 °C and extension at 72 °C, each for 30 s. The resulting PCR product was cloned in TA cloning vector (Invitrogen) and sequenced by dideoxynucleotide chain termination method using the Sequenase kit (USB), as described previously¹⁹.

The same mutation, an A→G transition at nucleotide 394, which causes a tyrosine-to-cysteine substitution at amino acid 100 (Tyr100→Cys), was found in six clones from patient G.M. (Fig. 1). This mutation results in the disappearance of an *Rsa*I site. To exclude a rare polymorphism, we analysed more than 150 chromosomes. As our investigation of the corresponding genomic region showed the presence of an intron at nucleotide 403 of the Jak-3 transcript, an upstream exonic primer (282F) and a downstream intronic primer (In-2) were used to amplify a 184-bp-long PCR product from genomic DNA, encompassing the mutated nucleotide.

FIG. 2 Restriction analysis of the genomic region around the mutation of patient G.M. a, Electrophoresis of PCR products obtained by DNA amplification with primers 282F and In-2. Lane 1, patient G.M.; lane 2, mother of patient G.M.; lanes 3–16, normal controls. b, Schematic representation of the *Rsa*I restriction sites in normal and mutated DNA around nucleotide 394. The arrowheads indicate *Rsa*I sites; the white one indicates the site eliminated by the A→G mutation at nucleotide 394 in the abnormal allele from patient G.M. family members. This results in a 141-bp band instead of the 99- and 42-bp bands, in addition to two other bands of 30 and 13 (the latter is not visible in the gel).

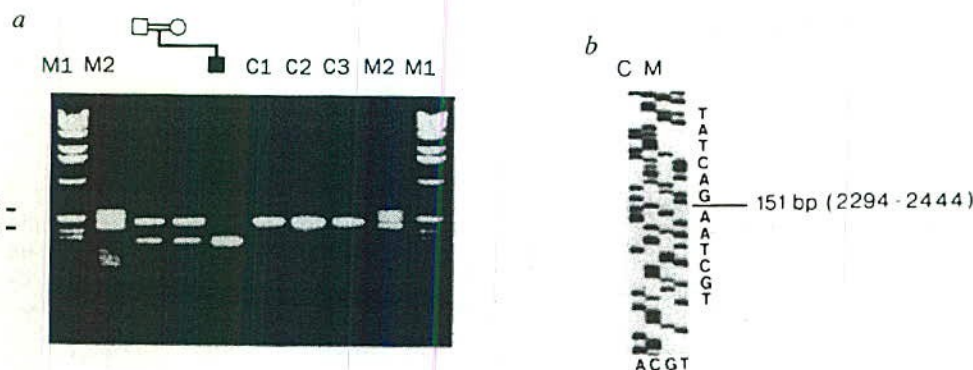
METHODS. For *Rsa*I digestion analysis, PCR was performed on DNA extracted from peripheral blood cells of the patient, his mother and normal controls, with the primer pair 282F/In-2 (282F: ATCCTGCCTGTGTACT; In-2: CCTCGAAATGCAAGGAGATG), the latter being located in the second intron. Amplification conditions were as described for Fig. 1. The products were digested according to the manufacturer's instructions and separated on an 8% polyacrylamide gel.



LETTERS TO NATURE

FIG. 3 Deletion of the region between nucleotides 2,294 and 2,444 in patient C.M. **a**, Electrophoresis of RT-PCR products obtained by mRNA amplification with primers 2130F and 2600R. M1, 1-kb marker; M2, marker V (Boehringer); lane 1 (white square), father of patient C.M.; lane 2 (circle), mother of C.M.; lane 3 (black square), patient C.M.; lanes 4–6 (C1–C3), normal controls. **b**, Sequence of the region surrounding the deletion, showing the absence of a region of 151 bp from nucleotides 2,294 and 2,444 included.

METHODS. mRNA extraction, subcloning in TA vector and sequencing was performed as described for Fig. 1. The primer pair used was:



2130F/2600R (2130: TTAAGCCTGGAGATGCTCAC; 2600R: GCTGCCAAAGTTGCCCTTGC). Conditions for the RT-PCR were as described for Fig. 1, except that the annealing step was at 60 °C.

RsaI digestion of specific PCR products from 154 independent chromosomes demonstrated, in all cases, the presence of the expected bands of 99, 42, 30 and 13 bp. In contrast, in patient G.M. the bands of 99 and 42 bp were replaced by one band of 141 bp (Fig. 2a, b). These data are in keeping with homozygosity for the mutation. Accordingly, in addition to the four normal digestion products, the abnormal 141-bp band was also present in the mother of patient G.M., showing her to be heterozygous for the mutation (DNA from the father was not available as he is deceased).

The amplification of the Jak-3 transcript in the second patient (C.M.) revealed a smaller-than-expected band when the amplification was performed with exonic primers 2130F and 2600R. As shown in Fig. 3a, the expected 471-bp band was detected in normal controls whereas only a shorter band was seen in patient C.M., suggesting that the patient was homozygous for a deletion in this transcript region. Accordingly, from both father and mother a shorter PCR product (of the same size as that found in the proband) was present in addition to the normal one. Sequencing of 5 clones showed that all the clones of patient C.M. bore a deletion of 151 nucleotides, from nucleotides 2,294 to 2,444 (inclusive), in agreement with the RT-PCR analysis (Fig. 3b). This deletion causes a shift in the reading frame after amino acid 733 (in the final portion of the kinase-like domain), leading to premature termination (a TAG stop codon is formed at nucleotides 2,626–2,628). It seems highly unlikely that the deleted PCR product represents a normal alternative transcript as we have not seen it previously described and it was never present in our controls, but it was present at the homozygous level in the proband and at the heterozygous levels in his parents. In addition, a normal-sized PCR product was absent in the proband.

To analyse the consequences of the patients' mutations of the Jak-3 gene on the function of Jak-3 protein, we first immunoblotted whole-cell lysates with anti-Jak-3 antibody³ to determine the level of expression of the mutated protein. As shown in Fig. 4 (top), EBV-transformed normal B cells (lane 2) had levels of Jak-3 protein comparable to those seen in the NK3.3 cell line (lane 1), which expresses high levels of Jak-3 (refs 4, 5). In sharp contrast, neither patient expressed substantial amounts of Jak-3 protein; patient G.M. had barely detectable Jak-3 protein (lane 3), and patient C.M. had too little to be detected (lane 4). Thus both of these mutations lead to marked reduction in the steady-state levels of Jak-3. To ascertain equal loading of protein from each of these samples, the filter was stripped and reprobed with anti-Jak-1 antibody. As shown in Fig. 4 (bottom), unlike Jak-3, Jak-1 was expressed at roughly comparable levels in all of the cells. Thus it appears that the mutant Jak-3 alleles in both patients generated protein products that are poorly expressed.

In future studies it will be important to determine the mechanism responsible for the poor expression of these mutants proteins. However, this lack of expression of Jak-3 protein is likely to be pathogenetically responsible for the immune deficiency in these patients.

In contrast to other Jaks that are ubiquitously expressed, Jak-3 has been shown to have limited tissue expression^{3,5,9}. Con-

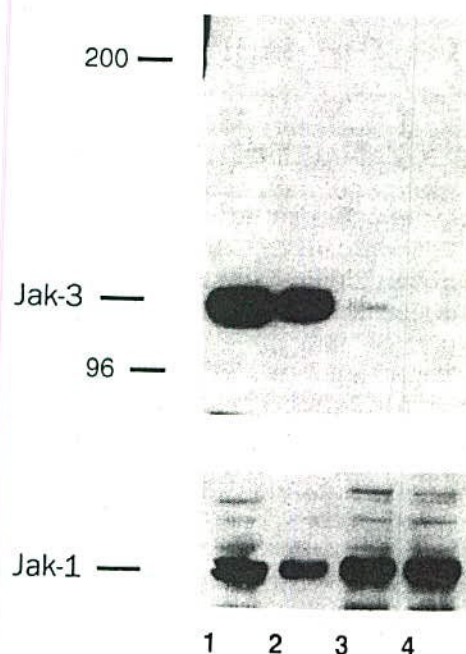


FIG. 4 Markedly reduced levels of Jak-3 protein in EBV-transformed B cells from patients with Jak-3 mutations. Lysates from the NK3.3 cell line (lane 1), EBV-transformed B cells from a normal individual (lane 2), and EBV-transformed B cells from patient G.M. (Tyr100→Cys mutation, lane 3) and patient C.M. (deletion of 2,294–2,444, lane 4) were electrophoresed and immunoblotted for Jak-3 expression (top). The filter was stripped and reprobed for Jak-1 expression (bottom). Jak-3 expression was found to be greatly reduced in patient cells, whereas the level of Jak-1 expression was similar for all of the cell lines.

METHODS. Cells (5×10^6) were lysed as described previously⁵. Post-nuclear supernatants were assayed for protein concentration (BCA reagent, Biorad), and 100 µg of each sample was electrophoresed on an 8% SDS-polyacrylamide gel, transferred to Immobilon and immunoblotted with anti-Jak-3 (top) antibody³ and developed by enhanced chemiluminescence (ECL, Amersham). The filter was stripped according to the manufacturer's recommendations and reblotted with anti-Jak-1 (bottom) antibody (Transduction Labs, Lexington, KY).

LETTERS TO NATURE

sistent with these previous observations is the present demonstration that mutation of Jak-3 does not appear to have developmental consequences other than the effects on the immune system, because, apart from the immune deficiency, the two patients are normal. Moreover, since bone-marrow transplantation, patient G.M. has developed normally (patient C.M. has not yet undergone transplantation). This indicates that the principal functions of Jak-3 relate to lymphoid development, and it is dispensable for the development of other systems.

Mutation of the γ_c gene in humans results in $T^+ B^+$ X-linked SCID^{1,2}, and knocking out the corresponding gene in mice heavily affects both T- and B-lymphoid development^{12,13}. The detection of Jak-3 mutations in two patients with $T^+ B^+$ SCID confirms that in humans the γ_c chain/Jak/STAT signalling path-

way is critical to early T-cell, but not B-cell, development. In addition, both patients examined here showed a lack of circulating natural killer (NK) cells (as detected by CD16 monoclonal antibody). Lack of circulating NK cells is not unique to Jak-3-deficient SCID patients, having also been reported in X-linked SCID¹⁴. Further, carrier females of X-linked SCID exhibit a non-random pattern of X-chromosome inactivation in NK cells¹⁵, as well as in T and B lymphocytes^{16,17}, indicating that mutations of the γ_c gene interfere with proliferation/differentiation of all these lymphoid lineages. As the γ_c chain and Jak-3 are elements of the same signal-transducing pathway and are both expressed in NK cells, it is tempting to speculate that the integrity of this pathway is strictly required for NK-cell development. □

Received 2 June; accepted 11 August 1995.

1. Noguchi, M. et al. *Cell* **73**, 147–157 (1993).
2. Schmaistieg, F. C. et al. *J. clin. Invest.* **95**, 1169–1173 (1995).
3. Kawamura, M. et al. *Proc. natn. Acad. Sci. U.S.A.* **91**, 6374–6378 (1994).
4. Witthuhn, B. A. et al. *Nature* **370**, 153–157 (1994).
5. Johnston, J. A. et al. *Nature* **370**, 151–153 (1994).
6. Boussiotis, V. A. et al. *Science* **266**, 1039–1042 (1994).
7. Russell, S. M. *Science* **266**, 1042–1045 (1994).
8. Miyazaki, T. et al. *Science* **266**, 1045–1047 (1994).
9. Musso, T. et al. *J. exp. Med.* **181**, 1425–1431 (1995).
10. Darnell, J. E., Kerr, I. M. & Start, G. R. *Science* **264**, 1415–1421 (1994).
11. Ihle, J. N. & Kerr, I. M. *Trends Genet.* **11**, 69–74 (1995).
12. Cao, X. et al. *Immunity* **2**, 223–238 (1995).
13. Di Santo, J. P., Muller, W., Guy-Grand, D., Fischer, A. & Rajewsky, K. *Proc. natn. Acad. Sci. U.S.A.* **93**, 377–381 (1995).

14. Gougeon, M.-L. et al. *J. Immun.* **145**, 2873–2877 (1990).
15. Wengler, G. S., Allen, R. C., Parolini, O., Smith, H. & Conley, M. E. *J. Immun.* **150**, 700–704 (1993).
16. Puck, J. M., Nussbaum, R. L. & Conley, M. E. *J. clin. Invest.* **79**, 1395–1400 (1987).
17. Conley, M. E. et al. *Proc. natn. Acad. Sci. U.S.A.* **85**, 3090–3094 (1988).
18. Chomczynski, P. & Sacchi, N. *Anal. Biochem.* **162**, 156–159 (1987).
19. Villa, A. et al. *Nature Genet.* **9**, 414–417 (1995).

ACKNOWLEDGEMENTS. P.M. and A.V. contributed equally to this work. We thank R. Dulbecco and D. Nelson for suggestions; L. Rossi Bernardi for encouragement; M. Littardi, D. Strina and L. Susani for technical assistance; S. Bevan for typing the manuscript; and J. Kornbuth for promoting the NK3.3 cell line. This work was supported by grants from CNR (P.F. Ingegneria Genetica to L.N. and P.V., and ACRO to A.V.), Biomed 1 (PL 1321 to L.N.), AIRC (to A.V.) and Telethon Italy (to A.U. and P.V.).

MIF as a glucocorticoid-induced modulator of cytokine production

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GLUCOCORTICOID hormones are important for vital functions and act to modulate inflammatory and immune responses^{1,2}. Yet, in contrast to other hormonal systems, no endogenous mediators have been identified that can directly counter-regulate their potent anti-inflammatory and immunosuppressive properties. Recent investigations of the protein macrophage migration inhibitory factor (MIF), which was discovered originally to be a T-lymphocyte-derived factor^{3,4}, have established it to be a pro-inflammatory pituitary and macrophage cytokine and a critical mediator of septic shock^{5–7}. Here we report the unexpected finding that low concentrations of glucocorticoids induce rather than inhibit MIF production from macrophages. MIF then acts to override glucocorticoid-mediated inhibition of cytokine secretion by lipopolysaccharide (LPS)-stimulated monocytes and to overcome glucocorticoid protection against lethal endotoxaemia. These observations identify a unique counter-regulatory system that functions to control inflammatory and immune responses.

Activation of the hypothalamic-pituitary-adrenal (HPA) axis is an essential feature of the systemic stress response, and leads ultimately to an increase in circulating levels of glucocorticoids, which are essential for modulating the host response to infection and tissue invasion^{1,2,8}. Because glucocorticoids are potent inhibitors of cytokine production^{2,9–13}, we examined their effect

on the production of MIF by LPS-activated macrophages *in vitro*. In sharp contrast to expectations, glucocorticoids alone were found to induce rather than inhibit MIF production. MIF secretion was induced by low concentrations of dexamethasone or hydrocortisone (cortisol), the principal glucocorticoid hormone in humans, and was detectable in culture medium 3 hours after glucocorticoid stimulation (Fig. 1). Glucocorticoid-induced macrophage MIF secretion showed a bell-shaped dose-response curve when assessed by a sensitive, MIF-specific enzyme-linked immunosorbent assay (ELISA). MIF release was triggered by concentrations of dexamethasone as low as 10^{-16} M, peaked at 10^{-14} to 10^{-12} M, and decreased at higher concentrations (10^{-8} to 10^{-6} M) (Fig. 1). A similar profile of glucocorticoid-induced MIF secretion was observed in primary mouse

FIG. 2 Glucocorticoid-induced MIF production *in vivo*. a, Western blot analyses of serum MIF in vehicle-injected control rats (top) and rats injected with 1 mg per kg (middle) and 10 mg per kg (bottom) dexamethasone.

METHODS. Female Sprague-Dawley rats (250–300 g) were prepared surgically with an indwelling catheter in the jugular vein 5–7 days before the experiment. The catheter was externalized via a headset and connected to a tethered line which allowed injection of drug and collection of blood with minimal disturbance to the animals. Rats were housed individually, had free access to food and water, and were maintained on a 12-h light/12-h dark cycle (lights on at 7:00). All experiments commenced at 8:00. Dexamethasone, diluted in sterile saline, was injected intravenously (0.1–20 mg per kg) when all rats were resting and had been undisturbed for at least 1 h. The injected materials were determined previously to be free of endotoxin contamination by the chromogenic *Limulus* amoebocyte assay (BioWhittaker, Walkersville, MD). Blood was withdrawn via the catheter at 0 (baseline), 1.5, 3.0, 4.5, 6.0, 7.5 and 9.0 h after injection of saline or dexamethasone and analysed for MIF content by western blotting or ELISA. For western blotting, portions of 6 μ l serum were analysed as described in Fig. 1, and the MIF bands visualized by a chemiluminescence reaction (ECL; Amersham) and exposure on X-OMAT-XAR film (Eastman Kodak, Rochester, NY). As a reference standard, rMIF was electrophoresed and transferred in lane 1. By ELISA, the serum concentration of MIF in resting, unstressed animals (serum-corticosterone concentrations <25 ng ml⁻¹) was 15.9 ± 6.5 ng ml⁻¹ (mean $\pm$ s.d. of 14 rats) and

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