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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
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Trámite: **11**
Evento: **378**
Actuación: **412**

Señor
SUPERINTENDENTE DE INDUSTRIA Y COMERCIO
DIVISIÓN DE NUEVAS CREACIONES
E. S. D.

Re: PI.-**INCYTE CORPORATION.**-Solicitud de Patente de Invención en Fase Nacional PCT para **"DERIVADOS DE PIRROLOPIRIDINA Y PIRROLOPIRIMIDINA SUSTITUIDOS CON CICLOBUTILO COMO INHIBIDORES DE JAK"**.-Clase **C07D 471/04.**-Expediente número **13-134.023**.-

Yo, **ALICIA LLOREDA RICAURTE**, mayor, vecina de esta ciudad, identificada con la cédula de ciudadanía número 39.690.713 de Usaquén, Representante Legal en Bogotá para todos los asuntos relacionados con la Propiedad Industrial de la sociedad **INCYTE CORPORATION**, organizada y existente de conformidad con las leyes del Estado de Delaware, Estados Unidos de América, domiciliada en Wilmington, Estado de Delaware, Estados Unidos de América, atentamente manifiesto a usted que interpongo **RECURSO DE REPOSICIÓN** contra la Resolución Número **3680** dictada por el señor Superintendente de Industria y Comercio el 2 de Febrero de 2015 y notificada por fijación en lista el 10 de Febrero de 2015, por medio de la cual se niega el privilegio de patente solicitado para la invención contenida en el expediente de la referencia.

I. FUNDAMENTOS DE DERECHO

Como fundamento de mi recurso, me permito manifestar ante ese Despacho que el contenido de la Resolución Número **3680** evidencia que durante el examen técnico realizado a la solicitud denegada se realizó una evaluación incorrecta del objeto de la invención allí reclamada, sin considerarse su real alcance e importancia. En este sentido, no se consideró el verdadero y valioso aporte técnico que representa para el arte en cuestión los derivados de pirrolopirimidinas y pirrolopiridinas sustituidos con ciclobutilo de fórmula I que modulan la actividad de las Janus quinasas (JAKs), revelados en la solicitud denegada.

Es así que, a pesar de los argumentos proporcionados por el solicitante en respuesta al Concepto Técnico emitido por ese Despacho el 23 de Mayo de 2014, en la Resolución impugnada este Despacho mantiene las objeciones frente al Nivel Inventivo de la solicitud, argumentando básicamente que:

"No existe efecto técnico sorprendente porque los valores de IC_{50} de los compuestos divulgados en el documento D2 (...) son mejores con respecto a los valores de IC_{50} de los compuestos de la solicitud (...)".

Además, el señor Examinador resalta que:

"(...) en el mismo D2 se mencionan compuestos que no mencionan la inclusión de un sustituyente L entre el grupo ciclobutil y un heterociclo, por lo cual la persona versada en la materia puede prescindir de L para obtener los compuestos de la solicitud. De manera que la reivindicación 1 de la solicitud se considera obvia y, en consecuencia, no tiene nivel inventivo".

Así como también que:

"Dado que las reivindicaciones 2 a 37 no presentan alguna característica adicional que conceda nivel inventivo al objeto de la reivindicación independiente, se considera que tampoco tienen nivel inventivo".

Por consiguiente, a continuación me permito brindar argumentos técnicos para no sólo demostrar ante ese Despacho que el nuevo capítulo reivindicatorio que se presenta junto con el presente recurso cumple a cabalidad con el nivel inventivo exigido por el artículo 18 de la Decisión 486 de 2000, sino además para establecer que la invención ha sido interpretada de manera errónea a la luz de los documentos del arte previo.

1. MODIFICACIONES AL CAPITULO REINVIDICATORIO.

Inicialmente y en razón de defender la patentabilidad de la presente solicitud denegada, me permito manifestar que el solicitante ha decidido realizar las siguientes modificaciones en el capítulo reivindicatorio de la invención actualmente presentada:

- **Nueva Reivindicación 1:** tiene como base la reivindicación 1 actualmente presentada, la cual ha sido modificada para restringir considerablemente el alcance de la materia reclamada y así diferenciar la invención del estado del arte previo. Específicamente, dichas modificaciones consisten en:

  - Restringir los sustituyentes X y Z a nitrógeno.
  - Restringir los sustituyentes R^1, R^2, Y R^3 a hidrógeno.
  - Restringir el sustituyente Y a ciano.
  - Restringir el sustituyente W a N o CH, y consecuentemente restringir el sustituyente L a $C(R^6)_2$, C(=O), C(=O)O, $C(=O)N(R^7)$, $S(=O)_2$, o $S(=O)_2N(R^7)$ o a O.
  - Restringir los sustituyentes R^6 a H y R^7 a H o metilo.
  - Definir con mayor especificidad el sustituyente A como metilo, etilo, propilo, isopropilo, isobutilo, sec–butilo, 1,2–dimetilpropilo, 1–(ter–butil)metilo, ciclopropilo, ciclobutilo, ciclopentilo, ciclohexilo, fenilo, un anillo tetrahidropirano, un anillo pirrolidina, un anillo piperidina, un anillo piridina, un anillo pirimidina, un anillo tiazol o un anillo pirazina; cada uno de los cuales no está sustituido o está sustituido con p sustituyentes R^8 seleccionados de modo independiente.
  - Restringir el sustituyente R^8 a halo, ciano, alquilo C_{1-6}, haloalquilo C_{1-6}, cicloalquilo C_{3-7}, heterocicloalquilo C_{2-7}, $-OR^a$, $-C(=O)OR^a$, o $-NR^eR^f$; en donde dicho alquilo C_{1-6} no está sustituido o está sustiuido con 1, 2, 3 ó 4 grupos R^9 seleccionados de modo independiente.
  - Restringir los sustituyentes R^a, R^e y R^f a H, alquilo C_{1-6} y haloalquilo C_{1-6}.
  - Restringir el sustituyente R^9 a heterocicloalquilo C_{2-7}, $-OR^{a1}$, $-NR^{e1}R^{f1}$; en donde dicho heterocicloalquilo C_{2-7} no está sustituido o está sustituido con 1 ó 2 grupos R^h seleccionados, de modo independiente, de fluoro, OH, alquilo C_{1-3}, alcoxi C_{1-3} e hidroxi–alquilo C_{1-4}.
  - Restringir los sustituyentes R^{a1}, R^{e1} y R^{f1} a H, cicloalquilo C_{3-7} y alquilo C_{1-6}.
  - Definir los valores para p (1,2 o 3), m (1), y n (0).

  Las anteriores modificaciones encuentran pleno sustento en la forma de realización de la página 23 líneas 19 a 30 y página 24 líneas 1 a 20.

- **Nuevas Reivindicaciones 2 y 3:** corresponden a las reivindicaciones 27 y 28 del capítulo actualmente presentado.

- **Nueva Reivindicación 4:** tiene como base la reivindicación 10 actualmente presentada, la cual ha sido modificada para constituir una nueva reivindicación dependiente de la nueva reivindicación 1, restringiendo aún más el alcance de la materia reclamada.

- **Nueva Reivindicación 5:** tiene como base las reivindicaciones 27 y 28 actualmente presentada, las cuales han sido combinadas bajo una misma reivindicación independiente con el fin de reclamar compuestos específicos de la presente solicitud.

- **Nueva Reivindicación 6:** constituye una nueva reivindicación independiente, la cual reclama los compuestos de los Ejemplos 7b (pág. 106), 157 (pág. 343), 161 (pág. 346) y 162 (pág. 347), restringiendo aún más el alcance del objeto de la solicitud denegada.

- **Nuevas Reivindicaciones 7 y 8:** corresponden a las reivindicaciones 31 y 32 del capítulo reivindicatorio actualmente presentado.

- **Nuevas Reivindicaciones 9 y 10:** constituyen nuevas reivindicaciones dependientes de la nueva reivindicación 6, las cuales reclaman específicamente los compuestos de los Ejemplos 161 y 162 de la solicitud denegada.

- **Nuevas Reivindicaciones 11 y 12:** corresponden a las reivindicaciones 35 y 36 del capítulo reivindicatorio actualmente presentado.

De acuerdo a lo anterior, me permito señalar que las reivindicaciones **2 a 26, 33, 34 y 37** del Capítulo Reivindicatorio actualmente presentado fueron **eliminadas** del documento modificado. Asimismo, me permito aclarar que el orden de enumeración y dependencia de las nuevas reivindicaciones se ha reajustado de conformidad con las modificaciones previamente descritas.

Adicionalmente, me permito señalar que dichas modificaciones cumplen con el propósito de restringir significativamente la materia reclamada y así permitir una mayor diferenciación de la invención de la solicitud denegada con el estado del arte previo.

De esta forma, el mencionado **capítulo reivindicatorio modificado queda constituido por 12 reivindicaciones**, la cuales no constituyen de ninguna manera una ampliación al alcance de la solicitud original y cuenta con pleno sustento en las enseñanzas de la descripción. Con lo anterior, me permito reiterar que dicho capítulo cumple a cabalidad con las disposiciones de patentabilidad establecidas en el Artículo 34 de la Decisión 486 de 2000.

2. CONSIDERACIONES TÉCNICAS FRENTE A LAS ENSEÑANZAS DEL ARTE PREVIO

Ahora, teniendo en cuenta el alcance del nuevo capítulo reivindicatorio de la invención en cuestión, me permito manifestar ante ese Despacho que los derivados de pirrolopirimidinas y pirrolopiridinas sustituidos con ciclobutilo de fórmula I en la solicitud de patente denegada presentan un inequívoco nivel inventivo frente a las enseñanzas del documento **WO 2009/9114512 (D2).**

En la Resolución aquí impugnada, el señor Examinador considera que la composición reclamada en la solicitud no tiene nivel inventivo puesto que, a la luz de las enseñanzas del documento D2, la persona del oficio normalmente versada en la materia podría derivar de manera evidente el objeto de la reivindicación 1 de la solicitud.

En relación con lo anterior, **me permito sostener que las enseñanzas del documento D2 de ninguna manera anticipan o sugieren de manera obvia los compuestos derivados de pirrolopirimidinas o pirrolopiridinas substituidos con ciclobutilo de fórmula I con todas y cada una de las definiciones específicas para sus sustituyentes, tal y como se reclaman en el capítulo reivindicatorio modificado que se presenta junto con este memorial.**

Con el ánimo de demostrar ante ese Despacho que la anterior afirmación es completamente correcta, a continuación me permito realizar un análisis comparativo detallado entre la información revelada en la presente solicitud y las enseñanzas brindadas por dicha anterioridad.

2.1. Las diferencias estructurales existentes entre los compuestos de la presente invención y aquellos divulgados en el documento D2 no son evidentes para la persona medianamente versada en la materia

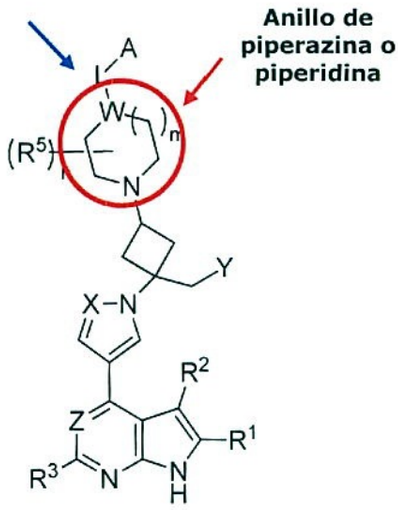
De conformidad con lo señalado por el señor Examinador en la Resolución aquí impugnada, el documento D2 divulga compuestos derivados de azetidina y ciclobutilo que presentan actividad inhibitoria de JAK y que son útiles en el tratamiento de

enfermedades asociadas a dicha enzima, incluyendo por ejemplo, desordenes autoinmunes e inflamatorios y cáncer.

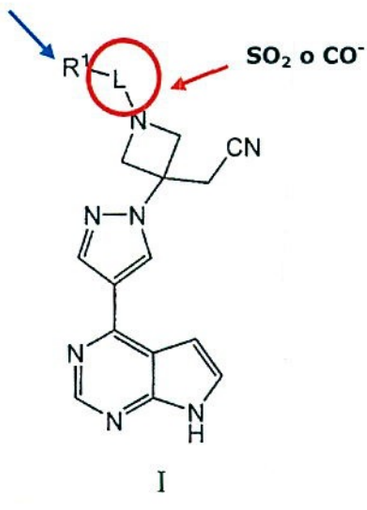
Específicamente, el documento D2 reclama compuestos de fórmula I (reivindicación 1) y fórmula II (reivindicación 18), los cuales comparten semejanzas estructurales con los compuestos de fórmula I de la presente invención. Sin embargo, tal y como señala el señor Examinador, los compuestos del documento D2 difieren de los compuestos reclamados en la presencia del sustituyente L entre el anillo de azetidina o ciclobutilo y el sustituyente A.

Ahora, tras la atenta lectura y revisión de las enseñanzas de D2, **me permito manifestar que el documento D2 no sólo difiere en la presencia de dicho sustituyente L sino también presenta otras diferencias estructurales con relación a la naturaleza de los sustituyentes en las fórmulas I y II de D2.**

En primer lugar y en comparación con los compuestos la fórmula I de D2 (reivindicación 1), los compuestos de la invención tienen directamente unido al grupo ciclobutilo un anillo de piperazina o piperidina mientras que los compuestos de fórmula I del documento D2 tienen directamente unido un sustituyente L que se define únicamente como SO_2 o CO^- (reivindicación 1):



Fórmula I de la presente invención



I

Fórmula I del documento D2

De las anteriores figuras, es evidente que el sustituyente L en ambas fórmulas es diferente y corresponde a una posición distinta en la estructura general de los compuestos. En este sentido, el sustituyente L de D2 correspondería con el anillo de piperazina o piperidina de la fórmula I de la presente invención (*ver flecha roja)* y el sustituyente R^1 de D2 correspondería con el sustituyente L de la presente invención (*ver flecha azul)*. De igual forma, se observa que el sustituyente A de la presente

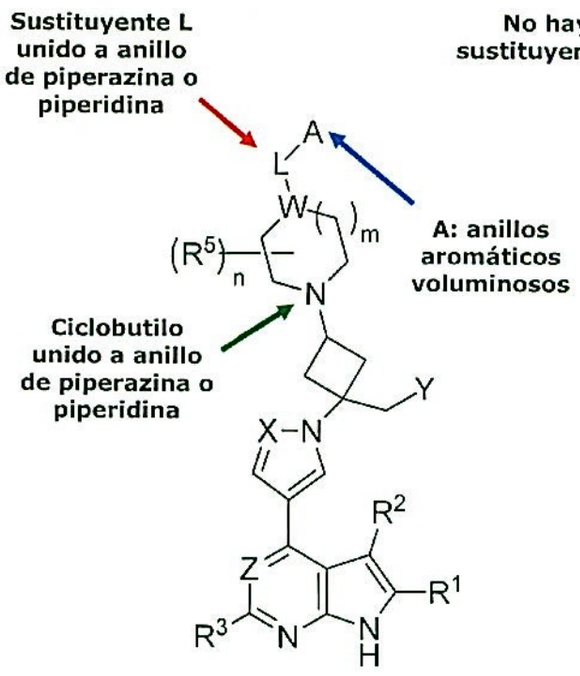
invención no tendría un grupo funcional equivalente en la fórmula I de D2, lo cual diferencia aún más las estructuras químicas de ambas fórmulas.

Por otro lado y en comparación con la fórmula II de D2 (reivindicación 18), dichos compuestos difieren significativamente en relación con lo siguiente:

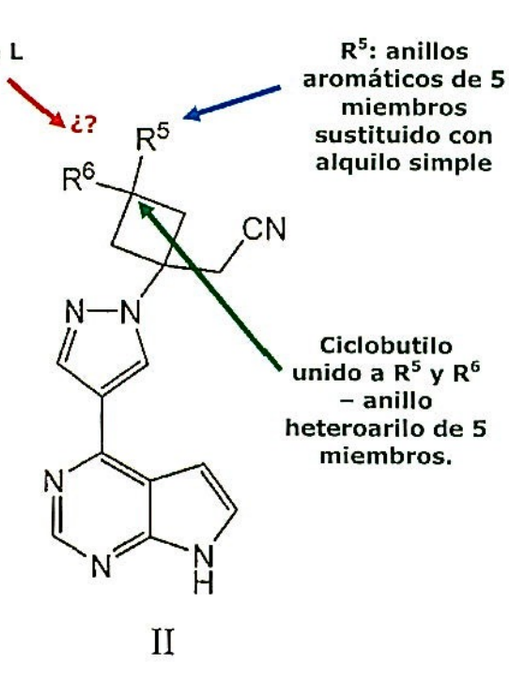
- Los compuestos de fórmula II de D2 no incluyen el grupo funcional L mientras que los compuestos de fórmula I de la presente invención sí lo incluyen (*ver flecha roja).*

- El anillo de ciclobutilo de la presente invención está exclusivamente unido a un anilllo heterocicloalquilo saturado de 6 miembros (piperazina o piperadina). Por otro lado, al anillo ciclobutilo en la fórmula II de D2 se une a los sustituyentes R^5 y R^6, los cuales sólo pueden incluir un anillo heteroarilo de 5 miembros (reivindicación 18), el cual es aromático en naturaleza (*ver flecha verde).*

- Los compuestos de la invención requieren en realidad un sustituyente L-A en el anillo de piperazina o piperidina mientras que la estructura de fórmula II de D2 termina en los sustituyentes R^5 y R^6. En este sentido, mientras que los 4 compuestos de la reivindicación 6 de la presente solicitud incluyen anillos aromáticos de gran tamaño unidos al anillo de piperazina o piperidina a través del sustituyente L, el anillo heteroarilo del sustituyente R^5 de la fórmula II de D2 solo puede ser sustituido por un simple grupo alquilo C_{1-4} (*ver flecha azul).*



Fórmula I de la presente invención



Fórmula II del documento D2

En vista de las diferencias más significativas encontradas entre los documentos del arte previo, es evidente que la persona del oficio medianamente versada en la materia tendría que realizar varios tipos de modificaciones a las moléculas del documento D2, para llegar a los compuestos que se reclaman en la presente solicitud. En este sentido, **dicho documento no brinda ninguna sugerencia acerca de que estas modificaciones deben realizarse con el fin de llegar a obtener compuestos más adecuados.**

En este punto, considero oportuno resaltar que la simple modificación de algunos compuestos de fórmula II del documento D2, basándose en la estructura de los compuestos de fórmula I del mismo documento, no es obvia ni evidente para la persona versada en la materia. En realidad, no es correcto afirmar que *"la persona versada en la materia puede prescindir de L para obtener los compuestos de la solicitud"* puesto que los compuestos de fórmula I reclamados *incluyen* dicho sustituyente L.

Por lo tanto, eliminar la inclusión de dicho sustituyente entre el grupo ciclobutilo y un heterociclo, tal y como señala el señor Examinador en la Resolución aquí impugnada, no llevaría a la obtención de los compuestos de fórmula I reclamados. Por otro lado, si dicha inclusión o eliminación de dicho sustituyente L se llevara a cabo, **los compuestos resultantes todavía serían diferentes a los aquí reclamados ya que aún carecerían de los específicos sustituyentes que se reclaman con el capítulo reivindicatorio modificado.**

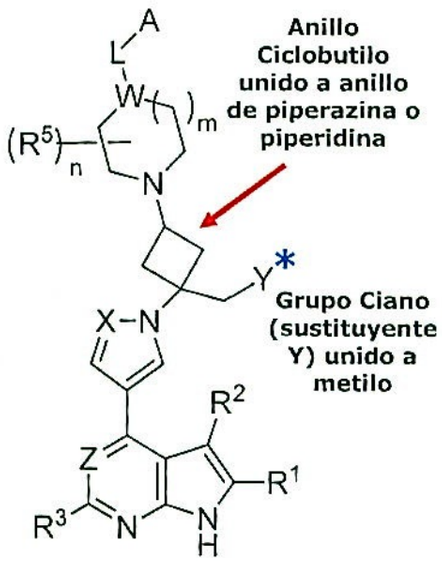
2.2. Las diferencias estructurales existentes entre los compuestos de la presente invención y aquellos divulgados en el documento D1 tampoco son evidentes para la persona medianamente versada en la materia

Ahora bien, a pesar que la evaluación de patentabilidad se hace exclusivamente a la luz de las enseñanzas del documento D2 (*tal y como se evidencia en la determinación del estado de la técnica y en la tabla comparativa de las características técnicas de la* Resolución Número **3680**), me permito reiterar que los compuestos de la presente invención tampoco pueden obtenidos de manera obvia a partir de las enseñanzas del documento D1, el cual corresponde al documento utilizado en el Concepto Técnico emitido por ese Despacho el 23 de Mayo de 2014 y que es también referenciado en la Resolución aquí impugnada.

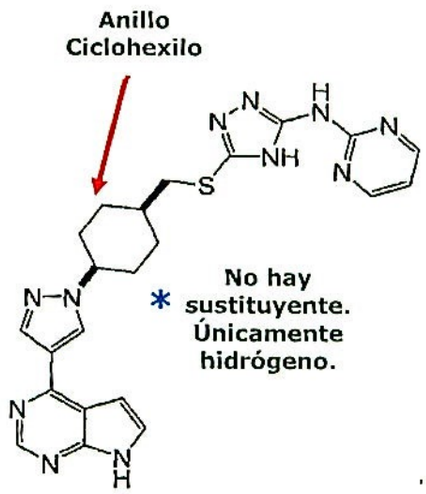
Frente a la afirmación del señor Examinador, de acuerdo con la cual

"los compuestos del documento D1 (...) si tienen el anillo ciclohexano (...) por lo cual dado que los compuestos del documento D2 sí lo enseñan, se considera que la persona versada, quien tiene a la vista los dos documentos, está en capacidad de incluirlo. Por lo tanto, el objeto de la solicitud no tiene nivel inventivo",

Considero de gran importancia destacar ante ese Despacho que no hay ningún compuesto en D1 que incluya un anillo ciclobutilo unido directamente a un anillo pirrol o pirazol, como en los compuestos reclamados en la presente solicitud, ni mucho menos un compuesto con un grupo ciclobutilo unido a un anillo de piperazina o piperidina (*ver flecha roja*):



Fórmula I de la presente invención



Ejemplo 682 documento D1

Ante lo anterior, muy amablemente me permito señalar que, contrario a lo que sostiene el señor Examinador, el reemplazo del anillo de ciclohexano de algunos compuestos del documento D1 por el anillo de ciclobutilo del documento D2 no es obvio ni evidente puesto que la persona versada en la materia tendría que además modificar la naturaleza de los otros sustituyentes de los compuestos del documento D1 (*ver asteriscos azules*) o del documento D2. **Ni en el documento D1 ni en el documento D2 existen las sugerencias o motivaciones correspondientes para realizar todas las modificaciones necesarias para obtener los compuestos que son objeto de la solicitud denegada.**

2.3. Los compuestos de la presente invención no son sólo diferentes estructuralmente a compuestos del arte previo sino también presentan un efecto técnico sorprendente y mejorado.

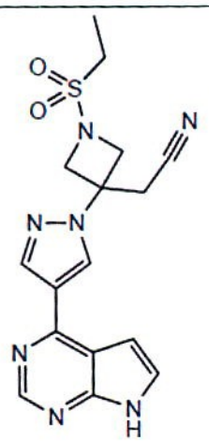
Tal y como se planteó en la respuesta al Concepto Técnico emitido por ese Despacho el 23 de Mayo de 2014, se encontró sorpresiva e inesperadamente que los compuestos reclamados en la solicitud denegada son inhibidores selectivos de JAK1 sobre JAK2 (Ver Ejemplo A de la página 358 y Tablas A a F de las páginas 359 a 373). Para ejemplificar dichos resultados, la siguiente tabla 1 presenta los valores IC_{50} para JAK1 y JAK2 de los 4 compuestos específicos reclamados en la nueva reivindicación 6. Dichos valores demuestran que los compuestos reclamados son más de 10-veces más selectivos por JAK1 sobre JAK2:

Tabla 1. Valores de CI_{50} para los compuestos de la nueva reivindicación 6.

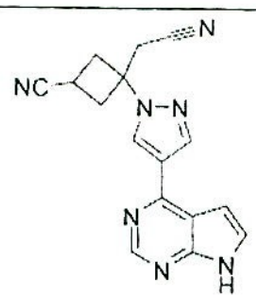
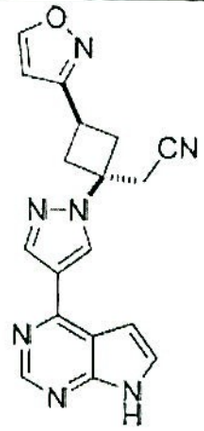
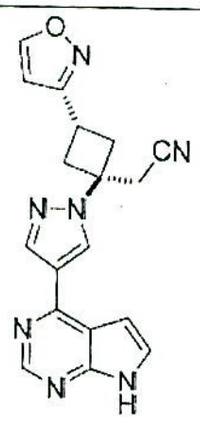
Ejemplo	CI_{50} (nM) para JAK1	CI_{50} (nM) para JAK2
7b	4.2	92
157	2.4	90
161	2.5	80
162	1.6	100

Frente a las enseñanzas de D1, dicho documento únicamente menciona que, en alguna realizaciones, los compuestos de la invención son: a) inhibidores selectivos de JAK1 o JAK2 sobre JAK3 y/o Tyk2, o b) son inhibidores selectivos de JAK2 sobre JAK1, JAK3 o Tyk2 (Página 54, líneas 9 a 12). Por otro lado, los compuestos reclamados en el documento D2 no exhiben selectividad para JAK1 sobre JAK2 (ver Tabla 3 Ejemplo G, página 155 líneas 25 a 26), tal y como se demuestra a continuación para valores CI_{50} de los compuestos identificados como el estado del arte más cercano por el señor Examinador:

Tabla 2. Valores de CI_{50} para algunos compuestos de D2

Compuesto de D2	CI_{50} (nM) para JAK1	CI_{50} (nM) para JAK2
	4.3	3.7

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	3.0	1.8
	3.5	<1.1
	2.2	1.9

Ahora, ante dicha selectividad inhibitoria, ¿qué ventaja confiere a un compuesto seleccionar la inhibición de JAK1 sobre la inhibición de JAK2?

De acuerdo a la página 51 líneas 27 a 30 y página 52 líneas 1 a 23, "*los inhibidores selectivos de JAK1, distinto de otras JAK quinasas, pueden tener múltiples ventajas terapéuticas con respecto a otros inhibidores menos selectivos*". En particular, inhibir selectivamente JAK1 sobre JAK2 puede reducir efectos secundarios no deseados que resultan de la inhibición de la señalización de JAK2. Específicamente, la inhibición de JAK2 puede resultar en efectos inesperados, tales como inmunosupresión, anemia y trombocitopenia, al reducir la señalización de la eritropoyetina (EPO) y la trombopoyetina (TPO), las cuales pertenecen a un grupo de citoquinas y factores de crecimientos que actúan a través de la JAK2 (Parganas, E. et al. Cell. 93:385-395, 1998).

En conexión con lo anterior, la EPO es un factor de crecimiento importante para la producción de glóbulos rojos en la sangre. Por lo tanto, una reducción o insuficiencia en la señalización mediad por EPOs puede resultar en un número reducido de glóbulos rojos en la sangre, y por lo tanto, incrementar el riesgo de sufrir anemia (Kaushansky K, NEJM, 354:2034-2045, 2006). Similarmente, la TPO también es un ejemplo de un factor de crecimiento dependiente de JAK2, el cual juega un rol central en el control de la proliferación y maduración de megacariocitos, o células que producen las plaquetas o trombocitos (Kaushansky K, NEJM, 354:2034-2045, 2006). Entonces, una reducción en la señalización de dichos TPOs causaría una reducción en el número de megacariocitos (condición conocida como *megacariocitopenia*), y así, un conteo reducido de las plaquetas circulantes en la sangre (*trombocitopenia),* lo cual resultaría en un sangrado no deseado y/o incontrolable.

Adicionalmente, reducir la inhibición de otras JAK quinasas, tales como JAK3 y Tyk2, puede ser un efecto conveniente puesto que se ha encontrado que individuos que no tienen una versión funcional de dichas quinasas sufren de numerosas condiciones, como por ejemplo inmunodeficiencia-combinada severa o síndrome de hiperinmunoglobulina E (Minegishi, Y., et al. Immunity 25:745-755, 2006; Macchi, P., et al. Nature 377:65-68, 1995).

En vista de las anteriores consideraciones, un inhibidor exclusivo de JAK1 con reducida afinidad por otras JAK quinasas presentaría ventajas significativas sobre un inhibidor menos selectivo en cuanto a efectos secundarios relacionados a la inmunosupresión, anemia y trombocitopenia. Consecuentemente, **me permito señalar ante ese Despacho que la inesperada y sorprendente selectividad de los compuestos reclamados en la solicitud denegada por JAK1 sobre JAK2 es directamente vinculada a un perfil terapéutico que no es atribuido a inhibidores no selectivos.**

Por lo tanto, me permito también afirmar que se descarta cualquier indicio de obviedad en las posibles modificaciones a los sustituyentes del compuesto de fórmula I y en la obtención de compuestos con el mismo efecto técnico sorprendente de inhibición selectiva de JAK1, evidenciando así el nivel inventivo de los compuestos derivados de pirrolopirimidinas y pirrolopiridinas substituidos con ciclobutilo de las nuevas reivindicaciones de la presente solicitud.

Ahora bien, el punto no es si un experto en la materia podría haber llegado a la invención mediante la adaptación o modificación de las enseñanzas del documento D2 sino que si lo hubiera hecho de esa manera, ¿por qué los compuestos del documento D2 le motivó a hacerlo con la expectativa de resolver el problema técnico objetivo?

Es más, **¿qué razón tendría la persona versada en la materia para que, a la luz del problema resuelto por la presente invención, decidiera modificar los compuestos de D2 de manera que las estructuras resultantes comprendan compuesto derivados de fórmula I, y no otro tipo de modificación?**

En conexión con lo anterior, muy respetuosamente me permito señalar que la única motivación que tendría la persona versada en la materia para hacer esta particular modificación sería la revelación hecha por la presente invención, por lo que el análisis de nivel inventivo realizado por el Señor Examinador está enmarcado dentro de lo que en el ámbito de las patentes se conoce como *"hindsight"* o análisis *"Ex post facto"*. Conociendo de antemano la invención, el Señor Examinador está seleccionando compuestos específicos dentro del documento D2, los cuales modifica de forma que permiten construir la materia reclamada por la nueva reivindicación 1 de la presente solicitud. Dichas acciones o modificaciones de ningún modo podrían ser evidentes para la persona versada en la materia en el momento en el cual se desarrolló la invención.

En este sentido, vale la pena resaltar que, al tener conocimiento de la invención, es de esperarse que dichas modificaciones particulares de las enseñanzas de D2 parezcan obvias, toda vez que ya se sabe de antemano que efectivamente estas sustituciones resuelven el problema planteado. Sin embargo, esta información no era conocida por el inventor en el momento de desarrollar la invención, por lo que no podría tener certeza de la utilidad de realizar estos pasos.

En vista de este señalamiento, y a la luz de lo discutido en el presente memorial, me permito resaltar que al no *"saltar a la vista de manera clara y directa"* a partir de la las enseñanzas de D2 compuestos de fórmula I, como los considerados en la presente invención, y al no tenerse *"certeza clara y manifiesta"* de que dichos compuestos inhibirían selectivamente la enzima JAK1, es claro que el documento D2 no puede de ninguna manera afectar el Nivel Inventivo de la solicitud en estudio.

II. CONCLUSIÓN

Con base en los argumentos técnicos señalados previamente, me permito manifestar ante ese Despacho que el nuevo capítulo reivindicatorio presentado junto

con este memorial goza de un inequívoco carácter novedoso frente a las enseñanzas del documento D1 y D2, razón por la cuales dicha materia es clara merecedora del privilegio de patente solicitado.

Así mismo me permito manifestar que el nuevo capítulo reivindicatorio cumple a cabalidad con las disposiciones de claridad exigidas por el artículo 30 de la Decisión 486 de 2000, respectivamente.

III. PETICIÓN

En vista de todo lo anterior, ruego a usted proceder a estudiar nuevamente la solicitud a la luz de las modificaciones y argumentaciones presentadas, y proceder a revocar la Resolución Número **3680**, con el fin de que se conceda el privilegio de patente solicitado.

IV. PODER

En este punto me permito mencionar ante ese Despacho, que el documento que acredita mi calidad de Representante Legal en Bogotá para asuntos relativos a la Propiedad Industrial de la sociedad peticionaria, fue presentado mediante mi memorial de 17 de julio de 2013.

V. ANEXOS

Anexo al presente recurso me permito presentar:

i) Recibo número 15-007221, expedido por la Superintendencia de Industria y Comercio, que acredita el pago de las modificaciones mencionadas;

ii) Copia de las páginas **382 a 400** que corresponden al nuevo capítulo reivindicatorio modificado, compuesto por **12** reivindicaciones, tal y como se describe en el numeral 1.1 del presente memorial;

iii) Copia del documento Parganas, E. et al. FCell. 93:385-395, 1998;

iv) Copia del documento Kaushansky K, NEJM, 354:2034-2045, 2006;

v) Copia del documento Minegishi, Y., et al. Immunity 25:745-755, 2006; y

vi) Copia del docuemnto Macchi, P., et al. Nature 377:65-68, 1995.

VI. DOMICILIO

Mi dirección es Calle 72 No. 5-83, Piso 6º, Bogotá D.C.

Del Señor Superintendente,

ALICIA LLOREDA RICAURTE

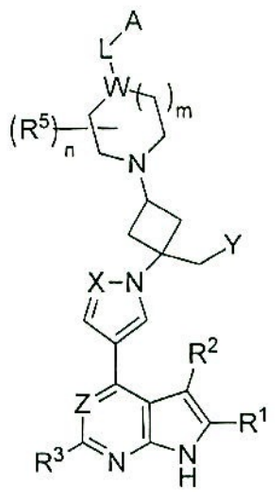
T.P. 53.215

Dirección para Notificaciones: notificacionespatentes@lloredacamacho.com

Incyte Corp.
Exp. 13-134.023

REIVINDICACIONES

1. Un compuesto de la fórmula I:



(I)

o una de sus sales farmacéuticamente aceptables; en donde:

X es N;

Z es N;

R^1, R^2, y R^3 son cada uno H;

Y es ciano;

W es N y L es $C(R^6)_2$, C(=O), C(=O)O, $C(=O)N(R^7)$, $S(=O)_2$, o $S(=O)_2N(R^7)$ o W es CH, entonces L es O,

R^6 es H;

R^7 es H o metilo;

A es es metilo, etilo, propilo, isopropilo, isobutilo, sec–butilo, 1,2–dimetilpropilo, 1–(ter–butil)metilo, ciclopropilo, ciclobutilo, ciclopentilo, ciclohexilo, fenilo, un anillo tetrahidropirano, un anillo pirrolidina, un anillo piperidina, un anillo piridina, un anillo pirimidina, un anillo tiazol o un anillo pirazina; cada uno de los cuales no está sustituido o está sustituido con p sustituyentes R^8 seleccionados de modo independiente;

cada R^8 está seleccionado, de modo independiente, de halo, ciano, alquilo C_{1-6}, haloalquilo C_{1-6}, cicloalquilo C_{3-7}, heterocicloalquilo C_{2-7}, $–OR^a$, $–C(=O)OR^a$, o $–NR^eR^f$; en donde dicho alquilo C_{1-6} no está sustituido o está sustiuido con 1, 2, 3 ó 4 grupos R^g seleccionados de modo independiente;

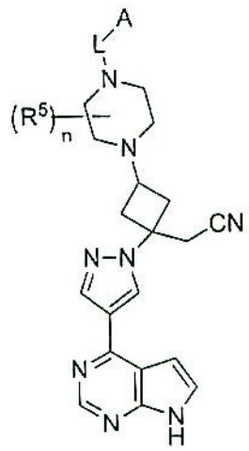
cada R^a, R^e y R^f está seleccionado, de modo independiente, de H, alquilo C_{1-6} y haloalquilo C_{1-6};

cada R^g está seleccionado, de modo independiente, de heterocicloalquilo C_{2-7}, $-OR^{a1}$, $-NR^{e1}R^{f1}$; en donde dicho heterocicloalquilo C_{2-7} no está sustituido o está sustituido con 1 ó 2 grupos R^h seleccionados, de modo independiente, de fluoro, OH, alquilo C_{1-3}, alcoxi C_{1-3} e hidroxi–alquilo C_{1-4};

cada R^{a1}, R^{e1} y R^{f1} están seleccionados, de modo independiente, de H, cicloalquilo C_{3-7} y alquilo C_{1-6};

p es 1, 2 ó 3;
m es 1; y
n es 0.

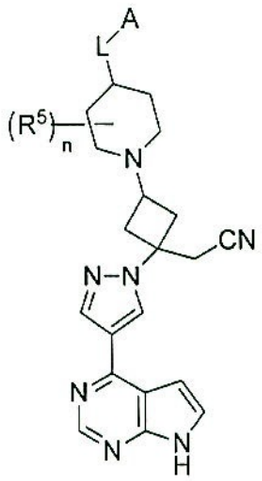
2. El compuesto de acuerdo con la reivindicación 1, en donde dicho compuesto tiene la fórmula IV:



(IV)

o una de sus sales farmacéuticamente aceptables.

3. El compuesto de acuerdo con la reivindicación 1, en donde dicho compuesto tiene la fórmula V:



(V)

o una de sus sales farmacéuticamente aceptables.

4. El compuesto de acuerdo con la reivindicación 3, donde L es O.

5. Un compuesto seleccionado de:

3–[(4–{3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}piperazin–1–il)metil]–5–fluorobenzonitrilo;

3–[(4–{3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}piperazin–1–il)metil]–6–(dimetilamino)–2–fluorobenzonitrilo;

4–{3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–[4–fluoro–2–(trifluorometil)fenil]piperazin–1–carboxamida;

{3–(4–{[(2S)–2–metilpirrolidin–1–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–(4–{[(2S)–2–etilpirrolidin–1–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–{4–[3–fluoro–2–(trifluorometil)isonicotinoil]piperazin–1–il}–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

[1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]–3–(4–{[2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)ciclobutil]acetonitrilo;

{3–[4–(3,5–difluorobenzoil)piperazin–1–il]–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–{4–[(2–cloro–5–fluoropiridin–3–il)carbonil]piperazin–1–il}–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–{4–[(5–fluoropiridin–3–il)carbonil]piperazin–1–il}–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–{4–[2–(difluorometil)–3–fluoroisonicotinoil]piperazin–1–il}–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

3–[(4–{3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}piperazin–1–il)carbonil]–5–fluorobenzonitrilo;

[1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]–3–(4–{[4–(trifluorometil)–1,3–tiazol–2–il]carbonil}piperazin–1–il)ciclobutil]acetonitrilo;

[1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]–3–(4–{[6–(trifluorometil)pirazin–2–il]carbonil}piperazin–1–il)ciclobutil]acetonitrilo;

{3–[4–(3,4–difluorobenzoil)piperazin–1–il]–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–[4–(2–cloro–3,6–difluorobencil)piperazin–1–il]–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–{4–[3–fluoro–5–(trifluorometil)benzoil]piperazin–1–il}–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–{4–[2–fluoro–4–(trifluorometil)benzoil]piperazin–1–il}–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–[4–(pirrolidin–1–ilcarbonil)piperazin–1–il]–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]–3–(4–{[6–

(trifluorometil)piridin–2–il]carbonil}piperazin–1–il)ciclobutil}acetonitrilo;

{3–(4–{[6–(difluorometil)piridin–2–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–{4–[2–fluoro–3–(trifluorometil)benzoil]piperazin–1–il}–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–{4–[(5–fluoropiridin–3–il)metil]piperazin–1–il}–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–{4–[(2–isopropilpirimidin–4–il)carbonil]piperazin–1–il}–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–[4–(piperidin–1–ilcarbonil)piperazin–1–il]–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–{4–[4–fluoro–3–(trifluorometoxi)benzoil]piperazin–1–il}–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–(4–{[3–fluoro–5–(trifluorometil)piridin–2–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–{4–[4–clorobenzoil]piperazin–1–il}–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–{4–[2–fluoro–4–(trifluorometil)benzoil]piperazin–1–il}–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

4–{3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N,N–dimetilpiperazin–1–carboxamida;

{3–(4–{3–[(dimetilamino)metil]–5–fluorobenzoil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–(4–{3–[(dimetilamino)metil]–5–fluorobencil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–[4–(etilsulfonil)piperazin–1–il]–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–[4–(ciclopropilsulfonil)piperazin–1–il]–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

4–{3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N,N–dimetilpiperazin–1–sulfonamida;

4–{3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–etil–N–metilpiperazin–1–carboxamida;

{3–{4–[3–[(dimetilamino)metil]–5–(trifluorometil)benzoil]piperazin–1–il}–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–[4–(3–fluoro–5–{[(2S)–2–metilpirrolidin–1–il]metil}fenoxi)piperidin–1–il]–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{3–(4–{3–[(dimetilamino)metil]–5–fluorofenoxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

[1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]–3–(4–{[2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)ciclobutil]acetonitrilo–d1;

3–[(4–{cis–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}piperazin–1–il)carbonil]–5–[(dimetilamino)metil]benzonitrilo;

3–[(4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}piperazin–1–il)metil]–5–[(dimetilamino)metil]benzonitrilo;

3–[(4–{cis–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}piperazin–1–il)metil]–5–[(dimetilamino)metil]benzonitrilo;

{trans–3–{4–[3–[(dimetilamino)metil]–5–(trifluorometil)bencil]piperazin–1–il}–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{cis–3–{4–[3–[(dimetilamino)metil]–5–(trifluorometil)bencil]piperazin–1–il}–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[6–[(etilamino)metil]–2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

6–[(4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}piperazin–1–il)carbonil]–2–(trifluorometil)pirimidin–4–carboxílico;

{trans–3–(4–{[6–(azetidin–1–ilmetil)–2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[6–[(metilamino)metil]–2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[6–(hidroximetil)–2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[6–[(dimetilamino)metil]–2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[6–(pirrolidin–1–ilmetil)–2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[6–(aminometil)–2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[6–[(isopropilamino)metil]–2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[6–[(ciclobutilamino)metil]–2–(trifluorometil)pirimidin–4–

il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[6–[(ter–butilamino)metil]–2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[6–(1–hidroxi–1–metiletil)–2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{cis–3–(4–{[6–(1–hidroxi–1–metiletil)–2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[6–(metoximetil)–2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[6–(1–aminociclobutil)–2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{cis–3–(4–{[6–(1–aminociclobutil)–2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[6–[1–(dimetilamino)ciclobutil]–2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{cis–3–(4–{[6–[1–(dimetilamino)ciclobutil]–2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–[(dimetilamino)metil]–6–(trifluorometil)piridin–2–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–(hidroximetil)–6–(trifluorometil)piridin–2–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–(aminometil)–6–(trifluorometil)piridin–2–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–[(metilamino)metil]–6–(trifluorometil)piridin–2–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[6–(1–metoxi–1–metiletil)–2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[6–[1–(metilamino)ciclobutil]–2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

2–[(4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}piperazin–1–il)carbonil]–6–(trifluorometil)isonicotinonitrilo;

{trans–3–(4–{[6–(1–hidroxiciclobutil)–2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–(4,5–dihidro–1H–imidazol–2–il)–6–(trifluorometil)piridin–2–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{3–(difluorometil)–5–[(dimetilamino)metil]benzoil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–(1–hidroxi–1–metiletil)–6–(trifluorometil)piridin–2–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–

1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–(metoximetil)–6–(trifluorometil)piridin–2–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[6–(1–hidroxi–1–metiletil)–2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)–1–[4–(1H–pirrolo[2,3–b]piridin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–isopropil–N–metilpiperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–metil–N–propilpiperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–etilpiperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–[3–(dimetilamino)propil]–N–metilpiperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–ciclopropil–N–metilpiperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–metil–N–(2,2,2–trifluoroetil)piperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–isopropilpiperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–(trans–4–hidroxiciclohexil)piperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–[(3R)–tetrahidrofuran–3–il]piperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–

1–il]ciclobutil}–N–(2–hidroxiciclopentil)piperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–[(1S,2R)–2–hidroxiciclopentil]piperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–ciclopentilpiperazin–1–carboxamida;

{trans–3–(4–{[(3S)–3–hidroxipirrolidin–1–il]carbonil}piperazin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–(ciclopropilmetil)piperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–[(1R)–1,2–dimetilpropil]piperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–[(1S)–1,2–dimetilpropil]piperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}piperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–[(1S)–1–ciclopropiletil]piperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–[(1R)–1–ciclopropiletil]piperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–ciclopropilpiperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–ciclobutilpiperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–(2,2–dimetilpropil)piperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–isobutilpiperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–[(1R)–1–metilpropil]piperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–[(1S)–1–metilpropil]piperazin–1–carboxamida;

4–{cis–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–ciclobutilpiperazin–1–carboxamida;

4–{cis–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–[(1R)–1–metilpropil]piperazin–1–carboxamida;

4–{cis–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–[(1S)–1–metilpropil]piperazin–1–carboxamida;

4–{cis–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–ciclopropilpiperazin–1–carboxamida;

4–{cis–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–isopropilpiperazin–1–carboxamida;

4–{cis–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–ciclopentilpiperazin–1–carboxamida;

4–{cis–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–[(1R)–1,2–dimetilpropil]piperazin–1–carboxamida;

4–{cis–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–[(1S)–1,2–dimetilpropil]piperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–metilpiperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–[(1R)–2,2,2–trifluoro–1–metiletil]piperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–[(1S)–2,2,2–trifluoro–1–metiletil]piperazin–1–carboxamida;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–[(1S)–1–(trifluorometil)propil]piperazin–1–carboxamida;

[trans–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]–3–(4–{[(2R)–2–(trifluorometil)pirrolidin–1–il]carbonil}piperazin–1–il)ciclobutil]acetonitrilo;

[trans–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]–3–(4–{[(2S)–2–(trifluorometil)pirrolidin–1–il]carbonil}piperazin–1–il)ciclobutil]acetonitrilo;

N'–ciano–4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N,N–dimetilpiperazin–1–carboximidamida;

{trans–3–[4–(metilsulfonil)piperazin–1–il]–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo ;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}piperazin–1–carboxilato de isopropilo;

{cis–3–(4–{[4–(metoximetil)–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–(metoximetil)–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–(hidroximetil)–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{cis–3–(4–{[4–(hidroximetil)–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–(1–hidroxi–1–metiletil)–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–[(ter–butilamino)metil]–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{cis–3–(4–{[4–[(ter–butilamino)metil]–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–(aminometil)–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–[(dimetilamino)metil]–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–[(etilamino)metil]–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–[(metilamino)metil]–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

2–[(1–{cis–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}piperidin–4–il)oxi]–6–(trifluorometil)isonicotinonitrilo;

2–[(1–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}piperidin–4–il)oxi]–6–(trifluorometil)isonicotinonitrilo;

{cis–3–{4–[3–[(dimetilamino)metil]–5–(trifluorometil)benzoil]piperazin–1–il}–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

3–[(4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}piperazin–1–il)carbonil]–5–[(dimetilamino)metil]benzonitrilo;

{cis–3–(4–{[6–(pirrolidin–1–ilmetil)–2–(trifluorometil)pirimidin–4–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{cis–3–(4–{[6–(morfolin–4–ilmetil)–2–(trifluorometil)pirimidin–4–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{cis–3–(4–{[6–(azetidin–1–ilmetil)–2–(trifluorometil)pirimidin–4–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

3–[(1–{cis–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}piperidin–4–il)oxi]–5–[(dimetilamino)metil]benzonitrilo;

{cis–3–{4–[3–[(dimetilamino)metil]–5–(trifluorometil)fenoxi]piperidin–1–il}–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{cis–3–{4–[3–[(dietilamino)metil]–5–(trifluorometil)fenoxi]piperidin–1–il}–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{cis–3–(4–{3–(difluorometil)–5–[(dimetilamino)metil]fenoxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{cis–3–[4–({6–cloro–4–[(dimetilamino)metil]piridin–2–il}oxi)piperidin–1–il]–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{cis–3–(4–{[6–[(dimetilamino)metil]–2–(trifluorometil)pirimidin–4–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{cis–3–(4–{[6–[(etilamino)metil]–2–(trifluorometil)pirimidin–4–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{cis–3–(4–{[6–[(3–hidroxiazetidin–1–il)metil]–2–(trifluorometil)pirimidin–4–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{cis–3–(4–{[6–metil–2–(trifluorometil)pirimidin–4–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{cis–3–(4–{[6–(hidroximetil)–2–(trifluorometil)pirimidin–4–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{cis–3–(4–{[6–(aminometil)–2–(trifluorometil)pirimidin–4–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

4–{trans–3–(cianometil)–3–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}–N–[2,2,2–trifluoro–1–(trifluorometil)etil]piperazin–1–carboxamida;

{trans–3–(4–{[4–{[(2–hidroxi–1,1–dimetiletil)amino]metil}–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–{[(2–hidroxietil)amino]metil}–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–{[(3–hidroxipropil)amino]metil}–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–(azetidin–1–ilmetil)–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–[(3–hidroxiazetidin–1–il)metil]–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–(pirrolidin–1–ilmetil)–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–(morfolin–4–ilmetil)–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–[(3,3–difluoropirrolidin–1–il)metil]–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–{[(2S)–2–(hidroximetil)pirrolidin–1–il]metil}–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–{[(2R)–2–(hidroximetil)pirrolidin–1–il]metil}–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–(1–hidroxi–1–metiletil)–6–(trifluorometil)piridin–2–il]carbonil}piperazin–1–il)–1–[4–(1H–pirrolo[2,3–b]piridin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{cis–3–(4–{[6–(2–hidroxietil)–2–(trifluorometil)pirimidin–4–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[6–(2–hidroxietil)–2–(trifluorometil)pirimidin–4–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{cis–3–(4–{[4–[(2–oxo–1,3–oxazolidin–3–il)metil]–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo; y

{trans–3–(4–{[4–[(2–oxo–1,3–oxazolidin–3–il)metil]–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

o una sal farmacéuticamente aceptable de cualquiera de los antes mencionados.

6. Un compuesto seleccionado de:

trans–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]–3–(4–{[2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)ciclobutil]acetonitrilo;

{trans–3–(4–{[4–[(3–hidroxiazetidin–1–il)metil]–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

{trans–3–(4–{[4–{[(2*S*)–2–(hidroximetil)pirrolidin–1–il]metil}–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo; y

{trans–3–(4–{[4–{[(2R)–2–(hidroximetil)pirrolidin–1–il]metil}–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo;

o una sal farmacéuticamente aceptable de cualquiera de los antes mencionados.

7. El compuesto de acuerdo con la reivindicación 6, en donde el compuesto es *trans*–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]–3–(4–{[2–(trifluorometil)pirimidin–4–il]carbonil}piperazin–1–il)ciclobutil}acetonitrilo, o una sal farmacéuticamente aceptable del mismo.

8. El compuesto de acuerdo con la reivindicación 6, en donde el compuesto es {trans–3–(4–{[4–[(3–hidroxiazetidin–1–il)metil]–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo, o una sal farmacéuticamente aceptable del mismo.

9. El compuesto de acuerdo con la reivindicación 6, en donde el compuesto es {trans–3–(4–{[4–{[(2*S*)–2–(hidroximetil)pirrolidin–1–il]metil}–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo, o una sal farmacéuticamente aceptable del mismo.

5

10. El compuesto de acuerdo con la reivindicación 6, en donde el compuesto es {trans–3–(4–{[4–{[(2*R*)–2–(hidroximetil)pirrolidin–1–il]metil}–6–(trifluorometil)piridin–2–il]oxi}piperidin–1–il)–1–[4–(7H–pirrolo[2,3–d]pirimidin–4–il)–1H–pirazol–1–il]ciclobutil}acetonitrilo, o una sal farmacéuticamente aceptable del mismo.

11. Una composición que comprende un compuesto de acuerdo con cualquiera de las reivindicaciones 1 a 5 o una de sus sales farmacéuticamente aceptables y al menos un portador farmacéuticamente aceptable.

12. Una composición que comprende un compuesto de acuerdo con cualquiera de las reivindicaciones 6 a 10, o una de sus sales farmacéuticamente aceptables y al menos un portador farmacéuticamente aceptable.

BG 10

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Jak2 Is Essential for Signaling through a Variety of Cytokine Receptors

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Summary

A variety of cytokines activate receptor-associated members of the Janus family of protein tyrosine kinases (Jaks). To assess the role of Jak2, we have derived Jak2-deficient mice. The mutation causes an embryonic lethality due to the absence of definitive erythropoiesis. Fetal liver myeloid progenitors, although present based on the expression of lineage specific markers, fail to respond to erythropoietin, thrombopoietin, interleukin-3 (IL-3), or granulocyte/macrophage colony-stimulating factor. In contrast, the response to granulocyte specific colony-stimulating factor is unaffected. Jak2-deficient fibroblasts failed to respond to interferon γ (IFNγ), although the responses to IFNα/β and IL-6 were unaffected. Lastly, reconstitution experiments demonstrate that Jak2 is not required for the generation of lymphoid progenitors, their amplification, or functional differentiation. Therefore, Jak2 plays a critical, nonredundant role in the function of a specific group of cytokines receptors.

Introduction

A variety of cytokines function through their ligation of members of the cytokine receptor superfamily. This family of receptors associates with and mediates ligand-dependent activation of members of the Janus family of protein tyrosine kinases (Jaks). The Jaks were first identified by screens to detect novel protein tyrosine kinases (Firmbach-Kraft et al., 1990; Wilks, 1991). The potential function of this family of protein tyrosine kinases was first indicated in studies of cell lines that were defective in interferon (IFN) signaling (Velazquez et al., 1992). Isolation of genomic clones, capable of reconstituting the response of one mutant to IFNα/β, resulted in the demonstration that Tyk2 was required in IFNα/β signaling in fibroblasts. Subsequently, it was shown that the ability of erythropoietin (Epo) receptor mutants to function correlated with their ability to activate another family member, Jak2 (Witthuhn et al., 1993), and that growth hormone activated Jak2 (Artgetsinger et al., 1993). Following these studies, additional IFN response mutant complementation classes were shown to involve the disruption of Jak1 in IFNα/β and IFNγ response–defective cells (Müller et al., 1993) and Jak2 in IFNγ response–defective cells (Watling et al., 1993). In a series of subsequent studies, it was demonstrated that all the cytokines that utilize receptors of the cytokine receptor superfamily were capable of activating members of the Jak family of protein tyrosine kinases (reviewed in Ihle, 1995).

The Jak family of protein tyrosine kinases consists of four members (Jak1, Jak2, Jak3, and Tyk2) that are differentially activated in the response to various cytokines (Ihle, 1995). Among these, gene disruptions have thus far only been reported for Jak3 (Nosaka et al., 1995; Thomis et al., 1995). Jak3 specifically associates with the common γ_c receptor chain that was first identified as an essential chain in the functional receptor for IL-2 (Leonard et al., 1995). The γ_c chain is also a critical component of the receptors for a variety of cytokines that affect lymphoid functions, including IL-7, a cytokine that is essential for the expansion of early lymphoid progenitors (von Freeden-Jeffry et al., 1995). Mutant mice lacking Jak3 were shown to be viable but had a phenotype of severe combined immunodeficiency (SCIDs) and functional deficiency of mature T and B cells consistent with a critical, nonredundant role for Jak3 in the function of all the receptors utilizing the γ_c chain. Similarly the absence of Jak3 in humans has been shown to be associated with SCIDs (Macchi et al., 1995; Russell et al., 1995). These results have demonstrated that Jak3 plays an essential, nonredundant role in the functioning of one group of cytokine receptors of the cytokine receptor superfamily that is characterized by the use of the γ_c chain.

In the studies presented here, we have focused on the role of Jak2 in cytokine signaling. Jak2 has been shown to be the predominant Jak activated in the response to a wide variety of cytokines, including Epo (Witthuhn et al., 1993), IL-3, and granulocyte/macrophage colony-stimulating factor (GM-CSF) (Silvennoinen et al., 1993), IL-5, thrombopoietin (Tpo) (Drachman et al., 1995; Miyakawa et al., 1995; Tortolani et al., 1995), growth hormone (Artgetsinger et al., 1993), and prolactin (Campbell et al., 1994; reviewed in Ihle, 1995). In addition, it is activated, along with other Jaks, in the responses to G-CSF (Shimoda et al., 1994), cytokines related to IL-6 (Stahl et al., 1994) and IFNγ (Guschin et al., 1995). However, in each of these cases, other cytoplasmic protein tyrosine kinases are activated, and therefore it is unclear which have unique or redundant roles in receptor signaling. To assess further the specific role of Jak2 in these responses and to determine whether Jak2 might be essential for other unknown receptor systems, we have developed mutant mice that

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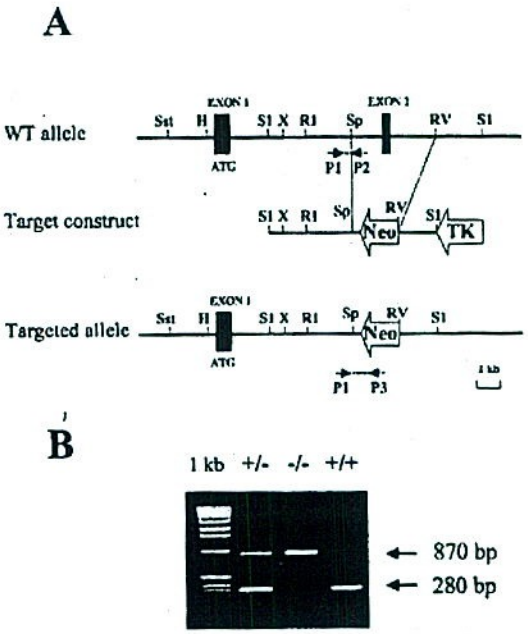


Figure 1. Disruption of the *Jak2* Locus by Homologous Recombination

(A) To obtain mice in which the *Jak2* locus was functionally inactivated, a targeting construct was developed that would remove the second coding exon and flanking sequences as indicated. The construct contained 4.5 kb 5' flanking sequences and 2.5 kb 3' flanking sequences. The restriction maps of the *Jak2* locus and the targeting construct are indicated. The restriction enzymes include Sst (SacI), H (HindIII), S1 (SalI), X(XbaI), R1 (EcoRI), Sp (SpeI), and RV (EcoRV). The construct contained a neomycin gene and the Herpes virus thymidine kinase (TK) gene for selection. The location of primers sequences used in the characterization of the locus are shown.

(B) PCR analysis of tissues from 10- to 12-day-old embryos. Tissues from the embryos were examined by PCR for the presence of the disrupted locus as described in Experimental Procedures.

are deficient in Jak2 through gene disruption in embryonic (ES) cells. The results demonstrate that Jak2 plays an essential, nonredundant role in the function of a number of these cytokines.

Results

To disrupt the *Jak2* gene, a genomic targeting vector was constructed that would delete the second coding exon of the *Jak2* gene and would be anticipated to generate a protein null mutation (Figure 1A). The construct was used to obtain nine clones of ES cells that contained an appropriately disrupted allele. Individual clones of targeted ES cells were injected into C57Bl/6 blastocysts and implanted into pseudopregnant females. Chimeric mice were bred to obtain germ line transmission. Four independently derived clones of ES cells gave rise to mutant strains, all of which had identical phenotypes. Heterozygous animals were phenotypically normal and were bred to obtain mice homozygous for the disrupted allele. Typing of the progeny of such crosses resulted in the absence of viable homozygous mice indicating embryonic lethality. Analysis of timed pregnancies demonstrated that homozygous embryos could be identified by PCR analysis at 10–12 days (Figure 1B) but that such embryos were not present at later times. Analysis of tissues from 10- to 12-day-old embryos demonstrated that homozygously deficient embryos lacked detectable Jak2 by immunoprecipitation and Western blotting (data not shown).

The potential basis for the lethality was evident from the appearance of the homozygous embryos (Figure 2, top). At approximately 10 days of development, definitive erythropoiesis initiates in the fetal liver and is evident from the bright red appearance of the organ as illustrated in Figure 2 (top left) for wild-type animals. In contrast, homozygous Jak2-deficient embryos lacked detectable definitive erythropoiesis. By histological analysis of 11.5 day fetal livers, wild-type embryos contained numerous hematopoietic elements (Figure 2, bottom left). In contrast, fetal livers from mutant mice consisted primarily of hepatic cells and, infrequently, nucleated, primitive red cells or hematopoietic progenitors (Figure 2, bottom right). These aspects of the phenotype are comparable to those observed in mice lacking Epo or the receptor for Epo (Wu et al., 1995b) and demonstrate that Jak2 plays an essential, nonredundant role in Epo signal transduction.

To examine further the potential effects of the absence of Jak2 on erythropoiesis, as well as other cytokines responses, fetal liver cells from 10-day-old embryos were examined for their ability to respond in colony formation to several cytokines (Table 1). Consistent with the above results, erythroid colony-forming units (CFU-E) were not detected in fetal liver cells from mutant mice but were readily detectable with cells from wild-type embryos or heterozygous embryos (data not shown). Fetal liver cells from mutant mice also failed to respond to IL-3, in the presence or absence of Epo, in the formation of mixed lineage colonies or erythroid lineage burst forming units (BFU-E). In addition, fetal liver cells failed to respond to Tpo in the formation of megakaryocytic colonies. Lastly, the cells failed to respond to GM-CSF or IL-5 (data not shown). The results therefore demonstrate that Jak2 has a critical, nonredundant role in the function of IL-3, GM-CSF, IL-5, and Tpo.

G-CSF has been shown to cause the activation of Jak1, Jak2, and Tyk2 (Shimoda et al., 1997). Studies with mutant cell lines have suggested that the presence of Jak1 was critical for the responses to G-CSF, whereas the presence of Jak2 was not required. As illustrated in Table 1, fetal liver cells from mutant mice responded to G-CSF in the formation of granulocytic colonies at levels comparable to wild-type animals, indicating that in the granulocytic lineage, Jak2 is either not required or is redundant to the activation of other Jaks. The presence of G-CSF responsive cells also indicates that hematopoietic lineage precursor cells are present in Jak2-deficient mice.

The above cytokines utilize receptors of the cytokine receptor superfamily. In contrast, CSF-1 and SCF utilize protein tyrosine kinase receptors. It was therefore important to assess the effect of Jak2 deficiency on their responses. As illustrated in Table 1, fetal liver cells from both wild-type and mutant mice were capable of forming macrophage colonies in response to CSF-1, although the numbers were reduced relative to wild-type mice.

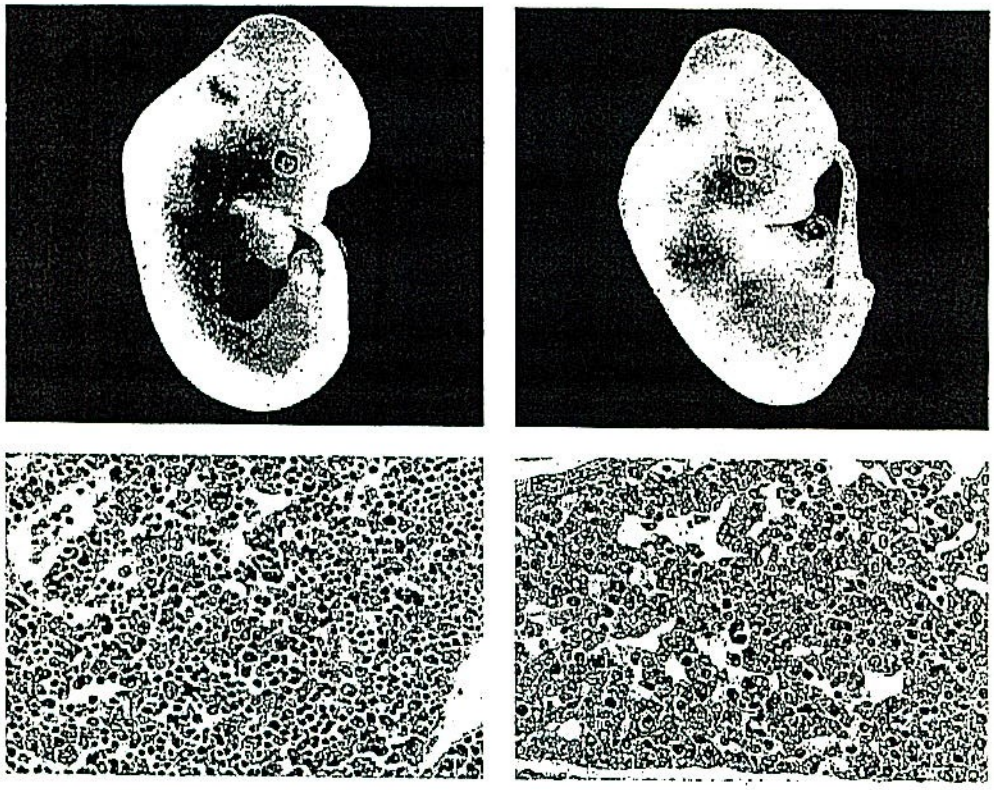


Figure 2. Morphological Phenotype of Jak2-Deficient Embryos

Embryos were dissected from the embryo sacks and were photographed. Top left panel shows the morphology of a wild-type embryo, while the morphology of the Jak2-deficient embryo is illustrated on the top right. The mutant embryo is characterized by the visually obvious absence of red cells in the fetal liver and surrounding tissues. The histological appearance of the wild-type and mutant fetal livers are shown on the bottom left and right, respectively. The wild-type fetal liver contains a mixture of fetal hepatocytes and myeloid cells, including erythroblasts and red cells. The mutant fetal livers contain predominantly fetal hepatocytes with few myeloid lineage cells. A few nucleated, primitive red cells are present.

The reduction may reflect a requirement for Jak2 in the amplification of progenitors as discussed below. Similarly, the number of mixed colonies formed in response to SCF was reduced, but colonies were present. It should be noted that in the response to SCF, no mature red cells were observed in the colonies (data not shown), demonstrating that SCF alone is not capable of supporting full erythroid differentiation.

The absence of Epo, IL-3, and Tpo responsive cells could be due to an essential role for Jak2 in their function or could be due to the absence of responsive progenitors. To address the later possibility, fetal liver cells were examined for the expression of a variety of genes associated with hematopoietic progenitors (Figure 3). Consistent with the hypothesis that hematopoietic progenitors were present was the ability to detect the expression of a number of genes characteristic of early hematopoiesis. In particular, transcripts for the Epo receptor were present, although at a reduced level, as were transcripts for EKLF, which is essential for globin expression during red cell development (Nuez et al., 1995; Perkins et al., 1995). Other genes that are required for definitive erythropoiesis and hematopoiesis were detectable, including AML1 (Okuda et al., 1996), rhombotin-2 (Warren et al., 1994), Gata-1 (Pevny et al., 1991), and Gata-2 (Tsai et al., 1994). Interestingly, a few genes were expressed at reduced levels and were at levels comparable to the levels of the Epo receptor, including c-myb and Pu.1, both of which are required for early myeloid lineages (Mucenski et al., 1991; Scott et al., 1994). We also determined the presence of globin gene transcripts that are differentially regulated during development (Whitelaw et al., 1990). Transcripts for all the globin genes were detectable, including the predominantly embryonic ϵy^2, ζ, and $\beta h1$ genes as well as the α gene that is expressed embryonically and in definitive erythropoiesis. Lastly, expression of the β^{maj} gene, whose expression is initiated in fetal liver definitive erythropoiesis, was also detected, although at levels lower than controls. This is consistent with the lack of expansion of definitive erythrocytes.

Another approach to assessing the presence of erythroid lineage progenitors involved the use of retroviral vectors to attempt to rescue erythroid lineage cells. In these experiments, fetal liver cells from 10- to 12-day-old embryos were obtained and infected in cultures with a retroviral construct capable of expressing Jak2. The cells were subsequently plated in colony assays with Epo to assess their ability to generate erythroid colonies. As shown in Table 1, CFU-E could be rescued from

Table 1. Colony Formation Ability of Hematopoietic Progenitors in Liver Cells from Jak2 Wild-Type and Mutant Mice in Response to Various Cytokines

	Colony Type	Genotype	
		Jak2$^{+/+}$	*Jak2*$^{-/-}$
Cytokine			
Epo[a]	CFU-E	739 ± 35 (n = 6)	0 ± 0 (n = 6)
IL-3 + Epo	BFU-E	9 ± 1 (n = 6)	0 ± 0 (n = 6)
IL-3 + Epo	CFU-Mix	62 ± 4 (n = 6)	0 ± 0 (n = 6)
IL-3	CFU-Mix	213 ± 12 (n = 6)	0 ± 0 (n = 6)
TPO	CFU-Meg	30 ± 3 (n = 6)	0 ± 0 (n = 6)
G-CSF	CFU-G	62 ± 6 (n = 4)	60 ± 6 (n = 4)
CSF-1	CFU-M	420 ± 45 (n = 4)	143 ± 9 (n = 4)
SCF	CFU-Mix	101 ± 4 (n = 4)	31 ± 4 (n = 4)
Retroviral Transduction[b]			
Epo + IL-3	CFU-Mix	92/105	20/31
Epo	CFU-E	776/841	95/112

[a] Mean ± standard deviation of number of colonies/10^5 liver cells.

[b] Fetal liver cells were obtained from either wild-type or Jak2-deficient embryos at 10–11 days of gestation. The cells were cultured with a Jak2-expressing retrovirus overnight and plated in colony assays as described in Experimental Procedures. The results indicate the colonies per 10^5 cells plated, and the results from two animals are given.

Jak2-deficient fetal liver cells by infection with the Jak2 retrovirus, although the numbers of colonies present per 10^5 cells were reduced relative to the number seen with fetal liver cells from wild-type embryos. This reduction suggests that, although committed progenitors can be generated, they cannot be expanded or maintained in the absence of Jak2.

Fetal liver cells from normal 10- to 12-day-old embryos also contain progenitors for the lymphoid lineages. It was therefore important to determine whether such progenitors could be generated in mice that lack Jak2. For these experiments, fetal liver cells from 10- to 12-day-old wild-type and Jak2-deficient embryos were used to reconstitute sublethally irradiated Jak3-deficient mice. As we have previously shown (Nosaka et al., 1995), Jak3 is essential for the amplification of early lymphoid progenitors and for the function of a number of cytokine receptors that utilize the γ_c receptor chain and regulate a variety of lymphoid functions. Four weeks following reconstitution of Jak3-deficient mice, the mice were examined for the presence of peripheral, B220-positive B cells (Figure 4A). As shown, normal mice have readily detectable peripheral B cells (panel 1), while Jak3-deficient mutant mice are dramatically reduced in the numbers of B220-positive cells (panel 2). Reconstitution of sublethally irradiated Jak3-deficient mutant mice with fetal liver cells from either wild-type embryos (panel 3) or Jak2 mutant embryos (panel 4) resulted in the appearance of peripheral B cells. To assess the contribution of the Jak2-deficient cells to peripheral lymphoid cells, PCR analysis was done to detect the mutant cells. As shown in Figure 4B, the mutant cells from the Jak2-deficient embryos were readily detectable in the peripheral lymphocytes of the reconstituted Jak3-deficient mice.

The above results demonstrate that Jak2 is not required for the generation of lymphoid progenitors during embryonic development and that it is not required for the expansion of B cell progenitors in mice. To assess the potential contribution to other lymphoid functions, we examined lymphocytes from the reconstituted mice. The ability to reconstitute the T cell lineage was evident from the number of thymocytes and peripheral T cells. As illustrated in Figure 5B, few $CD4^+$ $CD8^+$, double positive cells were present in the thymus of a Jak3 deficient, nonreconstituted mouse. In contrast, the frequencies of double positive cells in thymi from reconstituted mice (Figures 5C and 5D) were similar to those seen in the

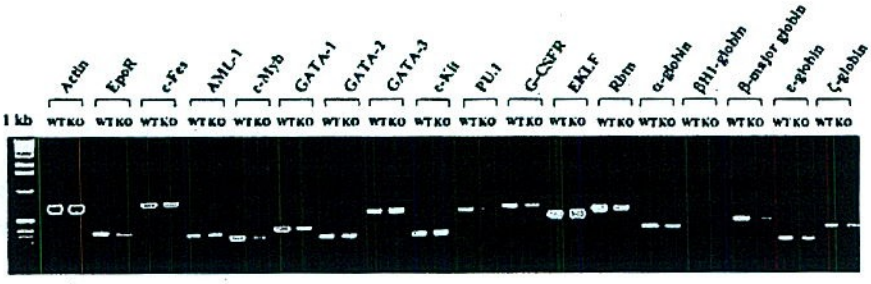


Figure 3. Expression of Myeloid Lineage Marker Genes in Fetal Livers of Jak2-Deficient Mice

Polymerase chain reactions were used to assess the presence of a variety of genes that are involved in myeloid differentiation. RNA was obtained from the fetal livers of 10- to 12-day-old embryos of either wild-type (WT) or Jak2-deficient (KO) embryos. The RNA was used to make cDNA, using random primers and an oligo(dT) primer, and the cDNA was used in reactions to amplify segments of the indicated genes. The primers for the various reactions have been previously described (Okuda et al., 1996).

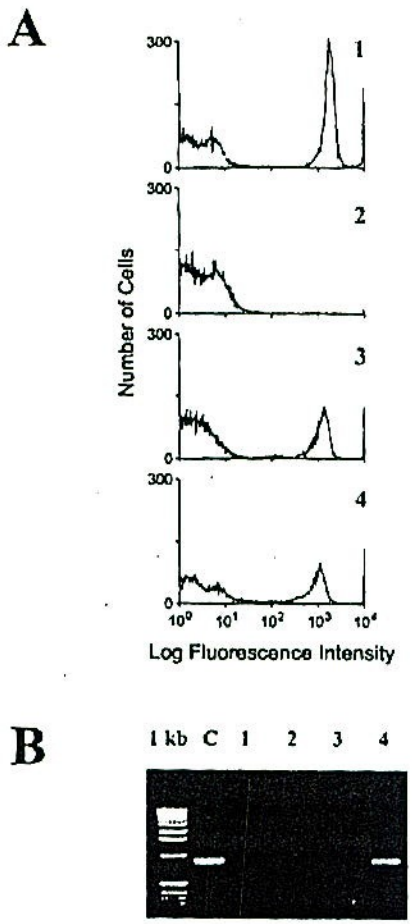


Figure 4. Reconstitution of the Lymphoid Lineages of Jak3-Deficient Mice by Fetal Liver Cells from Jak2-Deficient Embryos

(A) Jak3-deficient mice were sublethally irradiated and reconstituted by injection of fetal liver cells from wild-type embryos or Jak2-deficient embryos. Four weeks after reconstitution, blood was obtained by retroorbital bleeding and analyzed by fluorescence activated cell sorting for the presence of B220-positive, peripheral B cells. The samples shown include wild-type mice (1), Jak3-deficient mice (2), and Jak3-deficient mice reconstituted with wild-type fetal liver cells (3) or with fetal liver cells from Jak2-deficient embryos (4).

(B) To assess the contribution of cells from Jak2-deficient fetal liver cells, a PCR reaction was used to detect the presence of the altered allele. The samples examined include a control (C) and samples from peripheral blood as in (A).

thymus from a wild-type mouse (Figure 5A). The functionality of these T cells was examined by assessing their ability to respond by proliferation to engagement of the T cell receptor in the presence or absence of IL-2. As shown, T cells from Jak3-deficient mice that had been reconstituted with fetal liver cells from Jak2-deficient embryos proliferated following stimulation with increasing concentrations of anti-CD3 (E) or IL-2 (F), whereas cells from nonreconstituted mice failed to respond under either conditions. In addition, B cells from Jak3-deficient mice, reconstituted with fetal liver cells from Jak2-deficient embryos, were capable of responding to anti-IgM or LPS (data not shown). Together the results demonstrate that Jak2 is not required for the embryonic development of progenitors capable of reconstituting the lymphoid lineages and is not required for the cytokine functions examined.

In addition to a number of cytokines that affect myeloid lineages, Jak2 activation is observed in the responses to cytokines of the IL-6 family as well as IFNγ but not in the response to IFNα/β. Consistent with the latter observations, previous studies with mutant cell lines (Watling et al., 1993) had suggested a critical role for Jak2 in IFNγ but not IFNα/β responses. To assess the role of Jak2 in these responses, fibroblasts were derived from wild-type and Jak2-deficient embryos and examined for their ability to respond to various cytokines. As illustrated in Figure 6A, cells from both Jak2-deficient and wild-type embryos responded to IL-6 as assessed by tyrosine phosphorylation of Jak1 and Tyk2, and gp130 and Stat3. Moreover, IL-6 was able to induce the expression of a characteristic response gene (IRF-1) as illustrated in Figure 6E. Similarly, fibroblasts from both wild-type and mutant mice responded comparably to IFNα/β as assessed by tyrosine phosphorylation of Jak1, Tyk2, and Stat1 (Figure 6B), as well as by the ability to induce IRF-1 gene expression (Figure 6E). However, unlike fibroblasts from wild-type embryos, fibroblasts from Jak2-deficient embryos were unable to respond to IFNγ as assessed by tyrosine phosphorylation of Jak1 or Stat1 (Figure 6C) or by the induction of IRF-1 or SOCS-1, another IFN response gene (Figure 6D).

As an additional assay for Jak2 function in IFNγ signaling, we assessed the ability of IFNγ to protect against the cytopathic effects of encephalomyocarditis virus (EMCV). Cells were treated with various concentrations of IFNγ for 16 hr, removed from IFNγ, and infected with a dilution of EMCV that would produce a 100% cytopathic effect in 24 hr of untreated cells. Wild-type cells were protected from EMCV-induced cytopathy with increasing doses of IFNγ. However, fibroblasts from Jak2-deficient embryos were not protected even at 1000 U/ml of IFNγ (data not shown). Therefore, Jak2 is required for the antiviral response mediated by IFNγ.

Discussion

The results demonstrate that Jak2 is required for the function of a variety of cytokine receptors. A potential role for Jak2 in cytokine signaling was initially suggested in studies of the receptors for growth hormone (Artgetsinger et al., 1993), Epo (Witthuhn et al., 1993), and IL-3 (Silvennoinen et al., 1993). However, in the case of the Epo or IL-3 receptors, a number of kinases have been implicated in signaling. For example, Tec (Machide et al., 1995) (Mano et al., 1995), fes (Hanazono et al., 1993), syk (Tsubokawa et al., 1997), and lyn (Torigoe et al., 1992a; 1992b) have all been reported to be activated in the response to IL-3 and/or Epo and have been speculated to play a critical role. Moreover, as discussed below, it has been hypothesized that c-kit associates with the Epo receptor and was speculated to mediate Jak2 independent activation of Epo receptor signaling. Because of the possible redundant involvement of other

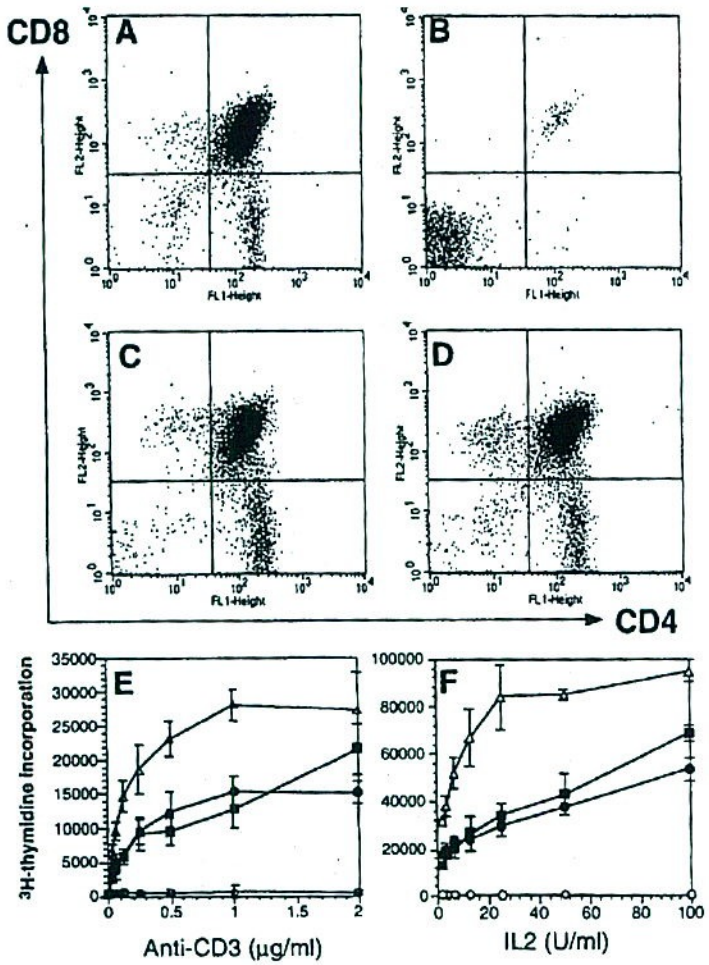


Figure 5. Functional Reconstitution of the Lymphoid Lineages by Jak2-Deficient Fetal Liver Cells

Jak3-deficient mice were sublethally irradiated and reconstituted with fetal liver cells from Jak2-deficient embryos as in Figure 4. The ability to reconstitute the thymocyte populations was examined by analysis of the expression of the T lineage markers, CD4 and CD8. Thymocytes from normal mice express both CD4 and CD8 (A), while the thymuses of Jak3-deficient mice are greatly reduced in this subset of cells (B). Reconstitution of Jak3-deficient mice with wild-type (C) or Jak2-deficient fetal liver cells (D) resulted in a comparable reconstitution of the double positive population. The functional capabilities of peripheral T cells was examined by assessing their ability to proliferate to increasing concentrations of anti-CD3 (E) or to proliferate to a limiting concentration of anti-CD3 in the presence of increasing concentrations of IL-2 (F). The samples shown include cells from wild-type mice (open triangles), Jak3-deficient mice (open circles), Jak3 mice reconstituted with fetal liver cells from wild-type mice (filled circles), or Jak2-deficient embryos (filled rectangles).

cytoplasmic tyrosine kinases, it was possible that deletion of Jak2 would not have had a demonstrable phenotype like the deletion of the other cytoplasmic protein tyrosine kinases implicated in signaling.

The phenotype of Jak2-deficient embryos is very similar to that of the Epo or Epo receptor mutant mice (Wu et al., 1995b). However, the reduction in erythropoiesis in Jak2-deficient mice appears somewhat more profound than that reported for Epo- or Epo receptor–deficient mice. This could be due to the inactivation of Tpo function, since Tpo contributes to the expansion of early erythroid lineage cells and can rescue in vitro erythroid colony formation from mouse embryos lacking the Epo receptor (Kieran et al., 1996). Importantly, however, progenitors for the erythroid lineage are present as indicated by the expression of genes that are associated with lineage committed cells as well as by the ability to rescue erythroid lineage cells by infection with a retrovirus expressing Jak2.

The responses to other cytokines including Tpo, GM-CSF, IL-5, and IL-3 are also deficient in Jak2-deficient mice. The later cytokines utilize a common signaling chain, termed the β chain, which has been shown to associate with Jak2. Since the functions for all these cytokines are absent, the results would suggest that none of these cytokines is essential, even in a potentially redundant manner, for the generation of early hematopoietic progenitors.

Previous studies (Wu et al., 1995a) suggested that SCF, through its receptor, c-kit, could induce tyrosine phosphorylation of the Epo receptor and that c-kit associates with the Epo receptor as assessed by GST-fusion proteins. Based on these results, it was hypothesized that c-kit transduced a mitogenic or differentiation signal through the Epo receptor that was independent of Jak2 activation. This model was evoked to explain the reduction of erythroid lineage cells seen in c-kit mutant mice. Our results are not consistent with this hypothesis and demonstrate that Jak2 is essential for either Epo receptor function in the induction of erythroid lineage expansion and differentiation, independent of whether the receptor is activated by Epo or by SCF through c-kit. The effects of c-kit mutations are therefore likely to be upstream of the Epo signaling in the expansion of the progenitors that become Epo responsive.

The development of the embryos until day 10–12 is normal and suggests that Jak2 is not required, in a nonredundant manner, for any early developmental

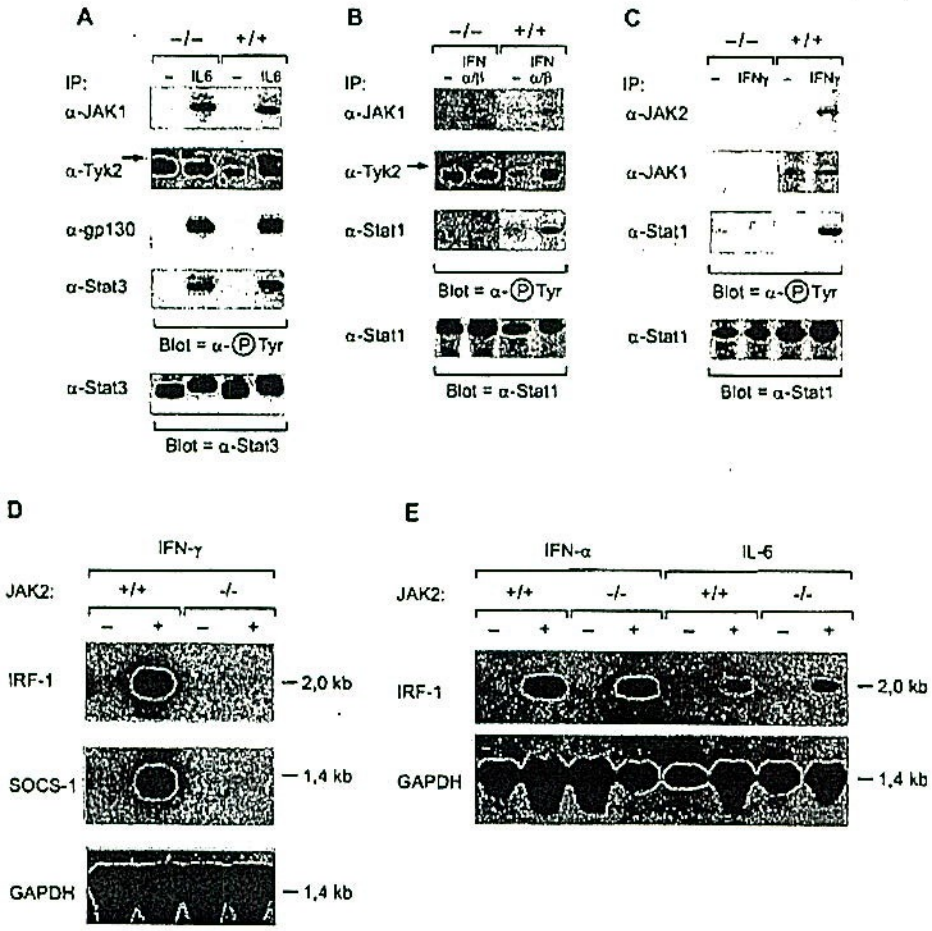


Figure 6. Fibroblasts from Jak2-Deficient Embryos Respond to IL-6 or IFNα but Not to IFNγ

Fibroblasts were derived from either wild-type embryos (+/+) or Jak2-deficient embryos (−/−) as described in Experimental Procedures.

(A) To examine the response to IL-6 fibroblasts cultures were not stimulated (−) or were stimulated with a mixture of IL-6 and the soluble IL-6 receptor α chain (IL6). Following 10 min of stimulation, the cells were collected and analyzed for the induction of tyrosine phosphorylation of Jak1 or Tyk2, gp130 or Stat3 by immunoprecipitation with the indicated antisera and Western blotting with a monoclonal antibody against phosphotyrosine. To control for protein loading, the anti-Stat3 immunoprecipitations were analyzed for Stat3 protein by Western blotting.

(B) The responses of the fibroblasts to IFNα/β were determined comparably except that the induction of tyrosine phosphorylation of Stat1 was examined.

(C) The response to IFNγ was also comparably analyzed with the same exception.

(D) To assess further the ability to respond to IFNα, the induction IRF-1 or SOCS-1 was examined by Northern analysis 1 hr following stimulation of fibroblasts.

(E) The ability of IFNα or IL-6 to induce IRF-1 expression was examined by Northern analysis as above. The levels of GAPDH RNA were used to control for RNA loading.

events (Neubauer et al., 1998 [this issue of *Cell*]). We have, however, noticed a morphologically detectable delay in heart development in the Jak2-deficient embryos (data not shown). This was of potential interest because previous studies described a defect in the development of the heart of gp130-deficient mice (Yoshida et al., 1996) that may be due to the loss of function of cardiotropin, a cytokine that utilizes the gp130 cytokine receptor chain (Hirota et al., 1995; Wollert and Chien, 1997). However, the defect seen in gp130-deficient mice occurs at 16.5 days of development and later. Moreover, the results with fibroblasts suggest that the absence of Jak2 does not detectably affect gp130-mediated signaling by IL-6. Therefore, it is unlikely that the defects are related to gp130 signaling. The possibility exists that effects on the heart seen in Jak2-deficient mice may be related to the anoxia as a consequence of the profound lack of definitive erythropoiesis and reduced primitive erythropoiesis. Alternatively, red cells may produce cytokines that contribute to myocardial development.

Fetal liver cells from Jak2-deficient embryos were able to numerically reconstitute the lymphoid lineages in Jak3-deficient mice. In addition, all the T and B cell functions examined were functionally reconstituted.

These results demonstrate that Jak2, unlike Jak3, is not required for the embryonic development of lymphoid lineage progenitors, for the IL-7 dependent expansion lymphoid lineage committed progenitors, or for the functional differentiation of peripheral T and B cells. In contrast to these results, reconstitution of Jak3-deficient mice with AML1-deficient fetal liver cells does not result in reconstitution (unpublished data), consistent with a critical role for AML1 in early hematopoietic stem cell function (Okuda et al., 1996). Although not addressed, one potentially interesting cytokine that affects T cell function is IL-12, which has been shown to activate Jak2 (Bacon et al., 1995).

G-CSF activates several of the Jak family members, including Jak1, Jak2, and Tyk2, although most studies have observed that Jak2 is the most significantly activated (Nicholson et al., 1994, 1995; Rauprich et al., 1995; Barge et al., 1996). Previous studies (Shimoda et al., 1997) using mutant cell lines that are deficient in Jak2, Jak1, or Tyk2 suggested that the absence of Jak2 or Tyk2 did not affect G-CSF receptor function but that in the absence of Jak1, signaling was lost. The results obtained with fetal liver cells from Jak2-deficient mice support the conclusion that Jak2 is not essential for a G-CSF response. Interestingly, studies with Jak1-deficient mice also suggest that Jak1 is not essential for a G-CSF response (Rodig et al., 1998 [this issue of *Cell*]). It is possible that the various Jaks activated by the G-CSF receptor fulfill redundant functions. Alternatively, it is possible that other cytoplasmic kinases are involved.

Similar to the studies with G-CSF, the results with fibroblasts from Jak2-deficient embryos demonstrated that Jak2 is not essential for the response to IL-6. The IL-6 family of cytokines utilizes a common signaling chain, referred to as gp130 or the highly related LIFRβ chain, that associates with and activates multiple Jaks (Andus et al., 1988; Narazaki et al., 1994; Stahl et al., 1994). As with G-CSF, studies with mutant cell lines lacking various Jaks indicated that Jak1 was critical for IL-6 signaling (Guschin et al., 1995). The results with Jak2-deficient fibroblasts demonstrate that Jak2 is not essential for an IL-6 response. However, as indicated by Rodig et al. (1998), Jak1 is essential for the response of cytokines that utilize gp130.

Among the cytokines that have been shown to activate Jak2 are the growth hormone (Artgetsinger et al., 1993) and prolactin (Campbell et al., 1994). Because of the embryonic lethality of the phenotype and the lack of expression of the receptors for these cytokines in fibroblasts, we have not had the opportunity to determine whether Jak2 is essential for their function. However, since the receptors are highly related structurally and functionally to the receptors for Epo and Tpo, we would anticipate that their functions would also be absent in Jak2-deficient animals. We are currently attempting to use fetal liver rescues to obtain mice in which the hematopoietic system is rescued but would be otherwise Jak2 deficient to address specifically the role of Jak2 in the response to growth hormone or prolactin.

Together the results demonstrate that Jak2 is essential for the function of a variety of cytokine receptors. Equally striking is the lack of an effect in any of the signaling pathways required for normal development to the stage when definitive erythropoiesis is required. The results are consistent with the hypothesis that cytokines, cytokine receptors, the Jaks, and the Stats have evolved to fulfill very specific functions acquired during evolution.

Experimental Procedures

Construction of Jak2 Targeting Vector

The *Jak2* gene was isolated from a 129/SvE mouse genomic library in λEMBL3 using a 5′ *Jak2* cDNA probe. Positive clones were restriction mapped, and the exon/intron structure of the 5′-end of the gene was determined by hybridization using 5′ specific cDNA oligonucleotides or sequencing. For the Jak2 targeting vector, a 12.5 kb SalI fragment was subcloned, followed by replacement of a 5 kb SpeI/EcoRV fragment containing the second *Jak2* coding exon with a neomycin resistance cassette (van Deursen et al., 1991). A herpes simplex thymidine kinase (HSV-tk) cassette mediating negative selection was inserted in the 3′-end of the Jak2-neo construct.

Culturing and Electroporation of ES Cells

E14 (129/Ola mouse strain) ES cells were cultured in Dulbecco's modified Eagle medium (D-MEM) containing 15% fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 55 μM 2-mercaptoethanol, 10 μg/ml gentamicin, and 1,000 U/ml LIF (all from GIBCO-BRL). Mitotically inactivated SNLH9 were used as feeder cells for the E14 ES cells. Twenty-five micrograms of NotI linearized Jak2 plasmid construct was electroporated into the ES cells using a gene pulser set at 0.23 kV, 500 μF (Bio-Rad). Selection 24 hr after electroporation was in 350 μg/ml geneticin (G418 [GIBCO-BRL]) and 2 μM gancyclovir. ES clones were picked and expanded 7–10 days after electroporation.

Generation of Jak2-Deficient Mice

Conditions for blastocyst injection of correctly targeted and karyotypically normal ES clones and breeding to generate mice homozygous for the mutated *Jak2* gene were performed essentially as described (van Deursen et al., 1993). Nine independent ES clones were injected, of which four gave germline transmission. The mice were maintained under specific pathogen-free (SPF) conditions. Mouse-tail DNA was prepared by using QIAamp tissue kit (Qiagen) or from approximately 1 mm tail snips of neonatal pups as described (Hogan et al., 1994). Genotyping of mice was performed by Southern blot analysis or by PCR of tail DNA. For the PCR assay, approximately 200 ng of tail DNA was amplified per 50 μl reaction using 2.5 U AmpliTaq Gold (Perkin-Elmer) in PCR buffer II with a final concentration of each dNTP at 0.2 mM, $MgCl_2$ at 1.5 mM. Multiplex PCR with three primers per reaction were used in the Jak2 assay. For the assay, JF1 primer (5′-TGCCAGGTGGACGTGTTTAAAT-3′) at 0.4 μM, JR1 primer (5′-AGGCATGCAATACCGAGTGTTG-3′) at 0.4 μM, and NEOF1 primer (5′-ATCTCCTGTCATCTCACCTTG-3′) at 0.4 μM produced an 870 bp and 280 bp fragment from the mutant and wild-type alleles, respectively. The PCR cycle profile on a PTC-200 (MJ Research) was as follows: 1 cycle at 94°C for 10 min followed by 30 cycles at 94°C for 1 min, 55°C for 30 s, 72°C for 1 min with 5 s autoextension in every cycle.

Colony Assays

Cells were prepared from livers of d10-12 embryos in α-MEM medium (GIBCO/BRL) containing 2% fetal bovine serum (FBS) (StemCell Technologies) and counted in the presence of 3% acetic acid to lyse the erythrocytes. Diluted cell suspensions and recombinant cytokines specific for each of the assays (see below) were mixed with MethoCult M3230 (StemCell Technologies). This gave a final concentration of 0.9% methylcellulose, 30% FBS, 1% bovine serum albumin, 0.1 mM 2-mercaptoethanol, and 2 mM L-glutamine. Assays were plated in 35 mm culture dishes in duplicate and cultured at 37°C, 5% CO_2. For the CFU-E assay, 5×10^4 cells/dish were cultured in 0.2 U/ml recombinant human erythropoietin, rhEpo (Amgen), and benzidine-positive CFU-E colonies were scored at day 3. For the BFU-E assay, 1×10^5 cells/dish were cultured in 3 U/ml rhEpo and 10 ng/ml recombinant murine IL-3, rmIL-3 (R&D Systems), and

benzidine-positive BFU-E colonies were scored at day 8. For the CFU-Mix assay, 5×10^4 cells/dish were cultured in 10 ng/ml rmIL-3 or 1×10^5 cells/dish were cultured in 50 ng/ml rmSCF (R&D Systems), and colonies were scored at day 12. For the CFU-Meg assay, 5×10^5 cells/dish were cultured in 50 ng/ml recombinant human thrombopoietin, rhTpo (Genzyme), and colonies were scored at day 8. For the CFU-GM and CFU-M assay, 5×10^4 cells/dish were cultured in 10 ng/ml rmGM-CSF or 10 ng/ml rmCSF-1 (R&D Systems), and colonies were scored at day 12. For the CFU-G assay, 5×10^4 cells/dish were cultured in 10 ng/ml rhG-CSF (Amgen), and colonies were scored at day 12.

Reconstitution

Fetal liver cells from wild-type or Jak2 d10-12–deficient embryos were used to reconstitute sublethally irradiated Jak3-deficient mice. The Jak3-deficient mice were irradiated with a total dose of 385 rads over a period of 3 min. A total number of 5×10^6 Jak2-deficient liver cells in 150 μl of DMEM were injected retroorbitaly into Jak3-deficient mice.

Immunoprecipitation, SDS-PAGE, and Western Blotting

Embryonic fibroblasts from Jak2 wild-type, Jak2 heterozygous, or Jak2-deficient embryos were cultured in DMEM supplemented with 10% fetal bovine serum. The cells were starved for 14–16 hr in serum-free DMEM before treated with IFNα (1000 IRU/ml), IFNγ (500 IRU/ml), or IL-6 (500 ng/ml) plus soluble IL-6 receptor (500 ng/ml) for 10 min. Cells were lysed in lysis buffer as previously described (Wang et al., 1996). Cell lysates were centrifuged at 12,000 × g for 15 min to remove debris, and the supernatants were incubated with the indicated antisera for 2 hr. Immune complexes were precipitated with protein A-Sepharose, washed three times in lysis buffer, and then eluted with sample buffer for SDS-PAGE. Eluted proteins were resolved by SDS-7.5% PAGE and transferred to nitrocellulose. Membranes were probed by using designated sera or antibodies and visualized with the ECL detection system (Amersham).

Northern Blot Analysis

Wild-type and Jak2-deficient primary fibroblasts were starved of serum overnight and consequently stimulated with IFNα (1000 IRU/ml), IFNγ (500 IRU/ml), or IL-6 (500 ng/ml) plus soluble IL-6 receptor (500 ng/ml) for 1 hr. Total RNA from both wild-type and Jak2-deficient primary embryonic fibroblasts was prepared using the RNAzol-B purification method (Tel-test), and 20 μg RNA per lane was separated on 1% formaldehyde agarose gels according to Sambrook et al (1989). The gels were blotted onto Nytran plus membranes (Schleicher & Schuell). Hybridization was performed in a modified church buffer containing 600 mM phosphate buffer, 8% SDS, 1 mM EDTA, 1% BSA, at 65°C overnight. Interferon regulatory factor 1 (IRF-1) and suppressor of cytokine signaling 1 (SOCS-1) cDNAs were used as probes. All probes were labeled using a random priming kit readiprime (Amersham). The membranes were washed in 50 mM phosphate buffer, 5% SDS, 1 mM EDTA at 65°C for 45 min. Exposure time for autoradiography ranged from 1–3 days.

RT-PCR

Total RNA was isolated from livers of wild-type and Jak2-deficient d10-12 embryos (see above method for total RNA isolation). For cDNA synthesis, 5–10 ug of total RNA in a final volume of 36 μl of DEPC-treated water was heated to 65°C for 5 min and chilled on ice for 5 min. Either 3 μl of oligo (dT) primer (100 ng/μl) or random primers (100 ng/μl) was added to the reaction mix. Components added to reaction mix included 5 μl of 10× first-strand buffer, 1 μl of RNase block ribonuclease inhibitor (40 U/μl), 2 μl of 100 mM dNTPs, and 1 μl of MMLV-RT (50 U/μl). Incubated reaction was at 37°C for 1 hr. The primers and the conditions for the PCR have been previously described (Okuda et al., 1996).

Histology

For sectioning, tissues were fixed in 10% phosphate-buffered formalin (Fisher) and B-5 solution, paraffin embedded, sectioned, and stained in hematoxylin and eosin.

Generation of Ecotropic LJAK2SN Retroviral Producer Cells

High titer ecotropic LJAK2SN producer cell lines were obtained by transducing GP+E86 packaging cells with high titer amphotrophic LJAK2SN a total of six times over 3 days. The transduced GP+E86 cells were then plated and placed under G418 (500 μg/ml) selection for 14 days. The clones were subsequently pooled and used to produce LJAK2SN retrovirus containing supernatants. The high titer amphotropic LJAK2SN clones were obtained by transient transfection of 293 T cells with the plasmid containing the LJAK2SN construct and a plasmid containing the retroviral *gag*, *pol*, and *env* genes, whose expression is driven by the Moloney Leukemia Virus LTR (D. Persons and E. F. V., unpublished data).

Flow Cytometry

Thymus cells obtained from individual mice were made into single-cell suspensions in PBS supplemented with 1% bovine serum albumin (PBS/BSA). Nonspecific staining was minimized by blocking with 10% normal mouse serum in PBS/BSA. The cells were then stained with a cocktail of fluorescein isothyocyanate (FITC)-conjugated rat anti-mouse CD4 (clone RM-4-4) and phycoerythrin (PE)-conjugated rat anti-mouse CD8 (clone 53-6.7) in PBS/BSA for 30 min at 4°C. The mAbs were purchased from Pharmingen (San Diego, CA) and can be referenced in their current catalog. Cell surface expression of the markers was subsequently analyzed in a Becton-Dickinson FACScan in two-color mode using CellQuest software.

Proliferation Responses

Single-cell suspensions of spleen cells in SMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), L-glutamine, penicillin, streptomycin, gentamycin (all from Life Technologies), sodium bicarbonate (Sigma, St. Louis, MO), essential and nonessential amino acids (Life Technologies), and 2-mercaptoethanol (Sigma) were plated in 96-well round-bottomed plates (Corning, Corning, NY) at a density of 1×10^5 per well in 100 μl. The stimuli were anti-CD3 alone (clone 2C11, Pharmingen, San Diego, CA) at a range of concentrations as indicated or at a fixed concentration of 0.2 μg/ml in combination with several dilutions of recombinant human IL2 (Chiron, Emeryville, CA). Proliferation was assessed by measuring radioactivity using a Matrix 9600 beta counter (Packard, Meriden CT) 18 hr after adding 1 μCi/well ^{3}H-thymidine (Amersham Life Sciences, Arlington Heights, IL).

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REVIEW ARTICLE

MECHANISMS OF DISEASE

Lineage-Specific Hematopoietic Growth Factors

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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 or 10 or more when the demand for blood cells increases.

In 1906, Carnot[1] 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 colleagues[2] 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 leukocytes[4] and platelets[5] 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 1α (HIF-1α). Once hydroxylated, HIF-1α binds the von Hippel–Lindau (VHL) protein, thereby targeting it for ubiquitin-mediated de-

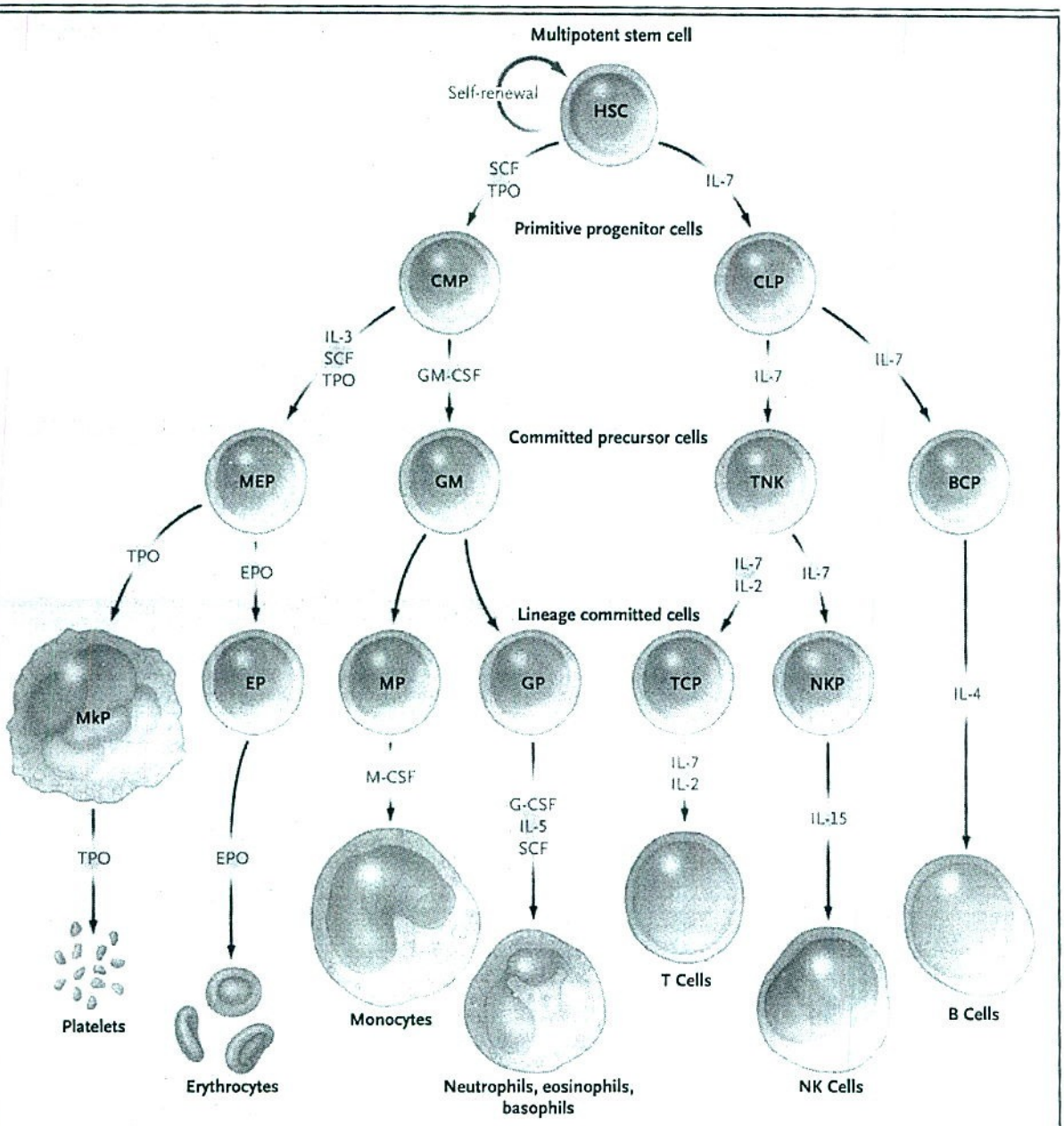


Figure 1. A General Model of Hematopoiesis.

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 unilineage 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.

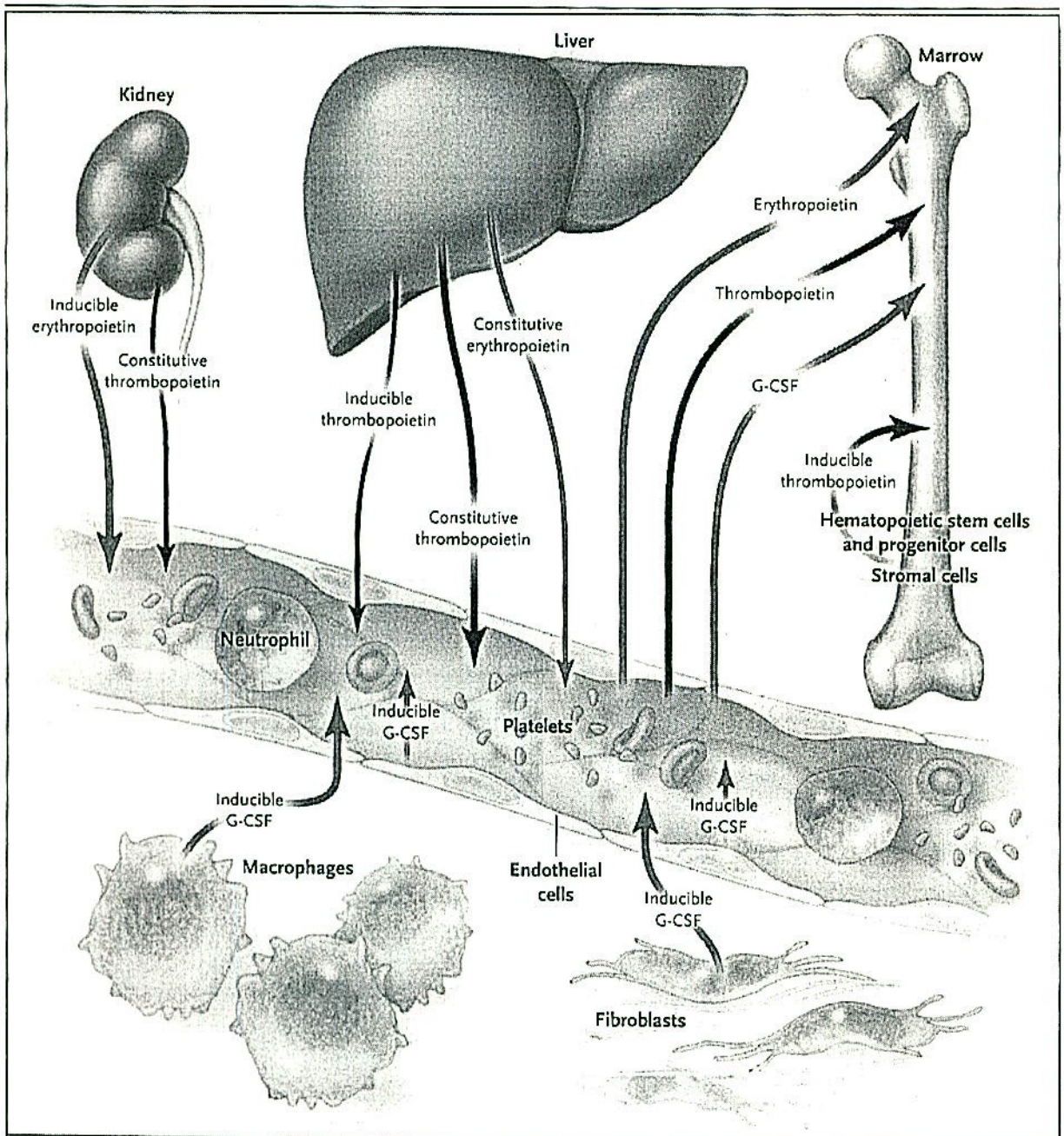


Figure 2. Regulation of the Production of Hematopoietic Growth Factors.

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-1α 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, Chebokshary), 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 stimuli[12] 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 semisolid 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 nonredundant 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 marrow[5]; 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 homodimer[37]; 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

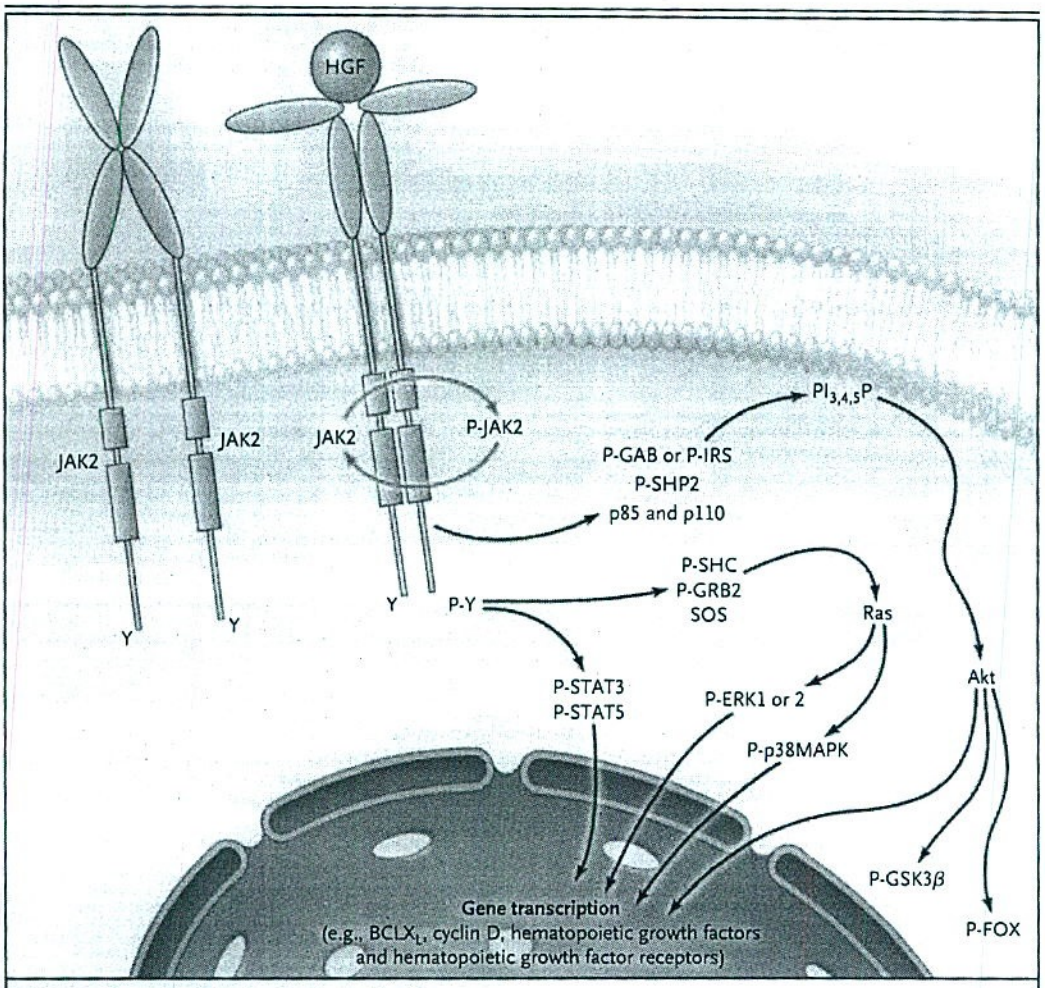


Figure 3. Hematopoietic Growth Factor Signaling.

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 3β (P-GSK3β). Eventually, most such signals affect nuclear transcription of antiapoptosis molecules ($BCLX_L$), 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 (cyclins[45]) and inhibitors ($p27^{Kip}$),[46] anti-

apoptosis molecules ($BCLX_L$ and inhibitor of apoptosis[47]), and other growth factors (transforming growth factor β[48] and vascular endothelial growth factor[49]) 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 leukemia[52]; 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 diagnostic 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 Oncology[56] 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-CSF[76] 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 hematopoiesis 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.

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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 (Velazquezet 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 IFNα, but a high concentration of IFNα 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 λ5 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 IFNα/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 IFNα 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-12Rβ1, 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 IFNγ when stimulated with IL-12 plus IL-18, a potent IFNγ inducer for T cells (Figure 1A). The failure could not be attributed to a total defect in the machinery for IFNγ production, because the patient's T cells produced considerable amounts of IFNγ 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 IκBα 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 IFNα (Figure 1C). This finding was in sharp contrast to human IL-12Rβ1 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 IFNα (Fieschi et al., 2004).

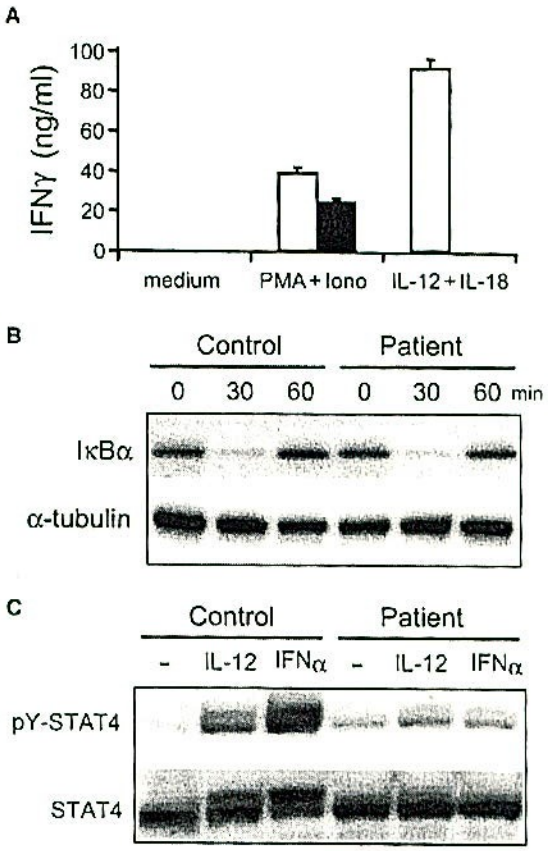


Figure 1. Impaired Responses to IL-12 and IFNα in a Patient with HIES

(A) The patient's T cells were defective in IFNγ production in response to IL-12 and IL-18. The concentration of IFNγ 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. IκBα 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 IFNα 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 IFNα for 15 min. Results are representative of at least three independent experiments.

A Homozygous Mutation in the Patient's Tyk2 Gene

The defect in both IL-12 and IFNα 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).

The second candidate we investigated was Tyk2, because a mutant human cell line unresponsive to IFNα turned out be deficient in Tyk2 (Velazquez et al., 1992), even though Tyk2 appeared to be not absolutely essential for IL-12 and IFNα 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

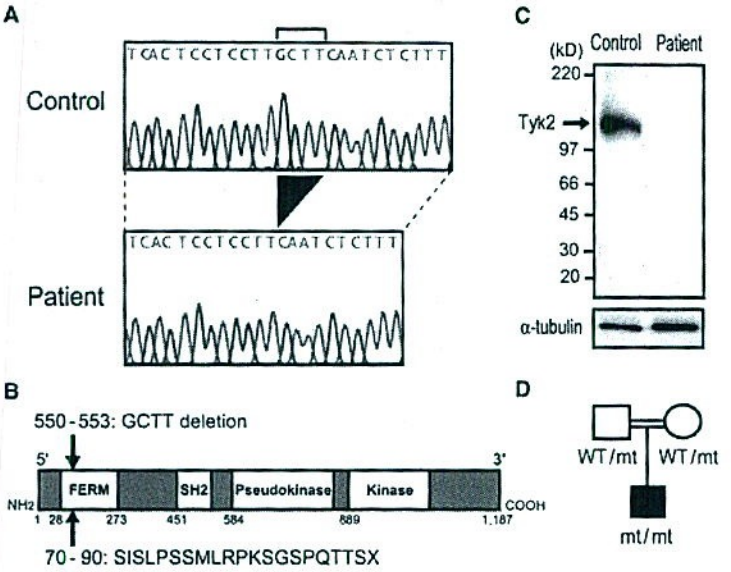


Figure 2. A Homozygous Mutation of the Tyk2 Gene in the Patient

(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 frameshift 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 pseudokinase 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 IFNα or IFNβ 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 IFNα or IFNβ, 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 IFNα 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 IFNα nor IFNβ 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 IFNγ in the patient (Figure 3F). Stimulation with IFNγ upregulated the transcription of two IFNγ-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 IFNγ 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 IFNγ 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 IFNγ secretion even though the background amount of

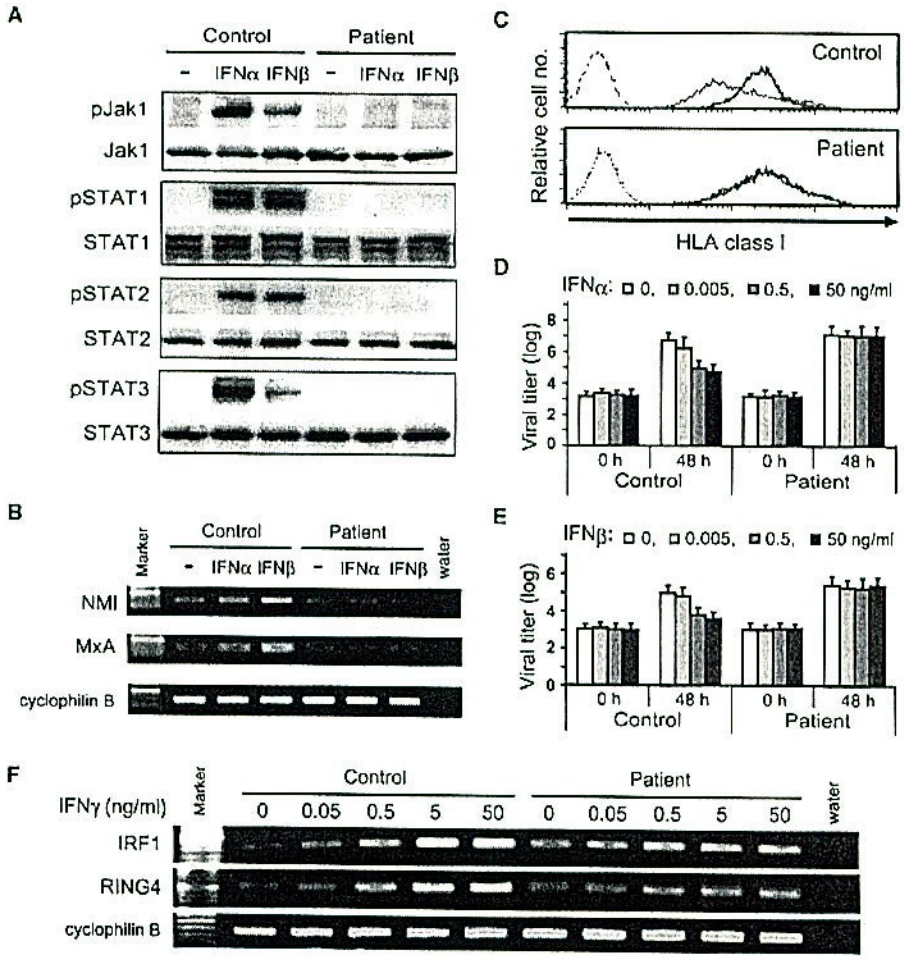


Figure 3. Defects in the Type I and II IFN Signals in the Patient's T Cells

(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 IFNα or IFNβ 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 IFNα or IFNβ 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) IFNα 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 IFNα (D) or IFNβ (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 IFNγ 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 IFNγ for 2 hr.

IFNγ 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 TNFα 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

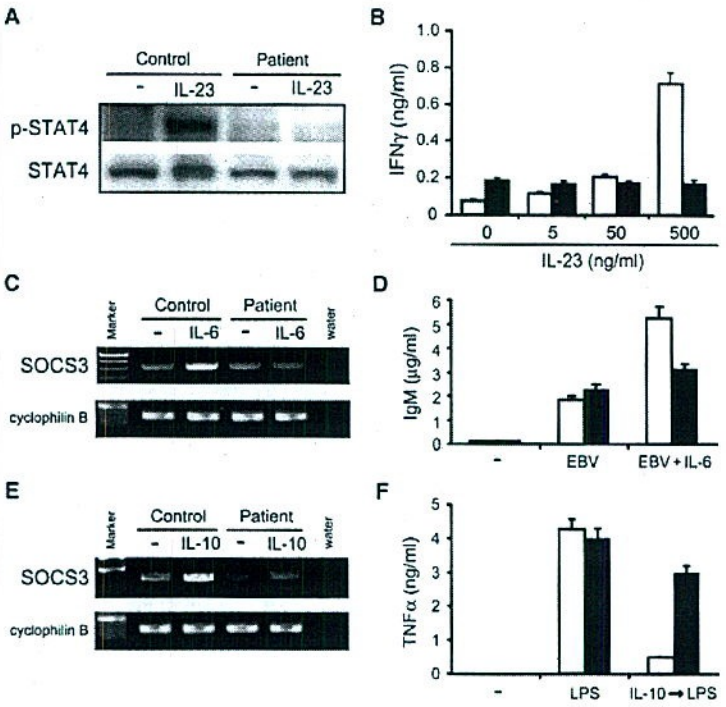


Figure 4. Defects in IL-23, IL-6, and IL-10 Signals in the Patient's Cells

(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 IFNγ production. The concentration of IFNγ 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 TNFα production by IL-10 in the patient's macrophages. The concentration of TNFα 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 IFNγ-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 IFNγ 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 Tyk-2-expressing cells produced IFNγ, 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 IFNγ 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 IFNα (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 IFNα 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.

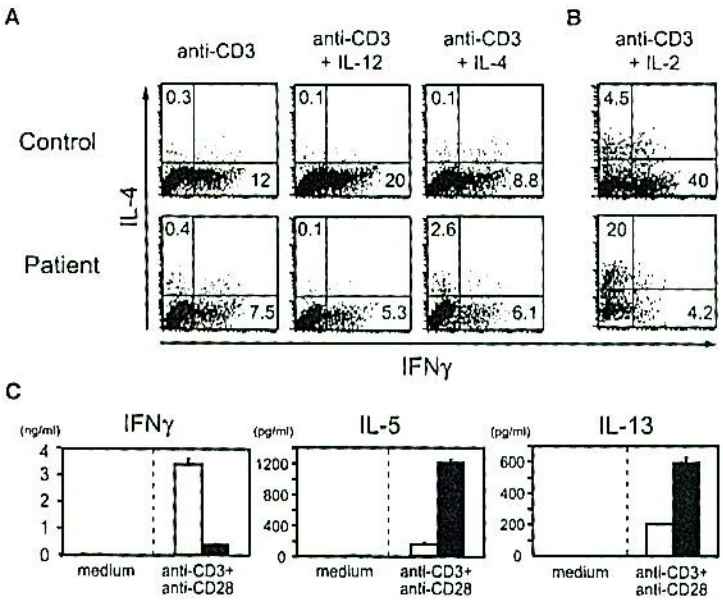


Figure 5. Impaired Th1 and Accelerated Th2 Differentiation in the Patient's T Cells

(A) Intracellular staining with anti-IFNγ 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 IFNγ- 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 IFNγ, 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 IFNα 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 (IFNγ) 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 IFNγ signaling could be explained, at least in part, by the reduced amounts of STAT1 protein or by the observation that IFNγ 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

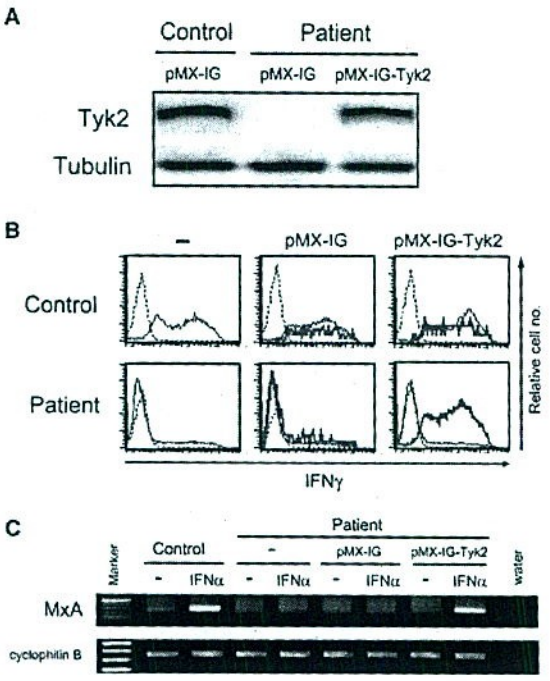


Figure 6. Transduction of the Intact Tyk2 Gene Rescued the Patient's T Cells from Defects in the IL-12 and Type I IFN Signals

(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 IFNγ production in response to IL-12 and IL-18 in the patient's T cells. The production of IFNγ was detected by intracellular staining with an IFNγ 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 IFNα 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 IFNα 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-12Rβ1 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 bacterial 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 pneumonia 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-12Rβ1 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-12Rβ1 deficiency (Altare et al., 1998; de Jong et al., 1998). The immunological work-up performed at this occasion revealed mild eosinophilia (700–800/mm^3) 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-12Rβ1, IL-12Rβ2, IκBα, IL-4, and IFNγ 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, IFNα, IFNβ, IFNγ, 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 μg/ml plate-bound CD3 mAb and 300 U/ml IL-2 in RPMI 1640 (Sigma-Aldrich) containing 10% fetal bovine serum (FBS), 50 μM 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 μg/ml plate-bound CD3 mAb and 300 IU/ml IL-2. Cells (1 × 10^6/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′ → 3′) were as follows: Tyk2 siRNA1, CCCAGAGAUGCAAGCCU 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 IFNα (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′-ACTTTTTCATTTGCTTCCTT-3′; NMI, sense, 5′-TTAAGGAGCATTCGCCAGAT-3′, antisense, 5′-TTCGAGC TCACTTGAAACGA-3′, MxA, sense, 5′-GAGGTGCAGGAGAACAG CTC-3′, antisense 5′-CTCCTCATACTGGCTGCACA-3′; cyclophilin B, sense, 5′-GCCCAAAGTCACCGTCAAGG-3′, antisense, 5′-GGAA GCGCTCACCGTAGATG-3′; IRF1, sense, 5′-GCTGGGACATCAACA AGGAT-3′, antisense, 5′-GTGGAAGCATCCGGTACACT-3′; RING4, sense, 5′-AGGGCTGGCTGGCTGCTTTGA-3′, antisense, 5′-ACGTG GCCCATGGTGTTGTTAT-3′; and SOCS3, sense, 5′-TTCAGCTCCA AGAGCGAGTA-3′, antisense, 5′-CGGAGTAGATGTAATATGGCTC-3′. Sequencing was performed with an 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 μg/ml), IL-12 (10 ng/ml), IL-18 (50 ng/ml), IFNα (50 ng/ml), IFNβ (50 ng/ml), IL-6 (10 ng/ml), or IL-10 (100 ng/ml). The concentration of IFNγ in the culture supernatants was determined by ELISA (BD-PharMingen) according to the manufacturer's instructions, after the cells (1 × 10^6/ml) were cultured in 96-well plates at 37°C for 24 hr with or without stimulation. The concentration of TNFα in the culture supernatants was determined by ELISA (BD-PharMingen), after the cells (1 × 10^5/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 μg/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 IFNγ, 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 μM 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 IFNα, or 50 ng/ml IFNβ. 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 μg/ml aprotinin, 100 μg/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 IFNα or IFNβ 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/.

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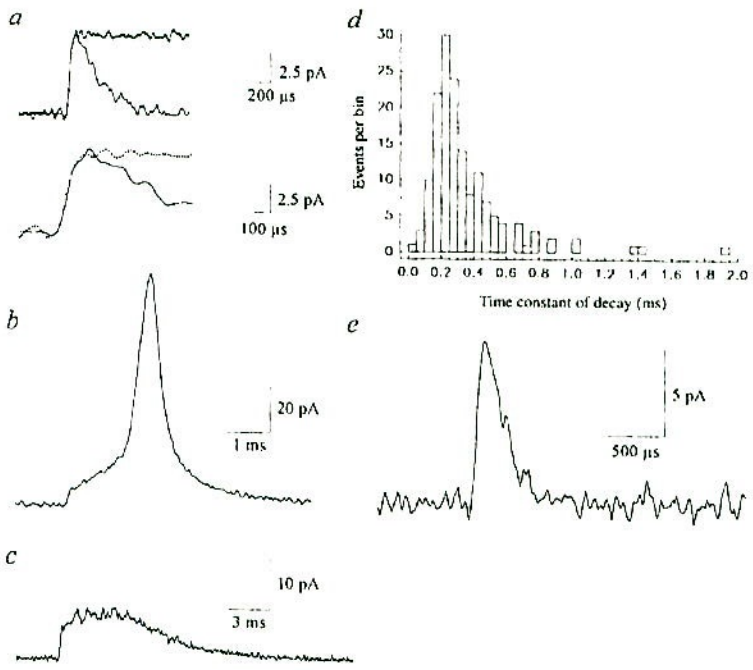


FIG. 3 Kinetic characteristics of transmitter discharge from SSV and LDCV. *a*, Initiation of transmitter discharge from SSV. Ten individual small events were averaged after alignment with respect to the midpoint of their rising phase. The same procedure was followed to obtain the instrument's step response, 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 µs. 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 µs, maximum digitizing rate). *b*, 'Foot' signals often precede the main phase of transmitter discharge from LDCV. The foot had a duration of 540 ± 40 µs and a maximal amplitude of 10 ± 1 pA. The integral of the foot signal reflects on average 4 ± 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 µs (median, 60 µs; 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 granules[12]), 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 µs, the mean was 360 ± 30 µs (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 µs.

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 membrane[18]. □

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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 heterogenous 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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(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 γᶜ-chain-containing cytokine receptors[4–9]. Therefore Jak-3 or other STAT proteins with which it interacts[10,11] are candidate genes for autosomal recessive T⁻ B⁺ SCID[7]. 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 immunoglobulines, 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).

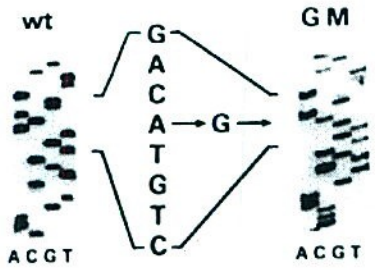


FIG. 1 Tyr100→Cys mutation in the Jak-3 gene of patient G.M. with autosomal SCID. The sequence of the region around nucleotide 394 of the Jak-3 sequence (Accession Number U09607), demonstrating an A→G transition (arrow) at this nucleotide, is shown.
METHODS. RNA was extracted from an EBV-transformed lymphoblastoid cell line obtained from peripheral blood cells of patient GM, according to standard techniques[18]. RT–PCR was performed with primer pair 96F/600R (96F:ATGGCACCTCCAAGTGAAG; 600R:CCAACACGGCCAGGCTGAGA). 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 previously[19].

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.

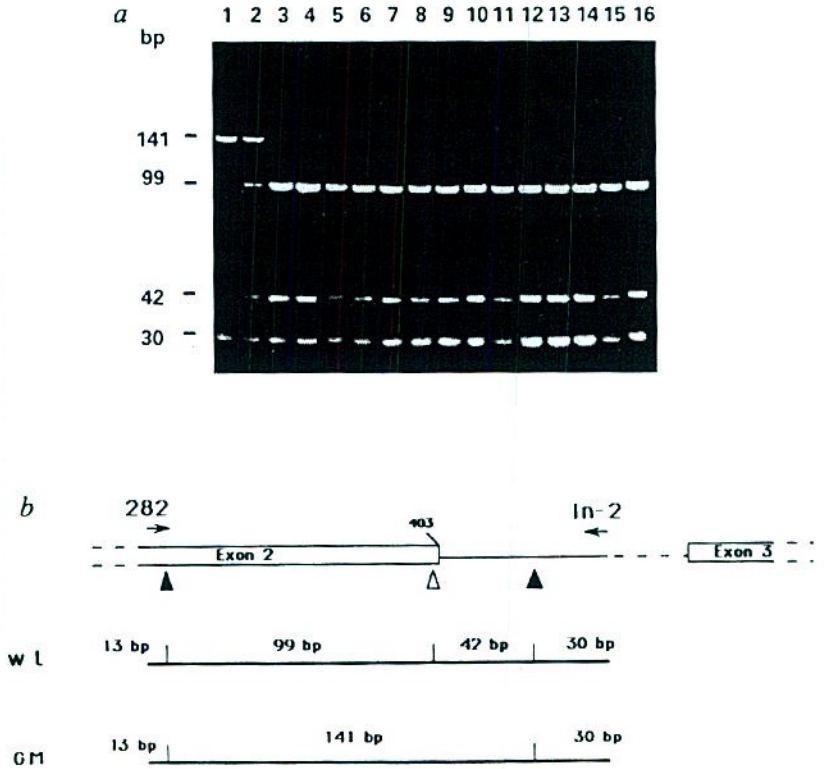


FIG. 2 Restriction analysis of the genomic region around the mutation of patient G.M. *a*, Electrophoresis of PCR products obtained by DNA amplification with primers 282F and In-2. Lane 1, patient G.M.; lane 2, mother of patient G.M.; lanes 3–16, normal controls. *b*, Schematic representation of the *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:ATCCTGCCTGTGTACACT; 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.

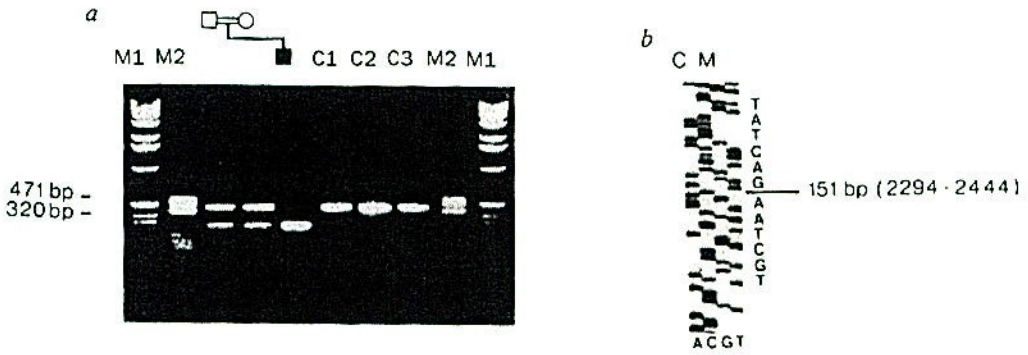


FIG. 3 Deletion of the region between nucleotides 2,294 and 2,444 in patient C.M. *a*, Electrophoresis of RT–PCR products obtained by mRNA amplification with primers 2130F and 2600R. M1, 1-kb marker; M2, marker V (Boehringer); lane 1 (white square), father of patient C.M.; lane 2 (circle), mother of 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: GCTGCCAA-AGTTGCCCTTGC). Conditions for the RT–PCR were as described for Fig. 1, except that the annealing step was at 60 °C.

*Rsa*I 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. 2*a*, *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. 3*a*, 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. 3*b*). 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 antibody[3] 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-

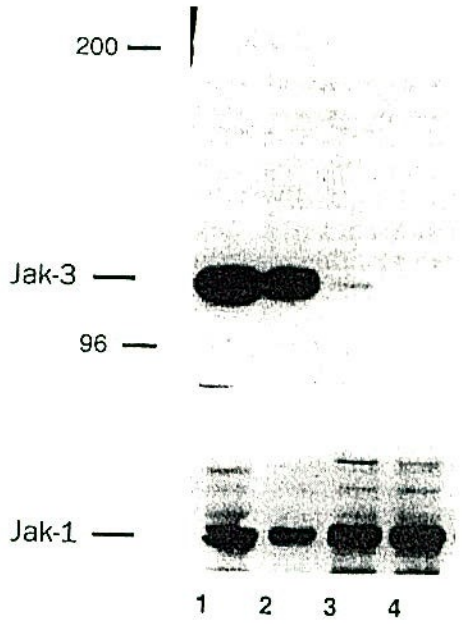


FIG. 4 Markedly reduced levels of Jak-3 protein in EBV-transformed B cells from patients with Jak-3 mutations. Lysates from the NK3.3 cell line (lane 1), EBV-transformed B cells from a normal individual (lane 2), and EBV-transformed B cells from patient G.M. (Tyr100→Cys mutation, lane 3) and patient 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 previously[5]. 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) antibody[3] 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).

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 pathway 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 SCID[14]. Further, carrier females of X-linked SCID exhibit a non-random pattern of X-chromosome inactivation in NK cells[15], 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. □

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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 μl 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 ± s.d. of 14 rats) and

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