Immunity, Vol. 13, 561–571, October, 2000, Copyright ©2000 by Cell Press

Tyk2 Plays a Restricted Role in IFNα Signaling, Although It Is Required for IL-12-Mediated T Cell Function

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Summary

Janus kinases (Jaks) play an important role in signal transduction via cytokine receptors. Tyk2 is a Janus kinase, and we developed tyk2-deficient mice to study the requirement for tyk2 in vivo. Tyk2-deficient mice show no overt developmental abnormalities; however, they display a lack of responsiveness to a small amount of IFNα, although a high concentration of IFNα can fully transduce its signal even in the absence of tyk2. Furthermore, IL-12-induced T cell function is defective in these mice. In contrast, these mice respond normally to IL-6 and IL-10, both of which activate tyk2 in vitro. These observations demonstrate that tyk2 plays only a restricted role in mediating IFNα-dependent signaling while being required in mediating IL-12-dependent biological responses.

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Introduction

Many cytokines bind to their specific cell surface receptors and activate members of the Janus family of protein tyrosine kinases (Jaks), which are associated with cytokine receptors (Ihle, 1995). Activated Jaks phosphorylate both cytokine receptors and the Jaks themselves. Through recognition of a phosphorylated receptor complex, signal transducers and activators of transcriptions (Stats), which are latent cytoplasmic transcription factors, are recruited to that complex and are activated by Jaks (Darnell et al, 1994; Ihle, 1996). Subsequently, these active Stats translocate to the nucleus to affect gene expression. This Jak-Stat signal pathway is widely used by members of the cytokine receptor superfamily (Ihle et al., 1995).

The Jaks were first identified as novel protein tyrosine kinases (Firmbach-Kraft et al., 1990; Wilks et al., 1991), and their function was initially discovered by generating cell lines that were resistant to the effects of interferon (IFN) (Velazquez et al., 1992). Isolation of genomic clones, capable of reconstituting the response of one mutant to IFNα, resulted in the demonstration that tyk2 is required for IFNα signaling in fibroblasts. Using additional IFN response mutant complementation classes, it was shown that cellular responses to IFNα require Jak1 and Tyk2, whereas a response to IFNγ requires Jak1 and Jak2 (Muller et al., 1993; Watling et al., 1993).

There are four mammalian Jaks: Jak1, Jak2, Jak3, and tyk2. *Jak1*, *Jak2*, and *tyk2* are ubiquitously expressed, whereas the expression of *Jak3* appears to be restricted to hematopoietic cells (Witthuhn et al., 1994; Ihle, 1995). They are differentially activated in response to various cytokines. The essential role of Jaks in mediating cytokine signaling was recently confirmed by targeted disruption of each of the Jaks. Jak1-deficient mice exhibited perinatal lethality because of defective lymphoid development and defective neural function. Jak1-deficient cells fail to respond to cytokines that utilize class II cytokine receptors, the common γ chain of IL-2 receptor, or gp130 subunit for signaling (Rodig et al., 1998). Jak2-deficient mice show an embryonic lethality due to the absence of definitive erythropoiesis. Jak2-deficient fetal liver myeloid progenitors fail to respond to Epo, Tpo, and IL-3, while Jak2-deficient fibroblasts fail to respond to IFNγ (Neubauer et al., 1998; Parganas et al., 1998). Jak3-deficient mice show a marked reduction in the numbers of functional T and B cells (Nosaka et al., 1995; Park et al., 1995; Thomis et al., 1995).

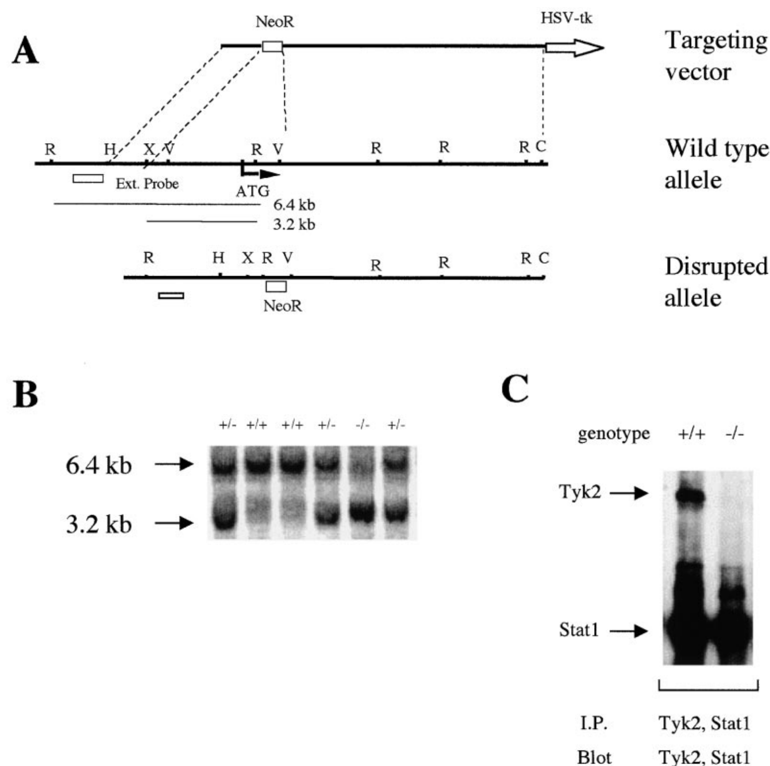

Figure 1. Targeted Disruption of the Murine tyk2 Gene

(A) The genomic locus of the wild-type *tyk2* gene, the targeting vector, and the disrupted *tyk2* gene. The targeting vector was designed to replace part of the first coding exon of the tyk2 protein by the neomycin-resistant cassette (*NeoR*). Locations of probes for hybridization of ES cells are indicated. The locations of restriction sites for EcoRI (R), EcoRV (V), HindIII (H), XbaI (X), and ClaI (C) are indicated.

(B) Southern blotting analysis of EcoRI-digested DNA isolated from the tails of mice derived from the mating of heterozygote mice. The bands at 6.4 and 3.2 kb correspond to the endogenous and mutant *tyk2* genes, respectively.

(C) Spleen cells (1×10^8) of each genotype were lysed and analyzed by immunoprecipitation and Western blotting for tyk2 and Stat1.

In this report, we have focused on the role of tyk2 in cytokine signaling. Tyk2 is a molecule initially identified as essential for mediating IFNα signaling, and it is also activated in response to IL-6 (Stahl et al., 1994), IL-10 (Finbloom and Winestock, 1995), and IL-12 (Bacon et al., 1995). In each of these cases, several Jak kinases including tyk2 are phosphorylated, and additional tyrosine kinases besides Jaks are also activated. This makes it difficult to clarify which of the kinases bears unique or redundant roles in cytokine signaling. To assess the specific and nonredundant role of tyk2, we developed mutant mice that are deficient in tyk2 through gene disruption in embryonic (ES) cells. In contrast to the predictions arising from the analysis of Jak kinase-deficient mutant cell lines, a high amount of IFNα can transduce its own signal and biological response in the absence of tyk2, although tyk2 is essential for an antiviral effect under a low concentration of IFNα. Furthermore, tyk2 was revealed to play an essential role in IL-12-induced T cell functions.

Results

Generation of tyk2-Deficient Mice

To ablate the *tyk2* gene, a targeting construct was designed to replace the first coding exon with a *neo* resistant gene cassette. It contains a 1.0 kb short arm and a 8.4 kb long arm of homologous sequences (Figure 1A). After electroporation and drug selection, five out of 196 ES cell clones were identified by PCR and confirmed by Southern blotting analysis as having undergone successful gene targeting. Chimeric mice were bred to obtain germline transmission, and two of these clones gave rise to mutant strains. Breeding of heterozygote mice resulted in wild-type, heterozygote, and homozygote mice, as identified by Southern blotting analysis (Figure 1B). Homozygote mice, which displayed no obvious abnormalities, were bred at the expected Mendelian frequency. Immunoprecipitation and Western blotting with a tyk2 carboxy-terminal polyclonal antibody showed the presence of 135 kDa tyk2 protein in lysates of spleen cells derived from wild-type mice but not homozygous mice for the disrupted *tyk2* gene, although 91 kDa Stat1 protein was identified in both wild-type and homozygous mice at comparable levels (Figure 1C). No differences were observed with regard to lymphoid, monocyte, or myeloid cell numbers, or the expression of cell surface markers, including B220, IgM, CD3, CD4, CD8, CD14, or Gra-1 (data not shown).

Tyk2 Has Only a Restricted Function and Does Not Play a Major Role in IFNα Signaling

Tyk2 was originally cloned as an essential molecule for IFNα signaling (Velazquez et al., 1992). We therefore first analyzed whether or not tyk2 deficiency affects IFNα signaling. To assess the role of tyk2 in these responses, fibroblasts were derived from wild-type and tyk2-deficient embryos and examined for their ability to respond to IFNα. As illustrated in Figure 2, cells from both wild-type and tyk2-deficient embryos respond to IFNα as assessed by the tyrosine phosphorylation of Jak1, Stat1, and Stat2. In cells from both wild-type and tyk2-deficient mice, Jak2 is not phosphorylated in response to IFNα, whereas it is phosphorylated by the stimulation of IFNγ (Figure 2B). Moreover, IFNα was able to induce *IRF-1* gene expression in tyk2-deficient fibroblasts at nearly equal levels compared to wild-type cells, as illustrated in Figure 2E. We next examined the level of expression of cell surface IFNα receptors. Functional IFNα receptors are composed of two chains, IFNAR1 and IFNAR2.

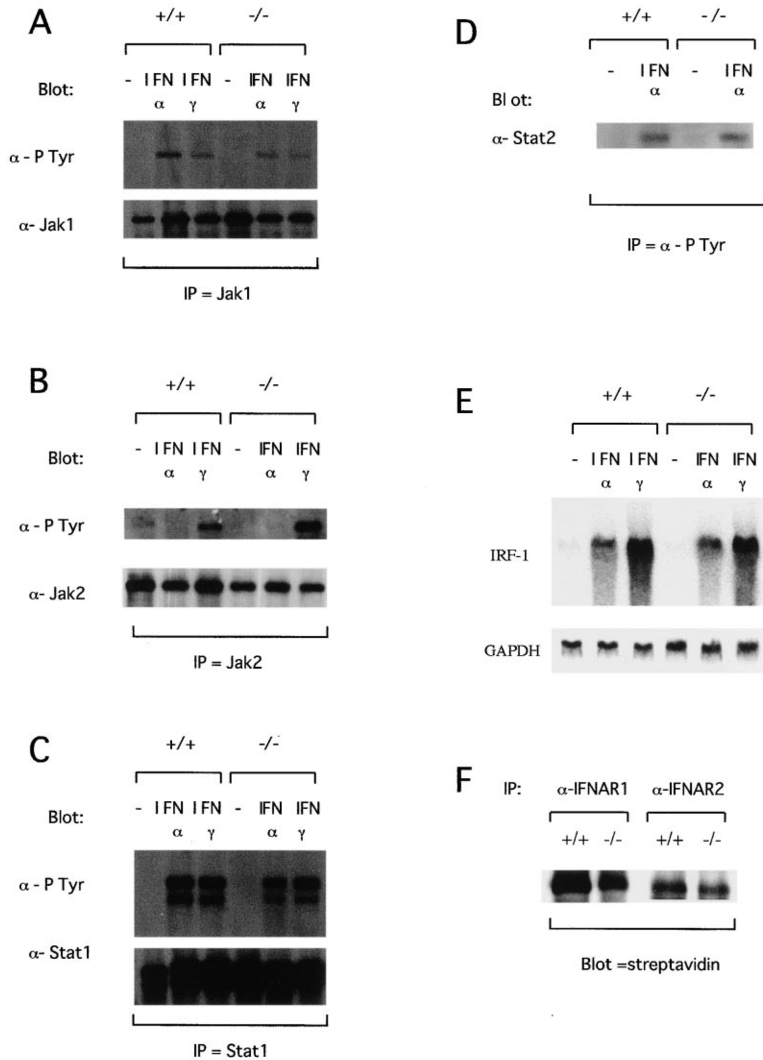

Figure 2. Fibroblasts from tyk2-Deficient Embryos Respond to IFNα

Embryonic fibroblasts (EF cells) were derived from either wild-type embryos (+/+) or from tyk2-deficient embryos (−/−), as described in Experimental Procedures.

(A) To examine the response to IFNα or IFNγ, EF cells were either not stimulated (-) or were stimulated with IFNα or IFNγ. Following 15 min of stimulation, the cells were harvested to analyze the induction of tyrosine phosphorylation of Jak1 by immunoprecipitation with anti-Jak1 polyclonal antibody and Western blotting with a monoclonal antibody against phosphotryrosine (top) or Jak1 polyclonal antibody (bottom).

(B) The phosphorylation of Jak2 in response to IFNα or IFNγ was examined.

(C) The phosphorylation of Stat1 was examined and comparably analyzed with anti-Stat1 polyclonal antibody.

(D) To examine the phosphorylation of Stat2 in response to IFNα, stimulated or not stimulated cells were lysed. Immunoprecipitation was carried out with anti-phosphotyrosine mAb, and Western blotting was performed with anti-Stat2 polyclonal antibody.

(E) To assess further the ability to respond to IFNα, the induction of *IRF-1* was examined by Northern blotting 1 hr following the stimulation of EF cells.

(F) To examine the IFNα receptors, cell surface molecules were biotinylated. Cells were harvested to analyze the expression level of IFNα receptors by immunoprecipitation with anti-IFNAR1 or IFNAR2 antibodies and Western blotting with HRP-conjugated biotin (top) or IFNα receptor antibodies (bottom).

Cell surface proteins were biotinylated using sulfo-NHS-biotin. The cells were harvested to analyze the level of expression of IFNα receptors by immunoprecipitation with anti-IFNAR1 or IFNAR2 antibodies and Western blotting with HRP-conjugated streptoavidin. As illustrated in Figure 2F, the level of expression of IFNα receptors (IFNAR1 and IFNAR2) on cells from tyk2-deficient mice were almost equal to those on cells from wild-type mice.

IFNα has some biological functions on cells, such as the induction of the expression of MHC class I antigen and of the resistance to viral infections. We monitored the ability of IFNα to enhance MHC class I expression on spleen cells. In contrast to the complete loss of this function reported in Jak1- or Stat1-deficient mice (Meraz et al., 1996; Rodig et al., 1998), tyk2-deficient splenocytes exposed to a high concentration of IFNα expressed enhanced levels of MHC class I, comparable to the level of wild-type spleen cells (Figure 3A). When spleen cells were treated with a low concentration of IFNα, such as 10 U/ml, those derived from wild-type mice showed an enhancement of MHC class I expression, which was slightly less than that seen when cells were treated with 1000 U/ml of IFNα, although spleen cells from tyk2-deficient mice were unable to respond to IFNα to induce MHC class I expression under such a low concentration of IFNα. As an additional assay for tyk2 function in IFNα signaling, we assessed the ability of IFNα to protect against the cytopathic effects of vesicular stomatitis virus (VSV). EF cells were treated with various concentrations of IFNα for 48 hr, removed from IFNα, and then infected with sufficient VSV to produce a 100% cytopathic effect within 24 hr in untreated cells. EF cells from both wild-type and tyk2-deficient mice responded to IFNγ in a dose-dependent manner and resisted the cytopathic effects of VSV (Figure 3B, b). High concentrations of IFNα (more than 100 U/ml) can protect both wild-type and tyk2-deficient EF cells. Similar to the result of the induction of MHC class I expression, a low concentration of IFNα such as 1–10 U/ml had no antiviral effect on EF cells from tyk2-deficient mice, although a small amount of IFNα is enough to protect wild-type EF cells from VSV infection (Figure 3B, a).

The Absence of tyk2 Does Not Affect IL-6 or IL-10 Signaling

In addition to IFNα, tyk2 activation is observed in response to IL-6 and IL-10.

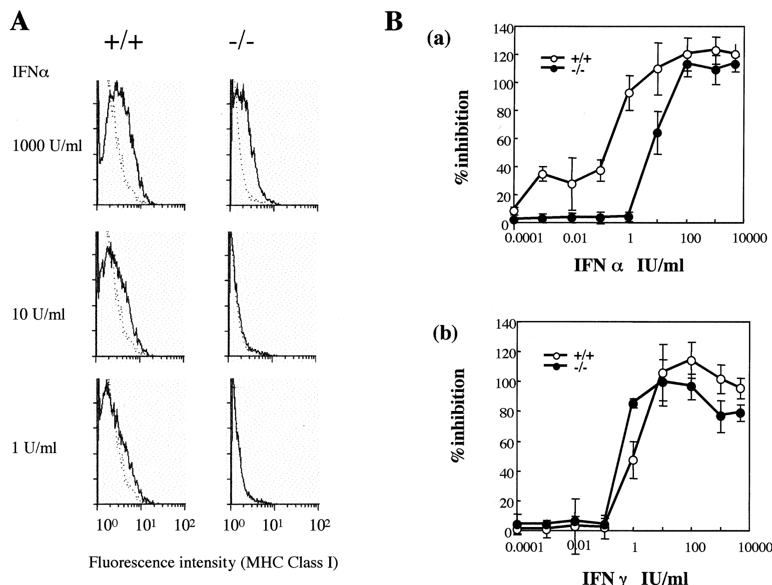

Figure 3. Biological Response to IFNα

(A) MHC class I induction in spleen cells. Spleen cells from wild-type or tyk2-deficient mice were stimulated with PBS (dot line) or the indicated concentration of IFNα (solid line) for 72 hr and analyzed for the expression of MHC class I by flow cytometry.

(B) Antiviral response of fibroblasts. Primary EF cells from wild-type or tyk2-deficient mice were stimulated with IFNα (a) or IFNγ (b) and cultured with vesicular stomatitis virus. After 48 hr, surviving cells were quantified by vital dye staining.

IL-6 has been shown to cause an activation of Jak1, Jak2, and tyk2 (Stahl et al., 1994). Previous studies with mutant cell lines have suggested that the presence of Jak1 is critical for IL-6 signaling (Guschin et al., 1995). Moreover, from an analysis of Jak1- and Jak2-deficient mice, Jak1 but not Jak2 was demonstrated to play a critical role in IL-6 signaling (Parganas et al., 1998; Rodig et al., 1998). Consistent with these observations, EF cells from both wild-type and tyk2-deficient mice respond to IL-6 as assessed by tyrosine phosphorylation of Stat1 and Stat3 (Figure 4A, top). Furthermore, thymocytes from tyk2-deficient mice proliferate in the stimulation with IL-6 and PHA at levels comparable to wild-type mice (Figure 4B, left), indicating that tyk2 is either not required or else is redundant in IL-6 signaling.

IL-10 affects both monocytes and T cells by the activation of Jak1 and tyk2. It has been shown that monocytes from Jak1-deficient embryos did not respond to IL-10 by inhibiting LPS-induced TNFα production, thus suggesting that Jak1 plays an obligatory role in mediating IL-10 signaling (Rodig et al., 1998). We assessed whether tyk2 is also essential for the response to IL-10. IL-10 stimulates tyrosine phosphorylation of Stat3 in $CD3^-$ spleen cells derived from wild-type mice, and the phosphorylation of Stat3 by IL-10 is also observed in $CD3^-$ spleen cells from tyk2-deficient mice (Figure 4A, bottom). As shown in Figure 4B (right), there is no difference in proliferation between T cells from wild-type mice and T cells from tyk2-deficient mice induced by a combination of IL-2 and IL-10. In addition, the upregulation of MHC class II antigens induced by IL-10 is also observed in cells from tyk2-deficient mice (Figure 4C). Furthermore, macrophages derived from tyk2-deficient mice, as well as cells from wild-type mice, respond to IL-10 in a dose-dependent manner by inhibiting LPS and IFNγ-induced TNFα production (Figure 4D). In contrast to the absence of a low dose-IFNα-inducible biological response in tyk2-deficient cells, a low dose of IL-10 has an inhibitory effect on tyk2-deficient macrophages, as well as on macrophages from wild-type mice. These observations indicate that tyk2 has no obligatory role to play in IL-10 signaling.

Tyk2 Is Essential for IL-12-Mediated T Cell Response

IL-12 is important in the control of cell-mediated immunity and induces tyrosine phosphorylation of Jak2 and tyk2 (Bacon et al., 1995). Since Stat4 is only tyrosine phosphorylated following the stimulation of T cells with IL-12 (Jacobson et al., 1995), we assessed the ability of tyrosine phosphorylation of Stat4 in tyk2-deficient T cells. IL-12 activates Stat4 in spleen cells from wild-type mice. In contrast, Stat4 activation was decreased but still detectable in tyk2-deficient cells stimulated with IL-12 (Figure 5A). To determine whether this decrease of Stat4 activation by IL-12 in tyk2-deficient mice is or is not due to the alteration of IL-12 receptor expression, we examined the level of expression of IL-12 receptor β1 chain on cells from tyk2-deficient mice. As shown in Figure 5B, the levels of expression of IL-12 receptor β1 chain on spleen cells are almost equal between wild-type and tyk2-deficient mice. Since tyk2-deficient cells displayed only a limited capacity to activate Stat4 through IL-12 stimulation, we examined whether partial signaling was of sufficient magnitude to induce biological responses in the treated cells. Stat4-deficient mice showed that Stat4 bears all IL12 functions, including mitogenesis, induction of IFNγ from T cells, enhancement of natural killer cytolytic function, and Th1 differentiation (Kaplan et al., 1996; Thierfelder et al., 1996).

To examine whether tyk2 is required for IL-12-induced proliferation, T lymphocytes were activated with anti-CD3 for 4 days, washed, and then stimulated with IL-12 for an additional 48 hr. IL-12 stimulation of activated lymphocytes from both wild-type and tyk2-deficient mice leads to an equal level of increase in proliferation (Figure 5C).

One major activity of IL-12, in synergy with CD3 or IL-2, is the induction of IFNγ by resting and activated NK and T cells. We therefore examined the ability of splenocytes from wild-type and tyk2-deficient mice to

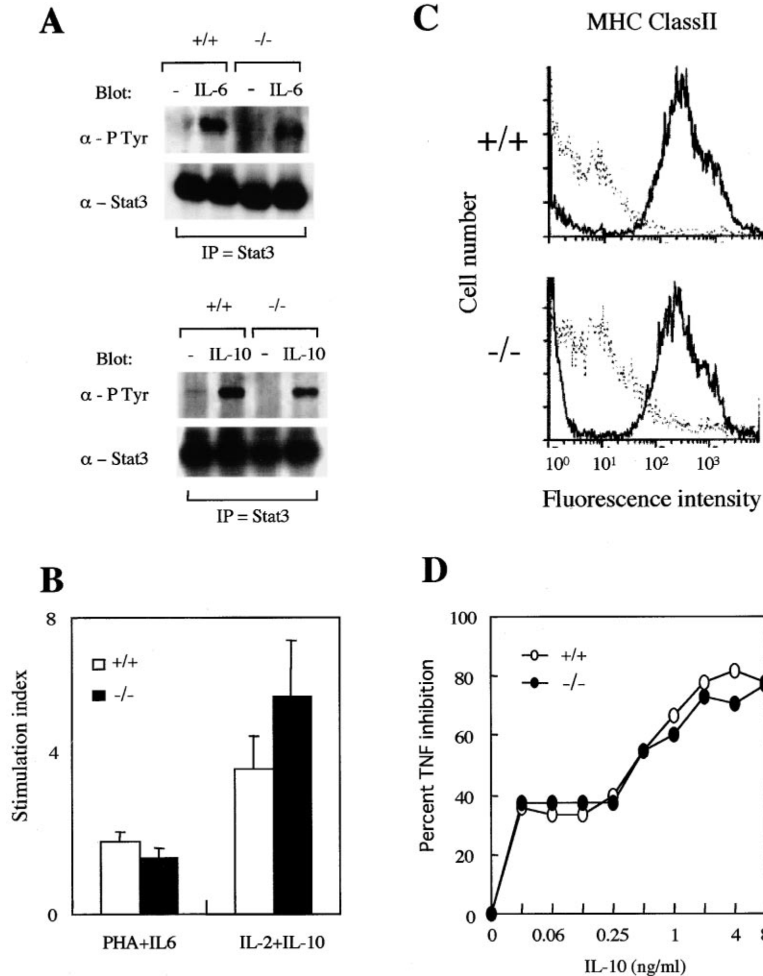

Figure 4. The Absence of tyk2 Does Not Affect IL-6 or IL-10 Signaling

(A) Stat3 phosphorylation in tyk2-deficient EF cells by IL-6 (top) or IL-10 (bottom). To examine the response to IL-6, EF cells were either not stimulated (−) or else were stimulated with a mixture of IL-6 and soluble IL-6 receptor for 15 min. To examine the response to IL-10, $CD3^-$ spleen cells derived from wild-type or tyk2-deficient mice were cultured with PHA (2 μg/ml) for 72 hr, starved for 12 hr, and stimulated with or without IL-10 (50 ng/ml) for 15 min. The cells were harvested to analyze the induction of tyrosine phosphorylation of Stat by immunoprecipitation with anti-Stat3 polyclonal antibody and Western blotting with a monoclonal antibody against phosphotyrosine or Stat3 polyclonal antibody.

(B) T cell proliferation by IL-6 or IL-10. Thymocytes from wild-type and tyk2-deficient mice were cultured for 96 hr with PHA (10 μg/ml) and IL-6 (10 ng/ml) (left) or with IL-10 (10 ng/ml) and 1000 U/ml of IL-2 (right), and the cells were labeled with tritiated thymidine. In each case, the extent of incorporation relative to only PHA stimulation control (for IL-6-induced proliferation) or IL-2 stimulation control (for IL-10-induced proliferation) is plotted as the stimulation index.

(C) MHC class II antigen expression on $CD3^-$ spleen cells. $CD3^-$ spleen cells were obtained by negative selection using magnetic beads from wild-type or tyk2-deficient mice. Cells were stimulated with PBS or IL-10 (10 ng/ml) for 24 hr and analyzed for the expression of MHC class II by flow cytometry.

(D) Inhibition of LPS-induced TNF production by IL-10. Macrophages (1×10^5) from wild-type and tyk2-deficient mice were incubated with IL-10 for 12 hr followed by treatment with LPS (10 ng/ml) and IFNγ (100 U/ml). After 48 hr, TNF in the culture supernatants was quantified by ELISA.

produce IFNγ. Strikingly, the stimulation of spleen cells from tyk2-deficient mice with IL-12 resulted in no detectable IFNγ production, whereas IFNγ was readily detected in the supernatants of IL-12-stimulated splenocytes from wild-type mice (Figure 6A). Spleen cells contain T cells and NK cells, both of which can produce IFNγ by IL-12 stimulation. We then examined the numbers of T cells and NK cells in the spleen. The population of CD3-positive T cells is 42% in wild-type mice and 44.5% in tyk2-deficient mice, while the population of NK cells is 3.1% in wild-type mice and 3.0% in tyk2-deficient mice. Since there is no difference in T and NK cell numbers of spleen cells from wild-type and tyk2-deficient mice, we isolated T cells to examine the ability of producing IFNγ in response to IL-12.

As shown in Figure 6B, small amounts of IFNγ were produced by CD3-negative cells (those that contain NK cells and B cells) from wild-type mice, while CD3-negative cells from tyk2-deficient mice did not produce IFNγ by the stimulation of IL-2 plus IL-12. On the other hand, large amounts of IFNγ were produced from CD3-positive T cells from wild-type mice, whereas T cells from tyk2-deficient mice with IL-2 and IL-12 resulted in no detectable IFNγ production. To determine whether or not the reduction in IFNγ production from tyk2-deficient T cells by IL-12 occurs at the transcriptional level, we analyzed the expression of IFNγ mRNA by the stimulation of IL-2 and IL-12 in spleen cells from wild-type and tyk2-deficient mice (Figure 6C). IFNγ mRNA was only detected in spleen cells from wild-type mice by the stimulation of IL-2 plus IL-12 and not detected in those from tyk2-deficient mice. Then, the reduction in IFNγ production from tyk2-deficient T cells by IL-12 is thought to occur at the transcriptional level as a consequence of the reduction in Stat4 activation by IL-12.

The defect in IFNγ production by IL-12 stimulation was observed under the costimulation with anti-CD3 or IL-2. Anti-CD3 stimulation of T cells produces IL-2. Then, we examined whether the activation of Stat5 by IL-2 stimulation was affected on spleen cells from tyk2-deficient mice. As illustrated in Figure 6D, IL-2 stimulated tyrosine phosphorylation of Stat5 in spleen cells from both wild-type and tyk2-deficient mice. Then, the defect in IFNγ production under the stimulation of anti-CD3 plus IL-12 or IL-2 plus IL-12 is thought to be due to the abolishment of IL-12 signaling.

IL-12 facilitates the differentiation of naive $CD4^+$ cells into Th1 cells that secrete IFNγ after antigen stimulation.

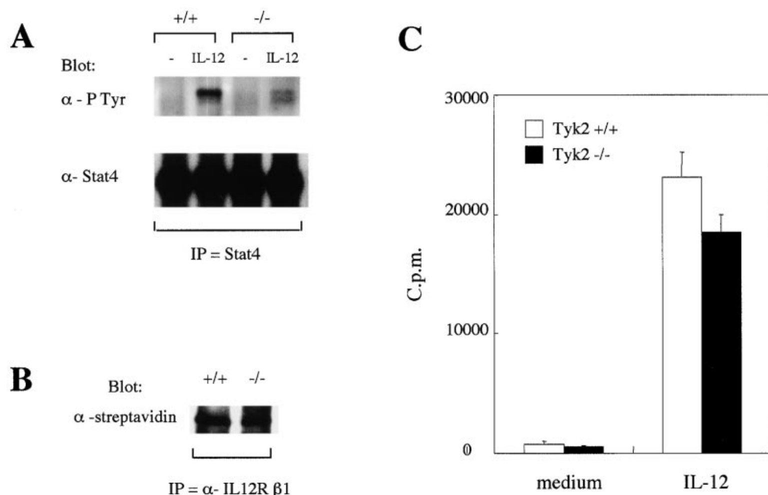

Figure 5. IL-12 Signaling in tyk2-Deficient Mice

(A) IL-12-induced Stat4 phosphorylation in spleen cells from tyk2-deficient mice was almost completely abolished, although it remained detectable. Spleen cells from wild-type and tyk2-deficient mice were cultured with 500 U/ml of IL-2 for 72 hr, starved for 12 hr, and stimulated with or without IL-12 (20 ng/ml) for 15 min and lysed. Stat4 was immunoprecipitated and analyzed by Western blotting with a monoclonal antibody against phosphotyrosine.

(B) Equal level of expression of IL-12 receptor on spleen cells from tyk2-deficient mice. To examine the level of expression of cell surface IL-12 receptor, biotinylated spleen cells from wild-type or tyk2-deficient mice were lysed, and immunoprecipitation with IL-12 receptor β1 chain antibody was carried out. Cell surface IL-12 receptor β1 chain was analyzed by Western blotting with HRP-conjugated streptavidin.

(C) Normal mitogenetic response of activated lymphocytes from tyk2-deficient mice. $CD3^+$ splenic lymphocytes were obtained by the immunomagnetic cell isolation method. Cells were cultured for 72 hr in the presence of anti-CD3. Cells were washed and cultured with or without IL-12 (7 ng/ml) for 24 hr, and the cells were labeled with tritiated thymidine for a further 24 hr.

This activity requires Stat4 because T cells from Stat4-deficient mice fail to differentiate into Th1 cells (Kaplan et al., 1996; Thierfelder et al., 1996). We therefore used an in vitro assay to assess the consequence of tyk2 deletion on helper T cell differentiation. Enriched splenic naive $CD4^+$ cells were cultured for 2 days with immobilized anti-TCR in the presence of IL-12 and anti-IL4 to promote Th1 differentiation or in the presence of IL-4 to promote Th2 differentiation. The cells were then cultured with only cytokines for another 3 days. As illustrated in Figure 7A, IFNγ-producing Th1 cells were generated from naive $CD4^+$ cells from wild-type mice in the conditions that promote Th1 differentiation. In contrast, the generation of Th1 cells in the Th1-skewed conditions was not dramatically enhanced from naive $CD4^+$ cells from tyk2-deficient mice. In cultures supporting Th2 differentiation, restimulation resulted in the generation of IL-4-producing Th2 cells, and there was no difference between wild-type and tyk2-deficient mice.

Our results demonstrate that tyk2 is critical for mediating IL-12-induced IFNγ production from T cells and the differentiation into Th1 cells from naive helper T cells, although it does play a redundant role in T cell proliferation by IL-12.

Discussion

Tyk2 was originally cloned as an essential molecule for the transduction of IFNα signaling (Velazquez et al., 1992). When cells are treated with IFNα, Jak1 and tyk2 are initially phosphorylated, followed by Stat1 and Stat2 activation. The activated Stat1 and Stat2 form ISGF-3 (IFN-stimulated gene factor 3) complexes and bind to the ISRE (IFN stimulated response element) of the IFNα inducible gene. On the other hand, Jak1 and Jak2 are phosphorylated in response to IFNγ, followed by the activation of Stat1, which binds to the GAS sites (gamma activated sites) of the IFNγ inducible gene by making Stat1 homodimerizations (Darnell et al., 1994). According to an analysis of Stat1-deficient mice, it has been shown that Stat1 plays an essential role in mediating IFN-dependent biological responses (Durbin et al., 1996; Meraz et al., 1996).

Previous studies using mutant human fibroblast cell lines that are deficient in Jak1, Jak2, or tyk2 have shed light on IFN signaling (Muller et al., 1993, Watling et al., 1993). Cell lines deficient in Jak1 are incapable of mediating IFNα and IFNγ, while those deficient in tyk2 (U1D cells) are incapable of mediating IFNα, and those deficient in Jak2 are incapable of responding to IFNγ. These observations demonstrate the reciprocal interdependence between Jak1 and tyk2 activities in the IFNα pathway and between Jak1 and Jak2 activities in the IFNγ pathway. They suggest that Jak1 and tyk2 or Jak1 and Jak2 would make a correct assembly of IFN receptor complexes and may be juxtaposed on receptor dimerization and cross-phosphorylate in response to IFN treatment.

Consistent with this hypothesis, cells from Jak1-deficient mice failed to respond to IFNα or IFNγ and lost all IFN-inducible biological activities (Rodig et al., 1998). Fibroblasts from Jak2-deficient embryos failed to respond to IFNγ for the phosphorylation of Jak1 or Stat1 or the induction of *IRF-1* (Parganas et al., 1998). Furthermore, cells from Jak2-deficient embryos were not protected against EMCV infection under a high concentration of IFNγ. Therefore, it was clearly demonstrated that Jak1 and Jak2 are both independently required for the biological responses mediated by IFNγ.

For IFNα signaling, the story is a little bit different. As shown in Figure 2A, Jak1 is phosphorylated by IFNα in EF cells from tyk2-deficient embryos. In addition, the tyrosine phosphorylation of Stat1 and Stat2 and the induction of the *IRF-1* gene are also observed in EF cells from tyk2-deficient mice in response to IFNα (Figures 2C–2E). Regarding the biological responses induced by IFNα, a high concentration of IFNα showed antiviral effects and the induction of MHC class I antigen on fibro-

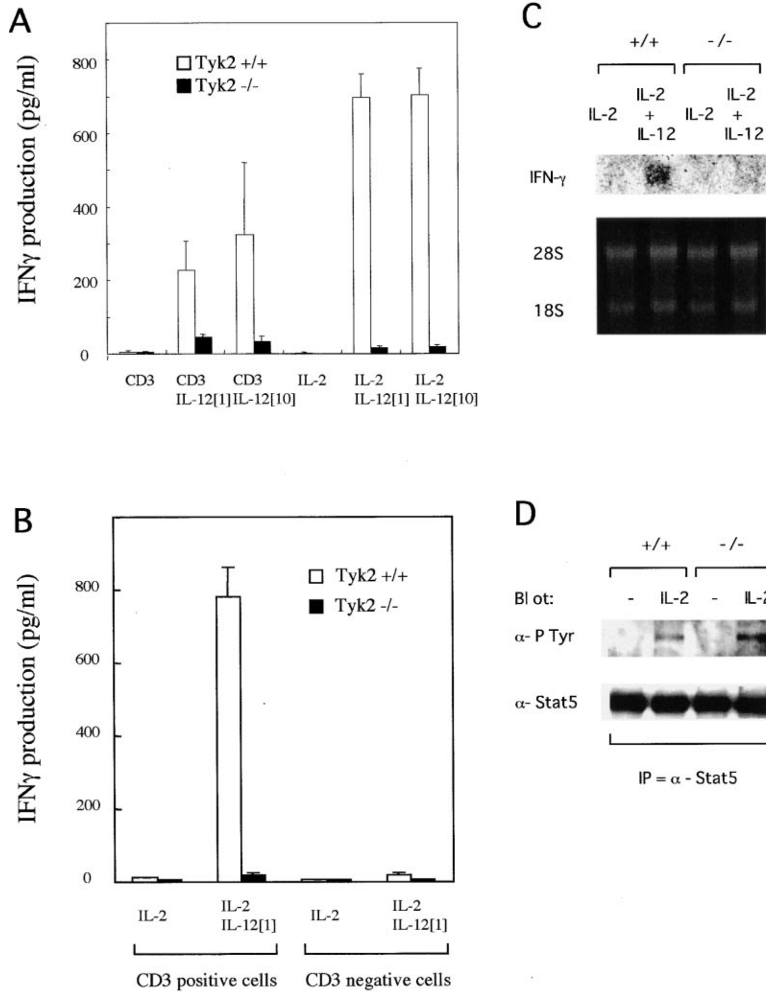

Figure 6. Lack of IL-12-Induced T Cell Functions in tyk2-Deficient Mice

(A) Lack of IFNγ production following IL-12 stimulation of splenocytes from tyk2-deficient mice. Splenocytes were obtained from either wild-type or tyk2-deficient mice and plated in wells containing anti-CD-3 (10 μg/ml) or IL-2 (50 U/ml), either alone or with the addition of the indicated amount of IL-12. Supernatants were collected after 48 hr, and the amount of IFNγ was quantified by ELISA.

(B) Lack of IFNγ production from T cells in response to IL-12 from tyk2-deficient mice. $CD3^+$ cells and $CD3^-$ cells from nonadherent splenocytes were stimulated with IL-2 only or with IL-2 plus IL-12. Supernatants were collected after 48 hr, and the amount of IFNγ was quantified by ELISA.

(C) Decreased levels of expression of *IFNγ* mRNA in splenocytes by IL-12 stimulation from tyk2-deficient mice. Splenocytes from wild-type or tyk2-deficient mice were stimulated with IL-2 only or IL-2 plus IL-12 for 20 hr. The induction of *IFNγ* mRNA was examined by Northern blotting.

(D) IL-2-induced Stat5 phosphorylation in spleen cells from tyk2-deficient mice was not affected. Spleen cells from wild-type and tyk2-deficient mice were stimulated with or without IL-2 (1000 U/ml) for 15 min and lysed. Stat5 was immunoprecipitated and analyzed by Western blotting with a monoclonal antibody against phosphotyrosine.

blasts and spleen cells from tyk2-deficient cells, as well as cells from wild-type mice. This means that tyk2 is not essential for mediating IFNα signaling, and the biological functions induced by IFNα are transduced by means of Jak1 only.

Our finding is in contrast to the previous conception that Jak1 and tyk2 are reciprocally independent in the IFNα pathway (Muller et al., 1993) and suggests the importance of Jak1 in IFNα signaling. Since it is reported that tyk2 is required for the correct formation of the IFNα receptor and that the number of high-affinity IFNα binding sites on U1D cells (tyk2-deficient human fibroblasts) is markedly decreased (Pellegrini et al., 1989), this may be the reason why U1D cells could not respond to IFNα. In contrast to U1D cells, the expression levels of IFNα receptors (IFNAR1 and IFNAR2) on cells from tyk2-deficient mice were comparable to those on cells from wild-type mice (Figure 2F).

Under stimulation by a low concentration of IFNα, such as 1–10 U/ml, tyk2-deficient EF cells or splenocytes could not respond to IFNα to show any antiviral effect or the induction of HLA class I antigen, respectively. However, such a low concentration of IFNα is enough for the cells from wild-type mice to mediate the above IFNα-inducible biological activities. Since the expression levels of IFNα receptors are not affected on cells from tyk2-deficient mice, the reduction in IFNα signaling at low concentrations may be due to events that occurred after IFNα binds to its receptors. Takaoka recently reported the presence of cross-talk between IFNγ and IFNα signaling components (Takaoka et al., 2000). This cross-talk is unidirectional in that IFNγ signaling was dependent on IFNα signaling, while IFNα signaling was not affected by IFNγ signaling. Consistent with this, Jak2, which is an essential molecule for the IFNγ signaling, is not involved in the stimulation of IFNα in cells that are deficient in tyk2, and so IFNα signaling is transduced by means of Jak1 only (Figure 2B). Since cells from Jak1-deficient mice could not respond to IFNα completely under high concentrations (Rodig et al., 1998), our findings suggest two possibilities. One is that only Jak1 phosphorylates Stat1, while tyk2 is unable to directly activate Stat1, although the binding of IFNα to its cell surface receptor activates both Jak1 and tyk2. In this situation, the role of tyk2 is probably to activate Jak1 for the augmentation of Jak1 function in IFNα signaling. The other is that when IFNα binds to its receptor, Jak1 is phosphorylated. After this, the phosphorylated Jak1 activates tyk2. Finally, both activated Jak kinases phosphorylate Stat1. The latter could explain our observation that there is a difference between wild-type and tyk2-deficient cells regarding the antiviral effect and the

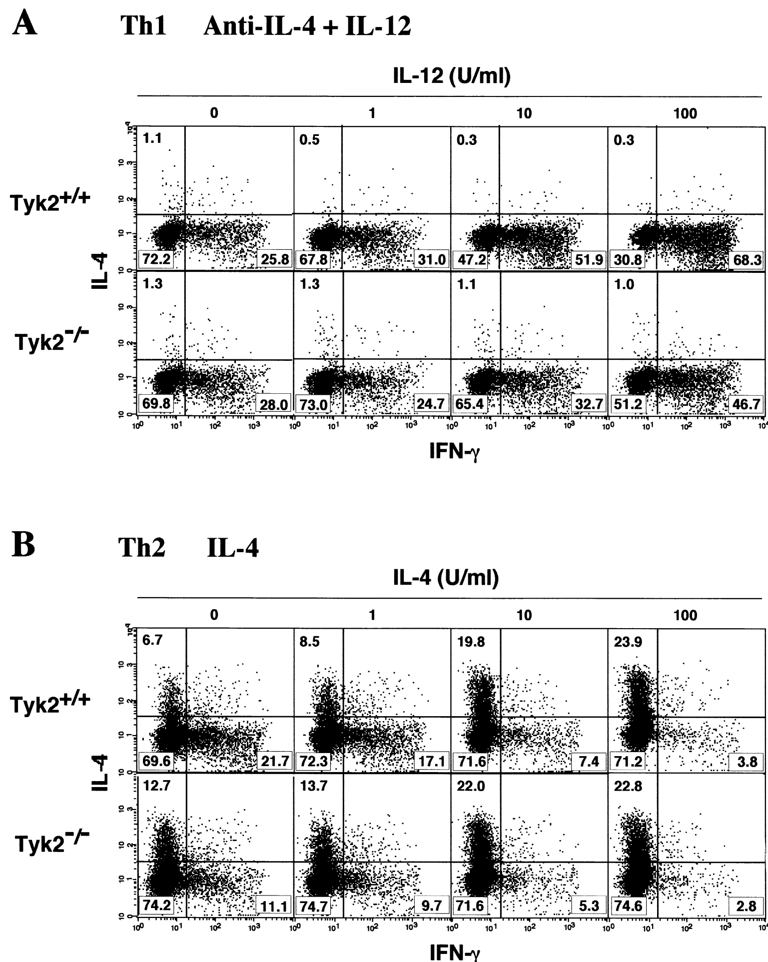

Figure 7. In Vitro T Helper Cell Differentiation

(A) Naive CD4 T cells from wild-type and tyk2-deficient mice were stimulated with immobilized anti-TCR mAb plus IL-12 and anti-IL-4 (Th1-skewed). The cultured cells were subjected to intracellular staining with anti-IFNγ and anti-IL-4. The numbers of the cells harvested (yield) are almost same among experiments. The percentage of the cells in each quadrant are shown as boxed numbers.

(B) Naive CD4 T cells from wild-type and tyk2-deficient mice were stimulated with immobilized anti-TCR mAb plus IL-4 (Th2-skewed).

induction of the HLA-class I antigen expression in the presence of only a small amount of IFNα.

In the IL-6- and IL-10-signaling pathway, it has been reported that Jak1 plays an essential role in promoting biological responses (Rodig et al., 1998). The IL-6 family of cytokines utilizes a common signaling chain, gp130, that activates several of the Jak family members, including Jak1, Jak2, and tyk2 (Stahl et al., 1994). Previous studies using mutant cell lines that are deficient in Jak1, Jak2, or tyk2 suggested that the absence of Jak2 or tyk2 did not affect gp130 function, except in the absence of Jak1 (Guschin et al., 1995). An analysis of Jak1-deficient and Jak2-deficient mice revealed an essential role of Jak1, but not of Jak2, in IL-6-promoting biological responses (Parganas et al., 1998; Rodig et al., 1998). The absence of tyk2 does not affect the phosphorylation of Stat3 or the proliferation of thymocytes by IL-6, consistent with the observation in Jak2-deficient mice, indicating the redundant role of tyk2 in IL-6 signaling. IL-10 acts on macrophages to inhibit the production of reactive intermediates and results in the inhibition of cytokine synthesis by activated Th1 T cells and natural killer cells. The stimulation of cells with IL-10 induces the activation of Jak1 and tyk2. Since it has been reported that macrophages derived from Jak1-deficient mice do not respond to IL-10 by inhibiting LPS-induced TNFα production, Jak1 is thought to play an essential role in the IL-10-signaling pathway (Rodig et al., 1998). We assessed whether tyk2 is also essential for the response to IL-10. All examined IL-10 functions, including Stat3 phosphorylation, T cell proliferation, induction of MHC class II antigen, and the inhibition of LPS and IFNγ-induced TNF production, are conserved in cells from tyk2-deficient mice. Cells derived from tyk2-deficient mice do not respond to low-dose IFNα and thus do not show the induction of the MHC class I antigen or an antiviral effect. In contrast to this IFNα-signaling pathway, a low dose of IL-10 has the same effect on macrophages from tyk2-deficient mice, as well as cells from wild-type mice, indicating that tyk2 does not have an obligatory role to play in IL-10 signaling.

IL-12 is a cytokine that controls cell-mediated immunity, including the induction of IFNγ from T and NK cells, the stimulation of T cell proliferation as a costimulant, the enhancement of cytolytic activity of T and NK cells, and the differentiation of Th1 cells from progenitors. IL-12 induces the tyrosine phosphorylation of Jak2 and tyk2, followed by that of Stat4. Since Stat4-deficient mice were reported to be deficient in all the IL-12 biological activities, Stat4 is thought to bear all IL-12 functions. In tyk2-deficient cells (Figure 5A), the phosphorylation of Stat4 by IL-12 was decreased. This was not due to the decrease in IL-12 receptor expression levels, because there is no difference between wild-type and tyk2-defi-

cient mice in the expression levels of IL-12 receptor β1 chain detected by Western blotting (Figure 5B). In addition, a deficiency in tyk2 greatly inhibited the IFNγ production from T cells (Figure 6B). Although the production of IFNγ from T cells induced by IL-12 requires costimulation with anti-CD3 or IL-2, the phosphorylation of Stat5, which is a major stat protein activated by IL-2, in response to IL-2 is not affected in the absence of tyk2 (Figure 6D). Then, the defect in IFNγ production under the stimulation of anti-CD3 plus IL-12 or IL-2 plus IL-12 is thought to be due to the abolishment of IL-12 signaling. IFNγ mRNA was only detected in spleen cells from wild-type mice by the stimulation of IL-2 plus IL-12 and not detected in those from tyk2-deficient mice (Figure 6C). Then the reduction of IFNγ production from tyk2-deficient T cells by IL-12 occurred at the transcriptional level as a consequence of the reduction of Stat4 activation by IL-12.

Th1 and Th2 helper T cells play unique roles in immune responses. IL-12 facilitates differentiation of naive $CD4^+$ cells into Th1 cells, the process of which is regulated by Stat4. As shown in Figure 7A, $CD4^+$ splenic helper T cells from tyk2-deficient mice failed to differentiate to IFNγ-producing-Th1 cells.

Our observations demonstrate that tyk2 plays a major role in the phosphorylation of Stat4, and the absence of tyk2 reduces Stat4 activation, resulting in the loss of IL-12 biological responses, such as IFNγ production from T cells and the differentiation to Th1 cells from naive helper T cells, although we cannot deny the possibility that other molecules, which might also be essential for IL-12 signaling, just as Stat4 is, are affected by the absence of tyk2. The presence of Jak2 does not compensate for this tyk2 function. On the other hand, T cell proliferation induced by IL-12 is not affected by a deficiency in tyk2. Since it is also regulated by Stat4, a very small amount of activated Stat4 that is observed in tyk2-deficient cells may be of sufficient magnitude to induce the proliferation response in treated cells.

The Jak family of protein tyrosine kinases consists of four members (Jak1, Jak2, Jak3, and tyk2) that are differentially activated in response to various cytokines (Ihle, 1995). Deficiency in Jak1 results in perinatal lethality and impaired lymphocytic development. Jak1-deficient cells fail to manifest biological responses to cytokines that bind to three distinct families of cytokine receptors, all class II cytokine receptors, cytokine receptors that utilize the common γ chain, and gp130 (Rodig, 1998). Jak2 deficiency causes an embryonic lethality due to the absence of definitive erythropoiesis, and fetal liver myeloid progenitors fail to respond to Epo, IL-3, and TPO, while Jak2-deficient fibroblasts fail to respond to IFNγ (Neubauer et al., 1998; Parganas et al., 1998). Mutant mice lacking Jak3 had a phenotype of severe combined immunodeficiency and functional deficiency of mature T and B cells, indicating a nonredundant role for Jak3 in the function of all the receptors utilizing the common γ chain. Here, we report the phenotype of tyk2-deficient mice. In contrast to the other Jak kinases, the absence of tyk2 does not affect cell proliferation regardless of the cell lineage. In spite of previous speculation that tyk2 is an essential molecule in IFNα signaling, tyk2 was revealed to be a redundant molecule that mediates the IFNα response, although it modulates the IFNα signaling pathway. Moreover, tyk2 was revealed to play an essential role in IL-12-induced T cell functions.

Experimental Procedures

Targeting Vector

Genomic clones containing portions of the murine *tyk2* gene were isolated from a Lambda Fx II murine 129/Sv genomic library (Stratagene). To generate the *tyk2*-targeting vector, a 4.2 kb XbaI-EcoRV fragment containing exon 1 was replaced with a neomycin-resistant cassette. A herpes simplex thymidine kinase (*HSV-tk*) cassette that mediated negative selection was inserted in the 3′-end of the *tyk2-neo* construct.

Transfection of ES Cells and Generation of tyk2-Deficient Mice

E14 (129/Ola mouse strain) ES cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 15% FCS (Hyclone), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acid, 50 μM 2-mercaptoethanol, 100 U/ml of Penicillin-G, 100 μg/ml of streptomycin, and 1000 U/ml LIF (all obtained from GIBCO-BRL, with the exception of FCS). Irradiated primary embryonic fibroblasts expressing the neomycin-resistant gene were used as feeder cells for ES cells. Eighty micrograms of NotI-linearized *tyk2* plasmid construct was electroporated into the 4×10^7 ES cells using a gene pulser set at 0.3 kV, 125 μF (BioRad). Selection 24 hr after electroporation was in 300 μg/ml geneticin (G418)(GIBCO-BRL) and 1 μM gancyclovir. ES clones were picked up and expanded 7–10 days after electroporation. Five positive clones were identified by PCR, confirmed by Southern blotting, and microinjected into blastocysts. Two clones gave germline transmission.

Genotyping by Southern Blotting and PCR

For PCR screening, the following primers were used: gtyk2F1 (5′-CAAGCACTGAGCTACAGCCTATTC-3′) annealing upstream of the targeting construct and PJL (5′-TGCTAAAGCGCATGCTCCAGACTG-3′) annealing in the neomycin-resistant cassette. Southern blotting analysis was performed on 20 μg of EcoRI-digested genomic DNA. Filters were probed with a ^{32}P-labeled external probe.

Tyk2 Western Blotting

Spleen cells from wild-type or tyk2-deficient mice were lysed in lysis buffer as previouly described (Shimoda et al., 1997). Cell lysates were centrifuged at 12,000 × g for 15 min to remove debris, and the supernatants were incubated with anti-tyk2 polyclonal antibody and anti-Stat1 polyclonal antibody (Santa-Cruz) overnight. 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-10% PAGE and transferred to a nitrocellulose membrane. Membranes were probed using anti-tyk2 and anti-Stat1 antibodies and visualized with the ECL detection system (Amersham).

Preparation of Fractionated Spleen Cells

$CD3^+$ and $CD3^-$ spleen cells derived from wild-type and tyk2-deficient mice were obtained by the immunomagnetic cell isolation method using Dynabeads coated with a rat mAb specific for each mouse cell surface antigen (Dynal). Spleen cells were cultured on plastic plates for 1 hr at 37°C to remove macrophages. Nonadherent spleen cells were incubated with Dynabeads mouse CD3 for 20 min at 4°C with gentle rotation. The rosetted $CD3^+$ cells were collected by placing the test tube containing cells in Dynal MPC for 2 min. Unrosetted cells in the culture with Dynabeads mouse CD3 were also used as $CD3^-$ cells.

Biotinylation of Cell Surface Proteins

Cell surface molecules were biotinylated by the incubation of Sulfo-NHS-biotin (Pierce) with cells in PBS (pH 8.0) for 30 min at room temperature according to the directions of the manufacturer.

Immunoprecipitation, SDS-PAGE, and Western Blotting

Embryonic fibroblasts from wild-type or tyk2-deficient embryos were cultured in DMEM supplemented with 10% fetal bovine serum. The cells were starved for 12–14 hr in serum-free DMEM prior to

cytokine stimulation. Spleen cells from wild-type or tyk2-deficient mice were cultured with 25 μg/ml of LPS for 24 hr or 2 μg/ml of PHA for 72 hr. Prior to cytokine stimulation, cells were washed in PBS and allowed to sit for 24 hr in RPMI with 2% FCS. The cells were treated with IFNα (1000 U/ml), IFNγ (100 ng/ml), IL-6 (500 ng/ml) plus soluble IL-6 receptor (500 ng/ml), IL-10 (50 ng/ml), or IL-12 (20 ng/ml) for 15 min, and lysed in lysis buffer as previously described (Shimoda et al., 1997). The supernatants were incubated with the indicated antibodies for 12 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 a nitrocellulose membrane. Membranes were probed using designated antibodies and visualized with the ECL detection system (Amersham).

Northern Blotting Analysis

Wild-type and tyk2-deficient primary fibroblasts were starved of serum overnight and consequently stimulated with IFNα (1000 ng/ml) and IFNγ (100 ng/ml) for 1 hr. Total RNA was prepared using Isogen (Nippon Gene), and 10 μg RNA were fractionated on 1% formaldehyde agarose gels, blotted onto Nytran-plus membranes (Schleicher & Schuell), and hybridized with ^{32}P-labeled cDNA probes. Interferon regulatory factor 1 (*IRF-1*) cDNAs were used as probes.

Induction of Cell Surface Immunoregulatory Proteins and FACS Analysis

Spleen cells from mice were cultured for 72 hr in vitro in the presence of PBS or serial dilutions of IFNα. Cells were stained with fluorescein isothiocyanate-conjugated mouse anti-mouse H-2k^b mAb, and MHC class I expression on spleen cells in the cultures was assessed by FACS. Splenic CD3$^-$ lymphocytes were obtained by negative selection using magnetic beads. CD3$^-$ cells were stimulated with IL-10 (10 ng/ml) or PBS for 24 hr and were stained with phycoerythrin-conjugated rat anti-mouse I-A^b and assessed for the expression of MHC class II antigen by FACS.

Antiviral Activity Assay

The ability of primary EF cells to resist VSV infection was determined using a cytopathic effect assay. EF cells were seeded into 96-well tissue culture plates at a density of 8×10^4 cells per well and incubated with serial dilutions of IFNα or IFNγ. After 48 hr, plates were washed, and the cells were exposed to VSV. Cell viability was determined by crystal violet staining and quantified by spectroscopy at 540 nm.

Proliferation Assay

Thymocytes from wild-type and tyk2-deficient mice were resuspended in RPMI 1640, supplemented with 2% FCS, PHA (10 μg/ml), and IL-6 (10 ng/ml) as described by Lotz et al. (1988). Cells (1×10^5) in 200 μl medium were added per well and cultured for 96 hr. During the final 4 hr, [^{3}H] thymidine (1 μCi/well) was added. The cells were harvested by filtration, and radioactivity was counted by scintillation spectrophotometry. For the proliferation assay of IL-10, thymocytes from wild-type and tyk2-deficient mice were stimulated with IL-10 (10 ng/ml) in the presence of 1000 U/ml of IL-2 for 96 hr. Cultures were pulsed for the last 12 hr with [^{3}H] thymidine. For the proliferation assay of IL-12, CD3$^+$ spleen cells from wild-type and tyk2-deficient mice were activated with anti-CD3 (10 μg/ml) for 3 days and then restimulated with IL-12 (7 ng/ml) or PBS for 48 hr. Cultures were pulsed for the final 24 hr with [^{3}H] thymidine.

Induction of Cytokine-Dependent Cellular Responses in Macrophages and Lymphocytes

Peritoneal macrophages (1×10^5 cells) were plated on a 96-well plate with sequential concentrations of IL-10 for 12 hr followed by treatment with LPS (10 ng/ml) and IFNγ (100 U/ml). After 48 hr, supernatants were harvested and the TNF concentrations were quantified by ELISA (Genzyme). Spleen cells (5×10^5 cells) from wild-type and tyk2-deficient mice were activated with IL-2 (50 U/ml) either alone or with the addition of the indicated amount of IL-12. Plates were cultured for 48 hr at 37°C, and the IFNγ released into the medium was quantified by ELISA (Genzyme).

Th1/2 Cell Differentiation In Vitro

Naive (CD44^{low}) CD4 T cells from the spleen were prepared by panning with anti-CD8 and anti-CD44 mAbs. The enriched naive CD4 T cells (5×10^5) were stimulated for 2 days with immobilized anti-TCR in the presence of IL-12 and anti-IL-4 mAb (Th1-skewed condition) or IL-4 (Th2-skewed condition). The cells were then cultured with only cytokines for another 3 days. Intracellular staining of IFNγ and IL-4 was performed as described (Yamashita et al., 1999).

Acknowledgments

We thank Drs. T. Otsuka, H. Nakashima, T. Maruyama, Y. Nemoto, Y. Inoue, and Y. Fukumaki for discussion, and A. Koutate, A. Tomioka, Y. Yamada, and K. Shimoharada for their excellent technical assistance. This work was supported by Grants-in-Aid for Scientific Research (numbers 11770577 and 11307015) from the Ministry of Education, Science, Sports, and Culture in Japan.

Received May 19, 2000; revised September 18, 2000.

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