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Partial Impairment of Cytokine Responses in Tyk2-Deficient Mice

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Summary

To assess the role of the Janus kinase (Jak) family member Tyk2, we have generated *Tyk2*$^{-/-}$ mice. In contrast to other Jaks, where inactivation leads to a complete loss of the respective cytokine receptor signal, *Tyk2*$^{-/-}$ mice display reduced responses to IFNα/β and IL-12 and a selective deficiency in Stat3 activation in these pathways. Unexpectedly, IFNγ signaling is also impaired in *Tyk2*$^{-/-}$ mice. *Tyk2*$^{-/-}$ macrophages fail to produce nitric oxide upon lipopolysaccharide induction. *Tyk2*$^{-/-}$ mice are unable to clear vaccinia virus and show a reduced T cell response after LCMV challenge. These data imply a selective contribution of Tyk2 to the signals triggered by various biological stimuli and cytokine receptors.

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Introduction

Cytokines and their receptors can be divided into subgroups with distinct structural/functional features and common principles of intracellular signal transduction (Ihle, 1995). The non-receptor tyrosine kinases of the Jak (Janus) family play a pivotal role in signaling via ligand-activated cytokine receptors. At present, the Jak family consists of four members in mammals, Jak1 to 3 and Tyk2. Jaks associate with intracellular domains of cytokine receptors and become phosphorylated after ligand binding and aggregation of the respective receptor chains. Activated Jaks phosphorylate tyrosine residues of the receptor chains, thereby recruiting Stats (signal transducers and activators of transcription) and other signaling molecules into the activated receptor complex (Briscoe et al., 1996). Stats are in turn specifically phosphorylated on tyrosines, subsequently homo- or heterodimerize, and are translocated to the nucleus, where they bind to response elements of cytokine-dependent genes (Darnell, 1997; Schindler, 1999).

The potential function of the Jaks was indicated by genetic complementation of human cell lines selected for defective interferon (IFN) signal transduction (Velazquez et al., 1992; Müller et al., 1993; Watling et al., 1993). Biochemical studies demonstrated Jak-mediated immediate phosphorylation events upon ligand binding to many cytokine receptors in various cell lines (Ihle, 1995). For Jak1 (Rodig et al., 1998), Jak2 (Neubauer et al., 1998; Parganas et al., 1998), and Jak3 (Nosaka et al., 1995; Park et al., 1995; Thomis et al., 1995), these in vitro data have been completed by studies in gene-targeted mice.

In the present studies, we have focused on the role of Tyk2 in the biological response to cytokines. Following the initial characterization in the IFNα/β signaling pathway (Velazquez et al., 1992), subsequent studies showed Tyk2 to be activated by various other cytokines (Schindler, 1999). The Jak family members have a C-terminal catalytic domain (JH1) adjacent to a pseudokinase domain (JH2). They share five additional Jak homology (JH3–7) domains toward their N terminus (Pellegrini and Dusanter-Fourt, 1997). Studies employing a Tyk2-deficient human cell line revealed that Tyk2 also plays a structural role at the IFNα receptor (IFNAR) because binding of certain type I IFNs is abrogated and the cell surface level of IFNAR1 is low in these cells. The N-terminal domains JH7 and JH6 of Tyk2 are sufficient for the interaction with IFNAR1, but additional JH regions were required for the correct in vivo assembly and stabilization of the receptor (see Pellegrini and Dusanter-Fourt, 1997; Yeh and Pellegrini, 1999).

To further assess the in vivo function of Tyk2, we generated mice deficient in this kinase. We show that Tyk2 does not play a crucial role in the structure and

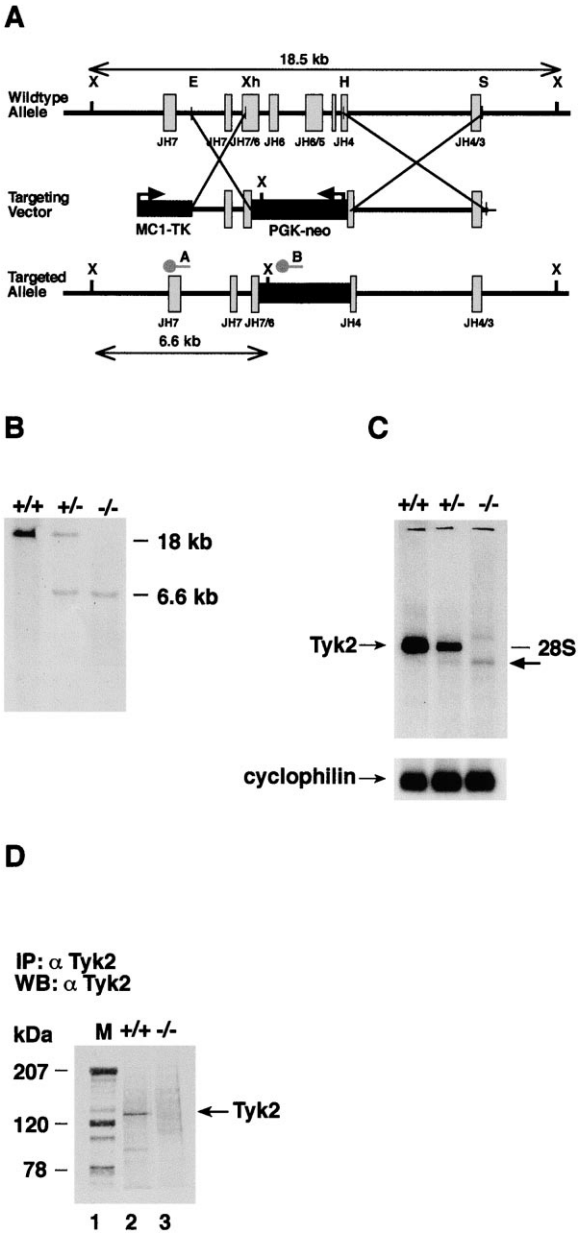

Figure 1. Targeted Disruption of the Murine *Tyk2* Gene

(A) Scheme of the targeting vector. The mouse *Tyk2* locus is shown at the top. In the targeting vector (middle), exons encoding the Jak homology (JH) domains JH7 (partly), JH6/5, and JH4 (partly) were replaced by a neomycin resistance (GK-neo) gene cassette. A thymidine kinase gene (MC1-TK) cassette was used for negative selection. The structure of the targeted allele is depicted at the bottom. The position of the flanking (A) and internal (B) probes used for Southern hybridization is marked.

(B) Southern blot analysis of genomic tail DNA from wild-type (+/+), heterozygous (+/-), and homozygous (-/-) Tyk2 mice. XbaI digested DNAs were hybridized to probe A. The wild-type allele is 18.5 kb in length, and the targeted allele is 6.6 kb. Hybridization to probe B confirmed the single integration event of the targeting vector (data not shown).

(C) Northern blot analysis of polyA$^+$-RNA (2 μg per lane) isolated from *Tyk2*$^{+/+}$, *Tyk2*$^{+/-}$, and *Tyk2*$^{-/-}$ embryonic fibroblasts (EFs) and hybridized to a mouse Tyk2 cDNA probe and as a control to cyclophilin. A minor aberrant transcript arising in *Tyk2*$^{+/-}$ and *Tyk2*$^{-/-}$ cells is indicated by an arrow. This message together with the wild-type murine Tyk2 mRNA was cloned and sequenced (B. M., unpublished data). The shorter transcript was found to be an alternatively spliced RNA lacking exons 3–9. An open reading frame would give rise to a putative 90 kDa polypeptide. Experiments performed in order to prove the possible biological activity of a putative truncated Tyk2 protein are described in the text.

(D) Immunopreciptiation (IP) of Tyk2 protein bone marrow macrophages (BMMs). Tyk2 was immunoprecipitated from cell homogenates of BMMs and detected by Western blotting (WB) using anti-Tyk2 antibodies (Abs). The size of the molecular mass markers in kDa is indicated.

assembly of the IFNAR in mice; however, it is required for a full response to type I and type II IFNs. Tyk2 plays an essential and nonredundant role in promoting selective immune responses, including innate host defense mechanisms and specific antiviral activities.

Results

Generation of *Tyk2*$^{-/-}$ Mice

In order to disrupt the *Tyk2* gene, translated exons 3–9 were targeted (Figure 1A). Homologous recombination deleted domains known to be crucial for Tyk2 function (Yeh and Pellegrini, 1999). Germline chimeric mice from two independently targeted embryonic stem (ES) cell clones were generated and mated to produce *Tyk2*$^{-/-}$ mice (Figure 1B). Northern analysis of primary embryonic fibroblasts (EFs) hybridized with a murine Tyk2 cDNA probe revealed a 4.7 kb band for wild-type cells. As expected, the Tyk2 signal intensity decreased in *Tyk2*$^{+/-}$ EFs and the Tyk2 full-length message was not detectable in *Tyk2*$^{-/-}$ cells (Figure 1C). A minor Tyk2 hybridizing transcript appeared in *Tyk2*$^{+/-}$ and *Tyk2*$^{-/-}$ EFs after lengthy exposure (Figure 1C, arrow). Immunoprecipitations (IP) and Western blotting (WB) with antibodies directed against the C terminus of Tyk2 were carried out. Tyk2-specific protein was not detectable in *Tyk2*$^{-/-}$ primary EFs or in bone marrow–derived macrophages (BMMs) (Figure 1D, left; Figure 2A, lanes 6–9). To further rule out the presence of functional Tyk2 in the gene-targeted mice, the Tyk2-deficient human cell line U1A (11.1) (Velazquez et al., 1992) was complemented by overexpressing murine wild-type and aberrant Tyk2 cDNAs and by fusing U1A cells with mouse *Tyk2*$^{+/+}$ and *Tyk2*$^{-/-}$ EFs. Functional complementation of U1A cells was assayed by two independent methods, namely IFNα-dependent growth during a highly sensitive drug selection and IFNα response of an endogenous inducible gene. Neither the overexpression of aberrant Tyk2 cDNA nor the fusion to *Tyk2*$^{-/-}$ EFs functionally restored the Tyk2 deficiency of U1A cells, whereas complementation with full-length Tyk2 cDNA and fusion to wild-type murine EFs reconstituted IFNα signaling (data not shown). On the basis of the results of these rigorous experiments, we conclude that Tyk2 was successfully inactivated in the *Tyk2*$^{-/-}$ mice. Generation of *Tyk2*$^{-/-}$ mice by heterozygous intercrosses revealed no statistically relevant deviation from the normal Mendelian inheritance. *Tyk2*$^{-/-}$ mice showed no gross abnormalities in development, fertility, and ontogeny of blood cells.

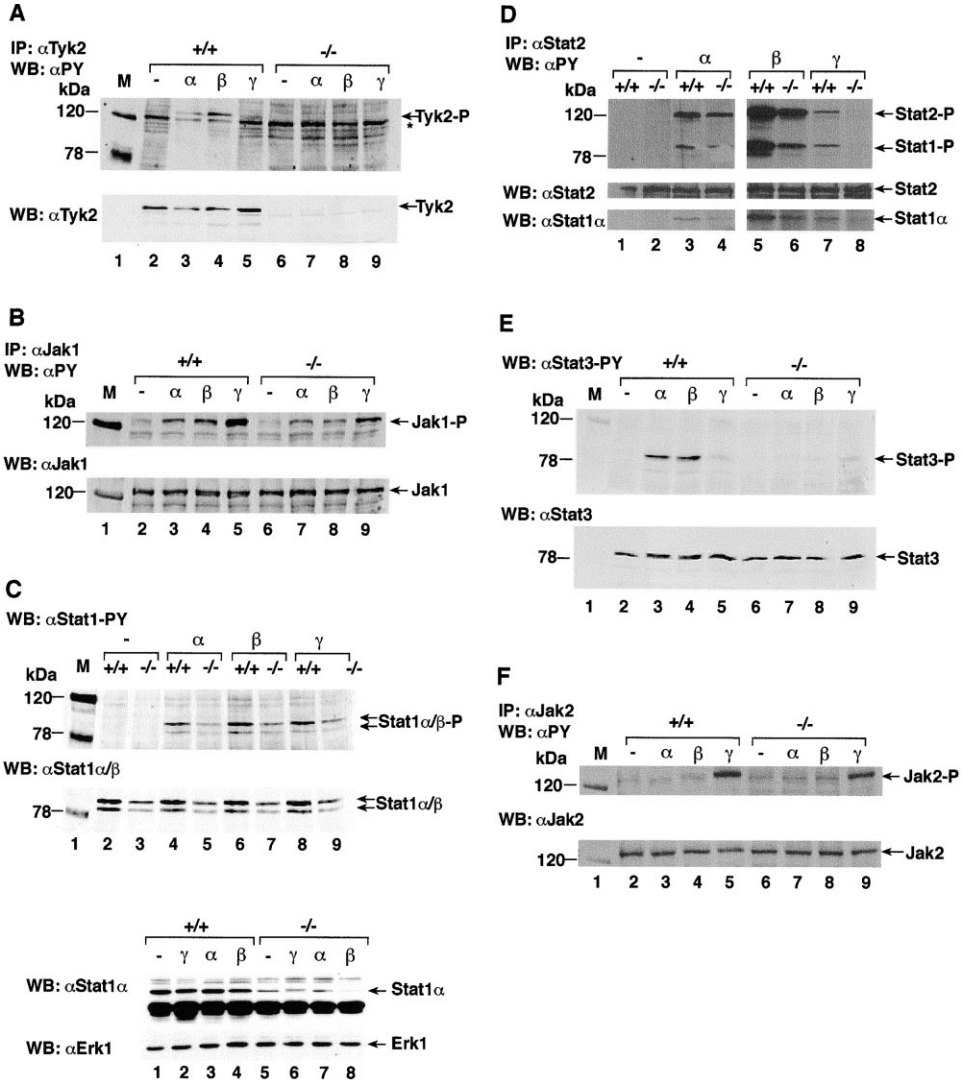

Figure 2. Diminished but Detectable Activation of Jaks and Stats in *Tyk2*$^{-/-}$ Cells after IFN Treatment

(A–F) EFs or BMMs derived from *Tyk2*$^{+/+}$ or *Tyk2*$^{-/-}$ mice were stimulated with the indicated IFNs (1000 U/ml) for 20 min or left untreated (-). Cells were analyzed for activation of Jaks and Stats by IP with the indicated antisera and WB with anti-phosphotyrosine Abs (αPY) or by direct WB with anti-phosphotyrosine-Stat (αStat-PY) Abs. Protein loading was controlled by reprobing with the indicated Abs. Activation of Tyk2 (A) and Jak1 (B) in EFs (asterisk indicates an unspecific band in Tyk2 IPs). Activation of Stat1α/β ([C], top) and reduced level of Stat1 protein in *Tyk2*$^{-/-}$ BMMs ([C], bottom). Activation of Stat2 and coprecipitation of Stat1 in EFs (D). Activation of Stat3 in BMMs (E). Activation of Jak2 in EFs (F).

Signaling through Type I and Type II IFN Receptors Is Compromised but Not Abolished in Tyk2-Deficient Mice

Tyk2 deficiency in U1A cells leads to a complete failure to respond to IFNα and a partial defect in the IFNβ response (Velazquez et al., 1992). Therefore, we first analyzed the activation of signaling molecules in the type I IFN pathway and as a control in the IFNγ pathway (Stark et al., 1998). Tyk2 was phosphorylated on tyrosines after treatment of wild-type EFs with IFNα or IFNβ but not with IFNγ (Figure 2A, lanes 3–5). Importantly, this could not be detected in *Tyk2*$^{-/-}$ EFs (Figure 2A, lanes 7–9). Jak1 tyrosine phosphorylation was slightly reduced in *Tyk2*$^{-/-}$ EFs after treatment with IFNα or IFNβ (Figure 2B, lanes 7 and 8). *Tyk2*$^{-/-}$ BMMs showed a readily detectable activation of Stat1α and Stat1β after treatment with type I and II IFNs (Figure 2C, lanes 5, 7, and 9). However, as assayed by standardized WB, the level of Stat1 protein was reduced in *Tyk2*$^{-/-}$ BMMs (Figure 2C, bottom) and EFs (data not shown). No difference could be detected between *Tyk2*$^{+/+}$ and *Tyk2*$^{-/-}$ EFs in the time course of Stat1 induction after IFNα/β treatment (data not shown). Stat2 was analyzed in a coprecipitation assay with Stat1 upon activation. Challenge of *Tyk2*$^{-/-}$ EFs with IFNα/β revealed a reduced but detectable phosphorylation of Stat2 and coprecipitation of phosphorylated Stat1 (Figure 2D, lanes 4 and 6). Strikingly, IFNα/β-treated *Tyk2*$^{-/-}$ BMMs showed a complete impairment of Stat3 activation (Figure 2E, lanes 7 and 8). In the absence of Tyk2, the activation of Jaks and Stats

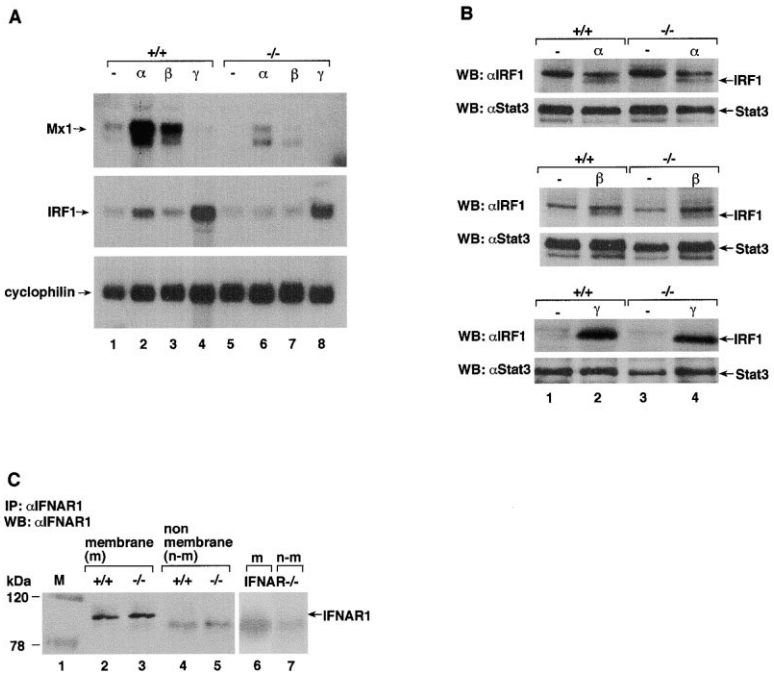

Figure 3. Expression of IFN-Responsive Genes and of IFNAR1 in Tyk2-Deficient Mice

(A) Transcriptional response to IFNs in EFs. *Tyk2*$^{+/+}$ and *Tyk2*$^{-/-}$ cells were treated with the indicated IFNs (1000 U/ml) for 6 hr or left untreated (-). Northern blot analyses were performed using 15 μg total RNA per lane and hybridizing to Mx1 and IRF-1 and as a control to cyclophilin.

(B) Expression of IRF-1 protein in peritoneal macrophages (PMs) after treatment with IFNs. Thioglycolate-elicited PMs were isolated and stimulated with type I and II IFNs (1000 U/ml) for 4 hr. Cells were lysed and WB was performed with anti-IRF-1 Abs (αIRF1). Protein loading was controlled by reprobing with anti-Stat3 Abs (αStat3).

(C) Presence of IFNAR1 in membrane fractions of murine EFs and BMMs. Whole cell extracts from *Tyk2*$^{-/-}$ and *Tyk2*$^{+/+}$ EFs, and as a negative control from *IFNAR1*$^{-/-}$ BMMs, were fractionated in nonsoluble membrane proteins and soluble cytoplasmic proteins by ultracentrifugation. The membrane pellet was dissolved, normalized to protein content and IP, and WB was performed with anti-IFNAR1 Abs (αIFNAR1).

after IFNγ treatment was also decreased. Initially, we detected a slight reduction of the activation of Jak1 after IFNγ stimulus (Figure 2B, lane 9). The ability of IFNγ to activate ISGF3, known to be predominantly involved in the type I IFN response, was reported (Matsumoto et al., 1999). Therefore, we analyzed the IFNγ-mediated activation of Stat2, a component of ISGF3. In *Tyk2*$^{-/-}$ EFs, it was not possible to detect activated Stat2 pulling down activated Stat1 in a coprecipitation assay (Figure 2D, lane 8). IFNγ stimulation resulted in a similar weak phosphorylation of Stat3 in *Tyk2*$^{-/-}$ and *Tyk2*$^{+/+}$ BMMs (Figure 2E, lanes 5 and 9; Figure 4A, lanes 4 and 5). Finally, we analyzed the RNA expression of IFN-inducible genes binding ISGF3, i.e., Stat1/Stat2 heterodimers associated with IRF-9 (e.g., Mx1) or Stat homodimers (e.g., IRF-1) or combinations thereof (e.g., GBP-1) (Stark et al., 1998). Consistent with the activation of Jaks and Stats, the induction of Mx1 RNA after IFNα and -β treatment and IRF-1 after IFNγ treatment was reduced although clearly detectable in *Tyk2*$^{-/-}$ cells (Figure 3A, top lanes 6 and 7 and middle lane 8, respectively). The same was observed in the response of GBP-1 to the type I and II IFNs (data not shown). To further investigate the apparent unresponsiveness of IRF-1 in *Tyk2*$^{-/-}$ cells to IFNα/β (Figure 3A, middle lanes 6 and 7), we performed WB for IRF-1 protein. IRF-1 accumulated at comparable levels in wild-type and targeted cells after stimulation with IFNs (Figure 3B). Possible differences in the time course of IRF-1 mRNA induction between *Tyk2*$^{-/-}$ and *Tyk2*$^{+/+}$ cells are currently under investigation. Thus, Tyk2 deficiency leads to a partial defect in the IFNα and -β response and, surprisingly, also to an impairment of IFNγ signaling.

Lack of Structural Requirement or Functional Compensation for Tyk2 at the Murine IFNAR

Detailed work with the Tyk2-deficient human cell line U1A established a kinase activity–independent role of Tyk2 for high-affinity IFNα binding and stable processing of IFNAR1 to the cell surface (Pellegrini and Dusanter-Fourt, 1997; Yeh and Pellegrini, 1999). In *Tyk2*$^{-/-}$ BMMs, high doses of ligand (up to 5×10^4 U/ml) did not overcome the IFNα signaling defects as assayed by Stat3 activation (data not shown). Moreover, membrane fractions from *Tyk2*$^{+/+}$ and *Tyk2*$^{-/-}$ EFs contained similar amounts of IFNAR1 (Figure 3C). Jak2 did not compensate for the absence of Tyk2 at the IFNAR because *Tyk2*$^{-/-}$ cells showed no activated Jak2 after the treatment with IFNα (Figure 2F). These data show that *Tyk2*$^{-/-}$ cells have no lowered affinity for IFNα and normal expression levels of IFNAR1. In addition, no compensatory effects of Jak2 for Tyk2 deficiency could be detected.

Lack of Stat3 Activation, Reduced Stat4 Activation, and Reduced IFNγ Production in Tyk2-Deficient Spleen Cells after IL-12 Treatment

IL-12 receptor signaling involves the Jak family members Tyk2 and Jak2 and the Stat proteins Stat3 and Stat4 (Bacon et al., 1995; Jacobson et al., 1995). To investigate the role of Tyk2 in the response to IL-12, Con A-activated splenocytes were treated with IL-12 and assayed for activation of Stats. The absence of Tyk2 led to a complete lack of Stat3 activation (Figure 4A, lanes 2 and 3) and a clearly reduced tyrosine phosphorylation of Stat4 (Figure 4B, lanes 5 and 6). IL-12 induces IFNγ production by activated T lymphocytes (Trinchieri, 1995). Con A-activated spleen cells of Tyk2-deficient mice were able to increase IFNγ production upon IL-12 stimulus; however, the level of IFNγ was clearly reduced in comparison to IL-12-treated *Tyk2*$^{+/-}$ cells. The production of IFNγ in response to Con A alone was not detectably altered (Figure 3C). These data establish the requirement of Tyk2 for the full cellular response to IL-12 and suggest that Stat3 activation selectively requires the presence of Tyk2 in IL-12 signaling.

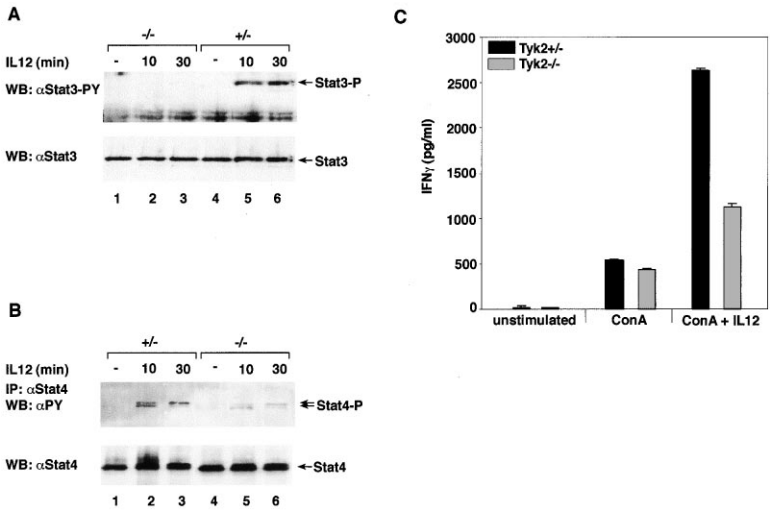

Figure 4. Tyk2 Deficiency Causes Lack of Stat3 Activation and Reduced IFNγ Production of Splenocytes after IL-12 Treatment

Concanavalin (Con) A-activated splenocytes of *Tyk2*$^{-/+}$ and *Tyk2*$^{-/-}$ mice were treated with IL-12 (5 ng/ml) for the indicated times or left untreated (-). Cell extracts were used for IP and WB. Analysis of Stat activation was performed as in Figure 2. (A) Activation of Stat3. (B) Activation of Stat4. (C) Production of IFNγ in Con A-activated splenocytes after IL-12 treatment. Splenocytes were isolated and cultured in the presence of Con A (1 μg/ml) or Con A + IL-12 (1 ng/ml) or left untreated. Culture supernatants were analyzed for IFNγ production by ELISA after 72 hr. The mean IFNγ production is shown for four *Tyk2*$^{+/-}$ and four *Tyk*$^{-/-}$ mice.

Tyk2 Is Not Required for IL-10 Response of Macrophages, IL-3-, G-CSF-, or TPO-Induced Colony Formation of Myeloid Progenitors and IL-6- or LIF-Induced Stat3 Activation in Embryonic Fibroblasts

Jak1, Stat1, and Stat3 have been described as part of the IL-10R signaling cascade in mouse macrophages (Riley et al., 1999). Using macrophages from gene-targeted mice, it was found that the downregulation by IL-10 of lipopolysaccharide (LPS)-stimulated TNFα production is strictly dependent on Jak1 and Stat3 (Rodig et al., 1998; Riley et al., 1999; Takeda et al., 1999). Tyk2 has been reported to be phosphorylated on tyrosines after treatment with IL-10 in responsive cell types (Finbloom and Winestock, 1995). We therefore measured the effect of IL-10 on the production of TNFα by BMMs. No differences in the suppression of TNFα by IL-10 were observed between wild-type and targeted BMMs (Figure 5B). Consistent with these data, the activation of Stat3 (Figure 5A) and Stat1 (data not shown) by IL-10 remained unaffected in *Tyk2*$^{-/-}$ BMMs. Tyk2 was shown to be activated by ligands using the gp130 family of cytokine receptors (Stahl et al., 1994). To test whether Tyk2 is required for gp130-mediated signaling, BMMs and EFs were stimulated with IL-6 and LIF, respectively. Assaying Stat3 activation revealed no gross differences between *Tyk2*$^{-/-}$ and *Tyk2*$^{+/+}$ cells (data not shown). In vitro data showed that IL-3 (Nagata and Todokoro, 1996), G-CSF (Shimoda et al., 1997), and TPO (Sattler et al., 1995) activated Tyk2. Absence of Tyk2 did not prevent colony formation in response to IL-3, G-CSF, and TPO (data not shown). This is consistent with observations that for all five cytokines other Jaks seem to play the major role (Guschin et al., 1995; Shimoda et al., 1997; Parganas et al., 1998; Drachmann et al., 1999). Therefore, in the biochemical and biological assays employed, Tyk2 is either not required or redundant for the response to G-CSF, IL-3, IL-6, IL-10, LIF, and TPO.

Impairment of Type I IFN and LPS-Induced NO Production in Tyk2-Deficient Macrophages

Type 2 nitric oxide synthase (NOS2 or iNOS) is induced by IFNγ, microbial products (e.g., LPS), and, in the pres-

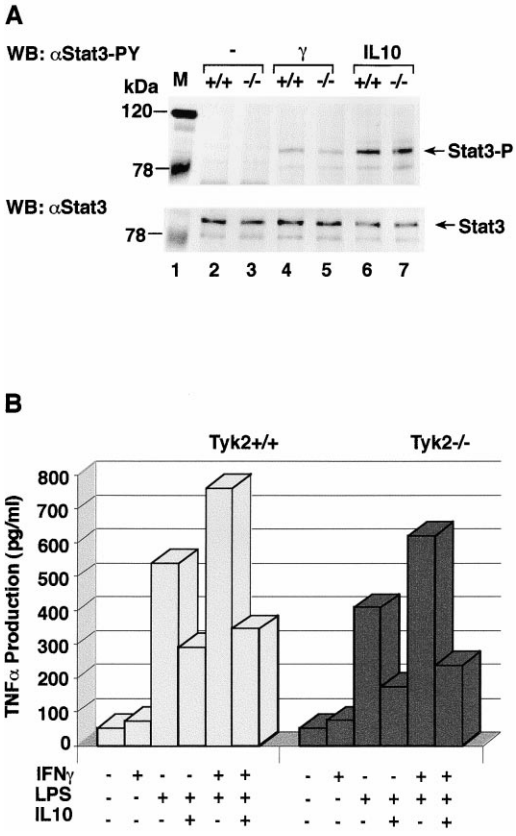

Figure 5. Redundant Role of Tyk2 in IL-10 Signaling of Macrophages

(A) Anti-phosphotyrosine-Stat3-specific WB of *Tyk2*$^{+/+}$ or *Tyk2*$^{-/-}$ BMMs stimulated with IL-10 (200 ng/ml) for 20 min or left untreated. Treatment with IFNγ (1000 U/ml, 20 min) was used as a stimulation control. The blot was reprobed with anti-Stat3 Abs.

(B) IL-10-mediated inhibition of TNFα production in BMMs. Cells were pretreated with IL-10 (100 ng/ml) for 12 hr and subsequently stimulated with IFNγ (1000 U/ml), LPS (50 ng/ml), a combination thereof, or left untreated (-) for an additional 24 hr. TNFα levels were quantified by ELISA in the supernatants. The values represent mean values of duplicates from two independent experiments.

ence of a costimulus, by type I IFNs. NOS2-derived NO is critical for the innate and adaptive immune responses to a wide variety of infectious pathogens (Bogdan et al., 2000). Wild-type and *Tyk2*$^{-/-}$ peritoneal macrophages (PMs) were compared for their ability to produce NO in response to IFNγ, IFNα/β, LPS, or combinations thereof. The nitrite levels after IFNγ treatment were comparable in both macrophage populations (Figure 6A). In accordance with previous data (Ding et al., 1988), type I IFN alone did not induce NO production in wild-type macrophages unless combined with LPS (Figure 6B). In contrast to *Tyk2*$^{+/+}$ cells, *Tyk2*$^{-/-}$ PMs failed to produce NO after treatment with LPS (Figure 6A) and LPS plus IFNα/β (Figure 6B). To elucidate the underlying mechanism(s), we assayed NOS2 expression after the stimuli described above by WB and RT-PCR. NOS2 could not be detected in *Tyk2*$^{-/-}$ PMs in response to LPS, IFNγ, or LPS plus IFNα/β (Figure 6C, lanes 9, 10, and 12), while wild-type PMs showed NOS2 synthesis (Figure 6C, lanes 2, 3, and 5). RT-PCR revealed that the lack of NOS2 protein is due to the lack of LPS-inducible mRNA in *Tyk2*$^{-/-}$ PMs (Figure 6D). Stat1 was shown to be a converging point for LPS and IFNγ stimuli in macrophages (Kovarik et al., 1998). Time course experiments in PMs showed that LPS alone activates Stat1, albeit in an indirect (cycloheximide sensitive) fashion (Figure 6E, bottom and top panels, lane 2; Gao et al., 1998). LPS also causes a significant increase in Stat1 levels (Figure 6E, top panel, lanes 1–5, and bottom panel, lanes 1–7). Absence of Tyk2 strongly reduces LPS-mediated Stat1 activation (Figure 6E, top panel, lanes 10–6, and bottom panel, lanes 8–14), as well as delays the onset (Figure 6E, top panel, compare lanes 4–7) and shortens the duration of the response (Figure 6E, bottom panel, compare lanes 4 and 11 and see note in figure legend). Earlier studies suggested that short time treatment of mouse macrophages with LPS did not result in detectable activation of any of the Jaks (Kitamura et al., 1996; Kovarik et al., 1999). Nevertheless, assaying for LPS-induced Tyk2 activation revealed a slight increase in tyrosine phosphorylation of Tyk2 after a 2 hr stimulus (Figure 6F), the time point when Stat1 activation occurred (Figure 6E), while no Tyk2 activation after 10 min treatment could be detected (data not shown). These data indicate that Tyk2 plays an indispensable and nonredundant role in the type I IFN- and/or LPS-mediated production of NO in macrophages.

Increased Susceptibility to Specific Viral Pathogens in the Absence of Tyk2

Inoculating *Tyk2*$^{-/-}$ and *Tyk2*$^{+/+}$ mice with vesicular stomatitis virus (VSV), lymphocytic choriomeningitis virus (LCMV), and vaccinia virus (VV), assessed the consequences of Tyk2-deficiency for host resistance to viral infections. No gross differences were detectable between *Tyk2*$^{-/-}$ and *Tyk2*$^{+/+}$ EFs or BMMs in the IFNα/β level-dependent appearance of cytopathic effects upon VSV infection (data not shown). To assess the effect of Tyk2 deficiency in vivo, we inoculated wild-type, *Tyk2*$^{-/-}$, and *IFNAR1*$^{-/-}$ (Müller et al., 1994) mice with VSV. All wild- type mice survived over 12 days after infection, while 80% of the *Tyk2*$^{-/-}$ mice and only 33% of the *IFNAR1*$^{-/-}$ mice were capable of surviving (Figure 7A). The challenge with LCMV revealed a significant (3- to 10-fold) reduction of the cytotoxic T lymphocyte (CTL) activity at day 8 after infection in *Tyk2*$^{-/-}$ mice (Figure 7B). The CTL activity was measured ex vivo with splenic T cells on target cells coated with LCMV-specific peptides. After inoculation with VV, virus was not found in the spleen of wild-type mice, while other organs such as ovary and lung carried a high load of infectious virus on day 4 after infection (Müller et al., 1994). In contrast, infection of *Tyk2*$^{-/-}$ mice with VV resulted in elevated viral replication in the spleen (Figure 7C). Hence, Tyk2 contributes selectively to host defense against differing viral infections. Depending on the pathogenicity of the virus, Tyk2 plays a redundant role in the resistance to infection or is absolutely required for particular or concerted signaling events leading to antiviral activity.

Discussion

Tyk2 was the first member of the Jak family of kinases to be completely isolated (Firmbach-Kraft et al., 1990) and originally described to be involved in IFNAR signaling (Velazquez et al., 1992). Our results employing Tyk2-deficient mice demonstrate an indispensable role of Tyk2 in the induction of innate and adaptive immune responses. In contrast to the obligate requirement for the presence of all other Jak family members for the general functioning of specific cytokine receptors, Tyk2 is redundant in the initial activation of the receptor complexes investigated.

Role of Tyk2 in Cytokine Class I and Class II Receptor Signaling

The type I IFN response is clearly impaired but not completely abolished in *Tyk2*$^{-/-}$ mice. Previous studies with the IFNα-unresponsive mutant human cell line U1A lacking Tyk2 (Velazquez et al., 1992) implied a prominent role of Jak1 in type I IFN signaling, and therefore a subordinated role of Tyk2 was suggested. A defect in the receptor assembly in U1A leads to loss of high-affinity binding of IFNα (Pellegrini and Dusanter-Fourt, 1997). In contrast, this function of Tyk2 appears absent in *Tyk2*$^{-/-}$ mice. Hence, the Tyk2-dependent IFNAR1 transport mechanism is species specific, or the *Tyk2*$^{-/-}$ phenotype of U1A is a peculiarity of this mutant or its parental cell lines. Nonetheless, the stochiometry of the IFNAR in human and mice remains to be fully elucidated. Another novel aspect is the impairment of parts of IFNγ signaling due to Tyk2 deficiency. This suggests a receptor-independent role of Tyk2 or alternatively places Tyk2 in the proximity of the IFNGR. The latter model is supported by experimental evidence suggesting an association of IFNAR1 and IFNGR2 and an IFNAR1-dependent full functional activation of IFNγ signaling components (Takaoka et al., 2000). The emerging model of a cross-talk of chains belonging to differing cytokine receptors would bring Tyk2 bound to IFNAR1 (Colamonici et al., 1994) into proximity of the IFNGR2-associated Jak2 (Pestka et al., 1997). Nevertheless, the role of Tyk2 in an IFNAR-IFNGR superstructure is indispensible, since neither Jak1 nor Jak2 is able to compensate for the signaling defects. In addition, we demonstrate that Tyk2 is required for normal levels of Stat1 protein. *Stat2*$^{-/-}$

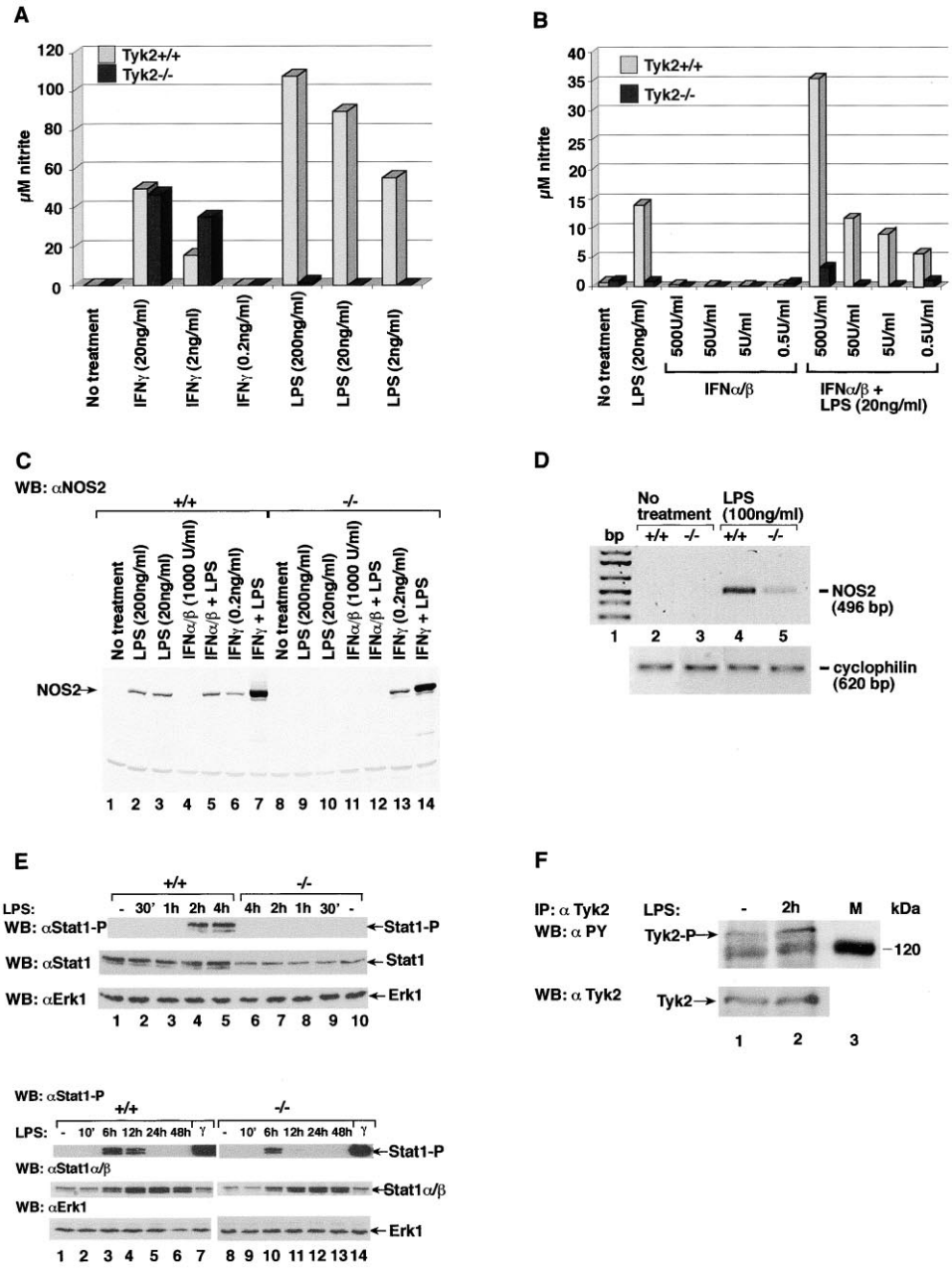

Figure 6. *Tyk2*$^{-/-}$ Macrophages Fail to Produce NO upon Stimulation with LPS or LPS Combined with Type I IFNs

Thioglycolate-elicited PMs were stimulated as indicated. The accumulation of nitrite in the culture supernatants was taken as an indicator of NO production and was determined by the Griess reaction.

(A) PMs were treated with IFNγ (titration 20 to 0.2 ng/ml) and LPS (titration 200 to 2 ng/ml) for 48 hr.

(B) PMs were stimulated with IFNα/β (titration 500 to 0.5 U/ml) alone or in combination with 20 ng/ml LPS for 38 hr.

(C) Expression of NOS2 protein in PMs stimulated as indicated for 48 hr. The protein concentration of the whole cell extracts was measured and normalized prior to loading. WB was performed as described (Bogdan et al., 1997).

(D) Induction of NOS2 mRNA in LPS-treated PMs. RT-PCR was performed from RNA extracted from cells treated with LPS (100 ng/ml) for 4 hr or left untreated. Equal amounts of input cDNA was controlled by simultaneous amplification of cyclophilin.

(E) Time course of Stat1 activation in PMs after LPS treatment. *Tyk2*$^{+/+}$ or *Tyk2*$^{-/-}$ PMs were stimulated with 100 ng/ml LPS for the times indicated. Whole cell extracts were analyzed for activation and protein level of Stat1 with the indicated Abs. Equal protein loading was controlled by probing of the WB for Erk1. Note that the blots are equalized to Stat1 levels. Without normalization, the Stat1-PY in *Tyk2*$^{-/-}$ PMs would be barely detectable.

(F) LPS-induced activation of Tyk2 in mouse macrophages. Bac1.2F5 cells were treated with LPS (100 ng/ml) for 2 hr. Tyk2 activation and protein levels were assayed as in Figure 2A.

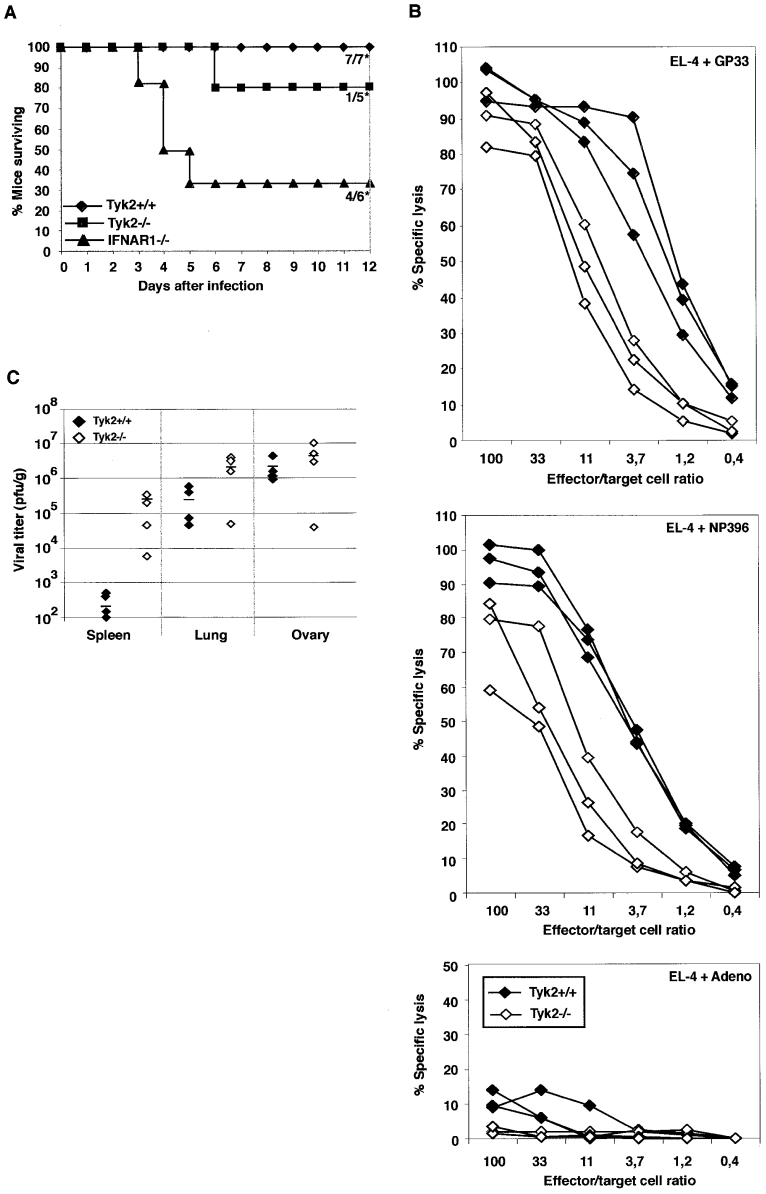

Figure 7. *Tyk2*$^{-/-}$ Mice Show Differing Susceptibility to Infection with Differing Viruses

(A) Survival rate of wild-type (rhombus) compared to *Tyk2*$^{-/-}$ (squares) and *IFNAR*$^{-/-}$ (triangles) mice after i.v. infection with 10^4 pfu of VSV. Survival was monitored for 12 days every 24 hr. Asterisk, number of surviving mice/total number of mice.

(B) Activity of CTLs after challenge with LCMV. CTLs from spleens of three member groups of *Tyk2*$^{-/-}$ (open symbols) and *Tyk2*$^{+/+}$ (closed symbols) mice were collected on day 8 after i.v. infection with 200 pfu of LCMV and cocultured with ^{51}Cr-labeled target cells loaded with the LCMV-specific peptides GP33 (top panel) or NP396 (middle panel) or an adenovirus-derived control peptide (bottom panel). Specific ^{51}Cr-release is shown.

(C) Clearance of virus from spleen after inoculation with VV. Four member groups of *Tyk2*$^{+/+}$ (closed symbols) and *Tyk2*$^{-/-}$ (open symbols) mice were challenged with 2×10^6 pfu of VV administered i.v., and viral titers in organs were determined 4 days postinfection. The values are compiled and shown for individual mice. Horizontal bars indicate mean values.

mice also show reduced Stat1 expression as well as an IFNγ signaling deficiency phenotype (C. Schindler, personal communication). In contrast, *IFNAR1*$^{-/-}$ cells have normal amounts of endogenous Stat1 protein (Takaoka et al., 2000). Thus, the Stat1 protein level appears to be regulated downstream of the IFNAR in a Tyk2/Stat2-dependent manner. Taken together, a possible explanation for our findings is the positive correlation of Stat1 protein level with the presence of active Tyk2 and Stat2 and/or the reduced cross-talk of the IFNR components in the membrane superdomains.

As observed for IFN signaling, Tyk2 deficiency only partially impairs the activation of the main signal transducer in the IL-12 response, i.e., Stat4. Previous studies have shown that the IL-12 promoted IFNγ production of differentiated T cells is completely disrupted in IL-12R-deficient (Magram et al., 1996) as well as in Stat4-deficient mice (Kaplan et al., 1996; Thierfelder et al., 1996). Therefore, an obvious explanation for the reduction of IL-12-mediated IFNγ production would be a direct causation with the reduced activation of Stat4 in *Tyk2*$^{-/-}$ mice. Deficiency of Tyk2 results in the complete impairment of phosphorylation of STAT3 after IFNα/β and IL-12 challenge. In accordance with these data, it has been shown that catalytically active Tyk2 is required for IFNα/β and IL-12-mediated phosphorylation of Stat3 (Bright et al., 1999; Rani et al., 1999). Stat3 deficiency leads to very early developmental stage lethality (Takeda et al., 1997). There is evidence for requirement of Stat3 for the full cellular response to IFNα/β (Yang et al., 1998). The biological role of Stat3 in IL-12 signaling is not yet clear. Mice with a Stat3 deficiency specifically in lymphoid and myeloid cells have not been analyzed in this context (Takeda et al., 1998, 1999). In vitro data suggested an involvement of Tyk2 and Stat3 in the IL-12-induced IFNγ production (Bright et al., 1999), although other work favored the importance of Tyk2 and Stat4 in this biological activity (see above and Ahn et al., 1998). At the IL-10R

and gp130 utilizing receptors, no Tyk2 dependency for Stat3 could be detected. This may be explained by receptor stochiometry, i.e., the proximity of Tyk2/Stat3 and/or the avaiablity of docking sites/molecules.

Role of Tyk2 in the Production of NO by Inflammatory Peritoneal Macrophages

Tyk2$^{-/-}$ PMs fail to produce NO after stimulation with LPS. Mouse macrophages treated with LPS produce a number of cytokines that are known to (co-)induce (e.g., IFNα/β, IFNγ, TNFα, IL-12, and IL-18) or to suppress the production of NO by NOS2 (e.g., IL-10 and TGFβ). For IFNα/β, it has been formally demonstrated that its neutralization ablates the induction of NOS2 by LPS or IFNγ/LPS (e.g., Gao et al., 1998). However, other endogenously produced cytokines that in some cases can cross-induce or antagonize each other might also affect the expression of NOS2 through autocrine/paracrine stimulation. Upon activation of the *NOS2* gene with the stimuli listed, the promoter has been described to engage the transcription factors IRF-1, NF-κB, HIF-1, Oct-1, and NF-IL6 in mouse macrophages (see Bogdan et al., 2000; MacMicking et al., 1997). In addition, a Stat1 binding site has been suggested to be required for optimal LPS/cytokine induction of the *NOS2* gene (Gao et al., 1997). The complete failure of LPS to induce NO accumulation (Figure 6) in the absence of Tyk2 (whereas the LPS-induced production of TNFα remained unimpaired [Figure 5B]) suggests that Tyk2 is the converging point for the signals controlling NOS2 activity. We show that the underlying mechanism is the affection of NOS2 expression on transcriptional level in the absence of Tyk2, with Stat1 being one central molecule downstream of Tyk2, albeit acting in a protein synthesis-dependent manner.

Selective Requirement of Tyk2 for Resistance to Viruses

The selective susceptibility to viruses in *Tyk2*$^{-/-}$ mice suggests that the defense against different types of infections depends on differing facets of Tyk2 action and/or their complexity. Resistance to VSV in mice has been shown to be mainly dependent on functioning IFNα/β signaling (van den Broek et al., 1995). The reduced expression of the IFNα/β inducible genes in Tyk2-deficient mice is apparently sufficient for establishing defense against VSV in vivo. IFNγ has been described to be important in protecting mice against VV infection (van den Broek et al., 1995, 2000). NO has been demonstrated to inhibit VV replication (Karupiah et al., 1993), although it may not suffice for virus clearance (Rolph et al., 1996; van den Broek et al., 2000). IL-12- and IFNβ-deficient mice were shown to be highly susceptible to VV (Deonarain et al., 2000; van den Broek et al., 2000). The elevated replication of VV observed in *Tyk2*$^{-/-}$ spleens may be due to observed defects in the IL-12 response alone, or the combination of impaired type I and II IFN signaling and NO production. The biological response to noncytopathogenic LCMV is dependent on specific and overlapping effects of the type I and type II IFN systems, since defects in either the IFNAR or the IFNGR lead to susceptibility to LCMV, which is increased in mice with a combined receptor defect (Müller et al., 1994; van den Broek et al., 1995). Despite the pivotal role of IFNs in the CTL activity induced by LCMV, it has been shown that in the absence of IFNα/β effects an alternative IL-12-dependent pathway was utilized (Cousens et al., 1999). NO seems to play, if any, only a minor role in the host response to LCMV (Bartholdy et al., 1999). Splenic leukocyte IFNγ responses were shown to be severely impaired during early LCMV infection; the underlying mechanisms appeared to be IL-12 independent (Pien and Biron, 2000). Recently, it was demonstrated that virus-induced type I IFNs, through activation of Stat1, negatively regulate IFNγ expression and that IFNα/β promotes IFNγ production in *Stat1*$^{-/-}$ mice (Nguyen et al., 2000). Therefore, the relatively mild LCMV-related phenotype in *Tyk2*$^{-/-}$ mice might be caused by the reduced availability of activated Stat1, which in turn leads to a diminished downregulation of the IFNγ defense mechanisms.

Role of Tyk2 in Immunity and Cytokine Signaling: Amplifier–Maintainer–Modulator–Selector?

The present study indicates an apparent indispensibility of Tyk2 for innate (such as the failure to produce NO after LPS exposure) and adaptive (such as the failure to develop a normal antiviral CTL activity after LCMV challenge) immune mechanisms. The results presented here support a model with Tyk2 playing an amplifying role in transducing signals by defined subgroups of cytokine receptors. Tyk2 as the amplifier rather than the initiator of signaling through the IFNα and gp130-containing receptors was suggested earlier in studies using human Jak-family member-deficient cell lines (Guschin et al., 1995; Pellegrini and Dusanter-Fourt, 1997). A selecting and modulating role of Tyk2 is supported by the specificity for Stat3 activation in IFNα/β and IL-12 signaling. In both response cascades, Stat3 is not considered to transduce the signals for the activation of the major biological responses. However, it can be envisaged that the Tyk2-Stat3 activation path accounts for specific subsets of functions mediated by these cytokines. Finally, Tyk2 seems to be responsible for the maintenance of normal Stat1 protein and activation levels.

Experimental Procedures

Cytokines, Antibodies, and Reagents

Purified recombinant, murine, or human cytokines were obtained from Calbiochem, Genzyme Corp., GIBCO-BRL, Insight Tech., and R&D Systems. For the NO assays, IFNs were kindly provided by G. Adolf, Ernst Boehringer Institut Vienna (rmIFNγ lot M3RD48) and I. Gresser, Institute Curie Paris(mIFNα/β lot T685). Cytokines were used at concentrations and times as indicated in the figures. Antibodies (Abs) were obtained from Santa Cruz Biotechnology, New England Biolabs, and Upstate Biotechnology. Anti-Stat1 Abs in Figure 2C and D, anti-Jak2 Abs in Figure 4B, and anti-NOS2 Abs in Figure 6C were as published (Müller et al., 1993; Bogdan et al., 1997; Kovarik et al., 1998). Anti-Ig peroxidase-conjugated Abs were obtained from Sigma Aldrich and Amersham Pharmacia Biotech. The TNFα and IFNγ ELISA Kits were purchased from R&D Systems.

Targeting Vector and Generation of *Tyk2*$^{-/-}$ Mice

A genomic clone containing exons 3–12 of the murine *Tyk2* gene was isolated from a λFixII murine 129/Sv genomic library (Stratagene) using the full-length human Tyk2 cDNA (GenBank Accession number X54637) as a probe. The isolated mouse *Tyk2* sequences are deposited in the GenBank (Accession number AF052607). A 2.4 kb EcoRI-

XhoI 5′ genomic fragment and a 5.3 kb HindIII-StuI genomic 3′ fragment were inserted into the pKOV920 vector (LexiconGenetics) containing the neomycin resistance expression cassette (GK-neo, derived from pKO SelectNeo, LexiconGenetics). The 5′ end of the targeting construct was fused to the herpes simplex virus thymidine kinase cassette (MC1-TK, derived from pKO SelectTK, LexiconGenetics). Twenty micrograms SalI linearized pTyk2KO was electroporated into 5×10^6 E14.1 ES cells (129/Sv/J) and grown under double selection as described (Neubauer et al., 1998). 500 colonies were screened for homologous recombination by PCR and targeting of four clones was confirmed by Southern blot analysis. Two independently isolated ES clones containing the correctly targeted *Tyk2* allele were injected into C57BL/6 blastocysts. Chimeric mice were backcrossed to C57BL/6 mice, and germline transmission of the mutant allele was confirmed.

Culture of Established Cell Lines and Primary Mouse Cells, Colony Forming Assays, and Mouse Strains

E14.1 ES cells (129/Sv/J) were cultured as described (Neubauer et al., 1998). The human 2fTGH cell line and the mutant U1A (11.1) derivative were cultured and selected as described (Müller et al., 1993). Bac1.2F5 mouse macrophages were cultured as described (Kovarik et al., 1999). Mouse embryonic fibroblasts (EFs) were prepared and cultured as described (Neubauer et al., 1998). EFs were immortalized by long time culture. Primary bone marrow macrophages (BMMs) were isolated from mice housed under specific pathogen free conditions and cultured as described (Takeda et al., 1999). Colony forming assays were performed as described (Parganas et al., 1998; Rodig et al., 1998). Thioglycolate-elicited peritoneal macrophages (PMs) were prepared and cultured as described (Bogdan et al., 1997). For the isolation of murine Concanavalin A (Con A) splenic blast cells, spleens were explanted from mice, and single cell suspensions were obtained by squeezing the organ using a cell strainer. Cells (5×10^7) were incubated in the presence of 2 μg/ml Con A (Amersham Pharmacia Biotech) in RPMI medium (Seromed) supplemented with penicillin/streptomycin (100 μg/ml, Seromed), 2-mercaptoethanol (0.05 mM, GIBCO-BRL), and FCS (10%, GIBCO-BRL). After 3 days of incubation, blast cells were purified performing a Ficoll density gradient (Amersham Pharmacia Biotech), and 1×10^7 cells were plated in supplemented RPMI medium. *IFNAR1*$^{-/-}$ mice were purchased from B&K Universal, Ltd., UK.

Immunoprecipitation and Western Blotting

For IPs and WB, 1–5 $\times$ 10^7 cells were lysed as described (Müller et al., 1993). The cell lysate was precleared for 1 hr, at 4°C in 40 μl protein-A sepharose (Amersham Pharmacia Biotech) and 10 μl (500 μg) of rabbit preimmune serum (Sigma). IP was performed with the indicated Abs overnight at 4°C. For Tyk2 IP, the lysis and preclear was performed at 250 mM NaCl while the IP reaction was done at 125 mM NaCl concentration. For crude extracts, the cells were lysed after stimulation directly in 2× Laemmli buffer or RIPA buffer. Fractionation of cellular extracts in vesicle-plasma membrane and soluble cytoplasmic protein fractions was performed as described (Eilers et al., 1996). Proteins were separated on SDS-8.0% PAGE and transferred to nitrocellulose (GeneScreen, NEN Life Science Products). Membranes were probed by using the indicated Abs and the ECL detection system (Amersham Pharmacia Biotech).

Northern Blot Analysis, RT-PCR, and cDNA Cloning

For the Northern blots experiments, total RNA was isolated from 1×10^7 cells using TRIzol (GIBCO-BRL). PolyA$^+$-RNA was isolated using the PolyATract mRNA Isolation System (Promega). RNA was fractionated on glyoxal/DMSO denaturing agarose gels, transferred to Hybond N$^+$ membrane (Amersham Pharmacia Biotech), and hybridized to ^{32}P-labeled probes. The following murine cDNA probes and primers (GenBank Accession number) were used: IRF-1 (M21065), GBP1 (M55544), Mx1 (M12279), cyclophilin (X52803), Tyk2 (AF173032), NOS2 (M84373) 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ and 5′GGCTGTCAGAGCCTCGTGGCTTTGG-3′, and cyclophilin (X52803) 5′-GACGCCACTGTCGCTTTTCG-3′ and 5′-CAGGACATTGCGAGC AGATGG-3′. For cloning of the full-length and aberrant mouse Tyk2 and for NOS2 analysis reverse transcription, PCR was employed using the SuperScript Preamplification System for First Strand cDNA Synthesis (GIBCO-BRL). The sequence of the murine Tyk2 cDNA is deposited in GenBank under Accession number AF173032.

Antiviral Responses

Lymphocytic choriomeningitis virus (LCMV strain WE) was propagated on L929 cells, vesicular stomatitis virus Indiana (VSV, Mudd-Summers isolate) was grown on BHK cells, and vaccinia virus strain WR (VV) was produced by infecting BSC 40 cells. Virus administration was intravenous (i.v.); the virus titers are indicated in pfu. To determine the primary LCMV-specific CTL activity, mice were infected; the lytic activity of spleen cells was tested in a 5 hr ^{51}Cr-release assay on LCMV peptide-pulsed EL-4 target cells as described (Zimmerman et al., 1996). The specific ^{51}Cr-release was calculated as percentage ratio of experimental minus specific release and total minus spontaneous release. To test susceptibility to VSV, infected mice were monitored for survival. Susceptibility to VV was examined as described (Binder and Kundig, 1991).

NO Assay, TNFα Assay, and IFNγ Assay

The accumulation of nitrite in the culture supernatants of PMs was taken as an indicator of NO production and was determined by the Griess reaction (detection limit1 μM) as described (Bogdan et al., 1997). For assaying TNFα, BMMs (5×10^4) were cultured in the presence of 100 ng IL-10. After 12 hr incubation, LPS (50 ng/ml) or IFNγ (1000U/ml) was added to the cultures for an additional 24 hr. Supernatants were harvested and used for measurement of TNFα production by ELISA. For the IFNγ assay, splenocytes were incubated alone, in the presence of 1 μg/ml Con A, or in the presence of 1 μg/ml Con A and 1 ng/ml IL-12. After 72 hr of incubation, the supernatant was collected, and the amount of IFNγ was measured by ELISA.

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