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J Immunol 2010; 185:3544-3553; Prepublished online 16 August 2010;
doi: 10.4049/jimmunol.0904000
http://www.jimmunol.org/content/185/6/3544
Tyrosine Kinase 2 Controls IL-1β Production at the Translational Level

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IL-1β is an important proinflammatory cytokine with a major role in several inflammatory diseases. Expression of IL-1β is tightly regulated at the level of transcription, mRNA stability, and proteolytic processing. In this study, we report that IL-1β expression in response to LPS is also regulated at the translational level. LPS-induced IL-1β protein levels in macrophages derived from murine bone marrow are markedly increased in the absence of tyrosine kinase 2 (Tyk2). Increased IL-1β is found intra- and extracellularly, irrespective of the efficiency of IL-1β processing. We show that the absence of Tyk2 results both in higher translational rates and in enhanced association of IL-1β mRNA with polysomes. Induction and stability of IL-1β mRNA are not affected by the lack of Tyk2. We show further that the Tyk2-dependent translational inhibition is mediated by autocrine/paracrine type I IFN signaling and requires signal transducer and activator of transcription 1. Enhanced IL-1β production in Tyk2- and IFN receptor 1-deficient macrophages is also observed following Listeria monocytogenes infection. Taken together, the data describe a novel mechanism for the control of IL-1β synthesis.


Tyrosine kinase 2 (Tyk2) is a member of the Janus kinase (Jaks) family of nonreceptor tyrosine kinases (1). Jak and signal transducers and activators of transcription (Stats) constitute the Jak/Stat signal transduction cascade that is used by many cytokines and some growth factors (2). Binding of ligands to their cognate receptors induces activation of JakS, subsequent auto- or transphosphorylation and phosphorylation of the signal transducing cytoplasmic receptor domains. These then serve as docking sites for Stats, which upon phosphorylation by JakS translocate as hetero- or homodimers to the nucleus, bind to specific consensus elements in promoter regions and activate transcription of target genes. Most prominently, Tyk2 is involved in signal transduction of type I IFNs and IL-12 (3, 4). Accordingly, mice lacking Tyk2 are more sensitive to most microbial infections studied so far (3, 5–10). By contrast, Tyk2 deficiency results in enhanced resistance against sterile inflammation and several inflammatory diseases (6, 11–15).

LPS is recognized by the TLR4 complex and leads to the activation of two main branches of signal transduction initiated at different intracellular sites. The myeloid MyD88-dependent pathway is essential for the activation of NF-kB and MAP kinases and the subsequent induction of proinflammatory cytokines, whereas the MyD88-independent pathway is required for the activation of IFN regulatory factor 3 and the expression of type I IFNs (16, 17). Type I IFNs bind to the receptor chains IFNAR1 and IFNAR2, thereby activating Tyk2, Jak1, and subsequently, mainly Stat1/Stat2 dimers. In combination with IFN regulatory factor 9 these form IFN-stimulated gene factor 3, which activates the transcription of a large number of IFN-responsive genes. Type I IFN receptor chains are widely expressed and thus autocrine/paracrine actions of type I IFNs contribute substantially to the complex biological responses to LPS treatment in most, if not all, cell types.

The central involvement of IL-1β in a wide range of inflammatory and autoimmune diseases makes it an attractive target for therapeutic interventions (18–20). IL-1β expression is believed to be regulated largely at the level of transcription and by its processing and release (21, 22). In addition, regulation of IL-1β mRNA stability via AU-rich elements (ARE) has been reported (23). Pro–IL-1β synthesis is induced by LPS through activation of the NF-kB and MAPK pathways (24, 25), although NF-kB can also negatively regulate IL-1β processing (26). Pro–IL-1β can be cleaved into the biologically active cytokine by several proteases in the extracellular space but caspase (casp) 1 is the main protease responsible for cleavage within macrophages (27–29). Recently, casp-8–dependent and casp-1–independent IL-1β maturation was reported in TLR2-primed, LPS-stimulated peritoneal macrophages (30). Casp-1 itself requires proteolytic processing for activation and this occurs within an activated inflammasome. LPS is a strong inducer of pro–IL-1β but a poor activator of the inflammasome and so only weakly induces the release of mature IL-1β unless a second stimulus triggers inflammasome activation (31–33).
By means of a two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) approach, we recently reported that lack of Tyk2 results in strong alterations of the macrophage proteome both before and after LPS stimulation (34). In the current study, we identify IL-1β as a protein that is strongly enhanced in the absence of Tyk2 upon LPS treatment in bone marrow-derived macrophages. Mechanistically, we show that Tyk2 limits IL-1β expression at the level of translation and provide evidence that this Tyk2 function is dependent on canonical IFN-α/β signaling.

Materials and Methods

Animals and cells
Tyk2, IFNAR1, and Stat1 knockout mice have been described previously (3, 35, 36), and were backcrossed to C57BL/6 background for at least 10 generations. Bone marrow-derived macrophages were isolated and grown in the presence of CSF-1 derived from L929 cells as described previously (37). Cells were cultivated for 6 d in DMEM supplemented with 10% FCS, 15% L929 cell conditioned medium, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM β-mercaptoethanol (complete medium [CM]) prior to the experiments. All cell culture reagents were from Invitrogen (Lofer, Austria). Mice were housed under specific pathogen-free conditions according to FELASA guidelines. All animal experiments were discussed and approved by the institutional ethics committee and conform with Austrian laws (68.205/0204-C/GT/2007 and 68.205/0233-II/10b/2009).

Reagents and preparation of whole cell lysates
Cells were treated with 100 ng/ml LPS (Escherichia coli serotype 055:B5, Sigma-Aldrich, St. Louis, MO) for the times indicated. IFN-β (Calbiochem, San Diego, CA) was used at the concentration of 100 U/ml. Actinomycin D (actD; Sigma-Aldrich) and cycloheximide (CHX; Sigma-Aldrich) were used at a concentration of 10 μg/ml. Whole cell lysates were prepared as described previously (34). Listeria monocytogenes (EGD) strain was kindly provided by Thomas Decker (Max F. Perutz Laboratories, University of Vienna, Vienna, Austria) and infections were performed as described (38).

2D-DIGE and protein identification by mass spectrometry
DIGE labeling, 2-DE separation, evaluation/statistical analyses and protein identification by MALDI-mass spectrometry (MS) and MS/MS have been performed as described (39) with minor modifications. Cells (1.5 × 10⁶) were lysed in 1 ml ice-cold buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM MgCl₂, 5% [v/v] NP-40, 500 U RiboLock) and nuclei removed by centrifugation at 3,000 × g, 4˚C for 2 min. The supernatant was supplemented with 20 mM DTT, 150 μg/ml CHX, and 1 mM PMSF and centrifuged at 15,000 × g, 4˚C for 5 min. Supernatants were layered onto 10 ml continuous 15–40% sucrose gradient (100 g/ml sucrose, 100 μg/ml CHX) and centrifuged at 38,000 rpm, 4˚C for 2 h (Sorvall SW41 rotor; ThermoFisher Scientific, Waltham, MA). Fractions (0.5 ml) were collected manually from top to bottom. Proteins were digested with 100 μg trypsin in the presence of 1% (w/v) SDS and 10 mM EDTA pH 8. RNA was extracted with 25:24:1 phenol-chloroform-isomyl alcohol (Invitrogen), supplemented with 1 μl glycogen (Sigma-Aldrich, ~20 mg/ml), 225 mM sodium acetate pH 5.2, and precipitated overnight with ethanol.

Reverse transcription-quantitative PCR
Reverse transcription-quantitative PCR (RT-qPCR) was performed as described previously (34). RT-qPCR of sucrose gradient-fractionated mRNA: two fractions each were pooled and 4 μl RNA was used for cDNA synthesis with iScript (Bio-Rad Laboratories, Vienna, Austria). Expression levels of the target genes (TNF-α, IL-1β, PAI2, NF-κB inhibitor α (NfκBia), and TATA box binding protein [TBP]) were determined by RT-qPCR in each pooled fraction and are given as percentages of the target mRNA in all fractions. Primers and probes: the primers and probes for TNF-α (12), Ube2d2 (7), and PAI2 (34) have been described previously. IL-1β was detected with a conventional gene expression assay (assay ID MM0043228_m1; Applied Biosystems). TBP and NfκBia were detected with EVAgreen (Biotium, Hayward, CA) using the following primers (5′–3′): TBP-fwd: 5′-GAA-TATAATCCCAAGGGAATTGC-3′ (Tm = 58˚C); TBP-rev: 5′-ACAGCAGGAGGAGAAGGCAC-3′ (Tm = 60˚C); NFκBia-fwd: 5′-TGGCCCATGTAGCAGTCTTTGAC-3′ (Tm = 60˚C); NFκBia-rev: 5′-GGGAGGAGGAGAAGGCAC-3′ (Tm = 59˚C), amplicon length: 117 bp.

Analysis of ELISA data
For the analysis of ELISA data of single treatments/time points t tests (Mann-Whitney) were used (Prism 5 software for Mac OS X; GraphPad Software, La Jolla, CA).

In vivo LPS challenge
Age- (8–10 wk) and sex-matched mice were injected i.p. with 1 mg/20 g body weight LPS. After 4 h, mice were anesthetized with ketamine-xylazine and blood was collected retrobulbarly. For peritoneal lavages, mice were sacrificed and peritonea flushed with 5 ml PBS.
Results

Intracellular pro–IL-1β in response to LPS treatment is increased in the absence of Tyk2 in bone marrow-derived macrophages

Using a 2D-DIGE and MALDI-MS approach, we recently reported a regulatory role for Tyk2 in the expression of a number of proteins before and after LPS treatment, whereby 21 of them were unambiguously identified (34). In a subsequent analysis, we identified IL-1β in a spot that was differentially expressed between WT and Tyk2−/− macrophages after LPS treatment (see Materials and Methods and Table I for details on MS). IL-1β was found in whole cell lysates in a spot with a Mr of ∼30 kDa and an isoelectric point around 4.6. The peptides identified were common to both mature (Mr of 17 kDa) and immature (Mr of 31 kDa) IL-1β but based on the Mr the spot could be assigned to the latter (pro–IL-1β). After 18 h, LPS treatment the levels of pro–IL-1β were found to be higher in Tyk2−/− cells than in WT cells (Fig. 1A, 1B). Spot volume ratios as determined by 2D-DIGE increased in WT cells upon 18 h LPS treatment by ∼3-fold, whereas a significantly stronger increase (∼14-fold) was found in the absence of Tyk2 (Fig. 1B). Enhanced levels of pro–IL-1β in whole cell lysates from Tyk2−/− macrophages were confirmed by Western blot time course experiments (Fig. 1C) and were detected from around 2 h after LPS stimulation onward. As reported previously (40–42), mature IL-1β was hardly detectable in cell extracts after LPS treatment (Fig. 1C).

IL-1β mRNA induction and stability are similar in WT and Tyk2−/− macrophages

Induction of IL-1β gene expression in response to LPS treatment in macrophages occurs via the MyD88-dependent pathway (43, 44), which is not believed to depend on Tyk2 (12, 34). Consistent with this notion, we found no significant differences in IL-1β mRNA levels between WT and Tyk2−/− macrophages in time-course experiments (Fig. 2A). IL-1β mRNA was induced rapidly within the first hour of LPS treatment in WT and Tyk2−/− cells, reached its maximal level expression around 12 h after LPS treatment and remained high for up to 20 h. As expected, TNF-α mRNA was induced to a similar extent in WT and Tyk2−/− macrophages (Fig. 2B). To determine also the effects of Tyk2 on mRNA stability, cells were treated with LPS for 4 h and subsequently incubated with the transcriptional inhibitor act D for the times indicated. mRNA levels of IL-1β and proinflammatory response genes as controls were determined at different times after treatment using RT-qPCR. IL-1β mRNA was stable (Fig. 2C) as described previously in the context of LPS responses (45) and no

Table I. Detailed MS data for the unambiguous identification of IL-1β (Swissprot accession no. P10749)

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FIGURE 1. Pro–IL-1β protein expression is elevated in the absence of Tyk2. Macrophages were treated with LPS for the indicated times and whole cell lysates were subjected to 2D-DIGE (A, B) or Western blot analysis (C). A. Selected regions from Cy3 and Cy5 images converted to gray scale showing pro–IL-1β spot positions (indicated by arrows). B. Protein expression levels are given as fold ratios relative to untreated WT cells. Mean values ± SD of three biological replicates are shown. ***p ≤ 0.01. C. Five microgram protein per lane were separated by 14%T SDS-PAGE and subjected to Western blot analysis. Membranes were probed with an anti–IL-1β Ab; protein loading was controlled by reprobing with an anti-panERK Ab.
difference was observed between WT and Tyk2\(^{-/-}\) cells. Consistent with other reports (45), mRNA of TNF-\(\alpha\) and NF\(\kappa\)Bia decayed rapidly. No differences between WT and Tyk2\(^{-/-}\) cells were observed for TNF-\(\alpha\) and NF\(\kappa\)Bia mRNA decay (Fig. 2D, 2E). Ubiquitin-conjugating enzyme E2 D2 (Ube2d2) mRNA, which was used as an endogenous control for most of the RT-qPCR analyses, was very stable and as expected its level was not affected by the absence of Tyk2 (Fig. 2F).

**Intra- and extracellular levels of IL-1\(\beta\) are enhanced in the absence of Tyk2**

IL-1\(\beta\) processing and release is tightly regulated. LPS stimulation alone massively induces pro–IL-1\(\beta\) production but processing and externalization occurs very inefficiently (31, 32, 46). Thus, IL-1\(\beta\) remains largely intracellular as unprocessed pro–IL-1\(\beta\) and only low levels of mature IL-1\(\beta\) can be detected in the extracellular space. Nevertheless, and to test whether the increased intracellular pro–IL-1\(\beta\) level in Tyk2\(^{-/-}\) cells is caused by reduced IL-1\(\beta\) processing and/or release, we determined the amount of extracellular IL-1\(\beta\) with ELISA. As shown in Fig. 3A, the amounts of IL-1\(\beta\) protein detected in the supernatants were low in WT cells with around 50–100 pg/ml at 24 h after LPS stimulation. The level of IL-1\(\beta\) was increased by \(\sim\)4- to 5-fold in Tyk2\(^{-/-}\) macrophage supernatants. Again consistent with previous studies (12), secreted levels of TNF-\(\alpha\) were similar in WT and Tyk2\(^{-/-}\) cells (Fig. 3B).

We next asked whether increased IL-1\(\beta\) production in Tyk2\(^{-/-}\) cells is also observed under conditions that promote maturation of IL-1\(\beta\) (32). Macrophages were treated for 4 h with LPS, followed by 30 min stimulation with the receptor agonist ATP (3 or 5 mM). In contrast to the 8 h time point (Fig. 3A), treatment with LPS alone for 4 h resulted in barely detectable levels of IL-1\(\beta\) and a slight, but not significant increase in Tyk2\(^{-/-}\) cells (Fig. 4A). As expected, extracellular levels of IL-1\(\beta\) in WT cells were dramatically higher (\(\sim\)1600 pg/ml) after addition of ATP (Fig. 4A). Importantly, significantly enhanced IL-1\(\beta\) levels were detected in the absence of Tyk2 and mature IL-1\(\beta\) was readily detectable at concentrations of 3 mM and 5 mM ATP, respectively. Importantly, mature 17 kDa IL-1\(\beta\)

**FIGURE 2.** IL-1\(\beta\) mRNA expression and stability is unaltered in Tyk2\(^{-/-}\) cells. Macrophages were treated with LPS for the times indicated and total RNA was subjected to RT-qPCR for IL-1\(\beta\) (A) or TNF-\(\alpha\) (B). mRNA expression levels were calculated relative to untreated WT cells, with Ube2d2 as endogenous control. Mean values \(\pm\) SE from at least three independent experiments are shown. No significant differences were found. Macrophages were treated with LPS for 4 h and act D was added for the indicated times (C–F). Total RNA was isolated and subjected to RT-qPCR analysis for IL-1\(\beta\) (C), TNF-\(\alpha\) (D), NF\(\kappa\)Bia (E), and Ube2d2 (F). Mean values \(\pm\) SD from three replicates derived from two independent experiments are depicted.
was also increased in supernatants of Tyk2-deficient cells (Fig. 4B). Intracellular, mature IL-1β was not detectable in WT cells treated with LPS/ATP (Fig. 4C). In contrast, mature IL-1β was detectable even intracellularly in Tyk2−/− cells after treatment with LPS and 5 mM ATP (Fig. 4C).

In summary, the data show that both intra- and extracellular levels of pro–IL-1β and mature IL-1β are increased in the absence of Tyk2 as compared with WT cells, independently of the presence of a trigger that promotes IL-1β processing.

**Limitation of IL-1β expression depends on the presence of IFNAR1 and Stat1**

In the context of macrophage LPS responses, Tyk2 is mainly associated with IFN-α/β signaling. Maximum levels of basal and/or induced expression of at least some genes that depend on functional IFN-α/β responses are only observed in the presence of Tyk2 (3, 7, 12). We thus asked whether the inhibitory role of Tyk2 in IL-1β expression may be extended to other signaling molecules of the type I IFN pathway. To this end, cells lacking IFNAR1 or Stat1 were treated with LPS for 4 or 18 h, with or without additional incubation with ATP. IL-1β expression was analyzed in supernatants by Western blotting (Fig. 5A). IL-1β levels were enhanced to a similar extent in IFNAR−/−, Tyk2−/−, and Stat1−/− cells at both times of treatment and, additionally, levels of processed IL-1β were also clearly higher than in WT cells. Similarly, enhanced pro-IL-1β and low levels of mature IL-1β were observed intracellularly in the absence of IFNAR1, Tyk2, or Stat1 after LPS treatment, independent of the presence of ATP (Fig. 5B). We note that our data on Stat1−/− cells contradict a previous report showing reduced extracellular levels of IL-1β protein upon LPS treatment in the absence of Stat1 in thioglycollate-elicited peritoneal macrophages (47). It seems likely that this discrepancy can be explained by differences in the macrophage populations used, namely, resting versus inflammatory.

To test whether similar effects occur in response to infection with a pathogen that readily activates the inflammasome (48), we infected macrophages with *Listeria monocytogenes* and monitored IL-1β levels. IL-1β concentrations in supernatants of infected cells were in the range of those found in LPS/ATP-treated cells. Again, increased IL-1β was found in whole cell extracts (Fig. 5C) and cell supernatants (Fig. 5D) in the absence of either Tyk2 or IFNAR1. IL-1β secretion occurred more efficiently in cells infected with *L. monocytogenes* than after LPS/ATP treatment, as we could not detect any mature IL-1β in whole cell extracts (data not shown). Extracellular IL-1β amounts as detected by ELISA were increased around 5- to 10-fold in both Tyk2−/− and IFNAR1−/− deficient as compared with WT cells (Fig. 5D). In contrast, the level of TNF-α was similar in WT and Tyk2−/− macrophages and interestingly decreased in IFNAR1−/− deficient cells (Fig. 5E).

Based on the conclusion that LPS-induced, autocrine/paracrine IFN-α/β signaling can inhibit IL-1β protein expression, we tested whether addition of exogenous IFN-β can decrease IL-1β production. For that purpose, WT and Tyk2−/− cells were pretreated with IFN-β for 1 h, or left untreated, and then subsequently treated with LPS for 24 h, or left untreated. A clear decrease in IL-1β protein expression could be observed in WT and Tyk2−/− cells that were pre- or cotreated with IFN-β, whereby pretreatment had a more pronounced effect (Fig. 6A, data not shown). These data confirm previous observations in human whole blood cells (49) where type I IFN treatment was found to decrease IL-1β protein production. IFN-β pretreatment decreased intracellular pro–IL-1β levels by ∼10-fold in WT cells as determined by quantification of Western blot data (Fig. 6B). As expected, and in accordance with
We next determined whether IL-1β enhanced protein synthesis. IL-1β for 4 h, the translational inhibitor CHX was added and IL-1β mRNA and protein levels were increased in Tyk2−/− cells compared with WT cells at 4 h of LPS stimulation. IL-1β protein levels declined after 6 h and 12 h addition of CHX in both WT and Tyk2−/− cells (Fig. 7A) and no significant differences could be observed.

Because the protein synthesis inhibitor CHX might introduce artifacts, for example, by blocking the expression of a labile protein, we confirmed the results by metabolic labeling with [35S]methionine/cysteine in pulse-chase experiments. Cells were treated with LPS for 4 h, labeled with [35S]methionine/cysteine (pulse), washed and further incubated in the presence of excess cold methionine/cysteine for various times (chase). We found clear differences in IL-1β synthesis between WT and Tyk2−/− cells during the 1 h and 2 h pulse periods (Fig. 7B, 7C). The observed half-life of IL-1β in WT cells was similar to that previously described (50) and not greatly different between WT and Tyk2-deficient cells (Fig. 7B, 7D). The data thus indicate that Tyk2 deficiency results in enhanced translation of IL-1β rather than in increased protein stability.

Association of IL-1β mRNA with polysomes is enhanced in the absence of Tyk2

To test directly whether Tyk2 influences the translation of IL-1β mRNA, we fractionated cytoplasmic RNA from LPS-treated WT and Tyk2−/− macrophages via sucrose gradients (Fig. 8A) and compared mRNA distributions among the different fractions (Fig. 8B–E). After 4 h LPS stimulation of WT cells, IL-1β mRNA was found in the ribosome-free fractions as well as in association with polysomes (Fig. 8B). In contrast, IL-1β mRNA was significantly enriched in the polysomal fractions in Tyk2−/− cells (p = 0.019). We also observed differences in the IL-1β polysome profiles after 14 h of LPS treatment, although IL-1β mRNAs were generally shifted toward the ribosome-free mRNA fractions (data not shown). As previously reported (51, 52), TNF-α mRNA was found in monosomal and polysomal fractions in WT cells treated with LPS (Fig. 8C). In accordance with the unchanged expression of TNF-α protein, TNF-α mRNA showed similar polysome profiles in WT and Tyk2-deficient cells (Fig. 8C). Furthermore, we observed no difference in mRNA profiles of the two housekeeping genes, Weilflog and Lysozyme, in WT and Tyk2−/− macrophages (Fig. 8D). These results suggest that the increased IL-1β mRNA synthesis in the absence of Tyk2 is not due to a general increase in protein synthesis but rather to a selective increase in the translation of IL-1β mRNA.

The increased amount of IL-1β in Tyk2−/− cells is caused by enhanced protein synthesis

We next determined whether IL-1β protein stability is influenced by the absence of Tyk2. Macrophages were stimulated with LPS for 4 h, the translational inhibitor CHX was added and IL-1β protein expression monitored over time. As described previously, IL-1β protein levels were increased in Tyk2−/− as compared with WT cells at 4 h of LPS stimulation. IL-1β protein levels declined after 6 h and 12 h addition of CHX in both WT and Tyk2−/− cells (Fig. 7A) and no significant differences could be observed.

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genes, TBP and Ube2d2 (Fig. 8D, data not shown). To assess the specificity of the translational effects, we monitored plasminogen activator inhibitor 2 (PAI2/serpinb2) mRNA. We had previously identified PAI2 as an LPS-inducible protein whose expression is regulated posttranscriptionally by Tyk2-dependent mechanisms (34). In support of and extending these results, we show that significantly more PAI2 mRNA associates with polysomes in the absence of Tyk2 (Fig. 8E, \( p = 0.008 \)).

Local IL-1\( \beta \) levels are increased in the absence of Tyk2 after LPS challenge in vivo

Previous studies have not reported differences in the in vivo IL-1\( \beta \) levels in Tyk2-deficient mice treated with LPS (12). Because this analysis was limited to systemic cytokine levels and to very early time points after LPS injection, we examined in more detail whether Tyk2 might have an inhibitory role on IL-1\( \beta \) production in vivo. WT and Tyk2\(^{-/-} \) mice were injected i.p. with LPS and IL-1\( \beta \) was measured in peritoneal lavages and in sera. As shown in Fig. 9A, IL-1\( \beta \) levels were increased ~2-fold in the peritonea of Tyk2\(^{-/-} \) as compared with WT mice at 4 h post injection. Although the increase in IL-1\( \beta \) was less pronounced than in macrophages in vitro, the result supports our model for an inhibitory role of Tyk2 on IL-1\( \beta \) production. In contrast, serum levels of IL-1\( \beta \) were slightly lower in Tyk2\(^{-/-} \) mice at 4 h after challenge (Fig. 9B), suggesting differences in the regulation of local versus systemic IL-1\( \beta \). Alternatively, kinetic differences in the regulatory pathways might lie behind the apparent differential effect of Tyk2 deficiency on IL-1\( \beta \) levels in the peritoneal cavity and systemically.

Discussion

We show that IL-1\( \beta \) protein expression in response to LPS is strongly increased in the absence of Tyk2 and that the effect does not stem from changes to IL-1\( \beta \) mRNA induction or stability (Figs. 1A–C, 2A, 2C). The lack of inhibition of IL-1\( \beta \) mRNA induction is in accordance with previous observations that activation of the NF-κB and MAPK pathways is normal in Tyk2-
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FIGURE 8. Polysome profile of IL-1β, TNF-α, TBP, and PAI2 mRNAs. Macrophages were treated with LPS for 4 h and cytoplasmic extracts separated in a continuous 15–40% sucrose gradient by ultracentrifugation. Fractions were manually collected from top to bottom and deproteinized and RNA was extracted. A. Polysomal fractions were separated on 0.8% agarose gels. Representative results are shown for WT and Tyk2−/− macrophages. B–E. Two fractions each were pooled and mRNA levels determined by RT-qPCR. Amounts of the mRNA per fraction for IL-1β (B), TNF-α (C), TBP (D), and PAI2 (E) are given as percentage of mRNA present in all fractions of each genotype. Mean values ± SD of three independent experiments are shown. *p ≤ 0.05; **p ≤ 0.01.

deficient macrophages (12, 34). We can eliminate the possibility that defects in processing/release of IL-1β lie behind the enhanced intracellular IL-1β levels, as IL-1β was increased in cell supernatants (Fig. 3A). Under conditions that efficiently activate the inflammasome and pro–IL-1β conversion (i.e., ATP treatment), the levels of mature IL-1β in Tyk2-deficient macrophages are enhanced (Figs. 4B, 4C, 5A, 5B). Similarly, intra- and extracellular IL-1β levels were enhanced in Tyk2−/− macrophages infected with L. monocytogenes (Fig. 5C, SD). By pulse–chase experiments and using the translational inhibitor CHX, we show that IL-1β protein stability does not depend on the presence of Tyk2 (Fig. 7A, 7B, 7D). In contrast, IL-1β protein synthesis within a given pulse-period is enhanced in Tyk2−/− cells (Fig. 7B, 7C), suggesting an increased rate of translation. In line with this finding, IL-1β mRNA association with polysomes is enhanced in the absence of Tyk2 (Fig. 8B). The association of PAI2 mRNA with polysomes also increases (Fig. 8E), providing a mechanistic explanation for the posttranscriptional upregulation of PAI2 in Tyk2−/− cells that has been reported previously (34). Importantly, TNF-α mRNA shows similar polysome profiles in WT and Tyk2−/− cells (Fig. 8C) and similar levels of TNF-α protein are found in cell supernatants (Fig. 3B). Hence, the translational inhibition mediated by Tyk2 is specific, at least to some degree.

An increase in IL-1β protein level is observed in IFNAR1−/− and Stat1−/− macrophages, both intra- and extracellularly and independent of ATP treatment (Fig. 5A, 5B). Furthermore, IFN-β pretreatment inhibits LPS-induced IL-1β protein expression in WT cells, and to a lesser extent in Tyk2−/− cells, without affecting TNF-α levels (Fig. 6). This argues that autocrine/paracrine canonical IFN-α/β signaling is the signaling cascade involved, although we have as yet no direct evidence for an effect of IFNAR1 and Stat1 on translational control of IL-1β.

IFN-mediated translational inhibition has been known for a long time (53–55) and the underlying mechanisms have been extensively studied. Activated IFN-inducible RNA-dependent protein kinase phosphorylates the α subunit of eukaryotic translation initiation factor 2A, which results in the inhibition of viral protein synthesis (56, 57). Interestingly, Tyk2 has been reported to activate protein kinase R directly (58). Two other closely related and IFN-inducible proteins, ISG54 (IFIT2) and ISG56 (IFIT1), can negatively regulate translation (59–62) by inhibiting the formation of the translation initiation complex, although they target different steps (63). Interestingly, the level of stimulation of both IFIT1 and IFIT3 (another member of the IFIT protein family) in response to LPS is reduced in the absence of Tyk2 (34). Little is known about the specificity of these pathways and it is difficult to explain the effects on the translation of IL-1β and PAI2 but not TNF-α and NFκBia mRNAs (Fig. 8). It is noteworthy that IL-1β and PAI2 mRNAs are both fairly stable, whereas TNF-α and NFκBia mRNAs

FIGURE 9. Effect of Tyk2 deficiency on IL-1β production in vivo. WT and Tyk2−/− mice were injected with LPS (1 mg/20 g body weight) i.p. IL-1β was measured 4 h after challenge in (A) peritoneal lavages and (B) sera by ELISA. Mean values ± SE for (A) 16 or (B) 15 mice from three independent experiments are depicted. *p ≤ 0.05.
Tyk2 exerts protective functions: Tyk2-induced arthritis (76). Tyk2 seems not to act protectively during induced local inflammation, contact hypersensitivity, and collagen-eases. In mice, IL-1β might have protective functions during IL-1β on IL-1β-resistant phenotype. Nevertheless, the suppressive role of Tyk2 is relevant in vivo, although its specific contribution to the complex regulation of IL-1β production, might override the effects of Tyk2 on IL-1β expression.

In summary, we report a hitherto undescribed negative regulatory function for Tyk2 on IL-1β expression in response to LPS. We show that this occurs at the level of translation and most probably depends on canonical type I IFN signaling.

Acknowledgments
We thank Claus Vogl for supervision of statistical analysis. We also thank Caroline Lassnig and Graham Tebb for critically reading the manuscript. We thank Thomas Kolbe for mouse breeding and coordination.

Disclosures
The authors have no financial conflicts of interest.

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