Cell Death & Differentiation (2021) 28:748–763
https://doi.org/10.1038/s41418-020-00621-x

Cell Death & Differentiation

ARTICLE

TYK2 licenses non-canonical inflammasome activation during endotoxemia

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Received: 7 January 2020 / Revised: 31 August 2020 / Accepted: 1 September 2020 / Published online: 14 September 2020

Abstract

The non-canonical inflammasome is an emerging crucial player in the development of inflammatory and neurodegenerative diseases. It is activated by direct sensing of cytosolic lipopolysaccharide (LPS) by caspase-11 (CASP11), which then induces pyroptosis, an inflammatory form of regulated cell death. Here, we report that tyrosine kinase 2 (TYK2), a cytokine receptor-associated kinase, is a critical upstream regulator of CASP11. Absence of TYK2 or its kinase activity impairs the transcriptional induction of CASP11 in vitro and in vivo and protects mice from LPS-induced lethality. Lack of TYK2 or its enzymatic activity inhibits macrophage pyroptosis and impairs release of mature IL-1β and IL-18 specifically in response to intracellular LPS. Deletion of TYK2 in myeloid cells reduces LPS-induced IL-1β and IL-18 production in vivo, highlighting the importance of these cells in the inflammatory response to LPS. In support of our data generated with genetically engineered mice, pharmacological inhibition of TYK2 reduced LPS-induced upregulation of CASP11 in bone marrow-derived macrophages (BMDMs) and of its homolog CASP5 in human macrophages. Our study provides insights into the regulation of CASP11 in vivo and uncovered a novel link between TYK2 activity and CASP11-dependent inflammation.

Introduction

Tyrosine kinase 2 (TYK2) is a member of the Janus kinase (JAK) family of receptor-associated tyrosine kinases, which mediates signalling by a large number of cytokines and some growth factors. TYK2 is ubiquitously expressed and transduces responses to cytokines that utilize the type I interferon (IFN) receptor chain 1 (IFNAR1), interleukin-10 receptor 2 (IL-10R2), IL-12Rβ1, and IL-13Rα1. TYK2 drives immunity to viral and bacterial infections in humans and mice [1–3]. By contrast, TYK2 aggravates inflammatory and autoimmune diseases, as established by studies with TYK2-deficient ($Tyk2^{-/-}$) mice and supported by genome-wide association studies (GWAS) in humans [4]. TYK2 inhibitors have been developed for the treatment of inflammatory diseases, with some of them being already investigated in clinical trials [5, 6]. In addition to its functions as signal-transducing kinase, TYK2 has functions that do not rely on its enzymatic activity, although these are still incompletely understood [3, 7–9]. A better understanding of how TYK2 integrates signalling inputs in different pathophysiological conditions and which of its functions depend on enzymatic activity is urgently needed.

Edited by C Borner.

Supplementary information The online version of this article (https://doi.org/10.1038/s41418-020-00621-x) contains supplementary material, which is available to authorized users.

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Inflammatory responses are essential for the host defence against pathogens but, if unbalanced, can lead to septic shock and multiorgan failure. The pathogenesis of severe systemic inflammation is not fully understood and remains a major problem in health care due to high mortality rates [10]. Lipopolysaccharide- (LPS-) induced endotoxemia is an experimental model of hyperinflammation induced by Gram-negative bacterial compounds [10, 11]. *Tyk2*$^{-/-}$ mice are protected from LPS-induced endotoxin shock [12–14]. The underlying mechanisms are largely unknown but have been linked to the role of TYK2 in IFNα/β signalling, including the induction of IL-27p28, and IL-12-mediated induction of IFNγ [12–14]. We have shown previously that TYK2 suppresses pro-IL1β production at the translational level by IFNα/β-dependent mechanisms, which correlates with an increase in IL-1β release upon activation of the canonical NLRP3 (NLR family pyrin domain containing 3) inflammasome in *Tyk2*$^{-/-}$ macrophages and increased local, but not systemic, IL-1β at early time points after LPS challenge in vivo [15].

In this study, we used *Tyk2*$^{-/-}$ mice and mice that express kinase-inactive TYK2 (*Tyk2*^{K923E} mice) to investigate the role of TYK2 and its enzymatic activity in the pathogenesis of endotoxemia. We uncovered a crucial role of TYK2 signalling in the induction of caspase-11 (CASP11) and the non-canonical inflammasome-dependent release of IL-1β and IL-18. We provide evidence that during endotoxemia this pro-inflammatory function of TYK2 is mainly exerted by myeloid cells (i.e. LysM-positive cells). In line with the genetic deletion or inactivation of TYK2, pharmacological inhibition of TYK2 in macrophages markedly inhibited LPS-induced upregulation of murine CASP11 and its human homolog CASP5. Our data suggest that the absence or inactivation of TYK2 protects from inflammation-associated mortality by impairing the upregulation of CASP11, the central component of the non-canonical inflammasome.

Methods

Mice and ethics statement

Wild-type (*WT*, C57BL/6N; purchased from Charles River Laboratories), *Tyk2*$^{-/-}$, *Tyk2*^{K923E}, *Tyk2*$^{fl/fl}$, *Tyk2*$^{\Delta LysM}$, *Ifnar1*$^{-/-}$ and *Ifngr1*$^{-/-}$ mice [16–20] were bred at the University of Veterinary Medicine Vienna under specific-pathogen-free conditions according to Federation of European Laboratory Animal Science Associations (FELASA) guidelines. All animal experiments were approved by the ethics and animal welfare committee of the University of Veterinary Medicine Vienna and the national authority (Austrian Federal Ministry of Science and Research) according to §§ 26ff. of Animal Experiments Act, Tierversuchsgesetz 2012—TVG 2012 (BMWFW-68.205/0032-WF/II/3b/2014) and conform to the guidelines of FELASA and ARRIVE (Animal Research: Reporting of In Vivo Experiments).

In vivo LPS challenge

Age-matched (8–12-weeks-old) female mice on C57BL/6N background were used for all experiments. Mice were injected intraperitoneally (i.p.) with 50 mg/kg LPS from *Escherichia coli O55:B5* (Sigma) in 200 µl PBS or with PBS only as control. Depending on the read-out, blood and organs were harvested after 2, 6, 16 or 24 h. Survival was monitored for 7 days, body weight and the body temperature of the mice (rectal temperature) was determined twice daily. Severity of disease was assessed on the basis of an established scoring system, including criteria such as appearance (eyes, coat/fur), mobility, behaviour, posture and response to external stimuli. Humane endpoint by cervical dislocation was conducted upon reaching the threshold sickness score. Animal experiments were performed by trained and authorized personnel.

Measurement of glucose and beta-ketones and adipose tissue isolation

Glucose and beta-ketone levels in the blood were measured directly before and 6 h after LPS application. Therefore, a tail vein was punctured and a small blood drop taken with the respective test strips (FreeStyle Precision, Abbott). 24 h after LPS treatment, mice were sacrificed and inguinal white adipose tissue (WAT) and the interscapular brown adipose tissue (BAT) were harvested and weighed.

Histopathology

Organs (lung, liver, spleen and kidney) were harvested 16 h after LPS injection and transferred immediately into 4% buffered formaldehyde for fixation. Lungs were inflated with 4% buffered formaldehyde prior to fixation. After 48 h at 4 °C in formaldehyde, organs were washed with and stored in 70% ethanol before dehydrating, embedding in paraffin and cutting 2 µm sections. Sections were stained with haematoxylin and eosin (H&E) and analysed by a certified pathologist in a blinded fashion. The lungs were scored for two pathological alterations, which characterize interstitial pneumonia: (1) alveolar septal thickening caused by infiltration of inflammatory cells, fibrin exudation and interstitial oedema and (2) oedema affecting the interstitium except the alveolar walls. Both changes were graded from 0 to 3 (0 = no alterations, 1 = mild, 2 = moderate, 3 = severe

alterations). The two scores were summed up and the total interstitial pneumonia score is given in the figure.

Immunohistochemistry

For immunohistochemical staining, sections were deparaffinized, rehydrated and endogenous peroxidase activity was blocked with 2% H_2O_2. Antigen retrieval was performed with 20 µg/ml proteinase K (for F4/80 staining), 0.01% pronase (for neutrophil staining with NIMP-R14) or citrate-based antigen unmasking solution (pH 6, Vector labs; for cleaved caspase-3 staining), followed by several blocking steps. Sections were incubated overnight at 4 °C (anti-cleaved caspase-3 antibody or anti-neutrophil antibody NIMP-R14) or for 1 h at room temperature (anti-F4/80 antibody). Antibodies and dilutions are given in Supplementary Table 1. After incubation with the respective secondary antibody, stainings were visualized with the Vectastain ABC kit and DAB substrate (Vector labs) and sections were counterstained with haematoxylin. For quantifications, 5 pictures per section were taken with the upright research microscope Axio Imager Z.2 (Carl Zeiss) with 20-fold magnification and positive signals from each picture were counted with the Fiji software (http://fiji.sc) and are shown as mean/section.

Luminex bead-based multiplex assay

Systemic cytokine levels were measured with the Luminex bead-based multiplex assay (Thermo Fisher Scientific). Blood was collected via the retrobulbar venous plexus and either serum or plasma was separated, depending on additional analysis needed from the samples. ProcartaPlex Simplex Kits from the cytokines of our interest were used and samples processed according to the manufacturer's instructions. Analysis was performed with the Luminex Bio-Plex 200 system and the Bio-Plex Manager software (Bio-Rad).

Isolation and stimulation of bone marrow-derived macrophages (BMDMs)

BMDMs were isolated and grown as described [15]. Briefly, bone marrow was flushed out from both hind legs and BMDMs were grown and differentiated at 37°C and with 5% CO_2 for 7 days in BMDM growth medium (high glucose DMEM, supplemented with 10% FCS, 100 U/ml penicillin/100 µg/ml streptomycin, 2 mM L-glutamine, 50 µM β-mercaptoethanol and 15% L929 cell-conditioned medium). On day 7, 1×10^6 cells/well were seeded into six-well cell culture plates and treated on day 8 in 1.2 ml Opti-MEM reduced serum medium (Gibco)/well as follows: unstimulated (medium only), mock transfection [FuGene® (Promega) 0.25% (v/v)], LPS transfection [FuGene® (Promega) 0.25% (v/v) and 2 µg/ml LPS] or LPS (LPS 100 ng/ml). For LPS + ATP, 3 mM ATP (Sigma) was added 16 h after LPS (LPS 100 ng/ml) treatment for 2 h.

For the pharmacological inhibition of TYK2, cells were treated with the indicated dose of the allosteric TYK2 inhibitor BMS-986165 (MedChemExpress) or with DMSO as control (0.01 % v/v) together with LPS (100 ng/ml) in growth medium for 18 h.

Culture, differentiation and stimulation of U937 cells

U937 cells were kindly provided by Thomas Decker (Max Perutz Labs, Vienna BioCenter, Vienna, Austria) and originally obtained from ATCC (ATCC® CRL-1593.2™). Cells were tested negative for mycoplasma and were cultured in U937 growth medium (RPMI-1640 medium, supplemented with 10% FCS and 100 U/ml penicillin/100 µg/ml streptomycin), at 37°C and 5% CO_2. Prior to the experiments, U937 cells were differentiated into macrophages as described [21] with minor modifications: cells were treated with 10 nM PMA (1×10^6 cells/well of six-well cell culture plates) for 48 h, washed with pre-warmed PBS and kept in growth medium for another 24 h. Stimulations were performed as described for BMDMs.

Cell lysis and trichloroacetic acid (TCA) precipitation

Cells were lysed in 150 µl lysis buffer containing 50 mM Tris/HCl pH 8, 0.5% NP-40 (Igepal CA-630), 10% (v/v) glycerol, 0.1 mM EDTA pH 8, 150 mM sodium chloride, 25 mM sodium fluoride, 2 mM DTT, 0.2 mM sodium orthovanadate, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 µg/ml pepstatin. U937 cells were directly lysed in 100 µl 1x Laemmli sample buffer and immediately incubated at 99 °C for 10 min (shaking at 1400 rpm).

Cell culture supernatant was collected for TCA precipitation: 900 µl were mixed with 13.5 µl 10% (w/v) sodium deoxycholate monohydrate and 300 µl ice-cold 6.1 M TCA and incubated for 1 h on ice. Samples were centrifuged for 15 min at 20,000 × *g* at 4 °C and pellets were washed 3 times with ice-cold acetone. After the last washing step, pellets were air-dried for 10 min on ice, dissolved in 20 µl 0.2 M NaOH and incubated for another 10 min on ice. The samples were vortexed, 30 µl of ultrapure water was added and again incubated for 10 min on ice. In all, 50 µl of 2x Laemmli sample buffer was added to each sample.

Organ homogenization and western blot

Organs were harvested 16 h after LPS injection, shock frozen in liquid nitrogen and stored at −80 °C. For protein

extraction, organs were homogenized with the 1600 MiniG tissue homogenizer at 1500 rpm for 30 s (Spex SamplePrep) in lysis buffer (as described above for BMDMs). Cell and organ lysates were centrifuged to remove cell debris (16,000 × *g*). Lysates were mixed with 2x Laemmli sample buffer and boiled for 5 min at 95 °C. In all, 15 µg of total cell lysate from spleens, 5–10 µg from BMDM lysates, 10 µl from U937 lysates or 15 µl of TCA-precipitated cell culture supernatants were loaded per lane. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose (for the detection of GSDMD, CASP4 and CASP5) or PVDF membranes. Membranes were probed with the primary antibodies overnight at 4 °C. Incubation with the secondary antibodies was for 1 h at room temperature. The detection of chemiluminescence was performed by incubating the membrane with Clarity Western ECL substrate (Bio-Rad) and using ChemiDoc Touch Imaging System (Bio-Rad). Antibodies and dilutions are given in Supplementary Table 1.

RNA extraction and RT-qPCR

For total RNA extraction, organs were homogenized (as described above) in peqGOLD TriFast reagent (Peqlab) and cDNA was generated with the iScript cDNA Synthesis Kit (Bio-Rad). qPCR for *Il1b* was performed on the Stratagene MX3000 instrument (Agilent Technology), all others were performed on the CFX Touch Real-Time PCR Detection System (Bio-Rad). *Ube2d2* was used as housekeeping gene. See Supplementary Table 1 for assays, primers and probes.

Propidium iodide and Hoechst staining

Propidium iodide (PI) and Hoechst (ThermoFisher) were mixed in PBS to concentrations of 2 µg/ml (PI) and 5 µg/ml (Hoechst). BMDMs were incubated with the staining solution for 10 min at 37 °C in the dark and washed twice with PBS. For quantification, pictures were taken with the inverted microscope Axio Observer Z1 (Carl Zeiss) from three different regions. Positive signals for PI and Hoechst were counted with the Fiji software (http://fiji.sc). Results are shown as % PI-positive cells (out of Hoechst-positive cells), mean values from three pictures are given.

Lactate dehydrogenase measurement

Lactate dehydrogenase (LDH) measurement in cell culture supernatants was performed as described [22, 23]. In brief, 25 µl sample was mixed with 25 µl assay substrate (12 mg/ml lactate, 0.66 mg/ml INT, 4.5 U/ml diaphorase, 0.01% BSA, 0.4% sucrose, 1 mg/ml NAD^+) and incubated at room temperature for 15 min. In all, 25 µl stop solution (1 M acetic acid) was added and the read-out performed at 490 nm (wavelength correction at 570 nm) on the Epoch Microplate Spectrophotometer (BioTek). Results are presented as % LDH release and mock transfected BMDMs served as control.

Reagents

For detailed information about chemicals, antibodies, assays and primers used in this study, see Supplementary Table 1.

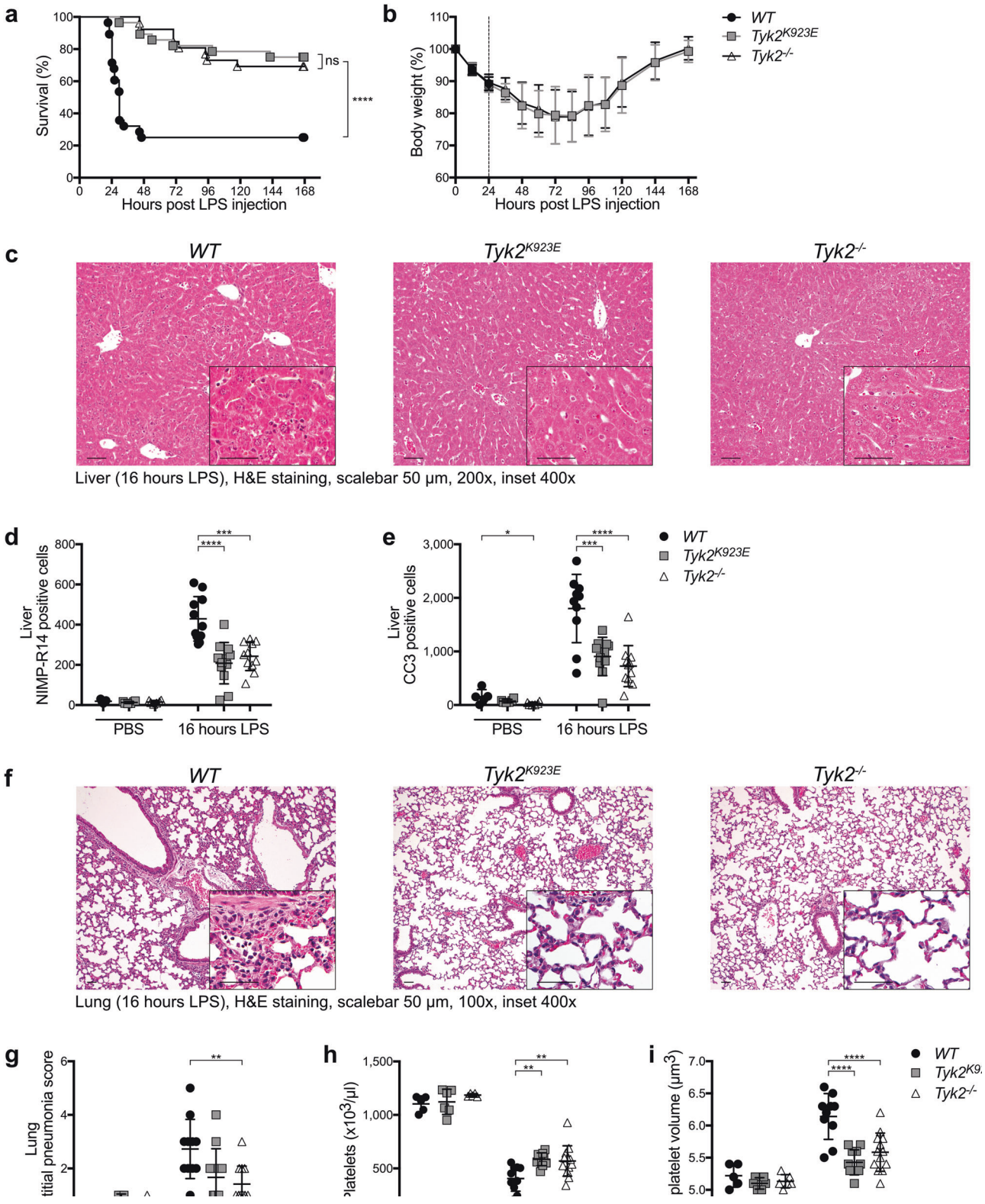
Statistical analysis

Log-rank (Mantel-Cox) test (Fig. 1a), Kruskal–Wallis test (Fig. 1g), unpaired two-tailed *t*-test with Welch correction (Figs. 4a and 6a, b), One-way ANOVA followed by Tukey's multiple comparisons test (Figs. 4j and 5e), Brown-Forsythe and Welch ANOVA followed by Dunett's multiple comparison test (Fig. 2a–c), or Two-way ANOVA followed by Bonferroni's (Fig. 6e) or Tukey's (Figs. 1b, h, i, 4i, 5d and 6f) multiple comparisons test. PBS and LPS groups were analysed separately (one-way ANOVA followed by Tukey's multiple comparisons test) when data did not meet the assumption of equal variance (Figs. 1d–e and 3a–d). Statistical analysis was performed using GraphPad Prism 8 for Mac (GraphPad Software). Sample size and number of experiments are given in the figure legends. Significance was defined as follows: $*p \le 0.05$, $**p \le 0.01$, $***p \le 0.001$, $****p \le 0.0001$.

Results

TYK2 aggravates LPS-induced endotoxin shock, thrombocytopenia and histopathological changes in liver and lung

To test whether kinase-independent functions of TYK2 contribute to LPS-induced pathogenesis, mice expressing kinase inactive TYK2 ($Tyk2^{K923E}$), $Tyk2^{-/-}$ and *wild-type (WT)* mice were challenged with high-dose LPS and survival was monitored over time. $Tyk2^{K923E}$ and $Tyk2^{-/-}$ mice showed strongly improved survival compared to *WT* mice (Fig. 1a). Weight-loss (Fig. 1b) and hypothermia (Supplementary Fig. 1a) were similar to *WT* mice until 24 h after LPS injection, the time point when the majority of *WT* mice reached humane endpoint criteria. *WT* and *Tyk2*-mutant mice also showed a comparable drop in glucose and increase in beta-ketone levels (Supplementary Fig. 1b, c), suggesting that TYK2 does not affect the ability to metabolize glucose and to gain energy from stored fat. Mass of white and brown adipose tissue (WAT and BAT) after LPS injection was similar between mice of all genotypes after LPS injection (Supplementary Fig. 1d, e), indicating that



mice do not differ in regard to the use of WAT and BAT to produce energy and heat. LPS-induced lethality occurs through multiple organ failure [24–26] but only a few reports exist on organ pathologies induced by high-dose LPS. The most prominent finding in liver sections of *WT* mice was diffuse infiltration of neutrophils into the sinusoids, which was substantially decreased in *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ mice (Fig. 1c). This was confirmed by

◀ **Fig. 1 TYK2 aggravates LPS-induced endotoxin shock, thrombocytopenia and histopathological changes in liver and lung.** *WT*, *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ mice were injected i.p. with 50 mg/kg LPS and monitored for 7 days with respect to **a** survival and **b** body weight. Data are pooled from two independent experiments ($n = 26$–28/genotype) and represented as mean ± SD. The vertical dotted line in **b** indicates the time point when the majority of *WT* mice reached humane endpoint criteria. **c**–**g** *WT*, *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ mice were injected i.p. with 50 mg/kg LPS or PBS as control and 16 h later, **c**–**e** liver and **f**, **g** lung were taken for histopathological analysis ($n = 5$–6 for PBS and $n = 10$–12/genotype for LPS samples). Representative pictures of H&E stained **c** liver and **f** lung sections. **d** Quantification of NIMP-R14 staining for infiltrating monocytes and neutrophils, representative pictures are shown in Supplementary Fig. 2a. **e** Quantification of cells positive for cleaved caspase-3 (CC3), representative pictures are shown in Supplementary Fig. 2b. **g** Interstitial pneumonia scores from lung sections. **h**, **i** Blood was taken 16 h after LPS injection and **h** the number of platelets and **i** the mean platelet volume was determined with a VetABC blood analyzer ($n = 5$–6 for PBS and $n = 10$–13/genotype for LPS samples). **d**, **e**, **g**–**i** Data are pooled from two independent experiments, horizontal lines and error bars represent mean ± SD. *$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.001$, ****$p \le 0.0001$.

immunohistochemical staining for neutrophils and inflammatory monocytes (NIMP-R14; Fig. 1d and Supplementary Fig. 2a). *Tyk2*-mutant mice had less cells positive for cleaved caspase-3 (CC3; Fig. 1e and Supplementary Fig. 2b), indicating a decrease in apoptosis in the absence of kinase-active TYK2, but no differences in the number of resident macrophages (i.e. F4/80^{+} cells; Supplementary Fig. 2c, d). In the lung, the interstitial pneumonia score was lower in *Tyk2*-mutant than in *WT* mice (Fig. 1f, g). Disseminated intravascular coagulation is a common feature of endotoxin shock, leading to thrombi formation, platelet consumption and subsequent organ damage [27]. The LPS-induced decrease in platelet numbers and the increase in mean platelet volume, which is indicative for an increase in the frequency of platelet precursors, was less pronounced in *Tyk2*-mutant mice (Fig. 1h, i). LPS treatment did not result in histopathological alterations in kidneys (data not shown).

Taken together, these data suggest that the absence or enzymatic inactivation of TYK2 protects from liver and lung pathology and reduces thrombocytopenia in response to high-dose LPS challenge.

TYK2 promotes the production of pro-inflammatory cytokines during endotoxemia

We next assessed systemic cytokine levels in *WT*, *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ mice at 2, 6 and 16 h after LPS injection. IL-6, TNFα and IL-1β levels were comparable in mice of all genotypes at 2 h after LPS challenge (Fig. 2a), confirming that immediate early pro-inflammatory responses do not depend on TYK2 [13, 14]. Against our expectations, IL-6, TNFα, IL-1β and IL-18, which was first detectable at 6 h after treatment, were strongly reduced in *Tyk2*-mutant mice at 6 and 16 h after LPS treatment (Fig. 2b, c). In line with reports on *Tyk2*$^{-/-}$ mice [12, 14], *Tyk2*^{K923E} mice failed to produce IFNγ and had strongly reduced systemic IL-27 levels (Supplementary Fig. 3a, b). Moreover, *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ mice failed to produce IL-17A (Supplementary Fig. 3a, b), extending the reports on the importance of TYK2 for IL-17A production [2, 3, 28–32] to LPS-induced endotoxemia.

TYK2 does not impact on *Il1b* and *Il18* mRNA levels but facilitates transcriptional upregulation of *Casp11*

Il18 mRNA is constitutively expressed in myeloid and epithelial cells, whereas *Il1b* is transcriptionally regulated [33]. *Il1b* mRNA was strongly upregulated by LPS treatment and did not grossly differ between *WT*, *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ mice in spleen (Fig. 3a), lung and liver (Supplementary Fig. 4a, b). *Il18* mRNA levels did not change upon LPS treatment and were only modestly lower in *Tyk2*-mutant mice 6 h after LPS treatment (Fig. 3b and Supplementary Fig. 4c). In contrast to other cytokines (e.g. IL-6, TNFα, IL-27 and IL-17A), IL-1β and IL-18 are produced as precursors that require proteolytic processing by CASP1, which is activated by the formation of an inflammasome, and release through pyroptosis [33, 34]. *Casp1* mRNA levels in spleen and liver modestly changed upon LPS treatment and were lower in *Tyk2*-mutant mice before and/or after LPS treatment (Fig. 3c and Supplementary Fig. 4d). However, this did not translate into gross differences in CASP1 protein levels (Fig. 3e). CASP11 is the central component of the non-canonical inflammasome, senses intracellular LPS and drives LPS-induced lethality in vivo [35–39]. *Casp11* mRNA and protein were strongly induced by LPS in spleen and liver of *WT* mice, which was profoundly decreased in *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ mice (Fig. 3d, e and Supplementary Fig. 4e).

Collectively, these data show that TYK2 is essential for the transcriptional induction of *Casp11* but dispensable for the upregulation of *Il1b* mRNA and only modestly affects the expression of *Il18*.

TYK2 and its kinase activity is required for the induction of CASP11 and the release of mature IL-1β and IL-18 in response to intracellular LPS

Monocytes and macrophages are among the main producers of IL-1β and IL-18 [40–42]. To test whether TYK2 in these cells contributes to the production of IL-1β and IL-18 during LPS-induced endotoxemia, we made use of mice that lack TYK2 specifically in monocytes, macrophages and granulocytes (*Tyk2*$^{fl/fl}$ × *LysMCre* mice, herein referred to as *Tyk2*$^{\Delta LysM}$). IL-1β and IL-18 levels were drastically reduced

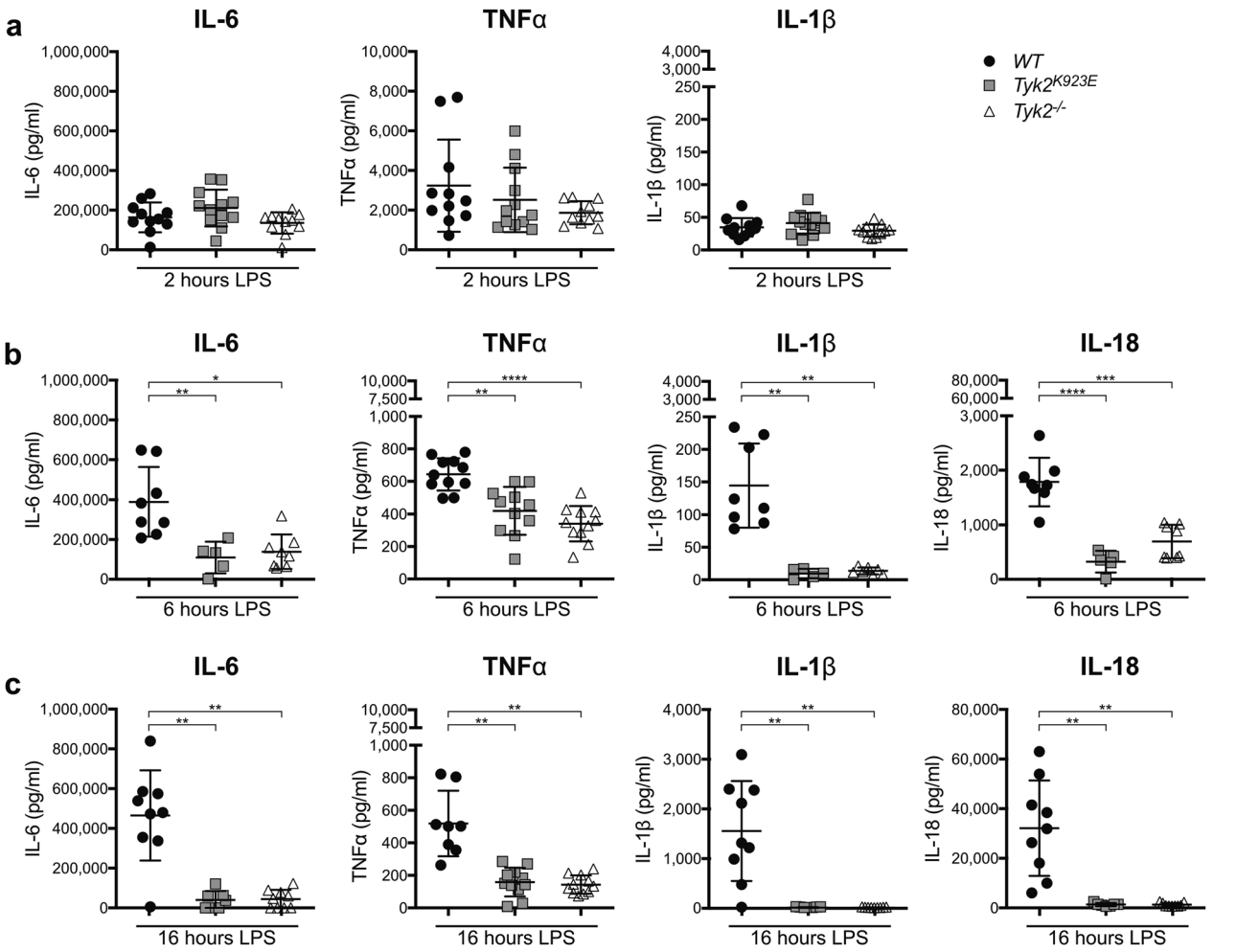


Fig. 2 TYK2 promotes the production of pro-inflammatory cytokines during endotoxemia. *WT*, *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ mice were injected i.p. with 50 mg/kg LPS and plasma levels of IL-6, TNFα, IL-1β and IL-18 were measured with Luminex bead-based multiplex assay at **a** 2 h ($n = 11$–13/genotype), **b** 6 h ($n = 5$–11/genotype) and **c** 16 h ($n = 7$–11/genotype). Data are pooled from two independent experiments, horizontal lines and error bars represent mean ± SD. *$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.001$, ****$p \le 0.0001$.

in *Tyk2*$^{\Delta LysM}$ mice compared to littermate controls, whereas IL-6 and TNFα levels did not significantly differ between mice of the two genotypes (Fig. 4a).

To test the hypothesis that TYK2 is required for non-canonical inflammasome activation, we analysed levels of CASP11, CASP1 and processing and release of IL-1β and IL-18 in BMDMs from *WT*, *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ mice in response to intracellular LPS. In addition, we treated cells with extracellular LPS with or without subsequent treatment with ATP, which activates the canonical NLRP3 inflammasome independently of CASP11. CASP11 was strongly upregulated in *WT* but not in *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ BMDMs upon LPS transfection (Fig. 4b) or treatment with extracellular LPS+/−ATP (Fig. 4c). CASP1 protein levels neither changed upon LPS treatment nor differed between *WT* and *Tyk2*-mutant BMDMs (Fig. 4b, c). Consistent with our previous study [15], pro-IL-1β levels were higher in *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ BMDMs, irrespective of whether LPS was delivered intra- or extracellularly (Fig. 4b, c). Pro-IL-18 was comparably upregulated in BMDMs of all genotypes (Fig. 4b, c). *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ BMDMs did not release IL-18 and showed reduced levels of IL-1β in supernatants upon LPS transfection, despite the strong increase of intracellular pro-IL-1β (Fig. 4d). In contrast to previous reports [35, 43], we did not detect cleaved CASP1 in cell culture supernatants of LPS-transfected cells (Fig. 4d). Upon treatment with extracellular LPS + ATP, IL-1β was increased in supernatants of *Tyk2*-mutant BMDMs (Fig. 4e), which is in line with our previous study [15]. Although intracellular pro-IL-18 was not increased in *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ BMDMs, we observed increased release of IL-18 and cleaved CASP1 (Fig. 4e), indicating enhanced NLRP3 inflammasome activity.

CASP11 does not directly process pro-IL-1β and pro-IL-18 but induces pyroptosis through the cleavage of gasdermin D (GSDMD) [44–46], which results in secondary activation of CASP1 through the NLRP3 inflammasome [37, 47]. LPS transfection induced considerably higher

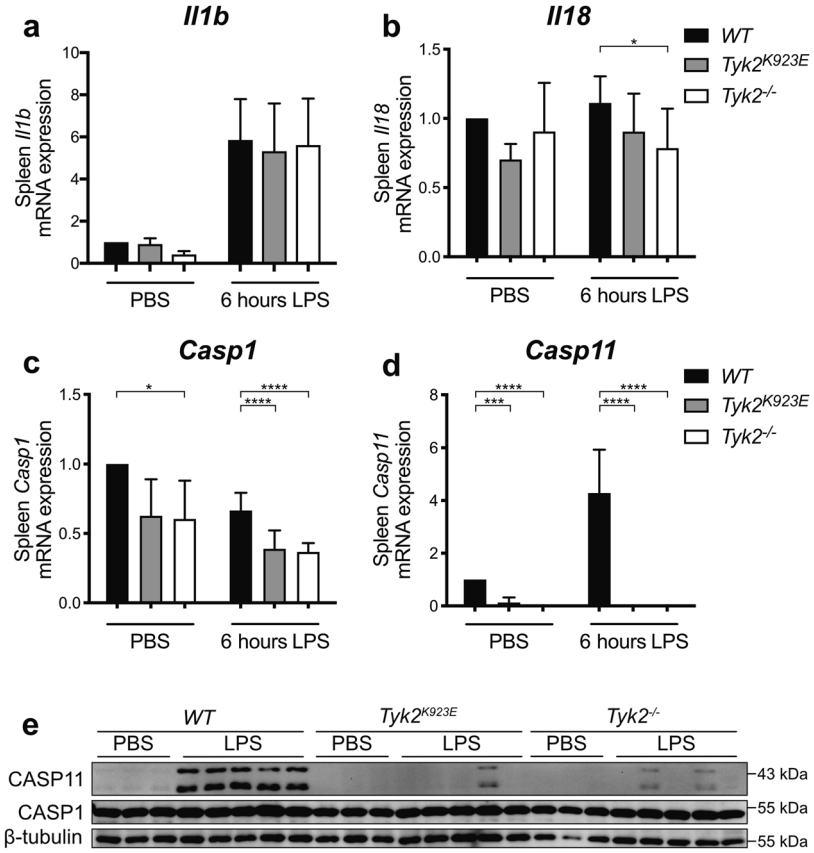


Fig. 3 TYK2 does not impact on *Il1b* and *Il18* mRNA levels but facilitates transcriptional upregulation of *Casp11*. *WT*, *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ mice were injected i.p. with 50 mg/kg LPS or PBS as control and **a**–**d** 6 h later spleen was harvested and mRNA levels of **a** *Il1b*, **b** *Il18*, **c** *Casp1* and **d** *Casp11* were measured by RT-qPCR (n = 2–5 for PBS and n = 8–9/genotype for LPS samples). Results are shown as relative expression compared to WT mice injected with PBS. Data are pooled from two independent experiments and represented as mean ± SD. *$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.001$, ****$p \le 0.0001$. **e** 16 h after LPS treatment CASP11 and CASP1 protein levels were determined in spleen lysates by western blot (n = 5/genotype). In all, 15 µg of total cell lysate was loaded per lane and β-tubulin was used as loading control. Molecular weights of the pre-stained protein marker bands are indicated. Data are representative of two independent experiments.

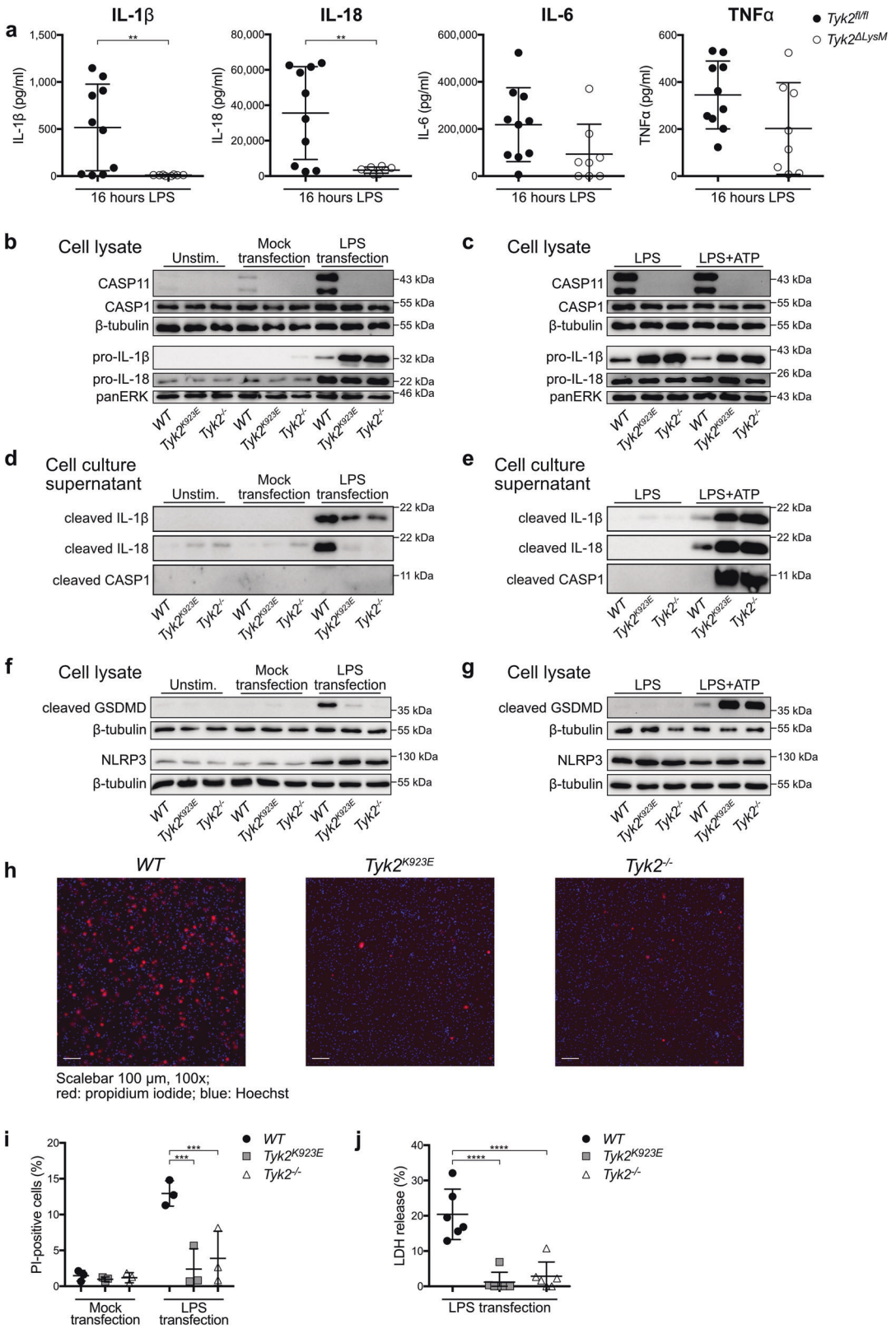
GSDMD cleavage in *WT* than in *Tyk2*-mutant BMDMs (Fig. 4f), whereas the opposite was observed in response to extracellular LPS + ATP (Fig. 4g). NLRP3 was upregulated upon treatment with intra- or extracellular LPS and levels did not grossly differ between *WT* and *Tyk2*-mutant BMDMs (Fig. 4f, g), supporting previous studies indicating that IFNα/β does not suppress LPS-induced upregulation of NLRP3 but enhances its activity [48, 49].

To further substantiate that non-canonical inflammasome activation and pyroptosis is impaired in the absence of enzymatically active TYK2, we analysed cell death by staining cells with PI, which only stains cells with a disrupted cell membrane. The percentage of PI-positive cells was significantly reduced in LPS-transfected *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ BMDMs (Fig. 4h, i). Consistently, we detected lower levels of lactate dehydrogenase (LDH), a cytosolic enzyme that is only released upon disruption of the plasma membrane, in the supernatant of transfected *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ BMDMs (Fig. 4j).

Collectively, these data show that TYK2 functions upstream of the induction of CASP11, the cleavage of GSDMD and the release of mature IL-1β and IL-18 in response to intracellular LPS. Furthermore, our data indicate that TYK2 has opposing effects on the release of biologically active IL-1β and IL-18 in response to non-canonical and canonical inflammasome activation.

Intact type I IFN signalling is required for the induction of CASP11 and the release of mature IL-1β and IL-18 in response to intracellular LPS

CASP11 can be induced by IFNβ and IFNγ [37, 50–52]. Macrophages lacking a functional type I IFN receptor (*Ifnar1*$^{-/-}$) fail to induce CASP11 in response to infection with Gram-negative bacteria or treatment with extracellular LPS [51], suggesting that the impaired induction of CASP11 in *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ BMDMs reflects their defects in type I IFN signalling. To confirm these finding in



the context of LPS transfection, we analysed CASP11, CASP1, cleaved GSDMD and NLRP3 levels, IL-1β and IL-18 processing and release and cell death in *Ifnar1*$^{-/-}$ BMDMs. To formally exclude effects of IFNγ in this experimental setting, we included BMDMs from mice that lack a functional IFNγ receptor (*Ifngr1*$^{-/-}$). Similar to

◀ **Fig. 4 TYK2 and its kinase activity is required for the induction of CASP11 and the release of mature IL-1β and IL-18 in response to intracellular LPS. a** Mice lacking TYK2 in myeloid cells ($Tyk2^{\Delta LysM}$) and littermate controls ($Tyk2^{fl/fl}$) were injected i.p. with 50 mg/kg LPS and levels of IL-1β, IL-18, IL-6 and TNFα were measured in the plasma with Luminex bead-based multiplex assay after 16 h of LPS treatment ($n = 8$–10/genotype). Data are pooled from two independent experiments. **b–j** BMDMs from *WT*, $Tyk2^{K923E}$ and $Tyk2^{-/-}$ mice were **b**, **d**, **f**, **h–j** transfected with 2 µg/ml LPS for 18 h, mock transfected or left untreated (Unstim.) or **c**, **e**, **g** stimulated with 100 ng/ml LPS for 16 h, followed by stimulation with 3 mM ATP for 2 h. **b**, **c** CASP11, CASP1, pro-IL-1β, pro-IL-18 and **f**, **g** cleaved GSDMD and NLRP3 levels were detected in cell lysates; **d**, **e** cleaved IL-1β, cleaved IL-18 and cleaved CASP1 were detected in the cell culture supernatants by western blot. **b**, **c**, **g** In all, 5 µg or **f** 10 µg of total cell lysate or **d**, **e** In all, 15 µl of TCA-precipitated supernatant was loaded per lane. **b**, **c**, **f**, **g** β-tubulin and panERK were used as loading controls. Molecular weights of the pre-stained protein marker bands are indicated. **b–g** Data are representative of two independent experiments. **h**, **i** LPS-transfected BMDMs were stained with propidium iodide (PI) and Hoechst and **h** pictures were taken from three different regions to calculate **i** the percentage of PI-positive cells. Quantifications are from three independent experiments. **j** Lactate dehydrogenase (LDH) was measured in cell culture supernatants after LPS transfection and data are presented as % LDH release relative to the values of mock-transfected BMDMs. Data are pooled from six independent experiments. **a**, **i**, **j** Horizontal lines and error bars represent mean ± SD. $*p \le 0.05$, $**p \le 0.01$, $***p \le 0.001$, $****p \le 0.0001$.

$Tyk2^{K923E}$ and $Tyk2^{-/-}$ cells, CASP11 and cleaved GSDMD were barely detectable in LPS transfected $Ifnar1^{-/-}$ BMDMs, whereas levels of CASP1 and NLRP3 were similar to *WT* cells (Fig. 5a, b). $Ifnar1^{-/-}$ BMDMs had increased intracellular levels of pro-IL-1β (Fig. 5a), which is in line with observations in response to extracellular LPS [15, 53]. However, secreted IL-1β levels were not increased in $Ifnar1^{-/-}$ BMDMs, indicating reduced IL-1β processing and release (Fig. 5c). In line with a previous study using extracellular LPS [54], $Ifnar1^{-/-}$ BMDMs had decreased upregulation of pro-IL-18 upon LPS transfection (Fig. 5a). We did not detect IL-18 in the supernatants of $Ifnar1^{-/-}$ BMDMs (Fig. 5c), indicating that the release of biologically active IL-18 in response to intracellular LPS is completely dependent on IFNAR1. The frequency of PI-positive cells and the levels of LDH in the cell culture supernatants were clearly lower in LPS transfected $Ifnar1^{-/-}$ BMDMs (Fig. 5d, e and Supplementary Fig. 5). $Ifngr1^{-/-}$ BMDMs did not differ from *WT* BMDMs (Fig. 5a–e and Supplementary Fig. 5).

Collectively, these data confirm that type I IFN signalling promotes the induction of CASP11 and non-canonical inflammasome-dependent IL-1β and IL-18 processing and release in macrophages.

Type I IFN and IFNγ promote the production of IL-1β and IL-18 in vivo

Type I IFNs promote the production of IL-1β during LPS-induced endotoxemia [38], whereas the contribution of IFNγ to CASP11-dependent IL-1β production in vivo is unknown. IL-1β and IL-18 levels were strongly reduced in LPS-treated $Ifnar1^{-/-}$ and $Ifngr1^{-/-}$ mice at 16 h after treatment (Fig. 6a, b), although these defects were less pronounced than in $Tyk2^{K923E}$ and $Tyk2^{-/-}$ mice (Fig. 2c). TNFα levels were reduced in $Ifnar1^{-/-}$ and $Ifngr1^{-/-}$ mice, whereas IL-6 levels were reduced in $Ifngr1^{-/-}$ but not $Ifnar1^{-/-}$ mice (Fig. 6a, b). CASP11 levels in spleens from LPS-treated $Ifnar1^{-/-}$ mice were lower (Fig. 6c) but did not differ between $Ifngr1^{-/-}$ and *WT* mice (Fig. 6d). CASP1 levels neither changed upon LPS nor differed between mice of the three genotypes (Fig. 6c, d). Recognition of intracellular LPS by CASP11 is facilitated by guanylate binding proteins (GBPs) [55, 56]. GBPs are classical IFN-stimulated genes (ISGs) and are among the most strongly induced genes in response to IFNγ [57]. Deletion of all GBPs on chromosome 3 (i.e. GBP1, GBP2, GBP3, GBP5 and GBP7) profoundly reduced the release of IL-1β during LPS-induced endotoxemia, whereas deletion of GBP2 alone had more modest effects [38, 58]. However, GBP2 has a prominent role in CASP11 activation during Gram-negative bacterial infections [59]. *Gbp2* mRNA levels were reduced in spleens from $Ifngr1^{-/-}$ mice after treatment with PBS or LPS (Fig. 6e). In line with the impaired production of IFNγ, LPS-induced upregulation of *Gbp2* mRNA was impaired in spleens from $Tyk2^{K923E}$ and $Tyk2^{-/-}$ mice (Fig. 6f).

Taken together, these data suggest that type I IFN, IFNγ and TYK2 increase systemic levels of IL-1β and IL-18 during endotoxemia and that this correlates with a positive regulatory role on CASP11 and/or GBP2 expression in the spleen.

Pharmacological inhibition of TYK2 in mouse and human macrophages leads to a dose-dependent reduction of CASP11 and CASP5 protein levels

To complement our genetic data, we tested whether pharmacological inhibition of TYK2 in BMDMs impairs LPS-induced upregulation of CASP11. Treatment with the allosteric TYK2 inhibitor BMS-986165 [60] inhibited the upregulation of CASP11 in a dose-dependent manner (Fig. 7a). There are two homologs of CASP11 in humans: CASP4 and CASP5. CASP4 is constitutively expressed in many cell types [34], including peripheral blood mononuclear cells (PBMCs), monocytes and the monocyte/macrophage cell lines U937 and THP-1, whereas CASP5 is transcriptionally upregulated by LPS, IFNα/β and IFNγ [61–64]. Similar to its effects on CASP11, BMS-986165 dose-dependently inhibited upregulation of CASP5 in response to LPS in U937 macrophages. CASP4 was constitutively expressed and not further upregulated by LPS (Fig. 7b).

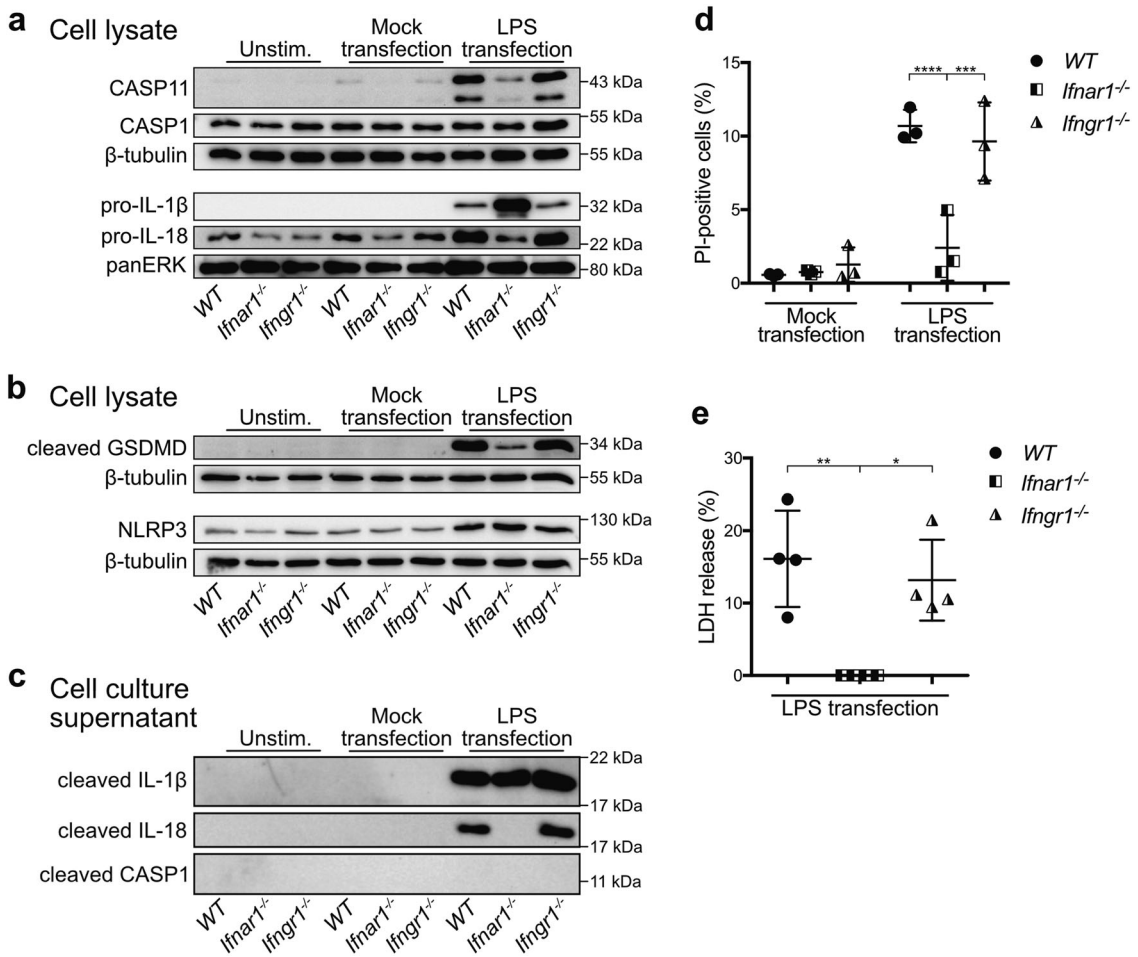


Fig. 5 Intact type I IFN signalling is required for the induction of CASP11 and the release of mature IL-1β and IL-18 in response to intracellular LPS. BMDMs from *WT, Ifnar1*$^{-/-}$ and *Ifngr1*$^{-/-}$ mice were transfected with 2 µg/ml LPS for 18 h. **a** CASP11, CASP1, pro-IL-1β, pro-IL-18 and **b** cleaved GSDMD and NLRP3 were detected in cell lysates; **c** cleaved IL-1β, cleaved IL-18 and cleaved CASP1 were detected in cell culture supernatants by western blot. **a** In all, 5 µg or **b** 10 µg of total cell lysate or **c** 15 µl of TCA-precipitated supernatant was loaded per lane. **a**, **b** β-tubulin and panERK were used as loading controls. **a**–**c** Molecular weights of the pre-stained protein marker bands are indicated. Data are representative of two independent experiments. **d** LPS-transfected BMDMs were stained with propidium iodide (PI) and Hoechst and pictures were taken from three different regions to calculate the percentage of PI-positive cells. Representative pictures are shown in Supplementary Fig. 5. Quantifications are pooled from three independent experiments. **e** Lactate dehydrogenase (LDH) was measured in cell culture supernatants after stimulation and data are presented as % LDH release relative to the values of mock transfected BMDMs. Data are pooled from four independent experiments. **d**, **e** Horizontal lines and error bars represent mean ± SD. $*p \le 0.05$, $**p \le 0.01$, $***p \le 0.001$, $****p \le 0.0001$.

Collectively, the data demonstrate that pharmacological inhibition of TYK2 impairs LPS-induced upregulation of CASP11 in murine and CASP5 in human macrophages.

Discussion

In this study we identified an essential role of kinase-active TYK2 in LPS-induced endotoxemia and non-canonical inflammasome activation in macrophages. Mechanistically, we show that TYK2 functions upstream of the induction of CASP11 and the release of mature IL-1β and IL-18. We propose that the impaired CASP11 availability contributes to the increased survival of *Tyk2*$^{-/-}$ mice following LPS-induced endotoxemia, which is supported by the decreased LPS-induced lethality of *Casp11*$^{-/-}$ and *Il1b*$^{-/-}$/*Il18*$^{-/-}$ mice [65, 66]. CASP11 has also been implicated in LPS-induced tissue injury [38, 67], suggesting that the decreased organ pathology in *Tyk2*$^{-/-}$ mice may also reflect impaired upregulation of CASP11.

In support of our in vivo findings, we show that *Tyk2*$^{-/-}$ BMDMs fail to upregulate CASP11 and show strongly reduced release of IL-1β and IL-18 in response to intracellular LPS. This is in stark contrast to the increased release of IL-1β and IL-18 in response to extracellular LPS

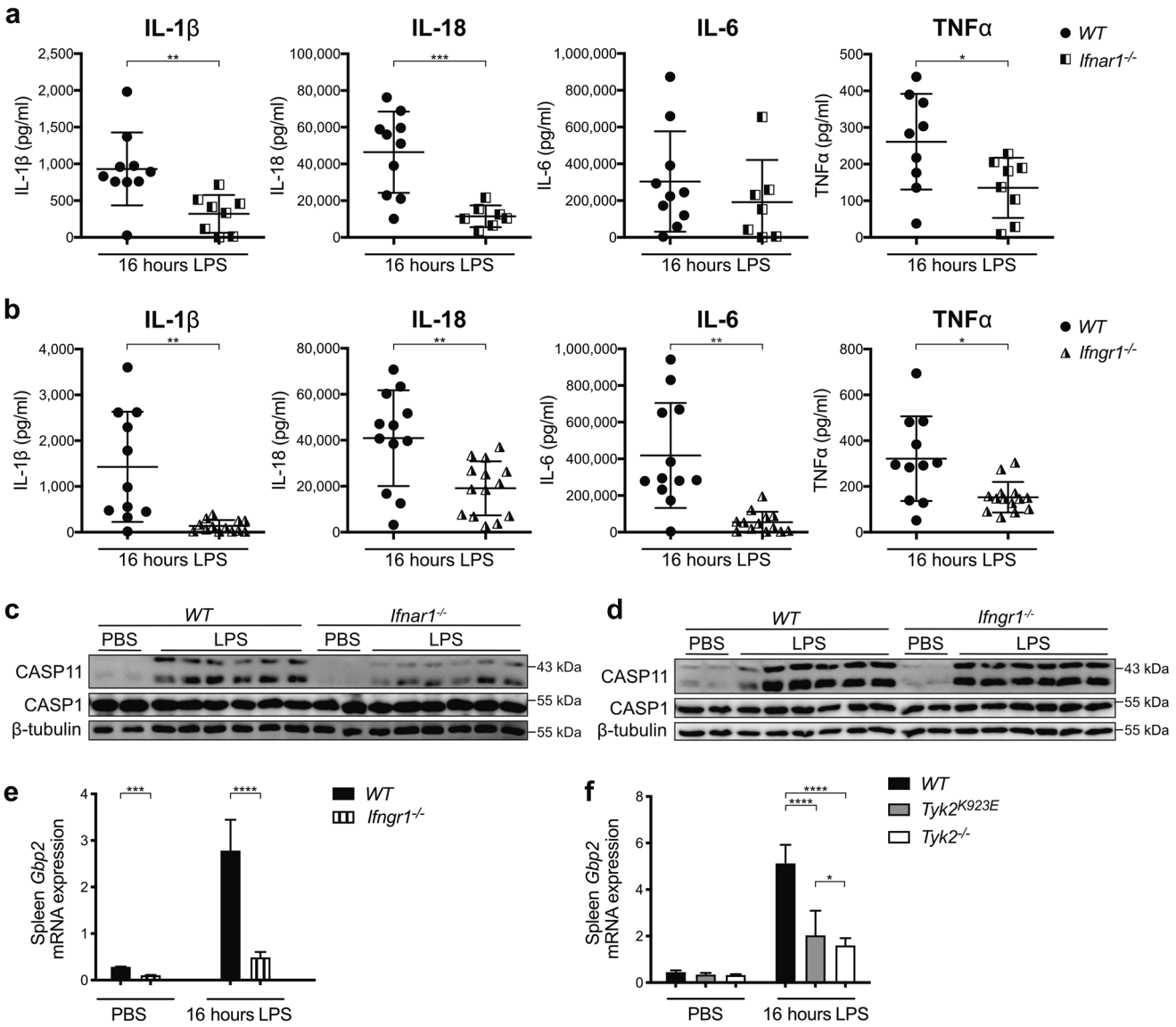


Fig. 6 Type I IFN and IFNγ promote the production of IL-1β and IL-18 in vivo. a, **c** *WT* and *Ifnar1*$^{-/-}$, **b**, **d**, **e** *WT* and *Ifngr1*$^{-/-}$ or **f** *WT*, *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ mice were injected i.p. with 50 mg/kg LPS. **a**, **b** Plasma levels of IL-1β, IL-18, IL-6 and TNFα were measured with Luminex bead-based multiplex assay after 16 h. Data are pooled from two independent experiments **a** ($n = 7$–10/genotype), **b** ($n = 11$–14/genotype). Horizontal lines and error bars represent mean ± SD. **c**, **d** 16 h after treatment, CASP11 and CASP1 levels were determined in spleen lysates by western blot. In all, 15 µg of total cell lysate was loaded per lane. β-tubulin was used as loading control. Molecular weights of the pre-stained protein marker bands are indicated. Data are representative of two independent experiments ($n = 6$/genotype). **e**, **f** Spleens were harvested at 16 h after treatment and mRNA levels of *Gbp2* were measured by RT-qPCR. Results are shown as relative expression compared to the housekeeping gene *Ube2d2* and are given as mean ± SD. Data are from **e** two independent experiments ($n = 12$–14/genotype) and **f** one representative out of two independent experiments ($n = 6$/genotype). $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$, $^{****}p \le 0.0001$.

and ATP [15] and clearly demonstrates that TYK2 positively and negatively impacts on the LPS-induced production of mature IL-1β and IL-18, depending on the type of inflammasome involved.

Previous studies with *Ifnar1*$^{-/-}$ BMDMs indicated that CASP11 is induced by autocrine/paracrine IFNα/β signalling in response to LPS and infection with Gram-negative bacteria [51]. Our study supports these findings and suggests that CASP11 upregulation depends on an intact IFNAR1-TYK2 axis, irrespective of whether LPS is applied intra- or extracellularly. Studies with *Casp11* promoter-reporter constructs and *Stat1*$^{-/-}$ BMDMs revealed a crucial role of STAT1 for the transcriptional induction of *Casp11* in response to IFNγ [52] and the IFNAR1-dependent upregulation of CASP11 upon infection with *Salmonella typhimurium* [68]. Thus, TYK2 most likely promotes the induction of *Casp11* by amplifying STAT1 activation in response to IFNα/β [17, 19, 69].

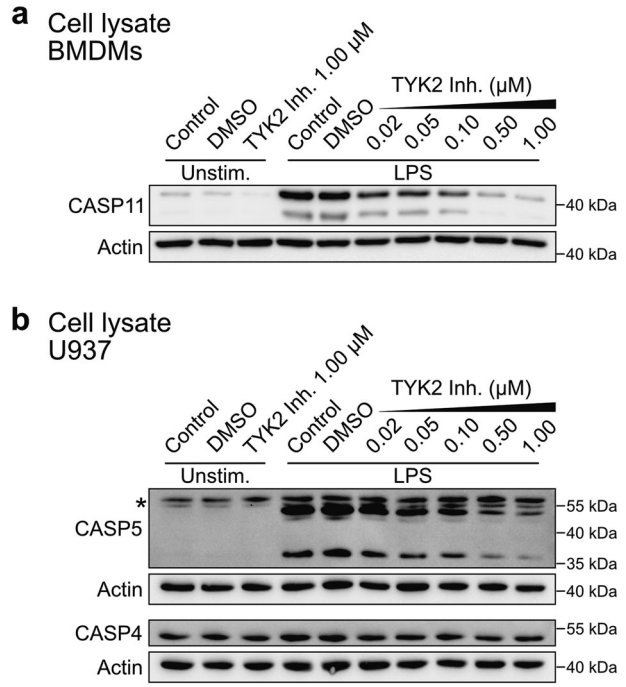


Fig. 7 Pharmacological inhibition of TYK2 in mouse and human macrophages leads to a dose-dependent reduction of CASP11 and CASP5 protein levels. **a** BMDMs from *WT* mice or **b** PMA-differentiated U937 macrophages were either stimulated with 100 ng/ml LPS for 18 h or left unstimulated (Unstim.). **a**, **b** Cells were treated with the allosteric TYK2 inhibitor BMS-986165 (TYK2 Inh.) or with DMSO (0.01% v/v) as control. **a** CASP11 or **b** CASP5 and CASP4 were detected in cell lysates by western blot. **a** In all, 10 µg or **b** 10 µl of total cell lysate was loaded per lane **a**, **b** Actin was used as loading control. Molecular weights of the pre-stained protein marker bands are indicated. Data are representative of two independent experiments. Asterisk indicates two unspecific bands in U937 cells.

In line with the findings in BMDMs, we show that IFNAR1-TYK2 signalling promotes the upregulation of CASP11 during endotoxemia in the spleen. This contrasts a previous report that showed unaltered CASP11 levels in splenocytes isolated from LPS-treated *Ifnar1*$^{-/-}$ mice [38]. The reason for this discrepancy is unclear, but might lie in the different dose or type of LPS used. In support of our data, this study also reported reduced systemic levels of IL-1β 16 h after LPS treatment in *Ifnar1*$^{-/-}$ and *Casp11*$^{-/-}$ mice.

Most of the studies on the non-canonical inflammasome have been conducted with macrophages. However, CASP11 and GSDMD are also expressed in non-myeloid cells, although some cell types, such as epithelial cells, lack a functional NLRP3 pathway and undergo pyroptosis without the release of mature IL-1β [47]. Using conditional *Tyk2*-knockout mice, we provide evidence that myeloid cells are of prime importance for the TYK2-dependent release of IL-1β and IL-18 during endotoxemia. In contrast, TYK2 activity in non-myeloid cells augments the production of TNFα and IL-6, underscoring that non-myeloid cells are relevant sources of these cytokines [70, 71].

Impaired IL-1β production in *Ifnar1*$^{-/-}$ mice during endotoxemia has been recently linked to an impaired induction of GBPs [38], although direct evidence was not provided. Our data suggest a role of IFNγ, at least for the induction of *Gbp2* mRNA in the spleen. This is also supported by the fact that GBPs are more potently induced by IFNγ than IFNα/β [57] and our finding that *Ifngr1*$^{-/-}$ mice have reduced levels of IL-1β and IL-18, despite an unimpaired upregulation of splenic CASP11. GBPs facilitate the binding of intracellular LPS to CASP11 [47, 55, 56], raising the possibility that *Ifngr1*$^{-/-}$ mice show reduced IL-1β and IL-18 production due to an impaired binding of LPS to CASP11. However, more detailed studies will be required to define the spatiotemporal contribution of type I IFNs and IFNγ to the induction of CASP11 and GBPs. Our findings that *Tyk2*$^{-/-}$ mice have strongly reduced serum levels of IFNγ and reduced IFNGR1-dependent upregulation of splenic *Gbp2* mRNA supports the concept that TYK2 also promotes the pathogenesis of endotoxemia through its involvement in the IL-12/IFNγ axis [13].

Only a few studies exist on the role of the non-canonical inflammasome during infections with Gram-negative bacteria in vivo [50, 72, 73]. As TYK2 differentially affects the release of mature IL-1β upon canonical and non-canonical inflammasome activation, it seems reasonable to speculate that in the context of bacterially-induced sepsis, the consequences of TYK2 deficiency on inflammatory responses may depend on the bacterial community.

Another important finding of our study is that *Tyk2*^{K923E} mice are phenotypically indistinguishable from *Tyk2*$^{-/-}$ mice, suggesting that kinase-active TYK2 drives LPS-induced pathogenesis. This goes in line with the requirement for enzymatically active TYK2 in the antiviral defense [19] but contrasts kinase-independent functions of TYK2 in NK cell-dependent tumour surveillance [8]. In support of our genetic data, we show that a TYK2-selective inhibitor [60] blocks LPS-induced upregulation of CASP11 in BMDMs and CASP5 in human macrophages. The second human CASP11 homolog CASP4 is constitutively expressed in most cell types [34] and not further upregulated by LPS [62]. Both CASP4 and CASP5 are activated by cytosolic LPS and capable of inducing pyroptosis [34]. However, the relative contribution to the pathogenesis of endotoxemia or bacterial sepsis is unclear [74]. Studies in mice have highlighted the involvement of CASP11-dependent inflammation in a wide spectrum of inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease and neurodegenerative diseases [75]. Although there might be considerable redundancies in the functions of CASP4 and CASP5, our results raise the interesting possibility that inhibition of TYK2 may be an attractive therapeutic target for the treatment of CASP5-driven diseases.

Collectively, we uncovered a fundamental role of TYK2 in the regulation of the non-canonical inflammasome. Our findings suggest that impaired upregulation of CASP11 and processing and release of IL-1β and IL-18 in the absence of TYK2 or its kinase activity contributes to the increased resistance against LPS-induced lethality.

Acknowledgements This work was supported by the Austrian Science Fund (FWF) project P25642-B22, DOC 32-B28 and the Special Research Program SFB-F6101, SFB-F6105 and SFB-F6106. AL is supported by the Deutsche Forschungsgemeinschaft (Heisenberg-Professorship, project number 24141047). We thank Marion Bokor for histological sample preparation, immunohistochemical staining and help with RT-qPCRs, Bettina Tutzer and Sara Lang for their help with in vivo experiments and Claus Vogl for help with the statistical analysis.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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