universität wien

DISSERTATION

Titel der Dissertation

Characterisation of cellular and systemic functions of Tyk2 in lipopolysaccharide-induced endotoxin shock

angestrebter akademischer Grad

Doktor der Naturwissenschaften (Dr. rer.nat.)

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Wien, im November 2010

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ABBREVIATIONS

Act1 ..... actin related gene 1
AHR ..... aryl hydrocarbon receptor
AP-1 ..... activating protein-1
ARE ..... AU-rich elements
BMDM ..... bone marrow-derived macrophages
CARD ..... caspase recruitment domain
Cardif ..... CARD adaptor inducing IFN-beta
Casp-1/Casp-8 ..... caspase-1/caspase-8
CCR6 ..... chemokine (C-C motif) receptor 6
CIA ..... collagen-induced arthritis
CIKS ..... connection to IKK and SAPK/JNK
CTL ..... cytotoxic T lymphocyte
DAI ..... DNA-dependent activator of interferon regulatory factors
DAMPs ..... danger-associated molecular patterns
DBD ..... DNA-binding domain
DC ..... dendritic cell
DHX ..... DEAH (Asp-Glu-Ala-His) box polypeptide
EAE ..... experimental autoimmune encephalomyelitis
EF2 ..... elongation factor 2
ERK ..... extracellular signal-regulated kinase
FCS ..... fetal calf serum
FERM ..... Four-point-one/Ezrin/Radixin/Moesin
γc ..... γ-chain
HIES ..... hyper IgE syndrome
IFN ..... interferon
IFNα/β ..... type I IFNs
IFNγ ..... type II IFN
IFNλs ..... type III IFNs
Ifnar ..... IFNα/β receptor
Ifngr ..... IFNγ receptor
IgE ..... immunoglobulin E
IKK ..... IκB kinase
IL ..... interleukin
IL-12Rβ1 ..... IL-12 receptor β1

iNKT.....invariant natural killer T cell
iNOS.....inducible nitric oxide synthase
i.p.....intraperitoneal
IPS-1.....IFN-beta promoter stimulator 1
IRAK.....interleukin-1 receptor-associated kinase
IRF.....interferon regulatory factor
ISG.....IFN-stimulated gene
ISGF3.....IFN-stimulated gene factor 3
i.v.....intravenous
Jak.....Janus kinase
JNK.....c-jun N-terminal kinase
LBP.....LPS-binding protein
LCMV.....lymphocytic choriomeningitis virus
LIF.....leukemia inhibitory factor
LPS.....lipopolysaccharide
LRR.....leucine rich repeat
LTi-like cell.....lymphoid tissue inducer-like cell
Mal.....MyD88 adaptor-like
MAPK.....mitogen-activated protein kinase
MAVS.....mitochondrial anti-viral signaling protein
MCMV.....murine cytomegalovirus
MD-2.....myeloid differentiation protein-2
MDA5.....melanoma differentiation associated gene 5
MHC.....major histocompatibility complex
mPDCA1.....murine pDC antigen-1
MS.....multiple sclerosis
MyD88.....myeloid differentiation primary response gene 88
NEMO.....NF-κB essential modulator
NF-κB.....nuclear factor-κB
NFκBia.....NF-κB inhibitor α
NLRs.....nucleotide-binding domain, leucine-rich repeat containing family
NK cell.....natural killer cell
NKT cell.....natural killer T cell
NO.....nitric oxide
NOD.....nucleotide-binding oligomerisation domain containing
NOS2.....nitric oxide synthase 2

OSM ..... oncostatin M
PAI2 ..... plasminogen activator inhibitor 2
PAMPs ..... pathogen-associated molecular patterns
pDC ..... plasmacytoid dendritic cell
PM ..... thioglycollate-elicited peritoneal macrophages
PRRs ..... pattern-recognition receptors
RIG-I ..... retinoic-acid-inducible gene 1 protein
RLH ..... RIG-like helicases
RORγt/RORα ..... retinoic acid receptor-related orphan receptor γt / α
ROS ..... reactive oxygen species
SARM ..... sterile alpha and Toll/IL-1R resistance motif containing protein
SCID ..... severe combined immunodeficiency
SDS-PAGE ..... sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH2 ..... Src homology 2
Siglec-H ..... sialic acid binding Ig-like lectin H
SLE ..... systemic lupus erythematosus
Stat ..... signal transducer and activator of transcription
SUMO ..... small ubiquitin-related modifier
TAB ..... TAK1 binding protein
TAD ..... transcriptional activation domain
TAK1 ..... TGF-β-activated kinase 1
TBP ..... TATA box binding protein
TCR ..... T cell receptor
TGF-β ..... transforming growth factor beta
Th1/Th2/Th17 ..... T helper cell type 1 / type 2 / type 17
TIR ..... toll-interleukin-1 receptor
TIRAP ..... TIR domain-containing adapter protein
TLR ..... Toll-like receptor
TNFα ..... tumour necrosis factor α
TRAF ..... TNF-receptor associated factor
TRAM ..... TRIF-related adaptor molecule
TRIF ..... TIR domain-containing adaptor protein inducing IFN-β
Tyk2 ..... tyrosine kinase 2
Ube2d2 ..... ubiquitin-conjugating enzyme E2 D2
VISA ..... virus-induced signalling adapter variant 1a
VSV ..... vesicular stomatitis virus

VV.....vaccinia virus

WT.....wildtype

ZBP1.....Z-DNA-binding protein 1

ABSTRACT

Once pathogens have entered an organism, the host immune system has to recognise and respond immediately to the microorganisms and their immunogenic products. Following recognition by pattern recognition receptors, the production and auto-/paracrine action of signalling molecules (e.g. cytokines) the Janus kinase and signal transducer and activator of transcription (Jak-Stat) signalling pathway becomes activated. In response to outside signals this pathway can alter nuclear gene expression programs within minutes, thereby modulating cellular processes like development, cell growth and homeostasis. As a result, deregulation of the Jak-Stat signalling pathway can lead to severe consequences for an organism. Tyrosine kinase 2 (Tyk2) is one of four mammalian Jaks that participate in the Jak-Stat signalling pathway and engagement of Tyk2 in response to a variety of cytokines and some growth factors has been demonstrated. Accordingly, Tyk2-deficient mice are more susceptible to most microbial infections, which is mainly attributed to their defects in type I interferon (IFNα/β) and interleukin (IL)-12 signalling. In contrast, Tyk2-deficient mice are resistant to lipopolysaccharide (LPS)- and intestinal ischemia/ reperfusion-induced shock. Additionally, Tyk2 has also been linked to the development of autoimmune and inflammatory diseases.

In order to study how Tyk2 globally influences the response to LPS we have recently conducted transcriptome and proteome studies in macrophages. Aim of this study was to confirm and further characterise the kinetics and mechanism(s) of induction of selected Tyk2-regulated genes/proteins. Furthermore, to substantiate our findings *in vivo*, we examined gene induction, organ and cell type specificity of the most interesting gene(s)/protein(s).

Two genes that we analysed in more detail are IL-17 and IL-17F. Both are important proinflammatory cytokines involved in the pathogenesis of several autoimmune and inflammatory diseases and are nonetheless essential for the host defence against many microbes. We could show that Tyk2 positively regulates expression of IL-17 and IL-17F in macrophages at the transcriptional level and demonstrate that this function of Tyk2 does not depend on IFNα/β signalling. Our study revealed that Tyk2 is also involved in IL-17 production in spleens following intraperitoneal administration of LPS. Additionally, we can show that IL-17 in spleens is almost exclusively produced by two cell types, i.e. $\gamma\delta$ T cells and plasmacytoid dendritic cells (pDCs). Interestingly, it has not been reported until yet that pDCs are capable of IL-17 production.

A third gene that we focussed on was IL-1β. The substantial contribution of IL-1β to a variety of autoimmune and inflammatory diseases is well known, but Tyk2 has not yet

been linked to the control of IL-1β expression. Regulation of IL-1β at the transcriptional level and by proteolytic processing from pro-IL1β to the mature form has already been described. Our study in macrophages revealed that Tyk2 suppresses IL-1β expression in response to LPS at the translational level, thereby uncovering a novel mechanism of IL-1β regulation. By showing increased IL-1β production in the absence of IFNα/β receptor 1 (Ifnar1) and Stat1, we provide evidence for the involvement of canonical IFNα/β signalling. We could demonstrate a similar Tyk2-dependent downregulation of IL-1β following *Listeria monocytogenes* infection in macrophages. In support of our *in vitro* data, IL-1β levels were increased in the peritoneal lavages of Tyk2-deficient mice after LPS challenge, highlighting the importance of Tyk2-mediated translational regulation of IL-1β. In contrast to local IL-1β expression, systemic IL-1β levels were decreased in the absence of Tyk2, further illustrating the complexity of IL-1β regulation.

In summary, this study provides novel insights in the diverse and non-redundant regulatory functions of Tyk2 *in vitro* and *in vivo*. Tyk2 can modulate processes as diverse as transcription and translation. In particular, we identified IL-17 as LPS-induced and Tyk2-dependent gene in inflammatory macrophage populations *in vitro* and in γδ T cells and pDCs in spleens. In addition, we identified a Tyk2-dependent inhibitory loop that limits excessive IL-1β production in response to LPS treatment and *Listeria monocytogenes* infection. We show that this suppressive effect occurs at the translational level, thereby identifying a novel mode of IL-1β regulation.

ZUSAMMENFASSUNG

Sind Pathogene erst einmal in einen Organismus eingedrungen, ist es die Aufgabe des Immunsystems diese zu erkennen und umgehend auf die Mikroorganismen und ihre immunogenen Produkte zu reagieren. Nach dem Erkennen durch Muster-Erkennungs-Rezeptoren, der Produktion und auto-/paracrinen Wirkung von Signalmolekülen (wie z.B. Zytokinen) wird die Jak-Stat (Janus Kinase - Signal Transducer and Activator of Transcription) Signaltransduktionskaskade aktiviert. Als Antwort auf äußere Signale kann diese Kaskade die Genexpression im Kern innerhalb von Minuten modifizieren und dadurch zelluläre Prozesse wie Entwicklung, Zellwachstum und Homeostase an äußere Gegebenheiten anpassen. Dementsprechend kann die Deregulierung der Jak-Stat Signaltransduktionskaskade mit erheblichen Konsequenzen für einen Organismus verbunden sein. Tyrosin Kinase 2 (Tyk2) ist eine der vier Säugetier-Jaks, von denen alle an der Jak-Stat Signaltransduktionskaskade beteiligt sein können. Es konnte gezeigt werden, dass Tyk2 an der Reaktion auf eine Vielzahl von Zytokinen und einigen Wachstumsfaktoren mitwirkt. Folglich sind Tyk2-defiziente Mäuse anfälliger gegenüber den meisten mikrobiellen Infektionen, was hauptsächlich auf die Defekte in der Typ I Interferon (IFNα/β) und Interleukin (IL)-12 Signaltransduktion zurückzuführen ist. Im Gegensatz dazu sind Tyk2-defiziente Mäuse resistent gegen Schock induziert durch Lipopolysaccharid (LPS) und intestinale Ischämie/Reperfusion. Zusätzlich wurde Tyk2 mit der Entstehung von Autoimmun- und entzündlichen Krankheiten in Zusammenhang gebracht.

Um herauszufinden, wie Tyk2 global die Antwort auf LPS beeinflusst, führten wir Transkriptom- und Proteom-Studien durch. Ziel dieser Arbeit war, die Kinetik und die Mechanismen der Induktion ausgewählter, Tyk2-regulierter Gene/Proteine zu bestätigen und im Detail zu charakterisieren. Außerdem untersuchten wir Geninduktion, Organ- und Zelltypspezifität der interessantesten Gene/Proteine *in vivo*, um unsere Ergebnisse zu bekräftigen.

Zwei Gene, die wir genauer analysierten sind IL-17 und IL-17F, bedeutende, entzündungsfördernde Zytokine, welche in die Pathogenese verschiedener Autoimmun- und entzündlicher Krankheiten involviert, jedoch essentiell für die Abwehr von Mikroben sind. Wir konnten zeigen, dass Tyk2 die Expression von IL-17 und IL17F auf transkriptioneller Ebene in Makrophagen positiv reguliert, und dass diese Tyk2-Funktion nicht von der IFNα/β Signaltransduktion abhängt. Unsere Studie ergab weiters, dass Tyk2 auch die IL-17 Produktion in der Milz, nach intraperitonealer Administration von LPS, beeinflusst. Zusätzlich konnten wir zeigen, dass IL-17 in der Milz fast ausschließlich von zwei Zelltypen, nämlich $\gamma\delta$ T Zellen und plasmazytoiden dendritischen Zellen (pDCs),

produziert wird. Interessanterweise war bis jetzt noch nicht bekannt, dass pDCs IL-17 produzieren können.

Das dritte Gen, auf welches wir uns fokussierten, war IL-1β. Es ist bekannt, dass IL-1β substantiell an einer Vielzahl von Autoimmun- und entzündlichen Krankheiten beteiligt ist, allerdings wurde Tyk2 bis jetzt noch nicht mit der Kontrolle der IL-1β Expression in Zusammenhang gebracht. Die Regulation von IL-1β auf transkriptioneller Ebene und durch proteolytische Prozessierung von pro-IL-1β zur reifen Form wurde bereits beschrieben. Unsere Studie in Makrophagen zeigte, dass Tyk2 die Expression von IL-1β auf translationeller Ebene hemmt, womit wir eine neuartige Form der IL-1β Regulation aufdeckten. Dadurch, dass wir in Abwesenheit von IFNα/β Rezeptor 1 (Ifnar1) und Stat1 erhöhte IL-1β Produktion zeigten, lieferten wir den Beweis für die Mitwirkung von kanonischer IFNα/β Signaltransduktion. Wir konnten eine gleichartige Tyk2-abhängige Reduktion von IL-1β nach *Listeria monocytogenes* Infektion in Makrophagen demonstrieren. Übereinstimmend mit unseren *in vitro* Daten, war die IL-1β Konzentration in peritonealen Spülungen Tyk2-defizienter Mäuse nach LPS erhöht, was die Wichtigkeit Tyk2-vermittelter translationeller Regulation von IL-1β weiter hervorhebt. Im Gegensatz zu lokaler IL-1β Expression war die Konzentration an systemischem IL-1β in der Abwesenheit von Tyk2 reduziert – ein weiterer Hinweis auf die komplexe Regulation von IL-1β.

Zusammenfassend bietet diese Studie neuartige Einblicke in die vielschichtigen und spezifischen regulatorischen Funktionen von Tyk2 *in vitro* und *in vivo*. Tyk2 kann Prozesse so unterschiedlich wie Transkription und Translation modulieren. Insbesondere identifizierten wir IL-17 als LPS-induziertes Tyk2-abhängiges Gen in entzündlichen Makrophagen-Populationen *in vitro* und in γδ T Zellen und pDCs in der Milz. Zusätzlich beschrieben wir einen Tyk2-abhängigen inhibitorischen Wirkungskreis, der übermäßige IL-1β Produktion nach LPS Behandlung oder *Listeria monocytogenes* Infektion limitiert. Wir zeigen, dass dieser hemmende Effekt auf translationeller Ebene stattfindet und stellen eine neuartige Form der IL-1β Regulation vor.

INTRODUCTION

1. The Immune System

1.1. Overview

Vertebrates are permanently colonised by and communicate with a variety of microorganisms. Interactions culminate in effects that may be either beneficial or pathogenic for the host. Microbial sensing mechanisms are needed to maintain host-microbial homeostasis and to induce antimicrobial defense mechanisms, where necessary. In order to combat pathogenic microorganisms two types of immunity have evolved in mammals: the innate and the adaptive immune system. The evolutionary older innate immune system can be regarded as the first line of defense against infectious invaders. Highly conserved and invariant features of microbes are recognised by a limited number of germline-encoded receptors to discriminate self from non-self. Important cells involved in innate immune responses are phagocytes (i.e. neutrophiles, dendritic cells and macrophages), natural killer (NK) cells and $\gamma\delta$ T cells. This is in contrast to the more specific adaptive immunity, which is mediated mainly by T and B lymphocytes. Specificity is achieved by random somatic rearrangement of gene segments and clonal expansion leading to a huge repertoire of antigen-specific receptors. Compared to the innate, the adaptive immune responses develop later and it can take days to weeks until responses are mounted entirely. One important feature of adaptive immunity is the development of an immunological memory, thereby providing a mechanism to respond more vigorously to repeated exposures to the same pathogens. Both branches of the immune system are interrelated, act coordinated and in concert to immune stimuli as the innate immune system stimulates and influences the character of adaptive immune responses and as effector functions of innate immune cells are utilised by the adaptive immunity. Finally, termination of responses and return to a basal state (homeostasis) is an important function of the immune system [1, 2].

1.2. Pattern Recognition Receptors (PRRs)

PRRs are microbial sensors utilised by the innate immune system, which are expressed almost ubiquitously and independent of immunological memory. PRRs recognise highly conserved structures that are often shared by all microorganisms of a given class, referred to as pathogen-associated molecular patterns (PAMPs), although they are not necessarily unique to pathogenic species. PAMPs are rather essential for microbial physiology and therefore difficult to alter. Endogenous danger or stress signals (so called danger-associated molecular patterns or DAMPs) are also detected by these receptors.

PRRs are located throughout the body either in body fluids, on cell membranes, in endosomal compartments or in the cytoplasm. They are essentially involved in mechanisms as diverse as opsonisation and phagocytosis, complement activation, presentation and transfer of PAMPs to other PRRs, and induction of major signalling pathways leading to the production of mainly proinflammatory cytokines, such as tumour necrosis factor α (TNFα) and interleukin(IL)-1β, but also type I interferons (IFNs). Different PRRs react with specific PAMPs and show distinct expression patterns. Important bacterial and fungal PAMPs like lipopolysaccharide (LPS) and β-glucan, respectively, are often components of the cell wall. In contrast, the main targets of sensing viruses are their nucleic acids. Discrimination between self (host) and non-self is based on the structure and specific chemical modifications that are unique to viral RNA and DNA, as well as on the cellular compartment in which the nucleic acids are located [2-5].

1.2.1. Toll-like Receptors (TLRs)

The best characterised family of PRRs are the TLRs. Up until now, 11 human and 13 mouse TLR members have been described. TLRs are expressed on various immune cells, including macrophages, dendritic cells (DCs), B cells, specific types of T cells, and even on certain types of non-immune cells. These type I integral membrane glycoproteins are characterised by an outside ligand binding domain consisting of leucine rich repeats (LRR) and an inside signalling Toll-/IL-1 receptor (TIR) domain. Following ligand binding the TIR domain interacts with one or more of the five TIR domain-containing adaptor molecules: MyD88, Mal, TRIF, TRAM and SARM. This leads to activation of various downstream signalling cascades and production of mainly proinflammatory cytokines and chemokines. PAMPs sensed by the TLR family range from lipids and lipopeptides, to proteins and nucleic acids. Based on their subcellular localisation TLRs can be further divided into two groups. The first group (TLR1, 2, 4, 5 and 6) is localised on the cell surface, therefore capable of sensing microbial cell wall components, and recognises lipid structures as well as flagellin. The endosome/lysosome membrane-located second group (TLR3, 7, 8 and 9) is specialised in the recognition of nucleic acids derived from viral or bacterial genomes. Nowadays it has become increasingly evident that TLR localisation and trafficking is an important regulatory mechanism and also prevents overactivation of TLR signalling pathways [3, 4, 6-8].

1.2.2. Cytoplasmic PRRs

More recently, intracellular microbial sensors have been identified. They can be roughly subdivided into the nucleotide binding oligomerisation domain (NOD)-leucine rich repeat (LRR) proteins and the caspase recruitment domain (CARD)-helicase proteins, which are implicated in the recognition of bacterial and viral components, respectively.

1.2.2.1. NOD-like Receptors (NLRs)

NLRs function in complementing and synergising with TLRs in innate immunity. Their tripartite structure is composed of a variable N-terminal domain essential for protein-protein interactions, a centrally located NOD domain facilitating self-oligomerisation after activation and a LRR domain at the C-terminus responsible for binding and detecting PAMPs. After oligomerisation so-called NOD signalosomes are assembled leading to the transcription of proinflammatory cytokines and chemokines following activation of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways. Some NLRs also sense DAMPs and form together with caspase-1 large cytoplasmic complexes, the inflammasomes, that are responsible for the proteolytic activation of the proinflammatory cytokines IL-1β and IL-18 [9, 10].

1.2.2.2. RIG-like Helicases (RLHs)

To date, two RNA helicases, namely retinoic-acid-inducible gene 1 protein (RIG-I) and melanoma differentiation associated gene 5 (MDA5) are described to sense viral RNAs. Viral stimulation leads to the activation of NF-κB and IRF3/7 via the CARD adaptor protein Cardif (also known as IPS-1, MAVS or VISA), which in turn induces the transcription of proinflammatory cytokines and type I IFNs, respectively. LGP2, a third RLH that lacks the CARD domain, can act either as negative or positive regulator in the antiviral response, depending on the stimulus [9, 11].

1.2.3. Other PRRs

A collectin family member, few complement components and several pentraxin family members constitute a group of PRRs residing in body fluids. They all play key roles in PAMP opsonisation and activation of the complement pathway [4]. A number of membrane bound PRRs, such as mannose receptor, scavenger receptors and the C-type lectin, and dectin-1, also promote phagocytosis. Dectin-1 additionally generates intracellular signals by itself and in conjunction with TLR2 and is the major sensor for antifungal immunity [4]. Recently, novel cytosolic DNA sensors have been uncovered including DNA-dependent activator of interferon regulatory factors (DAI, also known as

ZBP1 or DLM-1) and a HIN200 protein inflammasome containing AIM2 (absent in melanoma 2). At least in the murine system AIM2 is negatively regulated by p202, another HIN200 protein. These proteins sense dsDNA irrespective of the sequence but dependent on its length. However, additional evidence for the biological significance of DAI and the AIM2 inflammasome remains to be provided [4, 12]. Most recently, IFI16 and its mouse ortholog p204 have been added to the growing list of intracellular DNA sensors [13]. RNA polymerase III was identified as a cytosolic sensor for B-form dsDNA by two independent studies. Once recognised by cytosolic RNA polymerase III, B-form dsDNA is converted into a substrate for the RIG-I pathway that then induces type I IFN production [14, 15]. The cytosolic sensor LRRFIP1 has been reported to bind B- and Z-form dsDNA and to mediate type I IFN induction together with its downstream partner β-catenin via IRF3 [16]. The two DExD/H-box helicases DHX36 and DHX9 have been identified as specific sensors for CpG-A and CpG-B, respectively, in human pDCs. Both are critical for sensing viral DNA in the cytosol and trigger differential cytokine responses via MyD88-dependent activation of IFN regulatory factor 7 (IRF7) and NF-κB [17].

1.3. Lipopolysaccharide (LPS)

Bacterial LPS (or endotoxin), a component of the outer cell wall of gram-negative bacteria, is a potent stimulator of the immune system. The polysaccharide groups of LPS, which might vary considerably in length and composition amongst bacterial species, are recognised by the adaptive immune system. In contrast, innate immune responses are induced by a conserved lipid moiety of LPS (lipid A) that is responsible for most of the toxic and pyrogenic properties of endotoxin. Local and systemic inflammation along with many other features of infection by gram-negative bacteria can be mimicked by administration of LPS alone, although the variation in biological activities of LPS originating from different bacteria has to be taken into account. High levels and prolonged production of various cytokines, reactive oxygen and nitrogen intermediates as well as other inflammatory mediators can lead to a condition designated septic (or endotoxin) shock, which is accompanied by tissue injury and multiorgan failure and often ends lethally for the host. Macrophages are of primary importance in this context as they produce all these components in response to LPS [1, 3, 18].

1.3.1. LPS-induced TLR4 signalling pathways in macrophages

The nature of the LPS signalling pathway was originally identified by two spontaneous mutations, rendering C3H/HeJ and C57BL/10ScCr mice unresponsive to LPS, as both mutations were located in the TLR4 gene. LPS liberated from gram-negative bacteria

associates with LPS binding protein (LBP), which in turn binds to membrane-bound CD14. LPS is then transferred to MD-2, which associates with the extracellular domain of TLR4, culminating in homodimerisation of the receptor complex and initiating signal transduction by the recruitment of intracellular adaptor molecules via their TIR domains. At least two distinct pathways are activated by LPS, the MyD88-dependent and the MyD88-independent pathway. They modulate transcription, mRNA stability and/or translation of a number of proinflammatory cytokine genes such as TNFα, IL-12, IL-6, IL-1β and type I IFNs [3, 4, 18].

In MyD88-dependent signalling TIRAP/Mal serves as bridging adaptor between MyD88 and TLR4. MyD88 then recruits and activates IRAK-4, followed by IRAK-2, IRAK-1 and IRAK-M. IRAK activation leads to association with TRAF6. After polyubiquitination of TRAF6 itself, IRAK-1 and NEMO, a complex composed of TGF-β-activated kinase 1 (TAK1) and the TAK1 binding proteins (TAB1-3), is recruited to TRAF6. TAK1 then activates IκB kinase (IKK) and mitogen-activated protein kinases (MAPKs) such as p38, c-jun N-terminal kinase (JNK) and extracellular signal-regulated kinases (ERKs). This ultimately leads to nuclear translocation and early activation of the transcription factors NF-κB and activator protein-1 (AP-1). MyD88 also directly interacts with IRF5 and upon translocation into the nucleus, a set of proinflammatory cytokine genes are induced by IRF5. Generally spoken, the MyD88-dependent pathway is crucial for driving proinflammatory responses [3, 19, 20].

The MyD88-independent pathway is initiated by the adaptor TRIF. This adaptor is also linked to TRL4 by a membrane bound bridging adaptor, namely TRAM. Following internalisation and translocation to endosomes/lysosomes this receptor-complex associates with TRAF3. Most importantly, downstream of TRAF3, TBK1 and IKKi (IKKε) are activated, which in turn phosphorylate IRF3. Then IRF3 homo- or heterodimers, containing the p65 subunit of NF-κB, translocate to the nucleus where they activate transcription of a subset of genes, including IFNβ. TRAF6 is also activated by TRIF and that ultimately leads to the late activation of NF-κB, characterised by a second wave of proinflammatory cytokine expression [19, 21, 22]. In addition, TLRs 3, 4, 7, 8, 9 and in inflammatory monocytes also TLR2, induce type I IFN production through the activation of the transcription factors IRF3 and/or IRF7 [23]. In myeloid DCs IRF1 is activated by TLRs 7, 8, 9 to induce IFNβ production [8].

1.3.2. LPS *in vivo*

LPS directly stimulates macrophages to secrete a number of cytokines and other inflammatory mediators, like type I IFNs and IL-12. Type I IFN signalling results in the

expression of numerous IFN-stimulated genes (ISGs), which comprise transcriptional and translational regulators and pro-apoptotic genes. IL-12 induces the local expression of IFNγ by T cells and NK cells. IFNγ then activates macrophages for phagocytosis and also augments the synthesis of proinflammatory cytokines. The actions of IL-12 are further complemented by IL-18. Type I IFNs together with IL-18 also can induce IFNγ production. IL-10 acts as a negative feedback regulator, since it inhibits for instance IL-12 expression, and expression of co-stimulatory and class II MHC molecules, which are all produced by activated macrophages and DCs. Macrophage-derived cytokines, especially TNFα, IL-1β and IL-12 are responsible for the systemic manifestation of LPS challenge. Mild responses consist of neutrophilia, fever and a rise of acute-phase reactants in the plasma, mostly originating from the liver and upregulated by cytokines such as IL-6, IL-1β and TNFα. Upon presence of LPS in the blood (sepsis), circulating cytokine levels increase and the host response changes. Overproduction of cytokines and other harmful mediators (like nitric oxide) then can result in septic shock [1, 24]. The central role of type I IFNs in this context was demonstrated by the fact that IFNβ-deficient mice are highly resistant to LPS induced endotoxin shock [25].

2. Jak-Stat signalling pathway

2.1. Overview

One of the best characterised pathways by which cytokines transduce their signals from the cell surface to the nucleus is the Janus kinase and signal transducer and activator of transcription (Jak-Stat) signalling pathway. This pathway was discovered by studies of IFN signalling and is used by all type I and II cytokine receptors. The sequential events leading to the transcriptional regulation of responsive genes are depicted in figure 1 (as exemplified for type I IFNs). Upon ligand binding, oligomerisation and subsequent activation of the respective receptor chains is initiated. Thereby Jaks bound to their cytoplasmic portion come into close proximity, auto- and/or cross-phosphorylate and phosphorylate tyrosine residues on the receptor chains. These are then recognised by the Src homology 2 (SH2) domains of specific Stats leading to their tyrosine phosphorylation and activation. Activated Stat homo- and/or heterodimers translocate to the nucleus where they bind to the promoter regions of target genes. Although only four Jaks and seven Stats exist, highly specific and diverse responses can be mounted by the different usage of various combinations of one or more Jaks and Stats. In addition other signalling

cascades and transcription factors can be activated, thereby adding another level of complexity to the biological responses to a given cytokine or growth factor [26-28].

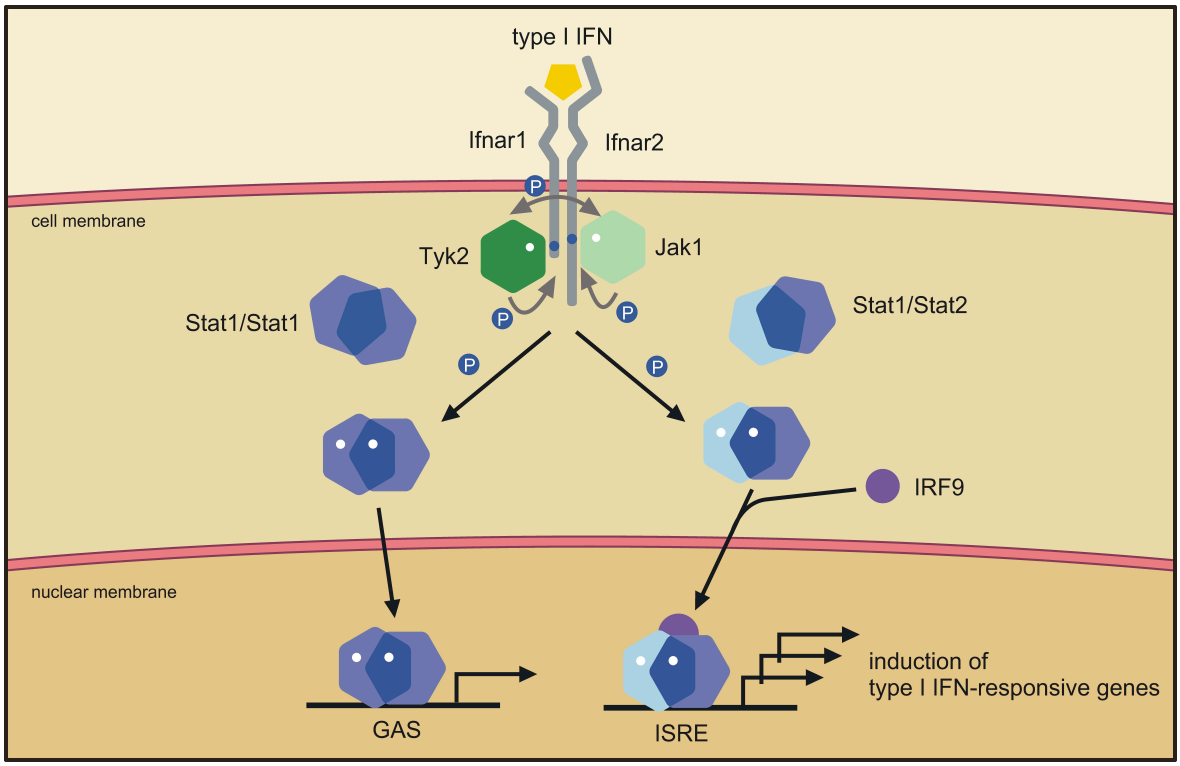

Figure 1: The Jak-Stat signalling pathway. Schematic overview as exemplified for type I IFNs (see text for details).

2.2. Janus Kinases (Jaks)

There are four Jak family members (Jak1-3 and Tyk2) in mammals that range in size from 120 to 140 kDa and, with the exception of Jak3, are expressed in most tissues. This family of non-receptor tyrosine kinases features seven conserved Jak homology (JH) domains. Notably, they possess a tandem set of C-terminal kinase domains, but only JH1 is catalytically active, whereas JH2, referred to as pseudokinase domain, serves an essential regulatory role. Activation is driven by phosphorylation of critical tyrosines in the “inactivation loop”, thereby releasing the blockade of the catalytical site. So far no function has been assigned to the SH2-related domain (JH3 and half of JH4) but the N-terminal Four-point-one/Ezrin/Radixin/Moesin (FERM) domain (half of JH4 and JH5-7) is important for stable association with cognate receptor chains.

Jak1 knock-out mice die perinatally due to defective leukemia inhibitory factor (LIF) receptor signalling. The critical role of this kinase in the response to IFNs, IL-10, IL-2/IL-4

and IL-6 cytokine families was clarified by analyses of cells and tissues from Jak1 deficient mice. Consistent with a critical role in erythropoiesis, Jak2 knock-out mice die of anaemia prenatally (E12.5). *Ex vivo* studies on embryonal Jak2-/- cells and tissues revealed an important role in directing responses to IFNγ and cytokines utilising the single-chain and IL-3 receptor families. Intriguingly, several myeloproliferative disorders observed in humans can be attributed to a single pointmutation in the JH2 domain of Jak2. Jak3 expression is mainly restricted to leukocytes, where this kinase exclusively associates with the IL-2 receptor γ-chain (γc), a receptor component for a number of lymphotrophic cytokines. Mutations in either Jak3 or γc are associated with severe combined immunodeficiency (SCID) in humans and similarly, Jak3 knock-out mice develop an immunodeficiency syndrome, although less severe. Therefore, Jak3 has become an important therapeutic target [26-28].

2.3. Tyk2

2.3.1. Tyk2 in cytokine signalling

The essential role of Jak kinases in cytokine signalling was originally revealed for Tyk2 in type I IFN responses. Tyk2 associates with receptors for type I and type III IFNs (also designated IFNλs), IL-12/23, IL-6 and IL-10 cytokine families and mediates signals in combination with Jak1 and Jak2 but not Jak3 (figure 2). The two receptor chains of the type I IFN receptor, Ifnar1 and Ifnar2, are permanently associated with Tyk2 and Jak1, respectively. Tyk2-deficient human cell lines are unresponsive to IFNα and display partial defects in IFNβ signalling [29]. Moreover steady-state expression of Ifnar1 is compromised in the absence of Tyk2 in these cells [30, 31]. Whereas type I IFN receptor signalling seems highly dependent on Tyk2 in humans, surprisingly modest effects were observed in the murine system and lack of responsiveness could be detected only with low concentrations of IFNα/β [32-34]. Although Tyk2 does not bind to the IFNγ receptor (neither Ifngr1 nor Ifngr2 chain), IFNγ signalling as well as production are both dependent on Tyk2, presumably due to reduced Stat1 protein levels and through impaired action of IL-12, respectively. Tyk2 is associated with the IL-12 receptor β1 (IL-12Rβ1) chain of the IL-12R and activates mainly Stat4, which is required for the activation of the IFNγ gene. Interestingly, the Tyk2-associated IL-12Rβ1 chain is also part of the IL-23R complex, but different DNA-binding Stat complexes are formed in response to IL-23 as compared to IL-12 [35, 36]. IL-23 is critically involved in the development of a growing number of autoimmune and inflammatory diseases, but the precise role of Tyk2 for IL-23-mediated responses remains to be determined [37]. Together with Jak1 and Jak2, Tyk2 is also

activated by cytokines that share the gp130 receptor chain, such as IL-6, IL-27, LIF and oncostatin M (OSM). As far as analysed, Tyk2 exerts rather ancillary or redundant functions in this context [32, 34, 38]. Some controversy exists about the role of Tyk2 in IL-10-mediated signalling. Although no effect has been observed in earlier studies [32, 34], partially impaired responses to IL-10 were reported in Tyk2-deficient peritoneal macrophages more recently [39]. Interestingly, IL-10R2 and Tyk2 are likely also involved in type III IFN signalling, albeit direct evidence remains to be provided. Despite sharing the same receptor chain with the IL-10 family of cytokines, type III IFNs are more related to and exert similar biological functions as type I IFNs [40].

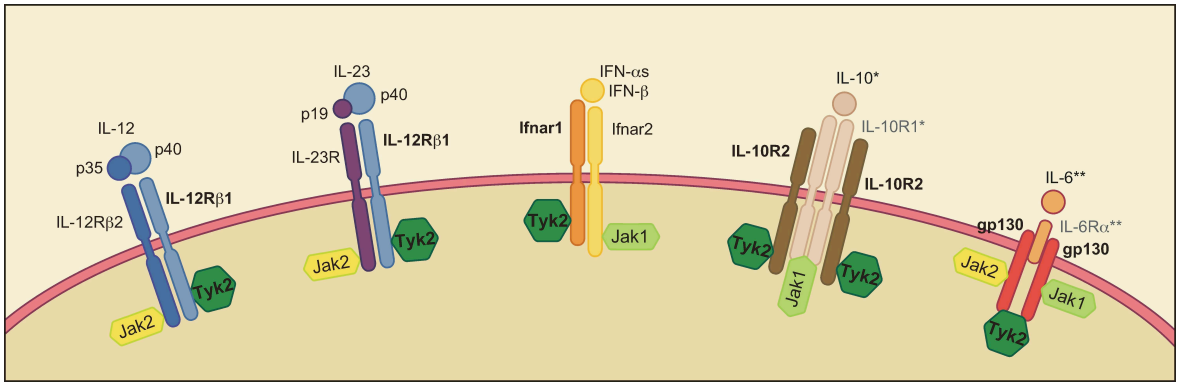

Figure 2: Cytokines and receptors that utilise Tyk2. Receptor complexes utilised by the IL-10 and gp130 family of cytokines consist of a common and a cytokine-specific receptor chain (*, **). Additional cytokines (and their respective cytokine-specific chains) that signal *via* these common chains are for (*): e.g. IL-22 (IL-22R) and IFNλ (IL-28R1) and for (**) e.g.: LIF (LIFR), oncostatin M (OSMR) and IL-27 (WSX-1). Therefore, Tyk2 might also affect signalling by these cytokines.

2.3.2. Consequences of Tyk2 deficiency

In order to analyse the functions of Tyk2, three Tyk2 knockout mice have been engineered independently from each other [32, 34, 41]. In addition, the B10.Q/J strain carries a naturally occurring mutation in the Tyk2 gene that was reported to result in the absence of Tyk2 protein [33]. Conflictingly, Tyk2 protein was detected in B10.Q/J mice very recently [42]. Tyk2-deficient mice are viable and fertile and do not show gross abnormalities in lymphocyte development and proliferation, but their innate and adaptive immune responses are impaired. Until now Tyk2-deficient mice have been challenged with a range of viral and bacterial pathogens and effects of Tyk2 in diverse disease models have been analysed. Tyk2 can be protective, neutral or even deleterious for the

host. Tyk2 is dispensable in the host defense against vesicular stomatitis virus (VSV). In contrast, prominent defects in cytotoxic T lymphocyte (CTL) activity following infection with lymphocytic choriomeningitis virus (LCMV), and elevated vaccinia virus (VV) replication in spleens of Tyk2-/- mice have been reported [32]. Strongly reduced survival and impaired clearance of virus from several organs of mice lacking Tyk2 were monitored after challenge with murine cytomegalovirus (MCMV) [43]. Tyk2-deficient mice are also highly susceptible to infections with the intracellular bacteria *Listeria monocytogenes* [44] and (as demonstrated with B10.Q/J mice) the protozoan parasite *Toxoplamsa gondii* [33, 45]. Tyk2 is differentially required for the defense against *Leishmania major*, as more severe and prolonged skin lesions develop in Tyk2-/- mice, but Tyk2 is not required for the ultimate control of the disease [46].

Apart from infectious diseases, Tyk2 involvement in several other disease models has been studied. For example, resistance of Tyk2-deficient mice against high-dose LPS-induced [25, 47] and also intestinal ischemia/reperfusion-induced shock [48] could be shown. Moreover B10.Q/J mice are completely resistant to collagen-induced arthritis (CIA), an animal model for inflammatory polyarthritis [33]. Tyk2-mediated signalling plays a critical role in pathogenic CD4 T cell responses involved in the development of experimental autoimmune encephalomyelitis (EAE) [49]. Mice lacking Tyk2 show increased B lymphoid leukaemia/lymphoma formation and impaired tumour surveillance [50, 51]. Nonetheless, Tyk2-dependent invasiveness of malignant cells was reported in human prostate cancer cells *in vitro* [52] and more importantly, in a murine model system for human Burkitt's lymphoma [53]. Tyk2-deficient mice exhibit a tendency towards Th2 responses, as exemplified by the use of a mouse asthma model, resulting in enhanced allergic lung inflammation, increased antibody production and recruitment of eosinophils [54].

Intriguingly, loss of Tyk2 has been associated with distinct phenotypes in humans and mice and a patient who had been clinically diagnosed with hyper-IgE syndrome (HIES), turned out to carry a homozygous Tyk2 mutation. This patient displayed an unusual susceptibility to a number of microorganisms including virus, fungi and mycobacteria and suffered from atopic dermatitis. In contrast to previous data obtained in mice, almost complete defects in type I IFN, IL-6 and IL-10 signalling has been observed in this patients T cells, peripheral blood monocytes and macrophages, respectively. Transduction of a functional Tyk2 gene completely rescued cytokine signalling in T cells from this patient [54, 55]. Intriguingly, evidence for an implication of Tyk2 in human diseases, like multiple sclerosis (MS), psoriasis, systemic lupus erythematosus (SLE) and Crohn's disease has been provided by genome-wide association studies [56-61].

2.4. Signal transducers and activators of transcription (Stats)

The seven mammalian Stat family members (Stat1-4, 5a, 5b and 6) largely reside in the cytoplasm of resting cells as inactive dimers. Stats range in size from 750 to 900 amino acids and constitute of seven structurally and/or functionally conserved domains. The amino-terminal domain is responsible for dimerisation of unphosphorylated Stats, whereas the adjacent coiled-coil domain associates with regulatory proteins. As indicated by its name, the DNA-binding domain (DBD) enables binding of Stats to DNA. The linker domain serves to achieve an appropriate conformation between the DNA-binding and the dimerisation domains. All of these domains have also been implicated in the regulation of nuclear import and/or export. The SH2 domain, which is the most highly conserved domain within the Stat family, mediates specific recruitment to receptor chains, and formation of active Stat dimers. In close proximity to the SH2 domain, the tyrosine activation motif is positioned, usually near residue 700, thereby precluding intramolecular SH2-phosphotyrosine association. The adjacent carboxy-terminal residues constitute the transcriptional activation domain (TAD), which varies considerably in length and sequence among different Stats. This divergence facilitates association with multiple transcriptional regulators and the TADs of all Stats (except Stat2) include conserved serine phosphorylation sites providing additional opportunities for regulation (see below). Intriguingly, a number of naturally occurring carboxy-terminally truncated Stat isoforms exist, i.e. Stat1β, Stat3β, and Stat4β [62-64]. The latter two of which have been shown to direct gene expression programs different to those mediated by their full-length counterparts (Stat3α and Stat4α) [65-68].

Apart from the canonical tyrosine phosphorylation, Stats can undergo additional covalent modifications, most notably serine phosphorylation. All Stats, with the exception of Stat2, can be phosphorylated on at least one serine residue in their TAD, and this promotes transcriptional activity [69-73]. Several additional, although poorly characterised, modifications that regulate Stat activity (e.g. acetylation and/or O-glycosylation, R-methylation, SUMOylation and ubiquitin-directed decay of Stats) have been described. However, their roles are still partially controversial [28]. Recently, it has been found that Stats 1 and 3 (and probably other Stats) also play important roles in mediating gene expression without tyrosine phosphorylation [74, 75].

Stat1 knock-out mice exhibit profound defects in their biological response to type I and type II IFNs and consequently are highly susceptible to microbial infections [28]. Furthermore, Stat1-null mice displayed increased susceptibility to chemically induced and spontaneous tumours and Stat1 deficiency has also been linked to impaired growth control [63, 64]. In general, Stat1 target genes appear to promote inflammation and

antagonise proliferation [28]. Stat2 is mainly active in a complex together with Stat1 and IRF9, the ISGF3 and Stat2-deficient mice showed severe defects in their biological response to type I IFNs and loss of IFN I autocrine activity [27, 28]. Stat3 deficiency results in early embryonic lethality (E6.5-7.5) and this is consistent with the involvement of Stat3 in signal transduction downstream of a wide range of cytokines and growth factors. For example biochemical and genetic studies have revealed a pivotal role of Stat3 in transducing signals of the entire IL-6 and IL-10 families of cytokines. Tissue-specific knock-outs have highlighted an important anti-inflammatory role for Stat3, whereas many cancers have been associated with constitutively active Stat3 [27]. Stat4 knock-out mice elucidated a critical role of this transcription factor in IL-12 signalling. Therefore, Stat4-deficient mice are not able to mount Th1 responses or activate NK cells and consequently show impaired IFNγ secretion [27, 28]. Two tandem genes encode Stat5a and Stat5b. Stat5a-deficient mice displayed impaired mammary gland development owing to the loss of prolactin responsiveness, whereas deletion of Stat5b resulted in impaired growth due to the loss of growth hormone responsiveness. Deletion of the entire Stat5a-Stat5b gene locus has revealed a substantial role of Stat5 in erythropoiesis and lymphopoiesis [27, 62]. Biochemical and genetic studies have demonstrated the importance of Stat5a and Stat5b in signalling of the IL-3, single chain and γc receptor families [28]. Stat6-null mice exhibited profound defects in Th2 cell polarisation due to impaired IL-4 and IL-13 signalling. Furthermore Stat6 deficiency has been associated with impaired mast cell activation and B-cell function, including defects in proliferation, maturation, and MHC II and IgE expression [27, 28].

3. Interleukin-17 (IL-17) and the IL-17 family of cytokines

IL-17 (also designated IL-17A) is the founding member of a novel cytokine family that has originally gained prominence due to its involvement in various autoimmune diseases in both men and mice. The mammalian IL-17 family comprises six members (IL-17A-F), amongst those IL-17F protein is most homologous to IL-17 (~50%). IL-17E (also known as IL-25) is most divergent and shares only 17% protein sequence similarity. Although cellular sources and expression patterns differ, all IL-17 cytokines have proinflammatory activities. Nevertheless, half of the IL-17 family members (IL-17B, IL-17C and IL-17D) are still poorly characterised and await future investigation [76].

Up until now IL-17 and IL-17F are the most intensively studied family members. Both form homodimers but also IL-17–IL-17F heterodimers. Homo- and heterodimers utilise the same receptor subunits for signalling, namely IL-17RA and IL-17RC, which together form heteromeric complexes. Although there is considerable overlap in their biological

functions, individual biological effects have been reported. IL-17 and IL-17F, as well as IL-21 and IL-22 are the lineage-defining cytokines of T helper type 17 (Th17) cells, the most recently discovered Th cell lineage [76-78]. Th17 cells are considered particularly important in host defense against bacterial, mycobacterial and fungal pathogens, especially at epithelial surfaces. However, Th17 functions extend beyond protection against extracellular pathogens, as demonstrated by infections with a number of intracellular bacteria. Like other effector T cells, dysregulated Th17 cells can cause immunopathology and they have been implicated in organ-specific and systemic autoimmune diseases like psoriasis, rheumatoid arthritis and multiple sclerosis, and also in animal models like EAE and CIA [79]. Moreover, it has become apparent that Th17 responses can also contribute to viral persistence and chronic inflammation associated with parasitic infection [80]. In mouse tumour models and in human cancers it has been shown that Th17 cells and associated cytokines have both anti- and pro-tumourigenic functions [81, 82].

Th17 cells depend on a specific cytokine milieu, composed of IL-6, TGFβ and, only in humans, IL-1β for their development from naïve T cells to effector T cells, while IL-23 is important for their maintenance. IL-21 drives Th17 development *via* a positiv feedback loop. Additionally, several specific transcription factors (Stat3, RORγt and RORα) are critically involved in Th17 development [83].

IL-17 and IL-17F induced signalling is mediated by Act1 (also known as CIKS), TRAF6 and NF-κB [78]. However, Act1-independent pathways are also induced, since ERK1 and ERK2 are activated in Act1-/- cells in response to IL-17, albeit with delayed kinetics [84]. Although IL-17 induces the activation of various MAPKs, ERK is generally most strongly and rapidly phosphorylated. Interestingly, pharmacological inhibitors of Jaks have been reported to limit IL-17-mediated signalling and weak activation of Stats and PI3K have also been shown [78].

IL-17 triggers downstream production of a number of proinflammatory cytokines (e.g. IL-6, IL-1β and TNFα), chemokines and metalloproteinases in various cell types, thereby co-ordinating neutrophil expansion, survival, and early recruitment to local sites of infection [79]. Additionally, target genes that have chemotactic activity for lymphocytes, DCs and other immune cells, as well as monocyte-recruiting chemokines are induced by IL-17. Direct killing of invading organisms is warranted by the upregulation of antimicrobial peptides and notably, some chemokines that also exhibit antimicrobial activity.

Besides being produced by Th17 cells, both IL-17 and IL-17F production by a growing number of cell types has been reported [83, 85]. For example, IL-23–dependent IL-17 production by $\gamma\delta$ T cells has been detected in mice following infection with *Escherichia*

coli, *Mycobacterium tuberculosis* or *Mycobacterium bovis* [86-88]. It has also been demonstrated that IL-1β and IL-23 induced IL-17 expression in γδ T cells independent of TCR engagement [89]. Peritoneal NK cells isolated from *Toxoplasma gondii* infected mice were shown to produce IL-17 in an IL-6–, IL-23– and TGF-β–dependent manner [90]. Invariant natural killer T (iNKT) cells and NKT cells secreted IL-17 either after TCR or IL-23R ligation, and engagement of both receptors has a synergistic effect. Unlike NK and naïve T cells, NKT and iNKT cells constitutively express IL-23R and RORγt [91-93]. In a mouse model of LPS-induced lung inflammation IL-17 secretion by neutrophils has been observed [94]. IL-17 was also secreted by eosinophils in the bronchoalveolar lavage from asthmatic patients [95]. However, in a mouse model mimicking allergic asthma, alveolar macrophages have been identified as the main source of IL-17 [96]. Interestingly, TLR2-dependent IL-17 production has been detected in murine peritoneal macrophages after treatment with *Porphyromonas gingivalis* LPS but not after challenge with live bacteria [97]. Furthermore, IL-17 expression by IL-10–deficient peritoneal macrophages has been demonstrated in response to *E. coli* LPS [98]. Stimulation with IL-23 or the TLR2 agonist zymosan, rapidly induced IL-17 production in lymphoid tissue inducer-like (LTi-like) cells, which was impaired in the absence of Stat3. These cells constitutively express the IL-23R, aryl hydrocarbon receptor (AHR) and CCR6 [99]. In a model of TNF-induced shock IL-17 was rapidly secreted by Paneth cells, which are highly specialised epithelial cells in the gut [100].

AIMS

The Janus kinase family member tyrosine kinase 2 (Tyk2) participates in numerous cytokine and growth factor signalling pathways. We and others have shown that Tyk2-/- mice have defects in the antiviral defense against selected viruses and are more susceptible to various other pathogens. In contrast, Tyk2-/- mice are resistant to lipopoly-saccharide(LPS)-induced endotoxin shock. Most of the defects described so far could be attributed to impaired IL-12 signalling and consequently, reduced IFNγ production, and to partial defects in IFNα/β responses.

In ongoing transcriptomics and proteomics studies comparing wildtype with Tyk2-/- macrophages, several genes/proteins were identified that showed Tyk2-dependent regulation in response to LPS. The first aim of my thesis was to further characterise a selection of these genes/proteins with respect to the kinetics of induction and the molecular mechanisms involved. The second major goal was to investigate the *in vivo* relevance of these findings. For this purpose, I focussed on gene induction *in vivo*, organ specificity and cell type distribution of the most interesting target gene(s)/protein(s). Based on the findings, further in depth *in vitro* analysis should be conducted in additional primary cell types (e.g. T cells, NK cells, DCs).

RESULTS I

Tyrosine kinase 2 is required for LPS-induced IL-17 production in inflammatory macrophages and spleens

Stiefvater *et al.*, *manuscript in preparation*

TYROSINE KINASE 2 (TYK2) IS REQUIRED FOR LPS-INDUCED IL-17 PRODUCTION IN INFLAMMATORY MACROPHAGES AND SPLEENS

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Abstract

The Janus kinase (Jak) family member tyrosine kinase 2 (Tyk2) is an integral part of various cytokine and growth factor signalling pathways. We have previously reported that macrophages from Tyk2-deficient mice fail to fully respond to lipopolysaccharide (LPS). We demonstrate herein, that LPS stimulation induces interleukin-17 (IL-17) production via a Tyk2-dependent pathway in thioglycollate-elicited peritoneal macrophages. IL-17 and IL-17F are upregulated upon LPS treatment with similar kinetics and both mRNAs are considerably reduced in the absence of Tyk2. Induction of IL-17 and IL-17F is only slightly reduced in type I interferon (IFNα/β) receptor 1 (Ifnar1) deficient macrophages, demonstrating that auto-/paracrine actions of IFNα/β do not strongly impact on IL-17 production in macrophages. Interestingly, signal transducer and activator of transcription 3 (Stat3) is not required for LPS-induced IL-17 production in macrophages. In the absence of Stat3, IL-17 and IL-17F mRNA and IL-17 protein are already produced constitutively and are strongly increased upon LPS treatment. Thus, in contrast to its essential role in the differentiation/maintenance of IL-17 producing T cells (Th17), Stat3 in macrophages exerts inhibitory rather than stimulatory effects on LPS-induced IL-17 production. We furthermore show that Tyk2 is indispensable for splenic IL-17 production following intraperitoneal LPS administration *in vivo*. However, we did not find evidence for a substantial contribution of $CD11b^+$ cells to the early IL-17 production. Instead, $\gamma\delta$ T cells and, to a lesser extent, plasmacytoid dendritic cells (pDCs) appear to be the main sources of innate IL-17 in spleens. Taken together we show a crucial role of Tyk2 for LPS-induced innate IL-17 production *in vitro* and *in vivo*.

Introduction

A large number of cytokines transduce their signals via the Janus kinase (Jak) - signal transducer and activator of transcription (Stat) signalling pathway [1, 2]. Tyrosine kinase 2 (Tyk2) is one of the four mammalian Jak family members and has been initially identified via its critical role in type I interferon (IFNα/β) signalling [3]. Tyk2 activation has been subsequently demonstrated for gp130-receptor chain-utilising cytokines, interleukin (IL)-10, IL-12, IL-13, IL-22 and IL-23 [4-6]. Cells from Tyk2-deficient mice show selective and partial defects in cytokine responses and Tyk2 deficiency leads to increased susceptibility to a number of different pathogens [7-12]. On the contrary, $Tyk2^{-/-}$ mice are resistant to high-dose lipopolysaccharide (LPS)-induced endotoxin shock [13, 14]. LPS signals through Toll-like receptor 4 (TLR4) and evokes various cell signalling pathways [15-17]. The induction of proinflammatory cytokines, like tumour necrosis factor α (TNFα) and IL-1β, occurs through the activation of the nuclear factor-κB (NF-κB) pathway via the adaptor molecule myeloid differentiation factor 88 (MyD88). MyD88-independent activation of interferon regulatory factor 3 (IRF3) induces expression of IFNβ, which subsequently leads in an auto-/paracrine way to the induction of numerous IFN-responsive genes. In addition, IFNα/β can amplify its own expression via the induction of IRF7 in the presence of activating stimuli [18]. Tyk2-deficient macrophages show impaired IFNα/β and inducible nitric oxide synthase (iNOS, NOS2) expression in response to LPS, while the production of TNFα remains unaffected [14]. This phenotype may be caused by reduced basal and induced availability of transcription factors in the absence of Tyk2, i.e. Stat1, IRF1 and IRF7 [8, 14], but the exact mechanisms are not clear to date.

IL-17 (also designated IL-17A) is the prototype of the IL-17 family, which consists of six members (IL-17A–F). Amongst those, IL-17F is most homologous to IL-17 and considerably overlapping yet distinct biological functions have been reported [19-21]. IL-17 is currently classified as proinflammatory cytokine, as it induces a number of mediators including those involved in chemotaxis of neutrophils. The T helper type 17 (Th17) cell lineage is considered to be the major source of IL-17, especially in autoimmune diseases, but also in immunity to infection [22]. In addition IL-17F, IL-22 and IL-21 are preferentially produced by this T cell subset [23, 24]. Transforming growth factor β (TGF-β) and IL-6 have been shown to drive Th17 development [25-27], whereas subsequent exposure to IL-23 and IL-1β is necessary for full effector differentiation and function [25, 28-31]. Furthermore, IL-23 mediated proliferation and IL-17 production have been reported in memory T cells [32, 33]. Only recently, more attention has been paid to IL-17 production and underlying mechanisms in innate immune responses. It has been demonstrated that

IL-17 can be rapidly produced by innate immune cells like γδ T cells, iNKT cells, lymphoid tissue inducer (LTi)-like cells and macrophages [34-38]. It has become clear, however, that although these cell populations share some common activating signals (e.g. IL-23, IL-23R, RORγt), unique yet ill-defined pathways are involved [39].

In this study we show that IL-17 mRNA and protein production in response to LPS are dependent on the presence of Tyk2 but independent of functional IFNα/β signalling in murine inflammatory peritoneal macrophages. We define a negative regulatory role for Stat3, as myeloid-specific Stat3 deletion results in enhanced IL-17 production. The importance of Tyk2 for LPS-induced IL-17 production is confirmed *in vivo*. Although γδ T cells appear to be the major source of innate IL-17 production in spleens, we provide for the first time evidence for the ability of pDCs to produce IL-17 and exclude a substantial contribution of $CD11b^+$ cells.

Materials and Methods

Mice

All mice were on C57BL/6 background. Tyk2$^{-/-}$, Ifnar1$^{-/-}$ and Stat3$^{fl/fl}$ mice were described previously [8, 40, 41] and were backcrossed over 10 generations into C57BL/6. LyzsCre (also designated LysMCre) mice were purchased from the Jackson Laboratory, Sacramento, CA (strain name: B6.129P2-*Lyzs*$^{tm1(cre)Ifo}$/J) and crossed with Stat3$^{fl/fl}$ mice to generate Stat3$^{LyzsCre-/-}$ mice. This strain expresses Cre recombinase from the endogenous *Lyzs* locus. Cre-mediated recombination results in deletion of the loxP site-flanked Stat3 gene in the myeloid cell lineage (including monocytes, mature macrophages, and granulocytes). 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 (BM.W_F^{a} ref. 68.205/0204-C/GT/2007 and ref. 68.205/0233-II/10b/2009).

Isolation of cells and LPS challenge

Thioglycollate-elicited peritoneal macrophages were prepared and cultured in DMEM (supplemented with 5 % FCS, 100 µg/ml penicillin, 100 U/ml streptomycin, 1 mM L-glutamine and 50 µM 2-mercaptoethanol) as described previously [42]. Macrophages were stimulated approximately 24 h after isolation with 100 ng/ml LPS (*E. coli* serotype 055:B5; Sigma-Aldrich, St. Louis, MO; dissolved in PBS) for the times indicated. Age- (8-10 weeks) and sex-matched mice were injected i.p. with LPS (1 mg per 20 g body weight). To assess *in vivo* intracellular IL-17 levels, 0.25 mg Brefeldin A (Sigma-Aldrich) was simultaneously injected i.v., as previously described [43]. At the indicated times mice were sacrificed. Single cell suspensions were prepared from spleens of LPS-challenged and control mice. Splenocytes were incubated with MACS beads conjugated with anti-Dx5, anti-CD11b, anti-CD19 and anti-PDCA1. Cells were purified by positive selection using MACS columns following the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany).

Antibodies

Anti-phospho-Stat3, anti-Stat3, anti-phospho-p44/p42 MAPK (ERK1, ERK2), anti-phospho-Akt, anti-Akt and anti-phospho-NF-κB-p65 antibodies (Abs) were purchased from Cell Signaling Technologies (Danvers, MA); anti-pan-ERK from BD Biosciences (San Diego, CA) and anti-NF-κB-p65 Ab from Upstate Biotechnology (Lake Placid, NY). Pacific

Blue-conjugated anti-CD3 (17A2) Ab was from BioLegend (San Diego, CA), PE-conjugated anti-IL-17 (TC11-18H10) and FITC-conjugated anti-TCR$\gamma\delta$ (GL3) from BD Biosciences. AlexaFluor® 647-conjugated Siglec-H (eBio440c) was purchased from eBioscience (San Diego, CA).

Cell lysis, Western blot analysis

Cells ($2x10^6$) were lysed in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 % Igepal CA-630 (v/v), 10 % glycerol (v/v), 1 mM DTT, 0.1 mM EDTA, 1 mM sodium orthovanadate, 25 mM sodium fluoride, 3 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 mM PMSF. Cell debris was removed by centrifugation, and whole-cell lysates were used for Western blot analysis. 10 µg of whole-cell lysates were separated on 6.5 % or 10 % SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were probed using the indicated Abs and the ECL detection system (GE Healthcare, Munich, Germany).

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated using TRIzol (Invitrogen Life Technologies, Lofer, Austria), and reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Vienna, Austria) each according to the manufacturer's instructions. The standard 20 µl reaction contained 1x PCR buffer (Reaction buffer B, Solis BioDyne, Tartu, Estonia), 200 µM dNTP, 4 mM $MgCl_2$, 300 nM primers, 100 nM probe, 1U HOT FIREPol (Solis BioDyne). For *IL-17 (IL-17A)* and *IL-17F* TaqMan Gene Expression Assays (Applied Biosystems by Life Technologies, Carlsbad, DA, assay IDs Mm00439619_m1 and Mm00521423_m1, respectively) were used for cDNA amplification. Primers and probes for *Ube2d2* and *TNFα* were submitted to the Real-Time PCR Primer and Probe Database (http://medgen.ugent.be/rtprimerdb/; RTPrimerDB identification numbers 3377 and 3747, respectively [44]). *IL-17, IL-17F* and *Ube2d2* qRT-PCRs were conducted with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). *TNFα* qRT-PCR was conducted with a Mastercycler realplex (Eppendorf, Vienna, Austria). All assays were performed under the same cycling conditions: 15 min at 95°C, 20 s at 95°C and 1 min at 60°C; steps 2 and 3 were repeated 40 cycles (*TNFα, Ube2d2*) or 45 cycles (*IL-17, IL-17F*). Fluorescence data were collected during the extension phase (step 3). Differential target gene expression was calculated as previously described [14], using *Ube2d2* as endogenous control. With the exception of MACSed spleen cells, n-fold expression levels were calculated relative to the untreated wildtype (WT 0h). Target gene expression in

MACSed spleen cells was normalised to the endogenous control (Ube2d2) and multiplied with 1000.

Measurements of cytokines

Cell culture supernatants were collected and centrifuged at 5000 × *g* for 3 min. IL-17 and IL-17F concentrations were determined by ELISA (R&D Systems, Minneapolis, MN; detection limit 10 pg/ml).

Intracellular cytokine staining and flow cytometric analysis

Single cell suspensions were prepared from spleens of LPS-challenged and control mice (all treated with Brefeldin A). Splenocytes were incubated with MACS beads conjugated with anti-CD19. Cells were depleted using MACS columns following the manufacturer's protocol (Miltenyi Biotec). Cell surface markers were stained with appropriate antibodies for 30 min at 4°C. Thereafter, intracellular staining was performed according to manufacturer's instruction (BD Biosciences). Briefly, 100 µl of BD Cytofix/Cytoperm solution (BD Biosciences) was added to cell suspension with mild mixing and incubated for 20 min at 4°C. Fixed cells were washed twice with 250 µl of BD Perm/Wash solution (BD Biosciences) and were stained intracellularly with PE-conjugated anti-mIL-17 mAb for 30 min at 4°C. Stained cells were analysed on a flow cytometer (FACSAria; BD Biosciences). Events were collected and analysed with FACSDiva software (BD Biosciences).

Statistical analysis

qRT-PCR gene expression data were investigated for differences among genotypes and time after challenge. Univariate regression was calculated with the log-transformed target to control gene ratio as dependent variable. Linear contrasts were encoded using dummy variables, such that i) for each time point the mutant genotypes were compared to the wildtype and ii) for each time point the mutant genotypes were compared to each other. In both cases, differences among experiments were taken into account. Analysis of ELISA data was performed using log-transformed data. As above linear contrasts were encoded using dummy variables such that genotypes were compared after 24 h of treatment. Analysis was done using SPSS 13 for Mac OS-X. For the evaluation of FACS data of single treatments/time points chi-squared tests were applied (R: a language and environment for statistical computing, http://www.R-project.org).

Results

IL-17 and IL-17F expression is induced by LPS in macrophages in a Tyk2-dependent and Ifnar1-independent manner

In order to further elucidate the impact of Tyk2 deficiency on LPS responses, we have conducted a qRT-PCR screening experiment and found IL-17F as Tyk2-dependent, LPS induced gene (E. Hofmann, unpublished data). In addition to IL-17F, we also found Tyk2 dependence for IL-17 induction in macrophages (Fig. 1A). IL-17 mRNA was rapidly and strongly induced by LPS. Induction could already be observed 1 h following treatment and peaked around 6 to 8 h. IL-17 mRNA expression was dependent on the presence of Tyk2 at all time points investigated. In the absence of the IFNα/β receptor 1 (Ifnar1), IL-17 induction occurred slightly delayed but reached similar mRNA levels as in wildtype cells at later time points. Detailed analysis of IL-17F mRNA expression revealed a similar transient induction and mRNA levels peaked at the same time but with a slightly higher amplitude than observed for IL-17 (Fig. 1B). Dependency on Tyk2 and induction in the absence of Ifnar1 was similar for IL-17 and IL-17F. In contrast, and consistent with previous data on TNFα protein secretion in Tyk2$^{-/-}$ macrophages [14], TNFα mRNA induction occurred independently of Tyk2 and Ifnar1 (Fig. 1C). We next examined whether IL-17 and IL-17F protein is secreted by macrophages. IL-17 in cell culture supernatants reached about 60 pg/ml on average at 24 h after LPS treatment (Fig. 1D). In the absence of Ifnar1, secretion was modestly but significantly lower than in wildtype cells and severely impaired in Tyk2$^{-/-}$ macrophages (Fig. 1D). Similar results were obtained for IL-17F (Fig. 1E). Thus, although IFNα/β signalling can inhibit IL-17 production under certain conditions [45, 46], auto-/paracrine IFNα/β acts modestly enhancing in LPS-treated macrophages. Of note, overall IL-17 protein levels were quite low but consistent with previous reports on LPS-treated macrophages [47, 48]. Comparable IL-17 amounts were also secreted by peritoneal macrophages in response to chitin [49] and by alveolar macrophages following exposure to supernatants derived from OVA/IgE-stimulated mast cells [50].

LPS-mediated Stat3 activation is impaired in the absence of Tyk2

Stat3 has been implicated in the development of IL-17–producing T cells [51, 52] and direct binding of Stat3 to the IL-17 and IL-17F promoters has been postulated [51]. We thus analysed the dependence of Stat3 tyrosine phosphorylation on Tyk2 in response to LPS. Stat3 phosphorylation could first be detected after 2 h LPS treatment, declined at 4 h

and was detectable for up to 12 h after treatment (Fig. 2A and data not shown). Stat3 phosphorylation was strongly dependent on the presence of Tyk2 at all time points investigated (Fig. 2A and data not shown). Apart from Stat3, the PI3K/Akt and NF-κB pathways have been implicated in the induction of IL-17 expression under certain conditions [53, 54]. As shown in Fig. 2B, Akt serine phosphorylation upon LPS treatment was relatively low in our experimental setup and clearly not affected by the absence of Tyk2. Similarly, LPS-induced activation of NF-κB was not dependent on Tyk2 (Fig. 2C). This is in accordance with previous findings showing unperturbed IκB degradation in Tyk2-deficient macrophages [14] and further underscores the redundant role of Tyk2 in the activation of the MyD88/NF-κB pathway.

Stat3 plays an inhibitory role for the induction of IL-17 by LPS

Given the reduced Stat3 activation observed in the absence of Tyk2, we examined the role of Stat3 for LPS-mediated IL-17 induction. For that purpose we used macrophages derived from $Stat3^{fl/fl}$LyzsCre ($Stat3^{Lyzs-/-}$) mice that lack Stat3 specifically in myeloid cells (i.e. macrophages and neutrophils). Surprisingly, IL-17 and IL-17F mRNA induction were strongly increased in cells from $Stat3^{Lyzs-/-}$ mice (Fig. 3A and 3B). Notably, even basal levels of both IL-17 and IL-17F mRNA were more than 10-fold higher in cells from $Stat3^{Lyzs-/-}$ mice as compared to $Stat3^{fl/fl}$. Enhanced TNFα production upon LPS treatment has been reported in macrophages from mice with a similar conditional Stat3 deletion [55] and this is attributable to a well-established impaired IL-10 response in the absence of Stat3 [56, 57]. However, under our experimental conditions, TNFα mRNA was induced similarly in macrophages derived from $Stat3^{fl/fl}$ or $Stat3^{Lyzs-/-}$ mice, respectively (Fig. 3C). In line with IL-17 mRNA expression data, IL-17 protein secretion was increased in cells derived from $Stat3^{Lyzs-/-}$ mice (Fig. 3D). Remarkably, constitutive IL-17 secretion in cells derived from $Stat3^{Lyzs-/-}$ mice reached similar levels as in LPS-treated cells derived from $Stat3^{fl/fl}$ (Fig. 3D) or wildtype (Fig. 1D) mice. Increased IL-17 expression was not due to a generally increased LPS response, since neither ERK1/2 nor NF-κB were constitutively active and their LPS-mediated activation was similar in cells derived from $Stat3^{fl/fl}$ or $Stat3^{Lyzs-/-}$ mice, respectively (data not shown).

IL-17 mRNA expression is reduced in the absence of Tyk2 following LPS challenge in vivo

To investigate whether IL-17 production is similarly dependent on Tyk2 *in vivo*, we injected wildtype, Tyk2- and Ifnar1-deficient mice intraperitoneally (i.p.) with LPS and examined IL-17 mRNA expression in spleens. As shown in Fig. 4A IL-17 mRNA levels

were 1000-fold increased in WT mice 12 h and 18 h following LPS challenge, and similar levels were detected in the absence of Ifnar1. In contrast, IL-17 mRNA expression was strongly reduced in Tyk2-deficient spleens at both time points analysed. Thus, analogous to what we found in macrophages, IL-17 mRNA expression was highly dependent on Tyk2 but independent of Ifnar1 in spleens. Since we additionally evidenced an inhibitory role of Stat3 for IL-17 production in macrophage cultures we tested whether Stat3 in myeloid cells likewise inhibits IL-17 production *in vivo*. We injected $Stat3^{fl/fl}$ and $Stat3^{Lyzs-/-}$ mice i.p. with LPS and quantified IL-17 mRNA expression in spleens. Although mRNA levels of IL-17 were modestly increased in $Stat3^{Lyzs-/-}$ compared to $Stat3^{fl/fl}$ mice, it was not statistically significant (Fig. 4B). Thus, unlike IL-17 production in peritoneal macrophage cultures, LPS-mediated IL-17 expression in spleens is not inhibited by myeloid Stat3.

IL-17 mRNA levels are not affected by the absence of mature T- and B-cells

We next examined the possibility that Th17 cells are responsible for IL-17 production after LPS challenge *in vivo*. Therefore, WT and Rag2-deficient mice, which lack mature T and B cells were challenged with LPS. As shown in Fig. 4C the level of IL-17 mRNA in spleens of untreated Rag2-deficient mice was 10-fold increased as compared to WT mice. Interestingly, following LPS challenge similar IL-17 mRNA levels were reached in WT and $Rag2^{-/-}$ mice. From these data we conclude, that neither mature T nor B cells are required for IL-17 production in response to LPS.

$CD11b^+$ cells do not substantially contribute to IL-17 mRNA expression in spleens after LPS challenge in vivo

In order to determine if macrophages produce IL-17 *in vivo*, we enriched cells by magnetic cell sorting for specific markers and determined IL-17 mRNA expression in these cell populations from LPS-challenged WT mice. Since NK cells also express CD11b, albeit to a lower extent, we depleted $DX5^+$ cells prior to the selection for CD11b-expressing cells. $CD19^+$ cells were selected as negative control, as at least to our knowledge B cells do not produce IL-17. As shown in Fig. 5A, low levels of IL-17 mRNA were found in $DX5^+$ and $CD11b^+$ cells, whereas IL-17 mRNA was hardly detectable in $CD19^+$ cells. However, only the flow population, i.e. DX5-, CD11b-, and CD19-depleted cells, displayed an increase in IL-17 mRNA expression relative to the expression levels determined in the total splenocyte population. In contrast, IFNγ mRNA was specifically increased in $DX5^+$ splenocytes relative to the levels in total spleen cells and was barely detectable in other populations (Fig. 5B). This is in accordance with previous reports identifying NK cells as

the major IFNγ-producing cell type in response to LPS treatment [58, 59]. Interestingly, IL-17 mRNA was also clearly detectable in cells selected for mPDCA1 (Fig. 5C), indicating a contribution of pDCs to splenic IL-17 production. However, mPDCA1$^-$ cells expressed clearly higher levels of IL-17 mRNA. As expected, IFNγ mRNA expression was higher in mPDCA1-depleted cells, but substantial amounts were also produced in cells selected for mPDCA1 (Fig. 5D). Importantly, IL-17 and IFNγ mRNA were barely detectable in mPDCA1$^+$ and mPDCA1$^-$ cells derived from Tyk2$^{-/-}$ mice. Neither IL-17 nor IFNγ mRNA was detectable in any of the selected populations of untreated mice (data not shown). In summary, these data indicated that CD11b$^-$DX5$^-$CD19$^-$ and mPDCA1$^-$ cells are the major IL-17 producers in spleens from LPS-treated WT mice, whereby mPDCA1$^+$ cells might also contribute.

γδ T cells and pDCs are the major sources of IL-17 in response to LPS in vivo

In order to confirm the results obtained with magnetic cell sorting and to further define the cell types involved, we next determined IL-17 protein producing cells by flow cytometry. CD19-depleted spleen cells of untreated and LPS-challenged WT mice were intracellularly stained for IL-17 in combination with various cell surface markers. We found a significant increase (around 2-fold) in IL-17$^+$CD3$^-$Siglec-H$^+$ cells upon LPS-treatment (Fig. 6A). Siglec-H was shown to be highly specific for pDCs [60]. Importantly, analysis of the pDC population utilising distinct markers (i.e. CD3$^-$CD4$^+$B220$^+$CD11c^{dim}) revealed similar results (data not shown). In addition to Siglec-H$^+$ cells, we found LPS-induced IL-17 production in CD3$^+$TCRγδ$^+$ cells (Fig. 6B). This population represents γδ T cells and showed a more pronounced increase in % positive cells than Siglec-H$^+$ cells (around 4-fold) (Fig. 6A and B). We could not detect any IL-17 and DX5 or CD11b double positive cells (data not shown).

Discussion

Although IL-17 production has been mainly described in the context of Th17 cells, numerous studies reported IL-17 production by non-Th17 cells, e.g. $CD8^+$ T cells and various subsets of innate immune cell populations [39, 61]. However, the mechanism(s) of IL-17 induction in these cells and their involvement in IL-17 production *in vivo* remain largely elusive. In this report we demonstrate that Tyk2 is indispensable for LPS-induced IL-17 production in peritoneal macrophage cultures and in spleens *in vivo*. We show that γδ T cells are the main source of IL-17 in spleens and demonstrate for the first time that pDCs contribute to IL-17 production in the early LPS response *in vivo*.

Our data showing IL-17 production by murine peritoneal macrophages in response to LPS are in accordance with previous studies [38, 47]. Although the mechanism of IL-17 induction in macrophages is unclear to date, it has been shown that many innate immune cells can directly respond to IL-23 and express the transcription factor RORγt [39]. Stat3 has been shown to be critically involved in both, IL-23 mediated signalling and regulation of RORγt expression [52, 62-65]. We show here reduced activation of Stat3 and decreased IL-17 and IL-17F production in Tyk2-deficient as compared to wildtype macrophages following LPS treatment. Interestingly, we found enhanced IL-17 and IL-17F production upon LPS treatment in macrophages derived from mice with a Stat3 deletion in myeloid cells, as compared to cells derived from $Stat3^{fl/fl}$ mice. Thus, our data illustrate a Tyk2-dependent but Stat3-independent pathway for LPS-mediated IL-17 and IL-17F production, thereby excluding the contribution of an impaired IL-23/Stat3 axis to the phenotype observed in Tyk2-deficient macrophages. In addition, treatment with exogenous IL-23 was not sufficient to induce IL-17 (data not shown). In contrast to the crucial role of Stat3 for IL-17 production in T cells, we propose a Stat3-dependent negative regulatory pathway acting on IL-17 and IL-17F gene induction in macrophages. This might be due to the role of Stat3 in IL-10 signalling [56], since enhanced levels of IL-17 have been reported in IL-10-deficient macrophages following LPS treatment [47]. It is notable, that IL-17 and IL-17F basal mRNA expression are already significantly increased in cells derived from mice with a Stat3 deletion in myeloid cells and thus the effect is not strictly LPS-dependent.

In contrast to peritoneal macrophages, we could not detect IL-17 and IL-17F mRNA or protein in bone marrow-derived macrophages (data not shown) and airway macrophages treated with LPS *in vitro* failed to produce detectable amounts of IL-17 mRNA [66]. This would suggest that the ability to respond to LPS with IL-17 and IL-17F production is dependent on the macrophage differentiation and/or activation state. However, we can not exclude the possibility that minor amounts of other cell types present in our macrophage

cultures [9] contribute to or are required for IL-17 and IL-17F production, as we were not able to detect intracellular IL-17 in peritoneal macrophage cultures by FACS analysis.

Over the last years it has become increasingly evident that innate immune cells, most prominently γδ T cells and LTi-like cells, are critically involved in IL-17 production *in vivo* [37, 67]. It is known that γδ T cells are an important source of IL-17 following infections with various bacteria [45, 68, 69]. The importance of Tyk2 for IL-17 production by peritoneal γδ T cells following *E. coli* infection has been shown recently [70]. We report herein drastically reduced IL-17 levels in the absence of Tyk2 in spleens of LPS-challenged mice, thereby providing new evidence for an essential role of Tyk2 in innate IL-17 production. Consistent with our results in peritoneal macrophage cultures, IL-17 mRNA expression *in vivo* is independent of Ifnar1. Conversely, augmented IL-17 production has been reported in γδ T cells in the spleens of Ifnar1$^{-/-}$ mice following infection with *Francisella tularensis* or *Listeria monocytogenes* [45]. Thus, the potential of IFNα/β to inhibit IL-17 production is context-dependent and it has to be determined if this is due to differences in the amounts of IFNα/β, kinetics of induction and/or cell type specificity.

Despite the dramatically increased IL-17 production observed in peritoneal macrophages derived from Stat3$^{Lyzs-/-}$ mice, only a slight and not significant increase in IL-17 mRNA levels was observed in spleens of Stat3$^{Lyzs-/-}$ mice upon LPS challenge. This further underscores the context dependence and complexity of regulatory networks controlling IL-17 production *in vivo*.

We show that γδ T cells are the main source of IL-17 in spleens following LPS challenge, further emphasising their crucial role for innate IL-17 production. In addition, we show herein that splenic pDCs are capable of IL-17 production in response to LPS. We detect high levels of IL-17 mRNA in splenocyte subpopulations enriched for the expression of the pDC marker mPDCA1 [71] and IL-17 protein expression by FACS analysis in cells expressing the highly specific pDC marker Siglec-H [60, 72] and, additionally, by gating for CD3^{-}CD4^{+}B220^{+}CD11c^{dim} cells (data not shown). Thus, we suggest that in contrast to conventional DCs (cDCs), which drive Th17 differentiation [23, 73], pDCs *per se* are an early source of IL-17. Although γδ T cells apparently dominate the IL-17 production in spleens, the contribution of pDCs has to be taken into account. Importantly, IL-17 production is dependent on Tyk2 in mPDCA1^{+} and mPDCA1^{-} splenocytes. Noteably, we did not find any evidence for substantial IL-17 production by macrophages in spleens of LPS-challenged mice. Similarly, IL-17 mRNA was not detectable in macrophages isolated from bronchoalveolar fluid after intranasal LPS application [74].

In summary, we have characterised for the first time LPS-induced requirements for the production of IL-17 and IL-17F in murine peritoneal macrophage cultures and in spleens. We show that Tyk2 exerts an essential function in response to LPS independent from its role in the IFNα/β signalling cascade. We establish a negative regulatory role of myeloid Stat3 in the induction of IL-17 production in peritoneal macrophage cultures as opposed to its positive regulatory role in the generation and maintenance of IL-17–producing T cells. We confirm the importance of $\gamma\delta$ T cells for innate IL-17 production and provide for the first time evidence for a contribution of pDCs to IL-17 production in spleens following LPS challenge. The further characterisation of the mechanism by which Tyk2 facilitates IL-17 production *in vivo* as well as the physiological importance of pDC-derived IL-17 will be interesting topics for future investigations.

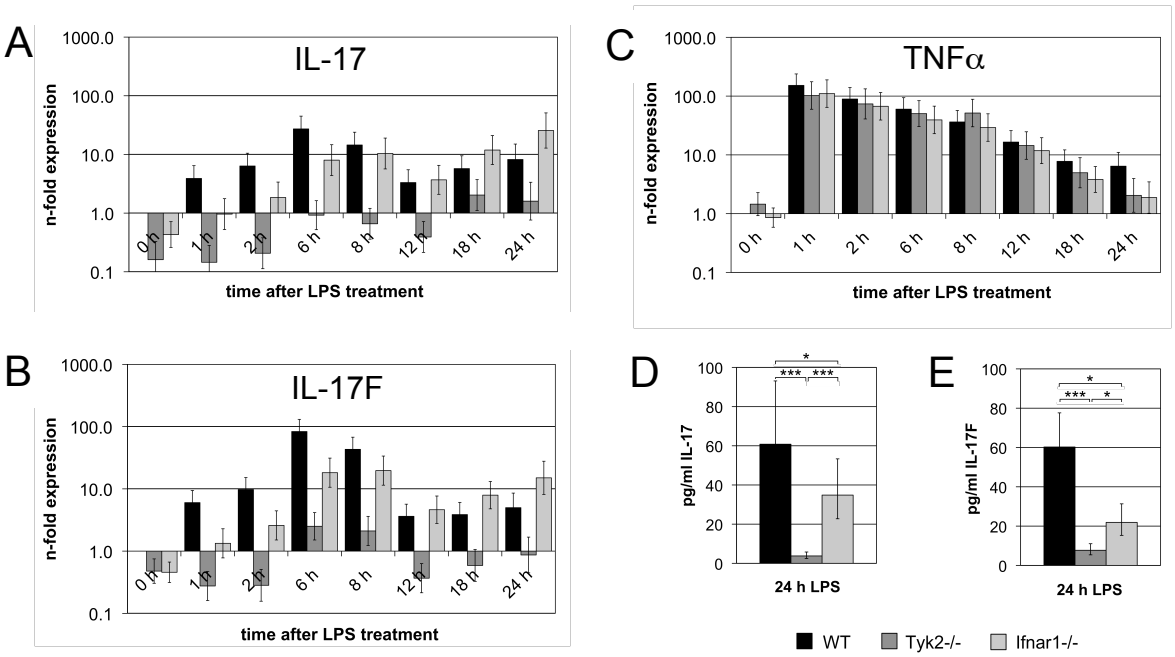

Fig. 1. WT, Tyk2$^{-/-}$ and Ifnar1$^{-/-}$ macrophages were treated with 100 ng/ml LPS for the times indicated or left untreated. The expression of (A) IL-17, (B) IL-17F and (C) TNFα mRNA was analysed by qRT-PCR. Ube2d2 was used for normalisation, and expression levels were calculated relative to untreated wildtype cells (WT 0 h). Data are depicted as mean ± SE derived from at least 3 independent experiments per time point; except for 0 h post treatment, mRNA expression levels in (A) and (B) were significantly different between Tyk2$^{-/-}$ and both other genotypes at all time points ($p \leq 0.01$), mRNA expression levels in Ifnar1$^{-/-}$ differed from those in WT cells from 0 h up to 6 h post treatment ($p \leq 0.05$) and no significant difference was obtained at later time points. (C) mRNA expression was not significantly different between the genotypes, except at 24 h post treatment between WT and both other genotypes ($p \leq 0.05$). (D) IL-17 and (E) IL-17F accumulation was measured in cell culture supernatants with ELISA. Data are depicted as mean ± SE (D) from 4 and (E) from 3 independent experiments (comprising cells from 10 and 3 individual mice respectively; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

Fig. 1

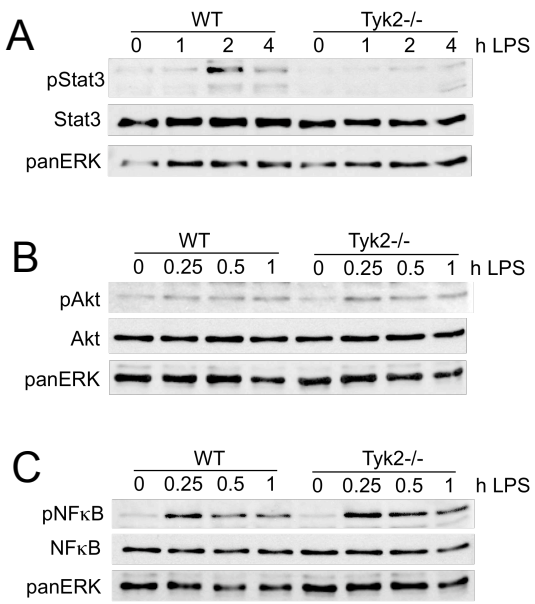

Fig. 2. WT and Tyk2$^{-/-}$ macrophages were treated with 100 ng/ml LPS or left untreated. At the times indicated, total cell lysates were prepared and subjected to Western blot analysis. Membranes were probed with Abs specific for (A) Tyr705-phosphorylated Stat3 (pStat3) or Stat3 protein (Stat3), (B) Ser473-phosphorylated Akt (pAkt) or Akt protein (Akt), (C) Ser536-phosphorylated NF-κB p65 (pNFκB) or NF-κB p65 protein (NFκB). Protein loading was controlled by reprobing with anti-pan-ERK Ab (panERK). Data are representative for at least two independent experiments.

Fig. 2

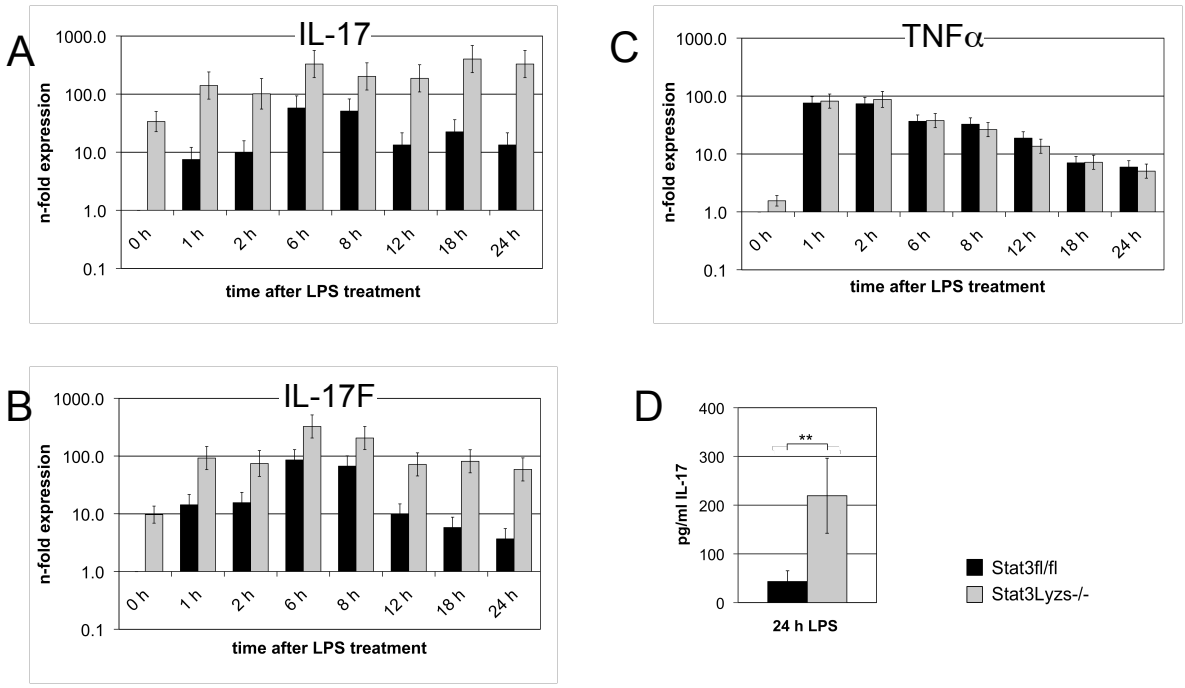

Fig. 3. Stat3$^{fl/fl}$ and Stat3$^{Lyzs-/-}$ macrophages were treated with 100 ng/ml LPS for the times indicated or left untreated. Expression of (A) IL-17, (B) IL-17F and (C) TNFα mRNA was analysed by qRT-PCR. Ube2d2 was used for normalisation and expression levels were calculated relative to untreated Stat3$^{fl/fl}$ cells (Stat3$^{fl/fl}$ 0 h). Data are depicted as mean ± SE from at least 3 independent experiments per time point; expression levels in (A) and (B) were significantly different between the genotypes at all time points ($p \leq 0.05$ for 8 h post treatment, all other time points $p \leq 0.01$); expression levels in (C) were only significantly different at 0 h ($p \leq 0.05$). (D) IL-17 accumulation was measured in cell culture supernatants by ELISA. Data are depicted as mean ± SE from 3 independent experiments (comprising cells from 6 individual mice; ** $p \leq 0.01$).

Fig. 3

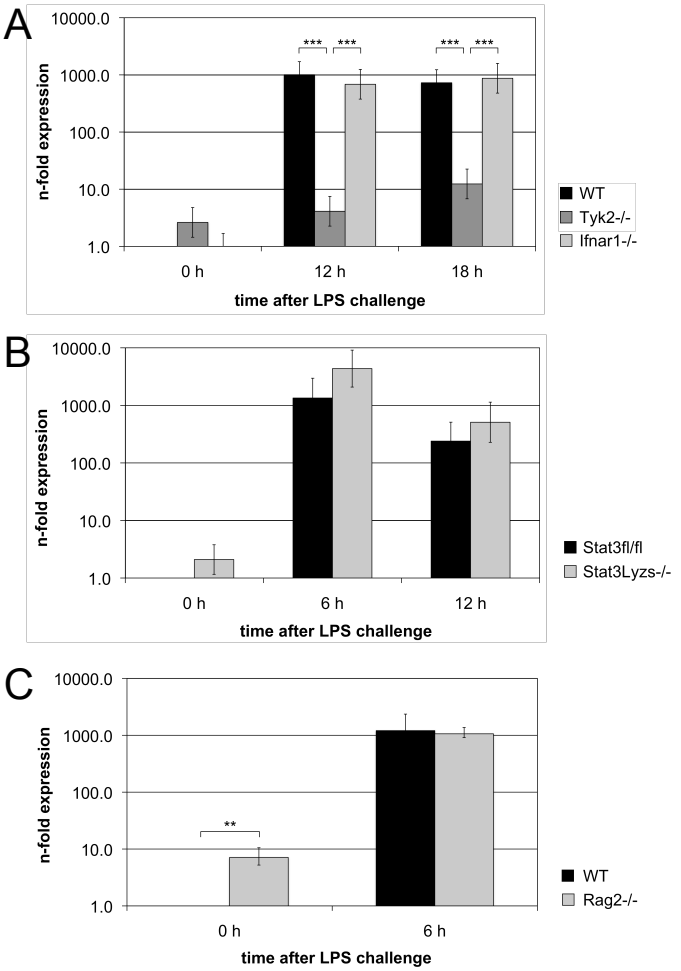

Fig. 4. (A) WT, Tyk2$^{-/-}$ and Ifnar1$^{-/-}$ or (B) Stat3$^{fl/fl}$ and Stat3$^{Lyzs-/-}$ or (C) WT and Rag2$^{-/-}$ mice were injected i.p. with LPS (1 mg per 20 g body weight) or left untreated. At the times indicated IL-17 mRNA expression in spleens was analysed by qRT-PCR. Ube2d2 was used for normalisation and expression levels were calculated relative to the untreated WT (WT 0 h, Stat3$^{fl/fl}$ 0 h). Data are depicted as mean ± SE from at least 4 mice of 3 independent experiments per time point; (** $p \leq 0.01$, *** $p \leq 0.001$).

Fig. 4

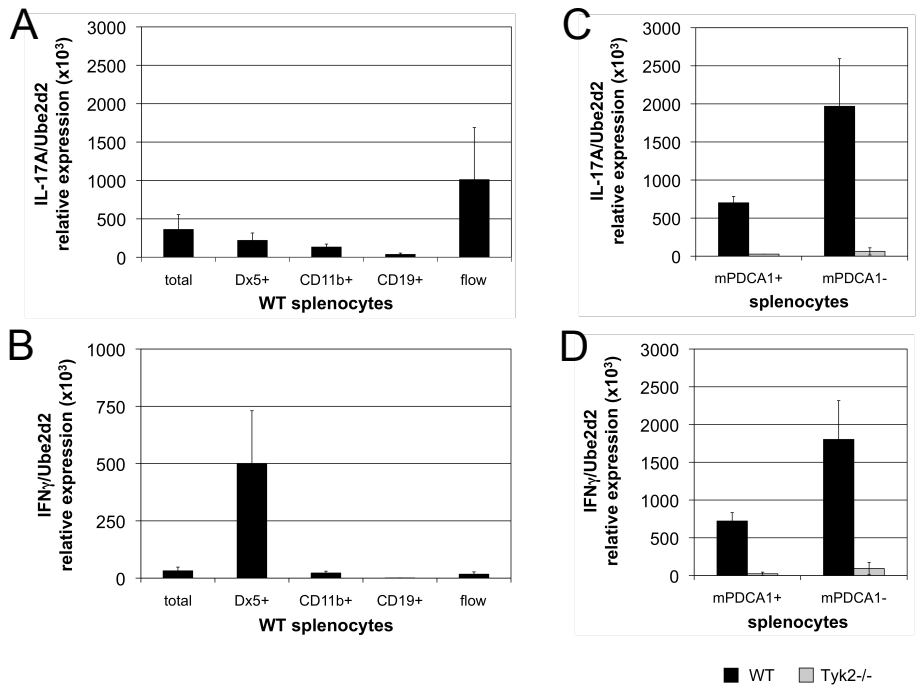

Fig. 5. (A, B) WT or (C, D) WT and Tyk2$^{-/-}$ mice were injected i.p. with LPS (1 mg per 20 g body weight) or left untreated. Following 6 h LPS challenge spleen cells were separated via successive MACS, as indicated. (A, C) IL-17 or (B, D) IFNγ mRNA expression in different spleen cell populations was analysed by qRT-PCR. Expression levels were calculated relative to the endogenous control (Ube2d2). Data are depicted as mean ± SE from 2 independent experiments.

Fig. 5

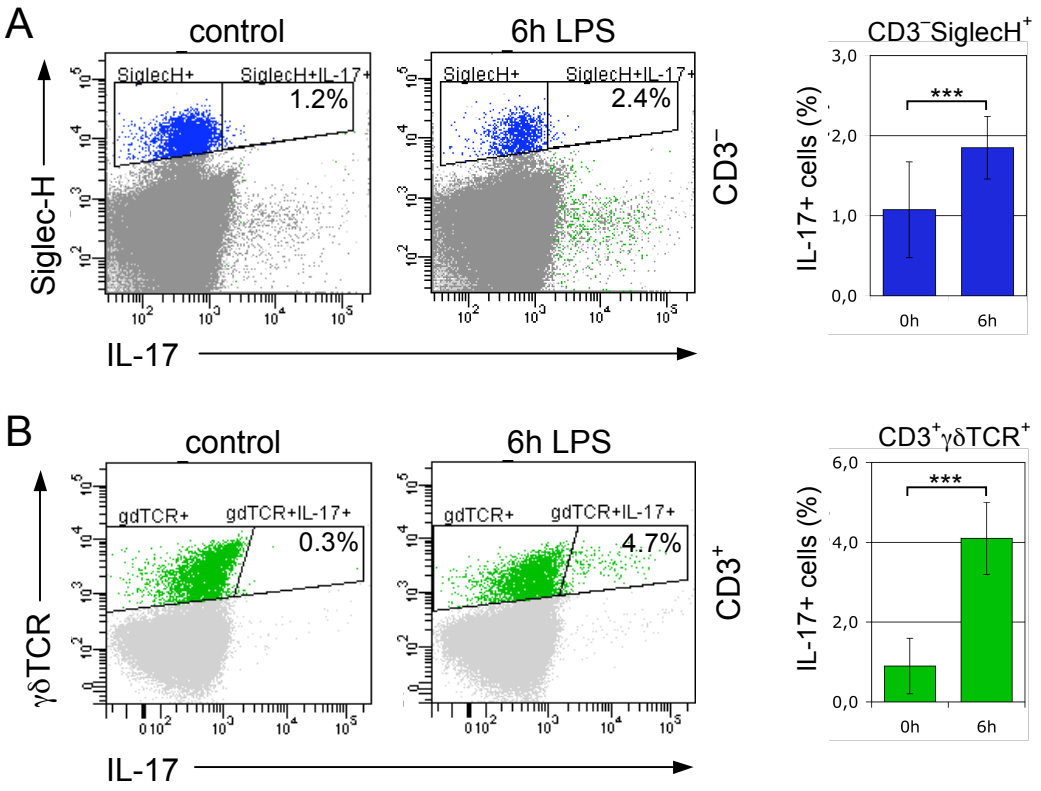

Fig. 6. WT mice were injected i.p. with LPS (1mg per 20g body weight) or left untreated. Subsequently, mice were injected i.v. with 0.25 mg Brefeldin A and 6 h following challenge spleen cells were depleted of $CD19^+$ cells by MACS and then FACSed. Dot plots show intracellular staining for IL-17 in (A) pDCs and (B) γδ T cells. Aggregates and dead cells were excluded based on FSC-H *versus* FSC-A plots. Among the resulting live cell singlet pDCs were determined as $CD3^-$Siglec-H^+ and γδ T cells as $CD3^+\gamma\delta TCR^+$. Numbers in the gates indicate the percentage of IL-17^+ cells in total (A) $CD3^-$Siglec-H^+ cells or (B) $CD3^+\gamma\delta TCR^+$ cells. In the right panels of (A) and (B) the IL-17^+ cells are depicted as mean ± SD from 3 independent experiments (*** $p \leq 0.001$).

Fig. 6

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RESULTS II

Tyrosine kinase 2 controls interleukin-1β production at the translational level

Radwan and Stiefvater *et al.*, *Journal of Immunology* 2010; 185; 3544-3553

The Journal of Immunology

Tyrosine Kinase 2 Controls IL-12 Production at the Translational Level

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J. Immunol. 2010;185;3544-3553; originally published online Aug 16, 2010;
doi:10.4049/jimmunol.0904000
http://www.jimmunol.org/cgi/content/full/185/6/3544

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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. *The Journal of Immunology*, 2010, 185: 3544–3553.

Tyrosine kinase 2 (Tyk2) is a member of the Janus kinase (Jak) family of nonreceptor tyrosine kinases (1). Jaks 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- and/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-κB 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-κB and MAPK pathways (24, 25), although NF-κB 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).

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Received for publication December 14, 2009. Accepted for publication July 8, 2010.

The work was supported by the Austrian Science Fund (SFB-F28 to M.M. and B.S.) and the Austrian Federal Ministry for Science and Research (GZ.200.191/1-II/1a/2008 to Biomodels Austria).

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Abbreviations used in this paper: actD, actinomycin D; ARE, AU-rich elements; casp, caspase; CHX, cycloheximide; CM, complete medium; 2D-DIGE, two-dimensional fluorescence difference gel electrophoresis; I, IFNAR1$^{-/-}$; IFIT, IFN-induced protein with tetratripeptide repeats; Jak, Janus kinase; MOI, multiplicity of infection; MS, mass spectrometry; nd, not detectable; NFκBia, NF-κB inhibitor α; PAI2, plasminogen activator inhibitor 2; RT-qPCR, reverse transcription-quantitative PCR; S, Stat1$^{-/-}$; Stat, signal transducers and activators of transcription; T, Tyk2$^{-/-}$; TBP, TATA-box-binding protein; TTP, tristetraprolin; Tyk2, tyrosine kinase 2; Ube2d2, ubiquitin-conjugating enzyme E2 D2; W, WT; WT, wild-type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0904000

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 described previously (34). See Table I for detailed MS data.

Western blot analysis

Western blots were performed as described previously (34). The following Abs were used: goat anti–IL-1β (R&D Systems, Minneapolis, MN), mouse anti-panERK (BD Biosciences, San Diego, CA), donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA), sheep anti-mouse IgG-HRP F(ab)′$_2$ fragment (GE Healthcare, Munich, Germany). Quantifications were performed using ImageJ software for Mac OS X (open source, http://rsb.info.nih.gov/ij/index.html).

ATP treatment

Cells (2×10^6) were stimulated with LPS in 1.5 ml CM (see above) for 4 h, washed once with PBS, 1.5 ml DMEM containing 3 mM, 5 mM ATP (Sigma-Aldrich), or without supplements were added and cells were further incubated for 30 min.

ELISA

Cell culture supernatants were collected and centrifuged at 14,000 × *g* for 3 min and concentrations of IL-1β and TNF-α were determined using ELISA (R&D Systems). Detection limits of ELISAs are 10 pg/ml and 25 pg/ml for IL-1β and TNF-α, respectively.

TCA precipitation of supernatants

Supernatants (900 μl) were mixed with 13.5 μl 10% (w/v) sodium deoxycholate monohydrate (Sigma-Aldrich) (final concentration 0.15%) and 300 μl ice-cold 6.1 M TCA solution was added. Extracts were incubated 1 h on ice to allow protein precipitation. Samples were centrifuged at 10,000 × *g* for 10 min at 4°C. Supernatants were discarded, pellets were washed three times with 1 ml ice-cold acetone and dissolved in 10 μl 0.2 M NaOH, diluted in 15 μl H_2O and mixed with 25 μl 2 × SDS-PAGE Laemmli sample buffer, followed by boiling for 5 min. For Western blotting, 15 μl sample was separated on a 15%T SDS-PAGE and probed with anti–IL-1β Abs.

Pulse/chase experiments and immunoprecipitation

Cells (3×10^6) were stimulated with LPS in CM (see above) for 2.5 h, then washed once with PBS and incubated in LPS containing starving medium (DMEM methionine/cysteine-free, 1% BSA, 2 mM L-glutamine, 15% conditioned medium, 100 U/ml penicillin, 100 μg/ml streptomycin) for 30 min. Medium was replaced by 1.5–2 ml pulse medium (starving medium supplemented with 70–100 μCi/ml [^{35}S]methionine/cysteine [Met-^{35}S-label], Hartmann Analytic, Braunschweig, Germany) and cells were incubated in the presence of LPS for the indicated pulse times. Cells were washed once with PBS and incubated in chase medium (CM supplemented with 15 mg/ml L-methionine and L-cysteine [Sigma-Aldrich]). IL-1β was immunoprecipitated from 400 μg protein with hamster anti–IL-1β Ab (BD Biosciences) using protein A-coupled Sepharose beads. Quantification of signal intensities was performed with ImageJ software as described for Western blots.

Polysome gradients

Sucrose gradient fractionation was performed as previously described (39) with minor modifications. Cells (1.5×10^7) were lysed in 1 ml ice-cold buffer (10 mM Tris-HCl pH 8, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.5% [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% (w/v) sucrose gradients (containing 10 mM Tris-HCl pH 7.5, 140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM DTT, and 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 proteinase K in the presence of 1% (w/v) SDS and 10 mM EDTA pH 8. RNA was extracted with 25:24:1 phenol-chloroform-isoamyl 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) of total RNA 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). RT-qPCR was performed in duplicate on Eppendorf realplex4 (Eppendorf, 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 TaqMan gene expression assay (assay ID Mm00434228_m1; Applied Biosystems). TBP and NFκBia were detected with EVAGreen (Biotium, Hayward, CA) using the following primers (5′–3′): TBP-fwd: 5′-GAATATAATCCCAAGCGATTTGC-3′ (Tm = 58°C); TBP-rev: 5′-CTGGATTGTTCTTCACTCTTGGCT-3′ (Tm = 60°C), amplicon length: 122 bp; NFκBia-fwd: 5′-TGGCCAGTGTAGCAGTCTTGAC-3′ (Tm = 60°C); and NFκBia-rev: 5′-CAGGTAGCCGTGAGTAGAGGCTAG-3′ (Tm = 59°C), amplicon length: 117 bp.

Statistical analysis of RT-qPCR and ELISA data

RT-qPCR gene expression data were investigated for differences among genotypes and time after challenge. Univariate regression was calculated with the log of the transformed target to endogenous control gene expression ratio as dependent variable. Linear contrasts were encoded such that for each time point, Tyk2$^{-/-}$ were compared with wild-type (WT) cells. Differences among experiments were taken into account. Analysis of ELISA data with multiple measurements was performed using the log of the data and as above. Data were analyzed with SPSS 17.0 for Mac OS-X. 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 anesthesized with ketamine-xylazine and blood was collected retrobulbarly. For peritoneal lavages, mice were sacrificed and peritonea flushed with 5 ml PBS.

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

Peptide mass fingerprint	Probability based MOWSE score (significant score > 55, $p < 0.05$)	47
	Sequence coverage	8%
	Matched/unmatched peptides	3/1
Sequencing	No. of peptides used for protein ID	3
	Probability based MOWSE score (significant ion score > 30, $p < 0.05$)	130
	Monoisotopic values of precursor ion(s) $[M+H]^+$ *m/z* (selected)	716.38
		916.48
		1263.74
	Peptide sequences obtained	QLHYR
		LRDEQQK
		SLVLSDPYELK

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 M_r of ~30 kDa and an isoelectric point around 4.6. The peptides identified were common to both mature (M_r of 17 kDa) and immature (M_r of 31 kDa) IL-1β but based on the M_r 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. 1*A*, 1*B*). 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. 1*B*). Enhanced levels of pro–IL-1β in whole cell lysates from Tyk2$^{-/-}$ macrophages were confirmed by Western blot time course experiments (Fig. 1*C*) 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. 1*C*).

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. 2*A*). 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. 2*B*). 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. 2*C*) as described previously in the context of LPS responses (45) and no

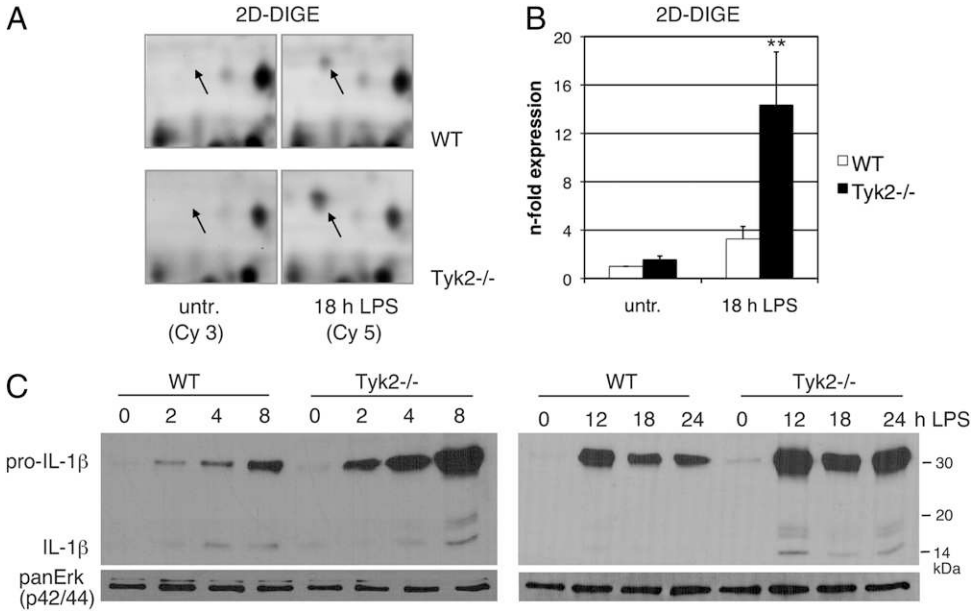

FIGURE 1. Pro–IL-1β protein expression is elevated in the absence of Tyk2. Macrophages were treated with LPS for the indicated times and whole cell lysates were subjected to 2D-DIGE (*A, B*) or Western blot analysis (*C*). *A*, Selected regions from Cy3 and Cy5 images converted to gray scale showing 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. $^{**}p \leq 0.01$. *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.

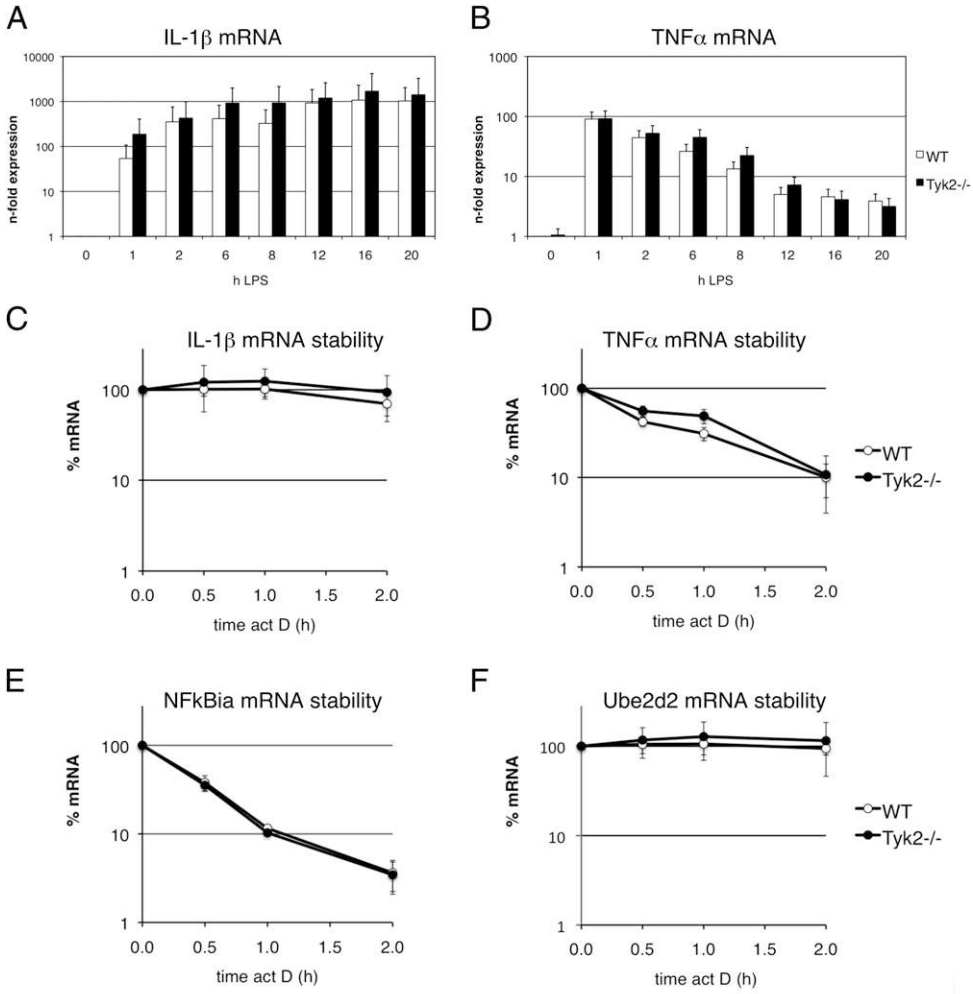

FIGURE 2. IL-1β mRNA expression and stability is unaltered in Tyk2$^{-/-}$ cells. Macrophages were treated with LPS for the times indicated and total RNA was subjected to RT-qPCR for IL-1β (*A*) or TNF-α (*B*). mRNA expression levels were calculated relative to untreated WT cells, with Ube2d2 as endogenous control. Mean values ± 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-1β (*C*), TNF-α (*D*), NFκBia (*E*), and Ube2d2 (*F*). Mean values ± SD from three replicates derived from two independent experiments are depicted.

difference was observed between WT and Tyk2$^{-/-}$ cells. Consistent with other reports (45), mRNA of TNF-α and NFκBia decayed rapidly. No differences between WT and Tyk2$^{-/-}$ cells were observed for TNF-α and NFκBia mRNA decay (Fig. 2*D*, 2*E*). Ubiquitin-conjugating enzyme E2 D2 (Ube2d2) mRNA, which was used as a 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. 2*F*).

Intra- and extracellular levels of IL-1β are enhanced in the absence of Tyk2

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

We next asked whether increased IL-1β production in Tyk2$^{-/-}$ cells is also observed under conditions that promote maturation of IL-1β (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. 3*A*), treatment with LPS alone for 4 h resulted in barely detectable levels of IL-1β and a slight, but not significant increase in Tyk2$^{-/-}$ cells (Fig. 4*A*). As expected, extracellular levels of IL-1β in WT cells were dramatically higher (~1600 pg/ml) after addition of ATP (Fig. 4*A*). Importantly, significantly enhanced IL-1β levels were detected in Tyk2$^{-/-}$ macrophages in the presence of 3 mM and 5 mM ATP (Fig. 4*A*).

As the ELISA might also detect unprocessed IL-1β, we used Western blot analysis to verify these results. Under our experimental conditions, IL-1β was not detectable in supernatants of cells treated with LPS alone (data not shown), whereas pro–IL-1β and mature IL-1β could be detected in supernatants of WT cells after treatment with 5 mM ATP (Fig. 4*B*). Strongly enhanced IL-1β levels were detected in the absence of Tyk2 and mature IL-1β was readily detectable at concentrations of 3 mM and 5 mM ATP, respectively. Importantly, mature 17 kDa IL-1β

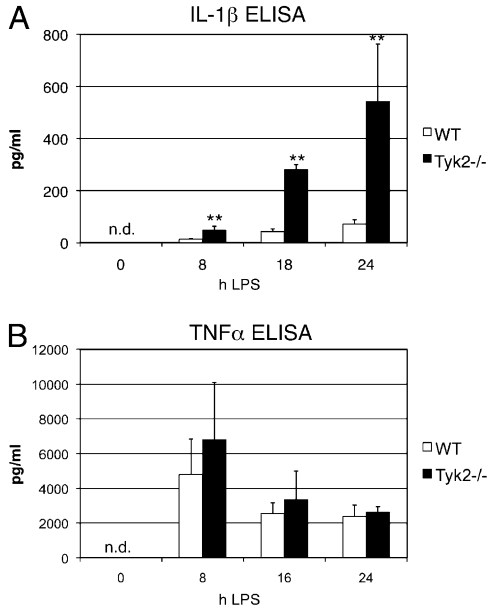

FIGURE 3. Extracellular protein levels of IL-1β are elevated in Tyk2$^{-/-}$ cells. Macrophages were treated with LPS for the times indicated and cell culture supernatants collected. Extracellular protein concentrations of IL-1β (*A*) and TNF-α (*B*) were measured by ELISA. Mean values ± SD from three independent experiments are shown; $**p \leq 0.01$. nd, not detectable.

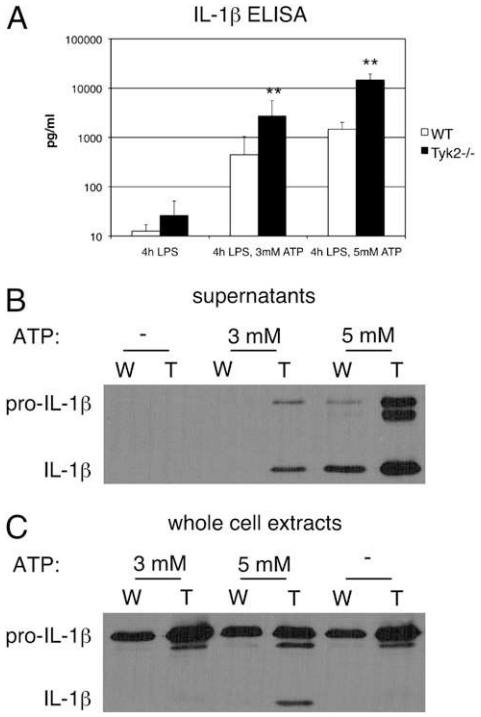

FIGURE 4. Pro- and mature IL-1β, and intra- and extracellular protein levels of IL-1β are higher in the absence of Tyk2, independent of the presence of ATP. Macrophages were treated with LPS for 4 h with or without subsequent addition of ATP at the concentrations indicated for 30 min. *A*, Extracellular IL-1β concentrations were measured by ELISA. Mean values ± SD from three independent experiments are shown, $**p \leq 0.01$. *B*, Proteins were precipitated from supernatants and 15 μl per lane were subjected to Western blot analysis by 15%T SDS-PAGE. *C*, Five microgram protein from whole cell lysates per lane were separated by 15%T SDS-PAGE. *B* and *C*, Membranes were probed with an anti–IL-1β Ab. Data are representative for three independent experiments. T, Tyk2$^{-/-}$; W, WT.

was also increased in supernatants of Tyk2-deficient cells (Fig. 4*B*). Intracellular, mature IL-1β was not detectable in WT cells treated with LPS/ATP (Fig. 4*C*). In contrast, mature IL-1β was detectable even intracellularly in Tyk2$^{-/-}$ cells after treatment with LPS and 5 mM ATP (Fig. 4*C*).

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. 5*A*). IL-1β levels were enhanced to a similar extent in IFNAR1$^{-/-}$, 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. 5*B*). 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-β 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. 5*C*) and cell supernatants (Fig. 5*D*) 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. 5*D*). In contrast, the level of TNF-α was similar in WT and Tyk2$^{-/-}$ macrophages and interestingly decreased in IFNAR1-deficient cells (Fig. 5*E*).

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. 6*A*, 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. 6*B*). As expected, and in accordance with

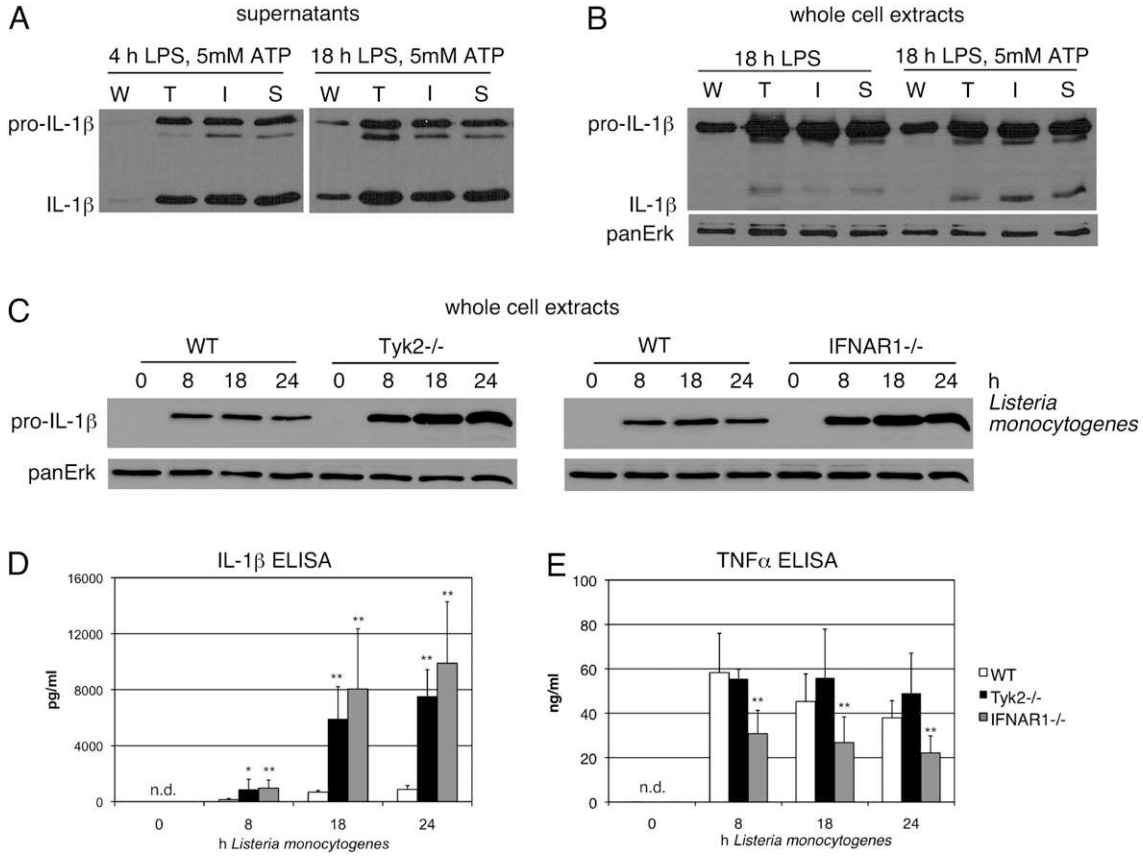

FIGURE 5. Effect of IFNAR1 and Stat1 deficiency on IL-1β production induced by LPS and *Listeria monocytogenes* infection. *A* and *B*, Macrophages were treated with LPS for the times indicated with or without subsequent addition of ATP for 30 min. *C–E*, Macrophages were infected with *L. monocytogenes* at an MOI of 10 for the indicated times. *A*, Proteins were precipitated from supernatants and 15 μl per lane separated by 15%T SDS-PAGE. *B*, Five microgram or (*C*) 10 μg protein from whole cell lysates per lane were separated by 15%T SDS-PAGE. *A–C*, Membranes were probed with an anti–IL-1β Ab. *B* and *C*, Protein loading of whole cell extracts was controlled by reprobing membranes with an anti-panERK Ab. *D* and *E*, Extracellular IL-1β and TNF-α concentrations were measured by ELISA. Mean values ± SD from three independent experiments are depicted. nd, not detectable; $*p \leq 0.05$, $**p \leq 0.01$ as compared with WT cells. Western blot results (*A–C*) are representative of at least two independent experiments. I, IFNAR1$^{-/-}$; MOI, multiplicity of infection; S, Stat1$^{-/-}$; T, Tyk2$^{-/-}$; W, WT.

the amplifying role of Tyk2 in IFN-α/β signaling (3, 4), inhibition by IFN-β still occurred but was less pronounced (~2-fold) in Tyk2$^{-/-}$ cells (Fig. 6*B*). IL-1β levels in supernatants from WT cells were decreased to below the detection limit of the ELISA (Fig. 6*C*) and by around 5-fold in Tyk2$^{-/-}$ cells. In WT and Tyk2$^{-/-}$ cells we were unable to observe any effect on TNF-α levels of pretreatment with IFN-β for 1 h (Fig. 6*D*).

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. 7*A*) 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 protease, we confirmed the results by metabolic labeling with [^{35}S]methionine/cysteine in pulse-chase experiments. Cells were treated with LPS for 4 h, labeled with [^{35}S]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. 7*B*, 7*C*). 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. 7*B*, 7*D*). 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. 8*A*) and compared mRNA distributions among the different fractions (Fig. 8*B–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. 8*B*). 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. 8*C*). In accordance with the unchanged expression of TNF-α protein, TNF-α mRNA showed similar polysome profiles in WT and Tyk2-deficient cells (Fig. 8*C*). Furthermore, we observed no difference in mRNA profiles of the two housekeeping

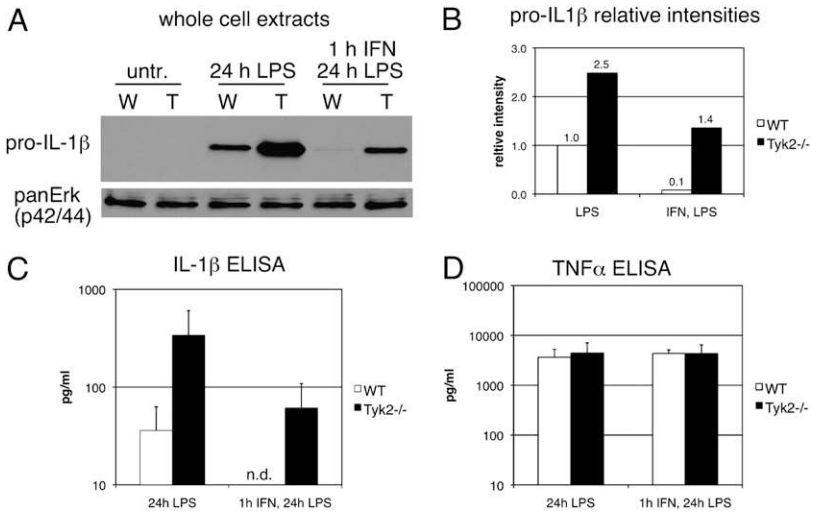

FIGURE 6. Effect of exogenous IFN-β on IL-1β protein levels. Macrophages were pretreated with IFN-β for 1 h or left untreated and subsequently treated with LPS or left untreated for 24 h. *A*, 5 μg protein from whole cell lysates per lane were separated by 15%T SDS-PAGE. Membranes were probed with an anti–IL-1β Ab and protein loading controlled by reprobing membranes with an anti-panERK Ab. W, WT; T, Tyk2$^{-/-}$. *B*, Mean values of pro–IL-1β signal intensities from three independent experiments. *C*, IL-1β and (*D*) TNF-α were measured in cell supernatants by ELISA, mean values ± SD from three independent experiments are depicted.

genes, TBP and Ube2d2 (Fig. 8*D*, 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. 8*E*, $p = 0.008$).

Local IL-1β levels are increased in the absence of Tyk2 after LPS challenge in vivo

Previous studies have not reported differences in the in vivo IL-1β 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-1β production in vivo. WT and Tyk2$^{-/-}$ mice were injected i.p. with LPS and IL-1β was measured in peritoneal lavages and in sera. As shown in Fig. 9*A*, IL-1β 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-1β was less pronounced than in macrophages in vitro, the result supports our model for an inhibitory role of Tyk2 on IL-1β production. In contrast, serum levels of IL-1β were slightly lower in Tyk2$^{-/-}$ mice at 4 h after challenge (Fig. 9*B*), suggesting differences in the regulation of local versus systemic IL-1β. Alternatively, kinetic differences in the regulatory pathways might lie behind the apparent differential effect of Tyk2 deficiency on IL-1β levels in the peritoneal cavity and systemically.

Discussion

We show that IL-1β 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-1β mRNA induction or stability (Figs. 1*A–C*, 2*A*, 2*C*). The lack of inhibition of IL-1β mRNA induction is in accordance with previous observations that activation of the NF-κB and MAPK pathways is normal in Tyk2-

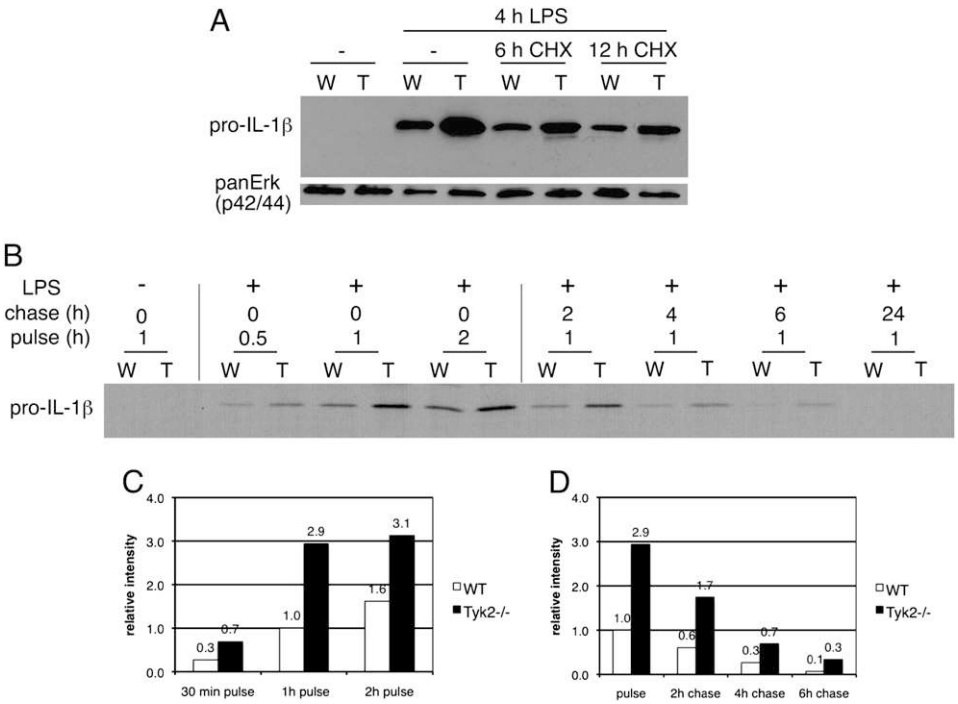

FIGURE 7. Pro–IL-1β protein stability and synthesis in the presence or absence of Tyk2. *A*, Macrophages were treated with LPS for 4 h, 10 μg/ml CHX was added and cells were further cultivated for the times indicated. Five microgram protein from whole cell lysates per lane were separated by 15%T SDS-PAGE. Membranes were probed with an anti–IL-1β Ab, protein loading was controlled by reprobing with an anti-panERK Ab. *B*, Macrophages were pulse labeled with [^{35}S] methionine/cysteine and chased for the indicated times. IL-1β was immunoprecipitated from 400 μg whole cell extracts and detected by autoradiography. *C* and *D*, Quantifications of signal intensities from *B* for (*C*) the three different pulse-periods and (*D*) 1 h pulse and the different chase periods. Results are representative of two independent experiments. T, Tyk2$^{-/-}$; W, WT.

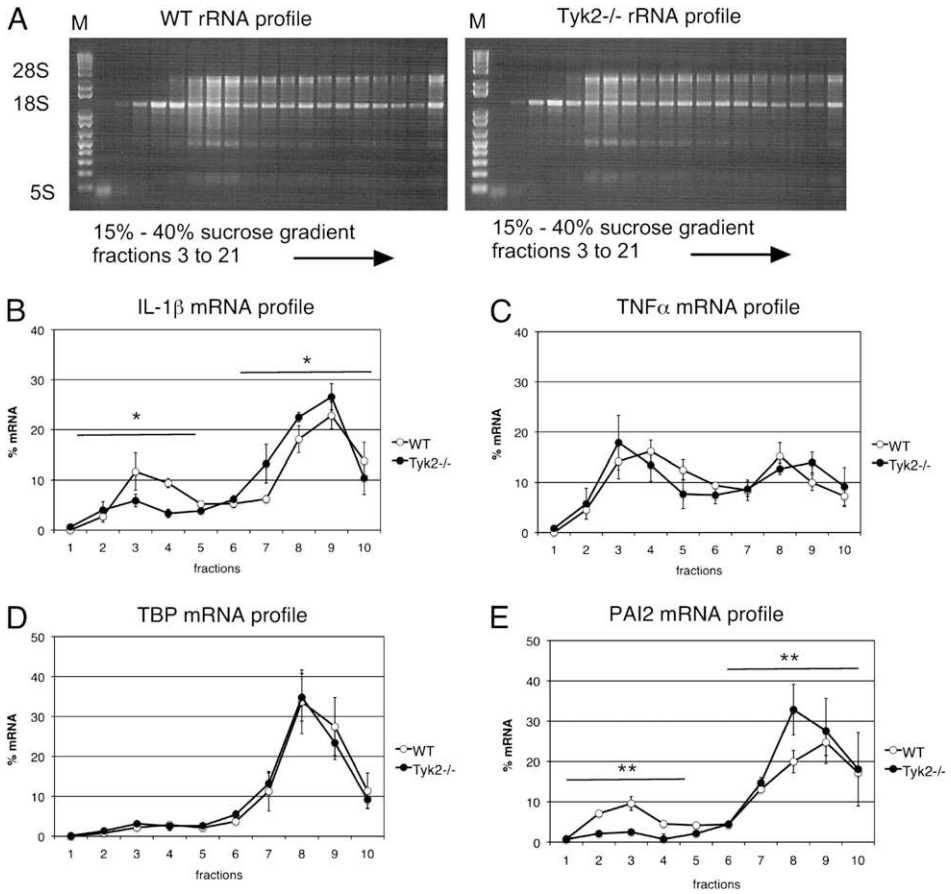

FIGURE 8. Polysome profile of IL-1β, TNF-α, TBP, and PAI2 mRNAs. Macrophages were treated with LPS for 4 h and cytoplasmic extracts separated in a continuous 15–40% sucrose gradient by ultracentrifugation. Fractions were manually collected from top to bottom and deproteinized and RNA was extracted. *A*, 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. $*p \leq 0.05$; $**p \leq 0.01$.

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. 3*A*). 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. 4*B*, 4*C*, 5*A*, 5*B*). Similarly, intra- and extracellular IL-1β levels were enhanced in Tyk2$^{-/-}$ macrophages infected with *L. monocytogenes* (Fig. 5*C*, 5*D*). 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. 7*A*, 7*B*, 7*D*). In contrast, IL-1β protein synthesis within a given pulse-period is enhanced in Tyk2$^{-/-}$ cells (Fig. 7*B*, 7*C*), 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. 8*B*). The association of PAI2 mRNA with polysomes also increases (Fig. 8*E*), 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. 8*C*) and similar levels of TNF-α protein are found in cell supernatants (Fig. 3*B*). Hence, the translational inhibition mediated by Tyk2 is specific, at least to some degree.

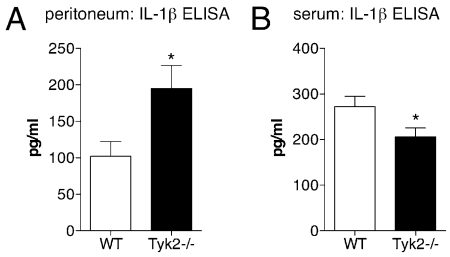

FIGURE 9. Effect of Tyk2 deficiency on IL-1β production in vivo. WT and Tyk2$^{-/-}$ mice were injected with LPS (1 mg/20 g body weight) i.p. IL-1β was measured 4 h after challenge in (*A*) peritoneal lavages and (*B*) sera by ELISA. Mean values ± SE for (*A*) 16 or (*B*) 15 mice from three independent experiments are depicted. $*p \leq 0.05$.

An increase in IL-1β protein level is observed in IFNAR1$^{-/-}$ and Stat1$^{-/-}$ macrophages, both intra- and extracellularly and independent of ATP treatment (Fig. 5*A*, 5*B*). 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

decay more rapidly [Fig. 2*C–F*, data not shown, and (45)]. It may be interesting to analyze whether stable mRNAs are more subject to translational regulation than unstable ones. High-throughput polysomal profiling experiments or ribosome sequencing approaches would be required to address these speculations. A recent report describes positive regulatory effects of IFNs on translation that appear to be specific to genes stimulated by IFN (64).

A large number of cytokines are known to be regulated at the posttranscriptional level (65). Regulation occurs via AREs in the 3′-UTRs of the mRNAs and several different ARE-binding proteins regulate mRNA stability positively or negatively and can also affect translational efficiency. Early studies reported that IL-1β can be regulated at the translational level under certain conditions (66–68) but the mechanism and stimuli involved remain largely unknown. More recently (52), steroid receptor coactivator-3 has been shown to be required for the translational repression of TNF-α and IL-1β in response to LPS in peritoneal macrophages, although the underlying mechanism was only described for TNF-α. IFN-α/β, and p38 MAPK synergistically induce expression of the ARE-BP tristetraprolin (TTP) (69). TTP destabilizes several cytokine and chemokine mRNAs, including IL-1β (70–72), and can also influence translation (73). Surprisingly, we find that TTP expression is increased in response to LPS in the absence of Tyk2 (data not shown), arguing against an involvement of TTP in the translational regulation of IL-1β. However, TTP activity is known to be regulated by phosphorylation (74) and it is possible that Tyk2 affects the phosphorylation status of TTP.

Tyk2$^{-/-}$ mice are highly resistant to high-dose LPS-mediated endotoxin shock, but serum levels of TNF-α and IL-1β have been reported to be normal in these mice at early stages (1.5 h) after LPS injection (12). In support of our in vitro data, we find increased IL-1β levels in the peritoneal lavages from Tyk2$^{-/-}$ mice after LPS injection (Fig. 9*A*). Interestingly, and extending previous results (12), systemic IL-1β levels are slightly but significantly lower in Tyk2$^{-/-}$ mice 4 h after LPS challenge (Fig. 9*B*). Our data thus point to differences in the regulation of local *versus* systemic IL-1β production. The underlying mechanisms are unclear but it is possible that IL-1β in the peritoneal cavity and the blood may stem largely from different sources and that positive/negative regulatory pathways might be differentially active at different times, in different tissues, cell types, and/or in response to activation by different stimuli. Consistent with this hypothesis, we see decreased IL-1β production in the lungs of Tyk2$^{-/-}$ mice after intranasal LPS administration (data not shown). We conclude that the inhibitory role of Tyk2 is relevant in vivo, although its specific contribution to the complex regulation of IL-1β is highly dependent on the cellular context and, presumably, the specific mode and state of activation.

IL-1β–deficient mice respond to systemic LPS challenge similar to WT mice (75), so it is unlikely that the enhanced local IL-1β expression in the absence of Tyk2 contributes to the LPS-resistant phenotype. Nevertheless, the suppressive role of Tyk2 on IL-1β expression that we describe raises the possibility that Tyk2 might have protective functions during IL-1β–driven diseases. In mice, IL-1β is involved in the pathogenesis of several autoimmune and inflammatory diseases, for example, turpentine-induced local inflammation, contact hypersensitivity, and collagen-induced arthritis (76). Tyk2 seems not to act protectively during autoimmune and inflammatory diseases but may actually exacerbate the symptoms. An exception is contact hypersensitivity, where Tyk2 exerts protective functions: Tyk2$^{-/-}$ mice show increased sensitivity (77). Enhanced IL-1β expression in the absence of Tyk2 would be consistent with these findings. However, it is important to bear in mind that other functions of Tyk2, in particular in IL-12 signaling/IFN-γ 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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RESULTS III

Transcriptome analysis reveals a major impact of JAK protein tyrosine kinase 2 (Tyk2) on the expression of interferon-responsive and metabolic genes

Vogl *et al.*, *BMC Genomics* 2010, 11:199

BMC Genomics

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Transcriptome analysis reveals a major impact of JAK protein tyrosine kinase 2 (Tyk2) on the expression of interferon-responsive and metabolic genes

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Abstract

Background: Tyrosine kinase 2 (Tyk2), a central component of Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling, has major effects on innate immunity and inflammation. Mice lacking Tyk2 are resistant to endotoxin shock induced by lipopolysaccharide (LPS), and Tyk2 deficient macrophages fail to efficiently induce interferon α/β after LPS treatment. However, how Tyk2 globally regulates transcription of downstream target genes remains unknown. Here we examine the regulatory role of Tyk2 in basal and inflammatory transcription by comparing gene expression profiles of peritoneal macrophages from Tyk2 mutant and wildtype control mice that were either kept untreated or exposed to LPS for six hours.

Results: Untreated Tyk2-deficient macrophages exhibited reduced expression of immune response genes relative to wildtype, in particular those that contain interferon response elements (IRF/ISRE), whereas metabolic genes showed higher expression. Upon LPS challenge, IFN-inducible genes (including those with an IRF/ISRE transcription factor binding-site) were strongly upregulated in both Tyk2 mutant and wildtype cells and reached similar expression levels. In contrast, metabolic gene expression was strongly decreased in wildtype cells upon LPS treatment, while in Tyk2 mutant cells the expression of these genes remained relatively unchanged, which exaggerated differences already present at the basal level. We also identified several 5'UR transcription factor binding-sites and 3'UTR regulatory elements that were differentially induced between Tyk2 deficient and wildtype macrophages and that have not previously been implicated in immunity.

Conclusions: Although Tyk2 is essential for the full LPS response, its function is mainly required for baseline expression but not LPS-induced upregulation of IFN-inducible genes. Moreover, Tyk2 function is critical for the downregulation of metabolic genes upon immune challenge, in particular genes involved in lipid metabolism. Together, our findings suggest an important regulatory role for Tyk2 in modulating the relationship between immunity and metabolism.

Background

The first crucial step in successfully fighting infections is the sensing of microbial products by innate immune cells, e.g. macrophages and dendritic cells [1]. Toll-like receptors (TLRs) sense microbial products and initiate a cascade of signaling events to alert the immune defense. Members of the TLR family recognize a range of pathogen-associated molecular patterns produced by bacteria, protozoa, fungi, or viruses [2,3]. One of the best studied examples for a pathogen associated pattern is bacterial lipopolysaccharide (LPS). Binding of LPS to the TLR4 complex of the host activates two signaling cascades via different adaptor proteins leading to the activation of two

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main transcription factors: NFκB, a key transcription factor regulating pro-inflammatory genes, and interferon (IFN) regulatory factor 3 (IRF3), which induces IRF3-responsive genes, most prominently IFNβ. IFNβ and other cytokines subsequently act in an autocrine/paracrine manner to induce the expression of immune responsive genes [4,5].

A major component of IFNα/β signaling is Tyrosine kinase 2 (Tyk2), a member of the Janus kinase (JAK) family [6]. Activated JAKs phosphorylate members of the signal transducer and activator of transcription (STAT) family, which mediate signals from a large number of cytokines and growth factors [7,8]. Activation of JAK1 and Tyk2 by IFNα/β stimulation leads to a series of phosphorylation events, causing the formation of IFN stimulated gene factor 3 (ISGF3) complexes that consist of STAT1, STAT2 and IFN regulatory factor 9 (IRF9). Subsequently, ISGF3 translocates to the nucleus and binds to a specific transcription factor binding-site (TFBS), the IFN stimulated response element (IRF/ISRE), which in turn activates transcription of several hundred IFN-responsive genes. To a lesser extent, these signaling events also lead to the formation of STAT1 and STAT3 homo- and heterodimers that bind another TFBS, the IFNγ activated sequence (GAS TFBS) [9].

In mice, Tyk2 is only partially required for IFNα/β signaling, where it mainly serves to amplify the immune response [10,11]. We and others have previously shown a surprisingly low susceptibility of Tyk2 knockout mice to LPS-induced endotoxin shock, despite normal production of proinflammatory cytokines (the interleukins IL6, IL1B) and tumor necrosis factor α (TNFα) [12,13]. *In vitro*, Tyk2 mutant peritoneal macrophages fail to efficiently induce IFNα/β and nitric oxide (NO) production upon LPS treatment. In addition, Tyk2 deficiency influences the basal level of several IFN-responsive genes [10,12]. However, despite the importance of Tyk2 in mediating immune and inflammatory responses, how this JAK regulates global transcription of downstream target genes remains largely unknown.

Here we compare the genome-wide expression profiles of Tyk2 mutant and wildtype mouse peritoneal macrophages with and without activation by LPS using microarrays. In particular, we focus on IFN-responsive genes and their putative regulators and use bioinformatic analysis to examine how the observed changes in gene expression relate to gene ontology categories and *cis*-regulatory elements (i.e., 5'UR TFBSs and 3'UTR elements) [14]. We find that Tyk2 is essential for mediating the full LPS response and for baseline expression of IFN-inducible genes, but not for the LPS-induced upregulation of IFN-responsive genes. Moreover, we show that LPS challenge suppresses the expression of genes involved in metabolism and we establish a critical role for Tyk2 function in this downregulation.

Results

Effects of Tyk2 and LPS on gene expression

To determine the effects of Tyk2 and LPS on gene expression we used CodeLink Mouse Whole Genome Arrays on mRNA isolated from Wt (C57BL/6) and Tyk2-/- (on a C57BL/6 background) macrophages, either stimulated with LPS for six hours or kept untreated. After filtering and quality control, we retained 7546 genes for statistical and bioinformatic analysis (see Methods).

At the basal level, in the absence of LPS stimulation, we found that 428 (6%) of 7546 genes analyzed were differentially expressed between Wt and Tyk2-/- macrophages (effect of genotype, Figure 1A; full dataset available at Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/geo/, GEO accession number GSE19733). Among the genes with the largest expression differences between genotypes, many are known to be involved in immune function. The majority of these genes was downregulated

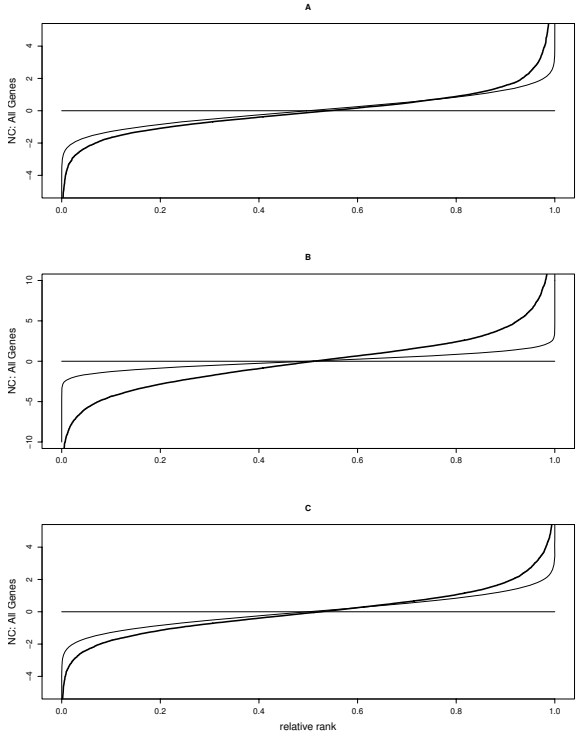

Figure 1 Effects of Tyk2 and LPS on overall gene expression. Effects of (A) Tyk2 genotype (Wt minus Tyk2-/-), (B) LPS treatment (6 hours of LPS minus control), and (C) genotype by treatment interaction (difference in LPS induction between Wt and Tyk2-/-) on expression levels of all genes, plotted as normed effect coefficients (NCs) (y-axis) against relative ranks (absolute rank divided by the number of genes; x-axis). Thick solid lines represent NCs; thin solid lines the values expected under the assumption of the null hypothesis and normality.

in Tyk2-/- relative to Wt cells, including for example IFNγ- inducible protein 47 (IFI47), IFN regulatory factor 7 (IRF7), and 2'-5' oligoadenylate synthetase-like genes 1 and 2 (OASL1, OASL2), as well as three out of five (STAT1, IRF1, and IRF7) IFN-responsive genes shown in Figure 2.

Consistent with this, gene ontology (GO) analysis showed that gene classes involved in immunity were significantly downregulated in Tyk2-/- relative to Wt cells (Table A1A). Interestingly, genes belonging to metabolic gene classes showed higher expression in Wt relative to Tyk2-/-, including genes in the classes "metabolism", "lipid biosynthesis", "sterol biosynthesis", and "cholesterol biosynthesis" (Table A1A). Overall, we observed a small but significant shift in gene expression towards lower values in Tyk2-/- cells, suggesting the involvement of the majority of metabolic genes in this shift (Figure 3A). This relatively small but consistent effect can also be seen at the level of individual genes, for example for the lipid genes shown in Figure 2. Thus, in macrophages, Tyk2 is not only required for basal expression of immune genes but also for the maintenance of metabolic genes at a low level of expression in an uninduced state.

In contrast to the relatively small number of differences in baseline expression between Wt and mutant cells, LPS treatment changed the transcriptional status of Wt macrophages dramatically: 3622 genes (48%) were either up- or downregulated upon exposure to LPS, including many genes involved in immunity (effect of LPS treatment, Figure 1B; GEO accession number GSE19733). This massive change in transcriptional status is also reflected in the large distance between the observed curve and the curve showing the theoretical expectation in Figure 1B. Among the genes upregulated by LPS, several are known to also be induced by cytokines, chemokines, or cellular stressors, including the interleukins IL1A, IL1B, IL12A, the IFN stimulated gene ISG20, and TNFα, as well as all five IFN-responsive genes shown in Figure 2. In contrast, genes involved in lipid metabolism were typically downregulated in Wt cells (see Figures 2 and 3B). Similar to the situation at the basal level, this downward shift in expression involved almost all genes (Figure 3B).

In total, LPS treatment influenced 19 GO classes, with strong upregulation of genes involved in inflammatory responses and chemotaxis and downregulation of genes involved in metabolism, indicating a negative relationship between immune response and metabolism (Table B1B; see Figure 3B for metabolic genes). In Tyk2 mutant cells, however, only 2297 genes (30%) were influenced by LPS, suggesting that Tyk2-/- cells respond overall much less to LPS than Wt cells (GEO accession number GSE19733).

The observation that Tyk2-/- macrophages might exhibit an impaired LPS response was confirmed when we analyzed differences in how genotypes react to LPS challenge: 1202 genes (16%) showed a difference in LPS induction between Wt and Tyk2 mutant (effect of genotype × treatment interaction, Figure 1C, GEO accession number GSE19733). For example, among the differentially induced immune genes, LPS caused a much weaker induction of the interferon-stimulated protein ISG20 and the NO synthase NOS2 in Tyk2-/- than in Wt cells, but a stronger induction of IFI47 and IRF7 relative to Wt (Figure 2).

Overall, we found that eleven classes of genes were differentially induced by LPS between genotypes, including weaker upregulation of genes involved in apoptosis and negative cell cycle control and weaker suppression of metabolic gene expression in Tyk2-/- relative to Wt cells (Table C1C; see Figures 2 and 3C for metabolic genes). Again, we found that this downward shift in gene expression involved the majority of all genes (Figure 3C). Most notably, genes involved in "cholesterol biosynthesis", "sterol biosynthesis", "steroid biosynthesis", and "lipid biosynthesis" were much less strongly suppressed upon LPS challenge in Tyk2-/- than in Wt macrophages. Metabolic genes were therefore downregulated by LPS in Wt cells, but their expression levels remained closer to the basal, uninduced state in Tyk2-/- macrophages. RT-qPCR broadly confirmed the effects of Tyk2 on immune and metabolic genes (Additional File 1). Thus, Tyk2 is required for LPS-induced upregulation of immune and other genes and for the suppression of metabolic genes upon LPS challenge (also see Additional File 1).

Transcription factor binding-sites

The expression of the about 25,000 genes in mammalian genomes is regulated by many fewer transcription and other regulatory factors. We therefore related our microarray expression data to information on TFBSs upstream of the transcription start site [14]. Since TFBSs are often short and relatively uninformative, and because many putative TFBSs are biologically irrelevant, we extracted sequences from the 5' upstream region (5'UR) that are conserved between human and mouse, as such phylogenetic conservation might indicate functional importance in transcriptional control. This bioinformatics analysis identified 174 classes of putative 5'UR TFBSs.

At the basal level, only genes that contain the IRF/ISRE TFBS were differentially regulated between genotypes; genes with GAS elements showed no significant difference between genotypes (Figure 4A, Table A2A, Additional File 2). Since the IRF/ISRE element is responsive to the ISGF3 complex, genes containing such an element are likely candidate targets of IFNα/β signaling. Tyk2-/- macrophages had on average an approximately 1.4 times reduced expression of genes with an IRF/ISRE TFBS as compared to Wt cells (Figure 4A, Table A2A, Additional File 2). Unlike the situation for metabolic genes, this dif-

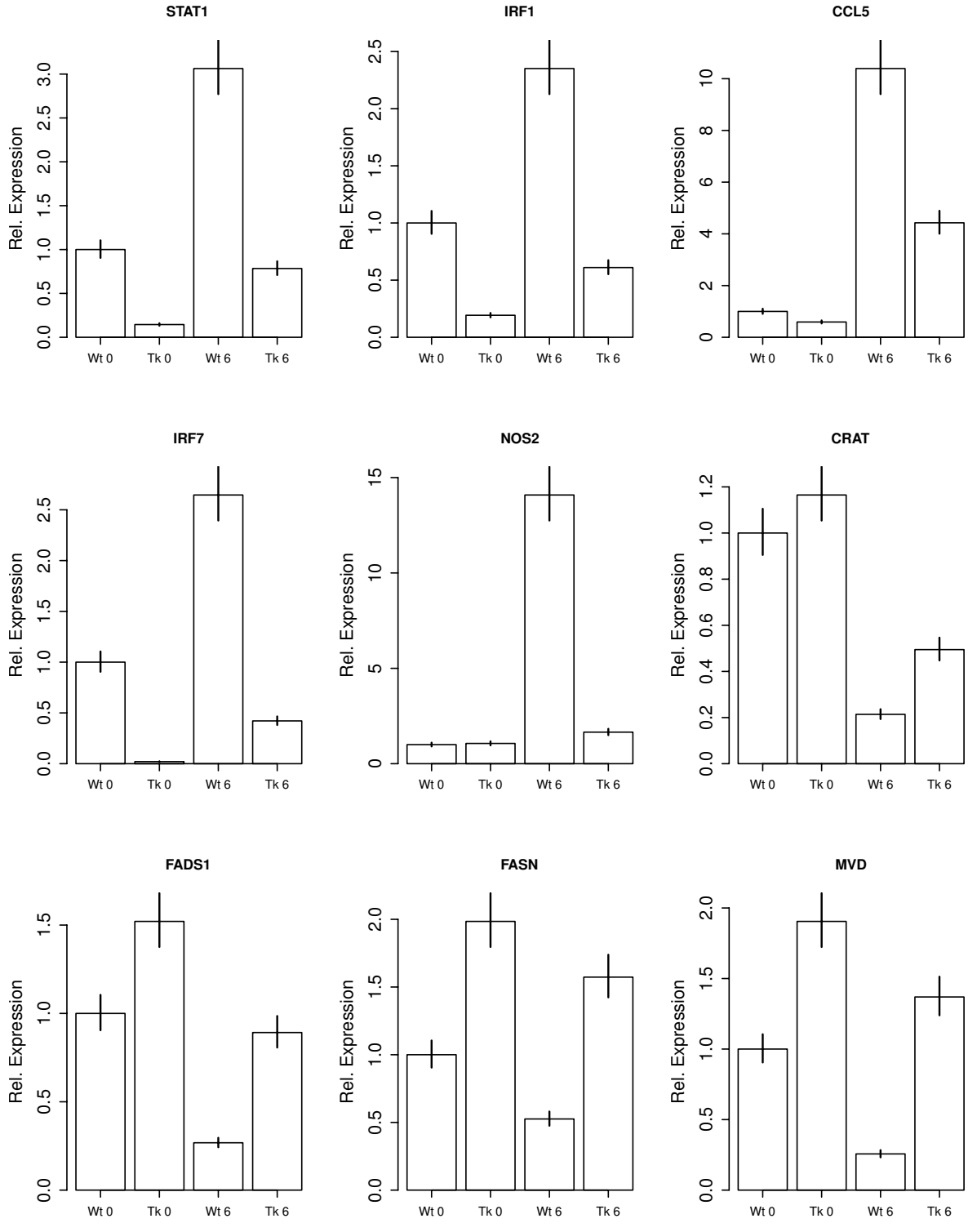

Figure 2 Effects of Tyk2 and LPS on relative expression levels of immune genes and lipid genes. Representative examples of the effects of genotype (Wt vs.Tyk2-/- (Tk)) at the basal level (0) or after six hours of LPS treatment (6). Five genes (STAT1, IRF1, CCL5, IRF7, NOS2) are immunity annotated and IFN-responsive genes; four genes (CRAT, FADS1, FASN, MVD) are annotated for lipid metabolism. Expression levels, as determined by microarray fluorescence intensities, are shown relative to the mean basal level in the Wt. Error bars represent standard errors. All genes were validated with RT-qPCR (see Materials and Methods).

Table 1: Results of gene ontology analysis using the "biological process" annotation

GO category	# genes	ER	NC	p-value
(A)				
humoral defense mechanism sensu Vertebrata	11	1.58	4.3	0
antigen presentation	11	1.54	4.02	0
defense response	46	1.34	2.72	0
immune response	102	1.28	2.31	0
positive regulation of transcription				
DNA-dependent	13	1.19	1.65	0.0013
regulation of apoptosis	45	1.11	0.94	0.0005
protein ubiquitination	78	1.06	0.58	0.0049
protein modification	71	1.06	0.57	0
transcription	449	1.04	0.34	0
regulation of transcription DNA.dependent	586	1.03	0.29	0
transport	576	0.97	-0.29	0.0002
electron transport	138	0.95	-0.49	0.002
metabolism	151	0.92	-0.8	0
sodium ion transport	22	0.88	-1.2	0.0025
biosynthesis	24	0.87	-1.26	0.0009
fatty acid biosynthesis	15	0.85	-1.57	0.0011
lipid biosynthesis	34	0.83	-1.77	0

Table 1: Results of gene ontology analysis using the "biological process" annotation (Continued)

steroid biosynthesis	20	0.83	-1.79	0
cholesterol biosynthesis	12	0.79	-2.21	0
sterol biosynthesis	13	0.77	-2.5	0
(B)				
inflammatory response	46	2.24	7.55	0
chemotaxis	30	2	6.48	0
immune response	102	1.93	6.18	0
sensory perception	42	1.64	4.66	0
defense response to bacteria	11	1.63	4.61	0.0001
defense response	46	1.37	2.97	0
cell surface receptor linked signal transduction	32	1.28	2.28	0.001
sensory perception of smell	70	1.25	2.08	0
cell proliferation	42	1.24	2.03	0.0008
signal transduction	222	1.21	1.79	0
regulation of apoptosis	45	1.2	1.73	0
G-rotein coupled receptor protein signaling pathway	166	1.15	1.32	0
regulation of transcription	119	1.1	0.87	0
regulation of transcription DNA.dependent	586	1.04	0.38	0

Table 1: Results of gene ontology analysis using the "biological process" annotation (Continued)

electron transport	138	0.87	-1.26	0
metabolism	151	0.82	-1.91	0
lipid metabolism	59	0.79	-2.21	0
lipid biosynthesis	34	0.77	-2.49	0
fatty acid metabolism	32	0.71	-3.15	0
(C)				
negative regulation of progression through cell cycle	26	1.17	0.95	0.0022
anti apoptosis	30	1.17	0.92	0.0015
metabolism	151	0.92	-0.47	0.0009
protein biosynthesis	180	0.92	-0.52	0
cell proliferation	42	0.87	-0.8	0.0021
fatty acid metabolism	32	0.85	-0.95	0.0015
rRNA processing	29	0.85	-0.97	0.0019
lipid biosynthesis	34	0.84	-1.02	0.0004
steroid biosynthesis	20	0.83	-1.09	0.0037
sterol biosynthesis	13	0.8	-1.34	0.0039
cholesterol biosynthesis	12	0.79	-1.43	0.003

Within the "biological process" annotation, gene classes (412 in total) were separately contrasted with all other classes using *t*-tests on standardized coefficients. (A) Basal differences between genotypes, (B) LPS induction in Wt, and (C) differences between genotypes in LPS induction (genotype by treatment interaction). ER, approximate mean expression ratio, NC, mean normed coefficients.

ference between genotypes was not caused by the majority of genes, but by a few genes with much higher expression in Wt than in Tyk2-/- (Figure 4A). Moreover, the expression of several immune genes, including Z-DNA binding protein 1 (ZBP1), guanylate binding protein 3 (GBP3), IFN-induced transmembrane protein 3 (IFITM3), and the interleukin 15 receptor α (IL15RA), was about three times higher in Wt than in Tyk2-/- cells (Additional File 1, GEO accession number GSE19733).

LPS treatment influenced seven classes of genes in Wt macrophages (Figure 4B, Table B2B, Additional File 2), with two of them containing putative binding-sites for

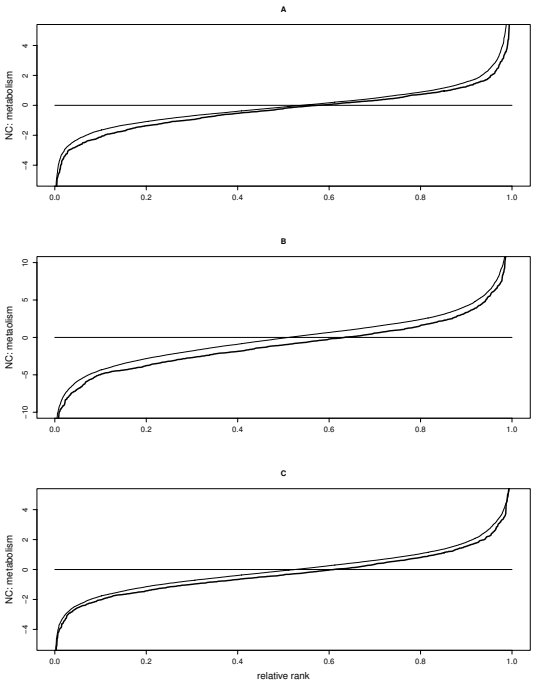

Figure 3 Effects of Tyk2 and LPS on metabolic gene expression. Effects of (A) Tyk2 genotype (Wt minus Tyk2-/-), (B) LPS treatment (6 hours of LPS minus control), and (C) genotype by treatment interaction (difference in LPS induction between Wt and Tyk2-/-) on expression levels of genes annotated for metabolism, plotted as NCs (y-axis) against relative ranks (absolute rank divided by the number of genes; x-axis). Thick solid lines represent NCs; thin solid lines the values of all genes (see Figure 1) in order to highlight effects on metabolic genes relative to all genes analyzed.

unknown transcription factors. Similar to the baseline differences in expression between genotypes, genes with the IRF/ISRE TFBS (e.g., gram negative binding protein 3 (GNBP3), cluster of differentiation 274 (CD274), and IL15RA) were strongly upregulated after six hours of LPS treatment. These genes were followed by an uncharacterized class of genes, either with CHX10 TFBS (e.g., β-site APP cleaving enzyme 1 (BACE1), the 14-3-3 gene Stratifin, the Myc associated factor X (MAX), and CD38) or containing homeodomain transcription factor IPF1/PDX1 motifs (e.g., Stratifin, the tripartite motif gene TRIM21, and the zinc finger transcription factor Zfp281). To our knowledge, neither CHX10 TFBS nor IPF1/PDX1 motifs have previously been implicated in the LPS response. Genes containing a TATA box were also significantly upregulated upon LPS exposure (especially the LIM homeodomain factor LHX4 and TNFα). Furthermore, we found evidence for weak upregulation of genes with conserved NF-AT motifs (Table B2B), with AP-1/Jun and c-REL consensus sequences (using a significance level of α = 0.01 - see Methods for definition of cutoff criteria; Additional File 2), and with NFκB motifs (using α = 0.05; Additional File 2). Interestingly, we failed to observe LPS-induced downregulation of any gene class (either using FDR = 0.05 or α = 0.01 as cutoffs; Table B2B, Additional File 2).

Six classes of genes differed in their induction by LPS between genotypes, and for all these classes induction by LPS was lower in Tyk2-/- than in Wt cells (Table C2C, Additional File 2). Genes containing the pituitary-specific transcription factor POU1F1 motif showed the most pronounced differences in mean expression, whereas genes with a IPF1/PDX1 motif (especially Stratifin) or a TATA box (especially LHX4 and NOS2) showed the highest statistical significance (Table C2C, Additional File 2). However, despite these genotypic differences in inducibility, LPS induced on average similar expression of genes with an IRF/ISRE TFBS in Wt and Tyk2-/- macrophages (Figure 4C, Table C2C, Additional File 2). Upon LPS challenge, Wt and Tyk2-/- cells therefore reached qualitatively and quantitatively similar expression levels of genes that contain the IRF/ISRE TFBS. These results suggest that Tyk2 is functionally required for the baseline expression of genes with an IRF/ISRE TFBS, but dispensable for their LPS-induced upregulation.

Since the above analysis of IRF/ISRE TFBSs was based on very stringent criteria and thus likely to miss many known IFN-inducible genes (see Methods), we also produced a list of 187 IFN-responsive genes from the literature and asked how many of those genes showed expression changes in our microarray experiment and contain IRF/ISRE or GAS TFBSs.

After filtering of lowly expressed genes, 137 genes remained in the analysis. At the basal level, we found that this class of IFN-inducible genes was on average downregulated relative to all other genes in Tyk2-/- versus Wt macrophages (Additional File 3). While the mean expression ratios relative to Wt were quite small, the average difference was caused by a few genes of large effect, with 23 genes being significantly differentially expressed (Additional File 3). The genes that showed the largest expression differentials were IRF7, IFN-inducible proteins 1 and 205 (IFI1, IFI205), and transporter associated with Ag processing 1 (TAP1), thereby confirming that Tyk2 is required for baseline expression of IFN-inducible genes. Upon LPS stimulation, IFN-inducible genes were on average much more strongly upregulated than other genes (74 genes in Wt; Additional File 3). Again, this pattern was mainly caused by a few genes with extreme effects, including IL1B, chemokine (C-C motif) ligands 2 and 7 (CCL2, CCL7), and chemokine (C-X-C motif) ligand 2 (CXCL2). As in our analysis of genes with the IRF/ISRE TFBS, the average induction of IFN-inducible genes by LPS was similar between Tyk2-/- and Wt macrophages, with both cell types reaching qualitatively identical expression levels (Additional File 3). To validate

Table 2: Analysis of putative 5'UR TFBSs

TFBS	# genes	ER	NC	p-value
(A)				
IRF/ISRE	40	1.41	3.20	0.0011
(B)				
IRF/ISRE	40	1.53	3.99	0.0001
127	35	1.19	1.60	0.0006
CHX10	160	1.09	0.80	0.0001
IPF1 = PDX1	108	1.08	0.72	0.0010
TATA	280	1.07	0.67	0.0005
174	176	1.06	0.56	0.0009
NF-AT	519	1.03	0.25	0.0011
(C)				
115	33	1.13	0.73	0.0006
POU1F1	49	1.12	0.65	0.0019
IPF1 = PDX1	108	1.08	0.45	0.0003
FOXF2	171	1.07	0.40	0.0018
CHX10	160	1.06	0.35	0.0010
TATA	280	1.06	0.33	0.0005

We assigned genes to 174 classes of putative TFBSs, either identified by their name, the major transcription factor binding to them, or their number in Xie et al. [14]. The table shows the number of genes in each TFBS class, approximate mean expression ratios (ER), mean normed coefficients (NC), and p-values from *t*-tests. (A) Basal differences between genotypes, (B) LPS induction in Wt, and (C) differences between genotypes in LPS induction (genotype by treatment interaction).

these microarray results we performed RT-qPCR on nine IFN-inducible genes and found a good agreement with respect to approximate fold induction (Additional File 1).

IFN-responsive genes contained significantly more IRF/ISRE and GAS TFBSs than other genes, although enrichment was only modest for the latter. The criteria of inclusion, i.e. the length of the 5'UR and the counting of only TFBSs conserved between human and mouse influenced the results considerably. As expected, conservation of TFBSs among species decreased the number of false positives, whereas a long 5'UR stretch or no conservation increased it (Additional File 3).

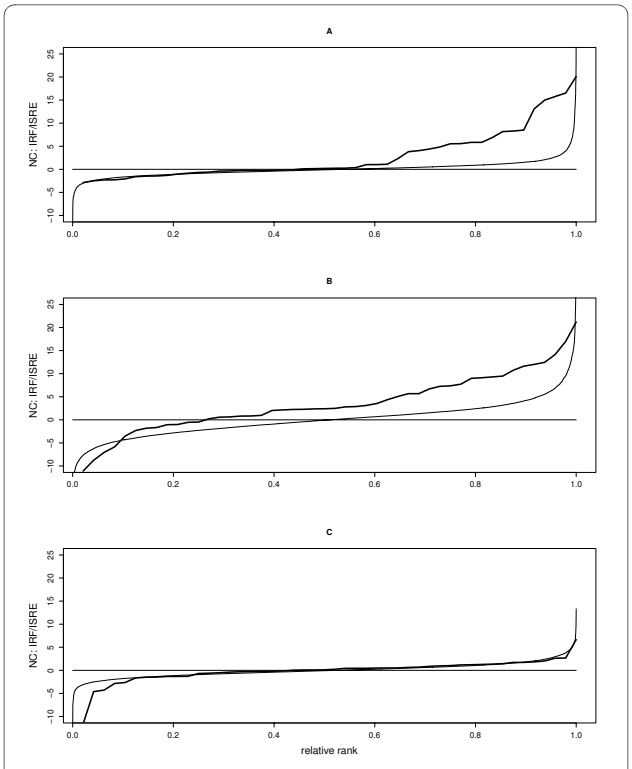

Figure 4 Effects of Tyk2 and LPS on genes with IRF/ISRE TFBS. Effects of (A) Tyk2 genotype (Wt minus Tyk2-/-), (B) LPS treatment (6 hours of LPS minus control), and (C) genotype by treatment interaction (difference in LPS induction between Wt and Tyk2-/-) on expression levels of genes containing the IRF/ISRE TFBS, plotted as NCs (y-axis) against relative ranks (absolute rank divided by the number of genes; x-axis). Thick solid lines represent NCs; thin solid lines the values of all genes (see Figure 1) in order to highlight the effects on genes with IRF/ISRE TFBS relative to all genes analyzed.

3' UTR regulatory elements

As mRNA levels are not only known to be regulated by 5'UR TFBSs, but also post-transcriptionally by regulatory elements in the 3'untranslated region (UTR), we were interested in relating the observed expression differences to putative 3'UTR regulatory sequences. We focused on two not mutually exclusive classes of regulatory sequences: (i) conserved 3'UTR motifs and (ii) 3'UTR microRNA (miRNA) cognate sequences.

To examine motifs in the 3'UTR we analyzed our data with respect to 175 classes of conserved putative 3'UTR regulatory sequences defined by Xie et al. [14] (Table 3, Additional File 4). As compared to 5'UR TFBSs, we found many more gene classes to be differentially expressed (Table 3, Additional File 4). This might simply be due to a larger number of genes containing specific 3'UTR motifs than 5'UR TFBSs. Alternatively, 3'UTR elements might be more conserved, or easier to detect, than 5'UR TFBSs. We observed four classes of motifs that were differentially regulated between genotypes at the basal level (Table A3A), 37 that were up- or downregulated after LPS treatment in Wt (Table B3B), and three that showed differences in LPS inducibility between genotypes (Table C3C). Genes with AU-rich elements (m2/ARE) were the most significantly upregulated upon LPS treatment (Table B3B, Additional File 5). Similar to what we observed for IFN-responsive genes or those with IRF/ISRE TFBSs, this pattern was mainly caused by a few genes with extreme effects on expression, such as IL1A, IL1B, CXCL2, and TNFα, which were among the most responsive genes (GEO accession number GSE19733). In contrast, LPS caused downregulation of a large class of genes containing poly-A signal (m1/poly-A) motifs (Table B3B). However, genes with m2/ARE or m1/poly-A motifs neither differed in baseline expression, nor in LPS inducibility between Wt and Tyk2-/- macrophages.

A special class of 3'UTR regulatory elements important in the regulation of gene expression are the targets of miRNAs, short single-stranded RNA molecules consisting of 21-24 nucleotides. To analyze miRNA cognate motifs we related known miRNAs from miRBase http://www.mirbase.org/[15-17] to the octameric sequences in [14]. Using this approach, we were able to uniquely define different cognate 3'UTR sequences (which might otherwise have been lumped into a single class in the analysis above). Out of 111 such sequences, we found three miRNA cognate motif classes to be differentially expressed at the basal level, three classes to be influenced by LPS in Wt cells, and three classes to differ in their LPS inducibility between genotypes (Additional File 6). As far as we know, none of these miRNA motifs has been previously implicated in the IFN or LPS response. However, since conserved 3'UTR elements and miRNA consensus sites are still poorly characterized, it is difficult to functionally interpret these results. Nonetheless, our observations provide strong evidence for an involvement of such regulatory sequences in the LPS response of Wt and Tyk2-/- macrophages.

Discussion

In this microarray study we have investigated the role of the JAK protein kinase family member Tyk2 in basal and LPS-induced gene expression in mouse peritoneal macrophages. While several previous studies have demonstrated that Tyk2 is an important regulator of immune and inflammatory responses [10,12,18], here we show how Tyk2 globally affects genome-wide transcript levels.

At the basal uninduced level, we found subtle but significant differences in gene expression between Wt and Tyk2-/- macrophages. Relative to Wt, Tyk2-deficient macrophages displayed reduced expression of many immune genes, in particular some genes that contain IFN response elements (IRF/ISRE) in their 5'UR, suggesting that Tyk2 deficiency compromises the type I IFN (i.e.,

Table 3: Analysis of 3'UTR regulatory sequences

3'UTR motif	gene #	ER	NC	p-value
(A)				
m57	110	1.04	0.39	0.0037
m11	623	0.98	-0.23	0.0026
m43	254	0.96	-0.33	0.0009
o7	207	0.96	-0.33	0.0058
(B)				
m53	42	1.30	2.60	0.0076
o34	75	1.13	1.22	0.0006
m35	54	1.13	1.18	0.0026
m103	41	1.11	1.05	0.0048
m33	119	1.11	1.04	0.0026
m95	56	1.11	1.03	0.0004
m16	213	1.10	0.99	0.0000
o25	105	1.09	0.86	0.0011
m85	82	1.09	0.84	0.0043
m2/ARE	675	1.09	0.82	0.0000
m67	164	1.07	0.68	0.0004
o18	278	1.07	0.65	0.0001
m50	108	1.07	0.64	0.0081
m100	70	1.06	0.60	0.0079
o43	142	1.05	0.49	0.0021

Table 3: Analysis of 3'UTR regulatory sequences (Continued)

m30	85	1.05	0.49	0.0133
m70	136	1.05	0.48	0.0108
o12	196	1.05	0.48	0.0012
m24	332	1.05	0.47	0.0001
o15	253	1.04	0.43	0.0011
m3	481	1.04	0.43	0.0000
m20	262	1.04	0.40	0.0024
o38	142	1.04	0.38	0.0063
m29	232	1.04	0.37	0.0013
m41	224	1.04	0.36	0.0025
o32	155	1.04	0.36	0.0067
m13	1015	1.03	0.30	0.0000
m4	625	1.03	0.29	0.0000
o21	363	1.03	0.26	0.0011
m19	308	1.02	0.25	0.0028
m9	942	1.02	0.24	0.0000
m31	347	1.02	0.21	0.0018
m25	488	1.02	0.21	0.0006
m8	337	1.02	0.17	0.0077
m26	383	1.02	0.15	0.0017
m5	1175	1.01	0.06	0.0000
m1/poly-A	1938	0.95	-0.54	0.0091

Table 3: Analysis of 3'UTR regulatory sequences (Continued)

(C)				
m91	66	1.1	0.56	0.0046
m13	1015	1.01	0.06	0.0016
m9	942	1.01	0.06	0.0016

A total of 175 3'UTR motis were identified as described in Xie et al. [14]. The table shows the number of genes in each class of motif, approximate mean expression ratios (ER), mean normed coefficients (NC), and p-values from *t*-tests. (A) Basal differences between genotypes, (B) LPS induction in Wt, and (C) differences between genotypes in LPS induction (genotype by treatment interaction).

IFNα/β) response. This observation confirms previous findings demonstrating that IFNα/β signaling is reduced in Tyk2-/- mice and that IFNα4 and IFNβ mRNA levels are diminished in Tyk2 null macrophages at the basal level [10-12].

In addition to the ISGF3 complex, type I IFNs also activate STAT1 homodimers, which induce gene expression via IFNγ activated sequence (GAS) elements. Furthermore, STAT1 homodimers are strongly activated by IFNγ, whose production and signaling is partially impaired in the absence of Tyk2 [10-12]. It is still controversial whether macrophages can produce significant amounts of biologically active IFNγ [19], however, cells might have been exposed to IFNγ during the generation procedure. We were therefore interested to determine if genes containing GAS TFBSs are also influenced by the absence of Tyk2. In contrast to the involvement of IRF/ISRE TFBS in the Tyk2 mediated response, we failed to find evidence for differential expression of genes containing GAS elements. Since the mouse genome contains very many such elements, not all of which are involved in gene regulation, it is possible that our bioinformatic analysis failed to find those GAS elements that are biologically relevant. Thus, while in our study reduced baseline expression of immunity related and IFN-responsive genes in Tyk2-deficient macrophages seems to be mainly mediated by IRF/ISRE TFBSs, the role of Tyk2 in GAS-driven gene regulation deserves further investigation.

We also found that Tyk2 is required for the full response of genes upon LPS challenge, as expected based on previous experiments [10,12]. LPS challenge affected the expression of about 48% of all genes in Wt cells, including many immune genes, but influenced only about 30% of all genes in Tyk2-/- cells. Consequently, Tyk2-deficient macrophages failed to induce many LPS-responsive transcripts, in particular immunity related genes. Surprisingly, however, IFN-responsive genes did not follow this trend: LPS challenge increased the expression of IFN-inducible genes to a similar extent in Wt and Tyk2-/- macrophages, suggesting that Tyk2 might not be critical for LPS-induced upregulation of IFN-responsive genes. Thus, although previous observations have found reduced levels of IFNα4 and IFNβ in Tyk2-/- macrophages [12], a high proportion of LPS-induced type I IFN signaling might be largely independent of Tyk2.

Several previous studies have examined IFN mediated immune and inflammatory transcription in bone marrow derived mouse macrophages (BMDMs) using microarrays [20,21]. Thomas et al. [20] studied Wt and IFNβ-/- cells at the basal level and after one and three hours of LPS treatment using Affymetrix microarrays. Many genes whose baseline levels were reduced by lack of IFNβ also had significantly lower levels in Tyk2-/- cells in our experiment. The overall correlation between expression differences for IFNβ-/- vs. Wt [20] and Tyk2-/- vs. Wt (our study) was $r = 0.41$ (see Additional File 7). Hence, the effect of IFNβ deficiency on baseline gene expression is similar to the lack of Tyk2. Thomas et al. [20] also identified many LPS-induced genes in Wt that we found to be induced in our study (overall correlation $r = 0.48$ - see Additional File 7). However, while IFNβ-/- macrophages showed an impaired LPS response for many IFN dependent genes [20], induction of these genes by LPS was generally unimpaired in Tyk2-/- macrophages in our experiment. A comparison of LPS inducibility between IFNβ-/- and Tyk2-/- macrophages only revealed a weak correlation of $r = 0.03$ (Additional File 7). Tyk2 therefore seems to be required for basal expression of IFNβ target genes but not for their induction by LPS. In another microarray study by Fleetwood et al. [21], genes whose basal expression levels depend on intact type I IFN signaling were characterized in BMDMs, either cultivated in the presence of macrophage colony stimulating factor (M-CSF, or CSF1) or granulocyte macrophage colony stimulating factor (GM-CSF). Most genes that were downregulated in IFNAR1-/- genotypes in both M-CSF and GM-CSF BMDMs were also decreased in Tyk2-/- relative to Wt cells in our experiment (Additional File 8), thus confirming our observation that Tyk2 is involved in basal IFNα/β signaling. Furthermore, the effects of basal

type I IFN signaling on gene expression in the thioglycolate-elicited peritoneal macrophages used in our study were similar to those observed in the BMDMs used in [20] and [21].

Interestingly, our study also provides evidence for a previously unknown role of Tyk2 in regulating metabolism. Tyk2 mutant macrophages showed enhanced baseline expression of metabolic genes relative to Wt cells, including genes involved in steroid, sterol, and lipid metabolism. Upon LPS treatment, metabolic gene expression was strongly decreased in Wt macrophages, while in Tyk2-/- macrophages expression of these genes remained relatively unchanged. Thus, the differential LPS response of Wt versus mutant macrophages increased differences in gene expression already present at the basal level: the relatively low expression of metabolic genes in Wt cells was strongly suppressed by LPS, whereas in Tyk2-/- cells LPS suppressed the relatively high expression of metabolic genes much less. This effect pertained to most genes in the GO class "metabolism", and in particular to genes belonging to the classes "cholesterol biosynthesis", "sterol biosynthesis", "steroid biosynthesis", and "fatty acid metabolism" Taken together, these results suggest an involvement of Tyk2 in the regulation of general metabolism and of lipid metabolism in particular. Tyk2 might thus be an important mediator of the connection between immunity and metabolism.

Two not mutually exclusive mechanisms might account for the effects of Tyk2 on metabolic gene expression. On the one hand, Tyk2/IFN signaling might directly lead to decreased metabolism, possibly because upregulation of immune function shuts down other energetically demanding processes [22,23]. On the other hand, Tyk2 might exhibit pleiotropic effects on immunity and metabolism that are largely independent: a canonical IFN signaling function of Tyk2 might be associated with an independent role in metabolic regulation. Potentially consistent with either model, we observed that LPS treatment of macrophages reduces the expression of mitochondrial genes and that this downregulation is impaired in Tyk2-/- cells relative to Wt (GO annotation "cellular component", GO class "mitochondrion"; results not shown). Remarkably, dysfunctional mitochondrial respiration has been shown for Tyk2-deficient pro-B cells [24]. In a similar vein, Pitroda et al. [25] found that STAT1 knockdown is associated with alterations in the expression of genes involved in energy metabolism, including glycolysis, oxidative phosphorylation, and the citrate cycle. Tyk2/IFN signaling might thus regulate metabolism by targeting mitochondrial processes via STAT1.

Notably, we observed a particularly pervasive effect of Tyk2 on lipid and fatty acid metabolism in macrophages. This result fits well with previous studies that have reported intricate but not well understood interactions between immunity, inflammation, and lipid metabolism [for reviews see [21,22]]. For example, lipids can be sensed by and act on Toll-like receptors (TLRs) such as TLR4, probably because TLR agonists like LPS contain a biologically active lipid moiety [see [23]]. Moreover, macrophages and adipocytes are derived from a common ancestral progenitor and share several transcriptional features, with macrophages expressing some adipocyte specific genes and adipocytes expressing several macrophage specific genes, including IL6 and TNFα [see [23], and references therein]. However, little is currently understood about the downstream effects of impaired IFN signaling on lipid metabolism, as we have observed them in our experiments.

Although the biological details remain unclear, several studies have established a link between IFN signaling, innate immunity, and lipid metabolism. Mice deficient for interleukin 1 receptor antagonist α (IL1Ra), for instance, are lean, have impaired body fat accumulation, and exhibit reduced lipoprotein lipase activity [26], and TNFα can increase lipolysis and promote apoptosis of adipocytes [27]. Moreover, Zwaferink et al. [28] have recently reported that key lipogenic enzymes, including fatty acid synthase (FASN), are suppressed in IFNβ treated mouse BMDMs. Since we have observed a similar downregulation in Tyk2 mutant macrophages, and because Tyk2 deficiency is known to decrease IFNβ levels [12], it might be possible that Tyk2 influences lipid metabolism through a basal feed-forward loop between IFNβ and Tyk2.

Our findings are also consistent with our previous observation that Tyk2 modulates metabolic proteins in BMDMs before and after LPS treatment [29]. However, it remains possible that isolated mouse macrophages display somewhat abnormal metabolic behavior. We found most metabolic genes to be suppressed upon LPS treatment in Wt macrophages, but *in vivo* results suggest that Wt mice become hypermetabolic ninety minutes after LPS injection [30]. Thus, future studies will need to examine the detailed role of Tyk2 in regulating metabolism, both in macrophages and other cell types as well as *in vivo*.

In addition to the IRF/ISRE TFBS already mentioned above, our study also identified several other TFBSs that might be involved in the immune and inflammatory response. In particular, we found several genes with 5'UR binding-sites for transcription factors that have not yet been implicated in immunity. For example, some genes with a TATA box were upregulated by LPS. Unfortunately, we cannot decipher this "regulatory code" in mammals with current bioinformatics means, although it might be conserved over quite large phylogenetic distances [31]. We also observed five known and one unknown TFBS to be correlated with induction differ-

ences between Wt and Tyk2-/- cells. While further analysis will be required to examine the effect of these motifs, we generally observed similar GO classes to be upregulated after six hours of LPS as Nilsson et al. [32] who studied the expression of genes and gene regulation *via* 5'UR TFBS in BMDMs after seven hours of LPS stimulation.

Finally, we related our results to information on putative 3'UTR elements. We found many more genes per 3'UTR element than per 5'UR TFBS, possibly because the 3'UTR is evolutionarily more conserved than the 5'UR or because regulation at the 3'UTR is more pleiotropic than at the 5'UR. Overall, our results provide strong evidence for the involvement of 3'UTR elements in the LPS response and in the differential regulation of expression between genotypes. Several classes of conserved 3'UTR regulatory sequences and 3'UTR miRNA cognate sequences were differentially regulated between genotypes, in response to LPS, and in terms of LPS inducibility between Wt and Tyk2 mutant cells. Most notably, LPS strongly induced expression of genes with AU-rich elements (m2/ARE), which have already previously been implicated in innate immunity [33,34]. In contrast, among the miRNA cognate motifs that were differentially regulated in our experiment, none has a known function. Thus, since 3'UTR conserved elements and miRNA consensus sites are still poorly annotated and characterized, it is difficult to biologically interpret these results. Nevertheless, it has become clear in recent years that 3'UTR elements and miRNA cognate sequences are highly important in regulating gene expression, and it will therefore be interesting to explore their potential role in immune and inflammatory transcription.

Conclusions

We conclude that Tyk2 function is required for the full transcriptional response upon LPS challenge, but dispensable for the LPS induction of IFN-responsive genes. Although it remains presently unclear why Tyk2 mainly mediates baseline but not LPS-induced type I IFN signaling, we suggest that Tyk2 maintains IFN-responsive genes in a primed, "ready to go" state, with factors other than Tyk2 mediating their induction upon immune challenge. Moreover, we conclude that Tyk2 plays a major but previously unknown role in the regulation of metabolism, especially lipid metabolism. Our data suggest that Tyk2 is not only critically required for the downregulation of baseline expression of many metabolic genes, but also for their further LPS-mediated suppression. While these results deserve in-depth future analysis, they clearly strengthen the previously made case for intricate connections between IFN signaling and metabolism and provide evidence for a role of Tyk2 in this connection. A better understanding of the relationship between immunity and metabolism will also likely be important for improving therapeutic interventions that target immune diseases as well as metabolic disorders.

Methods

Mice and macrophages

C57BL/6 wildtype (Wt) mice (*Mus musculus*) were purchased from Charles River Laboratories International, Inc. (Wilmington, MA, USA). Tyk2-/- mice are described in [10] and were backcrossed for ten generations into the C57BL/6 background. Mice were housed and bred under specific pathogen free conditions according to FELASA guidelines. All animal experiments were discussed with and approved by the institutional ethics committee and were carried out in accordance with protocols approved by Austrian laws (GZ 68.205/67-BrGT/2003; GZ 68.205/0204-C/GT/2007) and European directives.

Thioglycolate-elicited peritoneal macrophages were isolated from sex- and age-matched mice (males, 8-10 weeks old) as described in [35]. Cells from 2-3 mice per genotype were pooled for each of the three independent replicate experiments. 4×10^6 cells were plated out on 6 cm cell culture dishes (BD Falcon) and grown under standard conditions (37°C, 5% CO_2) in DMEM medium (Invitrogen) supplemented with 5% fetal calf serum (FCS), 100 mg/mL penicillin, 100 U/mL streptomycin, 2 mM L-glutamine and 50 μM β-mercaptoethanol. The day after isolation, cells were stimulated with 100 ng/mL LPS (*E. coli* serotype 055:B5, Sigma) or medium only (control), respectively, for six hours. For each combination of genotype (Wt versus Tyk2-/-) and treatment (control versus LPS) we carried out three independent replicate experiments.

Microarrays and RT-qPCR

For microarrays and RT-qPCR, total RNA was isolated using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration and integrity were determined by capillary electrophoresis (Agilent Technologies).

Total RNA (2 μg) was reverse transcribed using the iScript First Strand cDNA Synthesis Kit (Bio-Rad) and hybridized to the CodeLink Mouse Whole Genome Bioarray http://www.appliedmicroarrays.com/ according to the manufacturer's protocols. Arrays were scanned with a GenePix Array Scanner 4000B and GenePix Pro 4.0 software (Axon Instruments, Inc.). Data were prepared for statistical analysis (see below) with CodeLink Expression Analysis software http://www.appliedmicroarrays.com/. A gene was recorded as expressed if its unnormalized fluorescence intensity value exceeded 25 (see below). For these expressed genes, we log-transformed their intensity values and normalized them, separately for each array, to a mean of zero and a variance of one. Genes with missing

annotation and duplicate gene entries were removed before analysis;7546 genes of the approximately 30,000 genes on the microarray remained after filtering. The microarray dataset has been deposited at Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/ with accession number GSE19733).

To validate the expression data from the microarrays we performed reverse transcription-quantitative polymerase chain reactions (RT-qPCR) for about 170 genes including several IFN-inducible genes (Additional File 1). RNA (3 μg/60 μl) was reverse transcribed using the iScript First Strand cDNA Synthesis Kit (Bio-Rad). Taqman qPCR Low Density Custom Arrays (Applied Biosystems) were used to validate the microarray data, either in 48 well (samples in duplicates) or in 96 well (single samples) format. 5 μl cDNA (corresponding to 250 ng input RNA) were used in a 100 μl mastermix per sample-loading port, containing 5 mM $MgCl_2$, 200 μM of each dNTP (MBI Fermentas), 1 × ROX reference dye (Invitrogen), 4 Units HotFire DNA polymerase (Solis Biodyne), and 1 × reaction buffer B (Solis Biodyne). RT-qPCR was performed on a ABI PRISM 7900 HT machine (Applied Biosystems) using the following cycling conditions: initial denaturation at 95°C for 12 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min.

Validation of selected addional genes (mixed GO annotations) and genes involved in lipid metabolism was performed with single RT-qPCR assays. RNA (1 μg/20 μl) was reverse transcribed as described above. For assays using probes, we used 0.5 -2 μl cDNA (dependent on the gene analyzed) in a 25 μL mastermix, containing 2.5 mM $MgCl_2$, 200 μM of each dNTP (MBI Fermentas), 300 nM primer, 100 nM probe, 1 Unit HotFire DNA polymerase (Solis Biodyne), and 1 × reaction buffer B (Solis Biodyne). For Qiagen assays, we used 0.5-2 μl cDNA in a 25 μL mastermix, containing 2.5 mM $MgCl_2$, 200 μM of each dNTP (MBI Fermentas), 1 × QuantiTect primer assay (Qiagen), 0.2 × EvaGreen (Biotium), 1 Unit HotFire DNA polymerase (Solis Biodyne), and 1 × reaction buffer B (Solis Biodyne). RT-qPCR was performed on a Mastercycler realplex (Eppendorf) machine using the following cycling conditions: initial denaturation at 95°C for 15 min, 40 cycles of 95°C for 20 sec and 60°C for 1 min. For Qiagen assays, melting curve analyses were performed in order to check specificity. For details on RT-qPCR assays see Additional File 1.

The resulting data were analyzed using SDS 2.2 (Applied Biosystems) or realplex (Eppendorf) software and the statistical package R [36]. To compare microarray and RT-qPCR data we used log-transformed fluorescence intensity data. To standardize the RT-qPCR data we subtracted the mean Ct values of two endogenous control genes, ubiquitin-conjugating enzyme E2D 2 (UBE2D2) and hypoxanthine phosphoribosyltransferase (HPRT), from the RT-qPCR Ct values of the focal genes analyzed by Taqman qPCR Low Density Custom Arrays. For single RT-qPCR assays, the standard curve method was used to calculate the log expression levels, and values for the endogenous control gene UBE2D2 were subtracted from those of the focal genes. Thus, the microarray and RT-qPCR values were expected to be approximately proportional or inversely proportional, respectively, to the log of the mRNA concentrations of the focal genes. We found a good, approximately linear relationship between log microarray fluorescence intensities and normalized RT-qPCR delta Ct values for medium to highly expressed genes, while at lower levels we generally observed overwhelming background noise (Additional File 1). Importantly, we found a good agreement in terms of approximate fold induction between array and RT-qPCR data in general, and especially for IFN-inducible genes, genes involved in lipid metabolism, and five other genes (Additional File 1).

In order to check the experiment used for microarray analysis for effective LPS stimulation and for the expected differences between Wt and Tyk2-/- cells, IFNβ mRNA expression was monitored with RT-qPCR (data not shown). IRF7 and IRF1 were included in the validation experiments (Additional File 1). mRNA expression patterns of all three genes were as expected [12]. In addition, microarray results for TNFα and NOS2 expression confirmed our previous findings [10,12].

Analysis of single genes and gene ontology

To analyze the effects of genotype and LPS treatment on gene expression we used Analysis of Variance (ANOVA). For each gene, log-transformed normalized data were used in an univariate fully factorial two-way ANOVA, with genotype (Wt versus Tyk2-/-) and LPS treatment (untreated control versus LPS) as fixed factors:

$$y_{gti} = \mu + genotype_g + treatment_t + (genotype \times treatment)_{gt} + e_{gti},$$

where y_{gti} is the gti-th expression level for each gene, μ is the overall mean, g denotes the effect of genotype, t the effect of LPS treatment, gt the effect of the interaction between genotype and treatment, and e the residual error variance. Individual entered the analysis as a random effect.

Data inspection showed that the distribution of variances approximately follows the expectation (assuming all genes have equal variances), i.e. a scaled χ^2-distribution with four degrees of freedom. Since averaging over all genes gives a much better estimate of residual variation than single gene estimates of error variance, we estimated the effect sizes for each of the three effects

(genotype, treatment, and genotype × treatment) as standardized coefficients for each gene, i.e., by dividing the effects by the average of the square root of the residual variance over all genes. This approach is similar to the use of fold changes instead of *t*-values (see [37,38], but see [39] for a critique). To test the robustness of our method we also used moderated *t*-statistics [40]. Since the outcomes of both types of analysis were qualitatively and quantitatively similar (data not shown), we only report the former analysis.

Of the 7546 genes analyzed, $7546 \times 0.05 \approx 377$ genes are expected to be significantly different by chance alone, about half of them up and half of them down (assuming a type I error rate or statistical significance level of $\alpha = 0.05$). To address this multiple testing problem, we used a false discovery rate (FDR) of 0.05 to detect genes that are likely to be differentially regulated between genotypes, influenced by LPS treatment, or both. Results based on genes lists assuming $\alpha = 0.01$ did not change the qualitative outcome of our analyses (data not shown).

To refine our single gene analysis in terms of biological function we performed gene ontology (GO) analysis, using the classification developed by the GO Consortium http://www.geneontology.org/. This classification consists of three structured controlled vocabularies (or ontologies) that describe gene products in terms of their associated "biological processes", "cellular components", or "molecular functions", in a species-independent manner. We restricted our analysis to the "biological process" annotation, as this category is easiest to interpret and since we observed substantial overlap between this group of genes and the "molecular function" category. For GO analysis within the "biological process" category, genes were ordered into GO classes. We separately contrasted each class with all other classes by performing *t*-tests (assuming normality of the data) and nonparametric Kolmogorov-Smirnov tests (relaxing the assumption of normality) on standardized coefficients. Since data were approximately normally distributed, results differed little between the tests; we therefore only report *t*-tests.

Analysis of putative regulatory elements

To relate the gene expression effects of genotype and LPS treatment to putative regulatory regions, we used bioinformatic analysis to identify putative *cis*-regulatory elements in the 5'UR, i.e. TFBSs in the promoter region, and 3'UTR regulatory elements (including miRNA targets). We aimed to use the same alignment methods and similar methods for defining these different *cis*-regulatory regions.

Sequence information was obtained from the University of California Santa Cruz (UCSC) Genome Bioinformatics site http://genome.ucsc.edu; in particular, the mouse mm7-assembly and the human hg18-assembly data were used. For each gene, we extracted the region up to 8 kb upstream from the transcription start point (5'UR) and the 3'UTR, using the UCSC KnownGenes database, which has a higher coverage than the RefSeq database. For the few protein coding genes with multiple entries, we randomly kept a single entry. If an adjacent gene was closer than 8 kb in the UR, the recorded upstream sequence was truncated before the start of the transcribed region of the adjacent gene. We also performed our analyses with shorter regions and obtained similar results (data not shown).

Homologous human and mouse genes were acquired using the HomoloGene database http://www.ncbi.nlm.nih.gov/homologene (48.1) at the National Center for Biotechnology Information (NCBI). Only genes with reciprocal best BLAST hit e-values were selected. For each gene, we aligned the 5'UR and the 3'UTR separately using LAGAN [41]. To obtain a reasonable alignment of the 5'UR, we truncated the longer sequence so that it had the same distance from the presumed transcription start site as that of the shorter sequence, despite the fact that in one or the other species the 5'UR might be shortened by another gene. This truncation thus only affected the most upstream part of the region. For orthologous 3'UTRs, we allowed for different lengths because of the generally higher similarity and thus easier alignment. We validated this method extensively (results not shown, see B. Fuhrmann, unpublished diploma thesis) [42]. In particular, we found that pairwise alignments with LAGAN performed better than available multi-species alignments, because fewer regions were excluded and because placement of insertions and deletions in multi-species alignments is problematic.

To identify putative regulatory elements (TFBSs and 3'UTR regulatory sequences) we used a comparative genomics approach. Xie et al. [14] inferred conserved motifs in the 5'UR (putative TFBSs) and 3'UTR (containing many miRNA cognate sequences) using a comparison among mammalian genomes. While we are aware that regulatory sequences may lie outside of these regions (e.g., in the 5' untranslated region (5'UTR) or in introns, especially the first), we refrained from including such regions, for two reasons. First, as a transcribed region, the 5'UTR imposes more (and different) constraints on regulatory sequences than the untranscribed 5'UR that are difficult to account for. Second, the search space would have increased vastly with the inclusion of introns, which also would have caused alignment problems. We note that the list of Xie et al. [14] contains several unknown putative TFBSs, whereas some known TFBSs are not included. Furthermore, Xie et al. [14] do not distinguish between TFBSs of some related transcription factors (e.g., ISGF3 and most other IRF TFBSs). We used these putative regulatory sequences as raw data for our analysis. We

parsed each gene of the aligned human and mouse sequences for conserved motifs in the lists given in Xie et al. [14]: for putative TFBSs see Supplementary Material Table S3, for putative 3'UTR regulatory sequences Supplementary Material Table S4, and for conserved octamer motifs in the 3'UTR Supplementary Material Table S5, respectively. To exclude false positives, a motif was defined as conserved if it occurred in the homologous mouse and human sequence at the same position of the alignment in any of its forms (including the reverse complement for the 5'UR). To validate the ability of this comparative genomics approach to identify 5'UR TFBSs we used publicly available microarray data http://symatlas.gnf.org/SymAtlas/ on samples from diverse mouse tissues (see Additional File 9). We noticed some discrepancies between the annotation of Xie et al. [14] and the list of miRNAs in miRBase http://www.mirbase.org/[15-17]. Conserved octameric sequences in [14] were therefore re-annotated using the list in miRBase release 10.1. An association was called if the distal octamer of the mature miRNA matched perfectly to the octameric region.

To analyze TFBSs and 3'UTR regulatory elements, we contrasted the normalized effect coefficients of genes that had at least one conserved regulatory element with those of the rest of the genes. As for the GO analysis, we performed both *t*-tests Kolmogorov-Smirnov tests. Since the outcome of these tests was again qualitatively similar, we only report *t*-tests.

For our TFBS analysis (see above) we used a rather stringent definition [14], filtered out all non-conserved and non-alignable motifs, and excluded all weakly expressed genes, such that our analysis was based on only 40 genes containing an IRF/ISRE TFBS, with many known IFN-inducible genes missing. To extend this analysis and to explore different inclusion criteria for 5'UR TFBSs, we produced a list of 187 known IFN-responsive genes from the literature (without differentiating between type I IFN and IFNγ-responsive genes) and asked how many of these genes show expression changes in our experiment. As above, we analyzed the standardized effects of genotype, treatment, and genotype × treatment on gene expression (Additional File 3, Table 1). Furthermore, we compared IFN-responsive genes to all other genes with respect to IRF/ISRE or GAS TFBSs in their 5'UR. We used different criteria for including or excluding TFBSs, i.e. we report enrichments for conserved and nonconserved TFBSs that differ in the length of the upstream search region (Additional File 3, Table 2). We also lowered the specificity of our screening for TFBSs of known IFN-induced transcription factors by allowing for mismatches (data not shown).

Additional material

Additional file 1 RT-qPCR validation of microarray data. This file contains independent validation of the microarray expression data with RT-qPCR, with a particular emphasis on IFN-responsive genes and genes involved in lipid metabolism.

Additional file 2 Analysis of genes with putative TFBS. This file contains a list of gene classes grouped according to their putative 5'UR TFBS and a t-test analysis of their expression patterns.

Additional file 3 Analysis of IFN-inducible genes. This file contains a list of IFN-inducible genes and a t-test analysis of their expression patterns.

Additional file 4 Analysis of 3'UTR regulatory elements. This file contains a list of gene classes grouped according to putative 3'UTR regulatory sequence and a t-test analysis of their expression patterns.

Additional file 5 Expression of genes with the m2/ARE 3'UTR regulatory sequence. This file contains a plot of the effects on expression levels of genes containing the m2/ARE 3'UTR regulatory sequence.

Additional file 6 Analysis of 3'UTR miRNA cognate motifs. This file contains a list of genes with 3'UTR miRNA cognate sequences and a t-test analysis of their expression patterns.

Additional file 7 Comparison to microarray data of Thomas et al. [20]. This file contains a graph depicting the relationship between the effects on expression of genes examined by Thomas et al. [20] and our study.

Additional file 8 Comparison to microarray data of Fleetwood et al. [21]. This file contains a graph depicting the relationship between the effects on expression of genes examined by Fleetwood et al. [21] and our study.

Additional file 9 Validation of comparative approach to identify TFBS. This file contains a bioinformatic validation of the comparative approach to identify 5'UR TFBSs using publicly available microarray gene expression data.

Abbreviations

FDR: false discovery rate; IFN: interferon; IL: interleukin; IRF/ISRE: IFN-responsive factor/IFN-stimulated response element; ISGF3: interferon stimulated gene factor 3; JAK/STAT: Janus kinase/signal transducer and activator of transcription; LPS: lipopolysaccharide; miRNA: microRNA; TFBS: transcription factor binding-site; RT-qPCR: reverse transcription-quantitative polymerase chain reaction; 5'UR: 5' untranslated region upstream of transcription start site of protein coding genes; 3' UTR: 3' untranslated region of protein coding genes; Wt: wildtype.

Authors' contributions

MM, BS, and PK designed and supervised experiments; EH, BW, and RS performed experiments; CV supervised and carried out statistical and bioinformatic analyses, BF conducted bioinformatic analyses; MM, BS, BW, EH, PK, and CV wrote the initial version of the manuscript; TF, CV, BS, and MM rewrote and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank the members of the Müller group and the other F28 labs (groups Beug, Decker, Eferl, Kovarik, Mikulits, Moriggl, Müllner, and Sexl) for discussion, Andreas Futschik for statistical advice, and three anonymous reviewers for very helpful comments on a previous version of the manuscript. Our work was supported by a FWF (Austrian Science Foundation) Special Research Program grant (F28, JakStat Signalling: From Basics to Disease), a grant from the Austrian Federal Ministry of Science and Research (BM.W_F[a] GZ200.112/1-VI/1/2004), and a FWF Standalone Grant (P21948-B11).

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Received: 12 August 2009 Accepted: 25 March 2010
Published: 25 March 2010

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doi: 10.1186/1471-2164-11-199
Cite this article as: Vogl *et al.*, Transcriptome analysis reveals a major impact of JAK protein tyrosine kinase 2 (Tyk2) on the expression of interferon-responsive and metabolic genes *BMC Genomics* 2010, **11**:199

FINAL DISCUSSION

Tyk2 is a non-receptor tyrosine kinase that is involved in the Jak-Stat signalling pathway. The essential contribution of Tyk2 to various cytokine and growth factor signalling pathways has been reported [27] and, accordingly, Tyk2 has a broad spectrum of effects on host immunity, which range from protective to detrimental. During most microbial infections, Tyk2 acts beneficial for the host, mainly due to its contribution to IFNα/β and IL-12 signalling [32-34, 43-46, 101]. Conversely, deletion of Tyk2 confers resistance to LPS-induced endotoxin shock [25, 47]. Endotoxin shock is accompanied by the release of massive amounts of cytokines and proinflammatory mediators (e.g. TNFα, IL-6, IL-1β, IFNα/β, IFNγ and nitric oxide [NO]). Whereas the early systemic production of IL-6, IL-1β, TNFα and NO is unimpaired in Tyk2 knock-out mice, they fail to induce IFNγ after LPS challenge [25, 47]. Mice deficient for Tyk2 or IFNβ are similarly resistant to LPS-induced shock, demonstrating the crucial role of IFNα/β [25]. However, since Tyk2 is only partially required for IFNα/β signalling, additional Ifnar-independent pathways might contribute to the high resistance of Tyk2 knockout mice.

In order to examine the global influence of Tyk2 on transcription we performed expression profiling in response to LPS. Transcriptional analysis in thioglycollate-elicited peritoneal macrophages (PM) revealed that Tyk2 most prominently impacts on baseline expression of many IFN-stimulated genes (ISGs), whereas in response to LPS most of those genes were upregulated similar to wildtype cells. These data extend previous observations on the expression of specific ISGs [25] to a global scale and establish the importance of Tyk2 for constitutive, low-level IFNα/β signalling. Interestingly, we found that Tyk2 is critical for the downregulation of metabolic genes upon LPS treatment, in particular genes involved in lipid metabolism [102]. Consistently, Tyk2 modulates the expression of several metabolic proteins in bone marrow-derived macrophages ([103] and Grunert T., Strobl B. et al, manuscript in preparation). It is known for some time that immunity, inflammation, and lipid metabolism are interconnected [104]. Since the molecular mechanisms behind are not well understood, it will be interesting to further analyse the contribution of Tyk2 to these complex regulatory networks.

Transcription of many Tyk2-regulated genes has been observed to be even more compromised by the absence of Ifnar1 (unpublished data). Nevertheless, we can show that IL-17 and IL-17F are two genes, which are regulated by Tyk2 at the transcriptional level, largely independent of type I IFN signalling. During the last years IL-17 and IL-17F have gained prominence due to their involvement in several inflammatory and autoimmune diseases [85]. Besides Th17 cells, a growing number of cell types have been

reported to be critically involved in IL-17 secretion depending on the stimulus [83]. Although we can show that IL-17 is produced by peritoneal macrophage cultures following LPS treatment *in vitro*, we found no evidence for IL-17 producing macrophages in spleens after intraperitoneal LPS administration *in vivo*. We identified γδ T cells and pDCs as the cell types responsible for Tyk2-dependent IL-17 production in spleens. However, whether both cell types are similarly required and serve non-redundant functions has to be clarified. Furthermore, it is not yet clear if cell-intrinsic or -extrinsic signalling/responses require Tyk2. Lck- or CD11c-Cre recombinase-driven celltype-specific knockout of Tyk2 in T cells or DCs, respectively and adoptive transfer of Tyk2-deficient hematopoietic cells in wildtype mice or *vice versa* would be valuable tools to address these questions in future studies.

In contrast to its detrimental functions in autoimmunity, IL-17 has a vital role in protecting the host from infection. Important key functions of IL-17 are the induction of neutrophil recruitment and the increase of their microbicidal activity. Consistently, loss of IL-17R signalling during non-severe polymicrobial sepsis induced by cecal ligation and puncture (CLP) leads to increased mortality due to defective neutrophil recruitment and activity [105]. On the other hand, decreased neutrophil recruitment after blocking IL-17 or deleting the IL-17 receptor turned out to be beneficial for the host following lethal TNF challenge [100]. Blocking neutrophil recruitment to affected organs has mainly beneficial effects in TNF- and LPS-induced shock as it protects the host from excessive tissue damage [106-108]. We hypothesise therefore that Tyk2 contributes to LPS-induced shock by regulating IL-17 mediated neutrophil recruitment and tissue destruction. IL-17 also induces the expression of inducible NO synthase (iNOS, NOS2) and NO production in various cell types. NO has been shown to mediate the microbicidal activity of neutrophils, and organ-specific defects in iNOS induction in Tyk2-deficient mice following LPS challenge *in vivo* have been reported previously [109]. Similar to IL-17 (data not shown), iNOS expression in livers was induced by a Tyk2-dependent, but Ifnar1-independent mechanism. Nevertheless, whether IL-17 indeed induces iNOS expression in this context has to be clarified.

Using a proteomics approach, we identified several proteins that are differentially regulated in wildtype and Tyk2-deficient bone marrow-derived macrophages (BMDM) after LPS treatment. Further analyses revealed unperturbed mRNA expression in the absence of Tyk2 for some of these proteins, indicating the involvement of Tyk2 in posttranscriptional regulation. We show that Tyk2 specifically suppresses IL-1β expression in BMDM at the translational level, thereby unravelling a novel pathway of IL-1β regulation. Even though the precise mechanism remains unknown, we provide

evidence for the involvement of IFNα/β signalling. In line with our *in vitro* data we report increased levels of IL-1β in the peritoneal lavages of Tyk2-deficient mice following intraperitoneal LPS administration. However, serum IL-β levels were decreased and also intranasal application of LPS led to slightly but significantly reduced IL-1β levels in the lung of Tyk2-/- mice (data not shown). Taken together, our data clearly show that Tyk2 is intricately linked to the regulation of IL-1β. Since IL-1β is regulated at multiple levels, other pathways may counter-regulate Tyk2-mediated translational regulation. Whether IL-1β is augmented or suppressed by Tyk2 likely depends on several factors including the stimulus, the duration of treatment and the cell type(s) involved. Furthermore, local and systemic levels of IL-1β may be regulated differently. Delineating the possible protective functions of Tyk2 in IL-1β driven diseases (e.g. turpentine-induced local inflammation, contact hypersensitivity and collagen induced arthritis) will be an attractive target for future studies. Notably, Tyk2-deficient mice are more sensitive to contact hypersensitivity [110], and the underlying mechanisms are still unclear.

One recurrent issue that came up during our studies concerns the heterogeneity of different types of macrophages. It has been described that macrophages can differ in their cytokine profiles and transcription factor activities dependent on the isolation and/or differentiation procedure [111]. In our studies we show IL-17 expression in PM, while we could not detect IL-17 in BMDM. Conversely, Tyk2-mediated suppression of IL-1β protein could exclusively be monitored in BMDM but not PM. It is comprehensible that these two types of macrophages, which can be regarded as resident and inflammatory, respectively, respond differentially to a given stimulus. Nevertheless, it remains an important unresolved issue in macrophage biology to which degree those two types correlate to distinct macrophage populations *in vivo*.

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4. FEBS-Special Meeting 2010: Jak-Stat Signalling – from Basics to Disease, Vienna, Austria.
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Konferenzposter und publizierte Konferenz-Abstracts

1. R. Stiefvater, E. Hofmann, T. Kolbe, U. Reichart, C. Lassnig, C. Vogl, V. Poli, M. Müller, B. Strobl
   Tyk2 is required for IL-17 production by innate immune cells in response to LPS
   Cytokine, Volume 52, Issues 1-2, October-November 2010, Page 31
2. R. Stiefvater, B. Strobl, E. Hofmann, T. Kolbe, U. Reichart, C. Lassnig, C. Vogl, V. Poli, M. Müller
   Type I interferon-independent requirement of Tyk2 for LPS-mediated IL-17 expression in murine macrophages
   Wiener Klinische Wochenschrift, Volume 120, Supplement 1, August 2008, Page 88

Publikationen

1. Radwan M*, Stiefvater R*, Grunert T, Sharif O, Miller I, Marchetti-Deschmann M, Allmaier G, Gemeiner M, Knapp S, Kovarik P, Müller M, Strobl B.
   Tyrosine kinase 2 controls IL-1beta production at the translational level.
   J Immunol. 2010 Sep 15;185(6):3544-53. Epub 2010 Aug 16.
2. Vogl C, Flatt T, Fuhrmann B, Hofmann E, Wallner B, Stiefvater R, Kovarik P, Strobl B, Müller M.
   Transcriptome analysis reveals a major impact of JAK protein tyrosine kinase 2 (Tyk2) on the expression of interferon-responsive and metabolic genes.
   BMC Genomics. 2010 Mar 25;11:199.

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Meetings

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Published Abstracts

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2. R. Stiefvater, B. Strobl, E. Hofmann, T. Kolbe, U. Reichart, C. Lassnig, C. Vogl, V. Poli, M. Müller
   Type I interferon-independent requirement of Tyk2 for LPS-mediated IL-17 expression in murine macrophages
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Publications

1. Radwan M*, Stiefvater R*, Grunert T, Sharif O, Miller I, Marchetti-Deschmann M, Allmaier G, Gemeiner M, Knapp S, Kovarik P, Müller M, Strobl B.
   Tyrosine kinase 2 controls IL-1beta production at the translational level.
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2. Vogl C, Flatt T, Fuhrmann B, Hofmann E, Wallner B, Stiefvater R, Kovarik P, Strobl B, Müller M.
   Transcriptome analysis reveals a major impact of JAK protein tyrosine kinase 2 (Tyk2) on the expression of interferon-responsive and metabolic genes.
   BMC Genomics. 2010 Mar 25;11:199.

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ACKNOWLEDGEMENTS

First and foremost I want to thank Prof. Dr. Mathias Müller for giving me the opportunity to do my PhD at the Institute of Animal Breeding and Genetics at the Veterinary University Vienna. In particular I would like to thank him for his ongoing support and the fruitful discussions throughout my thesis as well as for correction of the manuscripts.

I am deeply grateful to my supervisor Dr. Birgit Strobl for her constant support and encouragement. I appreciate and admire her patience and understanding but at the same time her guidance whenever I needed to stay on course. No less I would like to thank her for the faith she had in me, even when things went not so well. – Many thanks for all!

Moreover I would like to thank all my former and current colleagues for their help and motivation. Thanks to all of you for making our institute such a nice place to work. Special thanks to Gitti and Inge for beeing such nice lab-mates over the last years. Many thanks go to Nicki and Caro for their advice and motivation, the many serious discussions, and for critically reading this thesis.

Last but not least, I would like to dedicate this work to you, Alfred. None of this would have been possible without your assistance and endless patience. – Thank you so much!

CONFERENCE POSTERS

1. ***Tyk2 is required for IL-17 production by innate immune cells in response to LPS***
   R. Stiefvater, E. Hofmann, T. Kolbe, U. Reichart, C. Lassnig, C. Vogl, V. Poli, M. Müller, B. Strobl
   Presented at the "Joint Annual Meeting of Immunology of the Austrian and German Societies (ÖGAI, DGfI)", Vienna Austria
   Abstract published in: Cytokine, Volume 52, Issues 1-2, October-November 2010, Page 31

2. ***Type I interferon-independent requirement of Tyk2 for LPS-mediated IL-17 expression in murine macrophages***
   R. Stiefvater, B. Strobl, E. Hofmann, T. Kolbe, U. Reichart, C. Lassnig, C. Vogl, V. Poli, M. Müller
   Presented at the "Tri-Society Annual Conference 2009 of the Society for Leukocyte Biology, the International Cytokine Society and the International Society for Interferon and Cytokine Research: Cellular and cytokine interactions in health and disease", Lisbon, Portugal.
   Abstract published in: Wiener Klinische Wochenschrift, Volume 120, Supplement 1, August 2008, Page 88

Type I interferon-independent requirement of Tyk2 for LPS-mediated IL-17 expression in murine macrophages

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INTRODUCTION

IL-17, which is the signature cytokine of the T helper cell lineage Th17, is known to play an essential role in host defense and autoimmunity (1). Amongst the IL-17 family, IL-17F has similar biological functions to IL-17. Although several cell types have been reported to produce IL-17, underlying mechanisms are still poorly characterized. Tyrosine kinase 2 (Tyk2), a member of the Janus kinase family, is involved in signal transduction of a number of different cytokines, most prominently type I interferons (IFNα/β) (2, 3). We have reported previously that Tyk2-deficient macrophages have selective defects in their response to lipopolysaccharide (LPS) and virus infection (2, 4). We report here Tyk2-specific defects in the IL-17 production in thioglycollate elicited peritoneal macrophages in response to LPS.

IL-17 and IL-17F mRNA expression is induced by LPS in a Tyk2-dependent and IFNα/β-receptor (IFNAR1)-independent manner

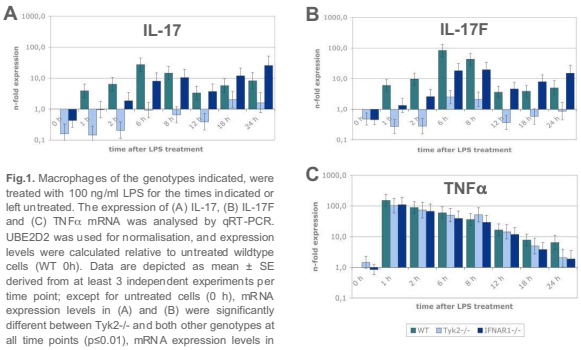


Fig.1. Macrophages of the genotypes indicated, were treated with 100 ng/ml LPS for the times indicated or left untreated. The expression of (A) IL-17, (B) IL-17F and (C) TNFα mRNA was analysed by qRT-PCR. UBE2D2 was used for normalisation, and expression levels were calculated relative to untreated wildtype cells (WT 0h). Data are depicted as mean ± SE derived from at least 3 independent experiments per time point; except for untreated cells (0 h), mRNA expression levels in (A) and (B) were significantly different between Tyk2-/- and both other genotypes at all time points (p≤0.01), mRNA expression levels in IFNAR1-/- differ from those in WT cells from 0 h up to 6 h post treatment (p≤0.05) and no significant difference is obtained at later time points. (C) mRNA expression was not significantly different between the genotypes, except at 24 h post treatment between WT and both other genotypes (p≤0.05).

Tyk2 is required for IL-17 protein production in response to LPS

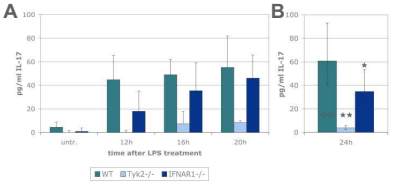


Fig. 2. Macrophages of the genotypes indicated, were treated with 100 ng/ml LPS for the times indicated or left untreated (untr.). IL-17 accumulation was measured in cell culture supernatants by ELISA. Data are depicted as mean ± SE (A) from 2 independent experiments and (B) from 10 independent experiments (* p=0.04, ** p<0.001).

LPS-mediated STAT3 activation is impaired in the absence of Tyk2

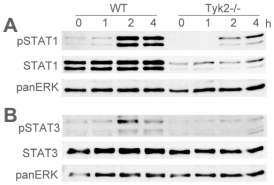
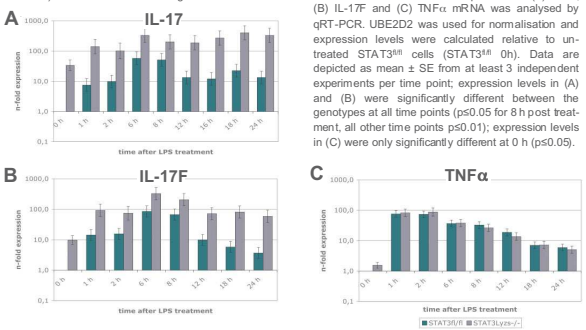


Fig. 3. Macrophages of the genotypes indicated, were treated with 100 ng/ml LPS or left untreated. At the times indicated, total cell lysates were prepared and subjected to Western blot analysis. Membranes were probed with Abs specific for (A) phosphorylated STAT1 (pSTAT1) or STAT1 protein (STAT1) and (B) phosphorylated STAT3 (pSTAT3) or STAT3 protein (STAT3). Protein loading was controlled by reprobing with anti-pan-ERK Ab (panERK).

Myeloid STAT3 plays an inhibitory rather than a stimulatory role for the induction of IL-17 and IL-17F mRNA expression by LPS

Fig. 4. Macrophages derived from Stat3$^{fl/fl}$ or STAT3$^{Lyzs-/-}$ mice (i.e. mice harbouring a myeloid specific STAT3 deletion) were treated with 100 ng/ml LPS for the times indicated or left untreated. The expression of (A) IL-17, (B) IL-17F and (C) TNFα mRNA was analysed by qRT-PCR. UBE2D2 was used for normalisation and expression levels were calculated relative to untreated STAT3$^{fl/fl}$ cells (STAT3$^{fl/fl}$ 0h). Data are depicted as mean ± SE from at least 3 independent experiments per time point; expression levels in (A) and (B) were significantly different between the genotypes at all time points (p≤0.05 for 8 h post treatment, all other time points p≤0.01); expression levels in (C) were only significantly different at 0 h (p≤0.05).



LPS-induced IL-17 protein production is increased in the absence of STAT3

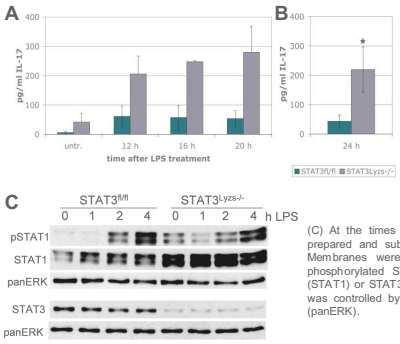


Fig. 5. Macrophages derived from STAT3$^{fl/fl}$ or STAT3$^{Lyzs-/-}$ mice (i.e. mice harbouring a myeloid specific STAT3 deletion) were treated with LPS (100 ng/ml) for the times indicated or left untreated (untr.). IL-17 accumulation was measured in cell culture supernatants by ELISA. Data are depicted as mean ± SE (A) from 2 independent experiments and (B) from 3 independent experiments (comprising cells from 4 to 6 individual mice; * p=0.012).

(C) At the times indicated, total cell lysates were prepared and subjected to Western blot analysis. Membranes were probed with Abs specific for phosphorylated STAT1 (pSTAT1), STAT1 protein (STAT1) or STAT3 protein (STAT3). Protein loading was controlled by reprobing with anti-pan-ERK Ab (panERK).

CD11b$^+$ cells produce IL-17 in response to LPS in peritoneal macrophage cultures

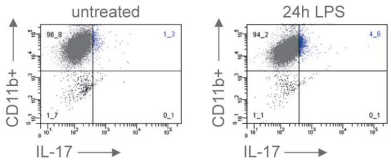


Fig. 6. Wildtype macrophages were treated for 24 h with 100 ng/ml LPS or left untreated. Brefeldin A was added to the cultures for the last 12 h. Cells were harvested and stained for CD11b and intracellular IL-17. Cells were analysed by flow cytometry. Numbers in the upper right quadrants indicate the percentage of CD11b$^+$IL-17$^+$ cells in peritoneal macrophage cultures.

SUMMARY

Here we show, that LPS induces IL-17 expression in peritoneal macrophages in a Tyk2-dependent manner. IL-17 and IL-17F mRNA and protein expression are upregulated upon LPS treatment and are strongly reduced in the absence of Tyk2. Induction is only slightly reduced in IFNAR1-deficient macrophages, demonstrating that Tyk2 does not exert the positive regulatory function through its involvement in IFNα/β signalling. We furthermore show, that, in contrast to the development/maintenance of Th17 cells, STAT3 is not required but rather inhibitory for LPS induced IL-17 expression in peritoneal macrophages.

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ACKNOWLEDGEMENT

MM is funded by the Austrian Science Fund (FWF) SFB F28 Jak-Stat Signalling. Biomodels Austria is supported by the Federal Austrian Ministry of Science and Research (BM.W_F^a GZ200.11/1-VI/1/2004)

Tyk2 is required for IL-17 production by innate immune cells in response to LPS

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INTRODUCTION

During the last years it became evident that in addition to Th17 cells interleukin 17 (IL-17) is also produced by innate immune cells (1). However, mechanisms of IL-17 induction in non-Th17 cells and the contribution of different innate immune cell types to IL-17 production *in vivo* are still largely elusive. The Janus kinase family member tyrosine kinase 2 (Tyk2) is mainly involved in IFNα/β and IL-12 signaling (2, 3). We have reported previously that macrophages from Tyk2-deficient mice exhibit selective defects in response to lipopolysaccharide (LPS) and virus infection (2, 4). We show here, that Tyk2 is required for LPS induced IL-17 production both in primary macrophages *in vitro* and in spleens *in vivo*.

IL-17 production in macrophages is induced by LPS in a Tyk2-dependent and largely IFNα/β-receptor (Ifnar1)-independent manner

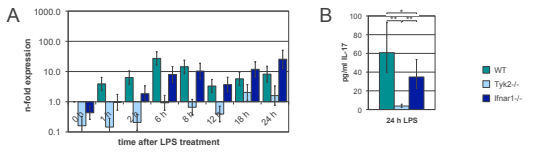

Fig. 1. WT, Tyk2-/- and Ifnar1-/- macrophages were treated with 100 ng/ml LPS for the times indicated or left untreated. The expression of (A) IL-17 mRNA was analyzed by qRT-PCR. Ube2d2 was used for normalization, and expression levels were calculated relative to untreated wildtype cells (WT 0 h). Data are depicted as mean ± SE derived from at least 3 independent experiments per time point; except for untreated cells (0 h, mRNA expression levels were significantly different between Tyk2-/- and both other genotypes at all time points (p≤0.01), mRNA expression levels in Ifnar1-/- differed from those in WT cells from 0 h up to 6 h post treatment (p≤0.05). (B) IL-17 accumulation was measured in cell culture supernatants with ELISA. Data are depicted as mean ± SE from 4 independent experiments (comprising cells from 10 individual mice; * p<0.05, ** p<0.001).

Stat3 has an inhibitory function in the induction of IL-17 by LPS

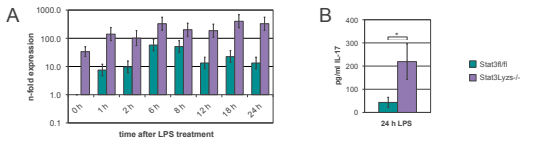

Fig. 2. WT (Stat3$^{fl/fl}$) and Stat3-deficient (Stat3$^{Lyzs-/-}$) macrophages were treated with 100 ng/ml LPS for the times indicated or left untreated. Expression of (A) IL-17 mRNA was analyzed by qRT-PCR. Ube2d2 was used for normalization and expression levels were calculated relative to untreated Stat3fl/fl cells (Stat3$^{fl/fl}$ 0 h). Data are depicted as mean ± SE from at least 3 independent experiments per time point; expression levels were significantly different between the genotypes at all time points (p≤0.05). (B) IL-17 accumulation was measured in cell culture supernatants by ELISA. Data are depicted as mean ± SE from 3 independent experiments (comprising cells from 6 individual mice; * p=0.012).

SUMMARY

Here we show that IL-17 induction is considerably reduced in the absence of Tyk2 but largely independent of functional IFNα/β signaling upon LPS treatment in thioglycolate-elicited peritoneal macrophages. Interestingly, signal transducer and activator of transcription 3 (Stat3) is not required for LPS-induced IL-17 production. In contrast to its essential role in the differentiation and maintenance of Th17 cells, Stat3 exerts inhibitory rather than stimulatory effects on LPS-induced IL-17 production in macrophages. Similarly, Tyk2 is indispensable for IL-17 production in spleens following LPS challenge *in vivo*, whereas absence of the interferon α/β receptor 1 (Ifnar1) did not affect splenic IL-17 levels. We furthermore show that γδ T cells, and to a lesser extent, pDCs are the main sources of IL-17 in spleens following intraperitoneal administration of LPS.

Splenic IL-17 mRNA expression is dependent on Tyk2 but not on the presence of mature T- and B-cells following LPS challenge *in vivo*

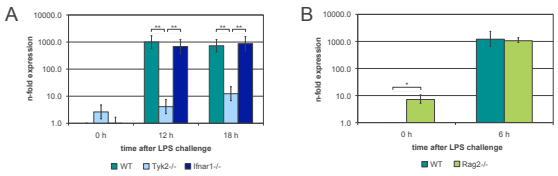

Fig. 3. (A) WT, Tyk2$^{-/-}$ and Ifnar1$^{-/-}$ or (B) WT and Rag2$^{-/-}$ mice were injected i.p. with LPS (1mg per 20g body weight) or left untreated. At the times indicated IL-17 mRNA expression in spleens was analyzed by qRT-PCR. Ube2d2 was used for normalization and expression levels were calculated relative to the untreated WT (WT 0 h). (A) Data are depicted as mean ± SD from at least 4 mice in 3 independent experiments per time point; no significant difference was observed between mRNA expression levels in Ifnar1$^{-/-}$ and those in WT spleens at any time point tested. (B) Data are depicted as mean ± SD from at least 5 mice in 3 independent experiments per time point; expression levels were only significantly different between untreated (0 h) Rag2$^{-/-}$ and WT mice; * p≤0.01, ** p≤0.001.

CD11b$^+$ cells do not substantially contribute to IL-17 mRNA expression in spleens after LPS challenge *in vivo*

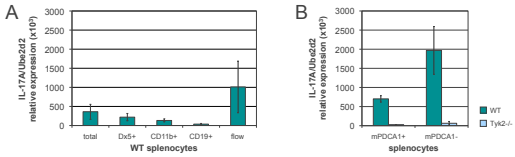

Fig. 4. (A) WT or (B) WT and Tyk2$^{-/-}$ mice were injected i.p. with LPS (1mg per 20g body weight) or left untreated. Following 6 h LPS challenge spleen cells were separated by successive magnetic cell sorting. (A, B) IL-17 mRNA expression in different spleen cell populations was analyzed by qRT-PCR. Expression levels were calculated relative to the endogenous control (Ube2d2). Data are depicted as mean ± SE from 2 independent experiments.

γδ T cells and pDCs are the major sources of IL-17 in spleens

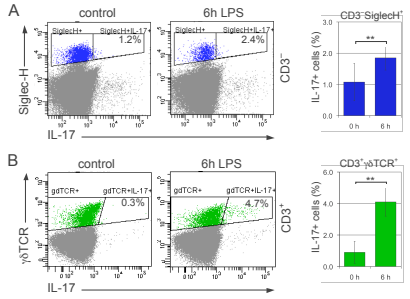

Fig. 5. WT mice were injected i.p. with LPS (1mg per 20g body weight) or left untreated. Subsequently, mice were injected i.v. with 0.25 mg Brefeldin A and after 6 h challenge CD19-depleted spleen cells were analyzed by FACS. Dot plots show intracellular staining for IL-17 in (A) pDCs and (B) γδ T cells. Aggregates and dead cells were excluded based on FSC-H versus FSC-A plots. Among the resulting live cell singlets, pDCs were gated for CD3$^-$Siglec-H$^+$ and γδ T cells for CD3$^+$γδTCR$^+$. Numbers in the gates indicate the percentage of IL-17$^+$ cells in total (A) CD3$^-$Siglec-H$^+$ cells or (B) CD3$^+$γδTCR$^+$ cells. Right panels show IL-17$^+$ cells as mean ± SD from 3 independent experiments; (** p≤0.001).

ACKNOWLEDGEMENT

This work is funded by the Austrian Science Fund (FWF) SFB F28 Jak-Stat Signalling to M.M. and B.S. Biomodels Austria is supported by the Austrian Federal Ministry for Science and Research (GZ 200.191/1-II/1a/2008).

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