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DISSERTATION

Titel der Dissertation

Proteomics in immunity: Regulatory functions of tyrosine kinase 2 on protein expression in macrophages

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ABSTRACT

Tyrosine kinase 2 (Tyk2) is a member of the Janus kinase (Jak) family and was originally identified by its essential role in type I interferon (IFN) signalling. Tyk2 is involved in signal transduction *via* various cytokines and some growth factors. Tyk2-deficient mice show increased sensitivity to infection with a number of different pathogens (e.g. Murine Cytomegalovirus, *Listeria monocytogenes*). In contrast, they show high resistance to lipopolysaccharide- (LPS) and ischemia/reperfusion-induced shock. Macrophages play a crucial role in the recognition of and response to LPS, and consequently, in the pathogenesis of endotoxin shock. In the absence of Tyk2, macrophages show a decreased activation of the IFN signalling cascade upon LPS treatment and, accordingly, reduced induction of at least some IFN target genes. Aim of this study was to investigate the molecular role of Tyk2 in macrophages in more detail with the main focus on the identification of novel Tyk2-dependent host cell responses.

A proteomic approach was chosen for the comparative analysis of protein patterns in lysates from wild-type (WT) *versus* Tyk2-deficient primary macrophages. The key method was two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) using minimal labelling with cyanine dyes. Experimental conditions for the specific sample type were optimised and reproducibility and the minimal detectable differences (effect size) were determined by statistical analyses. The method was applied to select candidates, which were then subjected to identification by mass spectrometry.

We could show that protein patterns in whole cell lysates and nuclear extracts are significantly different between WT and Tyk2-deficient bone marrow-derived macrophages (BMM) both in the LPS-treated and the untreated state. Twenty-three different proteins derived from 27 differentially expressed spots were identified by mass spectrometry. The identified proteins belong to distinct functional categories (e.g. immune response, oxidative stress response, apoptosis, metabolism, and transcription/translation) and their expression is either positively or negatively regulated by Tyk2. More detailed analysis of selected proteins revealed that Tyk2 influences protein expression at the mRNA and/or at the protein level. We show that Tyk2 influences LPS-dependent changes in the peroxiredoxin 1 (PRDX1) spot pattern but not the total PRDX1 expression level, as displayed by 1D and 2D western blot analysis. Subcellular distribution of elongation factor 2 (EF2) appeared dependent on Tyk2, since genotype specific differences were only detected in nuclear but not in whole cell extracts. We could furthermore show that Tyk2 negatively regulates plasminogen activator inhibitor 2 (PAI2) and pro-interleukin-1β (pro-IL-1β) protein but not mRNA expression. In

contrast, N-myc interactor (NMI), a protein known to be transcriptionally regulated by IFN, was dependent on the presence of Tyk2 for efficient mRNA and protein expression.

Since IL-1β plays an important role in immunity and Tyk2 has not yet been linked to IL-1β expression, we further analysed the mechanistic basis of enhanced pro-ILβ protein expression in Tyk2-deficient macrophages. We could show that enhanced levels of intracellular pro-IL-1β are not due to defective processing and/or secretion and that also extracellular IL-1β is enhanced in the absence of Tyk2. Similar protein stability in both genotypes could be demonstrated by using the translational inhibitor cycloheximide and monitoring pro-IL-1β degradation and pulse/chase labelling experiments. Interestingly, we found increased association of IL-1β mRNA with polysomes in the absence of Tyk2, arguing for differences in translational efficiency and suggesting a novel role of Tyk2 in translational control.

In summary, we show that high reproducibility can be achieved with 2D-DIGE technology with the developed experimental set-up. Detection of differences in protein expression in cell lysates derived from primary macrophage cultures of as low as 30% (on average) could be demonstrated. With respect to Tyk2, our results imply regulatory roles of Tyk2 at multiple levels of protein expression and establish novel connections between Tyk2 and several cellular proteins.

ZUSAMMENFASSUNG

Tyrosinkinase 2 (Tyk2) gehört zur Familie der Janus Kinasen (Jak). Tyk2 wurde ursprünglich als essentielle Komponente in der Typ-I Interferon (IFN) Signaltransduktions-Kaskade identifiziert und ist in die Signaltransduktion vieler weiterer Zytokine und einiger Wachstumsfaktoren involviert. Tyk2-defiziente Mäuse zeigen erhöhte Sensitivität bei Infektion mit vielen unterschiedlichen Pathogenen (z.B. Maus Cytomegalovirus, *Listeria monocytogenes*). Im Gegensatz dazu sind sie resistent gegen Lipopolysaccharid (LPS)- und Ischämie/Reperfusion-induzierten septischen Schock. Makrophagen spielen eine zentrale Rolle bei der Erkennung von und der Antwort auf LPS und somit in der Pathogenese des septischen Schocks. Makrophagen zeigen in Abwesenheit von Tyk2 eine reduzierte Aktivierung der IFN-Signaltransduktion nach Behandlung mit LPS und folglich eine erniedrigte Expression zumindest mancher IFN-induzierbarer Gene.

Ziel dieser Arbeit war es, die molekulare Rolle von Tyk2 in Makrophagen detaillierter zu untersuchen, wobei der Fokus auf die Ermittlung von neuen, Tyk2-abhängigen Antworten der Wirtszellen gelegt wurde. Ein Proteomics-Ansatz zur vergleichenden Analyse von Proteinmustern in Zelllysaten von Wildtyp- *versus* Tyk2-defizienter primärer Makrophagen wurde ausgewählt. Als zentrale Technik hierfür wurde zweidimensionale „Fluorescence Difference Gel Electrophoresis" (2D-DIGE) verwendet. Experimentelle Bedingungen für die spezifischen Probentypen wurden optimiert und die Reproduzierbarkeit sowie die minimal detektierbaren Unterschiede statistisch ermittelt. Diese optimierte Methode wurde dann eingesetzt, um Kandidaten für die massenspektrometrische Identifizierung zu ermitteln.

Wir konnten zeigen, dass die Proteinmuster in Gesamtzell- und Kern-Lysaten zwischen Wildtyp- und Tyk2-defizienten Knochenmark-Makrophagen, sowohl vor als auch nach der Behandlung mit LPS, signifikant unterschiedlich sind. In 27 differentiell exprimierten Spots wurden mittels Massenspektrometrie 23 verschiedene Proteine identifiziert. Diese Proteine gehören zu diversen funktionellen Klassen (z.B. Immunantwort, oxidative Stressantwort, Apoptose, Metabolismus und Transkription/Translation) und ihre Expression ist durch Tyk2 entweder positiv oder negativ reguliert. Weitere detaillierte Untersuchungen an ausgewählten Proteinen ergaben, dass Tyk2 die Proteinexpression auf mRNA- und/oder Proteinebene beeinflusst. Mittels 1D und 2D Westernblot Analysen fanden wir, dass Tyk2 die LPS-abhängigen Veränderungen im Proteinmuster von Peroxiredoxin 1 (PRDX1), nicht aber dessen Gesamtproteinmenge, beeinflusst. Die subzelluläre Verteilung von Elongationsfaktor 2 (EF2) scheint abhängig von Tyk2 zu sein, da Genotyp-spezifische Unterschiede nur in Kern- und nicht in Gesamtlysaten detektierbar sind. Wir konnten weiters zeigen, dass Tyk2 die Expression von Plasminogen Activator Inhibitor 2 (PAI2) und pro-

Interleukin-1β (pro-IL-1β) Protein, nicht aber deren mRNAs, negativ reguliert. Im Gegensatz dazu ist die Anwesenheit von Tyk2 für eine effiziente mRNA Expression von N-myc Interactor (NMI) erforderlich, dessen transkriptionelle Regulation durch IFNs bekannt ist.

Die Tatsache dass IL-1β eine wichtige Rolle in der Immunität spielt und seine Expression bisher nicht in direkten Zusammenhang mit Tyk2 gebracht wurde, hat uns veranlasst den molekularen Mechanismus der erhöhten pro-IL-1β Proteinexpression näher zu analysieren. Wir konnten zeigen, dass die erhöhte intrazelluläre Menge von pro-IL-1β nicht durch defekte Prozessierung und/oder Sekretion verursacht wird und dass in Abwesenheit von Tyk2 auch die extrazelluläre Menge von IL-1β erhöht ist. Die Analyse des Proteinabbaus nach der Applikation des translationellen Inhibitors Cycloheximid oder einer radioaktiven Protein-Markierung ergab, dass die Stabilität von pro-IL-1β in beiden Genotypen ähnlich ist. Interessanterweise beobachteten wir eine erhöhte Assoziation der IL-1β mRNA mit Polysomen in Abwesenheit von Tyk2. Dies lässt Differenzen in der translationellen Effizienz und eine neue Rolle von Tyk2 in der translationellen Kontrolle annehmen.

Zusammenfassend zeigen wir, dass mittels 2D-DIGE mit unserem experimentellen Aufbau eine hohe Reproduzierbarkeit erzielt wird. Durchschnittlich können signifikante Unterschiede in der Proteinexpression schon ab 30% Differenz in Zelllysaten primärer Makrophagen festgestellt werden. In Bezug auf Tyk2 weisen unsere Ergebnisse auf einen regulativen Einfluss auf mehrere Ebenen der Proteinexpression und auf neue Zusammenhänge zwischen Tyk2 und diversen zellulären Proteinen hin.

INTRODUCTION

I. Immunity

1. Immune system

Vertebrates have developed a potent defence mechanism, the immune system, against various infectious microorganisms. The immune system in jawed vertebrates consists basically of two components, the innate and the adaptive immunity. The main features of this system include recognition and elimination of dangerous agents, tolerance of self antigens and development of an immunological memory. Additionally, termination of responses and return to a basal state (homeostasis) is also an important function. The immune system comprises cellular and biochemical defence mechanisms distributed widely through the body. The classification into innate and adaptive immunity is mainly based on the receptor types which are used to recognise pathogens or components thereof. Innate immunity is the instantaneous, first line defence against infections and is evolutionary older. It initiates host defence responses and also stimulates and influences the character of adaptive immunity. Adaptive immunity is more specific and the development of its maximal efficiency takes several days or weeks. Both are linked and act in concert and coordinated in responses to immune stimuli. Important cells of the innate immunity are phagocytes such as macrophages, neutrophils, dendritic cells, and natural killer cells. The receptors of innate immunity are germline encoded and have broad specifities for conserved and invariant features of microorganisms. Adaptive immunity engages lymphocytes (T and B cells) and their secreted mediators (e.g. cytokines, antibodies). The receptors are generated by random somatic gene rearrangements that result in the generation of a broad diversity. These receptors are expressed clonally and recognise antigens, i.e. specific components of macromolecules. The recognition of antigens leads to proliferation and differentiation of the individual lymphocytes into effector and memory cells. The production of memory cells enables more effective responses to subsequent contact with the same antigens [1,2].

2. Macrophages

Cells of the mononuclear phagocyte system originate from pluripotent haematopoietic stem cells in bone marrow and develop into circulating monocytes. Monocytes migrate permanently and upon stimulus into tissues and maturate into macrophages. Macrophages are very dynamic and heterogeneous cells with the ability to adapt to the microenvironment [3,4]. The diverse phenotypes and specific functions of macrophages arise from their

anatomical tissue location and from their activation state, which is fully reversible. Activated macrophages are involved in the initiation, progress and the resolution of inflammation [3,4]. They play important regulatory and effector roles in innate and adaptive immunity. They secrete and respond to various cytokines and chemokines, and are able to direct cell-cell contact. These auto/paracrine interactions and cell contacts modulate responses and functions of other cells as well as of macrophages themselves [5]. Macrophages recognise, phagocytose and destroy pathogens, apoptotic cells and some tumor cells. As antigen presenting cells, macrophages process the phagocytosed particles for presentation to T cells on the major histocompatibility complex (MHC) molecules. Recognition and phagocytosis of microbial products activate macrophages to kill the pathogens by proteases, antimicrobial peptides, reactive oxygen species (ROS) and nitric oxide (NO). Their microbicidal activity is further enhanced after antigen presentation [2]. Tissue-resident macrophages play important roles in tissue homeostasis by removing senescent cells and remodelling and repair of tissues after injury or infection [4].

3. Pattern-recognition receptors in innate immunity (PRRs)

PRRs are receptors of innate immunity and recognise pathogen-associated molecular patterns (PAMPs) and molecules released from stressed or injured cells, called danger-associated molecular patterns (DAMPs) [6-9]. PPRs are present in three different compartments: body fluids, cell membranes and inside the cells. PRRs in body fluids are important for opsonisation of PAMPs, complement activation or for delivery of PAMPs to other PRRs (e.g. lipopolysaccharide (LPS)-binding protein (LBP)). PRRs on cell membranes initiate phagocytosis, present PAMPs to other PRRs or trigger signalling pathways. Intracellular PRRs recognise and initiate responses to intracellular pathogens and DAMPs [6-9]. PRRs and their ligands (or activators) are listed in Table 1. PRRs initiate inflammatory responses through activation of multiple signalling pathways and effector functions of the immune cells. The signalling pathways activate several transcription factors, which collaborate to induce expression of a large number of downstream genes important for host defence. These coordinate the local and systemic inflammation and also initiate adaptive immune responses. The genes include mainly proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, tumour necrosis factor α (TNFα), and type I interferons (IFNs). The induced response is important for an effective host defence; however, prolonged or hyperactivation may also be detrimental for host cells and tissues. Thus, expression of anti-inflammatory factors (e.g. IL-10, transforming growth factor-β (TGF-β), IL-1 receptor antagonist (IL-1ra) and glucocorticoids) and inhibitors of signalling cascades is of equal importance for the host defence. The balance between positive and negative regulation of

responses is crucial to successfully overcome pathogen invasion and to return to homeostasis [7-10].

3.1 Toll-like receptor (TLR) family

TLRs are widely expressed among immune and certain non-immune cells, respond to different PAMPs, and act as homo- or heterodimers and with other PRRs. Up until recently, 10 human and 13 murine TLRs have been identified (see Table 1). TLRs are evolutionary conserved type I integral membrane glycoproteins. The ligand sensing part of TLRs contains leucine-rich repeats (LRRs) and is localised extracellularly (TLRs 1, 2, 3, 4, 5, 6, 10 and 11) or inside endosomes (TLRs 3, 7, 8 and 9) [7-9]. The cytoplasmic part, toll-interleukin-1 receptor (TIR) domain, is involved in the initiation of signalling. TLRs trigger signalling pathways through different combinations of the five TIR-domain containing adaptor molecules: myeloid differentiation primary response gene 88 (MyD88), MyD88 adaptor-like (Mal, also known as TIRAP), TIR domain-containing adaptor protein inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM) and sterile α and armadillo-motif-containing protein (SARM), although SARM is a negative regulator. All TLRs (except TLR3) use the MyD88 adaptor, Mal is used by TLR2 and 4, TRIF by TLR3 and 4 and TRAM only by TLR4 [11]. All TLRs activate nuclear factor-κB (NFκB) and activating protein-1 (AP-1) transcription factors, which induce expression of mainly proinflammatory cytokines and chemokines. TLRs 3, 4, 7, 8 and 9 additionally activate IFN regulatory factor 3 (IRF3) and/or IRF7 transcription factors that induce the transcription of type I IFNs. Depending on the cell type other transcription factors may also be activated [7-9].

3.2 Nucleotide-binding domain, leucine-rich repeat containing family (NLR)

NLRs are intracellular sensors of pathogens and other stress signals. This family comprises about 20 known members, but the ligands and functions of many of these receptors are unknown at present [6,12].

3.2.1 Nucleotide-binding oligomerisation domain containing (NOD) proteins

NOD1 and NOD2 are cytoplasmic bacterial sensors (see Table 1), which have as effector domains the caspase-recruitment domains (CARDs). After ligand recognition NODs undergo oligomerisation, recruit and activate receptor-interacting protein 2 (RIP2) kinase. RIP2 is essential for activation of NFκB and mitogen-activated protein kinases (MAPKs). NOD signalling induces the expression of proinflammatory cytokines and chemokines [12,13].

3.2.2 NLRs and inflammasomes

Several NLR proteins, NLR family, pyrin domain containing 1-3 (NLRP1-3), NLR family, CARD domain containing 4 (NLRC4) and NLR family, apoptosis inhibitory protein 5 (NAIP5), are involved in inflammasome formation [6,14-16]. Inflammasomes are multiprotein complexes forming a molecular scaffold for caspase-1 (casp-1) activation, which is the central effector molecule of the inflammasome [14,16]. The activation of casp-1 is important for the processing of the precursors of the proinflammatory cytokines IL-1β and IL-18 into their biologically active (mature) forms. IL-33, which is involved in T helper cell type 2 (T$_H$2) responses [14] and Mal are also substrates for casp-1 [17]. Additionally, activation of inflammasomes may induce apoptosis of host cells [14].

3.3 RIG-like helicases (RLHs)

Retinoic-acid-inducible gene 1 protein (RIG-I, also known as Ddx58) and melanoma differentiation associated gene 5 (MDA5) are two cytoplasmic CARD helicases, and sensors of viral infections in most cell types. Both activate through interaction with IFN-β promoter stimulator-1 (IPS-1, also known as MAVS, VISA, and Cardif) the transcription factors IRF3/7 and NFκB. These induce the production of type I IFNs and proinflammatory cytokines, respectively [18,19].

3.4 Other PRRs

C-type lectin receptors (CLRs) are transmembrane receptors that recognise mainly carbohydrate structures. The best known CLR is dectin-1, which recognises beta-glucan from fungal cell walls [7,20,21]. Dectin-1 induces production of proinflammatory cytokines, phagocytosis and respiratory burst.

Recent studies have indicated a TLR-independent recognition system for DNA in the cytosol that results in the expression of type I IFNs and activation of other immune responses. Z-DNA binding protein 1 (ZBP1) was identified as a candidate and renamed as DNA-dependent activator of IRFs (DAI) [22]. However, studies with knockout mice have shown that production of type I IFNs after stimulation with DNA occurs also independently of DAI [9]. Very recently, HIN-200 protein family members (haematopoietic IFN-inducible nuclear proteins with characteristic 200 amino acid domains) were found to act as PRRs that mediate responses to cytoplasmic dsDNA [23].

Table 1. PRRs and their ligands/activators

Proteins	Location	Major ligands
Toll-like receptors (TLRs)		
TLR1	Cell surface	triacyl lipopeptides [7,24]
TLR2	Cell surface	peptidoglycan, lipoteichoic acid, lipoarabinomannan, lipopeptides (di- and tri-acyl lipopeptides, macrophage-activating lipopeptide 2), zymosan [7,24]
TLR3	Endosome	viral double-stranded RNA (dsRNA), polyinosine-polycytidylic acid (poly(I:C)) [7,24], siRNA, endogenous mRNA [24]
TLR4	Cell surface	LPS, viral envelope proteins from RSV and MMTV [7,24]; mannan, glycoinositolphospholipid [7]; heat-shock proteins 60 and 70, fusion protein of RSV, fibronectin, hyaluronan [24]
TLR5	Cell surface	bacterial flagellin [7,24]
TLR6	Cell surface	diacyl lipopeptides, lipoteichoic acid, zymosan [7,24]
TLR7	Endosome	imiquimod, resiquimod (R848), viral single-stranded RNA (ssRNA), certain siRNAs [7,24]; synthetic polyU RNA [7]
TLR8	Endosome	R848* and viral ssRNA [7,24]
TLR9	Endosome	unmethylated CpGs [7,24], sugar backbone of DNA [25]
TLR10**	Cell surface	unknown [7,24]
TLR11***	Cell surface	profilin, not yet identified ligand from uropathogenic bacteria [7,24]
TLR12*** TLR13***	Unknown	unknown [24]

MMTV - mouse mammary tumour virus, RSV - respiratory syncytial virus

* recognised by human TLR8, ** identified only in humans, *** identified only in mice

Table 1. PRRs and their ligands/activators (continued)

Proteins	Location	Major activators
Nucleotide-binding domain, leucine-rich repeat containing family (NLRs)		
NOD1	Cytoplasm	γ-D-glutamyl-*meso*-diaminopimelic acid [12]
NOD2	Cytoplasm	muramyl dipeptide (MDP) [12]
NLRP1 (Nalp1)	Cytoplasm / nucleus	MDP, anthrax lethal toxin [14]
NLRP3 (Nalp3)	Cytoplasm	PAMPs (e.g. MDP, bacterial RNA), microbial toxins (e.g. Nigericin), live bacteria (*Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*), viruses (Sendai and Influenza virus), DAMPs (e.g. adenosine triphosphate (ATP), monosodium urate crystals, ROS, UVB) [6]
NLRC4 (IPAF)	Cytoplasm	functional type III or IV secretion system from *Shigella, Salmonella, Legionella, Pseudomonas* species and flagellin from *Salmonella, Legionella, Pseudomonas* species [15]
NAIP5	Cytoplasm	*Legionella pneumophila* (by interaction with NLRC4) [14]
NLRX1	Mitochondria	unknown [12]
RIG-like helicases (RLHs)		
RIG-I	Cytoplasm	RNA viruses, ssRNA with 5'-triphosphate, short dsRNA, short poly(I:C) [19]
MDA5	Cytoplasm	picornaviruses, long dsRNA, long poly(I:C) [19]

4. Lipopolysaccharide (LPS)

LPS, also called endotoxin, is a major structural component of the outer membrane of gram-negative bacteria. It is a potent activator of immune responses. LPS consists of four major components: the O-specific chain, the outer core, the inner core and lipid A. Lipid A is the region, which is recognised by the innate immune system. The recognition of and response to LPS is the critical point in its toxicity and macrophages are key players in this process [26,27]. The important role of macrophages has been shown, for example, using an LPS non-responsive mouse strain (C3H/HeJ), which becomes sensitive to LPS after injection of macrophages from an LPS responsive mouse strain (C3H/HeN) [26].

LPS activates macrophages to produce a variety of cytokines, reactive oxygen and nitrogen species and other inflammatory mediators. These cytokines and mediators activate other immune and non-immune cells, and induce a local inflammatory response. High concentrations of LPS can lead to excessive and/or prolonged production of these cytokines. These combined with the actions of activated cells can culminate in a systemic disorder with septic shock, multiple organ dysfunction, and death [2,26]. Exogenous administration of LPS is one of the experimental models to study biological mechanisms and pathophysiology of sepsis [28].

5. LPS-induced TLR4 signalling pathways in macrophages

The TLR4 complex consists of cluster of differentiation 14 receptor (CD14) and myeloid differentiation factor-2 (MD-2), which are associated with TLR4. LBP binds to LPS and delivers it to the CD14 receptor, which transfers it to the TLR4/MD-2 receptor complex. Docking of LPS to this complex leads to homodimerisation and recruitment of adaptor proteins *via* interactions with TIR domains. This induces at least two distinct signalling pathways that modulate transcription, mRNA stability and/or translation of a number of proinflammatory cytokine genes such as IL-1β, IL-6, IL-12, TNFα, and type I IFNs [7,29]. A simplified overview of LPS-induced signalling pathways is illustrated in Fig. 1. LPS stimulation results in the recruitment of Mal and MyD88 to the TLR receptor complex. This complex recruits and activates the IL-1 receptor associated kinases (IRAKs) IRAK1, 2 and 4. IRAKs dissociate then from the complex, associate with and activate TNF-receptor associated factor 6 (TRAF6). Activated TRAF6 associates with a complex consisting of transforming growth factor-β-activated kinase 1 (TAK1) and TAK-binding proteins (TAB) TAB-1, 2 and 3. Activated TAK1 activates inhibitor of κB (IκB) kinases (IKKs), which phosphorylate IκBs. The phosphorylated IκBs are then ubiquitinated and degraded. This leads to release and translocation of NFκB to the nucleus. TAK1 activates also MAPKs such

as p38 and c-jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK). These kinases mediate the activation of AP-1. Another transcription factor, IRF5, associates with MyD88 and TRAF6, becomes activated and translocates into the nucleus. Activation of these transcription factors results mainly in the synthesis of proinflammatory cytokines [7,9,24].

TRIF interacts with TLR4 through TRAM. Association of TRAM and TRIF with TLR4 is followed by endocytosis of the TLR4 complex and the TRIF-dependent signalling pathway is successively induced from the endosomal compartment [30,31]. TRIF recruits TRAF3 and activates TRAF-family-member-associated NFκB activator (TANK) binding kinase (TBK1) and IKKí, which phosphorylate IRF3. Phosphorylated IRF3 form homo- and heterodimers with the p65 subunit of NFκB [32,33], translocate to the nucleus and induce the expression of responsive genes, most prominently IFN-β. TRIF interacts also directly with receptor-interacting protein 1 (RIP1) and this interaction is responsible for NFκB activation and expression of proinflammatory cytokines [7,9,24].
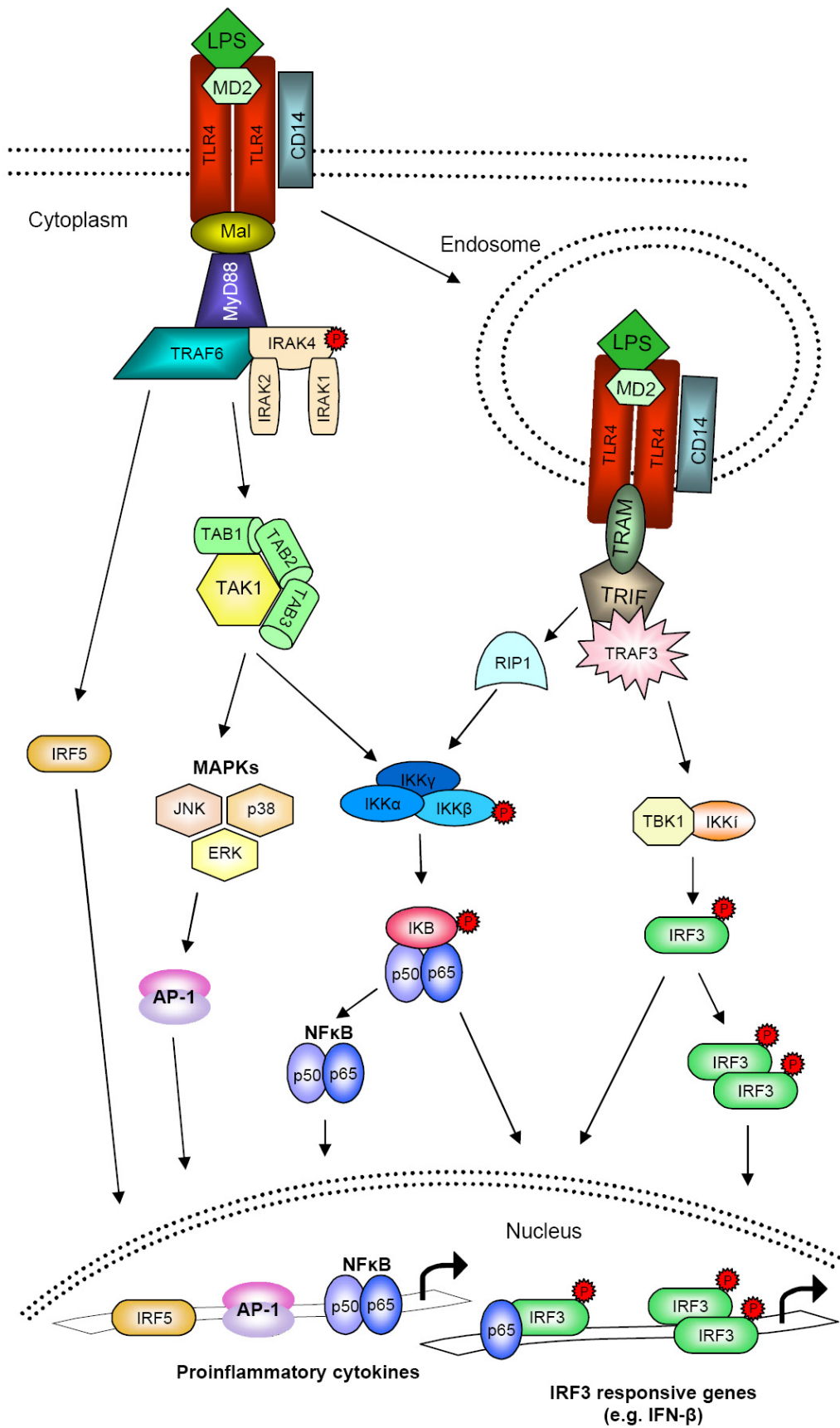
Figure 1. LPS-induced signalling pathways

6. Cross talk in PRR signalling

Different PRRs recognise pathogens simultaneously or sequentially and activate distinct and/or shared signalling pathways that determine the intensity and quality of responses. The interactions may be synergistic or antagonistic and result in high complexity of processes occurring after pathogen recognition. The collaboration of TLRs and NLRs inflammasomes to produce mature IL-1β is the best known crosstalk amongst the PRRs. TLRs induce expression of IL-1β precursor but its processing into the mature form depends on the activation of casp-1 by inflammasomes. PRRs form heterodimers with other PRRs to recognise distinct ligands and/or modulate the immune response. Moreover, stimulation of certain PRRs can influence expression levels of other PRRs and/or signalling molecules, and may enhance or attenuate responses. However, the interplay between signalling pathways and their integrated outcomes are still poorly defined [7,10,29,34].

II. Jak-Stat signalling pathway

1. Jak-Stat signalling pathway overview

The Janus kinase (Jak) and signal transducer and activator of transcription (Stat) signalling pathway is one of the best studied signalling pathways and transmits signals from the cell surface to the nucleus (see Fig. 2). A large number of cytokine and several non-cytokine receptors utilise this signalling pathway [35,36]. The Jak-Stat signalling pathway is activated by ligand binding to the corresponding receptors. This event induces oligomerisation of the receptor chains and their subsequent transition into an active conformation. The associated Jaks come to close proximity, auto- and/or cross-phosphorylate and phosphorylate the receptor. The phosphorylated tyrosine residues on the receptor chains allow recruitment of Stats which are then phosphorylated and activated. Activated homo- and/or heterodimerised Stats translocate to the nucleus where they modulate the transcription of several genes. Different receptors use different combinations of one or more Jaks and activate different Stat homo- and/or heterodimers. In addition to Jaks and Stats other signalling cascades can be activated. Activation of distinct Stats and other transcription factors as well as their synergistic/antagonistic actions mediate the differential and complex biological responses to a given cytokine or growth factor [36,37].
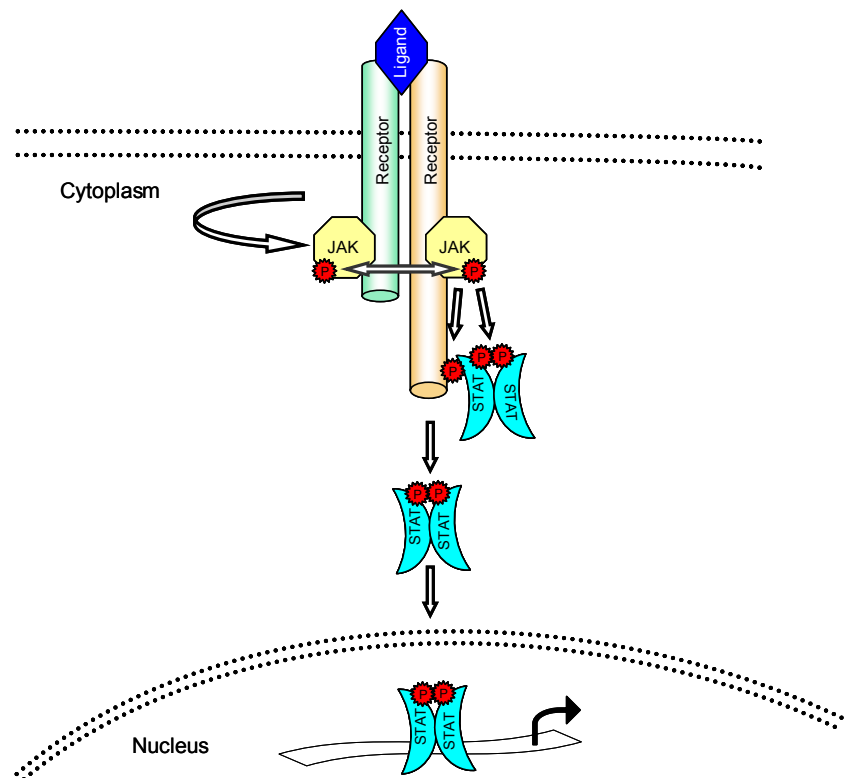

Figure 2. Jak-Stat signalling pathway

The cytokine superfamily of receptors (type I and II) comprises about 50 members including receptors for interleukins, IFNs and some hormones. These receptors are single membrane-spanning proteins that lack enzymatic activity in the cytosolic domains, and signal through associated kinases. The extracellular part is responsible for ligand binding [38,39].

In mammals, the Janus kinase family consists of the four known members Jak1-3 and tyrosine kinase 2 (Tyk2). Jaks are non-receptor tyrosine kinases which associate non-covalently with receptors. Jak1, Jak2 and Tyk2 are ubiquitously expressed, Jak3 predominantly in cells of haematopoietic origin, such as NK cells and lymphocytes. Jaks range in size from 120 to 140 kDa and feature seven Janus homology (JH) domains. JH1 is the catalytically active kinase domain containing tyrosine residues and a conserved aspartic acid residue involved in phosphotransfer reactions. JH2, the pseudokinase domain, is highly homologous to JH1 but is catalytically inactive and important for regulation of Jak activity. The presence of a pseudokinase domain in addition to the functional kinase domain was actually the basis to name this kinase family after the two-faced Roman god Janus. JH3 and half of JH4 form a Src-homology-2 (SH2) related domain of unknown function. Half of JH4 and JH5-JH7 domains contain a so-called Four-point-one, Ezrin, Radixin, Moesin (FERM) domain responsible for interactions of Jaks with receptors [40].

The Stat protein family comprises the seven members Stat1, 2, 3, 4, 5a, 5b and 6. These transcription factors consist of 7 conserved domains. The amino-terminal domain is responsible for protein-protein interaction and homodimerisation of unphosphorylated Stats. The coiled-coil domain mediates the interaction with other transcription factors and regulatory proteins. The DNA binding domain (DBD) allows binding to DNA, the linker domain is responsible for appropriate conformation between DBD and SH2. The SH2 domain enables binding of Stats to the specific phosphorylated receptor subunits and formation of active Stat dimers. The Stats are activated by phosphorylation on the tyrosine activation motif. The transcription activation domain (TAD) contributes to Stats specificity and is involved in interactions with transcriptional regulators. The transcriptional activity of Stats is further regulated by phosphorylation on the serine residue located within TAD in all Stats, except Stat2. Inactive Stats are predominantly localised in the cytoplasm and are translocated into the nucleus after activation [36,41,42].

2. Type I interferons

IFNs were the first cytokines discovered and studies of the mechanism by which IFNs induce the expression of various genes led to discovery of the Jak-Stat signalling pathway [43,44]. IFNs play important roles in the host defence by mediating the early immune responses to viral and to bacterial infections [45]. Moreover, IFNs exhibit anti-tumoural effects, inhibit cell

growth and angiogenesis, and regulate apoptosis [46]. The IFNs are classified into three types based on the utilisation of distinct cytokine receptors on the cell surface: type I (see below), type II (IFN-γ) and type III (IFN-λ, divided into three subtypes IFN-λ1, 2 and 3, also known as IL-29, IL-28A and IL-28B, respectively) [45,47,48].

Type I IFNs include IFN-α (13 subtypes in humans, 14 in mice), β, κ, ε, ω, and ζ. All type I IFNs signal through the IFN-α/β receptor (IFNAR) consisting of two subunits IFNAR1 and IFNAR2. Type I IFNs are expressed in almost all cells. IFNAR1 is constitutively associated with Tyk2 and IFNAR2 with Jak1. The major transcriptional complex activated by type I IFNs is the IFN-stimulated gene factor 3 (ISGF3). This complex consists of activated Stat1 and 2 heterodimer and IRF9. ISGF3 activates gene expression of IFN-stimulated genes (ISGs) containing IFN-stimulated response elements (ISRE) in their promoter regions [45,49]. Depending on the cell type, type I IFNs can also activate all other Stats, although their contributions to the overall response are less well-defined [50]. Moreover, type I IFNs activate several additional signalling pathways, e.g. MAPKs, and phosphoinositide-3 kinase (PI3K) [49]. These signalling cascades modify gene expression of ISGs either independent or cooperative with Stat homo/heterodimers [50] and can influence protein expression post-transcriptionally [51]. Type I IFN signalling leads to the expression of several hundreds of ISGs [52]. These participate in host defence against virus, bacteria and intracellular protozoa. ISGs are implicated in cell cycle regulation and differentiation, regulation of transcription, translation, apoptosis and angiogenesis. Moreover, type I IFNs induce also expression of genes that inhibit the Jak-Stat signalling pathway and hence act as negative feedback regulators [43,53,54]. Although there are large overlaps, the biological effects mediated by type I IFNs depend on cell type and the IFN subtype [50].

3. Tyk2

3.1 Tyk2 in cytokine signalling

Tyk2 was originally identified as essential in type I IFNs signalling [55]. Subsequent genetic and biochemical studies indicated involvement of Tyk2 in the response to IL-10, IL-12, IL-23, and several members of IL-6 receptor family [43]. Tyk2-deficient mice were generated by three independent groups [56-58] and are viable and fertile. Additionally, the B10.Q/J mouse strain exhibits Tyk2 deficiency because of a mutation in the Tyk2 gene [59].

The most unexpected finding by studies with Tyk2-deficient mice was the modest effect of Tyk2 deficiency on type I IFN responses. Cells derived from Tyk2-deficient mice show defects in IFN-α signalling although, in contrast to human Tyk2-deficient cell lines, the response was not completely abrogated [56,58]. The response to IFN-γ was also affected in

Tyk2-deficient cells, which is most likely not a direct effect but due to the reduced Stat1 protein level reported in these cells [58].

Stimulation of Tyk2-deficient cells with IL-12 results in impaired Stat3 and Stat4 activation and consequently, in reduced production of IFN-γ by NK and T cells [56,58-60]. Recently, it has been reported that murine Tyk2 is also critical for IL-23 signalling [59,61] and subsequent IL-17 production in peritoneal $\gamma\delta$T cells [61].

Murine Tyk2 is not essential for the responses to several IL-6 family members and IL-10 [56,58]. However, a recent study showed partial impairment of IL-10 signalling in Tyk2-deficient peritoneal macrophages [62].

3.2 Tyk2 in host defence and diseases

According to the importance of Tyk2 in multiple signalling pathways, Tyk2 has critical functions in the host defence against various viral, bacterial and parasitic pathogens. Tyk2-deficient mice exhibit inefficient cytotoxic T cell (CTL) responses against lymphocytic choriomeningitis virus (LCMV) and elevated replication of vaccinia virus (VV) in spleen [58]. Infection of Tyk2-deficient mice with murine cytomegalovirus (MCMV) leads to increased load of virus in organs and reduced survival [63]. In contrast, Tyk2 is not essential for the survival upon vesicular stomatitis virus (VSV) infection [58]. Mice lacking Tyk2 show increased susceptibility to infection with the intracellular bacteria *Listeria monocytogenes* [64] and the protozoan parasite *Toxoplasma gondii* [59,65]. Tyk2 is not essential for the ultimate control of an infection with *Leishmania major*, another protozoan parasite, but Tyk2-deficient mice develop more severe and prolonged skin lesions [66].

In addition to the important role of Tyk2 in the defence against infectious diseases, involvement of Tyk2 in several other disease models has been shown. Among those, resistance of Tyk2-deficient mice against high-dose LPS-induced shock was the first one described [67,68]. High resistance of Tyk2$^{-/-}$ mice was subsequently also shown in ischemia/reperfusion-induced shock [69] and collagen-induced arthritis [59]. Tyk2-deficient mice are more sensitive to leukaemias/lymphomas [70,71]. However, the invasiveness of cancer cells is reduced in Eµ-Myc mice, a model system for human Burkitt's lymphoma [72]. Mice lacking Tyk2 exhibit a tendency to develop allergic T$_H$2 responses and show enhanced T$_H$2 cell-mediated antibody production [60].

3.3 Tyk2 deficiency in humans

To date, only one human patient with Tyk2 deficiency has been reported [73]. The patient with a diagnosed primary immunodeficiency shows homozygous mutations in the Tyk2 gene

resulting in a premature stop codon. This patient exhibits a severe allergic phenotype and highly elevated IgE levels. In contrast to Tyk2-deficient mice, cells from this patient showed dramatically impaired responses to type I IFNs, IL-6 and IL-10, indicating that human Tyk2 has an obligatory role in responses to these cytokines. Furthermore, this patient shows high susceptibility to viral, mycobacterial and fungal infections. Levels of IFNAR1 receptor were reduced in the patient's T cells, which is consistent with previous observations in human cell lines, that Tyk2 is important for maintaining cell surface levels of IFNAR1 [74-76]. A recent study indicates that the association of Tyk2 with IFNAR1 masks a tyrosine-based linear endocytic motif in IFNAR1 and therefore prevents its internalisation [77]. However, this function of Tyk2 is absent in mice [56-58], presumably because murine IFNAR1 lacks this endocytic motif [77].

III. Proteomics

1. Proteomics overview

The set of all expressed proteins in a cell, tissue or an organism is called "proteome" and the study of the proteome became known as proteomics. The diversity of expressed proteins results from the regulation of their transcription and translation. In addition, several isoforms may exist due to alternative splicing and post-translational modifications, e.g. phosphorylation, glycosylation and ubiquitination. Moreover, protein expression changes dynamically in response to developmental and environmental stimuli. Basically, there are two classical proteomics approaches, namely gel-based and mass spectrometry (MS)-based; an overview of the experimental workflows is given in Fig. 3 (the approach used in this work is indicated in blue). A typical gel-based proteomics study includes separation of proteins by two-dimensional gel electrophoresis (2DE) and their visualisation, followed by evaluation of the obtained gel images and statistical analyses. The spots of interest are then excised from the gel and subjected to MS for identification. The methods used in this approach are described in more detail in the following sections.

The MS-based proteomics approach involves separation of peptides resulting from proteolytic digestion of proteins using liquid chromatography-tandem mass spectrometry (LC-MS-MS). In this case, the peptide mixtures are usually pre-fractionated by different electrophoretic and/or chromatographic separation techniques. In addition, the peptide mixtures may be obtained from proteins previously separated by one-dimensional electrophoresis (immobilised pH gradients (IPG) or sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)). Quantification is facilitated by stable isotope labelling of proteins or chemical modification of peptides with isobaric tags [78-81].
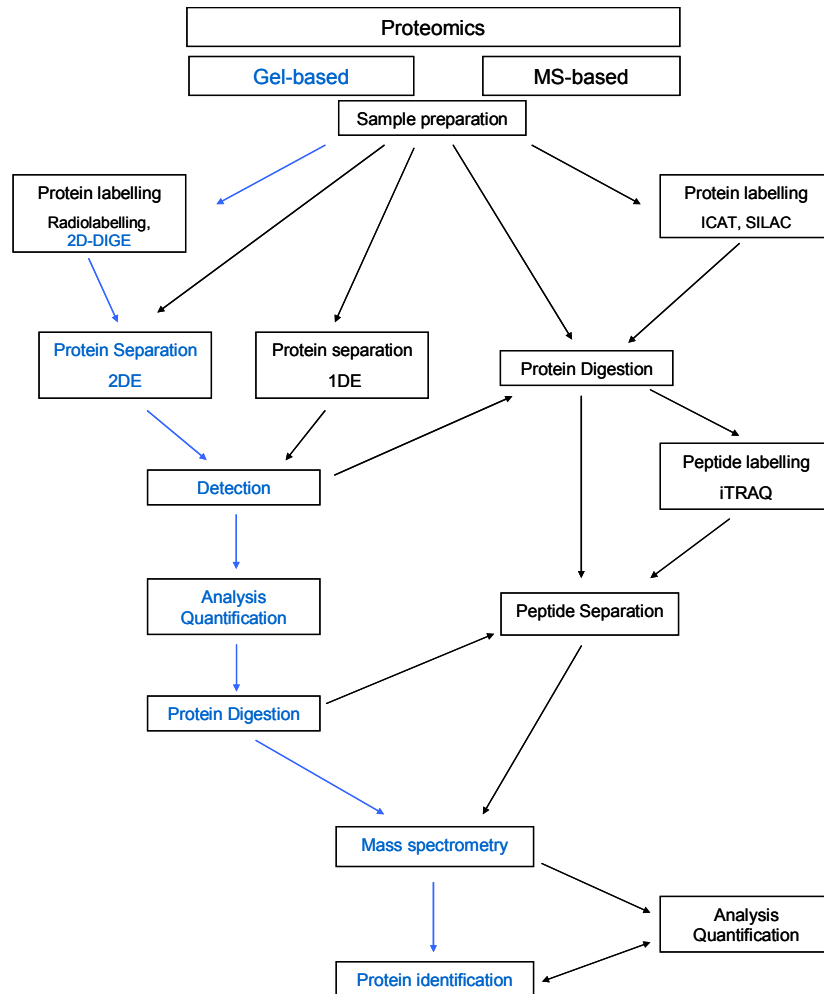
Figure 3. Classical proteomics approaches

2D-DIGE: two-dimensional fluorescence difference gel electrophoresis; ICAT - isotope-coded affinity tags; iTRAQ - isobaric tags for relative and absolute quantification SILAC - stable isotope labelling with amino acids in cell culture

2. Two-dimensional gel electrophoresis (2DE)

2DE is a powerful technique in proteomic studies enabling simultaneous separation and analysis of about thousands of proteins [82,83]. 2DE provides information about physicochemical properties and subunit structure of the separated proteins, allows detection of changes in protein expression levels, protein isoforms, post-translational modifications and of incomplete proteolysis. Protein separation by 2DE uses two independent parameters. In the first dimension proteins are separated in a pH gradient according to their isoelectric points (pIs) by isoelectric focusing (IEF), and the second dimension uses the principle of SDS-PAGE to separate the proteins according to their molecular mass (M_r). 2DE was first

described in 1975 [84,85], and many improvements have been made since this time, the most important being the introduction of IPG. Other modifications in the technology improved features like experimental variation, detection of low abundant proteins and resolution of highly hydrophobic proteins which had been regarded as weak points of 2DE [82,83].

2.1 2DE - sample preparation/separation

As in many other analytical methods sample preparation is a critical point in 2DE analyses and reproducibility is a prerequisite for a good proteomics study. Therefore, sample collection has to be done under well-defined conditions and its preparation should be as simple as possible. There is no single protocol for protein extraction suitable for all sample/protein types. However, many standard protocols have been published that can be adapted and optimised for an individual sample type or for a subset of proteins of interest [82,83].

The separation of proteins by 2DE is only possible if they are completely soluble under electrophoretic conditions. Protein solubilisation is usually performed in a buffer containing high concentrations of chaotropes, e.g. urea combined with thiourea, and other additives. Chaotropes unfold proteins and prevent protein-protein interactions. Zwitterionic or non-ionic detergents, e.g. CHAPS, Triton X-100 or NP-40 increase the solubility of hydrophobic proteins. To reduce and prevent re-oxidation of disulfide bonds, reducing agents like DTT or DTE are used. Additionally, the solubility of proteins is improved by carrier ampholytes or IPG buffers of the appropriate pH range. Degradation of proteins by proteolysis complicates the subsequent analysis [82,83,86] and addition of protease inhibitors during protein extraction is recommended [83,86]. Of importance is also a low content of interfering substances such as salt, charged molecules, nucleic acids, ionic molecules and insoluble material. Higher amounts may disturb the protein patterns by interfering either with the first or the second dimension of 2DE and should be removed (e.g. by precipitation, desalting). Similarly, application of standardised protein amounts assures quality of protein separation, especially in quantitative studies [82,83]. The first dimension of 2DE is performed in individual IPG gel strips, in different pH ranges from 2.5 to 12, length up to 24 cm, with a linear or non-linear pH gradient. The sample can be applied either by in-gel rehydration or by cup loading after the rehydration step. IEF conditions have to be optimised for each sample type empirically, according to existing guidelines. Insufficient focusing leads to horizontal streaks in the second dimension, overfocusing results in protein losses and distorted spot patterns. To improve the protein transfer into the second dimension the strips are equilibrated in urea/glycerol containing buffer, which reduces the electroendosmotic effects. The second dimension is usually performed with the discontinuous buffer system of Laemmli

in homogenous or gradient gels and a polyacrylamide concentration optimised for the specific M_r range [82,83].

It is usually not possible to display and analyse all proteins from one sample in a single gel. Due to the wide range in protein abundance levels (e.g. 6-8 orders of magnitude in human cells) low abundant proteins are usually not detected. Therefore, application of additional techniques to deplete highly abundant or enrich low copy number proteins is suggested to obtain better resolution and sensitivity. However, this may reduce the reproducibility [82,83,87,88].

Subcellular fractionation is very frequently used to enrich proteins from organelles and/or to obtain information about cellular localisation of proteins. Stretching of the protein patterns in the first dimension using narrow pH gradients (zoom-gels), particularly in combination with electrophoretic pre-fractionation of proteins according to their p*I*s, may greatly improve the detection of low abundant proteins. Chromatographic pre-fractionation enables selective retention of proteins with specific chemical or antigenic properties, and can be used for enrichment or depletion of specific protein classes [82,83,88]. Resolution of very basic proteins may be improved by enrichment with TCA/acetone precipitation and optimised conditions during the first dimension [82]. The poor solubility of very hydrophobic proteins can be increased by use of new detergents such as sulfabetaines [83]; sequential protein extraction with buffers of increasing solubilising power has also been propagated [82,83].

2.2 Spot detection/visualisation

After 2DE separation, several techniques are used for protein detection; the most common are briefly characterised below.

Two different varieties of Coomassie brilliant blue (CBB), R-250 and G-250, can be used in solutions with solvents or as colloidal CBB (cCBB) dyes. They interact with proteins through electrostatic and hydrophobic interactions. The main advantage of CBB stains is the compatibility with MS. The limitations are the low sensitivity, which lies in the range of approximately 30-100 ng for CBB and 8-10 ng for cCBB, and a low linear range of about 1-1.3 orders of magnitude [82,89,90].

By silver staining, Ag^+ ions bind to proteins and are starting points for silver crystallisation in the subsequent reduction process. Silver staining has no endpoint, the spot intensity depends on the duration of the development process. This reduces the reproducibility and makes it less suitable for quantitative analysis. Silver stain has a high sensitivity and, depending on the protocol used, can detect as little as 0.1-2 ng protein [82] and the dynamic range is approximately one order of magnitude. Classical silver stain is not compatible with

MS, however the omission of aldehydes in the fixative and impregnating buffers allows MS analysis, but makes it less sensitive [82,89,90].

Radiolabelling is commonly accomplished by the incorporation of radioactive amino acids such as ^{35}S methionine/cysteine or ^{14}C leucine into proteins during cell growth. This makes it possible to analyse newly synthesised proteins. Labelling after cell lysis with ^{131}I or ^{125}I is also possible but less accurate. The separated proteins can then be detected by X-ray films (dynamic range 10^3) or by more sensitive storage-phosphor screens (dynamic range 10^5) [82,90].

Non-covalent fluorescent stains can detect proteins with a wide dynamic range, which is over three orders of magnitude, and their sensitivity is in the range of 1-10 ng. The dyes interact with proteins either directly by electrostatic interactions with basic amino acid residues e.g. SYPRO Ruby or indirectly through intercalation into SDS micelles, e.g. SYPRO Red. Fluorescent stains allow more reproducible quantitative analysis and are compatible with MS, but need special equipment for detection/visualisation [82,89,90].

2.2.1 Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE)

For 2D-DIGE, proteins are labelled before electrophoretic separation through covalent binding of the dyes to specific amino acid residues in the molecules. 2D-DIGE is based on the specific properties of the fluorescent dyes, which are matched for charge and molecular weight but are spectrally distinct [91-93]. Currently there are two matched sets of CyDye DIGE fluors, CyDye fluor minimal and CyDye fluor saturation dyes [91,92].

CyDye fluor minimal dyes are available as Cy2, Cy3, and Cy5. These dyes have an N-hydroxysuccinimide (NHS) ester reactive group, which forms a covalent bond with an ε amino group of lysine in proteins *via* an amide linkage. The ratio of dye to protein is kept very low, as lysine is a very frequent amino acid. The dyes label approximately 1-2% of lysine residues so that, on average, each protein carries only one dye *per* molecule. Sensitivity of these CyDyes is 0.1-0.2 ng and the linear dynamic range up to five orders of magnitude (10^5) [89,91,92].

CyDye DIGE fluor saturation dyes Cy3 and Cy5 have a maleimide reactive group which forms a covalent bond with the thiol group of cysteine residues on a protein *via* thioether linkage. Usually, proteins have a low cysteine content and by maintaining a high dye to protein ratio the dyes label all available cysteine groups on each protein. Saturation labelling allows analyses in minute sample amounts, as little as 5 μg protein can be used for labelling reaction ("scarce sample labelling", e.g. from laser microdissection microscopy). Detection limit of these dyes is 5-10 pg and the dynamic range is 10^5. However, depending on the cysteine distribution in the analysed samples, this technique needs optimisation and may result in patterns different to other stains [89,91,92].

The experimental workflow of 2D-DIGE using CyDye fluor minimal dyes is illustrated in Fig. 4. The labelled protein samples are mixed and separated simultaneously on the same gel. The corresponding protein spot patterns are scanned individually with the excitation wavelengths specific for the respective dyes. This multiplexing enables to separate two to three different protein samples on one 2D gel, one sample can serve as a reference sample known as internal standard (usually labelled with Cy2 in minimal labelling experiments) [91-94]. The internal standard is a pool of equal amounts of proteins from each sample in an experiment, and is the same on each gel within the experiment. It contains, theoretically, each protein from all samples and is used for matching of protein patterns across gels and for normalisation. Volumes of protein spots are normalised against the internal standard and are given as volume ratios (sample spot volume divided by the corresponding internal standard spot volume) [91,92,94].

The 2D-DIGE approach reduces the gel to gel variation and the number of gels required in one experiment. The incorporation of the internal standard greatly improves the accuracy of protein spot quantification. This combined with the high sensitivity and linearity of the dyes allows highly reproducible, quantitative proteome studies. The reduced experimental variation considerably improves detection of small differences in the protein expression levels [91,92,94].

However, this technique has also some limitations. Preferential labelling of some proteins by the dyes has been observed and may lead to misinterpretation of the results. This may be overcome through reverse labelling of the samples [89]. The slight increase in M_r of the labelled proteins (approximately 500 Da), noticeable especially for low M_r proteins, could be problematic in spot excision for MS analysis. Therefore additional post-staining is recommended and, moreover, necessary if spots are picked manually. This may lead to difficulties, because some protein spots may not be detected due to the different properties and sensitivity of the applied stains [89,92].
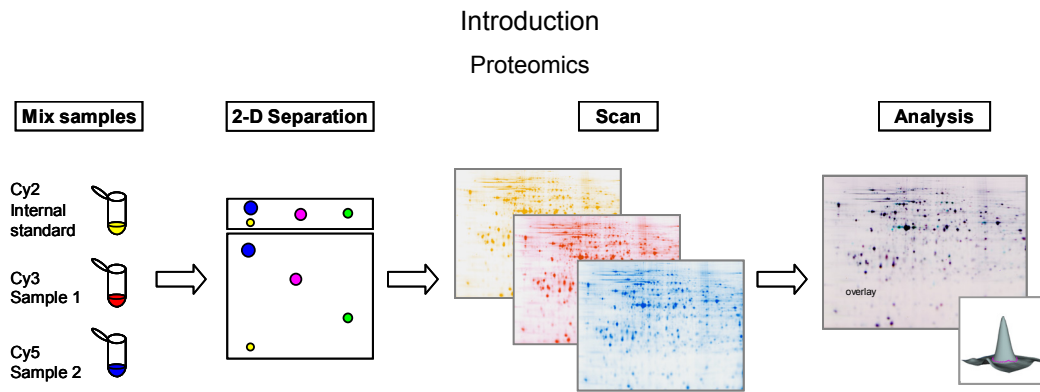
Figure 4. Principle of 2D-DIGE technology

2.3 Quantification

The separated proteins are visualised with one of the methods described and the obtained spot pattern images are converted into digital images. Ideally, the resulting 2DE protein image is free of streaks and background staining, the spots are well-distributed throughout the gel, do not overlap and have well-defined borders. Subsequent analysis is usually performed with the help of special 2DE software. The analysis involves background correction, spot detection, filtering and editing, gel matching and normalisation. The quantification is based on relative changes in protein amount between two or more samples [82,83]. The protein expression data obtained are complex, have many variables, and therefore need appropriate statistical methods of analysis [95,96].

2.4 Identification and verification

Protein spots of interest are excised from 2D gels, cleaved into peptides with a protease of known cleavage specificity (usually trypsin), extracted and subjected to MS. The peptides are separated according to their masses (mass to charge ratio). Most commonly used ionisation techniques by MS analyses are electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI). They usually are combined with tandem mass spectrometry, which provides information about the primary sequence of a selected precursor ion and therefore increases the confidence for the protein identification. Appropriate software enables protein identification on the basis of the obtained peptide masses by searching the existing protein databases [79,87]. The expression profiles of the identified proteins are usually further analysed by other techniques e.g. 1D and/or 2D western blot.

3. Analyses of macrophage proteomes using 2DE

The proteomes of macrophages and their precursors, monocytes, have been studied in human and murine cell lines as well as in primary cells of different sources. Several studies were performed with the focus on the generation of protein reference maps, i.e. identification of a number of proteins expressed under basal conditions (e.g. expression profiling of intracellular and secreted proteins in human macrophages [97], protein spot map of human monocytes including determination of their phosphorylation states [98], protein spot map of the phagosome [99]). Some spot maps are available in the gel-based proteomics databases (World-2DPAGE portal; http://www.expasy.ch/world-2dpage). Other studies aimed to analyse proteomic alterations in macrophages under different conditions. A large number of comparative proteomics analyses in macrophages involving various immunological stimuli (e.g. infection with bacteria, viruses, fungi or protozoa, challenge with bacterial toxins or IFN-γ activation) were performed in order to better understand the host immune responses to pathogens.

The macrophage responses to LPS using primary murine macrophages have been investigated in a few older proteomics studies, but at that time the 2DE protein patterns were characterised solely by physicochemical properties of the spots (pI, M_r) [100-102]. More recent studies that include MS identification of LPS responsive proteins employed cell lines rather than primary cells [103-105]. To date only one study in primary murine macrophages using 2D-DIGE technology (without internal standard) was published, however this study was applied to investigate responses to ionising radiation in macrophages from two different mouse strains [106].

AIMS

Tyk2 plays an important role in host defence against different pathogens. It has been shown that Tyk2-deficient mice are highly resistant to high-dose LPS-induced endotoxin shock and Tyk2-deficient macrophages show impaired responses to LPS. The importance of macrophages in the pathogenesis of endotoxic shock is well-documented in the literature. Based on these findings, this work was directed to investigate the role of Tyk2 in macrophages with the main focus on the identification of novel Tyk2-dependent host cell responses. For this purpose we chose 2D-DIGE as key technique. The first objective was to establish reproducible experimental conditions for the proteomics study of protein extracts from primary murine macrophages. With this optimised approach, protein expression patterns between wild-type (WT) and Tyk2-deficient cells before and after LPS treatment were compared in order to find proteins that show genotype specific differences in expression patterns. Dependent on the above results, protein expression, as determined by 2D-DIGE technology, should be validated for a subset of differentially expressed proteins and analysed in more detail using other techniques (e.g. 1D and 2D western blot). Further aims were to subsequently investigate the regulatory mechanisms responsible for the positive or negative impact of Tyk2 on the expression of selected proteins (e.g. transcriptional and translational analysis).

RESULTS

I. Published results

1. Contribution of cell culture additives to the two-dimensional protein patterns of mouse macrophages.

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Short Communication

Contribution of cell culture additives to the two-dimensional protein patterns of mouse macrophages

Low levels of fetal calf serum (FCS), used as protein supplement in cell culture medium, were traced in preparations of primary murine macrophages (bone-marrow-derived macrophages (BMM) and peritoneal macrophages (PM)). Main components of this common additive were mapped in 2-DE by means of differential image gel electrophoresis and immunoblotting. Additional washing steps in cell preparation helped to decrease the levels of the four highest abundance foetal serum proteins (serum albumin (SA), α_1-fetoprotein (AFP), α_1-antitrypsin (α_1AT) and transferrin (Tf)) to less than 1% of total protein. Macrophage spot pattern was recorded in parallel and showed little variation. Results presented are supposed to be of general interest for cell preparations with similar background.

Analysis of cellular proteomes (primary cells as well as established cell lines) is an important field of proteomics and 2-DE as reflected, *e.g.*, by the considerable number of 2-DE maps and databases compiled in WORLD-2DPAGE (http://www.expasy.org/ch2d/2d-index.html) and SWISS-2DPAGE (http://www.expasy.org/ch2d/publi/inside1995.html). Primary cells from organs and tissues are often kept in cell culture for propagation, differentiation or *ex vivo* experiments. Culture media are usually supplemented with up to 20% fetal calf serum (FCS), horse serum or homologous serum. Although serum-free media formulations have been developed for certain cell types and applications, they often also contain protein supplements (*e.g.*, serum albumin (SA), transferrin (Tf). During our proteomics analyses of primary murine macrophages the question arose whether those protein supplements are detected in protein patterns of cell lysates, to what extent and how and whether these contaminations can be avoided.

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Abbreviations: AFP, α_1-fetoprotein; **α_1AT**, α_1-antitrypsin; **BMM**, bone-marrow-derived macrophages; **FCS**, fetal calf serum; **PM**, peritoneal macrophages; **SA**, serum albumin; **Tf,** transferrin

Mice (strain C57BL/6) were housed under specific pathogen-free conditions. Bone-marrow-derived macrophages (BMM) and peritoneal macrophages (PM) were obtained as described [1, 2]. Both protocols include an incubation step of the isolated cells for several hours/days in cell culture medium with FCS as a supplement. Cells were washed three times with ice-cold PBS and lysed in 10 mM Tris-HCl (pH 8), 150 mM NaCl, 0.5% Nonidet-P40 v/v, 10% glycerol v/v, 1 mM DTT, 0.1 mM EDTA, 1 mM sodium orthovanadate, 25 mM sodium fluoride, 1 mM PMSF, 1 μg/mL aprotinin and 1 μg/mL leupeptin. Cell debris were removed by centrifugation at $1000 \times g$. Protein content was determined by Coomassie G-250 protein binding assay [3] and was in the range of 4–6 mg/mL. Samples were lyophilized and dissolved in labelling buffer (30 mM Tris-HCl, 9 M urea, 4% CHAPS w/v, pH 8.5) to a concentration of 5 mg protein/mL. Labelling with CyDyes® (GE Health Care, formerly Amersham Biosciences; Uppsala, Sweden) was performed according to the manufacturer, except for lower coupling rates (4 nmol dye/mg protein). Two labelled samples (Cy3, Cy5) and a sample pool (internal standard, Cy2) were mixed and run on one gel. The internal standard contained all samples present in one sample set.

Classical 2-DE was performed in accordance to existing protocols (http://www.expasy.ch/ch2d). Twenty-five micrograms *per* labelled-sample was applied to labora-

tory-made nonlinear IPGs pH 4–10 [4] of 10 cm length by in-gel rehydration for 7 h. Strips were run on a Multiphor system (GE Health Care) until 15 kVh was reached. After equilibration, strips were transferred to an SDS-PAGE gel ($T = 10$–15% linear gradient, $C = 2.7\%$) according to Laemmli [5] for the second dimension in a Hoefer SE 600 vertical electrophoresis chamber (GE Health Care). For fluorescence detection, 2-D differential gel electrophoresis (DIGE) gels were scanned on a Typhoon 9400 imager and evaluated with DeCyder Software V5.02. Quantification was based on standard ratios, *i.e.*, ratio between the spot volume of a particular single spot and the respective spot of the internal standard.

Figure 1A shows the 2-DE pattern of a typical macrophage sample, prepared as described above. Comparing different preparations, a faint, but distinct spot chain of about 67 kDa was noticed which varied between specimens. The concentration differed in samples, but was independent of investigated cell type (BMM, PM). Closer inspection and comparison with the FCS pattern (Fig. 1B) revealed that this was SA. In an overlay of Cy3-labelled FCS (shown in green) with a macrophage lysate pattern (Cy2-labelled, coded in red), overlapping spots appear yellow. There is complete overlap not only for SA but also for other major FCS proteins.

As a further proof for the presence of FCS traces, immunoblotting experiments were performed using unlabelled samples, either 50 µg of macrophage lysates or 0.05 µL of FCS. The 2-DE run was followed by semidry blotting onto

NC (Hybond ECL, GE Health Care) according to [6]. The membrane was stained for total protein pattern with ruthenium(II)tris(bathophenanthroline disulfonate), using the same method as described for SYPRO Ruby (product information from Sigma; St. Louis, MO). The fluorophore was synthesized according to [7]. After scanning on the Typhoon 9400 (excitation 488 nm, emission filter 610 nm), the blots were probed with the following antibodies: anti-FCS (DakoCytomation, Glostrup, Denmark), antibovine AFP (DakoCytomation), anti-BSA (Cappel Laboratories, Cochranville, PA) and a cross-reactive human anti-α_1-antitrypsin (anti-α_1AT) (DakoCytomation). The cross-reactivity of the latter had already been tested in a previous study [8]. Anti-rabbit IgG HRPO conjugate and ECL™ Western blotting detection reagents (both, GE Health Care) were used for detection of immunoreactive spots. With those antibodies, the most abundant proteins in FCS were identified (Fig. 1C) and detected in the macrophage lysates.

On the basis of these findings, experiments were undertaken to improve harvesting and washing of macrophages. Different numbers of washing steps with PBS were performed and will be called A1, A3 and A6 in accordance with the number of washing steps included. Volumes for washing were increased and special care was taken to quantitatively remove the washing solution. Protein patterns of cell lysates obtained by these procedures are shown in Fig. 2. In A1 samples, FCS proteins were clearly visible (Fig. 2A); upon further washing, levels dropped markedly. This can also be seen when evaluating the concentration of these spot groups: Fig. 3 depicts
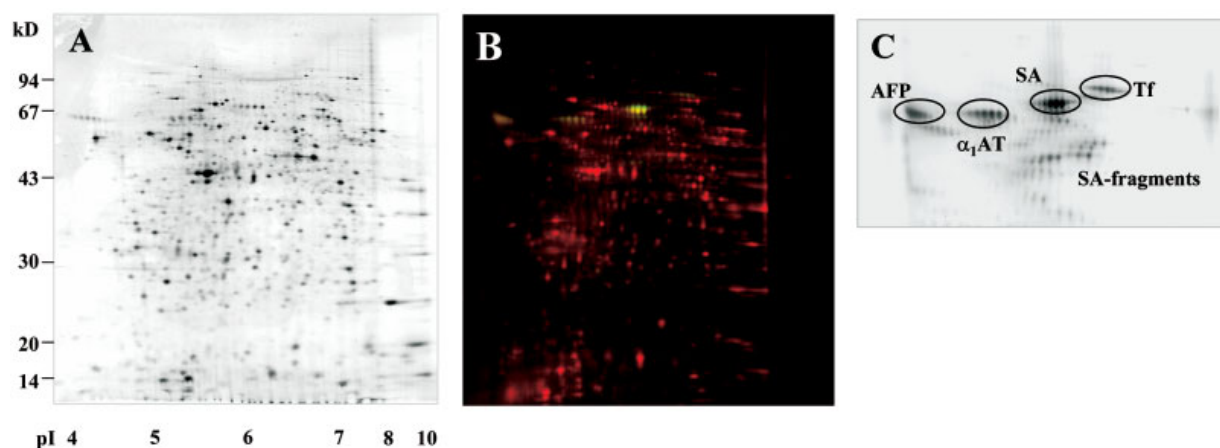
Figure 1. 2-DE using a PM preparation according to [2]. 2-DE as described (10–15% T, 2.7% C). (A) 25 µg PM, silver stain [6]. (B) DIGE-gel. Sample 1: 25 µg PM, labelled with Cy2 (4 nmol dye/mg protein); colour coding: red. Sample 2: corresponding to 0.1 µL undiluted FCS, labelled with Cy3 (4 nmol dye/mg protein); colour coding: green; yellow: overlap of samples 1 and 2. Color coding has been selected to give colors with best contrast. (C) Identification of main FCS components. (B) was split into single channels, and the image of sample 2 was inverted and turned into grey levels; only the part with the major FCS components is shown. AFP, α_1AT and SA were identified by immunoblotting, Tf by mass spectrometric methods [8].
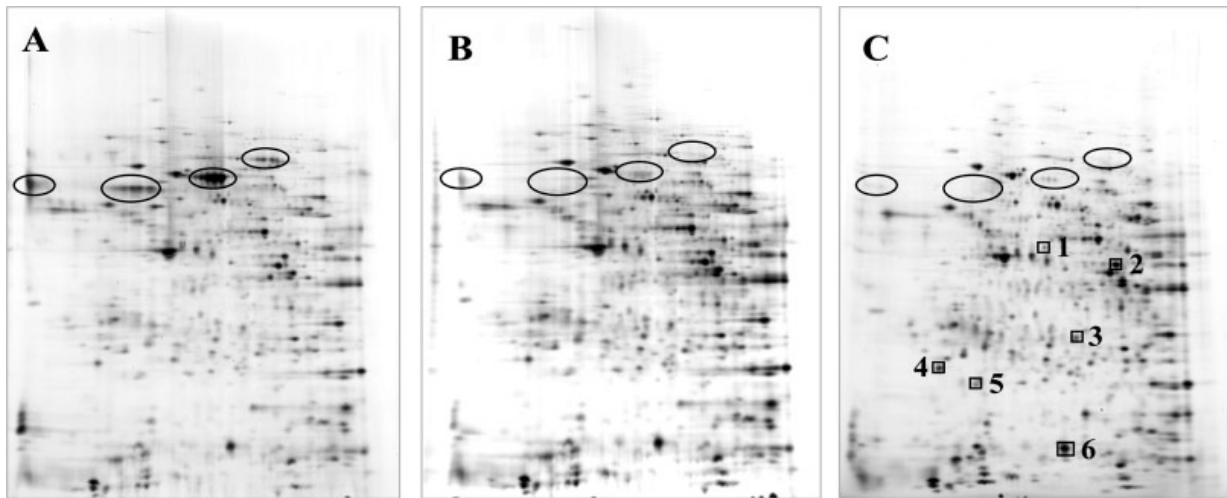
Figure 2. Varying the washing procedure for primary macrophages, primary PM were washed: (a) once (A1), (b) three times (A3) and (c) six times (A6) with cold PBS as described in the text. Twenty-five micrograms was labelled with CyDyes and subjected to 2-DE. Positions of AFP, α_1AT, SA and Tf are circled (identifications in Fig. 1C). Single channels were separated and are shown in grey levels. In Fig. 2C, six protein spots are marked with boxes; for their evaluation see Fig. 4B.
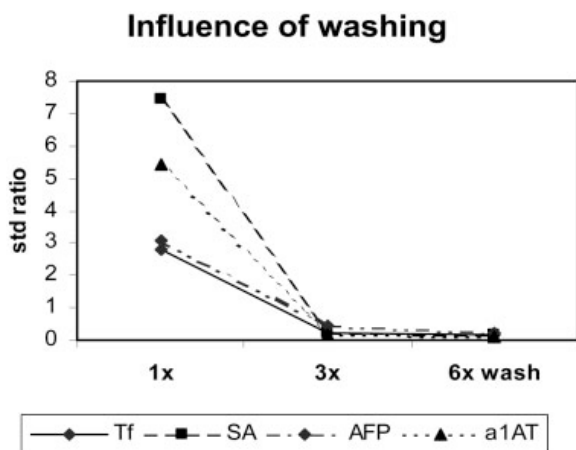

Figure 3. Detection of FCS components. Concentrations of the four main FCS components were monitored in differently washed cells (A1, A3 and A6) and expressed as standard ratios (std ratio).

standard ratios for one spot out of the respective spot trains of BSA, Tf, α_1AT and AFP (for each chain, the most intense spot is shown, but trends of the other spots of the same chain are similar). Standard ratios drop from 3.5–8 to less than 0.5, with only two additional washing steps (A1 *vs*. A3), and improve slightly further in A6. One additional preparation was produced by washing cells *via* centrifugation (three times) instead of in the culture dish. A first try yielded lower protein concentration and recovery, suggesting possible harm of the cells with this treatment. As our specific aim is to preserve macro-

phage functions during isolation in order to perform studies on specific pathways, we did not further follow this line.

For evaluation of sample preparation procedures, quantitation of residual FCS components was performed with DeCyder. Figure 4A compares BMM preparations obtained with protocols according to [1] and A6, focusing on the main FCS proteins BSA, α_1AT, AFP and Tf. The graph is based on relative percentages of those proteins in the sample (based on spot volumes, as described in the Figure caption). Average values are shown, and SDs are indicated as error bars. As data illustrate, more extensive washing considerably lowers FCS levels, but there are still traces of these essential medium components. When comparing DIGE patterns of once and three times washed cell lysates, 4–5% of overall spots were decreased to half or less of their volumes. Most of them belong to the main BSA, α_1AT, AFP and Tf spot chains, but there are also traces of some other spots known from the FCS pattern, *e.g.*, apolipoprotein A-I. The four main proteins we have focused on are by far the most intense. By adding up their spot volumes we estimated that those four proteins comprise less than 1% of total protein of the A6 and 1–2% for A3 lysates. Values were similar for BMM and PM preparations.

In contrast to the differences in FCS content displayed in Fig. 4A, in the same samples the overall distribution of macrophage protein spots is rather stable. Figure 4B shows this for six spots of different intensity, selected from different regions of the gel. Values were calculated in

Evaluation of FCS components

Evaluation of macrophage spots
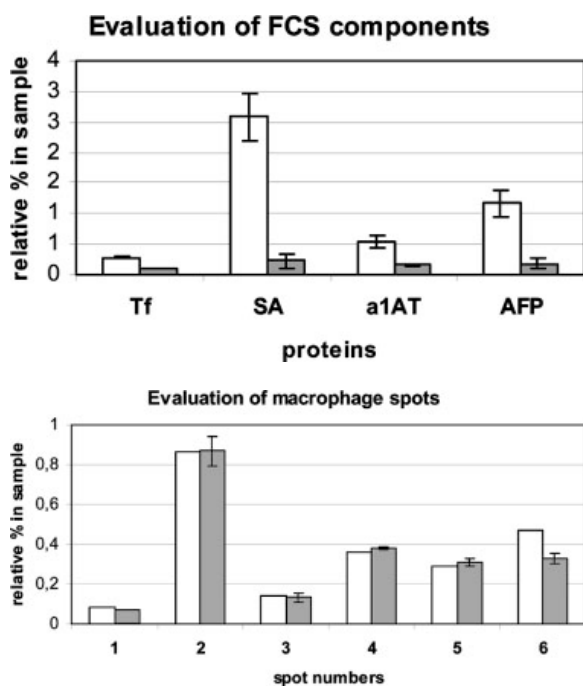
Figure 4. Comparison of BMM preparations with different protocols. (A) Evaluation of residual FCS components; samples: BMM, according to [1] (white bars; *n* = 4) *vs.* A6 (grey bars, *n* = 8). (B) Evaluation of some selected macrophage spots; spot positions are indicated in Fig. 2C; samples: BMM, according to [1] (white bars) *vs.* A6 (grey bars). Average values are given; error bars show SD. Samples were run within one set, with the same internal standard (a pool of all samples).

the same way as for Fig. 4A and give similar levels or variations for corresponding spots of the two preparations. Careful, but extensive washing does not seem to have a major effect on those spots and the majority of the other macrophage proteins detected by the present method.

In our opinion, residual FCS may be a general problem for samples derived from cell culture. This concerns all methods using detection based on total protein staining, and therefore also most proteomics applications. In 1-D methods like SDS-PAGE, contaminations may not be noticed, but – if variable – they contribute to the variation of the system. Due to its high resolution, 2-DE is likely to detect those traces as single spots or spot rows, provided that they do not overlap with other proteins in the sample. Residual medium supplements may be regarded as differentially regulated spots and potential new biomarkers, till they are positively identified by MS methods. The aim of our study was to prevent such pitfalls by detecting/locating spot positions of the main FCS components and to evaluate the actual contamination levels in cell lysates. Our experiments showed that even with extensive washing not all traces of FCS could be removed. We avoided more stringent washing or centrifugation steps on purpose, in order not to stress the cells and risk changes in protein expression patterns. Nevertheless, those could be appropriate alternatives for more robust cells or cell lines, but all steps would require proper evaluation. Even with the mild conditions chosen, we could show that careful washing reduces FCS contamination from about 3–5% to 1% or less of total protein content, for both macrophage populations described. At the same time, overall protein pattern and distribution stayed constant within the normal range of variaton. In addition, we learnt about the exact positions of the main spots in 2-DE. If necessary, they can now be excluded from evaluation (so far no overlap with macrophage proteins has been noticed).

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2. The impact of tyrosine kinase 2 (Tyk2) on the proteome of murine macrophages and their response to lipopolysaccharide (LPS).

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RESEARCH ARTICLE

The impact of tyrosine kinase 2 (Tyk2) on the proteome of murine macrophages and their response to lipopolysaccharide (LPS)

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Tyrosine kinase 2 (Tyk2) belongs to the Janus kinase (Jak) family and is involved in signalling *via* a number of cytokines. Tyk2-deficient mice are highly resistant to lipopolysaccharide (LPS)-induced endotoxin shock. Macrophages are key players in the pathogenesis of endotoxin shock and, accordingly, defects in the LPS responses of $Tyk2^{-/-}$ macrophages have been reported. In the present study, the molecular role of Tyk2 is investigated in more detail using a proteomics approach. 2-D DIGE was applied to compare protein patterns from wild-type and $Tyk2^{-/-}$ macrophages and revealed significant differences in protein expression patterns between the genotypes before and after LPS treatment. Twenty-one proteins deriving from 25 differentially expressed spots were identified by MALDI/ESI MS. Among them, we show for N-myc interactor that its mRNA transcription/stability is positively influenced by Tyk2. In contrast, LPS-induced expression of plasminogen activator 2 protein but not mRNA is strongly enhanced in the absence of Tyk2. Our data furthermore suggest an influence of Tyk2 on the subcellular distribution of elongation factor 2 and on LPS-mediated changes in the peroxiredoxin 1 spot pattern. Thus, our results imply regulatory roles of Tyk2 at multiple levels and establish novel connections between Tyk2 and several cellular proteins.

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Abbreviations: EF2, elongation factor 2; **ERK,** extracellular signal regulated kinase; **GAPDH,** glyceraldehyde-3-phosphate dehydrogenase; **IFIT,** interferon-induced protein with tetratripeptide repeats; **IFN,** interferon; **IN35,** interferon-induced protein 35 kD homologue; **Jak,** Janus kinase; **LPS,** lipopolysaccharide; **NMI,** N-myc interactor; **PAI2,** plasminogen activator inhibitor 2; **PRDX1,** peroxiredoxin 1; **RT-qPCR,** reverse transcription–quantitative PCR; **SP1,** transcription factor Sp1; **STAT,** signal transducer and activator of transcription; **Tyk2,** tyrosine kinase 2; **UBE2D2,** ubiquitin-conjugating enzyme E2 D2; **uPA,** urokinase plasminogen activator

1 Introduction

Tyrosine kinase 2 (Tyk2) belongs to the Janus kinase (Jak) family of nonreceptor tyrosine kinases. Jaks associate with the intracellular domains of cytokine receptors and are activated upon ligand binding by auto- and/or transphosphorylation. Activated Jaks phosphorylate signal transducers and activators of transcription (STATs), which then translocate to the nucleus as activated homo- or heterodimers where they affect the expression of responsive genes [1, 2]. Type I interferons (IFNs) were the first cytokines described to utilise Tyk2 for signal transduction and subse-

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quently activation of Tyk2 has been shown for a number of other cytokines and some growth factors [3–5]. Cells derived from mice lacking Tyk2 revealed a partial requirement for Tyk2 for IFNα/β and interleukin 12 (IL-12) signalling [6, 7]. *In vivo*, Tyk2 plays an important role in the host defence against microbial pathogens. Tyk2 deficient mice show increased susceptibility to murine cytomegalovirus [8], *Leishmania major* [9] and *Listeria monocytogenes* [10]. In contrast, a negative role of Tyk2 was shown for disease progression in lipopolysaccharide (LPS) and ischemia/reperfusion-induced shock models [11–13]. LPS is the major structural component of the outer membrane of Gram-negative bacteria and a potent activator of immune cells. Macrophages and monocytes are of primary importance for the recognition of and the response to LPS. Activated macrophages produce a variety of cytokines, ROS and other inflammatory mediators that contribute to the effective eradication of invading pathogens. Excessive or deregulated production of these cytokines and mediators, however, can be harmful for the host and lead to the endotoxic shock syndrome [14, 15]. The high resistance of Tyk2$^{-/-}$ mice to LPS prompted us to further investigate the molecular role of Tyk2 for macrophage functions. LPS signalling in macrophages is induced through the Toll-like receptor 4 (TLR4) complex. Basically, LPS treatment leads to the activation of two independent signalling cascades *via* distinct adaptor proteins bound to the cytoplasmic moiety of the receptor chains: one resulting mainly in the activation of nuclear factor-κB (NF-κB) and in the production of pro-inflammatory cytokines and mediators. The second cascade induces gene expression, most prominently IFNβ, *via* activation of members of the interferon regulatory factor (IRF) family. Subsequently, IFNβ activates Tyk2 and Jak1 in an autocrine or paracrine manner and triggers the expression of IFN responsive genes. The successive and concerted action of signalling cascades and their cross-influence determines the complex cellular responses initiated by LPS recognition [16–18].

Proteomics studies on human and murine macrophages have been performed since early days of 2-DE, using cell lines and primary cells of different sources. MS later on allowed the identification of differentially expressed spots when comparing 2-DE patterns under different conditions, and to attribute protein regulations to various stimuli. In a few studies primary murine macrophages (peritoneal or bone marrow-derived) were analysed, *e.g.* under ionising radiation [19], infection with bacteria or protozoa [20], IFNγ activation [21] or LPS stimulation [22, 23].

In our study, we focused on the influence of Tyk2 on the macrophage proteome both in the untreated and the LPS treated state. Using 2-D DIGE technology we show complex consequences of the lack of a single protein on the overall protein expression pattern. We identified by MALDI and ESI MS 21 different proteins from 25 spots, which are either up- or downregulated in the absence of Tyk2. Proteins from various functional categories were identified, thereby suggesting an influence of Tyk2 on various cellular processes. More

detailed analyses on selected proteins showed that this is exerted at mRNA and/or protein level further underscoring the importance of proteomics studies.

2 Materials and methods

2.1 Animals and cells

Tyk2 knockout mice have been described previously [6] and were on C57BL/6 background. Animals were housed under specific pathogen-free conditions and were sex- and age-matched (8–12 wk) for each experiment. Bone marrow-derived macrophages were isolated and grown in the presence of macrophage colony-stimulating factor 1 (CSF-1) derived from L929 cells as described previously [24]. After cultivation for 6 days cells were treated with 100 ng/mL LPS (*Escherichia coli* serotype 055:B5, Sigma, St. Louis, MO, USA).

2.2 Sample preparation

2.2.1 Whole cell lysates

Cells (5×10^6) were lysed in 100 μL lysis buffer (50 mM Tris-HCl pH 8.0; 10% glycerol v/v; 0.5% NP-40 v/v; 0.1 mM EDTA; 150 mM NaCl; 2 mM DTT; phosphatase inhibitors: 2 mM sodium orthovanadate; 25 mM sodium fluoride and protease inhibitors: 2 mM PMSF; 2 μg/mL aprotinin; 2 μg/mL leupeptin; 2 μg/mL pepstatin). Cell debris was removed by centrifugation at $14\,000 \times g$. For 2-D DIGE samples were lyophilised and dissolved in DIGE lysis buffer (7 M urea; 2 M thiourea; 4% CHAPS w/v; 30 mM Tris-HCl pH 8.5).

2.2.2 Nuclear extracts

Nuclear extracts were produced as previously described [25] with a few modifications. Cells (1.5×10^7) were collected in 1 mL PBS and pelleted by centrifugation at $1500 \times g$ for 5 min. The pellets were resuspended in 1.5 mL cold buffer A (10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 2 mM DTT and phosphatase/protease inhibitors as described above). They were allowed to swell on ice for 15 min, NP-40 was added to a final concentration of 0.6% v/v. The mixtures were vortexed vigorously for 10 s followed by spinning at $13\,000 \times g$ for 1 min at 4°C. The nuclear pellets were washed with cold PBS and then incubated with benzonase (1000 units; Sigma, purity >90%) in 15 μL H$_2$O *per* sample for 15 min at room temperature. Pellets were resuspended in 1 mL cold buffer B (20 mM HEPES pH 7.9; 400 mM NaCl; 1 mM EDTA; 1 mM EGTA; 2 mM DTT and protease inhibitors) and incubated for 45 min at 4°C with vigorous shaking. Insoluble material was removed by centrifugation at $13\,000 \times g$ for 5 min at 4°C, and the supernatants were kept for further analysis ('nuclear extracts').

2.2.3 TCA precipitation

Nuclear extracts (whole sample from 1.5×10^7 cells) were mixed with 0.25 volumes of ice-cold 6.1 M TCA solution. DTT was added to a final concentration of 20 mM. The extracts were incubated for 2 h on ice to allow complete precipitation. The samples were centrifuged at $10\,000 \times g$ for 10 min at 4°C. The supernatant was discarded and the pellet was washed three times with 1 mL ice-cold acetone containing 20 mM DTT. The pellet was air-dried and dissolved in DIGE lysis buffer (7 M urea; 2 M thiourea; 4% CHAPS w/v; 30 mM Tris) by shaking overnight at 4°C. The pH of the samples was adjusted to pH 8.5 with 100 mM Tris. Prior to separation on 2-DE, nuclear extracts were tested for reproducibility and quality with western blot analyses, using transcription factor Sp1 (SP1) as a nuclear and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a cytosolic marker protein. Protein concentration in both, whole cell and nuclear extracts was determined by the Coomassie G-250 protein binding assay [26].

2.3 DIGE labelling

Twenty-five micrograms of protein *per* sample were labelled with CyDye Fluor minimal dyes (GE Healthcare Life Sciences, Munich, Germany) according to the manufacturer's instructions, except for a lower coupling rate. For whole cell lysates 4 nmol dye/mg protein were used (as described previously [27]), for nuclear extracts 2 nmol dye/mg protein were found optimal to avoid artefacts due to multiple labelling of lysine residues. Three biological replicates were used *per* experiment. Untreated samples from wild-type and Tyk2$^{-/-}$ macrophages were labelled with Cy3, corresponding treated samples with Cy5. The internal standard, prepared by pooling 25 µg of protein from each sample included in an experimental set, was labelled with Cy2. Two samples (Cy3, Cy5) and the internal standard (Cy2) were separated on each gel.

2.4 2-DE separation

The first dimension was carried out on an IPGphor III system using 24 cm IPG Dry strips with linear pH gradients (all GE Healthcare). The IPG strips pH 4–7 were loaded with the samples through passive in-gel rehydration for 10 h at room temperature. The three labelled samples were mixed and the volume was adjusted to 450 µL with rehydration buffer (7 M urea; 2 M thiourea; 4% CHAPS w/v; 70 mM DTT; 1% IPG buffer 4–7 v/v). For IPG strips pH 6–9 samples were applied by anodic cup loading. The strips were rehydrated overnight in 450 µL rehydration buffer (7 M urea; 2 M thiourea; 4% CHAPS w/v; 150 mM DTT; 2% IPG buffer 6–11 v/v). The mixed samples were adjusted to 50 µL in rehydration buffer and loaded onto the strips *via* loading cups. The proteins were focused for 30 kV·h in a step gradient with a maximum of 3500 V. The focused IPG strips were reduced 10 min

(1.25% DTT w/v) and alkylated 10 min (2.5% w/v iodoacetamide) in equilibration buffer (6 M urea; 30% glycerol v/v; 75 mM Tris-HCl pH 8.8; 2% SDS w/v). The equilibrated strips were placed onto 10%T or 11.5%T polyacrylamide gels and sealed with 0.5% agarose in SDS running buffer. SDS-PAGE was performed according to Laemmli [28]. Gels were run overnight at 13 mA *per* gel in an Ettan Dalt Six electrophoresis chamber (GE Healthcare).

2.5 2-DE evaluation

All gels were scanned at 100 µm resolution on a Typhoon 9400 imager and analysed with DeCyder software V5.02 (all GE Healthcare). Evaluation was based on volume ratios (sample spot volume divided by the corresponding internal standard spot volume). Protein spots differentially expressed between groups were extracted, using volume ratios and Student's *t*-test as selection criteria. Based on reverse labelling experiments spots showing dye labelling bias were excluded from the candidate list. For figures and tables, expression levels were calculated relative to unstimulated wild-type cells $\pm$ SD.

2.6 Statistical analysis

For statistical analyses the volume ratios were used. A linear model was fitted with genotype (*gt*), LPS-treatment (*tr*), and the interaction between these two (*gt* $\times$ *tr*) as fixed effects. The influence of the individuals was considered as a random effect. Significance of the effects was tested using a moderated *t*-test [29]. The residual variances of all spots from one experiment were assumed to be prior F-distributed. In an empirical Bayes fashion, the parameters for these F-distributions were estimated from the data using maximum likelihood. The moderated variance of each gene was set to the posterior maximum. With this variance, a moderated *t*-value was calculated for the two relevant fixed effects *gt* and *gt* $\times$ *tr*. The moderated *t*-test method strikes a balance between using a *t*-test, where the interference of the variance is compromised by the low sample size for each gene, and using a fold-change approach, where differences among spots in variance are ignored entirely.

2.7 MS

Protein spots were detected by acidic silver nitrate staining as previously described [30], making it MS-compatible by omitting glutaraldehyde from the sensitiser and formaldehyde from the silver nitrate solution. Spots of interest were manually excised and pooled for MS analyses. Semi-preparative 2-DE gels with a protein load of 150–200 µg were used to recover enough material for MS-analysis of faint spots. All spots were destained as previously described [31].

2.7.1 Protein identification by MALDI-MS

In-gel digestion was performed according to [32] with slight modifications. Gel pieces were equilibrated with 25 mM ammonium bicarbonate, washed with ACN, dried in a vacuum centrifuge and rehydrated in 25 mM ammonium bicarbonate containing 12.5 ng/µL sequencing grade trypsin (Roche, Mannheim, Germany) at 4°C for 30 min. Excess of trypsin working solution was removed, samples were covered with 25 mM ammonium bicarbonate and incubated overnight at 37°C. Supernatant was collected and the elution of the peptides was performed twice with 66% ACN/33% 0.1% TFA. The combined aliquots were dried in a vacuum centrifuge and dissolved in 0.1% TFA. Samples were purified and concentrated with ZipTip$_{\mu-}$c$_{18}$ pipette tips (Millipore, Billerica, MA, USA) and analysed on a MALDI-TOF/reflectron TOF-instrument (TOF^2; Shimadzu Biotech Kratos Analytical, Manchester, UK) in the positive ion mode with delayed extraction applying a thin layer preparation technique as described earlier [33]. External calibration was performed using monoisotopic values of the singly charged ions of an aqueous solution of standard peptides (bradykinin fragment 1–7, human angiotensin II, P14R, ACTH fragment 18–39). Autolytic tryptic products, matrix clusters [34], keratin and gel blank artefacts [35] were removed from the mass spectra and the resulting mono-isotopic list of *m/z* values was submitted to search engines MASCOT (Revision 2.1.0 to 2.2.0) [36] and ProFound [37] searching the databases Swiss-Prot (Version 50.6 of 05-Sep-2006 to 54.5 of 04-Nov-2007) and NCBI (Sequence Release 19 of 10-Sep-2006 to 26 of 13-Nov-2007). Search criteria were: taxonomy, *Mus musculus*; mass accuracy, 200 ppm; fixed modifications, carbamidomethylation; variable modifications, methionine oxidation and acetylation at the protein N-terminus, maximum one missed cleavage site. PSD experiments were performed on selected peptides from the measured PMFs. MS/MS database searches have been performed using the same restrictions as for PMF, additionally a product ion tolerance of ± 1 Da was defined. A hit was considered significant, if the scores of at least two database searches, obtained for PMF data and/or PSD data, independently exceeded the algorithm's significance threshold ($p < 0.05$).

2.7.2 Protein identification by ESI-MS

Spot plugs were digested with trypsin as previously described [31]. All samples were run on an LTQ linear ion mass spectrometer (Thermo Scientific, San Jose, CA, USA) connected to a Surveyor chromatography system incorporating an autosampler as previously described [38]. Tryptic peptides were resuspended in 0.1% formic acid and were separated by means of a SurveyorLC system (Thermo Scientific) connected directly to the source of the LTQ. Each sample was loaded onto a Biobasic C18 Picofrit™ column (100 mm length, 75 µm id) at a flow rate of 30 nL/min. The samples were then eluted from the C18 Picofrit™ column by an increasing ACN gradient. The mass spectrometer was operated in positive ion mode with a capillary temperature of 200°C, a capillary voltage of 46 V, a tube lens voltage of 140 V and with a potential of 1800 V applied to the frit. All data were acquired with the mass spectrometer operating in automatic data-dependent switching mode. A zoom scan was performed on the five most intense ions to determine charge state prior to MS/MS analysis. All MS/MS spectra were analysed using BioworksBrowser (Version 3.2; Thermo) using the TurboSEQUEST® algorithm under default settings. The MS/MS spectra were searched against the Swiss-Prot database (Uniprot_sprot; Release 10.0). The following search parameters were used: precursor ion mass tolerance of 1.5 Da, fragment ion tolerance of 1.0 Da with methionine oxidation and cysteine carboxyamidomethylation specified as differential modifications and a maximum of two missed cleavage sites allowed.

2.8 Western blot analysis

1-D Western blots were performed on small-size SDS-PAGE gels as previously described [8].

2-D Western blot: 2-DE was performed as above except that we used 10 cm laboratory-made pH 6–10 IPG strips [39] with 50 µg protein applied by anodic cup loading. Focusing was carried out without oil for a total of 13 kV·h on a Multiphor II system (GE Healthcare). The second dimension on $14 \times 14 \times 0.15$ cm^3 gels and semidry blotting were performed as previously described [27].

The following antibodies were used: goat anti-NMI, rabbit anti-PAI2, rabbit anti-SP1, donkey anti-goat IgG-HRP (all Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit anti-EF2 (Cell Signalling, Danvers, MA, USA); goat anti-PRDX1 (R&D Systems, Wiesbaden-Nordenstadt, Germany); mouse anti-extracellular signal regulated kinase (ERK) (BD Biosciences Transduction Laboratories, Lexington, KY, USA); rabbit anti-GAPDH (Biotrend, Cologne, Germany); donkey anti-rabbit IgG-HRP F(ab)$_2'$ fragment; sheep anti-mouse IgG-HRP F(ab)$_2'$ fragment (all GE Healthcare).

2.9 Reverse transcription–quantitative PCR (RT-qPCR)

Total RNA was isolated from 10^6 cells using TRIzol (Invitrogen Life Technologies, Lofer, Austria) according to the manufacturer's instructions. RNA was treated with DNase I (Promega Corporation, Mannheim, Germany) to eliminate genomic DNA. Total RNA (1.5 µg) was used for cDNA synthesis using iScript (BioRad Laboratories, Vienna, Austria). Primers and probes were designed using Primer Express (Applied Biosystems, Vienna, Austria) software. Probes (Genexpress, Wiener-Neudorf, Austria) were labelled with 6-carboxyfluorescein (FAM) and Blackhole Quencher 1 (BHQ1) at the 5′ and 3′ ends, respectively. The following primers (Invitrogen Life Technologies) and probes were used, all 5′ to 3′ direction: *PAI2* fwd: ACTCAGATCCTAGAACTTCCGCAT; rev: AAAGTTTATTTCACTTTCCAGCAATTC; probe: CATGCTCCTGTTGCTTCCCGATGAGA;

NMI fwd: GCCAAGCGCTCATCACCTT; rev: CCTTCCATC TGCACGACATG; probe: AGAAGTCGCACAAAATGTGA- TATCGATGGG; *PRDX1* fwd: CTAGTCCAGGCCTTC- CAGTTCA; rev: TCAGGCTTGATGGTATCACTGC; probe: AACATGGTGAAGTGTGTCCAGCTGGCTG. RT-qPCR was performed in duplicate on Eppendorf realplex4 (Eppendorf, Vienna, Austria). Gene expression was calculated as pre- viously described [11] except that ubiquitin-conjugating en- zyme E2 D2 (*UBE2D2*) was used as endogenous control [8]. Expression levels were calculated relative to unstimulated wild-type cells ± standard error (SE).

3   Results

3.1   2-D DIGE protein patterns of primary murine macrophages are highly reproducible

We compared protein patterns of wild-type and Tyk2$^{-/-}$ bone marrow-derived macrophages before and after LPS treat- ment in whole cell lysates and in nuclear extracts, respec- tively. Both types of cell extracts were analysed in two differ- ent pH gradients (pH 4–7 and 6–9, respectively), thus yield- ing four 2-D DIGE experiments. Each experiment comprised three biological replicates *per* genotype and treatment resulting in six analytical gels. Representative 2-DE protein patterns are shown in Fig. 1. Depending on the specific experiment, we detected 478–792 spots that were present in all images (Table 1) and only those spots were included in the further examinations. Spot volume ratios of biological repli- cates showed low variability with mean SDs between ±0.073 and ±0.085 (Table 1 and Fig. S1 of Supporting Information). For a given statistical power of 0.9, a *p*-value of ≤0.05 (two- sided), the sample size of three, and the respective mean SD, the resulting minimal detectable differences (effect size) in expression levels were between 26.2 and 30.5%. Thus, on average, differences in expression values of around 30% can be detected with our experimental set-up.

Nuclear extracts comprised about 10% of total proteins found in whole cell lysates and protein patterns were clearly
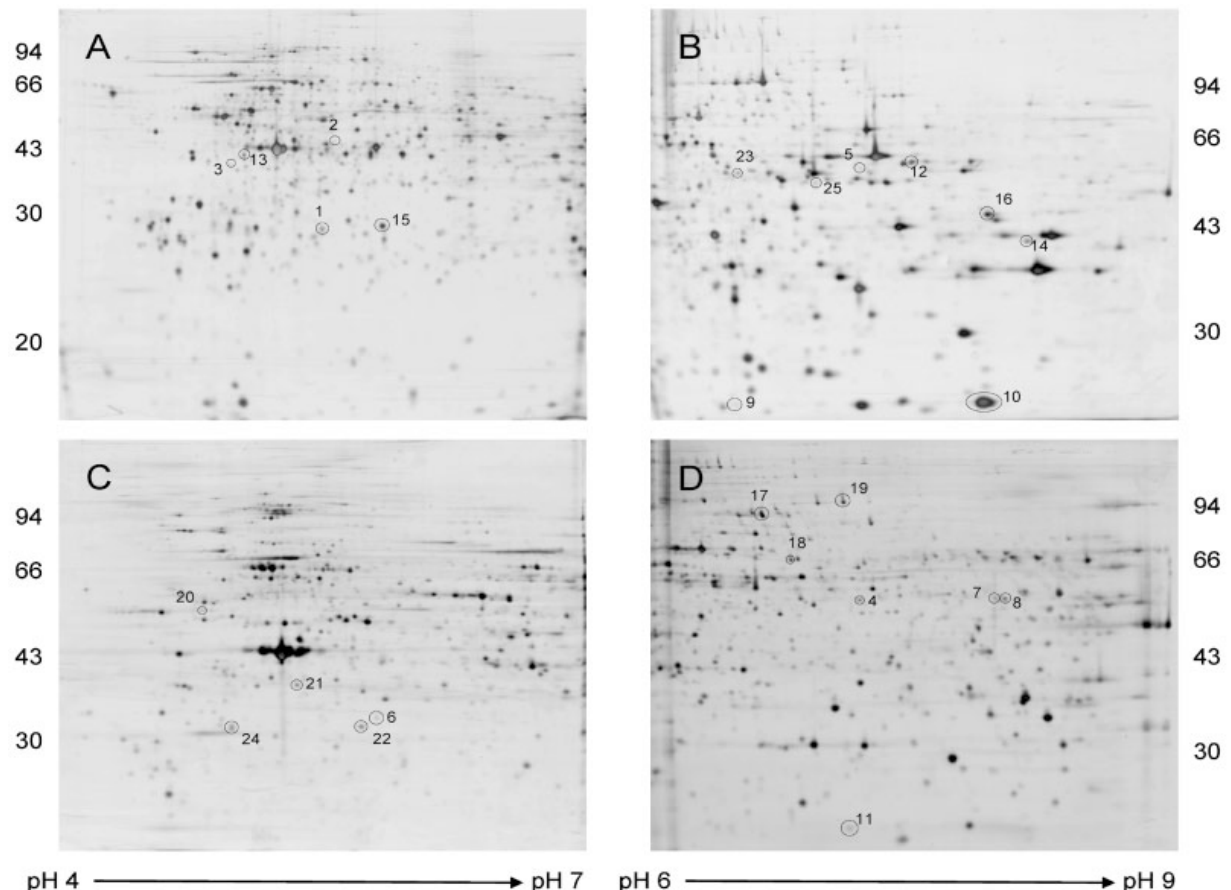
Figure 1. Representative 2-DE protein patterns of Tyk2$^{-/-}$ macrophages. Analytical gels (75 µg protein each) visualised by MS-compatible silver stain after 2-D DIGE. Identified proteins are indicated by the spot numbers used in Table 3. Whole cell lysates: (A) pH gradient 4–7, 11.5%T SDS-PAGE; (B) pH 6–9, 10%T SDS-PAGE. Nuclear extracts: (C) pH 4–7; (D) pH 6–9; both: 10%T SDS-PAGE.

Table 1. Experiments performed and their statistical properties

	Whole cell lysates ±18 h LPS		Nuclear extracts ±8 h LPS	
pH	4–7	6–9	4–7	6–9
Number of spots in all 18 images	579	478	731	792
Mean SD	0.073	0.078	0.085	0.085
Minimal detectable difference	26.2%	28.0%	30.5%	30.5%

different, although with some overlaps (Fig. 1). This standard protocol for the preparation of nuclear extracts does not result in the complete extraction of nuclear proteins and can also lead to copurification of proteins from other compartments, *e.g.* plasma membrane, ER, cytoskeletal fragments and large cytoplasmic protein complexes. Nevertheless, extracts showed good reproducibility and enrichment of a subset of cellular proteins, which might not be detected in whole cell extracts. Western blot analysis with specific marker proteins showed hardly detectable levels of GAPDH, a cytosolic marker protein, and high amounts of SP1, a nucleus specific protein (see Fig. S2 of Supporting Information).

3.2 Absence of Tyk2 significantly alters the protein expression pattern in macrophages

The analyses with respect to the impact of Tyk2 deficiency on the macrophage proteome were performed with an emphasis on the effects on basal protein expression (genotype effects) and on LPS mediated changes in expression levels (genotype × treatment effects). Statistically significant differences were found for a large number of spots in both cases (see Fig. S3 of Supporting Information). We filtered these spots for differences between wild-

type and Tyk2$^{-/-}$ cell extracts of at least 40% before and/ or after LPS treatment. Forty-six spots in whole cell lysates and seventy-three in nuclear extracts met these criteria (Table 2), this corresponds to 3–6% of spots examined, depending on the specific experiment. Positive as well as negative effects of Tyk2 deficiency on expression levels were found with similar distribution in all cases. Among the 119 differentially expressed spots, 75 spots displayed genotype effects, 13 spots genotype × treatment effects and 31 spots showed both effects. A similar predominant genotype effect was found if data were analysed without filtering for the minimal difference of 40% (see Fig. S3 of Supporting Information).

3.3 Differentially expressed proteins exert various functions

From among the 119 differentially expressed spots, 34 spots were selected for protein identification. The selection was based on a combination of the following criteria: expression pattern in the 2-D DIGE analysis (extent of difference between the genotypes before and after treatment), stainability of spots with silver (for colorimetric visualisation on the gels enabling manual spot cutting), spot quantity (spot density in silverstained patterns supplying sufficient amounts for MS analysis) and spot quality (shape and compactness of spots, nonoverlap with others). Usually selection criteria were used with equal emphasis, where impossible, the most common denominator of selectable criteria was used for decision making.

Using LC-ESI- and MALDI-MS/MS, 25 of the 34 selected spots gave interpretable MS spectra, which equates to a success rate of 73.5%. In these spots, 21 different proteins were identified (see Fig. 2 for 2-D DIGE spot images and Table S1 of Supporting Information for details of MS analysis). The differentially expressed proteins (listed in Table 3) were classified according to their gene ontology annotation. Identified proteins belong to various functional categories including

Table 2. Numbers of differentially expressed and identified spots[a]

Experiment	Total	*gt*[b]		*gt* × *tr*[c]		*gt* + *gt* × *tr*[d]		ID[e]
		Up[f]	Down[f]	Up	Down	Up	Down	
Whole cell lysates (pH 4–7)	17	2	–	3	–	7	5	5
Whole cell lysates (pH 6–9)	29	4	5	5	4	6	5	8
Nuclear extracts (pH 4–7)	33	22	14	–	1	1	2	5
Nuclear extracts (pH 6–9)	40	11	17	–	–	2	3	7
Total	119	39	36	8	5	16	15	25 (21 proteins)

a) At least 40% difference in expression ± LPS treatment between genotypes, $p \leq 0.05$.
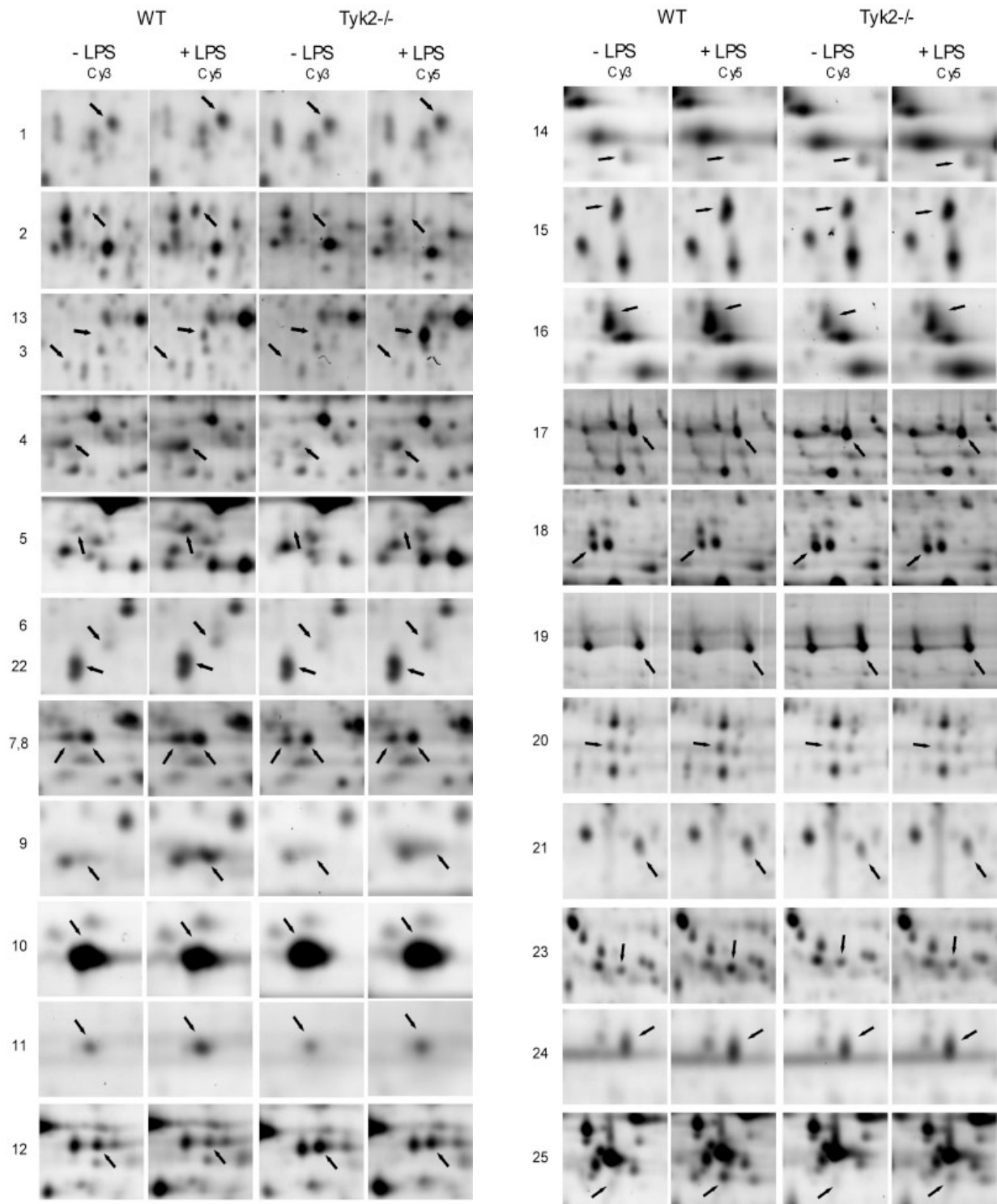b) Genotype effect (significant differences between genotypes under basal conditions).
c) Genotype × treatment effect (significant differences between genotypes in response to LPS treatment).
d) Genotype and genotype × treatment effect.
e) Identified spots.
f) Up/downregulated in the absence of Tyk2.

Figure 2. Comparison of proteome pattern in wild-type *versus* Tyk2$^{-/-}$ macrophages with or without LPS treatment. Selected regions from 2-D DIGE images are shown (compare Fig. 1 for exact spot location on the whole gel images). Cy3/Cy5 images converted to grey scale for all 25 spots identified (indicated by arrows and the spot numbers used in Fig. 1 and Table 3) are depicted. See legend to Fig. 1 and Table 3 for details on protein extracts and electrophoretic conditions.

Table 3. Differentially expressed proteins in Tyk2$^{-/-}$ macrophages

Spot no.	Protein name	Accession no. Swiss-Prot	WT	WT + LPS	Tyk2$^{-/-}$	Tyk2$^{-/-}$ + LPS	p (gt)[a]	p (gt × tr)[b]	M_r (kDa) theoret.	pI theoret.	MS method	Protein extract[c]
Immune response												
1	Proteasome activator complex subunit 2 (PSME2)	P97372	1	1.30	0.76	0.92	0.0033	ns[d]	27	5.5	ESI	A
2	Interferon-induced protein with tetratripeptide repeats 3 (IFIT3)	Q64345	1	3.12	0.51	0.65	0.0021	0.0021	47.2	5.5	ESI	A
3	N-myc-interactor (NMI)	O35309	1	1.27	0.59	0.48	0.0004	0.0025	35.2	5	ESI MALDI	A
4	Interferon-induced protein with tetratripeptide repeats 1 (IFIT1)	Q64282	1	1.65	0.38	0.73	0.0006	ns	53.7	7.2	MALDI	D
5	Interferon-induced protein with tetratripeptide repeats 1 (IFIT1)	Q64282	1	3.95	0.67	0.62	ns	0.0010	53.7	7.2	MALDI	B
6	Interferon-induced protein 35 kD homologue (IIN35)	Q9D8C4	1	1.26	0.54	0.73	0.0002	0.0468	31.8	5.6	MALDI	C
7	Interferon-activable protein 205 (IFI5) or interferon-activable protein 204 (IFI4)[e]	Q08619/P15092	1	1.31	0.60	1.03	0.0014	0.0129	47/71.6	8.4/9	MALDI	D
8	Interferon-activable protein 205 (IFI5) or interferon-activable protein 204 (IFI4)[e]	Q08619/P15092	1	1.27	0.56	0.97	0.0014	0.0129	47/71.6	8.4/9	MALDI	D
Oxidative stress response												
9	Peroxiredoxin-1 (PRDX1)	P35700	1	3.94	1.19	1.99	0.0221	0.0012	22.2	8.3	MALDI	B
10	Peroxiredoxin-1 (PRDX1)	P35700	1	0.68	1.08	1.28	ns	0.0044	22.2	8.3	MALDI	B
11	Peroxiredoxin-1 (PRDX1)	P35700	1	1.78	0.98	1.06	ns	0.0197	22.2	8.3	MALDI	D
12	Catalase (CATA)	P24270	1	0.60	1.48	1.13	0.0024	0.0257	59.8	7.7	MALDI	B
Apoptosis												
13	Plasminogen activator inhibitor 2 (PAI2)	P12388	1	4.73	1.97	30.0	0.0010	0.0006	46.3	5	MALDI	A
Metabolism												
14	3-Ketoacyl-CoA thiolase A (THIKA) or 3-ketoacyl-CoA thiolase B (THIKB)[e]	Q921H8/Q8VCH0	1	0.54	1.12	1.04	ns	0.0036	44	8.7	MALDI	B
15	Purine nucleoside phosphorylase (PNPH)	P23492	1	1.56	0.66	0.99	0.0013	ns	32.2	5.8	ESI	A
16	Argininosuccinate synthase (ASSY)	P16460	1	1.55	0.60	0.98	0.0024	ns	46.6	8.4	MALDI	B
Translation, transcription												
17	Elongation Factor 2 (EF2)	P58252	1	0.84	1.59	1.37	0.0016	ns	95.3	6.4	MALDI	D
18	Phenylalanyl-tRNA-synthase β chain (SYFB)	Q9WUA2	1	0.79	1.34	1.24	0.0064	ns	65.7	6.7	MALDI	D

Table 3. Continued

Spot no.	Protein name	Accession no. Swiss-Prot	WT	WT + LPS	Tyk2$^{-/-}$	Tyk2$^{-/-}$ + LPS	p (gt)[a]	p ($gt \times tr$)[b]	M_r (kDa) theoret.	p*I* theoret.	MS method	Protein extract[c]
19	Staphylococcal nuclease domain-containing protein 1 (SND1)	Q78PY7	1	0.87	1.54	1.38	0.0006	ns	102.1	7.1	MALDI	D
Cytoskeleton												
20	Vimentin (VIME)	P20152	1	1.35	0.55	0.60	0.0002	ns	53.7	5.1	MALDI	C
21	F-actin capping protein subunit α-1 (CAZA1)	P47753	1	1.29	0.70	0.77	0.0011	ns	32.9	5.3	MALDI	C
22	F-actin capping protein subunit β (CAPZB)	P47757	1	1.20	0.68	0.75	0.0014	ns	31.3	5.5	MALDI	C
23	Adenyl cyclase-associated protein 1 (CAP1)	P40124	1	1.92	1.23	1.19	0.0102	0.0007	51.6	7.2	MALDI	B
Others												
24	EF-hand domain containing protein 2 (EFHD2)	Q9D8Y0	1	1.38	0.73	0.78	0.0016	ns	26.8	5	MALDI	C
25	Immune-responsive protein 1 (IRG1)	P54987	1	4.42	0.75	1.90	ns	0.0014	53.8	7.1	MALDI	B

a) *p*-Values for the differences between genotypes under basal conditions.
b) *p*-Values for the differences between genotypes in response to LPS treatment.
c) Protein extracts: A, whole cell lysates pH 4–7; B, whole cell lysates pH 6–9; C, nuclear extracts pH 4–7; D, nuclear extracts pH 6–9.
d) Not significant (*p*-value>0.05).
e) Proteins with high sequence homology.

oxidative stress and immune responses, metabolism and cytoskeleton architecture. In several cases we identified the same protein in different spots on one gel or in both protein extracts. For instance, interferon-induced protein with tetratripeptide repeats 1 (IFIT1) was identified in whole cell lysates and nuclear extracts located at p*I* 7.2 and M_r of 60 kDa (spots 4 and 5 in Table 3, Figs. 1B, D and 2). Both spots showed upregulation by LPS treatment in wild-type cells, whereas the effect of Tyk2 deficiency on basal expression and LPS induction was different for the two spots. Interferon-activable protein 204 or 205 (IFI4/5) was found in two neighbouring spots showing identical expression patterns (spots 7 and 8 in Table 3, Figs. 1D and 2). We could not distinguish between IFI4 and IFI5 since they display high sequence homology and only peptides in common were found in MS. Six known IFN-inducible proteins (proteasome activator complex subunit 2 (PSME2), IFIT1, IFIT3, IFI4/5, interferon-induced protein 35 kD homologue (IN35), N-myc-interactor (NMI)) were identified. All of them showed upregulation upon LPS treatment in wild-type cells. Interestingly, all of them displayed reduced basal expression levels and, except for PSME2, reduced or even absent LPS-mediated upregulation in Tyk2-deficient cells in at least one of the corresponding spots (spots 1–8 in Table 3 and Fig. 2).

From the list of differentially regulated proteins (Table 3 and Fig. 2) we selected several candidates based on their regulation patterns or their molecular functions for further investigations. We performed time course studies of protein and mRNA expression using 1-D Western blotting and RT-qPCR, respectively. When a complex spot pattern was assumed for a particular protein 2-D Western blots were performed in addition.

3.3.1 Tyk2 is required for efficient expression of NMI protein and mRNA

According to the 2-D DIGE analysis, NMI showed approximately 0.6-fold expression levels before and after LPS treatment in Tyk2$^{-/-}$ as compared to wild-type cells (spot 3 in Table 3 and Figs. 2 and 3A). We subsequently used 1-D Western blot analysis for validation of expression patterns. The amount of NMI protein was clearly reduced in Tyk2$^{-/-}$ cells upon 0 and 18 h LPS treatment (Fig. 3B). The relatively modest upregulation of NMI protein upon LPS treatment in wild-type cells found with 2-D DIGE technology (1.3-fold) was only just about detectable with Western blot analysis. Similar results were obtained in a more extensive time course experiment (data not shown). We next examined by RT-qPCR whether the reduced protein level in Tyk2$^{-/-}$ cells
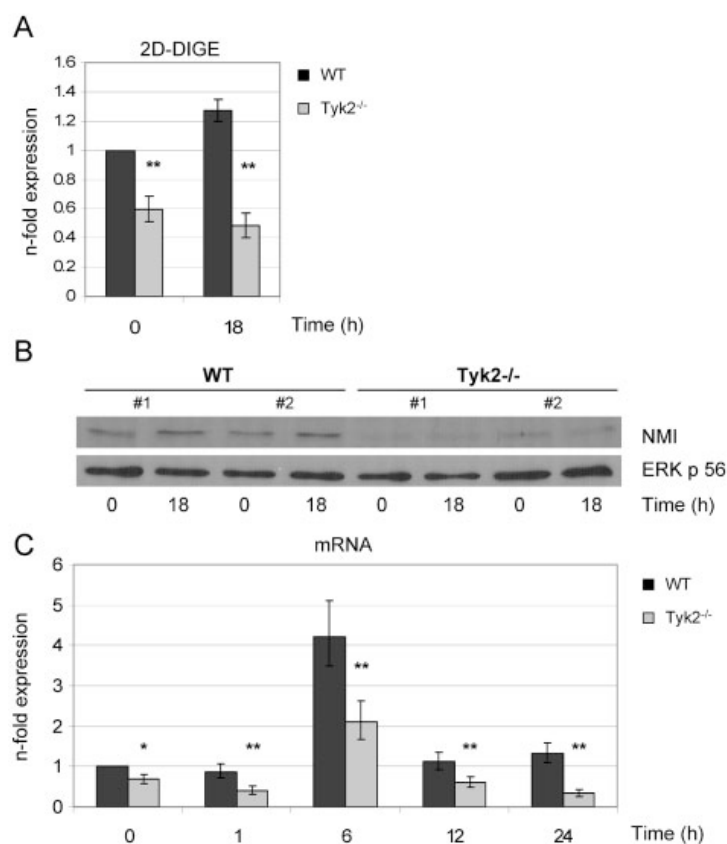
Figure 3. Effect of Tyk2 deficiency on the expression of NMI protein (A, B) and mRNA (C). Macrophages were treated with LPS for the indicated times and whole cell lysates or total RNA were subjected to 2-D DIGE analysis (A), Western blotting (B) or RT-qPCR (C). (A) 2-D DIGE: expression levels are given as fold ratios relative to unstimulated wild-type (WT) cells. Mean values ±SD of three biological replicates are shown. (B) Western blot analysis: 20 µg protein *per* lane were separated by 10%T SDS-PAGE. Protein loading was controlled by reprobing with an anti-panERK antibody. Data are representative of at least three independent experiments. Nos. 1 and 2 indicate that the cells were derived from independent mice. (C) mRNA expression was analysed by RT-qPCR. *UBE2D2* was used as endogenous control, expression levels were calculated relative to untreated WT cells. Mean values ±SE from at least three independent experiments are depicted. * $p \leq 0.05$, ** $p \leq 0.01$.

is due to lower mRNA expression. A modest and transient upregulation of mRNA was detectable upon 6 h of LPS treatment in both wild-type and Tyk2$^{-/-}$ macrophages (Fig. 3C). However, mRNA levels were consistently lower in the absence of Tyk2 throughout the time course investigated.

3.3.2 Tyk2 negatively regulates plasminogen activator inhibitor 2 (PAI2) protein but not mRNA expression

Expression levels of PAI2 were particularly interesting since Tyk2$^{-/-}$ macrophages displayed elevated basal expression as well as a strongly enhanced LPS mediated upregulation (spot 13 in Table 3 and Figs. 2 and 4A). Strong induction of PAI2 protein by LPS treatment was detectable in both genotypes and enhanced expression in the absence of Tyk2 could be clearly confirmed by 1-D Western blot analysis (Fig. 4B). PAI2 protein expression could be detected from 8 h LPS stimulation onwards (data not shown) with stable maximal expression from about 18–24 h of treatment (Fig. 4B). Increased PAI2 protein level in Tyk2$^{-/-}$ cells was observed throughout all time points with detectable PAI2 expression (Fig. 4B and data not shown). In both 2-D DIGE and 1-D Western blot analysis we detected PAI2 with the M_r of approximately 42 kDa, which resembles the nonglycosylated form of PAI2 reported in the literature [40]. PAI2 mRNA

expression was not affected by the absence of Tyk2 (Fig. 4C). Expression was rapidly induced in wild-type as well as Tyk2$^{-/-}$ macrophages, reached maximal expression levels at around 6–12 h LPS treatment and modestly declined thereafter (Fig. 4C).

3.3.3 Absence of Tyk2 affects peroxiredoxin 1 (PRDX1) spot patterns

PRDX1 was identified in three spots with similar molecular weight (23 kDa) but distinct p*I*s of about 6.5, 7.2 and 8 (Table 3, spots 9–11). Two spots were identified from whole cell lysates (spots 9 and 10 in Table 3, Figs. 1B and 2) and one from nuclear extracts (spot 11 in Table 3, Figs. 1D and 2). For validation experiments, we concentrated on whole cell lysates in order to define the total spot composition rather than to address possible differences in subcellular distributions. According to the 2-D DIGE data, spot 9 was upregulated and spot 10 was downregulated by LPS treatment in wild-type cells (Table 3 and Fig. 5A). In the absence of Tyk2, spot 9 showed slightly elevated basal expression and reduced upregulation upon LPS treatment. In contrast, spot 10 was only modestly but conversely regulated by LPS in wild-type and Tyk2$^{-/-}$ cells resulting in a 1.9-fold higher level in Tyk2$^{-/-}$ cells as compared to stimulated wild-type cells (Table 3 and Fig. 5A). Surprisingly, we could not find an
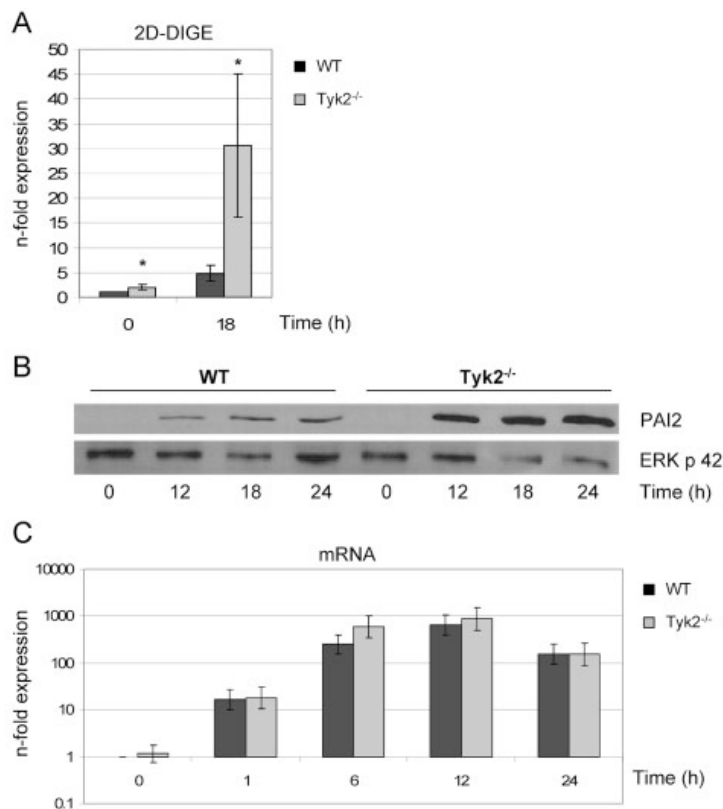
Figure 4. Effect of Tyk2 deficiency on the expression of PAI2 protein (A, B) and mRNA (C). Macrophages were treated with LPS for the indicated times and whole cell lysates or total RNA were subjected to 2-D DIGE analysis (A), Western blotting (B) or RT-qPCR (C). (A) 2-D DIGE: expression levels are given as fold ratios relative to unstimulated WT cells. Mean values ±SD of three biological replicates are shown. (B) Western blot analysis: 5 μg protein *per* lane were separated by 10%T SDS-PAGE. Protein loading was controlled by reprobing with an anti-panERK antibody. Data are representative of at least three independent experiments. (C) mRNA expression was analysed by RT-qPCR. *UBE2D2* was used as endogenous control, expression levels were calculated relative to untreated WT cells. Mean values ±SE from at least three independent experiments are depicted. * $p \leq 0.05$, ** $p \leq 0.01$.

A

B

C

D
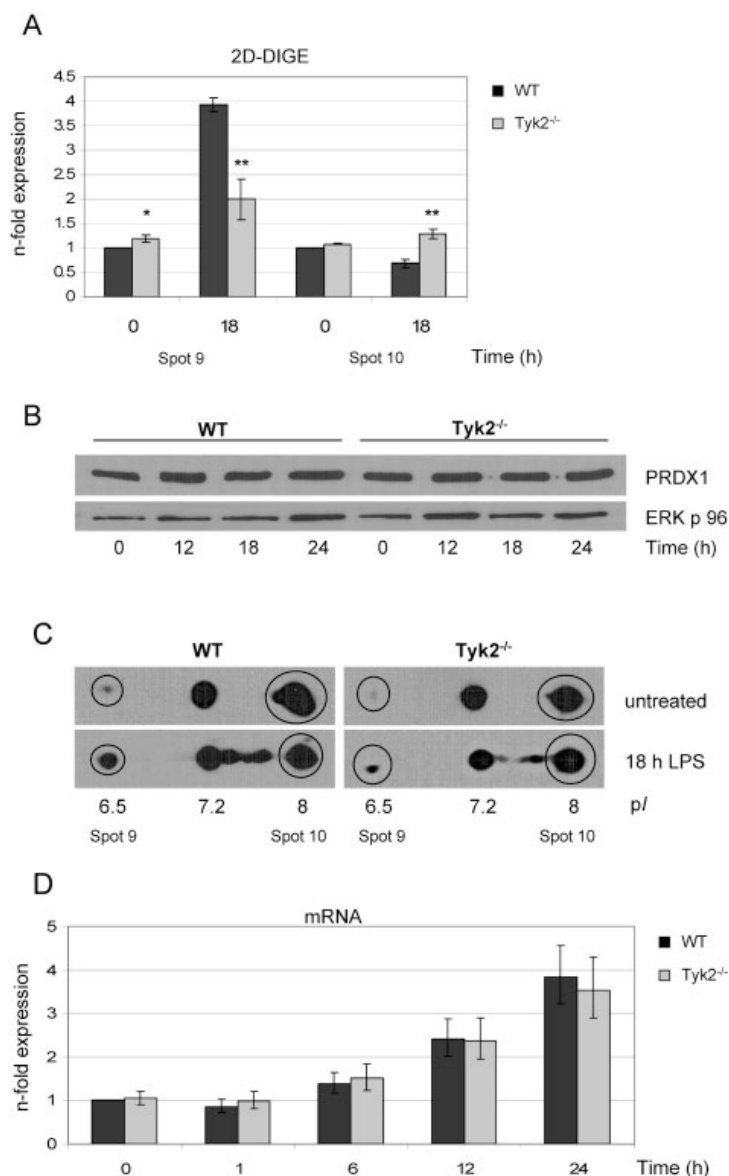
Figure 5. Effect of Tyk2 deficiency on the expression of PRDX1 protein (A–C) and mRNA (D). Macrophages were treated with LPS for the indicated times and whole cell lysates or total RNA were subjected to (A) 2-D DIGE analysis, (B, C) Western blotting or (D) RT-qPCR. (A) 2-D DIGE: expression levels are given as fold ratios relative to unstimulated WT cells. Mean values ±SD of three biological replicates are shown. (B) 1-D Western blot: 5 μg protein *per* lane were separated by 12%T SDS-PAGE. Protein loading was controlled by reprobing with an anti-panERK antibody. Data are representative of at least three experiments. (C) 2-D Western blot: 50 μg protein were separated by 2-DE using a pH 6–10 gradient for the first dimension, followed by the second dimension on a 12%T SDS-PAGE. Spots identified by MS are indicated in circles. Data are representative of three experiments. (D) mRNA expression was analysed by RT-qPCR. *UBE2D2* was used as endogenous control, expression levels were calculated relative to untreated WT cells. Mean values ±SE from three independent experiments are depicted. * $p \leq 0.05$, ** $p \leq 0.01$.

increase in total PRDX1 protein expression upon LPS treatment using 1-D Western blot analysis (Fig. 5B). Similarly, no difference in PRDX1 expression between wild-type and Tyk2$^{-/-}$ cells was detectable.

Figure 1B illustrates that PRDX1 is a multiple spot protein in 2-DE (spots 9 and 10), which all have a similar M_r, thus resulting in the detection of one single band on 1-D Western blots. To get more insight into the protein heterogeneity, we performed 2-D Western blot analysis to determine the number of detectable PRDX1 spots and their relative abundances. As shown in Fig. 5C, three spots were present before and five after LPS treatment in whole cell lysates from both genotypes. Consistent with the 2-D DIGE

data, spot 9 increased clearly after LPS treatment and this was considerably lower in Tyk2$^{-/-}$ than in wild-type cells. In contrast, differences in spot 10 intensities were not apparent. Apart from spots 9 and 10, one additional spot was detected in 2-D Western blot analysis in untreated cells (probably the counterpart of spot 11 identified in nuclear extracts, see Figs. 1B and D). Two further spots appeared upon LPS treatment (Fig. 5C), both with a more acidic p*I* than spot 10. As spot 9, both showed reduced increase in the absence of Tyk2. They were also calculated from 2-D DIGE data (Table 3 and data not shown) and results confirmed the partially Tyk2-dependent acidic spot shift. Although we could not see increased total PRDX1 protein (Fig. 5B) upon LPS treatment,

PRDX1 mRNA expression increased around four-fold with no differences between wild-type and Tyk2$^{-/-}$ cells (Fig. 5D). Thus, Tyk2 deficiency does neither influence total PRDX1 protein nor PRDX1 mRNA expression. Instead, our results indicate that Tyk2 influences the LPS-mediated changes in the PRDX1 spot pattern.

3.3.4 Tyk2 affects elongation factor 2 (EF2) subcellular levels

EF2 was found in nuclear extracts as differentially regulated spot with elevated amounts in Tyk2$^{-/-}$ macrophages independent of LPS treatment (spot 17 in Table 3, Figs. 1D, 2 and 6A). Enhanced expression of EF2 protein in the absence of Tyk2 was also readily detectable by Western blot analysis (Fig. 6B) and, also consistent with the 2-D DIGE data, LPS treatment did not result in any change of protein expression. Interestingly, differential EF2 protein expression between wild-type and Tyk2$^{-/-}$ cells was only found in nuclear extracts and not in whole cell lysates (Figs. 6B and C).

4 Discussion

In this report, we have used a proteomics approach in order to find novel functions of Tyk2 in the response to LPS in primary murine macrophages. Using 2-D DIGE technology for the comparative analysis of whole cell lysates as well as nuclear extracts from wild-type and Tyk2$^{-/-}$ cells, we achieved high reproducibility between biological replicates allowing us to detect differences in protein expression as low as 30%.

Proteomics studies on the LPS response of murine macrophages have been performed in a few older studies [22, 23, 41], but at that time 2-DE patterns were characterised solely by physicochemical properties of the obtained spots (pI, M_r). More recent studies including identification of intracellular LPS responsive proteins employed cell lines rather than primary cells and, in addition, used different comparative 2-DE approaches [42–44]. Accordingly, the sets of proteins identified show generally little overlap with ours. Interestingly, five of the proteins identified in our study (IFIT3, PRDX1, IRG1, vimentin and EF2) have also been described as LPS regulated in microglia cells, a macrophage-like cell type of the CNS [45].

Our results show that absence of Tyk2 significantly alters the overall protein expression pattern at basal conditions and affects changes in expression levels in response to LPS treatment, whereby the former effect predominates. We found 119 spots (corresponding to 3–6% of spots analysed) that showed at least 40% difference in expression values either before or after LPS treatment. We identified 21 different proteins in 25 of these spots which were either up- or downregulated in the absence of Tyk2. The proteins belong to several different functional categories suggesting that the absence of Tyk2 affects many distinct cellular processes. These include oxidative stress and immune response, metabolism, transcription, translation and cytoskeleton architecture.

All proteins involved in immune responses identified herein are known IFN regulated proteins. Consistent with the role of Tyk2 in IFN signalling, they showed reduced expression in the absence of Tyk2. Importantly, basal
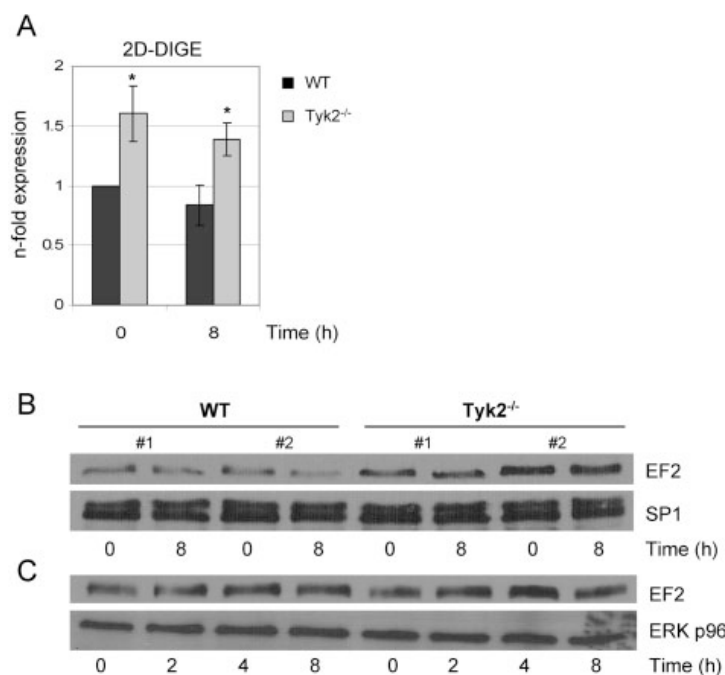
Figure 6. Effect of Tyk2 deficiency on the expression of EF2 protein. Macrophages were treated with LPS for the indicated times and nuclear extracts (A, B) or whole cell lysates (C) were subjected to DIGE analysis (A) or Western blotting (B, C). (A) 2-D DIGE: expression levels are given as fold ratios relative to unstimulated WT cells. Mean values ±SD of three biological replicates are shown. (B) Western blot analysis: 10 μg protein of nuclear extracts were separated by 6.5%T SDS-PAGE. Protein loading was controlled by reprobing with an anti-SP1 antibody. Nos. 1 and 2 indicate that the cells were derived from independent mice. (C) Western blot analysis: 5 μg protein of whole cell lysates were separated by 10%T SDS-PAGE. Protein loading was controlled by reprobing with an anti-panERK antibody. Data are representative of at least three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$.

expression was already reduced in the absence of Tyk2 in at least one of the spots identified, a phenomenon that has been described previously for two IFN-inducible proteins and some mRNAs [6, 8, 11]. Hence our data support the notion of Tyk2 as an essential component for the maintenance of low-level expression of at least a subset of IFN target proteins. Reduced basal expression of NMI in Tyk2$^{-/-}$ cells (approximately 0.6-fold according to the 2-D DIGE data) was confirmed by Western blot analysis and was also found at the mRNA level. NMI was originally found as a protein that interacts with members of the Myc family of proto-oncogenes [46] and subsequently interaction with several other transcription factors was reported. For example, NMI can interact with all STAT proteins (*e.g.* upon IL-2, IFNγ), except STAT2, and enhance their transcriptional activity by increasing their association with CREB-binding protein (CBP) and p300 co-activators [47]. NMI is homologous to IN35 and both proteins can stabilise each other through their interaction [48], thus crossinfluence might contribute to their similarly reduced expression levels in the absence of Tyk2.

We found several proteins differentially expressed in wild-type *versus* Tyk2$^{-/-}$ macrophages which have not yet been linked to either IFN or Tyk2 (Table 3). Three of them (EF2, PAI2 and PRDX1) were analysed in more detail. We found increased protein expression of EF2 in the absence of Tyk2 specifically in nuclear extracts but not in total lysates, suggesting a difference in subcellular localisation. Due to our relatively crude nuclear extract preparation, the exact localisation cannot be determined and further studies including highly purified organelle preparations will be required. EF2 is critically involved in protein translation where it catalyses elongation of polypeptide chains *via* promotion of ribosomal translocation. EF2 activity is inhibited by PTMs, *e.g. via* phosphorylation by the EF2 kinase [49], which itself is activated by phosphorylation *via* a number of different stimuli, *e.g.* cellular stress. However, we show by 1-D Western blot analysis differences in total EF2 protein levels in Tyk2$^{-/-}$ cells, thereby excluding a potential shift of spots in p*I* caused by phosphorylation as the sole cause of differential expression patterns found with 2-D DIGE technology.

Catalase and PRDX1 were identified as differentially expressed between wild-type and Tyk2$^{-/-}$ cells. Both enzymes are involved in the detoxification of hydrogen peroxide, which is produced as a result of normal cellular processes that involve oxygen and, importantly, in response to inflammatory stimuli including LPS [50, 51]. We show that although PRDX1 mRNA is modestly upregulated by LPS treatment an increase in PRDX1 protein is detectable neither by 1-D Western blot analysis nor by 2-D DIGE (by adding up spot volumes of all immunoreactive spots shown in Fig. 5C, data not shown). 2-DE reveals a noticeable change in the distribution of different PRDX1 spots, mainly a shift towards more acidic p*I* in response to LPS. Interestingly, the appearance of the spot with the lowest p*I* value was less prominent in the absence of Tyk2. Like the other members of the peroxiredoxin family, PRDX1 has a cysteine in its active site

which is reversibly oxidised to a disulfide in the normal catalytic cycle. It can also be hyperoxidised to sulphinic or sulphonic acid, which leads to inactivation of the enzyme [52]. Conway *et al.* [53] described three PRDX1 spots for the mouse macrophage cell line J774. In Western blots the authors proved that these spots correspond to the sulphonic, the sulphenic and the nonoxidised form of PRDX1 (from left to right in 2-DE pattern), and all were increased by chemical oxidation or by challenge with oxidised low density lipoprotein, without additional pattern changes. For human PRDX1, only doublet spots have been shown on 2-DE gels, a native form (basic spot) that shifts nearly completely to the overoxidised form (acidic spot) upon strong oxidative stress [54, 55]. Although under basal conditions our PRDX1 patterns look similar to those reported for the murine cell line [53], the noticed shift towards lower p*I* values upon LPS treatment would suggest oxidation of the protein as shown for the human homologue. Our data would then further suggest that lack of Tyk2 results in the suppression of LPS induced (hyper)-oxidation of PRDX1. However, a shift in p*I* might also be caused by other modifications (*e.g.* phosphorylation). In that context, it is interesting to note that interaction of PRDX1 with the tyrosine kinase c-Abl has been reported [56]. In order to define the exact nature of the modification(s), further and more detailed MS analysis of all five spots seen in our PRDX1 pattern would be required (*e.g.* digest with a different enzyme, high energy CID or multistage CID experiments).

The most dramatic effect on protein expression with respect to differences between genotypes was found for PAI2. Consistent with previous reports [57], PAI2 protein was strongly upregulated by LPS treatment in wild-type cells. Expression of PAI2 was dramatically increased in the absence of Tyk2 as determined by 2-D DIGE and 1-D Western blots analysis. Interestingly, PAI2 mRNA was similarly induced in Tyk2$^{-/-}$ and wild-type cells. This points to the existence of a Tyk2 dependent negative regulatory pathway that limits LPS-induced PAI2 expression at a post-transcriptional level. PAI1 and PAI2 belong to the serpin gene family of protease inhibitors and are the physiological inhibitors of tissue- and urokinase-type plasminogen activator (tPA, uPA) [40]. Although the main proportion of newly synthesised PAI2 remains in the cell, the intracellular function of PAI2 is poorly characterised. There is evidence that PAI2 is involved in multiple cellular processes like proliferation, differentiation, and most consistently, apoptosis [40, 58]. Another important function of PAI2 is the inhibition of cancer metastasis and high PAI2 expression is associated with good prognosis in human cancers [40]. Recently, involvement of Tyk2 in tumour cell invasiveness has been reported [59, 60] and reduced uPA signalling was proposed as the underlying mechanism [59]. The fact that we find increased PAI2 expression in Tyk2$^{-/-}$ cells under inflammatory conditions (*i.e.* LPS treatment) poses the question of whether Tyk2 limits PAI2 expression also during tumour metastasis and whether this contributes to the reduced uPA signalling

described above. Also of relevance in this context is that activation of Tyk2 by uPA has been reported [61–63].

In summary, our data are in line with and further support the role of Tyk2 in the basal and LPS-induced, autocrine/paracrine actions of IFNα/β. We furthermore describe novel positive as well as negative regulatory roles of Tyk2 on protein expression in macrophages and demonstrate that this occurs both at the level of mRNA transcription and post-transcriptionally. As yet unrecognised connections between Tyk2 and diverse cellular proteins reported in this study raise interesting new topics for future research on Tyk2 biology.

5 References

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PROTEOMICS

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The impact of tyrosine kinase 2 (Tyk2) on the proteome of murine

macrophages and their response to lipopolysaccharide (LPS)
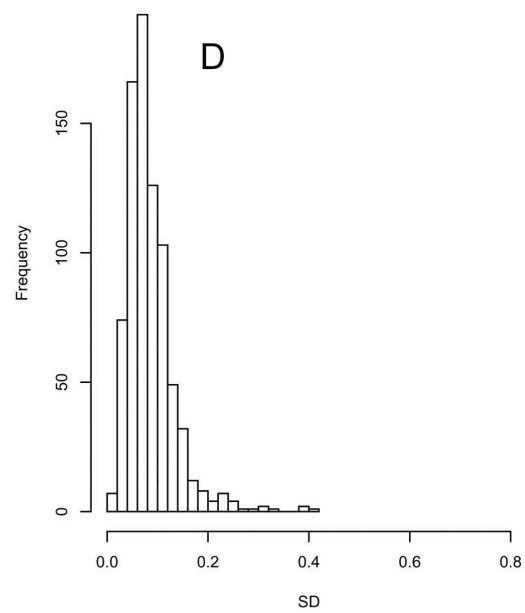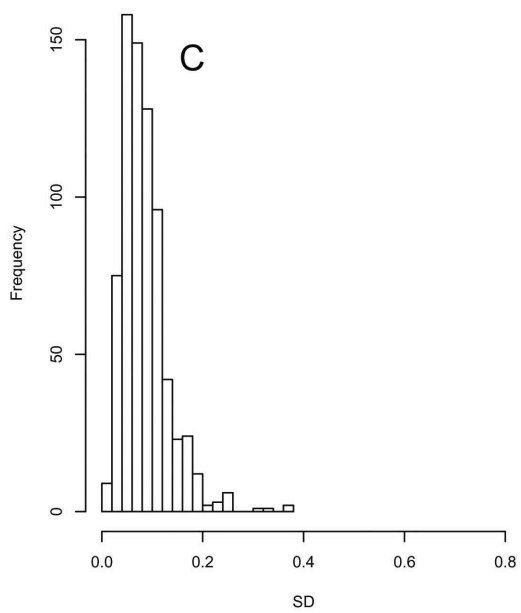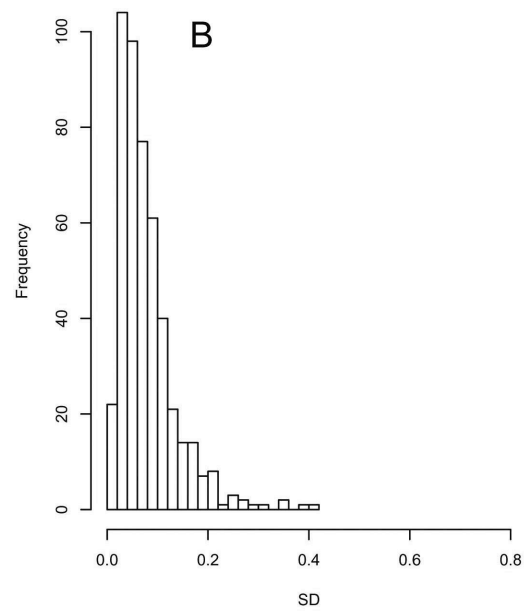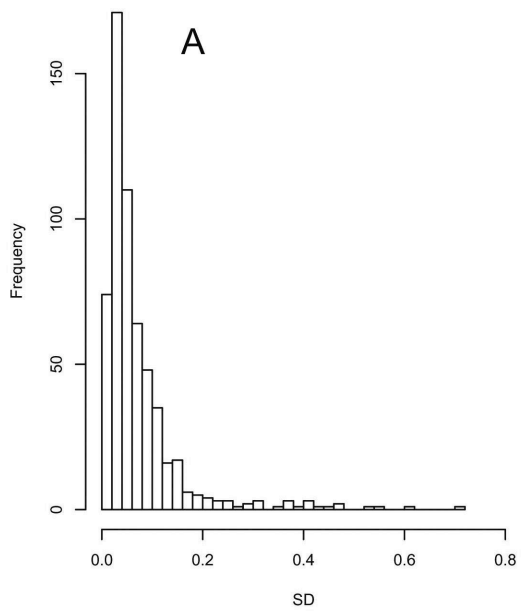
Figure S1

A
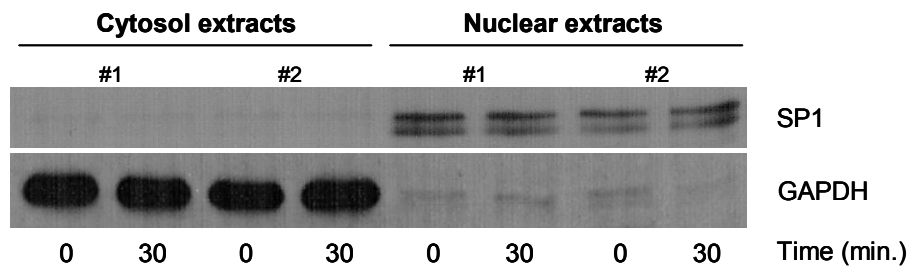
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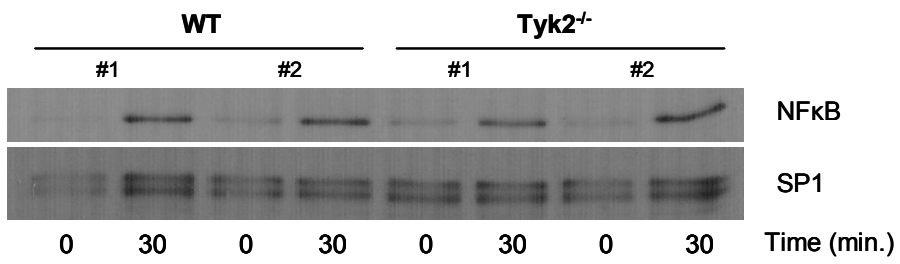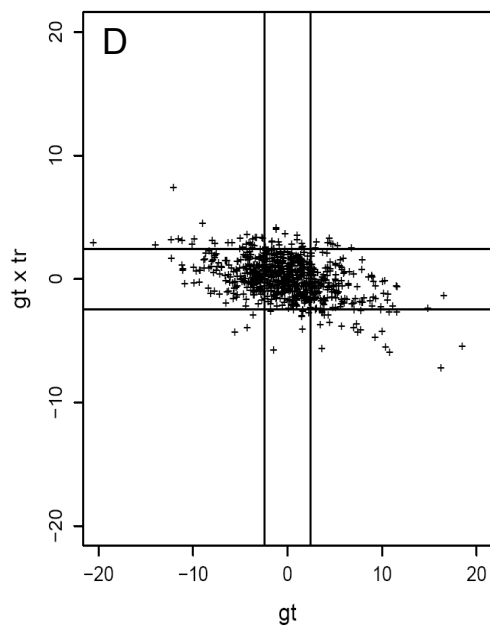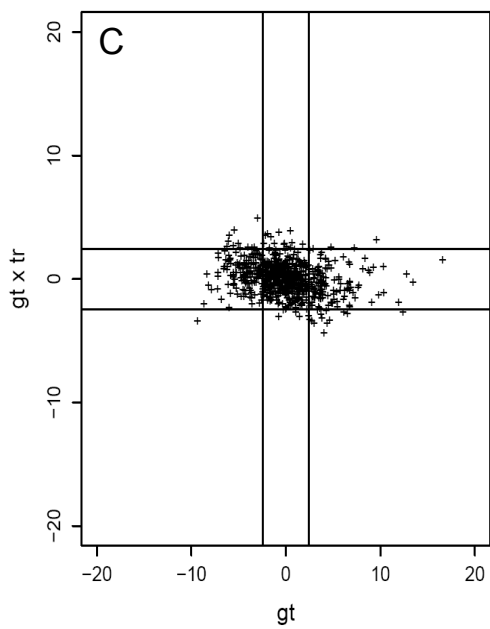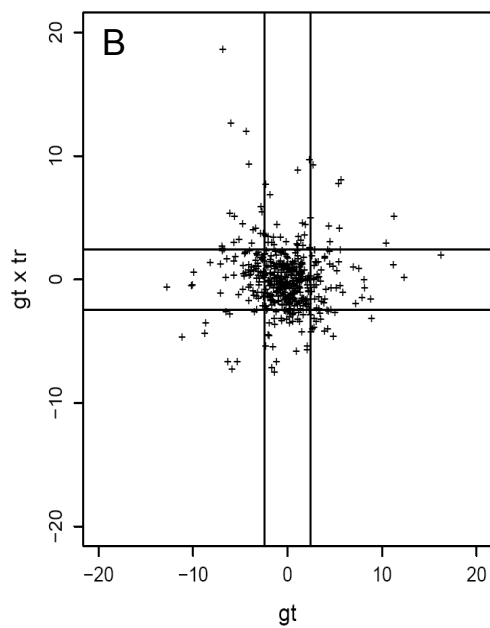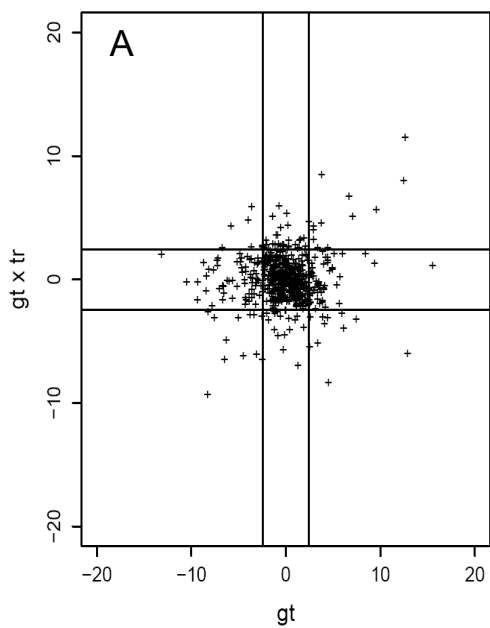
Figure S3

Supplements

Figure S1. 2D-DIGE data are highly reproducible. Histograms show the frequency distribution of SD values over the experiments performed. The SD of spot volume ratios from three biological replicates was calculated for each spot and was plotted against frequencies. (A) whole cell lysates pH 4-7 (n=579 spots), (B) whole cell lysates pH 6-9 (n=478 spots), (C) nuclear extracts pH 4-7 (n=731 spots), (D) nuclear extracts pH 6-9 (n=792 spots).

Figure S2. (A) Nuclear extracts show reproducible enrichment of nuclear proteins. Macrophages were treated with LPS for 30 minutes. 3 µg of nuclear or cytosolic extracts per lane were separated by 8%T SDS-PAGE. GAPDH was used as a cytosolic and SP1 as a nuclear marker. Data are representative of at least three experiments. (B) Translocation of NF-κB is unperturbed in Tyk2$^{-/-}$ macrophages. Macrophages were treated with LPS for 30 minutes. 3 µg of nuclear extract *per* lane were separated by 8%T SDS-PAGE. Protein loading was controlled by re-probing with an anti-SP1 antibody. #1 and #2 indicate that the cells were derived from independent mice.

Figure S3. Tyk2 deficiency significantly alters the proteome pattern of macrophages. The t-values of the genotype effect (*gt*) are plotted against the t-values of the genotype x treatment effect (*gt x tr*). Data include all spots (found in all images) without filtering for minimal differences in the expression values. Values above and below the critical t-values ± 2.306 (indicated by lines) are considered to be significant. Positive t-values indicate decreased and negative t-values increased expression / induction levels in the absence of Tyk2. (A) whole cell lysates pH 4-7 (n=579 spots), (B) whole cell lysates pH 6-9 (n=478 spots), (C) nuclear extracts pH 4-7 (n=731 spots), (D) nuclear extracts pH 6-9 (n=792 spots).

Table S1. Differentially expressed proteins in Tyk2$^{-/-}$ macrophages - detailed MS data.

Spot #	Differentially expressed proteins				MS Method	Peptide Mass Fingerprint			# of peptides for protein ID	Sequencing		
	Protein Name / Function	Accession #	M$_r$ (kDa)	pI		Probability based MOWSE Score	Sequ. Cov.	Matched peptides		Probability based MOWSE Score	Precursor Ion(s) m/z (observed) and charge state [a]	Peptide Sequence(s)
		Swissprot	theor.	theor.			%	matched/ unmatched				
1	Proteasome activator complex subunit 1 (PSME1)	P97371	28.7	5.7	ESI		28		6	237	684.3++ 873.7+++ 853.08+++ 810.32+++ 760.33++ 621.76++	VDVFREDLCSK, VDVFREDLCSKTENLLGSYFPK, KISELDAFLKEPALNEANLSNLK, ISELDAFLKEPALNEANLSNLK, IEDGNNFGVAVQEK, YFSERGDAVAK
2	Interferon-induced protein with tetratripeptide repeats 3 (IFIT3)	Q64345	47.2	5.5	ESI		11		6	164	563.179++ 604.2+ 557.204++ 641.719++ 660.23++	LSEAQAYVDK, QVCQK, APNQTDVLQK, YCNLQEYHR, SIEKEEVKEQT
3	N-myc-interactor (NMI)	O35309	35.2	5	ESI		4		1	36	763.24++	NGGGEVESVDYDRK,
					MALDI	84	30	6/2	2	100	1800.85+ 1921.88+	INVTGIPDELSEEQTR, CSLDQSFAAYFKEEAR

No.	Protein	Accession									Peptides
4	Interferon-induced protein with tetratripeptide repeats 1 (IFIT1)	Q64282	53.7	7.2	MALDI	21	6	2/2	162	1687.07+ 1944.15+	LLFENNDIPDLEVR, GENADGDQVMENLLQLR [b]
5	Interferon-induced protein with tetratripeptide repeats 1 (IFIT1)	Q64282	53.7	7.2	MALDI	147	32	13/4	83	1686.88+ 1943.93+	LLFENNDIPDLEVR, GENADGDQVMENLLQLR [b]
6	Interferon-induced protein 35kD homolog (IN35)	Q9D8C4	31.8	5.6	MALDI	58	17	4/2	181	756.48+ 2007.18+	QQVLLR; SVTLQTVLYSLQEEQAR [b]
7	Interferon-activable protein 205 (IFI5)	Q08619	47	8.4	MALDI	59	6	4/0	60	613.41+ 1181.61+	IVLLR, SQPQNQNIPR
	or Interferon-activable protein 204 (IFI4)	P15092	71.6	9		51	4	4/0	60	613.41+ 1181.61+	IVLLR, SQPQNQNIPR
8	Interferon-activable protein 205 (IFI5)	Q08619	47	8.4	MALDI	59	6	4/0	101	613.41+ 759.47+ 1181.61+	IVLLR, DLNLER, SQPQNQNIPR
	or Interferon-activable protein 204 (IFI4)	P15092	71.6	9		51	4	4/0	101	613.41+ 759.47+ 1181.61 +	IVLLR, DLNLER, SQPQNQNIPR
9	Peroxiredoxin-1 (PRDX1)	P35700	22.2	8.3	MALDI	112	28	7/3	124	1225.67+ 1359.81+	QITINDLPVGR, GLFIIDDKGILR
10	Peroxiredoxin-1 (PRDX1)	P35700	22.2	8.3	MALDI	153	41	9/2	99	831.46+ 1225.69+	SVDEIIR, QITINDLPVGR
11	Peroxiredoxin-1 (PRDX1)	P35700	22.2	8.3	MALDI	60	20	4/3	55	1225.67+ 1359.81+	QITINDLPVGR, GLFIIDDKGILR
12	Catalase (CATA)	P24270	59.8	7.7	MALDI	308	50	26/7	100	962.54+ 1276.63+	DAQLFIQK, LAQEDPDYGLR
13	Plasminogen activator inhibitor 2 (PAI2)	P12388	46.3	5	MALDI	70	15	7/5	105	1284.87+ 1698.07+	FKEEYIQLSK, VLQFNEIGSYGITTR

No.	Protein	Accession										Peptides
14	3-ketoacyl-CoA thiolase A (THIKA)	Q921H8	44	8.7	MALDI	165	44	14/6	2	212	1379.84+, 2042.12+	AEELGLPILGVLR, IAQFLSGIPETVPLSTVNR
	3-ketoacyl-CoA thiolase B (THIKB)	Q8VCH0				65	22	7/13	2	212	1379.84+, 2042.12+	AEELGLPILGVLR, IAQFLSGIPETVPLSTVNR
15	Purine nucleoside phosphorylase (PNPH)	P23492	32.2	5.8	ESI		59		16	769	935.5++, 907.3+++, 1360.6+++, 681.34+++, 718.4+, 840.96+++, 587.34++, 787.86++, 1221.14++, 814.36+++, 1157.1++, 958.93++, 1125.6+, 563.3++, 1239.62++, 620.3++, 593.32++, 1184.48++, 808.81++, 881.35++	MENEFTYEDYETTAK, EAQIFDYNEIPNFPQSTVQGHAGR, EAQIFDYNEIPNFPQSTVQGHAGR, FHMYEGYSLSK, VTFPVR, DHINLPGFCGQNPLR, FPAMSDAYDR, FPAMSDAYDRDMR, KLQEGTYVMLAGPNFETVAESR, KLQEGTYVMLAGPNFETVAESR, LQEGTYVMLAGPNFETVAESR, MLGADAVGMSTVPEVIVAR, VFGFSLITNK, VFGFSLITNK, VVMDYENLEK, VVMDYENLEK, ANHMEVLDAGK, ANHMEVLDAGK, FVSILMESIPLPDR, FVSILMESIPLPDRGS
16	Argininosuccinate synthase (ASSY)	P16460	46.6	8.4	MALDI	117	19	10/2	2	89	858.47+, 999.55+	IDIVENR, RQVEIAQR
17	Elongation Factor 2 (EF2)	P58252	95.3	6.4	MALDI	172	21	16/1	1	43	1091.64+	VNFTVDQIR
18	Phenylalanyl-tRNA-synthase beta chain (SYFB)	Q9WUA2	65.7	6.7	MALDI	57	7	5/2	3	117	744.46+, 921.47+, 1198.71+	LTELLR, TAEFQVAR, VRPFAVAAVLR
19	Staphylococcal nuclease domain-containing protein 1 (SND1)	Q78PY7	102.1	7.1	MALDI	170	17	18/5	3	96	757.34+, 786.29+, 957.51+	AGNLARR, FFTESR, QINLSNIR
20	Vimentin (VIME)	P20152	53.7	5.1	MALDI	124	24	11/4	1	72	1587.92+	TNEKVELQELNDR

No.	Protein	Accession									m/z	Peptide sequences
21	F-actin capping protein subunit alpha-1 (CAZA1)	P47753	32.9	5.3	MALDI	162	40	11/4	2	155	1197.72+ 2089.02+	LLLNNDNLLR, FITHAPPGEFNEVFNDVR
22	F-actin capping protein subunit beta (CAPZB)	P47757	31.3	5.5	MALDI	133	32	8/0	2	53	786.46+ 1108.64+	DIVNGLR, RLPPQQIEK
23	Adenyl cyclase-associated protein 1 (CAP1)	P40124	51.6	7.2	MALDI	110	21	9/4	2	55	1515.74+ 1958.08+	ADMQNLVERLER [b] LGLVFDDVVGIVEIINS
24	EF-hand domain containing protein 2 (EFHD2)	Q9D8Y0	26.8	5	MALDI	83	21	7/1	3	137	755.37+ 937.53+ 973.54+	NFFEAK, EFLLIFR, VQAINVSSR
25	Immune-responsive protein 1 (IRG1)	P54987	53.8	7.1	MALDI	126	30	12/12	2	64	1168.63+ 1852.97+	ALFPADHIER, RFHPPSVVGTLGSAAAASK

a) Monoisotopic; + singly; ++ doubly and +++ triply charged peptide

b) Acetylation at protein N-terminus

II. Unpublished results

1. Evidence for translational regulation of IL-1β by Tyk2-dependent mechanisms

1.1 Introduction

Within our 2D-DIGE experiments, designed to identify proteins that are differentially expressed between WT and Tyk2$^{-/-}$ bone marrow-derived macrophages (BMM), we have identified IL-1β as a protein that is strongly enhanced in the absence of Tyk2 upon LPS treatment. Since this is a novel phenotype with a potential impact on host defence mechanisms, we investigated the underlying mechanisms in more detail.

According to current knowledge, IL-1β expression is regulated mainly at the level of transcription and by its processing and release [107,108]. In addition, regulation of IL-1β mRNA stability *via* AU-rich elements (ARE) has been reported [109]. Pro-IL-1β synthesis is induced by LPS through activation of NFκB and MAPK pathways [110,111], although NFκB can also negatively regulate IL-1β processing [112]. Pro-IL-1β can be cleaved into the biologically active cytokine by several proteases in the extracellular space, but intracellularly, casp-1 is the main protease responsible for cleavage in macrophages [113-115]. Very recently, casp-8-dependent and casp-1-independent, IL-1β maturation was reported in TLR2-primed, LPS-stimulated peritoneal macrophages [116]. Casp-1 itself requires proteolytic processing to get activated and this occurs within an activated inflammasome. LPS is a strong inducer for pro-IL-1β production, but a poor activator of the inflammasome and, accordingly, weakly induces the release of mature IL-1β, unless a second stimulus triggers inflammasome activation [117-119].

In this part of the work, we provide evidence that Tyk2 negatively regulates IL-1β expression at the level of translation. Our data furthermore suggest that this occurs *via* auto/paracrine canonical IFN-α/β signalling.

1.2 Results

Intracellular pro-IL-1β is increased in the absence of Tyk2

IL-1β was identified in a spot with a M$_r$ of approximately 30 kDa and a p*I* around the theoretical p*I* 4.6 (see appendix, Fig. 2). The identified peptides are common to both mature and immature IL-1β, but based on the M$_r$ the spot could be assigned to the immature IL-1β (pro-IL-1β). Spot volume ratios as determined by 2D-DIGE increased in WT cells upon 18 h LPS treatment approximately 3-fold, whereas a significantly stronger increase (approximately 14-fold) was found in the absence of Tyk2 (Fig. 1A and B). Enhanced expression of pro-IL-1β in Tyk2$^{-/-}$ macrophages was confirmed by western blot time course

experiments (Fig. 1C) and was detected from very early upon LPS stimulation onwards. IL-1β is synthesised as biologically inactive procytokine (M_r of 31 kDa), that remains intracellular until a second stimulus induces its processing into the active cytokine (M_r of 17 kDa) and its release. As reported in the literature, mature IL-1β was hardly detectable intracellularly in WT cells [120-122].
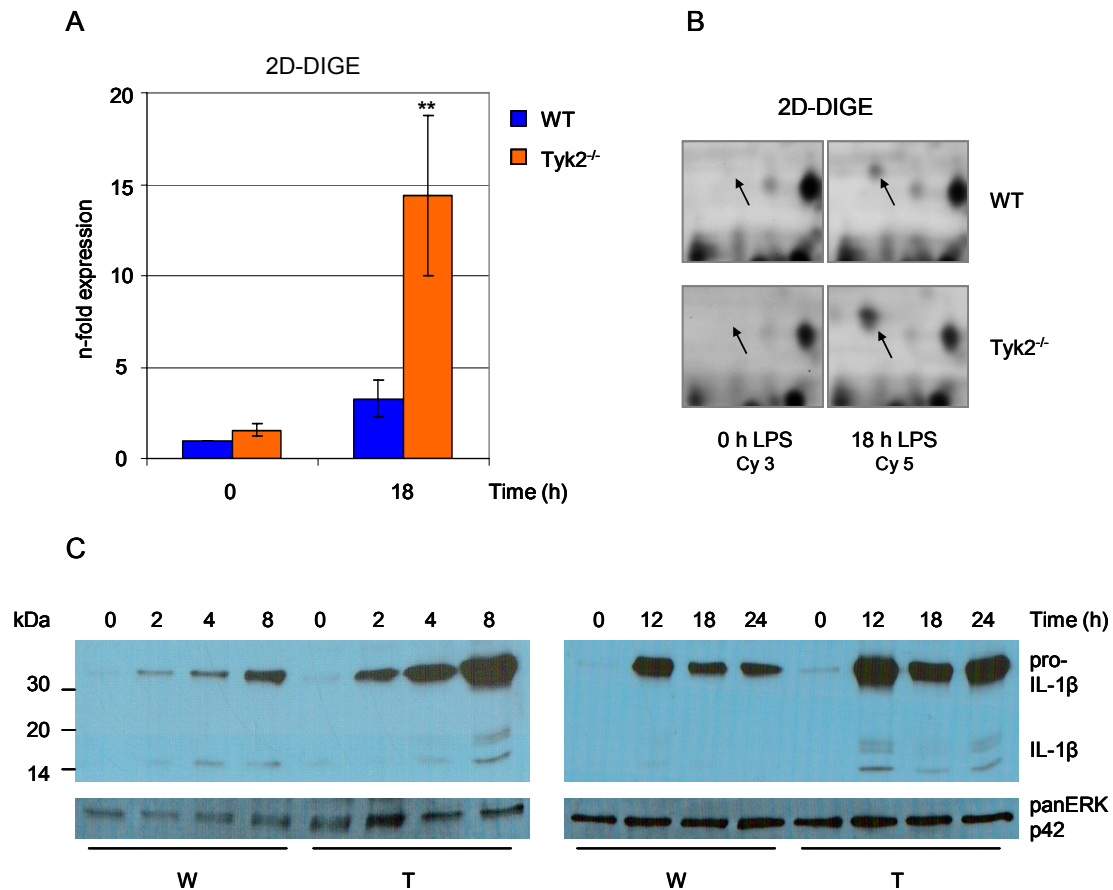

Figure 1. Effect of Tyk2 deficiency on pro-IL-1β protein expression. Macrophages were treated with LPS for the indicated times and whole cell lysates were subjected to 2D-DIGE (A, B) or western blot analysis (C). (A) Protein expression levels are given as fold ratios relative to unstimulated WT cells. Mean values ±SD of three biological replicates are shown. (B) Selected regions from Cy3 and Cy5 images converted to grey scale showing pro-IL-1β spot positions (indicated by arrows). (C) 5 μg protein *per* lane were separated by 14%T SDS-PAGE. Protein loading was controlled by reprobing with an anti-panERK antibody. W - WT, T - Tyk2$^{-/-}$, ** p≤ 0.01.

Tyk2 is not required for IL-1β mRNA induction

Induction of the IL-1β gene upon LPS treatment occurs *via* the MyD88-dependent pathway [123,124], which, according to current knowledge, does not depend on the presence of Tyk2 [67,125]. Consistently, we did not find any significant differences in IL-1β mRNA expression between WT and Tyk2^{-/-} macrophages (Fig. 2). We have also included IFNAR1-deficient cells in the analysis in order to determine potential contributions of auto/paracrine type I IFN signalling. IL-1β mRNA was induced rapidly and to a similar extent for up to 24 h after LPS treatment in WT, Tyk2^{-/-} and IFNAR1^{-/-} cells (Fig. 2).
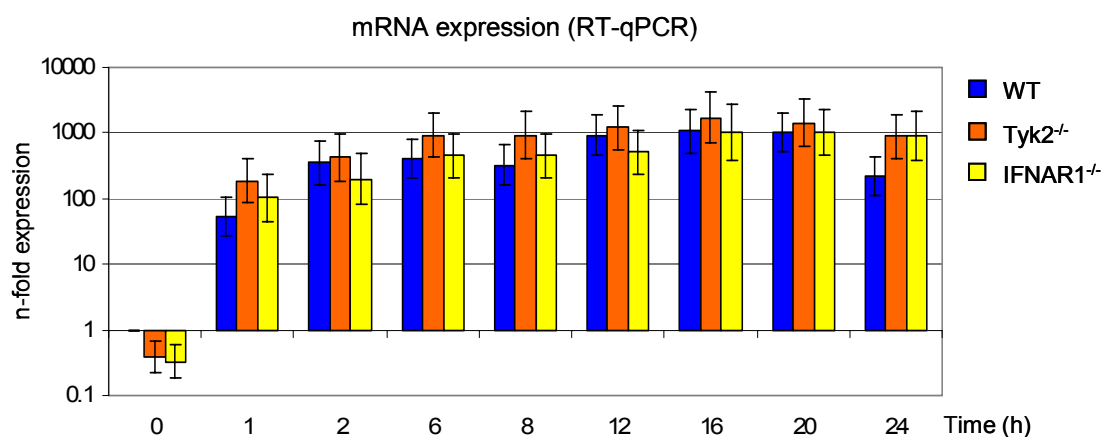
mRNA expression (RT-qPCR)

Figure 2. Effect of Tyk2 and IFNAR1 deficiency on IL-1β mRNA expression. Macrophages were treated with LPS for the indicated times and total RNA was subjected to RT-qPCR. mRNA expression levels were calculated relative to untreated WT cells, UBE2D2 was used as endogenous control. Mean values ±SE from at least three independent experiments are shown.

Tyk2 deficiency does not affect IL-1β processing and release

IL-1β processing and release is tightly regulated and increased intracellular pro-IL-1β might be caused by reduced processing/release. LPS stimulation alone massively induces IL-1β production, but IL-1β processing and externalisation occurs very inefficiently [117-119]. Thus, IL-1β mainly remains intracellularly as unprocessed pro-IL-1β and only low levels can be detected in the extracellular space. We nevertheless determined extracellular IL-1β with ELISA. As shown in Fig. 3A, IL-1β protein levels detected in the supernatants were expectedly low in WT cells with approximately 50-100 pg/mL at 24 h of LPS stimulation. However, IL-1β was approximately 4-5-fold increased in supernatants from Tyk2^{-/-} macrophages. Again, consistent with previous studies [67], data show that secreted levels of TNFα were similar in WT and Tyk2^{-/-} cells (Fig. 3B).

We next wanted to address whether increased IL-1β production in Tyk2$^{-/-}$ cells is also observed under conditions that promote maturation of IL-1β. Macrophages were treated 4 h or 18 h with LPS followed by 30 min stimulation with the P2X$_7$ receptor agonist ATP. As to be expected, extracellular levels of IL-1β in WT cells were dramatically higher under these conditions than upon stimulation with LPS alone (Fig. 4A and Fig. 3A). Importantly, enhanced IL-1β levels were still detected in Tyk2$^{-/-}$ macrophages at 4 h and at 18 h of LPS/ATP (Fig. 4A) or treatment with LPS alone (Fig. 3A). In order to exclude the possibility that ELISA also detects unprocessed IL-1β, we confirmed results with western blot analysis. Under the conditions used, 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 upon treatment with 5 mM ATP (Fig. 4B). Again, strongly enhanced IL-1β levels were detected in the absence of Tyk2. Importantly, also mature 17kDa IL-1β was increased in Tyk2-deficient cells. High concentrations of ATP can be toxic for the cells and thus pro-IL-1β might appear extracellularly *via* apoptosis. However, maturation of IL-1β can not be induced by this process [126]. Intracellularly, mature IL-1β was hardly detectable in WT cells treated with LPS alone (Fig. 4C and Fig. 1C) or with LPS + ATP (Fig. 4C). In contrast, mature IL-1β was detectable even intracellularly in Tyk2$^{-/-}$ cells upon LPS + 5 mM ATP (Fig. 4C).

In summary, these data show that IL-1β levels are increased in the absence of Tyk2 as compared to WT cells intra- and extracellularly, and independent of the presence of a trigger that promotes IL-1β processing.

A

B
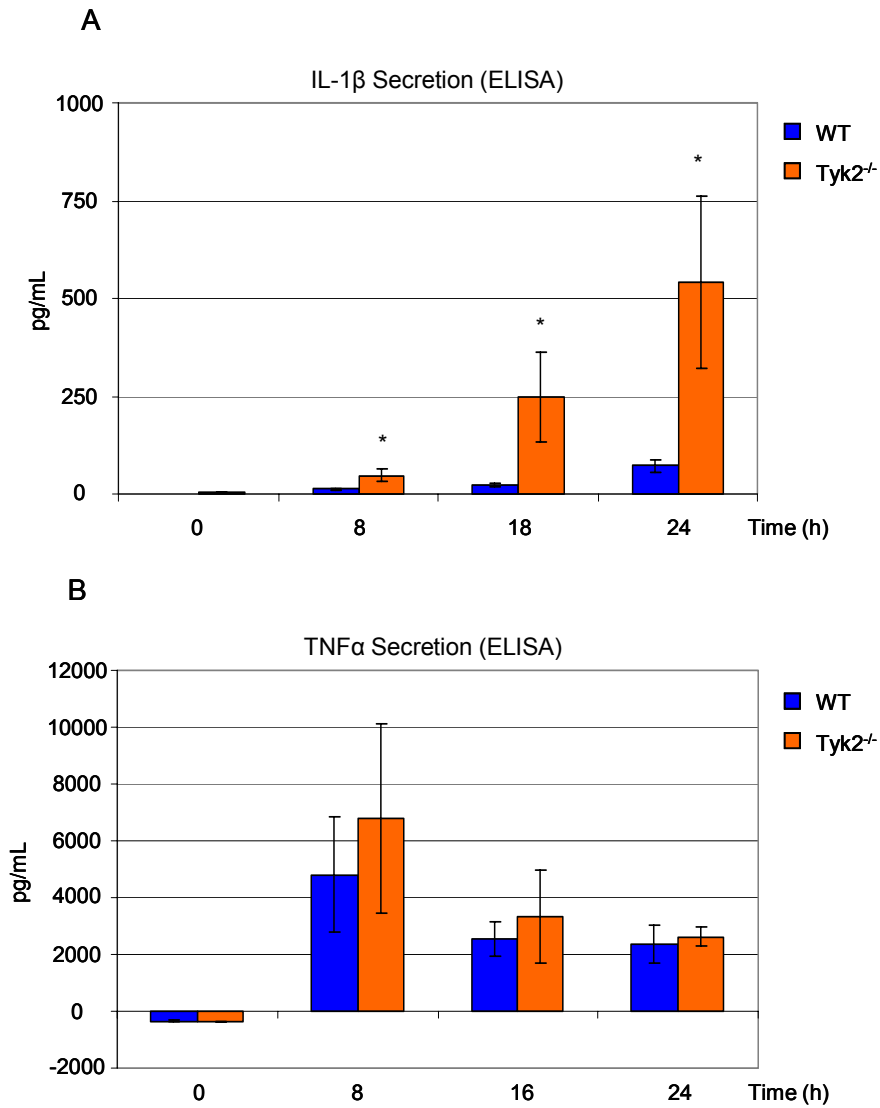

Figure 3. Effect of Tyk2 deficiency on extracellular protein levels of IL-1β and TNFα. Macrophages were treated with LPS for the indicated times and cell culture supernatants were collected. Exracellular protein concentrations of IL-1β (A) and TNFα (B) were measured by ELISA. Mean values ±SD from three independent experiments are shown. * p≤ 0.05.

A

IL-1β Secretion (ELISA)

- ■ WT
- ■ Tyk2⁻ᐟ⁻

ATP
5 mM

B

Supernatants

C

Whole cell lysates
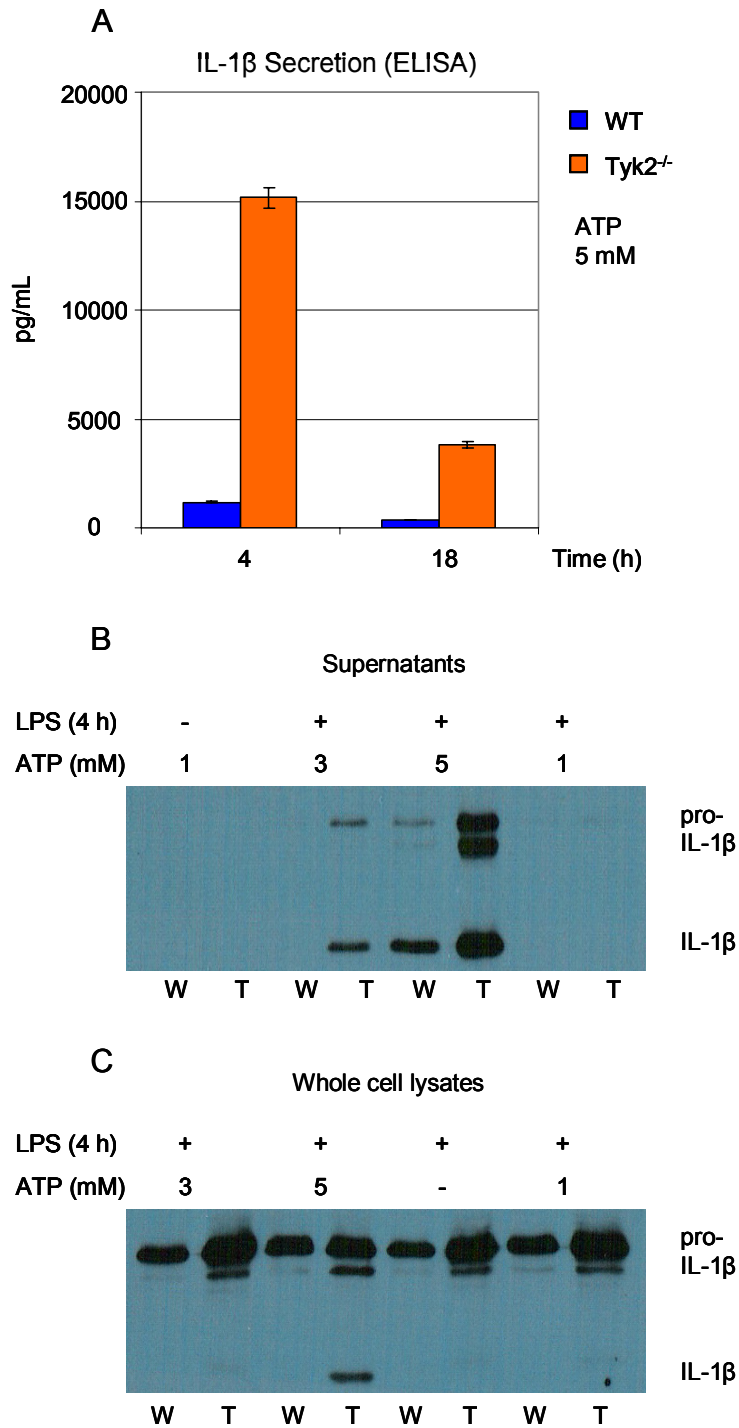

Figure 4. Effect of Tyk2 deficiency on IL-1β intra- and extracellular protein levels and processing after ATP treatment. Macrophages were treated with LPS for the indicated times with or without ATP at the concentrations indicated. (A) Extracellular protein concentrations in cell culture supernatants were measured by ELISA. Preliminary data (±SD of duplicates) from one experiment are shown. (B) Proteins were precipitated from supernatants and 10 µg *per* lane were subjected to western blot analysis by 15%T SDS-PAGE. (C) 5 µg protein from whole cell lysates *per* lane were separated by 15%T SDS-PAGE. W - WT, T - Tyk2⁻ᐟ⁻.

Limitation of IL-1β expression depends on the presence of Tyk2, IFNAR1 and Stat1

In the context of macrophage LPS responses, Tyk2 is mainly associated with IFN-α/β signalling. Basal and/or induced expression of some genes that depend on functional IFN-α/β responses have been shown to require Tyk2 for full expression [58,63,67]. Against this background, we tested whether the negative impact of Tyk2 on IL-1β expression is also observed in cells lacking IFNAR1 or Stat1. Cells were treated with LPS for 4 h (Fig. 5A) or 18 h (Fig. 5B), ATP or medium was added for 30 min, and IL-1β expression analysed in supernatants. 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 mature IL-1β were also observed intracellularly in the absence of IFNAR1, Tyk2 or Stat1 upon LPS treatment, irrespective of the presence of ATP (Fig. 5C).

Supernatants
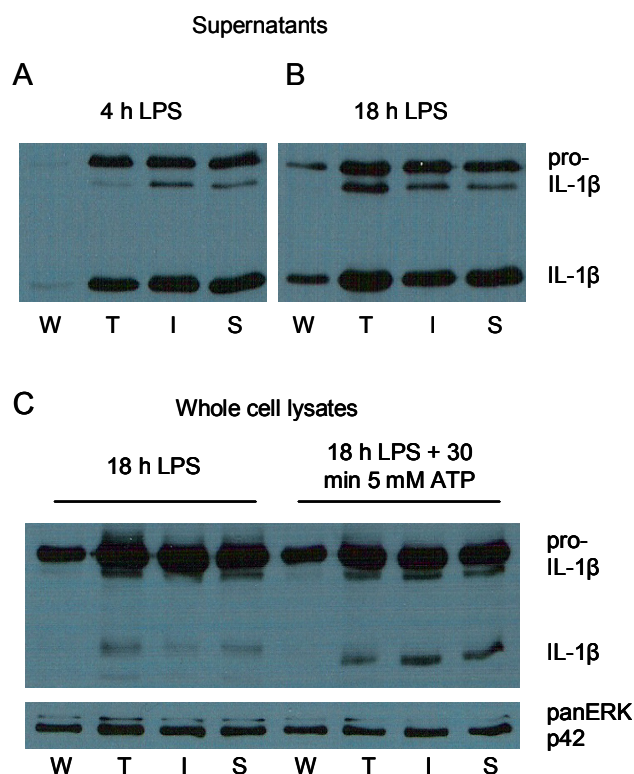
Figure 5. Effect of Tyk2, IFNAR1 and STAT1 deficiency on IL-1β protein levels and processing. Macrophages were treated with LPS for the indicated times with or without ATP at the concentrations indicated. Whole cell lysates and cell culture supernatants were collected and subjected to western blot analysis. (A, B) Proteins were precipitated from supernatants and 15 µL *per* lane were separated by 15%T SDS-PAGE. (C) 5 µg protein from whole cell lysates *per* lane were separated by 15%T SDS-PAGE. Protein loading was controlled by reprobing with an anti-panERK antibody. W - WT, T - Tyk2$^{-/-}$, I - IFNAR1$^{-/-}$, S - Stat1$^{-/-}$.

Enhanced IL-1β in Tyk2^{-/-} cells is not due to changes in protein stability

We next wanted to determine whether IL-1β protein stability is influenced by the absence of Tyk2. Macrophages were stimulated with LPS for 4 h, cycloheximide was added and IL-1β protein expression monitored over time. As shown in Fig. 6A, IL-1β protein levels were increased in Tyk2^{-/-} cells at 4 h of LPS stimulation, but declined with similar kinetics as in WT macrophages upon the translational block. Similar results were obtained using pulse-chase experiments (Fig. 6B). Cells were treated with LPS for 4 h, metabolically labelled with ^{35}S-methionine/cysteine, washed and further incubated in the presence of excess cold methionine/cysteine for various times. The observed half-life of IL-1β in WT cells was similar to what has been previously described [127] and not grossly different to what was seen in Tyk2-deficient cells. In contrast, clear differences were observed in IL-1β production during three different labelling periods. Thus, data suggest that IL-1β translation rather than stability is affected by the absence of Tyk2.

A

| LPS | 0 | 4 | 4 | 4 | Time (h) |

B
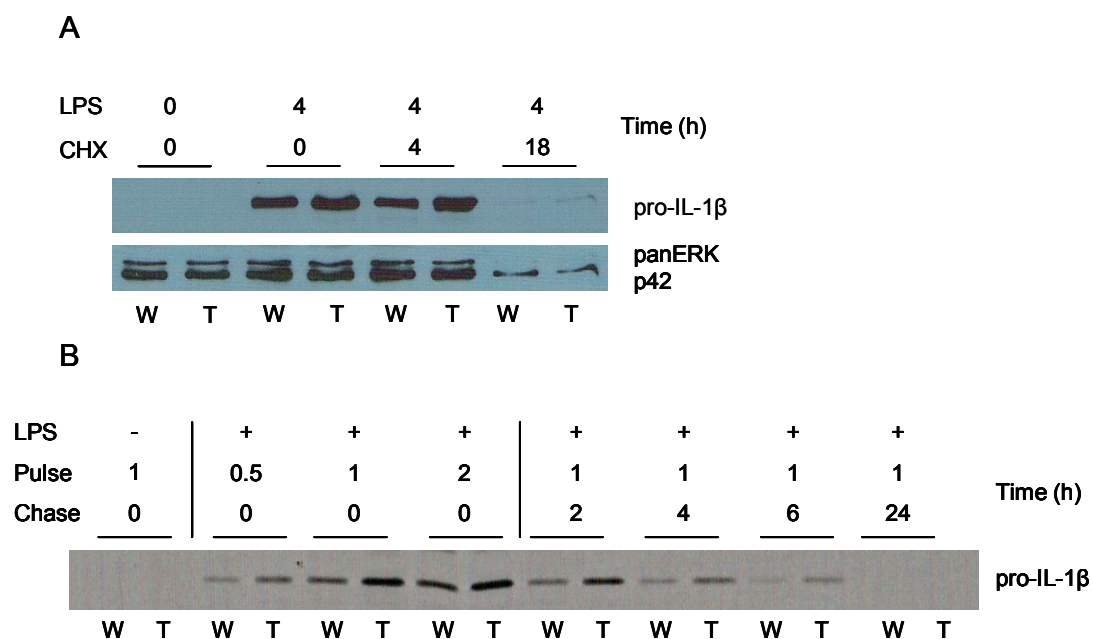
Figure 6. Effect of Tyk2 deficiency on pro-IL-1β protein stability and synthesis. (A) Macrophages were treated with LPS for 4 h, 20 µg/mL of CHX were added and cells were further cultivated for the times indicated. 5 µg proteins from whole cell lysates *per* lane were separated by 15%T SDS-PAGE. Protein loading was controlled by reprobing with an anti-panERK antibody. (B) Macrophages were pulse labelled with ^{35}S methionine/cysteine and chased for the indicated times. IL-1β was precipitated from 400 µg whole cell extracts and detected by autoradiography. W - WT, T - Tyk2^{-/-}.

Enhanced association of IL-1β mRNA with polysomes in the absence of Tyk2

In order to more directly test whether Tyk2 influences the translation of IL-1β mRNA, we fractionated cytoplasmic RNA from LPS-treated WT and Tyk2$^{-/-}$ macrophages *via* sucrose gradients (Fig. 7A and B) and compared mRNA distribution among the different polysomal fractions (Fig. 7C and D). Upon 4 h LPS stimulation, IL-1β mRNA was found in the ribosome-free fractions as well as associated with polysomes in WT cells (Fig. 7C). In contrast, IL-1β mRNA was nearly exclusively found in the polysome fractions in Tyk2$^{-/-}$ cells. In accordance with the unchanged expression of TNFα protein, TNFα mRNA showed similar polysome profiles in WT and Tyk2-deficient cells (Fig. 7D). Differences in the IL-1β polysome profiles were also found at 14 h upon LPS treatment, although IL-1β mRNAs were generally shifted towards the ribosome-free mRNA fractions.
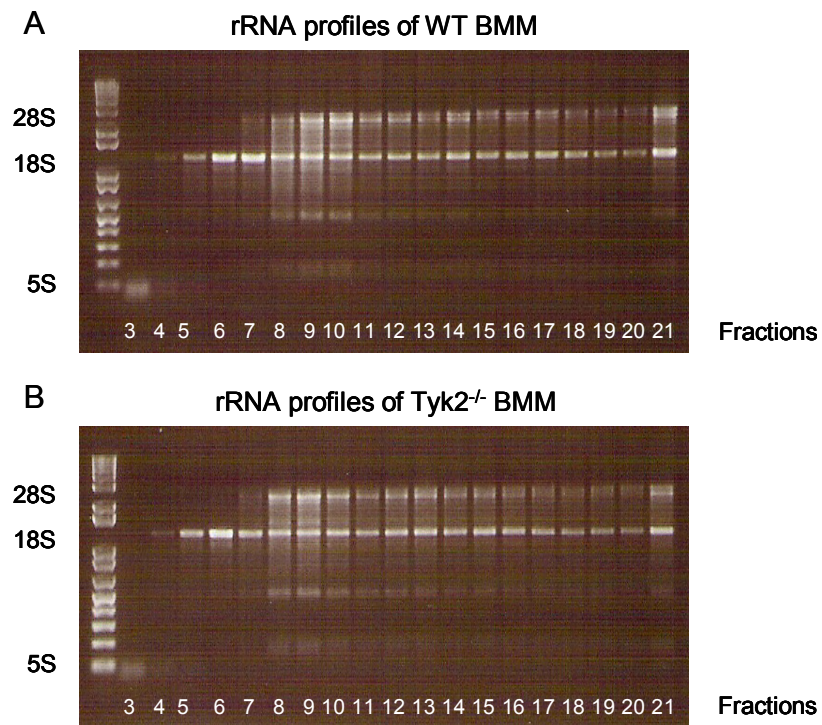
Figure 7. See next page for legend.

C

Distribution of IL-1β mRNA

D

Distribution of TNFα mRNA
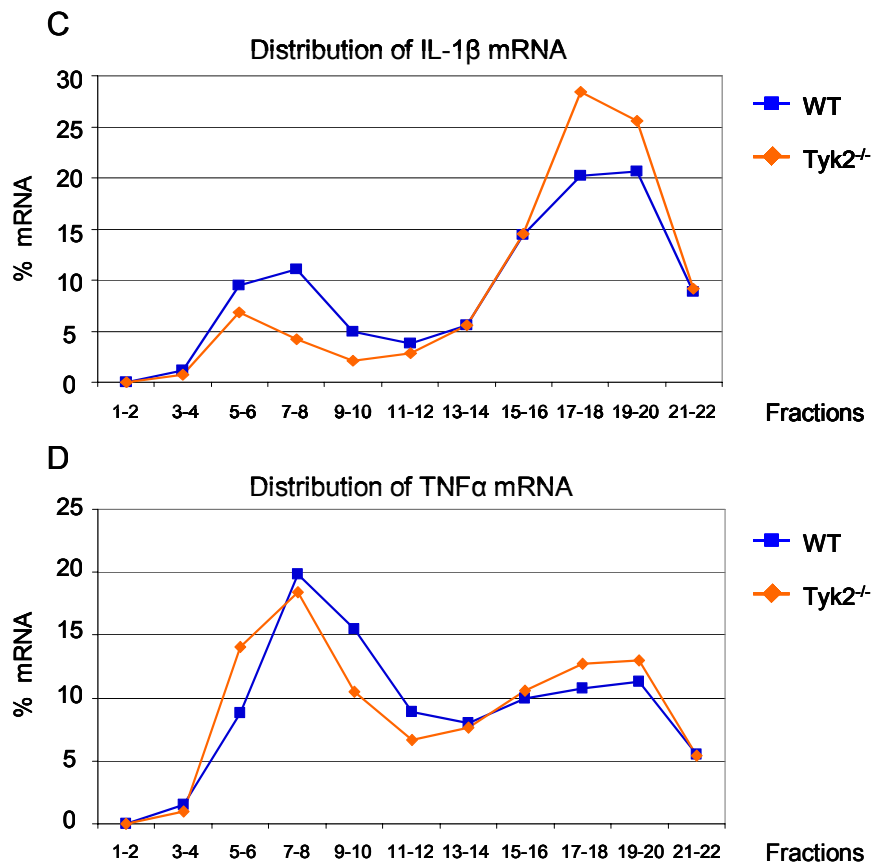
Figure 7. Effect of Tyk2 deficiency on polysome profiles of IL-1β and TNFα. Macrophages were treated with LPS for 4 h and cytoplasmatic extracts were separated in a continuous 15-40% sucrose gradient by ultracentrifugation. Fractions were manually collected from top to bottom, deproteinised and RNA extracted. (A, B) Polysomal fractions were separated on 0.8% agarose gel. (C, D) Two fractions each were pooled and mRNA levels were determined by RT-qPCR. TATA binding protein (TBP) was used as endogenous control. Amounts of the target mRNA of IL-1β (C) and TNFα (D) were calculated relative to the amount of TBP in each pooled fraction and are given as percentage of target mRNA present in all fractions. Representative results of three independent experiments are shown.

1.3 Discussion

Within this report we show that Tyk2 is critical for the limitation of IL-1β expression in macrophages in response to LPS. Our data suggest that this occurs *via* translational regulation, thus adding another level of complexity to the control of IL-1β expression.

We show herein that IL-1β protein expression in response to LPS is greatly increased in the absence of Tyk2, whereas its mRNA induction is unaltered. Unimpaired IL-1β mRNA induction is in accordance with the previously reported normal activation of NFκB and MAPK pathways in Tyk2-deficient macrophages [67,125]. We exclude defects in processing/release of IL-1β as potential reason for enhanced intracellular pro-IL-1β levels, since enhanced levels are also observed in the cell supernatants. We furthermore show that enhanced amounts of intra- and extracellular IL-1β (pro-IL-1β and mature IL-1β) are also observed under conditions that efficiently activate the inflammasome and pro-IL-1β conversion (i.e. ATP treatment). We show with pulse-chase experiments and by using the translational inhibitor cycloheximide, that IL-1β protein stability is unchanged in the absence of Tyk2. In contrast, IL-1β protein synthesis within a given pulse-period is enhanced in Tyk2$^{-/-}$ cells, arguing for an increased translational rate. In line with this finding, IL-1β mRNA association with polysomes is enhanced in the absence of Tyk2. Importantly, TNFα mRNA showed similar polysome profiles in WT and Tyk2$^{-/-}$ cells and, correspondingly, similar levels of TNFα protein were detectable in cell supernatants. Hence, the translational inhibition mediated by Tyk2 is not global but shows at least some degree of specificity.

With respect to the signalling pathways involved, a similar increase in IL-1β protein expression is observed in IFNAR1$^{-/-}$ and Stat1$^{-/-}$ macrophages (intra-/extracellular and with/without additional ATP treatment). Further, but preliminary data, suggest a similar effect of IFN-β but not IFN-γ deficiency. These data argue for auto/paracrine canonical IFN-α/β signalling as the signalling cascade involved, although direct evidence for an impact of IFNAR1, Stat1 and IFN-β on translational regulation of IL-1β in macrophages remains to be provided. We have to note, that these data are in contradiction to a previous report showing reduced intracellular IL-1β protein upon LPS treatment in the absence of Stat1 or IFN-β in thioglycollate-elicited peritoneal macrophages (PM) [128]. A potential reason for this discrepancy could be the different source of macrophages used in this study, but a direct comparison of the two different populations would be required to clarify this issue.

IFN-mediated translational inhibition is a long known phenomenon [129-131] and underlying mechanisms have been studied extensively. Activated IFN-inducible RNA-dependent protein kinase (PKR) phosphorylates the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α), which results in the inhibition of viral protein synthesis [132,133]. Two other IFN-

inducible and closely related proteins, ISG54 (IFIT2) and ISG56 (IFIT1), can negatively regulate translation [134-137]. Both proteins inhibit the formation of the translation initiation complex, although they target different steps [138]. Interestingly, IFIT1 and IFIT3, another member of the IFIT protein family, both show decreased LPS-induced expression in the absence of Tyk2 [125]. Furthermore, the pathways described above globally inhibit translation and thus, specificity for IL-1β but not TNFα is difficult to explain. Recently, the Akt pathway has been implicated in translation control upon IFNs, but in that case the influence is positive [139].

Post-transcriptional regulation has been shown for a large number of cytokines [140]. Most prominently, regulation occurs *via* AREs in the 3'-UTRs of the respective mRNAs. Several different ARE binding proteins (ARE-BPs) positively or negatively regulate mRNA stability, but can also affect translational efficiency. Although early studies show that IL-1β can be regulated at the translational level under certain conditions [141-143], mechanism and stimuli involved in regulating IL-1β translation are largely unknown. Within a more recent report [144], steroid receptor coactivator-3 (SRC-3) is shown to be required for translational repression of TNFα and IL-1β in response to LPS in peritoneal macrophages. For TNFα mRNA, this function was shown to be dependent on an ARE element and the ARE-BPs, T cell intracellular antigen 1 (TIA-1) and TIA-1-related protein (TIAR). The ARE binding protein tristetroprolin (TTP) is synergistically induced by IFN-α/β and p38 MAPK [145]. TTP destabilises several cytokine and chemokine mRNAs [145-147] and can also influence translation [148]. Surprisingly, TTP expression was even increased in response to LPS in the absence of Tyk2 (P. Kovarik, unpublished). TTP activity is also regulated by phosphorylation [149] and a potential impact of Tyk2 on the phosphorylation status of TTP remains to be determined.

Another interesting option is the involvement of Tyk2-dependent microRNAs in the observed translational regulation. A number of IFN and LPS-inducible microRNAs have been described during the last years [150], but to our knowledge none of these has so far been shown to target IL-1β.

IL-1β is critically involved in wide range of inflammatory and autoimmune diseases and is an attractive target for therapeutic interventions [151-153]. Many systemic inflammatory diseases are coupled to IL-1β expression [152], but its role in endotoxin shock is less clear. IL-1β-deficient mice do not show differences in survival in an LPS-induced endotoxin shock model [154], application of an IL-1ra reduced lethality upon LPS injection in rabbits [155], but clinical studies with IL-1ra on human septic patients did not show beneficial effects [156-158]. Tyk2$^{-/-}$ mice are highly resistant to high-dose LPS-mediated endotoxin shock. Serum TNFα and IL-1β are normal in these mice, at least early after LPS injection [67]. A potential

influence of Tyk2 on IL-1β expression at later time points and/or locally remains to be evaluated.

The potential anti-inflammatory role of Tyk2 described herein, raises the question whether Tyk2 has protective functions during IL-1β driven inflammatory diseases. Although the role of Tyk2 in diverse infection and tumour models has been extensively studied, its contribution to autoimmunity and acute/chronic inflammatory diseases is still poorly characterised.

1.4 Material and Methods

Animals and Cells

All mice were on C57BL/6 background, Tyk2, IFNAR1 and Stat1 knockout mice have been described previously [58,159,160]. BMM were isolated and grown in the presence of CSF-1 derived from L929 cells as described previously [161]. After cultivation for 6 days, cells were treated with 100 ng/mL LPS (*E.coli* serotype 055:B5, Sigma) for the times indicated. All animal experiments were discussed and approved by the institutional ethics committee and were carried out in accordance with protocols approved by the Austrian Laws (GZ 68.205/67-BrGT/2003; GZ 68.205/0204-C/GT/2007) and European Directives.

Preparation of whole cell lysates

Whole cell lysates were prepared as described previously [125].

Western blot analysis

Western blots were performed as described previously [125]. The following antibodies were used: goat anti-IL-1β (R&D Systems); mouse anti-panERK (BD Biosciences); donkey anti-goat IgG-HRP (Santa Cruz Biotechnology); donkey anti-rabbit IgG-HRP F(ab)'$_2$ fragment; sheep anti-mouse IgG-HRP F(ab)'$_2$ fragment (all GE Healthcare).

ATP-treatment

Cells (2×10^6) were stimulated with LPS in 1.5 mL complete medium (DMEM, 10% fetal calf serum (FCS), 15% conditioned medium (CM), 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µM β-mercaptoethanol) for 4 h followed by washing once with PBS and addition of 1.5 mL DMEM containing 1 mM, 3 mM or 5 mM ATP (Sigma) and incubated for 30 min.

ELISA

Cell culture supernatants were collected and centrifuged at 14000 $\times$ *g* for 3 min and concentrations of IL-1β and TNFα were determined using ELISA (R&D systems) according to manufacturer's instructions.

TCA precipitation of supernatants

Supernatants (900 µL) were mixed with 13.5 µL of 10% (w/v) sodium deoxycholate monohydrat (Sigma) (final conc. 0.15%) and 300 µL of ice-cold 6.1 M TCA solution were added. Extracts were incubated 1 h on ice to allow complete protein precipitation. Samples were centrifuged at 10000 $\times$ *g* for 10 min at 4ºC. Supernatants were discarded and 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$_2$O and mixed with 25 µL 2 $\times$ SDS-PAGE Laemmli sample buffer followed

by boiling for 5 min. For western blotting, 15-20 µL of sample were separated on a 15%T SDS-PAGE and probed with anti-IL-1β antibody.

Cycloheximide (CHX) treatment

Cells (1×10^6) were stimulated with LPS for 4 h and 20, 10 or 5 µg/mL of CHX (Sigma) were added. Cells were incubated in the presence of CHX and LPS for the indicated times.

Pulse/chase experiments and immunoprecipitation

Cells (3×10^6) were stimulated with LPS in complete medium (see above) for 2.5 h, followed by washing once with PBS and incubation in LPS containing starving medium (DMEM methionine/cysteine-free, 1% BSA, 200 mM L-glutamine, 15% CM, 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 GmbH) 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 (complete medium supplemented with 15 mg/mL L-methionine and L-cysteine (Sigma)). IL-1β was immunoprecipitated from 400 µg proteins with hamster anti-IL-1β antibody (BD Biosciences) using protein A-coupled sepharose beads.

Polysome gradients

Sucrose gradient fractionation was performed as previously described [162] 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 were removed by centrifugation at $3000 \times g$, 4 °C for 2 min. The supernatant was supplemented with 20 mM DTT, 150 µg/mL CHX and 1 mM PMSF and centrifuged at $15000 \times g$, 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, 100 µg/mL CHX) and centrifuged at 38000 rpm, 4 °C, for 2 h (Sorvall SW41 rotor). 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, approx. 20 mg/mL), 225 mM sodium acetate pH 5.2 and precipitated overnight with ethanol.

Reverse transcription-quantitative PCR (RT-qPCR)

RT-qPCR of total cellular mRNA was performed as described previously [125]. RT-qPCR of sucrose gradient-fractionated mRNA: Two fractions each were pooled and 4 µL RNA were used for cDNA synthesis with iScript (Bio-Rad Laboratories). RT-qPCR was performed in duplicate on Eppendorf realplex4 (Eppendorf). mRNA levels were determined by RT-qPCR using TBP as endogenous control. Expression levels of the target genes (TNFα and IL-1β)

were calculated relative to the amount of TBP in each pooled fraction and are given as percentage of the target mRNA present in all fractions.

Primers and probes: The primers and probes for TNFα were described previously [67]. IL-1β was detected with TaqMan Gene Expression Assay (assay ID Mm00434228_m1; Applied Biosystems). TBP was detected with EVAGreen using following primers (5' to 3' direction):

fwd: GAATATAATCCCAAGCGATTTGC (23 nts, Tm=58 °C);

rev: CTGGATTGTTCTTCACTCTTGGCT (24 nts, Tm=60 °C); Amplicon length: 122 nts.

CONCLUDING DISCUSSION

In our study we aimed to find novel functions of Tyk2 in macrophages and to analyse their responses to LPS on the protein level using 2D-DIGE. In the literature, 2DE was mostly applied for established cell lines, and only in a few reports primary cells were used. However, none of these used 2D-DIGE with included internal standard. Therefore, the first goal of the study was the establishment of reproducible experimental conditions for our approach (see appendix, section 1, and [163]). We analysed protein patterns in two different primary macrophage populations (BMM and PM) from WT and Tyk2-deficient mice with or without LPS treatment. During the optimisation we identified components of FCS in the protein patterns from macrophage cell lysates. These additives of standard cell culture media consistently appeared in 2DE analyses of protein lysates and could not be removed completely even with extensive washing steps. Spot positions of its main components (serum albumin, transferrin, α_1-fetoprotein, α_1-antitrypsin) were determined, which enabled us to eliminate spots derived from FCS from the list of candidates for identification by mass spectrometry [163].

Preliminary results showed that spot volume ratios obtained from protein extracts from PM exhibited higher variability than those from BMM. This may be explained by the fact that PM represent a more heterogeneous cell population than BMM [164]. Therefore, we performed our analyses in BMM [125]. We analysed whole cell lysates and nuclear extracts from WT and Tyk2-deficient cells before and after LPS treatment in two different pH gradients using a sample size of three. We detected between 478 and 792 spots present in all images *per* pH gradient and only those were subsequently analysed. We achieved highly reproducible expression patterns between the biological replicates in both types of protein extracts. Statistical analyses revealed that differences in protein expression as low as 30% (1.3-fold) can be detected with our experimental set-up.

We could show that absence of Tyk2 significantly alters the protein expression patterns before and after LPS treatment. However, genotype specific differences were more prevalent than differences in response to LPS. Using a cut-off of at least 40% difference in expression, we found 3-6% of the spots analysed with significant differences between the genotypes. In total, 119 spots were found to be differentially expressed before and after LPS treatment in the four experiments (whole cell lysates and nuclear extracts in two pH gradients, 4-7 and 6-9). Using MS we identified 27 spots, representing 23 different proteins, which were either positively or negatively regulated by Tyk2.

One important aspect of our study in relation to the identified proteins is that they belong to a wide spectrum of functional categories, e.g. oxidative stress and immune response,

metabolism, transcription/translation and cytoskeleton architecture. We identified seven proteins which are known to be regulated by IFNs and several proteins that, based on our present knowledge, have not been previously connected to Tyk2 and/or IFN signalling.

All of the IFN-inducible proteins (see also appendix, section 4) showed the expected up-regulation in response to LPS in WT cells. In the absence of Tyk2, for at least one of the corresponding spots expression levels were reduced after LPS treatment, and some showed already reduced basal expression levels. The requirement of Tyk2 for maintaining basal expression levels has been previously shown for two IFN-inducible proteins, Stat1 and Stat2, and some IFN-inducible mRNAs [58,63,67]. Thus, data are consistent with the reported amplifying role of Tyk2 for IFN signalling [56,58] and provide additional examples for proteins that require Tyk2 for the maintenance of their basal expression levels.

Two proteins involved in oxidative stress response were identified as differentially regulated, CATA (see also appendix, section 3) and PRDX1. Both are antioxidant enzymes and detoxify hydrogen peroxide (H_2O_2) [165]. H_2O_2 is produced during normal physiological processes under aerobic conditions. High amounts of H_2O_2 are produced in phagocytic cells during the "respiratory burst" in response to several stimuli including LPS [166]. H_2O_2 together with other ROS influences the pathogenesis of sepsis through modulation of signalling cascades and cellular injury/damage [167]. Therefore, maintenance of an oxidant/antioxidant balance is important for the appropriate function of immune cells. Changes in expression patterns of these proteins suggest an influence of Tyk2 on the redox balance in macrophages.

We identified three proteins involved in cellular metabolism, e.g. lipid metabolism (THIKA/B), arginine metabolism (ASSY) and purine metabolism (PNPH). Alteration of lipid metabolism during infection and inflammation is a known phenomenon [168] and macrophages are known modulators of immune responses as well as of lipid metabolism [169]. The implication of Tyk2 in the regulation of lipid metabolism in macrophages is further supported by a parallel proteomics study in our laboratory directed to test poly(I:C) responses in Tyk2-deficient macrophages (Grunert et al., unpublished). Two of these proteins are also interesting to mention with respect to their contribution to the immune response. ASSY has been shown to be a critical enzyme for NO-production [170], an important inflammatory mediator. PNPH deficiency leads to cellular immunodeficiency resulting from impaired T cell differentiation and reduced numbers of T cells [171]. Moreover, lower levels of PNPH were found in various types of leukemias and lymphomas [171].

In addition, several differentially expressed proteins play a role in transcription and translation. Although we did not find a direct connection between these and other differentially expressed proteins in our study, this provides further evidence for an impact of Tyk2 on the regulation of protein expression at multiple levels in macrophages.

Among the proteins that were not previously linked to Tyk2 expression, PAI2 showed the strongest difference in expression levels after LPS treatment, which was approximately 15-fold higher in Tyk2^{-/-} as compared to WT cells. PAI2 belongs to the serpin gene family of protease inhibitors and is the physiological inhibitor of tissue- and urokinase-type plasminogen activator (tPA, uPA) [172,173]. The most interesting interconnection between both molecules is their involvement in metastasis progression. Inhibition of metastasis progression *via* inhibition and clearance of extracellular or cell surface bound uPA is the best known function of PAI2 [172,173]. The good prognosis in human cancers associated with elevated level of PAI2 is presumably due to the fact that PAI2 inhibits and clears uPA without initiating downstream signalling events [173]. The involvement of Tyk2 in tumour cell invasiveness has been reported recently [72,174], and it has been shown that either genetic deletion or knock down of Tyk2 by siRNA, as well as inhibition of its activity, reduces metastatic progression. In addition, in the case of prostate cancer cells, the suggested underlying mechanism was reduced uPA signalling [174], by which Tyk2 activation has been reported [175-177]. Intracellular functions of PAI2 are less well-characterised, however, there is evidence that PAI2 is involved in multiple cellular processes including apoptosis [173]. Moreover, interactions of PAI2 with several cytosolic proteins (e.g. IRF3, retinoblastoma protein, proteasome subunit β type I) have also been reported [173]. In our work we demonstrate for the first time a link between Tyk2 and PAI2 expression and this offers an attractive topic for future research in regard to tumour biology as well as to other functions of PAI2.

A well-known property of 2DE is the detection of protein isoforms and/or spot shifts across pH gradients usually resulting from post-translational modifications. Accordingly, in some cases we identified the same protein in different spots on one gel or in both protein extracts at different p*I*s (e.g. PRDX1, IFI4/5). Among these proteins, we analysed PRDX1 spot patterns in more detail and could demonstrate a Tyk2-dependent acidic shift upon LPS treatment. PRDX1 catalyses the reduction of H_2O_2 and during this reaction a cysteine in the active site is oxidised to cysteine sulfenic acid that leads to formation of an intermolecular disulfide [165,178]. However, this reaction occurs slowly and PRDX1 can be further oxidised to sulfinic or sulfonic acid, which results in an inactivation of PRDX1. It has been reported that the (hyper)oxidation of PRDX1 results in a shift towards lower p*I* values in 2DE [179,180]. By comparison with the published findings (discussed in Radwan et al. [125]) we proposed that Tyk2 deficiency suppresses the (hyper)oxidation of PRDX1. However, an acidic shift may be also caused by other modifications, e.g. phosphorylation, which is also known for PRDX1 [165]. Therefore, further investigations should be performed in order to determine the exact nature of the modifications.

The most interesting protein identified within this work with respect to potential consequences for immunity, was IL-1β. We found strongly enhanced levels of pro-IL-1β in Tyk2-deficient as compared to WT macrophages after LPS treatment. IL-1β is an important proinflammatory cytokine produced mainly by monocytes and macrophages in response to inflammatory stimuli and is a key mediator of inflammatory and autoimmune diseases [151,181]. A connection between Tyk2 and IL-1β has not been previously described and we therefore analysed the underlying mechanisms in more detail. We found that Tyk2 is not necessary for IL-1β mRNA expression. In addition, protein stability, processing and secretion of IL-1β were not affected in the absence of Tyk2. In this context we should mention that a recent study showed a limiting effect of PAI2 expression on the secretion of IL-1β in macrophages [112]. However, we did not observe this cross-dependence and the secretion of IL-1β was unimpaired in Tyk2-deficient macrophages despite enhanced expression levels of PAI2. We could show that the translational efficiency of IL-1β mRNA is enhanced in Tyk2-deficient macrophages. This suggests a novel Tyk2 derived signal regulating mRNA translation and studies on the mechanism involved are still ongoing in our laboratory.

Interestingly, PAI2 mRNA expression was also similar in Tyk2$^{-/-}$ and WT cells, arguing for an effect of Tyk2 also on the translation or protein stability of PAI2. In contrast, NMI, a protein known to be transcriptionally regulated by IFNs, was also transcriptionally regulated by Tyk2. In addition to transcriptional and translational regulation, our results suggest that Tyk2 has an impact on the subcellular localisation of EF2. EF2 catalyses the translocation of tRNA and mRNA during the elongation step of protein synthesis. EF2 can be inactivated in response to several stimuli by post-translational modifications (e.g. phosphorylation) that result in a temporary inhibition of overall protein synthesis [182]. However, the observed higher amounts of total EF2 in nuclear extracts argue against a spot shift due to post-translational modifications. The consequence of the different subcellular localisation of EF2 on protein expression is not known at present. Moreover, owing to the facts discussed below further examinations to determine the exact localisation of EF2 should be performed.

Protein patterns of nuclear extracts were clearly different from those of whole cell lysates, although there were some overlaps. More importantly, nuclear extracts showed slightly reduced but still high reproducibility. The protocol used results in relatively crude nuclear extracts and may also lead to co-purification of proteins from other compartments. This was reflected to some extent in the identified proteins, which included, for instance, several cytoskeleton associated proteins. Nevertheless, this enrichment allowed us to detect proteins, which might not be detected by analysis of whole cell lysates. In order to more effectively analyse low abundant and/or compartment-specific proteins, pre-fractionated samples or highly purified organelles could be used. Taking our reproducible results into

account, we can assume that such analyses would be feasible, despite potentially higher technical variations caused by additional preparation procedures.

In summary, our results indicate new interconnections between Tyk2 and several cellular proteins as well as suggest an involvement of Tyk2 in a wide spectrum of cellular processes. The findings that Tyk2 influences multiple levels of protein expression highlight the relevance of proteomics approaches.

APPENDIX

1. Comparison of two different macrophage populations and optimisation of experimental conditions.

The initial work included the establishment of the experimental conditions for the proteomics study on primary murine macrophages using 2D-DIGE. We analysed the protein expression patterns derived from two different primary macrophage populations PM and BMM. For each 2D-DIGE experiment macrophages were isolated from three mice *per* genotype (WT, Tyk2$^{-/-}$) and were either treated with LPS or left untreated. The proteins from whole cell extracts of both populations were analysed in a 10 cm non-linear gradient pH 4-10 in the presence of urea followed by SDS-PAGE in 14 × 14 cm gels. Altogether, we obtained two 2D-DIGE experiments with six analytical gels *per* experiment. We detected 291 spots in PM and 365 spots in BMM, which were present in all images and these were submitted to statistical analysis. The average volume ratios of the three biological replicates and the standard deviation (SD) values were calculated for each spot. Mean SD values were ±0.183 in PM and ±0.122 in BMM. The frequency distribution of the SD values from both macrophage populations are shown in Fig. 1. The minimal detectable differences (calculated as described previously [125]) were 66% for PM and 44% for BMM. These results demonstrated that the spot volume ratios detected in samples from PM exhibit higher variability than those from BMM. Therefore, we decided to perform further analyses on BMM. We modified the composition of the 2DE buffers, used narrower pH gradients (4-7 and 6-9) and extended the separation distance in both dimensions. This resulted in mean SD values of ±0.073 (pH 4-7) and ±0.078 (pH 6-9) and minimal detectable differences of 26% and 28%, respectively.
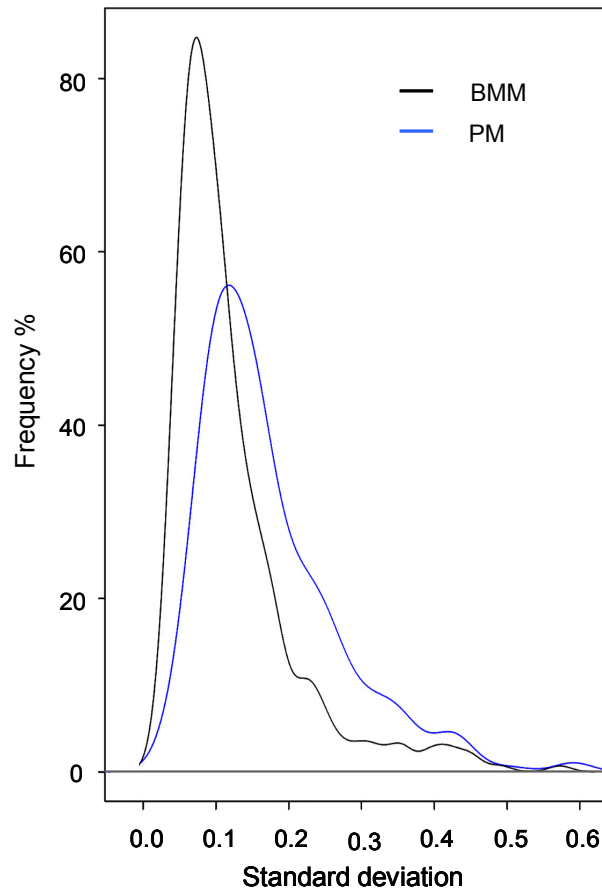
Figure 1. Frequency distribution of SD values in PM and BMM. SDs of spot volume ratios from three biological replicates were calculated for each spot and plotted against frequencies.

2. Other identified spots

Table 1 contains spots which were identified but were not included in the initial publication [125]. Positions of the spots on the corresponding gels are shown in Fig. 2. Most of the spots were identified as control spots for MS and accordingly, intensities in silver stained gels are high and, mostly   low (<30%) or no genotype specific differences were determined. In addition, two differentially expressed spots (pro-IL-1β and T-cell specific GTPase (TGTP)) were identified in the course of further MS-analyses.

Table 1. Other identified spots

Spot #	Protein Name	Accession# Swissprot	WT	WT +LPS	Tyk2-/-	Tyk2-/- +LPS	p gt [a]	p gt x tr [b]	M_r (kDa) theoret.	pI theoret.	MS Method	Protein extract [c]
	Proteins identified as control spots											
1	Proteasome activator complex subunit 1 (PSME1)	P97371	1	1.18	0.81	0.68	n.s. [d]	n.s.	28.7	5.7	ESI	A
2	D-3-phosphoglycerate dehydrogenase (SERA)	Q61753	1	0.91	0.68	0.60	0.0006	n.s	56.4	6.1	ESI	A
3	Aldehyde dehydrogenase mitochondrial, precursor (ALDH2)	P47738	1	0.73	1.31	0.98	0.0032	n.s.	56.5	7.5	ESI	A
4	Glutaredoxin-3 (GLRX3)	Q9CQM9	1	0.45	0.97	0.68	n.s.	0.0126	37.8	5.4	MALDI	A
5	Galectin-3 (LEG3)	P16110	1	0.90	0.70	0.56	0.0129	n.s.	27.5	8.5	MALDI	C
6	Plastin-2 (PLSL)	Q61233	1	1.27	1.06	1.54	n.s	n.s.	70.1	5.2	MALDI	B

Table 1. Other identified spots (continued)

Proteins identified in the course of further MS-analyses												
7	T-cell specific GTPase (TGTP)	Q62293	1	1.94	0.53	0.51	0.0003	0.0005	47.1	5.5	MALDI	A
8	Interleukin-1 beta, precursor (IL1B)	P10749	1	3.27	1.56	14.35	0.0083	0.0024	30.9	4.6	MALDI	A

[a] p-values for the differences between genotypes under basal conditions

[b] p-values for the differences between genotypes in response to LPS treatment

[c] Protein extracts: A: whole cell lysates pH 4-7; B: nuclear extracts pH 4-7; C: nuclear extracts pH 6-9
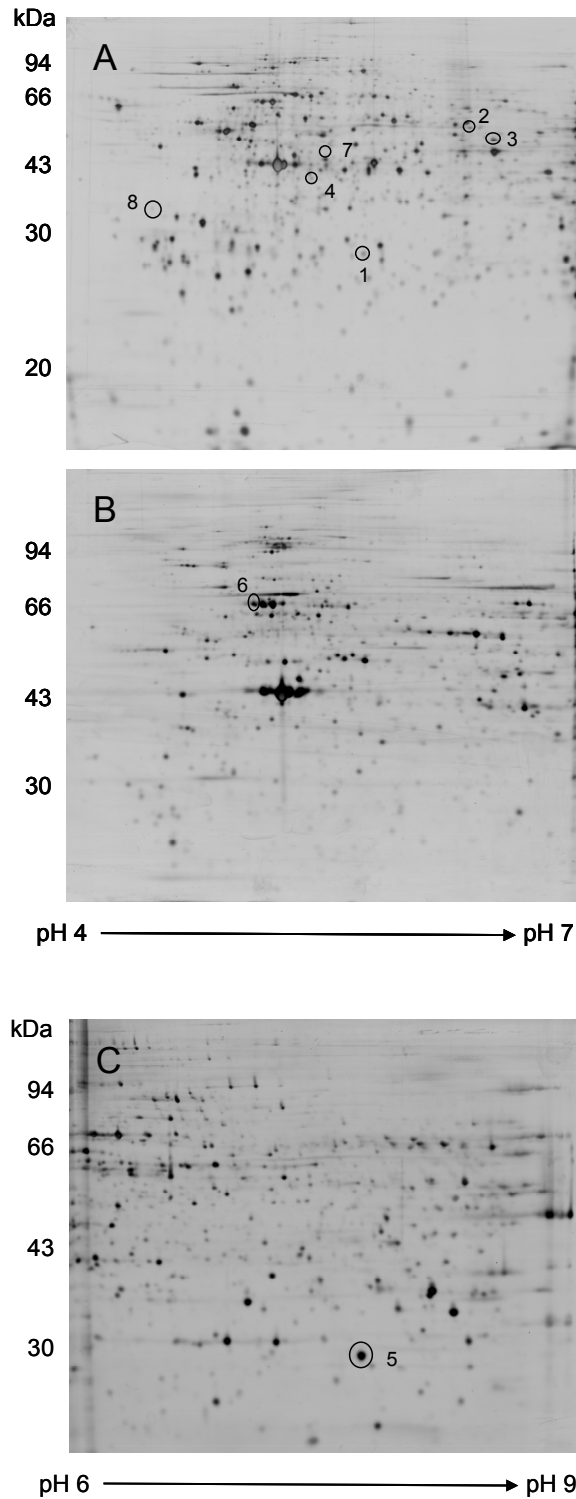
[d] Not significant (p-value >0.05)

Figure 2. Representative 2DE protein patterns of Tyk2^{-/-} macrophages. Analytical gels (75 µg protein each) visualised by MS-compatible silver stain after 2D-DIGE. Identified proteins are indicated by the spot numbers used in Table 1. Whole cell lysates: (A) pH gradient 4-7, 11.5%T SDS-PAGE. Nuclear extracts: (B) pH 4-7; (C) pH 6-9; both: 10%T SDS-PAGE.

3. Catalase (CATA) expression patterns

As determined by 2D-DIGE, CATA showed enhanced expression levels in Tyk2-deficient macrophages both before and after LPS treatment (spot 12 in Table 3 [125], and Fig. 3A). The expression level of CATA was increased in the absence of Tyk2 to similar degrees both before and after LPS treatment. By 1D western blot analyses we could not detect any differences in total CATA protein expression after LPS treatment and between the genotypes (Fig. 3B). Using 2D western blot we found four CATA spots (Fig. 3C), but could not explicitly determine which of the spots corresponds to the spot identified within the 2D-DIGE experiments. All of the detected spots displayed similar M_r but different pIs. However, there were no clear differences between genotypes and after LPS treatment for any of the spots.
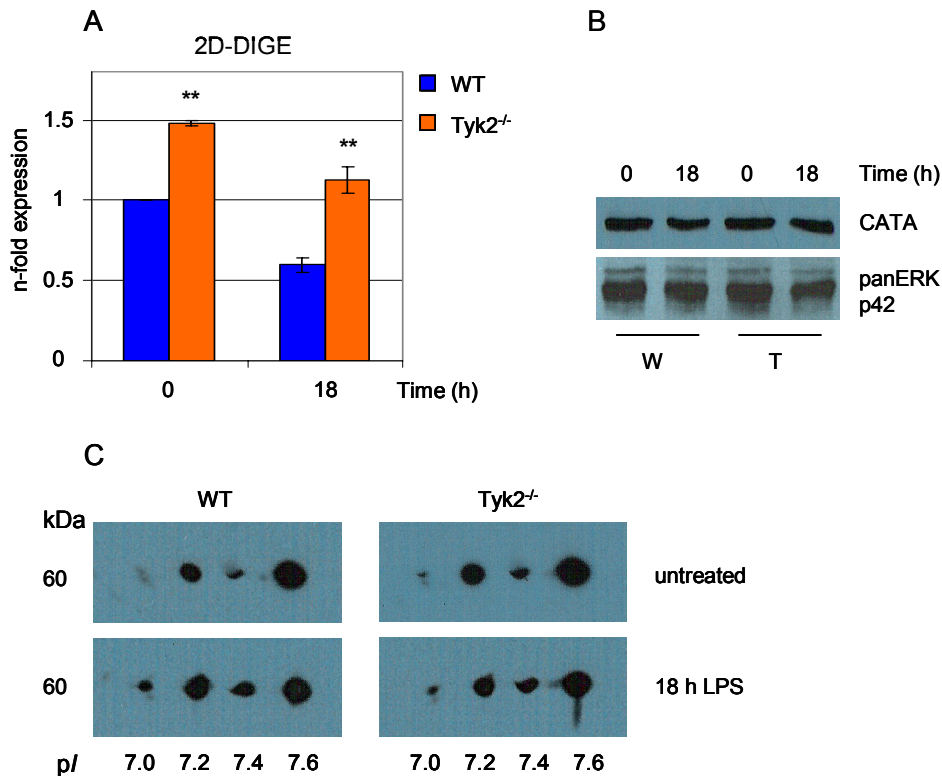

Figure 3. Effect of Tyk2 deficiency on the expression of CATA protein. Macrophages were treated with LPS for the indicated times and whole cell lysates were subjected to 2D-DIGE analysis (A) or western blotting (B, C). (A) 2D-DIGE: expression levels are given as fold ratios relative to unstimulated WT cells. Mean values ±SD of three biological replicates are shown. (B) 1D western blot: 5 µg protein *per* lane were separated by 10%T SDS-PAGE. Protein loading was controlled by reprobing with an anti-panERK antibody. Data are representative of three experiments. (C) 2D western blot: 50 µg protein were separated by 2DE using a pH 6-10 gradient for the first dimension, followed by the second dimension on a 12%T SDS-PAGE. Data are representative of three experiments. W - WT, T - Tyk2^{-/-}, ** p ≤ 0.01.

4. T-cell specific GTPase (TGTP) expression patterns

TGTP was found as differentially expressed spot with reduced expression levels in Tyk2-deficient macrophages both before and after LPS treatment (spot 7 in Fig. 2 and Table 1, and Fig. 4A). TGTP is up-regulated about 2-fold in WT cells after LPS treatment. In contrast, expression levels of TGTP remain unchanged in Tyk2$^{-/-}$ cells. Preliminary results from western blot analysis are shown in Fig. 4B. Up-regulation of TGTP protein after LPS treatment was clearly detectable in WT cells. TGTP is a known IFN-inducible protein and, as to be expected, TGTP could not be detected in untreated as well as LPS-treated IFNAR1-deficient cells. In the absence of Tyk2, TGTP could not be detected in untreated cells, which is in accordance with the reduced expression as determined by 2D-DIGE. In contrast to the 2D-DIGE data, a very faint TGTP band was detectable after LPS treatment in Tyk2$^{-/-}$ cells, arguing for LPS-mediated up-regulation in the absence of Tyk2. However, TGTP expression was still reduced as compared to WT cells.
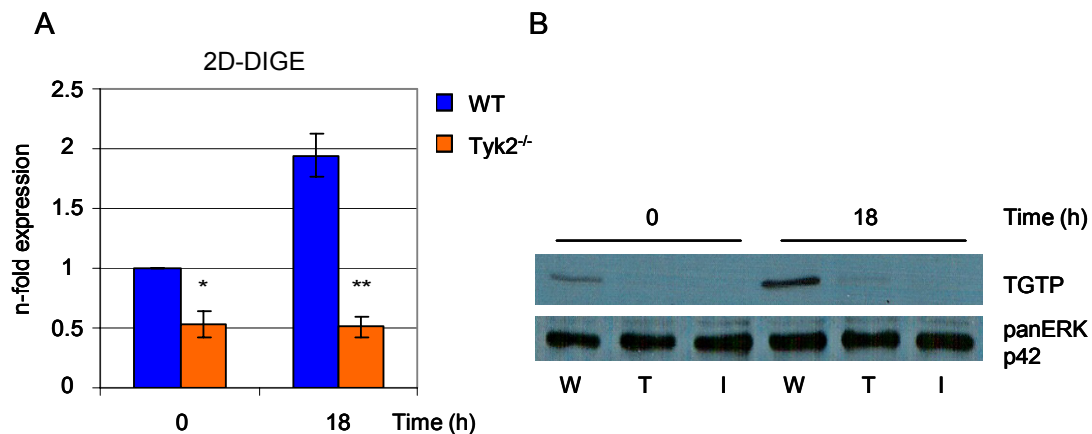
Figure 4. Effect of Tyk2 deficiency on the expression of TGTP protein. Macrophages were treated with LPS for the indicated times and whole cell lysates were subjected to 2D-DIGE (A) or western blotting (B). (A) 2D-DIGE: expression levels are given as fold ratios relative to unstimulated WT cells. Mean values ±SD of three biological replicates are shown. (B) Western blot analysis: 10 µg protein *per* lane were separated by 10%T SDS-PAGE. Protein loading was controlled by reprobing with an anti-panERK antibody. Preliminary data from one experiment are shown. W - WT, T - Tyk2$^{-/-}$, I - IFNAR1$^{-/-}$, * p ≤ 0.05, ** p ≤ 0.01.

5. Material and Methods

Animals, Cells and preparation of whole cell lysates

Mice, BMM, and preparation of whole cell lysates were described previously [125]. PM were isolated and cultivated as described previously [183].

Western blot analysis

1D and 2D Western blots were performed as described previously [125]. Antibodies used: goat anti-TGTP (Santa Cruz Biotechnology), rabbit anti-CATA (Calbiochem) mouse anti-panERK (BD Biosciences); donkey anti-goat IgG-HRP (Santa Cruz Biotechnology).

2D-DIGE pH 4-10 NL (10 cm)

The first dimension was carried out without oil for a total of 15 kVh on a Multiphor II system (GE Healthcare) using 10 cm laboratory-made pH 4-10 IPG strips with non-linear pH gradients. The three labelled samples were mixed and the volume was adjusted to 320 µL with rehydration buffer (8 M urea; 2% (w/v) CHAPS; 65 mM DTT; 2% (v/v) carrier ampholyte mix). The IPG strips pH 4-10 were loaded with the samples through passive in-gel rehydration for 8 h at room temperature. The focused IPG strips were reduced 10 min (1% DTT) and alkylated 5 min (1.25% IAA) in equilibration buffer (6M urea; 30% glycerol; 62.5 mM Tris-HCl pH 6.8; 2% SDS). The equilibrated strips were placed onto 10-15% gradient polyacrylamide gels (with 5% stacking gel) and sealed with 1% agarose in SDS running buffer. SDS-PAGE was performed according to Laemmli [184]. Gels (14 × 14 × 0.15 cm) were run for 5 h at 25 mA *per* gel in a Hoefer SE600 electrophoresis chamber (Hoefer Scientific Instruments).

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Publications

1. Radwan M, Miller I, Grunert T, Marchetti-Deschmann M, Vogl C, O'Donoghue N, Dunn J M, KolbeT, Allmaier G, Gemeiner M, Müller M, Strobl B.
   The impact of tyrosine kinase 2 (Tyk2) on the proteome of murine macrophages and their response to lipopolysaccharide (LPS).
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2. Miller I, Radwan M, Strobl B, Müller M, Gemeiner M.
   Contribution of cell culture additives to the two-dimensional protein patterns of mouse macrophages.
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Published Conference Abstracts

1. <u>Radwan M</u>, Miller I, Strobl B, Grunert T, Dunn J M, Vogl C, Marchetti M, Allmaier G, Gemeiner M, Müller M.

   The influence of Tyk2 deficiency on the murine macrophage proteome.

   Journal of Interferon & Cytokine Research 2007 Aug; 27(8): 727

Conference posters

1. 3rd ESF Functional Genomics Conference, Innsbruck, Austria, 2008

   <u>Radwan M</u>, Miller I, Marchetti-Deschmann M, Vogl C, O'Donoghue N, Dunn J M, Kolbe T, Allmaier G, Gemeiner M, Müller M, Strobl B.

   The impact of tyrosine kinase 2 (Tyk2) on the proteome of murine macrophages and their response to lipopolysaccharide (LPS).

2. 7th Annual World Congress Human Proteome Organisation, Amsterdam, the Netherlands, 2008

   Grunert T, Strobl B, Marchetti M, Miller I, <u>Radwan M</u>, Vogl C, Gemeiner M, Allmaier G, Müller M.

   Proteomics study of Tyk2-deficient murine macrophages in response to poly(I:C).

3. Annual International Society for Interferon and Cytokine Research Meeting, Oxford, UK, 2007

   <u>Radwan M</u>, Miller I, Strobl B, Grunert T, Dunn J M, Vogl C, Marchetti M, Allmaier G, Gemeiner M, Müller M.

   The influence of Tyk2 deficiency on the murine macrophage proteome.

4. 20th Annual Meeting of the European Macrophage and Dendritic Cell Society, Freiburg, Germany, 2006

   <u>Radwan M</u>, Miller I, Strobl B, Vogl C, O'Donoghue N, Dunn M, Gemeiner M, Müller M.

   The influence of Tyk2 deficiency on the murine macrophage proteome.

5. 3rd Summer School in Proteomics basic – Exploring the Diversity of Proteins, Brixen/Bressanone, Italy, 2006

   <u>Radwan M</u>, Miller I, Strobl B, Vogl C, O'Donoghue N, Dunn M, Gemeiner M, Müller M.

   The influence of Tyk2 deficiency on the murine macrophage proteome.

6. HUPO 4th Annual Word Congress, Munich, Germany, 2005

   Miller I, <u>Radwan M</u>, Strobl B, Vogl C, Müller M, Gemeiner M.

   Spot patterns of lysed mouse macrophages: What comes from cell culture medium?

7. 2nd International Symposium of the Austrian Proteomics Platform, Seefeld, Austria, 2005

Radwan M, Eliasen M, Winkler W, Seipelt J, Sommergruber W, Zehl M, Marchetti M, Allmaier G, Oehler R.

Fever-range hyperthermia induces specific proteome changes in human lymphocytes.

LEBENSLAUF

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2005 - present	Dissertation, Institut für Tierzucht und Genetik und Institut für medizinische Chemie, Veterinärmedizinische Universität Wien "Proteomics in immunity: Regulatory functions of tyrosine kinase 2 on protein expression in macrophages"
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Publikationen

1. Radwan M, Miller I, Grunert T, Marchetti-Deschmann M, Vogl C, O'Donoghue N, Dunn J M, KolbeT, Allmaier G, Gemeiner M, Müller M, Strobl B.
   The impact of tyrosine kinase 2 (Tyk2) on the proteome of murine macrophages and their response to lipopolysaccharide (LPS).
   Proteomics 2008 Sept; 8(17): 3469-3485

2. Miller I, Radwan M, Strobl B, Müller M, Gemeiner M.
   Contribution of cell culture additives to the two-dimensional protein patterns of mouse macrophages.
   Electrophoresis 2006 Apr; 27(8): 1626-9

Publizierte Konferenz-Abstracts

1. <u>Radwan M</u>, Miller I, Strobl B, Grunert T, Dunn J M, Vogl C, Marchetti M, Allmaier G, Gemeiner M, Müller M.

   The influence of Tyk2 deficiency on the murine macrophage proteome.

   Journal of Interferon & Cytokine Research 2007 Aug; 27(8): 727

Konferenzposter

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Fever-range hyperthermia induces specific proteome changes in human lymphocytes.

ABBREVIATIONS

1DE/2DE	one- or two-dimensional gel electrophoresis
2D-DIGE	two-dimensional fluorescence difference gel electrophoresis
AP-1	activating protein-1
ARE	AU-rich elements
ASSY	argininosuccinate synthase
BMM	bone marrow-derived macrophages
Casp-1/Casp-8	caspase-1/caspase-8
DAMPs	danger-associated molecular patterns
EF2	elongation factor 2
ERK	extracellular signal-regulated kinase
ESI	electrospray ionisation
FCS	fetal calf serum
IEF	isoelectric focusing
IFI4/5	interferon-activable protein 4 or 5
IFIT	interferon-induced protein with tetratripeptide repeats
IFN	interferon
IFNAR	IFN-α/β receptor
IL	interleukin
IL-1ra	IL-1 receptor antagonist
IPG	immobilised pH gradients
IRF	interferon regulatory factor
Jak	Janus kinase
LBP	LPS-binding protein
LC	liquid chromatography
LPS	lipopolysaccharide
Mal	MyD88 adaptor-like
MALDI	matrix-assisted laser desorption/ionisation
MAPK	mitogen-activated protein kinases
M_r	molecular mass
MS	mass spectrometry
MyD88	myeloid differentiation primary response gene 88
NAIP	NLR family, apoptosis inhibitory protein
NFκB	nuclear factor-κB
NLRC	NLR family, CARD domain containing
NLRP	NLR family, pyrin domain containing
NLRs	nucleotide-binding domain, leucine-rich repeat containing family
NLRX1	NLR family member X1

NMI	N-myc-interactor
NO	nitric oxide
NOD	nucleotide-binding oligomerisation domain containing
PAI2	plasminogen activator inhibitor 2
PAMPs	pathogen-associated molecular patterns
pI	isoelectric point
PM	thioglycollate-elicited peritoneal macrophages
PNPH	purine nucleoside phosphorylase
PRDX1	peroxiredoxin 1
PRRs	pattern-recognition receptors
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Stat	signal transducer and activator of transcription
TBP	TATA binding protein
T_H2	T helper cell type 2
THIKA/B	3-Ketoacyl-CoAthiolase A or B
TIR	toll-interleukin-1 receptor
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
WT	wild-type

CONFERENCE POSTERS

SPOT PATTERNS OF LYSED MOUSE MACROPHAGES: WHAT COMES FROM CELL CULTURE MEDIUM?

Ingrid Miller[1], Marta Radwan[1,2], Birgit Strobl[2], Claus Vogl[3], Mathias Müller[2,3], Manfred Gemeiner[1].

[1]) Institute of Medical Chemistry, Department of Natural Sciences, [2]) Austrian Centre for Biomodels and Transgenetics (OEZBT), [3]) Institute of Animal Breeding and Genetics; all: University of Veterinary Medicine Vienna

Macrophages play an important role in immunity. For *in vitro* analysis and proteomics, primary mouse macrophages were isolated from different origins by standard procedures. The protocols for preparation and growth/differentiation of peritoneal and bone marrow-derived macrophages differ in incubation time in cell culture, amount of fetal calf serum (FCS) in the culture medium (5-10%) and the requirement for conditioned medium (as a source for M-CSF).
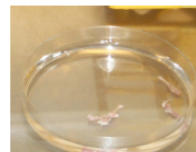
Preparation of macrophages

Bone marrow-derived macrophages were obtained from bone marrow cultures selected for 6 days in DMEM+10%FCS supplemented with L929 cell-derived M-CSF as described (Baccarini et al., J. Immunol. 134 (1985), 2658-2665).

Peritoneal macrophages were isolated from thioglycollate-pretreated mice (4 days after injection) as previously described (Bogdan et al., J. Immunol. 159 (1997), 4506-4513) and kept in DMEM+5%FCS overnight prior to treatment.

2-DE reveals that traces of these essential additives can be found in the overall protein pattern.

macrophage preparation

Standard preparation of peritoneal mouse macrophages; 2-DE: IPG 4-10NL, SDS-PAGE 10-15 % T, silverstain.

DIGE

red channel: peritoneal mouse macrophages
blue channel: FCS
violet (overlap): spots in common

FCS

Identification of main proteins

by immunoblotting:
SA, serum albumin
α_1AT, α_1-antitrypsin = Pi (antihuman, Dako)
AFP, α_1-fetoprotein (Dako)
Tf, transferrin
in addition: Wait et al, Electrophoresis 23 (2002), 3418-3427.

Optimization of cell washing

1x wash   3x wash   6x wash
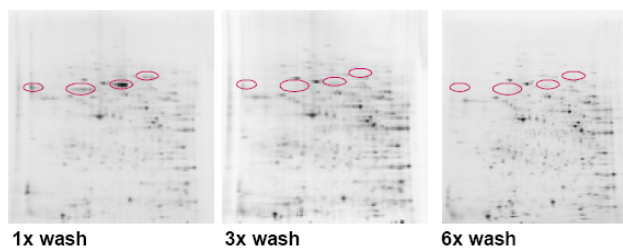
Macrophages are very sensitive to changes in the environment and to mechanical treatment. Therefore, special care has to be taken to avoid any procedures which might cause stress and – as a consequence – alter expression patterns.

Careful washing reduces FCS contamination from about 5 % to less than 1 % of overall protein content (for peritoneal macrophages).
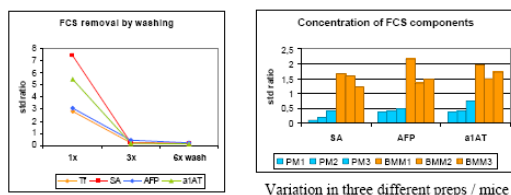
The residual traces of FCS components are rather stable in their levels and correlate with FCS concentration in original culture medium (slightly higher in bone-marrow derived macrophages). In addition, when knowing the exact positions of the main FCS spots in 2-DE, they can be excluded from evaluation.

Variation in three different preps / mice

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The influence of Tyk2 deficiency on the murine macrophage proteome

Marta Radwan[1,2,3], Ingrid Miller[2], Birgit Strobl[1,3], Claus Vogl[3], Niaobh O'Donoghue[4], Michael Dunn[4], Manfred Gemeiner[2] and Mathias Müller[1,3]

[1]Biomodels Austria, [2]Institute of Medical Chemistry, [3]Institute of Animal Breeding and Genetics, [1-3]Veterinary University of Vienna, Austria; [4]UCD Conway Institute of Biomolecular and Biomedical Research; University College Dublin, Ireland

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INTRODUCTION

The Janus kinase (Jak) family member Tyk2 is involved in signaling via a number of cytokines and growth hormones. It has been recently shown in our lab that Tyk2-deficient mice are highly resistant to lipopolysaccharide (LPS) induced endotoxin shock. Tyk2-deficiency also results in impaired production of nitric oxide and interferon-beta (IFNß) in LPS-treated macrophages. Macrophages play a major role in immunity and inflammatory responses. LPS is an integral structural component of the outer membrane of Gram negative bacteria and one of the most potent initiators of inflammation.

Aim of this project is to investigate in more detail the role of Tyk2 for macrophage function. Using Two-Dimensional Fluorescence Difference Gel Electrophoresis (2D-DIGE) we compared protein patterns in two different macrophage populations from Tyk2-deficient and wildtype mice before and after LPS treatment.

METHODS

Cells: Thioglycollate-elicited peritoneal (PM) and bone marrow-derived macrophages (BMM) from wildtype (WT) and Tyk2-deficient (Tyk2-/-) mice were isolated according to standard methods. Both were stimulated with LPS (100ng/ml for 8 h or 18 h, respectively). Each group comprised three animals.
Samples: Whole cell lysates or cytosolic/nuclear extracts were minimally labelled with CyDyes and subjected to 2D-DIGE.
2D: The first dimension was performed in IPG dry strips either laboratory made (11 cm) or commercial (24 cm). The second dimension was SDS-PAGE (10-15% or 10%, respectively).
Analysis: Gel images were evaluated with DeCyder in combination with univariate statistical analyses.
MS: Spots were tryptically digested and identified by nLC-MS/MS.

CONCLUSIONS

1. The absence of Tyk2 alters the expression level of a subset of proteins in whole cell lysates from two different macrophage populations.
2. Differences in expression levels are modest (usually <2-fold) but highly reproducible.
3. Differential expression is in most cases independent of LPS treatment.
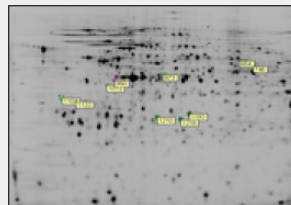4. Identification of candidate spots revealed several proteins involved in immunological processes.

Future perspectives

1. Extended separation distance and the use of more narrow pI ranges for 2D will allow better resolution.

2. Analysis of nuclear extracts will enable us to monitor expression levels in a distinct protein population (see Fig.5).
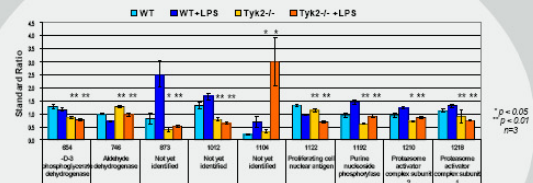
RESULTS

Fig. 1a.
Selected proteins exhibiting Tyk2 dependent expression levels.

*p < 0.05
**p < 0.01
n=3

Expression levels of the 9 proteins highlighted in Fig.1b are shown as a standard ratio, i.e. ratio of the sample compared to the internal standard (for each spot).

Differences in expression were analysed by the Student's test and ANOVA. Results are shown as mean values +-SD. Identified proteins are indicated.

Fig. 1b.
Master gel of 2D-DIGE experiment.

This image is an example for 2D-DIGE pattern of the BMM (pH 4-7; 24 cm). Histograms of highlighted spots are shown in Fig.1a. Overall spot number detected:1397; in all samples: 579.

Fig. 2.
Absence of Tyk2 significantly alters the overall protein pattern of macrophages.

Statistical analyses of the effect of Tyk2 on the overall protein pattern irrespective of LPS treatment (full factorial two-way ANOVA). Cumulative distribution of F-values of spots and their expectation under the null hypothesis.
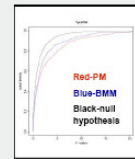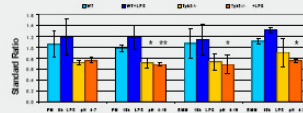
Red-PM
Blue-BMM
Black-null hypothesis

Fig. 3.
Expression levels are highly reproducible in both macrophage populations.

*p < 0.05
**p < 0.01
n=3

As an example the expression levels of proteasome activator complex subunit 1 from four different experiments are shown. The experiments were done in both cell populations and also with different treatments. In addition the lysates were separated on two gel formats and pH gradients.

Fig. 4.
Extended separation distance results in better resolution.

Statistical analyses of the effect of the gel system on the detection of differences in spot intensities between genotypes and treatment (a). Cumulative distribution of F-values of spots and their expectation under the null hypothesis.

Blue – b
Green – c
Black - null hypothesis
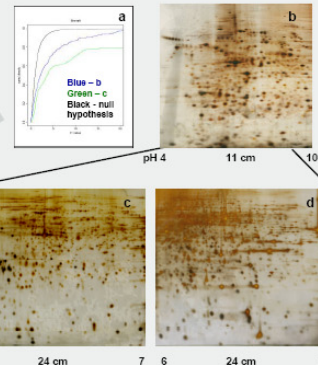
Silver stained 2D gel images of wildtype BMM, resolved over 3 pH ranges: 4-10 NL (b), 4-7 linear (c) and 6-9 linear (d)

Fig. 5.
Cytosolic versus nuclear extracts (2D-DIGE)
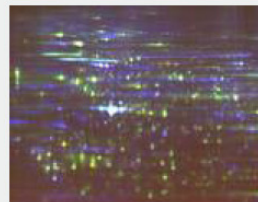
blue: nuclear extracts
green: cytosolic extracts
red: whole cell extracts

The influence of Tyk2 deficiency on the murine macrophage proteome

Marta Radwan[1,2,3], Ingrid Miller[2], Birgit Strobl[1,3], Claus Vogl[3], Niaobh O'Donoghue[4], Michael Dunn[4], Manfred Gemeiner[2] and Mathias Müller[1,3]

[1]Biomodels Austria, [2]Institute of Medical Chemistry, [3]Institute of Animal Breeding and Genetics, [1-3]Veterinary University of Vienna, Austria; [4]UCD Conway Institute of Biomolecular and Biomedical Research; University College Dublin, Ireland

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The Janus kinase (Jak) family member Tyk2 is involved in signaling via a number of cytokines and growth hormones. It has been recently shown in our lab that Tyk2-deficient mice are highly resistant to lipopolysaccharide (LPS) induced endotoxin shock. In macrophages Tyk2-deficiency results in impaired LPS induced production of nitric oxide and interferon-beta (IFNß).
Aim of this project is to investigate in more detail the role of Tyk2 for macrophage function. Using Two-Dimensional Fluorescence Difference Gel Electrophoresis (2D-DIGE) we compared protein patterns of bone marrow-derived macrophages from Tyk2-deficient and wildtype mice before and after LPS treatment.

METHODS

<u>Cells:</u> Bone marrow-derived macrophages (BMM) from wildtype (WT) and Tyk2-deficient (Tyk2-/-) mice were isolated according to standard methods. Both were stimulated with LPS (100 ng/ml for 8 h or 18 h, respectively). Each group comprised three animals.
<u>Samples:</u> Whole cell lysates and nuclear extracts were minimally labeled with CyDyes and subjected to 2D-DIGE.
<u>Analysis:</u> Gel images were evaluated with DeCyder in combination with univariate statistical analyses.
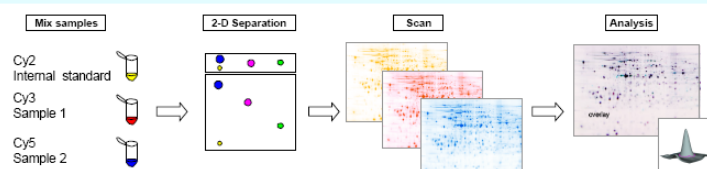<u>MS:</u> Spots were tryptically digested and identified by nLC-MS/MS.

Fig. 1. Principle of 2D-DIGE technology. Proteins are labelled through covalent attachment of fluorescent CyDyes to amino groups. CyDyes are mass and charge matched, have non overlapping emission and excitation spectra. On each gel two samples and an internal standard can be examined. The internal standard is a pool of all specimens within the respective study. This set up reduces gel to gel variation and allows more accurate quantification.

RESULTS

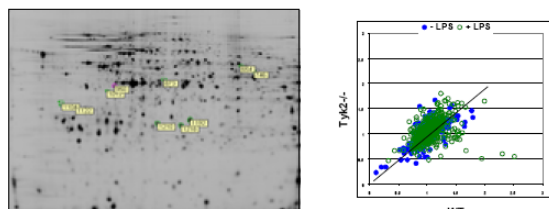### Absence of Tyk2 significantly alters the overall protein pattern of macrophages.

Fig. 2. This image is an example of a 2D pattern of whole cell extracts (pH 4-7; 24 cm). Histograms of highlighted spots are shown in Fig.4. Overall spot number detected:1397; in all samples: 579.

Fig. 3. Scatter plots of spots in 2D-DIGE of whole cell extracts. Protein expression in the two genotypes with and without LPS treatment are shown as mean values of standard ratios.
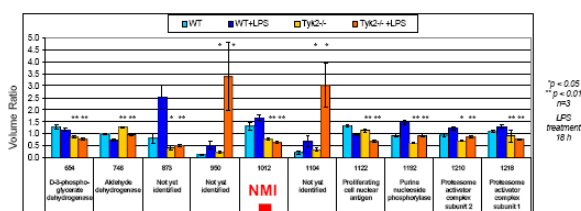
Fig. 4. Expression levels of the 10 proteins highlighted in Fig.2 are shown as standard ratios, i.e. ratio of the sample compared to the internal standard (for each spot). Differences in expression were analysed by the Student's test and ANOVA. Results are shown as mean values +/-SD. Identified proteins are indicated.
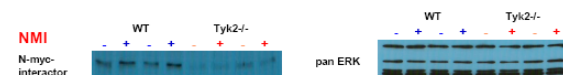
Fig. 5. Protein levels of NMI in whole cell lysates analysed by western blot. 20 µg protein were loaded per lane of a 10% SDS-PAGE. BMM were treated with LPS for 18h (+) or left untreated (-). pan ERK antibody was used as loading control.

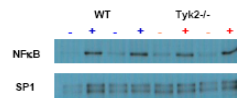### Translocation of NFκB upon LPS is not affected in Tyk2 deficient macrophages.

Fig.6. Western blot analyses of NFκB in nuclear extracts of BMM stimulated with LPS for 30 min (+) or left untreated (-). 3 µg of nuclear protein were loaded per lane of an 8% SDS-PAGE. SP1 antibody was used as loading control.

Absence of Tyk2 significantly alters the nuclear protein pattern.
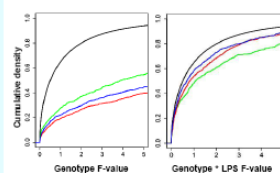
Fig.7. Statistical analysis of the effect of knocking out Tyk2 on the protein pattern (full factorial two-way ANOVA with genotype and LPS as factors), using whole cell extracts at pH 4-7 (green 579 spots), nuclear extracts at pH 4-7 (blue 731 spots) and nuclear extracts at pH 6-9 (red 794 spots). To the left, the deviation of spot intensity from the expectation due to genotype (cumulative density of the F-values), to the right, due to the interactions between genotype and LPS. The black line signifies the null hypothesis, i.e., the absence of an effect.

CONCLUSIONS

1. The present approach including 2D-DIGE technology shows highly reproducible expression values in macrophage cell extracts.
2. The absence of Tyk2 significantly alters the protein expression pattern in macrophage whole cell nuclear extracts.
3. With a few exceptions, genotype differences are only modest (2-fold or below) and independent of LPS treatment.
4. The LPS-TLR4-MyD88 dependent nuclear translocation of NFκB is unperturbed in the absence of Tyk2.
5. Seven differentially expressed proteins have been identified so far and several are involved in immunological processes.
6. Expression levels of one candidate, N-myc interactor (NMI), have additionally been analysed by western blotting and 2D-DIGE results were confirmed: expression of NMI is Tyk2 dependent both before and after LPS treatment.

The influence of Tyk2 deficiency on the murine macrophage proteome

Marta Radwan[1,2], Ingrid Miller[2], Birgit Strobl[1,3], Tom Grunert[1,4], Michael Dunn[5], Claus Vogl[1,3], Martina Marchetti[4], Günter Allmaier[4], Manfred Gemeiner[2] and Mathias Müller[1,3]

[1]Institute of Animal Breeding and Genetics; [2]Institute of Medical Chemistry; [3]University Center Biomodels Austria GmbH; [1-3] University of Veterinary Medicine Vienna, Austria; [4] Institute of Chemical Technologies and Analytics, Vienna University of Technology, Austria; [5] UCD Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Ireland

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INTRODUCTION

Tyrosine kinase 2 (Tyk2) belongs to the Janus kinase (Jak) family of non - receptor tyrosine kinases and was originally described to be essential for type I interferon signalling. It has been shown recently in our lab that Tyk2-deficient mice are highly resistant to lipopolysaccharide (LPS) induced endotoxin shock and that Tyk2-deficiency results in impaired macrophage responses.

Aim of this project is to investigate in more detail the role of Tyk2 for macrophage function. Using Two-Dimensional Fluorescence Difference Gel Electrophoresis (2D-DIGE) we compared protein patterns of bone marrow-derived macrophages (BMM) from Tyk2-deficient (Tyk2-/-) and wildtype (WT) mice before and after LPS treatment.
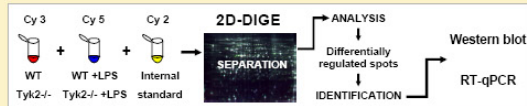
METHODS

Fig. 1. Experimental workflow: Whole cell and nuclear extracts, respectively, obtained from BMM (three mice per genotype) were labelled with CyDyes (mass and charge matched, spectrally resolvable fluorescent dyes). Two samples and an internal standard were loaded per gel. The internal standard is a pool of all specimens within the respective set and is used for normalisation of spot volumes. The gel images were analysed with DeCyder software. Differentially expressed proteins were identified by mass spectrometry (MS). Gene and protein expression profiles were further investigated by RT-qPCR and western blotting.

RESULTS

Absence of Tyk2 significantly alters the protein expression patterns in macrophages

B		Whole cell lysates		Nuclear extracts	
		18 h LPS		8 h LPS	
	pH	4-7	6-9	4-7	6-9
Number of spots in all 12 samples		579	467	731	795
Differentially [a)] expressed spots		44	53	66	51
Selected spots for MS		12	15	13	11
Identified spots/proteins		9/9	7/6	8/6	7/6

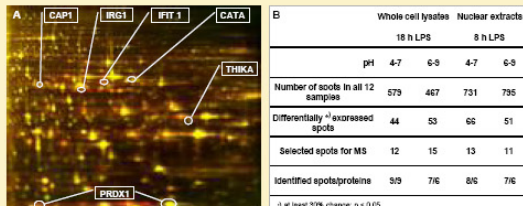a) at least 20% change; p < 0.05

Fig. 2. A) Representative 2D-DIGE gel of whole cell extracts from WT BMM (pH 6-9). Red: WT+LPS; green: WT untreated; yellow: overlap of the two samples. Protein names of identified spots within this experiment are indicated. B) Overview of analyses performed and spot numbers detected/identified.

Differentially expressed proteins exert various functions

Differentially expressed proteins				
Protein Name / Function	WT	WT +LPS	Tyk2-/-	Tyk2-/- +LPS
IMMUNE RESPONSE				
Proteasome activator complex subunit 1 (PA28α/PSME1)	1	1.2	0.8	0.7
Proteasome activator complex subunit 2 (PA28β/PSME2)	1	1.3	0.8	0.9
Interferon inducible proteins				
Interferon-induced protein with tetratricopeptide repeats 1 (IFIT1)	1	3.1	0.5	0.5
N-myc-interactor (NMI)	1	1.3	0.8	0.5
Interferon-induced protein with tetratricopeptide repeats 1 (IFIT1)	1	3.9	0.7	0.6
Interferon induced protein 35kD homolog (IFI35)	1	1.3	0.5	0.7
Interferon-activatable protein 205 or 204 (IFI205 or -4)	1	1.3	0.6	1
OXIDATIVE STRESS RESPONSE				
Peroxiredoxin-1 (PRDX1)	1	2.5	1.2	2
Peroxiredoxin-1 (PRDX1)	1	0.7	1.3	1.3
Catalase (CATA)	1	0.6	1.5	1.1
SKELETAL DEVELOPMENT				
Galectin-3 (LEG 3) *	1	0.9	0.7	0.6
APOPTOSIS				
Plasminogen activator inhibitor 2 (PAI2)	1	47	2	38

Differentially expressed proteins				
Protein Name / Function	WT	WT +LPS	Tyk2-/-	Tyk2-/- +LPS
METABOLISM				
3-ketoacyl-CoA thiolase A (THIKA)	1	0.5	1.1	1
Aldehyde dehydrogenase (ALDH2)	1	0.7	1.3	1
Purine nucleoside phosphorylase (PNPH)	1	1.6	0.7	1
D-3-phosphoglycerate dehydrogenase (SERA)	1	0.9	0.7	0.6
TRANSLATION, TRANSCRIPTION				
Elongation Factor 2 (EF2) *	1	0.8	1.5	1.4
Phenylalanyl-tRNA-synthase beta chain (FARSB) *	1	0.9	0.5	0.5
Staphylococcal nuclease domain-containing protein 1 (SND1) *	1	0.9	1.5	1.4
CYTOSKELETON				
Vimentin (VIME) *	1	1.4	0.4	0.3
F-actin capping protein subunit alpha-1 (CAZA1) *	1	1.3	0.7	0.8
F-actin capping protein subunit beta (CAPZB) *	1	1.2	0.7	0.7
Adenyl cyclase-associated protein 1 (CAP1)	1	1.9	1.2	1.2
OTHERS				
EF-hand domain containing protein 2 (EFHD2) *	1	1.4	0.7	0.8
Immune-responsive protein 1 (IRG1)	1	4.4	0.8	1.9

Fig. 3. Identified proteins that are differentially expressed in the absence of Tyk2. The expression levels are given as mean fold ratios relative to untreated WT cells (whole cell lysates or *nuclear extracts). Proteins are classified according to their gene ontology annotation. Proteins analysed in more detail are indicated in different colours.

LPS induces a shift of PRDX1 spots in dependence of Tyk2

Fig. 4. Protein levels of PRDX1 in whole cell lysates analysed by 2-DE western blotting. BMM were treated with LPS (100 ng/ml) or left untreated. Equal amounts of protein (50 µg) were separated by 2-DE using a pH 6-10 gradient for the first dimension, followed by the second dimension on a 12% SDS-PAGE. Spots indicated have been identified by MS.

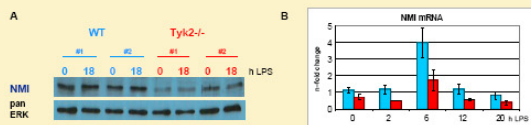### Tyk2 is required for efficient expression of NMI protein and mRNA

Fig. 5. NMI expression patterns at protein and mRNA level. WT and Tyk2-/- BMM were stimulated with LPS (100 ng/ml) for the indicated times. A) Western blot: Whole cell extracts (20 µg) were separated on a 10% SDS-PAGE. Membranes were probed with anti-NMI antibody, pan ERK was used as loading control. B) Total RNA was extracted and mRNA expression was analysed by RT-qPCR. UBE2D2 was used as endogenous control, the expression levels were calculated relative to untreated WT cells. Mean values ± SD from three independent experiments are depicted.
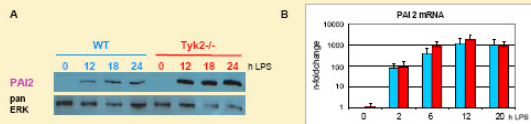
Tyk2 inhibits PAI2 protein but not mRNA expression

Fig. 6. PAI2 expression patterns at protein and mRNA level. WT and Tyk2-/- BMM were stimulated with LPS (100 ng/ml) for the indicated times. A) Western blot: Whole cell extracts (5 µg) were separated on a 12% SDS-PAGE. Membranes were probed with anti-PAI2 antibody, pan ERK was used as loading control. B) Total RNA was extracted and mRNA expression was analysed by RT-qPCR. UBE2D2 was used as endogenous control, the expression levels were calculated relative to untreated WT cells. Mean values ± SD from three independent experiments are depicted.

CONCLUSIONS

1. 2D-DIGE technology shows highly reproducible expression profiles in cell extracts from primary bone marrow-derived macrophages.
2. Absence of Tyk2 significantly alters the protein expression patterns in both whole cell and nuclear extracts.
3. Significant differences in expression profiles between genotypes are detectable before and after LPS treatment.
4. Tyk2 exerts positive and negative regulatory functions on protein expression.
5. The 27 differentially expressed proteins (31 spots) identified belong to distinct functional categories, e.g. interferon, immune and oxidative stress responses, metabolism and cytoskeleton architecture.
6. Tyk2 influences protein expression transcriptionally (e.g. NMI) and posttranscriptionally (e.g. PAI2).

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The impact of Tyk2 deficiency
on the proteome of murine macrophages and their response to LPS

INTRODUCTION

Tyrosine kinase 2 (Tyk2), a member of the Janus kinases (Jak) family, is involved in signalling via various cytokines and some growth hormones. Tyk2 plays an important role in host defence against different pathogens. It has been shown in our lab that Tyk2-deficient mice are highly resistant to lipopolysaccharide (LPS) induced endotoxin shock and that Tyk2 deficiency results in impaired macrophage responses [1].
The present study aimed to examine more closely the molecular role of Tyk2 for macrophage functions and responses to LPS. We applied two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) to compare protein patterns of bone marrow-derived macrophages (BMM) from Tyk2-deficient (Tyk2-/-) and wild type (WT) mice before and after LPS treatment [2]. We could demonstrate a significant effect of Tyk2 deficiency on the protein pattern in whole cell and nuclear extracts. Twenty-three of the differentially expressed proteins were identified by mass spectrometry [2]. Herein we present more detailed analyses of expression patterns for selected proteins, aiming to characterise the regulatory mechanisms underlying the positive or negative impact of Tyk2 on protein expression.
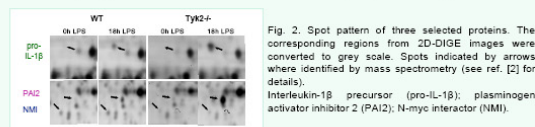
RESULTS

Absence of Tyk2 significantly alters the protein expression patterns in macrophages

Fig. 1. Overview of analyses performed, their statistical properties, and numbers of differentially expressed and identified spots. Analyses were performed with 2D-DIGE technology using minimal labelling with CyDyes. Internal standard, a pool of all samples in an experimental set, was used for normalisation. Volume ratios (sample spot volume divided by the corresponding internal standard spot volume) were tested for differential expression using a moderated t-statistic.

Differentially expressed spots were identified by MALDI/ESI MS. *) at least 40% difference in expression between genotypes, p≤0.05. b) identified spots (see ref. [2] for more details).

2D spot pattern of selected differentially expressed proteins

Fig. 2. Spot pattern of three selected proteins. The corresponding regions from 2D-DIGE images were converted to grey scale. Spots indicated by arrows where identified by mass spectrometry (see ref. [2] for details).
Interleukin-1β precursor (pro-IL-1β); plasminogen activator inhibitor 2 (PAI2); N-myc interactor (NMI).

Tyk2 positively influences the expression of NMI mRNA, and negatively regulates PAI2 expression post-transcriptionally
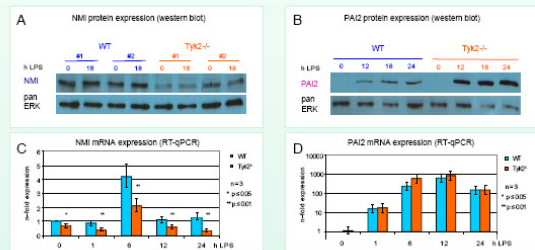

Fig. 3. NMI and PAI2 expression patterns at protein and mRNA level. WT and Tyk2-/- BMM were stimulated with LPS (100 ng/ml) for the indicated times. A) Whole cell extracts (20 µg) were separated on a 10% SDS-PAGE. Membranes were probed with anti-NMI antibody, pan ERK was used as loading control. B) Whole cell extracts (5 µg) were separated on a 10% SDS-PAGE. Membranes were probed with anti-PAI2 antibody, pan ERK was used as loading control. C) and D) Total RNA was extracted and mRNA expression was analysed by RT-qPCR. UBE2D2 was used as endogenous control, expression levels were calculated relative to untreated WT cells. Mean values ± SD from three independent experiments are depicted.

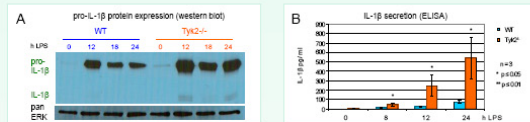### Intra- and extra-cellular levels of IL-1β protein are enhanced in the absence of Tyk2

Fig. 4. IL-1β protein expression. WT and Tyk2-/- BMM were stimulated with LPS (100 ng/ml) for the indicated times. A) Whole cell extracts (5 µg) were separated on a 15% SDS-PAGE. Membranes were probed with anti-IL-1β antibody, pan ERK was used as loading control. B) Cell culture supernatants were collected and extra-cellular concentrations of IL-1β were measured by ELISA. Mean values ± SD from three independent experiments are depicted.

IL-1β protein synthesis is enhanced in the absence of Tyk2, whereas IL-1β mRNA expression is not affected
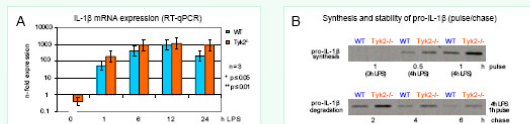

Fig. 5. IL-1β mRNA expression and protein synthesis/degradation. WT and Tyk2-/- BMM were stimulated with LPS (100 ng/ml) for the indicated times. A) Total RNA was extracted and mRNA expression was analysed by RT-qPCR. UBE2D2 was used as endogenous control, expression levels were calculated relative to untreated WT cells. B) BMM were pulse labelled with 35S methionine/cysteine and chased for the indicated times. IL-1β was immunoprecipitated from whole cell extracts and subjected to 15% SDS-PAGE and autoradiography.

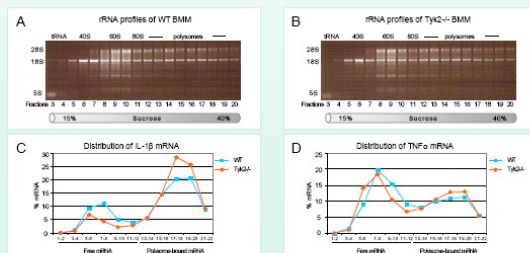### Association of IL-1β mRNA with polysomes is increased in the absence of Tyk2

Fig. 6. Polysome profiles of IL-1β and TNFα in BMM. WT and Tyk2-/- BMM were stimulated with LPS (100 ng/ml) for 4h. Cytoplasmic extracts were separated in a continuous 15-40% sucrose gradient by ultracentrifugation. 22 fractions were manually collected from top to bottom, deproteinised and RNA extracted. Representative rRNA profiles of WT (A) and Tyk2-/- (B) polysomal fractions separated on a 0.8% agarose gel. Two fractions each were pooled and mRNA levels determined by RT-qPCR using TATA binding protein (TBP) as endogenous control. Expression levels of the target genes were calculated relative to the amount of TBP in each pooled fraction. Representative results from three independent experiments are shown as percentage of total IL1-β (C) or TNFα (D) mRNA.

CONCLUSIONS

1. 2D-DIGE technology allows to detect minimal differences in protein expression in primary murine macrophages as low as 30% with our experimental set-up.
2. Absence of Tyk2 significantly alters the protein expression patterns in whole cell lysates and nuclear extracts.
3. Tyk2 enhances protein expression of N-myc interactor (NMI) at the transcriptional level.
4. Tyk2 inhibits expression of plasminogen activator inhibitor (PAI2) post-transcriptionally.
5. Tyk2 negatively regulates IL-1β protein synthesis by reducing association of IL-1β mRNA with polysomes.

➤ Tyk2 exerts positive and negative regulatory roles at multiple levels of protein expression in macrophages.

References
[1] "Central role for type I interferons and Tyk2 in lipopolysaccharide-induced endotoxin shock" Karaghiosoff et al. Nature Immunology 2003, 4 (471-477)
[2] "The impact of tyrosine kinase 2 (Tyk2) on the proteome of murine macrophages and their responses to lipopolysaccharide (LPS)" Radwan et al. Proteomics 2008, 8 (3469-3485)

Proteomic study of Tyk2 deficient murine macrophages in response to poly(I:C)

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Introduction

Tyrosine kinase 2 (Tyk2) is a member of the Janus kinase (Jak) family and involved, among others, in the intracellular signal transduction of type I interferons (Fig.1). We have recently shown a selective requirement of Tyk2 in the defense against certain virus infections *in vivo* (1, 2). Here we used synthetic polyinosinic acid-polycytidylic acid (poly(I:C)) to mimic the presence of double stranded RNA (dsRNA), a known (by-) product of virus replication.

Aim of the study is to advance our understanding of the molecular function of Tyk2 in macrophages in viral defense using a proteomic approach (based on 2D gel-electrophoresis and MALDI-RTOF-MS) and poly(I:C) treatment as a model system.
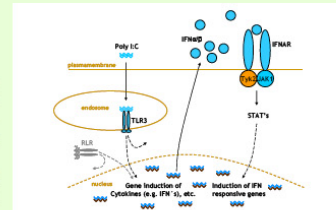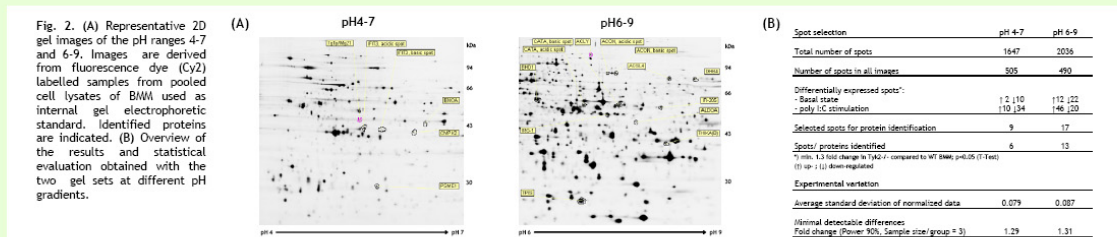

Fig. 1. Poly(I:C) induced signalling pathways.
Poly(I:C) is recognized by the endosomal pattern recognition receptor, Toll-like receptor 3 (TLR3), and the cytoplasmic RIG-1 like receptors (RLR). This leads to the production and release of type I interferon's (IFNα/β). Following binding of IFNα/β to the type I IFN receptor (IFNAR), the downstream JAK/STAT (Janus Kinase/ Signal Transducer and Activator of Transcription) pathway is activated and IFN responsive genes are induced.

Methods

Bone-marrow derived macrophages (BMM) were isolated from wild-type (WT, control) and Tyk2 deficient (Tyk2-/-) mice (triplicates). Following cultivation for 7 days, BMM were incubated with/without 50 µg/ml poly(I:C) and lysed after 18 h. Using two-dimensional difference gel electrophoresis (2D-DIGE), whole cell extracts were minimally labelled with CyDyes and separated on 24 cm IPG Dry strips with linear pH gradients of pH 4-7 and pH 6-9 (IPGphor III system) followed by the second dimension on a 10% SDS-PAGE (Ettan Dalt Six, all GE Healthcare). After software based quantitation of spot expression (DeCyder software, GE Healthcare), spots showing differences between WT and Tyk2-/- before and/or after poly(I:C) stimulation were selected and submitted to MS-based protein identification. Silver stained spots were manually excised, *in-gel* trypsin digested and peptide mass fingerprint (PMF) with PSD experiments were performed using MALDI-RTOF-MS (AXIMA CFR*plus*; Shimadzu Biotech Kratos Analytical) followed by searching with two independent algorithms (Mascot and ProFound) embedding SwissProt, NCBInr and IPIMouse as databases.

Results

The macrophage proteome is significantly affected by the absence of Tyk2.

Fig. 2. (A) Representative 2D gel images of the pH ranges 4-7 and 6-9. Images are derived from fluorescence dye (Cy2) labelled samples from pooled cell lysates of BMM used as internal gel electrophoretic standard. Identified proteins are indicated. (B) Overview of the results and statistical evaluation obtained with the two gel sets at different pH gradients.

Spot selection	pH 4-7	pH 6-9
Total number of spots	1647	2036
Number of spots in all images	505	490
Differentially expressed spots*:		
- Basal state	↑2 ↓10	↑12 ↓22
- poly·I:C stimulation	↑10 ↓34	↑46 ↓20
Selected spots for protein identification	9	17
Spots/ proteins identified	6	13

*) min. 1.3 fold change in Tyk2-/- compared to WT BMM; p≤0.05 (T-Test)
(↑) up ; (↓) down-regulated

Experimental variation		
Average standard deviation of normalized data	0.079	0.087
Minimal detectable differences		
Fold change (Power 90%, Sample size/group = 3)	1.29	1.31

Differentially expressed and unambiguously identified proteins are assigned to several distinct functional protein groups.

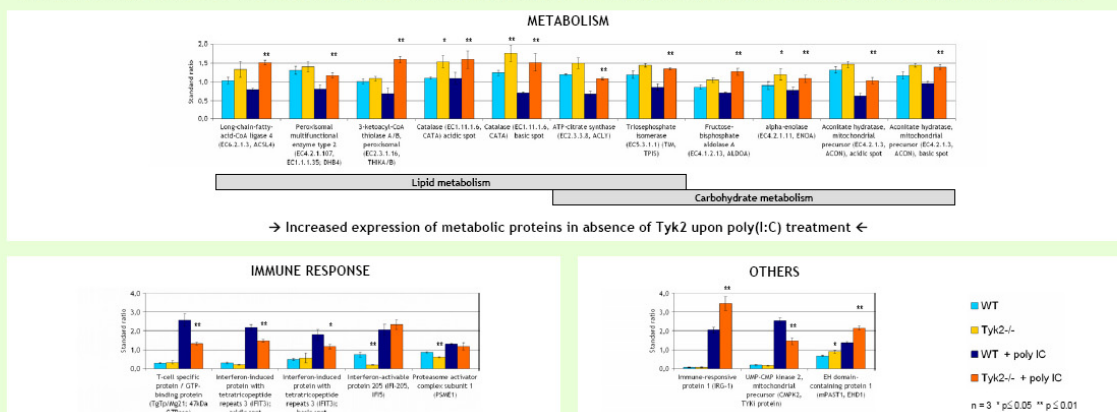→ Increased expression of metabolic proteins in absence of Tyk2 upon poly(I:C) treatment ←

Fig. 3. Relative expression levels obtained from 2D-DIGE are shown as standard ratios, i.e. ratio of the sample normalised to the inter-gel standard (for each spot). Differences in expression were analysed by the Student´s t-Test. Results are given as mean values ± SD. Proteins are assigned to their function according to Gene Ontology (GO) and UniProtKB/Swiss-Prot annotation.

Conclusion

- Overall differences in protein expression of more than 30% can be detected and are significant changes with our experimental setup.
- Tyk2 exerts both positive and negative regulatory functions of cellular protein expression with/without poly(I:C) stimulation.
- The proteins showing differential expression between WT and Tyk2-/- BMM can be assigned to different functional categories.
- The data further emphasize the essential role of Tyk2 for the regulation of cellular immune responses.
- The data suggest novel roles of Tyk2 in the regulation of cellular functions (i.e. lipid and carbohydrate metabolism).

Acknowledgements

References

1) Karaghiosoff et.al., Partial impairment of cytokine responses in Tyk2-deficient mice. Immunity. 2000; 13(4):549-60.
2) Strobl et.al., Novel Functions of Tyrosine Kinase 2 in the Antiviral Defense against Murine Cytomegalovirus. J. Immunology. 2005; 175(6):4000-8.

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