universität wien

# DISSERTATION

Titel der Dissertation

Responses of the Innate Immune System to the Human Pathogen

*Streptococcus pyogenes*

Verfasserin

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angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr. rer.nat.)

| | |
|---|---|
| Studienkennzahl (lt. Studienblatt) | A 091 441 |
| Dissertationsgebiet (lt. Studienblatt): | Dr. – Studium der Naturwissenschaften Genetik-Mikrobiologie |
| Betreuer: | Ao. Univ.-Prof. Dr. Pavel Kovarik |

Wien, im Juni 2010

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# 1. Summary

*Streptococcus pyogenes*, also known as group A streptococcus (GAS), is an important human pathogen responsible for a variety of diseases ranging from mild (e.g. tonsillitis) to life-threatening infections (e.g. invasive infections of skin and soft tissues that can develop into necrotizing fasciitis, sepsis or lethal toxic shock syndrome).

Bacterial pathogens are recognized by cells of the innate immune system through pattern recognition receptors (PRRs). Among them, the family of Toll-like receptors (TLRs) is the best characterized class of PRRs and virtually all pathogenic bacteria are recognized by at least one of them. However, PRRs and bacterial products responsible for the activation of an immune response against *S. pyogenes* are not known yet.

We were able to demonstrate that activation of an inflammatory response in murine bone marrow-derived macrophages (BMDMs) against *S. pyogenes*, involving the production of cytokines such as TNF-$\alpha$ and IL-6, depends on the adaptor molecule MyD88, but not on the prototype receptors for bacteria TLR22, TLR4 and TLR9. Further, streptococcal RNA has been identified to be an important factor for the induction of these cytokines in response against this pathogen.

Moreover, we could show that infection of innate immune cells with *S. pyogenes* causes the production of type I interferons (IFNs) and subsequent activation of the Jak/Stat signaling pathway. Experiments using BMDMs from mice deficient in components of the interferon pathway (IRF3, IRF1, STAT1, IFNAR1) and siRNA-mediated knock down of another important gene (TBK1) from this pathway revealed that IFN-production occurs downstream of an unknown PRR via IRF3, TBK1 and, but without the involvement of any described TLRs. A partial dependence on the universal adaptor MyD88 was observed. Surprisingly, IFN-$\beta$ is also induced, though to a lesser extent, by *S. pyogenes* lacking SLO and SLS, the genes encoding cytolysins that were shown to be required for IFN production in response to other Gram-positive bacteria. Furthermore, *S. pyogenes* extracts digested with either DNase I, RNase A or Proteinase K suggest that in macrophages bacterial DNA is required for the induction of IFN-$\beta$ production, whereas in dendritic cells RNA was identified as IFN-$\beta$ inducer. However, the receptor molecules which promote the induction of the interferon response remain elusive.

An experimental mouse model of skin infection using IFNAR1-deficient mice emphasized the important role of type I IFNs during the host immune response against *S. pyogenes*. IFNAR1-deficient mice showed a decreased survival and succumbed to infection earlier than control mice. Therefore it can be concluded that production of type I IFNs during streptococcal infection is beneficial for the host.

Taken together, our work demonstrates that infection of innate immune cells with *S. pyogenes* leads to the induction of multiple signaling pathways within the host leading to the production of inflammatory mediators such as TNF-$\alpha$ and IL-6 as well as type I IFNs. The production of type I IFNs in response to *S. pyogenes* is protective for the host and macrophages and dendritic cells use different signaling molecules for the induction of these cytokines. Further, we were able to show that *S. pyogenes* is able to avoid the standard arsenal of pattern recognition receptors deployed by the host, which may contribute to the known high capability to cause severe inflammatory diseases.

# 2. Zusammenfassung

*Streptococcus pyogenes*, auch bekannt als Gruppe A Streptokokkus (GAS), ist ein bedeutendes humanes Pathogen, das für eine Reihe von Krankheiten verantwortlich ist. Diese Krankheiten können relativ harmlos sein, wie zum Beispiel Mandelentzündung, aber auch lebensgefährlich, wie zum Beispiel invasive Infektionen der Haut und der darunterliegenden Gewebe, die zu einer nekrotisierenden Fasziitis, Sepsis oder zu einem streptokokkalen toxischen Schock-Syndrom führen können.

Bakterielle Pathogene werden von Zellen des angeborenen Immunsystems durch spezielle Rezeptoren erkannt, die auf die Erkennung von bestimmten bakteriellen Mustern von Bakterien spezialisiert sind. Die Toll-like Rezeptoren (TLRs) sind die wohl am besten charakterisierte Familie unter diesen Rezeptoren und jedes Pathogen wird von mindestens einem dieser Rezeptoren erkannt. Rezeptormoleküle, die für die Aktivierung einer Immunantwort gegen *S. pyogenes* verwendet werden, sind jedoch noch nicht bekannt.

Wir konnten zeigen, dass *S. pyogenes* zur Aktivierung einer inflammatorischen Antwort in aus dem Knochenmark von Mäusen gewonnenen Makrophagen führt, die durch die Produktion von Zytokinen wie TNF-$\alpha$ und IL-6 gekennzeichnet ist. Diese Aktivierung ist abhängig von dem Adapter-Molekül MyD88, jedoch unabhängig von den wichtigsten Rezeptoren für bakterielle Produkte wie TLR2, TLR4 und TLR9. Weiters wurde streptokokkale RNA als wichtiger Faktor für die Aktivierung einer inflammatorischen Antwort gegen dieses Pathogen identifiziert.

Zusätzlich konnten wir zeigen, dass die Infektion von Zellen des angeborenen Immunsystems mit *S. pyogenes* zu einer Produktion von Typ I Interferonen (IFNs) und einer anschließenden Aktivierung des Jak/Stat Signaltransduktionsweges führt. Experimente mit Makrophagen, denen bestimmte Komponenten des IFN- Signaltransduktionsweges fehlen (IRF3, IRF1, STAT1, IFNAR1) oder in denen die Signalkomponente TBK1 mittels siRNA inaktiviert wurde, führten zu dem Schluss, dass die IFN-Produktion unterhalb eines noch nicht bekannten Rezeptors von IRF3, TBK1 und IFNAR1 abhängig aber unabhängig von bekannten TLRs ist, obwohl eine partielle Abhängigkeit von MyD88 beobachtet wurde.

Überraschenderweise konnten auch Streptokokken, welchen die zwei Zytolysine SLO und SLS fehlten, eine IFN-$\beta$ Produktion hervorrufen, obwohl für andere Gram-positive Bakterien gezeigt wurde, dass diese Zytolysine für die Produktion von IFN-$\beta$ wichtig sind. Ferner deuteten mit Enzymen verdaute streptokokkale Extrakte darauf hin, dass in Makrophagen streptokokkale DNA und in dendritischen Zellen RNA für die Induktion der IFN-Produktion benötigt wird. Allerdings sind auch in diesem Fall die Moleküle die für diese Induktion verwendet werden, noch nicht bekannt.

Mittels eines kutanen Infektionsmodells, in dem IFNAR1-defiziente Mäuse mit *S. pyogenes* infiziert wurden, wurde die bedeutende Rolle der Typ I Interferone in der Immunantwort auf eine Streptokokken-Infektion untermauert. IFNAR1-defiziente Mäuse starben früher und in größerer Anzahl als Kontroll-Mäuse. Dieses Ergebnis lässt darauf schließen, dass die Produktion von Typ I Interferonen während eine streptokokkalen Infektion dem Wirt einen Vorteil verschafft.

Zusammengefasst wurde in dieser Studie gezeigt, dass die Infektion von Zellen des angeborenen Immunsystems mit *S. pyogenes* zur Produktion von inflammatorischen Zytokinen wie TNF-$\alpha$, IL-6 und Typ I Interferonen führt. Interferone schützen den Wirt vor einer streptokokkalen Infektion und in Makrophagen und dendritischen Zellen werden unterschiedliche Signalmoleküle für die Induktion der Interferon-Produktion verwendet. Weiters konnten wir zeigen, dass *S. pyogenes* die Fähigkeit besitzt, dem Standard-Arsenal an Pathogen-Rezeptoren des Wirts zu entkommen. Dies könnte zu der bekannten Eigenschaft des Bakteriums beitragen, sehr unterschiedliche und auch schwere Infektionskrankheiten hervorzurufen.

# 3. Introduction

## 3.1. Innate Immune Responses to Infection

The vertebrate immune system is divided into 2 major categories: the innate and the adaptive immunity. Innate immunity is defined as a rapid, non-specific response to infection, whereas the adaptive immunity involves slowly-developing, long-lasting and highly evolved antigen-specific responses such as cell-mediated immunity and antibody production (1).

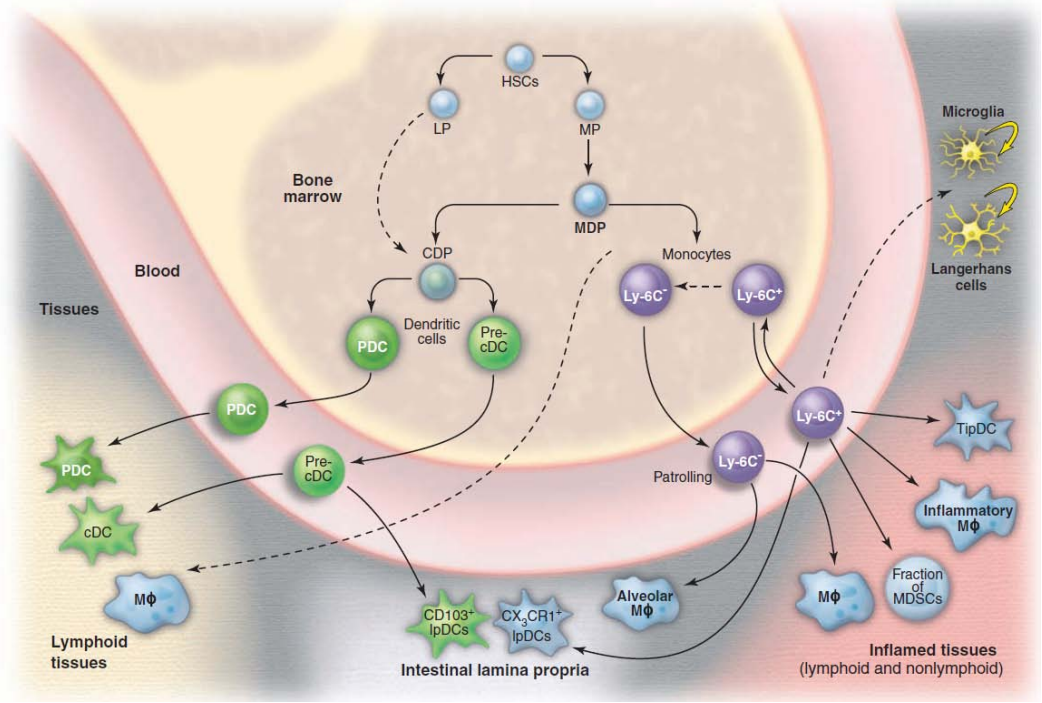
In addition to the barrier function of the epithelium and skin mucosa, several other cell types like macrophages, dendritic cells and neutrophils, contribute to innate immunity by patrolling through the body and killing the unwanted intruders. Further, soluble effectors such as complement proteins and antimicrobial peptides as well as pattern recognition receptors are acting together in order to defend the host against infection.

### 3.1.1. Phagocytes

Phagocytes are cells of the innate immune system which play an important role in the response to invading pathogens. The group of phagocytes includes macrophages, dendritic cells, neutrophils, mast cells and monocytes. These cells constitute the first line of defense since their major function is to ingest particles, which may be harmful for the body.

Leukocytes, also known as white blood cells, are a diverse group of cell types important for the host´s immune response. They share a common origin in hematopoietic stem cells (HSCs) and develop along distinct differentiation pathways in response to internal and external cues (see Fig. 1). HSCs produce myeloid (MP) and lymphoid (LM) committed precursors. MPs give rise to monocyte, macrophage, and DC precursors (MDPs) and these precursors then give rise to monocytes, some populations of macrophages and common DC precursors (CDPs). CDPs in turn develop into preclassical dendritic cells (pre-cDcs) and plasmacytoid dendritic cells (pDCs) (2).

**Figure 1: Differentiation of DCs and macrophages in mice** (from (2))




Macrophages are extremely versatile cells involved in a number of complex functions in health and disease. They are widely distributed throughout the body and show a remarkably diversity in their phenotype dependent on their anatomical location. Macrophages in the central nervous system are known as microglia, in the lung as interstitial or alveolar macrophages and in the liver as Kupffer cells (3). Differentiation and functional specialization is dependent on the tissue microenvironment, which consist of other cell types as well as growth factors and cytokines (4).Macrophages are involved in steady-state tissue homeostasis by removing apoptotic or possibly aberrant cells and by the production of growth factors (2). In addition, they express a large variety of receptors that mediate their function as professional phagocytes.

Scavenger receptors (SR), such as SR-A, CD36 and macrophage receptor with collagenous structure (MARCO), in addition to C-type lectins, are surface receptors and binding of microbes to these receptors leads to phagocytosis/ endocytosis of pathogens.

Complement receptors (Integrins) and Fc receptors (Ig superfamily) function in the endocytosis or phagocytosis of particles coated with serum complement proteins or antibodies (5) (6). Once a microbe has been taken up by a macrophage, it is trapped in the phagosome which fuses then with lysosomes that contain various hydrolytic enzymes. These function to digest the invading pathogens.

Further, macrophages express receptors that are not involved in mediating phagocytosis/ endocytosis but are important in mediating immune responses against microbes. These receptors

include the members of the Toll-like receptor (TLR) family, the NOD-like receptors (NLRs) and RIG-like helicases (RLHs). Ligand recognition by these receptors leads to the activation of signaling cascades resulting in the expression of proinflammatory cytokines, which enhance local antimicrobial defense systems.

The production of proinflammatory cytokines by macrophages also mediates a cross-talk between the innate and the adaptive immune system. Macrophages produce cytokines that help activating adaptive immune responses and in turn, interferon-gamma (IFN-$\gamma$), which is produced by T- and natural killer (NK) cells, was shown to be important for macrophage activation. This cytokine converts resting macrophages into powerful effector cells with increased antigen-presentation capacity and increased synthesis of proinflammatory cytokines such as TNF-$\alpha$, interleukin ( IL)-6 and IL-12, and toxic mediators such as nitric oxide (7).

Macrophages also play an important role in the adaptive immune response by the uptake of microbial antigens that are proteolytically processed and presented in forms that can activate T-cell responses (8).

Dendritic cells are highly specialized antigen-presenting cells (APCs), thereby linking the innate with the adaptive immune system by initiating and controlling the magnitude and quality of adaptive immune responses. This cell type was first described by Steinman and Cohn in the early 1970´s, and since then it became clear that there are many distinct subtypes existing, each with particular localization and functions in the immune system.

In their immature stage of development, DCs can be found in both peripheral tissues and lymphoid organs and also in the blood. They start out as immature cells with high endocytic activity and function to detect, capture and phagocytose pathogens, leading to TLR activation. Upon signal transduction through TLRs, DCs undergo a complex differentiation program termed collectively DC maturation (9).

This maturation process involves: (i) changes in morphology such as the loss of adhesive structures and the acquisition of high cellular motility; (ii) loss of endocytic/phagocytic receptors; (iii) secretion of chemokines in coordinated waves according to the type of immune cells that need to be attracted; (iv) upregulation of costimulatory molecules, such as CD40, CD80, and CD86; (v) translocation of MHC class II compartments to the cell surface and (vi) secretion of cytokines that differentiate and polarize the attracted immune effectors (10). Upon activation, DCs travel to lymphoid tissues such as the spleen and the lymph nodes, where they interact and activate antigen-specific T-cells (11).

As already mentioned, DCs are very heterogeneous and DC subtypes differ in location, migratory pathways and detailed immunological function (12).

Classical dendritic cells (cDCs) posses a high phagocytic activity as immature cells and a high cytokine-producing capacity when maturated. These cells can move from tissues to the T-cell and B-cell zones of lymphoid organs via afferent lymphatics and high endothelial venules and regulate T-cell responses in steady state and also during infection (2).

Plasmacytoid dendritic cells (pDCs) differ from cDCS in their surface phenotype, tissue localization, cytokine secretion and antigen presentation function. These cells are constantly produced in the bone-marrow and released into the blood stream, therefore they present in the bone-marrow and all peripheral organs. One major characteristic of pDCs is that they are professional type I interferon (IFN) producing cells. It has been shown, that human pDCs produce 200-1000 times more type I IFNs than any other blood cell type. However, pDCs can also act as APCs and control T-cell responses (13) (2).

### 3.1.2. Microbe Recognition

Mammalian organisms can be infected by a variety of microorganisms, including viruses, bacteria, fungi and protozoa. The innate immune response is responsible for the early detection and destruction of invading pathogens and relies on a limited set of germline-encoded pattern recognition receptors (PRRs). By contrast, the adaptive immune response relies on enormously diverse receptors generated by random somatic gene rearrangements to detect the microbes and also provides the host with immunological memory (14).

For the initiation of an immune response, pathogen-associated molecular patterns (PAMPs) have to be recognized by the PRRs. PAMPs are characterized as microbial structures that are evolutionarily conserved and therefore shared by different microorganisms. An important feature of PAMPs is that they are mainly expressed by pathogens but not by the host. Therefore PRRs can also discriminate between self and non-self (15).

After recognition of a PAMP by a PRR, multiple intracellular signaling pathways are triggered which cause activation of gene expression and synthesis of a broad range of molecules, including cytokines, chemokines, adhesion molecules and immunoreceptors resulting in proinflammatory and antimicrobial responses (16).

## Surface Receptors

Among the large group of PRR, the family of Toll-like receptors (TLRs) is best characterized. These receptors were initially discovered in *Drosophila melanogaster*, where the Toll protein is a critical component of host defense against fungal and Gram-positive bacterial infection (17). During the last 15 years, the mammalian homologues of Toll have been identified, extensively studied and functionally characterized as critical components of the innate immune system. At present, 10 TLRs have been described in humans (TLRs 1-10) and 12 TLRs in mice (TLRs 1-9 and 11-13). These receptors are expressed by a variety of cell types, but professional immune cells such as macrophages and dendritic cells are the most prominent cells in this respect (18).

TLRs are type I transmembrane proteins consisting of an ectodomain that contains varying numbers of leucine-rich-repeat (LRR) motifs , which are responsible for both, receptor dimerization and ligand recognition. Further, they own a cytoplasmic domain homologous to the interleukin 1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain that is required for the initiation of downstream signaling (19).

TLRs can be divided into subfamilies, dependent on their cellular distribution. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are expressed on the cell surface and mainly recognize bacteria-derived PAMPs like lipoteichoic acid (LTA), lipopolysaccharide (LPS), peptidoglycan (PGN) and lipoproteins.

Others (i.e. TLR3, TLR7; TLR8 and TLR9), are located in intracellular compartments, including endosomes and lysosomes, and are specialized in the recognition of nucleic acids, as virus-derived dsRNA and ssRNA as well as unmethylated 2´deoxyribo cytidine-phosphate guanosine (CpG) from both, viruses and bacteria (16). These intracellular TLRs are expressed on the ER in resting cells from where they get trafficked to the endosomal compartment in response to PAMP-mediated stimulation. This process is mediated by the ER protein UNC93B that interacts with the transmembrane regions of TLR3, TLR7, TLR9 and TLR13 (19). The intracellular location is important for the recognition of foreign nucleic acids and for the discrimination of self from non-self nucleic acids. Engagement of these receptors induces antiviral immune responses by producing proinflammatory cytokines and type I interferons (15). Table 1 shows a detailed list of TLRs and their ligands.

**Table 1: Toll-like receptors and their ligands** (from (20))

| Receptor | Ligand | Origin of ligand |
|---|---|---|
| TLR1 | Triacyl lipopeptides<br>Soluble factors | Bacteria and mycobacteria<br>*Neisseria meningitidis* |
| TLR2 | Lipoprotein/lipopeptides<br>Peptidoglycan<br>Lipoteichoic acid<br>Lipoarabinomannan<br>Phenol-soluble modulin<br>Glycoinositolphospholipids<br>Glycolipids<br>Porins<br>Atypical lipopolysaccharide<br>Atypical lipopolysaccharide<br>Zymosan<br>Heat-shock protein 70* | Various pathogens<br>Gram-positive bacteria<br>Gram-positive bacteria<br>Mycobacteria<br>*Staphylococcus epidermidis*<br>*Trypanosoma cruzi*<br>*Treponema maltophilum*<br>*Neisseria*<br>*Leptospira interrogans*<br>*Porphyromonas gingivalis*<br>Fungi<br>Host |
| TLR3 | Double-stranded RNA | Viruses |
| TLR4 | Lipopolysaccharide<br>Taxol<br>Fusion protein<br>Envelope protein<br>Heat-shock protein 60*<br>Heat-shock protein 70*<br>Type III repeat extra domain A of fibronectin*<br>Oligosaccharides of hyaluronic acid*<br>Polysaccharide fragments of heparan sulphate*<br>Fibrinogen* | Gram-negative bacteria<br>Plants<br>Respiratory syncytial virus<br>Mouse mammary-tumour virus<br>*Chlamydia pneumoniae*<br>Host<br>Host<br>Host<br>Host<br>Host |
| TLR5 | Flagellin | Bacteria |
| TLR6 | Diacyl lipopeptides<br>Lipoteichoic acid<br>Zymosan | *Mycoplasma*<br>Gram-positive bacteria<br>Fungi |
| TLR7 | Imidazoquinoline<br>Loxoribine<br>Bropirimine<br>Single-stranded RNA | Synthetic compounds<br>Synthetic compounds<br>Synthetic compounds<br>Viruses |
| TLR8 | Imidazoquinoline<br>Single-stranded RNA | Synthetic compounds<br>Viruses |
| TLR9 | CpG-containing DNA | Bacteria and viruses |
| TLR10 | N.D. | N.D. |
| TLR11 | N.D. | Uropathogenic bacteria |

Upon ligand binding, the TLR dimerizes and undergoes conformational changes required for the subsequent recruitment of different combinations of cytosolic TIR-domain containing adaptor molecules. These include myeloid differentiation primary response protein (**MyD88**), MyD88-adaptor-like (**MAL**, also known as Toll/interleukin-1 receptor domain-containing adapter protein (TIRAP)), TIR-domain-containing adaptor protein inducing IFN-β (**TRIF**, also known as TIR domain-containing adapter molecule 1 (TICAM1)), TRIF-related adaptor molecule (**TRAM**, also known as TICAM2) and sterile α- and armadillo-motif-containing protein (**SARM**) (21).

MyD88 is used by almost all TLRs except TLR3. It recruits the IL-1 receptor-associated kinase (IRAK) family of kinases, which further trigger the activation of nuclear factor-κB (NF-κB), p38 and ERK.

Mal gets recruited by the lipoprotein receptor TLR2 and the lipopolysaccharide (LPS) receptor TLR4 and appears to function as a stabilizer of MyD88 by acting as a bridging molecule (22). Since the surfaces of MyD88, TLR2 and TLR4 are largely positively charged, MyD88 is not able to interact with these receptors. Given the fact that Mal is mainly electronegative, TLR2 and TLR4 are able to bind, thereby allowing MyD88 to join the complex (23).

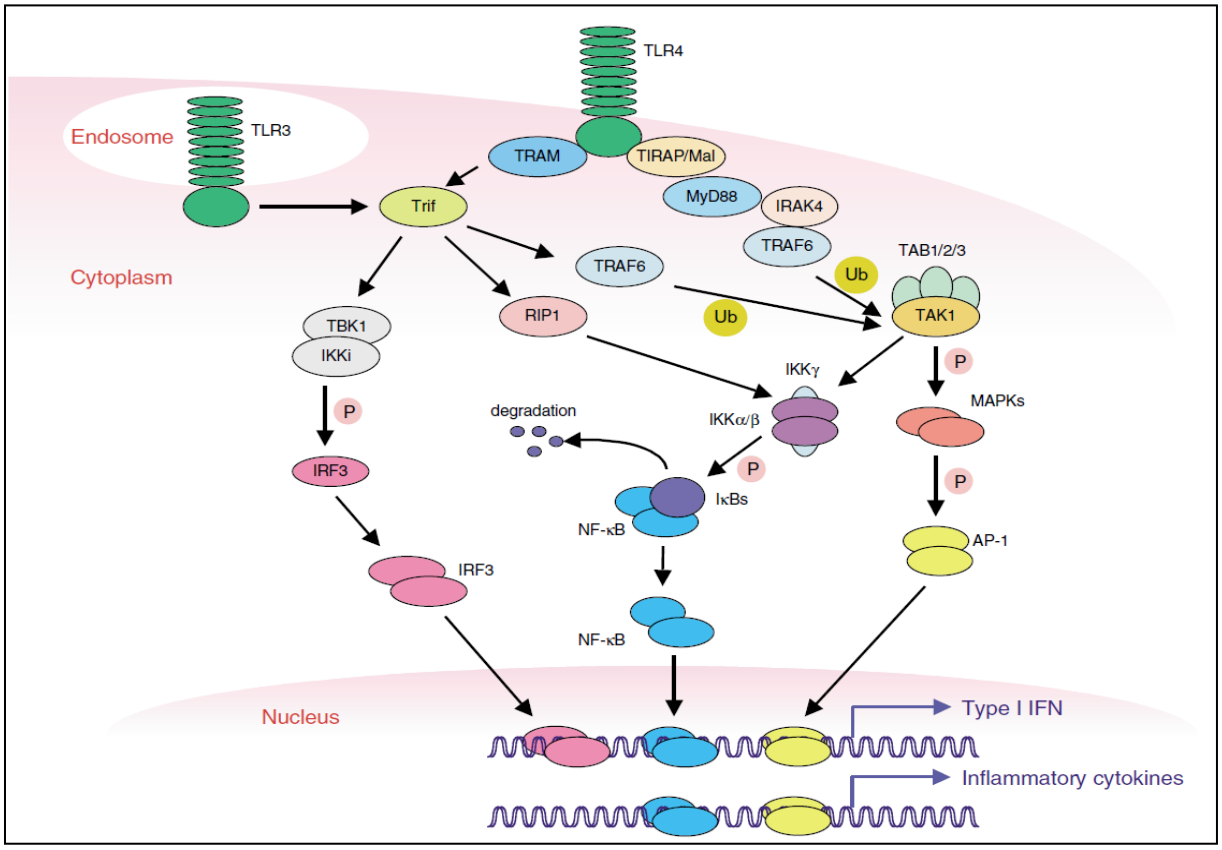
TRIF is the sole adaptor protein used by TLR3 and is needed for the induction of NF-κB.

Further, TRIF together with TRAM as bridging-molecule, serve as adaptor protein for TLR4. Since TLR4 is known to use also MyD88 for signaling, this receptor is very unique because it is the only one among the TLR family that uses two different adaptor molecules. It was not clear, how these two competing signaling molecules are coordinated by TLR4 until it has been recently shown that TLR4 first induces MyD88/Mal- dependent proinflammatory signaling at the plasma membrane, then becomes endocytosed and activates TRIF/TRAM signaling from the early endosome, resulting in the production of type I interferons (IFNs), such as IFN-β and IFN-α. Therefore, the subsequent use of two different adaptor molecules by TLR4 is providing access to distinct pool of signaling molecules (24) (25).

SARM was recently identified as the fifth member of the family of TLR-adaptor proteins. In contrast to the other four adaptor proteins, it was found to be a negative regulator of TLR signaling by inhibiting TRIF. The mechanism how SARM inhibits TRIF has yet not been elucidated, but there are two possibilities: either SARM prevents TRIF from accessing its downstream signaling molecules such as Tank-binding kinase 1(TBK1) or TNF receptor –associated factor 6 (TRAF6) or SARM recruits an inhibitory protein (26).

After recruitment of different combinations of adaptor proteins, TLRs activate three major signaling pathways: mitogen-activated protein kinases (MAPKs), interferon-regulatory factors (IRFs) and nuclear factor-κB (NF-κB). Activation of MAPKs results in the induction of the transcription factor activator protein-1 (AP-1) that, together with the transcription factor NF-κB, leads to the production of inflammatory cytokines. Further, IRF3 and/or IRF7, IRF1 and IRF5 get activated and these transcription factors are essential for the expression of type I IFNs and IFN-stimulated genes (18).

**Figure 2: TLR signaling pathways** (from (20))




To illustrate the principles of TLR signaling, TLR4 was chosen for description, since this receptor can initiate both of the major signaling pathways: the MyD88-dependent and the MyD88-independent (TRIF-dependent) pathway. The MyD88-dependent pathway downstream of TLR4 leads to the expression of inflammatory cytokines such as TNF-α, IL-6 and IL-12. The major role of the TRIF-dependent pathway is the expression of type I IFNs (14).

After ligand binding, MyD88 associates with the receptor and subsequently recruits members of the IL-1 receptor-associated kinase (IRAK) family, leading to a sequential activation of IRAK4, IRAK1 and TNF receptor-associated factor 6 (TRAF6). TRAF6 acts as an E3 ubiquitin protein ligase which, together with an ubiquitination complex (E2), ubiquinates TRAF6 itself and other substrates, including transforming growth factor-activated protein kinase 1 (TAK1) and the IκB kinase (IKK) subunit NF-κB essential modifier (NEMO). Ubiquitinated TRAF6 then recruits TAK1-binding protein 2 (TAB2) and TAB, which brings TAK1 into proximity of the signaling complex, leading to its activation. Activated TAK1 phosphorylates in turn members of the MAPK kinase (MKK) family, including MKK3, 4, 6 and 7. MKK3/6 subsequently phosphorylate and thereby activate p38, whereas MKK 4/7 activate c-Jun N-terminal kinase (JNK) (16).

Further, TAK1 mediates the activation of the IKK complex consisting of IKKα and IKKβ protein kinases and the regulatory molecule IKKγ/NEMO. The IKK complex mediates the phosphorylation of the inhibitor of NF-κB (IκB) proteins and this phosphorylation targets the IκBs for ubiquitination and degradation by the 26 S proteasome, releasing NF-κB into to nucleus where it binds to κB sites present in the promoters and enhancers of a broad range of proinflammatory genes (27).

The TRIF-dependent pathway requires the recruitment of TRAM and TRIF. The interaction of TRIF with receptor-interacting protein 1 (RIP1) is important for the activation of NF-κB (14).

Further, TRIF is responsible for the activation of TRAF-family-member-associated NF-κB activator (TANK) binding kinase 1 (TBK1, also known as NAK or T2K) via TRAF3. TBK1 comprises a family with inducible IκB kinase (IKK-i, also known as IKK-ε) and these kinases are able to phosphorylate interferon-regulatory factors (IRF) -3 and 7. Next, phosphorylated IRF3 and IRF7 form homodimers, translocate into the nucleus and bind to interferon stimulated response elements (ISREs), resulting in the expression of interferon-β (28).

## Cytoplasmic Receptors

Besides the TLRs, which are located either on the cell surface or the lumen of intracellular vesicles such as endosomes or lysosomes, the existence of cytosolic detection systems for intracellular pathogens have been reported. Pattern recognition receptors located in the cytosol include nucleotide-binding oligomerization domain (NOD)-like receptors and retinoic acid-inducible gene-I (RIG-I) like receptors. In addition, several studies indicate cytosolic DNA recognition. This occurs independently of TLRs, NLRs and RLRs and seems to be involved in the defense against both, bacteria and DNA viruses.

### Nucleotide-binding and oligomerization domain (NOD)-like Receptors (NLRs)

The mammalian NLR family consists of more than 20 members. They all share a modular domain organization of a C-terminal leucine-rich repeat (LRR) domain, a central nucleotide-binding NACHT domain and an N-terminal protein-protein-interaction domain composed of a caspase activation and recruitment domain (CARD), pyrin domain (PYD) or baculovirus inhibitor of apoptosis` repeat (BIR) domain (29). The LRR domain is involved in ligand recognition whereas the NACHT (also known as NOD) domain facilitates self-oligomerization and has ATPase activity. The CARD or pyrin domains interact with different downstream adaptor molecules. Upon activation of NOD molecules the recruitment of the downstream signaling molecules is induced thereby leading to upregulation of inflammatory genes (16).

NOD1 and NOD2 are the best characterized PRRs among the NLRs and have been shown to be involved in the detection of distinct structural motifs derived from peptidoglycan (PGN) of intracellular bacteria. They are mainly expressed by antigen-presenting cells and epithelial cells since it is very likely that these cell types come into contact with PGN during an infection (30).

NOD1 recognizes g-D-glutamyl-meso-diaminopimelic acid (iE-DAP), which can be found in the PGN structures of all Gram-negative as well as in several Gram-positive bacteria such as *Bacillus subtilis* and *Listeria monocytogenes*. NOD2 recognizes muramyl dipeptide (MDP), which is the largest component of the PGN motif that is present in both, Gram-positive and Gram-negative bacteria and is implicated in the recognition of S*treptococcus pneumoniae* and *Mycobacterium tuberculosis* (15).

Stimulation of NOD1 and NOD2 induces recruitment of the CARD-containing serine/threonine kinase RICK (also known as RIP2) to the receptors via CARD-CARD interactions, which leads to NF-κB activation and also to the activation of MAP kinase pathways. NOD1 was shown to activate JNK and NOD2 activates p38 and ERKs (14).

Other NLRs, such as NALP1, NALP3, IPAF and NAIP5 are components of a molecular complex called inflammasome. The inflammasome complex comprises one or some of the NLR proteins and caspase-1, which becomes activated and cleaves substrates such as IL-1β and IL-18 to produce mature proteins whose potent proinflammatory activities direct host responses to infection (31). Several families of inflammasomes have been described according to the recognition of different danger signals or PAMPs through their respective NLR. For example, NALP3 has been shown to be involved in the recognition of ATP, uric acid crystals, viral RNA and bacterial DNA (16).

**RIG-I like receptors (RLRs) RIG-I, MDA5, LGP2**

TLR3, TLR7, TLR8 and TLR9 are able to recognize viruses in the endosomal compartment and elicit antiviral responses such as the production of type I IFNs. However, viruses are known to enter the cytoplasm of a host cell and generate dsRNA during the course of replication. Therefore, intracellular receptors are required for efficient immune responses against viruses.

In this regard, the three members of the RIG-I-like receptor family have been reported to recognize viral RNA in the cytoplasm: retinoic-acid-inducible protein I (RIG-I, also known as DDX58)), melanoma differentiation associated gene 5 (MDA5, also known as helicard) and laboratory of genetics and physiology 2 (LGP2) (15).

RIG-I was shown to recognize ssRNAs that contain a triphosphate moiety in their 5´terminus as well as short dsRNAs. Relatively short poly (I:C) (approx. 300 bp, generated by RNase III digestion) can also serve as a RIG-I ligand, even without a 5`triphosphate.

In contrast to RIG-I, MDA5 preferentially recognizes longer dsRNAs, including synthetic poly (I:C). Long dsRNA is produced during replication of sense-strand ssRNA viruses, such as picornaviruses (31) (19).

RLRs are composed of two N-terminal caspase recruitment domains (CARDs), a central DEAD box helicase/ATPase domain and a C-terminal regulatory domain (RD). The helicase domain and the RD have been shown to interact with specific RNA species and CARDs are important for the initiation of downstream signaling (18) (32).

The receptors use the adaptor protein interferon promoter stimulator-1 (IPS-1) to induce the production of type I IFNs. This occurs via homotypic interaction with the CARD domain of IPS-1. Downstream of RIG-I/IPS-1, TBK1 and IKK-i become activated and thereby phosphorylate IRF-3 and IRF-7, leading to the expression of type I IFNs (28).

In contrast to RIG-I and MDA5, LGP2 does not have a CARD domain. While it has been demonstrated that LGP2 is a positive regulator of the MDA5-mediated immune response, the role of this protein in the RIG-I mediated response remains controversial (31) .

## Recognition of cytoplasmic DNA

Several studies indicate that when DNA enters the cytoplasm, it is not only recognized by TLR9 in endosomes but also by TLR9- and RLR-independent recognition machineries in the cytoplasm. Recognition of DNA in the cytoplasm results in the activation of the protein kinase TBK1, which phosphorylates the transcription factor IRF3, leading to a type I interferon response. This is in contrast to TLR9, which triggers an interferon response without the involvement of TBK1 (15).

Recently, DNA-dependent activator of IFN-regulatory factors (DAI, also known as DLM-1 and ZBP1) was identified as a DNA sensor and activator of immune responses. DNAs from various sources were shown to bind to DAI, thereby inducing type I IFN production and induction of other genes involved in innate immunity. However, it seems as if DAI is not the only cytosolic DNA sensor, since DAI knockdown using RNAi had little or no effect on the IFN response to B-DNA (33) (34).

Further, stimulator of IFN-genes (STING, also referred to as MITA and MPYS) was shown to form a complex with both, IPS-1 and RIG-I and is needed for virus-induced signal transduction through these molecules (35). Overexpression of STING upregulates type I IFN genes and suppresses viral replication, while STING-deficient cells show diminished type I IFN induction after stimulation with cytosolic dsDNA or infection with *Listeria monocytogenes* or HSV-1 (27).

Recently, a previously unknown cytosolic DNA-sensing pathway has been identified. In that pathway, RNA polymerase III transcribes microbial DNA into dsRNA containing a 5´-triphosphate, which is a

potent inducer of the RNA helicase RIG-I. Subsequent recognition of the dsRNA leads to an efficient type I IFN response (36).

Although cytosolic DNA recognition was mainly linked to the production of type I IFNs, absent in melanoma 2 (AIM2) has been recently described to be a cytosolic DNA sensor which is able to activate the inflammasome (31). Stimulation with TLR ligands alone does not lead to the activation of the inflammasome, but it has been shown that introduction of dsDNA induces proinflammatory signaling and maturation of pro-IL-1β. The AIM2 inflammasome is composed of AIM2, apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1, where AIM2 interacts with ASC to recruit procaspase-1 to the complex., leading to the production of IL-1β (32) (37).

## 3.1.3. Cytokines and Chemokines

### Inflammatory Cytokines

Inflammatory cytokines are primarily derived from mononuclear phagocytes, dendritic cells and other antigen-presenting cells. They are particularly effective in the generation of a potent immune response and provide signals that are contributing to the initiation and guidance of the nature of the adaptive immune response. Cytokines produced by antigen-presenting cells include TNF-α, interleukin (IL)-1, IL-6 (and other glycoprotein 130 (gp130)-utilizing factors), IL-12, IL-15, IL-18, IL-23, IL-27 and IL-32, CXCL8 (IL-8) and other members of the chemokine family (38).

IL-1, IL-6 and TNF-α are known to be the key cytokines involved in the acute inflammatory response.

TNF-α is the primary mediator of septic shock. Further it is a potent activator of neutrophils, mediating adherence, chemotaxis, degranulation and the respiratory burst. TNF is also responsible for severe cachexia in chronic inflammations and cancer and induces antitumor immunity by directly acting on cancerous cells (38).

It has been shown that disregulation of IL-6 signaling contributes to inflammation-associated disease condition such as inflammatory arthritis, sepsis, inflammatory bowel disease, obesity and insulin resistance (39)

Many cytokines that are released during an inflammation are able to further increase inflammation by enhancing the activation of immune cells at the site of inflammation or by acting on responsive cells throughout the body. Thereby, signaling cascades are activated which result in the enhancement or the prolongation of inflammatory responses (40).

### Interferons

In 1957, Isaacs and Lindenmann discovered a substance that protected cells from viral infection and named it interferon (IFN) (41). Over the years a whole family of these proteins was discovered and at present there are two main types of interferons existing: type I and type II IFNs and in addition, IFN-like cytokines.

The type I interferons are consisting of 7 classes: IFN-$\alpha$, IFN-$\beta$, IFN-$\varepsilon$, IFN-$\kappa$, IFN-$\omega$, IFN-$\delta$ and IFN-$\tau$. Type II interferon consists of a single IFN-$\gamma$ gene (42). Recently, a novel class of cytokines, the lambda IFNs (IFN-$\lambda$) have been identified and shown to functionally resemble type I IFNs (43).

Type I IFNs exert various effects on antibacterial immunity. They are the principle factors inducing the antiviral state, lead to cytokine and chemokine production and promote antibody isotype switching (40). Further, they lead to the activation and maturation of dendritic cells, thereby influence antigen-presentation, T-cell activation and the development of adaptive immunity.

To limit pathogen spread, these cytokines are able to decrease the survival of infected cells.

Moreover, type I IFNs lead to the activation of STAT4 in natural killer (NK)-cells and $T_H1$ cells, which together with IL-18-derived signals, activates IFN-$\gamma$ expression (44).

IFN-$\gamma$ is one of the most important cytokines responsible for cell mediated immunity.

It is produced by $T_H1$ cells, natural killer (NK) cells and cytotoxic T cells. This cytokine increases MHC class I and II expression, stimulates antigen presentation and production of cytokines by antigen-presenting cells (38).

Moreover, IFN-$\gamma$ was shown to activate genes with antimicrobial functions such as inducible nitric oxide synthase (iNOS, also known as NOS2) that generates nitric oxide and the phagocyte (NADPH) oxidase, which generates oxygen radicals (44).

## Chemokines

Chemokines are a group of small (8-12 kD) proteins that possess the ability to induce cell migration or chemotaxis in numerous cell types including neutrophils, monocytes, lymphocytes, eosinophils, fibroblasts and keratinocytes.

Chemokines were initially described as inflammatory mediators produced at sites of infection or injury or in response to proinflammatory stimuli. Their main feature is to recruit and activate leukocytes to mount an effective immune response and initiate wound healing (38). This is achieved by a process known as ´rolling´, where lymphocytes interact transiently with the vascular endothelium, searching for activating signals from chemokines. Once a cell has ceased rolling, it is

able to cross the endothelium and migrates along a concentration gradient to the source of the chemokine (38).

In addition, chemokines have been shown to be also involved in lymphoid organ development, $T_H1$ and $T_H2$ development, hematopoiesis, metastasis and angiogenesis (45).

Chemokines are divided into four main groups according to the number and relative position of their cysteine residues. The major chemokine families are termed CXC- and CC- chemokines (with 50 or more members in the 2 families), whereas the CX3C- and CX-chemokines represent the two smaller families of cytokines. The CXC-chemokines are further divided into two subfamilies, dependent on the presence or the absence of Glu-Leu-Arg (ELR) sequence preceding the first cysteine residue. ELR + chemokines preferentially attract neutrophils, whereas ELR- chemokines act on lymphocytes (46).

Each family of chemokines interacts with a reciprocal family of heterotrimeric, 7-transmembrane, G-protein-coupled receptors (GPCRs) that are almost exclusively expressed by lymphocytes (47). Chemokine signaling leads to the activation of several signaling pathways such as MAPK, protein kinase B (PKB), phospholipases A, C and D, phosphoinositol kinase (PI3K) and the JAK/STAT pathway (38) (47).

## Cytokine Signaling

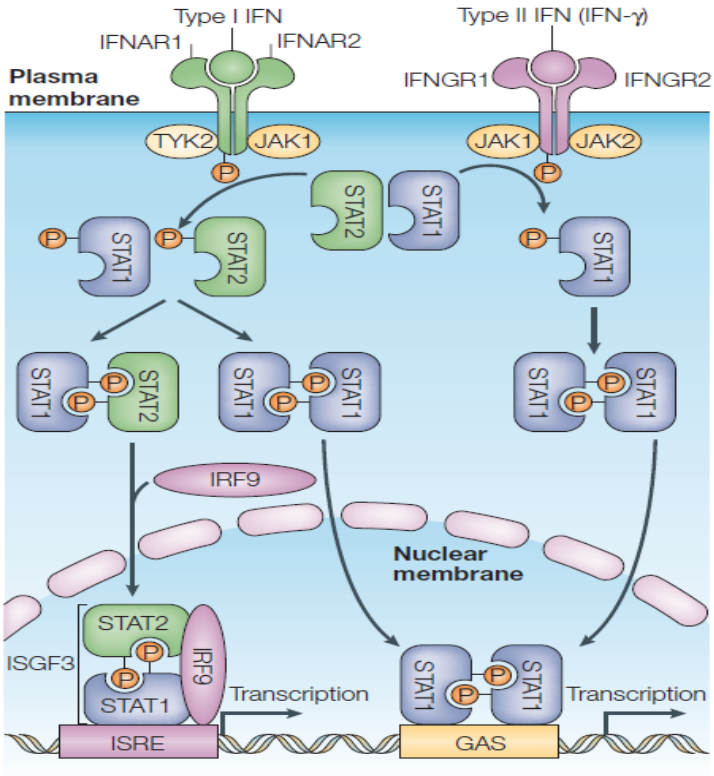
Cytokines act on cells through trans-membrane cell-surface receptors. After binding of a cytokine to its receptor, an intracellular signal transduction pathway is activated which ultimately leads to induction of gene transcription and synthesis of new proteins.

A large number cytokines, such as interleukins, interferons and hematopoietic growth factors, activate the Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signaling pathway.

The family of STAT proteins consists of latent cytoplasmic transcription factors that become tyrosine phosphorylated by the JAK enzymes in response to cytokine stimulation. The STAT family comprises seven members: STAT-1, STAT-2, STAT-3, STAT-4, STAT-5a, STAT-5b and STAT-6. The family of JAKs consists of: JAK-1, JAK-2, JAK-3 and tyrosine-kinase 2 (TYK2). The different members of the JAK and STAT families have different functions in cytokine signaling (48).

As an example for cytokine-induced signaling, signal transduction induced by interferons will be described in more detail (see also Figure 3).

**Figure 3: Signal transduction by the IFN receptors** (from (44))




Signal transduction by the IFN-receptors is activated by the binding of type I and type II IFNs to their respective receptors (type I IFNs: IFNAR, type II IFNs: IFNGR). Binding activates the Janus kinases (JAKs) that are permanently bound to these receptors (for IFNAR: JAK1 and TYK2 and for IFNGR: JAK1 and JAK2). The activated JAKs in turn phosphorylate tyrosine residues in the receptor. The phosphorylation creates then docking sites for STAT1 (for IFNGR) or a STAT1-STAT2 complex (for IFNAR). The receptor-associated STATs are phosphorylated on tyrosine residues by the receptor-associated JAKs. In the case of type I IFNs, phosphorylated STATs form STAT1-STAT2 heterodimers and to a lesser extend STAT1-STAT1 homodimers. After type II IFN stimulation only STAT1-STAT1 homodimers are formed. STAT1 homodimers translocate into the nucleus, where they bind to IFN-γ-activated-site (GAS) promoter sequences. STAT1-STAT2 heterodimers associate with a third protein, IFN regulatory factor 9 (IRF9) to form IFN-stimulated gene factor 3 (ISGF3) and to bind to the IFN-stimulated response element (ISRE) promoter sequence in the nucleus in order to stimulate gene transcription (44).

For full responses to both, type I and type II IFNs, the transactivation domain of STAT1 has to be phosphorylated on serine 727. This modification occurs only, when the protein is tightly bound to chromatin (49) (50) (51).

## 3.2. *Streptococcus pyogenes*

*Streptococcus pyogenes*, also known as Group A Streptococcus (GAS), is a gram-positive, non-motile, non-spore forming coccus. It occurs in pairs or in chains. Individual cells are round to ovoid and 0.6-1 µm in diameter. Due to the fact, that these bacteria are members of the lactobacilli, GAS is a fermentative, facultatively anaerobic organism that grows best in nutrient-rich environments.

A characteristic feature of these bacteria is a prominent zone of β-hemolysis when grown on solid agar containing intact red blood cells. β-hemolysis is described as a complete lysis of red blood cells by the bacterium resulting in a clear zone surrounding the colony grown on blood agar (52). *S. pyogenes* has been classified into serotypes by Rebecca Lancefield according to the variety of M virulence protein expressed on the cell surface.

Recently, a new approach for the identification of M-protein serotypes on a molecular basis has been developed. For this method, called *emm* typing, a large portion of the *emm* gene is amplified using highly specific primers. Located within this amplified region is the hypervariable sequence encoding M serospecificity, which allows direct sequencing. Genes that have the same *emm* type, share > 95% nt sequence identity (53, 54); ( ://www.cdc.gov/ncidod/biotech/strep/strepindex.htm).

Up to now, more than 200 different serotypes have been described and it was shown that these M-protein serotypes strongly correlate with the disease caused by a particular strain of *S. pyogenes* (55).

### 3.2.1. Streptococcal Pathogenesis and Clinical Manifestations

*S. pyogenes* is one of the most frequently found pathogens in humans and causes a wide spectrum of disease, ranging from uncomplicated mild infections (e.g. pharyngitis, tonsillitis, skin infections) to severe invasive disease such as sepsis, streptococcal toxic shock syndrome (STSS) and necrotizing fasciitis, which is also referred to as “flesh-eating disease”.

Before the invention of antimicrobials, diseases caused by *S. pyogenes* were very common and a huge medical problem in these days. It was reported, that this bacterium was the leading cause of puerperal infections, responsible for inestimable deaths of parturient women. Further, it was the major cause of deaths in burns patients.

With the introduction of penicillin into the clinical practice in the 1940ies, the number of streptococcus-caused diseases abated and it was believed that this pathogen is practically eliminated in the developed countries (54, 56).

In the mid-late 1980ies, clinics observed a resurgence of serious *S. pyogenes* infections, in Europe, in North America and other parts of the world (57, 58).

A current WHO study shows that diseases caused by *S. pyogenes* lead to 500.000 deaths each year worldwide and that the prevalence of severe diseases caused by this bacterium is at least 18 million cases (59).

Transmission of bacteria occurs directly from human-to-human, mostly via respiratory droplets or skin contact. The mucosal epithelium of the oropharynx and the epidermal layer of the skin represent the primary ecological niches of *S. pyogenes*, since it´s very likely that the bacterium enters the host via these sites (53).

Nevertheless, the bacterium can thrive in almost every tissue of the body including normally sterile tissues causing maladies like deep soft tissue infection, meningitis, pneumonia and pericarditis.

**Table 2: Diseases caused by *Streptococcus pyogenes*** (adopted from (54))

| **Category of Disease** | **Examples of Infection** |
|---|---|
| **Superficial Infections** | pharyngitis, skin and soft tissue infections, impetigo, erysipelas, vaginitis, post-partum infections |
| **Deep Infections** | bacteraemia, necrotizing fasciitis, deep soft tissue infections, cellulitis, myositis, puerperal sepsis, pericarditis, meningitis, pneumonia, septic arthritis |
| **Toxin-mediated Diseases** | scarlet fever, toxic-shock like syndrome |
| **Immunologically-mediated Diseases** | rheumatic fever, post-streptococcal glomerulonephritis, reactive arthritis |

Pharyngitis (also known as sore throat or strep throat) is the most common infection caused by *S. pyogenes* and is considered as a non-invasive infection.

It is defined as an inflammation of the throat and the pharynx and possibly the larynx and the tonsils. This disease occurs primarily in childhood (from 5-15 years), but is very rare in children younger than 3 years. 15-30% of cases are caused by *S. pyogenes*. Adults are most often affected in the late teens or early 20s (60). Pharyngitis is highly prevalent in late fall through early spring (55) (53).

In addition, *S. pyogenes* causes more severe invasive diseases such as sepsis, necrotizing fasciitis (NF) and streptococcal toxic shock syndrome (STSS).

Necrotizing fasciitis, also known as “flesh eating disease” is a rare but potentially fatal infection involving the skin, the subcutaneous tissue, fascia and muscles (52).

It is characterized by rapid destruction of tissue, systemic toxicity and with high morbidity and mortality (61). At the beginning of the disease, it is difficult to differentiate NF from other superficial infections of the skin. It often starts with a local, quite trivial injury (e.g. insect bites or scratches) and if diagnosis is delayed, worsens within hours or days. Studies show that rapid surgical intervention is important for the survival of the patient (52) (62).

Streptococcal toxic shock syndrome (STSS) often goes hand in hand with deep tissue infections like necrotizing fasciitis and cellulitis (63). This syndrome manifests as shock and organ failure and affects patients in all ages.

As a further complication, post-streptococcal sequelae such as acute rheumatic fever (ARF) and acute post-streptococcal glomerulonephritis (APSGN) can occur. These diseases are considered to be immunologically mediated.

Rheumatic fever usually follows a streptococcal pharyngitis and appears as an acute systemic febrile illness about 3 weeks after throat infection. The major outcomes of this disease include arthritis, carditis, the erythema marginatum rash, Sydenhams´s chorea and subcutaneous nodules (54, 60).

Arthritis, which is an inflammation of the joints, can be observed in 60-80% of ARF patients. It usually affects the large joints of the lower extremities, followed by the upper extremities.

Carditis, considered as one of the most serious complications of ARF, manifests as myocarditis, pericarditis and endocarditis (60). It occurs in 30-45% of ARF patients and leads to chronic rheumatic heart disease (RHD). It was estimated, that 60% of all ARF patients will develop RHD and that over 2 million children are affected with RHD worldwide (64) (55) (59).

Acute post-streptococcal glomerulonephritis (APSGN) is the second major sequel of streptococcal infections leading to an acute nephritic syndrome. It affects mainly children, young adults and also individuals over 40. Interestingly, males are affected twice as often as females (55). Patients suffer from hematuria (red or smoky urine), headache, anorexia, nausea, vomiting and due to the swelling of the renal capsule, flank or back pain (60).

APSGN usually occurs about 10 days after throat or skin infection by specific nephritogenic serotypes of *S. pyogenes*. Although it is now rare in the industrialized nations, it still is a concern in the developing countries with 9-29 new cases per 100.000 individuals per year (65).

Post-streptococcal sequelae have been linked to insufficient health care and poor hygienic conditions. For this reasons, poststreptococcal syndromes remain the diseases of underprivileged and developing countries (60). Due to the fact, that *S. pyogenes* is still causing high mortality rates, even in industrialized countries and reports showing the occurrence of macrolide-resistant strains of *S. pyogenes* (58, 66), the development of a vaccine against this bacterium is of great importance.

Several attempts have been made to design a vaccine against *S. pyogenes*. For example, the streptococcal M-protein is one of the most important virulence factors of *S. pyogenes* as it confers resistance to phagocytosis by cells of the host immune system. The M-protein consists of two polypeptide chains configured in an alpha-helical coiled coil complex that is anchored to the cell membrane via its C-terminus. The protein is composed of four repeat regions: the hypervariable (A), the variable (B) and two conserved regions (C, D) (67).Two regions of the M-protein have been targeted for vaccine development: the hypervariable N-terminal region and the conserved C-terminal region (68).

Due to the multiplicity of M-types expressed by *S. pyogenes* it is difficult to find a single antigen, which provides protection against all serotypes of this bacterium. Further, the development of M type-based vaccines is complicated by the fact that certain M types elicit both, protective antibodies and antibodies that cross-react with human tissues (heart, brain, kidney) (69).

Another approach to design an effective vaccine is the use of well described virulence factors of *S. pyogenes* as an antigen, including streptococcal C5a peptidase, cysteine protease, fibronectin-binding proteins, streptococcal carbohydrate and streptococcal pili. Further, reverse vaccinology was used to identify novel antigens (70). This approach for vaccine development is based on the *in silico* analysis of the whole genomic sequence of a pathogen in order to identify a suitable antigen (71). In these days, several vaccine candidates against *S. pyogenes* are in varying stages of preclinical and clinical development. But only one of the candidates, the multivalent N-terminal vaccine, has entered clinical trials during the last 30 years (70).

### 3.2.2. Interaction with the Host

The human immune system is a complex of biological structures and processes that protects the organism against unwanted intruders like bacteria or viruses. If microbes are somehow able to enter the organism, it is very likely that they will encounter cells of the innate immune system, which confer the first line of defense against invaders. Upon detection, these cells trigger an immune response resulting in the eradication of the pathogen.

The great success of *S. pyogenes* in establishing disease results from its ability to efficiently circumvent host defense mechanisms. The bacterium makes use of a huge armament of virulence factors, which helps the bacterium to colonize, multiply rapidly and spread within the host, thereby causing numerous, sometimes life-threatening, diseases.

Key components of the bacterium´s armament are surface bound M-proteins that inhibit phagocytotic defenses and promote invasion of host cells, capsules of hyaluronic acid (HA) mimic

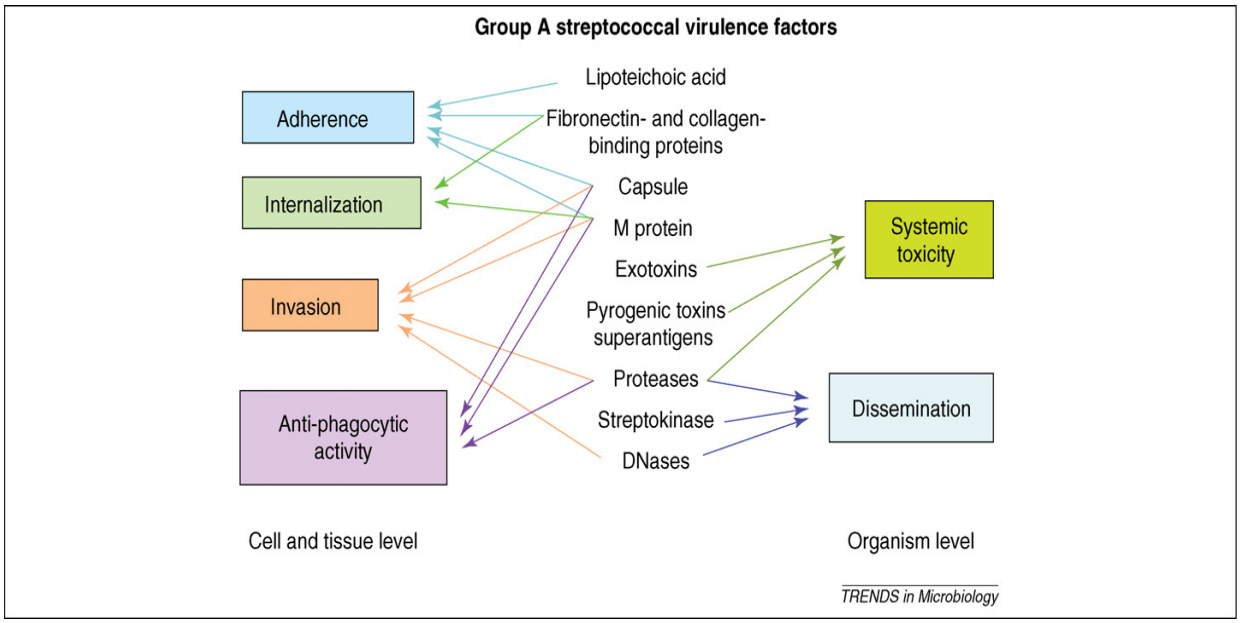
human tissue HA and proteases that specifically destroy chemotactic signals that should attract phagocytes (72).

Further, *S. pyogenes* produces pyrogenic exotoxins which act as bacterial superantigens. They include SpeC, SpeG, SpeH, SpeI, SpeJ, SpeK, SpeL, SpeM, SSA, the highly polymorphic SMEZ and the bacteriophage-encoded SpeA. SMEZ is the most potent superantigen and also the most immunoactive agent of *S. pyogenes* (73).

Superantigens produced by *S. pyogenes* are potent immunostimulators and systemic intoxication can lead to the life-threatening toxic shock syndrome (TSS). This is due to the ability of superantigens to bind simultaneously to major histocompatibility complex (MHC) class II molecules and the T-cell receptor (TCR). This binding leads to activation of a large number of T cells expressing specific V-beta subsets of the T cell repertoire resulting in a sudden and massive cytokine storm (74).

The presence of a superantigen in the blood stream rapidly elevates the amount of cytokines to toxic levels and it is believed that the production of interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) are the main responsible cytokines causing this massive toxicity.

**Figure 4: GAS virulence factors interact with the host at many levels** (from (75))




## Epithelial Cell Adherence

For a microbial pathogen, the initial step to cause a disease is the attachment to a mucosal or cutanous surface of the host. The attachment is usually hindered by the normal flora and numerous

electrostatic and mechanical forces. *S. pyogenes* uses multiple and complex strategies to attach and invade the human host, involving different adhesins and invasins.

Adherence of *S. pyogenes* is a two-step mechanism, consisting of unspecific and specific interactions with the host. First, the unspecific step allows a temporally limited, weak attachment via hydrophobic interactions on the surface of the bacterium with hydrophobic domains on the host cells, where lipoteichoic acid (LTA) is believed to be the responsible structure. The second step relies on the expression of specific adhesins on the surface of *S. pyogenes* which allow the bacterium to establish a firm adherence (76). So far, at least 17 adhesin candidates have been reported, but the most important and most extensively studied ones are the M-protein, fibronectin binding proteins and lipoteichoic acid (74).

In addition, several extracellular host cell proteins have been described in the context of attachment or adherence to *S. pyogenes*: collagen, fibronectin, vitronectin and the integral membrane proteins CD46 (membrane cofactor protein of keratinocytes) and CD44 (hyaluronate-binding receptor on keratinocytes) (55). These proteins are regarded as host extracellular matrix (ECM) and serve as prime target for interactions between bacteria and the host, because they are firmly bound to the surface of an eukaryotic cell and are present in a large number of species and types {Kreikemeyer, 2004 #227.

The M-protein mediates binding to collagen and fibronectin but it can also interact with the plasma proteins fibrinogen and plasminogen {Sanderson-Smith, 2008 #231}(77).

It was also shown that it is important for the adhesion of the bacteria to HEp-2 cells in tissue culture and to Keratinocytes, but not to buccal or tonsillar epithelial cells. All in all, the role of M-protein in context of adherence seems to be dependent on the M-protein serotype and on the host cell source, such as the skin or the phayrinx (55).

Fibronectin (Fn) binding proteins (FnBPs) are another group of proteins mediating adhesion of streptococcal cells to the host epithelium. These include protein F1, SfbI and the related proteins SfbII, FBP54, protein F2 and PFBP (74). *S. pyogenes* strains differ in the genetic potential to express FnBPs SfbI, SfbII and F2 and it was observed that strains possessing two or more genes for these FnBPs bound Fn significantly more than strains possessing one or none of the genes (78). Among them, protein F is the best studied fibronectin binding protein of *S. pyogenes*. Studies with protein F and the highly homologous protein SfbI (streptococcal fibronectin binding protein I) revealed that these proteins have the ability to inhibit binding of Fn to streptococcal cells when expressed in *Escherichia coli*. Further, inactivation of the gene (prtF), which codes for protein F, led to a mutant which was not longer able to adhere to respiratory epithelial cells (79, 80).

It was discovered recently, that *S. pyogenes* produce long, surface-exposed, pilus-like structures composed of members of a family of ECM- binding proteins. These pili are encoded by genes located in the FCT (fibronectin, collagen-binding, T-antigen) gene region and up to now, 4 major variants of the FCT region have been described. Each FCT region contains genes coding for pilus structural subunits (the backbone protein, which corresponds to the T antigen of the Lancefield T serotypes (and ancillary proteins) and for sortase enzymes required for pilus assembly (81, 82). Streptococcal mutants, where either pilus backbone structural protein or the sortase C1 have been deleted, show an impaired attachment to a pharyngeal cell line (83). Streptococcal pili mediate adhesion to human tonsil epithelium, primary human keratinocytes and skin but are not used by the bacterium for adhesion to HEp-2 and A549 cells (84). Recently it was reported that, in contrast to group B streptococcal and pneumococcal pili, pilus expression reduces virulence of *S. pyogenes* in murine models of necrotizing fasciitis, pneumonia and sepsis. The observed attenuated virulence associated with pilus expression was not due to differences in phagocytic uptake, complement deposition or antimicrobial peptide sensitivity (85). So it seems like that pili have not only an important role during attachment to the host and subsequent invasion but surprisingly also in the restriction of invasive disease pathogenesis.

## Intracellular Invasion and Spread through Tissues

The success of a pathogen depends on its ability to colonize host tissues and persist by ensuring its long-term survival. Therefore, adhesion to the host cell is a major prerequisite for colonization and invasion. Several proteins involved in streptococcal adherence are also involved in epithelial cell invasion.

Although *S. pyogenes* is predominantly an extracellular pathogen, several studies indicated that this bacterium invades and persist within human epithelial cells (86, 87). The reason for that might be that the intracellular status of the bacterium serves a niche, where the bacterium is protected against antibiotics and/or host defense mechanisms (78). Another possibility could be that the bacterium uses the entry into the host cell for invasion of deeper tissues.

Internalization is a complex process and two different pathways have been described.

Strains expressing the FnBP SfbI, use this protein for binding to Fn as a bridging molecule and the $\alpha5\beta1$ integrin as cellular receptor. In tonsils, the interaction of the bacterium with integrin-bound fibronectin leads to the induction of transforming growth factor-$\beta1$ (TGF-$\beta1$) from fibroblasts, which further upregulates the expression of $\alpha5$ integrin and Fn on epithelial cells and fibroblasts. This results in the formation of a higher density of $\alpha5\beta1$ receptors and a more efficient ingestion of bacteria (88).The thereby activated uptake process is characterized by the generation of large

membrane invaginations and a lack of actin recruitment or cell injury. This type of uptake is also known as “zipper mechanism” and involves the formation of an intimate contact between the host cell membrane and the bacterium leading to a tight engulfment of the pathogen by the cell membrane in a zipper-like fashion (89).
However, not all streptococcal strains express the SfbI protein, but many of those are still internalized by host cells. In that case, the uptake process involves major rearrangements of cytoskeletal proteins, leading to recruitment and fusion of microvilli and the generation of cellular leaflets (90).

The streptococcal capsule is an important virulence factor of *S. pyogenes* and plays a major role in the protection from phagocytosis by host immune cells. The capsule is composed of a polymer of hyaluronic acid containing repeating units of glucuronic acid and *N*-acetylglucosamine.
Streptococcal isolates vary in the amount of hyaluronic acid capsule they produce and variants showing increased encapsulation are linked to greater invasive disease potential (55) (91).
In contrast to M-protein and fibronectin-binding proteins, the hyaluronate capsule of *S. pyogenes* was shown to impair adherence to and invasion of epithelial cells and human keratinocytes (74, 92). On the other hand, a study using a murine model for necrotizing fasciitis shows that the loss of the hyaluronic acid capsule significantly attenuated local soft-tissue necrosis, systemic bacterial dissemination and mortality. This goes in line with another study that revealed that the streptococcal capsule is recognized by the hyaluronic acid-binding protein CD44 on human keratinocytes. Binding of *S. pyogenes* induced marked cytoskeletal rearrangements, resulting in the disruption of intercellular junctions thereby allowing the bacterium to remain extracellular when it penetrates the epithelium (93).

Hyaluronidase, DNases, Streptokinase and the streptococcal pyrogenic exotoxin B (SpeB) are extracellular products of *S. pyogenes* that are considered as spreading factors by promoting liquefaction of pus and helping the bacterium to get through tissue planes.
Hyaluronidase enzymatically degrades hyaluronic acid which is present in the connective tissue and DNases (DNases A, B, C and D) are responsible for the degradation of DNA (74).
Expression of DNase is crucial for *S. pyogenes* to avoid destruction by neutrophils, since these cells can kill invading microbes by engulfment of the bacteria within neutrophil extracellular traps which are consisting of DNA and chromatin (75).
A common defense mechanism to prevent invading pathogens from spreading involves the encapsulation of bacteria at confined sites by deposition of fibrin networks. This allows a directed inflammatory response at the affected area in order to eradicate the invaders.

*S. pyogenes* circumvents this defense mechanism by acquisition of the serine protease plasmin at the bacterial cell surface. Streptococcal Streptokinase is a plasminogen-activating protein that combines with plasminogen to make functional plasmin. This protease is then able to hydrolyze fibrin thereby promoting the spread of *S. pyogenes* through tissues (82, 94).

The cysteine protease SpeB is a crucial virulence factor of *S. pyogenes* and plays a major role during colonization of the host by modulation of functions of cell surface proteins. Further, it contributes to tissue destruction during necrotizing fasciitis. This toxin is produced by virtually all *S. pyogenes* strains, but the degree of expression varies from strain to strain. Although at the beginning believed as a superantigen, it is now thought that its contribution to disease results entirely from the protease activity (74). The functions of this virulence factor a diverse, including degradation of host ECM proteins (i.e. fibrin and fibronectin) and human immunoglobulins, activation of IL-1β and the release of proteins from the surface of *S. pyogenes* (95) (96). SpeB was also shown to degrade a variety of chemokines (i.e. IP10/CXCL10, I-TAC/CXCL11, PF4/CXCL4, GROα/CXCL1, GROβ/CXCL2, GROγ/CXCL3, ENA78/CXCL5, GCP-2/CXCL6, NAP-2/CXCL7, SDF-1/CXCL12, BCA-1/CXCL13, BRAK/CXCL14, SRPSOX/CXCL16, MIP-3α/CCL20, Lymphotactin/XCL1, and Fractalkine/CX3CL1), thereby destroying most of their signaling and antibacterial properties (97). Further a study shows that during localized infections, some *S. pyogenes* strains can lose SpeB activity, leading to an accumulation of plasmin activity on the cell surface of the bacterium. Loss of SpeB activity helps the bacterium to leave the local site of infection and to enter the blood stream, thereby promoting dissemination to other parts of the host (75).

## Immune System Avoidance

### Impairment of Phagocyte Recruitment

Once a pathogen managed to invade the host, the innate immune system induces an inflammatory response to clear the bacterial infection. Phagocytes recognize and kill the invading pathogen via a process called phagocytosis, therefore they are the most important effectors cells in this initial phase. These cells are recruited to the site of infection via chemoattractants, which are molecules that form a gradient in the tissues in order to guide the phagocytes to the site of infection and inflammation. Chemoattractants for phagocytes can be bacterial-derived products such as N-formyl peptides from the bacterial cell wall but can be also host-derived, like leukotriene B4, platelet-activating factor, the complement-derived anaphylotoxin C5a and chemokines such as CXCL8 (interleukin-8 (IL-8)) (46) .

*S. pyogenes* produces a serine protease (SpyCEP, also known as ScpC) that degrades human IL-8, growth regulated oncogene-alpha (Gro-$\alpha$ /CXCL1) and granulocyte chemoattractant protein-2 (GCP-2/CXCL6) (98). Further, it was shown to degrade also the murine chemokines keratinocyte chemoattractant (KC/CXCL1) and macrophage inflammatory protein 2 (MIP-2/CXCL2) (99). IL-8/CXCL8 is a chemokine which plays an important role in neutrophil transmigration and activation. SpyCEP/ScpC specifically cleaves the C-terminus of IL-8 leading to a functional inactivation of the chemokine (91). In a murine model of necrotizing fasciitis, SpyCEP/ScpC was shown to be important for *S. pyogenes* virulence since it was necessary and sufficient for systemic bacterial dissemination within soft tissues and the respiratory tract (98).

The C5a peptidase ScpA is another immunomodulating enzyme of *S. pyogenes* that inhibits recruitment of phagocytes to the site of infection. This cell-wall-anchored serine endopeptidase specifically cleaves the chemotactic complement factor C5a, which is known to be important for the activation of neutrophils and macrophages (100). The cleavage of C5a requires the expression of a plasmin receptor (Plr), also known as surface dehydrogenase (SDH) or glyceraldehydes-3-phosphate dehydrogenase (GAPDH) which binds C5a with a higher affinity than ScpA. It seems that the Plr/SDH/GAPDH mediates the recruitment of C5a to the streptococcal cell surface since it has no protease activity, while ScpA is responsible for the cleavage of C5a (46) (101).

ScpA mutants show a reduced ability to colonize the murine nasopharynx and active immunization with ScpA confers resistance to nasal colonization by *S. pyogenes* in a mouse model (102). Furthermore, it was demonstrated that the important cytolytic toxin streptolysin S (SLS) of *S. pyogenes* is also involved in the suppression of migration of phagocytes to the site of streptococcal infection during the early period of infection. This function seems to be independent of the lytic activity of SLS but dependent on the ability to inhibit production of chemotactic signals by the host cells (103).

## Avoidance of Phagocyte Killing

After internalization by a phagocyte, pathogens get trapped in intracellular membrane-bound compartments where an array of bactericidal mechanisms is deployed to destroy the pathogen. These mechanisms include generation of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI), the production of antimicrobial peptides including lysozyme and myeloperoxidase and the fusion of endocytic organelles with phagosomes, which are acidified and mature into phagolysosomes.

In order to generate a functional phagosome, neutrophils make use of specialized secretory organelles, the primary (azurophilic) and the secondary (specific) granules (104).

*S. pyogenes* was shown to survive within phagocytes and is also able to escape from the phagosome into the cytoplasm of polymorphonuclear neutrophils (PMNs) (105). The ability to survive within these cells is associated with the expression of the streptococcal M- and M-like surface proteins, since mutants for the respective proteins are rapidly killed by the neutrophils (106). Further, strains of *S. pyogenes* that express the M-protein, inhibit the secretion of azurophilic granules and the formation of pinosomes (107). To cope with hydrogen peroxide and organic peroxides during an inflammatory response, *S. pyogenes* expresses the gluthathione peroxidase GpoA. Using several different murine models, it was shown that this peroxidase is important for the bacterium to adapt to oxidative stress and for streptococcal virulence (108).

The two pore-forming cytotoxins Streptolysin O (SLO) and Streptolysin S (SLS) are key virulence factors of *S. pyogenes* and are, among other things, also involved in promoting resistance to phagocyte killing by triggering the death of phagocytes before bacterial killing is accomplished. These two haemolysins are produced by almost all *S. pyogenes* strains.

SLO derives its name from its oxygen lability and belongs to a large family of highly conserved pore-forming toxins, which can be found in more than 20 different species, including the genera *Clostridium*, *Streptococcus*, *Listeria*, *Bacillus* and *Arcanobacterium* (109).

It is reversibly inhibited by oxygen and irreversibly inhibited by cholesterol. It is toxic to a variety of cell types and fractions, including erythrocytes, polymorphonuclear leukocytes, platelets and tissue culture cells (74). SLO has been shown to impair the phagocytotic capacity of polymorphonuclear leukocytes (110). Further, it protects the bacterium from host cell killing by preventing the internalization of the bacteria into the lysosome (111).

A recent study revealed that SLO is also the responsible virulence factor for the induction of apoptosis in macrophages and neutrophils, thereby contributing to streptococcal immune evasion and virulence. Apoptosis appeared to be caspase- dependent and was promoted by release of cytochrome c and permeabilization of mitochondrial outer membranes caused by SLO (112).

Like SLO, SLS damages the membranes of polymorphonuclear leukocytes, platelets and subcellular organelles and is responsible for the hemolytic zone around *S. pyogenes* colonies grown on blood agar plates (113).This hemolysin exists in intracellular, cell-surface-bound and extracellular forms and is produced by *S. pyogenes* growing in presence of serum, serum-albumin, alpha-lipoprotein and ribonucleic acid (74).

SLS was shown to excert cytocidal activity on host neutrophils, thereby promoting resistance of *S. pyogenes* to killing by phagocytes (91).

SLS-negative mutant strains, which were obtained by insertion of the Tn916 transposon in the sag operon promoter of M1 or M18 serotype strains, show reduced virulence in a murine model of soft

tissue infection (114). In contrast to these findings, studies using other streptococcal M-types (M3, M5) with a sagA deletion revealed that there was no effect on virulence in similar animal models (110)(115).

Moreover, the streptococcal DNase Sda1 has been reported to be deployed by *S. pyogenes* to avoid destruction by neutrophils. This cell type has three different strategies to eradicate invading microbes. As professional phagocytes, they are able to recognize invaders and kill them by phagocytosis. In addition, they secrete highly reactive antimicrobial effector molecules. Another way to kill pathogens is the release of neutrophil extracellular traps (NETs), which are composed of decondensed chromatin and antimicrobial proteins (116).
The streptococcal DNase Sda1 has been shown to degrade NETs, thereby maintaining bacterial survival in neutrophils and mouse blood. Further, this DNase is necessary and sufficient to promote virulence in a murine model of necrotizing fasciitis (117).

### Interference with the Complement System

The plasma proteins of the complement system are important mediators of the innate immune response against pathogens. The major functions of the complement cascade are to label immunogenic particles or pathogens with C3b and iC3b to facilitate phagocytic uptake via complement receptors, to generate chemoattractants (e.g. C5a) to recruit phagocytes to the site of infection and to directly lyse gram-negative bacteria through the membrane attack complex (MAC) (67). On the other hand, bacterial pathogens evolved various strategies to circumvent the complement response.
The complement system is divided into three different initiation pathways: the classical (CP), the lectin (LP) and the alternative (AP) pathway. All of them converge at the cleavage of the central complement protein C3 by the two C3 convertases C4b2a and C3bBb.
The host expresses complement regulators such as factor H (FH), factor H-like 1 (FHL1), C4b-binding protein (C4BP) and membrane cofactor protein (MCP, CD46), which function to downregulate convertase activity to prevent tissues from excessive complement activity (67).
*S. pyogenes* is able to interact with these proteins via the M- and M-like proteins.
C4BP is a plasma glycoprotein that is involved in the regulation of the classical complement pathway by accelerating the decay of the C3 convertase and by acting as a cofactor in the FI-mediated proteolytic inactivation of C4b. *S. pyogenes* was shown to bind C4BP via the hypervariable N-terminal region in the M-protein to the C4b- binding site in C4BP. This interaction occurs with a high affinity

and confers bacterial protection against classical complement activation and resistance to phagocytosis (118).

The hypervariable region and/or the conserved C-repeat region of many streptococcal M-proteins have also been shown to bind factor H and its splice variant factor H-like 1 for immune evasion. However the significance of M-protein binding to complement regulators is controversial since a study analyzing the capacity of several clinical isolates of *S. pyogenes* to bind to regulators of complement activators revealed that the most virulent M1 and M3 strains did not bind to these proteins (119).

FH regulates the activation of complement by acting as a cofactor for factor I-dependent cleavage of C3b and by disrupting the alternative pathway C3 convertase. FHL-1 was identified as an alternative splicing product of the FH gene and was shown to have FH complement regulatory protein activity (67). Aquisition of FH and FHL-1 can limit the deposition of the opsonin C3b on bacteria, thereby decreasing the bacteriums susceptibility to phagocytosis. However, it has been demonstrated that binding of factor H to a certain M1 strain is not mediated by the M-protein or M-like proteins but by the cell surface protein Fba. This protein prevents deposition of C3b on the streptococcal surface and promotes survival of *S. pyogenes* in human whole blood (120).

The ultimate step of the complement cascade is the cleavage of C5 by the C5 convertases, which results in the formation of C5b, the molecule that is responsible for the formation of the membrane attack complex (MAC), which consists of proteins C5b-C9. This complex functions to destroy target cells, like for example gram-negative bacteria.

*S. pyogenes* expresses the streptococcal inhibitor of complement (SIC), which was found to specifically block the formation of the MAC. This is achieved by the prevention of insertion of C5b67 into the cell membrane (67). Since gram-positive bacteria are anyway protected from lysis by the complement system by their peptidoglycan-containing cell walls and hyaluronic acid capsules, the relevance of inhibition of the MAC complex is questionable.

The SIC molecule has also been shown to inhibit a variety of antimicrobial peptides and proteins including lysozyme, secretory leucocyte proteinase inhibitor (SLPI), LL-37, human neutrophil peptide-1 (hNP-1), and the human β-defensins 1, 2, and 3 (121). This function might be more relevant for the protection of *S. pyogenes* from the host immune system.

### Evasion of Antibody-mediated Opsonization

A second form of opsonization is provided by the production of immunoglobulins (Igs) against specific bacterial epitopes during the humoral immune response. Immunoglobulins can be divided

into four different classes (IgG, IgM, IgA, IgD, IgE) based on differences in the amino acid sequences in the constant region of the heavy chains.

They consist of antigen-recognizing Fab regions, which are linked through a flexible hinge region to the constant Fc effector region.

The Fc effector region is responsible for the activation of the classical pathway of complement and mediates the contact with Fc receptors on phagocytes.

Immunoglobulins have various functions during the humoral and inflammatory immune response against a pathogen: they (1) interact with Fc receptors (FcRs) on phagocytes and complement components, (2) activate phagocytes, (3) modulate B-cell functions (e.g. memory induction, activation and/or feedback inhibition of antibody production), (4) neutralize toxins, (5) mediate antibody-dependent cellular cytotoxicity, (6) activate natural killer cells and eosinophils, (7) induce mast cell degranulation and mediate opsonization of antigens, thereby causing enhanced phagocytosis (122).

*S. pyogenes* evolved several virulence factors to circumvent or destroy components of this important branch of host defense.

The streptococcal cysteine proteases SpeB and IdeS (also designated as Mac-1) have been shown to cleave IgG in the hinge region. SpeB has been reported to cleave also the carboxy-terminal parts of the heavy chains of IgM, IgA, IgD, IgE antibodies, whereas IdeS has a high specificity to IgG.

These two proteases act at different phases of the immune response against *S. pyogenes*. Whereas IdeS protects the bacterium in the early phase of infection (i.e. during colonization and growth), SpeB is important during later phases, when the infection is losing momentum (123)(124).

Mac-2, a variant of IdeS (Mac-1), has a weak endopeptidase activity against IgG compared to IdeS, but it is able to bind to the immunoglobulin receptors FcγRII and FcγRIII. Binding results in the inhibition of IgG recognition by Fc receptors, leading to an enhanced survival of the bacterium (125).

Further, the endoglycosidase EndoS, cleaves the asparagine-linked glycan on IgG, thereby impairing opsonophagocytosis, activation of the classical complement pathway and recognition of IgG by phagocyte Fc receptors (123).

The function of antibodies is also hindered, when the pathogen is able to bind to the Fc region of IgG, resulting in the decoration of the bacterial surface with the host molecule. This mechanism of immune invasion is for example applied by the streptococcal fibronectin binding protein I (SfbI), thus preventing antibody-dependent cell-cytotoxity and phagocytosis by macrophages (126). Moreover, several types of M-protein and protein H of *S. pyogenes* have been reported to bind to the Fc domains of IgG, thereby inhibiting complement activation on the bacterial cell surface (91).

Another strategy of *S. pyogenes* to avoid antibody-mediated recognition is the upregulation of hyaluronic acid capsule expression. The capsule then shields the bacterial cell-wall attached

immunogenic determinant G-related alpha2-macroglobulin-binding protein (GRAB) from detection by antigen-specific antibodies (127).

### 3.2.3. Innate Immune Responses to *Streptococcus pyogenes*

Activation of the immune system upon any type of infection is fundamentally dependent on the recognition of the pathogen by the cells of the innate immune system.

In case of the important human pathogen *S. pyogenes*, the molecules needed for the pathogen recognition and downstream signaling events that mediate an effective immune response are largely not known.

It has been shown that macrophages play an important role during an infection with *S. pyogenes*, since depletion of macrophages in mice using carrageenan significantly increased susceptibility to *S. pyogenes* infection (128). Further it has been demonstrated that also dendritic cells are important contributors to the host defense against *S. pyogenes* (129).

Recently it has been shown by our group that this bacterium induces an inflammatory response in murine bone-marrow derived macrophages (BMDMs), resulting in the production of proinflammatory cytokines such as TNF-$\alpha$ and IL-6. Surprisingly, this response appeared to be independent of the main bacterial receptors TLR2, TLR4 and TLR9 but dependent on the important TLR-adaptor protein MyD88 (130).

The importance of this protein during *S. pyogenes* infection was also proofed using a murine in vivo model. *S. pyogenes*-infected, MyD88-deficient mice harbored significantly more bacteria in their organs and succumbed much earlier to infection compared to C57BL/6 animals (129). Further, a recent report shows that in dendritic cells, streptococcus-mediated production of proinflammatory cytokines such as IL-12 and IL-6 is, like in macrophages, also dependent on MyD88 (131).

*S. pyogenes* has also been shown to activate Stat1, Stat3 and IRF1 in human macrophages (132).

Our group was the first to demonstrate that mouse innate immune cells are able to produce IFN-$\beta$ in response to an extracellular Gram-positive bacterium (*S. pyogenes*) (130). In addition, it was shown that group B streptococcus (GBS) is able to induce IFN-β production and that IFN-β is critical for host defense against these bacteria (133). The IFN-$\beta$ production in response to GBS appears to be induced in dendritic cells by bacterial RNA in a TLR7-dependent way whereas in macrophages bacterial DNA, recognized by a so far unidentified receptor, is the IFN inducer (134) (135). Overall, reports on the molecular mechanism underlying IFN-$\beta$ induction by pathogens suggest that individual pathogens developed specific and often unique ways how to elicit IFN production in innate immune cells.

Recently, *S. pyogenes* was also shown to activate the inflammasome, which is a multiprotein-complex known to mediate the activation of caspase-1 that leads to the production of the

proinflammatory cytokines IL-1β and IL-18 (136). In the case of *S. pyogenes*, the NLR family pyrin domain-containing 3 (NLRP3) and the adaptor protein apoptosis-associated speck-like protein (ASC) were crucial for caspase-1 activation and IL-1β secretion. Inflammasome activation during streptococcal infection proceeds independently of TLR signaling but is depending on NF-κB and the streptococcal cytolysin Streptolysin O (SLO) (136).

# 4. Aims

Although *S. pyogenes* is an important pathogen causing a wide range of diseases, very little is known about the molecules and processes which are fundamental for its recognition and the activation of the innate immune system.

It was decided to address this issue by investigation of *S. pyogenes* infection in murine bone-marrow-derived macrophages (BMDMs), since these cells confer, among others, the first line of defense against bacterial infections.

One aim of this thesis was the identification of the molecule responsible for the recognition of *S. pyogenes* and the characterization of signaling pathways leading to a proinflammatory response against this bacterium. Therefore, primary macrophages from several TLR-deficient mouse lines have been analyzed for their potential to initiate proinflammatory responses against *S. pyogenes*, since it is likely that these sensor molecules are involved in its recognition.

The second aim was the identification of mechanisms responsible for type I interferon (IFN) production in BMDMs in response to *S. pyogenes*. We were able to show that *S. pyogenes* induces an IFN response in macrophages by a yet unknown receptor. Therefore, we wanted to investigate the molecule responsible for the initiation of IFN signaling and also the signaling events occurring after induction of IFN production.

Further, we were interested in the nature of the streptococcal ligand, inducing the proinflammatory (i.e. TNF-$\alpha$ production) as well as the type I IFN response to *S. pyogenes*. We assumed that the identification of the ligand would eventually facilitate the search for the receptor molecule. Therefore, streptococcal fractions were treated with either RNase A, DNase I or Proteinase K or the combination of them. The potential of streptococcal DNA, RNA or proteins to induce immune responses was assessed by the measurement of TNF-$\alpha$ and IFN-$\beta$ production.

The third aim of this thesis was the establishment of an *in vivo* mouse model to identify the role of type I IFN production in the host defense against invasive infection with *S. pyogenes*. Therefore, a subcutaneous infection model was established which resembles invasive infections in humans. Applying this model, we assessed the role of type I IFN production after *S. pyogenes* infection *in vivo* using IFNAR1-deficient mice.

# 5. Results

## 5.1. Group A streptococcus activates type I interferon production and MyD88-dependent signaling without involvement of TLR2, TLR4, and TLR9

**Publication, The Journal of Biological Chemistry,2008; 283(29):19879-87.**

⌘ *Author's Choice*

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 29, pp. 19879–19887, July 18, 2008


# Group A Streptococcus Activates Type I Interferon Production and MyD88-dependent Signaling without Involvement of TLR2, TLR4, and TLR9*[S]

Received for publication, April 14, 2008 Published, JBC Papers in Press, May 14, 2008, DOI 10.1074/jbc.M802848200

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**Bacterial pathogens are recognized by the innate immune system through pattern recognition receptors, such as Toll-like receptors (TLRs). Engagement of TLRs triggers signaling cascades that launch innate immune responses. Activation of MAPKs and NF-κB, elements of the major signaling pathways induced by TLRs, depends in most cases on the adaptor molecule MyD88. In addition, Gram-negative or intracellular bacteria elicit MyD88-independent signaling that results in production of type I interferon (IFN). Here we show that in mouse macrophages, the activation of MyD88-dependent signaling by the extracellular Gram-positive human pathogen group A streptococcus (GAS; *Streptococcus pyogenes*) does not require TLR2, a receptor implicated in sensing of Gram-positive bacteria, or TLR4 and TLR9. Redundant engagement of either of these TLR molecules was excluded by using TLR2/4/9 triple-deficient macrophages. We further demonstrate that infection of macrophages by GAS causes IRF3 (interferon-regulatory factor 3)-dependent, MyD88-independent production of IFN. Surprisingly, IFN is induced also by GAS lacking *slo* and *sagA*, the genes encoding cytolysins that were shown to be required for IFN production in response to other Gram-positive bacteria. Our data indicate that (i) GAS is recognized by a MyD88-dependent receptor other than any of those typically used by bacteria, and (ii) GAS as well as GAS mutants lacking cytolysin genes induce type I IFN production by similar mechanisms as bacteria requiring cytoplasmic escape and the function of cytolysins.**

Group A streptococcus (GAS[4]; *Streptococcus pyogenes*) is an important human Gram-positive pathogen responsible for a wide spectrum of infections, ranging from mild diseases (*e.g.* tonsillitis) to serious illness (*e.g.* necrotizing fasciitis, sepsis, or severe poststreptococcal sequelae) (1). The persistence of GAS in the human population and the severity of some GAS diseases are the result of activities of a number of virulence factors that enable the pathogen to escape immune surveillance or, on contrary, induce an overreaction of the immune system (2, 3). Although GAS is generally regarded as an extracellular pathogen, recent findings suggest that GAS can survive (although not multiply) within various host cells, such as neutrophils, macrophages, epithelial cells, and fibroblasts (4–7). The surviving bacteria may serve as a reservoir for recurrent GAS diseases.

Immune responses to bacteria are initiated by recognition of bacterial components called pathogen-associated molecular patterns through host cell-encoded pattern recognition receptors (PRRs) (8, 9). Typically, pathogen-associated molecular patterns are components of the bacterial cell wall (*e.g.* lipopolysaccharide and lipoteichoic acid), but they may also be derived from the inside of bacteria (*e.g.* DNA). The primary function of PRRs is to trigger signaling cascades that activate antimicrobial defense programs. The best studied class of PRRs is the Toll-like receptor (TLR) family, which consists of 13 transmembrane glycoproteins in mammals (8). Virtually all pathogenic bacteria are recognized by one or more TLRs, with TLR2 being the receptor for lipoteichoic acid of Gram-positive bacteria, TLR4 the receptor for lipopolysaccharide of Gram-negative bacteria, and TLR9 the receptor for bacterial DNA containing unmethylated CpG sequences. Ligand binding to TLR3, TLR4, TLR7, and TLR9 but not to TLR2 launches two distinct signaling pathways that result in the production of proinflammatory cytokines (*e.g.* TNFα and IL-1) and type I IFNs, respectively. Five TIR domain-containing adaptor molecules are involved in signaling downstream of TLRs: MyD88, TIRAP (or MAL), TRIF (or TICAM1), TRAM (or TICAM2),

* This work was supported in part by Austrian Research Foundation Grants P16726-B14, I27-B03, and SFB F28 (to P. K.) and Grant P17238-B09 (to E. C.); the University Jubilee Foundation of the City of Vienna (Hochschuljubiläumsstiftung der Stadt Wien, HJST) Grant H-1020-2004 (to E. C.); and the European Science Foundation under the EUROCORES Programme EuroDYNA through Contract ERAS-CT-2003-980409 of the European Commission, DG Research, FP6 (to the laboratory of P. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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[4] The abbreviations used are: GAS, group A streptococcus; BMDM, bone marrow-derived macrophages; IFN, interferon; IRF, interferon regulatory factor; MAPK, mitogen-activated protein kinase; PRRs, pattern-recognition receptors; TLR, Toll-like receptor; TNFα, tumor necrosis factor α; IL, interleukin; MOI, multiplicity of infection; ELISA, enzyme-linked immunosorbent assay; qRT, quantitative reverse transcription.

and SARM, a negative regulator of TRIF (10). MyD88, an essential signaling component of all TLRs except TLR3, is required for activation of MAPKs and NF-κB that cause production of proinflammatory cytokines. Signaling components that are required for the production of type I IFNs are less uniform, and they are partially TLR- and cell type-specific. Recently, much attention has been paid to the elucidation of pathways leading to TLR-independent production of type I IFN in response to intracellular bacteria and/or intracellular DNA (11–14). Although the identity of the critical PRR remains unknown, it is generally believed that it is a cytoplasmic molecule that recognizes bacterial products (possibly bacterial DNA) in the cytoplasm. Signaling downstream of this unknown PRR employs the serine threonine kinase TBK1 or, less frequently, its relative IKK (15–18), which phosphorylates primarily the transcription factor IRF3, causing the activation of the IFN-β gene, the first type I IFN to be expressed (19, 20). Other cytoplasmic PRRs, such as the NOD proteins, recognize bacterial products in the cytoplasm and activate MAPKs and NF-κB but not IFN production (21).

Despite a considerable knowledge of GAS virulence factors, the PRRs that are responsible for recognition of this pathogen by the host are unknown. Remarkably little information is available also about the signaling pathways triggered by GAS in infected innate immune cells that are known to be required for defense against GAS (22). Here we show that GAS activates p38 MAPK, NF-κB, TNFα, and IL-6 production in infected bone marrow-derived mouse macrophages (BMDMs). Similar to other bacteria, these responses depend on MyD88. However, in contrast to most other pathogenic bacteria, the MyD88-mediated signaling was independent of TLR2, the receptor for Gram-positive bacteria, and the other bacterial receptors TLR4 and TLR9. We also rule out the involvement of IL-1 receptor signaling. We further demonstrate that GAS also elicits MyD88-independent signaling that results in type I IFN production. The IFN production did not require the presence of the GAS-encoded cytolysins SLO and SLS. This finding is surprising, since during infections with other Gram-positive bacteria, either the cytolysin itself or cytolysin-mediated cytoplasmic escape of bacteria from phagocytic vesicles was implicated in triggering IFN production (12, 23–25). However, the requirement for IRF3 in the IFN production suggested that other aspects of the GAS-induced IFN production resembled the well established TBK1/IRF3 signaling pathway. This is the first description of activation of MyD88-dependent and -independent pathways upon GAS infection. Our data implicate that GAS is recognized by a yet unknown receptor upstream of MyD88 and establish GAS as a Gram-positive pathogen capable of inducing type I IFN synthesis without molecules usually required for entry of bacterial products into the cytoplasm of infected cells.

## EXPERIMENTAL PROCEDURES

*Bacterial Strains—Escherichia coli* DH5-α and TOP10 were used as hosts for cloning. *S. pyogenes* serotype M1 (ATCC 700294) is a clinical strain originally isolated from an infected wound. The isogenic *sagA*- and *slo*-deficient mutants were constructed using a thermosensitive strategy described previously (26). First, using wild-type genomic DNA as template and primers containing flanking restriction sites, a 1111-bp *sagA* upstream fragment (primers OLEC120/OLEC143), a 1200-bp *sagA* downstream fragment (primers OLEC123/OLEC144), a 1159-bp *slo* upstream fragment (primers OLEC248/OLEC249), and a 1033-bp *slo* downstream fragment (primers OLEC250/OLEC251) were amplified. After digestion with the respective restriction enzymes, the upstream and downstream fragments were cloned into thermo-sensitive plasmids pRDN18 (*sagA*-deficient mutant) and pEC84 (*slo*-deficient mutant) (26). After introduction of the recombinant plasmids into *S. pyogenes* ATCC 700294, a series of temperature shifts with appropriate antibiotic selection was performed, thus leading to the final deficient mutants (strain EC548 (*sagA*-deficient mutant) and EC997 (*slo*-deficient mutant)) in which the entire coding sequence of the gene was deleted in a nonpolar fashion. To create the *sagA*/*slo*-deficient strain (EC1142), the deletion of *slo* was performed as mentioned above in the background of the *sagA*-deficient strain. The correct deletion event in the mutants was checked by PCR, Southern blot, and sequencing analysis. SLS and SLO hemolysis assays further confirmed that the *sagA*-, *slo*-, and *sagA*/*slo*-deficient mutants were defective in SLS and/or SLO activity as described (27, 28). DNA manipulations are described in the supplemental materials.

*Bacterial Culture—S. pyogenes* strains were grown at 37 °C with 5% $CO_2$ without agitation in Todd-Hewitt broth (BD Biosciences) supplemented with 0.2% yeast extract and on tryptic soy agar supplemented with 3% defibrinogated sheep blood. Transformation of *E. coli* and *S. pyogenes* was performed as previously described (29, 30). Whenever required, antibiotics were added to the medium at the following final concentrations: erythromycin, 300 μg/ml for *E. coli* and 3 μg/ml for *S. pyogenes*; spectinomycin, 100 μg/ml for both *E. coli* and *S. pyogenes*. Bacterial cell growth was turbidimetrically monitored at 620 nm with a microplate reader.

*Macrophage Cell Culture*—Primary BMDMs were obtained from the femur bone marrow of 6–10-week-old mice. Cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in the presence of L cell-derived CSF-1, as described (31). MyD88$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$, TLR9$^{-/-}$, TLR2/4/9$^{-/-}$, IFNAR1$^{-/-}$, IRF3$^{-/-}$, IL1-RI$^{-/-}$, and control WT mice, all on a C57Bl/6 background, were housed under specific pathogen-free conditions (32–36).

*GAS Infections*—For infection assays, BMDMs were seeded at $5 \times 10^6$ cells/dish in 10-cm dishes containing medium without antibiotics. The next day, *S. pyogenes* cultures grown in THY were harvested at midlogarithmic phase, washed with phosphate-buffered saline, and added to the BMDM monolayers at a multiplicity of infection (MOI) of 100. After 30 min of incubation at 37 °C, nonadherent extracellular bacteria were eliminated by removing the culture medium, and adherent extracellular bacteria were subsequently killed by incubation with fresh medium (without fetal calf serum) containing 60 μg/ml penicillin. At specific time points after infection, supernatants were collected for ELISA, and whole cell extracts were prepared for Western blot analysis. At least three mice of each genotype were used in all infection experiments.

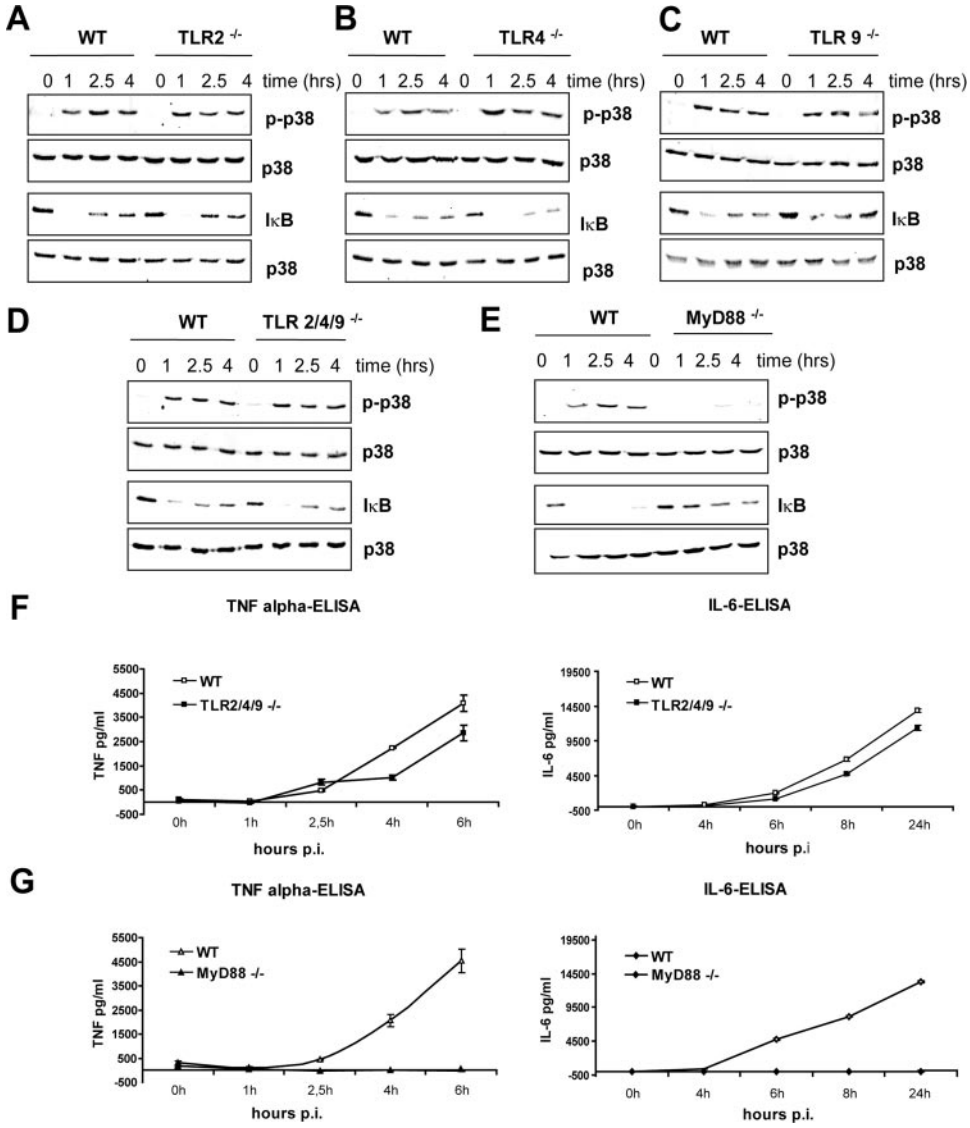


FIGURE 1. **GAS-induced inflammatory signaling and the production of TNF$\alpha$ and IL-6 depends on MyD88 but not on TLR2, TLR4, and TLR9.** *A–E*, BMDMs from TLR2$^{-/-}$ (*A*), TLR4$^{-/-}$ (*B*), TLR9$^{-/-}$ (*C*), TLR2/4/9$^{-/-}$ (*D*), MyD88$^{-/-}$ (*E*), and WT control mice were infected with GAS (MOI 100). At the indicated time points, whole cell extracts were prepared and analyzed by Western blotting using antibodies to phosphorylated p38 MAPK (*pp38*; *top*) and to IκB (*bottom*). Equal loading was controlled by reprobing the membrane with antibody to total p38 MAPK. *F* and *G*, BMDMs derived from TLR2/4/9 triple-deficient (TLR2/4/9$^{-/-}$) (*F*), MyD88$^{-/-}$ (*G*), and control mice were infected with GAS (MOI 100). At the indicated time points, supernatants were collected, and TNF$\alpha$ (*left*) and IL-6 release (*right*) was measured by ELISA. The data represent one of at least three infection experiments carried out independently with different mice of each genotype.

*Antibodies*—Antibodies to Tyr$^{701}$-phosphorylated Stat1 (pY701-S1) and phosphorylated p38 (pp38) were purchased from Cell Signaling (Frankfurt/Main, Germany). Antibodies to IκB-$\alpha$ and p38 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody to Stat1-$\alpha$ C terminus was previously described (37).

*ELISA and Western Blot Analysis*—For ELISAs, supernatants of infected macrophages were collected and diluted 1:5 in reagent diluent. TNF$\alpha$ and IL-6 were assayed using DuoSET ELISA kits (R&D Systems, Minneapolis, MN). Western blot analysis was performed using fluorophore-linked secondary antibodies (Molecular Probes-Invitrogen (Lofer, Austria) and Rockland (Gilbertsville, PA)) and an Odyssey infrared imaging system (LI-COR Bioscience, Lincoln, NE).

*qRT-PCR*—Total RNA was isolated using Trizol® LS reagent (Invitrogen). Reverse transcription of total RNA was performed using oligo(dT)$_{18}$ as primer and Moloney murine leukemia virus reverse transcriptase (Fermentas, St. Leon-Rot, Germany). The cDNA of the IFN-$\beta$ and Mx2 genes was subsequently analyzed by qRT-PCR as described in the supplemental materials.

## RESULTS

*GAS Activates Inflammatory Signaling Independently of TLR2, TLR4, and TLR9*—To investigate the role of bacteria-recognizing TLRs in responses to GAS infection, we examined the activation of the transcription factor NF-κB by assaying the degradation of its inhibitor (IκB) and the phosphorylation of p38 MAPK in infected BMDMs. Activation of both NF-κB and p38 MAPK was shown in numerous studies to be essential for production of proinflammatory cytokines (38–42). Furthermore, macrophages express all components of the TLR signaling cascade and are known to be required for defenses against GAS infection (22). We first compared the activation of p38 MAPK and NF-κB in WT and TLR2$^{-/-}$ BMDMs, since many Gram-positive pathogens are recognized by this receptor. GAS induced in both WT and TLR2$^{-/-}$ BMDMs a rapid and sustained activation of p38 MAPK (Fig. 1*A*). The degradation of IκB was maximal after 1 h of infection. IκB gradually reappeared at later time points but did not reach the original level during the time frame of observation (up to 4 h). The internalization of GAS (monitored by fluorescence microscopy) was equally efficient in cells of both genotypes (data not shown). These data demonstrate that TLR2 was not required for GAS-induced signaling. Other TLRs frequently engaged by bacteria are TLR4 and TLR9. TLR4 is the receptor for lipopolysaccharide of Gram-negative bacteria, but it has been shown to recognize also cell wall components of mycobacteria and several cytolysins of Gram-positive bacteria (25, 43, 44). TLR9, a CpG DNA receptor localized in endosomal and lysosomal membranes of macrophages and, most prominently, dendritic cells, can bind DNA from both Gram-positive and Gram-negative species (35, 45). Similar to TLR2$^{-/-}$ BMDMs, the activation of p38 MAPK and degradation of IκB in TLR4$^{-/-}$ and TLR9$^{-/-}$ BMDMs was

indistinguishable from WT BMDMs (Fig. 1, *B* and *C*). To examine whether a combination of TLR2, TLR4, and TLR9 was used for GAS recognition, we analyzed GAS-induced signaling in TLR2/4/9 triple-deficient BMDMs. As depicted in Fig. 1*D*, the signaling events in triple-deficient BMDMs were comparable with WT cells. These data exclude a redundant function of TLR2, TLR4, and TLR9 in GAS recognition and demonstrate that macrophages sense GAS using a PRR other than the most common bacterial receptors.

*GAS-induced Inflammatory Signaling Depends on MyD88*—All TLRs except for the double-stranded RNA receptor TLR3 require MyD88 for activation of MAPKs, NF-κB, and the subsequent proinflammatory cytokine production. To investigate whether MyD88 was involved in GAS-induced inflammatory signaling, we examined the activation of p38 MAPK and degradation of IκB in infected MyD88$^{-/-}$ BMDMs. The experiment revealed that GAS-induced activation of p38 MAPK was almost completely abolished, and the degradation of IκB was strongly diminished in MyD88$^{-/-}$ cells when compared with WT cells (Fig. 1*E*). A weak p38 MAPK activation and IκB degradation were observed in MyD88$^{-/-}$ cells at later time points of infection. Thus, the GAS-induced inflammatory signaling was markedly reduced and delayed in MyD88$^{-/-}$ cells. The activation of MAPKs and NF-κB plays a fundamental role in induction of proinflammatory cytokines, such as TNFα or IL-6. To estimate the effect of reduced GAS-induced inflammatory signaling in MyD88$^{-/-}$ BMDMs, we determined the amounts of secreted TNFα and IL-6 in supernatants of GAS-infected MyD88$^{-/-}$ and control WT cells. In parallel, we also measured TNFα and IL-6 production by infected TLR2/4/9 triple-deficient cells. Infection of WT BMDMs with GAS resulted in a robust cytokine production that was only slightly reduced (by less than 20%) in TLR2/4/9 triple-deficient cells (Fig. 1*F*). In marked contrast, TNFα and IL-6 production was completely abolished in GAS-infected MyD88$^{-/-}$ BMDMs (Fig. 1*E*). These data show that GAS-induced proinflammatory cytokine production is absolutely dependent on MyD88. The slight but reproducible reduction of cytokine production by TLR2/4/9 triple-deficient BMDMs indicates that one of these TLRs or a combination of them plays a minor role in GAS-induced proinflammatory cytokine production. However, the largest part of GAS-induced inflammatory cytokine production is mediated by a receptor (or receptors) different from the most common bacteria-specific TLRs. Importantly, this receptor signals via MyD88. The only other known pathways that use the adaptor MyD88 for signaling are the IL-1/IL-18 pathways (32). Although IL-18 is most relevant for the activation and IFN-γ production by Th1 cells (46), IL-1 has been reported be released by innate immune cells upon infection with various pathogens that are sensed in the cytoplasm by components of the inflammasome (47–49). To investigate whether IL-1 release by GAS-infected macrophages is responsible for the MyD88-dependent inflammatory signaling, we infected cells deficient in IL1-RI, the most important IL-1 receptor chain (50). As shown in Fig. 2, the activation of p38 MAPK, the degradation of IκB, and TNFα production were not affected by the deficiency in IL-1 signaling. These data further strengthen our notion that GAS is sensed by a yet unidentified receptor upstream of MyD88.

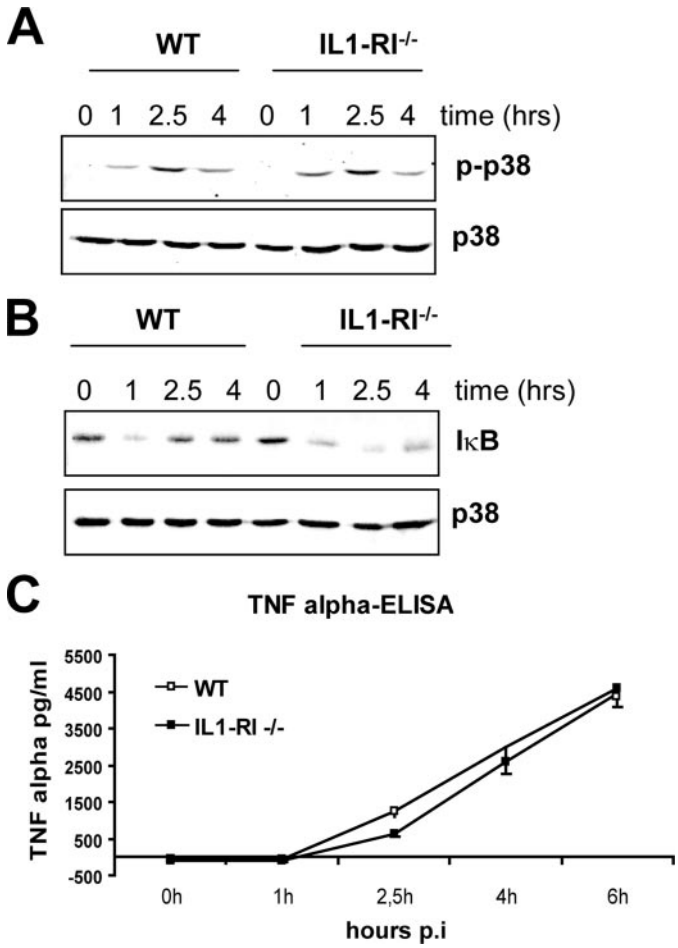


FIGURE 2. **IL-1 signaling is not required for responses of BMDM to GAS infection.** *A* and *B*, BMDMs from IL-1RI$^{-/-}$ and control (WT) mice were infected with GAS (MOI 100) for the indicated periods. p38 MAPK activation (*pp38*) (*A*) and degradation of IκB (*B*) were determined by Western blot analysis. Equal protein loading was confirmed by reprobing the membrane with antibody to total p38 MAPK. *C*, BMDMs derived from IL-1RI$^{-/-}$ and control mice were infected with GAS (MOI 100). At the indicated time points, supernatants were collected, and TNFα release was measured by ELISA. The data represent one of at least three infection experiments carried out independently with different mice of each genotype.

*GAS Induces Type I Interferon*—Gram-negative and intracellular bacteria are known to elicit MyD88-independent signaling that causes activation of the transcription factor IRF3 and subsequent transcription of the type I IFN-β. The mechanisms of IFN production induced by these two types of bacteria differ upstream of IRF3. Whereas Gram-negative bacteria use TLR4 to stimulate IFN production, intracellular bacteria are recognized by a still unknown cytoplasmic receptor. Engagement of TLR9 can also trigger IRF3/IRF7-mediated type I IFN expression. GAS has been previously reported to induce IFN production in primary human macrophages (51). We asked whether GAS can exert similar effects in mouse macrophages and what the role of MyD88 or the bacteria-specific TLRs therein is. First, tyrosine phosphorylation of the crucial IFN-activated transcription factor Stat1 was analyzed. Tyrosine phosphorylation of Stat1 is a good indicator for autocrine IFN-β production, since this cytokine is the first IFN to be synthetized upon challenge of macrophages with bacteria or their products (52–54). Infection of BMDMs with GAS revealed induction of Stat1

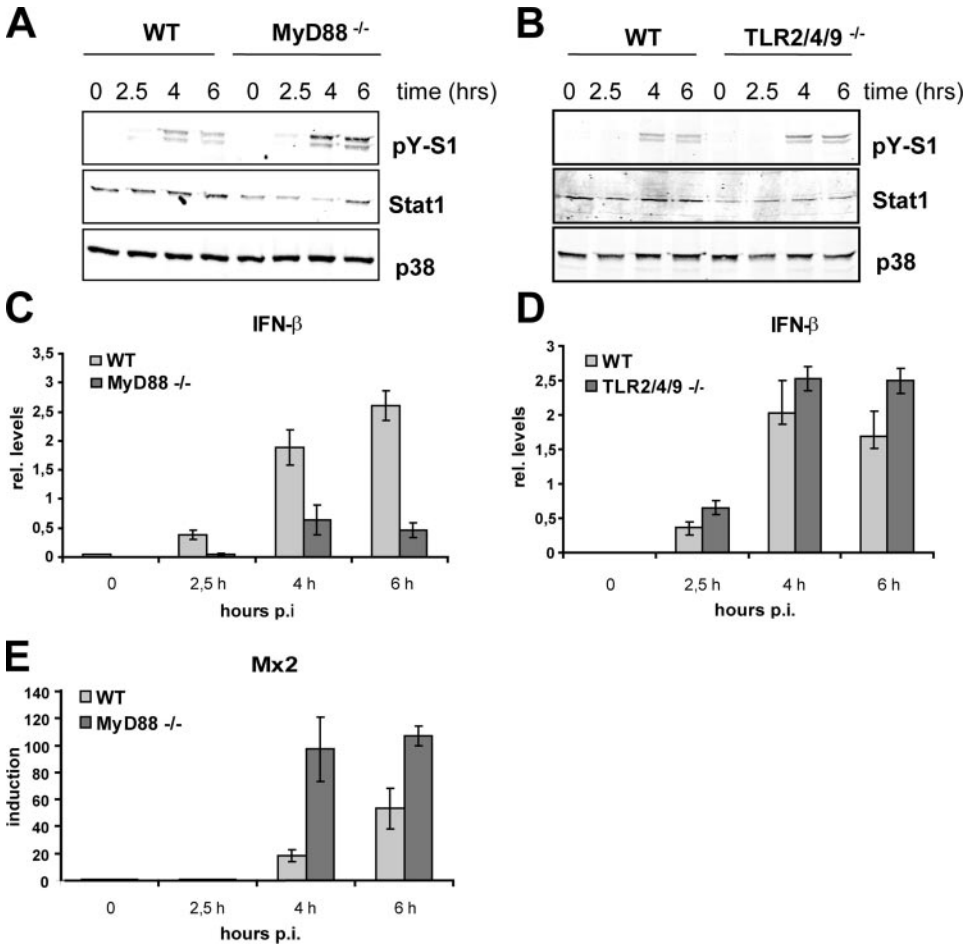


FIGURE 3. **GAS induces IFN-$\beta$ production and Stat1 activation independently of MyD88, TLR2, TLR4, and TLR9.** *A* and *B*, BMDMs from control (WT), MyD88$^{-/-}$ (*A*), and TLR2/4/9 triple-deficient (TLR2/4/9$^{-/-}$) (*B*) mice were infected with GAS (MOI 100), and whole cell extracts were prepared after the indicated time periods. Stat1 Tyr$^{701}$ phosphorylation and expression were determined by Western blotting using antibody to phosphorylated Stat1 (pY-S1) and total Stat1. Differences in Stat1 expression levels were revealed by reprobing the membrane with antibody to total p38 MAPK (p38). Note the double band on the pY-S1 blot represents the phosphorylated forms of both Stat1 splicing isoforms Stat1-$\alpha$ and Stat1-$\beta$. Loading control (S1) was performed with antibody directed to the C terminus of Stat1, which is absent in the Stat1-$\beta$ isoform. *C* and *D*, BMDMs from wild type, MyD88$^{-/-}$ (*C*), and TLR2/4/9 triple-deficient (TLR2/4/9$^{-/-}$) mice (*D*) were exposed to GAS (MOI 100). After the indicated time points, total mRNA was extracted, reverse-transcribed, and analyzed by qRT-PCR for IFN-$\beta$ and GAPDH (for normalization) expression. Note that the data show relative IFN-$\beta$ mRNA levels normalized to GAPDH but not to uninfected samples. *E*, GAS-induced IFN-$\beta$ stimulates transcription of type I IFN target gene *Mx2*. BMDMs from MyD88$^{-/-}$ and control (wild type) mice were exposed to GAS, and 2.5, 4, and 6 h postinfection (*hours p.i.*), total mRNA was extracted, reverse-transcribed, and analyzed by qRT-PCR for *Mx2* after normalizing to GAPDH and uninfected samples. *Bars*, S.D. of three experiments.

tyrosine phosphorylation after 4 h of infection of WT BMDMs (Fig. 3, *A* and *B*). This late tyrosine phosphorylation is similar to challenges with other bacteria (*e.g. Listeria monocytogenes*) that do not induce IFN synthesis through direct engagement of an IFN-inducing TLR (23). Consistently, Stat1 activation proceeded independently of MyD88 and TLR2, TLR4, and TLR9 signaling, as shown by GAS infection of BMDMs from MyD88$^{-/-}$ (Fig. 3*A*), TLR2$^{-/-}$, TLR4$^{-/-}$, TLR9$^{-/-}$ (all in Fig. S1), or TLR2/4/9 triple-deficient (Fig. 3*B*) mice. In fact, Stat1 tyrosine phosphorylation was slightly but reproducibly increased in MyD88$^{-/-}$ and TLR2/4/9 triple-deficient BMDMs (Fig. 3, *A* and *B*) when normalized to total Stat1 levels. Since the Stat1 levels were generally slightly lower in BMDMs derived from the gene-targeted mice compared with WT controls, the Western blots were reprobed with p38 antibody to allow a more accurate normalization. Reduced Stat1 levels have been already previously observed in cells that are deficient in IFN or TLR/MyD88 signaling (11, 55, 56). To directly assess IFN-$\beta$ expression, we performed qRT-PCR to determine IFN-$\beta$ mRNA levels in cells infected with GAS. The data shown represent relative IFN-$\beta$ mRNA levels normalized to GAPDH mRNA. We did normalize to uninfected cells, since the IFN-$\beta$ mRNA was very often below the levels that could be reliably detected by qRT-PCR. The experiment confirmed that the IFN-$\beta$ expression was independent of TLR2, TLR4, and TLR9 (Fig. 3*D*). The IFN-$\beta$ mRNA was also induced upon infection of MyD88$^{-/-}$ cells with GAS, albeit the amount was reduced if compared with WT cells (Fig. 3*C*). The reduced IFN-$\beta$ mRNA levels in MyD88$^{-/-}$ did not result in diminished IFN signaling, since Stat1 activation was increased in these cells if compared with the controlled BMDMs (Fig. 3*A*). The functional activation of IFN/Jak/Stat signaling in MyD88$^{-/-}$ and WT cells was further confirmed by the induction of the type I IFN target gene *Mx2* (57). The transcription of *Mx2* was induced in both the control and MyD88$^{-/-}$ BMDMs with a kinetics that correlated well with the activation profile of Stat1 (Fig. 3*E*). Moreover, *Mx2* transcription was more strongly induced in MyD88$^{-/-}$ cells that also display increased Stat1 activation. Thus, despite the decreased IFN-$\beta$ mRNA, the IFN/Jak/Stat1 signaling was more efficiently turned on in MyD88$^{-/-}$ cells, suggesting that the increased Stat1 phosphorylation was caused by a more efficient phosphorylation or a lower dephosphorylation rate. To prove the autocrine/paracrine role of the type I IFN in Stat1 activation, we examined Stat1 activation and IFN responses using BMDMs lacking the type I IFN receptor chain 1 (IFNAR1$^{-/-}$). As shown in Fig. 4*A*, Stat1 tyrosine phosphorylation was completely abolished in GAS-infected IFNAR1$^{-/-}$ cells despite robust induction of IFN-$\beta$ gene transcription in these cells (Fig. 4*A*). Consistently, the induction of the type I IFN-responsive gene *Mx2* was entirely dependent on signaling by the IFNAR1 receptor (Fig. 4*C*). In most cell types, activation of the transcription factor IRF3 launches a feed forward type I IFN amplification loop through induction of IFN-$\beta$ that drives the expression of

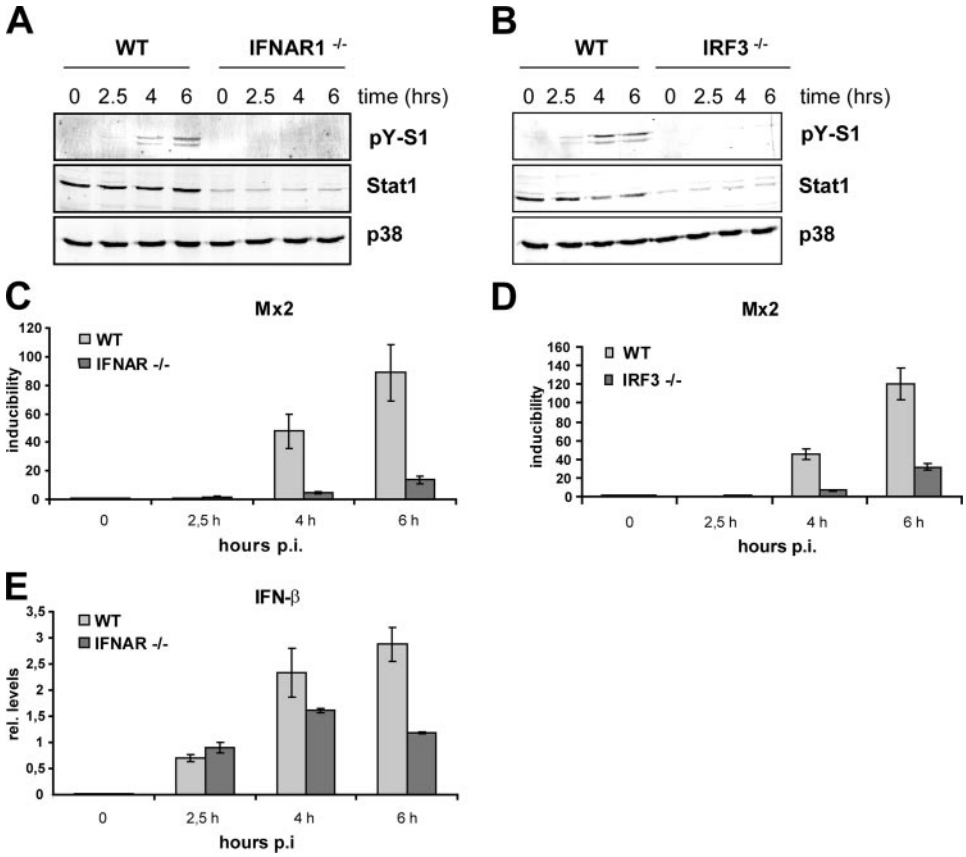


FIGURE 4. **GAS-induced IFN signaling depends on IRF3 and IFNAR1.** *A* and *B*, BMDMs from control (WT), IFNAR1$^{-/-}$ (*A*), and IRF3-deficient (*IRF3*$^{-/-}$) (*B*) mice were infected with GAS (MOI 100), and whole cell extracts were prepared 2.5, 4, and 6 h postinfection. Stat1 Tyr$^{701}$ phosphorylation and expression were determined by Western blotting using antibody to phosphorylated Stat1 (pY-S1) and total Stat1. Differences in Stat1 expression levels were revealed by reprobing the membrane with antibody to total p38 MAPK (p38). *C* and *D*, BMDMs from IFNAR1$^{-/-}$ (*C*), IRF3$^{-/-}$ (*D*), and control (WT) mice were exposed to GAS for the indicated times, and total mRNA was extracted, reverse-transcribed, and analyzed by qRT-PCR for *Mx2* after normalization to GAPDH. *E*, BMDMs from IFNAR1$^{-/-}$ and control (WT) mice were infected with GAS (MOI 100). IFN-$\beta$ expression was analyzed by qRT-PCR after normalization to GAPDH. *Bars*, S.D. of three experiments.

IFN-$\alpha$s (19, 20, 58). Thus, a failure in activation of IRF3 results in a deficient production of both the immediate IFN-$\beta$ and the late phase IFN-$\alpha$s. To further elucidate the mechanism of GAS-induced IFN production, we infected IRF3$^{-/-}$ BMDMs with GAS and examined the activation of the transcription factor Stat1 and induction of the Stat1 target gene *Mx2*. Both the tyrosine phosphorylation of Stat1 and the transcription of the type I IFN-inducible gene *Mx2* were diminished in IRF3$^{-/-}$ (Fig. 4, *B* and *D*), whereas the MyD88 pathway was not affected by the IRF3 deletion, as revealed by p38 MAPK activation. These data demonstrate that GAS has the ability to induce IFN-$\beta$ synthesis and IFN signaling in a way that mechanistically resembles the IFN-$\beta$ activation by intracellular Gram-positive pathogens.

*GAS Stimulates IFN Signaling Independently of Cytolysins*—The TLR-independent IFN-$\beta$ induction by GAS was surprising, since GAS is generally regarded as an extracellular pathogen that has only a limited capacity to survive in host cells (4–7). For the so far characterized induction of IFN-$\beta$ production by Gram-positive bacteria, the expression of cytolysins was required (12, 23, 24). Cytolysins are thought to enable cytoplasmic escape of phagocytosed bacteria. In addition, cytolysins were also shown to directly stimulate IFN production (25). The GAS genome contains genes for two cytolysins. The *slo* gene encodes SLO (streptolysin O), which resembles other known cytolysins (*e.g.* listeriolysin, pneumolysin, and anthrolysin) in terms of sequence, sensitivity to oxygen, and cholesterol binding (59, 60). The other cytolysin, SLS (streptolysin S), is encoded by the *sagA* gene, which is unrelated to the cholesterol-binding cytolysins (60). To examine whether the GAS-derived cytolysins play a role in GAS-induced IFN signaling, *sagA* (SLS)-deficient, *slo* (SLO)-deficient, and double *sagA*/*slo*-deficient mutants were generated and used in infection assays. Analysis of Stat1 activation revealed that neither the deletion of the single cytolysin genes (data not shown) nor the double deletion (Fig. 5*A*) caused a reduction of GAS-induced IFN signaling. The inflammatory signaling was also not dependent on the expression of the GAS cytolysins, as shown by p38 MAPK activation (Fig. 5*B*). These findings suggest that the ability of GAS to induce IFN-$\beta$ synthesis may not require a contact of GAS components with the still unknown cytoplasmic receptor that is used by other Gram-positive bacteria for IFN-$\beta$ induction.

## DISCUSSION

GAS is the etiological agent of a variety of human diseases. The heterogeneity of GAS diseases arises in part from the high diversity of GAS-mediated host-pathogen interactions in which virulence factors, GAS genome composition, prophage DNA, and plasticity of the GAS transcriptome play key roles (2, 61–64). A major factor influencing the severity of GAS infections is also the genetic inventory of the host. In humans, the differences in susceptibility to severe GAS diseases were mapped to the major histocompatibility complex locus (65). More severe infections and a generalized toxic shock syndrome appear to be associated with higher expression of inflammatory cytokines in both humans and mice (66). Cytokine production in GAS responses is regulated by T cells through GAS superantigens and the as yet poorly understood activation of innate immune cells. Our findings demonstrate that GAS is able to induce cytokines in macrophages through MyD88-dependent and -independent pathways. The identity of the GAS-recognizing receptor upstream of MyD88 poses an intriguing question,

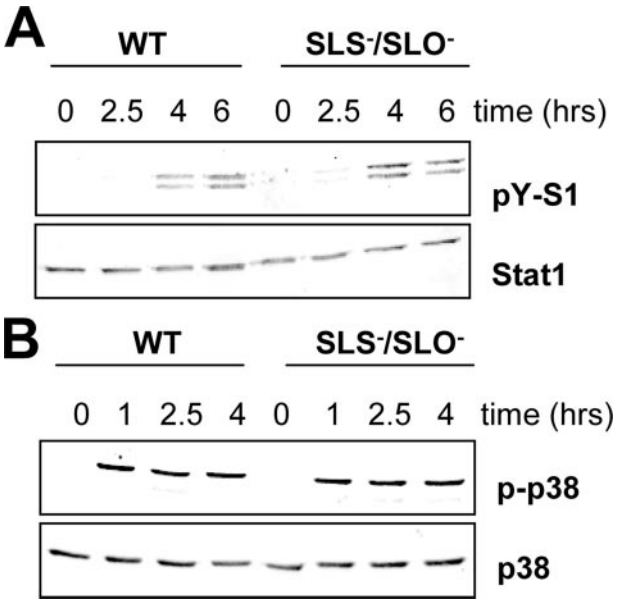


FIGURE 5. **GAS-derived cytolysins SLO and SLS are not required for induction of IFN and stress signaling.** *A* and *B*, BMDMs from wild-type mice were infected with either *S. pyogenes* WT strain or the isogenic mutant strain *sls/sagA* lacking both *sagA* (SLS) and *slo* (SLO) genes. At the indicated time points, activation of Stat1 (*A*) and p38 MAPK (*B*) was detected by Western blot analysis using antibodies to $Tyr^{701}$-phosphorylated Stat1 (pY-S1) and phosphorylated p38 MAPK (pp38). Equal protein loading was confirmed by reprobing the membranes with antibody to total Stat1 and p38 MAPK (p38), respectively. The data shown represent one of at least three independent infection experiments.

since our study rules out the exclusive involvement of the most prominent bacteria-recognizing receptors, TLR2, TLR4, and TLR9, or their combination. Other MyD88-dependent TLRs are the flagellin receptor TLR5 and the single-stranded RNA receptors TLR7 and TLR8 (8). Involvement of these receptors in GAS-induced MyD88-dependent signaling is not likely, since flagellin or a similar protein has not been found in GAS, and single-stranded RNA is associated with viral recognition. However, the role of these receptors cannot be entirely excluded, because PRRs are, in general, not specific for a single molecule. For example, TLR2 was reported to cooperate with dectin-1 in recognition of fungi (67). We ruled out the role of signaling through the IL-1R, which is known to be MyD88-dependent. IL-1 can be released by infected cells as a result of inflammasome-mediated caspase 1 activation, which is required for IL-1$\beta$ processing (68). Although we did not address the GAS-mediated IL-1 production, a role for IL-1 as a possible mediator of the MyD88-dependent responses can be excluded. A function of IL-18 as another molecule requiring MyD88 for signaling is rather unlikely, since the IL-18 receptor is predominantly expressed on Th1 cells, whereas a prolonged treatment with *e.g.* IL-18 plus IL-12 is required for stronger expression in monocytic cells (69, 70). The ability of GAS to induce MyD88-dependent signaling independently of TLR2/4/9 may not be unique, since other Gram-positive pathogens have been reported to initiate inflammatory responses in the absence of multiple TLRs. For example, heat-inactivated group B streptococci and *Streptococcus pneumoniae* as well as spores of *Bacillus anthracis* induce MyD88-dependent responses in TLR2-, TLR4-, and TLR9-deficient macrophages or splenocytes (71–73). Despite the fact that TLR2/4/9 triple-deficient cells were not used in these studies, they support our hypothesis that some Gram-positive pathogenic bacteria are recognized by an as yet unidentified receptor upstream of MyD88.

The surprising finding that GAS is able to induce IFN-$\beta$ independently of cytolysins represents another so far unique feature of GAS-induced responses in innate immune cells. In this regard, GAS-derived streptolysins are different from several other cytolysins that have been reported to induce IFN-$\beta$ synthesis through activation of TLR4 (25). Another proposed mechanism of IFN-$\beta$ induction by Gram-positive bacteria (*e.g. L. monocytogenes*) involves listeriolysin O-dependent liberation of bacteria and/or bacterial components from phagosomes (12, 23, 24). Although GAS is a prototype extracellular pathogen, it can be efficiently internalized by many phagocytic and nonphagocytic cells. However, GAS survives only for a short period of time in host cells due to degradation in lysosomal and autophagosomal compartments (5, 6). Interestingly, *Bacillus subtilis* engineered to express streptococcal SLO was not able to survive or multiply in infected cells, as opposed to listeriolysin O-expressing *B. subtilis* (74). This observation suggests that SLO, which displays a high degree of similarity with listeriolysin O, cannot mediate cytoplasmic escape. Thus, the reported data together with our findings support the hypothesis that GAS stimulates IFN-$\beta$ synthesis by a novel mechanism that does not require cytoplasmic escape. Interestingly, the signaling events that culminate in Stat1 activation and transcription of IFN-responsive genes follow the well established pathway involving the transcription factor IRF3 and the type I IFN receptor but independent of MyD88. The increased IFN signaling in $MyD88^{-/-}$ cells may be explained by reduced expression of the inhibitor of IFN signaling SOCS1 (75). SOCS1 expression is known to be dependent on p38 MAPK (76), which we show in our study to be strongly reduced in GAS-infected $MyD88^{-/-}$ cells.

Our work demonstrates the ability of GAS to induce multiple inflammatory responses via in part novel recognition mechanisms. MyD88-dependent TLR2/TLR4/TLR9-independent signaling and cytolysin-independent IFN-$\beta$ induction are so far exceptional characteristics of host cell responses to infection. Since GAS induces proinflammatory cytokine and IFN production in human macrophages (51), we assume that our findings are not restricted to the murine system, which, in the context of the whole organism, is considerably more resistant to GAS infection (66). The efficient stimulation of multiple signaling pathways may contribute to the known high capability of GAS to cause severe inflammatory diseases.

*Acknowledgments—We thank Silvia Stockinger for critically reading the manuscript and Birgit Rapp for help with bacterial cultures.*

## REFERENCES

1. Carapetis, J. R., Steer, A. C., Mulholland, E. K., and Weber, M. (2005) *Lancet* **5,** 685–694
2. Bisno, A. L., Brito, M. O., and Collins, C. M. (2003) *Lancet* **3,** 191–200
3. Chhatwal, G. S., and McMillan, D. J. (2005) *Trends Mol. Med.* **11,** 152–155
4. Staali, L., Morgelin, M., Bjorck, L., and Tapper, H. (2003) *Cell. Microbiol.* **5,** 253–265

5. Nakagawa, I., Amano, A., Mizushima, N., Yamamoto, A., Yamaguchi, H., Kamimoto, T., Nara, A., Funao, J., Nakata, M., Tsuda, K., Hamada, S., and Yoshimori, T. (2004) *Science* **306,** 1037–1040
6. Hakansson, A., Bentley, C. C., Shakhnovic, E. A., and Wessels, M. R. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 5192–5197
7. Thulin, P., Johansson, L., Low, D. E., Gan, B. S., Kotb, M., McGeer, A., and Norrby-Teglund, A. (2006) *PLoS Med.* **3,** e53
8. Akira, S., Uematsu, S., and Takeuchi, O. (2006) *Cell* **124,** 783–801
9. Lee, M. S., and Kim, Y. J. (2007) *Annu. Rev. Biochem.* **76,** 447–480
10. O'Neill, L. A., and Bowie, A. G. (2007) *Nat. Rev.* **7,** 353–364
11. Stockinger, S., Reutterer, B., Schaljo, B., Schellack, C., Brunner, S., Materna, T., Yamamoto, M., Akira, S., Taniguchi, T., Murray, P. J., Muller, M., and Decker, T. (2004) *J. Immunol.* **173,** 7416–7425
12. Stetson, D. B., and Medzhitov, R. (2006) *Immunity* **24,** 93–103
13. Takaoka, A., Wang, Z., Choi, M. K., Yanai, H., Negishi, H., Ban, T., Lu, Y., Miyagishi, M., Kodama, T., Honda, K., Ohba, Y., and Taniguchi, T. (2007) *Nature* **448,** 501–505
14. Ishii, K. J., Kawagoe, T., Koyama, S., Matsui, K., Kumar, H., Kawai, T., Uematsu, S., Takeuchi, O., Takeshita, F., Coban, C., and Akira, S. (2008) *Nature* **451,** 725–729
15. Fitzgerald, K. A., McWhirter, S. M., Faia, K. L., Rowe, D. C., Latz, E., Golenbock, D. T., Coyle, A. J., Liao, S. M., and Maniatis, T. (2003) *Nat. Immunol.* **4,** 491–496
16. Sharma, S., tenOever, B. R., Grandvaux, N., Zhou, G. P., Lin, R., and Hiscott, J. (2003) *Science* **300,** 1148–1151
17. Hemmi, H., Takeuchi, O., Sato, S., Yamamoto, M., Kaisho, T., Sanjo, H., Kawai, T., Hoshino, K., Takeda, K., and Akira, S. (2004) *J. Exp. Med.* **199,** 1641–1650
18. Perry, A. K., Chow, E. K., Goodnough, J. B., Yeh, W. C., and Cheng, G. (2004) *J. Exp. Med.* **199,** 1651–1658
19. Levy, D. E. (2002) *J. Exp. Med.* **195,** F15–18
20. Taniguchi, T., and Takaoka, A. (2002) *Curr. Opin. Immunol.* **14,** 111–116
21. Strober, W., Murray, P. J., Kitani, A., and Watanabe, T. (2006) *Nat. Rev.* **6,** 9–20
22. Goldmann, O., Rohde, M., Chhatwal, G. S., and Medina, E. (2004) *Infect Immun.* **72,** 2956–2963
23. Stockinger, S., Materna, T., Stoiber, D., Bayr, L., Steinborn, R., Kolbe, T., Unger, H., Chakraborty, T., Levy, D. E., Muller, M., and Decker, T. (2002) *J. Immunol.* **169,** 6522–6529
24. O'Riordan, M., Yi, C. H., Gonzales, R., Lee, K. D., and Portnoy, D. A. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 13861–13866
25. Park, J. M., Ng, V. H., Maeda, S., Rest, R. F., and Karin, M. (2004) *J. Exp. Med.* **200,** 1647–1655
26. Mangold, M., Siller, M., Roppenser, B., Vlaminckx, B. J., Penfound, T. A., Klein, R., Novak, R., Novick, R. P., and Charpentier, E. (2004) *Mol. Microbiol.* **53,** 1515–1527
27. Ruiz, N., Wang, B., Pentland, A., and Caparon, M. (1998) *Mol. Microbiol.* **27,** 337–346
28. Sierig, G., Cywes, C., Wessels, M. R., and Ashbaugh, C. D. (2003) *Infect. Immun.* **71,** 446–455
29. Caparon, M. G., Stephens, D. S., Olsen, A., and Scott, J. R. (1991) *Infect. Immun.* **59,** 1811–1817
30. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, pp. 1.74–1.84, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
31. Baccarini, M., Bistoni, F., and Lohmann-Matthes, M. L. (1985) *J. Immunol.* **134,** 2658–2665
32. Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K., and Akira, S. (1998) *Immunity* **9,** 143–150
33. Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999) *J. Immunol.* **162,** 3749–3752
34. Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., and Akira, S. (1999) *Immunity* **11,** 443–451
35. Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. (2000) *Nature* **408,** 740–745
36. Yasuda, K., Yu, P., Kirschning, C. J., Schlatter, B., Schmitz, F., Heit, A., Bauer, S., Hochrein, H., and Wagner, H. (2005) *J. Immunol.* **174,** 6129–6136
37. Kovarik, P., Stoiber, D., Novy, M., and Decker, T. (1998) *EMBO J.* **17,** 3660–3668
38. Alcamo, E., Mizgerd, J. P., Horwitz, B. H., Bronson, R., Beg, A. A., Scott, M., Doerschuk, C. M., Hynes, R. O., and Baltimore, D. (2001) *J. Immunol.* **167,** 1592–1600
39. Senftleben, U., Li, Z. W., Baud, V., and Karin, M. (2001) *Immunity* **14,** 217–230
40. Kim, D. H., Feinbaum, R., Alloing, G., Emerson, F. E., Garsin, D. A., Inoue, H., Tanaka-Hino, M., Hisamoto, N., Matsumoto, K., Tan, M. W., and Ausubel, F. M. (2002) *Science* **297,** 623–626
41. Suzuki, N., Suzuki, S., Duncan, G. S., Millar, D. G., Wada, T., Mirtsos, C., Takada, H., Wakeham, A., Itie, A., Li, S., Penninger, J. M., Wesche, H., Ohashi, P. S., Mak, T. W., and Yeh, W. C. (2002) *Nature* **416,** 750–756
42. Matsuzawa, A., Saegusa, K., Noguchi, T., Sadamitsu, C., Nishitoh, H., Nagai, S., Koyasu, S., Matsumoto, K., Takeda, K., and Ichijo, H. (2005) *Nat. Immunol.* **6,** 587–592
43. Abel, B., Thieblemont, N., Quesniaux, V. J., Brown, N., Mpagi, J., Miyake, K., Bihl, F., and Ryffel, B. (2002) *J. Immunol.* **169,** 3155–3162
44. Ferwerda, G., Girardin, S. E., Kullberg, B. J., Le Bourhis, L., de Jong, D. J., Langenberg, D. M., van Crevel, R., Adema, G. J., Ottenhoff, T. H., Van der Meer, J. W., and Netea, M. G. (2005) *PLoS Pathog.* **1,** 279–285
45. Latz, E., Schoenemeyer, A., Visintin, A., Fitzgerald, K. A., Monks, B. G., Knetter, C. F., Lien, E., Nilsen, N. J., Espevik, T., and Golenbock, D. T. (2004) *Nat. Immunol.* **5,** 190–198
46. Wei, X. Q., Leung, B. P., Niedbala, W., Piedrafita, D., Feng, G. J., Sweet, M., Dobbie, L., Smith, A. J., and Liew, F. Y. (1999) *J. Immunol.* **163,** 2821–2828
47. Gavrilin, M. A., Bouakl, I. J., Knatz, N. L., Duncan, M. D., Hall, M. W., Gunn, J. S., and Wewers, M. D. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 141–146
48. Mariathasan, S., Weiss, D. S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., Lee, W. P., Weinrauch, Y., Monack, D. M., and Dixit, V. M. (2006) *Nature* **440,** 228–232
49. Ozoren, N., Masumoto, J., Franchi, L., Kanneganti, T. D., Body-Malapel, M., Erturk, I., Jagirdar, R., Zhu, L., Inohara, N., Bertin, J., Coyle, A., Grant, E. P., and Nunez, G. (2006) *J. Immunol.* **176,** 4337–4342
50. Glaccum, M. B., Stocking, K. L., Charrier, K., Smith, J. L., Willis, C. R., Maliszewski, C., Livingston, D. J., Peschon, J. J., and Morrissey, P. J. (1997) *J. Immunol.* **159,** 3364–3371
51. Miettinen, M., Lehtonen, A., Julkunen, I., and Matikainen, S. (2000) *J. Immunol.* **164,** 3733–3740
52. Gao, J. J., Filla, M. B., Fultz, M. J., Vogel, S. N., Russell, S. W., and Murphy, W. J. (1998) *J. Immunol.* **161,** 4803–4810
53. Toshchakov, V., Jones, B. W., Perera, P. Y., Thomas, K., Cody, M. J., Zhang, S., Williams, B. R., Major, J., Hamilton, T. A., Fenton, M. J., and Vogel, S. N. (2002) *Nat. Immunol.* **3,** 392–398
54. Decker, T., Muller, M., and Stockinger, S. (2005) *Nat. Rev.* **5,** 675–687
55. Karaghiosoff, M., Neubauer, H., Lassnig, C., Kovarik, P., Schindler, H., Pircher, H., McCoy, B., Bogdan, C., Decker, T., Brem, G., Pfeffer, K., and Muller, M. (2000) *Immunity* **13,** 549–560
56. Park, C., Li, S., Cha, E., and Schindler, C. (2000) *Immunity* **13,** 795–804
57. Hug, H., Costas, M., Staeheli, P., Aebi, M., and Weissmann, C. (1988) *Mol. Cell. Biol.* **8,** 3065–3079
58. Panne, D., Maniatis, T., and Harrison, S. C. (2007) *Cell* **129,** 1111–1123
59. Alouf, J. E. (1980) *Pharmacol. Ther.* **11,** 661–717
60. Nizet, V. (2002) *Trends Microbiol.* **10,** 575–580
61. Beres, S. B., Sylva, G. L., Sturdevant, D. E., Granville, C. N., Liu, M., Ricklefs, S. M., Whitney, A. R., Parkins, L. D., Hoe, N. P., Adams, G. J., Low, D. E., DeLeo, F. R., McGeer, A., and Musser, J. M. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 11833–11838
62. Beres, S. B., Richter, E. W., Nagiec, M. J., Sumby, P., Porcella, S. F., Deleo, F. R., and Musser, J. M. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 7059–7064
63. Cho, K. H., and Caparon, M. G. (2005) *Mol. Microbiol.* **57,** 1545–1556
64. Virtaneva, K., Porcella, S. F., Graham, M. R., Ireland, R. M., Johnson, C. A., Ricklefs, S. M., Babar, I., Parkins, L. D., Romero, R. A., Corn, G. J., Gardner, D. J., Bailey, J. R., Parnell, M. J., and Musser, J. M. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 9014–9019

65. Kotb, M., Norrby-Teglund, A., McGeer, A., El-Sherbini, H., Dorak, M. T., Khurshid, A., Green, K., Peeples, J., Wade, J., Thomson, G., Schwartz, B., and Low, D. E. (2002) *Nat. Med.* **8,** 1398–1404
66. Medina, E., and Lengeling, A. (2005) *Brief. Funct. Genomics Proteomics* **4,** 248–257
67. Rogers, N. C., Slack, E. C., Edwards, A. D., Nolte, M. A., Schulz, O., Schweighoffer, E., Williams, D. L., Gordon, S., Tybulewicz, V. L., Brown, G. D., and Reis e Sousa, C. (2005) *Immunity* **22,** 507–517
68. Delbridge, L. M., and O'Riordan, M. X. (2007) *Curr. Opin. Immunol.* **19,** 10–16
69. Xu, D., Chan, W. L., Leung, B. P., Hunter, D., Schulz, K., Carter, R. W., McInnes, I. B., Robinson, J. H., and Liew, F. Y. (1998) *J. Exp. Med.* **188,** 1485–1492
70. Gerdes, N., Sukhova, G. K., Libby, P., Reynolds, R. S., Young, J. L., and Schonbeck, U. (2002) *J. Exp. Med.* **195,** 245–257
71. Henneke, P., Takeuchi, O., Malley, R., Lien, E., Ingalls, R. R., Freeman, M. W., Mayadas, T., Nizet, V., Akira, S., Kasper, D. L., and Golenbock, D. T. (2002) *J. Immunol.* **169,** 3970–3977
72. Lee, K. S., Scanga, C. A., Bachelder, E. M., Chen, Q., and Snapper, C. M. (2007) *Cell. Immunol.* **245,** 103–110
73. Glomski, I. J., Fritz, J. H., Keppler, S. J., Balloy, V., Chignard, M., Mock, M., and Goossens, P. L. (2007) *Cell. Microbiol.* **9,** 502–513
74. Portnoy, D. A., Tweten, R. K., Kehoe, M., and Bielecki, J. (1992) *Infect. Immun.* **60,** 2710–2717
75. Alexander, W. S., Starr, R., Fenner, J. E., Scott, C. L., Handman, E., Sprigg, N. S., Corbin, J. E., Cornish, A. L., Darwiche, R., Owczarek, C. M., Kay, T. W., Nicola, N. A., Hertzog, P. J., Metcalf, D., and Hilton, D. J. (1999) *Cell* **98,** 597–608
76. Vazquez, N., Greenwell-Wild, T., Rekka, S., Orenstein, J. M., and Wahl, S. M. (2006) *J. Leukocyte Biol.* **80,** 1136–1144

## 5.2. Type I Interferon production induced by *S. pyogenes*-derived nucleic acids is required for host protection

**Manuscript**

**Type I interferon production induced by *Streptococcus pyogenes*-derived nucleic acids is required for host protection.**

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Financial Disclosure:
This study was supported by Austrian Science Fund (FWF) through Grants SFB F28 and EraNet Pathogenomics I 287-B09 to P.K. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Abstract**

*Streptococcus pyogenes* is a Gram-positive human pathogen that is recognized by so far unknown pattern recognition receptors (PRRs). Engagement of these receptor molecules during *S. pyogenes* infection causes innate immune cells to produce inflammatory mediators such as Tnf, but also type I interferon (IFN) which was unexpected for a Gram-positive bacterium with an extracellular life cycle. Here we show that signaling elicited by type I IFN is required for successful defense of mice against lethal subcutaneous cellulitis caused by *S. pyogenes*. Mechanistic analysis revealed that macrophages and conventional dendritic cells (cDCs) employ different signaling pathways leading to IFN-beta production. Macrophages required IRF3, TBK1 and only partially MyD88 whereas in cDCs the IFN-beta production was dependent on IRF1 and fully required MyD88. Furthermore, in macrophages the IFN-beta production was dependent on the endosomal delivery of streptococcal DNA while in cDCs streptococcal RNA was identified as the IFN-beta inducer. Despite a role of MyD88 in both cell types no known TLR was required for generation of IFN-beta response. These results demonstrate that the innate immune system employs several strategies to efficiently recognize *S. pyogenes*, a pathogenic bacterium that succeeded in avoiding recognition by the standard arsenal of TLRs.

**Introduction**

*Streptococcus pyogenes*, also known as group A streptococcus (GAS), is a Gram-positive human pathogen causing an exceptionally broad range of infectious diseases [1]. The bacterium colonizes the throat and the skin where it can evoke usually mild illness such as pharyngitis (strep throat) or scarlet fever (rash). Systemic infections with *S. pyogenes* are less frequent but they can develop into life-threatening diseases such as necrotizing fasciitis and streptococcal toxic shock syndrome that occur in ~10000 cases in the US annually resulting in ~1500 deaths [2]. The wide spectrum of *S. pyogenes*-related diseases arises from diversity in the genetic inventory of both the host and the pathogen [3]. For example, in a recent Europe-wide epidemiology survey of the streptococcal M-protein, a key virulence factor occurring in over 100 variants, the M-protein types 1 and 3 (M1 and M3 serotypes) were found to be associated with particularly high mortality rates in invasive streptococcal diseases [4]. On the host side, certain variants of the HLA class II and the TNF microsatellite haplotypes are implicated in increased susceptibility to severe invasive infections [5,6]. Interestingly, a genetic linkage analysis in mouse strains displaying different susceptibilities to *S. pyogenes* infections demonstrated a

dominant contribution of the genetics of the innate immune system [7]. Further animal studies confirmed an essential role of macrophages and dendritic cells (DCs) in protection against *S. pyogenes* infections [8,9]. Cells of the innate immune system can combat the pathogen directly by phagocytosis, and indirectly, by launching the primary immune response characterized by the production of inflammatory mediators that results from the recognition of the pathogen by the pattern recognition receptors (PRRs) [10]. PRRs recognize various pathogen-derived products commonly referred to as pathogen-associated molecular patterns PAMPs. Binding of PAMPs to their cognate PRRs triggers signaling cascades that culminate in the production of cytokines, chemokines and antimicrobial compounds. The recognition of bacteria is achieved by membrane-bound PRRs of the TLR family and by several classes of cytoplasmic PRRs. In mouse, 12 TLRs (TLR1 - TLR9, TLR11 - TLR13) are known [10,11]. They all signal using the adaptor MyD88 except of TLR3 which employs the adaptor TRIF. TRIF is used also by TLR4, together with MyD88. Further downstream events include the activation of MAPKs and transcription factors of the NF-κB and IRF families. TLRs can be classified also by their subcellular localization. TLR3, TLR7, TLR8 and TLR9 are localized in the endosomal compartments whereas the remaining TLRs reside at the cell membrane and are exposed to the outside environment [12]. However, this static view of TLR localization is becoming increasingly challenged by recent findings revealing that TLR2 and TLR4 acquire different signaling properties after internalization triggered by ligand binding [13,14]. Thus, the dynamic trafficking of TLRs is an important component of the regulation of TLR function. Signaling from the cell membrane-bound TLRs generally leads to the production of NF-κB driven cytokines e.g. Tnf and IL-6 while signaling from the endosomal TLRs triggers the induction of both, the IRF-driven interferon beta (IFN-β) and the NF-κB-driven cytokines. The initially cell surface-localized TLR2 and TLR4 turn into IFN-β inducers after becoming compartmentalized into endosomes. Bacterial products that reach the cytoplasm (from e.g. intracellular bacteria), thereby escaping detection by cell surface- or endosome-associated TLRs, can be recognized in MyD88-independent way by cytoplasmic PRRs. They include the nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and the DNA-sensors DAI, AIM2 and LRRFIP1 [10,15]. NLRs and AIM2 cause activation of the inflammasome-dependent IL-1β release although the non-inflammasome function of the NLR members NOD1 and NOD2 in NF-κB-driven cytokine production is also critical for the immune response [16]. RLRs, DAI and LRRFIP1 are involved in type I IFN production in response to bacteria-derived nucleic acids.

Despite the knowledge about the critical contribution of the innate immune system to the severeness and diversity of diseases caused by *S. pyogenes* the recognition of this bacterium by the innate immune cells is still not understood. Only recently studies by us and others demonstrated that murine bone marrow-derived macrophages (BMDMs) and conventional DCs (cDCs) responded to infection with *S. pyogenes* by a TLR2-, TLR4- and TLR9-independent production of the NF-κB-driven cytokines TNF and IL-6 [17,18]. The lack of requirement for TLR2 is surprising since *S. pyogenes* is a Gram-positive bacterium that contains the TLR2 ligand lipoteichoic acid [19]. The *S. pyogenes*-elicited TNF and IL-6 production was strictly dependent on the adaptor MyD88 that was demonstrated by Medina and coworkers to be required for survival of mice in subcutaneous infection models [20].

Our previous study showed that *S. pyogenes* was capable to induce IFN-β in BMDMs which was the first evidence for a Gram-positive bacterium with an extracellular life cycle to do so [17]. Subsequently, the Group B Streptococcus was also shown to induce IFN-β in BMDMs and cDCs [21,22]. The detailed mechanism of IFN-β induction by *S. pyogenes* and the role of type I IFN signaling in host defense in a valid infection model remained to be elucidated. Depending on the pathogen, type I IFN signaling can have protective or deleterious effects in models for bacterial infectious diseases [23]. Here we show that mice deficient in type I IFN signaling ($IFNAR1^{-/-}$) are susceptible to invasive *S. pyogenes* infection in a model for innate immune system-controlled subcutaneous infectious disease. Infection studies revealed that cDCs require MyD88 and IRF1 for IFN-β production while BMDMs possess MyD88-dependent and independent pathways that however both require IRF3. In both cell types the IFN-β induction was not regulated by any known TLR. Analysis of bacterial components indicates that *S. pyogenes*-derived RNA is the IFN-β inducer in cDCs whereas bacterial DNA is the inducer in BMDMs. Our data also propose that the *S. pyogenes*-mediated induction of IFN-β is achieved by different sensing mechanisms than the previously described induction by Group B Streptococcus which further illustrates the diversity of these streptococcal species.

## Results

### Type I IFN signaling is required for host protection against *S. pyogenes* infection in lethal cellulitis

*S. pyogenes* was recently reported by us to induce IFN-β in bone marrow-derived macrophages (BMDMs) [17]. Depending on the pathogen, type I IFNs may be beneficial or harmful to the host [23]. To test how type I IFNs contribute to the host defense against *S. pyogenes* we examined the survival of type I IFN receptor 1 (IFNAR1)-deficient and control (WT) mice in a lethal subcutaneous infection model. This mode of infection resembles human skin infections with *S. pyogenes* which result in cellulitis and may develop into severe streptococcal toxic shock syndrome [24]. The survival of animals was monitored for 6 days in order to examine the host response during the innate immune system-dominated phase of infection. IFNAR1-deficient mice displayed 75% lethality rate whereas only 25% WT controls died demonstrating that type I IFNs were essential for a full blown host defense (Fig. 1A).

**Macrophages and dendritic cells employ different mechanisms for IFN-β induction by *S. pyogenes***

Macrophages (BMDMs) and cDCs, two cell types critically contributing to the innate immune responses, were shown to be required for protection of mice against *S. pyogenes* infections and for preventing dissemination of bacteria from the primary subcutaneous inoculation site [8,9]. Both cell types are known to respond to *S. pyogenes* infection by a robust Tnf and IL-6 production that is MyD88-dependent and involves a so far unindentified receptor different from the most common receptors for bacterial components i.e. TLR2, TLR4 and TLR9 [17,18]. In addition, both cell types were also reported to induce IFN-β although the precise mechanism remained unknown [17,22]. We directly compared the requirement for MyD88 in *S. pyogenes*-induced IFN-β production by infecting BMDMs and cDCs derived from MyD88$^{-/-}$ and WT mice. IFN-β amounts were measured in supernatants collected 4 and 6 hrs after infection of cDCs, whereas for BMDMs the 6 and 8 h time points were assayed since the response was consistently slower and less strong in these cells compared to cDCs (data not shown). In BMDMs the amount of IFN-β was reduced to 50% in MyD88-deficient cells (Fig. 1B) while in cDCs the IFN-β production was fully dependent on MyD88 (Fig. 1C). We observed that the IFN-β-driven activation of the transcription factor Stat1 was stronger in MyD88$^{-/-}$ as in WT BMDMs (Fig. 1D and [17]). We reasoned that the increased Stat1 activation despite of a reduced IFN-β production in MyD88$^{-/-}$ cells might be caused by the inability of MyD88$^{-/-}$ cells to efficiently activate expression of the *Socs1* gene, an inhibitor of IFN signaling. Full expression of *Socs1* requires activation of the p38 MAPK [25] that was found to be reduced in *S. pyogenes*-infected MyD88$^{-/-}$ cells [17]. Consistently, *Socs1* mRNA was reduced in *S. pyogenes*-infected MyD88$^{-/-}$ BMDMs (Fig. 1E). This result also illustrates that direct measurements of IFN levels by ELISA

more accurately reflect the intensity of IFN production than indirect assays such as activation of Stat1.

In cDCs, IFN-β production induced by a variety of stimuli is often regulated by the transcription factors IRF1 and IRF7 downstream of MyD88, whereas in BMDMs so far only IRF3-dependent MyD88-independent induction of IFN-β has been described [10]. To address the role of IRFs in *S. pyogenes*-induced IFN-β production we examined BMDMs and cDCs derived from IRF1$^{-/-}$ and IRF3$^{-/-}$ mice. In BMDMs, IRF1 was dispensable while IRF3 was absolutely essential for IFN-β induction (Fig. 2A, C). In contrast, IFN-β production in cDCs was strongly dependent on IRF1 but not on IRF3 (Fig. 2B, D). The data in cDCs are consistent with a study of Mancuso and colleagues describing partial dependency of *S. pyogenes*-induced IFN-β on IRF1 [22]. Interestingly, in that study Group B Streptococcus-induced IFN-β in cDCs was completely dependent on IRF1 indicating that the two streptococcal species employ different mechanisms for IFN-β induction in cDCs. In BMDMs, the complete dependency of *S. pyogenes*-induced IFN-β on IRF3 (Fig. 2C) resembled the reported Group B Streptococcus-stimulated IFN-β in the same cell type [21]. However, Group B Streptococcus-induced IFN-β did not require MyD88 in BMDMs [21] whereas our data show that upon *S. pyogenes* infection about 50% of IFN-β depends on MyD88 (Fig. 1). Together, the data illustrate that IFNβ-production in response to *S. pyogenes* requires MyD88 and partially IRF1 in cDCs, while in BMDMs IRF3 fully controls IFN-β generation 50% of which is MyD88-dependent. In addition, *S. pyogenes* and Group B Streptococcus, two streptococcal pathogens, significantly differ in the requirement for MyD88 and IRFs in IFN-β induction.

### *S. pyogenes* induces IFN-β by using receptors that differ from PRRs typically engaged by bacterial products

Our findings that cDCs fully and BMDMs partially require MyD88 for *S. pyogenes*-induced IFN-β production prompted us to test the involvement of the IFN-β-inducing TLRs, namely TLR7 that signals via the MyD88 adaptor and the MyD88-independent TLR3. A role of TLR9 was excluded in our previous study and the study by Mancuso et al. [17,22]. TLR7$^{-/-}$ BMDMs and cDCs infected with *S. pyogenes* did not differ in their ability to generate IFN-β if compared to WT controls (Fig. 3A, B). Similarly, deficiency in TLR3 did not result in changes in IFN-β induction in *S. pyogenes*-infected BMDMs compared to WT cells (Fig. 3C). The role of TLR3 in cDCs has been ruled out previously [22]. Thus, neither TLR7 that was shown to be critically involved in Group B Streptococcus-induced IFN-β produced by cDCs [22] nor TLR3 could account for the

recognition mechanism causing IFN-β production by *S. pyogenes*-infected BMDMs and cDCs. To further characterize pathways engaged in *S. pyogenes* recognition we examined the function of the IRF3 activating kinase TBK1. Since IRF3 is essential for IFN-β generation by *S. pyogenes*-infected BMDMs but not cDCs (Fig. 2C, D) we silenced TBK1 in BMDMs using siRNA. This approach reduced TBK1 protein levels to approximately 20% of the non-target siRNA control (Fig. 3E) and resulted in a 50% drop of IFN-β (Fig. 3F). The result revealed that TBK1 played an important role in *S. pyogenes*-elicited IFN-β induction in BMDMs despite the lack of evidence for the involvement of any upstream TLR known to signal via TBK1. TBK1 can be activated in a MyD88-independent way by the adaptor TRIF [10]. To examine whether the MyD88-independent part of *S. pyogenes*-induced IFN-β in BMDMs was regulated by TRIF we tested $TRIF^{-/-}$ cells. As depicted in Fig. 3D, TRIF deficiency did not result in a decrease of IFN-β production which was consistent with the lack of any effect of TLR3 (Fig. 3C), the TLR upstream of TRIF.

Recently, the nucleotide-binding oligomerization domain (NOD) 1 and 2, two members of the intracellular PRRs of the nucleotide-binding domain- and leucine-rich-region-containing cytoplasmic receptors (known as NLRs), were unexpectedly found to be required for IFN-β induction under certain conditions. Binding of the NOD1 ligand, a peptide derived from bacterial peptidoglycan, to NOD1 in epithelial cells resulted in a TBK1-dependent production of IFN-β [26]. In the case of NOD2, binding of virus-derived single stranded RNA (ssRNA) to NOD2 caused activation of IRF3 and induction of IFN-β [27]. The novel functions of NOD1 and NOD2 prompted us to test the involvement of these PRRs in *S. pyogenes*-induced IFN-β. However, BMDMs from $NOD1^{-/-}$ and $NOD2^{-/-}$ mice produced similar amounts of IFN-β and displayed comparable activation of Stat1 as WT cells (Fig. S1A, B)

Cumulatively, the MyD88-dependent production of IFN-β in response to *S. pyogenes* infection does not result from activation of TLR7, the TLR utilizing MyD88. Furthermore, the MyD88-independent IFN-β induction in BMDMs cannot be explained by the activation of TLR3, NOD1 or NOD2, the PRRs employing TBK1 and IRF3 signaling for IFN-β induction despite the requirement of these two signaling components for IFN-β caused by *S. pyogenes* infection.

**Endosomal delivery of *S. pyogenes*-derived RNA and DNA activate IFN-β production in cDCs and BMDMs, respectively**

It is becoming increasingly clear that the recognition of nucleic acids by innate immune cell receptors is not limited to viral infections but appears to be a more universal way of sensing danger signals which may originate from viruses, bacteria or even mammalian cells [10]. The

response to nucleic acids by PRRs occurs either in the endosomal compartment or in the cytoplasm ensuring detection in both the phagosome and, in case phagosomal escape, in the cytoplasm. We assumed that the *S. pyogenes*-derived PAMPs were recognized primarily during phagocytosis since *S. pyogenes* has no intracellular life cycle, although streptolysin-mediated escape from phagosome to autophagosome with a short-lived cytoplasmic intermediate has been reported [28,29]. This assumption was supported by our findings that *S. pyogenes*-induced IFN-β is in cDCs completely and in BMDMs to 50% dependent on MyD88 signaling (Fig. 1) since the cytoplasmic PRRs do not signal via the MyD88 adaptor [10]. Furthermore, heat-killed *S. pyogenes* that is supposed to lack the ability to deliver bacterial products into the cytoplasm [30], retained, albeit in BMDMs to a lesser extent, its capacity to induce IFN-β (Fig. S2 and data not shown). To address the role of *S. pyogenes*-derived nucleic acids in the induction of IFN-β, extracts of sonicated *S. pyogenes* were treated with DNase I, RNase A, proteinase K or left untreated (control extract). The extracts devoid of *S. pyogenes*-derived DNA, RNA or protein as well as control extracts were delivered to the endosomes of cDCs and BMDMs using DOTAP that is known to be taken up together with the bound cargo by the endocytic pathway [31]. cDCs responded most robustly by IFN-β induction to extracts containing streptococcal RNA (Fig. 4A), whereas BMDMs required DNA for IFN-β induction (Fig. 4D). IFN-β induced by RNA from Group B Streptococcus as well as by live Group B Streptococcus was reported to be dependent in cDCs on TLR7 [22]. However, similar to infection with live *S. pyogenes* (Fig. 3) TLR7 was not needed for IFN-β production in response to RNA from *S. pyogenes* (Fig. 4A) indicating a distinct mode of IFN-β induction. To further substantiate the critical role of *S. pyogenes*-derived RNA in IFNβ induction by cDCs, we examined responses of $IRF1^{-/-}$ and $MyD88^{-/-}$ cells to extracts from *S. pyogenes*. The responses of cDCs to the bacterial RNA-containing extracts were reduced in $IRF1^{-/-}$ cells and completely abolished in $MyD88^{-/-}$ cells (Fig. 4B, C). This result virtually mirrored the infection of cDCs with live *S. pyogenes* (Fig. 2B), confirming that the extracts of *S. pyogenes* administered using DOTAP represented simplified yet still correct delivery of the *S. pyogenes*-derived PAMPs to their authentic PRRs. Importantly, DOTAP-mediated delivery did not result in the activation of cytoplasmic nucleic acid sensors NLRs, RLRs (RIG-I, Mda5 and LGP2), DAI or the recently reported LRRFIP1 since these PRRs signal independently of MyD88 [15,26,27,32,33]. This notion is of a particular relevance also for induction of IFN-β in BMDMs since direct delivery of Group B Streptococcus extracts containing DNA into the cytoplasm using lipofectamine was found to elicit IFN-β response [21]. To directly test the role of endosomes in the recognition of DNA from *S. pyogenes* we employed dynasore, an inhibitor of endocytosis used to assess the contribution of endosomes to various cellular responses [34,35].

Pretreatment of BMDMs with dynasore inhibited the streptococcal extracts-induced IFN-β, confirming that the *S. pyogenes*–derived DNA was functionally sensed only after endosome-mediated uptake (Fig. 4D). The finding that in BMDMs the *S. pyogenes*-derived DNA rather than RNA was the IFN-β inducer (Fig. 4C) prompted us to re-examine the function of TLR9 that is known to recognize, in endosome-dependent way, unmethylated DNA containing CpG motives frequently present in bacterial genomes. Treatment of TLR9$^{-/-}$ BMDMs with *S. pyogenes* extracts revealed that TLR9 did not mediate the recognition of *S. pyogenes*-derived DNA (Fig. 4E) confirming also our previous study showing no requirement for TLR9 in IFN-β induction by BMDMs infected with live *S. pyogenes* [17].

Together, our data reveal that bacterial RNA and DNA are key players in induction of IFN-β by *S. pyogenes*-infected cDCs and BMDMs, respectively. Although RNA and DNA were reported to be involved in IFN-β induction by Group B Streptococcus in cDCs and BMDMs, respectively, the mode of recognition and the PRRs involved are distinct for *S. pyogenes*-derived nucleic acids.

**Discussion**

Elucidating the mechanisms of pathogen recognition and the subsequent host immune response improves not only our understanding of the basic principles of host-pathogen interactions but has also practical implications for the development of new therapies of infectious diseases. Blockade or augmentation of the recognition process may be beneficial in cases of exacerbated or insufficient immune response, respectively. Agonists and antagonists of pathogen recognition by e.g. TLRs are currently one of the most promising therapeutic strategies [36]. Recognition of Gram-positive extracellular bacteria was long thought to be primarily driven by interactions between TLR2 and bacteria-derived LTA or lipoproteins [37]. It was therefore surprising that the recognition of *S. pyogenes* and Group B Streptococci was found to be independent of TLR2 although both species produce TLR2 ligands [17,18,19,38,39]. In addition, both streptococcal species induce NF-κB-driven cytokines (e.g. TNF and IL-6) in a completely MyD88-dependent way employing so far unidentified receptors upstream of the essential MyD88 adaptor protein. Subsequently, studies by our lab and others demonstrated that both *S. pyogenes* and Group B Streptococcus were also able to induce IFN-β in innate immune cells. Despite these similarities, *S. pyogenes* and Group B Streptococcus share only about 200 out of ~2000 genes present in their genomes as judged by sequence comparison [40]. The homologous genes are mostly limited to housekeeping genes whereas the virulence factor genes are dissimilar. Hence, it is not surprising that *S. pyogenes* and Group B Streptococcus cause different set of infectious diseases [40]. *S. pyogenes* causes an exceptionally wide range of diseases and is regarded as

one of the most versatile pathogens known. Genetic data support the hypothesis that the responses of the host innate immune system contribute to the morbidity and diversity of the *S. pyogenes*-caused diseases [5,6,7]. Type I IFNs belong to the most potent modulators of the host response during the initial phase of infection that is controlled largely by the innate immune system [41]. The so far unknown role of type I IFNs in a relevant model for infection with *S. pyogenes* and the detailed mechanism of IFN-β induction in BMDMs and cDCs were for the first time addressed in this study.

We provide evidence that type I IFN signaling is required for survival of mice challenged with *S. pyogenes* in a model for invasive subcutaneous infection. This finding should stimulate clinical research in this area since no epidemiology data on type I IFN production and responses in patients are available to date. During the innate immune system-controlled phase of infection type I IFNs, which are produced directly by cells encountering the pathogen, regulate in multiple ways the host response. They increase chemokine expression at the site of infection and thereby promote recruitment of effector cells such as neutrophils [42]. Type I IFNs influence also cell autonomous functions of immune cells by e.g. regulating anti-microbial products or controlling cell death [23]. These issues should be addressed in future studies of *S. pyogenes*-caused infections.

In bacterial infections the type I IFN-producing cells are predominantly macrophages and cDCs rather than plasmacytoid DCs [22,43]. In our study we therefore focused on macrophages and cDCs which were also previously shown to be crucial cell types for successful defense of mice against *S. pyogenes* infection [8,9]. We examined IFN-β production since this IFN is the first type I IFN to be synthesized during infections, and it drives the expression of other type I IFN species [44]. BMDMs and cDCs displayed common as well as unique features with regard to the induction of IFN-β. In both cell types MyD88 fulfilled an important role although in BMDMs about one half of the generated IFN-β was independent of MyD88. In cDCs the complete dependence on MyD88 was accompanied with a key function of IRF1 which was consistent with the current view of the IFN-β-inducing pathway positioning IRF1, IRF5 and IRF7 downstream of MyD88 [10]. However, the canonical MyD88/IRF axis is triggered by TLR7 or TLR9 which were ruled out as the *S. pyogenes*-sensing TLRs in cDCs in this and a previous study [22]. The involvement of TLR8, the only IFN-β-inducing MyD88-dependent TLR not experimentally tested by us, is unlikely since TLR8 may not be functional in the mouse as suggested by complete lack of TLR7-deficient mice to respond to R-848 and ssRNA commonly used as TLR7 and TLR8 agonists in human cells [10,45]. Thus, the identity of the PRR responsible for triggering the MyD88/IRF pathway in response of cDCs to *S. pyogenes* remains to be determined.

In BMDMs, about half of the induced IFN-β was MyD88-independent yet it fully required IRF3, and the IRF3 kinase TBK1 was also critically involved. TBK1/IRF3 can be activated by TLR3 and TLR4 via the adaptor TRIF rather than MyD88. Our data demonstrate that TRIF-deficient cells respond normally to *S. pyogenes* suggesting that TLR3 and TLR4 were not the upstream activators of the TBK1/IRF3 module. We confirmed this conclusion by the analysis of $TLR3^{-/-}$ BMDMs. TLR4 was disqualified already in our previous study [17]. For the MyD88-dependent IFN-β production we could exclude also the involvement of TLR7 (this study) and TLR9 [17]. Thus, both the MyD88-dependent and -independent IFN-β production is induced by *S. pyogenes* independently of TLRs. TBK1/IRF3 can be activated by various cytoplasmic sensors [10]. The signaling by these cytoplasmic PRRs does not require MyD88 so that only the MyD88-independent part of IFN-β induction could be potentially explained by these molecules. Our experiments using BMDMs deficient in NOD1 and NOD2, two cytoplasmic PRRs recently shown to activate the TBK1/IRF3 pathway [26,27] and known to recognize peptidoglycan from both Gram-positive and –negative bacteria [46], ruled out the role of these PRRs in *S. pyogenes*-induced IFN-β induction.

The nature of the IFN-β-inducing bacterial product was tested by delivering complete *S. pyogenes* extracts or extracts depleted of DNA, RNA or protein to the endosomes. The endosomal delivery was justified by the fact that IFN-β production in *S. pyogenes*-infected cells was reduced by the endocytosis inhibitor dynasore. Our data showing *S. pyogenes*-derived RNA as the principle IFN-β inducer in cDCs are consistent with a model wherein the *S. pyogenes*-derived RNA is liberated from the bacterial cells upon phagocytosis. The bacterial RNA is then sensed by an endosomal receptor that triggers the IFN-β-inducing MyD88/IRF pathway. This model is further supported by our findings that in cDCs signaling by the *S. pyogenes* extracts was dependent on MyD88 and IRF1 thereby fully recapitulating signaling upon infection with live bacteria. Thus, we could rule out DOTAP-mediated misguiding of bacterial RNA to the cytoplasm where it could generate artificial IFN response due to the activation of cytoplasmic i.e. MyD88-independent PRRs. Thus, a signaling from cytoplasmic PRRs is not the dominant mode of IFN-β induction in cDCs infected with *S. pyogenes*. Bacterial RNA as the endosomal inducer of IFN-β in cDCs has been proposed for Group B Streptococcus [22]. However, *S. pyogenes* and Group B Streptococcus differ in terms of their requirement for TLR7 that is needed for sensing Group B Streptococcus but not *S. pyogenes*. Thus, the still unknown endosomal PRR for *S. pyogenes*-derived PAMPs and the PRR for Group B Streptococcus are different despite sharing same signaling components downstream of the receptors. In BMDMs the IFN-β-inducing *S. pyogenes* extracts required the presence of bacterial DNA rather than RNA. Since DOTAP

delivers to the endosomal compartment and taking into account that lack of any effect of TLR9 we conclude that an endosomal PRR distinct from TLR9 is able to detect DNA from *S. pyogenes*. This PRR signals via TBK1 and IRF3 in a partially MyD88-dependent way. Comparison with Group B Streptococcus-induced IFN-β by BMDMs reveals some parallels but also important differences [21]. Both pathogens signal via bacterial DNA and employ the TBK1/IRF3 pathway. For Group B Streptococcus it was proposed that the bacterial DNA escapes in a pore-forming-toxin-dependent way from the phagosomes into the cytoplasm of infected cells where it is sensed by an unknown IFN-β-inducing PRR. However, the production of IFN-β was independent of MyD88 and required live bacteria, which is in marked difference to *S. pyogenes*. Currently, we cannot rule out that the MyD88-independent part of *S. pyogenes*-induced IFN-β is caused by bacterial products liberated from the phagosome to the cytoplasmic PRRs. However, we do not favor this hypothesis since *S. pyogenes* lacking both pore-forming streptolysins SLO and SLS was still able to activate type I IFN signaling in BMDMs [17].

This study delineates the importance of type I IFN induction by innate immune cells for successful defense against *S. pyogenes*. In a similar infection model, MyD88 was recently found to be required for full blown immune response and survival [20]. Since our data reveal that MyD88 plays a critical role in the induction of IFN-β it appears conceivable that the decreased IFN-β production contributed to the high susceptibility of MyD88-deficient mice to the infection. The comparison of BMDMs and cDCs revealed different mechanism of IFN-β induction. Both cell types employ unknown endosomal nucleic acid-sensing PRRs for IFN-β induction. It is striking that the PRRs required for the NF-κB-driven induction of TNF and IL-6 in *S. pyogenes*-infected BMDMs and cDCs have so far also not been identified [17,18]. Thus, in general, it appears that *S. pyogenes* evolved mechanisms allowing this pathogen to avoid the standard set of pathogen sensing molecules. This strategy may not be unique since Group B Streptococcus-induced TNF and IL-6 production is also independent of the typical PRRs for bacteria [38]. It remains to be elucidated whether other extracellular Gram-positive bacteria display similar properties. This study and other recently published reports [17,18,22] clearly demonstrate that innate immune cells posses yet not well understood backup strategies for a highly efficient TLR-independent detection of bacteria such as *S. pyogenes*.

## Materials and Methods

### Bacterial strains and culture

The *Streptococcus pyogenes* serotype M1 strain ISS 3348 (provided by Roberta Creti, Instituto Superiore di Sanita, Italy) was used for *in vitro* as well for *in vivo* experiments [47]. Bacteria were grown at 37 °C with 5% $CO_2$ without agitation in Todd-Hewitt-Broth (BD Biosciences) supplemented with 0.2% yeast extract and on tryptic soy agar supplemented with 3% defibrinogated sheep blood. Bacterial cell growth was turbidimetrically monitored at 620 nm with a microplate reader until mid-log phase was reached. For preparation of heat-killed Streptococci, bacteria were grown to mid-log phase, washed twice with sterile PBS and incubated for 20 min at 70° C in a water bath.

### Mice

IFNAR1$^{-/-}$, MyD88$^{-/-}$, IRF1$^{-/-}$, IRF3$^{-/-}$, TLR3$^{-/-}$, TLR7$^{-/-}$, TRIF$^{-/-}$ , TLR9$^{-/-}$ mice on C57BL/6 background were housed under specific pathogen-free conditions. C57BL/6 WT mice were purchased from Charles River Laboratories.

### Experimental model of *S. pyogenes* infection

Age- and sex-matched, pathogen free, 8-10 week old WT and IFNAR1$^{-/-}$ mice (all C57BL/6) were used in all experiments. *S. pyogenes* strain ISS 3348 was grown for 5 hours to mid-logarithmic phase at 37°C using Todd-Hewitt-Broth (BD Biosciences), harvested by centrifugation at 6.500 rpm for 8 min, and washed twice in sterile isotonic saline. Bacteria were then resuspended at a concentration of 3x10$^8$ CFU per 50 µl as determined by plating serial 10-fold dilutions on sheep blood agar plates (Biomerieux). Mice were lightly anesthetized by inhalation of isoflurane (Baxter) and the fur at the flank was partially removed by shaving. The inoculum was injected subcutaneously in one flank of each mouse. Survival of mice was monitored every 4 - 8 hours. All experiments were approved by the Animal Care and Use Committee of the Medical University of Vienna and the Ministry of Science through the permission BMWF-66.009/0031-II/10b/2008. Survival curves were analyzed by the Logrank Test in GraphPad Prism 4 (GraphPad Software). All data are presented as mean SD. Comparison between two groups was performed using t-test. P values ≤ 0.05 were considered as significant.

### Cell culture

Primary bone-marrow derived macrophages (BMDMs) and conventional dendritic cells (cDCs) were obtained from the femur and tibia bone-marrow of 6-10-week old mice (all C57BL/6).

Macrophages were cultivated in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% fetal calf serum (GIBCO) in the presence of L929-cell derived CSF-1, as described [17]. Dendritic cells were cultivated in DMEM supplemented with 10% fetal calf serum in the presence of X-6310 cell derived GM-CSF as described [48].

### *S. pyogenes* infection *in vitro*

For infection assays, BMDMs and cDCs were seeded on 6-cm dishes (2 x $10^6$ cells/ dish) in DMEM containing 10% FCS and L-cell-derived CSF-1 or X-6310 cell derived GM-CSF, respectively, without antibiotics. The cells were then infected as described previously [17]. In experiments with heat-killed *S. pyogenes* equal amounts of heat-killed and live bacteria (MOI 100) were used for infection. Infections under conditions of blocked endocytosis were performed by pretreatment of cells with dynasore (Sigma-Aldrich, 80 µM) for 45 min prior to infection with *S. pyogenes*.

### Preparation and transfection of *S. pyogenes* extracts

*S. pyogenes* was grown to $OD_{620}$=0.3 and 5 mL aliquots of the bacterial suspensions were spun at 6.500 rpm for 8 min. The bacteria were resuspended in 400 µL sterile PBS followed by disruption using the Bandelin Sonopuls GM70 sonicator for 3 × 2 min. Remaining debris was removed by centrifugation at 13.200 rpm for 15 min at 4°C. The supernatants were pooled, adjusted to contain 2mM $MgCl_2$, 50 mM KCl and 20mM Tris-HCl (pH 8) and divided into four aliquots. The same was done with sterile PBS for the reagent control. Remaining extract was frozen at -20 °C for later usage. Aliquots were digested with DNase I (Roche, 100U/mL), RNase A (Roche, 333 µg/mL) or proteinase K (Sigma-Aldrich, 1mg/mL) or left-untreated (control extract). Extracts treated with either DNase I or RNase A were incubated at 37 °C for 45 min, extracts digested with proteinase K were incubated for 1 hour at 37 °C. After incubation, EGTA for proteinase K (final 2 mM) treatment or EDTA (pH 8, final 2.5 mM) for DNase I and RNase A treatment were added to extracts. In case of the reagent control and control extract, both EGTA and EDTA were used. The extracts were then incubated at 70 °C for 10 min and centrifuged for 5 min at 13.200 rpm at 4 °C. Supernatants were collected and subsequently frozen at -20 °C until usage. 10 µl of these extracts were then allowed to form complexes with DOTAP (Roche) and transfected according to the manufacturer´s instructions.

**Cytokine measurement**

IFN-β in supernatants of infected macrophages and dendritic cells was measured by ELISA using the VeriKine Mouse Interferon Beta Kit (PBL Biomedical Laboratories), according to the manufacturer´s instructions. Samples were applied undiluted.

**Antibodies**

Antibodies to Tyr701-phosphorylated Stat1 (pY701-S1) and TBK1 were purchased from Cell Signalling. The antibody against p38 MAPK was purchased from Santa Cruz Biotechnology. The antibody to Stat1-C-terminus was previously described [49].

**Western blot analysis**

After treatment, whole cell extracts were prepared and assayed by Western blotting as described previously [49]. Detection and quantification of signals were performed using the infrared imaging system Odyssey (LI-COR Biosciences) using fluorophore-linked secondary antibodies (Molecular Probes and Rockland).

**Quantitative RT-PCR (qRT-PCR)**

Total RNA was isolated using Trizol® LS reagent (Invitrogen). Reverse transcription of total RNA was performed using oligo $(dT)_{18}$ as primer and Mu-MLV reverse transcriptase (Fermentas) and the cDNA was subsequently used to amplify SOCS1 by qRT-PCR. qRT-PCR was run on iCycler (BioRad). For SOCS1 the Quantitect ®Primer Assay (QIAGEN) was used. For HPRT, the housekeeping gene for normalization, the following primers were used: HPRT-fwd 5'-GGATTTGAATCACGTTTGTGTCAT-3', and HPRT-rev 5'-ACACCTGCTAATTTTACTGGCAA-3'. Amplification of DNA was monitored by SYBR Green (Molecular Probes) [50].

**siRNA–mediated silencing**

BMDMs were seeded on 6-cm dishes (2 x $10^6$ cells) in DMEM containing 10 % FCS and L-cell-derived CSF-1 without antibiotics. Mouse TBK1 (ON-TARGETplus, Dharmacon) siRNA (6 pmol) or a non-target control-siRNA were mixed with Opti-MEM I reduced serum medium (GIBCO) and transfected into BMDMs using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer´s instructions. 48 hrs after transfection, medium was replaced by medium without siRNA. After additional 24 hrs cells were infected with *S. pyogenes* (MOI=100) as described [17].

**Acknowledgements**

We thank Shizuo Akira for the permission to use MyD88$^{-/-}$, TLR3$^{-/-}$, TLR7$^{-/-}$ , TLR9$^{-/-}$ and TRIF$^{-/-}$ mice. We thank Roberta Creti and Giuseppe Teti for providing bacterial strains, and Ivo Boneca for providing bone marrow from NOD1$^{-/-}$ and NOD2$^{-/-}$ mice. We appreciate the help of Birgit Rapp with bacterial cultures.

**References**

1. Bisno AL, Brito MO, Collins CM (2003) Molecular basis of group A streptococcal virulence. Lancet Infect Dis 3: 191-200.
2. O'Loughlin RE, Roberson A, Cieslak PR, Lynfield R, Gershman K, et al. (2007) The epidemiology of invasive group A streptococcal infection and potential vaccine implications: United States, 2000-2004. Clin Infect Dis 45: 853-862.
3. Randall J. Olsen SASJMM (2009) Molecular mechanisms underlying group A streptococcal pathogenesis. Cellular Microbiology 11: 1-12.
4. Luca-Harari B, Darenberg J, Neal S, Siljander T, Strakova L, et al. (2009) Clinical and Microbiological Characteristics of Severe Streptococcus pyogenes Disease in Europe. J Clin Microbiol.
5. Kotb M, Norrby-Teglund A, McGeer A, El-Sherbini H, Dorak MT, et al. (2002) An immunogenetic and molecular basis for differences in outcomes of invasive group A streptococcal infections. Nat Med 8: 1398-1404.
6. Kotb M, Norrby-Teglund A, McGeer A, Green K, Low DE (2003) Association of human leukocyte antigen with outcomes of infectious diseases: the streptococcal experience. Scand J Infect Dis 35: 665-669.
7. Goldmann O, Lengeling A, Bose J, Bloecker H, Geffers R, et al. (2005) The role of the MHC on resistance to group a streptococci in mice. J Immunol 175: 3862-3872.
8. Goldmann O, Rohde M, Chhatwal GS, Medina E (2004) Role of macrophages in host resistance to group A streptococci. Infect Immun 72: 2956-2963.
9. Loof TG, Rohde M, Chhatwal GS, Jung S, Medina E (2007) The contribution of dendritic cells to host defenses against Streptococcus pyogenes. J Infect Dis 196: 1794-1803.
10. Kawai T, Akira S The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. (2010) Nat Immunol 11: 373-384.

11. O'Neill LA (2008) The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress. Immunol Rev 226: 10-18.
12. McGettrick AF, O'Neill LA (2010) Localisation and trafficking of Toll-like receptors: an important mode of regulation. Curr Opin Immunol 22: 20-27.
13. Kagan JC, Su T, Horng T, Chow A, Akira S, et al. (2008) TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. Nat Immunol.
14. Barbalat R, Lau L, Locksley RM, Barton GM (2009) Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. Nat Immunol 10: 1200-1207.
15. Yang P, An H, Liu X, Wen M, Zheng Y, et al. (2010) The cytosolic nucleic acid sensor LRRFIP1 mediates the production of type I interferon via a beta-catenin-dependent pathway. Nat Immunol 11: 487-494.
16. Ting JP, Duncan JA, Lei Y (2010) How the noninflammasome NLRs function in the innate immune system. Science 327: 286-290.
17. Gratz N, Siller M, Schaljo B, Pirzada ZA, Gattermeier I, et al. (2008) Group A Streptococcus Activates Type I Interferon Production and MyD88-dependent Signaling without Involvement of TLR2, TLR4, and TLR9. J Biol Chem 283: 19879-19887.
18. Loof TG, Goldmann O, Medina E (2008) Immune recognition of Streptococcus pyogenes by dendritic cells. Infect Immun.
19. Courtney HS, Ofek I, Penfound T, Nizet V, Pence MA, et al. (2009) Relationship between expression of the family of M proteins and lipoteichoic acid to hydrophobicity and biofilm formation in Streptococcus pyogenes. PLoS ONE 4: e4166.
20. Loof TG, Goldmann O, Gessner A, Herwald H, Medina E (2009) Aberrant Inflammatory Response to Streptococcus pyogenes in Mice Lacking Myeloid Differentiation Factor 88. Am J Pathol.
21. Charrel-Dennis M, Latz E, Halmen KA, Trieu-Cuot P, Fitzgerald KA, et al. (2008) TLR-independent type I interferon induction in response to an extracellular bacterial pathogen via intracellular recognition of its DNA. Cell Host Microbe 4: 543-554.
22. Mancuso G, Gambuzza M, Midiri A, Biondo C, Papasergi S, et al. (2009) Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells. Nat Immunol.
23. Decker T, Muller M, Stockinger S (2005) The yin and yang of type I interferon activity in bacterial infection. Nat Rev Immunol 5: 675-687.
24. Medina E Murine model of cutaneous infection with Streptococcus pyogenes. (2010) Methods Mol Biol 602: 395-403.

25. Sahay B, Patsey RL, Eggers CH, Salazar JC, Radolf JD, et al. (2009) CD14 signaling restrains chronic inflammation through induction of p38-MAPK/SOCS-dependent tolerance. PLoS Pathog 5: e1000687.
26. Watanabe T, Asano N, Fichtner-Feigl S, Gorelick PL, Tsuji Y, et al. (2010) NOD1 contributes to mouse host defense against Helicobacter pylori via induction of type I IFN and activation of the ISGF3 signaling pathway. J Clin Invest.
27. Sabbah A, Chang TH, Harnack R, Frohlich V, Tominaga K, et al. (2009) Activation of innate immune antiviral responses by Nod2. Nat Immunol 10: 1073-1080.
28. Nakagawa I, Amano A, Mizushima N, Yamamoto A, Yamaguchi H, et al. (2004) Autophagy defends cells against invading group A Streptococcus. Science 306: 1037-1040.
29. Yamaguchi H, Nakagawa I, Yamamoto A, Amano A, Noda T, et al. (2009) An initial step of GAS-containing autophagosome-like vacuoles formation requires Rab7. PLoS Pathog 5: e1000670.
30. Harder J, Franchi L, Munoz-Planillo R, Park JH, Reimer T, et al. (2009) Activation of the Nlrp3 Inflammasome by Streptococcus pyogenes Requires Streptolysin O and NF-{kappa}B Activation but Proceeds Independently of TLR Signaling and P2X7 Receptor. J Immunol.
31. Zabner J, Fasbender AJ, Moninger T, Poellinger KA, Welsh MJ (1995) Cellular and molecular barriers to gene transfer by a cationic lipid. J Biol Chem 270: 18997-19007.
32. Takaoka A, Wang Z, Choi MK, Yanai H, Negishi H, et al. (2007) DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. Nature 448: 501-505.
33. Yoneyama M, Fujita T (2008) Structural mechanism of RNA recognition by the RIG-I-like receptors. Immunity 29: 178-181.
34. Macia E, Ehrlich M, Massol R, Boucrot E, Brunner C, et al. (2006) Dynasore, a cell-permeable inhibitor of dynamin. Dev Cell 10: 839-850.
35. Stetson DB, Medzhitov R (2006) Recognition of Cytosolic DNA Activates an IRF3-Dependent Innate Immune Response. Immunity 24: 93-103.
36. Kanzler H, Barrat FJ, Hessel EM, Coffman RL (2007) Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. Nat Med 13: 552-559.
37. Draing C, Sigel S, Deininger S, Traub S, Munke R, et al. (2008) Cytokine induction by Gram-positive bacteria. Immunobiology 213: 285-296.
38. Henneke P, Takeuchi O, Malley R, Lien E, Ingalls RR, et al. (2002) Cellular activation, phagocytosis, and bactericidal activity against group B streptococcus involve parallel myeloid differentiation factor 88-dependent and independent signaling pathways. J Immunol 169: 3970-3977.

39. Henneke P, Dramsi S, Mancuso G, Chraibi K, Pellegrini E, et al. (2008) Lipoproteins are critical TLR2 activating toxins in group B streptococcal sepsis. J Immunol 180: 6149-6158.
40. Mitchell TJ (2003) The pathogenesis of streptococcal infections: from tooth decay to meningitis. Nat Rev Microbiol 1: 219-230.
41. Theofilopoulos AN, Baccala R, Beutler B, Kono DH (2005) Type I interferons (alpha/beta) in immunity and autoimmunity. Annu Rev Immunol 23: 307-336.
42. Kelly-Scumpia KM, Scumpia PO, Delano MJ, Weinstein JS, Cuenca AG, et al. (2010) Type I interferon signaling in hematopoietic cells is required for survival in mouse polymicrobial sepsis by regulating CXCL10. J Exp Med.
43. Stockinger S, Kastner R, Kernbauer E, Pilz A, Westermayer S, et al. (2009) Characterization of the interferon-producing cell in mice infected with Listeria monocytogenes. PLoS Pathog 5: e1000355.
44. Levy DE, Marie I, Prakash A (2003) Ringing the interferon alarm: differential regulation of gene expression at the interface between innate and adaptive immunity. Curr Opin Immunol 15: 52-58.
45. Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, et al. (2002) Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat Immunol 3: 196-200.
46. Boneca IG (2005) The role of peptidoglycan in pathogenesis. Curr Opin Microbiol 8: 46-53.
47. Mora M, Bensi G, Capo S, Falugi F, Zingaretti C, et al. (2005) Group A Streptococcus produce pilus-like structures containing protective antigens and Lancefield T antigens. Proc Natl Acad Sci U S A 102: 15641-15646.
48. Reutterer B, Stockinger S, Pilz A, Soulat D, Kastner R, et al. (2008) Type I IFN are host modulators of strain-specific Listeria monocytogenes virulence. Cell Microbiol 10: 1116-1129.
49. Kovarik P, Stoiber D, Novy M, Decker T (1998) Stat1 combines signals derived from IFN-gamma and LPS receptors during macrophage activation [published erratum appears in EMBO J 1998 Jul 15;17(14):4210]. Embo J 17: 3660-3668.
50. Morrison TB, Weis JJ, Wittwer CT (1998) Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Biotechniques 24: 954-958, 960, 962.

## Figure Legends

**Figure 1. Type I IFNs are required for efficient host immune response against *S. pyogenes* and MyD88 plays a key role in type I IFN production.**

(**A**) Kaplan-Meier survival curves of C57BL/6 and IFNAR1$^{-/-}$ mice (15 mice per genotype) after subcutaneous infection with 3x10$^{8}$ CFU of *S. pyogenes*. Survival was monitored for 6 days. Significance: ** = P value < 0.01. (**B, C**) IFN-β production is in BMDMs partially (B) and cDCs completely (C) dependent on MyD88. Cells from wild-type (WT) and MyD88$^{-/-}$ mice were infected with *S. pyogenes* (MOI100) or left untreated. After indicated periods, supernatants were collected and IFN-β release was measured by ELISA. Values represent mean ± SD; n = 3. (**D, E**) MyD88 deficiency results in stronger Stat1 activation and reduced SOCS1 expression in response to *S. pyogenes*. Control (WT) and MyD88$^{-/-}$ BMDMs were infected with *S. pyogenes* (MOI100) or left untreated. At indicated time-points, either whole cell extracts were prepared or total mRNA was extracted. In (D), Stat1 activation was determined by Western blotting using antibody to phosphorylated Stat1 (pY-S1). For loading control the membrane was reprobed using antibodies to total Stat1 (Stat1) and p38MAPK (p38). Note the double band on the pY-S1 blot represents the phosphorylated forms of both Stat1 splicing isoforms Stat1-α and Stat1-β. Loading control (Stat1) was performed with an antibody directed to the C-terminus of Stat1, which is absent in the Stat1-β isoform. In (E), total RNA was reverse-transcribed and analyzed by qRT-PCR for SOCS1 expression. For normalization expression of the house-keeping gene HPRT was measured. These data represent one of at least three independent infection experiments with different mice from each genotype.

**Figure 2. BMDMs and cDCs differ in their requirements for IRF1 and IRF3 in *S. pyogenes*-induced IFN-β production.**

BMDMs from IRF1$^{-/-}$ (**A**) and IRF3$^{-/-}$ (**C**) mice or cDCs derived from IRF1$^{-/-}$ (**B**) and IRF3$^{-/-}$ (**D**) (as well as control mice (WT) were infected with *S. pyogenes* (MOI100) or left untreated. At indicated time-points, supernatants were collected and IFN-β release was measured by ELISA. The data represent at least three independent infection experiments. SDs (n ≥ 3) are shown.

**Figure 3. *S. pyogenes*-mediated induction of IFN-β occurs independently of TLR3, TLR7 or TRIF and in BMDMs requires TBK1.**

BMDMs (**A**) and cDCs (**B**) and from control (WT) and TLR7$^{-/-}$ mice or BMDMs from control (WT), TLR3$^{-/-}$ (**C**) and TRIF$^{-/-}$ (**D**) mice were infected with *S. pyogenes* (MOI100) for the indicated

periods or left untreated. Supernatants were collected and IFN-β release was measured by ELISA. The data represent at least three independent infection experiments. SDs (n ≥ 3) are shown. (**E, F**) BMDMs were transfected with siRNA specific for TBK1 or non-target control siRNA. siRNA-treated cells were then infected with *S. pyogenes* (MOI 100) or left uninfected. At indicated time-points, whole-cell extracts were prepared and supernatants were collected. In (E), silencing of TBK1 was controlled by Western Blot using an antibody against TBK1. Equal loading was determined by re-probing the membrane with an antibody to p38 MAPK. In (F), IFN-β production was measured using ELISA. The data represent one of at least three independent experiments.

**Figure 4. *S. pyogenes*-derived RNA and DNA delivered to endosomes induce IFN-β in cDCs and BMDMs, respectively.**

*S. pyogenes* cells were sonicated and treated with either DNase I, RNase A, Proteinase K or left untreated (control extract). These extracts and a reagent control were delivered into cDCs derived from control (WT), TLR7$^{-/-}$ (**A**), IRF1$^{-/-}$ (**B**) or MyD88$^{-/-}$ (**C**) mice using DOTAP. After stimulation for 6 h supernatants were collected and IFN-β release was measured using ELISA. (**D**) *S. pyogenes* extracts were delivered into BMDMs pretreated (for 45 min) or untreated with dynasore. After stimulation for 8 h supernatants were collected and IFN-β production was measured using ELISA. Values represent mean ± SD; n = 3. (**E**) BMDMs from control (WT) and TLR9$^{-/-}$ mice were transfected with streptococcal extracts using DOTAP and after 8 h supernatants were collected for IFN-β measurements by ELISA. Values represent mean ± SD; n = 3.

**Supporting Information**

**Figure legends**

**Figure S1. NOD1 and NOD2 are not required for IFN-β induction by *S. pyogenes*.**

BMDMs from control (WT), NOD1$^{-/-}$ or NOD2$^{-/-}$ mice were infected with *S. pyogenes* (MOI100) and after indicated time-points whole cell extracts were prepared and supernatants were collected. In (**A**), Stat1 activation was determined by Western blotting using an antibody to phosphorylated Stat1 (pY-S1) and total Stat1 (Stat1). In (**B**), IFN-β release after 6 h of infection

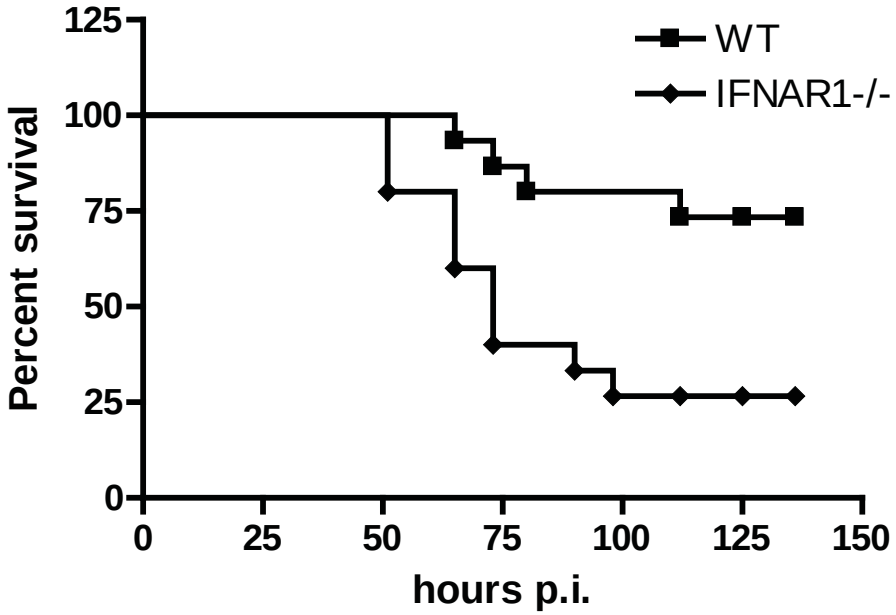
was measured in three independent infection experiments using ELISA. Values represent mean ± SD; n = 3.

**Figure S2. Heat-killed *S. pyogenes* causes induction of IFN-β in BMDMs.**
BMDMs were infected with equal amounts of live and heat-killed *S. pyogenes* (MOI100) or left untreated. After 4 and 6 h, supernatants were collected and IFN-β release was measured using ELISA. Mean ± SD; n = 3.

**Supporting information Figures**

Figure 1

A)

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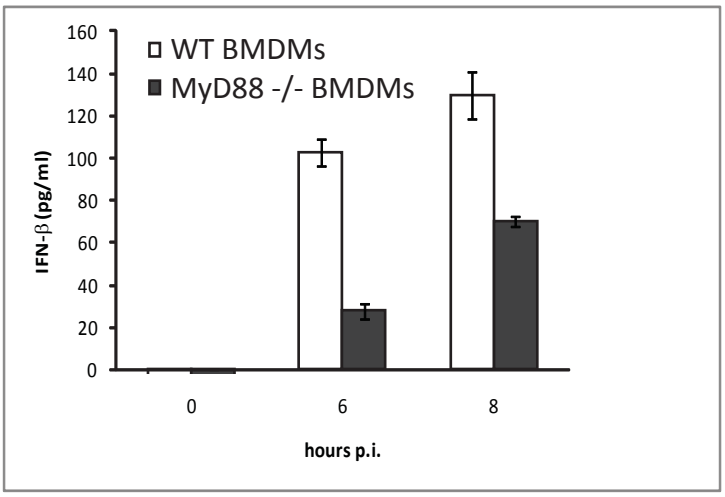


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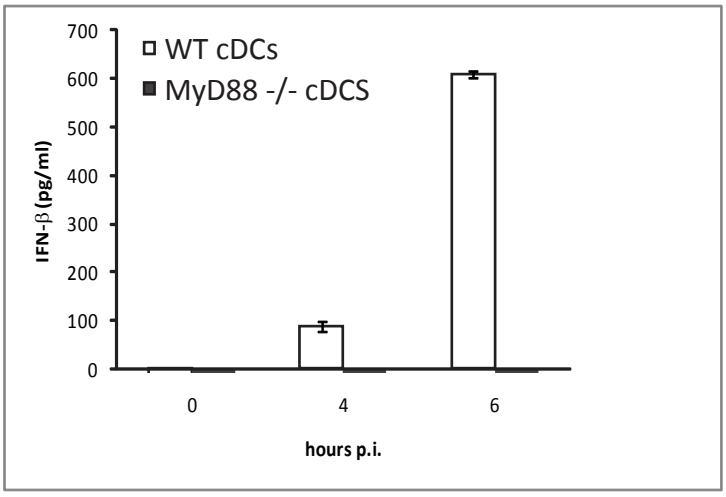


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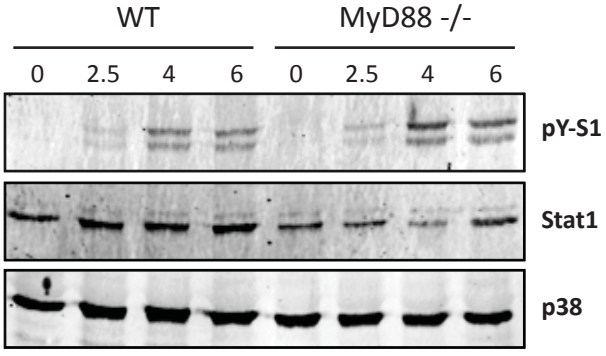


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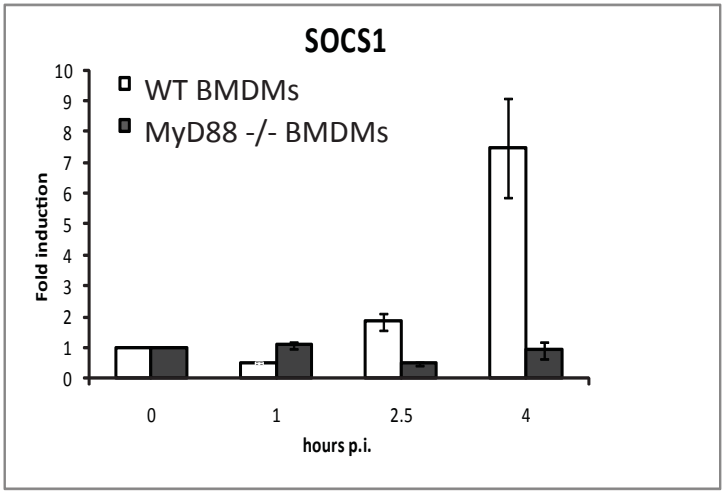
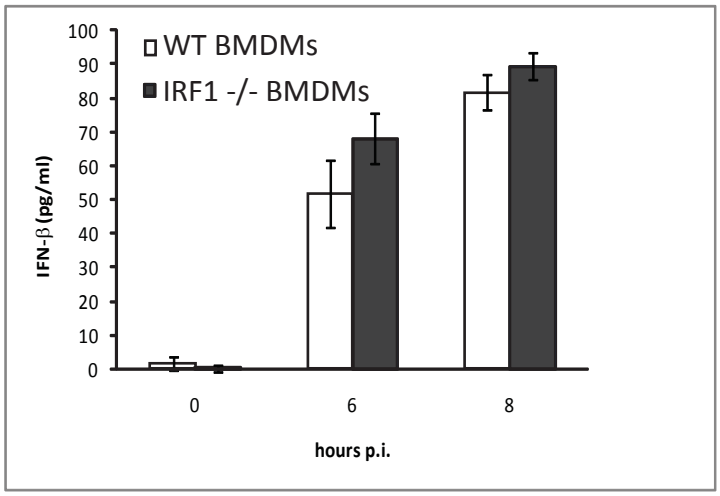


Figure 2

**A)**

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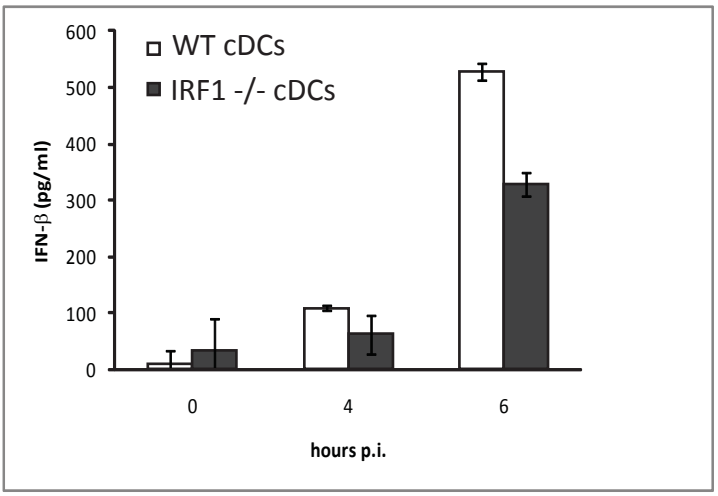


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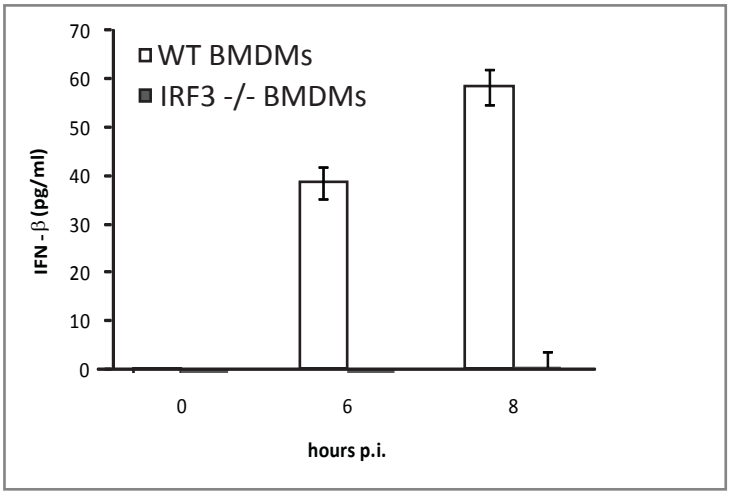


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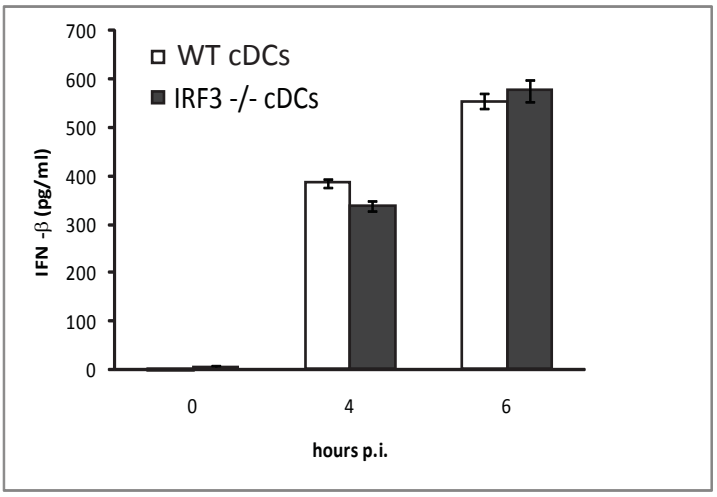
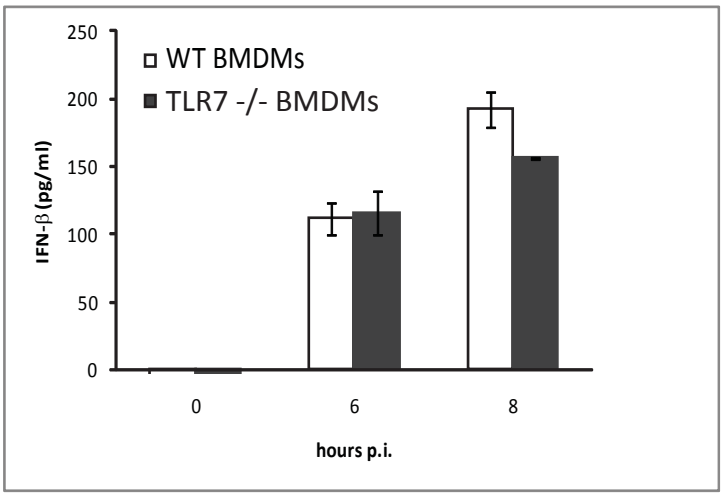


Figure 3

A)

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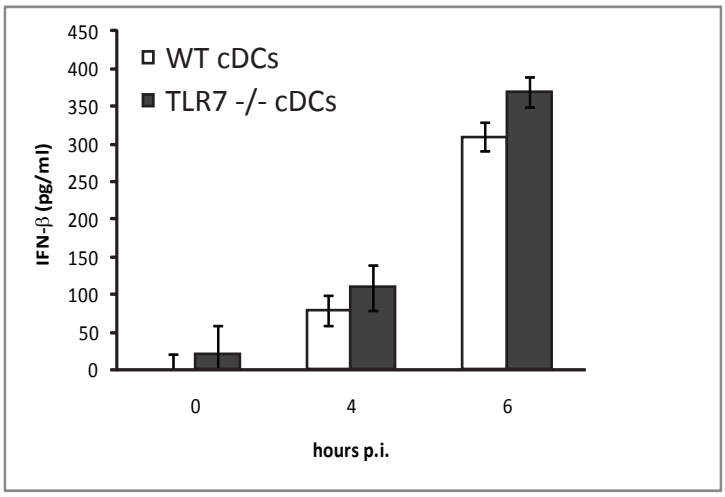


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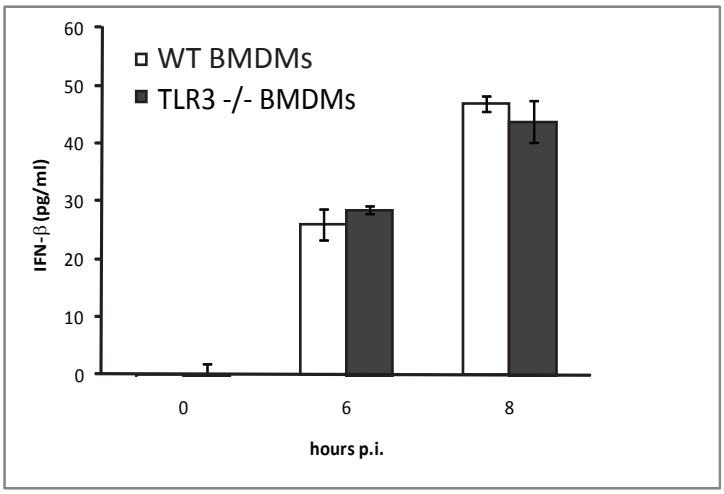


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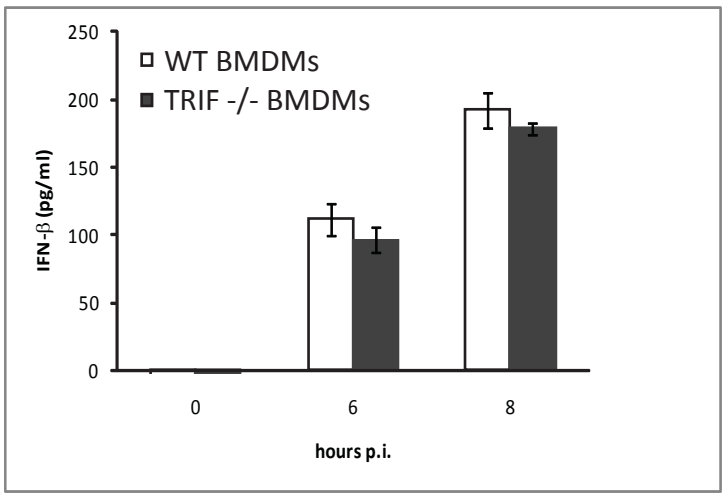


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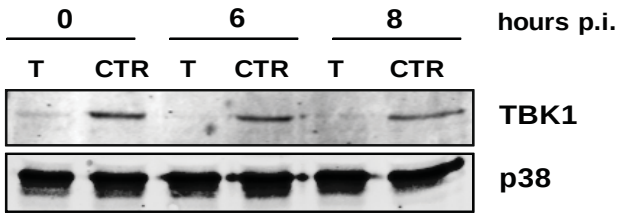


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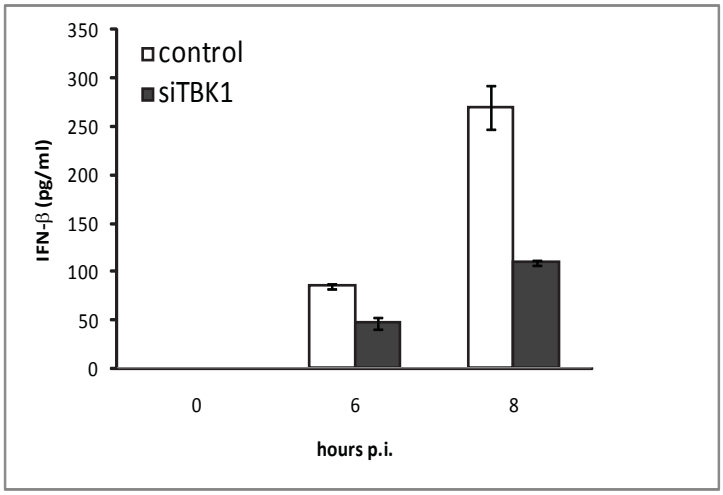
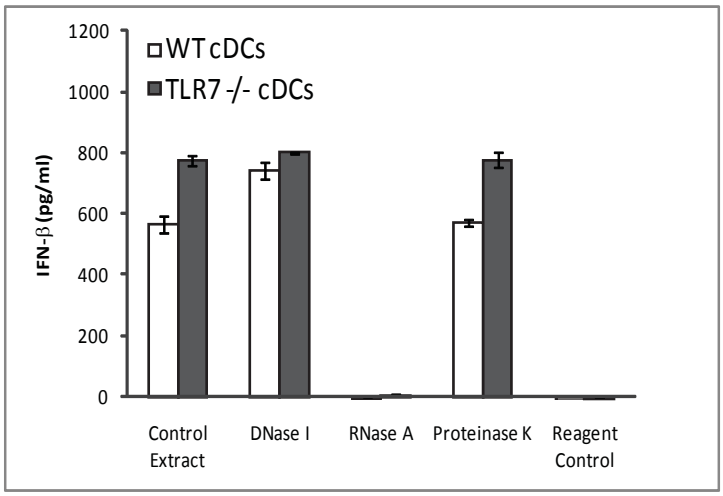


Figure 4

A)

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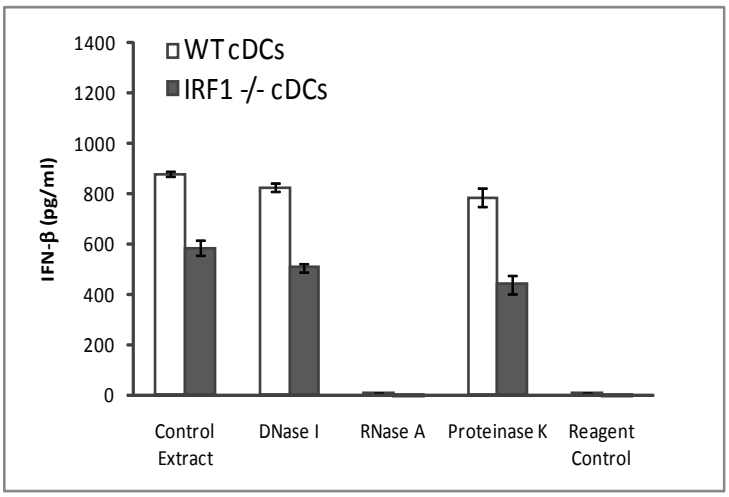


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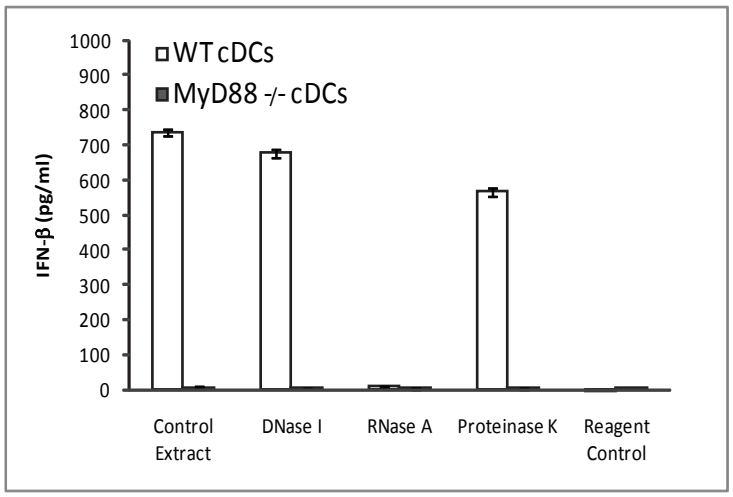


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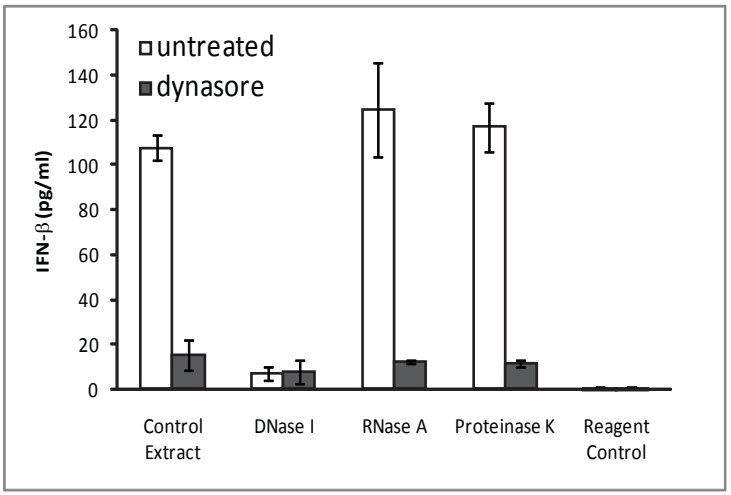


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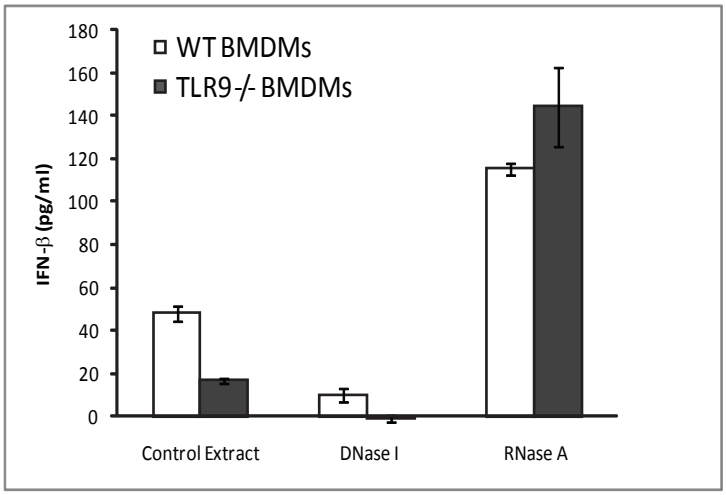
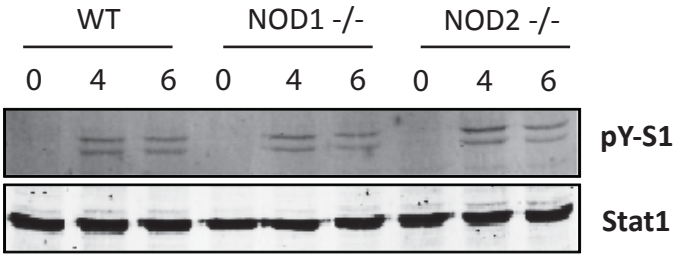


Figure S1

**A)**


WT
NOD1 -/-
NOD2 -/-
0 4 6 0 4 6 0 4 6
pY-S1
Stat1

**B)**

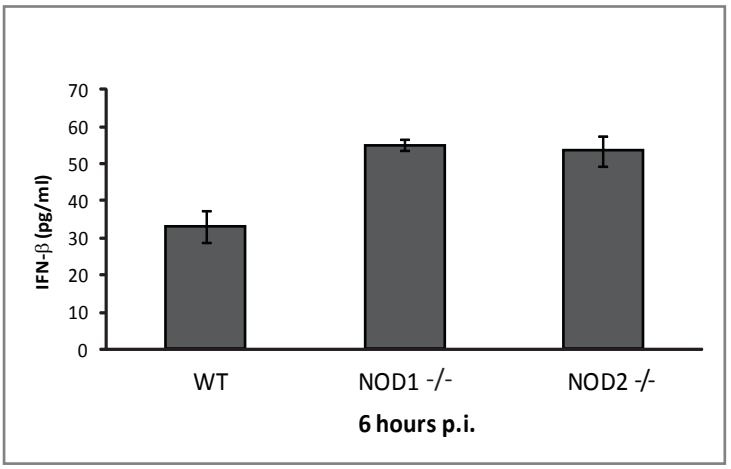

IFN-β (pg/ml)
70
60
50
40
30
20
10
0
WT
NOD1 -/-
NOD2 -/-
6 hours p.i.

Figure S2

**A)**


120
100
80
60
40
20
0
IFN-β (pg/ml)
live S. pyogenes
heat-killed S. pyogenes
0
4
6
hours p.i.

## 5.3. Unpublished Data

### 5.3.1. NOD-Proteins are not involved in the Recognition of *S. pyogenes* by BMDMs

Besides Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are known to be important pattern recognition receptors (PRRs). Among them, NOD1 and NOD2 are the best understood members of this family and have been shown to be important sensors of bacterial infection in the cytoplasm.

To assess the role of these two proteins during streptococcal infection, bone marrow derived-macrophages (BMDMs) from wild-type mice and mice deficient for NOD1 or NOD2 were infected with *S. pyogenes* with a multiplicity of infection (MOI) of 100.

After indicated time-points, whole cell extracts for Western blot were prepared and supernatants were taken for subsequent ELISA analysis. The activation of p38 and Stat1 as well as the production of TNF-α and interferon-β (IFN-β) served as read-out for the induction of an inflammatory response against *S. pyogenes* in these macrophages.

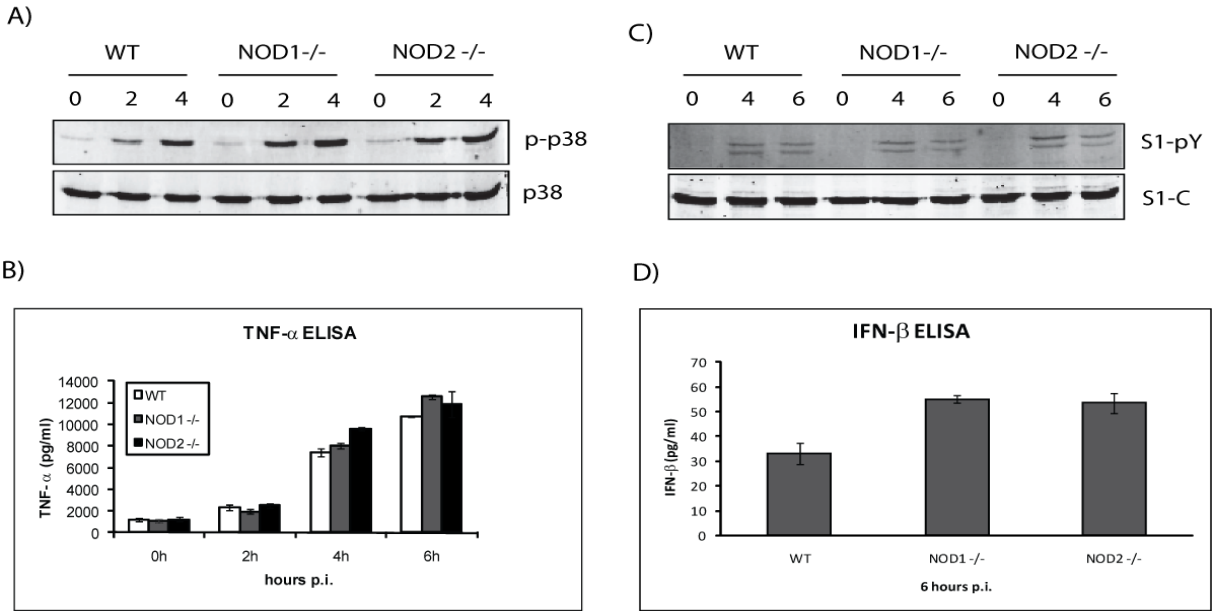



**Figure 5: BMDMs from NOD1 and NOD2-deficient mice display normal response to infection with *S. pyogenes*** BMDMs derived from NOD1-and NOD2-deficient and wild-type (WT) mice were infected with *S. pyogenes* (EC700, MOI 100) or left untreated. At indicated time-points, supernatants were collected and whole cell extracts were prepared. (**A**) Activation of p38 MAPK (p-p38) and (**C**) activation of Stat1 (S1-pY) were analyzed by Western Blot. Equal loading was determined by re-probing the membrane with an antibody specific for (**A**) total p38 or (**C**) to the C-terminus of Stat1. TNF-α (**B**) and interferon-β (**D**) production was measured by ELISA.

Infection of NOD1-, NOD2- deficient BMDMs with *S. pyogenes* led to the activation of p38 (p-p38) similar to wild-type macrophages. TNF-α production was also observed in both, NOD1- and NOD2-deficient macrophages as well as in wild-type macrophages.

IFN-β production after 6 hours of infection with *S. pyogenes* was detectable in wild-type, NOD1- and NOD2- deficient macrophages. The levels of IFN-β were higher in the NOD1- and NOD2-deficient macrophages compared to the wild-type ones.

These results indicate that NOD1 and NOD2, which are known to be important bacterial sensors within the cytoplasm, are not involved in the sensing and the induction of an immune response against *S. pyogenes*.

### 5.3.2. Streptococcal RNA induces TNF-α Production

The receptor molecule(s), which are inducing the inflammatory response against *S. pyogenes,* are not known yet. We wanted to address the question for the receptor of *S. pyogenes* not only by analyzing the host side, but also the pathogen site.

Therefore it was decided to use bacterial fractions in order to elucidate possible ligands recognized by macrophages. For this purpose, Streptococci grown to mid-log phase were sonicated and subsequently treated with DNase I, Proteinase K, RNase A or the combination of them. These fractions were then transfected into wild-type BMDMs using the transfection reagent DOTAP. This reagent is used for the transfer of negatively charged molecules (e.g. RNA, oligonucleotides, proteins) into cells by the engagement of the endosome/lysosome system (137) (138). DOTAP-mediated transfection of the control extract (i.e. sonicated *S. pyogenes*), the DNase I-, the Proteinase K- and a double-treated extract (DNase I and Proteinase K) led to the production of TNF-α, whereas macrophages transfected with RNase A-treated extracts did not show any TNF-α production. This was also observed when double-treated extracts (i.e. DNase I and RNase A or RNase A and Proteinase K) were used for transfection. Stimulation with heat-killed streptococci led to induction of TNF-α, which was higher than TNF-α levels obtained with transfection. Contamination of the buffers or DOTAP was excluded, since the reagent control did not induce any observable TNF-α production.

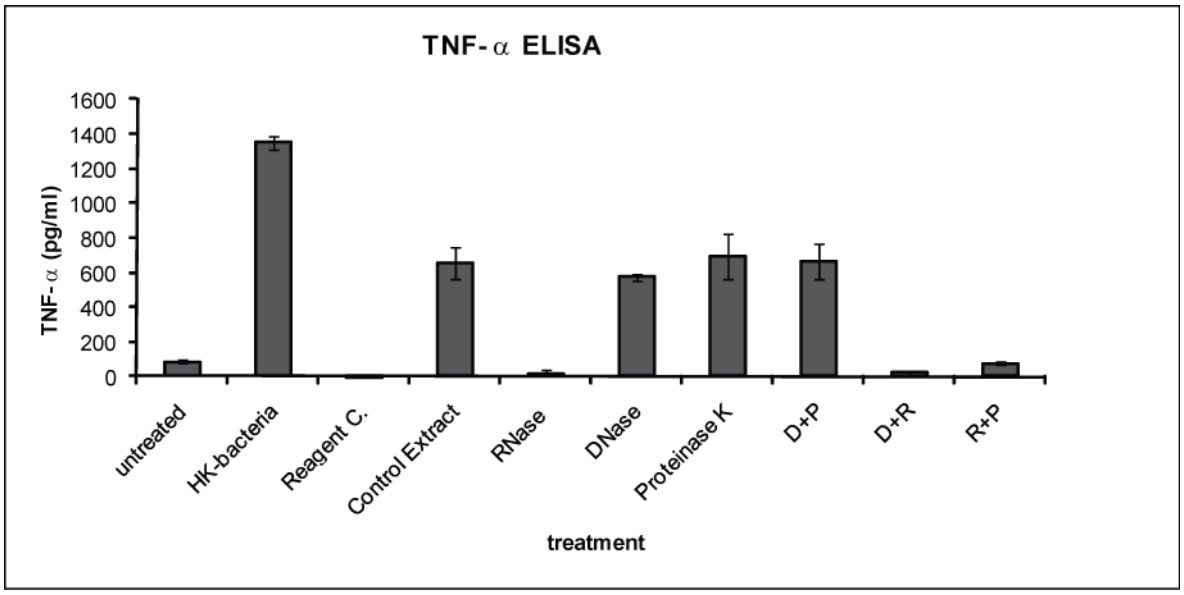



**Figure 6: TNF-alpha production requires the presence of streptococcal RNA in the bacterial extracts**
Streptococcal fractions were treated with either RNase A, DNase I, Proteinase K or combinations of them or left untreated (control extract). The fractions and a reagent control were transfected into wild-type BMDMs using DOTAP. Heat-killed bacteria served also as a control and were applied directly to the BMDMs. After 8 hours, supernatants were collected and TNF-α secretion was measured using ELISA.

These data suggest that streptococcal RNA is the ligand for a yet unknown receptor which is inducing an inflammatory response against *S. pyogenes*. Since this finding is only observable for extracts complexed with DOTAP that is known to deliver complexed material mainly into the endosome/lysosome system, and not when the extracts are directly added to the cells (data not shown), it is very likely that the streptococcal RNA is recognized by a pattern recognition receptor (PRR) within the endosome. However, using this method, one cannot exclude the possibility that parts of the bacterium enter compartments of the cells which cannot be reached during normal infection. Further, sonication of bacteria may also expose parts of the bacteria normally not accessible for recognition by cells of the innate immune system.

## 5.3.3. Strain Comparison

During *in vivo* challenges of mice with *S. pyogenes*, we realized that the ATCC 700294 strain (for simplicity EC700) was inducing lesions at the site of infection, but was not able to cause a lethal infection. The ISS 3348 strain (3348), which is like EC700 a streptococcal M1-serotype, was shown to be more virulent and was therefore chosen for the subsequent experiments (81).

Pilot-experiments revealed that the 3348 strain had a higher capacity to kill mice compared to EC700 (data not shown). Both strains share the same streptococcal M-type, namely M1, which is among the

most prevalent ones in terms of involvement in severe invasive diseases in humans (139). This finding raised the question, why the 3348-strain is more efficient in killing infected mice.

On that account, we wanted to compare these two strains for their ability to induce proinflammatory responses in BMDMs.

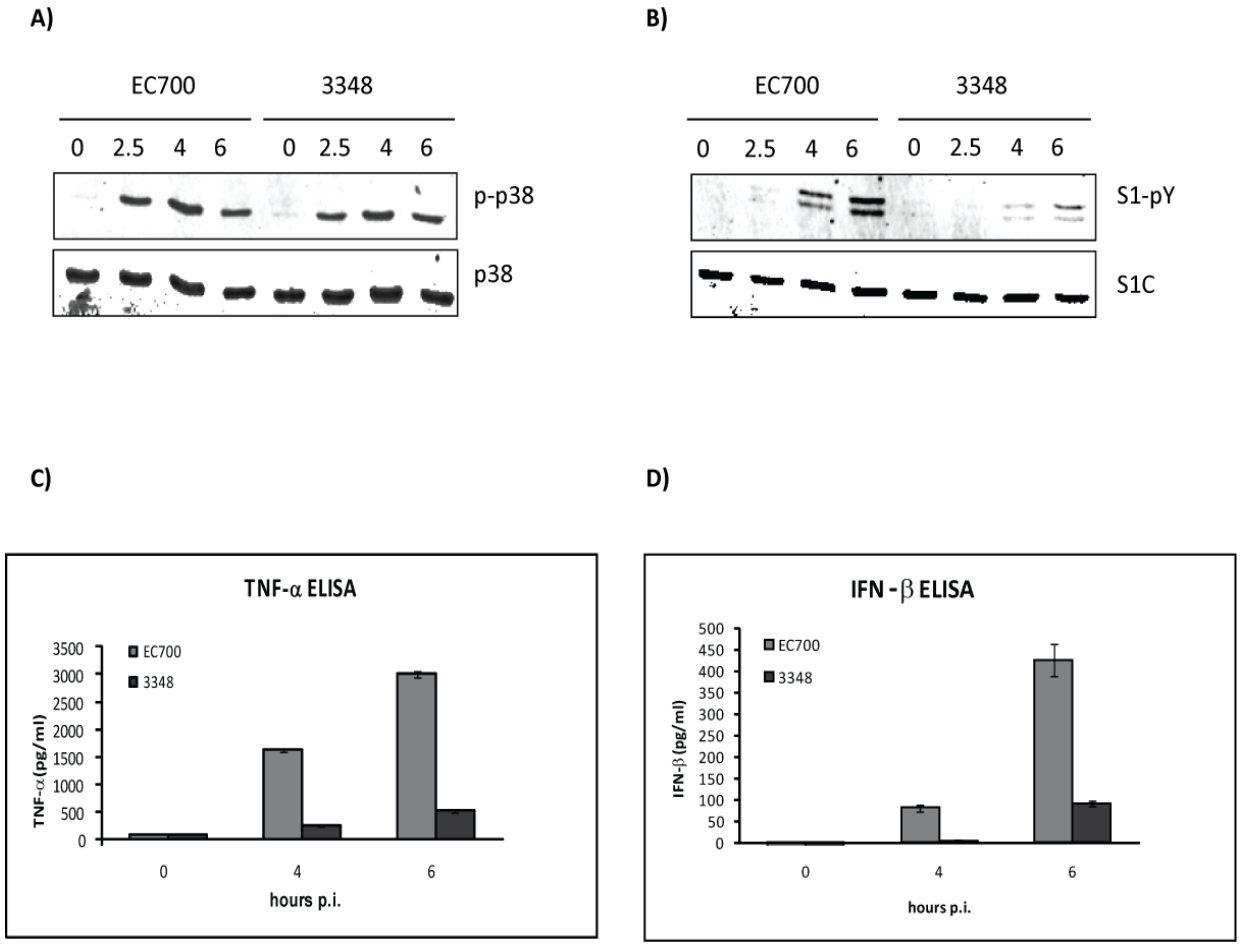



**Figure 7: Two Streptococcal strains of the same M1-serotype induce different levels of inflammatory cytokines** BMDMs were infected with either the *S. pyogenes* EC700 or the 3348 strain. At indicated time-points, whole cell extracts for Western Blot analysis were prepared and cell-culture supernatants were collected. (**A**) Activation of p38 MAPK (p-p38) and (**B**) activation of Stat1 (S1-pY) were analyzed using Western Blot. Equal loading was determined by re-probing the membrane with an antibody specific for (**A**) total p38 or to (**B**) the C-terminus of Stat1. TNF-α (**C**) and IFN-β (**D**) secretion was measured using ELISA.

Analysis of activation of the p38 MAPK using an antibody specific for the phosphorylated and therefore activated form of p38 revealed that both strains (EC700 and 3348) were able to induce similar activation of p38 MAPK in infected macrophages (Fig. 7, A).

The Stat1 phosphorylation at tyrosine 701, which serves as a read-out for activation of the JAK/STAT signaling pathway, was also observable, but in response to 3348, the phosphorylation was strongly reduced compared to the EC700 strain (Fig.7, B).The production of TNF-α and IFN-β induced by 3348

was much lower compared to EC700 (Fig. 7, C and D). The lower IFN-β production is consistent with the reduced Stat1 activation in 3348-infected BMDMs.

We thus conclude that the EC700 strain is a more potent activator of inflammatory responses in macrophages than the 3348 strain. The molecular reasons why these two strains differ in regard to the induction of proinflammatory cytokines and type I IFNs are not known yet. Therefore, it would be valuable to gain further information about the microbial features of these two streptococcal strains. For example, analysis of differences in the expression of virulence factors such as capsule, Streptolysins or analysis of cell wall composition would be important.

### 5.3.4. Heat-killed Streptococci

In this part we wanted to investigate, if induction of innate immune responses by *S. pyogenes* is dependent on living bacteria, which would suggest that the streptococcal ligand sensed by macrophages has to be actively secreted by the bacterium. Therefore heat-killed with living Streptococci were compared for their ability to induce TNF-α and IFN-β production, since these are the two major cytokines that have been shown to be produced in response to by *S. pyogenes*.

Bacteria were grown to mid-log phase, washed with PBS and were then heat-inactivated for 20 minutes at 70 °C. Equal amounts of living and heat-killed bacteria were then applied to macrophages (MOI 100). We analyzed the induction of immune responses in macrophages by heat-killed bacteria from both, the EC700 and the 3348 strain, since we were able to observe differences in the potential to activate inflammatory responses by living EC700 and 3348 (see results 5.3.3.).

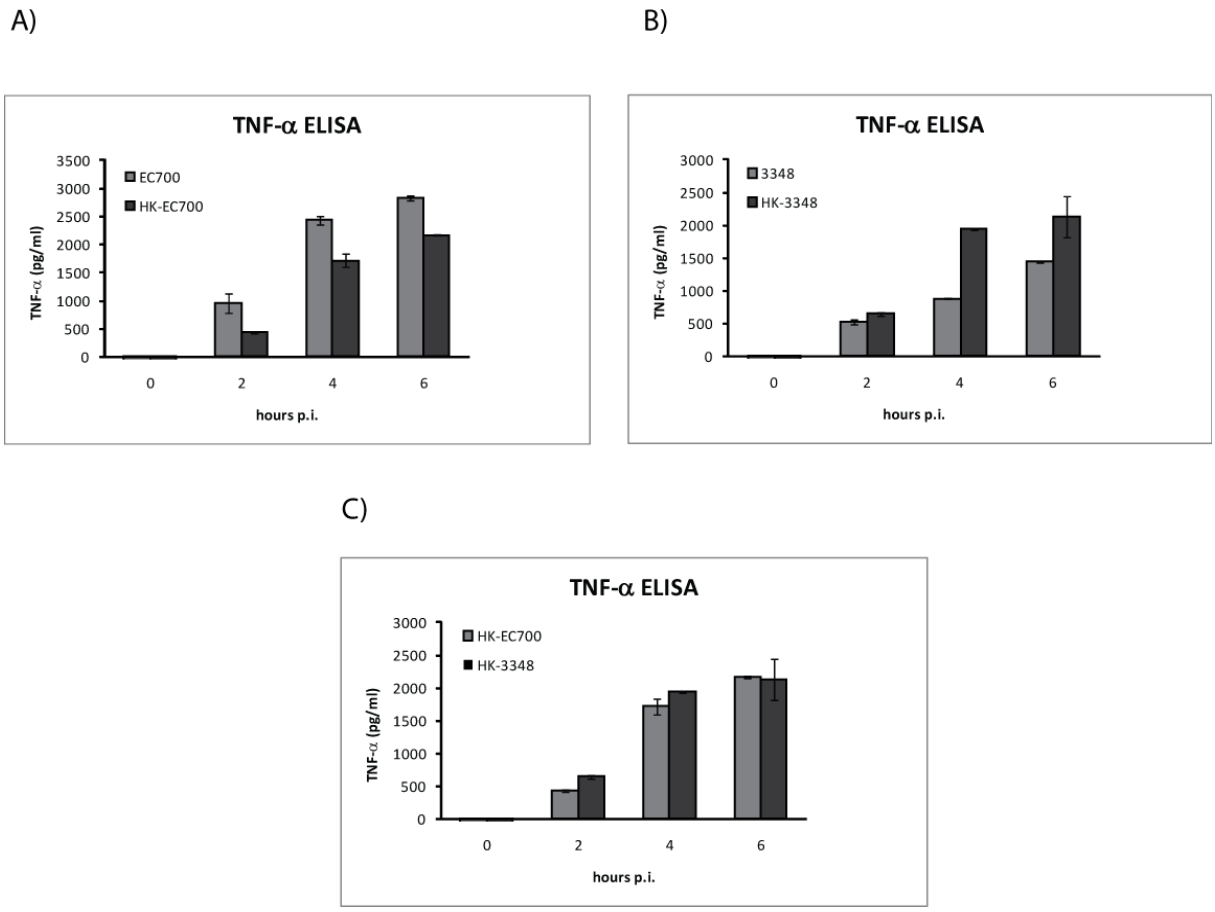



**Figure 8: Heat-killed Streptococci induce production of TNF-α**

BMDMs were infected with equal amounts (MOI 100) of living or heat-killed (HK) EC700 and 3348 strains. After indicated time-points, cell culture supernatants were collected and TNF-α release by BMDMs was measured using ELISA. TNF-α production induced by (**A**) the living EC700 strain was compared to heat-killed (HK) EC700, in (**B**) the living 3348 strain with HK-3348 and in (**C**) HK-EC700 with HK-3348.

Infection of macrophages with both, the streptococcal EC700 strain and heat-killed (HK) EC700 led to the production of TNF-α, although TNF-α levels were lower in macrophages treated with heat-inactivated bacteria (Fig. 8, A).

Surprisingly, comparison of the living and heat-killed 3348 strain revealed that TNF-α production in response to the heat-killed bacteria was higher than after infection with living bacteria (Fig. 8, B). Direct comparison of the heat-killed strains revealed that induced TNF-α levels in macrophages appeared quite similar (Fig. 8, C).

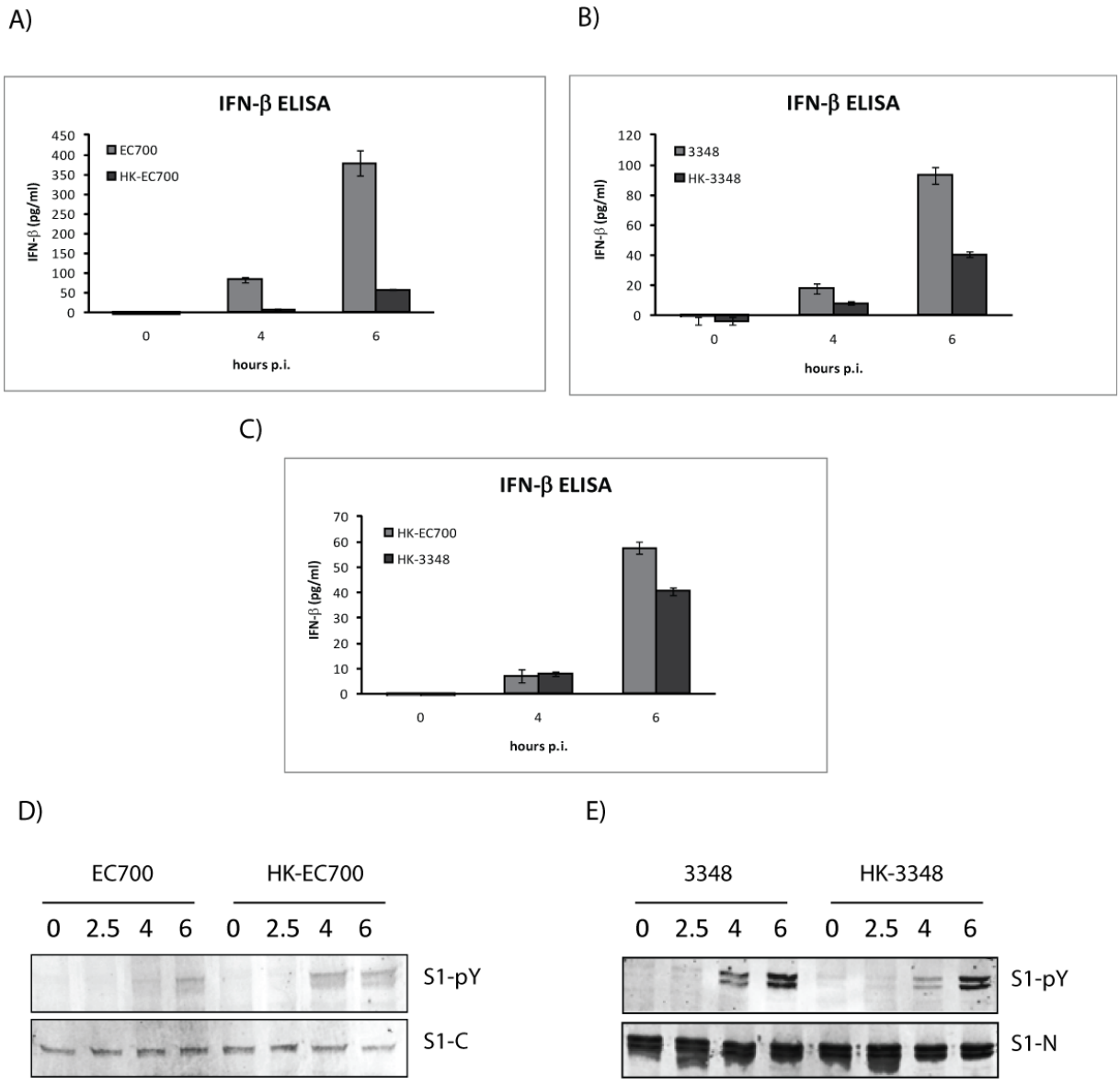



**Figure 9: Heat-killed Streptococci cause type I IFN production, although less than live bacteria, and the activation of Stat1**

BMDMs were infected with equal amounts (MOI 100) of living or heat-killed (HK) EC700 and 3348 strains. After indicated time-points, cell culture supernatants were collected and IFN-β release by BMDMs was measured using ELISA. TNF-α secretion from (**A**) the living EC700 strain was compared to heat-killed (HK) EC700, in (**B**) the living 3348 strain was compared with HK-3348 and in (**C**) HK-EC700 was compared with HK-3348. (**D**) Stat1 activation in response to living and HK-EC700 as well as to (**E**) living and HK-3348 in BMDMs was analyzed using Western Blot with a specific antibody for Tyrosine701-phosphorylated Stat1. Equal loading was checked by re-probing the membrane with an antibody specific for (**D**) the C-terminus or (**E**) the N-terminus of Stat1.

Infection with heat-killed Streptococci also led, as during infection with living bacteria, to the production of type I IFNs. The IFN-β production was lower in macrophages treated with HK-EC700 (see Fig. 9, A) or HK-3348 (B) than with the respective living streptococcal strains. Direct comparison of IFN-β secretion induced by the HK-strains revealed that after 4 hours of infection both strains

produced nearly the same amount of IFN-β whereas after 6 hours, the HK-EC700 strain was more efficient in IFN-β induction (Fig. 9, D and E).

Further, the capacity of living or heat-killed bacteria of both strains to activate the Jak/Stat signaling pathway was assessed. Therefore the activation of the Stat1 protein was determined by Western blotting with an antibody specific for the phosphorylated form of Stat1. Analysis of Stat1 activation in macrophages infected with living and heat-killed bacteria from the EC700 strain revealed that Stat1-phosphorylation appeared similar (Fig. 9, D). This was also the case when macrophages were infected with either living or the HK-3348 streptococci (Fig. 9, E).

Given these results, we conclude that TNF-α and IFN-β secretion occurs also in response to HK bacteria derived from both strains. The fact that HK-streptococci from the 3348 strain are able to induce a higher TNF-α secretion than living bacteria was an unexpected finding. A possible explanation for this phenomenon could be that during the heat-killing process, specific parts of the bacteria present in the 3348 strain only get exposed, which are not accessible under normal conditions.

# 6. Final Discussion

## 6.1. Production of TNF-α and IL-6 in Response to *S. pyogenes*

Recognition of an invading pathogen by a pattern-recognition receptor (PRR) expressed by the host and the subsequent activation of an effective immune response in order to eradicate the unwanted intruder is a fundamental process in innate immunity.

In the case of the human pathogen *S. pyogenes*, the molecules responsible for its recognition by cells of the innate immune system have still to be identified.

Thus, the main aim of this thesis was the analysis of the involvement of the known PRRs in *S. pyogenes* recognition and the investigation of the signaling pathways that are initiated after recognition.

For this purpose, it was decided to use bone marrow-derived macrophages (BMDMs) for investigations, since these cells constitute the first line of defense against invading pathogens.

Macrophages are important mediators of innate immunity. Once activated, they kill pathogens by phagocytosis, become efficient antigen-presenting cells (APCs) and are known to produce inflammatory cytokines and growth factors that trigger a strong inflammatory response.

It is very likely that during an infection with *S. pyogenes* this cell type is among the first encountered by the bacterium. Further, it has been shown using an experimental mouse model of streptococcal infection that macrophages play an important role during the early immune responses against *S. pyogenes* (128). Dendritic cells have also been shown to contribute to host defenses against *S. pyogenes* by preventing bacterial dissemination and production of IL-12 (129).In contrast, natural killer (NK)-cells are participating in the progression of septic shock in *S. pyogenes*-infected mice by amplification of the immune response (140).

We were able to show that infection of BMDMs with *S. pyogenes* leads to the activation of p38 mitogen-activated protein kinase (MAPK), NF-κB and the production of proinflammatory cytokines such as tumor necrosis factor- α (TNF-α) and interleukin (IL)-6 (130). These proteins are well known for being activated during the inflammatory response after the recognition of microbial pathogens. Activation of p38 MAPK and NF-κB in response to *S. pyogenes* has also been observed in human respiratory epithelial HEp-2 cells (141). Since both pathways are activated after Toll-like receptor (TLR) engagement, we decided to analyze murine macrophages deficient for the prototype bacterial receptors TLR2, TLR4 and TLR9. Infection of these macrophages led to a normal activation of p38 MAPK and NF-κB and an undisturbed production of TNF-α and IL-6 in response to *S. pyogenes* (130). Redundancy of these three receptors was excluded by analysis of TLR2/4/9-deficient BMDMs. These

results led to the assumption that the main bacterial TLRs are not involved in the recognition of *S. pyogenes* and are dispensable for the induction of an inflammatory response against this bacterium. It is therefore elusive why TLR2, known to sense peptidoglycan (PGN) from Gram-positive bacteria, is not involved in the detection of the Gram-positive bacterium *S. pyogenes* (142).

Moreover, the involvement of the important TLR-adaptor protein myeloid differentiation primary response gene 88 (MyD88) in *S. pyogenes*-induced inflammatory signaling was analyzed
This protein is used for initiation of downstream signaling by almost all TLRs, except TLR3 (21). We found that initiation of inflammatory signaling during streptococcal infection fully depends on this adaptor protein, since MyD88-deficient BMDMs neither activate p38 MAPK and NF-κB nor they produce TNF-α and IL-6 in response to *S. pyogenes* infection.
These findings were supported by a recent *in vivo* study, where MyD88-deficient mice were infected with *S. pyogenes*. The MyD88-deficient mice showed diminished levels of proinflammatory cytokines such as TNF-α, IL-12 and IFN-γ in response to infection with *S. pyogenes* and succumbed much earlier to infection as wild-type control mice (143) .
Members of the IL-1R family (IL-1RI, IL-18R and ST2) also have been described to signal via MyD88 leading to the activation of NF-κB, p38 MAPK and JUN amino-terminal kinase (JNK) (144). To exclude the involvement of IL-1 in the induction of the inflammatory signaling during streptococcal infection, BMDMs from IL-1R-deficient mice were analyzed. Since we were not able to observe any differences in the activation of proinflammatory signaling in the IL-1RI-deficient BMDMs, we came to the conclusion that the signaling events induced by *S. pyogenes* are also not dependent on the IL-1 receptor (130).

NOD1 and NOD2, both members of the nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR) family, have been reported to be important cytosolic sensors of muropeptides from bacterial cell walls (18). Upon recognition of an intracellular pathogen, NOD1 and NOD2 get activated thus leading to the activation of NF-κB, MAPKs such as p38 MAPK, JNK and extracellular-signal-regulated kinase (ERK) (30).
Although *S. pyogenes* is an extracellular bacterium and is therefore not able to replicate within the cytosol, there is evidence that *S. pyogenes* is able to evade lysosomal killing in epithelial cells with the help of Streptolysin O (SLO) (111). Studies using nonphagocytic cells showed that *S. pyogenes* escapes from the endosomes into the cytoplasm where it is trapped in autophagosome-like compartments and degraded upon fusion with lysosomes (145) (146).

Further, it has also been shown that *S. pyogenes* is able to survive within the cytoplasm of phagocytic cells by escaping from the phagosome by a yet unknown mechanism (147). However, it is very likely that also in this case Streptolysin O might be involved in evasion.

To investigate the involvement of NOD1 and NOD2 in sensing of *S. pyogenes*, NOD1-and NOD2-deficient BMDMs were infected with *S. pyogenes* and induction of proinflammatory signaling was monitored. Our experiments revealed that the two NOD-proteins are dispensable for the induction of an inflammatory response against *S. pyogenes*, since NOD1-and NOD2-deficient macrophages show a similar activation of p38 MAPK as well as production of TNF-$\alpha$ and IL-6 as wild-type BMDMs (see figure 5).The fact that initiation of signaling in response to *S. pyogenes* absolutely requires the adaptor protein MyD88 also strengthens the assumption that NOD proteins are not involved in sensing this bacterium, since it is known that MyD88 is not required for NOD-induced signaling. The dependency on MyD88 leads to the further assumption that a member of the TLR-family could be involved in sensing of *S. pyogenes*.

It is also of general interest, whether the ligands of the PRRs are produced by the bacterial cells upon infection. To analyze this question, Streptococci were grown to mid-log phase and then heat-inactivated for 20 min at 70° C. Living and heat-killed (HK) bacteria were then used to infect BMDMs at equal ratios (MOI 100) and production of TNF-$\alpha$ by BMDMs was measured.
It turned out that HK bacteria were also able to induce an inflammatory response in BMDMs.
When the EC700 strain was used, TNF-$\alpha$ levels in response to HK bacteria were lower than with living bacteria. Surprisingly, we observed the opposite after infection with HK-3348 bacteria, since TNF-$\alpha$ levels were higher compared to the production initiated by the living 3348 strain. Additional experiments will be needed to find out the reason for this observation. One possible explanation for this observed phenomenon is that the 3348 strain somehow might be able to actively prevent the production of TNF-$\alpha$.
The fact that heat-killed bacteria from both strains trigger an inflammatory response in BMDMs leads to the assumption that recognition of bacteria does not depend on the active secretion of the potential ligand. So the recognition of a secreted streptococcal virulence factor serving as ligand for the unknown receptor can be excluded.

The analysis of the receptor molecules responsible for the recognition of *S. pyogenes* can be addressed from two sites. In the first part of my thesis we aimed to identify the host receptor sensing *S. pyogenes*. However, we were also interested in the bacterial ligand that elicits the host response.

Since it is known that Toll-like receptors recognize various different molecules including nucleic acids, sugars and lipoproteins, we decided to perform experiments using streptococcal fractions.

Streptococci were grown to mid-log phase, were sonicated and afterwards treated with DNase I, Proteinase K, RNase A or the combination of them.

Stimulation of BMDMs with these extracts did not lead to a detectable production of TNF-α (data not shown). Therefore it was decided to transfect the streptococcal extracts into the BMDMs using DOTAP. DOTAP is a liposomal transfection reagent which delivers the associated complexes mainly to endosomes. BMDMs transfected with extracts enzymatically treated with RNase A did not secrete TNF-α, whereas extracts treated with DNase I, Proteinase K as well as an untreated control extract, led to production of TNF-α (Fig. 6).

These results lead to the conclusion that streptococcal RNA is the ligand that is recognized by a yet unknown receptor probably located in the endosomes of BMDMs.

Recognition of streptococcal RNA then leads to a proinflammatory response as indicated by the production of TNF-α. Four TLRs have been reported to be located within endosomes: TLR3, TLR7, TLR8 and TLR9 (18). All of them have been described to recognize nucleic acids derived from viruses and bacteria. TLR3 is known to recognize double-stranded RNA derived from viruses. TLR7 recognizes synthetic imidazoquinoline-like molecules, guanosine analogs such as loxoribine, single stranded RNA (ssRNA) derived from various viruses and small interfering RNA. Human TLR8 shares a high homology to TLR7 and has been shown to sense ssRNA and imidazoquinoline. However, ligands and function of murine TLR8 still have to be determined. CpG DNA motifs present in bacterial and viral genomes are detected by TLR9 (27).

We were able to exclude the involvement of TLR9 (130) and TLR7 (preliminary data, not shown) in the detection of *S. pyogenes* by BMDMs under normal infection conditions. Recognition of *S. pyogenes* by TLR3 can also be excluded, since this receptor uses the adaptor protein TRIF for downstream signaling upon activation, whereas our data show that induction of inflammatory signaling is fully dependent on MyD88.

The treatment of BMDMs with enzymatically modified streptococcal extracts and the use of DOTAP are not comparable to a normal infection experiment for following reasons: (i) sonication of Streptococci can expose PAMPs which are not accessible for PRRs under normal conditions; (ii) transfection using DOTAP might deliver the extracts not only to the endosomes but also to the cytoplasm, where other receptors than TLRs are recognizing them; (iii) DOTAP is known to form large complexes. Therefore, DOTAP could complex large amounts of ligand leading to an artificial receptor clustering.

Taken together, our results suggest that streptococcal RNA is recognized by BMDMs leading to induction of a proinflammatory response. As this response is fully dependent on the TLR-adaptor protein MyD88, the involvement of a RNA-recognizing TLR in detection of *S. pyogenes* is likely.
During this thesis, we were able to exclude all RNA-sensing TLRs (TLR3, TLR7) with exception of TLR8. This receptor has been shown to respond to the TLR7 ligand imidazoquinoline resiquimod (R848) in the human system, but is believed to be non-functional in mice, since HEK293 cells transiently transfected with murine TLR8 did not respond to R848 (148). A further proof for the lacking functionality of TLR8 in mice is the finding that TLR7-deficient mice did not respond to R848, although TLR8 was present (149). A more recent study suggests that murine TLR8 is indeed functional, since imidazoquinoline immune response modifiers (IRMs) in combination with oligodeoxynucleotides (ODNs) led to a NF-$\kappa$B response in HEK293 cells transfected with murine TLR8. In addition, stimulation of cells derived from TLR7-and TLR9-deficient mice responded to the IRM/ODN combination, whereas MyD88-deficient cells did not (150). However, there is no data available showing the involvement of murine TLR8 in host responses against pathogens.
Although it cannot excluded that this receptor is sensing *S. pyogenes* in BMDMs, it is rather likely that this bacterium is recognized by a yet unidentified receptor molecule.

## 6.2. Mechanisms of Induction of Type I Interferon Signaling by *S. pyogenes*

The production of interferons is a mechanism of the innate immune system to control infections caused by intracellular pathogens. Although initially described to be produced in response to viral infection, an increasing body of evidence shows that these cytokines play also an important role in the host response against bacterial infection (151).
Type I IFNs are produced in response to both, Gram-positive and Gram-negative bacteria, such as *Chlamydia* spp*., Salmonella typhimurium, Shigella flexneri* and *Escherichia* spp. (44). Type I IFN production in response to Gram-positive bacteria, is best studied in *Listeria monocytogenes.* This pathogen has a predominantly intracellular lifestyle, which is achieved by the escape from phagocytic vacuoles into the cytoplasm using Listeriolysin O (LLO), a pore-forming cytolysin. Once in the cytoplasm, the bacterium is recognized by a yet unknown receptor, leading to the induction of the IFN-$\beta$ gene and also initiation of JAK/STAT signaling depending on TBK1 and IRF3 in BMDMs (152).

We were able to show that the extracellular, Gram-positive bacterium *S. pyogenes* also induces the activation of Stat1 in murine BMDMs and that this event occurs without the involvement of the two streptococcal cytolysins Streptolysin O (SLO) and Streptolysin S (SLS)(130). Further, preliminary data

generated by us suggest that IFN-β production in response to *S. pyogenes* also occurs in the absence of SLS and SLO, albeit to a lesser extent. The finding that stimulation of BMDMs with heat-killed streptococci also leads to an IFN-β production, albeit to a lesser extent, supports the fact that these cytolysins, which are secreted by living cells only, are not needed for IFN-production (Fig. 9). The reason why heat-killed streptococci induce a reduced IFN-β production compared to living bacteria cannot be explained by us yet and was not expected, since Stat1 activation in response to heat-killed *S. pyogenes* was similar to the activation induced by living bacteria (see Fig. 9).

Further we could demonstrate that in BMDMs, type I IFN production is absolutely dependent on IRF3 and TBK1. Interestingly, we did not observe this IRF3-dependency in *S. pyogenes*-infected conventional dendritic cells (cDCs). This goes in line with a study that shows that in cDCs, infected either with *S. pyogenes* or Group B Streptococcus (GBS), production of IFN-β does not depend on IRF3 (134). Further we were able to show that in cDCs infected with *S. pyogenes*, IFN-β production is partially dependent on IRF1, whereas infection of IRF1-deficient BMDMs does not lead to any observable differences in the production of IFN-β (see manuscript, Fig. 2A,B).

In addition, we found that the presence of the adaptor protein MyD88 is crucial for the production of IFN-β in *S. pyogenes*-infected cDCs, but not in BMDMs, where just a partial dependency is observable. Similar results were obtained when GBS was used to infect these two cell types (134). TRIF, the second major adaptor protein involved in TLR signaling was found to be dispensable for production of IFN-β in BMDMs (see manuscript, Fig. 3D) and as well in cDCs, as shown by a recent report (134).

Obviously, the TBK1/IRF3 axis plays an important role in the transcriptional regulation of type I IFN production upon bacterial infection in *S. pyogenes*-infected macrophages. However, little is known about the receptor upstream of TBK1.

We wanted to obtain more information about the nature of the ligand that is inducing the IFN production in response to *S. pyogenes*, therefore streptococcal extracts either depleted for DNA, RNA or Proteins were transfected into BMDMs and cDCs with DOTAP.

Our experiments revealed that streptococcal DNA is launching an IFN-β response in BMDMs, whereas in conventional DCs (cDCs) streptococcal RNA seems to trigger IFN-β production (see manuscript, Fig. 4A). These results are consistent with the finding that IFN-β production in response to GBS appears to be induced in DC by bacterial RNA, whereas in macrophages bacterial DNA is the IFN inducer (135) (134). DOTAP employs the endosomal pathway for the delivery of molecules into the cell. To investigate if TLR9, one of the TLRs located in the endosomes and known to recognize bacterial DNA, is involved in the recognition of streptococcal DNA, experiments using TLR9-deficient BMDMs were performed. It turned out that recognition of streptococcal DNA occurs without the

involvement of TLR9, since TLR9-deficient BMDMs showed an undisturbed IFN production in response to streptococcal DNA (see manuscript, Fig. 4E).

Since we were able to exclude TLR9, the only DNA-sensing receptor within the endosomes, as possible receptor inducing an IFN-response in BMDMs, other DNA-sensing PRR might be involved in the recognition of *S. pyogenes*.

One possibility is that streptococcal DNA is recognized by DNA-dependent activator of IFN-regulatory factors (DAI), which has been shown to recognize various DNAs, including synthetic B- or Z-form DNA, or bacterial, viral or mammalian DNA, albeit with different efficiencies(33). Binding of DNA to DAI results in an increased interaction of DAI with IRF3 and TBK1, leading to type I IFN and proinflammatory cytokine production. However, studies using DAI-deficient cells revealed that those cells responded normally to poly(dA:dT) and plasmid DNA and they also showed an unimpaired response to a DNA virus. These findings lead to the conclusion that DAI has just a redundant role in sensing of DNA (153).

Recently, a new DNA-sensing pathway has been identified, in which RNA polymerase III efficiently triggers the production of type I IFN by transcribing microbial DNA templates into dsRNA containing 5'-triphosphate, which is a potent activator of RIG-I (154) (155). It has been shown that RNA Pol III converts the DNA within the cytoplasm, making it a true cytoplasmic pathway (155). The authors applied the RNA Pol III inhibitor ML-60218 to test, if infection with several pathogens is still inducing IFN-β production. They observed that Pol-III inhibition leads to a reduced induction of IFN-β by *Legionella pneumophila*, DNA viruses such adenovirus, HSV-1 and EBV, but not with RNA viruses (Sendai virus) (155). Experiments using the ML-60218 inhibitor might also provide new insights into the recognition of *S. pyogenes*. Previously, stimulator of IFN genes (STING) has been described to be a potent activator of IRF3 and seems to function downstream of both, the RNA and DNA recognition pathways, since STING-deficient cells lose their ability to mount an efficient immune response against intracellular B-DNA and IFN-stimulatory DNA (ISD, a synthetic 45-mer dsDNA of random sequence lacking contiguous CpG motifs) or to infection with *L. monocytogenes* and herpes simplex virus type 1 (HSV-1) (153). It is possible, that one of these three cytoplasmic DNA-receptors (STING, DAI, RIG-I) are involved in the sensing of streptococcal DNA in BMDMs. Thus, further experiments might reveal the mechanism by which the *S. pyogenes*-induced IFN response is induced in BMDMs.

In the case of cDCs, where RNA seems to be the ligand responsible for the induction of IFN-β production, TLR7-deficient cells were analyzed. TLR7 is also located within endosomes and known to recognize RNA. Our experiments revealed that TLR7 is dispensable for the recognition of streptococcal RNA in cDCs. In addition TLR3, a sensor for double-stranded RNA located in the

endosomes, was shown in this thesis to be not relevant for IFN-β production in *S. pyogenes* infected BMDMs and also in cDCs, being in accordance with other studies (134).

In cDCs, other RNA-sensing PRRs can serve as possible candidates, such as the family of RIG-like receptors (RLRs) consisting of RIG-I, MDA5 and LGP2. They have been described to sense intracellular RNA species leading to an IFN-response. But these molecules can be ruled out as receptors for *S. pyogenes* in cDCs, since they do not rely on the MyD88 adaptor protein for their signaling.

As already discussed in the previous section, the experiments using DOTAP-transfected streptococcal fractions may not resemble a normal infection experiment. Sonication might produce PAMPs that would not be recognized during normal infection and DOTAP might delivers PAMPs not only into endosomal compartments but also into the cytosol where other receptors might recognize the ligand. However, the DOTAP experiments are in good agreement with results obtained with HK so that all in all, these experiments provide a good hint in the search for possible receptors and molecules that might serve as ligands.

## 6.3. The Role of Type I Interferons during Streptococcal Infection

Interferon production during viral infection has been shown to be protective for the host but the role of type I interferons during bacterial infection is more complex as documented in extensive studies during the last years.

Upon infection of mice with *Salmonella typhimurium* for example, production of type I IFNs has been shown to be beneficial for survival of infected mice (156), whereas in the case of *Listeria monocytogenes*, type-I IFN production is detrimental (157) (158, 159). Both bacteria have an intracellular lifestyle and are able to replicate within the host cell which is known to be important for IFN-β production.

However, an increasing body of evidence suggests that type I IFN production is also induced in response to bacteria with an extracellular lifestyle (i.e. bacteria that are not able to replicate within the host cell). Our group was able to demonstrate that mouse innate immune cells produce IFN-β in response to an extracellular Gram-positive bacterium (*S. pyogenes*)(130). Subsequently, it has been shown that group B streptococcus (GBS) is able to induce IFN-β production in BMDMs and cDCs, and that IFN-β is critical for host defense against these bacteria *in vivo* (133) (135).

To investigate the role of type I IFN-production during *S. pyogenes* infection, it was decided to establish a mouse *in vivo* model. The subcutaneous infection route was chosen since it is the most physiologically relevant model for invasive streptococcal infections (160) (161) (105) (108). To study

the contribution of the innate immune response in this infection model, the survival of mice was monitored not longer than day 6 after infection.

*S. pyogenes* is a strictly human pathogen and only few strains can establish an infection in mice (105), therefore a murine model does not reflect natural infection by this bacterium. This limited host specificity is explained by the existence of several virulence factors which were shown to function only in human cells. To circumvent this problem, a human CD46 transgenic (Tg) mouse model of skin and soft-tissue infection with *S. pyogenes* has been established recently. This model closely resembles human infection, since the complement regulatory protein CD46 is implicated as receptor for several pathogens including *S. pyogenes*. Human CD46-expressing Tg mice are more susceptible to *S. pyogenes* infection as non-Tg ones (162).

Another transgenic mouse model has been established by inducing human plasminogen into mice in order to bypass the restricted host specificity of *S. pyogenes*. Plasminogen is a human blood-clot dissolving protein, which can be activated by the *S. pyogenes*-derived virulence factor streptokinase. Streptokinase is highly specific for human plasminogen, exhibiting little or no activity against other mammalian species, including mouse. These Tg mice showed an increased mortality when infected with *S. pyogenes* and this susceptibility was dependent on bacterial streptokinase expression (163).

The genetic background of mice can also influence the susceptibility to infection with *S. pyogenes*. A study where different genetic backgrounds were analyzed for the ability to control streptococcal infection revealed that BALB/c and C57BL/10 mouse strains are more resistant to infection with *S. pyogenes* as for example C3H/HeN and CBA/J mice (164). Further the age of the mice used for the experiments might influence the susceptibility to streptococcal infection, since it has been shown that aged-mice (20 months and older) show a faster progression of disease than young mice (2-3 months)(165).

We observed the ATCC 700294 strain (for simplicity: EC700) did not have the capacity to kill wild-type mice, although they developed lesions at the site of injection. Therefore, the ISS 3348 strain was used in further experiments, since this one is also able to kill infected mice.

Comparing these two strains, by monitoring the capacity to induce an inflammatory response, led to the conclusion that the 3348 strain is inducing a lower inflammatory response than the EC700 strain (see results 5.3.3.). One possible explanation for the observed differences could be that during infection of mice, the 3348 strain-induced immune response is lower. Lower amounts of cytokines are produced and so less cells of the innate immune system get attracted to the site of infection. This might be of advantage for the bacterium since it can proliferate and invade tissues more easily leading to a more severe systemic infection which is subsequently lethal.

The role of type I IFNs during *in vivo* infection of mice with *S. pyogenes* was studied by subcutaneous infection of wild-type and IFNAR1-deficient mice with the 3348 strain. The IFNAR1-deficient mice succumbed earlier to infection and also died at higher numbers than control mice. This finding led to the conclusion that defective type I IFN signaling results in impaired host responses against the extracellular bacterium *S. pyogenes*. Therefore, production of IFN-β is essential for an effective host response against *S. pyogenes*. This is in accordance to a study provided by Mancuso et al. where it has been shown that the production of IFN-β in response to other extracellular Gram-positive bacteria, such as GBS and *S. pneumoniae*, is critical for the clearance of infection by the host (133).

# 7. References


1. Tosi MF (2005) Innate immune responses to infection. (Translated from eng) *J Allergy Clin Immunol* 116(2):241-249; quiz 250 (in eng).
2. Geissmann F*, et al.* (2010) Development of monocytes, macrophages, and dendritic cells. (Translated from eng) *Science* 327(5966):656-661 (in eng).
3. Stout RD & Suttles J (2004) Functional plasticity of macrophages: reversible adaptation to changing microenvironments. (Translated from eng) *J Leukoc Biol* 76(3):509-513 (in eng).
4. Gordon S & Taylor PR (2005) Monocyte and macrophage heterogeneity. (Translated from eng) *Nat Rev Immunol* 5(12):953-964 (in eng).
5. Gordon S (2007) The macrophage: past, present and future. (Translated from eng) *Eur J Immunol* 37 Suppl 1:S9-17 (in eng).
6. Taylor PR*, et al.* (2005) Macrophage receptors and immune recognition. (Translated from eng) *Annu Rev Immunol* 23:901-944 (in eng).
7. Martinez FO, Helming L, & Gordon S (2009) Alternative activation of macrophages: an immunologic functional perspective. (Translated from eng) *Annu Rev Immunol* 27:451-483 (in eng).
8. Chaplin DD (2010) Overview of the immune response. (Translated from eng) *J Allergy Clin Immunol* 125(2 Suppl 2):S3-23 (in eng).
9. Parker LC, Prince LR, & Sabroe I (2007) Translational mini-review series on Toll-like receptors: networks regulated by Toll-like receptors mediate innate and adaptive immunity. (Translated from eng) *Clin Exp Immunol* 147(2):199-207 (in eng).
10. Ueno H*, et al.* (2007) Dendritic cell subsets in health and disease. (Translated from eng) *Immunol Rev* 219:118-142 (in eng).
11. Banchereau J & Steinman RM (1998) Dendritic cells and the control of immunity. (Translated from eng) *Nature* 392(6673):245-252 (in eng).
12. Shortman K & Naik SH (2007) Steady-state and inflammatory dendritic-cell development. (Translated from eng) *Nat Rev Immunol* 7(1):19-30 (in eng).
13. Lande R & Gilliet M (2010) Plasmacytoid dendritic cells: key players in the initiation and regulation of immune responses. (Translated from eng) *Ann N Y Acad Sci* 1183:89-103 (in eng).
14. Lee MS & Kim YJ (2007) Signaling pathways downstream of pattern-recognition receptors and their cross talk. (Translated from eng) *Annu Rev Biochem* 76:447-480 (in eng).

15. Kawai T & Akira S (2009) The roles of TLRs, RLRs and NLRs in pathogen recognition. (Translated from eng) *Int Immunol* 21(4):317-337 (in eng).
16. Mogensen TH (2009) Pathogen recognition and inflammatory signaling in innate immune defenses. (Translated from eng) *Clin Microbiol Rev* 22(2):240-273, Table of Contents (in eng).
17. Leulier F & Lemaitre B (2008) Toll-like receptors--taking an evolutionary approach. (Translated from eng) *Nat Rev Genet* 9(3):165-178 (in eng).
18. Rasmussen SB, Reinert LS, & Paludan SR (2009) Innate recognition of intracellular pathogens: detection and activation of the first line of defense. (Translated from eng) *APMIS* 117(5-6):323-337 (in eng).
19. Ishii KJ, Koyama S, Nakagawa A, Coban C, & Akira S (2008) Host innate immune receptors and beyond: making sense of microbial infections. (Translated from eng) *Cell Host Microbe* 3(6):352-363 (in eng).
20. Akira S & Takeda K (2004) Toll-like receptor signalling. *Nat Rev Immunol* 4(7):499-511.
21. O'Neill LA & Bowie AG (2007) The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. (Translated from eng) *Nat Rev Immunol* 7(5):353-364 (in eng).
22. O'Neill LA (2008) When signaling pathways collide: positive and negative regulation of toll-like receptor signal transduction. (Translated from eng) *Immunity* 29(1):12-20 (in eng).
23. Kenny EF & O'Neill LA (2008) Signalling adaptors used by Toll-like receptors: an update. (Translated from eng) *Cytokine* 43(3):342-349 (in eng).
24. Kagan JC*, et al.* (2008) TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. (Translated from eng) *Nat Immunol* 9(4):361-368 (in eng).
25. Watts C (2008) Location, location, location: identifying the neighborhoods of LPS signaling. (Translated from eng) *Nat Immunol* 9(4):343-345 (in eng).
26. Carty M*, et al.* (2006) The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling. (Translated from eng) *Nat Immunol* 7(10):1074-1081 (in eng).
27. Kawai T & Akira S (2006) TLR signaling. *Cell Death Differ* 13(5):816-825.
28. Akira S, Uematsu S, & Takeuchi O (2006) Pathogen recognition and innate immunity. *Cell* 124(4):783-801.
29. Fritz JH, Ferrero RL, Philpott DJ, & Girardin SE (2006) Nod-like proteins in immunity, inflammation and disease. (Translated from eng) *Nat Immunol* 7(12):1250-1257 (in eng).
30. Strober W, Murray PJ, Kitani A, & Watanabe T (2006) Signalling pathways and molecular interactions of NOD1 and NOD2. (Translated from eng) *Nat Rev Immunol* 6(1):9-20 (in eng).
31. Kumagai Y & Akira S (2010) Identification and functions of pattern-recognition receptors. (Translated from Eng) *J Allergy Clin Immunol* (in Eng).

32. Takeuchi O & Akira S (2010) Pattern recognition receptors and inflammation. (Translated from eng) *Cell* 140(6):805-820 (in eng).
33. Takaoka A*, et al.* (2007) DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. (Translated from eng) *Nature* 448(7152):501-505 (in eng).
34. Wang Z*, et al.* (2008) Regulation of innate immune responses by DAI (DLM-1/ZBP1) and other DNA-sensing molecules. (Translated from eng) *Proc Natl Acad Sci U S A* 105(14):5477-5482 (in eng).
35. Bowzard JB, Ranjan P, Sambhara S, & Fujita T (2009) Antiviral defense: RIG-Ing the immune system to STING. (Translated from eng) *Cytokine Growth Factor Rev* 20(1):1-5 (in eng).
36. Cao X (2009) New DNA-sensing pathway feeds RIG-I with RNA. (Translated from eng) *Nat Immunol* 10(10):1049-1051 (in eng).
37. Schroder K & Tschopp J (2010) The inflammasomes. (Translated from eng) *Cell* 140(6):821-832 (in eng).
38. Commins SP, Borish L, & Steinke JW (2010) Immunologic messenger molecules: cytokines, interferons, and chemokines. (Translated from eng) *J Allergy Clin Immunol* 125(2 Suppl 2):S53-72 (in eng).
39. Naugler WE & Karin M (2008) The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer. (Translated from eng) *Trends Mol Med* 14(3):109-119 (in eng).
40. Kovarik P, Sauer I, & Schaljo B (2007) Molecular mechanisms of the anti-inflammatory functions of interferons. (Translated from eng) *Immunobiology* 212(9-10):895-901 (in eng).
41. Isaacs A & Lindenmann J (1957) Virus interference. I. The interferon. (Translated from eng) *Proc R Soc Lond B Biol Sci* 147(927):258-267 (in eng).
42. Pestka S, Krause CD, & Walter MR (2004) Interferons, interferon-like cytokines, and their receptors. (Translated from eng) *Immunol Rev* 202:8-32 (in eng).
43. Kotenko SV*, et al.* (2003) IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. (Translated from eng) *Nat Immunol* 4(1):69-77 (in eng).
44. Decker T, Muller M, & Stockinger S (2005) The yin and yang of type I interferon activity in bacterial infection. *Nat Rev Immunol* 5(9):675-687.
45. Rossi D & Zlotnik A (2000) The biology of chemokines and their receptors. (Translated from eng) *Annu Rev Immunol* 18:217-242 (in eng).
46. Bestebroer J, de Haas CJ, & van Strijp JA (2009) How microorganisms avoid phagocyte attraction. (Translated from Eng) *FEMS Microbiol Rev* (in Eng).
47. Ono SJ*, et al.* (2003) Chemokines: roles in leukocyte development, trafficking, and effector function. (Translated from eng) *J Allergy Clin Immunol* 111(6):1185-1199; quiz 1200 (in eng).

48. Samuel CE (2001) Antiviral actions of interferons. (Translated from eng) *Clin Microbiol Rev* 14(4):778-809, table of contents (in eng).
49. Kovarik P*, et al.* (2001) Specificity of signaling by STAT1 depends on SH2 and C-terminal domains that regulate Ser727 phosphorylation, differentially affecting specific target gene expression. *Embo J* 20(1-2):91-100.
50. Varinou L*, et al.* (2003) Phosphorylation of the Stat1 transactivation domain is required for full-fledged IFN-gamma-dependent innate immunity. (Translated from eng) *Immunity* 19(6):793-802 (in eng).
51. Sadzak I*, et al.* (2008) Recruitment of Stat1 to chromatin is required for interferon-induced serine phosphorylation of Stat1 transactivation domain. (Translated from eng) *Proc Natl Acad Sci U S A* 105(26):8944-8949 (in eng).
52. Olsen RJ & Musser JM (2009) Molecular Pathogenesis of Necrotizing Fasciitis. (Translated from Eng) *Annu Rev Pathol* (in Eng).
53. Bessen DE (2009) Population biology of the human restricted pathogen, Streptococcus pyogenes. (Translated from eng) *Infect Genet Evol* 9(4):581-593 (in eng).
54. Efstratiou A (2000) Group A streptococci in the 1990s. *J Antimicrob Chemother* 45 Suppl:3-12.
55. Cunningham MW (2000) Pathogenesis of group A streptococcal infections. *Clin Microbiol Rev* 13(3):470-511.
56. Nathan L & Leveno KJ (1994) Group a streptococcal puerperal sepsis: historical review and 1990s resurgence. (Translated from eng) *Infect Dis Obstet Gynecol* 1(5):252-255 (in eng).
57. Veasy LG*, et al.* (1987) Resurgence of acute rheumatic fever in the intermountain area of the United States. (Translated from eng) *N Engl J Med* 316(8):421-427 (in eng).
58. Hoiby EA, Gaustad P, Aasen S, & Martin PR (1990) [Diseases caused by Streptococcus pyogenes in Norway, 1975-1989. A microbiologic and epidemiologic survey]. (Translated from nor) *Tidsskr Nor Laegeforen* 110(20):2625-2628 (in nor).
59. Carapetis JR, Steer AC, Mulholland EK, & Weber M (2005) The global burden of group A streptococcal diseases. *Lancet Infect Dis* 5(11):685-694.
60. Hahn RG, Knox LM, & Forman TA (2005) Evaluation of poststreptococcal illness. *Am Fam Physician* 71(10):1949-1954.
61. Puvanendran R, Huey JC, & Pasupathy S (2009) Necrotizing fasciitis. (Translated from eng) *Can Fam Physician* 55(10):981-987 (in eng).
62. Green RJ, Dafoe DC, & Raffin TA (1996) Necrotizing fasciitis. (Translated from eng) *Chest* 110(1):219-229 (in eng).
63. Lappin E & Ferguson AJ (2009) Gram-positive toxic shock syndromes. (Translated from eng) *Lancet Infect Dis* 9(5):281-290 (in eng).

64. Guilherme L & Kalil J (2010) Rheumatic fever and rheumatic heart disease: cellular mechanisms leading autoimmune reactivity and disease. (Translated from eng) *J Clin Immunol* 30(1):17-23 (in eng).
65. Rodriguez-Iturbe B & Musser JM (2008) The current state of poststreptococcal glomerulonephritis. (Translated from eng) *J Am Soc Nephrol* 19(10):1855-1864 (in eng).
66. Robinson DA, Sutcliffe JA, Tewodros W, Manoharan A, & Bessen DE (2006) Evolution and global dissemination of macrolide-resistant group A streptococci. (Translated from eng) *Antimicrob Agents Chemother* 50(9):2903-2911 (in eng).
67. Laarman A, Milder F, van Strijp J, & Rooijakkers S (2010) Complement inhibition by gram-positive pathogens: molecular mechanisms and therapeutic implications. (Translated from eng) *J Mol Med* 88(2):115-120 (in eng).
68. Batzloff MR, Pandey M, Olive C, & Good MF (2006) Advances in potential M-protein peptide-based vaccines for preventing rheumatic fever and rheumatic heart disease. (Translated from eng) *Immunol Res* 35(3):233-248 (in eng).
69. McNeil SA*, et al.* (2005) Safety and immunogenicity of 26-valent group a streptococcus vaccine in healthy adult volunteers. (Translated from eng) *Clin Infect Dis* 41(8):1114-1122 (in eng).
70. Steer AC, Batzloff MR, Mulholland K, & Carapetis JR (2009) Group A streptococcal vaccines: facts versus fantasy. (Translated from eng) *Curr Opin Infect Dis* 22(6):544-552 (in eng).
71. Rappuoli R (2001) Reverse vaccinology, a genome-based approach to vaccine development. (Translated from eng) *Vaccine* 19(17-19):2688-2691 (in eng).
72. Cleary PP (2006) Streptococcus moves inward. *Nat Med* 12(4):384-386.
73. Sriskandan S, Faulkner L, & Hopkins P (2007) Streptococcus pyogenes: Insight into the function of the streptococcal superantigens. (Translated from eng) *Int J Biochem Cell Biol* 39(1):12-19 (in eng).
74. Bisno AL, Brito MO, & Collins CM (2003) Molecular basis of group A streptococcal virulence. *Lancet Infect Dis* 3(4):191-200.
75. Tart AH, Walker MJ, & Musser JM (2007) New understanding of the group A Streptococcus pathogenesis cycle. (Translated from eng) *Trends Microbiol* 15(7):318-325 (in eng).
76. Hasty DL, Ofek I, Courtney HS, & Doyle RJ (1992) Multiple adhesins of streptococci. (Translated from eng) *Infect Immun* 60(6):2147-2152 (in eng).
77. Ringdahl U & Sjobring U (2000) Analysis of plasminogen-binding M proteins of Streptococcus pyogenes. (Translated from eng) *Methods* 21(2):143-150 (in eng).
78. Passali D, Lauriello M, Passali GC, Passali FM, & Bellussi L (2007) Group A streptococcus and its antibiotic resistance. (Translated from eng) *Acta Otorhinolaryngol Ital* 27(1):27-32 (in eng).

79. Hanski E & Caparon M (1992) Protein F, a fibronectin-binding protein, is an adhesin of the group A streptococcus Streptococcus pyogenes. (Translated from eng) *Proc Natl Acad Sci U S A* 89(13):6172-6176 (in eng).

80. Ozeri V*, et al.* (1996) A two-domain mechanism for group A streptococcal adherence through protein F to the extracellular matrix. (Translated from eng) *Embo J* 15(5):989-998 (in eng).

81. Mora M*, et al.* (2005) Group A Streptococcus produce pilus-like structures containing protective antigens and Lancefield T antigens. (Translated from eng) *Proc Natl Acad Sci U S A* 102(43):15641-15646 (in eng).

82. Nobbs AH, Lamont RJ, & Jenkinson HF (2009) Streptococcus adherence and colonization. (Translated from eng) *Microbiol Mol Biol Rev* 73(3):407-450, Table of Contents (in eng).

83. Manetti AG*, et al.* (2007) Streptococcus pyogenes pili promote pharyngeal cell adhesion and biofilm formation. (Translated from eng) *Mol Microbiol* 64(4):968-983 (in eng).

84. Abbot EL*, et al.* (2007) Pili mediate specific adhesion of Streptococcus pyogenes to human tonsil and skin. (Translated from eng) *Cell Microbiol* 9(7):1822-1833 (in eng).

85. Crotty Alexander LE*, et al.* (2009) M1T1 group A streptococcal pili promote epithelial colonization but diminish systemic virulence through neutrophil extracellular entrapment. (Translated from Eng) *J Mol Med* (in Eng).

86. LaPenta D, Rubens C, Chi E, & Cleary PP (1994) Group A streptococci efficiently invade human respiratory epithelial cells. (Translated from eng) *Proc Natl Acad Sci U S A* 91(25):12115-12119 (in eng).

87. Hagman MM, Dale JB, & Stevens DL (1999) Comparison of adherence to and penetration of a human laryngeal epithelial cell line by group A streptococci of various M protein types. (Translated from eng) *FEMS Immunol Med Microbiol* 23(3):195-204 (in eng).

88. Wang B, Li S, Southern PJ, & Cleary PP (2006) Streptococcal modulation of cellular invasion via TGF-beta1 signaling. (Translated from eng) *Proc Natl Acad Sci U S A* 103(7):2380-2385 (in eng).

89. Ozeri V*, et al.* (2001) De novo formation of focal complex-like structures in host cells by invading Streptococci. (Translated from eng) *Mol Microbiol* 41(3):561-573 (in eng).

90. Molinari G, Rohde M, Guzman CA, & Chhatwal GS (2000) Two distinct pathways for the invasion of Streptococcus pyogenes in non-phagocytic cells. (Translated from eng) *Cell Microbiol* 2(2):145-154 (in eng).

91. Nizet V (2007) Understanding how leading bacterial pathogens subvert innate immunity to reveal novel therapeutic targets. (Translated from eng) *J Allergy Clin Immunol* 120(1):13-22 (in eng).

92. Kawabata S*, et al.* (1999) Capsular hyaluronic acid of group A streptococci hampers their invasion into human pharyngeal epithelial cells. (Translated from eng) *Microb Pathog* 27(2):71-80 (in eng).

93. Cywes C & Wessels MR (2001) Group A Streptococcus tissue invasion by CD44-mediated cell signalling. (Translated from eng) *Nature* 414(6864):648-652 (in eng).

94. McArthur JD*, et al.* (2008) Allelic variants of streptokinase from Streptococcus pyogenes display functional differences in plasminogen activation. (Translated from eng) *FASEB J* 22(9):3146-3153 (in eng).

95. Hytonen J, Haataja S, Gerlach D, Podbielski A, & Finne J (2001) The SpeB virulence factor of Streptococcus pyogenes, a multifunctional secreted and cell surface molecule with strepadhesin, laminin-binding and cysteine protease activity. (Translated from eng) *Mol Microbiol* 39(2):512-519 (in eng).

96. Chhatwal GS & McMillan DJ (2005) Uncovering the mysteries of invasive streptococcal diseases. (Translated from eng) *Trends Mol Med* 11(4):152-155 (in eng).

97. Egesten A*, et al.* (2009) SpeB of Streptococcus pyogenes differentially modulates antibacterial and receptor activating properties of human chemokines. (Translated from eng) *PLoS One* 4(3):e4769 (in eng).

98. Kurupati P*, et al.* (2010) Chemokine-cleaving Streptococcus pyogenes protease SpyCEP is necessary and sufficient for bacterial dissemination within soft tissues and the respiratory tract. (Translated from Eng) *Mol Microbiol* (in Eng).

99. Hidalgo-Grass C*, et al.* (2006) A streptococcal protease that degrades CXC chemokines and impairs bacterial clearance from infected tissues. (Translated from eng) *Embo J* 25(19):4628-4637 (in eng).

100. Collin M & Olsen A (2003) Extracellular enzymes with immunomodulating activities: variations on a theme in Streptococcus pyogenes. (Translated from eng) *Infect Immun* 71(6):2983-2992 (in eng).

101. Terao Y, Yamaguchi M, Hamada S, & Kawabata S (2006) Multifunctional glyceraldehyde-3-phosphate dehydrogenase of Streptococcus pyogenes is essential for evasion from neutrophils. *J Biol Chem* 281(20):14215-14223.

102. Olsen RJ, Shelburne SA, & Musser JM (2009) Molecular mechanisms underlying group A streptococcal pathogenesis. (Translated from eng) *Cell Microbiol* 11(1):1-12 (in eng).

103. Lin A, Loughman JA, Zinselmeyer BH, Miller MJ, & Caparon MG (2009) Streptolysin S inhibits neutrophil recruitment during the early stages of Streptococcus pyogenes infection. (Translated from eng) *Infect Immun* 77(11):5190-5201 (in eng).

104. Borregaard N & Cowland JB (1997) Granules of the human neutrophilic polymorphonuclear leukocyte. (Translated from eng) *Blood* 89(10):3503-3521 (in eng).
105. Medina E, Goldmann O, Toppel AW, & Chhatwal GS (2003) Survival of Streptococcus pyogenes within host phagocytic cells: a pathogenic mechanism for persistence and systemic invasion. (Translated from eng) *J Infect Dis* 187(4):597-603 (in eng).
106. Staali L, Morgelin M, Bjorck L, & Tapper H (2003) Streptococcus pyogenes expressing M and M-like surface proteins are phagocytosed but survive inside human neutrophils. (Translated from eng) *Cell Microbiol* 5(4):253-265 (in eng).
107. Bauer S & Tapper H (2004) Membrane retrieval in neutrophils during phagocytosis: inhibition by M protein-expressing S. pyogenes bacteria. (Translated from eng) *J Leukoc Biol* 76(6):1142-1150 (in eng).
108. Brenot A, King KY, Janowiak B, Griffith O, & Caparon MG (2004) Contribution of glutathione peroxidase to the virulence of Streptococcus pyogenes. (Translated from eng) *Infect Immun* 72(1):408-413 (in eng).
109. Tweten RK (2005) Cholesterol-dependent cytolysins, a family of versatile pore-forming toxins. *Infect Immun* 73(10):6199-6209.
110. Sierig G, Cywes C, Wessels MR, & Ashbaugh CD (2003) Cytotoxic effects of streptolysin o and streptolysin s enhance the virulence of poorly encapsulated group a streptococci. *Infect Immun* 71(1):446-455.
111. Hakansson A, Bentley CC, Shakhnovic EA, & Wessels MR (2005) Cytolysin-dependent evasion of lysosomal killing. (Translated from eng) *Proc Natl Acad Sci U S A* 102(14):5192-5197 (in eng).
112. Timmer AM*, et al.* (2009) Streptolysin O promotes group A Streptococcus immune evasion by accelerated macrophage apoptosis. (Translated from eng) *J Biol Chem* 284(2):862-871 (in eng).
113. Wessels MR (2005) Streptolysin S. *J Infect Dis* 192(1):13-15.
114. Betschel SD, Borgia SM, Barg NL, Low DE, & De Azavedo JC (1998) Reduced virulence of group A streptococcal Tn916 mutants that do not produce streptolysin S. *Infect Immun* 66(4):1671-1679.
115. Fontaine MC, Lee JJ, & Kehoe MA (2003) Combined contributions of streptolysin O and streptolysin S to virulence of serotype M5 Streptococcus pyogenes strain Manfredo. (Translated from eng) *Infect Immun* 71(7):3857-3865 (in eng).
116. Papayannopoulos V & Zychlinsky A (2009) NETs: a new strategy for using old weapons. (Translated from eng) *Trends Immunol* 30(11):513-521 (in eng).

117. Buchanan JT*, et al.* (2006) DNase expression allows the pathogen group A Streptococcus to escape killing in neutrophil extracellular traps. *Curr Biol* 16(4):396-400.

118. Oliver MA, Rojo JM, Rodriguez de Cordoba S, & Alberti S (2008) Binding of complement regulatory proteins to group A Streptococcus. (Translated from eng) *Vaccine* 26 Suppl 8:I75-78 (in eng).

119. Perez-Caballero D, Alberti S, Vivanco F, Sanchez-Corral P, & Rodriguez de Cordoba S (2000) Assessment of the interaction of human complement regulatory proteins with group A Streptococcus. Identification of a high-affinity group A Streptococcus binding site in FHL-1. (Translated from eng) *Eur J Immunol* 30(4):1243-1253 (in eng).

120. Pandiripally V, Gregory E, & Cue D (2002) Acquisition of regulators of complement activation by Streptococcus pyogenes serotype M1. (Translated from eng) *Infect Immun* 70(11):6206-6214 (in eng).

121. Fernie-King BA, Seilly DJ, & Lachmann PJ (2006) Inhibition of antimicrobial peptides by group A streptococci: SIC and DRS. (Translated from eng) *Biochem Soc Trans* 34(Pt 2):273-275 (in eng).

122. Nandakumar KS & Holmdahl R (2008) Therapeutic cleavage of IgG: new avenues for treating inflammation. (Translated from eng) *Trends Immunol* 29(4):173-178 (in eng).

123. Collin M & Olsen A (2001) Effect of SpeB and EndoS from Streptococcus pyogenes on human immunoglobulins. (Translated from eng) *Infect Immun* 69(11):7187-7189 (in eng).

124. von Pawel-Rammingen U & Bjorck L (2003) IdeS and SpeB: immunoglobulin-degrading cysteine proteinases of Streptococcus pyogenes. (Translated from eng) *Curr Opin Microbiol* 6(1):50-55 (in eng).

125. Agniswamy J, Lei B, Musser JM, & Sun PD (2004) Insight of host immune evasion mediated by two variants of group a Streptococcus Mac protein. (Translated from eng) *J Biol Chem* 279(50):52789-52796 (in eng).

126. Guzman CA, Talay SR, Molinari G, Medina E, & Chhatwal GS (1999) Protective immune response against Streptococcus pyogenes in mice after intranasal vaccination with the fibronectin-binding protein SfbI. (Translated from eng) *J Infect Dis* 179(4):901-906 (in eng).

127. Dinkla K*, et al.* (2007) Upregulation of capsule enables Streptococcus pyogenes to evade immune recognition by antigen-specific antibodies directed to the G-related alpha2-macroglobulin-binding protein GRAB located on the bacterial surface. (Translated from eng) *Microbes Infect* 9(8):922-931 (in eng).

128. Goldmann O, Rohde M, Chhatwal GS, & Medina E (2004) Role of macrophages in host resistance to group A streptococci. (Translated from eng) *Infect Immun* 72(5):2956-2963 (in eng).

129. Loof TG, Rohde M, Chhatwal GS, Jung S, & Medina E (2007) The contribution of dendritic cells to host defenses against Streptococcus pyogenes. (Translated from eng) *J Infect Dis* 196(12):1794-1803 (in eng).

130. Gratz N*, et al.* (2008) Group A streptococcus activates type I interferon production and MyD88-dependent signaling without involvement of TLR2, TLR4, and TLR9. (Translated from eng) *J Biol Chem* 283(29):19879-19887 (in eng).

131. Loof TG, Goldmann O, & Medina E (2008) Immune recognition of Streptococcus pyogenes by dendritic cells. (Translated from eng) *Infect Immun* 76(6):2785-2792 (in eng).

132. Miettinen M, Lehtonen A, Julkunen I, & Matikainen S (2000) Lactobacilli and Streptococci activate NF-kappa B and STAT signaling pathways in human macrophages. *J Immunol* 164(7):3733-3740.

133. Mancuso G*, et al.* (2007) Type I IFN signaling is crucial for host resistance against different species of pathogenic bacteria. (Translated from eng) *J Immunol* 178(5):3126-3133 (in eng).

134. Mancuso G*, et al.* (2009) Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells. (Translated from eng) *Nat Immunol* 10(6):587-594 (in eng).

135. Charrel-Dennis M*, et al.* (2008) TLR-independent type I interferon induction in response to an extracellular bacterial pathogen via intracellular recognition of its DNA. (Translated from eng) *Cell Host Microbe* 4(6):543-554 (in eng).

136. Harder J*, et al.* (2009) Activation of the Nlrp3 inflammasome by Streptococcus pyogenes requires streptolysin O and NF-kappaB activation but proceeds independently of TLR signaling and P2X7 receptor. (Translated from eng) *J Immunol* 183(9):5823-5829 (in eng).

137. Zabner J, Fasbender AJ, Moninger T, Poellinger KA, & Welsh MJ (1995) Cellular and molecular barriers to gene transfer by a cationic lipid. (Translated from eng) *J Biol Chem* 270(32):18997-19007 (in eng).

138. Honda K*, et al.* (2005) Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. (Translated from eng) *Nature* 434(7036):1035-1040 (in eng).

139. Ferretti JJ*, et al.* (2001) Complete genome sequence of an M1 strain of Streptococcus pyogenes. (Translated from eng) *Proc Natl Acad Sci U S A* 98(8):4658-4663 (in eng).

140. Goldmann O, Chhatwal GS, & Medina E (2005) Contribution of natural killer cells to the pathogenesis of septic shock induced by Streptococcus pyogenes in mice. (Translated from eng) *J Infect Dis* 191(8):1280-1286 (in eng).

141. Tsai PJ*, et al.* (2006) Streptococcus pyogenes induces epithelial inflammatory responses through NF-kappaB/MAPK signaling pathways. (Translated from eng) *Microbes Infect* 8(6):1440-1449 (in eng).

142. Kumar H, Kawai T, & Akira S (2009) Toll-like receptors and innate immunity. (Translated from eng) *Biochem Biophys Res Commun* 388(4):621-625 (in eng).
143. Loof TG, Goldmann O, Gessner A, Herwald H, & Medina E (Aberrant inflammatory response to Streptococcus pyogenes in mice lacking myeloid differentiation factor 88. (Translated from eng) *Am J Pathol* 176(2):754-763 (in eng).
144. O'Neill LA (2008) The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress. (Translated from eng) *Immunol Rev* 226:10-18 (in eng).
145. Nakagawa I*, et al.* (2004) Autophagy defends cells against invading group A Streptococcus. *Science* 306(5698):1037-1040.
146. Amano A, Nakagawa I, & Yoshimori T (2006) Autophagy in innate immunity against intracellular bacteria. (Translated from eng) *J Biochem* 140(2):161-166 (in eng).
147. Medina E, Rohde M, & Chhatwal GS (2003) Intracellular survival of Streptococcus pyogenes in polymorphonuclear cells results in increased bacterial virulence. (Translated from eng) *Infect Immun* 71(9):5376-5380 (in eng).
148. Jurk M*, et al.* (2002) Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. (Translated from eng) *Nat Immunol* 3(6):499 (in eng).
149. Hemmi H*, et al.* (2002) Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. (Translated from eng) *Nat Immunol* 3(2):196-200 (in eng).
150. Gorden KK, Qiu XX, Binsfeld CC, Vasilakos JP, & Alkan SS (2006) Cutting edge: activation of murine TLR8 by a combination of imidazoquinoline immune response modifiers and polyT oligodeoxynucleotides. (Translated from eng) *J Immunol* 177(10):6584-6587 (in eng).
151. Bogdan C, Mattner J, & Schleicher U (2004) The role of type I interferons in non-viral infections. (Translated from eng) *Immunol Rev* 202:33-48 (in eng).
152. Stockinger S & Decker T (2008) Novel functions of type I interferons revealed by infection studies with Listeria monocytogenes. (Translated from eng) *Immunobiology* 213(9-10):889-897 (in eng).
153. Hornung V & Latz E (2010) Intracellular DNA recognition. (Translated from eng) *Nat Rev Immunol* 10(2):123-130 (in eng).
154. Ablasser A*, et al.* (2009) RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. (Translated from eng) *Nat Immunol* 10(10):1065-1072 (in eng).
155. Chiu YH, Macmillan JB, & Chen ZJ (2009) RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. (Translated from eng) *Cell* 138(3):576-591 (in eng).

156. Freudenberg MA*, et al.* (2002) Cutting edge: a murine, IL-12-independent pathway of IFN-gamma induction by gram-negative bacteria based on STAT4 activation by Type I IFN and IL-18 signaling. (Translated from eng) *J Immunol* 169(4):1665-1668 (in eng).

157. Carrero JA, Calderon B, & Unanue ER (2006) Lymphocytes are detrimental during the early innate immune response against Listeria monocytogenes. (Translated from eng) *J Exp Med* 203(4):933-940 (in eng).

158. Auerbuch V, Brockstedt DG, Meyer-Morse N, O'Riordan M, & Portnoy DA (2004) Mice lacking the type I interferon receptor are resistant to Listeria monocytogenes. (Translated from eng) *J Exp Med* 200(4):527-533 (in eng).

159. O'Connell RM*, et al.* (2004) Type I interferon production enhances susceptibility to Listeria monocytogenes infection. (Translated from eng) *J Exp Med* 200(4):437-445 (in eng).

160. Ashbaugh CD, Warren HB, Carey VJ, & Wessels MR (1998) Molecular analysis of the role of the group A streptococcal cysteine protease, hyaluronic acid capsule, and M protein in a murine model of human invasive soft-tissue infection. (Translated from eng) *J Clin Invest* 102(3):550-560 (in eng).

161. Datta V*, et al.* (2005) Mutational analysis of the group A streptococcal operon encoding streptolysin S and its virulence role in invasive infection. (Translated from eng) *Mol Microbiol* 56(3):681-695 (in eng).

162. Matsui H*, et al.* (2009) CD46 transgenic mouse model of necrotizing fasciitis caused by Streptococcus pyogenes infection. (Translated from eng) *Infect Immun* 77(11):4806-4814 (in eng).

163. Sun H*, et al.* (2004) Plasminogen is a critical host pathogenicity factor for group A streptococcal infection. (Translated from eng) *Science* 305(5688):1283-1286 (in eng).

164. Medina E, Goldmann O, Rohde M, Lengeling A, & Chhatwal GS (2001) Genetic control of susceptibility to group A streptococcal infection in mice. (Translated from eng) *J Infect Dis* 184(7):846-852 (in eng).

165. Goldmann O, Lehne S, & Medina E (2010) Age-related susceptibility to Streptococcus pyogenes infection in mice: underlying immune dysfunction and strategy to enhance immunity. (Translated from eng) *J Pathol* 220(5):521-529 (in eng).

# 8. Acknowledgements

First of all I want to sincerely thank Dr. Pavel Kovarik not only for giving me the opportunity to work on this exciting project but also for his constant support, motivation and his believe in me.

I further want to thank my PhD committee, Dr. Sylvia Knapp, Dr. Eszter Nagy and Dr. Thomas Decker for their valuable comments, advice and discussions.
Many thanks also go to my nominated thesis referees Dr. Giuseppe Teti and Dr. Mathias Müller for their prompt willingness to examine my thesis.

A big thank also goes to Biomodels Austria, especially to Thomas Kolbe and Susanne Gratzl for their continuous help regarding my mice.

I want to thank my ´amazingly awesome` diploma student Harald Hartweger, for supporting me in my project, the interesting discussions and also for becoming a friend.
I´m also greatly thankful to the current and former members of my lab: Asia, Ivana, Christian, Barbara, Iwona, Ines, Jakob and Stefan for their advice, helpful discussions and for incredibly funny times in the lab.
Furthermore I want to thank the members of Thomas Deckers Lab, Emmanuelle Charpentiers Lab and Manuela Baccarinis Lab for the very nice working atmosphere.

Big thanks also go to my friends Gundula, Claudia, Sarah und Martha for their friendship, for listening to my mourning and for cheering me up when I was frustrated.

Last but not least, my very special thanks go to my parents Franz and Christine, to my sister Verena and her family and Franz for their endless support in every sense. Without them this all would not have been possible!

# 9. Curriculum Vitae

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**Education:**

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| September 2009 | Research stay in the Lab of Prof. Giuseppe Teti at the University of Messina, Italy |
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|---|---|
| **2009** | Talk at the Interferon workshop „IFN and Infection", Bern |
| **2008** | Poster presentation at the TOLL 2008 Meeting in Lisbon, Portugal |
| **2007** | Poster presentation at the Meeting on Microbial Pathogenesis & Host Response, Cold Spring Harbor, NY |
| **2006** | Poster presentation at the Cytokine Conference 2006, Vienna |
| | Poster presentation at the 20 th annual Meeting of the European Macrophage & Dendritic Cell Society (EMDS), Freiburg, Germany |

**Publications:**

Roetzer A, Gratz N, Kovarik P, Schüller C.: **Autophagy supports *Candida glabrata* survival during phagocytosis.**, Cell Microbiol. 2010 Feb;12(2):199-216.

Schaljo B, Kratochvill F, Gratz N, Sadzak I, Sauer I, Hammer M, Vogl C, Strobl B, Müller M, Blackshear PJ, Poli V, Lang R, Murray PJ, Kovarik P.: **Tristetraprolin is required for full anti-inflammatory response of murine macrophages to IL-10.**, J Immunol. 2009 Jul 15;183(2):1197-206

Gratz N, Siller M, Schaljo B, Pirzada ZA, Gattermeier I, Vojtek I, Kirschning CJ, Wagner H, Akira S, Charpentier E, Kovarik P.: **Group A streptococcus activates type I interferon production and MyD88-dependent signaling without involvement of TLR2, TLR4, and TLR9.**, J Biol Chem. 2008 Jul 18;283(29):19879-87.

# 10. Lebenslauf

**Personalien:**

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| Name: | Nina Gratz |
| Geburtsdatum: | 18.08.1981 |
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| Staatsbürgerschaft: | Austrian |

**Ausbildung:**

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| Oktober 2006-present | Dissertation im Labor von Prof. Pavel Kovarik mit dem Titel: Responses of the innate immune system to the human pathogen *Streptococcus pyogenes* |
| September 2006 | Diplomprüfung (Mag.rer.nat) |
| August 2005 – Juli 2006 | Diplomarbeit im Labor von Prof. Pavel Kovarik am Department für Mikrobiologie und Immunbiologie an der Universität Wien<br>Titel: Host Responses to Infection with *Streptococcus pyogenes* |
| 2000 - 2006 | Studium der Biologie an der Universität Wien, Studienzweig: Mikrobiologie und Genetik |
| 1995 - 2000 | Bundesoberstufenrealgymnasium Innsbruck, Naturwissenschaftlicher Zweig |

**Praktikas:**

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| September 2009 | Forschungsaufenthalt im Labor von Prof. Giuseppe Teti an der Universität Messina, Italien |

| | |
|---|---|
| September 2004 | Praktikum im Labor von Prof. Lukas A. Huber Lab am Department für Histologie und Molekularer Zellbiologie der Universität Innsbruck |

**Wissenschaftliche Konferenzen:**

| | |
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| **2009** | Vortrag am Interferon Workshop „IFN and Infection", Bern |
| **2008** | Poster-Präsentation TOLL 2008 Meeting in Lissabon, Portugal |
| **2007** | Poster-Präsentation Meeting on Microbial Pathogenesis & Host Response, Cold Spring Harbor, NY |
| **2006** | Poster-Präsentation Cytokine Conference 2006, Wien |
| | Poster-Präsentation 20 th annual Meeting of the European Macrophage & Dendritic Cell Society (EMDS), Freiburg, Germany |

**Publikationen:**

Roetzer A, Gratz N, Kovarik P, Schüller C.: **Autophagy supports Candida glabrata survival during phagocytosis.**, Cell Microbiol. 2010 Feb;12(2):199-216.

Schaljo B, Kratochvill F, Gratz N, Sadzak I, Sauer I, Hammer M, Vogl C, Strobl B, Müller M, Blackshear PJ, Poli V, Lang R, Murray PJ, Kovarik P.: **Tristetraprolin is required for full anti-inflammatory response of murine macrophages to IL-10.**, J Immunol. 2009 Jul 15;183(2):1197-206

Gratz N, Siller M, Schaljo B, Pirzada ZA, Gattermeier I, Vojtek I, Kirschning CJ, Wagner H, Akira S, Charpentier E, Kovarik P.: **Group A streptococcus activates type I interferon production and MyD88-dependent signaling without involvement of TLR2, TLR4, and TLR9.**, J Biol Chem. 2008 Jul 18;283(29):19879-87.

# 11. Appendix (other publications)

11.1. Publication: Tristetraprolin is required for full anti-inflammatory response of murine macrophages to IL-10

11.2. Publication: Autophagy supports *Candida glabrata* survival during phagocytosis

# Tristetraprolin Is Required for Full Anti-Inflammatory Response of Murine Macrophages to IL-10[1]

**Barbara Schaljo,[2]* Franz Kratochvill,[2]* Nina Gratz,* Iwona Sadzak,* Ines Sauer,* Michael Hammer,[‖#] Claus Vogl,[†] Birgit Strobl,[†‡] Mathias Müller,[†‡] Perry J. Blackshear,[§] Valeria Poli,[¶] Roland Lang,[‖#] Peter J. Murray,[**] and Pavel Kovarik[3]***

**IL-10 is essential for inhibiting chronic and acute inflammation by decreasing the amounts of proinflammatory cytokines made by activated macrophages. IL-10 controls proinflammatory cytokine and chemokine production indirectly via the transcription factor Stat3. One of the most physiologically significant IL-10 targets is TNF-$\alpha$, a potent proinflammatory mediator that is the target for multiple anti-TNF-$\alpha$ clinical strategies in Crohn's disease and rheumatoid arthritis. The anti-inflammatory effects of IL-10 seem to be mediated by several incompletely understood transcriptional and posttranscriptional mechanisms. In this study, we show that in LPS-activated bone marrow-derived murine macrophages, IL-10 reduces the mRNA and protein levels of TNF-$\alpha$ and IL-1$\alpha$ in part through the RNA destabilizing factor tristetraprolin (TTP). TTP is known for its central role in destabilizing mRNA molecules containing class II AU-rich elements in 3′ untranslated regions. We found that IL-10 initiates a Stat3-dependent increase of TTP expression accompanied by a delayed decrease of p38 MAPK activity. The reduction of p38 MAPK activity releases TTP from the p38 MAPK-mediated inhibition, thereby resulting in diminished mRNA and protein levels of proinflammatory cytokines. These findings establish that TTP is required for full responses of bone marrow-derived murine macrophages to IL-10.** *The Journal of Immunology,* **2009, 183: 1197–1206.**

One of the key players in immune homeostasis is IL-10, a cytokine that was discovered 18 years ago as a T cell-secreted factor that inhibited cytokine production by Th1 cells (1). Over the years, it became clear that IL-10 is produced by many cell types including T and B cells, macrophages, dendritic cells, mast cells, keratinocytes, or epithelial cells (2). It is generally considered that the main biological function of IL-10 is to limit or shut down inflammatory responses. This notion is supported by the phenotype of IL-10-deficient mice that develop severe inflammatory bowel disease due to spontaneous chronic inflammation and are susceptible to endotoxin treatment because of acute overproduction of proinflammatory cytokines (3, 4). Many of these phenotypes can be recapitulated by T cell-specific deletion of the IL-10 gene, indicating that T cell-derived IL-10 is primarily responsible for chronic inflammation (5). Under conditions of acute inflammation, the main source of IL-10 are macrophages and dendritic cells (6). On the cellular level, IL-10 inhibits production of proinflammatory cytokines and regulates differentiation and proliferation of various immune cells. These effects depend entirely on the activation of the transcription factor Stat3 by IL-10 (7–11). The events downstream of Stat3 activation that mediate the anti-inflammatory functions of IL-10 remain an area of active research. It is becoming increasingly clear that multiple mechanisms mediate the IL-10 function. On the one hand, IL-10 inhibits transcription of a subset of proinflammatory genes (12, 13). In a mouse model lacking 3′ untranslated regions (UTR)[4] in the *Tnf* gene, the transcriptional mechanism was found to play a major role in IL-10 responses (13). On the other hand, IL-10 was also reported to act posttranscriptionally by increasing the rate of mRNA decay of inflammatory cytokines such as TNF-$\alpha$ or by inhibiting translation (12, 14, 15). The posttranscriptional regulation depends on 3′ UTR containing AU-rich elements (AREs) (16). However, far less is known about the IL-10-regulated effector genes that control the anti-inflammatory response. Several candidates have been described, but so far none have been shown to account for the majority of the anti-inflammatory effects (17). IL-10 was also demonstrated to up-regulate the dual-specificity phosphatase 1 (DUSP1) in LPS-stimulated macrophages. causing a more rapid inactivation of p38 MAPK (18). The reduction of p38 MAPK activity may lead to a decreased stability of ARE-containing mRNAs and/or reduced transcription by transcription factors that depend on p38 MAPK. Recently, the transcriptional repressor ETV3 and

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Received for publication November 20, 2008. Accepted for publication May 11, 2009.

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[1] This study was supported by Austrian Science Fund (Fonds zur Förderungder Wissenschaftlichen Forschung) Grants SFB F28 and P16726-B14, European Science Foundation Grant I27-B03 (to P.K.), Austrian Science Fund Fonds zur Förderungder Wissenschaftlichen Forschung Grant SFB F28 and the Austrian Federal Ministry for Science and Research Grant Bundesministerium für Wissenschaft und Forschung GZ200.112/1-VI/1/2004 (to M.M.), National Institutes of Health Grant AI062921 (to P.J.M.), and Deutsche Forschungsgemeinschaft Grants Sonderforschungsbereich 576/TP-A11 and LA1262/4-1 (to R.L.).

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[4] Abbreviations used in this paper: UTR, untranslated region; ARE, AU-rich element; DUSP1, dual-specificity phosphate 1; BMDM, bone marrow-derived macrophage; TTP, tristetraprolin; WT, wild type; qRT-PCR, quantitative RT-PCR; fwd, forward; rev, reverse; SOCS, suppressor of cytokine signaling; SB, SB203580.

the corepressor SBNO2 were characterized as IL-10/Stat3-induced genes that may contribute to the anti-inflammatory IL-10 effects (19). An important aspect of the above-mentioned studies that remains to be resolved is how IL-10 inhibits specifically only a subset of inflammatory genes. These studies indicate that yet unknown IL-10/Stat3 target genes need to be discovered to complement the current knowledge about the anti-inflammatory effects of IL-10.

In this study, we demonstrate that IL-10-mediated reduction of TNF-$\alpha$ and IL-1$\alpha$ production by LPS-treated mouse bone marrow-derived macrophages (BMDMs) is less efficient in cells lacking the RNA-destabilizing factor tristetraprolin (TTP). TTP is known to bind and destabilize mRNAs of several proinflammatory cytokines (e.g., TNF-$\alpha$) containing class II AREs in their 3′ UTRs (20). TTP facilitates degradation of the bound mRNA by initiating the assembly of RNA decay machinery (21, 22). The RNA degradation activity of TTP is negatively regulated by p38 MAPK-dependent signaling (23–25). Mice lacking TTP (encoded by the *Zfp36* gene) develop multiple chronic inflammatory syndromes ranging from arthritis to cachexia to dermatitis that can all be relieved by reduction of TNF-$\alpha$ levels (26). By comparing LPS-treated wild-type and TTP-deficient BMDMs, we show that IL-10 accelerates, in a TTP-dependent way, the decay of TNF-$\alpha$ mRNA, resulting in a reduction of secreted TNF-$\alpha$. Furthermore, IL-10 increases TTP expression in LPS-treated wild-type (WT) but not Stat3-deficient BMDMs. However, we show that the increased TTP levels are not sufficient to mediate the IL-10 effects. Instead, the IL-10- mediated reduction of p38 MAPK activity in LPS-treated BMDMs that is known to be caused by up-regulation of the p38 MAPK phosphatase DUSP1 (18) is required to act in concert with TTP to reduce mRNA levels of proinflammatory cytokines such as TNF-$\alpha$ and IL-1$\alpha$. We propose that the sustained activation of Stat3 by IL-10 causes both an increased TTP expression and a reduction in p38 MAPK activity. The combination of these effects results in a reduction of mRNA stability and attenuation of cytokine production.

## Materials and Methods

### *Reagents*

Stat3, p38 MAPK, and DUSP1 (sc-1102) Abs were from Santa Cruz Biotechnology, the ERK Ab was from BD Transduction Laboratories, and the phosphotyrosine-Stat3 Ab (pY-Stat3) was from Cell Signaling and New England Biolabs. Rabbit Ab to TTP was obtained by immunizing rabbits with 44 C-terminal amino acids of TTP fused to GST. IL-10 and IL-6 (Sigma-Aldrich) were used at a concentration of 10 ng/ml, LPS from *Salmonella minnesota* (Alexis) was used at a concentration of 5–10 ng/ml, and anisomycin and actinomycin D (both Sigma-Aldrich) were used at a concentration of 100 ng/ml and 5 $\mu$g/ml, respectively. SB203580 (SB; Sigma-Aldrich) was dissolved in DMSO and used at final concentration 4 $\mu$M. ATP (1 $\mu$M; Sigma-Aldrich) was added 1 h before collecting the samples.

### *Cell culture*

Primary macrophages were grown in L cell-derived CSF-1 as previously described (27). *Zfp36*$^{-/-}$ and Dusp1$^{-/-}$ mice (26) were on the C57BL/6 background. The *Zfp36* gene encodes TTP, which is the better known name and therefore is used throughout. For experiments with cells derived from TTP$^{-/-}$ and TTP$^{+/+}$ mice, littermates originating from the TTP$^{+/-}$ colony were used. The LysMcre-Stat3$^{fl/fl}$ mice were obtained by crossing Stat3$^{fl/fl}$ mice (28) with LysMcre, strain B6.129P2-Lyzs$^{tml(cre)Ifo}$ (The Jackson Laboratory), all on the C57BL/6 background. Mice, 8–12 wk old at the time of bone marrow collection, were housed under specific pathogen-free conditions. Mouse experiments were conducted in compliance with national laws. Mouse macrophage line RAW 264.7 was grown in DMEM supplemented with 10% FCS.

### *Inducible expression of TTP*

The open reading frame of the mouse TTP was PCR-amplified from cDNA. The PCR was used to add Flag tag DYKDDDDK at the C terminus. The TTP-Flag fragment was cloned into the pGL2-basic (Promega) containing a tetracycline-responsive element (TRE) in the promoter. The resulting plasmid pTRE-TTPfl was transfected into HeLa-Tet-off cells (BD Clontech) using nucleofection (Amaxa). After transfection, the cells were incubated overnight in medium with (no TTP expression) or without (TTP expression) tetracycline (1 $\mu$g/ml).

### *ELISA*

For ELISA, BMDMs were seeded the day before use at $2 \times 10^5$ cells/well in a 24-well plate. Supernatants were diluted 1/8 in DMEM (for TNF-$\alpha$) or 1/2 (for IL-1$\alpha$) and cytokines were assayed using ELISA kits (R&D Systems) according to the manufacturer's instructions.

### *Quantitative Western blot*

After treatment, whole cell extracts were prepared and assayed by Western blotting as described elsewhere (29). Detection and quantitation of signals were performed using the infrared imaging system Odyssey (LI-COR Biosciences).

### *Quantitation of gene expression by quantitative RT-PCR (qRT-PCR)*

Total RNA was isolated using TRIzol reagent (Invitrogen). Reverse transcription was performed with Moloney murine leukemia virus reverse transcriptase (Fermentas). The following primers were used: for HPRT, the housekeeping gene used for normalization, HPRT forward (fwd) 5′-GGATTTGAATCACGTTTGTGTCAT-3′ and HPRT reverse (rev) 5′-ACACCTGCTAATTTTACTGGCAA-3′; for TTP, TTP fwd 5′-CTCTGCCATCTACGAGAGCC-3′ and TTP rev 5′-GATGGAGTCCGAGTTTATGTTCC-3′; for TNF-$\alpha$, TNF-$\alpha$ fwd 5′-CAAAATTCGAGTGACAAGCCTG-3′ and TNF-$\alpha$ rev 5′-GAGATCCATGCCGTTGGC-3′; for suppressor of cytokine signaling (SOCS) 3, SOCS3 fwd 5′-GCTCCAAAAGCGAGTACCAGC-3′ and SOCS3 rev 5′-AGTAGAATCCGCTCTCCTGCAG-3′; and for TTP primary transcript, TTPpt fwd 5′-GACTGGCAAGCTCGTGAAGT-3′, pt-TTP-rev 5′-CAGTCAGGCGAGAGGTGA-3′. For determination of mRNA decay by qRT-PCR, the primers TNF-$\alpha$ fwd 5′-TTCTGTCTACTGAACTTCGGGGTGATCGGTCC-3′ and TNF-$\alpha$ rev 5′-GTATGAGATAGCAAATCGGCTGACGGTGTGGG-3′ and for IL-1$\alpha$, the primer set QT00113505 from Qiagen was used. Amplification of DNA was monitored by SYBR Green (Molecular Probes (30).

### *RNA EMSA*

To prepare extracts, cells were washed with cold PBS and lysed in buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 30 mM $NaPP_i$, 50 mM NaF, 2 mM EDTA, 1% Triton X-100, and a protease inhibitor mixture (Roche). Extracts were cleared by centrifugation at 15,000 rpm. Twelve-microliter cell extracts (30 $\mu$g of protein) from RAW 264.7 cells or 5-$\mu$l cell extracts (15 $\mu$g of protein) from pTRE-TTPfl-transfected HeLa-Tet-off cells were incubated with 0.5 $\mu$l of poly(U) RNA (100 ng/$\mu$l), 0.5 $\mu$l of Cy5.5-labeled TNF-$\alpha$ ARE (1 pmol/$\mu$l), 1 $\mu$l of RiboLock RNase Inhibitor (Fermentas), and 2.5 $\mu$l of 5× Gelshift buffer (200 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 2.5 mM DTT, 100 mM HEPES-KOH (pH 7.9), and glycerin 50% (v/v)) for 20 min at room temperature. For supershift assays, 0.5 $\mu$l of TTP antiserum was added. Samples were then separated on a 6% polyacrylamide gel. The Cy5.5 signal was detected and quantified using the infrared imaging system Odyssey (LI-COR Biosciences). Poly(U) RNA and Cy5.5′-labeled TNF-$\alpha$ ARE RNA were purchased from Microsynth. The sequence for Cy5.5′-labeled TNF-$\alpha$ ARE was as follows: 5′-AUUAUUUAUUAUUUAUUUAUUAUUUA-3′.

### *Statistical analysis*

Data from independent experiments were analyzed using univariate linear regression models and the SPSS program. For qRT-PCR normalized copy numbers and for ELISA pg/ml were log-transformed. Residuals were plotted, visually inspected, and tested for normality. Design matrices were specified such that the coefficients for the relevant comparisons could be calculated, e.g., between the baseline and induced states and between genotypes. Only the significance levels are reported.

## Results

### *IL-10 increases TTP expression in LPS-treated macrophages in a Stat3-dependent manner*

TTP expression has been reported to be controlled by the transcription factors Stat1 (in response to IFNs) and Stat6 (in response to IL-4) (27, 31). Affymetrix analysis revealed that LPS-induced TTP expression is further enhanced by IL-10 (data not shown) at

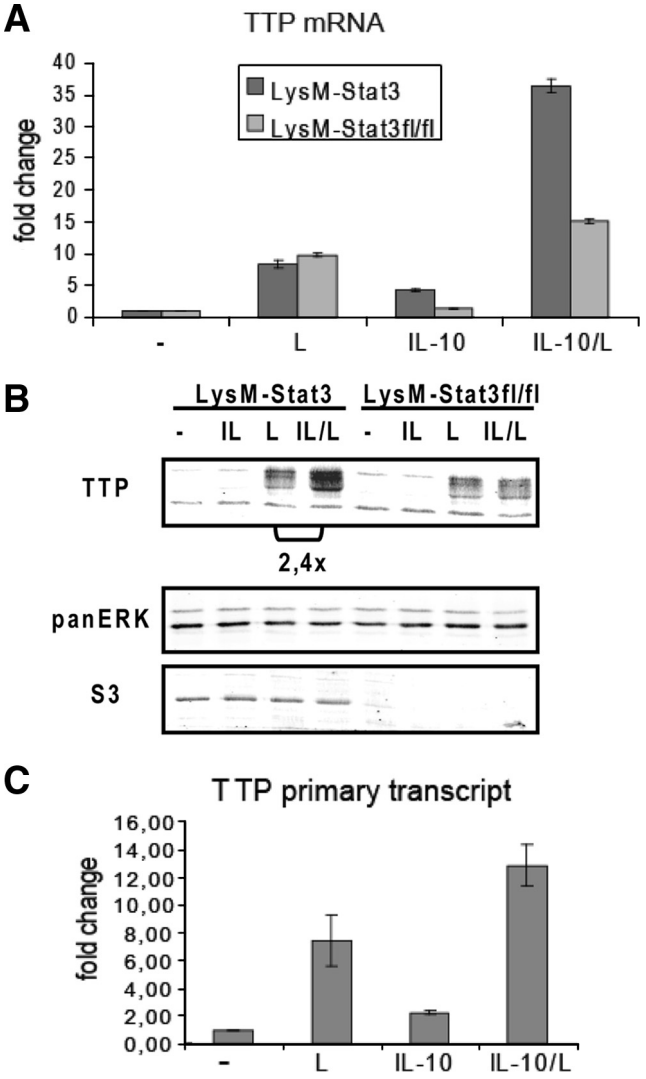


**FIGURE 1.** IL-10-mediated increase of TTP expression in LPS-treated macrophages depends on Stat3. *A*, BMDMs from LysMcre-Stat3 (LysM-Stat3) and LysMcre-Stat3$^{fl/fl}$ (LysM-Stat3fl/fl) mice were treated for 1 h with LPS (L), IL-10, or both (IL-10/L). Induction of TTP mRNA was quantified using qRT-PCR. Bars indicate SDs, $n = 3$. *B*, BMDMs from LysMcre-Stat3 (LysM-Stat3) and LysMcre-Stat3$^{fl/fl}$ (LysM-Stat3fl/fl) animals were left untreated or treated with IL-10 (IL), LPS (L), or both (IL/L) for 3 h. TTP protein levels were analyzed by Western blotting of cell extracts using TTP Ab. TTP appears in multiple bands representing phosphorylated forms. Blot was reprobed with Stat3 Ab to control for Stat3 deletion in LysMcre-Stat3$^{fl/fl}$ cells and panERK Ab for loading control. Differences in TTP levels (normalized to panERK and quantitated using infrared imaging system Odyssey) in cells treated with IL-10/LPS compared with LPS alone are indicated. *C*, BMDM (WT) were stimulated for 30 min with LPS (L), IL-10, or both (IL-10/L), total RNA was isolated, DNase treated, and the amount of primary transcript was determined by qRT-PCR. Bars indicate SDs, $n = 3$.

30 min after treatment of *Il10*$^{-/-}$ BMDMs with IL-10 and LPS, suggesting that TTP may represent a Stat3 target gene involved in the anti-inflammatory responses to IL-10. To examine the effect of IL-10 on TTP expression in more detail, we investigated TTP gene expression in BMDMs conditionally deleted for Stat3 (LysMcre-Stat3$^{fl/fl}$) and control LysMcre-Stat3 cells. LysMcre is known to delete loxP-flanked alleles in macrophage and neutrophil lineages with >90% efficiency (7). Treatment of BMDMs with IL-10 and LPS caused a 2- to 3-fold increase in TTP mRNA (Fig. 1*A*) and protein levels (Fig. 1*B*) compared with LPS treatment alone. The IL-10-mediated increase in TTP expression required Stat3 since in LysMcre-Stat3$^{fl/fl}$ cells the TTP levels remained unchanged upon IL-10 treatment. The expression of Stat3 was reduced by >90% in LysMcre-Stat3$^{fl/fl}$ cells (Fig. 1*B*), thereby confirming the deletion efficiency by LysMcre. Analysis of TTP primary transcripts by PCR (Fig. 1*C*) and nuclear run-on assays (data not shown) revealed that the IL-10-mediated induction of TTP was caused by increased transcription.

These findings document that IL-10-activated Stat3 increases expression of TTP in LPS-stimulated macrophages. IL-10 alone only weakly increased TTP transcription, and this induction was not sufficient to generate detectable levels of TTP protein. This way of regulation resembles that of IFN-induced TTP expression, which was shown to be activated by IFNs and the IFN-activated Stat1 only if p38 MAPK signaling (by e.g., LPS) is stimulated in parallel (27). p38 MAPK is known to increase by yet unclear mechanisms the transcriptional activity of several Stat members (32–34).

### *TTP is required for full inhibitory effect of IL-10 on TNF-α and IL-1α production*

To test whether TTP was an effector of the IL-10 anti-inflammatory responses, we measured the amount of secreted TNF-α by ELISA in BMDMs from TTP$^{-/-}$ and control WT littermates that were treated with LPS with or without pretreatment with IL-10. We measured TNF-α production at 6, 8, and 10 h after LPS stimulation. After 10 h, TNF-α was diminished by IL-10 to ~20% in control cells, whereas in TTP$^{-/-}$ cells only a reduction to 60% was achieved (Fig. 2*A*). Similar results were obtained if cells were treated simultaneously with LPS and IL-10 (Fig. 2*B*), although the contribution of TTP to the IL-10 response was higher in the pretreatment protocol (Fig. 2A). These data show that TTP contributes to IL-10-mediated inhibition of TNF-α cytokine production.

To investigate whether the incomplete IL-10-mediated inhibition of TNF-α production in TTP$^{-/-}$ BMDMs resulted from differences in mRNA amounts or in mRNA decay, we analyzed the amounts of TNF-α mRNA in LPS-stimulated TTP$^{-/-}$ and control BMDMs with or without pretreatment with IL-10. In WT cells, IL-10 caused a reduction of TNF-α mRNA to 40% of the amount present in cells treated with LPS alone, whereas in TTP$^{-/-}$ cells IL-10 caused a reduction to only 80% of the level in cells treated with LPS alone (Fig. 2*C*). In these experiments, TNF-α mRNA was measured after 2 h of LPS treatment since the amount of TNF-α mRNA peaks at this time point (Ref. 13 and data not shown). To further illustrate the role of TTP in IL-10-mediated decrease of TNF-α mRNA, we analyzed the rate of TNF-α mRNA decay in LPS-stimulated TTP$^{-/-}$ and WT BMDMs with or without IL-10 pretreatment. Transcription was stopped after 3 h of LPS stimulation by addition of actinomycin D, and the degradation of TNF-α mRNA was followed in 15- min intervals for a total of 45 min after imposing the transcriptional stop (Fig. 3*A*). In LPS-treated control BMDMs, the decay rate of TNF-α mRNA was increased 2.5-fold by IL-10 treatment (half-life $t_{½}$ without IL-10 = 32 min; $t_{½}$ with IL-10 = 13 min). In LPS-treated TTP$^{-/-}$ BMDMs, the residual TNF-α mRNA decayed with a 2.5-fold longer half-life ($t_{[1/2]}$ = 82 min) compared with WT. This difference is similar to the one reported previously (35). Importantly, IL-10 treatment of LPS-stimulated TTP$^{-/-}$ cells did not increase the decay rate and the remnant mRNA levels were comparable to those in cells treated with LPS alone. LPS-stimulated macrophages produced endogenous IL-10 that is known to mask to some extent the effect of added IL-10 (see Ref. 18). Since TTP was recently

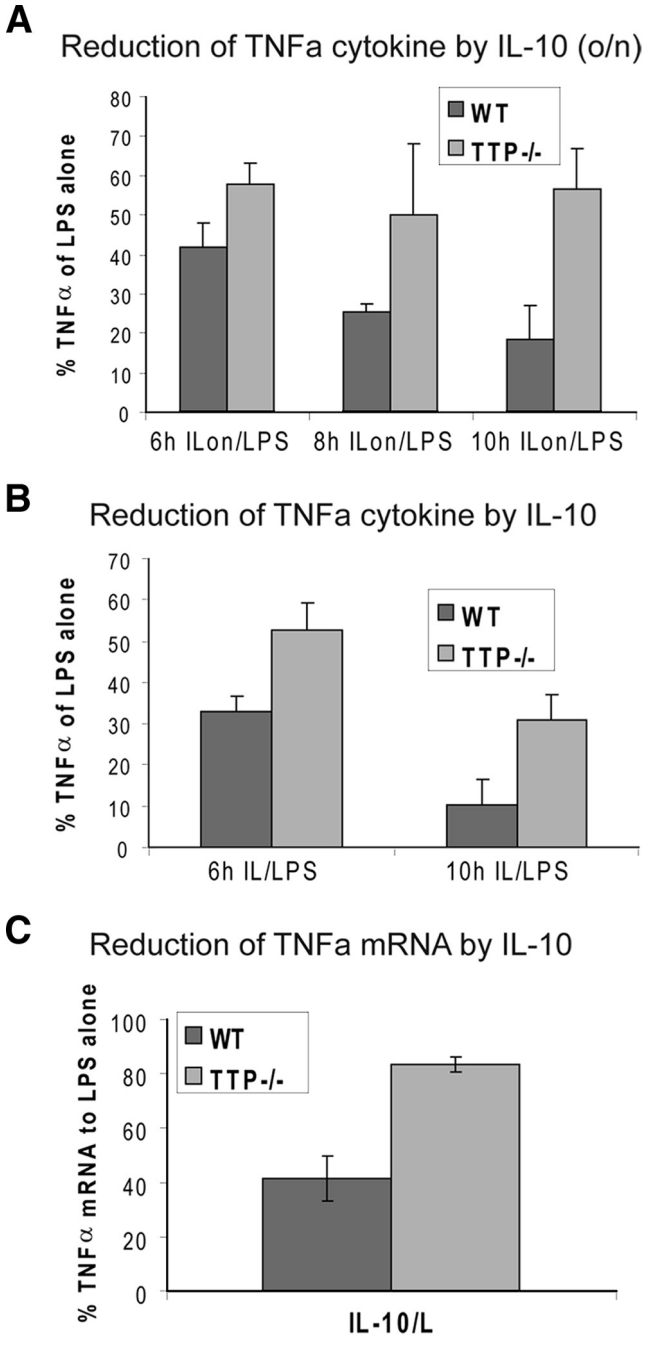


**FIGURE 2.** TTP is required for full IL-10-mediated inhibition of TNF-$\alpha$ production. *A*, Reduction of LPS-induced TNF-$\alpha$ cytokine production in cells pretreated with IL-10. BMDMs from WT and TTP$^{-/-}$ mice were treated with LPS or pretreated overnight (o/n) with IL-10 followed by stimulation with LPS (ILon/LPS) for indicated time points. Supernatants were collected and analyzed for TNF-$\alpha$ cytokine levels by ELISA. Reduction of TNF-$\alpha$ cytokine levels by IL-10 pretreatment (IL-10 o/n) relative (in percent) to LPS-alone treatment (100%) is depicted. SDs ($n = 4$) are indicated. *B*, Effect of simultaneous treatment with IL-10 and LPS on TNF-$\alpha$ cytokine production performed as described in *A*. Relative amounts of TNF-$\alpha$ cytokine secreted by LPS-treated WT and TTP$^{-/-}$ BMDMs compared with cells simultaneously treated with IL-10/LPS are shown. SDs ($n = 3$) are indicated. *C*, Reduction of LPS-induced TNF-$\alpha$ mRNA in cells pretreated with IL-10. WT-BMDMs and TTP-deficient BMDMs (TTP$^{-/-}$) were treated 2 h with LPS or pretreated overnight with IL-10 followed by LPS addition (IL-10/L) and analyzed for expression of TNF-$\alpha$ by qRT-PCR, normalized to HPRT mRNA. Shown is the reduction of TNF-$\alpha$ mRNA levels after IL-10/LPS treatment in relation to the sample treated with LPS alone. SDs ($n = 3$) are indicated.

shown to target IL-10 mRNA for degradation (36), we asked whether TTP$^{-/-}$ BMDMs produce more IL-10 after stimulation with LPS. Cytokine measurement revealed that TTP-deficient cells secrete 2- to 3-fold more IL-10 than the WT cells (supplemental Fig. S1[5]). To assess the contribution of endogenous IL-10 to the IL-10-mediated TNF-$\alpha$ mRNA decay, we compared the TNF-$\alpha$ mRNA levels and decay rates in IL-10$^{-/-}$ and WT cells. IL-10$^{-/-}$ BMDMs expressed 2-fold more TNF-$\alpha$ than WT cells (Fig. 3*B*). Importantly, the reduction of TNF-$\alpha$ mRNA by exogenous IL-10 was more pronounced in IL-10$^{-/-}$ cells compared with WT cells. The decay rate of TNF-$\alpha$ mRNA was not affected by IL-10 treatment of WT cells stimulated for 1 or 2 h with LPS (Fig. 3*C*). However, IL-10 accelerated the decay rate in IL-10$^{-/-}$ cells treated for 2 h with LPS, but not for 1 h (Fig. 3*D*). Therefore, endogenous IL-10 can mask to a certain extent the effect of exogenous IL-10 on the mRNA decay. In addition, the IL-10-mediated increase in mRNA decay rate becomes more apparent at later time points of LPS treatment, consistent with the need for new protein synthesis (e.g., TTP). Note that the duration of LPS treatment in Fig. 3*A* was 3 h and that the differences in IL-10-imposed inhibition of TNF-$\alpha$ production in TTP$^{-/-}$ vs WT cells increased with time of LPS treatment (Fig. 2, *A* and *B*).

To further substantiate the role of TTP in the anti-inflammatory effects of IL-10, we measured the IL-10-mediated reduction of IL-1$\alpha$ protein and mRNA in TTP$^{-/-}$ and WT cells (Fig. 3, *E* and *F*). Efficient LPS-stimulated production of IL-1$\alpha$, a known IL-10 target (13), depends on a second stimulus, such as ATP, that activates the IL-1-processing function of the inflammasome (37). Although IL-1$\alpha$ is not a direct substrate of caspase 1, production of mature IL-1$\alpha$ has been shown to be inflammasome/caspase 1 dependent (37). BMDMs from WT and TTP$^{-/-}$ animals were stimulated with LPS (10 h) and ATP (1 h before the collection of supernatants) in the presence or absence of IL-10. IL-10 caused a decrease of IL-1$\alpha$ protein to 20% of LPS plus ATP-treated WT cells, whereas in TTP$^{-/-}$ cells the IL-1$\alpha$ production was reduced only to 60% of the LPS plus ATP-treated samples (Fig. 3*E*). Similar differences between WT and TTP$^{-/-}$ cells were determined also for the IL-10-mediated decrease in IL-1$\alpha$ mRNA (Fig. 3*F*).

To rule out that the absence of TTP affected activation of Stat3 by IL-10 that might result in reduced IL-10 responsiveness of TTP$^{-/-}$ cells, the IL-10-induced tyrosine phosphorylation of Stat3 was determined in LPS-stimulated TTP$^{-/-}$ and WT BMDMs in the presence or absence of IL-10. The level of tyrosine-phosphorylated Stat3 was under all conditions comparable in both genotypes (supplemental Fig. S2*A*). In addition, the stimulatory effect of LPS was similar in TTP$^{-/-}$ and WT cells as judged by the activation of p38 MAPK (supplemental Fig. S2*B*). Thus, a different activation of the critical proinflammatory (p38 MAPK) and anti-inflammatory (Stat3) components in the WT and TTP$^{-/-}$ cells could be excluded as a reason for the observed differences in IL-10 responses.

These data establish that TTP plays an important role in IL-10-mediated down-regulation of two critical inflammatory cytokines (TNF-$\alpha$ and IL-1$\alpha$).

### *TTP function in IL-10 responses depends on IL-10-mediated reduction of p38 MAPK activity at later phase of inflammation*

IL-10 and IL-6 are both known to activate Stat3 yet only IL-10 exhibits anti-inflammatory properties (38–40). To examine whether and to what extent IL-6 was able to stimulate TTP expression, BMDMs were treated with LPS with or without IL-6.

[5] The online version of this article contains supplemental material.

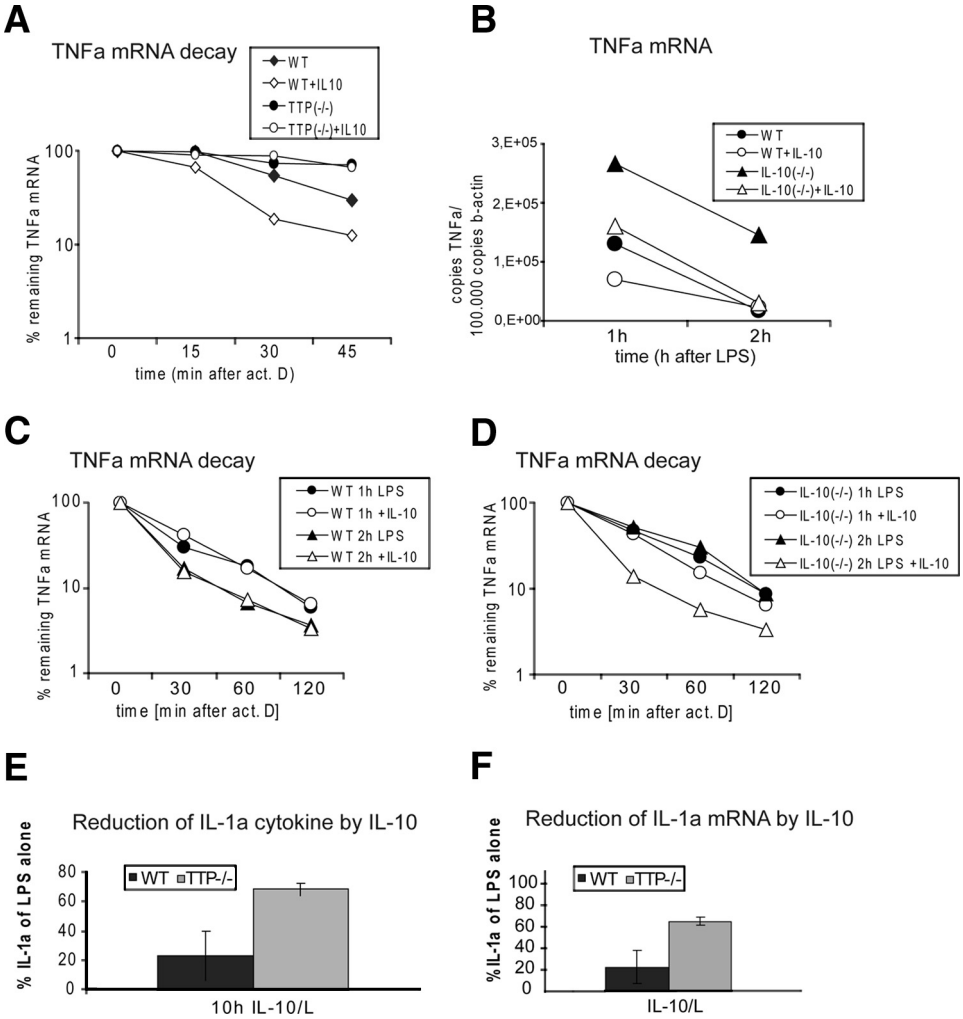


**FIGURE 3.** TTP is required for IL-10-mediated acceleration of TNF-$\alpha$ mRNA decay and for full IL-10-dependent reduction of IL-1$\alpha$. *A*, IL-10-induced changes in decay of TNF-$\alpha$ mRNA. WT-BMDMs and TTP-deficient BMDMs ($TTP^{-/-}$) were pretreated with IL-10 overnight and stimulated for 3 h with LPS followed by addition of actinomycin D (act D; 5 $\mu$g/ml) to stop transcription. After the indicated time points, TNF-$\alpha$ mRNA was quantified using qRT-PCR. Values were normalized against the housekeeping gene HPRT. Remnant TNF-$\alpha$ mRNA in percentage of the amount at the time point 0 of actinomycin D treatment is depicted. SDs ($n = 3$) are indicated. *B*, Effects of endogenous IL-10 on TNF-$\alpha$ mRNA induction in LPS- or LPS/IL-10-treated BMDMs. BMDMs derived from WT or $IL\text{-}10^{-/-}$ animals were treated for 1 or 2 h with LPS in the presence or absence of IL-10. The amount of TNF-$\alpha$ mRNA in these cells was determined by qRT-PCR. *C* and *D*, TNF-$\alpha$ mRNA decay in WT (*C*) and $IL\text{-}10^{-/-}$ (*D*) BMDMs. BMDMs were treated for 1 or 2 h with LPS in the presence or absence of IL-10. Thereafter, the transcription was stopped by actinomycin D and the remaining TNF-$\alpha$ mRNA was determined at the indicated times by qRT-PCR. Remnant TNF-$\alpha$ mRNA in percentage of the amount at the time point 0 of actinomycin D treatment is depicted. *E*, Reduction of IL-1$\alpha$ cytokine by IL-10. BMDMs from WT and $TTP^{-/-}$ mice were treated with LPS for 10 h in the presence or absence of IL-10. ATP was added 1 h before the collection of supernatants. Supernatants were collected and analyzed for IL-1$\alpha$ cytokine levels by ELISA. Reduction of IL-1$\alpha$ cytokine levels by IL-10 pretreatment relative (in percent) to LPS alone treatment (100%) is depicted. SDs are indicated, $n = 3$. *F*, Reduction of IL-1$\alpha$ mRNA by IL-10. BMDMs from WT and $TTP^{-/-}$ mice were treated with LPS for 2 h in the presence or absence of IL-10. IL-1$\alpha$ expression was analyzed by qRT-PCR. Reduction of IL-1$\alpha$ mRNA by IL-10 treatment relative (in percent) to LPS alone treatment (100%) is depicted.

Interestingly, IL-6 was able to increase TTP expression in LPS-treated macrophages almost to the same extent as IL-10 (Fig. 4*A*). Yet, consistent with the known properties of IL-6, IL-6 was not able to inhibit TNF-$\alpha$ (Fig. 4*B*) and IL-1$\alpha$ (Fig. 4*C*) in either WT or $TTP^{-/-}$ BMDMs. These data also implicate that the up-regulation of TTP expression by IL-10 cannot solely explain the role of TTP in the anti-inflammatory effects of this cytokine. We speculated that the role of TTP in IL-10 responses might be explained by an IL-10-dependent increase in TTP activity. TTP function is known to be negatively regulated by p38 MAPK and its downstream kinase MK2 (41). IL-10 has been reported to modestly inhibit p38 MAPK in the later phases of LPS treatment (18). The reduction of p38 MAPK activity is caused by the IL-10-mediated up-regulation of the dual-specificity phosphatase DUSP1 (Ref. 18 and Fig. 4*F*). We reasoned that such a reduction in p38 MAPK could relieve the p38 MAPK-dependent inhibition of TTP, thereby increasing the ability of TTP to down-regulate its target mRNAs. To investigate whether IL-6 was also able to reduce p38 MAPK activity, BMDMs were stimulated with LPS alone or along with either IL-10 or IL-6 and p38 MAPK activity was monitored after 2, 4, and 6 h. Treatment of LPS-stimulated cells with IL-10 caused a modest (3-fold) but consistent inhibition of p38 MAPK at 2, 4, and 6 h compared with the samples treated with LPS alone (Fig. 4*D*). To better document the IL-10 effect on p38 MAPK phosphorylation, the complete time course of IL-6- and IL-10-treated samples was run on a single gel (supplemental Fig. S3*A*). The LPS-induced activation of p38 MAPK at an early time point (30 min) was not affected by IL-10 cotreatment (Fig. 4*E*). Importantly,

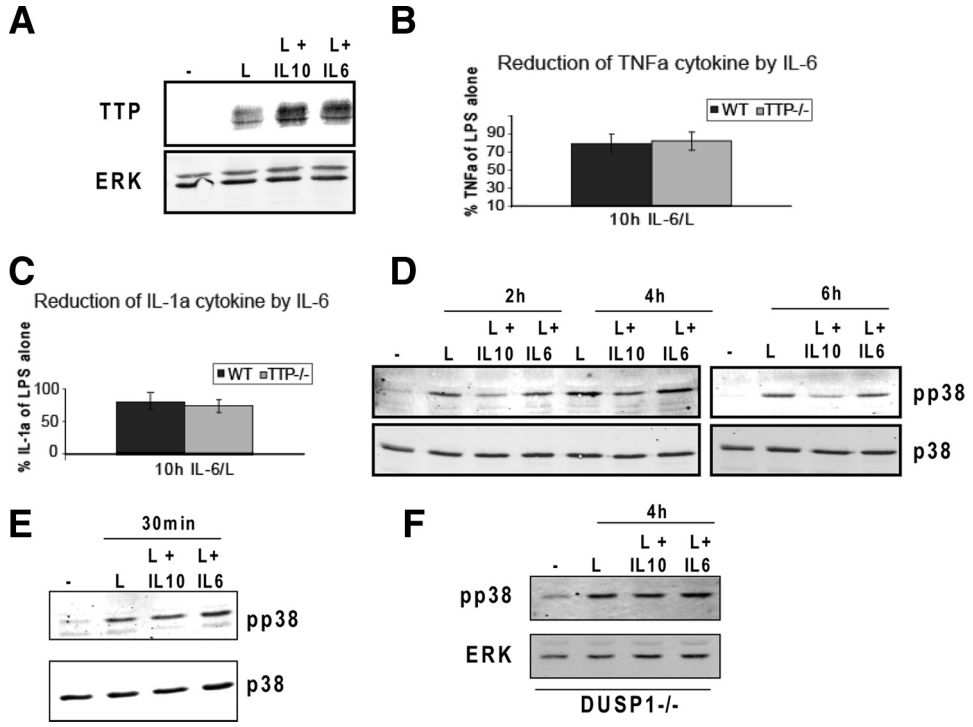


**FIGURE 4.** Effects of IL-6 on TTP expression, TNF-$\alpha$ and IL-1$\alpha$ production, and p38 MAPK activation in LPS-treated BMDMs. *A*, IL-6 and IL-10 increase TTP expression to similar levels in LPS-treated BMDMs. BMDMs were stimulated for 2 h with LPS alone or along with IL-10 or IL-6, and whole cell extracts were prepared. Expression of TTP was analyzed by Western blotting. panERK Ab was used for loading control. *B* and *C*, IL-6 does not reduce TNF-$\alpha$ production (*B*) or IL-1$\alpha$ (*C*) production in LPS-treated TTP$^{-/-}$ and control WT BMDMs. WT and TTP$^{-/-}$ BMDMs were stimulated for 10 h with LPS with or without cotreatment with IL-6. Amounts of TNF-$\alpha$ and IL-1$\alpha$ in supernatants were determined by ELISA. SDs of three representative experiments ($n = 3$) are indicated. *D*, IL-10 but not IL-6 decreases p38 MAPK activity in LPS-treated BMDMs. Whole cell extracts of BMDMs treated for 2, 4, and 6 h with LPS alone or cotreated with IL-10 or IL-6 were analyzed for activation of p38 MAPK by Western blotting using Ab to activated p38 MAPK (pp38). Ab to total p38 MAPK (p38) was used for loading control. *E*, p38 MAPK is not inhibited by IL-10 or IL-6 after 30 min of treatment. BMDMs were treated for 30 min with LPS with or without cotreatment with IL-10 or IL-6. p38 MAPK activation was determined as in *D*. *F*, IL-10 no longer reduces p38 MAPK phosphorylation in DUSP1$^{-/-}$ BMDMs. DUSP1$^{-/-}$ BMDMs were treated for 4 h with LPS alone, LPS + IL-10 or LPS + IL-6, and the activation of p38 MAPK was examined as in *D*. Figures are representative of at least three experiments.

p38 MAPK activity was not reduced by treatment with IL-6 at any time point examined (Fig. 4, *D* and *E*, and supplemental Fig. S3*A*). These findings are in agreement with the induction of mRNA for the MAPK phosphatase DUSP1 by IL-10 but not IL-6 in LPS-treated macrophages (18). To further support the role of DUSP1 in the IL-10-mediated effect on p38 MAPK activity, we examined p38 MAPK phosphorylation in DUSP1$^{-/-}$ BMDMs treated with LPS alone or along with either IL-10 or IL-6 for 4 h. In DUSP1$^{-/-}$ cells, IL-10 was no longer able to reduce p38 MAPK phosphorylation after treatment (Fig. 4*F*). Consistent with the IL-10-mediated decrease in p38 MAPK phosphorylation, at the 4 h-time point, the induction of DUSP1 by IL-10 plus LPS compared with LPS alone was more apparent than after a shorter treatment (1 h; supplemental Fig. S3*B*). The different effect of IL-10 and IL-6 on p38 MAPK is in agreement with the anti-inflammatory properties that are exhibited by IL-10 but not IL-6. The difference between IL-10 and IL-6 with regard to their anti-inflammatory properties has been attributed to SOCS3 that, as part of the negative feedback loop, binds to the gp130 subunit of the IL-6 receptor but not to the IL-10 receptor (38–40). Thus, SOCS3, a Stat3 target gene, is able to inhibit signaling elicited by IL-6 but not by IL-10. Consequently, IL-10 induces a prolonged Stat3 activation, whereas IL-6-mediated Stat3 activation is rapidly shut down. Consistently, in LPS-treated BMDMs IL-10 and IL-6 caused a comparable Stat3 activation after 30 min of cytokine treatment, whereas after 2 h Stat3 remained active only in cells stimulated with IL-10 but not IL-6 (supplemental Fig. 3*C*). These data suggest that a sustained Stat3 activation is needed for inhibition of p38 MAPK.

Although p38 MAPK is needed for TTP expression and protein stability (24, 25, 42), the kinase negatively regulates TTP activity at least at two levels. First, phosphorylation of TTP at Ser$^{52}$ and Ser$^{178}$ by MAPK-activated protein kinase kinase 2 (MK2), a kinase downstream of p38 MAPK, provides binding sites for 14-3-3 proteins that reduce the destabilizing activity of TTP (23, 43). Second, phosphorylation of TTP by MK2 was reported to negatively regulate its binding to AREs (25). To show that the IL-10-mediated decrease in p38 MAPK activity was able to change TTP properties in terms of its phosphorylation and binding to AREs, we analyzed the mobility of TTP in SDS-PAGE and binding of TTP to AREs in EMSA experiments. TTP appears in SDS-PAGE in the form of multiple bands that reflect various degree of predominantly p38 MAPK-dependent phosphorylation (24). To demonstrate p38 MAPK effects on TTP phosphorylation and binding to AREs, we first used an inducible expression of TTP in HeLa-Tet-off cells. This system allows manipulation of p38 MAPK activity without affecting the transcription of the TTP gene that is known to require p38 MAPK activity (27, 42). Stimulation of HeLa-Tet-off cells expressing TTP (i.e., without tetracycline) with anisomycin (a p38 MAPK agonist (44)) resulted in a more pronounced appearance of a slower migrating hyperphosphorylated TTP band, whereas the p38 MAPK inhibitor SB reduced the amount of the hyperphosphorylated TTP and also the total TTP level due to TTP protein destabilization (Fig. 5*A*). The analysis of

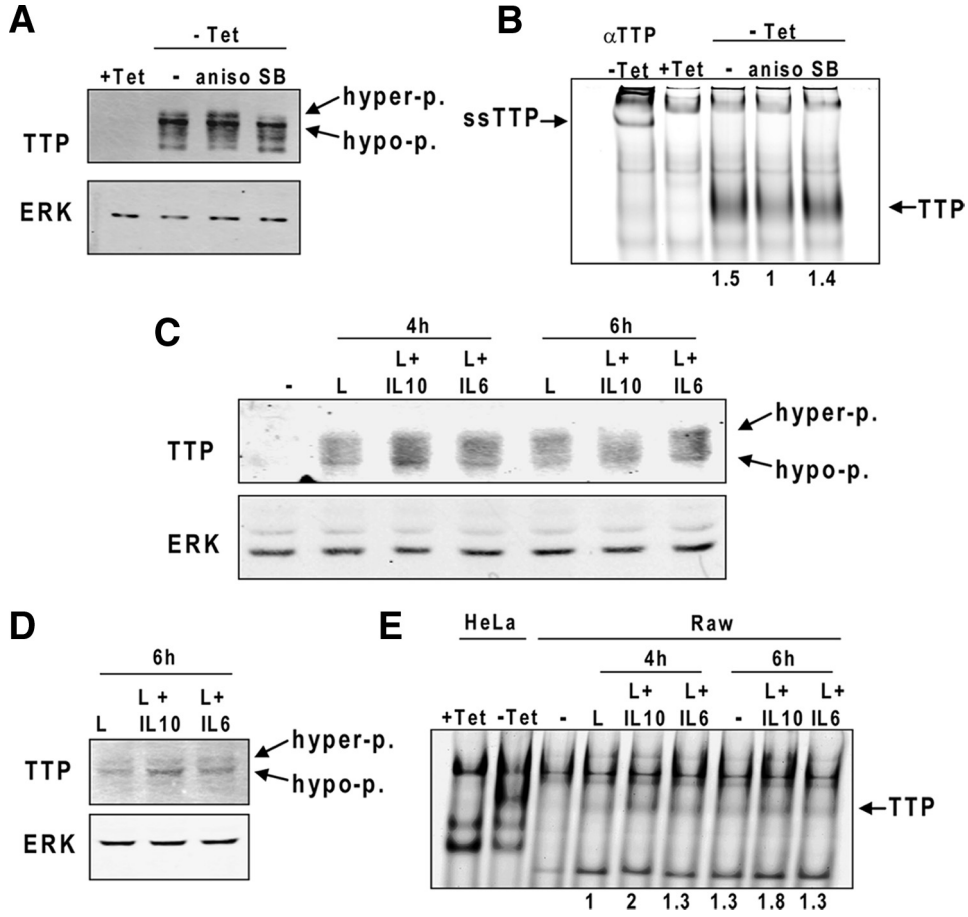


**FIGURE 5.** IL-10 reduces TTP phosphorylation and increases in vitro binding of TTP to ARE. *A* and *B*, HeLa-Tet-off cells were transiently transfected with pTRE-TTPfl and equally split into four 6-cm dishes. In three dishes, the expression of TTP was allowed overnight in medium without tetracycline (−Tet), whereas in one dish the TTP expression was blocked by tetracycline (+Tet). The (−Tet) cells were treated for 60 min with anisomycin (aniso) or SB. Whole cell extracts were prepared and split into one part for Western blot analysis (*A*) and a second part for RNA EMSA (*B*). The position of hyperphosphorylated (hyper-p.) and hypophosphorylated (hypo-p.) TTP is marked in *A*. *B*, The TTP-ARE complexes (TTP) were identified by a supershift (ssTTP) using a TTP Ab (αTTP). The relative intensity of the TTP-ARE complexes (as indicated be the numbers 1.5, 1, and 1.4) was quantitated using the LI-COR Odyssey software (supplementary Fig. 4*A*). *C*, Whole cell extracts of BMDMs treated for 4 and 6 h with LPS alone or cotreated with IL-10 or IL-6 were analyzed for TTP expression and SDS-PAGE mobility by Western blotting using Ab to TTP. Equal protein loading was controlled by reprobing with an anti-ERK Ab. The position of hyperphosphorylated (hyper-p.) and hypophosphorylated (hypo-p.) TTP is marked. *D*, Whole cell extracts of RAW 264.7 treated for 6 h with LPS alone or cotreated with IL-10 or IL-6 were analyzed as in *C*. *E*, Whole cell extracts of RAW 264.7 cells treated for 4 or 6 h with LPS alone or cotreated with IL-10 or IL-6 were assayed for in vitro binding of TTP to TNF-α ARE using RNA EMSA. The relative intensity of the TTP-ARE complexes (as indicated by the numbers 1, 2, 1.3, 1.3, 1.8, and 1.3) was quantitated using the LI-COR Odyssey software (supplementary Fig. 4*B*). To control the position of the TTP-ARE complexes extracts from HeLa-Tet-off cells expressing TTP (−Tet) or without TTP expression (+Tet) were used. The data are representative of three independent experiments.

the same extracts in a RNA EMSA experiment revealed that anisomycin reduced binding of TTP to the TNF-α ARE by 50% (Fig. 5*B*), whereas inhibition of p38 MAPK resulted in a similar amount of ARE-bound TTP as in untreated cells (Fig. 5*B*) despite a reduced TTP protein level present in that sample (compare SB-labeled lanes in Fig. 5*A* and 5*B*). Consistent with the IL-10-mediated p38 MAPK inhibition, the treatment of BMDMs with LPS plus IL-10 resulted in a shift to less phosphorylated TTP bands in SDS-PAGE if compared with cells treated with LPS alone or with LPS plus IL-6 (Fig. 5*C*). This shift to faster migrating TTP bands was more pronounced after 6 h of treatment. To address the IL-10 effect on TTP binding to AREs, we used the murine macrophage cell line RAW 264.7 since we were not able to detect TTP-ARE complexes in RNA EMSA experiments if BMDMs were used. LPS-stimulated RAW 264.7 cells express at least 3- to 5-fold more TTP than BMDMs (data not shown). Similar to BMDMs, LPS plus IL-10 treatment resulted in a more pronounced appearance of the hypophosphorylated TTP if compared with treatment with LPS alone or LPS plus IL-6 (Fig. 5*D*). Consistently, LPS plus IL-10 treatment caused an ~50–80% increase in the formation of TTP-ARE complexes in RNA EMSA experiments compared with LPS alone or LPS plus IL-6 treatments (Fig. 5*E*). These data show that IL-10 decreases the phosphorylation of TTP and enhances the in vitro binding of TTP to TNF-α ARE in a manner similar to the pharmacological inhibition of p38 MAPK. These findings are in agreement with an increased TTP activity under conditions of reduced p38 MAPK activation as is the case in IL-10-treated cells. Interestingly, despite the reduced p38 MAPK activity and hence increased proteasome-mediated TTP degradation in IL-10-treated cells, the TTP protein levels were not diminished compared with LPS plus IL-6-treated cells (Fig. 5, *C* and *D*). We explain this observation by the sustained Stat3 activation and, hence, a strong Stat3-driven transcription of the TTP gene in IL-10-treated cells (supplemental Fig. 3*C*).

To test whether p38 MAPK activity protects mRNA from TTP-mediated decay, we examined the stability of TNF-α and IL-1α

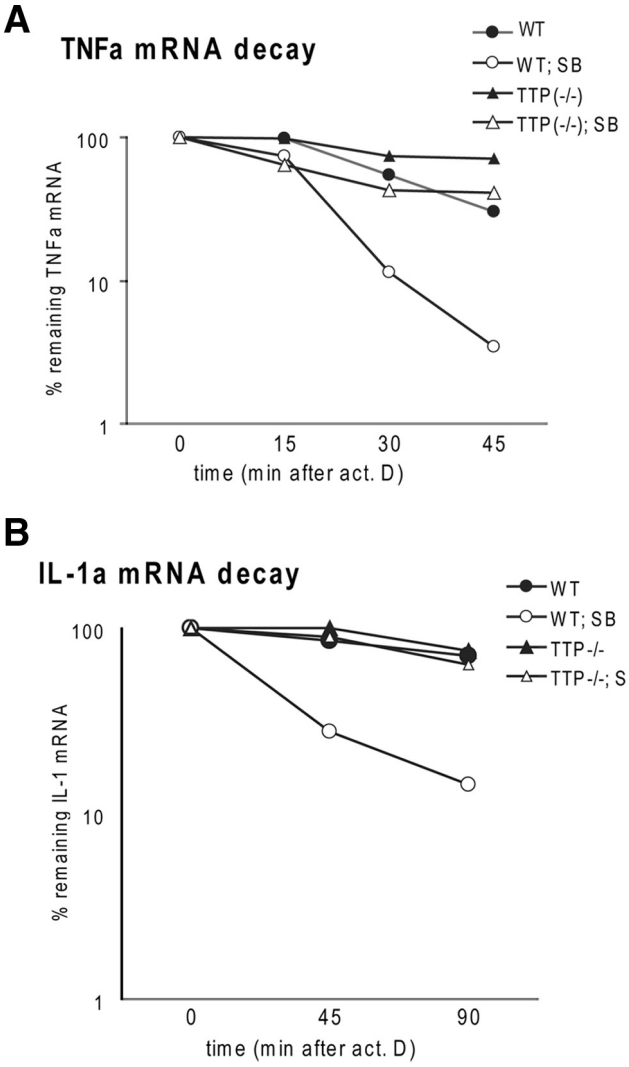


**FIGURE 6.** Effects of p38 MAPK inhibition on TTP-dependent reduction of mRNA for TNF-$\alpha$ and IL-1$\alpha$. *A* and *B*, WT and TTP$^{-/-}$ BMDMs were treated with LPS for 2 h, followed by treatment with SB or solvent control. Actinomycin D was added simultaneously with SB. The decay rates of TNF-$\alpha$ (*A*) and IL-1$\alpha$ (*B*) were monitored by qRT-PCR for the times indicated. Remnant TNF-$\alpha$ or IL-1$\alpha$ mRNA in percentage of the amount at the time point 0 of actinomycin D treatment is depicted.

mRNAs in the presence of the p38 MAPK inhibitor SB in TTP$^{-/-}$ and control WT BMDMs. Two hours after LPS stimulation, SB was added in combination with actinomycin D. p38 MAPK inhibition caused a 4-fold decrease of mRNA stability of TNF-$\alpha$ (TNF-$\alpha$ without SB: $t_{½}$ = 31 min; TNF-$\alpha$ with SB: $t_{½}$ = 8 min) and an ~8- to 10-fold decrease in IL-1$\alpha$ mRNA stability (IL-1$\alpha$ without SB: $t_{½}$ >2 h; IL-1$\alpha$ with SB: $t_{½}$ = 14 min; Fig. 6). In TTP$^{-/-}$ cells, the inhibition of p38 MAPK caused only a modest (2-fold) decrease in mRNA stability of TNF-$\alpha$ mRNA (TNF-$\alpha$ without SB: $t_{½}$ = 80 min; TNF-$\alpha$ with SB: $t_{½}$ = 37 min with SB) and no detectable decrease of IL-1$\alpha$ mRNA stability (IL-1$\alpha$ without SB: $t_{½}$ = >2 h; IL-1$\alpha$ with SB: $t_{½}$ = >2 h). These data indicate that the augmentation of mRNA decay by inhibition of p38 MAPK is to a large part TTP dependent. The contribution of TTP to the effects of p38 MAPK inhibition depends on the nature of the target mRNA.

We conclude that the IL-10-mediated reduction of p38 MAPK activity increases TTP-dependent mRNA decay. The combined effect of IL-10 on p38 MAPK activity and TTP expression contributes to the anti-inflammatory properties of IL-10.

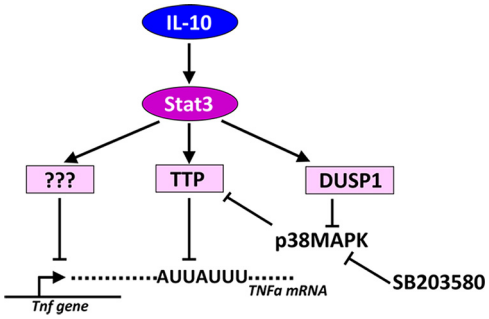


**FIGURE 7.** Model of IL-10-mediated anti-inflammatory effects. IL-10 activates the transcription factor Stat3 that drives the expression of at least three effector genes. The still unidentified repressors of transcription are depicted by question marks. The other effector is TTP that requires the activity of a third effector, the DUSP1 phosphatase, that reduces the activity of p38 MAPK, thereby increasing the TTP-destabilizing activity toward specific ARE-containing mRNAs. The activity of DUSP1 can be mimicked by, for example, the p38 MAPK inhibitor SB.

## Discussion

This study provides evidence that the mRNA- destabilizing factor TTP plays an important role in anti-inflammatory effects of IL-10. BMDMs deficient in TTP show a strongly reduced anti-inflammatory response to IL-10. Two cytokines, TNF-$\alpha$ and IL-1$\alpha$, were here demonstrated to be inhibited by IL-10 in a TTP-dependent manner. IL-10 appears to regulate TTP function in two ways (Fig. 7). First, IL-10 increases TTP expression in LPS-treated BMDMs. This augmented expression depends on Stat3, the key immediate effector of IL-10 effects. Second, IL-10 reduces at later time points the activity of p38 MAPK in LPS-treated BMDMs, hereby releasing TTP from the p38 MAPK-mediated inhibition. Since the increased expression of TTP is not sufficient to initiate an anti-inflammatory response (as demonstrated by IL-6-mediated up-regulation of TTP), we conclude that the TTP-dependent IL-10 effects can be best explained by the combination of higher TTP expression and reduction of p38 MAPK activity.

TTP is known for its mRNA-destabilizing activity most notably, but not exclusively, toward TNF-$\alpha$ mRNA via AREs in 3′ UTR. Thus, the IL-10-mediated rise in TNF-$\alpha$ decay rate is consistent with the elevated TTP levels and activity. In addition to TNF-$\alpha$, our data suggest that IL-1$\alpha$ is also a TTP target. The 3′ UTR of the murine IL-1$\alpha$ (GenBank accession no. NM_010554; http://www.ncbi.nlm.nih.gov/GenBank/) contains three elements (UAUUUAUA, AUAUUUAU, and UAUUAUUUAU) with similar sequences as the nonameric canonical sequence (UUAUUUAUU) recognized by TTP (45, 46). IL-1$\alpha$ has so far not been described as TTP target despite of several recently published global screens for novel TTP targets (36, 47). However, these searches revealed that in such global screens important targets might be missed (e.g., TNF-$\alpha$ in the study of Stoecklin et al. (36)). In addition, the finding that TTP regulates also mRNAs with no obvious AREs in 3′ UTR adds another degree of complexity to searches for TTP targets (47). The role of TTP in IL-10 responses is also in agreement with a study reporting IL-10-mediated ARE-dependent destabilization of CXCL1 mRNA (16). Importantly, in that study, the IL-10-induced mRNA decay was not detectable before 2 h of LPS/IL-10 treatment. This time point correlates well with the appearance of TTP expression (42) and with our data showing that IL-10-mediated decay of TNF-$\alpha$ mRNA is not apparent before 2 h of LPS treatment.

TTP-deficient cells are known to produce 2- to 3-fold more TNF-$\alpha$ (both mRNA and cytokine) than WT cells (26, 48). At the same time, IL-10 mRNA was shown to be targeted by TTP for degradation (36) and we found that the TTP-deficient BMDMs produce more IL-10 cytokine (supplemental Fig. S1). Thus, TTP cells produce more of both the proinflammatory TNF-$\alpha$ and the anti-inflammatory IL-10. Yet, the TTP cells still respond to IL-10 treatment (by e.g., Stat3 activation) and LPS treatment (by e.g., p38 MAPK activation) similarly as the WT cells (Fig. 4). We conclude that despite the 2- to 3-fold higher production of pro- and anti-inflammatory cytokines the TTP-deficient cells display comparable immediate-early response to pro- (LPS) or anti-inflammatory (IL-10) stimuli as WT cells.

We were not able to detect any differences in the TTP expression or p38 MAPK activity, the two key factors influencing the TTP function, when we compared the two protocols for IL-10 treatment. Thus, the higher absolute contribution of TTP to IL-10 responses in the pretreatment protocol suggests that IL-10 induces a cofactor of TTP during the pretreatment period. TTP serves as an adaptor protein linking mRNA to the mRNA processing and degradation machinery located in stress granules and processing bodies (21, 22). Some of the components of these complexes may be up-regulated by IL-10 during the pretreatment phase to enhance TTP function. Alternatively, IL-10-activated Stat3 may increase transcription of micro-RNAs such as micro-R16 that was shown to assist TTP in degradation of ARE-containing mRNAs (49). The influence of the experimental protocol on the IL-10 effects may also partially explain the discrepancy between our study and the study of Kontoyiannis et al. (14) who found no evidence for a role of TTP in IL-10 responses in the simultaneous treatment protocol. Instead, they proposed a rapid (within 15 min of IL-10 treatment) inhibition of p38 MAPK as the main mechanism of IL-10 action. We did not detect any inhibition of p38 MAPK activity by IL-10 at early time points regardless of applied protocol for IL-10 treatment (supplemental Fig. S2, *A* and *B*). These data are in agreement with several other studies showing no effect of IL-10 on p38 MAPK activity at early time points (2, 12, 50, 51). In a kinetics analysis of p38 MAPK activity, IL-10 was found to exhibit a modest inhibitory effect on p38 MAPK activity at later time points (after 3 h of LPS plus IL-10 treatment) (18). The IL-10-mediated inhibition of p38 MAPK correlated with the induction of the dual-specificity phosphatase DUSP1, a MAPK phosphatase (18). We observed a similar reduction in p38 MAPK phosphorylation in terms of both, the magnitude and kinetics, and found that DUSP1 was required for this effect. The inhibition of p38 MAPK was caused specifically by IL-10 but not by IL-6 and correlated with the ability of IL-10 (but not IL-6) to induce sustained Stat3 activation. Consistently, IL-10 but not IL-6 was reported to increase expression of DUSP1 in LPS-treated macrophages (18). DUSP1 function as a critical p38 MAPK-inactivating enzyme provides the most likely explanation for the susceptibility of DUSP1$^{-/-}$ mice to LPS-mediated toxic shock (52, 53). We speculate that the inhibition of the proinflammatory p38 MAPK by IL-10 at the later stages of macrophage stimulation is a key factor in immune homeostasis since p38 MAPK influences inflammation-related transcription, RNA stability, translation, as well as secretion. To mimic the inhibition of p38 MAPK by IL-10 at the late phase of stimulation, we used the p38 MAPK inhibitor SB and examined the effect of p38 MAPK on the TTP-dependent decrease of target mRNA molecules. Interestingly, whereas in the case of TNF-$\alpha$ a different decay rate (i.e., different decay rate in TTP$^{+/+}$ and TTP$^{-/-}$ cells) was observed also without p38 MAPK inhibition, for IL-1$\alpha$ mRNA the TTP-dependent decay was detectable only if p38 MAPK was inhibited (Fig. 5). A similar requirement for p38 MAPK inhibition has been recently described also for the TTP target CXCL1 (KC) mRNA (54). These findings indicate that the p38 MAPK-mediated control of TTP activity has a differential impact on target mRNAs. Although the TTP-dependent decay of some mRNAs (e.g., TNF-$\alpha$) proceeds in the presence of p38 MAPK activity, the degradation of other mRNAs (e.g., IL-1$\alpha$ and CXCL1) is strongly dependent on the kinase inhibition. This mechanism may play an important role in the specificity of TTP-mediated RNA decay: depending on the activation status of p38 MAPK or other kinases implicated in regulation of TTP activity (e.g., ERK of MK2), TTP would discriminate between various targets. The critical role that p38 MAPK plays in TTP-mediated RNA decay also suggests that the time point that is taken to determine RNA stability has a decisive effect on the outcome of the assay. The activation/inactivation profile of p38 MAPK is likely to vary between different experimental settings (e.g., amount and quality of LPS, the use of primary or immortalized cells, the origin of primary cells such as bone marrow or peritoneum) so that this aspect may also contribute to the variability of published data. For example, we did not observe an IL-10-mediated increase in TNF-$\alpha$ mRNA decay in peritoneal-derived macrophages.

Suppression of inflammatory responses by IL-10 is one of the key features in immune homeostasis. Despite many years of research, the question of how a single cytokine can specifically inhibit various inflammatory reactions with such a high efficiency remains unresolved. Recent studies suggest that known as well as yet unknown IL-10 effectors interfere on different levels and by different mechanisms with the intracellular inflammatory networks. This multitasking system employed by IL-10 is likely to be essential for the efficiency and specificity of the anti-inflammatory properties of IL-10. For example, the efficient and dominant inhibition of *Tnf* gene transcription by IL-10 still requires the remaining TNF-$\alpha$ mRNA to be removed from the system, i.e., by TTP. Although the primary IL-10-elicited signaling events that involve the IL-10 receptor, Jak1 and Tyk2 kinases, as well as the transcription factor Stat3, are to a large part common to all cell types, the more complex effects downstream of Stat3 may be cell type dependent. Thus, the function and activity of the Stat3-induced IL-10 effectors may be regulated by the environment within the particular cell type, hereby helping to explain the still incoherent and sometimes contradictory studies of the anti-inflammatory effects of IL-10.

## Acknowledgments

We thank S. Badelt for excellent technical help and Bristol-Myers Squibb for the permission to use Dusp1$^{-/-}$ mice.

## Disclosures

The authors have no financial conflict of interest.

## References

1. Fiorentino, D. F., M. W. Bond, and T. R. Mosmann. 1989. Two types of mouse T helper cell: IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med.* 170: 2081–2095.
2. Williams, L. M., G. Ricchetti, U. Sarma, T. Smallie, and B. M. Foxwell. 2004. Interleukin-10 suppression of myeloid cell activation–a continuing puzzle. *Immunology* 113: 281–292.
3. Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Muller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75: 263–274.
4. Berg, D. J., R. Kuhn, K. Rajewsky, W. Muller, S. Menon, N. Davidson, G. Grunig, and D. Rennick. 1995. Interleukin-10 is a central regulator of the response to LPS in murine models of endotoxic shock and the Shwartzman reaction but not endotoxin tolerance. *J. Clin. Invest.* 96: 2339–2347.
5. Roers, A., L. Siewe, E. Strittmatter, M. Deckert, D. Schluter, W. Stenzel, A. D. Gruber, T. Krieg, K. Rajewsky, and W. Muller. 2004. T cell-specific inactivation of the interleukin 10 gene in mice results in enhanced T cell responses but normal innate responses to lipopolysaccharide or skin irritation. *J. Exp. Med.* 200: 1289–1297.

6. Siewe, L., M. Bollati-Fogolin, C. Wickenhauser, T. Krieg, W. Muller, and A. Roers. 2006. Interleukin-10 derived from macrophages and/or neutrophils regulates the inflammatory response to LPS but not the response to CpG DNA. *Eur. J. Immunol.* 36: 3248–3255.
7. Takeda, K., B. E. Clausen, T. Kaisho, T. Tsujimura, N. Terada, I. Forster, and S. Akira. 1999. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity* 10: 39–49.
8. Lang, R., D. Patel, J. J. Morris, R. L. Rutschman, and P. J. Murray. 2002. Shaping gene expression in activated and resting primary macrophages by IL-10. *J. Immunol.* 169: 2253–2263.
9. Williams, L., L. Bradley, A. Smith, and B. Foxwell. 2004. Signal transducer and activator of transcription 3 is the dominant mediator of the anti-inflammatory effects of IL-10 in human macrophages. *J. Immunol.* 172: 567–576.
10. Maritano, D., M. L. Sugrue, S. Tininini, S. Dewilde, B. Strobl, X. Fu, V. Murray-Tait, R. Chiarle, and V. Poli. 2004. The STAT3 isoforms $\alpha$ and $\beta$ have unique and specific functions. *Nat. Immunol.* 5: 401–409.
11. Williams, L. M., U. Sarma, K. Willets, T. Smallie, F. Brennan, and B. M. Foxwell. 2007. Expression of constitutively active STAT3 can replicate the cytokine-suppressive activity of interleukin-10 in human primary macrophages. *J. Biol. Chem.* 282: 6965–6975.
12. Denys, A., I. A. Udalova, C. Smith, L. M. Williams, C. J. Ciesielski, J. Campbell, C. Andrews, D. Kwaitkowski, and B. M. Foxwell. 2002. Evidence for a dual mechanism for IL-10 suppression of TNF-$\alpha$ production that does not involve inhibition of p38 mitogen-activated protein kinase or NF-$\kappa$B in primary human macrophages. *J. Immunol.* 168: 4837–4845.
13. Murray, P. J. 2005. The primary mechanism of the IL-10-regulated antiinflammatory response is to selectively inhibit transcription. *Proc. Natl. Acad. Sci. USA* 102: 8686–8691.
14. Kontoyiannis, D., A. Kotlyarov, E. Carballo, L. Alexopoulou, P. J. Blackshear, M. Gaestel, R. Davis, R. Flavell, and G. Kollias. 2001. Interleukin-10 targets p38 MAPK to modulate ARE-dependent TNF mRNA translation and limit intestinal pathology. *EMBO J.* 20: 3760–3770.
15. Rajasingh, J., E. Bord, C. Luedemann, J. Asai, H. Hamada, T. Thorne, G. Qin, D. Goukassian, Y. Zhu, D. W. Losordo, and R. Kishore. 2006. IL-10-induced TNF-$\alpha$ mRNA destabilization is mediated via IL-10 suppression of p38 MAP kinase activation and inhibition of HuR expression. *FASEB J.* 20: 2112–2114.
16. Biswas, R., S. Datta, J. D. Gupta, M. Novotny, J. Tebo, and T. A. Hamilton. 2003. Regulation of chemokine mRNA stability by lipopolysaccharide and IL-10. *J. Immunol.* 170: 6202–6208.
17. Kuwata, H., Y. Watanabe, H. Miyoshi, M. Yamamoto, T. Kaisho, K. Takeda, and S. Akira. 2003. IL-10-inducible Bcl-3 negatively regulates LPS-induced TNF-$\alpha$a production in macrophages. *Blood* 102: 4123–4129.
18. Hammer, M., J. Mages, H. Dietrich, F. Schmitz, F. Striebel, P. J. Murray, H. Wagner, and R. Lang. 2005. Control of dual-specificity phosphatase-1 expression in activated macrophages by IL-10. *Eur. J. Immunol.* 35: 2991–3001.
19. El Kasmi, K. C., A. M. Smith, L. Williams, G. Neale, A. Panopolous, S. S. Watowich, H. Hacker, B. M. Foxwell, and P. J. Murray. 2007. Cutting edge: a transcriptional repressor and corepressor Induced by the STAT3-regulated anti-inflammatory signaling pathway. *J. Immunol.* 179: 7215–7219.
20. Carrick, D. M., W. S. Lai, and P. J. Blackshear. 2004. The tandem CCCH zinc finger protein tristetraprolin and its relevance to cytokine mRNA turnover and arthritis. *Arthritis Res. Ther.* 6: 248–264.
21. Kedersha, N., G. Stoecklin, M. Ayodele, P. Yacono, J. Lykke-Andersen, M. J. Fritzler, D. Scheuner, R. J. Kaufman, D. E. Golan, and P. Anderson. 2005. Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. *J. Cell Biol.* 169: 871–884.
22. Franks, T. M., and J. Lykke-Andersen. 2007. TTP and BRF proteins nucleate processing body formation to silence mRNAs with AU-rich elements. *Genes Dev.* 21: 719–735.
23. Stoecklin, G., T. Stubbs, N. Kedersha, S. Wax, W. F. Rigby, T. K. Blackwell, and P. Anderson. 2004. MK2-induced tristetraprolin:14-3-3 complexes prevent stress granule association and ARE-mRNA decay. *EMBO J.* 23: 1313–1324.
24. Brook, M., C. R. Tchen, T. Santalucia, J. McIlrath, J. S. Arthur, J. Saklatvala, and A. R. Clark. 2006. Posttranslational regulation of tristetraprolin subcellular localization and protein stability by p38 mitogen-activated protein kinase and extracellular signal-regulated kinase pathways. *Mol. Cell. Biol.* 26: 2408–2418.
25. Hitti, E., T. Iakovleva, M. Brook, S. Deppenmeier, A. D. Gruber, D. Radzioch, A. R. Clark, P. J. Blackshear, A. Kotlyarov, and M. Gaestel. 2006. Mitogen-activated protein kinase-activated protein kinase 2 regulates tumor necrosis factor mRNA stability and translation mainly by altering tristetraprolin expression, stability, and binding to adenine/uridine-rich element. *Mol. Cell. Biol.* 26: 2399–2407.
26. Taylor, G. A., E. Carballo, D. M. Lee, W. S. Lai, M. J. Thompson, D. D. Patel, D. I. Schenkman, G. S. Gilkeson, H. E. Broxmeyer, B. F. Haynes, and P. J. Blackshear. 1996. A pathogenetic role for TNF$\alpha$ in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. *Immunity* 4: 445–454.
27. Sauer, I., B. Schaljo, C. Vogl, I. Gattermeier, T. Kolbe, M. Muller, P. J. Blackshear, and P. Kovarik. 2006. Interferons limit inflammatory responses by induction of tristetraprolin. *Blood* 107: 4790–4797.
28. Alonzi, T., D. Maritano, B. Gorgoni, G. Rizzuto, C. Libert, and V. Poli. 2001. Essential role of STAT3 in the control of the acute-phase response as revealed by inducible gene activation in the liver. *Mol. Cell. Biol.* 21: 1621–1632.
29. Kovarik, P., D. Stoiber, M. Novy, and T. Decker. 1998. Stat1 combines signals derived from IFN-$\gamma$ and LPS receptors during macrophage activation. [Published erratum appears in *1998 EMBO J.* 17: 4210]. *EMBO J.* 17: 3660–3668.
30. Morrison, T. B., J. J. Weis, and C. T. Wittwer. 1998. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *BioTechniques* 24: 954–958, 960, 962.
31. Suzuki, K., H. Nakajima, K. Ikeda, Y. Maezawa, A. Suto, H. Takatori, Y. Saito, and I. Iwamoto. 2003. IL-4-Stat6 signaling induces tristetraprolin expression and inhibits TNF-$\alpha$ production in mast cells. *J. Exp. Med.* 198: 1717–1727.
32. Ramsauer, K., I. Sadzak, A. Porras, A. Pilz, A. R. Nebreda, T. Decker, and P. Kovarik. 2002. p38 MAPK enhances STAT1-dependent transcription independently of Ser-727 phosphorylation. *Proc. Natl. Acad. Sci. USA* 99: 12859–12864.
33. Pesu, M., S. Aittomaki, K. Takaluoma, A. Lagerstedt, and O. Silvennoinen. 2002. p38 Mitogen-activated protein kinase regulates interleukin-4-induced gene expression by stimulating STAT6-mediated transcription. *J. Biol. Chem.* 277: 38254–38261.
34. Li, Y., A. Sassano, B. Majchrzak, D. K. Deb, D. E. Levy, M. Gaestel, A. R. Nebreda, E. N. Fish, and L. C. Platanias. 2004. Role of p38$\alpha$ Map kinase in type I interferon signaling. *J. Biol. Chem.* 279: 970–979.
35. Carballo, E., W. S. Lai, and P. J. Blackshear. 1998. Feedback inhibition of macrophage tumor necrosis factor-$\alpha$ production by tristetraprolin. *Science* 281: 1001–1005.
36. Stoecklin, G., S. A. Tenenbaum, T. Mayo, S. V. Chittur, A. D. George, T. E. Baroni, P. J. Blackshear, and P. Anderson. 2008. Genome-wide analysis identifies interleukin-10 mRNA as target of tristetraprolin. *J. Biol. Chem.* 283: 11689–11699.
37. Sutterwala, F. S., Y. Ogura, M. Szczepanik, M. Lara-Tejero, G. S. Lichtenberger, E. P. Grant, J. Bertin, A. J. Coyle, J. E. Galan, P. W. Askenase, and R. A. Flavell. 2006. Critical role for NALP3/CIAS1/cryopyrin in innate and adaptive immunity through its regulation of caspase-1. *Immunity* 24: 317–327.
38. Yasukawa, H., M. Ohishi, H. Mori, M. Murakami, T. Chinen, D. Aki, T. Hanada, K. Takeda, S. Akira, M. Hoshijima, et al. 2003. IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. *Nat. Immunol.* 4: 551–556.
39. Lang, R., A. L. Pauleau, E. Parganas, Y. Takahashi, J. Mages, J. N. Ihle, R. Rutschman, and P. J. Murray. 2003. SOCS3 regulates the plasticity of gp130 signaling. *Nat. Immunol.* 4: 546–550.
40. Croker, B. A., D. L. Krebs, J. G. Zhang, S. Wormald, T. A. Willson, E. G. Stanley, L. Robb, C. J. Greenhalgh, I. Forster, B. E. Clausen, et al. 2003. SOCS3 negatively regulates IL-6 signaling in vivo. *Nat. Immunol.* 4: 540–545.
41. Sandler, H., and G. Stoecklin. 2008. Control of mRNA decay by phosphorylation of tristetraprolin. *Biochem. Soc. Trans.* 36: 491–496.
42. Mahtani, K. R., M. Brook, J. L. Dean, G. Sully, J. Saklatvala, and A. R. Clark. 2001. Mitogen-activated protein kinase p38 controls the expression and post-translational modification of tristetraprolin, a regulator of tumor necrosis factor $\alpha$ mRNA stability. *Mol. Cell. Biol.* 21: 6461–6469.
43. Johnson, B. A., J. R. Stehn, M. B. Yaffe, and T. K. Blackwell. 2002. Cytoplasmic localization of tristetraprolin involves 14-3-3-dependent and -independent mechanisms. *J. Biol. Chem.* 277: 18029–18036.
44. Fukunaga, R., and T. Hunter. 1997. MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. *EMBO J.* 16: 1921–1933.
45. Worthington, M. T., J. W. Pelo, M. A. Sachedina, J. L. Applegate, K. O. Arseneau, and T. T. Pizarro. 2002. RNA binding properties of the AU-rich element-binding recombinant Nup475/TIS11/tristetraprolin protein. *J. Biol. Chem.* 277: 48558–48564.
46. Blackshear, P. J., W. S. Lai, E. A. Kennington, G. Brewer, G. M. Wilson, X. Guan, and P. Zhou. 2003. Characteristics of the interaction of a synthetic human tristetraprolin tandem zinc finger peptide with AU-rich element-containing RNA substrates. *J. Biol. Chem.* 278: 19947–19955.
47. Emmons, J., W. H. Townley-Tilson, K. M. Deleault, S. J. Skinner, R. H. Gross, M. L. Whitfield, and S. A. Brooks. 2008. Identification of TTP mRNA targets in human dendritic cells reveals TTP as a critical regulator of dendritic cell maturation. *RNA* 14: 888–902.
48. Lai, W. S., E. Carballo, J. R. Strum, E. A. Kennington, R. S. Phillips, and P. J. Blackshear. 1999. Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor $\alpha$ mRNA. *Mol. Cell. Biol.* 19: 4311–4323.
49. Jing, Q., S. Huang, S. Guth, T. Zarubin, A. Motoyama, J. Chen, F. Di Padova, S. C. Lin, H. Gram, and J. Han. 2005. Involvement of microRNA in AU-rich element-mediated mRNA instability. *Cell* 120: 623–634.
50. Donnelly, R. P., H. Dickensheets, and D. S. Finbloom. 1999. The interleukin-10 signal transduction pathway and regulation of gene expression in mononuclear phagocytes. *J. Interferon Cytokine Res.* 19: 563–573.
51. Murray, P. J. 2006. STAT3-mediated anti-inflammatory signalling. *Biochem. Soc. Trans.* 34: 1028–1031.
52. Abraham, S. M., T. Lawrence, A. Kleiman, P. Warden, M. Medghalchi, J. Tuckermann, J. Saklatvala, and A. R. Clark. 2006. Antiinflammatory effects of dexamethasone are partly dependent on induction of dual specificity phosphatase 1. *J. Exp. Med.* 203: 1883–1889.
53. Hammer, M., J. Mages, H. Dietrich, A. Servatius, N. Howells, A. C. Cato, and R. Lang. 2006. Dual specificity phosphatase 1 (DUSP1) regulates a subset of LPS-induced genes and protects mice from lethal endotoxin shock. *J. Exp. Med.* 203: 15–20.
54. Datta, S., R. Biswas, M. Novotny, P. G. Pavicic, Jr., T. Herjan, P. Mandal, and T. A. Hamilton. 2008. Tristetraprolin regulates CXCL1 (KC) mRNA stability. *J. Immunol.* 180: 2545–2552.

Cellular Microbiology (2010) **12**(2), 199–216
doi:10.1111/j.1462-5822.2009.01391.x
First published online 26 October 2009

# Autophagy supports *Candida glabrata* survival during phagocytosis

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## Summary

**The opportunistic human fungal pathogen *Candida glabrata* is confronted with phagocytic cells of the host defence system. Survival of internalized cells is thought to contribute to successful dissemination. We investigated the reaction of engulfed *C. glabrata* cells using fluorescent protein fusions of the transcription factors CgYap1 and CgMig1 and catalase CgCta1. The expression level and peroxisomal localization of catalase was used to monitor the metabolic and stress status of internalized *C. glabrata* cells. These reporters revealed that the phagocytosed *C. glabrata* cells were exposed to transient oxidative stress and starved for carbon source. Cells trapped within macrophages increased their peroxisome numbers indicating a metabolic switch. Prolonged phagocytosis caused a pexophagy-mediated decline in peroxisome numbers. Autophagy, and in particular pexophagy, contributed to survival of *C. glabrata* during engulfment. Mutants lacking *CgATG11* or *CgATG17*, genes required for pexophagy and non-selective autophagy, respectively, displayed reduced survival rates. Furthermore, both CgAtg11 and CgAtg17 contribute to survival, since the double mutant was highly sensitive to engulfment. Inhibition of peroxisome formation by deletion of *CgPEX3* partially restored viability of Cg*ATG11* deletion mutants during engulfment. This suggests that peroxisome formation and maintenance might sequester resources required for optimal survival. Mobilization of intracellular resources via autophagy is an important virulence factor that supports the viability of *C. glabrata* in the phagosomal compartment of infected innate immune cells.**

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## Introduction

*Candida glabrata* belongs to the diverse group of human fungal pathogens and is phylogenetically closely related to *Saccharomyces cerevisiae* (Kaur *et al*., 2005, Marcet-Houben and Gabaldon, 2009). The high similarity of *C. glabrata* to *S. cerevisiae* suggests that also for fungi, relatively small genetic changes may be sufficient for adaptation to a pathogenic lifestyle (Dujon *et al*., 2004). *C. glabrata* is a common commensal, but can turn into an opportunistic pathogen with a rising frequency of isolates among immunocompromised patients and elder people (Li *et al*., 2007; Pfaller and Diekema, 2007; Presterl *et al*., 2007). In the host environment, *C. glabrata* has to evade or survive attacks of the cell-mediated immune system (Nicola *et al*., 2008). Counterstrategies of fungal pathogens differ between species. *Candida albicans* destroys macrophages by hyphal outgrowth. Alternatively, *Cryptococcus neoformans* either lyses macrophages or escapes via phagosomal extrusion (Alvarez and Casadevall, 2006; Ma *et al*., 2006). *C. glabrata* engulfed by macrophages do not undergo morphological transitions such as *C. albicans* (Leberer *et al*., 2001). An open question concerns how *C. glabrata* is coping with cells of the immune system, such as macrophages.

The phagosome is a hostile environment for fungi (reviewed in Nicola *et al*., 2008). After internalization of microbial cells, the organelle maturates into the phagolysosome containing mature hydrolytic enzymes and a more acidic pH 4.5–5.5 (Geisow *et al*., 1981; Levitz *et al*., 1999). Additionally, the NADPH oxidase complex generates reactive oxidative species to attack internalized microorganisms (for review see Romani, 2004; Segal, 2005). Thus, commensal and pathogenic fungi are exposed to reactive oxygen species (ROS) produced by polymorphonuclear leucocytes, macrophages and dendritic cells

(Miller and Britigan, 1997; Missall *et al*., 2004; Gildea *et al*., 2005). On the fungal side, antioxidant defence enzymes such as catalase, superoxide dismutase, thioredoxin- and glutathione-dependent peroxidases and reductases guard against oxidative stress and are thus considered virulence factors (Johnson *et al*., 2002; Cox *et al*., 2003; Missall *et al*., 2004; Chaves *et al*., 2007). Oxidative stress as defence strategy is not restricted to combat fungal infections. Invading bacteria, such as *Staphylococcus aureus*, or the malaria parasites *Plasmodium* sp., face ROS stress upon engulfment (Becker *et al*., 2004; Voyich *et al*., 2005). ROS sensed by microbes act also as signalling molecules. The *C. albicans* catalase, an enzyme which decomposes hydrogen peroxide, has been investigated more closely. *C. albicans* induces catalase when engulfed in neutrophils or in macrophages (Rubin-Bejerano *et al*., 2003; Lorenz *et al*., 2004; Enjalbert *et al*., 2007). Moreover, hydrogen peroxide promotes the morphological transition of *C. albicans* cells to hyphal growth, a form invading the host tissue (Nakagawa, 2008; Nasution *et al*., 2008). Finally, *C. albicans* devoid of catalase was eliminated more efficiently in a mouse infection model (Nakagawa *et al*., 2003). The filamentous fungus *Aspergillus fumigatus* lacking the catalases expressed in the mycelium exhibited delayed infection in a rat model of invasive aspergillosis (Paris *et al*., 2003). In contrast, *C. glabrata* catalase was not a virulence determinant in an immunocompromised mouse model (Cuellar-Cruz *et al*., 2008). In mice infected with a *C. neoformans* mutant devoid of all four catalases, mortality was unchanged (Giles *et al*., 2006). Thus the relative importance of individual ROS scavenging enzymes varies between fungal pathogens.

Besides being exposed to oxidative stress, cells engulfed by macrophages adjust their metabolic programme (Fan *et al*., 2005; Barelle *et al*., 2006). Engulfed *C. albicans* cells induce many genes involved in non-fermentative carbon metabolism (Prigneau *et al*., 2003; Lorenz *et al*., 2004). Phagocytosed *C. glabrata* induces genes encoding enzymes involved in β-oxidation, the glyoxylate cycle and gluconeogenesis (Kaur *et al*., 2007). Moreover, the glyoxylate cycle, which is required to channel fatty acid-derived two carbon units into metabolism, was early recognized as a virulence determinant for *C. albicans* (Lorenz and Fink, 2001). Other human fungal pathogens also induce glyoxylate cycle components during infection conditions (Rude *et al*., 2002; Canovas and Andrianopoulos, 2006; Derengowski *et al*., 2008). However, *A. fumigatus* and *C. neoformans* do not require the glyoxylate cycle for virulence (Idnurm *et al*., 2007; Schöbel *et al*., 2007). Some of the enzymes of the glyoxylate cycle are localized in the peroxisomal matrix (for review see, e.g. Kunze *et al*., 2006). Peroxisomes are inducible, single-membrane organelles which harbour enzymes for the oxidative catabolism of fatty acids, the glyoxylate cycle and others. Generally, peroxisome number and size vary according to metabolic needs (for review see Yan *et al*., 2005; Platta and Erdmann, 2007).

Autophagy continuously recycles almost all constituents of the cell (for review see Mizushima and Klionsky, 2007; Kraft *et al*., 2009). Different types of autophagy help organisms to overcome periods of nutrient starvation by recycling intracellular components to sustain vital cellular functions. It seems to be linked to the unique niches and morphology of fungal pathogens (for review see Palmer *et al*., 2008). For certain pathogenic fungi, autophagy has been identified as a virulence factor. *C. neoformans* requires an intact autophagy pathway during infection (Hu *et al*., 2008). Peroxisomes and their contents are delivered to the vacuole by the pexophagy pathway, a specialized form of autophagy (Guan *et al*., 2001; Kim *et al*., 2001; Farre and Subramani, 2004). In *S. cerevisiae* selective pexophagy is dependent on Atg11 and partly on Atg17 which is also important for non-selective autophagy (Cheong *et al*., 2005; Yorimitsu and Klionsky, 2005).

Here we investigated responses of *C. glabrata* during its encounter with the macrophage phagosome compartment from which it cannot escape. We developed *in vivo* reporters to track fungal responses to this environment. To detect oxidative and glucose starvation stress of cells, we used fluorescent protein fusions of the *C. glabrata* orthologues of the *S. cerevisiae* transcription factors Yap1 and Mig1 (Kuge *et al*., 1997; Carlson, 1999). We found the *C. glabrata* catalase gene *CgCTA1* and catalase activity regulated by oxidative stress and glucose starvation. Additionally, we demonstrated GFP–CgCta1 localization to peroxisomes. *C. glabrata* peroxisomes have not been described so far and were here defined by several independent criteria. We found that *C. glabrata* cells engulfed by mouse macrophages experience a mild oxidative stress and sustained carbon starvation. Additionally, peroxisomes became transiently induced in engulfed cells. We explored the role of peroxisomes with various mutants lacking peroxisome biogenesis or autophagy pathways mediating destruction of peroxisomes. We report here that autophagy and, surprisingly, pexophagy is a likely virulence factor for *C. glabrata.* Mutants lacking CgAtg11 and/or CgAtg17 were killed more efficiently by macrophages during engulfment. Thus, for engulfed *C. glabrata* cells, nutrient deprivation represents a serious challenge and mobilization of intracellular resources via autophagy is a major contributor to sustain viability.

## Results

### C. glabrata *catalase CgCta1 is induced by hydrogen peroxide and carbon starvation*

*Candida glabrata* harbours one catalase gene (*CgCTA1*, CAGL0K10868g), related to the yeast peroxisomal cata-

lase *CTA1* gene. The two catalase genes of *S. cerevisiae* are regulated differently. *CTT1*, coding for the cytoplasmic catalase, is induced by stress conditions (Marchler *et al.*, 1993). The *CTA1* gene is expressed only during growth on non-fermentable carbon sources (Cohen *et al.*, 1985; Hartig and Ruis, 1986). To find out the regulatory pattern of the *C. glabrata* catalase, we assayed its activity in crude protein extracts from cells grown either on glucose or on a non-fermentable carbon source. Cells adapting to ethanol as carbon source showed a substantial induction of catalase activity (Fig. 1A). Moreover, mild oxidative stress of 0.4 mM $H_2O_2$ induced *C. glabrata* catalase activity about 10-fold suggesting regulation by both glucose starvation and oxidative stress (Fig. 1B). To verify if the *CgCTA1* gene encodes the only catalase activity in *C. glabrata*, we replaced the open reading frame (ORF) with the *S. cerevisiae URA3* gene (Fig. S1). Catalase activity was undetectable in extracts derived from the mutant strain (Fig. 1A and B). A centromeric plasmid (p*CgCTA1*) harbouring the *CgCTA1* ORF including a 1.8 kb upstream region fully restored wild-type level catalase activity to the *cta1*Δ mutant (Fig. 1A and B). To demonstrate that the regulation of catalase activity occurs at the level of transcription, *CgCTA1* mRNA levels were analysed from cells shifted to medium lacking glucose or exposed to 0.4 mM $H_2O_2$. Glucose-starved cells displayed a continuous increase of *CgCTA1* mRNA immediately after shift to glucose-free medium (Fig. 1C). Hydrogen peroxide stress caused a rapid increase within 10 min. Taken together, regulation of the *C. glabrata* catalase gene *CgCTA1* by carbon source availability and oxidative stress combines elements of both *S. cerevisiae* catalases.

### *CgCta1 confers hydrogen peroxide stress resistance*

The similarity of the *CgCTA1* gene to the *S. cerevisiae CTA1* gene suggested its peroxisomal localization. To clarify the intracellular localization, we fused a green fluorescent protein (GFP) to the CgCta1 N-terminus (*GFP–CgCTA1*) to preserve the putative peroxisomal targeting sequence 1 (PTS1) (Fig. 1C, lower panel). The preceding 1.8 kb of the *CgCTA1* 5′ untranslated region conferred a wild type-like expression pattern in hydrogen peroxide-stressed cells (Fig. 1C). Basal catalase activity of GFP–CgCta1 was detectable in unstressed cells, whereas hydrogen peroxide stress-induced activity was reduced to about 20% of the wild-type level (Fig. 1B).

We assessed if the reduced activity of GFP–Cta1 interferes with hydrogen peroxide stress resistance. *C. glabrata cta1*Δ mutant cells transformed with either p*GFP–CgCTA1*, p*CgCTA1* or the empty plasmid (p*ACT*) were grown in synthetic medium, the cultures were split and one part treated with 0.4 mM $H_2O_2$. Both were subsequently exposed to higher doses of hydrogen peroxide. Growth was scored after 24 h (Fig. 1D). The *cta1*Δ mutant cells containing the empty plasmid failed to grow in medium containing 5 mM $H_2O_2$. In contrast, the strain carrying the p*CgCTA1* plasmid was resistant to medium supplemented with up to 20 mM $H_2O_2$, whereas pre-incubation with 0.4 mM $H_2O_2$ pushed the growth limit to 40 mM $H_2O_2$, similar to the wild-type parent strain (ΔHTU). Cells expressing the GFP–CgCta1 derivative displayed lower basal resistance. However, naïve cells without pre-treatment tolerated 5 mM $H_2O_2$ and failed to grow only at about 20 mM $H_2O_2$. Pre-treatment with 0.4 mM $H_2O_2$ shifted tolerance to about 30 mM $H_2O_2$. Thus, the GFP-tagged CgCta1 derivative, when compared with the untagged version, conferred resistance to oxidative stress to reduced but overall high level. These results suggested that $H_2O_2$ stress resistance of strains carrying the plasmid-encoded catalase derivatives encompasses the oxidative stress load of 0.4 mM $H_2O_2$ determined for the *in vivo* situation (Enjalbert *et al.*, 2007). Our data also showed that the *C. glabrata* strains tolerated a substantial higher oxidative stress load compared with *S. cerevisiae* laboratory strains, which failed to grow at concentrations higher than 3 mM $H_2O_2$ (Davies *et al.*, 1995; Cuellar-Cruz *et al.*, 2008).

### *Localization of GFP–CgCta1 is dependent on the carbon source*

Cells expressing *GFP–CgCTA1* were exposed to different stress conditions. In rich medium, GFP–CgCta1 fluorescence was hardly detectable, reflecting its low basal expression of *CgCTA1*. Oxidative stress caused induction of the GFP–CgCta1 fluorescence signal. To compare different expression levels directly, unstressed cells were marked by staining their nucleic acids with DAPI. For microscopy these marked unstressed cells were mixed to cells from the same culture treated for 1 h with 0.4 mM $H_2O_2$. The micrograph demonstrates induction of the fusion protein by oxidative stress and its initial localization in the cytoplasm (Fig. 2A). GFP–*CgCTA1* became also induced after the glucose concentration in the growth medium dropped below 0.03% (Fig. S2A). We then investigated GFP–CgCta1 distribution in cells growing on non-fermentable carbon sources. Cells expressing *GFP–CgCTA1* were grown in medium supplemented with 0.5% glucose and 1.5% ethanol. After 5 h, glucose was exhausted, and cells were switching to the non-fermentable carbon source. (Fig. 2B, left panel). Although the vast majority of GFP–CgCta1 was still located in the cytoplasm, small vesicles accumulating catalase became visible (see insert). After 20 h, almost all GFP–CgCta1 was accumulated in vesicular structures (Fig. 2B, middle panel).

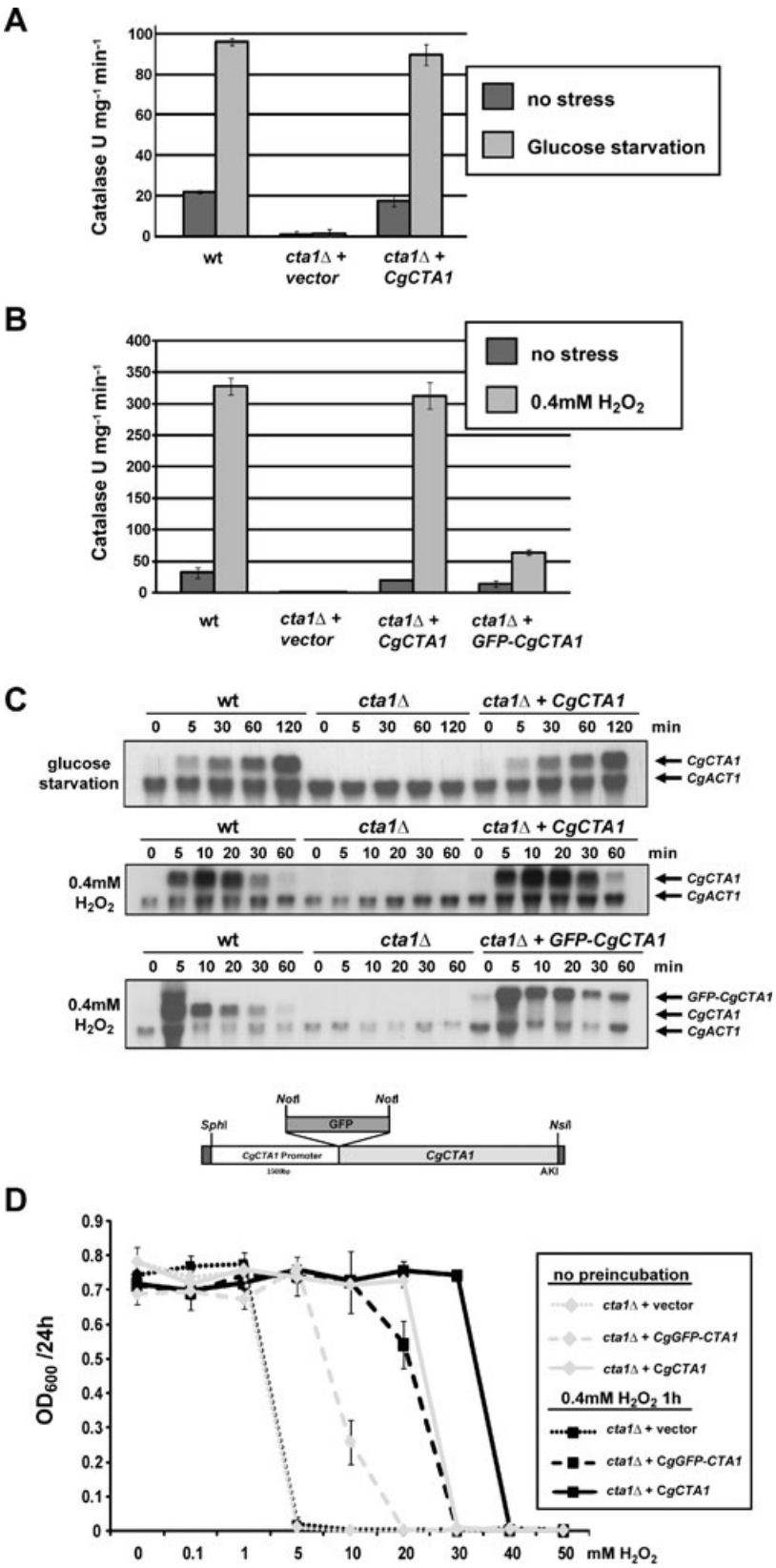


**Fig. 1.** Oxidative stress and carbon source stress regulate *C. glabrata* catalase *CgCTA1*.
A. To measure catalase activity upon glucose depletion, cells were grown to log phase in YPD and shifted to medium with 2% or 0.1% glucose and grown for 4 h. Catalase activity was determined as described in *Experimental procedures*.
B. Cells were incubated in YPD with 0.4 mM $H_2O_2$ for 45 min. Crude cell extracts were prepared and then assayed for catalase activity.
C. Northern blot analysis of *CgCTA1* mRNA levels from wild type, ARCg *cta1*Δ mutant and complemented mutant strain was performed under stress conditions (glucose starvation, 0.4 mM $H_2O_2$). Samples were taken at the indicated time points. *CgACT1* mRNA levels were used as loading control. mRNA levels were visualized by hybridization of radioactive probes and autoradiography. The p*CgC–GFP–CgCTA1* construct with GFP inserted at the N-terminus is illustrated.
D. *C. glabrata* ARCg *cta1*Δ mutant complemented with p*GFP–CgCTA1*, p*CgCTA1* or an empty plasmid was grown in synthetic medium to log phase, adjusted to $10^5$ cells $ml^{-1}$ and exposed to indicated doses of hydrogen peroxide. Optical density after 24 h of incubation at 37°C is indicated.

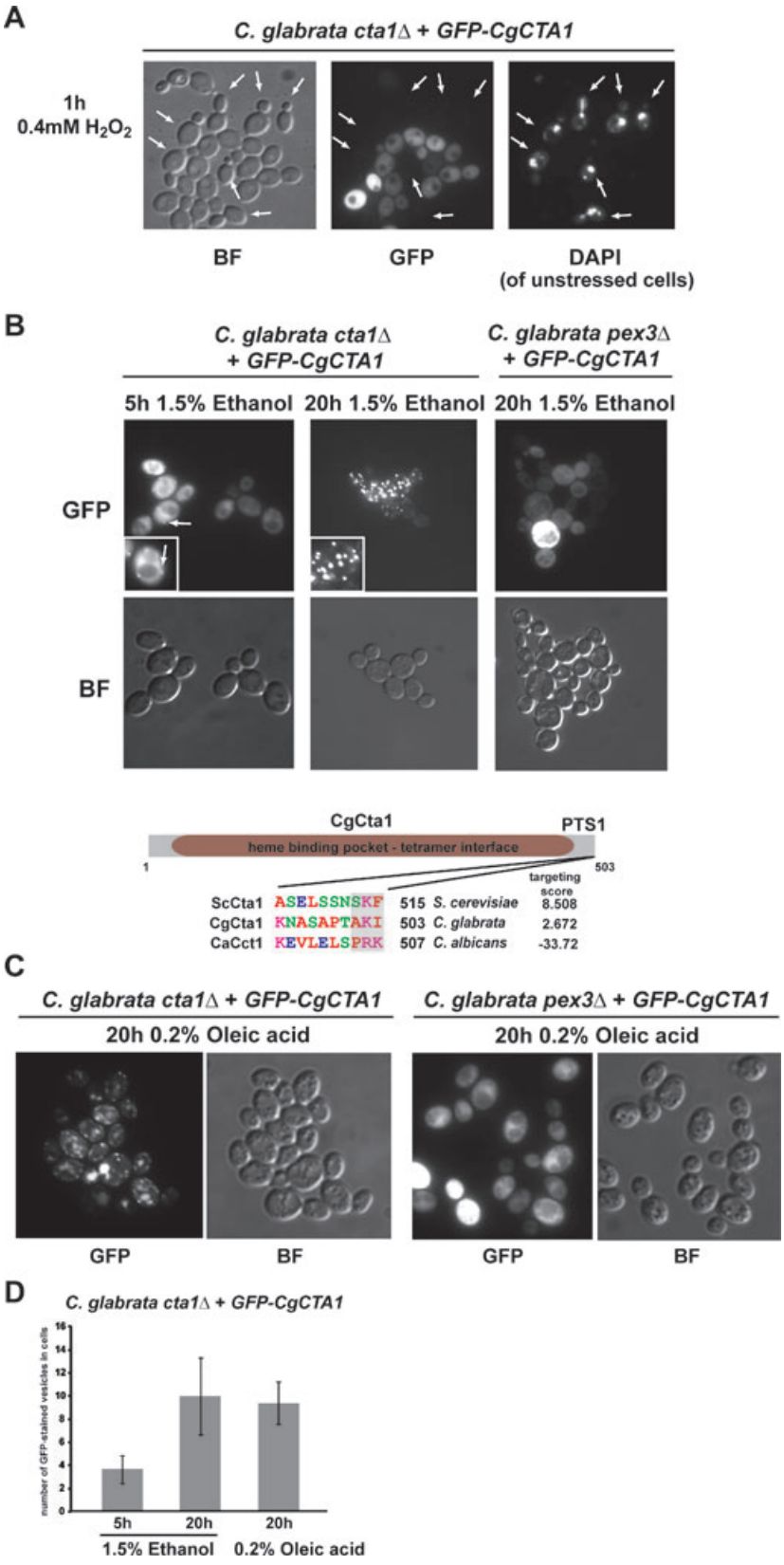


**Fig. 2.** Intracellular localization of *C. glabrata* catalase.
A. Localization of GFP–CgCta1 was determined by fluorescence microscopy in ARCg *cta1*Δ cells transformed with p*CgCTA1–GFP–CgCTA1*. Cells were incubated for 1 h after induction of oxidative stress with 0.4 mM $H_2O_2$. Unstressed cells were stained with DAPI (2 μg $ml^{-1}$) for 10 min. Aliquots of both cultures were pooled prior to microscopy. White arrows indicate unstressed cells.
B. ARCg *cta1*Δ and ARCg *pex3*Δ mutant strains transformed with p*CgC–GFP–CgCTA1* were grown in synthetic medium with 0.5% glucose and 1.5% ethanol for 20 h. White arrows indicate vesicular structures. Inserts show enlarged pictures of single cells. Possible peroxisomal targeting signals 1 (PTS1) detected at the C-terminus of CgCta1, ScCta1 and CaCct1 (Q6FM56, P15202, Q5AAT2; Neuberger *et al.*, 2003).
C. Fluorescence signals of strains as in (B) after growth in medium with 0.2% oleic acid for 20 h.
D. Number of peroxisomes in *C. glabrata* cells during growth with ethanol (1.5%) and oleic acid (0.2%) as main carbon source.

*CgCta1 can localize to peroxisomes*

We suspected that the vesicles accumulating GFP–CgCta1 were peroxisomes. The PTS1 of CgCta1 was a boundary case compared with *S. cerevisiae* Cta1 (Fig. 2B, lower panel). To interfere with *C. glabrata* peroxisome assembly, we chose to eliminate the *CgPEX3* gene (CAGL0M01342g). The *S. cerevisiae* orthologue Pex3 has an essential function for peroxisome biogenesis (Hohfeld *et al*., 1991). The *CgPEX3* ORF was replaced with the *ScURA3* gene and the correct integration was tested by Southern blot (Fig. S1). In these *pex3*Δ mutant cells, GFP–CgCta1 remained distributed in the cytoplasm, even in 1.5% ethanol grown cells (Fig. 2B, right panel). With oleic acid as sole carbon source, *S. cerevisiae* cells increase number and size of peroxisomes (Thieringer *et al*., 1991). GFP–CgCta1 accumulated in vesicles in cells growing in medium containing 0.2% oleic acid, whereas in *pex3*Δ mutant cells fluorescence was dispersed in the cytoplasm (Fig. 2C). The number of stained vesicles also increased substantially in cells growing on a non-fermentative carbon source (1.5% ethanol) (Fig. 2D). These data suggest that *C. glabrata* accumulates GFP–CgCta1 in CgPex3-dependent structures resembling peroxisomes.

To visualize peroxisomal structures in *C. glabrata*, we fused a generic peroxisomal targeting signal peptide (KNIESKL) derived from the *S. cerevisiae* citrate synthase to the C-terminus of YFP (Lewin *et al*., 1990; Kragler *et al*., 1993). The *YFP–KNIESKL* fusion gene expression was driven by the strong *CgADH1* promoter. YFP fluorescence marked peroxisomes, which increased their number during growth on ethanol (Fig. 3A, upper panel) and were absent in *pex3*Δ mutant cells (Fig. 3A, lower panel). This result confirmed the requirement of CgPex3 for *C. glabrata* peroxisome biogenesis.

The above results indicated a partial organellar localization of catalase, depending on the type of carbon source. To show this, we prepared cell extracts of *cta1*Δ mutant cells expressing *CgCTA1.* We separated these in an organellar pellet and a cytosolic supernatant fraction by centrifugation and tested the fractions for catalase activity. After oxidative stress, the entire induced catalase activity was found in the cytoplasmic supernatant (Fig. 3B). In extracts from cells growing with ethanol as main carbon source, catalase activity was found in the cytosolic supernatant, but about one-fourth of total activity was present in the pellet fraction (Fig. 3C, left panel). To confirm that the pellet fraction contained organelles, we used cytochrome *c* oxidase activity as marker enzyme for mitochondria. Most of the cytochrome *c* oxidase activity was found in the pellet fraction (Fig. 3C, right panel). Separation of extracts derived from cells grown in medium containing 0.2% oleic acid showed a further shift of catalase activity towards the pellet fraction (Fig. 3D). Activity of CgCta1 in the various fractions was distributed corresponding to the previously observed intracellular localization of GFP–CgCta1. Together, these results showed a dual localization of *C. glabrata* catalase depending on the presence of peroxisomes.

*Phagocytosis induces GFP–CgCta1 expression*

Fungal pathogens are exposed to a stressful environment, when they come into contact with phagocytic cells (Nicola *et al*., 2008). The regulation and localization of GFP–CgCta1 made it useful to report the environmental conditions during phagocytosis. *C. glabrata cta1*Δ mutant cells expressing *GFP–CgCTA1* grown to exponential phase were used for infection of primary mouse macrophages. We used time-lapse live microscopy to follow the fate of individual engulfed cells (Fig. 4A). Freshly phagocytosed *C. glabrata* cells reacted to this environment with a detectable GFP–CgCta1 fluorescence signal within 40 min (Fig. 4A and Fig. S2C). Furthermore, during prolonged phagocytosis, GFP–CgCta1 accumulated in peroxisomes. To support the idea of peroxisome proliferation during phagocytosis, we followed localization of the YFP–KNIESKL fusion protein during infection of macrophages. Cells were fixed and stained for microscopy immediately after infection and after 2.5, 5, 10 and 24 h (Fig. 4B). We counted cells with visible peroxisomes per macrophage at various time points (Fig. 4C, left panel; Fig. S3). The number of cells with peroxisomes and the number of peroxisomes within these cells transiently increased, reaching a peak after 5 h (Fig. 4C). After 24 h, the vast majority of cells displayed a cytoplasmic/vacuolar YFP–KNIESKL fluorescence signal. Thus, engulfed cells show transient proliferation of peroxisomes.

*GFP–CgYap1 and CgMig1–CFP localization changes in phagocytosed cells*

The localization of CgCta1 suggested that engulfed *C. glabrata* cells might experience oxidative stress and/or carbon source starvation. To confirm this independently, we created additional fluorescent reporter constructs. In *S. cerevisiae*, the glucose-regulated transcriptional repressor Mig1 is rapidly exported from the nucleus in cells starved for glucose (De Vit *et al*., 1997). *S. cerevisiae* Yap1 accumulates rapidly in the nucleus of cells exposed to mild oxidative stress (Kuge *et al*., 1997). To preserve the localization signals of the orthologous transcription factors, CgYap1 was N-terminally fused to GFP whereas CgMig1 was C-terminally fused to CFP. To be detectable, both fusion genes were expressed from centromeric plasmids and driven by the

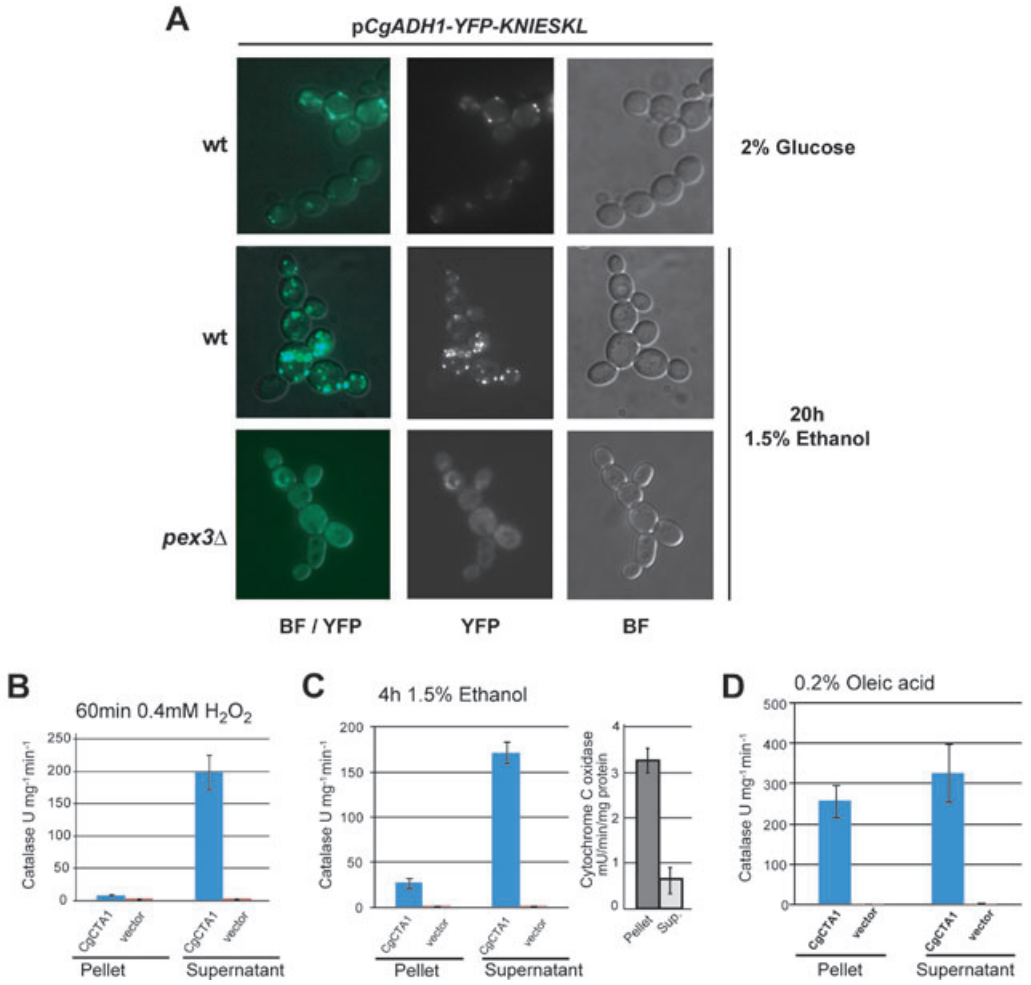


**Fig. 3.** CgCta1 localizes to peroxisomes upon glucose depletion.
A. *C. glabrata* ΔHTU and ARCg *pex3*Δ mutant cells expressing YFP–KNIESKL driven by the *CgADH1* promoter were grown in synthetic medium with 2% glucose or 1.5% ethanol for 20 h. Localization of YFP was recorded by fluorescence microscopy and bright field (BF) microscopy. An overlay of YFP and BF microscopy is shown in the left panel.
B. The ARCg *cta1*Δ strain carrying pCgC–*CgCTA1* was exposed for 1 h to oxidative stress (0.4 mM $H_2O_2$). Pellets containing mitochondria and small organelles and post-mitochondrial supernatants were assayed for catalase activity.
C. The same strain was grown in synthetic medium with 1.5% ethanol for 20 h. Catalase activity was measured in pellets and supernatants. Activity of cytochrome *c* oxidase was measured in pellet and supernatant fractions as described in *Experimental procedures* (right panel).
D. Catalase activity in pellets and supernatant fraction collected from ARCg *cta1*Δ containing p*CgC–CgCTA1* grown in synthetic medium with 0.2% oleic acid for 20 h.

*CgADH1* promoter. Nuclear localization was confirmed by simultaneous staining of nucleic acids with DAPI (Fig. 5A and B).

GFP–CgYap1 was located in the cytoplasm in unstressed *C. glabrata* ΔHTU cells. Upon exposure to mild oxidative stress (0.4 mM $H_2O_2$), GFP–CgYap1 rapidly accumulated in the nucleus (Fig. 5A, upper panel). The fusion gene could complement the transcription defects of the corresponding deletion mutant (our unpublished observation). Within the first hour upon engulfment, cells with nuclear GFP–CgYap1 were visible (Fig. 5A, middle panel). We determined the percentage of yeast cells with nuclear GFP–Yap1 per macrophage after 30 min, 1 h and 5 h (Fig. 5A, lower panel) and found a peak at about 1 h. The CgMig1–CFP fluorescence signal accumulated in the nucleus after addition of glucose (2%) to the medium of glucose-starved cells, and was also nuclear in the glucose-rich environment of the macrophage culture medium (DMEM) (Fig. 5B, upper panel). Immediately after phagocytosis, CgMig1–CFP accumulated in the cytoplasm and remained there constantly, indicating glucose starvation (Fig. 5B, lower panel). These data showed that within the phagosome oxidative stress is transient, whereas macrophages are highly effective in depriving the carbon source.

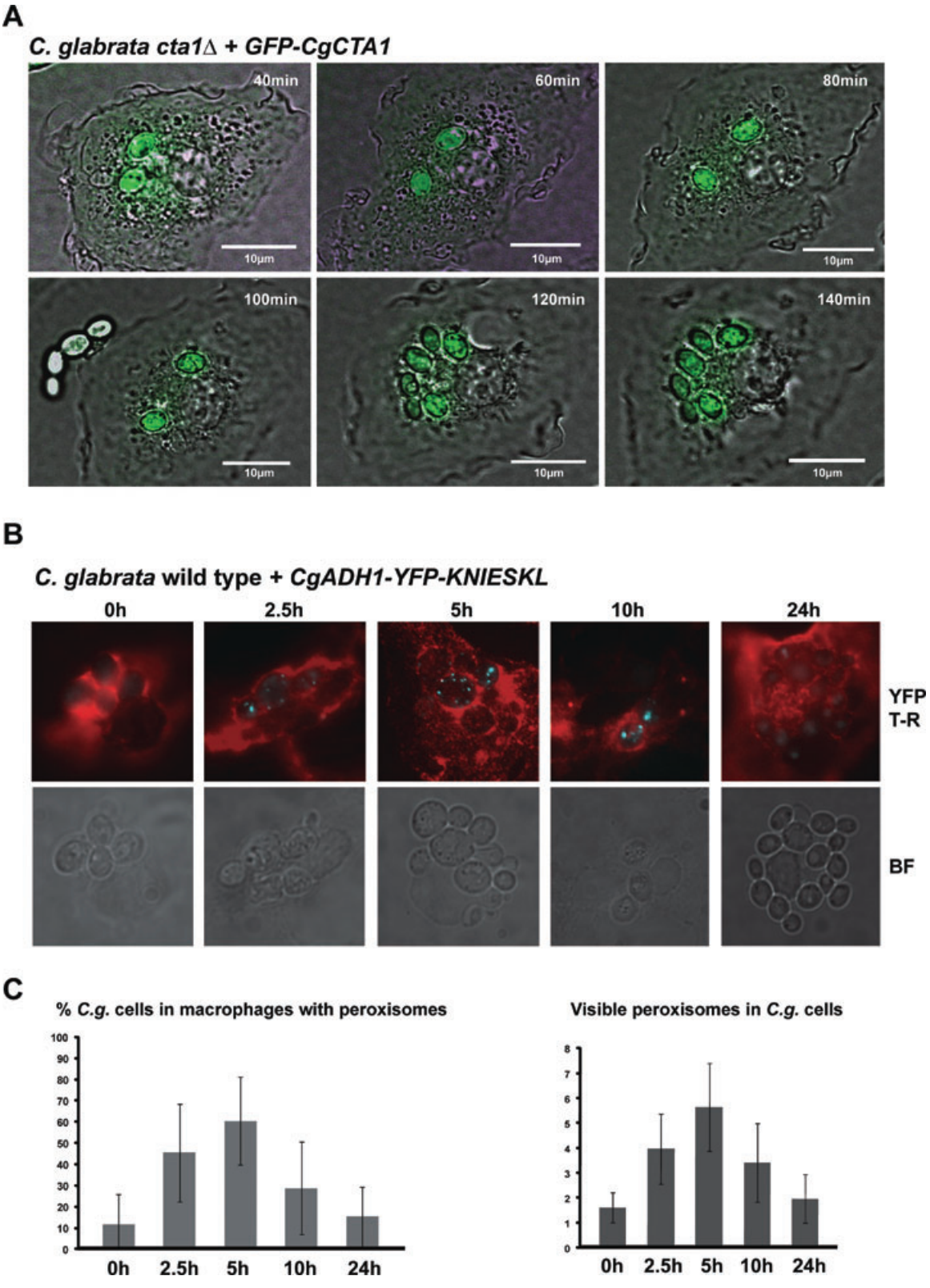
A
C. glabrata cta1Δ + GFP-CgCTA1
40min
60min
80min
100min
120min
140min
10µm
B
C. glabrata wild type + CgADH1-YFP-KNIESKL
0h
2.5h
5h
10h
24h
YFP T-R
BF
C
% C.g. cells in macrophages with peroxisomes
100
90
80
70
60
50
40
30
20
10
0
0h 2.5h 5h 10h 24h
Visible peroxisomes in C.g. cells
8
7
6
5
4
3
2
1
0
0h 2.5h 5h 10h 24h

**Fig. 4.** *GFP–CgCTA1* is induced upon phagocytosis and is located in both cytoplasm and peroxisomes.
A. *C. glabrata* cells before and after being phagocytosed. Exponentially growing ARCg *cta1*Δ cells transformed with p*CgC–GFP–CgCTA1* were washed in PBS containing 0.1% glucose and added to macrophages in a 4:1 ratio at 37°C. Still pictures at the indicated times are shown as overlay of bright-field and fluorescence signals.
B. Exponentially growing wild-type cells transformed with p*CgADH1–YFP–KNIESKL1* were washed in PBS containing 0.1% glucose and added to macrophages in a 4:1 ratio at 37°C. Cells were fixed and stained with Phalloidin Texas-Red after 0, 2.5, 5, 10 and 24 h for fluorescence microscopy.
C. Percentage of phagocytosed *C. glabrata* cells with visible peroxisomes per macrophage from the total cell number of *C. glabrata* cells per macrophage after 0, 2.5, 5, 10 and 24 h (left panel). Number of visible peroxisomes within phagocytosed *C. glabrata* cells after 0, 2.5, 5, 10 and 24 h (right panel).

### *Peroxisomes are transiently induced during phagocytosis*

Peroxisome numbers declined at later stages of engulfment (Fig. 4B). In *S. cerevisiae*, key factors for pexophagy are Atg11 (Yorimitsu and Klionsky, 2005) and Atg17, which is also essential for non-selective autophagy (Cheong *et al.*, 2005). We deleted the *C. glabrata CgATG11* and *CgATG17* homologues (CAGL0H08558g, CAGL0J04686g) in wild-type (ΔHTU) and *pex3*Δ cells (Fig. S1). We investigated engulfed *C. glabrata cta1*Δ, *pex3*Δ, *atg11*Δ, *atg17*Δ, *pex3*Δ *atg17*Δ, *pex3*Δ *atg11*Δ and *atg11*Δ *atg17*Δ mutant cells expressing GFP–CgCta1 after 5 and 24 h (Fig. 6A and E). After 5 h, GFP–Cta1 was located in peroxisomes in the *cta1*Δ, *atg11*Δ and *atg17*Δ mutant cells. In contrast it accumulated in the cytoplasm of *pex3*Δ, *pex3*Δ *atg11*Δ and *pex3*Δ *atg17*Δ mutant cells. However, after 5 h, wild type, *atg11*Δ and *atg17*Δ had similar numbers of cells with peroxisomes, whereas after 24 h, peroxisomes were more abundant in *atg11*Δ and *atg17*Δ mutants (Fig. 6B). In *atg11*Δ mutants, peroxisome numbers remained constant between 5 and 24 h engulfment. *C. glabrata atg17*Δ cells displayed a slight reduction of peroxisomes after 24 h of engulfment, similar to *S. cerevisiae atg17*Δ cells during prolonged starvation conditions (Cheong *et al.*, 2005), Upon internalization, the cytoplasmic localization of CgMig1–CFP demonstrated the same glucose starvation status in the *atg11*Δ, *pex3*Δ *atg11*Δ mutants and wild type (Fig. S2B).

We investigated if the turnover of peroxisomes and mobilization of internal resources are relevant for survival during engulfment. Indeed, the *atg11*Δ and *atg17*Δ mutants had a significantly reduced viability after 24 h compared with wild type, *cta1*Δ and *pex3*Δ strains. Furthermore, in *pex3*Δ *atg11*Δ and *pex3*Δ *atg17*Δ double mutants, the absence of pexophagy might be compensated by absence of peroxisome biogenesis. Consistently, we found that the loss of Pex3 partially reversed the effect of *atg11*Δ with respect to survival during engulfment (Fig. 6C). In contrast, the double mutant *pex3*Δ *atg17*Δ did not show this phenotype, indicating a broader function for CgAtg17-dependent non-selective autophagy during engulfment. Strikingly, the *atg11*Δ *atg17*Δ double mutant, lacking both selective and non-selective autophagy, was highly sensitive to phagocytosis.

To simulate the phagosome environment *in vitro* we combined nutrient starvation and acidic pH. We incubated *C. glabrata* wild type, *atg11*Δ, *pex3*Δ *atg11*Δ, *atg17*Δ, *pex3*Δ *atg17*Δ and *atg11*Δ *atg17*Δ mutant cells in medium lacking nitrogen and carbon sources at pH 3.5 at 37°C for 24 h. The survival was determined by counting colony-forming units (cfu) after 24 h relative to 2 h treatment (Fig. 6D). In comparison with the wild type, all mutants showed diminished survival. Intriguingly, the *pex3*Δ *atg11*Δ strain survived better than the *atg11*Δ strain. Furthermore, the double mutant *atg11*Δ *atg17*Δ displayed the lowest survival rate, similar to the macrophage model. In the macrophage, after 24 h, most of the engulfed *atg11*Δ *atg17*Δ cells had lost GFP–CgCta1 fluorescence presumably due to cell death (not shown). However, after 5 h the GFP–CgCta1 fluorescence signal indicated numerous peroxisomes (Fig. 6E). These results indicated that autophagy is beneficial for survival of *C. glabrata* during engulfment in macrophages, possibly counteracting acute nutrient starvation.

## Discussion

Phagocytic cells internalize microbial cells and attack them with a range of microbicidal strategies (Chauhan *et al.*, 2006; Nicola *et al.*, 2008). Microbial pathogens have developed a number of strategies to improve their survival in the host environment (Urban *et al.*, 2006). Here we used three reporters (CgCta1, CgYap1 and CgMig1) to visualize aspects of the response of the human fungal pathogen *C. glabrata* to macrophage engulfment. We found that *C. glabrata* cells engulfed by primary mouse macrophages suffer from transient oxidative stress, show signs of carbon source starvation, and transiently induce peroxisomes. Our results revealed that the recycling of internal resources, especially peroxisomes, plays an important protective role for *C. glabrata* during engulfment in the phagosome.

The presence and/or proliferation of peroxisomes in fungal cells points to adjustment of carbon metabolism. We demonstrated accumulation of peroxisomes in *C. glabrata* during growth on non-fermentable carbon sources and during engulfment in macrophages. Peroxisomes were visualized using two fluorescent reporter constructs

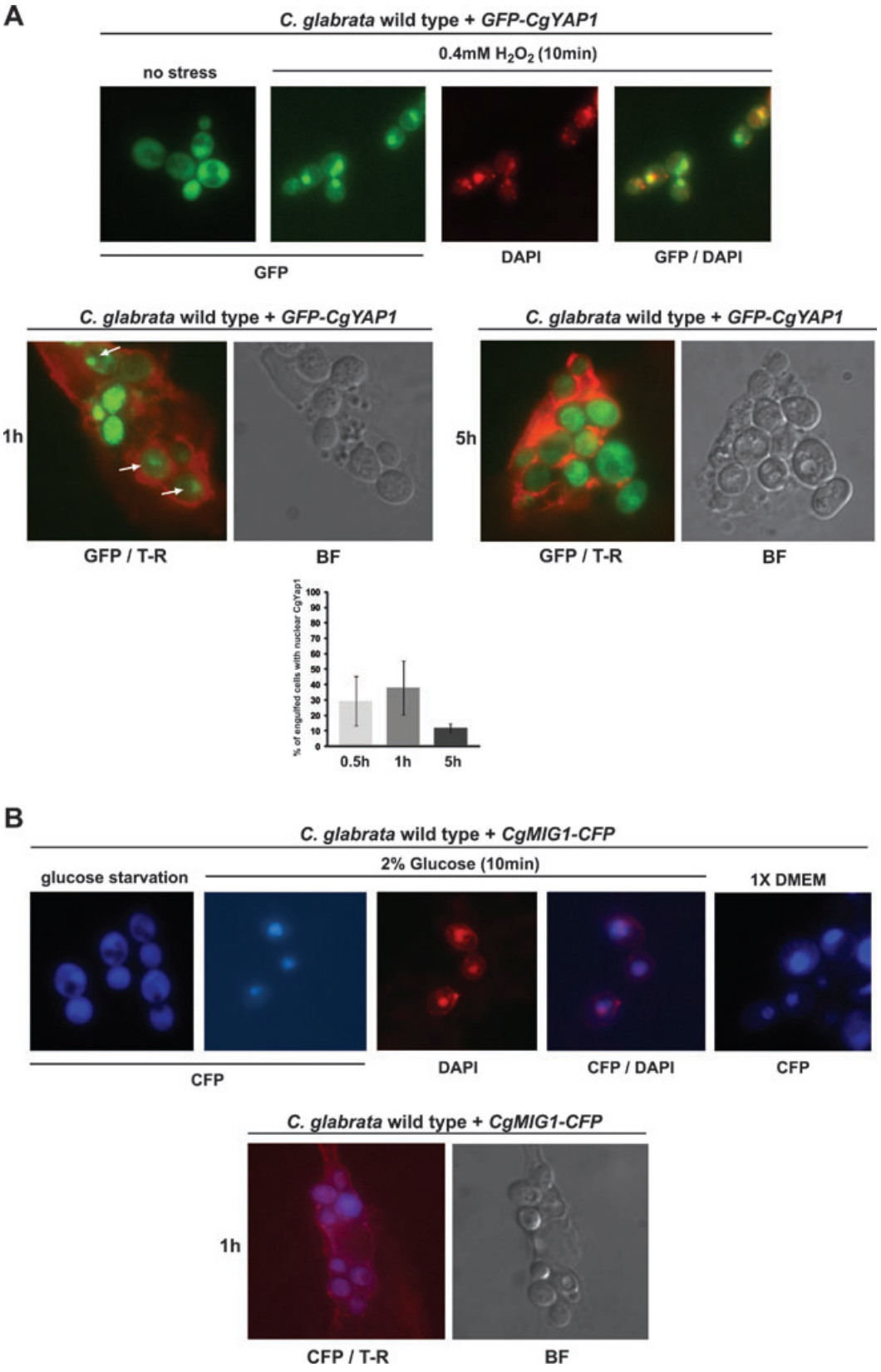
A
C. glabrata wild type + GFP-CgYAP1
0.4mM H2O2 (10min)
no stress
GFP
DAPI
GFP / DAPI
C. glabrata wild type + GFP-CgYAP1
1h
GFP / T-R
BF
C. glabrata wild type + GFP-CgYAP1
5h
GFP / T-R
BF
% of engulfed cells with nuclear CgYap1
0.5h
1h
5h
B
C. glabrata wild type + CgMIG1-CFP
glucose starvation
2% Glucose (10min)
1X DMEM
CFP
DAPI
CFP / DAPI
CFP
C. glabrata wild type + CgMIG1-CFP
1h
CFP / T-R
BF

**Fig. 5.** Localization of GFP–CgYap1 and CgMig1–CFP during early stage of phagocytosis.
A. *C. glabrata* wild-type cells transformed with p*CgADH1–GFP–CgYAP1* were grown in synthetic medium. Cells were stressed by addition of 0.4 mM $H_2O_2$ for 10 min. Nuclei were stained with DAPI. An overlay of GFP and DAPI staining is shown in the right panel. GFP–CgYap1 visualized by fluorescence microscopy under phagocytosis conditions (lower panel). Cells were washed in PBS 0.1% glucose and added to macrophages in a 4:1 ratio and incubated at 37°C for 10 min to follow the route of tagged transcription factors. Samples were fixed and stained with Phalloidin Texas-Red. Percentage of cells with nuclear GFP–Yap1 was calculated after 30 min, 1 h and 5 h. White arrows point to nuclear GFP–CgYap1 in yeast inside the phagosome.
B. *C. glabrata* wild-type cells transformed with p*CgADH1–CgMIG1–CFP* were grown in synthetic medium until glucose depletion. Cells were incubated in fresh medium containing 2% glucose for 10 min or 1× DMEM. Lower panel depicts localization of CgMig1–CFP under phagocytosis conditions. Cells were treated as described in (A).

GFP–CgCta1 and YFP–KNIESKL and further confirmed by other criteria. They were induced on medium containing ethanol and oleic acid as carbon source. Furthermore, peroxisomes were dependent on the *CgPEX3* gene, a peroxisomal integral membrane protein, whose orthologue in *S. cerevisiae* is essential for peroxisomal biogenesis (Hohfeld *et al*., 1991). Peroxisomal catalases such as *S. cerevisiae* Cta1 (Simon *et al*., 1991) are scavengers of hydrogen peroxide generated during peroxisomal β-oxidation. We find that *C. glabrata* catalase expression is regulated by oxidative stress and carbon source, and its intracellular localization correlates with the presence of peroxisomes. This combines the regulation of both yeast catalases. The *CgCTA1* gene lacks synteny with the yeast *CTA1* gene and other fungal catalases (Gordon *et al*., 2009). It is tempting to speculate that the shuffling of the *C. glabrata* genome fostered the accumulation of regulatory elements for oxidative stress and carbon source response.

In a phagocytosis model using bone marrow-derived mouse macrophages, GFP–CgCta1 expressed under the control of the *CgCTA1* promoter was induced in the earliest stages after internalization. This could be due to oxidative stress or acute carbon starvation. Intracellular localization of two other fluorescent reporters (CgYap1 and CgMig1) supported rather low oxidative stress load and starvation for glucose of engulfed *C. glabrata* cells. High-level expression was necessary for detection of GFP–transcription factor fusions and could potentially interfere with signalling. However, both factors are tightly regulated by post-translational modifications and thus buffered for expression level. We found that in a population of engulfed cells a minor fraction displayed signs of acute oxidative stress. This is consistent with other reports. Only a small portion of *C. albicans* cells derived from mouse kidneys displayed an acute oxidative stress response when examined for *CaCTA1* expression (Enjalbert *et al*., 2007).

The *C. glabrata* transcriptional response might have been selected to the specific conditions of phagocytosis. Microarray data indicated induction of a group of about 30 genes by both oxidative stress and glucose starvation (Roetzer *et al*., 2008). Moreover, phagocytosed *C. glabrata* cells induce genes involved in gluconeogenesis, β-oxidation, glyoxylate cycle, and transporters for amino acids and acetate (Kaur *et al*., 2007). Induction of peroxisomes after internalization by macrophages indicated adjustment of metabolism within the phagosome. Cells utilizing non-fermentable carbon sources, e.g. fatty acids or ethanol, require peroxisomal β-oxidation and the partly peroxisomal glyoxylate cycle. The induction of non-fermentative carbon metabolism genes is beneficial for the survival of *C. albicans* (Lorenz *et al*., 2004; Barelle *et al*., 2006). In a mouse infection model, Fox2, the second enzyme of the β-oxidation pathway, and isocitrate lyase (Icl1) an enzyme of the glyoxylate cycle, were required for *C. albicans* virulence (Lorenz and Fink, 2001; Piekarska *et al*., 2006). However, *C. albicans* mutants defective in the import receptor of PTS1-targeted peroxisomal proteins, CaPex5, displayed no attenuation of virulence (Piekarska *et al*., 2006). The survival of *C. glabrata* devoid of peroxisomes in a *pex3*Δ mutant was not compromised in our infection model. Also, *C. neoformans pex1*Δ deletion mutants were not attenuated for virulence (Idnurm *et al*., 2007). These data support the view that peroxisomes are not a major virulence determinant. Instead, the peroxisomal metabolic pathways, which can function to sufficient extent in the cytosol, appear to contribute to virulence.

In engulfed *C. glabrata* cells peroxisome numbers declined at later time points. Also at later time points GFP–CgCta1 accumulated partly in the cytosol. Peroxisomes are not known to export proteins, thus the cytosolic fluorescence was most probably due to *de novo* synthesis or peroxisome turnover. Peroxisomes are degraded by pexophagy, a selective autophagic pathway (Hutchins *et al*., 1999; Farre and Subramani, 2004). In *S. cerevisiae*, mutants lacking Atg11 and Atg17 had a severe delay of pexophagy (Kim *et al*., 2001; Cheong *et al*., 2005; 2008). In *C. glabrata*, we found that mutants lacking *atg11*Δ or *atg17*Δ had reduced survival in macrophages and *in vitro* during starvation. Moreover, the *C. glabrata* double mutant *atg11*Δ *atg17*Δ displayed a striking additive decrease of survival. In *S. cerevisiae*, the *atg11*Δ *atg17*Δ double mutant strain did not contain any detectable autophagic bodies and had a severe autophagy defect (Cheong *et al*., 2008). We suggest that *C. glabrata atg11*Δ *atg17*Δ is unable to induce autophagic processes in order

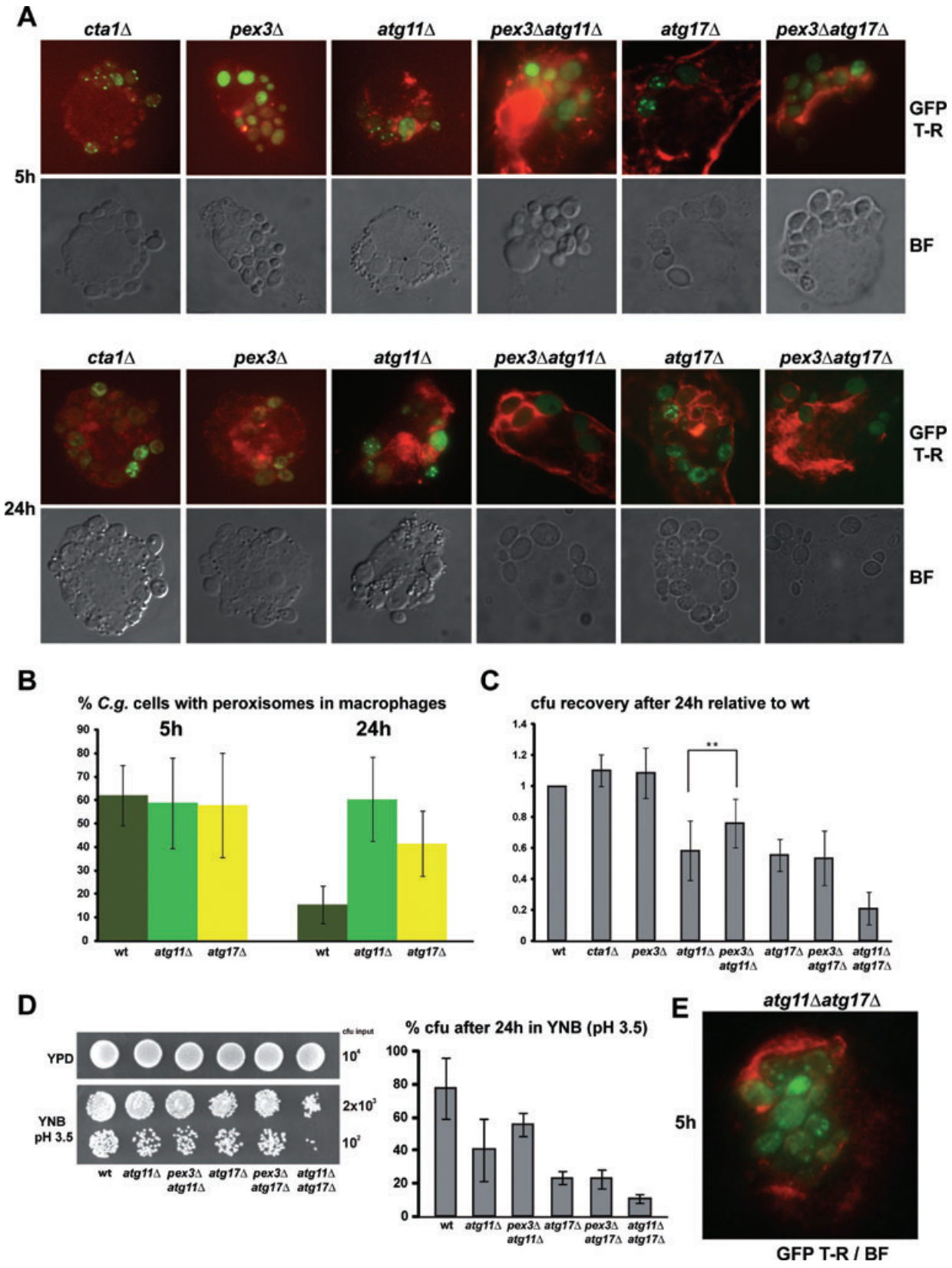
A
cta1Δ
pex3Δ
atg11Δ
pex3Δatg11Δ
atg17Δ
pex3Δatg17Δ
GFP T-R
BF
5h
24h
B
% C.g. cells with peroxisomes in macrophages
5h
24h
wt
atg11Δ
atg17Δ
C
cfu recovery after 24h relative to wt
**
wt
cta1Δ
pex3Δ
atg11Δ
pex3Δ atg11Δ
atg17Δ
pex3Δ atg17Δ
atg11Δ atg17Δ
D
YPD
YNB pH 3.5
cfu input
10⁴
2x10³
10²
% cfu after 24h in YNB (pH 3.5)
E
atg11Δatg17Δ
5h
GFP T-R / BF

**Fig. 6.** Induction and pexophagy of peroxisomes upon phagocytosis.
A. Log-phase *C. glabrata* ARCg *cta1*Δ, ARCg *pex3*Δ, ARCg *atg11*Δ, ARCg *pex3*Δ *atg11*Δ, ARCg *atg17*Δ and ARCg *pex3*Δ *atg17*Δ mutant cells transformed with p*GFP–CgCTA1* were used to infect mouse macrophages in a 4:1 ratio at 37°C. Cells were fixed for microscopy after 5 and 24 h.
B. Percentage of cells with visible peroxisomes after phagocytosis in macrophages after 5 and 24 h.
C. Log-phase *C. glabrata* ARCg *cta1*Δ, ARCg *pex3*Δ, ARCg *atg11*Δ, ARCg *pex3*Δ *atg11*Δ, ARCg *atg17*Δ and ARCg *pex3*Δ *atg17*Δ and ARCg *atg11*Δ *atg17*Δ mutant cells were used to infect mouse macrophages in a 1:2 ratio at 37°C. The viability of the engulfed cells was assessed by hypotonic lysis of the macrophages and quantification of colony formation (cfu) on rich medium. Assays were performed in triplicate. A one-way ANOVA was performed and *P*-values were calculated comparing the numbers of recovered colonies of the indicated strains (**$P < 0.005$).
D. *C. glabrata* wild type, ARCg *atg11*Δ, ARCg *pex3*Δ *atg11*Δ, ARCg *atg17*Δ, ARCg *pex3*Δ *atg17*Δ and ARCg *atg11*Δ *atg17*Δ mutant cells were grown to exponential phase in rich medium; after washing with PBS supplemented with 0.1% glucose, $2 \times 10^5$ cells were incubated in selective medium without nitrogen sources and glucose and pH 3.5 at 37°C. After 24 h colony formation (cfu) of mutant cells was determined. Percentage of viable cells was calculated relative to 2 h treatment.
E. Log-phase ARCg *atg11*Δ *atg17*Δ mutant cells transformed with p*GFP–CgCTA1* were used to infect mouse macrophages in a 4:1 ratio at 37°C. Cells were fixed for microscopy after 5 h. Overlay of GFP/Texas-Red and BF is shown.

to sustain prolonged phagocytosis. Notably, homologues of proteins of the autophagy core machinery have been found from yeast to mammals, but both Atg11 and Atg17 are not conserved and might be a target for antifungal drugs (reviewed by Xie and Klionsky, 2007).

Autophagy is required for *C. neoformans* virulence (Hu *et al*., 2008). Furthermore, *C. neoformans* genes involved in autophagy, peroxisome function and lipid metabolism became also induced during infection (Fan *et al*., 2005). *C. neoformans* could escape from macrophages through extrusions of the phagosome, without killing the phagocytic cell (Alvarez and Casadevall, 2006). It has been suggested that this is a pathway for dissemination within the host. Therefore, survival in the macrophage indirectly contributes to virulence. A *C. albicans* mutant lacking Ca*ATG9* was defective for autophagy, but nevertheless was able to kill macrophages (Palmer *et al*., 2007). In contrast to *C. albicans*, *C. glabrata* is trapped inside the phagosome. In *C. glabrata pex3*Δ *atg11*Δ cells, we found the sensitivity of *atg11*Δ partially reversed. We suggest from this genetic observation that autophagy of peroxisomes is beneficial for engulfed *C. glabrata* cells. *C. glabrata pex3*Δ *atg17*Δ mutants did not display this effect. Selective pexophagy, which is affected in both *atg11*Δ and *atg17*Δ mutants, might help to mobilize intracellular resources during prolonged engulfment. *S. cerevisiae* uses autophagy to recycle proteins to overcome nitrogen starvation (Onodera and Ohsumi, 2005). Autophagic processes, such as pexophagy, are contributing to virulence of important fungal plant pathogens (Veneault-Fourrey *et al*., 2006; Asakura *et al*., 2009). However, an *A. fumigatus* mutant strain lacking Atg1 also remained virulent (Richie *et al*., 2007). Thus the role of autophagy for fungal pathogens is also dependent on their morphology (Palmer *et al*., 2008).

Beside the carbon and nitrogen starvation conditions inside the phagosome, other restrictions, such as pH, hydrolytic enzymes and antimicrobial peptides, might act in a synergistic manner. We infer from the phenotype of our autophagy mutants that macrophage engulfment is essentially a starvation situation in combination with acidic pH. Acidification of the phagosome aids the destruction of some microbes, but it might also contribute to the escape of others. For example, lysosomal acidification induced germ tube formation of *C. albicans* and therefore contributed to its escape from the macrophage (Kaposzta *et al*., 1999). The observed oxidative stress response of *C. glabrata* might result from a switch of metabolism rather than a macrophage-derived oxidative burst. It has been reported that in *S. cerevisiae*, a shift to oleic acid as carbon source induced a specific Yap1-dependent subset of oxidative stress response genes (Koerkamp *et al*., 2002). However, we believe that the importance of autophagy for survival suggests a starvation situation. Furthermore, in our model system, the transient induction and degradation of peroxisomes is not supporting substantial metabolism in the phagosome.

Our results demonstrate that monitoring of the intracellular localization of proteins tagged with fluorescent reporters is a highly informative tool to reveal intracellular signalling and metabolic conditions. Here we show that the macrophage is efficiently depriving engulfed *C. glabrata* cells from nutrient sources. Autophagic processes, prolonging the survival of engulfed cells, are potentially aiding the dissemination of *C. glabrata* and the establishment of infection.

## Experimental procedures

### *Yeast strains and plasmids*

Yeast strains used in this study are listed in Table 1. Rich medium (YPD), synthetic medium (SC) and yeast nitrogen base medium (YNB) without amino acids and ammonium sulfate were prepared as described elsewhere (Current Protocols in Molecular Biology; Wiley). All strains were grown at 30°C or 37°C as indicated. Oleate medium contained 0.2% oleic acid, 0.3% yeast extract, 0.5% peptone and 0.5% $KH_2PO_4$ (pH 6). Oleate plates were incubated at 37°C for 7 days. Glucose concentration between 0.5% and 0.03% (w/v) was determined using the Freestyle mini (Abbott). To assess viability of cells during starvation (Fig. 6D), cfu were determined by spreading on rich medium, usually after 2 h of incubation at 37°C and after the indicated time (24 h). Oligonucleotides used in this study are listed in Table S1. *C. gla-*

**Table 1.** Strains used in this study.

| *C. glabrata* strain | Genotype | Source |
|---|---|---|
| ΔHTU | *his3*Δ *trp1*Δ *ura3*Δ | Kitada *et al.* (1996) |
| ΔHT6 | *his3*Δ *trp1*Δ | Kitada *et al.* (1996) |
| ARCg *cta1*Δ | *his3*Δ *trp1*Δ *ura3*Δ *cta1*Δ::*ScURA3* | This study |
| ARCg *pex3*Δ | *his3*Δ *trp1*Δ *ura3*Δ *pex3*Δ::*ScURA3* | This study |
| ARCg *atg11*Δ | *his3*Δ *trp1*Δ *ura3*Δ *atg11*Δ::*ScURA3* | This study |
| ARCg *pex3*Δ *atg11*Δ | *his3*Δ *trp1*Δ *ura3*Δ *pex3*Δ::*ScURA3 atg11*Δ::*ScHIS3* | This study |
| ARCg *atg17*Δ | *his3*Δ *trp1*Δ *ura3*Δ *atg17*Δ::*ScURA3* | This study |
| ARCg *pex3*Δ *atg17*Δ | *his3*Δ *trp1*Δ *ura3*Δ *pex3*Δ::*ScURA3 atg17*Δ::*ScHIS3* | This study |
| ARCg *atg11*Δ *atg17*Δ | *his3*Δ *trp1*Δ *ura3*Δ *atg11*Δ::*ScURA3 atg17*Δ::*ScHIS3* | This study |

*brata* strains AR*Cg cta1*Δ, AR*Cg pex3*Δ, AR*Cg atg11*Δ, AR*Cg pex3*Δ *atg11*Δ, AR*Cg atg17*Δ, AR*Cg pex3*Δ *atg17*Δ and ARCg *atg11*Δ *atg17*Δ were obtained by replacing the ORFs with the *S. cerevisiae URA3* gene or *HIS3* gene generated by genomic integration. Knockout cassettes were synthesized using fusion PCR according to Noble (Noble and Johnson, 2005) from the plasmids pRS316 and pRS313 (Sikorski and Hieter, 1989) with the oligonucleotides CTA1-1 to 6, PEX3-1 to 6, ATG11-1 to 6 and ATG17-1 to 6. Correct genomic integration was verified by genomic PCR (primer series Ctrl) followed by Southern analysis using probes generated with primers CTA1-4/CTA1-6, PEX3-1/PEX3-3, ATG11-4/ATG11-6 and ATG17-1/ATG17-3 or ATG17-4/ATG17-6. Probes for Southern and also for Northern analysis (CTA1-5/CTA1-3 and ACT1-5/ACT1-3) were amplified by PCR from genomic DNA.

Plasmids used in this study are listed in Table 2. To generate pGEM–ACT–CgCTA1, 1800 base pairs of the CgCTA1 promoter were inserted as a SphI/NotI PCR product obtained with primers CTAPro-up and CTAPro-down into the plasmid pGEM–ACT (Gregori *et al.*, 2007). The coding sequence for CgCTA1 was amplified from genomic DNA using primers CTA-up-Not and CTA-down-Nsi, cut and inserted as a NotI/NsiI fragment. GFP was inserted as a NotI/NotI fragment at the N-terminus of CgCTA1. To generate pYFP–KNIESKL YFP was inserted as a NotI/NotI fragment obtained by PCR with primers YFP–Not-Start and YFP–SKL-Stop into the plasmid pGEM–ACT–CgADH1 (Roetzer *et al.*, 2008). CgYAP1 was amplified using primers CgYap5/CgYap3 containing a NotI or a NsiI site; GFP was inserted as NotI/NotI fragment into the plasmid pGEM–ACT–CgADH1 at the N-terminus of CgYAP1. CgMIG1 was amplified using primers Mig1-5sac/Mig1-3nco and inserted into NcoI and SacII cut pGEM–ACT–CgADH1–MSN2–CFP (Roetzer *et al.*, 2008). All cloned PCR fragments used in this study were controlled by sequencing.

### *Catalase and cytochrome* c *oxidase assay*

Crude extracts were prepared by breakage of yeast cells with glass beads. Catalase activity was assayed spectrophotometrically at 240 nm as described in Durchschlag *et al.* (2004); protein concentrations were assayed at 280 nm. For the cytochrome *c* oxidase assay, 0.5 g $l^{-1}$ Sodium dithionite was added to reduce cytochrome *c* (0.1 mg $ml^{-1}$) solution. Cytochrome *c* has a sharp absorption band at 550 nm in the reduced state. Absorption spectra of cytochrome *c* were recorded between 410 and 570 nm. Five minutes after addition of crude extracts, spectra were measured to determine the oxidized state of cytochrome *c* (Lemberg, 1969).

### *Separation of organelles*

Cells were re-suspended in washing buffer (20 mM Hepes pH 7.4, 50 mM NaCl, 0.6 M sorbitol), incubated with protease inhibitor PMSF and broken using glass beads. The supernatant was centrifuged for 12 min at 6900 rcf to separate (post-mitochondrial) supernatant and the organellar pellet.

### *Northern and Southern blot analysis*

RNA extraction and separation followed essentially the described protocol (Current Protocols in Molecular Biology; Wiley). Hybrid-

**Table 2.** Plasmids used in this study.

| Plamid | Genotype | Source |
|---|---|---|
| p*RS316* | CEN6, ARSH4, *ScURA3* | Sikorski and Hieter (1989) |
| p*RS313* | CEN6, ARSH4, *ScHIS3* | Sikorski and Hieter (1989) |
| p*ACT14* | ARS, CEN and *TRP1* marker from *C. glabrata* | Kitada *et al.* (1996) |
| p*GEM–ACT* | ARS, CEN and *TRP1* marker from *C. glabrata* | Gregori *et al.* (2007) |
| p*CgC–GFP–CgCTA1* | CgCTA1–GFP–*CgCTA1* (*CgCTA1p*: SphI/NotI, *CgCTA1* ORF NotII/NsiI, GFP NotI/NotI) *CgTRP1* | This study |
| p*CgC–CgCTA1* | CgCTA1–*CgCTA1* (SphI/NotI and NotII/NsiI); *CgTRP1* marker | This study |
| p*CgCADH1–YFP–KNIESKL* | CgCADH1–*YFP–KNIESKL* (NotI/NotI fragment); *CgTRP1* | This study |
| p*CgADH1–CgMSN2–CFP* | *CgADH1–CgMSN2–CFP* (*CgADH1p*: SphI/SacII and *CgMSN2*: SacII/NsiI); *CgTRP1* | Roetzer *et al.* (2008) |
| p*CgADH1–CgMIG1–CFP* | *CgADH1–CgMIG1–CFP* (*CgMIG1*: SacII/NcoI); *CgTRP1* | This study |
| p*CgADH1–GFP–CgYAP1* | *CgADH1–GFP–CgYAP1* (*CgYAP1*: NotII/NsiI); *CgTRP1* | This study |

ization of [$\alpha$-$^{32}$P]-dATP-labelled probes occurred overnight in hybridization buffer (0.5 M Sodium phosphate buffer pH 7.2/7% SDS/1 mM EDTA) at 65°C. For DNA extraction, 10 ml yeast cells (grown to an $OD_{600} = 6$) were collected, washed once and re-suspended in Lysis buffer (2% Triton X-100/1% SDS/100 mM NaCl/10 mM Tris pH 8/1 mM EDTA). Genomic DNA was isolated by PCI (phenol/chloroform/isoamyl alcohol) extraction. Digestion of 10 $\mu$g of genomic DNA was performed overnight with XcmI for *CgPEX3*, EcoRV for *CgCTA1* and ClaI/NcoI for *CgATG11* (5 U $\mu g^{-1}$ DNA). The labelled probes were hybridized overnight in hybridization buffer at 65°C. Signals were visualized by autoradiography.

### *Microscopy*

GFP-fluorescence microscopy was performed as described previously (Görner *et al*., 1998). GFP was visualized in live cells without fixation. All cells were monitored using a Zeiss Axioplan 2 fluorescence microscope. Images were captured with a Spot Pursuit (Sony) CCD camera using Spotbasic software. Time-lapse microscopy was performed on an Olympus cell-imager system (IX81 inverted microscope) equipped for cell culture observation. Cells were incubated in a glass chamber at 37°C connected to an active gas mixer (Ibidi, Martinsried, Germany). Pictures were taken with a Hamamatsu ORCA-ER camera and analysed using cell$^{M}$&cell$^{R}$ software (Olympus). Nomarski contrasted, bright-field microscopy pictures are indicated as BF. Quantification and statistical analysis of peroxisomes in *C. glabrata* cells (Figs 2D, 4C and 6B) have been added in Fig. S3.

### *Macrophage cell culture*

Primary bone marrow-derived macrophages (BMDMs) were obtained from the femur bone marrow of 6- to 10-week-old C57Bl/6 mice. Cells were cultivated in DMEM supplemented with 10% FCS in the presence of L cell-derived CSF-1 as described (Baccarini *et al*., 1985). Mice were housed under specific pathogen-free conditions. For infection assays, BMDMs were seeded at $5 \times 10^5$ cells per dish in 3.5 cm dishes containing medium without antibiotics. Log-phase *C. glabrata* cells were washed with PBS supplemented with 0.1% glucose and added to macrophages in a 4:1 ratio and incubated at 37°C. For microscopy, cells were fixed with 2% formaldehyde for 5 min. After washing with PBS, cells were incubated in 1% Triton X-100 for 1 min. After washing with PBS, cells were dyed with Phalloidin Texas-Red for 30 min. Coverslips were fixed to slides with Mowiol. For cfu assays, BMDMs were seeded at $2 \times 10^5$ cells per dish. Exponentially growing *C. glabrata* cells were washed with PBS supplemented with 0.1% glucose and added to macrophages in a 1:2 ratio and incubated at 37°C. After 45 min, cells were washed three times with PBS to remove not phagocytosed yeast cells and fresh medium was added. At the indicated times, deionized water was added to lyse macrophage cells. *C. glabrata* cells were spread on YPD plates, colonies were counted after incubation at 37°C for 2 days.

## Acknowledgements

We thank Andreas Hartig, Fabian Rudolf, Claudine Kraft, Christa Gregori, Tobias Schwarzmüller and especially Wolfgang Reiter for discussions and advice, Christophe d'Enfert for critical reading, Josef Gotzmann and Doris Mayer for technical support. C.S. wishes to dedicate this article in memory of his father. C.S. was supported by the Herzfelder Foundation, and the Vienna Hochschuljubiläumsstiftung. This work was supported by the Austrian Research Foundation (FWF) through Grants P16726-B14, I27-B03 and SFB F28 to P.K., Grant I031-B from the University of Vienna (to C.S. and P.K.), and P19966-B12 to C.S.

## References

Alvarez, M., and Casadevall, A. (2006) Phagosome extrusion and host-cell survival after *Cryptococcus neoformans* phagocytosis by macrophages. *Curr Biol* **16:** 2161–2165.

Asakura, M., Ninomiya, S., Sugimoto, M., Oku, M., Yamashita, S., Okuno, T., *et al.* (2009) Atg26-mediated pexophagy is required for host invasion by the plant pathogenic fungus *Colletotrichum orbiculare*. *Plant Cell* **21:** 1291–1304.

Baccarini, M., Bistoni, F., and Lohmann-Matthes, M.L. (1985) *In vitro* natural cell-mediated cytotoxicity against *Candida albicans*: macrophage precursors as effector cells. *J Immunol* **134:** 2658–2665.

Barelle, C.J., Priest, C.L., Maccallum, D.M., Gow, N.A., Odds, F.C., and Brown, A.J. (2006) Niche-specific regulation of central metabolic pathways in a fungal pathogen. *Cell Microbiol* **8:** 961–971.

Becker, K., Tilley, L., Vennerstrom, J.L., Roberts, D., Rogerson, S., and Ginsburg, H. (2004) Oxidative stress in malaria parasite-infected erythrocytes: host–parasite interactions. *Int J Parasitol* **34:** 163–189.

Canovas, D., and Andrianopoulos, A. (2006) Developmental regulation of the glyoxylate cycle in the human pathogen *Penicillium marneffei*. *Mol Microbiol* **62:** 1725–1738.

Carlson, M. (1999) Glucose repression in yeast. *Curr Opin Microbiol* **2:** 202–207.

Chauhan, N., Latge, J.P., and Calderone, R. (2006) Signalling and oxidant adaptation in *Candida albicans* and *Aspergillus fumigatus*. *Nat Rev Microbiol* **4:** 435–444.

Chaves, G.M., Bates, S., Maccallum, D.M., and Odds, F.C. (2007) *Candida albicans GRX2*, encoding a putative glutaredoxin, is required for virulence in a murine model. *Genet Mol Res* **6:** 1051–1063.

Cheong, H., Yorimitsu, T., Reggiori, F., Legakis, J.E., Wang, C.W., and Klionsky, D.J. (2005) Atg17 regulates the magnitude of the autophagic response. *Mol Biol Cell* **16:** 3438–3453.

Cheong, H., Nair, U., Geng, J., and Klionsky, D.J. (2008) The Atg1 kinase complex is involved in the regulation of protein recruitment to initiate sequestering vesicle formation for nonspecific autophagy in *Saccharomyces cerevisiae*. *Mol Biol Cell* **19:** 668–681.

Cohen, G., Fessl, F., Traczyk, A., Rytka, J., and Ruis, H. (1985) Isolation of the catalase A gene of *Saccharomyces cerevisiae* by complementation of the *cta1* mutation. *Mol Gen Genet* **200:** 74–79.

Cox, G.M., Harrison, T.S., McDade, H.C., Taborda, C.P., Heinrich, G., Casadevall, A., and Perfect, J.R. (2003) Superoxide dismutase influences the virulence of *Cryptococcus neoformans* by affecting growth within macrophages. *Infect Immun* **71:** 173–180.

Cuellar-Cruz, M., Briones-Martin-del-Campo, M., Canas-Villamar, I., Montalvo-Arredondo, J., Riego-Ruiz, L.,

Castano, I., and De Las Penas, A. (2008) High resistance to oxidative stress in the fungal pathogen *Candida glabrata* is mediated by a single catalase, Cta1p, and is controlled by the transcription factors Yap1p, Skn7p, Msn2p, and Msn4p. *Eukaryot Cell* **7:** 814–825.

Davies, J.M., Lowry, C.V., and Davies, K.J. (1995) Transient adaptation to oxidative stress in yeast. *Arch Biochem Biophys* **317:** 1–6.

De Vit, M.J., Waddle, J.A., and Johnston, M. (1997) Regulated nuclear translocation of the Mig1 glucose repressor. *Mol Biol Cell* **8:** 1603–1618.

Derengowski, L.S., Tavares, A.H., Silva, S., Procopio, L.S., Felipe, M.S., and Silva-Pereira, I. (2008) Upregulation of glyoxylate cycle genes upon *Paracoccidioides brasiliensis* internalization by murine macrophages and *in vitro* nutritional stress condition. *Med Mycol* **46:** 125–134.

Dujon, B., Sherman, D., Fischer, G., Durrens, P., Casaregola, S., Lafontaine, I., *et al.* (2004) Genome evolution in yeasts. *Nature* **430:** 35–44.

Durchschlag, E., Reiter, W., Ammerer, G., and Schüller, C. (2004) Nuclear localization destabilizes the stress-regulated transcription factor Msn2. *J Biol Chem* **279:** 55425–55432.

Enjalbert, B., MacCallum, D.M., Odds, F.C., and Brown, A.J. (2007) Niche-specific activation of the oxidative stress response by the pathogenic fungus *Candida albicans*. *Infect Immun* **75:** 2143–2151.

Fan, W., Kraus, P.R., Boily, M.J., and Heitman, J. (2005) *Cryptococcus neoformans* gene expression during murine macrophage infection. *Eukaryot Cell* **4:** 1420–1433.

Farre, J.C., and Subramani, S. (2004) Peroxisome turnover by micropexophagy: an autophagy-related process. *Trends Cell Biol* **14:** 515–523.

Geisow, M.J., D'Arcy Hart, P., and Young, M.R. (1981) Temporal changes of lysosome and phagosome pH during phagolysosome formation in macrophages: studies by fluorescence spectroscopy. *J Cell Biol* **89:** 645–652.

Gildea, L.A., Ciraolo, G.M., Morris, R.E., and Newman, S.L. (2005) Human dendritic cell activity against *Histoplasma capsulatum* is mediated via phagolysosomal fusion. *Infect Immun* **73:** 6803–6811.

Giles, S.S., Stajich, J.E., Nichols, C., Gerrald, Q.D., Alspaugh, J.A., Dietrich, F., and Perfect, J.R. (2006) The *Cryptococcus neoformans* catalase gene family and its role in antioxidant defense. *Eukaryot Cell* **5:** 1447–1459.

Gordon, J.L., Byrne, K.P., and Wolfe, K.H. (2009) Additions, losses, and rearrangements on the evolutionary route from a reconstructed ancestor to the modern *Saccharomyces cerevisiae* genome. *PLoS Genet* **5:** e1000485.

Görner, W., Durchschlag, E., Martinez-Pastor, M.T., Estruch, F., Ammerer, G., Hamilton, B., *et al.* (1998) Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes Dev* **12:** 586–597.

Gregori, C., Schüller, C., Roetzer, A., Schwarzmüller, T., Ammerer, G., and Kuchler, K. (2007) The high-osmolarity glycerol response pathway in the human fungal pathogen *Candida glabrata* strain ATCC 2001 lacks a signaling branch that operates in baker's yeast. *Eukaryot Cell* **6:** 1635–1645.

Guan, J., Stromhaug, P.E., George, M.D., Habibzadegah-Tari, P., Bevan, A., Dunn, W.A., Jr, and Klionsky, D.J. (2001) Cvt18/Gsa12 is required for cytoplasm-to-vacuole transport, pexophagy, and autophagy in *Saccharomyces cerevisiae* and *Pichia pastoris*. *Mol Biol Cell* **12:** 3821–3838.

Hartig, A., and Ruis, H. (1986) Nucleotide sequence of the *Saccharomyces cerevisiae CTT1* gene and deduced amino-acid sequence of yeast catalase T. *Eur J Biochem* **160:** 487–490.

Hohfeld, J., Veenhuis, M., and Kunau, W.H. (1991) *PAS3*, a *Saccharomyces cerevisiae* gene encoding a peroxisomal integral membrane protein essential for peroxisome biogenesis. *J Cell Biol* **114:** 1167–1178.

Hu, G., Hacham, M., Waterman, S.R., Panepinto, J., Shin, S., Liu, X., *et al.* (2008) PI3K signaling of autophagy is required for starvation tolerance and virulenceof *Cryptococcus neoformans*. *J Clin Invest* **118:** 1186–1197.

Hutchins, M.U., Veenhuis, M., and Klionsky, D.J. (1999) Peroxisome degradation in *Saccharomyces cerevisiae* is dependent on machinery of macroautophagy and the Cvt pathway. *J Cell Sci* **112** (Part 22)**:** 4079–4087.

Idnurm, A., Giles, S.S., Perfect, J.R., and Heitman, J. (2007) Peroxisome function regulates growth on glucose in the basidiomycete fungus *Cryptococcus neoformans*. *Eukaryot Cell* **6:** 60–72.

Johnson, C.H., Klotz, M.G., York, J.L., Kruft, V., and McEwen, J.E. (2002) Redundancy, phylogeny and differential expression of *Histoplasma capsulatum* catalases. *Microbiology* **148:** 1129–1142.

Kaposzta, R., Marodi, L., Hollinshead, M., Gordon, S., and da Silva, R.P. (1999) Rapid recruitment of late endosomes and lysosomes in mouse macrophages ingesting *Candida albicans*. *J Cell Sci* **112** (Part 19)**:** 3237–3248.

Kaur, R., Domergue, R., Zupancic, M.L., and Cormack, B.P. (2005) A yeast by any other name: *Candida glabrata* and its interaction with the host. *Curr Opin Microbiol* **8:** 378–384.

Kaur, R., Ma, B., and Cormack, B.P. (2007) A family of glycosylphosphatidylinositol-linked aspartyl proteases is required for virulence of *Candida glabrata*. *Proc Natl Acad Sci USA* **104:** 7628–7633.

Kim, J., Kamada, Y., Stromhaug, P.E., Guan, J., Hefner-Gravink, A., Baba, M., *et al.* (2001) Cvt9/Gsa9 functions in sequestering selective cytosolic cargo destined for the vacuole. *J Cell Biol* **153:** 381–396.

Kitada, K., Yamaguchi, E., and Arisawa, M. (1996) Isolation of a *Candida glabrata* centromere and its use in construction of plasmid vectors. *Gene* **175:** 105–108.

Koerkamp, M.G., Rep, M., Bussemaker, H.J., Hardy, G.P., Mul, A., Piekarska, K., *et al.* (2002) Dissection of transient oxidative stress response in *Saccharomyces cerevisiae* by using DNA microarrays. *Mol Biol Cell* **13:** 2783–2794.

Kraft, C., Reggiori, F., and Peter, M. (2009) Selective types of autophagy in yeast. *Biochim Biophys Acta* **1793:** 1404–1412.

Kragler, F., Langeder, A., Raupachova, J., Binder, M., and Hartig, A. (1993) Two independent peroxisomal targeting signals in catalase A of *Saccharomyces cerevisiae*. *J Cell Biol* **120:** 665–673.

Kuge, S., Jones, N., and Nomoto, A. (1997) Regulation of yAP-1 nuclear localization in response to oxidative stress. *EMBO J* **16:** 1710–1720.

Kunze, M., Pracharoenwattana, I., Smith, S.M., and Hartig, A. (2006) A central role for the peroxisomal membrane in glyoxylate cycle function. *Biochim Biophys Acta* **1763:** 1441–1452.

Leberer, E., Harcus, D., Dignard, D., Johnson, L., Ushinsky, S., Thomas, D.Y., and Schroppel, K. (2001) Ras links cellular morphogenesis to virulence by regulation of the MAP kinase and cAMP signalling pathways in the pathogenic fungus *Candida albicans*. *Mol Microbiol* **42:** 673–687.

Lemberg, M.R. (1969) Cytochrome oxidase. *Physiol Rev* **49:** 48–121.

Levitz, S.M., Nong, S.H., Seetoo, K.F., Harrison, T.S., Speizer, R.A., and Simons, E.R. (1999) *Cryptococcus neoformans* resides in an acidic phagolysosome of human macrophages. *Infect Immun* **67:** 885–890.

Lewin, A.S., Hines, V., and Small, G.M. (1990) Citrate synthase encoded by the *CIT2* gene of *Saccharomyces cerevisiae* is peroxisomal. *Mol Cell Biol* **10:** 1399–1405.

Li, L., Redding, S., and Dongari-Bagtzoglou, A. (2007) *Candida glabrata*: an emerging oral opportunistic pathogen. *J Dent Res* **86:** 204–215.

Lorenz, M.C., and Fink, G.R. (2001) The glyoxylate cycle is required for fungal virulence. *Nature* **412:** 83–86.

Lorenz, M.C., Bender, J.A., and Fink, G.R. (2004) Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot Cell* **3:** 1076–1087.

Ma, H., Croudace, J.E., Lammas, D.A., and May, R.C. (2006) Expulsion of live pathogenic yeast by macrophages. *Curr Biol* **16:** 2156–2160.

Marcet-Houben, M., and Gabaldon, T. (2009) The tree versus the forest: the fungal tree of life and the topological diversity within the yeast phylome. *PLoS One* **4:** e4357.

Marchler, G., Schüller, C., Adam, G., and Ruis, H. (1993) A *Saccharomyces cerevisiae* UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. *EMBO J* **12:** 1997–2003.

Miller, R.A., and Britigan, B.E. (1997) Role of oxidants in microbial pathophysiology. *Clin Microbiol Rev* **10:** 1–18.

Missall, T.A., Pusateri, M.E., and Lodge, J.K. (2004) Thiol peroxidase is critical for virulence and resistance to nitric oxide and peroxide in the fungal pathogen, *Cryptococcus neoformans*. *Mol Microbiol* **51:** 1447–1458.

Mizushima, N., and Klionsky, D.J. (2007) Protein turnover via autophagy: implications for metabolism. *Annu Rev Nutr* **27:** 19–40.

Nakagawa, Y. (2008) Catalase gene disruptant of the human pathogenic yeast *Candida albicans* is defective in hyphal growth, and a catalase-specific inhibitor can suppress hyphal growth of wild-type cells. *Microbiol Immunol* **52:** 16–24.

Nakagawa, Y., Kanbe, T., and Mizuguchi, I. (2003) Disruption of the human pathogenic yeast *Candida albicans* catalase gene decreases survival in mouse-model infection and elevates susceptibility to higher temperature and to detergents. *Microbiol Immunol* **47:** 395–403.

Nasution, O., Srinivasa, K., Kim, M., Kim, Y.J., Kim, W., Jeong, W., and Choi, W. (2008) Hydrogen peroxide induces hyphal differentiation in *Candida albicans*. *Eukaryot Cell* **7:** 2008–2011.

Neuberger, G., Maurer-Stroh, S., Eisenhaber, B., Hartig, A., and Eisenhaber, F. (2003) Prediction of peroxisomal targeting signal 1 containing proteins from amino acid sequence. *J Mol Biol* **328:** 581–592.

Nicola, A.M., Casadevall, A., and Goldman, D.L. (2008) Fungal killing by mammalian phagocytic cells. *Curr Opin Microbiol* **11:** 313–317.

Noble, S.M., and Johnson, A.D. (2005) Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen *Candida albicans*. *Eukaryot Cell* **4:** 298–309.

Onodera, J., and Ohsumi, Y. (2005) Autophagy is required for maintenance of amino acid levels and protein synthesis under nitrogen starvation. *J Biol Chem* **280:** 31582–31586.

Palmer, G.E., Kelly, M.N., and Sturtevant, J.E. (2007) Autophagy in the pathogen *Candida albicans*. *Microbiology* **153:** 51–58.

Palmer, G.E., Askew, D.S., and Williamson, P.R. (2008) The diverse roles of autophagy in medically important fungi. *Autophagy* **4:** 982–988.

Paris, S., Wysong, D., Debeaupuis, J.P., Shibuya, K., Philippe, B., Diamond, R.D., and Latge, J.P. (2003) Catalases of *Aspergillus fumigatus*. *Infect Immun* **71:** 3551–3562.

Pfaller, M.A., and Diekema, D.J. (2007) Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* **20:** 133–163.

Piekarska, K., Mol, E., van den Berg, M., Hardy, G., van den Burg, J., van Roermund, C., *et al.* (2006) Peroxisomal fatty acid beta-oxidation is not essential for virulence of *Candida albicans*. *Eukaryot Cell* **5:** 1847–1856.

Platta, H.W., and Erdmann, R. (2007) Peroxisomal dynamics. *Trends Cell Biol* **17:** 474–484.

Presterl, E., Daxbock, F., Graninger, W., and Willinger, B. (2007) Changing pattern of candidaemia 2001–2006 and use of antifungal therapy at the University Hospital of Vienna, Austria. *Clin Microbiol Infect* **13:** 1072–1076.

Prigneau, O., Porta, A., Poudrier, J.A., Colonna-Romano, S., Noel, T., and Maresca, B. (2003) Genes involved in beta-oxidation, energy metabolism and glyoxylate cycle are induced by *Candida albicans* during macrophage infection. *Yeast* **20:** 723–730.

Richie, D.L., Fuller, K.K., Fortwendel, J., Miley, M.D., McCarthy, J.W., Feldmesser, M., *et al.* (2007) Unexpected link between metal ion deficiency and autophagy in *Aspergillus fumigatus*. *Eukaryot Cell* **6:** 2437–2447.

Roetzer, A., Gregori, C., Jennings, A.M., Quintin, J., Ferrandon, D., Butler, G., *et al.* (2008) *Candida glabrata* environmental stress response involves *Saccharomyces cerevisiae* Msn2/4 orthologous transcription factors. *Mol Microbiol* **69:** 603–620.

Romani, L. (2004) Immunity to fungal infections. *Nat Rev Immunol* **4:** 1–23.

Rubin-Bejerano, I., Fraser, I., Grisafi, P., and Fink, G.R. (2003) Phagocytosis by neutrophils induces an amino acid deprivation response in *Saccharomyces cerevisiae* and *Candida albicans*. *Proc Natl Acad Sci USA* **100:** 11007–11012.

Rude, T.H., Toffaletti, D.L., Cox, G.M., and Perfect, J.R. (2002) Relationship of the glyoxylate pathway to the pathogenesis of *Cryptococcus neoformans*. *Infect Immun* **70:** 5684–5694.

Schöbel, F., Ibrahim-Granet, O., Ave, P., Latge, J.P., Bra-

khage, A.A., and Brock, M. (2007) *Aspergillus fumigatus* does not require fatty acid metabolism via isocitrate lyase for development of invasive aspergillosis. *Infect Immun* **75:** 1237–1244.
Segal, A.W. (2005) How neutrophils kill microbes. *Annu Rev Immunol* **23:** 197–223.
Sikorski, R.S., and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122:** 19–27.
Simon, M., Adam, G., Rapatz, W., Spevak, W., and Ruis, H. (1991) The *Saccharomyces cerevisiae ADR1* gene is a positive regulator of transcription of genes encoding peroxisomal proteins. *Mol Cell Biol* **11:** 699–704.
Thieringer, R., Shio, H., Han, Y.S., Cohen, G., and Lazarow, P.B. (1991) Peroxisomes in *Saccharomyces cerevisiae*: immunofluorescence analysis and import of catalase A into isolated peroxisomes. *Mol Cell Biol* **11:** 510–522.
Urban, C.F., Lourido, S., and Zychlinsky, A. (2006) How do microbes evade neutrophil killing? *Cell Microbiol* **8:** 1687–1696.
Veneault-Fourrey, C., Barooah, M., Egan, M., Wakley, G., and Talbot, N.J. (2006) Autophagic fungal cell death is necessary for infection by the rice blast fungus. *Science* **312:** 580–583.
Voyich, J.M., Braughton, K.R., Sturdevant, D.E., Whitney, A.R., Said-Salim, B., Porcella, S.F., *et al.* (2005) Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *J Immunol* **175:** 3907–3919.
Xie, Z., and Klionsky, D.J. (2007) Autophagosome formation: core machinery and adaptations. *Nat Cell Biol* **9:** 1102–1109.
Yan, M., Rayapuram, N., and Subramani, S. (2005) The control of peroxisome number and size during division and proliferation. *Curr Opin Cell Biol* **17:** 376–383.
Yorimitsu, T., and Klionsky, D.J. (2005) Atg11 links cargo to the vesicle-forming machinery in the cytoplasm to vacuole targeting pathway. *Mol Biol Cell* **16:** 1593–1605.

## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Southern blot analysis of *cta1*Δ, *pex3*Δ, *atg11*Δ and *pex3*Δ *atg11*Δ deletion strains. Both *CgCTA1* and *CgPEX3* were replaced by *ScURA3. CgATG11* was replaced by *ScURA3* in the single mutant and by *ScHIS3* in the double mutant ARCg *pex3*Δ *atg11*Δ. *CgATG17* was replaced by *ScURA3* in the single mutant and by *ScHIS3* in the double mutants ARCg *pex3*Δ *atg17*Δ and ARCg *atg11*Δ *atg17*Δ. Amplified probes and chromosomal restriction enzyme locations are indicated. Chromosomal DNA derived from ARCg *cta1*Δ digested with EcoRV and ARCg *pex3*Δ with XcmI resulted in shortened fragments relative to wild type. Digestion of chromosomal DNA from the ARCg *atg11*Δ strain with NcoI and from the ARCg *pex3*Δ *atg11*Δ double mutant strain with ClaI led to shorter fragments in both cases, since *CgATG11* contains neither a NcoI site nor a ClaI site. Picture is a composite of two exposures of the same blot, due to different amounts of DNA (lower panel). Chromosomal DNA derived from ARCg *atg17*Δ digested with AflIII and ARCg *pex3*Δ *patg17*Δ or ARCg *atg11*Δ *atg17*Δ with NdeI resulted in shortened fragments.

**Fig. S2.** Glucose depletion leads to induction of *GFP–CgCTA1* and cytoplasmic localization of CgMig1–CFP.

A. *GFP–CgCTA1* is induced upon glucose depletion and is located in the cytoplasm. *C. glabrata* ARCg *cta1*Δ cells transformed with p*CgC–GFP–CgCTA1* were grown in rich medium with glucose to exponential phase and washed twice and incubated in rich medium including 0.5% glucose. Every 10 min concentration of glucose was determined (see *Experimental procedures*) and samples were fixed for microscopy. GFP fluorescence was visible at about 40 min after glucose exhaustion.

B. Localization of CgMig1–CFP in ARCg *atg11*Δ and ARCg *pex3*Δ *atg11*Δ mutants during internalization by macrophages. CgMig1–CFP was visualized by fluorescence microscopy under phagocytosis conditions. *C. glabrata* wild-type cells transformed with p*CgADH1–CgMIG1–CFP* were grown to exponential phase, washed in PBS 0.1% glucose and added to macrophages in a 4:1 ratio and incubated at 37°C for 1 h. Samples were fixed and stained with Phalloidin Texas-Red.

C. *GFP–CgCTA1* is induced upon phagocytosis. Still pictures from time-lapse analysis are shown as overlay of bright-field and fluorescence signals. Exponentially growing ARCg *cta1*Δ cells transformed with p*CgC–GFP–CgCTA1* were washed in PBS containing 0.1% glucose and added to macrophages in a 4:1 ratio at 37°C.

**Fig. S3.** Quantification details.

**Table S1.** Oligonucleotides used.

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