Towards understanding bacterial induced sepsis:
RNA recognition by murine TLR13 and human TLR8

Inaugural-Dissertation

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geboren in Luckenwalde

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<tr>
<td>-/-</td>
<td>unchallenged</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BMM</td>
<td>bone marrow derived macrophage</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
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<tr>
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<tr>
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<tr>
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<td>endoplasmic reticulum</td>
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<tr>
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<td>erythromycin</td>
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<td>ethanol</td>
</tr>
<tr>
<td>Ig</td>
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</tr>
<tr>
<td>hiEc</td>
<td>heat inactivated Escherichia coli</td>
</tr>
<tr>
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<td>heat inactivated Staphylococcus aureus</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>kb</td>
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<tr>
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<td>lipopolysaccharide</td>
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<tr>
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<tr>
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</tr>
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<td>oligoribonucleotide</td>
</tr>
<tr>
<td>Pam3CSK4</td>
<td></td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PGN</td>
<td>peptidoglycan</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
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<tr>
<td>r</td>
<td>ribosomal</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
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<td>Svedberg</td>
</tr>
<tr>
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<td>supernatant</td>
</tr>
<tr>
<td>ss</td>
<td>single stranded</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>UV</td>
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</tr>
<tr>
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<td>volume per volume</td>
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<tr>
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1 Introduction

1.1 The immune system - innate and adaptive

The immune system immediately protects every organism from disease upon infection. It thus detects a wide range of pathogens and distinguishes between foreign and self-derived compounds. Besides an innate immune system, advanced species evolved an adaptive immune system, which can be divided into a humoral and a cell-mediated branch (Janeway 1989).

Physical barriers such as skin or mucosae and fluids such as lacrimation, saliva and gastric fluid prevent pathogens like bacteria, viruses and parasites as well as toxins from invading host tissues. Upon invasion, pathogens face innate immune defense first. Macrophages, dendritic cells (DCs), mast cells, neutrophile granulocytes, eosinophile and basophile granulocytes as well as the complement system are activated. The innate immune response is triggered upon detection of pathogens by pattern recognition receptors (PRRs). These bind to highly conserved pathogen associated molecular patterns (PAMPs) or signals sent out by damaged or stressed cells (Janeway 1989, Medzhitov et al. 1997).

Early vertebrates additionally developed an adaptive immune system, mediating a more efficient immune response, including an immunological memory, based on the presentation and recognition of pathogen specific signature antigens (Litman et al. 2010, Kasamatsu 2013). Cells of the adaptive immune system consist of B and T lymphocytes derived from hematopoietic stem cells. They express clonal antigen specific receptors. T cell pathogen recognition depends on antigen processing and presentation by major histocompatibility complex (MHC) molecules. Natural killer T cells (NKT) recognize antigens coupled to class I MHC, while helper T cells (T_h1 & T_h2) only recognize antigens coupled to class II MHC molecules. A third type of T lymphocytes the regulatory T cells (T_reg) modulate immune responses, maintaining tolerance and abrogating autoimmunity. B lymphocytes either turn into plasma cells secreting specific antibodies or memory B cells storing antigen information, with each lineage of B cells expressing a different antibody (Murphy et al. 2008).

1.2 Pattern recognition receptors

In contrast to clonally expressed receptors of adaptive immunity, cells of the innate immune system express cell type specific sets of pattern recognition receptors (PRRs). These mediate phagocytosis of pathogens, induction of chemokine secretion attracting cells of the adaptive system to the source of infection as well as induction of pro- and anti-inflammatory cytokines. There are membrane bound and cytoplasmic receptors but also soluble PRRs circulating in blood plasma or secreted into epithelial fluids. Binding of pathogenic components to the corresponding receptor leads to opsonization and internalization by phagocytes. Membrane bound PRRs comprise complement receptors, C-type lectins (CLR), scavenger receptors and Toll-like receptors (TLR) (Hawlisch and Köhl 2006, Greaves and Gordon 2009, Takeuchi and Akira 2010, Hoving et al. 2014, Underhill and Ozinsky 2002, Jiang et al. 2014, Netea and van der Meer 2011). The complement system is evolutionarily one of the first in-
stances of innate immunity containing membrane bound as well as soluble proteins (Montz et al. 1991).

Figure 1: Pattern recognition receptors. Members of the TLR family are localized in the cytoplasmic or endosomal membrane, whereas CLRs are anchored in the cytoplasmic membrane. RLHs and NLRs are cytosolic receptors. Upon activation PRRs induce proinflammatory cytokines, chemokines and type-I IFNs. **TLRs recruit common adaptor molecules.** Following activation, TLR5, 7, 8, 9, 10, 11, 12 and 13 as well as IL-1 receptors exclusively interact with MyD88. TLR2 and TLR4 additionally involve the adaptors MAL and TRAM, while TRIF is recruited by TLR3 and TLR4.

### 1.2.1 C-type lectins

C-type lectin receptors (CLR, Fig.1) are characterized by the presence of one or more C-type lectin-like domains (CTLD). They are Ca\(^{2+}\)-dependent (or -independent) carbohydrate binding proteins, functionally diverse and have been implicated in processes like cell adhesion, tissue integration and remodeling, platelet activation, complement activation, pathogen recognition as well as endo- and phagocytosis. According to phylogeny and domain organization, they are divided into 17 groups. C-type lectins recognize ligands derived from pathogen envelope carbohydrate residues, such as β-glucans, mannose, fucose, N-acetylglucosamine (GlcNAc) or N-acetylmannosamine (ManNAc) (Weis and Drickamer 1996, Zelensky and Gready 2005). They recognize microbial pathogens like fungi and bacteria, organizing their uptake. Dectin-1 for example, containing a single CTLD and an immunoreceptor tyrosine-based activation motif (ITAM) is highly expressed in macrophages, DCs, monocytes and neutrophils. Mannose receptor (MR) however consists of 8 CTLDs and apart from recognizing microbes was implicated in phagocytic uptake of apoptotic cells in chronic obstructive pulmonary disease (COPD). Soluble mannose binding lectin (MBL) containing one CTLD forming oligomers upon
ligand binding in blood serum activates the complement system (Kerrigan and Brown 2009, Dambuza and Brown 2015, Gál and Ambrus 2001).

### 1.2.2 Scavenger receptors

Scavenger receptors are mainly expressed by myeloid cells. They have diverse functions, such as endocytosis, adhesion, lipid transport, antigen presentation, and pathogen clearance. According to their sequence and structure, they are divided into 8 classes and were originally described as low density lipoprotein receptors. In addition to their crucial role in maintaining host homeostasis, they have been implicated in pathogenesis of several diseases, like atherosclerosis and Alzheimer's disease (Canton et al. 2013, Yu et al. 2015).

### 1.2.3 NOD-like receptors & inflammasome

NOD-like receptors (nucleotide-binding domain leucine-rich repeat containing receptor, NLRs) are localized within the cytoplasm (Fig.1). There are 22 (murine) or 34 (human) NLRs, divided into five subfamilies. They consist of three distinctive domains, a central nucleotide-binding domain (NACHT), a C-terminal leucine-rich repeat (LRR) domain and a variable N-terminal protein-protein interaction domain (Ting et al. 2008, Lamkanfi and Dixit 2012).

NOD and inflammasome-forming NLRs are subdivided based on differences in the N-terminal domain. The former are composed of a central NACHT domain to which an N-terminal caspase recruitment domain (CARD), a pyrin domain (PYD) or Baculovirus inhibitor repeats (BIRs) are attached (Kanneganti et al. 2006). NOD1 and NOD2, the first NLRs described, intracellularly recognize microbial peptides and activate transcription factors leading to the induction of proinflammatory mediators through RIP2 (receptor-interacting protein 2) signaling (Girardin et al. 2003a, 2003b, Philpott et al. 2014).

Inflammasome forming NLRs and AIM2-like receptors comprise heterogeneous multi-protein complexes activating proinflammatory caspase 1 (Martinon et al. 2002). They are organized in ASC (apoptosis associated speck-like protein containing CARD) recruiting, e.g., NLRP3 and AIM2 (absent in melanoma 2) as well as ASC independent inflammasomes such as NLRC4 and NLRP1. Upon activation ASC, via its CARD, recruits pro-caspase-1, which is then activated autocatalytically to cleave cytosolic pro-forms of the cytokines IL-1β, IL-18 and IL-33 (Schröder and Tschopp 2010, Latz et al. 2013). ASC-independent inflammasomes directly interact via CARD-CARD binding of pro-caspase-1 (Van Opdenbosch et al. 2014). Viral dsRNA, imidazoquinolines, bacterial RNA and toxins like nigericin or listeriolysin O have been described as inflammasome ligands (Gurcel et al. 2006, Kanneganti et al. 2006, Allen et al. 2009, Sha et al. 2014).

### 1.2.4 RIG-I like helicases

RIG-like helicases (RLHs) represent another group of receptors localized within the cytoplasm (Fig.1). They include RIG-I (retinoic acid-inducible gene 1), MDA5 (melanoma differentiation-associated protein 5) and LGP2 (laboratory of genetics and physiology 2). While RIG-I and MDA5 consist of two N-terminal CARDs, a central RNA helicase DEAD-box and a regulatory C-terminal domain
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(CTD), LGP2 is devoid of CARD. Following activation through ss and ds viral RNA the adaptor protein MAVS (mitochondrial antiviral-signaling protein, synonyms are IPS-1, CARDIF or VISA) and downstream transcription factors IRF3 and 7 (interferon regulatory factor) induce type-I interferons (IFN) and further proinflammatory cytokines (Kawai et al. 2005, Takeuchi and Akira 2008, Freaney et al. 2013, Liu et al. 2013).

1.2.5 Toll-like receptors

Toll-like receptors (TLRs) activate the innate immune system in response to bacterial, fungal, viral and parasitic infections. Toll genes encode members of the TLR family. In 1985, mutations of the Toll gene have been described in Drosophila melanogaster by Christiane Nüsslein-Volhard and Eric Wieschaus and cloned by Kathryn Anderson in 1988 (Hansson and Edfeldt 2005, Hashimoto et al. 1988). In D. melanogaster Toll was first found to be involved in embryogenesis, in which it mediates dorsoventral polarization. A decade later its importance as PRR in innate immunity expressed not only in invertebrates but also in mammals has been reported (Lemaitre et al. 1996, Mitcham et al. 1996, Medzhitov et al. 1997, Kirschning et al. 1998, Poltorak et al. 1998, Qureshi et al. 1999, Yang et al. 1998). Thirteen mammalian toll-like receptors (TLRs) are expressed in mice and humans, whereof mice lack expression of TLR10, while humans lack expression of TLR11, -12 and -13. TLRs are either localized at the cell surface within the cytoplasmic membrane (TLR1, -2, -4, -5, -6 and -10) or in the endolysosomal membrane (TLR3, -7, -8, -9, -11, -12 and -13) (Akira and Takeda 2004) (Fig.1). TLRs are expressed in vertebrate immune cells like monocytes, macrophages, natural killer cells, mast cells and DCs, as well as in highly differentiated tissue specific cells like intestinal epithelial, liver and kidney cells and in neurons (Bell et al. 2003).

1.2.5.a Structure of TLRs

TLRs are type-I transmembrane proteins consisting of an extracellular/endosome-luminal N-terminal ligand specific recognition domain (ECD), a single transmembrane domain and an intracellular/cytosolic C-terminal signaling domain. TLRs are glycoproteins of 550-800 amino acids in size (Fig.1) (Bell et al. 2003).

The ECD forms a characteristic horse-shoe like solenoidal structure, due to multiple conserved leucine rich repeats (LRRs; LXXLXXLXLXN, L: leucine, X: arbitrary amino acid, N: asparagine). Single LRRs form turns stabilized by inward facing conserved hydrophobic residues (XΩXXΩX4FXXLX motif; X: arbitrary, Ω: hydrophobic amino acid, F: phenylalanine). Some irregular loops protruding out of the LRR backbone are important for ligand binding (Kobe and Kajava 2001, Bell et al. 2006). The intra- and extracellular domains of a TLR are connected by a membrane spanning α-helix of 20 nonpolar amino acids. The intracellular domains of all TLRs share a TIR (Toll/Interleukin-1 receptor) domain which resembles the cytosolic domain of IL-1 receptors. Its structure is highly conserved while the ligand specific ECD variates to a large degree (Burch-Smith and Dinesh-Kumar 2007). The TIR domain is composed of five alternating α-helices and β-sheets (Xu et al. 2000, Bell et al. 2003, Chan et al. 2010). It consists of 150 amino acids forming three conserved regions named box 1, 2 and 3. Box 2 includes the so called BB-loop, which is of importance for signal transduction (Brikos and O’Neill 2008) in that it binds TLR specific adaptor molecules. A TIR domain is also part of specific
signaling adaptor molecules. Receptor activation by ligand binding to the ECD induces interaction of its TIR domain with the TIR domain of adaptor molecules. Five such adaptor molecules are known: myeloid differentiation factor 88 (MyD88), Myd88-adaptor-like protein (MAL), TIR domain-containing adaptor inducing interferon-β (TRIF/Ticam)-1, TRIF related adaptor protein (TRAM/Ticam)-2 and sterile-α and HEAT/armadillo motifs-containing protein (SARM) (Takeda and Akira 2004). Recently, a sixth adaptor molecule BCAP (B cell adaptor for phosphoinositid-3-kinase (PI3K)) has been identified (Troutman et al. 2012).

1.2.5.b Accessory proteins & signaling pathways
TLRs dimerize as homodimers after specific ligand binding, except for TLR2, which forms heterodimers with either TLR1 or TLR6 (Ozinsky et al. 2000). Dimerization leads to allosteric conformational change of the TIR domain (Colonna 2007), upon which the adaptor molecule TIR domain is recruited to induce signal transduction toward the nucleus. Only TLR3 and TLR4 utilize TRIF, whereas all other TLRs and TLR4 utilize MyD88 to mediate phosphorylation and thereby activation of interleukin-1 receptor-associated kinases (IRAKs, Fig.1) (Takeda and Akira 2004). The complex ubiquitinylates TNF receptor associated factor 6 (TRAF6) inducing phosphorylation of mitogen-activated kinases (MAPKs) through the TAK-complex, as well as phosphorylation of the IKK-complex consisting of IKK-α, IKK-β and IKK-γ, leading to NF-κB activation via phosphorylation and dissociation of the inhibitor IκB. This results in induction of gene expression of proinflammatory cytokines like IL-6, IL-12, TNF and IL-1β. Unphosphorylated IκB is bound to the NF-κB subunits p50, p52 and p56 thereby blocking its translocation into the nucleus (Häcker et al. 2011). The second pathway also recruits members of the interferon regulatory factor (IRF)-family to induce expression of type-I IFNs, via IRF1 and IRF7, as well as of proinflammatory cytokines, via IRF5 (Colonna 2007, Coban et al. 2010). In contrast, IFN-β induction through IRF3 and IRF7 as well as induction of proinflammatory cytokines through NF-κB are the results of merely two distinct TRIF dependent signaling cascades following activation of TLR3 or TLR4 (Kawai and Akira 2010). One further TRIF dependent pathway resulting in NF-κB induction demands recruitment of a C-terminally localized RIP (receptor interacting protein) and an N-terminally localized TRAF6.

MyD88 and TRIF are thus essential for signal transduction following TLR activation, while MAL, also called TIR domain containing adaptor (TIRAP), and TRAM function as bridging molecules between TLR and adaptor molecule (Fig.1). MAL is involved in MyD88 driven induction of NF-κB downstream of TLR2 and TLR4 (Horng et al. 2002). MAL interacts with the intracellular domains of TLR1 and TLR2 as well as of TLR4. It was also implicated in inhibition of TLR3 signaling (O’Neill and Bowie 2007, Brikos and O’Neill 2008, Kenny et al. 2009). On the other hand, TRAM acts as a switch for internalized TLR4 and TRIF to induce type-I IFNs such as IFNα/β IRF3-dependently. This second signaling pathway of TLR4 is induced via activation of TRIF-MyD88 leading to endocytosis of TLR4 and endosomal activation of the TRAM-TRIF pathway (Kagan et al. 2008, Tanimura et al. 2008, Zanoni et al. 2011). Consequently, inflammatory responses to infection by viral or bacterial pathogens are largely abrogated in MyD88 or TRIF deficient mice (Adachi et al. 1998).
Another protein very important for an appropriate innate immune response to invading pathogens via TLRs is Unc93B1 (a polytopic endoplasmic reticulum-resident membrane protein). It acts as a shuttle protein directing endosomal TLRs from the ER to their subcellular localization in the endosomal/lysosomal membrane. Unc93B1 is a highly conserved protein consisting of 12 trans-membrane domains and two N-terminal glycosylation motifs (Kashuba et al. 2002, Brinkmann et al. 2007)). TLR3,-7,-8,-9,-11,-12 and -13 depend on functional Unc93B1 to reach the endosome (Itoh et al. 2011, Lee et al. 2013), which in case of a missense point mutation in Unc93B1, called 3D (H412R), results in absence of endosomal nucleic acid recognition (Tabeta et al. 2006). Subcellular compartmentalization of membrane bound innate immune receptors enables distinction between pathogen/foreign and self-derived nucleic acids permanently present in the cytosol and extracellular space. Phagocytosed microorganisms become digested within the endolysosomes due to acidification and presence of degrading enzymes, their components such as nucleic acids are recognized by endosomal TLRs. These are transformed into their functional conformation via cleavage in case of TLR7, -8 and -9, to further initiate signaling cascades inducing an innate immune response to the invading pathogens (Ewald et al. 2008, Ishii et al. 2014).

1.2.5.5 Recognition of specific ligands

Receptors localized within the cytoplasmic membrane are specialized in sensing bacterial cell wall and membrane components and proteins, while endosomally localized TLRs show an affinity to nucleic acid sensing (Table 1).

**Table 1:** TLR specific ligands and their origin

<table>
<thead>
<tr>
<th>TLR</th>
<th>Ligand</th>
<th>Origin</th>
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<tbody>
<tr>
<td>TLR1/2</td>
<td>triacylated lipopeptides</td>
<td>bacteria</td>
</tr>
<tr>
<td>TLR2</td>
<td>lipoproteins, peptidoglycan, LTA, LPS, zymosan</td>
<td>Gram-positive bacteria, fungi</td>
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<td>TLR2/6</td>
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<td>TLR3</td>
<td>dsRNA, Poly I:C</td>
<td>viruses, bacteria</td>
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<td>TLR4</td>
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<td>TLR5</td>
<td>flagellin</td>
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<td>ssRNA, R848, Loxoribine, Imiquimod</td>
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<td>TLR8</td>
<td>ssRNA, R848, Loxoribine</td>
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<td>TLR9</td>
<td>DNA, unmethylated CpG DNA</td>
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<td>TLR10</td>
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<td>viruses, <em>Plasmodium</em></td>
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<td>profilin, flagellin</td>
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<tr>
<td>TLR12</td>
<td>profilin</td>
<td><em>Toxoplasma gondii</em></td>
</tr>
<tr>
<td>TLR13</td>
<td>23S rRNA segment &quot;Sa19&quot;</td>
<td>bacteria</td>
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**TLR3** binds dsRNA such as from viral replicates (Alexopoulou et al. 2001). TLR3 consists of two dsRNA binding domains, one N-terminal and a second one proximal to the C-terminus (Wang et al. 2010). Upon activation by viral RNA or the synthetic analog Poly(I:C) (polynosine-polycytidylic acid) TLR3 induces expression of type-I and type-III IFNs via TRIF, subsequently resulting in maturation of DCs (Zhou et al. 2009, Pantel et al. 2014).

**TLR4**, first characterized as NF-κB activator in 1997 (Medzhitov et al. 1997), complexes with the accessory protein MD-2 (lymphocyte antigen 96) to sense Gram-negative bacterial LPS (Poltorak et al. 1998, Qureshi et al. 1999, Shimazu et al. 1999, Imler and Hoffmann 2001, Latz et al. 2002). Further TLR4 ligands have been reported, such as lipid A analogs (LPS, lipopolysaccharide), mannan, viral fusion proteins and the synthetic trimyristylated acylhexapeptide Myr3CSK4 (Kurt-Jones et al. 2000, Netea et al. 2004, Spiller et al. 2007, Wong et al. 2009).

In contrast to other TLRs, **TLR5** is not expressed in conventional (c)DCs or murine macrophages, but in intestinal epithelial cells, neutrophilic granulocytes, classical monocytes and a specific class of DCs (Uematsu et al. 2006, Shibata et al. 2012). Flagellin, a major flagellum component of all amphibitrichous bacteria, is the known TLR5 ligand (Hayashi et al. 2001).

The endosomal TLRs -7, -8 and -9 share high sequence homology and similar crystal structure (Kawai and Akira 2006, Wei et al. 2009). TLR7 and -8 together with TLR3 recognize RNA, while TLR9 binds DNA. **TLR7** is specifically activated by ssRNA derived from bacteria as well as from viruses. Studies with **TLR7**−/− mice have identified ssRNA from *Vesicular stomatitis virus* (VSV) and also of *Influenza virus* as stimulatory (Lund et al. 2004). Further studies point out the involvement of TLR7 in recognizing endogenous Retroviruses (Yu et al. 2012). Synthetic ligands are short molecular derivatives like R837 (imiquimod), R848 (resiquimod), CL075 (3-M-001) (Hemmi et al. 2002) and loxoribine (Heil et al. 2003). TLR7 is expressed mainly by dendritic cells and to a lower degree by monocytes and macrophages, mediating induction of type-I IFNs upon activation (Gorden et al. 2005, Hornung et al. 2005).
Introduction

**TLR8**, just like TLR7, shows diverging functions in mice and humans. Even though the murine and human receptors are sequentially very similar, the first is considered inactive due to its unresponsiveness to confrontation with either natural viral ssRNA or synthetic imidazoquinoline R848 (Jurk et al. 2002, Heil et al. 2004, Forsbach et al. 2008). This was partially refuted as murine PBMCs have been shown to sense CL075 if combined with a poly(dT) oligodeoxynucleotide (ODN) (Gorden et al. 2006). Human TLR8 in plasmacytoid dendritic cells (pDCs) is activated by *Vaccinia virus* DNA (Martinez et al. 2010). In contrast, another study shows TLR7 and -9 but not TLR8 to be highly expressed in pDCs (Bauer et al. 2010). Human TLR8 has been shown to be expressed in myeloid DCs, monocytes and macrophages to induce type-I IFN as well as TNF and IL-12 production (Lombardi et al. 2009, Han et al. 2012, Guiducci et al. 2013). Conversely to human TLR7, TLR8 is activated by CL075 as well as by ssRNA40 and R848, the last two of which are not recognized by murine TLR8 (Jurk et al. 2002, Heil et al. 2004, Gorden et al. 2005). Further studies have implied TLR8 in the recognition of *Borellia burgdorferi*, *Mycobacterium bovis* and *Helicobacter pylori* RNA (Davila et al. 2008, Gantier et al. 2010, Cervantes et al. 2013).

TLR9 deficient mice confronted with unmethylated CpG-DNA (*Cytosine-phosphatidyl-Guanine*) lacked responsiveness, implying **TLR9** as receptor (Hemmi et al. 2000). Activation of macrophages and DCs using DNA of *Babesia bovis*, *Trypanosoma cruzi* and *T. brucei* is also mediated by TLR9 (Shoda et al. 2001, Bafica et al. 2006). Synthetic oligonucleotides (ODN) used as TLR9 ligands can be divided into two categories. The first group of ODNs consists of a palindromic phosphodiester-backbone and a phosphorothiate modified 3'-poly-G end, including ODN 1585, 2216 and 2336. These are capable of inducing type-1 IFN production as well as maturation of pDCs. The second group of ODNs like ODN 1668, 2006 and 2007 is characterized by a monomeric phosphorothioate structure with a hexamer CpG motif. TLR9 induces increased IL-6 levels in PBMCs and splenocytes, besides serving as adjuvant in combating tumor development (Dalpke et al. 2002, Vollmer and Krieg 2009).

Human **TLR10** is expressed in kidney and lymph nodes, bone marrow and endothelial cells. A number of potential ligands has been proposed (Chuang and Ulevitch 2001, Verma et al. 2014). Its role in apoptosis induction by Gram-positive bacteria has been examined in 2013 (Mulla et al. 2013). TLR10 expression levels in human macrophages are upregulated upon confrontation with *Influenza virus*. Therefore, RNA-protein complexes have been implied as TLR10 activating PAMP (Lee et al. 2014). The murine Tlr10 gene has been inactivated by retroviral insertion and amplification of repeat regions disrupting the gene (Hasan et al. 2005).

The following receptors, not expressed by humans but by mice, TLR11, -12 and -13 have lately been assigned with ligands. **TLR11** has first been assumed to be incorporated within the cytoplasmic membrane, but multiple studies and Unc93B1-dependence indicate its localization in the endosomal membrane (Pifer et al. 2011). TLR11 is the only endosomal receptor recognizing a protein, namely the profilin-like protein of *Toxoplasma gondii*. **TLR11**− mice are highly susceptible to kidney infection by uropathogenic bacteria and *Salmonella typhimurium* and *S. typhi* infection (Zhang et al. 2004, Yarovinsky et al. 2005, Mathur et al. 2012). **TLR12** and TLR11 interact and form heterodimers following *T. gondii* profilin challenge (Andrade et al. 2013, Koblansky et al. 2013).
An orphan receptor status of TLR13 lasted until 2012 (Kawai and Akira 2011). It is located in the endosomal membrane and therefore has been supposed to sense nucleic acids. Our group and others identified TLR13 as sensor of a highly conserved sequence motif within bacterial 23S ribosomal RNA located within the peptidyl-transferase-loop, containing the catalytically active center of ribosomes (Hidmark et al. 2012, Li and Chen 2012, Oldenburg et al. 2012).

1.3 Immune stimulatory bacterial components

The innate immune system identifies pathogens through recognizing pathogen associated molecular patterns (PAMPs). These are highly conserved within groups of similar pathogens, but not by the host. Numerous components of the bacterial cell envelope activate TLRs.

1.3.1 Bacterial cell envelope components

Prokaryotes are faced with an unpredictable and often hostile environment such as the site of infection within a host. To survive, many bacteria have evolved a sophisticated and complex cell envelope protecting them, but allowing selective passage of nutrients from the outside and waste products as well as toxins and proteins from the inside.

In general, bacteria can be differentiated by the chemical and physical properties of their cell walls using Gram staining (Gram 1884). Gram-positive bacteria retain the crystal violet dye in their thick layer of peptidoglycan, whereas Gram-negative ones do not. However, not all bacteria can be classified by this technique. E.g., Mycobacteria stain Gram-positive although they have a distinct diderm-mycolate cell wall structure reminiscent of Gram-negative bacteria (Oertel et al. 2015, Sutcliffe 2010).

**Figure 2:** The Gram-positive and Gram-negative cell envelopes: CAP, covalently attached protein; IMP, integral membrane protein; LP, lipoprotein; LPS, lipopolysaccharide; LTA, lipoteichoic acid; OMP, outer membrane protein; WTA, wall teichoic acid.
Introduction

1.3.1.a The Gram-negative cell envelope

The Gram-negative bacterial cell envelope is built up of three principal layers (Fig. 2), namely the outer membrane (OM), the peptidoglycan cell wall and the cytoplasmic or inner membrane (IM). The OM and IM define an aqueous cellular compartment termed the periplasm (Mitchell 1961, Silhavy et al. 2010).

The OM is a distinctive feature of Gram-negative bacteria. It is a lipid bilayer containing phospholipids confined to the inner leaflet, while the outer leaflet is composed of glycolipids, dominated by lipopolysaccharide (LPS) (Kamio and Nikaido 1976). LPS challenge induces endotoxic shock associated with septicemia (Raetz and Whitfield 2002). The innate immune system is sensitized by LPS as robust indicator of infection. LPS is a glucosamine disaccharide carrying six or seven acyl chains, a polysaccharide core and an extended polysaccharide chain called O-antigen (Raetz and Whitfield 2002). Tightly packed LPS molecules forming a nonfluid continuum pose an effective barrier for hydrophobic molecules. Porins limiting diffusion of hydrophilic molecules larger than 700 Da, next to the LPS layer make the OM a very effective, yet selective, permeability barrier (Nikaido 2003).

Gram-negative peptidoglycan (PGN) is made up of repeating units of N-acetyl glucosamine-N-acetyl muramic acid, cross-linked by penta-peptide side chains (Vollmer 2008). The OM is connected to the underlying PGN by the lipoprotein Lpp, also called murein or Braun's lipoprotein. Lipids attached to its N-terminus embed Lpp into the OM. It is the most abundant protein in *E. coli* (Braun 1975).

The periplasm is densely packed with proteins and more viscous than the cytoplasm (Mullineaux et al. 2006, McNulty et al. 2006). Cellular compartmentalization allows Gram-negative bacteria to sequester potentially harmful degradatory enzymes such as RNase or alkaline phosphatase. Therefore, it has been called an evolutionary precursor of eukaryotic lysosomes (De Duve and Wattiaux 1966). It contains proteins involved in sugar and amino acid transport and chemotaxis as well as chaperone-like molecules involved in envelope biogenesis (Ehrmann 2007).

Unlike eukaryotes, bacteria lack intracellular organelles. Consequently, many membrane proteins involved in energy production, lipid biosynthesis, protein secretion and transport are localized in the IM (Silhavy et al. 2010). It is a phospholipid bilayer. In *E. coli*, it is constituted of phosphatidyl ethanolamine and phosphatidyl glycerol as well as lesser amounts of phosphatidyl serine and cardiolipin (Raetz and Dowhan 1990).

Gram-negative trans-envelope protein complexes are made up of protein components spanning the peptidoglycan layer. These include surface appendages such as flagella, required for bacterial motility (DePamphilis and Adler 1971, Macnab 2003); type III secretion systems, injecting toxins into host cells during infection (Kubori et al. 1998); and efflux pumps, excreting toxic molecules, e.g., antibiotics, into the surrounding medium. Efflux pumps also mediate antibiotic resistance in pathogenic bacteria (Koronakis et al. 2000, Eswaran et al. 2004, Murakami et al. 2006, Symmons et al. 2009).
1.3.1. b The Gram-positive cell envelope

Gram-positive bacteria, not possessing an outer membrane, are surrounded by layers of PGN, substantially thicker than that of Gram-negative bacteria, to withstand the turgor pressure exerted on the plasma membrane. Long anionic polymers, called teichoic acids, thread through the PGN (Fig. 2).

Gram-positive peptidoglycan, 30-100 nm thick, contains multiple layers, whereas Gram-negative PGN is only a few nanometers thick (Silhavy et al. 2010). Peptides cross-link glycan strands in Gram-positive PGN (Vollmer et al. 2008, Vollmer 2008). These branched stem peptides serve as attachment sites for covalently-attached proteins like transpeptidases and have been implicated in beta lactam resistance (Chambers 2003). Beta lactam antibiotics inhibit PGN synthesis through binding and inactivating transpeptidases (Waxman and Strominger 1983).

Adhesins are surface proteins of Gram-positive bacteria recognizing components of the host extracellular matrix such as fibronectin, fibrinogen and elastin (Clarke and Foster 2006). They enable adherence to host tissues crucial for effective colonization. Adhesins are either attached to PGN or teichoic acids via non-covalent ionic interactions, or attached covalently to stem peptides within PGN (Dramsi et al. 2008, Sjöquist et al. 1972, Fischetti et al. 1990). Proteins destined for covalent surface display contain an N-terminal signal sequence enabling export through the plasma membrane and a C-terminal pentapeptide cell wall sorting motif (LPXTG) (DeDent et al. 2008). The latter is processed mainly by sortase A. Covalently attached surface proteins are involved in immune evasion, internalization and phage binding as well as iron acquisition (Silhavy et al. 2010).

Teichoic acids are anionic cell surface polymers. Wall teichoic acids (WTAs) are covalently attached to PGN and lipoteichoic acids (LTAs) are anchored to plasma membrane lipids (Neuhaus and Baddiley 2003). Thus, the latter extend from the membrane into PGN layers. Teichoic acids, accounting for a significant fraction of the cell wall, have various species-dependent functions. Due to their anionic charge they bind cations, thus contributing to cation homeostasis (Marquis et al. 1976). Networks of metal cations between WTAs influence rigidity and porosity of the cell wall. Hence, WTAs are involved in scaffolding as well as cell wall synthesis and degradation. The net anionic charge of WTAs is modulated by esterification with D-alanine moieties. For example, S. aureus strains lacking D-alanine esters are more susceptible to cationic antimicrobial peptides and lytic enzymes produced by host neutrophils (Collins et al. 2002, Peschel et al. 1999, 2000).

1.3.2 Bacterial nucleic acids

Nucleic acids are essential for all forms of life. The two kinds of nucleic acids, DNA and RNA, encode genetic information and mediate its expression through transcription and translation. DNA and RNA are built up of nucleotides, each composed of a nucleobase, a sugar moiety and at least one phosphate group. Adenine, guanine (purines) as well as cytosine and uracil (pyrimidines) are the main nucleobases found in RNA, while DNA contains thymine instead of uracil. The different types of cellular RNA are messenger (m)RNA, ribosomal (r)RNA, transfer (t)RNA and further small noncoding or regulatory RNAs like miRNA. They are single stranded (ss), or form double stranded (ds) secondary and tertiary structures.
Introduction

Prokaryotic 70S ribosomes consist of two subunits, the large 50S ribosomal subunit containing 23S and 5S rRNA, and the small 30S ribosomal subunit containing the 16S rRNA, as well as ribosomal proteins. Most bacteria have multiple rrr operons encoding the rRNAs as well as ribosomal proteins (Evguenieva-Hackenberg 2005). 30S rRNA, a precursor transcript is processed into 16S and pre-23S rRNA by RNase III, a ds specific endoribonuclease (Allas et al. 2003).

Fragmentation of bacterial rRNA and removal of rRNA segments during rRNA maturation has been reported. Three fragmentation sites at the 5'-terminus of 16S rRNA and five in the 5'-half of 23S rRNA have been identified (Noller 1984, Springer et al. 1993). Fragmentation of rRNA does not affect ribosomal function (Evguenieva-Hackenberg 2005). But, it creates monophosphorylated 5' and 3'-end targets for degradation by ribonucleases like RNase E and RNase R, respectively, thereby enhancing the efficiency of rRNA decay and turnover of degradation products (Mackie 1998, Bessarab et al. 1998, Cheng and Deutscher 2003). Regulation of ribosome concentration provides a selective advantage to bacteria during altering growth phases and adaptation to changing environmental conditions (Klein et al. 2002, Cheng and Deutscher 2003).

Bacterial nucleic acids constitute an important group of PAMPs that, depending on their subcellular localization, are sensed by either endosomal or cytoplasmic receptors (Dixit and Kagan 2013, Wu and Chen 2014). All nucleic acid sensing TLRs utilize intracellular trafficking via Unc93B1 and are activated by pH-dependent processing in the endosomal lumen (Tabeta et al. 2006). Transfection of bacterial but not eukaryotic total RNA has been demonstrated to induce IL-12 release in human monocyte-derived DC precursors (Koski et al. 2004), representing one of the first implications of bacterial RNA as immune stimulatory PAMP. Human PBMCs and murine bone marrow derived macrophages (BMMs) respond to transfection with total bacterial RNA with TNF production (Deshmukh et al. 2011, Eberle et al. 2009, Kariko et al. 2005, Mancuso et al. 2009). Next to NF-kB dependent induction of cytokines, bacterial RNA has been reported as IRF-dependent trigger of IFNα in plasmacytoid (p)DCs and IFNβ in myeloid (m)DCs (Eberle et al. 2009, Mancuso et al. 2009, Cervantes et al. 2013, Gehrig et al. 2012, Gratz et al. 2011, Jöckel et al. 2012). This implicates a differentially regulated and cell type specific recognition of bacterial RNA.

Selective discrimination between host (self) and bacterial (foreign) RNA was suggested to involve subcellular compartmentalization, sequence composition, secondary/tertiary structures, and specific nucleotide modifications (Koski et al. 2004). Approximately 100 RNA modifications at either the nucleobase or the ribose have been identified that are introduced post-transcriptionally (Motorin and Helm 2011). In general, prokaryotic RNA is modified less abundantly than eukaryotic RNA. Known RNA modifications include methylation, thiolation and incorporation of pseudouridine (Eigenbrod and Dalpke 2015). Bacterial mRNAs are absent of or have relatively short 3'-poly(A) tails (14-60 nt) compared to eukaryotes (80-200 nt) (Sarkar 1997). Immune activation inversely correlates with the number of modified nucleotides in RNA. Highly modified eukaryotic RNA and broadly modified bacterial tRNA do not or induce less TNF release in human monocyte derived DCs as compared to bacterial total RNA (Kariko et al. 2005).
1.4 Pathogenesis of sepsis

Sepsis is not a disease but a syndrome, which was defined in 1991 as a documented or suspected infection combined with two or more abnormalities in body temperature (<36, >38°C), heart rate (>90/min), respiratory rate (tachypnea >20/min) or leukocyte count (<4,000/mm³, >12,000/mm³) (Bone et al. 1992, Vincent et al. 2013). Moreover, patients with additional organ dysfunction are termed "severe septic" while those with cardiovascular dysfunction not responsive to fluids and inadequate tissue perfusion are in "septic shock". Sepsis most often occurs in immuno suppressed patients. The outcome of sepsis treatment – survival or succumbing – is yet poorly understood (Angus and van der Poll 2013).

Initially, the focus of understanding and fighting sepsis pathogenesis lay in controlling pathogens of spreading in the host. Later on, the perspective shifted to the immune system. Macrophage activation and release of TNF and other mediators have been implicated in pathogenesis of acute septic shock (Deutschman and Tracey 2014). Bacterial infections are a major cause of septic shock, with Gram-positive or polymicrobial infections accounting for 30-50% and 25% of cases, respectively (Martin et al. 2003, Annane 2005). Bacterial PAMPs and toxins are recognized by PRRs mainly in mononuclear cells. TLR signaling activates transcription factors like NF-κB resulting in induction of proinflammatory response triggering genes, such as TNFα, IL-6, IL-1 and IL-12. TLR-activation triggers the innate immune system to eradicate invading microbes, but those cytokines also affect endothelial cells, e.g., by reducing synthesis of anticoagulation factors. Production of anti-inflammatory agents like IL-10 and cortisol is induced as compensatory response (Antonopoulou and Giamarellos-Bourboulis 2011).

Both pro- and anti-inflammatory reactions determine the course of septic shock, resulting in systemic vasodilation (hypotension), diminished heart rate, endothelial injury and activation causing leukocyte adhesion and alveolar lung damage, as well as activation of the coagulation system (Deutschman and Tracey 2014). It can harm tissue and organ function and may lead to death if untreated. Thus, early diagnosis and treatment of sepsis are of immense importance. Its treatment is nonspecific and limited mostly to organ function support, intravenous fluid, antibiotic and oxygen administration (Angus and van der Poll 2013, Bone et al. 1992). There is no approved therapy specifically targeting sepsis but an immense necessity for effective therapeutic approaches. Antibiotic resistance formation via genomic mutations or horizontal and vertical gene transfer impedes antibiotic treatment of bacterially induced sepsis (Giedraitienė et al. 2011).

1.5 Bacterial killing activity of macrophages

Macrophages belong to the first line of defense against invading pathogens (Houghton et al. 2009). Their ability to phagocytose and clear microorganisms has been documented (Gree and Kass 1964, Gordon 2007). Macrophages possess several effector molecules utilized to kill bacteria, including reactive oxygen species, nitric oxide, and antimicrobial proteins (Shiloh et al. 1999, Ganz 1999, Selsted and Ouellette 1995, Biggar and Sturgess 1977, del Cerro-Vadillo et al. 2006, Hiemstra et al. 1999,
1.5.1 Reactive oxygen species

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen. They include peroxides, superoxide (\(\cdot O_2^-\)), hydroxyl radicals (\(\cdot OH\)) and singlet oxygen (\(O_2^*\)). ROS are formed as byproducts of oxygen metabolism being important for cell signaling and homeostasis. However, during environmental stress (e.g., UV or heat) ROS levels increase. This may result in cell structure damage, termed oxidative stress (Devasagayam et al. 2004). Endogenous ROS are produced intracellularly in a cell type and tissue specific manner. NADPH oxidase (NOX) complexes in cell membranes, mitochondria, peroxisomes, and the endoplasmic reticulum are major sources of ROS (Muller 2000, Han et al. 2001). Mitochondria convert energy into ATP via oxidative phosphorylation involving transport of protons across the inner mitochondrial membrane. Oxygen molecules are normally reduced along the electron transport chain. However, in rare cases oxygen is instead prematurely and incompletely reduced to a superoxide radical (Li et al. 2013). If too much damage is caused in mitochondria, a cell undergoes apoptosis. Bcl-2 proteins are layered on the surface of mitochondria and detect damage, activating Bax proteins to perforate the mitochondrial membrane, causing cytochrome C to leak out into the cytoplasm. Cytochrome C binds to Apaf-1 (apoptotic protease activating factor-1), forming apoptosomes. These bind and activate caspase-9, which then cleaves mitochondrial membrane proteins, causing mitochondrial disintegration and initiating apoptosis (Czabotar et al. 2014).

Effects of ROS also include the induction of host defense genes. In particular, platelets release ROS to recruit additional platelets to sites of injury during wound repair. ROS are also implicated in cellular activity to a variety of inflammatory responses including cardiovascular disease. ROS is induced as an antimicrobial defense effector mechanism. Individuals having deficiencies in ROS generation, as in chronic granulomatous disease, are highly susceptible to infection by Salmonella enterica and Staphylococcus aureus. The exact mechanism underlying ROS driven defense of the host towards invading pathogens is unclear. In general, harmful effects of reactive oxygen species include DNA damage, lipid peroxidation, oxidation of amino acids in proteins, and deactivation of specific enzymes by cofactor oxidation (Rada and Leto 2008, Conner et al. 2002). Studies have shown that Salmonella require DNA repair mechanisms to withstand killing by ROS (Fang 2011, Slauch 2011). More recently, a role for ROS in antiviral defense has been implicated for RIG-I and MAVS (Jacobs and Coyne 2013). Increased levels of ROS induce signaling through MAVS to activate IRF-3, -7, and NF-\(\kappa\)B (West et al. 2011). Respiratory epithelial cells have been demonstrated to induce mitochondrial ROS in response to Influenza virus infection, leading to type III IFN induction (Kim et al. 2013). ROS are also involved in host defense against Mycobacteria, although ROS rather affect signaling, such as toward cytokine production, autophagy, and granuloma formation instead of direct killing (Deffert et al. 2014).

1.5.2 Nitric oxide

Phagocytes produce and secrete nitric oxide (NO) radicals as part of the immune response (Green et al. 1990b). The inducible nitric oxide synthase (iNOS) is activated by IFN-\(\gamma\) or TNF upon infection
Aims of this work

Infection with pathogenic bacteria poses a major global cause of death. Antibiotic therapy kills invading bacteria upon which immune stimulatory PAMPs like LPS, lipoproteins and nucleic acids are released into the bloodstream. These are recognized by PRRs and may cause an overamplified immune reaction causing multi-organ failure associated to a high mortality rate. Antibiotic therapy combined with blockade of both TLR2 and TLR4 in Gram-negative bacterial infection has been shown to prevent development of sepsis in mice (Spiller et al. 2008). Gram-positive bacteria are aggressive sepsis causing pathogens. Their recognition by specific PRRs however, required further analysis. Former studies in the lab showed MyD88-dependent yet IL-1 receptor 1 (IL1R1) independent recognition of S. aureus, implicating merely TLRs as Gram-positive bacteria receptor candidate molecules (Ferstl 2009).

The aim of the first part of this thesis was to narrow down the receptors and effector-mechanism(s) and/or -molecules activated by murine macrophages in response to Gram-positive bacterial infection in order to sense and clear the bacteria from their supernatants, respectively. A method of infecting macrophages with viable Gram-positive bacteria needed be established. Supernatants were analyzed for bacterial survival, cytokine and NO content upon short term infection. This allowed the analyses of the involvement of PRRs as well as the bacterial killing mechanisms utilized by murine bone marrow derived macrophages (BMMs) from specific TLR knockout mice.

Since TLR2 has been considered as a major receptor for lipoproteins, we focused on analyzing the role of bacterial RNA recognition. Specifically, we analyzed the interaction of murine TLR13 with bacteria derived ssRNA aiming at identification of a consensus ligand motif. Molecular subfractions of bacterial total RNA needed to be purified and individually analyzed for immune stimulatory potential. Further on, the respective RNA subspecies was analyzed to attain more detailed insight of the receptor ligand interaction. Confirmation of the latter had to be investigated by gain-and-loss of TLR13 function analyses.

Subsequently, it was unclear if humans lacking TLR13 expression express an equivalent receptor to sense bacterial ssRNA and if yes which PRR could partake this role. Human PBMCs had to be analyzed for their responsiveness to the TLR13 stimulatory ligand and derivatized RNA ligands. A responsive human cell line needed to be identified to further characterize the respective human receptor and its ligand. Further gain-and-loss of function analyses should verify the proposed receptor candidate molecule. Its ligand consensus motif was to be characterized and infection studies should point out its importance in immune activation upon recognition of viable Gram-positive and -negative bacteria.
2 Material and Methods

2.1 Materials

2.1.1 Equipment

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2.1.2 Kits and enzymes

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<tr>
<td>OptEIA TMB Substrate Reagent Set</td>
<td>substrate for ELISA detection</td>
<td>BD Biosciences Wizard Plus</td>
</tr>
<tr>
<td>PCR purification kit</td>
<td>PCR fragment purification</td>
<td>Stratec</td>
</tr>
<tr>
<td>peqGOLD Plasmid Miniprep Kit I</td>
<td>plasmid preparation</td>
<td>peqLab</td>
</tr>
</tbody>
</table>
Material

QIAquick Gel Extraction  DNA purification from agarose gel  Qiagen
RNeasy Mini Kit  preparation of RNA  Qiagen
SV Minipreps DNA Purification Systems  plasmid preparation  Promega
ZymocleanTM Gel RNA Recovery Kit  RNA agarose purification  Zymo Research
ZR small-RNATM PAGE Recovery Kit  RNA PAGE purification  Zymo Research
DNasel, RNase-free  specific degradation of DNA  Sigma Aldrich
HRP  horse radish peroxidase  R&D
M-MuLV  reverse transcriptase  New England Biolabs
Plusion HF polymerase  DNA polymerization  New England Biolabs
Proteinase K  broad-spectrum serine protease  Qiagen
Restriction enzymes (fast digest)  specific DNA cutting  Thermo Fisher Scientific
RNA 5'-Polyphosphatase  removal of 5'-tri-phosphate from RNA  epicentre (Illumina)
RNaseA  specific degradation of ssRNA  Sigma Aldrich
RNase III  specific degradation of dsRNA  New England Biolabs
 TerminatorTM-5'-Phosphate-Dependent Exonuclease  digestion of 5'-monophosphorylated RNA (rRNA removal)  epicentre (Illumina)

2.1.3 Oligonucleotides, primers & vectors

<table>
<thead>
<tr>
<th>Oligonucleotide / Primer</th>
<th>Sequence (sense 5’-3’) / ID</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biot3-23S</td>
<td>biotin-AAGGTTAACCCCTCAGGGTTTATAGTACCGGTT</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>pJET1.2 forward sequencing primer</td>
<td>5’-CGACTCACTATAGGGAGAGCGGC-3’</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>pJET1.2 reverse sequencing primer</td>
<td>5’-AAGAACATCGATTTTCCATGGCAG-3’</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>F_pET24a+/ERMC</td>
<td>5’-CGAATTCCAGCTCCGTCGACAAATGAACGAGGAGAGC-3’</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>R_pET24a+/ERMC</td>
<td>5’-TCGAGTGGCCGCCGATATTAAATATTTATAGCTATTGAAAAGAGATAA-3’</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>murine β-actin</td>
<td>Mm_Actb_1 SG QuantiTect Primer Assay</td>
<td>Qiagen</td>
</tr>
<tr>
<td></td>
<td>Mm_Actb_2 SG QuantiTect Primer Assay</td>
<td>Qiagen</td>
</tr>
<tr>
<td>human GAPDH</td>
<td>Hs_GAPDH_1 SG QuantiTect Primer Assay</td>
<td>Qiagen</td>
</tr>
<tr>
<td>murine/human MapK1</td>
<td>Hs_MAPK1_1 SG QuantiTect Primer Assay</td>
<td>Qiagen</td>
</tr>
<tr>
<td>murine Tlr13</td>
<td>Mm_TLR13_1 SG QuantiTect Primer Assay</td>
<td>Qiagen</td>
</tr>
<tr>
<td>human Th7 sense</td>
<td>5’-CCACAACCACTGACCACTG-3’</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>human Th7 antisense</td>
<td>5’-CCACCAGACACACACACACAG-3’</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>18S rRNA sense</td>
<td>5’-GTAACCCTGGTAGACAACCACACAT-3’</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>18S rRNA antisense</td>
<td>5’-CCATCCAATCGGTAGTGACCG-3’</td>
<td>MWG Eurofins</td>
</tr>
</tbody>
</table>
## Material and Methods

<table>
<thead>
<tr>
<th>Vector</th>
<th>Resistance</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJET1.2</td>
<td>ampicillin</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>pET-24a(+)</td>
<td>kanamycin</td>
<td>Novagen</td>
</tr>
<tr>
<td>pNF8</td>
<td>erythromycin</td>
<td>(Fortinea et al. 2000)</td>
</tr>
<tr>
<td>pAT18</td>
<td>erythromycin</td>
<td>(Trieu-Cuot et al. 1991)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>siRNA</th>
<th>ID</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR13</td>
<td>SI01449518</td>
<td>Qiagen</td>
</tr>
<tr>
<td>MapK1</td>
<td>0001022564</td>
<td>Qiagen</td>
</tr>
<tr>
<td>TLR7</td>
<td>SI02642402</td>
<td>Qiagen</td>
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### 2.1.4 Stimulants & Inhibitors

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Description</th>
<th>Receptor</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL075 (3M002)</td>
<td>thiazoloquinolone derivative</td>
<td>TLR7 &amp; TLR8</td>
<td>Invivogen</td>
</tr>
<tr>
<td>CpG-DNA (1668)</td>
<td>synthetic ODN containing unmethylated CpG dinucleotides</td>
<td>TLR9</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>Flagellin</td>
<td>isolated from bacteria</td>
<td>TLR5</td>
<td>Invivogen</td>
</tr>
<tr>
<td>Lipopolysaccharide, <em>E. coli</em> 0111:B4</td>
<td>isolated from bacteria</td>
<td>TLR4 (&amp; TLR2)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Loxoribine</td>
<td>guanosine analog derivatized at N7 and C8</td>
<td>TLR7 &amp; TLR8</td>
<td>Invivogen</td>
</tr>
<tr>
<td>Pam2CSK4</td>
<td>synthetic diacylated lipopeptide</td>
<td>TLR2/TLR6</td>
<td>Invivogen</td>
</tr>
<tr>
<td>Pam3CSK4</td>
<td>synthetic triacylated lipopeptide</td>
<td>TLR2/TLR1</td>
<td>Invivogen</td>
</tr>
<tr>
<td>Polynosinic:Polyctidylic acid (poly I-C) LMW</td>
<td>synthetic dsRNA analog (average size 0.2-1 kb)</td>
<td>TLR3, MDA5</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Polynosinic:Polyctidylic acid (poly I-C) HMW</td>
<td>synthetic dsRNA analog (average size 1.5-8 kb)</td>
<td>TLR3, MDA5</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Resiquimod (R848)</td>
<td>imidazoquinoline compound</td>
<td>TLR8, TLR7</td>
<td>Invivogen</td>
</tr>
<tr>
<td>ssRNA40/LyoVec</td>
<td>20-mer phosphorothioate protected ss ORN</td>
<td>TLR8, TLR7</td>
<td>Invivogen</td>
</tr>
<tr>
<td>Uridine</td>
<td>nucleoside</td>
<td>TLR8</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Zymosan</td>
<td>isolated from yeast</td>
<td>Dectin-1 &amp; TLR2</td>
<td>Invivogen</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Description</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>79i</td>
<td>TLR7 and TLR9 inhibitory ODN</td>
<td>TIB MolBiol</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>endosomal inhibitor (prevents endosomal acidification)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Cytochalasin D</td>
<td>phagosomal inhibitor (prevents actin polymerization)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>T2.5</td>
<td>TLR2 blocking monoclonal antibody (species: mouse)</td>
<td>Dynavax</td>
</tr>
<tr>
<td>Transfection Reagent</td>
<td>Description</td>
<td>Supplier</td>
</tr>
<tr>
<td>----------------------</td>
<td>-------------</td>
<td>----------</td>
</tr>
<tr>
<td>DOTAP Liposomal Transfection Reagent</td>
<td>cationic liposome-forming compound for transfection of negatively charged molecules</td>
<td>Roche</td>
</tr>
<tr>
<td>Lipofectamine® 2000 Transfection Reagent</td>
<td>formulation for transfection of nucleic acids</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Lipofectamine® RNAiMAX Transfection Reagent</td>
<td>formulation for delivery of siRNA</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>LyoVec™</td>
<td>cationic phospholipid-based transfection reagent</td>
<td>Invivogen</td>
</tr>
<tr>
<td>poly-L-arginine (pLA)</td>
<td>cationic liposome-forming polymer</td>
<td>Sigma Aldrich</td>
</tr>
</tbody>
</table>

### 2.1.5 Media and culture additives

<table>
<thead>
<tr>
<th>Medium / culture additive</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Antibiotic-Antimycotic 100X solution (10,000 u/ml of penicillin, 10,000 µg/ml of streptomycin, 25 µg/ml of amphotericin B)</td>
<td>Gibco</td>
</tr>
<tr>
<td>Beta-mercaptoethanol</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>BHI (brain heart infusion)</td>
<td>Roth</td>
</tr>
<tr>
<td>Columbia-agar (5% sheep blood)</td>
<td>Oxoid</td>
</tr>
<tr>
<td>DMEM (Dulbecco's Modified Eagle Medium)</td>
<td>Gibco</td>
</tr>
<tr>
<td>Fetal calf serum (FCS)</td>
<td>Gibco</td>
</tr>
<tr>
<td>G418 Geneticin (Neomycin resistance gene neo)</td>
<td>PAA</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Roth</td>
</tr>
<tr>
<td>Glycine</td>
<td>Roth</td>
</tr>
<tr>
<td>GM-CSF containing cell culture supernatant</td>
<td>own production</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>Stratagene</td>
</tr>
<tr>
<td>LB (Luria Bertani)</td>
<td>BD</td>
</tr>
<tr>
<td>NAD</td>
<td>Roth</td>
</tr>
<tr>
<td>Opti-MEM® Reduced Serum Medium</td>
<td>Gibco</td>
</tr>
<tr>
<td>PBS, Dulbecco, sterile</td>
<td>Gibco</td>
</tr>
<tr>
<td>Penicillin-Streptomycin solution (10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin)</td>
<td>Gibco</td>
</tr>
<tr>
<td>Phorbol 12-myristate 13-acetate (PMA)</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>RPMI-1640 (Roswell Park Memorial Institute medium)</td>
<td>Gibco</td>
</tr>
<tr>
<td>StemPro® Accutase® Cell Dissociation Reagent</td>
<td>Gibco</td>
</tr>
<tr>
<td>0.5% Trypsin-EDTA (10X)</td>
<td>Gibco</td>
</tr>
</tbody>
</table>
Material and Methods

2.1.6 Chemicals and solutions

All further chemicals and solutions used in this work were obtained from the companies Carl Roth GmbH (Karlsruhe), Sigma Aldrich Chemie GmbH (Steinheim), Thermo Fisher Scientific Inc. (Schwerte), PJK GmbH (Kleinblittersdorf), Promega (Mannheim), BD (Heidelberg), VWR (Darmstadt), Peptidech (Hamburg), Merck (Darmstadt), Invitrogen (Schwerte), Biochrom (Darmstadt), Qiagen (Hilden), R&D Systems (Abingdon, UK), Peqlab (Erlangen).

<table>
<thead>
<tr>
<th>Solution / Reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Coelenterazin</td>
<td>PJK</td>
</tr>
<tr>
<td>Coenzyme A</td>
<td>PJK</td>
</tr>
<tr>
<td>Deoxyribonucleotides (dNTP)</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>D-Luciferin</td>
<td>PJK</td>
</tr>
<tr>
<td>Disodium EDTA (Na₂EDTA x 2H₂O)</td>
<td>Sigma</td>
</tr>
<tr>
<td>FACS clean</td>
<td>BD</td>
</tr>
<tr>
<td>Interferon γ, recombinant human</td>
<td>Peprotech</td>
</tr>
<tr>
<td>Interferon γ, recombinant murine</td>
<td>Peprotech</td>
</tr>
<tr>
<td>Lucigenin</td>
<td>Enzo Life Science</td>
</tr>
<tr>
<td>Oligo (dT) 18 primer</td>
<td>fermentas</td>
</tr>
<tr>
<td>PBS, Dulbecco, powder</td>
<td>Biochrom</td>
</tr>
<tr>
<td>RNase Away Spray</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>RNase Inhibitor, RiboLock</td>
<td>Thermo Fisher (Fermentas)</td>
</tr>
<tr>
<td>Roti-Aqua-Phenol</td>
<td>Roth</td>
</tr>
<tr>
<td>Roti Store Cryo tubes</td>
<td>Roth</td>
</tr>
<tr>
<td>Rotiphores® NF-Acrylamid/Bis-Lösung 30% (29:1)</td>
<td>Roth</td>
</tr>
<tr>
<td>Rotiphores® NF-Acrylamid/Bis-Lösung 40% (29:1)</td>
<td>Roth</td>
</tr>
<tr>
<td>Streptavidin-HRP (horse raddish peroxidase)</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>SYBR® Safe DNA Gel Stain</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>TMB ELISA Substrate Reagent Set</td>
<td>BD</td>
</tr>
<tr>
<td>Tri Reagent</td>
<td>Sigma</td>
</tr>
<tr>
<td>TriFast, peqGold</td>
<td>Peqlab</td>
</tr>
</tbody>
</table>

2.1.7 Buffers and reagents

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA loading dye (5X)</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>DNA ladder 1 kb</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Reporter Lysis Buffer (5X)</td>
<td>Promega</td>
</tr>
<tr>
<td>RiboRuler High Range RNA Ladder</td>
<td>Thermo Scientific</td>
</tr>
</tbody>
</table>
### 2.1.8 Bacterial strains

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Strain</th>
<th>ID</th>
<th>Growth medium</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>168</td>
<td>DSMZ 402</td>
<td>BHI</td>
<td>(Spizizen 1958)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>clinical isolate</td>
<td>-</td>
<td>LB</td>
<td>(Spiller et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>BL21</td>
<td>-</td>
<td>LB</td>
<td>NEB, (Studier and Moffatt 1986)</td>
</tr>
<tr>
<td></td>
<td>XL.10 Gold</td>
<td>-</td>
<td>LB</td>
<td>Stratagene</td>
</tr>
<tr>
<td></td>
<td>DH5a</td>
<td>-</td>
<td>LB</td>
<td>(Hanahan 1985)</td>
</tr>
<tr>
<td></td>
<td>EHEC O104:H4</td>
<td>-</td>
<td>LB</td>
<td>clinical isolate, UKE, (Oldenburg et al. 2012)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>533 R4</td>
<td>DSMZ 20231</td>
<td>BHI</td>
<td>(Schleifer and Kocur 1973)</td>
</tr>
<tr>
<td>MRSA</td>
<td>-</td>
<td>BHI</td>
<td></td>
<td>clinical isolates, UKE, (Oldenburg et al. 2012)</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>D39</td>
<td>serotype 2</td>
<td>BHI</td>
<td>(Noske et al. 2009)</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>SF 130</td>
<td>DSMZ 20565</td>
<td>BHI</td>
<td>(Skerman et al. 1989)</td>
</tr>
</tbody>
</table>

### 2.1.9 Cell lines & primary cells

Raw264.7 (murine, ATCC: TIB-71), HEK293 (human, ATCC: CRL-1573)

Table 2: List of human cell lines tested:

<table>
<thead>
<tr>
<th>Cell</th>
<th>Phenotype</th>
<th>Cell type/line</th>
<th>Cell type/line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ma-Mel-65</td>
<td>adherent</td>
<td>melanoma</td>
<td>provided by A. Paschen (UKE)</td>
</tr>
<tr>
<td>Ma-Mel-119</td>
<td>adherent</td>
<td>melanoma</td>
<td>provided by A. Paschen (UKE)</td>
</tr>
<tr>
<td>Ma-Mel-47</td>
<td>adherent</td>
<td>melanoma</td>
<td>(Heinemann et al. 2012)</td>
</tr>
<tr>
<td>K562</td>
<td>suspension</td>
<td>erythromyeloblastoid leukemia</td>
<td>ATCC: CCL-243</td>
</tr>
<tr>
<td>HepG2</td>
<td>adherent</td>
<td>hepatocellular liver carcinoma</td>
<td>ATCC: HB-8065</td>
</tr>
<tr>
<td>HEL</td>
<td>suspension</td>
<td>erythroid leukemia</td>
<td>DSMZ No. ACC 11/ATCC TIB-180</td>
</tr>
<tr>
<td>Chang Liver</td>
<td>adherent</td>
<td>derived from HeLa</td>
<td>ATCC: CCL-13</td>
</tr>
<tr>
<td>Jurkat</td>
<td>suspension</td>
<td>T cell lymphoblast-like</td>
<td>ATCC: TIB-152</td>
</tr>
<tr>
<td>UM22b</td>
<td>adherent</td>
<td>head and neck squamous cell carcinoma</td>
<td>provided by S. Brandau (UKE)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ajay Verma (Merck &amp; Co., North Wales, PA)</td>
</tr>
<tr>
<td>FaDu</td>
<td>adherent</td>
<td>hypopharyngeal carcinoma</td>
<td>ATCC: HTB-43</td>
</tr>
<tr>
<td>DLD-1</td>
<td>adherent</td>
<td>colon adenocarcinoma</td>
<td>ATCC: CCL-221</td>
</tr>
<tr>
<td>MonoMac 6</td>
<td>suspension</td>
<td>acute monocytic leukemia derived</td>
<td>DSMZ No. ACC124</td>
</tr>
<tr>
<td>5637</td>
<td>adherent</td>
<td>bladder carcinoma</td>
<td>ATCC: HTB-9</td>
</tr>
</tbody>
</table>
Material and Methods

<table>
<thead>
<tr>
<th>Cell</th>
<th>Phenotype</th>
<th>Cell type/line</th>
<th>Cell type/line</th>
</tr>
</thead>
<tbody>
<tr>
<td>KG1a</td>
<td>suspension</td>
<td>acute myeloid leukemia</td>
<td>ATCC: CCL-246.1</td>
</tr>
<tr>
<td>KG1</td>
<td>suspension</td>
<td>acute myeloid leukemia</td>
<td>ATCC: CCL-246</td>
</tr>
<tr>
<td>THP-1</td>
<td>suspension</td>
<td>acute monocyctic leukemia</td>
<td>ATCC: TIB-202</td>
</tr>
<tr>
<td>U937</td>
<td>suspension</td>
<td>leukemic monocyte lymphoma</td>
<td>ATCC: CRL-1593.2</td>
</tr>
<tr>
<td>Fibroblast C3</td>
<td>adherent</td>
<td>transformation with an origin-defective mutant of SV-40</td>
<td>(Picard et al. 2011)</td>
</tr>
<tr>
<td>Fibroblast Tirap^-</td>
<td>adherent</td>
<td>transformation with an origin-defective mutant of SV-40</td>
<td>(Picard et al. 2011)</td>
</tr>
<tr>
<td>B-cells</td>
<td>suspension</td>
<td>mortilized with Epstein-Barr virus (EBV)</td>
<td>(Picard et al. 2011)</td>
</tr>
<tr>
<td>Huh-7</td>
<td>adherent</td>
<td>Differentiated hepatocyte derived cellu-</td>
<td>Cell Bank: JCRB0403J</td>
</tr>
<tr>
<td></td>
<td></td>
<td>lar carcinoma</td>
<td></td>
</tr>
</tbody>
</table>

Primary murine bone marrow derived macrophages (BMMs) and human peripheral blood mononuclear cells (PBMCs) as well as human whole blood preparations were prepared as described on page 24.

2.1.10 Software & analysis tools

List of software and tools, application, supplier

<table>
<thead>
<tr>
<th>Software/tool</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene5</td>
<td>Bio-Tek</td>
</tr>
<tr>
<td>Prism</td>
<td>GraphPad</td>
</tr>
<tr>
<td>Illustrator, Photoshop, Acrobat</td>
<td>Adobe</td>
</tr>
<tr>
<td>Simplicity 4.2</td>
<td>Berthold</td>
</tr>
</tbody>
</table>

2.2 Methods

2.2.1 Cell biology

2.2.1.a Culture of cell lines

Cultivation of the following cell lines was performed at 37°C, 5% CO₂ and 95% humidity. Media and buffers were prewarmed. Adherent cells were detached from plastic surfaces using an appropriate method. After centrifugation (1000 rpm, RT, 5’) viable cells were counted using trypan blue solution in a Neubauer hemocytometer. For cryopreservation, cells were resuspended in FCS or freeze medium (autologous serum supplemented with 10% DMSO), aliquoted, frozen slowly at 1°C/minute and...
stored at -80°C or in liquid nitrogen for long-term purposes. Cells were thawed quickly at 37°C and transferred to prewarmed medium. After centrifugation they were resuspended and cultivated in fresh medium.

**I) RAW264.7**

Murine RAW264.7 macrophagoid cells were cultured in 150 mm tissue culture dishes to 60-70% confluence, for approximately 2-3 days. The semi-adherent cells were rinsed from the surface, centrifuged and resuspended in fresh RAW264.7 medium. 1/10 of the suspension was transferred to a new dish containing fresh medium.

**RAW264.7 medium**

500 ml RPMI 1640
50 ml FCS
5 ml Penicillin-Streptomycin solution (Gibco)
5 ml Antibiotic-Antimycotic 100X solution (Gibco)
50 µM β-mercaptoethanol

**II) THP-1**

THP-1 is a human monocytic cell line, derived from an acute leukemia patient. THP-1 cells were cultured in 175 cm² cell culture flasks. Cells were split when reaching 60-70% confluence by transfer of 2 ml cell suspension to fresh medium.

THP-1 monocytic cells were differentiated to macrophages with 200 nM phorbol 12-myristate 13-acetate (PMA) (1x10⁷ cells per 96-well plate or per well of 6-well plate) for either 24 h followed by 3 days culture in PMA-free medium (3ddi), or 72 h followed by 5 days culture in PMA-free medium (8ddi). Finally the cells were challenged in medium containing 2% FCS for 16 h.

**THP-1 medium**

500 ml RPMI 1640
50 ml FCS
5 ml Penicillin-Streptomycin solution (Gibco)
5 ml Antibiotic-Antimycotic 100X solution (Gibco)

For siRNA mediated knockdown of TLR7, THP-1 cells were transfected with the respective siRNA or scrambled variant (Qiagen) 48 h upon PMA differentiation. Lipofectamine RNAiMAX Transfection Reagent (Life Technologies) was used according to the manufacturer’s instructions. 24 h upon transfection cells were challenged in fresh medium. Supernatants were analyzed by ELISA. RNA was isolated and analyzed via RT–PCR.
Material and Methods

(III) HEK293

The human embryonic kidney cell line HEK293 was applied for ectopic protein overexpression and functional analysis. HEK293 cells were cultured as adherent monolayer. The cells were grown to 70% confluence, for passaging they were washed with PBS and detached in 5 ml (per 10 cm dish) of 1% (w/v) trypsin-EDTA (Gibco) for 5 min. Trypsin activity was inhibited by addition of 1 volume of medium, the cells were thoroughly resuspended. 1/10 of the suspension was transferred to a new dish with fresh medium.

HEK293 medium

500 ml DMEM 1640
50 ml FCS
5 ml Penicillin-Streptomycin solution (Gibco)
5 ml Antibiotic-Antimycotic 100X solution (Gibco)

2.2.1.b Preparation and culture of primary cells

(I) Murine bone marrow derived macrophages

For generation of bone marrow derived macrophages (BMM), mice were sacrificed by cervical dislocation. Their legs were removed, the bones separated. Bone marrow was flushed from femora, tibiae and humeri with medium using a syringe. After centrifugation, the cells were resuspended and seeded in 25 ml BMM differentiation medium, containing 15% L-cell supernatant (L-cell conditioned medium, LCCM) as source of M-CSF into 145 mm (uncoated) petri dishes. Bone marrow cells differentiated to macrophages within 6-8 days, with addition of 10 ml fresh BB differentiation medium after three days. Differentiated macrophages were washed with PBS before being detached with 5 ml accutase, a mixture of proteolytic and collagenolytic enzymes. Addition of 5 ml BMM medium neutralized the enzymatic activity. The BMM were rinsed from the surface, centrifuged (1100 rpm, RT, 5’), resuspended in fresh BMM medium, counted and plated (2x10^7 cells per well in 200 µl medium) to reach the desired density.

BMM medium

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 ml</td>
<td>DMEM</td>
</tr>
<tr>
<td>50 ml</td>
<td>FCS</td>
</tr>
<tr>
<td>5 ml</td>
<td>Penicillin-Streptomycin solution (Gibco)</td>
</tr>
<tr>
<td>5 ml</td>
<td>Antibiotic-Antimycotic 100X solution (Gibco)</td>
</tr>
<tr>
<td>50 µM</td>
<td>β-mercaptoethanol</td>
</tr>
</tbody>
</table>

BMM differentiation medium

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 ml</td>
<td>DMEM</td>
</tr>
<tr>
<td>50 ml</td>
<td>FCS</td>
</tr>
<tr>
<td>75 ml</td>
<td>L-cell supernatant</td>
</tr>
<tr>
<td>5 ml</td>
<td>Penicillin-Streptomycin solution (Gibco)</td>
</tr>
<tr>
<td>5 ml</td>
<td>Antibiotic-Antimycotic 100X solution (Gibco)</td>
</tr>
<tr>
<td>50 µM</td>
<td>β-mercaptoethanol</td>
</tr>
</tbody>
</table>
(II) Human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood of healthy donors (buffy coats provided by the blood bank of University Hospital Essen, approved by the ethics committee: 14-5804-B0) via differential centrifugation. For this, 12 ml of blood were layered on 40 ml Ficoll solution (GE Healthcare) and centrifuged (400 g, RT, 30', deceleration 0). Differential migration during centrifugation results in the formation of layers containing different cell types. The bottom layer containing erythrocytes that have been aggregated, the layer immediately above them containing mostly granulocytes. Because of their lower density, the lymphocytes are found at the interface between the plasma and the Ficoll solution, forming a visible whitish ring together with platelets and monocytes. The plasma (upper phase) was transferred to a fresh tube and heat inactivated (55°C, 30') to supplement the PBMC medium. The thin cell layer was aspirated carefully and washed twice in PBS. After pelleting (400 g, 30', deceleration 9) the cells were resuspended in PBMC medium, strained through a 100 µm mesh, counted and seeded (5x10^7 per 96-well plate) or frozen in PBMC freeze medium at -80°C.

<table>
<thead>
<tr>
<th>PBMC medium</th>
<th>PBMC freeze medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>autologous serum (hi)</td>
</tr>
<tr>
<td>5% autologous serum (hi)</td>
<td>10% DMSO</td>
</tr>
<tr>
<td>1% Penicillin-Streptomycin solution (Gibco)</td>
<td></td>
</tr>
<tr>
<td>1% Antibiotic-Antimycotic 100X solution (Gibco)</td>
<td></td>
</tr>
</tbody>
</table>

(III) Human whole blood

Whole blood was drawn into monovettes (Braun, 8 ml) prefilled with Bivalirudin (5.3 mg) and aliquoted in 200 µl portions in 96-well plates (Coch et al. 2013). After pretreatment and challenge for 24 h, blood cells were centrifuged (1500 rpm, RT, 30'), the serum was transferred to new 96-well plates and stored at -20°C for cytokine measurement.

2.2.2 Immunology

2.2.2.a Griess assay (nitric oxide)

The concentration of nitric oxide (NO) in supernatants of challenged cells was measured using the Griess-Reagent (Green et al. 1982). 50 µl (Griess Reagent A equally mixed with B) were added to 50 µl of sample. After incubation at RT for 10', the absorbance was measured in an ELISA reader at 540 nm, and analyzed using Gen5 software (Bio-Tek). The NO concentration of samples was calculated by comparing their absorbance to that of a range of standard concentrations of sodium nitrite (NaNO₂).


Material and Methods

Griess Reagent A

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-(1-Naphtyl) Ethylene diamide dihydrochloride</td>
<td>0.4 g</td>
</tr>
<tr>
<td>Sulphamilamide</td>
<td>4 g</td>
</tr>
<tr>
<td>H₃PO₄ (5%)</td>
<td>10 g</td>
</tr>
</tbody>
</table>

ad. 200 ml water

Griess reagents A and B were protected from light and stored at 4°C.

2.2.2.2 Enzyme linked immuno sorbent assay (ELISA)

Cytokine concentrations in cell culture supernatants or sera were analyzed by enzyme linked immunosorbent assay with enzyme-mediated colorimetry. All proteins were detected by DuoSet ELISA Development System (R&D Systems) or IFNα ELISA (eBioscience) following the manufacturer’s protocols. Briefly, ELISA plates were coated with capture Ab (diluted in PBS, 50 µl/well), shaking o/n at RT (over the weekend at 4°C). Plates were washed (ELISA-washer, 3x 300 µl PBT/well). Blocking buffer was applied to prevent unspecific binding to the plastic surface for 1h (150 µl/well, shaking at RT). Meanwhile the samples were thawed. The plates were washed. Samples and standard curve dilutions of the respective cytokine applied (5; 2.5; 1.25; 0.63; 0.31; 0.16; 0.08 ng recombinant cytokine; H₂O as blank; samples were diluted in RD if applicable). Plates were incubated shaking (65 rpm) for 90' at 37°C (or 2h at RT). Plates were washed. Biotin-coupled detection Ab was added (diluted in RD, 50 µl/well) followed by 90' of incubation at 37°C with shaking (or 2h at RT). Plates were washed. Streptavidin-coupled horse radish peroxidase (HRP) was added (1:200, RD, 50 µl/well, 5 µl Streptavidin in 1 ml RD). The plates were shaken for 20' at 37°C in the dark. After washing, freshly prepared TMB substrate (reagent A : reagent B equally mixed, BD) was applied to the plates (50 µl/well). The enzyme reaction was kept in the dark to develop (blue colored product). The reaction was stopped by H₂SO₄ (25 µl/well, color switch to yellow). Cytokine concentrations were measured in an ELISA reader at 450 nm, and analyzed using Gene5 software (supplier).

<table>
<thead>
<tr>
<th>Blocking buffer</th>
<th>Reagent Diluent (RD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 g Sucrose</td>
<td>10 g BSA</td>
</tr>
<tr>
<td>5 g BSA</td>
<td>ad. 1 l PBS</td>
</tr>
<tr>
<td>250 mg NaN₃</td>
<td>ad. 1 l PBS</td>
</tr>
</tbody>
</table>

Blocking buffer and Reagent Diluent were filter sterilized and stored at 4°C.

<table>
<thead>
<tr>
<th>Stop solution</th>
<th>PBT (washing buffer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2N (1M) H₂SO₄</td>
<td>0,05 % (v/v) Tween 20</td>
</tr>
<tr>
<td>ad. 1 l water</td>
<td>ad. 1 l PBS</td>
</tr>
</tbody>
</table>
2.2.2.c **Luciferase reporter gene assay**

The luciferase reporter gene system was used to measure the activation of a transcription factor and promotor coupled to a luciferase gene which was thereby induced in a receptor specific signaling dependent manner. The regulatory sequence to be examined was cloned upstream of the luciferase gene from the firefly (*Photinus pyralis*) as well as the *Renilla reniformis* luciferase gene as control for transfection efficiency and for normalization. The luciferase enzyme activity enabled quantifying the activation of the coupled promotor.

HEK293 cells were seeded onto 96-well plate (2 x 10^6) and incubated at 37°C for at least 4 h. The receptors, of which the activation was to be quantified, were transfected into the cells via calcium-phosphate transfection.

**Transfection mix**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>660 µl H₂O</td>
<td></td>
</tr>
<tr>
<td>90 µl 2 M CaCl₂</td>
<td></td>
</tr>
<tr>
<td>0.2 µg NfκB</td>
<td>2 ng/well</td>
</tr>
<tr>
<td>IFNβ</td>
<td>15 ng/well</td>
</tr>
<tr>
<td>0.63 µg phRL-null</td>
<td>6.3 ng/well</td>
</tr>
<tr>
<td>receptor</td>
<td>2-50 ng</td>
</tr>
<tr>
<td>pRK5 (empty vector)</td>
<td>ad. 50 ng</td>
</tr>
</tbody>
</table>

1:1 with 2x HBS, vortexing

The transfection mix was incubated at RT for 20', 15 µl were added to the cells of each well for transfection o/n. Subsequently, medium was changed to 2% FCS and the cells were challenged for 16 h. Cell culture supernatants were removed and 40 µl 1x Reporter Lysis Buffer (Promega) were added to each well. Lysis was carried out shaking (800 rpm) for 1 h at RT. 20 µl of lysates were transferred to a white opaque microtiter plate. Conversion of the substrate D-luciferin by induced luciferase produced chemi-luminescence which was measured in an Orion II luminometer (Berthold).
Material and Methods

**Luciferase substrate**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>470 µM</td>
<td>D-Luciferin</td>
</tr>
<tr>
<td>270 µM</td>
<td>Coenzyme A A</td>
</tr>
<tr>
<td>33.3 mM</td>
<td>DTT</td>
</tr>
<tr>
<td>530 µM</td>
<td>ATP</td>
</tr>
<tr>
<td>1.07 mM</td>
<td>(MgCO₃)₂Mg(OH)₂</td>
</tr>
<tr>
<td>2.67 mM</td>
<td>MgSO₄</td>
</tr>
<tr>
<td>20 mM</td>
<td>Tricine</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>Na₂EDTA</td>
</tr>
</tbody>
</table>

pH 7.8

**Renilla substrate**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 M</td>
<td>NaCl</td>
</tr>
<tr>
<td>2.2 M</td>
<td>Na₂EDTA</td>
</tr>
<tr>
<td>220 mM</td>
<td>KHPO₄</td>
</tr>
<tr>
<td>1.3 M</td>
<td>NaN₃</td>
</tr>
<tr>
<td>440 µg/ml</td>
<td>BSA</td>
</tr>
<tr>
<td>1.43 µM</td>
<td>Coelenteracine</td>
</tr>
</tbody>
</table>

pH 5.0

2.2.3 Bacteria

**2.2.3.a Bacteria culture**

Bacteria were grown in the respective medium shaking (180 rpm) at 37°C.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>BHI, supplemented with 15 µg/ml erythromycin if indicated</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>LB, supplemented with 200 µg/ml erythromycin and/or 50 mg/l kanamycin, if indicated</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>BHI, supplemented with 10 µg/ml erythromycin, when indicated.</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>BHI, anaerobic conditions</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>BHI</td>
</tr>
</tbody>
</table>

**2.2.3.b Growth curves and harvest of bacteria**

Bacterial pre-cultures were inoculated from cryopreserved stocks or single colonies from agar plates and incubated at 37°C, 180 rpm, o/n. Subsequently, the optical density was measured at 600 nm (OD₆₀₀) to calculate the inoculation volume for the main-culture to attain a start OD₆₀₀ = 0.07 for standardized conditions. OD₆₀₀ was documented every 30’ from then until growth stagnated for several consecutive measuring points (stationary growth phase), and again after 24 h.

Bacteria were harvested either during the mid-exponential growth phase or when they reached the stationary phase. They were pelleted, washed once and resuspended in PBS for further application.
2.2.3.c Anaerobic culture
Anaerobic or microaerophilic bacteria, such as *Streptococcus pneumoniae*, require to be cultivated under anaerobic conditions for optimal growth. This was enabled using air-tight containers for anaerobic culture equipped with BD GasPak™ EZ Anaerobe Sachets (BD).

2.2.3.d Heat inactivation of bacteria suspension
To prepare a heat inactivated bacterial suspension, bacteria were grown to mid-exponential growth phase and harvested by centrifugation (6000 rpm, 4°C, 15'). After washing, they were resuspended in PBS (0.01 volume) and titrated in serial dilutions on appropriate agar plates. The suspension was boiled for 20' to inactivate and disintegrate viable bacteria before being stored at -80ºC.

2.2.3.e Cryo preservation
For long-term storage bacteria were grown until visible turbidity was reached, and 1 ml of the bacteria suspension was added to glycerol containing Roti®-Store cryo-vials, inverted, SN removed and stored at -80ºC. For short-term storage and direct application bacteria were harvested at the desired growth phase, centrifuged, resuspended in PBS, aliquoted, and stored at -80ºC.

2.2.3.f Bacterial infection and bacterial clearance assay
Bacteria were cultured in the appropriated culture medium to the desired growth phase (usually exponential phase), determined by measuring the optical density. They were harvested by centrifugation (6000 rpm, 4°C, 15') followed by resuspension in PBS. These suspensions were titrated and directly used to infect eukaryotic cells or they were frozen at -80ºC for storage. The bacterial suspensions were always titrated after thawing and before using them for infection assays, to maintain constant conditions. Serial dilutions of bacteria were prepared with sterile PBS.

2.2.3.g Bacterial clearance assay
To analyze the capacity of cells to clear bacteria from their supernatants, BMMs were differentiated as described, counted and plated into 96-well plates (1x10⁵ cells per well in 200 µl antibiotic-free medium) for o/n incubation. Viable bacteria (frozen aliquots in PBS) were thawed, serially diluted in PBS and seeded onto the BMMs for infection. The bacterial aliquots were always titrated and plated on blood agar to verify the infection doses. 6 h post infection (37°C, 5% CO₂), the 40 µl of the supernatants were sampled for serial dilution (titration in PBS) and subsequent plating on blood agar. After 16 h of incubation (37°C), the colonies were counted to quantify bacterial survival in the BMM supernatants. The supernatants were also sampled for Griess Assay and ELISA, 16 h post infection.
2.2.4 Molecular biology

2.2.4.a DNA isolation from eukaryotic tissue
Mouse DNA for genotyping was isolated from amputated tail tips. The tissue was incubated in 500 µl of tail lysis buffer, shaking at 55°C o/n. Next, the suspension was centrifuged (max. speed, 10') to pellet cell debris and remaining hair. The supernatant was mixed with an equal volume of 2-propanol for precipitation of nucleic acids. After centrifugation, the pellet was washed twice with 500 µl of 70% EtOH, dried and solubilized in water at 55°C for 10'. The DNA was stored at 4°C.

2.2.4.b Plasmid DNA isolation from bacteria
For purification of plasmid DNA silica gel membranes were used that separate DNA from interfering substances like proteins or RNA. For bacterial plasmid DNA purification mini- and maxi-preparations were performed using the respective kits. The DNA was eluted in nuclease free water. The concentration of purified plasmid DNA was measured in a NanoDrop cuvette (Eppendorf) at 260 nm. The ratio A260/A280 determined the purity of DNA, with values of about 1.8 indicating pure DNA.

For mini-scale plasmid preparation, single bacterial colonies were picked from agar plates and transferred to 3 ml of medium (complemented with the appropriate selective antibiotic) and grown at 37°C and 220 rpm o/n. Following the manufacturer's instructions the peqGODL Plasmid Miniprep KIT I (Peqlab) was used.

For maxi-scale plasmid preparation, bacteria were cultivated in a volume of 500 ml culture medium, inoculated from colonies growing on agar plates. The plasmid preparation was performed using the NucleoBond Xtra Maxi System (Macherey-Nagel).

2.2.4.c Polymerase chain reaction (PCR)
The polymerase chain reaction enables amplification of a specific DNA sequence, defined by flanking complementary oligonucleotides (primers). These also represent the initiation and termination sites for the DNA polymerase. After heat-denaturation the primers anneal to their template within the ssDNA, the polymerase then utilizes nucleoside triphosphates to elongate the complementary DNA strand. The annealing temperature of primers depends on their melting temperature. The duration of elongation depends on the length of the DNA fragment to be synthesized and the polymerase applied. PCR was carried out in a thermocycler with one fixed or a gradient of slightly differing annealing temperatures. PCR was also performed to verify the genotype of knockout mice using Taq-polymerase, the Thermus aquaticus derived DNA polymerase lacking proofreading activity.
PCR mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-200 ng template DNA</td>
<td></td>
</tr>
<tr>
<td>0.5 µM forward primer</td>
<td></td>
</tr>
<tr>
<td>0.5 µM reverse primer</td>
<td></td>
</tr>
<tr>
<td>200 µM dNTPs</td>
<td></td>
</tr>
<tr>
<td>1-2.5 units DNA polymerase</td>
<td></td>
</tr>
<tr>
<td>1x PCR buffer</td>
<td></td>
</tr>
<tr>
<td>ad. 50 µl water</td>
<td></td>
</tr>
</tbody>
</table>

PCR parameters (exemplary)

1. initial denaturation 95°C 2 min
2. denaturation 95°C 30 sec
3. annealing 48-58°C 45 sec 30-40 cycles
4. elongation 72°C 1 min/kb
5. final elongation 68-72°C 5 min
6. 4°C pause

PCR products were subsequently purified either from the reaction itself or from excised agarose gel slices after separation by agarose gel electrophoresis. Both methods removed primers, salt, enzymes and remaining nucleotides from the PCR product which is essential for further applications. The purification methods both based on column purification kits, for PCR purification (Stratec) as well as for PCR gel extraction (Qiagen). According to the length of the PCR product, the gel contained 0.5-1.5% agarose for adequate separation conditions.

Apart from agarose gel electrophoretic visualization of the PCR product, its DNA sequencing was performed utilizing the sequencing service offered by MWG-Biotech.

**2.2.4.d DNA agarose gel electrophoresis**

Agarose gel electrophoresis was used to separate DNA fragments according to size. 1% agarose was melted in 1x TAE buffer and supplemented with SYBR® Safe (Thermo Fisher Scientific) for DNA staining. DNA samples were mixed with 5x sample buffer (Thermo Fisher Scientific) and separated at constant 100 V. For size reference and to estimate DNA concentration of the samples 5 µl of a 1 kb DNA ladder were separated next to the samples. DNA bands were visualized using UV illumination (254 nm).

**TAE buffer (50x)**

- 242 g Tris base
- 57.1 ml Acetic acid
- 100 ml 0.5 M EDTA

ad. 1 l water

pH 8.5

**2.2.4.e Restriction digest, dephosphorylation and ligation**

DNA can be cleaved by use of specific endonucleases. These restriction enzymes detect sequence specific recognition sites, cut DNA highly specific and generate DNA fragments with defined overlaps. These enable specific ligation, for example to insert genetic elements into expression vectors. All re-
Material and Methods

Restriction enzymes were used according to the manufacturer’s instructions with appropriate reaction buffers for about 15'-2h at 37°C.

To avoid spontaneous religation of cleaved DNA fragments the 5'-phosphates were removed hydrolytically by shrimp alkaline phosphatase (SAP) activity at 37°C for 2 h. Only cleaved plasmids were de-phosphorylated in such a way, DNA fragments to be inserted had to stay phosphorylated.

The ligation of insert DNA fragments into a cleaved DNA vector required ester binding of the phosphate residues and was performed using T4 DNA ligase according to the manufacturer's instructions.

2.2.4.f Transformation of bacteria

Chemically competent bacteria are able to take up plasmid DNA, enabling overexpression of exogenous genes. E. coli strains DH5α (Invitrogen) or XL-10 Gold (Stratagene) were heat transformed according to the supplier's guidelines. Briefly, bacteria were mixed with 10 ng to 1 µg plasmid DNA and incubated on ice for 30’ before heat-shock treatment at 42°C for 40''. After cooling off on ice, 900 µl of LB liquid medium were added. The bacteria were shaken at 37°C for 1 h, centrifuged (5000 rpm, 5’) and resuspended in a smaller volume of the supernatant. The suspension was plated on prewarmed agar plates containing the desired selective antibiotic and incubated at 37°C o/n.

2.2.4.g RNA isolation from eukaryotic tissue

Cultured cells in a 6-well or 96-well plate were covered with 1 ml or 100 µl TRI-Reagent per well, respectively (Sigma Aldrich). It contains guanidinium thiocyanate, a strong protein denaturing agent inactivating RNases, whereas acidic phenol-chloroform separates RNA partitions into the aqueous phase (Chomczynski and Sacchi 1987, 2006). The cells were lysed at RT for 15’ or frozen immediately at -80°C. 200 µl chloroform were added, mixed thoroughly and incubated at RT for 5’. Phase separation was accomplished by centrifugation at 13,300 rpm for 15’. The RNA containing aqueous upper phase was transferred to a new tube and mixed with an equal volume of isopropanol by vortexing for precipitation at -20°C o/n. Precipitated RNA was pelleted (4°C, max. speed for 30’), washed twice in 70% EtOH, dried at RT and finally solubilized in DEPC water.

2.2.4.h Gene expression array

For gene expression analysis of undifferentiated versus 3ddi- versus 8ddiTHP-1 cells RNA was prepared using TRI-Reagent (as described above). Total RNA concentration and purity was measured with a NanoDrop 1000 spectrophotometer (NanoDrop Technologies/Thermo scientific). RNA integrity was assessed using an Agilent Bioanalyzer nano chip. Biotinylated cRNAs was prepared according to the Affymetrix ExpressKit protocol starting from 200 ng total RNA. Following fragmentation, 10 µg of cRNA were hybridized for 16 h at 45°C on GeneChip HG-U133Plus_2. GeneChips were washed and stained in the Affymetrix Fluidics Station 450 using the GeneChip® Hybridization, Wash, and Stain Kit (Affymetrix). Arrays were scanned in a GeneChip 3000 scanner with G7 update. Data were analyzed with MAS5 using Affymetrix default analysis settings and global scaling to a target intensity
of 1000 as normalization method. The microarray data were submitted to the NCBI GEO database http://www.ncbi.nlm.nih.gov/gds/GSE67264.

### 2.2.4.i Reverse transcription

To synthesize cDNA from isolated total RNA, potentially contaminating DNA was removed via DNase I digest at 37°C (DNase I digest mix). The DNase I was then inactivated by addition of 1 µl 12.5 mM EDTA and incubation at 65°C for 15'. After addition of 0.25 mg/ml oligo (dT) primer to each reaction the RNA was denatured at 70°C for 10'. The reverse transcription mixes were prepared on ice.

<table>
<thead>
<tr>
<th>DNase I digest mix</th>
<th>Reverse transcription mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 µg RNA</td>
<td>12 µl DNase I digested RNA</td>
</tr>
<tr>
<td>1 µl 10X buffer (-MgCl2)</td>
<td>1 µl 10X buffer (-MgCl2)</td>
</tr>
<tr>
<td>0.4 µl MgCl$_2$ (50 mM)</td>
<td>1 µl MgCl$_2$ (50 mM)</td>
</tr>
<tr>
<td>0.5 µl RiboLock RNase Inhibitor (40 U/µl)</td>
<td>1 µl dNTPs (10 mM)</td>
</tr>
<tr>
<td>0.5 µl DNase I (0.5 U)</td>
<td>2 µl DTT (0.1 M)</td>
</tr>
<tr>
<td>ad. 10 µl DEPC water</td>
<td>ad. 20 µl DEPC-water</td>
</tr>
</tbody>
</table>

### 2.2.4.j RT-PCR

Real time quantitative PCR (RT-qPCR) was carried out to quantify expression levels of specific genes. Fluorescence labeling (SYBR Green I, Thermo Scientific) enabled real time measurement of newly amplified ds DNA. Measurement was carried out using an ABI 7500Fast Cycler (Thermo Scientific).

<table>
<thead>
<tr>
<th>RT-qPCR mix</th>
<th>Reaction parameters (according to manufacturer's instructions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 µl cDNA</td>
<td>95°C 10 min</td>
</tr>
<tr>
<td>10 µl qPCR Master Mix (2x)</td>
<td>95°C 15 sec</td>
</tr>
<tr>
<td>0.6 µl Primer (20 µM)</td>
<td>60°C 20 sec</td>
</tr>
<tr>
<td>1 µl SYBR-Green (1:500)</td>
<td>72°C 30 sec</td>
</tr>
<tr>
<td>ad. 20 µl DEPC water</td>
<td>RampRate (3.5 °C/sec)</td>
</tr>
</tbody>
</table>

Gene expression was quantified on the basis of the cycle threshold ($C_t$) value, the region of the curve in which the fluorescence initially exceeds the background exponentially. Gene expression levels were normalized to constitutively expressed "housekeeping" genes, such as *Gapdh*, *β-Actinin* (mouse) or 18S rRNA (human). Relative RNA accumulation was calculated as follows.

\[ \Delta C_t = C_t^{\text{gene}} - C_t^{\text{housekeeping-gene}} \]
\[ \Delta \Delta C_t = \Delta C_t^{\text{challenged}} - \Delta C_t^{\text{unchallenged}} \]

relative mRNA accumulation = 2-$\Delta \Delta C_t$
2.2.4.4 RNA isolation from bacteria

Bacteria were grown in the appropriate medium to mid-exponential phase, then harvested into 50 ml tubes containing 100 μl of 2 M NaCl on ice. After centrifugation (8,500 rpm, 4°C, 10'), the supernatant was discarded and the pellet frozen immediately. Subsequently, it was resuspended in 1 ml cold Killing-Buffer, centrifuged (13,000 rpm, 4°C, 5’) and the pellet was stored at -80°C until further use. For lysis of bacterial cells, the pellet was resuspended in 500 μl cold lysis-Buffer and transferred to a screw-top tube with 500 μl silica spheres of 0.1 mm in diameter (FastPrep® Lysing Matrix B, MP Biomedicals). 500 μl of phenol-chloroform-isoamylalcohol (PCI) was added. Bacteria were lysed in a FastPrep®-24 Instrument (MP Biomedicals) three times 20”, 6.5 m/s, chilled intermittent. Bacterial lysates were centrifuged (13,000 rpm, RT, 5’). The upper aqueous phase, containing RNA, was transferred to 600 μl of PCI. The mixture was shaken rigorously for 5’, centrifuged and the upper phase was then transferred to a new tube with 600 μl of chloroform-isoamylalcohol (CI). The washing was repeated one more time with CI. Finally, for precipitation the upper phase was added to a fresh tube containing 3 M NaOAc (pH 5.2) (0.1 volume) and ice cold 96% EtOH was added (2.5 volumes). The tube was inverted and kept at -20°C for 2 h (or -80°C o/n). The precipitated RNA was centrifuged (15'; max. rpm; 4°C), the supernatant was discarded and 500 μl 70% EtOH were added for washing the pellet. After centrifugation (15'; max rpm; 4°C) the supernatant was aspirated thoroughly and the pellet dried under the fume hood. The RNA was solubilized in DEPC water (4°C, o/n) and stored at -80°C.

<table>
<thead>
<tr>
<th>Killing buffer</th>
<th>Lysis buffer</th>
<th>sodium acetate (3 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM Tris/HCl (pH 7.5)</td>
<td>3 mM EDTA</td>
<td>1.06 M acetic acid</td>
</tr>
<tr>
<td>5 mM MgCl₂</td>
<td>200 mM NaCl</td>
<td>3 M sodium acetate (NaOAc)</td>
</tr>
<tr>
<td>20 mM NaCl</td>
<td>water</td>
<td>water</td>
</tr>
</tbody>
</table>

**phenol-chloroform-isoamylalcohol (PCI)**

- 25 parts (v/v) Aqua-Roti-Phenol (pH 4.5-5)
- 24 parts (v/v) chloroform (CHCl₃)
- 1 part (v/v) isooamylalcohol

**chloroform-isoamylalcohol (CI)**

- 24 parts (v/v) chloroform (CHCl₃)

2.2.4.1 RNA agarose gel electrophoresis & purification

RNA agarose-gel electrophoresis was utilized to separate the high molecular weight fraction of bacterial total RNA into 23S and 16S rRNA.

The equipment to cast and run the agarose gels was cleaned thoroughly before use. The RNA and RNA ladder were equally mixed with gel loading buffer, then denatured at 72°C for 5’ before loading onto the gel (1% in 1x MOPS). The electrophoresis was carried out at constant 75 V. The RNA species were purified from excised gel slices using the Zymoclean™ Gel RNA Recovery kit (ZymoResearch) but using Zymo-Spin™ IIIC instead of IC columns for larger amounts of input RNA.
**Methods**

**MOPS buffer (10x)**

<table>
<thead>
<tr>
<th>0.2 M</th>
<th>MOPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM</td>
<td>NaOAc</td>
</tr>
<tr>
<td>10 mM</td>
<td>EDTA</td>
</tr>
</tbody>
</table>

ad 1 l DEPC water

pH 7 (NaOH)

---

**2.2.4.m RNA denaturing PAGE (poly-acrylamide gel electrophoresis) & purification**

Denaturing RNA polyacrylamide-gel electrophoresis was utilized to separate the low molecular weight fraction of bacterial total RNA into 5S rRNA and tRNA.

The equipment to cast and run the polyacrylamide gels was cleaned thoroughly before use.

**15 % polyacrylamide gel, 8 M urea**

<table>
<thead>
<tr>
<th>7.2 g</th>
<th>urea</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 ml</td>
<td>10x TBE</td>
</tr>
<tr>
<td>5.6 ml</td>
<td>Acrylamide-, Bis-Acrylamide-solution 40% (29:1)</td>
</tr>
</tbody>
</table>

ad. 15 ml DEPC water

75 µl 10 % ammonium persulfate

15 µl TEMED

mix briefly

**TBE (10x)**

<table>
<thead>
<tr>
<th>0.9 M</th>
<th>109 g</th>
<th>Tris base</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9 M</td>
<td>55 g</td>
<td>boric acid</td>
</tr>
<tr>
<td>20 mM</td>
<td>40 ml (0.5 M)</td>
<td>EDTA</td>
</tr>
</tbody>
</table>

ad. 1 l DEPC water

The gel was pre-run in 1x TBE for 45' at 15-25W. Subsequently, the gel slots were rinsed. Meanwhile, the RNA and RNA ladder were equally mixed with gel loading buffer, then denatured at 72°C for 5' before loading onto the gel. Electrophoretic separation was carried out at 30-45 mA, constant 250V, 80W. After disassembly, the gel was stained with 1 µg/ml ethidium bromide in 1x TBE.

If desired, the RNA was purified from cut gel slices using the ZR small-RNATM PAGE Recovery Kit (ZymoResearch) according to the manufacturer's instructions.

---

**2.2.4.n Capture of large rRNA & mRNA (MICROBExpress)**

The large ribosomal RNAs or enriched mRNA were isolated from total bacterial RNA using the MICROBExpress kit (Life Technologies). According to the manufacturer's instructions magnetic beads capturing large rRNAs enabled the recovery of mRNA. The rRNA was heat-eluted from the magnetic beads in TE buffer (pH 8) twice at 95°C for 5’, precipitated and solubilized.
2.2.4.o 23S rRNA fishing

23S rRNA was isolated from total bacterial RNA using biotinylated DNA oligonucleotides complementary to the 3' end of the ribosomal RNA (Biot3-23S) according to a modified protocol by (Tsurui et al. 1994). 500 µg total RNA were incubated with 25 µg of biotinylated DNA-ODN Biot3-23S in 2.4 M tetraethylammonium chloride (TEA-Cl) for 5' at 70°C. After incubation at 30°C for 30', 50 µl of equilibrated streptavidine-beads (Pierce Streptavidine UltraLink® Resin, Thermo Scientific) in 2.4 M TEA-Cl were added and incubated for further 30'. After three washing steps with 300 µl 2.4 M TEA-Cl for 5', the rRNA was eluted twice with 50 µl 2.4 M TEA-Cl for 5' at 70°C, followed by 1' on ice. It was dialyzed against DEPC water on 25 nm filter (Millipore) for 30'-2h, precipitated using sodium acetate (0.1 vol.) and 96% EtOH (2.5 vol.) for 2 h at -20°C, washed with 70% EtOH and solubilized in DEPC water.

2.2.4.p DNase I, RNase A and DRNase III digest

To eliminate either DNA, ssRNA or dsRNA from a preparation DNase I (Sigma Aldrich), RNase A (Sigma Aldrich) or RNase III (New England Biolabs) were applied, respectively, according to the supplier's instructions.

2.2.5 Mice

Wildtype (wt) C57BL/6, single and multiple TLR knockout mice, the latter of which were derived by cross breeding of single TLR knockout mice, were used as sources of in vitro generated macrophages. For in vivo analysis mice were sensitized with IFNγ (i.v.; 50 µg/kg; Peprotech) or α-D-galactosamine (i.v.; 0.8 g/kg). 45' later 4 mg/kg of oligoribonucleotide were injected i.v. as challenge. Blood was sampled by retrobulbar dotting. Animal experiments were approved by the State Office for Nature, Environment, and Consumerism of North Rhine-Westphalia, Recklinghausen, Germany (G1230/11).

2.2.6 Statistical analysis

Results were analyzed from at least three biological replicates (n = 3), unless indicated otherwise. The graphs show mean ± standard deviation (SD) of triplicate data points of one out of at least three independent experiments. The level of significance was calculated using the Student's t-test for unconnected samples, with p-values indicated as "*" (p ≤ 0.05) or "**" (p ≤ 0.01).
3 Results

3.1 Characterization of macrophage bacterial killing activity

Macrophages are first line immune cells that sense, phagocytose and kill host invading pathogens (Houghton et al. 2009, Gree and Kass 1964, Gordon 2007). Therefore, my aim was to investigate whether the recognition of Gram-positive bacteria is a prerequisite for proactive defense in murine bone marrow derived macrophages (BMM) as well as involvement of bacterial killing mechanisms. *Streptococcus pneumoniae* D39 (*S. pneumoniae*), Gram-positive cocci, found as diplococci or short chains, were chosen as an infection model, due to strong clinical relevance. They are alpha-hemolytic, facultative anaerobic, non-motile bacteria that do not form spores. *S. pneumoniae* colonize the upper respiratory tract as well as the nasopharynx of healthy carriers. They were first isolated by Louis Pasteur in 1881 (Watson et al. 1993). *S. pneumoniae* cause bacteremia, otitis media, meningitis and also sepsis in humans, though they are best known for causing pneumonia (Todar 2008). They are armed with a set of virulence factors such as a polysaccharide capsule which enables immune evasion, as well as autolysin, a toxin that induces the release of another toxin pneumolysin during host infection (AlonsoDeVelasco et al. 1995).

*S. pneumoniae* D39 were grown under anaerobic conditions and harvested in the mid-exponential growth phase (OD₆₀₀ = 0.5), according to a robust growth curve (Fig. 3 A). Cultured macrophages were infected with the pathogenic streptococci to mimic Gram-positive bacterial infection within hosts. The recognition of the bacteria by the macrophages was analyzed. I also examined whether the macrophages phagocytosed the bacteria followed by intracellular killing or if the release of effector molecules like NO or ROS caused bacterial clearance of the cell culture supernatants (Fig. 3 B).

![Figure 3: Infection of macrophages with *Streptococcus pneumoniae*. (A) *S. pneumoniae* D39 growth curve; OD₆₀₀, optical density at 600 nm. The red arrow indicates OD₆₀₀ = 0.5. (B) Hypothetical model of macrophage *S. pneumoniae* D39 infection and consequent macrophage responses (ROS, reactive oxygen species; AMP, antimicrobial peptides; NO, nitric oxide).](image)

3.1.1 RAW264.7 macrophagoid cells and THP-1 monocytoid cells kill infecting *S. pneumoniae*

To measure macrophage bactericidal activity, titers of viable bacteria in the supernatants of the infected macrophages were quantified by plating of serial dilutions on blood agar. RAW264.7 (murine) or THP-1 (human) cells cultured in 96-well plates were infected with increasing doses of viable *S.
Results

*Pneumococci* D39 (serial dilutions). After 6 hours of infection the supernatants were sampled and plated on blood agar. The number of bacteria that survived the confrontation with the macrophagoid RAW264.7 cells or the monocytoid THP-1 cells was calculated to get colony forming units per ml (cfu/ml) counted on blood agar. RAW264.7 cells killed bacteria to a significant degree. Specifically, bacterial survival was operative merely at infection doses of $10^2$ cfu/ml or higher, whereas bacteria were cultivatable (medium only, no cells) from an infection dose of $10^1$ cfu/ml upwards (Fig. 4 A). The THP-1 cells also abrogated bacterial propagation up to an infection dose of $10^2$ cfu/ml comprehensively. However, these cells were incapable of controlling bacterial growth at higher inoculation doses, (Fig. 4 B).

![Graph](image)

**Figure 4: Infection of macrophagoid and monocytoid cell lines.** (A) Murine Raw264.7 or (B) Human THP-1 cells were infected with serially diluted viable *S. pneumoniae* D39 for 6 h (no cells, only bacteria in 200 µl of cell culture medium without cells). Supernatants were analyzed for their content of viable bacteria (cfu/ml). The graphs show mean ± SD; ***, p ≤ 0.01; n = 3.**

### 3.1.2 Bacterial killing depends on TLR/IL1R1 signal protein expression

Next, I analyzed the bacterial killing activity of primary murine bone marrow derived macrophages (BMMs). BMMs from mice lacking both MyD88 and TRIF expression were applied to evaluate the involvement of pattern recognition receptors (PRRs) such as TLRs in sensing of the infecting bacteria. MyD88 and TRIF are major adaptor proteins that mediate the signal transduction upon ligand recognition by TLRs and also IL-1 receptor, type I (IL1R1) (Takeda and Akira 2004, Kawai and Akira 2010).

![Graph](image)

**Figure 5: Recognition and killing of streptococci depends on MyD88/TRIF expression.** Wild type (wt) and *Myd88/Trif*- BMMs were infected with serially increasing doses of viable *S. pneumoniae* D39 (infection doses) for 6 h. Supernatants were analyzed for their bacteria content (bacterial load) as well as nitric oxide (Nitrite) and cytokine concentration (mIL-6, mTNF); no cells, only bacteria in 200 µl of cell culture medium without BMMs. The graphs show mean ± SD, n = 3.
Characterization of macrophage bacterial killing activity

The Myd88/Trif<sup>−/−</sup> macrophages significantly lacked bacteria killing capacity. I observed S. pneumoniae growth at the infection dose of 10<sup>3</sup> cfu/ml, while wild type macrophages cleared their supernatants. The Myd88/Trif<sup>−/−</sup> macrophages produced neither nitric oxide nor proinflammatory cytokines like IL-6 or TNF while wild type (wt) controls did (Fig. 5). I controlled viability of all cells microscopically and by application of an MyD88/TRIF independent challenge, namely Zymosan, which is recognized by Dectin-1 apart from TLR2 (Fig. 6) (Dillon et al. 2006). This phenotype indicates involvement of TLRs and/or Il-1 cytokine receptor rather than other PRRs in the recognition of the live streptococci.

**Figure 6: Control of cell viability in MyD88/Trif<sup>−/−</sup> BMMs.** Wild type (wt) and Myd88/Trif<sup>−/−</sup> cells were challenged with Zymosan (5 and 10 μg/ml) for 16 h. The supernatants were analyzed via mKC ELISA. The graph shows mean ± SD.

### 3.1.3 Bacterial killing depends on recognition by TLR2 and an endosomal TLR

_Tlr379<sup>−/−</sup>_ macrophages lacking the endosomal nucleic acid sensing TLRs -3, -7 and -9, failed to clear streptococci from their supernatants (Fig. 7). The _Tlr379<sup>−/−</sup>_ cells cleared _S. pneumoniae_ from their supernatants up to an infection dose of 10<sup>3</sup> cfu/ml. TLR2 largely considered as "the" major receptor for Gram-positive bacteria (Takeuchi et al. 1999) as well as TLR13 are expressed in these cells. Nitric oxide and cytokine production were not impaired, implicating a rather minor involvement of the endosomal TLRs -3, -7 and -9 in recognition and mediation of killing the infecting streptococci (Fig. 7).

**Figure 7: Recognition and killing of streptococci is largely independent of the endosomal TLRs -3, -7 and -9.** Wild type (wt) and _Tlr379<sup>−/−</sup>_ BMMs were infected with serially diluted viable _S. pneumoniae_ D39 (infection doses) for 6 h. Supernatants were analyzed for their content of surviving bacteria (bacterial load) as well as nitric oxide (Nitrite) and cytokine concentration (mIL-6, mTNF); no cells, only bacteria in 200 μl of cell culture medium without BMMs. The graphs show mean ± SD, n = 3.

_Tlr23479<sup>−/−</sup>_ BMMs did not lack bacterial killing capacity even though they do not express TLR2, a major Gram-positive bacterial lipopeptide receptor. They even released NO and cytokines to a similar or even higher degree as compared to their wt counterparts (Fig. 8). TLRs share common signaling adap-
tor molecules and signaling cascades to induce proinflammatory responses upon activation. The exaggerated response might be due to excess of free signaling molecules not employed by TLR2, -3, -4, -7 and -9. Hence, the deficiency of five out of twelve TLRs in the Tlr23479⁻/⁻ mice might ensue higher signaling upon activation of the remaining TLRs.

Results

Macrophages from 3D/Tlr2⁻/⁻ mice lack both Unc93B1 mediating ER-endosome transition of endosomal TLRs (TLR3, -7, -9, -11, -12 and -13) (Tabeta et al. 2006) and TLR2 known to sense Gram-positive bacteria. Both 3D/Tlr2⁻/⁻ and 3D/Tlr24⁻/⁻ cells were largely unable to clear bacteria from their supernatants. They cleared 10⁴ cfu/ml bacteria from their supernatants while wild type macrophages cleared 10⁴ cfu/ml bacteria from their supernatant. This indicates involvement of endosomal TLRs beyond the TLR3, -7 and -9. The 3D/Tlr2⁻/⁻ and the 3D/Tlr24⁻/⁻ cells also did not produce NO or proinflammatory cytokines upon infection (Fig. 9 and 10).

Figure 8: Recognition and killing of Streptococci is independent of TLR2, -3, -4, -7 and -9. Wild type (wt) and Tlr23479⁻/⁻ BMMs were infected with serially diluted viable *S. pneumoniae* D39 (infection doses) for 6 h. Supernatants were analyzed for their content of surviving bacteria (bacterial load), nitric oxide (Nitrite) and cytokine concentration (mIL-6, mTNF); no cells, only bacteria in 200 µl of cell culture medium without BMMs. The graphs show mean ± SD, n = 3.

Figure 9: Recognition and killing of Streptococci depends on endosomal TLR and TLR2 ligand recognition. Wild type (wt) and 3D/Tlr2⁻/⁻ BMMs were infected with serially diluted viable *S. pneumoniae* D39 (infection doses) for 6 h. Supernatants were analyzed for their content of surviving bacteria (bacterial load) as well as nitric oxide (Nitrite) and cytokine concentration (mIL-6, mTNF); no cells, only bacteria in 200 µl of cell culture medium without BMMs. The graphs show mean ± SD, n = 3.
Characterization of macrophage bacterial killing activity

Figure 10: Additional deficiency of TLR4 in 3D/Tlr24− BMMS did not enhance the 3D/Tlr2− phenotype. Wild type (wt) and 3D/Tlr24− BMMS were infected with serially diluted viable S. pneumoniae D39 (infection doses) for 6 h. Supernatants were analyzed for their content of surviving bacteria (bacterial load) as well as nitric oxide (Nitrite) and cytokine concentration (mIL-6, mTNF); no cells, only bacteria in 200 µl of cell culture medium without BMMS. The graphs show mean ± SD, n = 3.

3.1.4 Bacterial killing occurs largely independent of ROS and NO production

Reactive oxygen species (ROS) are assigned to the antimicrobial effector molecules released by immune cells to kill bacteria. ROS are produced by a phagocyte NADPH-oxidase (Phox). Phox is a protein complex assembled by the membrane-bound flavocytochrome b558, and three cytosolic proteins p47phox, p67phox and the GTP-binding protein Rac (Jackson et al. 1995). p47phox deficient macrophages (p47phox−) cleared Streptococcus pneumoniae D39 as effectively as wt macrophages from their supernatants. Also, their production of proinflammatory cytokines was not impaired (Fig. 11). These results indicate that ROS production is probably not employed in bacteria killing by murine BMMS.

Figure 11: Deficiency of the phagosomal NADPH oxidase subunit p47phox did not impair recognition and killing of S. pneumoniae. Wild type (wt) and p47phox− BMMS were infected with serially diluted viable S. pneumoniae D39 (infection doses) for 6 h. Supernatants were analyzed for their content of surviving bacteria (bacterial load) as well as nitric oxide (Nitrite) and cytokine concentration (mIL-6, mTNF); no cells, only bacteria in 200 µl of cell culture medium without BMMS. The graphs show mean ± SD, n = 3.
Results

Besides its smooth muscle relaxant function, nitric oxide (NO) kills infecting bacteria and is thus an antimicrobial effector molecule (Wheeler et al. 1997). I infected macrophages of mice lacking inducible nitric oxide synthase expression (iNOS⁻) to analyze the role of NO in bacterial killing. The abrogated NO production was associated with a mild drop of cytokine release and bacteria killing (Fig. 12). Thus, NO was ascribed a minor role as bacteria killing effector molecule for S. pneumoniae D39 infected murine BMMs in vitro.

![Graph](image)

**Figure 12:** Killing of Streptococci was mildly impaired in macrophages devoid of nitric oxide synthase expression. Wild type (wt) and iNOS⁻ BMMs were infected with serially diluted viable S. pneumoniae D39 (infection doses) for 6 h. Supernatants were analyzed for their content of surviving bacteria (bacterial load) as well as NO concentration (Nitrite) and cytokine concentration (mIL-6, mTNF); no cells, only bacteria in 200 µl of cell culture medium without BMMs. The graphs show mean ± SD, n = 3.

### 3.1.5 Can macrophages kill bacteria without direct pathogen host cell interaction?

Asking whether bacteria were killed by macrophages with or without prior physical contact, I seeded macrophages into the wells of a 96-well plate and challenged them with heat inactivated Gram-positive (S. aureus, hiSa) and -Gram-negative bacteria (E. coli, hiEc) or TLR specific ligands as well as their combinations for 16 hours. The synthetic triacylated lipoprotein Pam3CSK4 (P;C) and LPS activate TLR2 and TLR4, respectively. Sa19, a bacterial 23S ribosomal RNA derived synthetic oligoribonucleotide (ORN) was used as a TLR13 ligand. These challenges activated the macrophages to produce mediators and effector molecules and release them into their supernatant. Cultured bacteria seeded into a separate plate were then challenged with these supernatants and their survival was monitored. Fresh medium containing the respective TLR ligands was added to bacteria as controls (Fig.13 A).
Figure 13: Extracellular killing of *S. pneumoniae* by murine macrophages is also independent of direct pathogen-host-cell interaction. (A) Model for bacterial clearance mediated by supernatants from macrophages pre-activated with TLR-specific ligands. (B) Survival of bacteria confronted with the supernatants (SN) of TLR ligand pre-stimulated macrophages (200 µl per 1x10^6 cfu; ctrl, only bacteria in fresh unchallenged medium with addition of the respective stimulus that was used for the challenged supernatants) for 6 h. TLR specific ligands were applied for pre-stimulation (16 h) as follows: heat inactivated *E. coli* (hiEc, 10^7 cfu/ml) or *S. aureus* (hiSa, 10^7 cfu/ml), P,C (0.1 µg/ml), Sa19 (100 pmol per 200 µl, complexed with LyoVec, LV) or and LPS (0.1 µg/ml). The graph shows mean ± SD; *, p ≤ 0.05; **, p ≤ 0.01; unpaired t-test; n = 2.

The supernatants of challenged macrophages contained a bactericidal or at least bacteriostatic activity that impaired the survival or propagation of *S. pneumoniae* D39, respectively. For example, supernatants from macrophages that were challenged with either hiSa or P,C limited the division of initially 10^4 *S. pneumoniae* D39 and killed the infecting bacteria to a significant degree (Fig.13 B). Supernatants from macrophages challenged with LPS or Sa19 reduced *S. pneumoniae* viability by 50% as compared to those Streptococci that were confronted with unchallenged medium. Supernatants from macrophages challenged with hiEc, as well as those challenged with the combination of TLR2, 13 and 4 ligands (P,C + Sa19 + LPS) or the combination of TLR2 and 13 ligands (P,C + Sa19) inhibited bacterial multiplication to a lesser extent. Application of ligands to otherwise unchallenged medium (ctrl) did not affect bacterial growth. These results implicate extracellular effector molecules in the killing of live *Streptococcus pneumoniae*.

I also used *S. aureus* as another clinically relevant and pathogenic Gram-positive bacterium to infect cultured macrophages. They, in contrast to *S. pneumoniae*, grew more aggressively in the presence of murine macrophages in that they were hardly killed by them. Wild type BMMs hardly produced NO upon infection and the highest concentration of proinflammatory cytokines was induced in response to the lowest infection doses, as if the macrophage response was attenuated at the higher bacterial infection doses. (Fig. 14).
Results

Figure 14: Macrophages confronted with viable S. aureus were incapable of bacterial clearance. Wild type BMMs were infected with serially diluted viable S. aureus (infection doses) for 6 h. Supernatants were analyzed for their content of surviving bacteria (bacterial load; cfu, colony forming units) as well as nitric oxide (Nitrite) and cytokine concentration (mIL-6, mTNF); no cells, only bacteria in 200 µl of cell culture medium without BMMs. The graphs show mean ± SD; *, p ≤ 0.05; n = 3.

Bacterial overgrowth of the macrophages usually did not occur during the analyses carried out with S. pneumoniae. Only the highest infection dose occasionally induced a bacterial overgrowth. The macrophages evidently were incapable of controlling the high amount of bacteria. Their morphological decline could be monitored under the microscope. This might have been due to hypoxic conditions in the medium upon bacterial overgrowth inhibiting the production of nitric oxide, ROS and or proinflammatory cytokines.

As shown, the macrophages derived from wild type mice produced cytokines at varying amounts upon challenge with the same dose of S. pneumoniae D39 in different experiments. Variables such as fitness of the primary cells as well as of the bacteria used for infection or the culture conditions did influence the reproducibility of repeated analyses.

Taken together, the results of this project implicate TLR2 as well as another endosomal TLR besides TLR3, -7 or -9 in the recognition and killing of infecting Streptococci by murine BMMs. The bacterial killing mechanism involves effector mechanisms beyond ROS or NO. Antimicrobial peptide release upon recognition of the infecting bacteria might be the effector mechanism in place. This statement is supported by the observation that bacteria do not necessarily need to be phagocytosed to be killed by the macrophages.
3.2 TLR13 recognizes a sequence within bacterial 23S ribosomal RNA

Recognition of Gram-negative bacteria is predominantly mediated by TLR2 and TLR4 (Spiller et al. 2008) and to a lesser degree by other PRRs. In contrast, Gram-positive bacterial immune recognition has not been as clear yet. Even macrophages from mice that lack TLR2, -3, -4, -7 and -9 responded like wild type macrophages to infection with Gram-positive *Streptococcus pneumoniae* D39 (see page 40). Challenge of *Tlr23479*−/− BMMs with heat inactivated pathogenic Gram-positive *Staphylococcus aureus* was also not remarkable. Macrophages lacking MyD88 expression but not those lacking C-type lectin, NOD or IL-1 cytokine signaling function were phenotypically striking (Ferstl 2009). Which implicated a TLR rather than other PRR in Gram-positive bacteria recognition. Accordingly, the recognition of viable streptococci depended on an endosomal TLR as by the 3D/TLR2−/− BMM phenotype (see page 40).

Thus, I embarked on identifying the receptor as well as its ligand recognizing and produced by Gram-positive bacteria. Nucleic acid PRRs are typically localized in the cytoplasm or within the endolysosomal membrane. Unc93B1 is a 12 α-helices containing membrane spanning ER-resident protein that mediates trafficking of the endosomal TLRs (TLR3, -7, -9 and -13) from the ER via the Golgi apparatus to the endolysosome. Unc93B1 defective 3D mice (Tabeta et al. 2006) cross bred with TLR2−/− or TLR24−/− mice were analyzed. TLR2 is broadly considered as major PRR for Gram-positive bacteria (Takeuchi et al. 1999).

*Tlr23479*−/− mice still express endosomal TLRs other than TLR3, -7 and -9, namely TLR13, -11, -12. These endosomal TLRs are not functional in mice of the 3D/TLR24−/− genotype. De facto, the latter are devoid of TLR2, -3, -4, -7, -9, -11, -12 and -13 functions. Comparison of the responsiveness of *Tlr23479*−/− with 3D/TLR24−/− macrophages enabled observation of the activity of TLR 13, -11 and -12. Notably, the latter two have been assigned with profilin and flagellin as their ligands recently (Yarovinsky 2014).

![Figure 15: Responsiveness of BMMs from mice lacking specific TLR function to specific ligands](image)

Bone marrow cells from wt, *Tlr23479*−/−, 3D/TLR2−/− or 3D/TLR24−/− mice were differentiated toward primary macrophages and challenged with TLR specific ligands for 16 h. The supernatants were sampled and subsequently analyzed for their content of cytokines released by the activated macrophages. The synthetic triacylated lipopetide Pam3CSK4 (P,C) activates TLR2. A synthetic analog of dsRNA polyinosinic-polycytidylic acid (poly I:C) activates TLR3, and LPS is sensed by TLR4. The imidazoquinol-
Results

line R848 activates TLR7. The synthetic unmethylated CpG oligodeoxynucleotide 1668 is recognized by TLR9 (Fig. 15).

3D/Tlr2−/−, 3D/Tlr24−/− and wild type BMMs were challenged with heat inactivated (hi) bacteria for 16 h, upon which supernatants were subjected to ELISA. Lysates of Gram-positive Bacillus subtilis or S. aureus as well as of Gram-negative Escherichia coli activated IL-6 release from wild type macrophages in a dose-dependent manner, whereas 3D/Tlr24−/− macrophages remained unresponsive (Fig. 16 A). Only hi E. coli activated cytokine production in 3D/Tlr2−/− BMMs through the LPS contained via TLR4.

Figure 16: Recognition of Gram-positive bacteria via endosomal TLRs. (A) Murine BMMs of indicated genotypes were challenged with heat inactivated bacteria (B. subtilis, S. aureus, E. coli; 10⁸ and 10⁷ cfu/ml, triangle) for 16 h. Supernatants were analyzed via ELISA (n.d., not detected). The graph shows mean ± SD, n = 3. (B) Mice were challenged by i.p. injection with hiSa (1.6x10⁹ cfu/kg body weight) and α-D-galactosamine (800 mg/kg body weight) 45' after i.v. injection of IFNγ (50 mg/kg body weight). Survival was monitored, all deaths occurred within 16 h of treatment (n = 6 per wt and 3D/Tlr24−/− groups, n = 4 for Tlr23479−/− mice).

In vivo, wild type, Tlr23479−/− and 3D/Tlr24−/− mice were immune-sensitized by injection with IFNγ and α-D-galactosamine and challenged with hiSa. Only the 3D/Tlr24−/− mice survived, whereas the wt and also the Tlr23479−/− mice succumbed to septic shock like syndrome (Fig. 16 B). I concluded that an endosomal receptor which is active in wt and Tlr23479−/−, but not in the 3D/Tlr24−/− mice mediates recognition of hiSa and elicitation of the overamplified immune response of which the mice died. Accordingly, a receptor which is functional in Tlr23479−/− but neither in the 3D/Tlr2−/− nor 3D/Tlr24−/− mice, recognizes hiSa.

3.2.1 Bacterial single stranded RNA is recognized by murine macrophages

TLRs are involved in the recognition of pathogen derived nucleic acids (Akira et al. 2006). Hence, suspensions hiSa were incubated with nucleases to specifically digest single stranded (ss)RNA (RNase A), DNA (DNase I) or double stranded (ds)RNA (RNase III). Yeast derived tRNA as well as plasmid DNA were treated equally to control the nucleolytic enzyme activities (Fig. 17 B, agarose gel image).

Surprisingly, Tlr23479−/− macrophages, although they were deficient for the nucleic acid sensors TLR3, TLR7 and TLR9, responded like wt and Tlr2−/− macrophages upon challenge with untreated hiSa. RNase A treatment however abrogated even TLR2 deficient cell activation (Fig. 17 A). The digestion of DNA (DNase I) as well as dsRNA (RNase III) did not affect the stimulatory capacity of hiSa (Fig. 17 B).
These results implied ssRNA and TLR2 ligands as the major Gram-positive bacterial immune stimulatory PAMPs in mice.

Figure 17: Gram-positive bacterial RNA activates Tlr23479\(^+\) macrophages. (A) BMMs of indicated genotypes were challenged with untreated or RNase A treated hiSa (10\(^8\) and 10\(^9\) cfu/ml, triangles) for 16 h and supernatants were analyzed by ELISA (B) Responsiveness of Tlr23479\(^+\) BMMs to challenge with hiSa preparations (10\(^7\) cfu/ml) nuclease-treated as indicated. The hiSa suspension labeled with an asterisk (*) remained undigested, but was incubated at 37\(^\circ\)C like the nuclease reaction mixes. Supernatants were analyzed by ELISA 16 h upon challenge. Agarose gel image of a RNA (top) and a DNA (bottom) 1% agarose gel (M, DNA size marker). Yeast tRNA (S. cerevisiae, Sigma Aldrich) and plasmid DNA (pRK5) were treated like the hiSa preparation as controls for the nuclease activities. The graphs show mean ± SD, n = 3.

3.2.2 The immune stimulatory capacity of bacterial RNA is localized in the 23S ribosomal RNA

Next, I fractionated total bacterial RNA to identify the stimulatory RNA species therein. The major portion of it (~ 90%) is made up of prokaryotic 70S ribosomes which in turn contain ribosomal RNA and protein complexes. The large subunit (50S) contains 23S and 5S rRNA, while the small subunit (30S) contains 16S rRNA. Messenger (m)RNAs and transfer (t)RNAs together with other small non-coding RNA species such as transfer-messenger (tm)RNAs and CRISPR RNAs, represent the remaining part of total RNA (~ 10%) (Lalaouna et al. 2014, Gottesman and Storz 2011). Total RNA sub-species were separated and subsequently the resulting RNA fractions were transfected into murine macrophages.

Total RNA of S. aureus and E. coli was split into a high molecular (hmw) and a low molecular (lmw) weight fraction via HPLC (anion-exchange chromatography) by collaborators (S. Bauer, Marburg). The hmw fraction is mainly comprised of 23S and 16S rRNA. The lmw fraction consists of 5S rRNA, tRNA and small RNAs such as short mRNAs. The purified RNA fractions were separated via agarose gel electrophoresis (Fig. 18 A). Prokaryotic and eukaryotic total RNA (from 3T3 or HEK293 cells) were loaded as controls.

Wild type, Tlr23479\(^+\) and 3D/Tlr2\(^-\) BMMs were transfected with total RNA as well as with the high and low molecular weight S. aureus RNA fractions (Fig. 18 B). IL-6 production in wt and Tlr23479\(^+\) BMMs was induced by total and high molecular weight RNA exclusively, the low molecular RNA did not induce cytokine production. 3D/Tlr2\(^-\) BMM did not respond to any RNA challenge. Transfection of E. coli total, hmw and lmw RNA into Tlr23479\(^+\) BMMs mirrored these results, although the total RNA from Gram-negative E. coli was not nearly as stimulatory as their purified high molecular weight fraction (Fig. 18 C). Total eukaryotic RNA did not induce production of proinflammatory cytokines (data not shown).
Results

Figure 18: Hmw bacterial RNA activates Tlr23479−/− BMMs. (A) Agarose gel image of total RNA, hmw (high molecular weight) and lmw (low molecular weight) RNA fractions as well as purified 23S and 16S rRNA from E. coli and S. aureus, as well as eukaryotic total RNA isolated from 3T3 and HEK293 cells. (B) Responsiveness of BMMs which were transfected with total S. aureus RNA, lmw or hmw RNA fraction (2 and 0.2 µg per 200 µl, depicted by triangle, complexed with Dotap) for 16 h. (C) Tlr23479−/− BMMs were challenged with E. coli total RNA, hmw or lmw RNA (2 µg per 200µl, complexed with LV). Supernatants were analyzed via ELISA after 16 h. The graphs show mean ± SD, n = 3.

Next, 5'-monophosphate specific depletion of the large rRNAs from total RNA was carried out. The enzyme "Terminator™ 5'-Phosphate-Dependent Exonuclease", a processive 5' → 3' exonuclease, digests 5'-monophosphate RNA and thus specifically large rRNAs, while it is di/triphosphate, 5'-cap and 5'-hydroxyl group ignorant. mRNA as well as small RNA species remained in the RNA preparation (Fig. 19 A, lanes labeled: dig.). The depletion of the large rRNAs from the total bacterial RNA preparation abrogated its stimulatory capacity, as shown by mIL-6 ELISA (Fig. 19 B).

Total RNA from S. aureus and E. coli was also treated with a RNA 5' polyphosphatase (Fig. 19 A, lanes labeled: dephosph.). The enzyme sequentially cleaves the γ and β phosphates from 5'-tri- as well as 5'-diphosphorylated RNA. 5'-monophosphorylated RNAs thus remains. Dephosphorylated S. aureus total RNA activated wt and Tlr23479−/− macrophages equally well, while 3D/Tlr24−/− cells were unresponsive (Fig. 19 B). Dephosphorylated E. coli total RNA did not activate cytokine production in Tlr23479−/− or 3D/Tlr24−/− BMMs.

Application of the MICROBExpress™ Kit enabled removal of the large rRNAs via a magnetic bead based capture hybridization procedure also from total RNA. The removed large rRNAs were applied as purified fraction (Fig. 19 A, lanes labeled: pur.) Like the "Terminator Exonuclease" activity, this procedure enriched mRNA together with 5S rRNA and tRNA (Fig. 19 A, lanes labeled: mRNA). Purified large rRNAs of both S. aureus and E. coli induced a high mIL-6 response in Tlr23479−/− BMMs, whereas transfection of their enriched mRNA did not (Fig. 19 B and C).
TLR13 recognizes a sequence within bacterial 23S ribosomal RNA

Figure 19: Purified large rRNAs of bacterial total RNA activates Tlr23479−/− BMMs. (A) Agarose gel image of bacterial RNA preparations upon incubation of total RNAs with RNA 5′-polynucleotidase (dephos.), 5′-phosphate specific exonuclease targeting large rRNAs (dig.; Terminator®-Phosphate-Dependent Exonuclease) or precipitation of both large rRNAs (pur.; MICROBExpress™). Eukaryotic total RNAs isolated from 3T3 and HEK293 cells were used as size controls. (B) RNA preparations were transfected into BMMs, for supernatant cytokine concentration measurement after 16 h. (C) mRNA as well as large ribosomal RNAs (pur.) were purified from total bacterial RNA via magnetic beads coupled to mRNA-specific DNA-oligonucleotides or precipitation, respectively (both MICROBExpress™ Kit). Responsiveness of Tlr23479−/− BMMs to transfection with the resulting RNA preparations was monitored as in (A). The graphs show mean ± SD, n = 3.

Next, I separated the large ribosomal RNAs from each other. Therefore a DNA oligonucleotide complementary to the 3′-terminus of the 23S rRNA and coupled to biotin (Biot3-23S) hybridized to 23S rRNA and was bound to streptavidin beads (23S rRNA fishing). The 23S RNA fraction was released from the bead bound Biot3-23S ODNs by heat denaturation (Fig. 20 A). To also isolate 16S rRNA, I separated total RNA via agarose gel electrophoresis. Subsequently, I excised the 23S and 16S rRNA containing agarose fragments from the gel for purification (Zymoclean™ Gel RNA Recovery kit, Zymoresearch; Fig. 20 B). Transfection of the purified 23S and 16S rRNAs into Tlr23479−/− macrophages identified 23S rRNA as the immune stimulatory total RNA moiety (Fig. 20 B).

Figure 20: 23S but not 16S rRNA activated Tlr23479−/− macrophages. (A) Agarose gel image of total RNA as well as purified 23S rRNA of E. coli and S. aureus. The respective total RNA was incubated with a 23S rRNA 5′-terminus complementary biotinylated ODN (Biot3-23S) and precipitated via streptavidin-biotin binding. The 23S rRNA was released from the ODNs via heat denaturation followed by RNA precipitation. (B) S. aureus total RNA was separated via agarose gel electrophoresis and subsequently purified from gel slices. S. aureus or E. coli total RNA, 23S and 16S rRNA were transfected (LyoVec, LV) into Tlr23479−/− BMMs. Supernatants were sampled for ELISA after 16 h. The graphs show mean ± SD, n = 3.
Results

Investigation of another Gram-positive pathogen, namely Streptococcus pyogenes was used to verify the collected data. S. pyogenes is usually part of the skin flora causing skin and respiratory tract infections such as impetigo or pharyngitis, but also severe invasive soft tissue infections. Diagnostic failure of S. pyogenes infections can result in sepsis (Reglinski and Sriskandan 2014). Total RNA as well as separated 23S and 16S rRNA or the small RNA fraction of S. pyogenes RNA were transfected into BMMs. Also, heat inactivated S. pyogenes (hiSp) were used to challenge the cells. An IL-6 induction upon challenge with total RNA or 23S rRNA as well as hiSp in an endosomal TLR function dependent manner but independent of TLR3, -7 and -9 replicated the previous findings in other Gram-positive bacteria (Fig. 21).

Figure 21: Streptococcus pyogenes 23S rRNA activated Tlr23479+ macrophages. BMMs were transfected with S. pyogenes total RNA, purified 23S or 16S rRNA, or the small RNA fraction (1, 0.8, 0.8 and 0.4 µg per 200 µl, respectively; all complexed with LyoVec, LV). HiSp (10^5 cfu/ml) were also applied as challenge. Supernatants were sampled for ELISA 16 h post challenge. The graph shows mean ± SD; *, p ≤ 0.05; **, p ≤ 0.01; n = 3.

Successive fractionation of total bacterial RNA into RNA subspecies and their subsequent transfection into Tlr23479+ macrophages identified 23S rRNA as a stimulatory active compound. The 23S rRNA was recognized by an endosomal TLR expressed in Tlr23479+ macrophages, but is nonfunctional in 3D/Tlr24-/- cells.

3.2.3 Antibiotic resistance-conferring methylation interferes with 23S rRNA recognition

Bacterial 23S rRNA is highly abundant and conserved, which qualifies it as a pathogen associated molecular pattern (PAMP) candidate molecule. Bacteria adapt to changing environmental conditions to successfully colonize specific ecological niches. Mechanisms like mutations or modifications of ribosomal RNA, such as methylation and phosphorylation can improve bacterial fitness (Karikó and Weissman 2007). These modifications also impose antibiotic resistances. The bacterial 23S rRNA is 3 kb in size and encompasses six domains (Fig. 22). Together with the 16S and 5S rRNA as well as ribosomal proteins it complexes to form ribosomes and performs protein biosynthesis. Modification of highly conserved regions – as such good candidates for immune stimulatory PAMPs – within 23S rRNA modulates ribosomal activity. We focused on 23S rRNA segments known to be methylated to establish antibiotic resistance or improvement of bacterial fitness.
TLR13 recognizes a sequence within bacterial 23S ribosomal RNA

Figure 22: Schematic representation of the molecular domains of 23S rRNA. Sequences of Sal as well as SaII (ψ, pseudouridine) and SaIII are found within domains III and V respectively (red rectangles). These sequences contain methylation sites that mediate antibiotic resistance or enhance bacterial fitness (Sergiev et al. 2008; Kehrenberg et al. 2005; Toh et al. 2008; Kaminska et al. 2010; LaMarre et al. 2011; Vester and Douthwaite 1994; Kovalic et al. 1995; Pfister et al. 2005). Domain V forms the peptidyl transferase loop.

The first segment analyzed and named oligoribonucleotide (ORN) "Sal" (Sa, S. aureus) is located in domain III of the 23S rRNA of bacteria, while the ORNs "SaII" and "SaIII" are both located in domain V (Fig. 22). Domain V contains the peptidyl transferase loop (PTL), the catalytically active site of the bacterial ribosome. The "Sal" sequence includes adenosine (A)1662 in S. aureus and A1616 in E. coli 23S rRNA. N6-methylation of the respective A increases bacterial fitness (Sergiev et al. 2008). "SaII" represents the ribosomal catalytic center (the PTL) and includes A2530 in S. aureus and A2503 in E. coli 23S rRNA. Methylation of the respective A mediates bacterial resistance against antibiotics such as chloramphenicol, clindamycin or tiamulin (Kehrenberg et al. 2005, Toh et al. 2008, Kaminska et al. 2010, LaMarre et al. 2011). "SaIII" includes A2085 in S. aureus and A2058 in E. coli 23S rRNA. Mutation or methylation of these mediate bacterial resistance against macrolide, lincosamide and streptogramin B (MLS) antibiotics (Vester and Douthwaite 1994, Kovalic et al. 1995, Pfister et al. 2005).

Wild type, Tlr23479<sup>−</sup> and 3D/Tlr24<sup>−</sup> macrophages were transfected with synthetic analogs of these three ORNs. Sal and SaII induced nitric oxide (NO) release neither from wt nor from Tlr23479<sup>−</sup> or 3D/Tlr24<sup>−</sup> BMMs. However, SaIII challenge induced NO production in wt and in an even stronger manner in Tlr23479<sup>−</sup> macrophages. The 3D/Tlr24<sup>−</sup> cells, lacking endosomal TLR function, remained inactive upon transfection with SaIII (Fig. 23).

Figure 23: Analysis of three 23S rRNA segments containing adenosines known to be methylated by methyltransferases to modulate fitness, e.g. upon antibiotic pressure. BMMs were challenged with the ORNs Sal, SaII and SaIII (1, 10 100 pmol per 200 µl, depicted by triangles; complexed with Dotap). Nitric oxide production was quantified by Griess assay (Nitrite) 16h post challenge. The graph shows mean ± SD, n = 3.

Our result implicated the 23S rRNA segment SaIII, highly conserved in bacteria, as immune stimulatory PAMP within bacterial 23S rRNA. Heat inactivated bacteria, total RNA and thereof purified bacterial 23S rRNA as well as the ORN SaIII activated Tlr23479<sup>−</sup> macrophages as strongly as wt controls. In contrast, 3D/Tlr24<sup>−</sup> macrophages lacking endosomal TLR function did not recognize the stimulatory RNA motif. Since TLR11 and 12 have been shown to recognize bacterial flagellin or profilin (Mathur et al. 2012, Andrade et al. 2013), TLR13 remained as our most promising SaIII RNA recognizing PRR candidate molecule.
Results

3.2.4 Gain- and loss-of-function analyses implicating TLR13 as bacterial 23S rRNA SalIII receptor

To validate the previous findings, gain- and loss-of-function analyses were carried out in cooperation with Marina Oldenburg (Oldenburg 2015). The endosomal localized TLR13 was over-expressed in HEK293 cells. The transfected cells were challenged with hiSa or transfected with SaIII. The synthetic ORN SaIII specifically and exclusively induced NF-xB mediated luciferase activity in TLR13 over-expressing cells (Fig. 24 A). Consequently, primary Tlr24 macrophages were transfected with siRNA targeting TLR13 mRNA to knock down TLR13 (Oldenburg 2015). The efficiency of the treatment was controlled by Real-Time PCR. The cells were transfected with a scrambled siRNA and a siRNA targeting MapK1 mRNA as controls. Tlr23479+ macrophages produced IL-6 upon challenge with SaIII when transfected with the control siRNAs but not if TLR13 had been silenced (Fig. 24 B).

Figure 24: Gain and loss of function experiments implicate TLR13 as receptor for bacterial 23S rRNA segment. (A) HEK293 cells were transfected with empty vector (15 ng/200 µl), TLR2 (2 ng/200 µl) or TLR13 expression plasmids as well as a NF-xB promotor-coupled luciferase reporter gene plasmid. After 24 h the cells were challenged with hiSa (106 cfu/ml) or transfected with SaIII (100, 10 and 1 pmol/well, depicted by triangle) for 16 h. NF-xB-promotor-driven relative luciferase activity (Rel. lucif. activ.) was analyzed (n.p., not performed). (B) Knock down of TLR13 and MAPK1 mRNAs via siRNA treatment (scram, scrambled control siRNA; 50 pmol/well of each siRNA) of Tlr23479+ BMMs. Levels of corresponding mRNAs were determined by RT-PCR (left and middle). BMMs were challenged for 16 h with SaIII (100 pmol/200 µl) and supernatants were analyzed by ELISA. The graphs show mean ± SD, n = 3.

TLR13 over-expression mediating 23S rRNA responsiveness and knock down of TLR13 abrogating 23S rRNA responsiveness supplement the findings of the experiments utilizing macrophages from 3D/ Tlr24+ and Tlr23479+ mice. We concluded, that TLR13 is a cellular receptor of SaIII, a highly conserved sequence segment within the peptidyl transferase loop of bacterial 23S rRNA.

3.2.5 Clinical MRSA isolate 23S rRNA fails to activate TLR13 upon bacterial culture with specific antibiotic

In 1959 methicillin was licensed to treat penicillin resistant S. aureus infections. But in short time, first methicillin resistant S. aureus strains (MRSA) had been identified (Barber 1961, Parker and Jevons 1964), followed by the discovery of adenosine specific N6-methyltransferase (Erm). Erm is an enzyme that catalyzes methylation of the bacterial 23S rRNA adenosine A2085 in S. aureus and A2058 in E. coli localized within the peptidyl transferase loop (PTL) (Fig. 25) (Eady et al. 1993, Weisblum 1995, Skinner and Cundliffe 1982). Erythromycin is an antibiotic targeting the PTL causing ribosome stalling. However N6-adenosine-methylation abrogates binding of erythromycin, thus conferring antibiotic resistance.
TLR13 recognizes a sequence within bacterial 23S ribosomal RNA

Figure 25: Erythromycin resistance-associated methyltransferases like ErmC catalyze methylation of adenine at N of C6 of the purine (A2085 in S. aureus and A2058 in E. coli) within bacterial 23S rRNA domain V (SalIII).

Next, I isolated total RNA and purified the 23S and 16S rRNA. Total RNA and 23S rRNA from MRSA strain 5454 grown in presence of erythromycin was barely recognized by Tlr23479<sup>−</sup> macrophages. While erythromycin free culture resulted in substantial Tlr23479<sup>−</sup> BMM activating capacity of 23S rRNA (Fig. 26 C).

Figure 26: Culture in presence of erythromycin abrogates Tlr23479<sup>−</sup> BMM immune stimulatory capacity of MRSA RNA. (A) BMMs were challenged with heat inactivated (hi) MRSA strains which were grown in erythromycin supplemented medium (10 µg/ml), or with erythromycin sensitive hiSa (each 10<sup>6</sup> cfu/ml). (B) Responsiveness of Tlr23479<sup>−</sup> BMMs to hi MRSA that were grown with (+ ery) or without (− ery) erythromycin (10<sup>6</sup> cfu/ml). (C) Total RNA, 23S or 16S rRNA from clinical MRSA strain (5454), grown in absence or presence of erythromycin, was applied to Tlr23479<sup>−</sup> BMMs by transfection (total RNA, 1 µg/200 µl; 23S and 16S rRNA, 400 ng/200 µl; all complexed with LV). (A-C) Cytokine production or nitric oxide release (Nitrite) were measured 16 h post challenge. The graphs show mean ± SD, n = 3.

Consequently, we i.v. infected Tlr23479<sup>−</sup> mice with live bacteria (10<sup>8</sup> cfu of MRSA isolate 5454 per animal) grown in absence or in presence of erythromycin. Serum was drawn from the infected mice after 2 h and analyzed for the content of secreted proinflammatory cytokines. Mice infected with MRSA grown in the presence of erythromycin produced significantly less serum cytokines as compared to those infected with MRSA grown without erythromycin (Fig. 27).

Figure 27: In vivo infection of mice with erythromycin resistant MRSA. Tlr23479<sup>−</sup> mice were i.v. infected with 10<sup>8</sup> cfu erythromycin resistant clinical MRSA isolate 5454 grown in the presence (+) or absence (−) of erythromycin (10 µg/ml) and harvested in the mid-exponential growth phase. Serum was drawn from the mice 2 h post infection and analyzed for cytokine content. Graphs represent mean ± SD for n = 6 mice per group; *, p ≤ 0.05.
Results

Our results indicated erythromycin resistance conferring methylation of *S. aureus* 23S RNA to also disrupt TLR13 binding.

3.2.6 Over-expression of ErmB & ErmC in Gram-negative and Gram-positive bacteria blocks RNA TLR13 interaction

The methyltransferases Erm C and ErmB catalyze N6-adenosine methylation of the target binding site of erythromycin, thereby conferring antibiotic resistance. Consequently, we cloned the coding sequence (cds) of the methyltransferase ErmC from the clinical MRSA isolate 5454, to analyze the involvement of methyltransferase activity in shielding 23S rRNA from being recognized by TLR13. Therefore, total RNA was reverse transcribed into cDNA. The cDNA served as template for a PCR with specific primers to amplify the ermC cds. The resulting PCR product was purified and illustrated by agarose gel electrophoresis and DNA-sequencing (Fig. 28). The PCR product was ligated into a cloning vector (pJET1.2, Thermo Scientific) and subsequently sub cloned into an *E. coli* expression vector (pGEX2T, GE lifesciences). The expression strain *E. coli* BL21 codon plus (Strategene) is susceptible to erythromycin. Hence, specifically vector transformants grew on agar in the presence of erythromycin (10 µg/ml). *E. coli* BL21 was also transformed with plasmids coding for another erythromycin related methyltransferase *ermB* or tRNA specific methyltransferase *trmH*.

![Figure 28: Amino acid sequences of ErmC.](image)

I isolated total RNA from these bacteria harvested in mid-exponential growth phase. The total RNA was separated via agarose gel electrophoresis to purify 23S and 16S rRNA. *Tlr23479*−/− macrophages were then transfected with the RNA preparations and the supernatants were analyzed for cytokine content 16 h upon challenge. The 23S rRNA from *E. coli* expressing ErmB or ErmC did not induce cell activation while their untransformed parental counterparts (ctrl.) did so (Fig. 29 A). Expression of the tRNA methyltransferase TrmH in *E. coli* impaired the TLR13 activating capacity of their 23S rRNA.

*ErmB* expression plasmids were also transferred into erythromycin susceptible apathogenic Gram-positive strain *B. subtilis* 168 via electroporation. Expression and enzymatic activity of ErmB (encoded by pAT18 as well as pNF8 plasmid) resulted in a decreased IL-6 induction in *Tlr23479*− BMMs by the
B. subtilis total RNA, when the bacteria were grown in erythromycin supplemented (15 µg/ml) medium (Fig. 29 B).

Figure 29: Expression of methyltransferases ErmB or ErmC in E. coli abrogates 23S rRNA stimulatory capacity. (A) E. coli BL21 was transformed with empty plasmid (ctrl.) or ermB or ermC expression plasmids, two erythromycin resistance conferring RNA methyltransferases. E. coli transformed with a trmH expression plasmid was another control. The 23S and 16S rRNAs were transfected into Tlr23479° BMMs. Supernatants were analyzed by ELISA. (B) B. subtilis 168 was subjected to electro transformation with expression plasmids pAT18 (pAT) or pNF8 (pNF) mediating constitutive expression of ErmB. Their total RNA was transfected into Tlr23479° BMMs, 16 h upon which the supernatants were sampled for ELISA. B. subtilis 168 was subjected to the transformation procedure without plasmid DNA (ctrl.). Graphs show mean ± SD, n = 3.

Over-expression of methyltransferases conferring erythromycin resistance in initially sensitive Gram-negative or -positive bacteria mirrored the inertness of 23S rRNA from erythromycin resistant clinical MRSA isolates that were grown in presence of erythromycin. N6-adenosine methylation blocked binding of bacterial 23S rRNA to both erythromycin and TLR13.

3.2.7 Not merely methylation but also point mutation of Sa19 affects TLR13 stimulatory capacity

The sequence motif SaIII within the peptidyl transferase loop of bacterial 23S rRNA remained to be characterized. Therefore, a range of ORNs were designed and synthesized to analyze the length requirement and sequence specificity of TLR13 (Table 3). The ORNs were applied to Tlr23479− BMMs to comparatively analyze their TLR13 activating potential. Initially, we narrowed down the minimum length of the stimulatory motif within 23S rRNA. Reduction of the length of the stimulatory ORN SaII (48 nt) to 19 nt (Sa19) did not decrease its stimulatory activity. The even shorter variant Sa12 (12 nt) also induced IL-6 production in Tlr23479− macrophages. The 9 nt variant Sa9 however, failed to activate Tlr23479− BMMs (Table 3, Fig. 30 A). Next, Sa12 was scanned by inserting adenosine point mutations (Table 3). None of the altered ORNs induced IL-6 production (Fig. 30 A, and data not shown), thus indicating the extraordinary sequence specificity of TLR13. The only exceptions from this observation were the Sa12 variants Sa12s1 and Sa12s2 which were immune stimulatory, despite being mutated in the first and second residue, respectively (Table 3, data not shown). Thus, we identified a segment of 10 highly conserved nucleotides (CGGAAAGACC) to comprise the TLR13 ligand motif.
Table 3: Synthetic ORNs based on bacterial 23S rRNA segment SaIII. The critical adenosine is typed bold, point mutations are labeled in red and methylated adenosines in blue. ORNs shown otherwise also within this thesis are underlined. The ORNs were applied to Tlr23479−/− BMMs to comparatively analyze their TLR13 activating potential. TLR13 activation (+) and failure to activate (−) are indicated.

<table>
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<th>Name</th>
<th>Sequence (5’–3’)</th>
<th>TLR13 stimulatory activity</th>
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<tr>
<td>Sa19</td>
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<td>+</td>
</tr>
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In parallel, Sa19 was modified by synthetic N6-adenosine methylation to resemble the integrity of the Sa19 segment of 23S rRNA in erythromycin grown and resistant bacteria as a result of the activity of a specific methyltransferase such as ErmB or ErmC. While Sa19 induced dose dependent cytokine production in Tlr23479−/− BMMs, its specifically methylated variant Sa19mA7 was nonstimulatory (Table 3, Fig. 30 B). Notably, N6-methyladenosine driven camouflage from TLR13 was residue specific. Activation of Tlr23479−/− BMMs upon transfection with Sa12 was strong. Methylation of the sixth adenosine (Sa12m6, equivalent to Sa19mA7) abrogated stimulation, whereas methylation of the seventh adenosine (Sa12m7) merely mildly affected the stimulatory capacity of the ORN. Consequently, double N6-adenosine methylated Sa12m6m7 also lacked TLR13 activating property (Table 3, Fig. 30 C).
TLR13 recognizes a sequence within bacterial 23S ribosomal RNA

Figure 30: Sa19 derived variant ORNs of reduced length and/or carrying mutations or methylations display altered TLR13 specific activity. (A) Tlr23479⁻ BMMs were challenged with ORNs Sa19, Sa12 and Sa9 that vary in length, as well as with the Sa12 variants Sa12s7C, Sa12s6G and Sa12s5A (see Table 3, each 100 pmol/200 µl, complexed with LV). (B) Tlr23479⁻ BMMs were transfected with Sa19 or specifically N6-methylated Sa19 (10 and 100 pmol/200 µl, depicted by triangle, complexed with LV). (C) Tlr23479⁻ BMMs were transfected with Sa12 methylated at position 6 (Sa12m6) or at position 7 (Sa12m7) or double methylated at both positions (Sa12m6m7) (each 100 pmol/200 µl complexed with pLA. (A-C) Supernatants were sampled 16 h upon challenge and analyzed via mL-6 ELISA. The graphs show mean ± SD.

Taken together, the major immune stimulatory activity of bacterial 23S rRNA was shown to be confined to a specific segment within the peptidyl transferase loop that has a length of ten nucleotides and can be corrupted to be recognized by TLR13 by N6-adenosine methylation at a specific residue.

In order to illustrate methylation of 23S rRNA, I purified it from E. coli that had been transformed with ermC or ermB expression plasmids and grown in the presence of erythromycin (see section 3.2.6). These 23S rRNAs were then subjected to HPLC analysis by S. Bauer (Marburg). Standard oligonucleotides containing N6-methyladenosine only or N6,N6-dimethyladenosine were used as positive controls. A peak representing the N6-methyladenosine A2058 was detected in 23S rRNA from ErmB and ErmC expressing bacteria (Fig. 31). An additional peak, visualizing N6,N6-dimethyladenosine A2058 was detected in 23S rRNA from ErmB expressing E. coli. Accordingly, a N6-methyladenosine peak was absent from both parental E. coli BL21 strain and the TrmH over-expressing bacteria derived 23S rRNA.
Figure 31: HPLC based analysis of ErmC or ErmB over-expressing E. coli. An oligonucleotide containing N6-methyl-A and N6,N6-dimethyl-A was used as standard (std). E. coli over-expressing ErmB or ErmC were grown in erythromycin-complemented medium, while wild type and TrmH over-expressing E. coli were grown without erythromycin. Subsequently, bacterial RNA was isolated and 23S rRNA purified for HPLC analysis, representative results of which are shown.

According to our results, ErmB dimethylates E. coli 23S rRNA presumably at A2058 while ErmC merely mono-methylated it. In contrast, TrmH failed to mediate a detectable N6-methylation of 23S rRNA in E. coli.

These results supplement our functional data by demonstrating molecular modification, namely N6-adenosine mono- and N6,N6-adenosine dimethylation, upon over-expression of erythromycin resistance conferring methyltransferases. Apparently, erythromycin or other MLS antibiotic resistant bacteria do escape TLR13 dependent immune surveillance.
3.3 Human TLR8 is a functional homolog of murine TLR13 with broad specificity

While mice express 12 TLRs, humans express 10 TLR in total. We identified bacterial 23S rRNA as the murine TLR13 activating PAMP. Next, we analyzed humans, not expressing TLR13, for bacterial RNA responsiveness.

3.3.1 Human PBMCs are activated by ribosomal RNA of bacteria and mitochondria

Human peripheral blood mononuclear cells (PBMCs) responded to challenge with bacterial 23S rRNA derived synthetic ORN Sa19 (Fig. 32 A). They, however, also recognized its N6-adenosine methylated variant Sa19m which is not recognized by murine TLR13 (Fig. 30 B).

I applied a monoclonal anti-TLR2 antibody (mAb T2.5) (Meng et al. 2004) to the PBMCs 30’ prior to challenge to block TLR2 as a known Gram-positive bacteria sensor. PBMCs were responsive towards hiSa (Fig. 32 B). Upon TLR2 blockade, recognition of P3C was significantly decreased, while cell activation by hiSa and Sa19 was unimpaired. However, combined TLR2 blockade and RNase A treatment abrogated stimulation of the cells by hiSa (Fig. 32 B). Thus, Gram-positive bacterial ssRNA and TLR2 ligands are the human immune stimulatory activities in hiSa.

![Figure 32: PBMCs respond to Gram-positive bacterial RNA, unless it is digested by RNase A.](image)

In 2010, immune stimulatory mitochondrial danger associated molecular patterns (DAMPs) were implicated. Mitochondrial DNA was identified as causative activity (Zhang et al. 2010). Mitochondria evolved from prokaryotes, according to the endosymbiotic theory. Specifically, prokaryotic Archaea phagocytosed prokaryotic Rickettsiales, which became endosymbionts and remained as mitochondria (Thrash et al. 2011, Williams et al. 2013). Mitochondria have distinct double membranes. Like bacteria, they contain a circular DNA genome and have their own ribosomes. The mitochondrial ribosome consists of a large and a small subunit. These subunits contain 16S and 12S rRNA in contrast to the bacterial 23S and 16S rRNA. My coworker Chiranjeevi Chebrolu chose a segment within mitochondrial 16S rRNA of *Bos taurus*, which was named BtmtD3_4 due to its localization in the transition region between domains 3 and 4 of the mitochondrial 16S rRNA (Fig. 33 A). It shares an identical core sequence with Sa19 in bacteria (Fig. 33 A).
Results

PBMCs challenged with BtmtD3_4 released more TNF as compared to Sa19 (Fig. 33 B). The methylated Sa19mA7 or the mutated Sa19A7G (see Table 3, page 56) also activated the cells. It has been shown that TLR7 ligands such as RNA40 and bacterial tRNA induce type I interferon (IFN) production only by activating TLR7 in the plasmacytoid DC subset of human PBMCs (Hornung et al. 2005, Heil et al. 2004). Since PBMCs did not release IFNα upon challenge with the ORNs, TLR7 is not involved in their recognition (Fig. 33 B). RNA40 applied as a control induced both TLR7 and TLR8 dependent TNF as well as TLR7 dependent IFNα release.

Figure 33: Activity of ORNs carrying a common sequence motif within mitochondrial 16S and bacterial 23S rRNA. (A) Schematic of domains I-VI of both bacterial 23S rRNA comprising Sa19 (red rectangle) and mitochondrial 16S rRNA comprising BtmtD3_4 (blue rectangle). Alignment of Sa19 and BtmtD3_4 (Bt, Bos taurus; mt, mitochondrial; D, domain; _ transition region) with common core sequence (bold). (B) PBMCs were transfected with RNA40 (5 μg/ml) or Sa19, methylated (Sa19mA7) and mutated (Sa19A7G) Sa19 variants or BtmtD3_4 (each 100 pmol per 200 μl, complexed with LyoVec). Supernatants were analyzed by ELISA after 16 h. The graph shows mean ± SD, n = 3.

I separated the ribosomal RNA molecular subspecies of S. aureus and E. coli to analyze their stimulatory potential in human cells. 23S and 16S rRNA were purified from excised agarose and the 5S rRNA from polyacrylamide gel fragments (Fig. 34 A). Transfection of total RNA and 23S rRNA from S. aureus and E. coli as well as 16S and 5S rRNA activated PBMCs (Fig. 34 B). These results indicate a broader ligand specificity of the human receptor in contrast to TLR13 specificity.

Figure 34: PBMC activation by bacterial RNA depends upon endosomal function. (A) Polyacrylamide gel electrophoresis image of total S. aureus RNA comprising tRNA and 5S rRNA (left panel) or isolated 5S rRNA (right panel). M, RNA size marker; tot, total; S, Svedberg; hmw, high molecular weight; r, ribosomal; t, transfer. (B and C) Responsiveness of PBMCs challenged with Loxoribine (0.6 mM) or transfected with RNA40 (5 μg/ml) or bacterial RNA preparations (total RNA, 1 μg/200 μl; 23S and 16S rRNA, 600 ng/200 μl; 5S rRNA, 200 ng/200 μl; all transfected with L2K). Supernatants were analyzed by ELISA 16 h post challenge. The graphs show mean ± SD, n = 3. (B only) PBMCs were left untreated or pretreated with chloroquine (5 μg/ml) 1 h before challenge.

Nucleic acid sensing TLRs are localized in endosomes. For this reason, we blocked endosomal PRR function in PBMCs. Pretreatment of PBMCs with chloroquine prevents endosomal acidification and therefore activation and function of endosomal TLRs (Ma et al. 2012). PBMC activation upon chal-
Human TLR8 is a functional homolog of murine TLR13 with broad specificity. These results implicate endosomal TLRs in the recognition of bacterial RNA.

Human TLR7 recognizes bacterial RNA, tRNA of which induces TLR7 dependent type I interferon production in monocytes (Jöckel et al. 2012, Gehrig et al. 2012). Accordingly, we analyzed separated bacterial rRNAs for their potential to induce IFNα release. PBMC challenge with total RNA (including the tRNA) from S. aureus or E. coli induced IFNα production similar to challenge with Loxoribine or RNA40 (synthetic TLR7 ligands). However, none of the purified ribosomal RNA fractions induced responsiveness (Fig. 34 C). The residual IFNα production upon S. aureus but not E. coli 5S rRNA challenge, might be due to contamination of S. aureus 5S rRNA with tRNA purified from polyacrylamide gel fragments (Fig. 34, left panel).

Next, we applied PBMCs from an individual expressing a defective MyD88 mutant (MyD88<sup>d/d</sup>) (in collaboration with P. Hennecke). These as well as wt PBMCs (control) were challenged with S. aureus RNA, Sa19 or BtmtD3_4 and analyzed for IL-8 secretion. Bacterial RNA as well as Sa19 and BtmtD3_4 activated the cells in a MyD88 dependent manner (Fig. 35). LPS driven IL-8 release was unremarkable (positive control, not shown).

![Graph showing IL-8 production](image)

**Figure 35:** PBMCs from a human individual expressing a non-functional Glu53Δ MyD88 mutant fail to recognize S. aureus RNA, Sa19 and BtmtD3_4. Control (healthy individual) or MyD88 defective PBMCs (Myd88<sup>d/d</sup>) were transfected with total S. aureus (Sa) RNA (1 µg/ml) or Sa19 and BtmtD3_4 (each 100 pmol per 200 µl) using LyoVec (LV) as transfection reagent, with R848 (10 µg/ml) as control. Supernatants were analyzed by ELISA 16 h post challenge. The graph shows mean ± SD, n = 2.

### 3.3.2 Differentiated THP-1 cells respond to RNA

Aiming at establishing a genome wide screening system for human RNA/Sa19 sensing PRRs, we analyzed 22 human cell lines including T- and B-cells, melanoma cells, moncytoid, epithelial and endothelial cells as well as fibroblasts (see Table 2, page 21). None of them produced TNF, IL-6 or IL-8 upon challenge (data not shown).

PBMCs contain B-, T- and NK-cells, monocytes and granulocytes. We focused on monocytes and macrophages. Accordingly, monocytic THP-1 cells were differentiated in phorbol 12-myristate 13-acetate (PMA) supplemented medium for 24 h, followed by culture in fresh medium for 72 h and subsequent medium exchange prior to challenge by transfection for 16 h (3ddi). 3ddiTHP-1 cells were responsive to challenge with Sa19 and BtmtD3_4. In contrast, undifferentiated (undiff) THP-1 cells as well as eight days PMA differentiated (8ddi) cells were unresponsive to ORN challenge Fig. 36 A).
Results

Figure 36: 3ddiTHP-1 cells were responsive to ribosomal RNA derived ORN challenge. (A) Responsiveness of undifferentiated (undiff) and 3 or 8 days PMA (200 nM) differentiated (ddi)THP-1 cells to LPS (0.1 μg/ml) challenge or transfection with Sa19 or BtmtD3_4 (100 pmol per 200 μl each, transfected with L2K or pLA, respectively). Supernatants were analyzed by ELISA 16 h after transfection. The graphs show mean ± SD, n = 3. (B) Ratios of constitutive mRNA amounts in unchallenged 3ddi versus 8ddi (grey bar) and of each of both versus undiff THP-1 cells (black or white bar, respectively) according to a transcriptome profiling result. Receptor mRNAs increased ≥ 2-fold in 3 ddi as compared to undiff and 8ddiTHP-1 cells, except for data framed (depicted for comparison; dual TLR7 probe set: CLEC7A, C-type lectin domain family 7A; FPR3, formyl peptide receptor 3; DDX58, DEAD box polypeptide; CLEC4A, C-type lectin domain family 4A; FPR1, formyl peptide receptor; SIGLEC1, sialic acid binding Ig-like lectin) according to comparative gene array based transcriptome profiling (n = 1).

We isolated the RNA from unchallenged undiff, 3ddi and 8ddi THP-1 cells for comparative transcriptome analysis via gene expression array. We considered genes that were induced at least two-fold (mRNA level) in 3ddi as compared to both undiff and 8ddi THP-1 cells as significant. Membrane spanning or cytoplasmic endogenous PRRs we found activated were FPR (formyl peptide receptor) 1 and FPR3, G-protein coupled receptors sensing bacterial formyl peptides. SIGLEC1 (sialic acid binding IG-like lectin 1) recognizes glyco-conjugates. DDX58 (Asp-Glu-Ala-Asp box polypeptide 58) is involved in viral dsRNA recognition. CLEC4A and CLEC7A are C-type lectin domain family members (annotations according to NCBI database: ncbi.nlm.nih.gov). Based on our implication of MyD88 involvement, we focused on TLR8 (Fig. 36 B). We analyzed supernatants from 3ddi THP-1 cells challenged with BtmtD3_4 for proinflammatory cytokine production via Luminex bead-based multiplex assay. TNF, IL-6, IL-12 and IL-8 were produced in response to the stimulus (data not shown).

Figure 37: Evaluation of different transfection reagents in THP-1 cells. Sa19 or BtmtD3_4 (100 and 50 pmol per 200 μl each, triangles) were complexed with LyoVec (LV), poly L-arginine (pLA), Lipofectamine 2000 (L2K) or Dotap for transfection into 3ddiTHP-1 cells. Supernatants were analyzed by ELISA 16 h after transfection. The graph shows mean ± SD, n = 3.

Transfection of ORNs was necessary for cell activation. Therefore, we evaluated different transfection conditions. Sa19 or BtmtD3_4 complexed with the transfection reagents LyoVec (LV), poly-L-arginine hydrochloride (pLA), Lipofectamine 2000 (L2K) and Dotap were applied to challenge 3ddiTHP-1 cells. While Sa19 transfected with L2K yielded the highest IL-8 induction, pLA transfection of BtmtD3_4 was most efficient (Fig. 37).
3.3.3 Endosome function is essential for RNA recognition, conceptually implicating TLR8

Next, we analyzed involvement of endosomal functionality in the recognition of Sa19 and derivative ORNs. 3ddi THP-1 cells were incubated with specific endosomal inhibitors. Cytochalasin D, a cell-permeable alkaloid mycotoxin, inhibits actin-polymerization mediating endocytosis (Rubtsova et al. 1998). Chloroquine was discovered in 1934 and has been widely applied as prophylactic and therapeutic drug against malaria since 1947 (Solomon and Lee 2009). It inhibits maturation of the endolysosome and endosomal TLR function by blocking endosomal acidification (Ma et al. 2012). Tlr79i (DV056) is an oligodeoxynucleotide (ODN) antagonizing TLR7 and -9 as well as TLR8 function (Kader et al. 2013). We treated 3ddiTHP-1 cells either with cytochalasin D, chloroquine or Tlr79i and subsequently challenged them with LPS (TLR4), ssRNA40 (RNA40, TLR7 and TLR8) as well as BtmtD3_4 for 16 h. While LPS activated the cells independently of endosomal inhibition, TNF induction upon challenge with RNA40 or BtmtD3_4 was broadly diminished after inhibitor pretreatment (Fig. 38).

Chloroquine and Cytochalasin D abrogated cell activation upon RNA40 challenge. The response to P3C and LPS challenge was not affected (Fig. 39 A). Chloroquine abrogated PBMC responsiveness to BtmtD3_4 as well (Fig. 39 B).

Figure 38: Inhibition of endocytosis or function of endosomal TLRs blocks the recognition of BtmtD3_4 and Sa19 in 3ddi THP-1 cells. 3ddi THP-1 cells were pretreated with Cytochalasin D (1 µg/ml), chloroquine (5 µg/ml) or Tlr79i (10 µM) for 1 h or left untreated. Subsequently cells were challenged with LPS (0.1 µg/ml) or transfected with ssRNA40 (5 µg/ml), BtmtD3_4 or Sa19 (50 pmol per 200 µl), complexed with LyoVec, pL.A or L2K, respectively. Supernatants were sampled for ELISA 6 h post challenge. The graph shows mean ± SD, n = 3.

Unc93B1 shuffles endosomal TLRs from the ER to the endosome. THP-1 cells deficient for Unc93B1 (Unc93b1<sup>−/−</sup>) were provided by V. Hornung (Bonn). A frameshift mutation has been introduced into the Unc93b1<sup>−/−</sup> orf via targeted application of the CRISPR/Cas9 system, resulting in loss of functional
Results

Unc93B1 expression (Schmid-Burgk et al. 2014). We differentiated Unc93b1\(^{-/}\)-THP-1 cells as well as unaltered parental cells with PMA (3ddi) and challenged them. P\(_2\)C induced proinflammatory cytokine production independently of Unc93B1 expression in both genotypes. However, Unc93b1\(^{-/}\)-3ddiTHP-1 cells remained silent upon ORN challenge (Fig. 40 A).

We also transfected total, 23S and 16S rRNA from *S. aureus* and *E. coli* into parental and Unc93b1\(^{-/}\) -3ddiTHP-1 cells. As expected, the latter largely failed to respond. Furthermore, Gram-positive and Gram-negative bacterial RNA were equally immune stimulatory (Fig. 40 B). Total RNA of *S. aureus* and *E. coli* induced TNF production in Unc93b1\(^{-/}\)-3ddiTHP-1 cells, albeit to a lower degree than in parental cells.

Figure 40: *Unc93B1*\(^{-/}\)-3ddiTHP-1 cells fail to respond to specific bacterial/mitochondrial ribosomal RNAs. Cytokine production of 3ddiTHP-1 cells transfected with ORNs (A) or bacterial RNA preparations (B) (P\(_2\)C (1 µg/ml), RNA40 (5 µg/ml transfected with LyoVec), Sa19 or BtmtD3 4 (50 pmol per 200 µl each, transfected with L2K or pLA, respectively), R848 (10 µg/ml) *S. aureus* or *E. coli* total RNA (1 µg/well), 23S or 16S rRNA (600 ng/well)). The bacterial RNAs were complexed with L2K (Lipofectamine 2000). Supernatants were analyzed by ELISA 16 h after challenge. The graphs show mean ± SD, n = 3.

A possible contamination of *E. coli* total RNA with LPS would activate both parental and Unc93b1\(^{-/}\) -3ddiTHP-1 cells (as seen in Fig. 40 B). Polymyxin B (PmnB), a peptide antibiotic, binds to LPS thereby permeabilizing the outer membrane. PmnB bound LPS is not recognized by TLR4 (Cardoso et al. 2007). In contrast to 23S and 16S rRNA purified from agarose gel, total RNA of *E. coli* was not further purified and thus treated with PmnB. Application of PmnB did not diminish the stimulatory activity of the *E. coli* total RNA, excluding an LPS contamination. Furthermore, this experiment included chloroquine pretreatment of PBMC\(_s\), strongly decreasing cell activation upon bacterial RNA challenge. The response to RNA40 or ORNs was completely abrogated. HiSa or LPS control challenges in contrast, induced cell activation independent of endosomal function and LPS was blocked by PmnB (Fig. 41).
Human TLR8 is a functional homolog of murine TLR13 with broad specificity

Figure 41: Polymyxin B incubation of Gram-negative bacterial RNA ruled out cell activation by contaminating LPS. PBMCs were pretreated with chloroquine (5 mg/ml) or left untreated. *S. aureus* and *E. coli* total RNA or LPS were incubated with PmnB (50 µg/ml). PBMCs were challenged with hiSa (10^8 cfu/ml), RNA40 (5 µg/ml transfected with LyoVec), pure and PmnB-treated LPS (0.1 µg/ml), PmnB only, BtmtD3_4 or Sa19 (50 pmol per 200 µl each, transfected with L2K or pLA, respectively). *S. aureus* or *E. coli* total RNA (1 µg/well, untreated and PmnB-treated), 23S RNA (600 ng/well). Bacterial RNAs were complexed with L2K (Lipofectamine 2000) for transfection. Supernatants were sampled for ELISA after 16 h. The graph shows mean ± SD, n = 3.

Challenge of *Unc93bl-/-*3ddiTHP-1 cells with hiSa or hiEc containing all bacterial components induced cell activation (Fig. 42). We incubated *Unc93bl-/-*3ddiTHP-1 cells with TLR2 blocking mAb (T2.5) prior to challenge demonstrating involvement of TLR2. Responsiveness to P,C as well as to hiSa and hiEc was inhibited by TLR2 blockade (Fig. 42). Accordingly, a lack of endosomal TLR function renders THP-1 cells largely unresponsive to bacterial challenge and narrows the THP-1 cell stimulatory PAMPs down to nucleic acids and TLR2 ligands.

Figure 42: Bacteria driven THP-1 cell activation largely depends upon endosomal TLR and TLR2 function. *Unc93bl-/-*3ddiTHP-1 cells were treated with mAb T2.5 (20 µg/ml) if indicated and challenged with hiSa or hiEc (10^8, 10^7, 10^6 cfu/ml; triangle). Supernatants were collected after 16 h for ELISA. The graphs show mean ± SD; **, p ≤ 0.01; n=3.

### 3.3.4 TLR8 recognizes bacterial and mitochondrial RNA in contrast to TLR13 which only recognizes one segment in bacterial 23S rRNA

The previous results suggested TLR8 involvement in bacterial or mitochondrial rRNA derived ORN sensing in human cells, which do not express TLR13. Therefore, we performed gain- and loss of function analyses.

We transfected HEK2393 cells with a human (h)TLR8 expression plasmid (or an empty vector as control) in combination with a plasmid encoding a luciferase reporter gene coupled to the NF-κB target promotor. Sa19 and to a higher degree BtmtD3_4 challenge induced NF-κB promoter driven luciferase activity in hTLR8- HEK293 cells (Fig. 43 A). *S. aureus* or *E. coli* total RNA or RNA40 (TLR8 ligand) induced cell activation as well (Fig. 43 A). Notably, over expression of nucleic acid sensors TLR3, -7 and -9 either alone or in combination did not mediate HEK293 cell responsiveness to ORN challenge (data not shown).
Results

Figure 43: TLR8 over expression in HEK293 cells conferred responsiveness to Sa19 and BtmtD3_4. HEK293 cells were transfected with an empty vector (vector) or hTLR8 expression plasmid (hTLR8, 30 ng/well) and a plasmid coding for a NFκ-B-promoter coupled luciferase. After 16 h, these cells were transfected with RNA40 (5 µg/ml), Sa19 or BtmtD3_4 (100 pmol per 200 µl each, complexed with L2K or pLA, respectively) as well as total RNA (1 µg/200 µl, complexed with L2K) from S. aureus (Sa) or E. coli (Ec). The cells were lysed for luciferase activity measurement after 16 h. The graph shows mean ± SD, n = 3.

Thereupon, we applied TLR8 deficient (Tlr8⁻⁻) THP-1 cells generated by CRISPR/Cas9 technology (V. Hornung, Schmid-Burgk et al. 2014). Tlr8⁻⁻ and parental 3ddiTHP-1 cells responded to P, C challenge. The former, however, lacked responsiveness towards confrontation with BtmtD3_4 or Sa19. Synthetic TLR8 ligands R848 and RNA40 also failed to activate Tlr8⁻⁻-3ddiTHP-1 cells (Fig. 44 A). Their responsiveness to S. aureus or E. coli total RNA challenge was merely decreased, as if other PRRs are also involved in total RNA recognition. Lack of TLR8 expression resulted in impaired responsiveness to purified 23S or 16S rRNA challenge (Fig. 44 B).

Figure 44: Recognition of bacterial/mitochondrial ribosomal RNA is abrogated in Tlr8⁻⁻ 3ddi-THP-1 cells. Responsiveness of parental or Tlr8⁻⁻-3ddi THP-1 cells 16 h upon challenge with ORNs (A) or bacterial RNA fractions (B). Challenges were applied as follows: P, C (1 µg/ml), R848 (10 µg/ml), RNA40 (5 µg/ml), total RNA (1 µg per 200 µl) as well as S. aureus or E. coli 23S and 16S rRNA (600 ng per 200 µl, complexed with L2K). Supernatants were analyzed by ELISA. The graphs show mean ± SD, n = 3.

Parental and Tlr8⁻⁻-3ddiTHP-1 cells were equally activated by hiSa or hiEc challenge, whereas Loxorbine (TLR7 and TLR8 ligand) did not induce TNF release in absence of TLR8 (Fig. 45 A). We applied T2.5 to Tlr8⁻⁻-3ddiTHP-1 cells to specifically detect TLR8 dependent cell activation (Fig. 45 B). The induction of TNF upon T2.5 treatment was decreased to a greater extent following hiSa as compared to hiEc challenge driving TLR4 activation (Fig. 45 B).
Human TLR8 is a functional homolog of murine TLR13 with broad specificity

Figure 45: THP-1 cells respond to hi bacteria in a TLR8 and TLR2 dependent manner. Responsiveness of 3ddiTHP-1 cells (A) challenged with decreasing doses (10³, 10⁴, 10⁵ cfu/ml; triangles) of hiSa or hiEc; or of Tlr8⁻/⁻ genotype (B) upon TLR2 blockade (T2.5, 20 µg/ml). Loxo, loxoribine (0.6 mM). Supernatants were sampled for ELISA after 16 h. The graphs show mean ± SD; n = 3; *, p ≤ 0.05; **, p ≤ 0.01; unpaired t-test.

We treated hiEc with RNase A next to specifically digest its ssRNA. Parental, Tlr8⁻/⁻ and Unc93b1⁻/⁻-3ddiTHP-1 cells were comparatively analyzed upon challenge with untreated or RNase A treated hiEc. RNase A treatment slightly decreased the dose dependent stimulatory capacity of hiEc in parental 3ddiTHP-1 cells. Responsiveness of Tlr8⁻/⁻ and Unc93b1⁻/⁻-3ddiTHP-1 cells to untreated hiEc challenge was reduced as compared to parental cells. However, digestion of ssRNA abrogated it dose dependently, in an even more pronounced manner in Unc93b1⁻/⁻-3ddiTHP-1 cells (Fig. 46). These results indicate TLR8 driven recognition of E. coli RNA while not excluding nucleic acid sensing by other endosomal TLRs.

Figure 46: HiEc driven activation of 3ddiTHP-1 cells depends on RNA recognition. THP-1 cells of indicated genotypes were challenged with untreated or RNase A treated hiEc (10³, 10⁴ and 10⁵ cfu/ml, triangle) as well as with P,C (0.1 µg/ml) and RNA40 (5 µg/ml). Responsiveness was measured 16 h post challenge via ELISA of supernatants. The graphs show mean ± SD, n = 3.

Human TLR7 and TLR8 sense ssRNA (Diebold et al. 2004, Heil et al. 2004). We knocked down TLR7 mRNA by specific siRNA transfection in parental or Tlr8⁻/⁻-3ddiTHP-1 cells to characterize the immune stimulatory activity of BtmtD3_4. Efficiency of siRNA treatment was analyzed by RT-PCR (Fig. 47). Recognition of R848 (an imidazoquinoline, TLR7/TLR8 agonist) and loxoribine (a guanosine analog, TLR7 ligand) were weak in Tlr8⁻/⁻ as compared to parental 3ddiTHP-1 cells. However, it was completely abrogated by TLR7 mRNA silencing in Tlr8⁻/⁻-cells. Parental 3ddiTHP-1 cell activation upon BtmtD3_4 challenge was not affected by TLR7 silencing, indicating its TLR8 specificity (Fig. 47). E. coli tRNA applied as TLR7 specific control, however, was still stimulatory upon TLR7 knockdown in parental but not in Tlr8⁻/⁻-3ddiTHP-1 cells as if tRNA was recognized by TLR8 (Fig. 47).
Results

Figure 47: TLR7 is not involved in the recognition of BmtD3\_4. Knockdown upon transfection of control (scramble) or TLR7 mRNA-specific siRNA in and cytokine release of respective 3ddiTHP-1 cells upon challenge. Rel., relative; Loxoridine (0.6 mM), R848 (10 µg/ml), E. coli tRNA (200 ng per 200 µl). Parental (left), Tlr8\(^{-}\) (right). Supernatants were analyzed by ELISA, 16 h after challenge. The graphs show mean ± SD, n = 3.

Our results thus implicate a dominance of TLR8 over other nucleic acid sensors in the detection of bacterial ssRNA.

3.3.5 Characterization of the TLR8 ligand motif

TLR7 and TLR8 bind U/G or U/A rich viral-, si-, and self-RNA sequences (Heil et al. 2004, Lund et al. 2004, Barrat et al. 2005, Hornung et al. 2005, Forsbach et al. 2008). RNA specificity of TLR8 might be guided by the U and G content of ssRNA. According to TLR8 structure analysis, two binding sites interact with degradation products of ssRNA to synergistically induce an immune response. The first site has been suggested to bind uridine mononucleosides, whereas the second site has been shown to bind to UG/UGG ssRNA motifs (Tanji et al. 2015). Identification of further Sa19 like ORNs in mitochondrial ribosomal RNAs as TLR8 stimulatory thus implicated their URR motifs as most prevalently TLR8 ligand consensus motif indicative. The *mtPTL* segment is located within the rRNA peptidyl transferase loop of the mitochondrial ribosome, conserved among specific species. HsmtD3\_4 and HsmtD4 are located in between domains 3 and 4 or in domain 1 of *H. sapiens* mitochondrial rRNA, respectively. The ORNs differ in their uridine (U) content (Fig. 48 A). We transfected respective ORNs into HEK293 cells over expressing TLR8 to measure NF-κB promoter driven relative luciferase activity and into hPBMCs to analyze the TLR8 stimulatory potential. Moreover, uridine nucleoside was admixed to potentially enhance TLR8 activation, according to previous findings (Tanji et al. 2015, Heil et al. 2004) (Fig. 48 B and C).

The stimulatory capacity of low U content ORNs such as Sa19, Sa19A7G or Sa19mA7 (UGG) substantially increased if uridine was added (Fig. 48 B and C). Notably, the ORN HsmtD1, like Sa19 containing only one U (UGA), strongly activated HEK293 cells overexpressing hTLR8 on its own – yet more strongly if uridine was admixed (Fig. 48 B). The same was true for *mtPTL* containing two Us (UA and UGG). In PBMCs however, HsmtD1 as well as *mtPTL* both induced strong immune activation independent of uridine addition (Fig. 48 C). BmtD3\_4 and HsmtD3\_4 containing four Us (UC, UAA and UUAA) activated a strong immune response no matter if uridine was admixed (Fig. 48 B and C). The capacity of bacterial total RNA to induce TNF production in hPBMCs was not enhanced by uridine addition (Fig. 48 C).
Human TLR8 is a functional homolog of murine TLR13 with broad specificity.

According to the data reported above, hTLR8 qualifies as a major Gram-positive bacteria and bacterial RNA sensor. Blockade of receptors, specifically TLR2 and TLR4, in combination with antibiotic treatment prevent septic shock upon Gram-negative bacterial infection in mice (Spiller et al. 2008). Simul-
Results

taneous blockade of TLR2 and TLR8 in human immune cells might thus inhibit their proinflammatory response to Gram-positive bacterial infection.

We incubated 3ddiTHP-1 cells with varying doses of T2.5 for 30' and challenged them with the synthetic triacylated lipopeptide Pam3CSK4 (ligand for TLR2/TLR1 dimers, Jin et al. 2007) or the diacylated Pam2CSK4 (ligand of TLR2/TLR6 dimers, Kang et al. 2009) to determine optimum conditions of TLR2 blockade. Supernatants were sampled for ELISA after 8 and 24 h (Fig. 50 A). In parallel, we preincubated PBMCs with T2.5, while challenging them with hiSa (Fig. 50 B). Both kinds of cells released higher amounts of IL-6 when sampled after 24 h of challenge as compared to 8 h. This was mirrored by the stronger inhibition of TLR2 after 24 h (Fig. 50 A and B). The reduced cytokine production in TLR2 blocked THP-1 cells was more pronounced upon P3CSK4 than upon Pam2CSK4 challenge (Fig. 50 A). T2.5 was applied at a concentration of 20 µg/ml in subsequent experiments.

![Figure 50: TLR2 blocking effect of the monoclonal antibody T2.5.](image)

(A) Parental 3ddiTHP-1 cells were pretreated with T2.5 (50 and 25 µg/ml, depicted by triangle) for 30' if indicated. Subsequently, cells were challenged with Pam2CSK4 or Pam3CSK4 (each 1 µg/ml). (B) Human PBMCs were pretreated with T2.5 (20 µg/ml) if indicated for 30'. Subsequently the cells were challenged with R848 (10 µg/ml) or hiSa (10^7 cfu/ml). The supernatants were analyzed by ELISA after 8 or 24 h. The graphs show mean ± SD, n = 3.

Next, we analyzed the impact of TLR8 on the outcome of bacterial infection. Therefore, we pretreated parental or Tlr8−/−3ddi-THP-1 cells with T2.5 (20 µg/ml) to block TLR2 before seeding them with serial dilutions of viable S. aureus or E. coli. TLR specific ligands or hi bacteria were applied as controls. The infection was stopped by addition of gentamicin (50 µg/ml) to prevent excessive bacterial growth after 1 h. Gentamicin is a broad spectrum aminoglycoside antibiotic irreversibly binding to the small ribosomal subunit thereby interrupting protein biosynthesis. The supernatants were sampled 24h post infection for ELISA (Fig. 51).

![Figure 51: Immune responsiveness to bacterial infection in 3ddiTHP-1 cells depends on TLR2 and TLR8 activity.](image)

Cytokine release of 3ddiTHP-1 cells of indicated genotypes upon TLR2 blockade (T2.5, 20 µg/ml, 30' pretreatment) and challenge with TLR ligands (P.C, 0.1 µg/ml; R848, 10 µg/ml; RNA40, 5 µg/ml), hi S. aureus or E. coli (10^7 cfu/ml) or viable bacteria (S. aureus, 10^8, 10^9 and 10^7 cfu/ml; E. coli 10^9, 10^8 and 10^7 cfu/ml, triangle). The graph shows mean ± SD; *, p ≤ 0.05; **, p ≤ 0.01; unpaired t-test, n = 3.
T2.5 application also to Tlr8<sup>-/-</sup>-3ddiTHP-1 cells did not affect TLR8 dependent recognition of R848 or RNA40. TLR2 blockade reduced the recognition of hiSa in parental and more efficiently in Tlr8<sup>-/-</sup>-3ddiTHP-1 cells. It equally strongly reduced hiEc recognition in both genotypes (Fig. 51). Cell activation by confrontation with viable bacteria was TLR8 dependent, since cytokine production was decreased in Tlr8<sup>-/-</sup>-3ddiTHP-1 cells. Blockade of TLR2 inhibited activation of parental and more profoundly of Tlr8<sup>-/-</sup>-3ddiTHP-1 cells (Fig. 51).

Next, we analyzed the responsiveness of primary human PBMCs to S. aureus or E. coli infection. We focused on TLR2 blockade (T2.5) combined with inhibition of endosomal maturation using chloroquine. PBMCs were pretreated with chloroquine (5 µg/ml) for 1h and/or T2.5 (20 µg/ml) for 30'. P,C, RNA40 and BtmtD3_4 were applied as controls for TLR2 blockade, cell viability and efficient blockade of endosomal TLRs including TLR8, respectively. Infection was performed by seeding bacteria in serial dilutions harvested during the mid-exponential growth phase. It was stopped by addition of gentamicin 1 h post infection. Supernatants were sampled after a total of 24 h infection for analysis of cytokine production (Fig. 52).

Recognition of viable bacteria was significantly decreased in PBMCs in which endosomal TLRs were blocked. Moreover, dual application of T2.5 and chloroquine completely abrogated cell activation upon infection with the lowest dose of S. aureus or the highest dose of E. coli, in respect to TNF release (Fig. 52 A). BtmtD3_4 (TLR8 ligand) like RNA40 (TLR8 and TLR7 ligand) equally activated PBMCs in absence of chloroquine (Fig. 52 A). However, BtmtD3_4, in contrast to RNA40, failed to induce IFNα (Fig. 52 B). Infection with viable bacteria induced TNF as well as IFNα release, which was abrogated upon chloroquine pretreatment (Fig. 52 A and B). These results indicate involvement of other PRRs than only TLR8 and TLR2 in recognition of bacterial infections, such as TLR7 as sole IFNα inducing TLR in human monocytes (Diebold et al. 2004, Heil et al. 2004, Hornung et al. 2005).

![Figure 52: Inhibition of endosomal maturation inhibits PBMC activation more efficiently as compared to TLR2 blockade](image-url)

PBMC responsiveness upon inhibition of endosomal function (chloroquine, 5 µg/ml, 1 h pretreatment) and/or TLR2 blockade (T2.5, 20 µg/ml, 30' pretreatment) followed by TLR ligand challenge (P,C, 1 µg/ml; RNA40, 5 µg/ml; BtmtD3_4, 100 pmol per 200 µl, transfected with pLA) or bacterial infection (S. aureus 1x10<sup>5</sup>, 5x10<sup>5</sup>, 1x10<sup>6</sup> and 1x10<sup>7</sup> cfu/ml (A), 1x10<sup>6</sup> cfu/ml (B); E. coli 5x10<sup>5</sup>, 1x10<sup>6</sup>, 5x10<sup>7</sup> and 1x10<sup>8</sup> cfu/ml (A), 5x10<sup>6</sup> cfu/ml (B); triangle, decreasing doses). The infection was terminated by addition of gentamicin (10 µg/ml) 1 h post infection. The supernatants were sampled for hTNF (A) or hIFNα (B) ELISA 24h post infection. The graphs show mean ± SD; n = 3; *, p ≤ 0.05; **, p ≤ 0.01; unpaired t-test.
Results

We extended our experimental setting to treatment and infection of whole blood in order to mimic a clinically relevant context, such as a bacterially induced sepsis in patients. Previous reports such as Coch et al. (2013) were taken into consideration. Accordingly, blood sampled in conventional tubes supplemented with heparin/EDTA as anti-coagulation agents lacks a substantial portion of stimulatory capacity. We used hirudin instead, an anticoagulant of leeches (Hirudo medicinalis). Blood was drawn into monovettes (Braun) pre-filled with hirudin (Bivalirudin, 0.5 mg/ml). It was pretreated with chloroquine (20 μg/ml, for 30’) to inhibit endosomal function and seeded into 96-well plates (200 μl per well). Subsequently, TLR ligands were applied and the blood was seeded with S. aureus or E. coli at multiple infection doses. The infection was stopped by addition of gentamicin after 1 h. 24 h post challenge the sera were sampled and analyzed for proinflammatory cytokine content (Fig. 53).

Figure 53: Inhibition of endosomal maturation in whole human blood decreases cell activation upon bacterial infection. Activity of whole blood culture upon endosome function inhibition (chloroquine, 5 mg/ml, 1 h pretreatment) and challenge with TLR ligands (P,C, 1 μg/ml; RNA40, 5 μg/ml; BtmtD3_4 and Sa19, each 100 pmol per 200 μl transfected with pLA and L2K), heat-inactivated (hiSa or hiEc, 10^7 cfu/ml) or viable bacteria (S. aureus 1x10^7, 1x10^8 and 1x10^9 cfu/ml; E. coli 1x10^7, 1x10^8 and 1x10^9 cfu/ml; triangle, decreasing doses). 24 h post infection the sera were analyzed for cytokine concentration by ELISA. The graphs show mean ± SD; n = 3; *, p ≤ 0.05; **, p ≤ 0.01; unpaired t-test.

Chloroquine inhibited RNA40, BtmtD3_4 and Sa19 driven cell activation (Fig. 53). P,C challenge strongly induced IL-6 production (Fig. 53, right), whereas TNF induction was merely mild (Fig. 53, left). The infection of whole blood with both viable S. aureus and E. coli induced an immune response. However, chloroquine inhibited TNF release more strongly upon infection with Gram-positive as compared to Gram-negative bacteria (Fig. 53, left).

Our findings from bacterial infection of whole blood were largely in line with the data collected from infection of either differentiated THP-1 cells (considered as macrophages) or isolated PBMCs. Our data identify TLR8 as a major host immune sensor of bacteria. They further indicate an anti-inflammatory potential of TLR8 blockade in acute infection. Combinatory blockade of TLR8 and TLR2, in conjunction with antibiotic therapy, might prevent bacterially induced acute sepsis syndrome.

3.3.7 Evolutionary context of TLR8 responsiveness

According to genomic and proteomic analyses carried out by D. Beisser, Sus scrofa (swine) and Macaca mulatta (macaque) lack TLR13 yet express TLR8 (Krüger et al. 2015).

Sa19, but not Sa19mA7 (N6-methylation of A at position 7) activated murine macrophages (Fig. 30 B) expressing TLR13 and a nonfunctional TLR8 (Krüger et al. 2015). In contrast, both variants of Sa19 activated human cells (as seen in Fig. 32 A) expressing functional TLR8 but not TLR13 (Krüger et al. 2015). These findings implicate that bacterial immune evasion via RNA methylation in mice is absent in humans. We transfected PBMCs of S. scrofa and M. mulatta with Sa19 and Sa19mA7. Both ORNs
activated PBMCs of *S. scrofa* or *M. mulatta* to similar degrees (Fig. 54). Like humans, swine and macaque are less specific in respect to RNA motif recognition, likely through TLR8, as compared to murine TLR13.

**Figure 54:** PBMCs of swine and macaque are activated by Sa19 and its N6-adenosine methylated variant Sa19m. Cytokine release of *Sus scrofa* and *Macaca mulatta* PBMCs upon transfection with ORN variants Sa19 and Sa19m (each 100 pmol per 200 µl, complexed with LyoVec). Supernatants were sampled for ELISA after 16 h. The graphs show mean ± SD, n = 3.
4 Discussion

4.1 Bacteria recognition by and killing capacity of murine macrophages

The antimicrobial activity of phagocytes such as macrophages and neutrophils is crucial for host innate immunity against infection. Upon recognition of pathogens like bacteria, these are engulfed and phagocytosed. Phagosomes mature to phagolysosomes upon acidification and vesicle-mediated delivery of antimicrobial effectors such as proteases, antimicrobial peptides and lysozyme (Garin et al. 2001). Harmful reactive oxygen and nitrogen species are also produced in phagolysosomes (Fang 2011). Phagocytosed bacteria find themselves in a nutrient-limited hostile situation facing an armament of bactericidal effectors. Most bacteria are rapidly disintegrated and thus killed inside phagolysosomes – unless they have evolved mechanisms to overcome the attack inside macrophages. However, it was still unclear how Gram-positive bacteria are recognized by the innate immune system and which effector mechanisms are induced following recognition and phagocytosis to efficiently kill infecting bacteria.

4.1.1 Recognition of Gram-positive bacteria depends on an endosomal TLR and TLR2 function

The importance of TLR2 in the recognition of Gram-positive bacterial derived PAMPs has been well documented. Accordingly, di- and triacylated lipopeptides as components of the Gram-positive bacterial cell envelope activate heterodimers of TLR2/TLR6 and TLR2/TLR1, respectively. Gram-positive bacterial peptidoglycan and lipoteichoic acids (LTA) have been demonstrated to activate TLR2/TLR6 heterodimers (Müller et al. 2004, Jin et al. 2007, Schwandner et al. 1999, Ozinsky et al. 2000, Takeuchi et al. 2001, Kawai and Akira 2010, Kang et al. 2009). Furthermore, it has been suggested that streptococcal pneumolysin, a cytolytic enzyme released by dead bacteria, is also recognized by TLR4 (Malley et al. 2003).

In this work I could demonstrate that bone marrow derived macrophages from mice lacking MyD88/TRIF expression were significantly impaired in killing the infecting S. pneumoniae D39. Production of nitric oxide or proinflammatory cytokines was absent from these cells (Fig. 5), indicating involvement of TLRs (TIR domain containing PRRs) in sensing of these Gram-positive bacteria. Surprisingly, macrophages from mice lacking endosomal nucleic acid sensing receptors TLR3, -7 and -9 (Tlr379-), or from mice additionally lacking TLR2 and TLR4 (Tlr23479-) cleared streptococci from their supernatants almost as efficiently as macrophages from wt controls (Fig. 7 and Fig. 8). Accordingly, production of proinflammatory cytokines or NO was not impaired in Tlr23479- macrophages. They even produced increased amounts of IL-6 and NO as compared to wt cells. TLRs recruit common adaptor molecules, such as MyD88, and trigger respective signaling pathways upon ligand interaction to induce proinflammatory immune responses (see section 1.2.5.b). Possibly, a remaining receptor activated in Tlr23479- cells recruits increased amounts of signaling molecules, thereby inducing an even
Bacteria recognition by and killing capacity of murine macrophages

stronger immune response as compared to wt cells. In Tlr23479+/ cells these remaining functional TLRs include TLR5, -8, -11, -12 and -13. Hence, macrophages from 3D/Tlr2+/ mice were infected, lacking function of all endosomal TLRs (TLR3, -7, -8, -9, -11, -12 and -13), due to a defective Unc93B1 chaperone, as well as expression of TLR2. 3D/Tlr2+ cells were largely impaired in bacterial killing, proinflammatory cytokine and NO production (Fig. 9). An additional TLR4 deficiency (3D/Tlr24-) did not intensify the observed phenotype (Fig. 10) indicating involvement of an endosomal TLR beyond TLR3, -7 and -9. It also hints at a minor involvement of TLR4 in the recognition and consequent killing of infecting S. pneumoniae. Taking into account the observed phenotypes of S. pneumoniae killing from wild type, Tlr23479+, and 3D/Tlr2+ macrophages together with our data collected on TLR13 and its ligand in the second part of this work, TLR13 can most likely be suggested as the major PRR that senses infecting Gram-positive bacteria and induces bacterial killing.

TLR13+/- mice would be advantageous to validate this assumption, although cross breeding with Tlr23479+/- mice would be necessary to gain further insight. Another approach could include blockade of TLR2 with the mAb T2.5 in TLR13+/- macrophages. Infection of 3D mice with functional TLR2 would clarify the specific role of TLR2 activation in the recognition of S. pneumoniae.

4.1.2 Involvement of effector mechanisms in bacterial killing

Upon recognition of infecting bacteria by innate immunity, invading pathogens are killed. The complement system is one innate immune defense system clearing pathogens from the host organism. It encompasses about 30 serum proteins and cell membrane receptors. Their interactions enable opsonization, agglutination and membrane rupture of pathogens as well as chemotaxis to attract immune cells such as macrophages and neutrophils to the site of infection (Serruto et al. 2010). We excluded a possible involvement of the complement system in macrophage bacterial killing by comparative analyses upon supplementation of BMM culture medium with native or heat inactivated fetal calf serum (FCS). Heat denaturation of complement components within the serum did not abrogate the bactericidal capacity of wt BMMs (data not shown).

Reactive oxygen species (ROS) and nitric oxide (NO) are broadly considered as major effector mechanisms in phagocyte bacterial killing. Low levels of ROS however, regulate cell growth, apoptosis, immune responses, tumorigenesis and prevent tissue colonization by microorganisms. High ROS production during an acute infection facilitates pathogen clearance, signaling and immune responses but also results in cellular death (Circu and Aw 2010, Forman et al. 2010, Kowaltowski et al. 2009, Sareila et al. 2011). A NADPH oxidase complex associated with the mitochondrial electron transport chain produces ROS. NLRX1, an intracellular Nod-like receptor, is localized in the proximity of mitochondria (Spooner and Yilmaz 2011). It triggers ROS production upon pathogen contact, which has been shown to be critical in defense against infection with Shigella (Tattoli et al. 2008). The phagocytic oxidase (Phox) also rapidly generates ROS (respiratory burst) upon infection. It is localized within membranes of phagosomes and endosomes as well as in the cell membrane of professional phagocytes. It is a protein complex of cytosolic phox subunits p40, p47 and p67 as well as membrane bound subunits p22 and gp9. The latter form the catalytic superoxide-generating flavocytochromeo558 (Jackson et al. 1995). Phox pumps electrons into the phago-/endosome reducing oxygen to superoxide anion (O2−)
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while also decreasing the phagolysosomal pH (Fang 2011). Superoxide anions reduce ferric iron (III) and undergo dismutation to hydrogen peroxide (H₂O₂). H₂O₂ oxidizes ferrous iron (II), thereby forming highly reactive hydroxyl radicals (OH·). Free oxygen radicals are highly toxic. Mechanisms of bacterial clearance via ROS activity include DNA base oxidation causing potentially lethal mutations, lesions and strand breaks (Fang 2011), as well as mobilization of iron from iron-sulfur clusters, thereby impairing crucial metabolic pathways in bacteria (Imlay 2006). Activation of ROS production via Phox is essential for fighting microbial invasion, such as by Listeria monocytogenes, Salmonella enterica, Staphylococcus aureus, Serratia marcescens and Escherichia coli (Fang 2011, Spooner and Yilmaz 2011). A variety of microorganisms have evolved mechanisms to withstand ROS inside of host cells and thus cause persistent infections. Some limit ROS production, e. g., by inhibiting assembly of the NADPH oxidase complex or by directly modulating ROS levels (Spooner and Yilmaz 2011). Salmonella for example express catalases and hydroperoxide reductases to scavenge and deactivate H₂O₂ (Slauch 2011). Human individuals with inherited deficiencies of Phox components show enhanced susceptibility to infection, such as in chronic granulomatous disease (CGD) (Fang 2011). We showed, that BMMs from p47phox−/− deficient mice (p47phoc−) are not impaired in killing Streptococcus pneumoniae D39 or producing proinflammatory cytokines upon infection (Fig. 11). Therefore we exclude ROS production to be a major bacterial killing effector mechanism employed by BMMs in vitro. Possibly, S. pneumoniae D39 express ROS resistance mediating genes.

NO is a toxic radical, synthesis of which results from L-arginine metabolism by nitric oxide synthases such as the inducible nitric oxide synthase (iNOS). Resting cells do not express iNOS. Cytokine or LPS challenge induces iNOS expression in phagocytes (Nussler and Billiar 1993). iNOS has been implicated as critical bacteria killing effector molecule, such as in Listeria monocytogenes infection (Ohya et al. 1998, Wheeler et al. 1997). According to our data, a deficiency of iNOS in BMMs correlated with a barely detectable decrease in bacterial killing and cytokine production upon infection with S. pneumoniae D39 (Fig. 12), implicating a rather minor role of NO in S. pneumoniae D39 killing.

In line with our findings, IFNγ primed peritoneal macrophages have been shown to release large amounts of NO upon LPS challenge. However, inhibition of NO production has not impaired their L. monocytogenes killing capacity (Ohya et al. 1998). This finding also excludes NO as primary mediator of bacterial killing in macrophages. NO together with superoxide anion (·O₂⁻) forms highly reactive peroxynitrite (ONOO⁻) (Slauch 2011). ONOO⁻ is capable of oxidizing sulphydryl groups (SH), lipids and DNA (Chakravortty and Hensel 2003) and is bactericidal towards Rhodococcus equi, demonstrating a ROS and NO synergy. Thus, a two-step model for efficient killing of R. equi by activated macrophages has been suggested. In a first step macrophages are activated by IFNγ and TNFα inducing iNOS activity. Phagocytosed bacteria, as second signal, stimulate a respiratory burst. High levels of ·O₂⁻ interact with NO to form ONOO⁻ efficiently killing R. equi (Darrah et al. 2000). ONOO⁻ mediated bactericidal activity of macrophages has also been shown for S. enterica and E. coli infections (Chakravortty and Hensel 2003). Antimicrobial peptides (AMP) are known to disintegrate the cell envelopes of bacteria and allow access of phagocyte degrading enzymes. Possibly, the activity of AMPs is also required to facilitate ROS and NO access to their targets inside pathogens. Alternatively, ROS mediated damage could also render bacteria susceptible to other antimicrobial effectors (Slauch 2011).
This could explain why single knockouts of either iNOS or p47/Phox did not significantly impair BMMs from killing bacteria. This indicates, that neither NO nor ROS production alone were the singular effector mechanisms mediating bacterial killing in murine BMMs. A double deficiency of both, the production of NO via iNOS and the production of ROS via Phox would probably not render the BMMs more susceptible to bacterial infection and decrease their bacterial killing capacity to a larger extent. However, it is most likely that other effector mechanisms such as the expression and secretion of antimicrobial peptides may also play a role in efficient killing of invading bacteria in host cells.

4.1.3 Bacterial killing independent of direct bacteria-macrophage contact - a potential role of antimicrobial peptides

As we observed, supernatants from TLR-activated BMMs killed or inhibited growth of *S. pneumoniae* D39 in the absence of cells. Thus direct bacteria-host cell contact was obsolete. Supernatants from macrophages that had been challenged either with single TLR ligands such as P,C (TLR2), LPS (TLR4) or Sa19 (TLR13), or with suspensions of hiSa or hiEc were bactericidal or bacteriostatic towards viable *S. pneumoniae* D39 (Fig. 13). The supernatants from hiSa or P,C challenged BMMs exerted the most efficient bacterial killing. We suggest that further extracellular effector mechanisms such as antimicrobial peptide release could also play a role in killing of infecting *S. pneumoniae* by murine macrophages.

Antimicrobial peptides (AMP) destroy bacteria, fungi and viruses. They are one element of the antimicrobial activity of phagocytes, inflammatory body fluids and epithelial secretions (Hancock and Diamond 2000, Zasloff 2002, Bals 2000). As a diverse group of molecules AMPs are categorized by homologous structural motifs such as amino acid composition and secondary structure. They are subdivided into anionic peptides, linear cationic α-helical peptides, cationic peptides enriched for specific amino acids (e.g., proline or arginine), anionic and cationic peptides containing cysteine to form disulfide bonds as well as peptide fragments of larger proteins (Brogden 2005, Ganz et al. 1985). AMPs interact with bacterial membranes creating ion-permeable channels via perturbation, leading to loss of membrane function, bacterial disintegration and killing (Brogden 2005). Initial binding depends on electrostatic interactions between positively charged AMPs and negatively charged pathogen surfaces (Bals and Wilson 2003). Other mechanisms of AMP activity include inhibition of protein and/or RNA synthesis upon obtaining access to the bacterial cytosol (Bals and Wilson 2003).

AMPs have also been shown to boost, inhibit or complement cellular functions such as chemotaxis, apoptosis, gene transcription and cytokine production, thereby also promoting bacterial clearance although not via direct killing (Finlay and Hancock 2004, Salzet 2002). AMPs also suppress bacteria-induced cytokine production (anti-inflammatory) and stimulate wound healing and angiogenesis (Brown and Hancock 2006). They contribute to homeostasis maintenance, particularly in commensal rich compartments like the intestine (Muniz et al. 2012). Their expression is tightly controlled by engagement of PRRs and their impairment is linked to abnormal host responses to infection and inflammatory bowel diseases (IBD) (Muniz et al. 2012). Accumulating evidence suggests regulation of AMP production by TLRs. *MyD88*− mice express Paneth cell-derived α-defensins and RegIIIγ at decreased levels (Brandl et al. 2007, Gong et al. 2010). AMP production also depends on TLR-activity. For instance, in vivo stimulation of TLR3 (poly I:C) and TLR9 (CpG-ODN) triggers rapid release of AMPs.
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by Paneth cells (Rumio et al. 2012). It has been shown that β-defensins are up-regulated in intestinal epithelial cells (IEC) following TLR2, -3, and -4 activation (Vora et al. 2004, Omagari et al. 2011).

The contribution of AMPs to antibacterial immunity and bacterial killing is likely to be of importance for the observed property of supernatants of TLR-activated BMMs to kill S. pneumoniae D39. Analysis of published gene expression profiles of murine macrophages infected with bacteria or challenged with TLR ligands might in future enable the identification of AMPs involved in S. pneumoniae D39 intra- as well as extracellular killing. Furthermore, AMP specific Western blotting or protein profiling might identify them in the supernatants and lysates of infected mBMMs. This would enable detection of AMP production and secretion. However, AMPs are known to be active at very low concentrations (Bals and Wilson 2003). Besides transcriptome profiling via gene expression array, analysis of AMP mRNA accumulation upon infection via RT-PCR could also enable elucidation of specific AMP contributions to bacterial killing of macrophages. This could be further tested in the context of specific TLR deficient mice to shed light on the mechanisms of AMP regulation upon pathogen recognition in macrophages. Analysis of AMP deficient BMMs would validate any respective implications.

4.2 TLR13 - an endosomal receptor recognizing a specific sequence within bacterial 23S rRNA

Sepsis, in most cases induced by bacterial infection, poses an immense clinical problem. Extensive application of antibiotics has led to development of resistances impeding antibacterial therapy. Sepsis caused by Gram-negative bacteria has already been intensively investigated. Its pathology is probably induced by activation mainly of TLR2 and TLR4. An experimentally induced septic shock in mice has been prevented by blockade of these TLRs (Spiller et al. 2008). The major PRRs sensing Gram-positive bacteria were not yet as well characterized. TLR2 is generally considered as the major Gram-positive bacteria sensor (Takeuchi et al. 2000, Yimin et al. 2013). Clinically relevant S. aureus colonize the mucosa of the respiratory tract as commensal (Peacock et al. 2001, Stokholm et al. 2014). Invasive infections of immunocompromised patients with S. aureus cause abscess, pneumonia, endocarditis and also sepsis formation (Lowy 1998). Next to TLR2, cytoplasmic PRRs were implicated as important sensors for Gram-positive bacteria. The relevance of the TLR family of receptors in sensing Gram-positive bacteria was validated by a study from our group. MyD88 dependent, but IL-1 receptor independent responsiveness of murine macrophages to confrontation with Gram-positive bacteria has been observed (Ferstl 2009). Furthermore, single stranded (ss)RNA has been implicated as PAMP (Deshmukh et al. 2011).

4.2.1 An endosomal TLR recognizes ssRNA of Gram-positive bacteria

Unc93B1-mutant (3D) macrophages, lacking ER-endosome TLR trafficking and thereby endosomal TLR function (Tabeta et al. 2006, Brinkmann et al. 2007), also lacking TLR2 (3D/Tlr2−/−) and TLR4 (3D/Tlr24−/−) did not react to challenge with hi S. aureus or B. subtilis. We thus considered involvement of an endosomal TLR in Gram-positive bacteria recognition (Fig. 16). The response of 3D/Tlr2−/− cells
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to hi E. coli was due to LPS. Application of Tlr23479− mice (de facto lacking function of TLR1, -2, -3, -4, -6, -7 and -9) allowed exclusion of the nucleic acid sensing receptors TLR3 (dsRNA), TLR7 (ssRNA) and TLR9 (Cpg DNA) (Chi and Flavell 2008). While all endosomal TLRs are nonfunctional in 3D mice, Tlr23479− mice carry unimpaired TLR8, -11, -12 and -13. TLR11 and TLR12 have been excluded as receptors of Gram-positive bacteria, since over-expression of either one in HEK293 cells did not confer responsiveness to hiSa (Oldenburg 2015). Also, sensing of profilin by TLR11 and TLR12 has been described (Raetz et al. 2013, Yarovinsky 2014, Mathur et al. 2012, Andrade et al. 2013). According to these findings, the yet uncharacterized endosomal receptor TLR13 remained as candidate receptor molecule, even though it has been linked to the recognition of Vesicular stomatitis virus a (-)ssRNA virus (Shi et al. 2011). In line with our BMM data, only 3D/Tlr24− mice survived in vivo confrontation with hiSa, whereas wt and Tlr23479− mice succumbed to septic shock like syndrome (Fig. 16). The results from 3D macrophages are affirmed by the unresponsiveness of Tlr2− macrophages to hiSa, in which endosomal function is interrupted by bafilomycin treatment (data not shown, Oldenburg et al. 2012). Unexpectedly, Tlr23479− macrophages responded like wt cells to hiSa challenge, unless the bacterial lysates were subjected to ssRNA specific RNase A digest. TLR2 activation was not impaired by this treatment. RNase A abrogated the Tlr2− BMM stimulatory activity of hiSa, but not the incubation with dsRNA specific RNase III or DNA specific DNase I (Fig. 17). These findings qualify ssRNA as immune stimulatory PAMP produced by Gram-positive bacteria, in line with a recent report (Deshmukh et al. 2011). Our data suggest another endosomal RNA sensor beyond TLR3 and TLR7 in the recognition of hiSa.

By the way, murine TLR8 is considered inactive (Forsbach et al. 2008). Further studies however described a key function of TLR8 in the inhibition or activation of TLR7 (Cervantes et al. 2012, Tran et al. 2015). Thus, the absence of TLR8 leads to increased expression of TLR7 in macrophages, DCs and monocytes, therefore to an increased risk to develop the autoimmune disease Lupus erythematoses. The over expression of murine TLR8 in HEK293 cells does not confer sensitivity for hiSa or their RNA (Oldenburg 2015).

4.2.2 TLR13 recognizes a specific sequence within bacterial 23S ribosomal RNA

The stimulatory capacity of bacterial ssRNA that activated Tlr23479− BMMs had to be narrowed down to identify the specific ligand motif. HPLC, carried out by S. Bauer, enabled separation of high-(hmw) and low-molecular weight (lmw) RNA fractions. The hmw RNA including 23S and 16S rRNA as well as hmw transcripts of Gram-positive and Gram-negative bacteria activated Tlr23479− macrophages, while their lmw RNA fractions lacked stimulatory activity (Fig. 18). S. aureus hmw RNA also triggered immune activation of TLR13 expressing cDCs (Oldenburg et al. 2012). 5′-dephosphorylated S. aureus total RNA activated wt and Tlr23479− macrophages equally well, while 3D/Tlr24− cells were refractory. Untreated or dephosphorylated E. coli total RNA did not activate cytokine production in Tlr23479+ or 3D/Tlr24− BMMs. Digestion of the large rRNAs (23S and 16S), via 5′-monophosphate specific exonuclease activity leaving mRNA intact, as well as small RNA species abrogated the stimulatory potential of S. aureus total RNA. Removal of the large rRNAs from both S. aureus and E. coli total RNA by magnetic bead mediated precipitation rendered the remaining en-
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riched mRNA (together with 5S rRNA and tRNA) unstimulatory, whereas the purified large rRNAs triggered activation of Tlr23479+ BMMs (Fig. 19). These findings suggested that a subspecies of large bacterial rRNAs activates macrophages and cDCs in a MyD88-dependent manner. Separately purified 23S but not 16S rRNA activated Tlr23479+ BMMs (Fig. 20). Successive fractionation of bacterial total RNA into RNA subspecies and their subsequent transfection into Tlr23479− macrophages identified 23S rRNA as the immune stimulatory total RNA moiety in Gram-positive and Gram-negative bacteria. The 23S rRNA is recognized by an endosomal TLR that is expressed in Tlr23479− macrophages, but nonfunctional in 3D/Tlr24− cells.

Bacterial 23S RNA has a size of 2.9 kb and contains six domains (Fig. 22) (Petrov et al. 2013). Ribosomal RNAs are highly abundant and conserved and therefore used as phylogenetic markers to distinguish bacterial relationships. Important genomic regions of high conservation were inherited during evolution mediating survival of the fittest (Pei et al. 2009, Karikó and Weissman 2007). Gene products which modify rRNAs, e.g., to impose resistance to antibiotics, are phylogenetically traced back to over four million years ago (Bhullar et al. 2012). 24 known motifs of modification are localized in one of the highly conserved regions of 23S rRNA (Dunkle et al. 2010, Branlant et al. 1981). Out of these, we chose three sequences that contain an adenosine enhancing bacterial fitness or conferring antibiotic resistance when modified by methylation (Sergiev et al. 2008, Long et al. 2006, Skinner and Cundliffe 1982, Weisblum 1995, Pfister et al. 2005, Kehrenberg et al. 2005, Toh et al. 2008, Kaminska et al. 2010, LaMarre et al. 2011). Synthetic oligoribonucleotides (ORNs) of these three sequences were analyzed for their immune stimulatory potential as PAMP and TLR13 ligand (Fig. 22). "SaIII" was the only ORN that activated wt and Tlr23479− macrophages (Fig. 23). It mirrors a 23S rRNA segment containing A2085 in S. aureus and A2058 in E. coli 23S rRNA. Interestingly, its mutation or methylation mediates bacterial resistance against macrolide, lincosamide and streptogramin B (MLS) antibiotics (Vester and Douthwaite 1994, Kovalic et al. 1995, Pfister et al. 2005). We observed an even increased activation of Tlr23479− BMMs as compared to wt cells upon RNA challenge, assuming that this reflects a lack of TLRs competing for downstream signaling molecules.

MyD88 dependent yet ASC, IL1R1 and IL-18 independent recognition of Gram positive bacteria implicated involvement of a TLR or TLRs beyond TLR2. A failure of Une93B1 defect 3D macrophages to respond to Gram-positive bacterial total and 23S rRNA challenge implicated endosomal TLR involvement. Tlr23479− macrophages were activated by bacterial 23S rRNA and a synthetic analog of a specific segment of it. Nonresponsiveness of 3D/Tlr24− cells on the one hand and responsiveness of Tlr23479− counterparts toward this challenge on the other hand implicated TLR13 as the specific 23S rRNA segment receptor.

4.2.3 Methyltransferases mask the 23S rRNA from being recognized by the immune system as well as mediate MLS antibiotic resistances

SaIII contains an adenosine at position 6 representing A2085 in S. aureus (Fig. 22) localized in the active center of the bacterial ribosome, called peptidyl transferase loop (PTL). Newly synthesized nascent polypeptide chains leave the ribosome through this tunnel structure. N6-methylation of A2085 within 23S RNA of S. aureus is catalyzed by the erythromycin resistance mediating methyltransferases ErmA, B or C. Such modification of the 23S rRNA alters its conformation and prevents binding of
TLR13 - an endosomal receptor recognizing a specific sequence within bacterial 23S rRNA

macrolide, licosamide or streptogramin B (MLS) antibiotics thereby conferring specific resistance (Lai and Weisblum 1971, Small et al. 2013, Skinner and Cundliffe 1982, Weisblum 1995). Macrolide antibiotics such as erythromycin inhibit protein biosynthesis through binding to the PTL. Binding of the antibiotic to the A2085 containing segment blocks the tunnel through which the nascent peptides exit the ribosome. This causes ribosome stalling, pausing of protein biosynthesis, and ribosomal decay (Tenson and Ehrenberg 2002). Erythromycin challenge induces ErmC expression in ermT strains (Bechhofer and Zen 1989, Weisblum 1995, Eady et al. 1993) and consequent 23S rRNA segment SaIII methylation.

Since the discovery of pathogenic methicillin resistant S. aureus (MRSA), the description of new antibiotic resistant strains has not come to a halt (David et al. 2012). MRSA usually also express MLS resistance conferring enzymes. We used six clinical MRSA isolates displaying various resistance phenotypes, including erythromycin resistance, to investigate whether rRNA modifications induced in antibiotic-resistant bacterial strains by antibiotic treatment have an impact on the immunostimulatory potential of rRNA. MRSA isolates grown in the presence of erythromycin largely lacked the capacity to activate Tlr23479+ macrophages. On the contrary, the same MRSA isolates grown in erythromycin-free medium activated wt as well as Tlr23479+ cells like erythromycin sensitive hiSa. Accordingly, total RNA and 23S rRNA from MRSA grown in presence of erythromycin failed to stimulate Tlr23479+ macrophages. Absence of erythromycin preserved the immune stimulatory potential of 23S rRNA of the same strain (Fig. 26). Tlr23479+ mice produced lower amounts of serum cytokines early (2h) upon infection with MRSA when the bacteria had been grown in the presence of erythromycin (Fig. 27). Later upon infection (16 h) their serum cytokine production increased independent of antibiotic presence during bacterial growth (data not shown, Oldenburg et al. 2012). An explanation could be the absence of erythromycin within the host during the infection. This could down-regulate the expression of resistance conferring methyltransferases, thereby restoring the immune stimulatory capacity of bacterial 23S rRNA. Our data demonstrate that an erythromycin-driven RNA modification mediates "camouflage" of bacterial 23S rRNA from innate immune recognition.

In S. aureus, the erythromycin resistance methyltransferases ErmB and ErmC specifically catalyze N6-methylation of adenosine 2058 in 23S rRNA, the target binding site of erythromycin, thereby conferring MLS group antibiotic resistance. The sequence of our synthetic ORN SaIII is highly conserved in 99.4% of all sequenced bacterial genomes (Pfister et al. 2005). E. coli however expresses a mutated variant of ErmC, which cannot mediate erythromycin resistance (Thakker-Varia et al. 1985). Hence, we investigated 23S rRNA of E. coli. It is alsoTLR2, -3, -4, -7 and -9 independently immune stimulatory (Fig. 20). We introduced erythromycin resistance into susceptible E. coli via transformation with plasmids encoding either ermB or ermC, which we cloned from an erythromycin resistant MRSA isolate. 23S rRNA from E. coli expressing ErmB or ErmC did not induce Tlr23479+ macrophage activation, given that they were grown in presence of erythromycin. Also, over expression of ErmB in Gram-positive B. subtilis susceptible to erythromycin not only conferred resistance, but also ablated the stimulatory activity of their 23S rRNA (Fig.29). Our data indicate that resistance to MLS group antibiotics like erythromycin, mediated by site-specific methylation targeting A2085 in S. aureus and A2058 in E. coli 23S rRNA, had a terminating impact on the ligand-receptor interaction.
Discussion

4.2.4 TLR13 recognizes a 10 nucleotide segment of 23S rRNA

Upon identification of the stimulatory segment in bacterial 23S rRNA, we validated the specific ligand interaction with the implicated receptor, namely TLR13, by gain- and loss-of-function analyses. Ec- topic over expression of TLR13 conferred specific responsiveness of human embryonic kidney (HEK)293 cells toward the ORN SaIII (Fig. 24) and also toward heat inactivated bacteria and total RNA thereof (data not shown, Oldenburg 2015). We excluded other PRRs like CD14, TLR2, -3, -7, -8, -9, or -12 from the recognition of SaIII similarly (data not shown, Oldenburg et al. 2012). TLR13 si- lencing via siRNA-mediated suppression of TLR13 mRNA accumulation in Tlr23479−/− macrophages also impaired the recognition of SaIII (Fig. 24). Sensing of hiSa has also been strongly impaired by TLR13 silencing, although high doses of bacterial lysates activated TLR13 siRNA treated cells, indicating incompleteness of mRNA knock down (data not shown, Oldenburg et al. 2012). In line with our data, a subsequent study applying siRNA mediated receptor silencing in murine DCs also reports TLR13 dependent S. aureus total RNA recognition (Hidmark et al. 2012). Unresponsiveness to SaIII of macrophages transfected with MapK1 mRNA specific siRNA, implicated activation of TLR associated proinflammatory cytokines downstream of TLR13 via the MapK1 (ERK2) signaling pathway (Fig. 24). The data from these gain- and loss-of-function analyses validated our hypothesis, that TLR13 is a cellular receptor of SaIII, a highly conserved sequence segment within the peptidyl transferase loop of bacterial 23S rRNA. Generation and over expression of a nonfunctional TLR13 mutant in HEK293 cells also strengthened this implication (Oldenburg 2015).

Dendritic cell (DC) subsets express different sets of TLRs (Luber et al. 2010). Analysis of DC subsets from Tlr23479−/− mice (H. Hochrein) substantiated our BMM analyses results. Plasmacytoid (p)DCs not expressing TLR13 lack responsiveness to SaIII and hiSa, while classical (c)DCs expressing TLR13 were activated by the challenge (data not shown, Oldenburg et al. 2012). Investigations of Hidmark et al. (2012) as well as of Li and Chen (2012) also implicated TLR13 as the receptor for bacterial 23S rRNA, utilizing myeloid DCs and siRNA mediated knockdown of TLR13 as well as Tlr13−/− mice.

We further characterized the stimulatory ligand motif within SaIII of the peptidyl transferase loop of bacterial 23S rRNA. While reduction in length of SaIII (48 nt) toward Sa19 (19 nt) and Sa12 (12 nt) did not extensively impair their stimulatory activity, an ORN as short as Sa9 (9 nt) failed to activate Tlr23479−/− BMMs. The highly sequence specific ligand recognition of TLR13 could be confirmed by consecutive single substitutions of Sa12 resulting in loss of its stimulatory capacity. We therefore suggest a consensus TLR13 ligand motif of 10 nt (CGGAAGACC) within bacterial 23S rRNA. An almost simultaneous study has reported similar results suggesting a 12 nt segment as minimal stimulatory lig- and motif (Li and Chen 2012). Specific N6-adenosine methylation of the consensus motif, correspond- ing to A2085 in S. aureus 23S rRNA and mimicking Erm mediated erythromycin resistance, abrogated its TLR13 stimulatory capacity (Table 3, Fig. 30). However, this methylation driven camouflage was residue specific, since methylation of the adjacent adenosine (A2086 in S. aureus) did not corrupt TLR13 activation.

N6-methylated A2058 in E. coli, conferring erythromycin resistance and shielding from TLR13 recogni- tion, could be illustrated by us utilizing HPLC analysis of 23S rRNA from E. coli over expressing ErmB or ErmC. Our results indicate that ErmB dimethylated, while ErmC mono-methylated A2058
(Fig. 31). Accordingly, N6-methyl-adenosine was absent from 23S rRNA of E. coli over expressing TrmH a tRNA specific methyltransferase.

We considered possession of TLR13 stimulatory capacity of other pathogenic Gram-positive bacteria like Streptococcus pyogenes as well. We observed activation of Tlr23479 but not 3D/Tlr24− BMMs upon challenge with hi S. pyogenes, total RNA and 23S rRNA thereof (Fig. 21). These data as well as a recent report, showing a TLR2 and TLR13 activity dependent murine immune response to viable S. pyogenes (Fieber et al. 2015), validate our hypothesis.

Taken together, the results of this project revealed both a highly conserved natural ligand of TLR13 within bacterial 23S rRNA and a specific mechanism of antibiotic resistance also conferring immune evasion of bacteria from being recognized by the strictly sequence specific TLR13.

Consequently, blockade of TLR2 and TLR13 with simultaneous antibiotic therapy should protect mice from Gram-positive bacterial induced sepsis pathology. Bacterial adaptation to evade host immune recognition by modification, such as methylation of A2085 (S. aureus), implicates that TLR13 as species specific innate immune receptor could not largely protect the host from bacterial infections. Consequent phylogenetic analyses (in cooperation with D. Beisser) suggest that TLR13 is evolutionary old and its expression has been discontinued in certain phyla explaining the absence of TLR13 expression in primates as well as other tetrapods (Oldenburg et al. 2012).

### 4.3 TLR8 as functional TLR13 homolog in humans

Following our identification of the TLR13 ligand interaction, we set out to investigate whether humans equivalently recognize bacterial 23S rRNA. We observed PBMCs, containing all immune cells but erythrocytes and polymorphonuclear cells, responsive to hiSa and not merely to Sa19, but also to its N6-adenosine methylated variant Sa19mA7 and its mutated variant Sa19A7G (Table 3, Fig. 2, Fig. 33) the latter of which do not activate TLR13. Recognition of hiSa and Sa19 upon TLR2 blockade (by cross-reactive mAb T2.5) was unimpaired unless ssRNA was subjected to RNase A digest. Thus, Gram-positive bacterial ssRNA and TLR2 ligands are major human immune stimulatory activities within hiSa. In contrast to mBMMs, hPBMCs were activated not only by total RNA but also by all purified rRNA molecular subspecies from S. aureus and E. coli. Application of chloroquine blocked endosomal nucleic acid sensing TLR function in PBMCs and thereby abrogated cell activation upon challenge with bacterial RNA preparations (Fig. 34). Our results indicate replacement of TLR13 in humans by an endosomal PRR with broad ligand specificity. The same conclusion has been drawn by triggering human monocytes by challenge with total RNA of erythromycin resistant clinical S. pneumoniae isolates. These are either methylated at A2058 or carry a resistance conferring A2058G point mutation, which renders them non-stimulatory in murine macrophages (Eigenbrod et al. 2015). Apart from S. aureus or E. coli total RNA none of the purified rRNA subspecies induced IFNα release in PBMCs (Fig. 34). Total RNA contains tRNA, which has been shown to induce TLR7 dependent IFNα production by pDCs (Jöckel et al. 2012, Gehrig et al. 2012, Hornung et al. 2005, Heil et al. 2004). Therefore, we exclude TLR7 dependent recognition of the bacterial ribosomal RNA.
Discussion

Self-RNA antimicrobial peptide complexes and also mitochondrial DNA have been implicated as immune stimulatory danger associated molecular patterns (DAMPs) (Zhang et al. 2010, Ganguly et al. 2009). Mitochondria evolved from prokaryotes, as described by the endosymbiotic theory. They accordingly derived from chemotrophic prokaryotic Rickettsiales phagocytosed by prokaryotic Archaea. They became endosymbionts and lost most of their then redundant genetic material (Thrash et al. 2011, Williams et al. 2013). This might explain the size reduction of mitochondrial ribosomes, which contain 16S and 12S rRNA in contrast to bacterial 23S and 16S rRNA. With regard to the prokaryotic origin of mitochondria we analyzed their ribosomal RNA for Sa19 like sequence patterns. C. Chebrolu considered a segment in mitochondrial 16S rRNA of cattle (Bos taurus, Bt) and later also of human, mouse and rat sharing an identical core sequence (GGAAAGA) with bacterial Sa19. The segment was named BtmtD3_4, due to its localization in between domains 3 and 4 of the 16S rRNA. It proved to activate human PBMCs even stronger as compared to Sa19, but not murine BMMs when applied as 19-mer ORN challenge (Fig. 33 and data not shown, Krüger et al. 2015). PBMCs however did not release IFNα upon challenge with ORNs, indicating lack of TLR7 specificity in their recognition (Fig. 33 B). These results strengthened our hypothesis of a broader ligand specificity of the human ssRNA receptor. Analysis of single receptors like in knockout mice is not possible by investigation of PBMCs isolated from healthy blood donors. Yet, the rare occurrence of genomic mutations such as in MyD88 allows the analysis of TLR involvement. Individuals expressing defective mutations of MyD88 are usually prone to inflammatory infections early in life due to an impaired innate immune system (Alsina et al. 2014). PBMCs isolated from a blood sample of a three year old girl expressing a nonfunctional Glu53D MyD88 mutant (MyD88<sup>GW</sup>) failed to respond to S. aureus RNA or the ORNs Sa19 and BtmtD3_4 (Fig. 35). We therefore consider a TLR as respective PRR.

4.3.1 Human endosomal TLRs in the recognition of bacterial and mitochondrial ribosomal RNA

In order to establish a genome wide screening system for the receptor in search, we applied human cell lines for ORN responsiveness evaluation. First, immortalized B-cells and fibroblasts from individuals lacking IRAK4 or TIRAP expression (Picard et al. 2011) remained silent upon Sa19 challenge. Next, we analyzed 22 human cell lines, among them B- and T-cells, fibroblasts and monocytes (Table 2). None of these were responsive to ORN Sa19 confrontation (data not shown). Focusing on major innate immune cells like macrophages, we differentiated monocytoid THP-1 as well as U937 cells prior to ORN confrontation. While undifferentiated (undiff) and 8 days PMA differentiated (8ddi) cells remained silent, THP-1 cells differentiated for 3 days (3ddi) were responsive to Sa19 and BtmtD3_4 (Fig. 36). U937 cells however, did not produce any cytokines even when PMA differentiated (data not shown). The unresponsiveness in 8ddiTHP-1 cells is probably due to down regulation of the respective receptor after a longer differentiation period. We excluded decreased viability of 8ddiTHP-1 cells, since LPS induced similar cytokine levels in undiff, 3ddi and 8ddiTHP-1 cells. We comparatively analyzed transcriptome profiles of undiff, 8ddi and 3ddiTHP-1 by gene expression array. Among at least 2-fold induced genes in 3ddi as compared to both undiff and 8ddiTHP-1 cells we identified seven non-
cytokine-receptors (Fig. 36). We focused on TLR13-like TLR8 as Sa19 sensing PRR, implication of which could also be explained by the observed MyD88 dependence.

The choice of transfection reagent for the translocation of stimulatory ORNs into cellular endosomes was of importance. While Sa19 complexed with lipofectamine 2000 (L2K) had the strongest impact on 3ddiTHP-1 cells, BtmtD3_4 was most immune stimulatory upon complex formation with poly-L-arginine (pLA) (Fig. 37). Transfection reagents complex nucleic acids thereby neutralizing their electric charge to improve cellular uptake via endocytosis (Dean 2005). Diverse application of cationic liposomes (lipoplex), polymers (polyplex), combinations of both (lipopolyplex) or calcium phosphate illustrates the clinical significance of complex formation, e.g., in application of pharmaceuticals or introduction of nucleic acids or proteins into eukaryotic cells (Shabani et al. 2010). Enzymatic degradation, intake efficiency and intracellular biological half-life are important parameters to be considered. The latter can be enhanced by addition of sugar or phosphate residues (phosphorothioate, PSO) to the nucleic acid (Furdon et al. 1989, Deleavey and Damha 2012). Lipoplexes (L2K, LyoVec, Dotap) are internalized via clathrin, while polyplexes (pLA) additionally recruit caveolin. These proteins localized in the cell membrane bind nucleic acid complexes mediating endocytosis (Shabani et al. 2010, Moghaddam et al. 2011). Transfection efficiency depends on nucleic acid integrity, successful complex formation as well as on caveolin and clathrin expression (Shabani et al. 2010).

3ddiTHP-1 cell and PBMC responsiveness to stimulatory ORNs and bacterial RNA was diminished upon application of chloroquine or cytochalasin D (Fig. 38, Fig. 39, Fig. 41). This implicates endosomal TLR involvement, particularly TLR8, in the recognition of bacterial and mitochondrial ribosomal RNA. This assumption is supported by expression analyses of B-, T-, NK-cells, plasmacytoid DCs and monocytes implicating TLR8 expression (mRNA) exclusively in monocytes (Hornung et al. 2002). The report also indicates absence of TLR3, -7 and -9 expression from monocytes, explaining the nonresponsiveness of the tested cell lines to confrontation with a TLR13 stimulatory ORN, given that TLR8 expression was up-regulated only upon differentiation of a specific monocyte cell line conferring responsiveness.

Unc93b1^−/-3ddiTHP-1 cells lacking endosomal TLR function (Schmid-Burgk et al. 2014) failed to respond towards Sa19 and BtmtD3_4. They also remained silent upon challenge with bacterial 23S and 16S rRNA (Fig. 40). Total RNA however also activated Unc93b1^−/-3ddiTHP-1 cells, albeit to a lower degree. We excluded a residual LPS contamination of the Gram-negative bacterial E. coli total RNA via application of polymyxin B (Fig. 41). Challenge of Unc93b1^−/-3ddiTHP-1 cells with hiSa or hiEc containing all bacterial components induced cell activation via TLR2 (Fig. 42). We therefore concluded that endosomal TLR function is indispensable for recognition of Gram-positive and -negative bacterial as well as mitochondrial ribosomal RNA. Partly in line with our data, a recent study also applying Unc93b1^−/-THP-1 cells reports abrogated cell activation upon S. pyogenes infection (Eigenbrod et al. 2015). However, they do not need to neutralize TLR2, which might be due to a lesser contribution of TLR2 to an immune response towards a S. pyogenes infection in humans. This, however, contradicts another report on synergistic TLR2 and TLR13 dependent immune activation by S. pyogenes in mice (Fieber et al. 2015), pointing out species-specific differences in the recognition of bacteria.
Discussion

Endosomal receptors like TLR3, -7, -8 and -9 have been ascribed with specific nucleic acid ligands. Specifically, TLR3 is activated by dsRNA and TLR9 recognizes unmethylated CpG DNA. TLR7 and TLR8 are activated by ORNs like ssRNA40 and synthetic small molecules such as imidazoquinolones (Gorden et al. 2005, Gantier et al. 2008). TLR7 and TLR8 display sequence similarity in human and mouse, in which both the genes are located on the X chromosome (Gantier et al. 2008). They are expressed cell type specific. TLR7 is predominantly expressed in pDCs and to a lower degree in B-cells and monocytes. TLR8 in contrast, is mainly expressed in monocytes and macrophages as well as myeloid DCs (Hornung et al. 2002, Gantier et al. 2008, Alexopoulou et al. 2012). However, compiled transcriptome data together with other studies have confirmed significantly lower expression of TLR7 in monocytes or macrophages as compared to that of TLR8 (Su et al. 2004).

We analyzed gain- and loss-of TLR8 function in order to substantiate our implication based on comparative transcriptome profiling. All Sa19 like ORNs as well as bacterial total RNAs activated HEK293 cells over expressing hTLR8 (hTLR8⁺) (Fig. 43, Fig. 48). However, ectopic expression of TLR3, -7 and -9 either alone or in combination did not confer ORN responsiveness to HEK293 cells (data not shown, Oldenburg 2015). Considering murine TLR8 as nonfunctional (Jurk et al. 2002, Forsbach et al. 2008), we observed activation of hTLR8⁺ murine RAW264.7 macrophagoid cells upon BtmtD3_4 challenge. Sa19 however, inherently induced cell activation independent of hTLR8 over expression, probably due to endogenous TLR13 activity (data not shown, Krüger et al. 2015).

Moreover, we confirmed exclusive TLR8 recognition of bacterial and mitochondrial ribosomal RNA in TLR8 deficient (Tlr8⁻) THP-1 cells. Total bacterial RNA recognition, in contrast, was largely operative in these cells (Fig. 44) indicating involvement of other PRRs besides TLR8. Responsiveness of Tlr8⁻ THP-1 cell towards hiSa and hiEc was unimpaired as compared to parental THP-1 cells, but largely TLR2 driven (Fig. 45). This observation was more profound regarding hiSa than hiEc, probably due to hiEc driven TLR4 activity. Accordingly, ssRNA digest of hiEc dose dependently inhibited cell activation in Tlr8⁻ and more so in Unc93b1⁻ THP-1 cells (Fig. 46). Furthermore, siRNA mediated knockdown of TLR8 in parental THP-1 cells abrogated sensitivity for BtmtD3_4, confirming our findings from a genomic TLR8 knockout (data not shown, Krüger et al. 2015). Others recently also reported siRNA mediated TLR8 mRNA silencing to show TLR8 but not TLR7 dependence of cytokine production in monocyte derived macrophages upon S. aureus infection or bacterial RNA challenge (Eigenbrod et al. 2015, Bergström et al. 2015). They are in line with our findings indicating TLR8 driven recognition of mitochondrial, S. aureus and E. coli rRNA while not excluding total bacterial RNA sensing by other endosomal TLRs.

Therefore we addressed involvement of human TLR7, also generally considered in ssRNA sensing (Diebold et al. 2004, Heil et al. 2004). BtmtD3_4 driven cell activation was not affected by siRNA mediated TLR7 mRNA knockdown (Fig. 47). TLR7 knockdown mildly inhibited parental THP-1 cell activation byloxoridine (a guanosine analog, TLR7 ligand), while not impairing R848 (TLR7 and TLR8 ligand) driven cell activation. Yet, Tlr8⁻-THP-1 cell responsiveness towardsloxoridine and R848 was decreased, but abrogated completely upon TLR7 silencing. We observed similar TLR7 and -8 dependent stimulatory capacity of purified E. coli tRNA, which we expected to induce TLR7 specific cell activation. These findings could be interpreted as stronger induction of NF-κB dependent TNF production upon TLR8 than upon TLR7 activation in 3ddi THP-1 cells. One might also argue
that TLR8 deficiency went along with lower TLR7 activity, which would however contradict a report showing increased TLR7 activation in absence of TLR8 in pDCs and monocytes (Tran et al. 2015). Nevertheless, our findings allowed exclusion of TLR7 activation upon BtmtD3_4 challenge in 3ddiTHP-1 cells, although TLR7 mRNA was also induced according to our transcriptome profiling results (Fig. 47, Fig. 36). Western blot analysis of parental, Unc93b1^−/− and Tlr8^−/−-3ddiTHP-1 cell lysates allowed semi-quantitative visualization of TLR7 and -8 protein levels. Absence of TLR8 in Tlr8^−/−-3ddiTHP-1 cells as well as decreased levels of both TLRs in Unc93b1^−/−-3ddiTHP-1 cells confirm the respective genotypes, in cooperation with M. Oldenburg (Krüger et al. 2015).

Investigations concerning the binding capacity of human TLR8 explain the inactive state of murine TLR8 which cannot be activated by ssRNA or small molecule agonists. A five aa-sequence (RQSYA) missing in mTLR8 has been described to be essential for the functionality of hTLR8 (Liu et al. 2010). A furin-like pro-protein convertase and the endoprotease cathepsin bind to the Z-loop localized between LRR domains 14 and 15, as well as to the RQSYA sequence. Through proteolytic cleavage, TLR8 is converted into its active form (Tanji et al. 2013, Ishii et al. 2014, Geyer et al. 2015).

TLR7 and TLR8 have been implicated as sensors for U/G or U/A rich viral-, si-, bacterial and self-RNA sequences (Heil et al. 2004, Lund et al. 2004, Barrat et al. 2005, Hornung et al. 2005, Forsbach et al. 2008, Cervantes et al. 2013). Structure analysis of TLR8 indicate specific binding of ssRNA dependent on its U and G content. Two ligand binding sites have been identified in TLR8, one binding uridine mononucleosides and the other interacting with ssRNA degradation products rich in UG/UGG motifs (Geyer et al. 2015, Tanji et al. 2015). Uridine interacts with amino acids within LRRs 9 to 13 localized in the Z-loop, whereas the second binding site (also interacting with RNA40) is found within LRR 11 to 14 as well as LRR 16 to 18 (Tanji et al. 2015). It has also been reported that both binding sites synergistically mediate TLR8 homo dimerization and activation of their TIR domains to further induce an immune response. Based on these reports we analyzed the stimulatory potential of BtmtD3_4 and further Sa19 like ORNs regarding their U content. TLR8^+ HEK293 cell or PBMC activation upon ORN challenge with low U content (Sa19) was increased by addition of U mononucleosides. However higher U content incorporated in sequence motifs like UG/UGG, UA/UAA or other combinations of U followed by a purine (R) proved to be stimulatory without additional uridine mononucleoside application (BtmtD3_4, HsmtD3_4), yet the latter resulted in further enhanced cell activation (HsmtD1, *mtPTL) (Fig. 48). Bacterial total RNA stimulatory potential was not increased by uridine mononucleoside addition (Fig. 48). We suggest a sufficient release of uridines during RNA degradation within the endolysosome serving both binding sites to activate TLR8. We introduced a U into the U-less TLR8 silent ORN Sa12 to form a UAA motif (Sa12s6U) thereby conferring strong stimulatory capacity (Fig. 49). Thus, we confirmed and expanded the previous implications of Tanji et al. (2015) in that U-content of ssRNA is essential for TLR8 recognition and that not merely UG/UGG but rather UR/URR constitute the RNA ligand consensus motif. It would be interesting to evaluate if pathogenicity of bacterial species correlated with the uridine content of their ribosomal RNA.
4.3.2 Relevance of TLR8 in sepsis caused by Gram-positive and Gram-negative bacterial infection

During this project human TLR8 was shown not only to represent a human functional homolog of murine TLR13, but also that its ligand sequence specificity is far broader to enable recognition of mutated and modified bacterial and mitochondrial ribosomal RNA. Our data qualify hTLR8 as a major bacterial RNA sensor. Earlier studies have indicated a contribution of RNA recognition to innate immune responses against live bacteria (group B streptococci, GBS; Borrelia burgdorferi) in human cells (Deshmukh et al. 2011, Cervantes et al. 2013). We implicate TLR13 next to TLR2 as a major innate immune sensor of Gram-positive bacteria in mice. Therefore, we hypothesized inhibition of proinflammatory immune overreaction upon bacterial infection in human cells by simultaneous blockade of TLR8 and TLR2 in combination with antibiotic treatment. We applied the TLR2 neutralizing mAb T2.5 to parental or Tlr8<sup>-/-</sup>-3ddiTHP-1 cells prior to confrontation with heat inactivated or viable S. aureus and E. coli. Tlr8<sup>-/-</sup>-3ddiTHP-1 cells were per se substantially less responsive to viable bacteria as compared to parental cells. Responsiveness of cells of both genotypes was significantly inhibited upon TLR2 blockade, but even more profoundly in the absence of TLR8 expression (Fig. 51). PBMC infection with viable bacteria induced cell activation, which was abrogated upon chloroquine - a well established endosomal function inhibitor - pretreatment. Dual application of T2.5 and chloroquine significantly inhibited PBMC activation upon infection with viable S. aureus or E. coli. Infection with both S. aureus and E. coli triggered release of TNF and IFNα in absence of chloroquine, while the TLR8 control stimulus BtmtD3_4 failed to induce IFNα release (Fig. 52). This indicates involvement of further endosomal pattern recognition activity upon bacterial infection, such as tRNA driven TLR7 activation. In line with our data, a recent study reports cytochalasin D mediated abrogation of PBMC activation upon group B streptococci (GBS) and S. pyogenes infection. In this study however, cytochalasin D treatment did not decrease cell activation upon infection with S. aureus (Eigenbrod et al. 2015). This discrepancy to our data might be due to inefficient endosomal inhibition by cytochalasin D in contrast to chloroquine regarding uptake and recognition of viable S. aureus or/and absence of TLR2 blockade.

TLR8 polymorphisms are associated with susceptibility to pulmonary tuberculosis, caused by Mycobacterium tuberculosis infection (Davila et al. 2008). Our implication of bacterial and mitochondrial ribosomal RNA as TLR8 ligands, corresponding with other studies, suggest a role of TLR8 in bacterially induced sepsis pathogenesis and probably also in trauma induced sterile inflammation.

Therefore we set out to analyze the clinical relevance of our in vitro infection studies. We infected whole blood from healthy donors with viable S. aureus or E. coli to mimic sepsis in patients. Our specific aim was to evaluate inhibition of proinflammatory cytokine production through blockade of TLR8. Chloroquine in combination with antibiotic treatment efficiently inhibited whole blood cell activity upon bacterial infection (Fig. 53). This observation was in line with our data from THP-1 cells and PBMC analyses. However, TLR2 neutralization with mAb T2.5 was less efficient in whole blood (data not shown) as compared to that in THP-1 cell and PBMC cultures, possibly due to interfering molecular interactions within whole blood containing erythrocytes and thrombocytes as well as complement system components coagulating in the 24 h period of the assay. Altogether, our data imply
TLR8 as a major bacteria and mitochondria sensor and hint at an anti-inflammatory therapeutic potential of TLR8 blockade in sepsis.

Preliminary results from murine macrophage infection with Gram-positive or Gram-negative bacteria indicate higher efficiency of TLR2 blockade (mAb T2.5) yet less efficiency of TLR13 blockade (chloroquine) in mBMMs as compared to hPBMCs (data not shown). Possibly, these data suggest a greater importance of human endosomal bacterial nucleic acid sensing through TLR8 than bacterial cell envelope component recognition via TLR2 in comparison to mice.

During the time of our manuscript submission on TLR8 driven RNA recognition, other studies reporting on human TLR8 as Gram-positive bacterial RNA sensor have been published (Bergstrøm et al. 2015, Eigenbrod et al. 2015, Nishibayashi et al. 2015). Accordingly, total RNA of S. aureus, S. pyogenes, Streptococcus agalactiae and Listeria monocytogenes specifically activate human TLR8 (Bergstrøm et al. 2015, Eigenbrod et al. 2015). Furthermore, total RNA, 23S and 16S rRNA of probiotic lactic acid bacteria such as Enterococcus faecalis (EC-12) induce IL-12 release in human monocytes dependent on TLR8 but independent of TLR7 (Nishibayashi et al. 2015). However, our results extended these and earlier reports as we implicate 5S beyond 23S and 16S rRNA of S. aureus and also of Gram-negative E. coli as well as mitochondrial ribosomal RNA as hTLR8 activating immune stimulatory PAMPs or DAMPs. Our implication of UR/URR as TLR8 activating ssRNA consensus motif provided further insight to the molecular basis of pathogen host interaction in innate immunity.

Defect TLR8 expression in mice might underlie continuation of TLR13 expression, since numerous TLR8 expressing species lack TLR13 expression. Hence, bacterial immune escape within mice upon bacterial N6-adenosine methylation (as in Sa19mA7) was not observed in other species expressing TLR8 but not TLR13 such as human, macaque and swine (Fig. 30, Fig. 32, Fig. 54). Evolvement of TLR8 as a bacteria sensor upon fish-tetrapod evolutionary transition might have resulted from persistent confrontation with MLS antibiotic producing soil bacteria, resistance conferring methyltransferase expressing bacteria or bacteria carrying a resistance conferring 23S rRNA mutation (Forsberg et al. 2014). Promiscuous bacterial ssRNA recognition via TLR8 was advantageous, as bacterial immune escape might have impaired host survival. The disadvantage however, was reactivity towards endogenous ssRNA, as observed in hTLR8 transgenic mice (Guiducci et al. 2013, Snyder et al. 2014).
5 Summary

Immune defense against infection depends on recognition of invading pathogens by innate pattern recognition receptors (PRRs) localized in the plasma- or endosomal membrane and the cytoplasm. Toll-like receptors (TLR) are an important family of PRRs detecting bacterial pathogen associated molecular patterns (PAMPs). Blood borne bacteria tend to over-amplify inflammatory signals upon infections, which often causes septic shock. Blockade of TLR2 and -4 in combination with antibiotic therapy efficiently inhibits sepsis pathology upon Gram-negative bacterial infection in mice. Major host receptors of Gram-positive bacteria beyond TLR2 had not been identified previously.

Here, I show that recognition and killing of Gram-positive bacteria by macrophages depends on an endosomal TLR beyond TLR3, -7, -8, -9, -11 and -12. Immune activation upon *Staphylococcus pneumoniae* D39 infection or heat inactivated *Staphylococcus aureus* (hiSa) challenge was abrogated in murine MyD88/Trif\(^{--}\) bone marrow derived macrophages (BMMs). In contrast, *Tlr23479\(^{--}\)* BMMs recognized and killed these bacteria like wt cells, yet 3D/Tlr24\(^{--}\) BMMs lacking endosomal TLR function were impaired in both. According to common view, the bactericidal activity of host cells involves production of reactive oxygen species (ROS) or nitric oxide (NO). However, *p47/Phox\(^{--}\)* and iNOS\(^{--}\)* BMMs were unremarkably competent in *S. pneumoniae* D39 killing. Bacterial recognition and killing capacity was carried by cell culture supernatants since bacteria were killed when confronted with cell free supernatant from pre-stimulated wt BMMs, indicating extracellular killing independent of phagocytosis. My results suggest involvement of other effector molecules such as antimicrobial peptides in macrophage bactericidal activity.

I also show that murine TLR13 recognizes a conserved bacterial 23S ribosomal (r)RNA segment of 10 nt (5\(^{--}\)CGGAAAGACC-3\(^{--}\)) which also binds macrolide, lincosamide and streptogramin (MLS) antibiotics (including erythromycin). RNase A treatment of hiSa abrogated *Tlr23479\(^{--}\)* cell activation. Consequent analysis of bacterial ssRNA molecular subspecies indicated 23S rRNA as TLR13 ligand. Detection of *S. aureus* RNA was conferred by TLR13 upon its over expression in HEK293 cells, while its mRNA knockdown upon siRNA transfection into BMMs abrogated it. The TLR13 activating 23S rRNA segment contains adenosine (A)2085 in *S. aureus*. Its N6-adenosine methylation or mutation mediated by specific methyltransferases (ErmC and ErmB) is known to confer antibiotic resistance and at once abrogated activation of TLR13. Hence, 23S rRNA from erythromycin resistant clinical *S. aureus* isolates grown from the presence of erythromycin as well as synthetic oligoribonucleotides (ORN) methylated at the respective adenosine or mutated towards guanosine mimicking resistance conferring modifications failed to activate TLR13. Over expression of ErmB or ErmC in erythromycin susceptible bacteria resulted in N6-adenosine methylation as shown by HPLC and masking towards TLR13. Thus, my data identify both TLR13 and its highly conserved ligand, as well as a mechanisms of antibiotic resistance qualifying as bacterial immune escape strategy to avoid TLR13 driven recognition.

Furthermore, I show that human TLR8 not only replaces TLR13 but also exceeds its specificity by promiscuously sensing bacterial and mitochondrial rRNA in a merely URR motif dependent manner. RNase A treated hiSa activated hPBMCs only via TLR2, implying ssRNA as major immune stimulant. PBMCs were MyD88 dependently activated by bacterial 23S (Sa19) or mitochondrial Sa19-like 16S rRNA (BtmtD3\(_{-4}\)) derived ORNs also when methylated or mutated, as well as by all bacterial rRNAs. Comparative transcriptome profiling of Sa19 responsive differentiated human monocytoid THP-1 cells indicated correlation of TLR8 expression with Sa19 responsiveness. Accordingly, *Unc93b1\(^{--}\)* and *Tlr8\(^{--}\)*-THP-1 cells as well as siRNA driven knockdown and ectopic TLR8 expression confirmed this statement. Responsiveness of *Tlr8\(^{--}\)* and *Unc93b1\(^{--}\)* THP-1 cells towards bacterial infection was abrogated upon TLR2 blockade (mAb T2.5). Additional endosomal inhibition (chloroquine) resulted in a substantial decrease of PBMC activation. Endosome inhibition by chloroquine and simultaneous antibiotic treatment efficiently inhibited bacterial infection driven inflammatory activity of human whole blood. Therapy at the early phase of bacterial sepsis might thus benefit from TLR8 and -2 blockade besides antibiotic therapy.
6 Zusammenfassung


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10 Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.
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12 Erklärung

Erklärung:

Essen, den ____________________ ________________________________ Prof. Dr. C. Kirschning

Erklärung:
Hiermit erkläre ich, gem. § 7 Abs. (2) d) und f) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient, bei der Abfassung der Dissertation nur die angegeben Hilfsmittel benutzt und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

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