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Detection of bacterial infection: Analysis of specificity and regulation as well as therapeutic blockade of Toll-like receptor 2 and Toll-like receptor 4

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ABBREVIATIONS

-/-	knock-out
α-	anti-
°C	Centigrade
µg	microgram
µl	microlitre
µM	micromolar
AP-1	Activator Protein 1
APC	Antigen-Presenting Cell
APS	Ammonium Persulphat
ASC	Apoptosis-Associated Speck-Like Protein Containing a CARD
ATF-2	Activating Transcription Factor 2
ATP	Adenosine tri-phosphate
BMDMo	Bone marrow-derived macrophage
bp	base pair
BSA	Bovine Serum Albumin
CARD	Caspase Recruitment Domain
cDC's	common Dendritic Cells
CD	Cluster of Differentiation
cDNA	complementary DNA
CpG	Cytosine-guanosine oligonucleotide
CTL	Cytotoxic T cells
d	Days
d/d	defective
DAMPs	Danger Associated Molecular Patterns
DC	Dendritic Cell
DD	Death Domain
ddH2O	double distilled water
DEX	Dexamethasone
D-GalN	D-Galactosamine
dKO	double Knock-out
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

dNTP	deoxynucleotide tri-phosphate
ds	double-stranded
DTT	Dithiothreitol
E.	*Escherichia*
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated protein kinase
EtBr	Ethidium bromide
EtOH	Ethanol
FITC	Fluorescent Isothiocyanate
GAPdH	Glyceraldehyde-3-Phosphate dehydrogenase
GPI	Glycosylphosphatidylinositol
h	hours
HEK	Human Embryonic Kidney
HRP	Horseradish Peroxidase
Ig	Immunogloblin
IPAF	Ice Protease-Activating Factor
i.p.	intraperitoneal
i.v.	intravenous
IFN	Interferon
IFNγR	Interferon-γ-Receptor
IKK	Inhibitor of κB kinase
IκB	Inhibitor of kappa Light Chain
IL	Interleukin
IPS-1	IFNβ-Promotor Stimulator
IRAK	IL-1 Receptor-Associated Kinase
IRF	Interferon Regulatory Factor
JAK	Janus Kinase
JNK	C-JUN N-terminal kinase
kb	kilobase
kDa	kilodalton
KO	knock-out
l	litre
LBP	LPS Binding Protein
LPS	Lipopolysaccharide
LRR	Leucine-Rich Repeats
LTA	Lipoteichoic Acid
mA	milliampere

mAb	monoclonal Antibody
MAL	MyD88-adaptor-like
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
Mda5	Melanoma-Differentiation Associated Gene 5
M-CSF	Macrophage colony stimulating factor
MHC	Major Histocompatibility Complex
min	minutes
ml	millilitre
mm	millimeter
mM	millimolar
MOF	Multiple Organ Failure
mRNA	messenger RNA
mV	millivolt
MyD88	Myeloid Differentiation primary response Gene 88
Myr_1GSK_4	Myristoyl-glcyl-seryl-tetralysine
Myr_3CSK_4	Tri-myristoyl-cysteinyl-seryl-tetralysine
NAIP	Neuronal Apoptosis Inhibitory Protein
NALP	NACHT Domain-, Leucine-Rich Repeat-, and PYD-Containing Protein
NF-κB	Nuclear factor-κB
ng	nanogram
NK	Natural Killer
NLR	NOD-Like Receptor
nm	nanometre
NO	Nitric Oxid
NOD	Nucleotide oligomerization domain
NP-40	Nonidet P-40
ODN	Oligodeoxynucleotide
ORF	Open Reading Frame
PAGE	Polyacrylamid gel electrophoresis
Pam_3CSK_4	Tri-palmitoyl-cysteinyl-seryl-tetralysine
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
pDC's	plasmacytoid Dendritic Cells
PE	Phycoerythrin

PGN	Peptidoglycan
PKC	Protein kinase C
RIG-I	Retinoic acid Inducible Gene I
RLH	RIG-like Helicase
PRR	Pattern Recognition Receptor
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rpm	rounds per minute
RT	Room Temperature
s	seconds
SARM	Sterile Alpha and TIR-motifs-containing Protein
SDS	Sodiumdodecylsulfate
SIGIRR	Single Immunoglobulin IL-1R-Related protein
SOCS	Suppressor of cytokine signalling
sTLR	soluble TLR
ST2	Suppressor of Tumorgenicity 2
TAB	TAK-1-binding protein
TAK	Transforming growth factor-β-Activated Kinase 1
TBS	Tris buffered saline
TBK-1	TANK-binding Kinase 1
TCR	T cell receptor
TEMED	N, N, N’, N’-Tetramethylethyleneamine
TGF	Transforming growth factor
Th	T helper
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adaptor molecule
TLR	Toll-like receptor
TMB	Tetramethylbezine
TNFα	Tumour Necrosis Factor-α
TOLLIP	Toll-interacting protein
TRAF	Tumor necrosis factor receptor-associated factor
TRAM	TRIF-related adaptor molecule
TREM	Triggering receptor expressed on myeloid cells
TRIF	TIR domain-containing adaptor inducing interferon-β
V	Volt

1 INTRODUCTION

1.1 Innate and adaptive immunity

The fascination of mankind for the idea of immunity is already in existence for thousands of years. From the prehistoric view disease, illness and especially epidemics were thought to be caused by supernatural forces as a form of theurgic punishment for “bad deeds” or “evil thoughts”. Today we know that the fact that a wide majority of people is healthy most of their lifetime, and that severe infections are normally rare, is due to a well-developed defence system but not to graciousness of upper powers. From time immemorial, pathogens and the host defending immune system are competing against each other and adapt persistently to the advance of the component. So vertebrates, in the course of time, have evolved a manifold immune system to eliminate infective pathogens in the body. The immune system consists of a mechanical barrier and a complex network of specific organs, cell types and molecules. However, body fluids with relatively low pH and the mucosa, which gets first in contact with pathogens, provide a striking mechanical barrier for invaders (Janeway, Travers et al. 2001). Furthermore production of antimicrobial peptides and punctual increased mucus secretion protect the host from pathogenic infiltration. Although these measures are very effective, bacteria, viruses and parasites developed efficient strategies to cross these first barriers and infect the host. Upon infection specific immune cells recognize invading pathogens and a multifarious response to combat ongoing infection and to provide long-dated pathogen-specific protection is initiated. The entirety of this manifold defence system is comprised of two branches: the innate and the adaptive immunity. The innate immune system lies behind most inflammatory processes and is evolutionarily and phylogenetically conserved in almost all animals as well as in plants. Innate immunity is mediated by a limited number of germline encoded pattern recognition receptors expressed mainly by phagocytes like macrophages and dendritic cells (DCs) (Akira, Uematsu et al. 2006). Aside of macrophages and DCs many other cell types like mast cells, neutrophils, eosinophils and Natural Killer (NK)-cells are activated upon microbial challenge and are therefore considered as innate immune cells. Furthermore production of antimicrobial peptides by polymorphonuclear lymphocytes, activation of the complement system via mannose-binding lectin and induction of acute phase proteins are measures of an innate immune response. Moreover the innate immune system can also be considered to be a property of the skin and the epithelia that line our internal organs such as the gut and the lungs. In contrast, the highly sophisticated adaptive immune system is observed only in vertebrates and is restricted to B- and T-lymphocytes, which develop and mature in the bone marrow and the thymus respectively and populate the lymph nodes, the spleen and other lymphatic organs. Because of the mechanism of generating receptors, which involves great variability and rearrangement

of receptor gene segments, the adaptive immune system can provide specific recognition of foreign antigens, immunological memory of infection and pathogen-specific adaptor molecules like antibodies (Janeway and Medzhitov 2002). However, aside of counteracting infectious diseases the adaptive immunity also inhibits tumour formation but in case of its dysregulation leads to the unfortunate effects of autoimmune disease, allergy and allograft rejection. Finally, both the innate and the adaptive immune system are linked together and consequently use the same activated effector cells. Cells characteristic for the innate immune system like DCs and macrophages, which guard against infection in virtually all tissues, not only detect invading pathogens via their encoded pattern recognition receptors (PRRs) and initiate inflammatory processes, they also present pathogen-specific peptides on their surfaces. Upon phagocytosis pathogenic proteins are processed to specific peptides and are embedded in self-major histocompatibility complex (MHC) molecules, which are presented to T-cells (Janeway, Travers et al. 2001). In these cases antigen-presenting cells (APCs) contact specific adaptive immune cells and so instruct the adaptive system about the nature of pathogenic challenge. Recognition of the MHC molecule presenting the antigen is mediated by B- and T-cell receptors, which are expressed on the respective cell type. In order to respond to a wide range of potential antigen, B- and T-cells rearrange their receptor genes to generate over 10^{11} different species of antigen receptors thus ensuring that the appropriate receptor specific for any pathogenic peptide presented on MHC class complexes is available (Takeda, Kaisho et al. 2003). Especially for T-cells, representing one main branch of the adaptive immunity, interaction for further processing with APCs is indispensable. Depending on the expression of their co-receptor, T-cells can be subdivided into two major functional subsets, the $CD4^+$ T-helper (Th) cells and the $CD8^+$ cytotoxic T-cells (CTLs). Peptides derived from cytosolic pathogenic proteins are loaded on MHC class I molecules and are recognized exclusively by CTLs whereas MHC class II molecules bind peptides derived from endocytosed proteins, which are recognized by Th cells. Notably, in the latter case in addition to the recognition of the antigen alone, the cell-cell interaction is further supported through costimulatory molecules such as CD80 and CD86 that are expressed on APCs and are upregulated upon cell activation (Janeway, Travers et al. 2001). Once T cells have received both signals they expand and differentiate into effector T cells. The main function of fully activated CTLs is the killing of infected target cells by secretion of proteases bearing lytic activity. Based on the type of antigen and the predominant cytokines, Th cells differentiate into two major subtypes of cells known as Th1 and Th2 cells. These subtypes are defined on the basis of the specific cytokines they produce. Th1 cells produce mainly interferon-gamma (IFNγ) (Romagnani 1996), which acts on macrophages and B-cells, stimulating phagocyte-mediated defence against infections, especially with intracellular pathogens. Th2 cells on the other hand produce interleukin (IL)-4, IL-5, IL-10 and IL-13 (Morel and Oriss 1998; Lee, Kim et al. 2006), cytokines important to fight infections with parasites and for the down-regulation of Th1 responses.

In contrast to T-cells, B-cells can be activated directly and are not restricted to interact with APCs. When a B-cell recognizes its target antigen by the B-cell receptor (BCR) it is internalized and processed. Subsequently, the B-cells move to specialized regions of the secondary lymphatic organs where they undergo differentiation and proliferation to become antibody producing plasma cells, a process called clonal expansion. After secretion and binding to their specific antigens, these antibodies perform various effector functions, including neutralizing antigens, activating complement, and promoting phagocytosis and destruction of microbes (Janeway, Travers et al. 2001). The B-cells are responsible for the so called humoral immunity, including production of highly specific antibodies and development of memory effector cells, which provide a long lasting immunity against specific viral or pathogenic infections (Manz, Hauser et al. 2005).

1.2 Innate immunity and the pattern recognition receptors (PRRs)

The precedent condition for the induction of every immune response is the recognition of a potentially infectious agent. This major decision to respond or not is made by the innate immune system and is of prime importance. The fact that defects in innate immunity are very rare and almost always lethal imposingly shows the inalienability of its functionality (Janeway and Medzhitov 2002). Microorganisms such as bacteria and viruses as well as parasites are initially recognized via pattern recognition receptors (PRRs) of the host innate immune system. PRRs are highly conserved, germline encoded, nonclonal and expressed on all cells of a given type. Different PRRs recognize specific microbial components, activate specific signalling pathways and induce an antipathogenic rapid inflammatory response (Akira, Uematsu et al. 2006). The targets of PRRs are often referred to as pathogen-associated molecular patterns (PAMPs) although they are present in pathogenic as well as in non-pathogenic microorganisms. PAMPs are mainly components of the cell wall of microorganisms such as lipopolysaccharide (LPS), lipoproteins, lipoteichoic acid (LTA) and peptidoglycan (PGN), and are well suited to be ligands for PRRs. They represent invariant components of specific classes of microbes. They are products of unique pathways of the microorganisms enabling the innate immune system to discriminate between self and non-self molecules. And last but not least PAMPs usually play an essential role in the microbial physiology, which limits the probability that its structure will adapt and change through evolution and therefore enables the microbe to evade innate immune recognition. In case of viral recognition, which replicate within the host cell, the targets recognized by PRRs are viral nucleic acids. Specific chemical modification and structural feature that are unique for viral RNA and DNA provide for discrimination between self and viral nucleic acids (Medzhitov 2007). The family of PRRs includes the Toll-like receptors (TLRs), the RNA-helicases (RIG-like helicases, RLHs) and the NOD-like receptors (NLRs). PRR subclasses are divided concerning their structure, the signalling pathways they induce and the compartment of the cell in which they are

located and its activation upon microbial challenge takes place. Whereas TLRs due to their transmembrane domain are located either on the cell surface or in endosomal compartments the RNA-helicases and the NLR proteins are located in the cytosol to recognize PAMPs from microbes, which invaded the cell (Takeuchi and Akira 2007).

1.2.1 Toll-like receptors (TLRs)

A prominent and already well-studied family of PRRs is the Toll-like receptor (TLR)-family. Toll, the founding member of the TLRs was initially identified as a gene product essential for the development of embryonic dorsoventral polarity in *Drosophila*. Further investigation identified a Toll-mediated pathway in *Drosophila* including the protein Spaetzle as the Toll-ligand, an adaptor protein Tube, a protein kinase Pelle, an Nuclear factor-κB (NF-κB) family transcription factor named dorsal and the dorsal inhibitor cactus, an Inhibitor of kappa Light Chain (IκB)-homologue (Belvin and Anderson 1996). Because the transcription factor NF-κB was known to be initiated during inflammatory processes a function of Toll in *Drosophila* immunity was assumed. Indeed analysis of *Drosophila* strains carrying loss of function mutations of Toll demonstrated that Toll plays a crucial role in anti-fungal immune responses in *Drosophila* (Hashimoto, Hudson et al. 1988). For instance it was shown that Toll-activation mediated secretion of several anti-fungal peptides with lytic activity, such as Dorsomycin (Lemaitre, Nicolas et al. 1996; Lemaitre, Reichhart et al. 1997). Furthermore challenge of *Drosophila* with Gram-negative bacteria Toll-independently induces a different subset of antimicrobial peptides. Consequently nine TLRs in *Drosophila* were identified and further characterized (Janeway and Medzhitov 2002). Meanwhile, through database researches Toll homologues in humans and other mammalian species were found. So far 13 Toll-like receptors in mice and 11 TLRs in humans were identified (Beutler 2004). TLRs 1-9 are conserved between mice and humans, whereas TLR10 can be found exclusively in humans. TLR11 was shown to be involved in recognition of Toxoplasma profilin but is only functionally active in mice and is because of a stop codon within the TLR11 open reading frame (ORF) not expressed in humans. (Chuang and Ulevitch 2001; Zhang, Zhang et al. 2004). Moreover, the biological role of TLRs 10, 12, and 13 still needs to be identified. TLRs are type I integral transmembrane receptors carrying a single α-helix that spans the cell membrane. The N-terminal extracellular domain contains 19-25 leucine-rich repeats (LRRs), which are responsible for the direct binding to the specific ligand (Akira and Takeda 2004). Each LRR shares the typical 24-29 amino acid long XLXXLXLXX motifs and finally the repeats comprise a β-strand and a α-helix connected by loops creating a horseshoe-like shape, which was found upon formation and analysis of crystallized single LRRs and later human TLR3 (Marino, Braun et al. 1999; Choe, Kelker et al. 2005). Meanwhile TLR4 as well as TLR2 in complex with antagonistic or agonistic ligands respectively have been crystallized and these reports substantially expand the

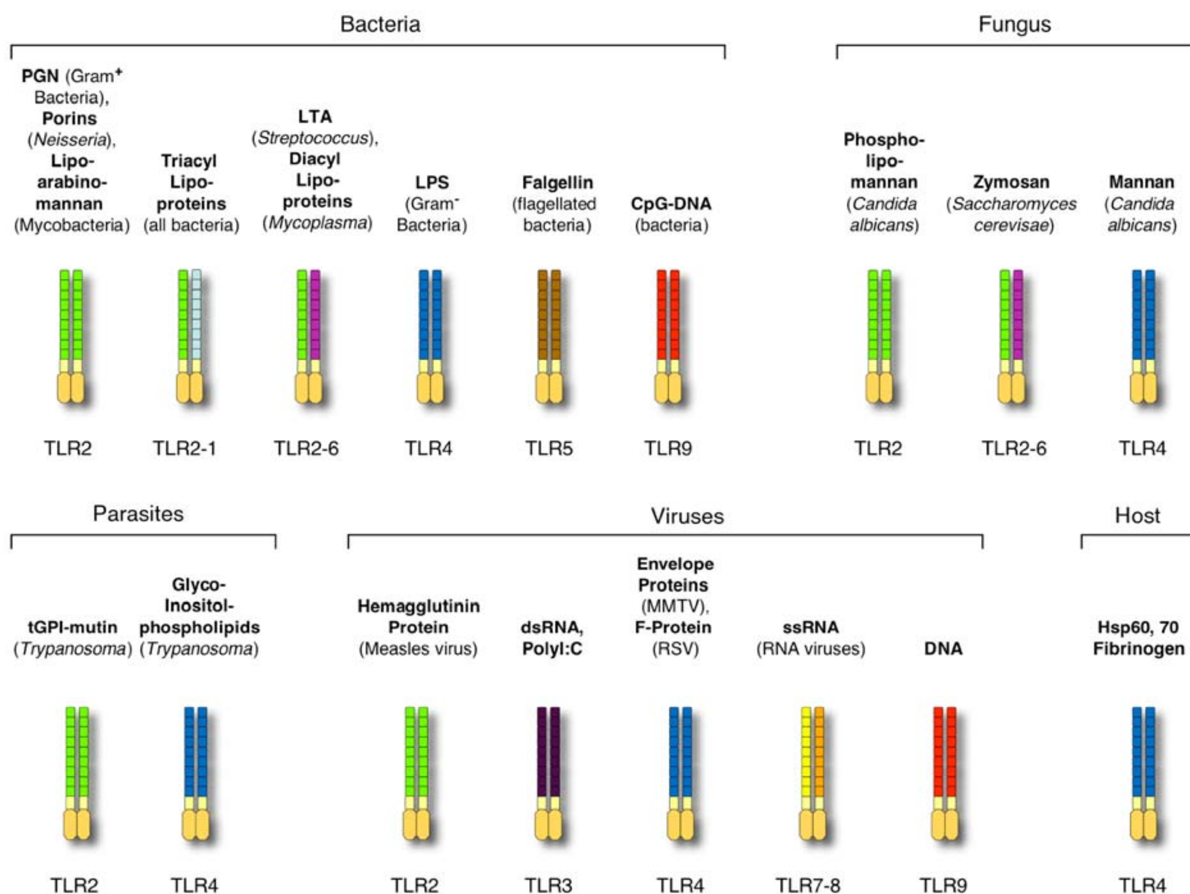


FIG.1: Toll-like receptors and their ligands

Bacteria, viruses, parasites, fungi and even products from inflamed tissue are sensed via Toll-like receptors (TLRs). Thereby TLR2 and TLR4 recognize components from all forms of microbes; TLR2 forms heterodimers with TLR1 or TLR6 supporting recognition of tri-acylated and di-acylated lipoproteins respectively. Although TLR4 is regarded as the lipopolysaccharide (LPS) receptor it is implicated to sense also viral, fungal and even host components. TLR3, TLR7 and TLR8 are restricted to recognition of viral nucleic acids whereas TLR5 is a receptor described to promote signalling upon flagellin challenge. TLR9 is the receptor for DNA. Therefore almost all pathogens are recognized via this repertoire of TLRs. (Gram$^+$, Gram-positive; Gram$^-$, Gram-negative)

understanding how TLR-ligand binding on the structural level occurs (Jin, Kim et al. 2007; Kim, Park et al. 2007). Whereas the extracellular domains of TLRs define the ligand specificity the intracellular domain is responsible for all further signalling processes initiated. The identification of TLRs was performed by database research on analysis of homology to the intracellular domain of *Drosophila* Toll. This intracellular domain matches to the cytosolic domain of the human IL-1 receptor and was hereby named as the Toll/Interleukin-1 receptor (TIR) domain. TIR domains are conserved in TLRs, IL-1 receptors and also IL-18 receptors as well as in several cytoplasmic adaptor molecules. Ligand binding is assumed to cause di- or oligomerization (formation of homo- or heterodimers) of the receptor, resulting in close proximity of the intracellular TIR-domains and subsequent recruitment of downstream adapter molecules by interaction of the TIR domains (Ozinsky, Underhill et al. 2000). They contain 150-200 amino acid residues and sequence identity between any pair of TIR domains is generally about 25% (Khan, Brint et al. 2004). This diversity might be crucial for the specificity of signal transduction among the different TLRs by ensuring specific complex formation with the proper adaptor molecules. The substantial role of the TIR domain in TLR signalling was previously

demonstrated by publications showing that single point mutations within the TIR-domains of human TLR2 and TLR4 disrupts the signalling cascade upon stimulation with specific ligands (Poltorak, He et al. 1998; Underhill, Ozinsky et al. 1999). In the year 2000 the structure was furthermost elucidated upon crystallization of TIR domains of human TLR1 and TLR2 (Xu, Tao et al. 2000; Khan, Brint et al. 2004).

Regarding the ligand specificity TLRs can be simplistically subdivided into several families. TLR1, TLR2 and TLR6 recognize lipids, TLR4 is regarded as the receptor specific for lipopolysaccharides (LPS), TLR3, TLR7, TLR8 and TLR9 are specific for nucleic acid-like structures and TLR5 was described as the receptor activated by flagellin (Akira and Takeda 2004). TLR9 was established as the receptor specific for DNA containing CpG-rich motifs specific for bacterial nucleic acids. Notably it was recently published that the base-free sugar backbone of DNA is sufficient to determine DNA recognition by TLR9 concluding that TLR9 is probably not capable to differentiate foreign-DNA from self-DNA (Haas, Metzger et al. 2008). A more detailed summary displaying TLR-specificity in terms of their ligands is shown in **Figure 1**. Immune cells expressing TLRs are DC's, macrophages, B-cells, specific types of T-cells and also nonimmune cells such as fibroblast, endothelial and epithelial cells (Hijiya, Miyake et al. 2002; Hornung, Rothenfusser et al. 2002; Zarember and Godowski 2002; Pasare and Medzhitov 2005). Furthermore TLRs are expressed either intra- or extracellularly. While certain TLRs (TLR1, 2, 4, 5 and 6) are located on the cell surface, others (TLR3, 7, 8 and 9) are restricted intracellularly within endosomal compartments. Consequently ligands of intracellular TLRs, which are mainly nucleic acids, require internalization and translocation to the endosome before signalling is possible (Akira, Uematsu et al. 2006).

1.2.1.1 TLR signalling

Upon recognition of PAMPs, activated TLRs initiate a downstream signalling cascade resulting in the activation of transcription factors, including NF-κB and interferon regulatory factors (IRFs). Both regulate expression of genes that encode proinflammatory cytokines and chemokines as well as type I interferons, which mediate innate immune responses as well as induce adaptive immunity. It is assumed that upon ligand binding TLRs form homodimers enabling association of the cytosolic TIR domains creating a platform for building a signalling complex to induce further downstream signalling. However recent reports demonstrate that TLR9 is constitutively expressed in form of an inactive homodimer and that allosteric conformational changes within the TIR domain upon ligand binding finally induce its activation and signalling (Latz, Verma et al. 2007). So crystal structures of receptor-adaptor interaction are required to elucidate how TLR signalling is initiated. Whereas the majority of TLRs form homodimers, TLR2 can function as heterodimer with TLR1 or TLR6, each dimer responding to different ligands (Ozinsky, Underhill et al. 2000). Signal transduction by the TLRs is achieved through the recruitment of adaptor molecules to the cytoplasmic TIR region of the

receptor (Takeda and Akira 2005). All TLRs use intrinsic pathways and recruit different adaptor molecules to propagate a signal, leading to different expression profiles. Dependent on which adaptor molecules are recruited either NF-κB activation alone, mediating expression of proinflammatory cytokines, or additional IRF activation, which results in production of type I interferons are promoted. For example, activation of TLR2, TLR2-TLR1 and TLR2-TLR6 heterodimers and TLR5 process NF-κB activation and TLR3 and TLR4 are capable to induce type I interferons synchronously. Although TLR7, 8 and 9 also induce type I interferons, the employed pathway is distinct from that used by TLR3 and 4 (Kawai and Akira 2007; Uematsu and Akira 2007). Up to now five adaptor molecules that directly interact with the TIR domain of the receptors and therefore represent the earliest phase of signal transduction are identified. Notably all of them, like the receptors themselves carry a TIR domain, which interacts with the TIR domain of the receptor. These adaptors are myeloid differentiation marker (MyD88), MyD88-adaptor-like (MAL, also known as TIRAP), TIR-domain-containing adaptor protein inducing IFNβ (TRIF, also known as TICAM1), TRIF-related adaptor protein TRAM (also known as TICAM2) and sterile α-and armadillo-motif-containing protein SARM. Whereas all the other adaptor molecules positively regulate TLR sensing, SARM functions as an inhibitor of TRIF (O'Neill and Bowie 2007).

MyD88-dependent pathway

MyD88 is the most prominent TIR-domain containing adaptor protein because it is recruited by all TLRs except of TLR3. The myeloid differentiation (MyD) marker MyD88 was first characterized during a study of the early genetic responses of murine myeloid cells to various differentiation and growth inhibitory stimuli (Lord, Hoffman-Liebermann et al. 1990). Upon identification of homology to the IL-1 receptor MyD88 was thought to play a role in immunity. Upon characterisation of MyD88-deficient mice it was observed that these mice were unable to produce IFNγ and to mediate NK-cell activity in response to IL-18. NF-κB activation in response to IL-1 or IL-18 was also impaired indicating that MyD88 is a critical component in the IL-1R and IL-18R signalling cascades (Adachi, Kawai et al. 1998). Further analysis extended these studies and showed that responses to ligands for TLR2, TLR4, TLR5, TLR7 and TLR9 were lost or delayed in MyD88-deficient mice, establishing MyD88 as a key player in the TLR signalling cascade (Kawai, Adachi et al. 1999; Takeuchi, Hoshino et al. 2000; Skerrett, Liggitt et al. 2004). The encoded protein consists of a C-terminal TIR domain that can interact with the TIR domain of TLRs and an N-terminal death domain (DD), which binds to the death domain of the interleukin-1 receptor-associated kinase (IRAK) family (Muzio, Ni et al. 1997). Upon stimulation, MyD88 binds to the TLR and then recruits IRAK4 to this complex through interaction of the death domains of both molecules. Subsequently IRAK1 is recruited and phosphorylated by IRAK4. Tumour-necrosis-factor-receptor-associated factor 6 (TRAF6) associates to the complex and phosphorylated IRAK1 together with TRAF6 dissociate and form a complex with

TAK1 (transforming-growth factor-β-activated kinase), TAB1 (TAK1-binding protein 1) and TAB2 at the plasma membrane. IRAK1 is degraded at the plasma membrane and the remaining complex translocates to the cytosol. Cytosolic ubiquitin-ligases ubiquitinylate TRAF6 leading to autophosphorylation and activation of TAK1. Active TAK1 phosphorylates components of the Inhibitor of κB kinase (IKK) complex (IKKα, IKKβ, NEMO) ending up in phosphorylation of IκB, which is associated with NF-κB in the cytosol. Thereupon IκB gets degraded and this allows NF-κB to translocate into the nucleus and start the expression of its target genes (Akira and Takeda 2004; Chen 2005; O'Neill and Bowie 2007). Aside of activating NF-κB, TAK1 is able to phosphorylate mitogen-activated protein kinase kinases (MAPKKs), which in turn phosphorylate mitogen-activated kinases (MAPKs) p38 and c-JUN N-terminal kinase (JNK) leading to activation of transcription factors like activator protein 1 (AP-1) and activating transcription factor 2 (ATF-2) (Wang, Deng et al. 2001). NF-κB as well as AP-1 and ATF-2 pathways ultimately result in the transcription of pro-inflammatory chemokines and cytokines like tumour necrosis factor-α (TNFα), IL-6, IL-1β, IL-12 and CXCL8 (Hayden and Ghosh 2004; Takeda and Akira 2005)

Another important function of MyD88 is its capacity to induce type I interferons. MyD88 interacts with IRF1, IRF5 and IRF7. Whereas IRF1 and IRF7 are main inducers of type I interferons IRF5 induces production of several proinflammatory molecules but no interferons. MyD88-dependent activation of IRF1 and IRF7 is initiated only upon stimulation of TLR7, 8 and 9 recognizing nucleic acids within endosomal compartments and is reported to occur only in specific cell types. Upon TLR-mediated cell activation of plasmacytoid DCs, which are specialized to produce large amounts of type I interferons during viral infections MyD88 forms a complex involving IRAK4, IRAK1, TRAF6 and IRF7 in the cytoplasm. TRAF6 activates IRF7 through its ubiquitin E3 ligase activity and IRF7 translocates into the nucleus to induce expression of IFNα and IFNβ (Honda, Yanai et al. 2004; Kawai, Sato et al. 2004; Honda, Yanai et al. 2005). Furthermore demonstrated only in myeloid DCs MyD88 interacts with IRF1 and translocates directly associated with IRF1 into the nucleus to initiate IFNβ secretion (Negishi, Fujita et al. 2006) . Kinases activating IRF1 within this cascade are not yet identified. MyD88 also interacts with IRF5 that had been identified as being crucial for the induction of TNFα by all TLRs tested (Takaoka, Yanai et al. 2005).

Notably MyD88-dependent signalling upon TLR2 and TLR4 activation is supported by another TIR-domain-containing adaptor protein, which is called MAL. Mice lacking MAL showed impaired responses towards TLR2 or TLR4 ligands, indicating that the role of MAL is restricted to these receptors (Fitzgerald, Palsson-McDermott et al. 2001; Yamamoto, Sato et al. 2002). Upon further experimentations MAL was identified as a bridging protein supporting the interaction of MyD88 and the TIR domains of TLR2 and TLR4. MAL directly binds to MyD88 and this complex further associates with the specific TIR-domain of the activated receptor (Kagan and Medzhitov 2006).

MyD88-independent pathways

The finding that mice lacking MyD88 are still responsive to specific TLR ligands arose the question for a MyD88-independent TLR pathway. Even though activation of MAPK and NF-κB through most TLRs is impeded in MyD88-deficient cells, they can still be activated by TLR4 and TLR3. Upon

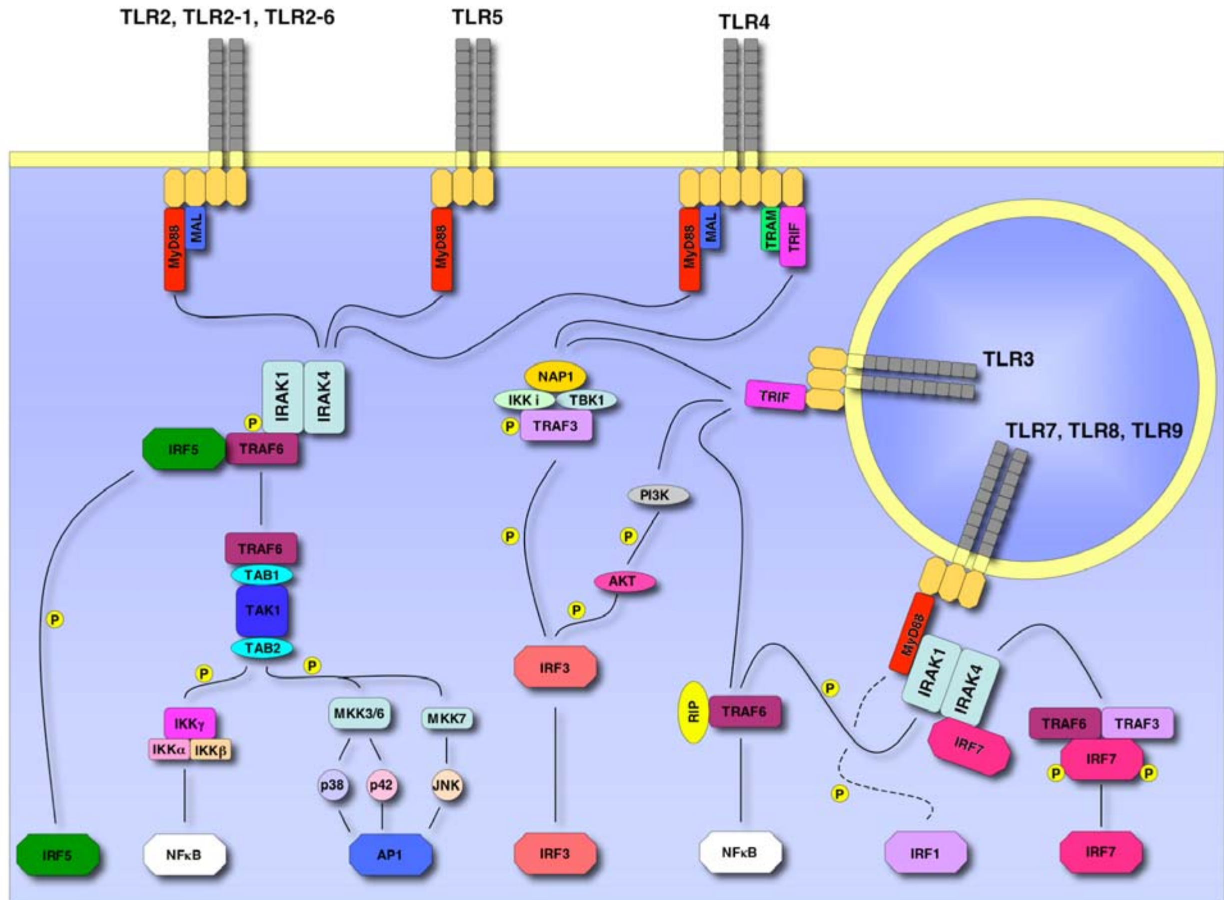


FIG.2: Structure of the main signalling pathways of TLRs

Via the intracellular conserved Toll/IL-1R (TIR) domain Toll-like receptors (TLRs) mediate proinflammatory signalling resulting in the activation of specific transcription factors including NF-κB, AP-1, IRF1, IRF3, IRF5 and IRF7. Furthermore activation of TLR2, TLR2 heterodimers, TLR4 and TLR5 induce activation of MAP kinases. The extracellular located TLRs recruit MyD88 as the initial TIR-domain containing adaptor molecule and thus activate IRF5, NF-κB and AP-1. Notably TLR4 recruits TRIF aside of MyD88 and additionally initiates type I interferon production upon IRF3 activation. TLR3 is located in endosomal compartments and exclusively recruits TRIF upon activation and mediates IRF3 and NF-κB activation. TLR7, TLR8 and TLR9, which are also located intracellularly, induce activation of NF-κB, IRF1 and IRF7 in a MyD88-dependent manner.

MyD88, myeloid differentiation primary-response protein 88; TRIF, TIR-domain containing adaptor protein inducing IFNβ; MAL, MyD88 adaptor-like protein; TRAM, TRIF-related adaptor molecule; IRAK, IL-1R associated kinase; TRAF, tumour-necrosis-factor-receptor-associated factor; TAK1, transforming-growth-factor-β-activated kinase; TAB, TAK1 binding protein; IKK, Inhibitor of NF-κB kinase, MKK, MAP kinase kinase; JNK, JUN N-terminal kinase; NAP-1, NAK-associated protein; TBK1, TANK-binding kinase; PI3K, Phosphatidylinositol-3 kinase; RIP, receptor interacting protein; IRF, Interferon regulatory factor.

stimulation of both receptors up-regulation of co-stimulatory molecules in MyD88-deficient dendritic cells as well as production of IFN-β is normal (Kaisho, Takeuchi et al. 2001). The adaptor molecule responsible for alternative TLR4 and initial TLR3 signalling was identified by database screening for TIR-domain-containing proteins and independently in a yeast-two-hybrid screen with TLR3 and was

called TRIF (Yamamoto, Sato et al. 2002). In contrast to MyD88-dependent signal transduction TRIF leads to the induction of type I interferons aside of proinflammatory cyto- and chemokines. TRIF-deficient mice were unable to initiate TLR3- and TLR4-induced IFN-β production and activation of IRF3 and inflammatory cytokine production was impaired in signalling through TLR4 but not TLR2, TLR7 or TLR9 (Oshiumi, Matsumoto et al. 2003; Yamamoto, Sato et al. 2003). Notably, cells deficient in MyD88 and TRIF are totally unresponsive to LPS showing that TRIF is the second key player in TLR4-mediated cell activation. The current model for the MyD88-independent pathway suggests that stimulation of TLR3 or TLR4 recruits TRIF to their TIR domains resulting in the activation of the transcription factor IRF3 *via* an IKK-like kinase termed TBK-1 (Fitzgerald, McWhirter et al. 2003). IRF3 induces transcription of IFN-β, which can itself induce several IFN-inducible genes, like CXCL10, GARG-16, or IRG-1 leading to an effective antimicrobial response (Kawai, Takeuchi et al. 2001; Toshchakov, Jones et al. 2002; Yamamoto, Sato et al. 2002; Oshiumi, Matsumoto et al. 2003). Main structural TLR-pathways are illustrated in **Figure 2**.

1.2.1.2 Bacterial recognition through TLR4 and TLR2

Gram-negative and -positive bacterial cell wall

The Gram-staining is a classical method to differentiate between distinct classes of bacteria. Due to positive or negative staining dependent on different cell wall constitutions bacteria were classified in the two specific groups: The Gram-negative and the Gram-positive bacteria. Some of their unique cell-wall components stimulate innate immune cells and display PAMPs recognized by distinct TLRs and other PRRs (**Figure 3**). A major group within the Gram-negative bacteria are the Proteobacteria which include members of the commensal flora (*Escherichia coli*) and also many pathogenic bacteria (*Salmonella, Enterobacter, Pseudomonas*). LPS also known to be an endotoxin is the most potent immune stimulatory component of the Gram-negative bacterial cell wall and consists of a unique Lipid A backbone that carries the characteristic polysaccharide chains. Different bacteria produce structurally different forms of LPS, which vary in their phosphate patterns, numbers of acyl-chains and organisation of their polysaccharide chains. These variations define the toxicity and the immune stimulatory potential of specific bacterial LPS. Notably toxicity of monophosphorly Lipid A is extremely reduced. The family of Gram-positive bacteria includes all strains of Actinobacteria (*Mycobacterium, Streptomyces*) and Firmicutes (*Streptococcus, Enterococcus, Staphylococcus, Listeria, Bacillus,* and *Lactobacillus*). Components of Gram-positive cell walls can also stimulate innate immunity although their cell wall does not contain LPS. Lipoteichoic acid (LTA), Peptidoglycan (PGN) and especially lipoproteins are common components of Gram-positive cell walls and also represent potent immuno-stimulants. Notably the latter two are also represented in Gram-

negative bacteria but in lower amounts in comparison to Gram-positive bacteria (Medzhitov and Janeway 1998; Medzhitov 2007). The acylation of lipoproteins is different among specific strains of bacteria. For instance total fatty acids within the lipoprotein fraction isolated from *E. coli* consists 53% of fatty acids carrying 16 carbon atoms (palmitic acid), 44% carrying up to 19 carbon atoms and 3% of fatty acids are shorter acylated and carry 14 carbon atoms (myristic acid) (Hantke and Braun 1973; Melchers, Braun et al. 1975). Consequently, according to their different cell wall assembly containing altered PAMPs Gram-negative and Gram-positive bacteria are described to be recognized via distinct PRRs.

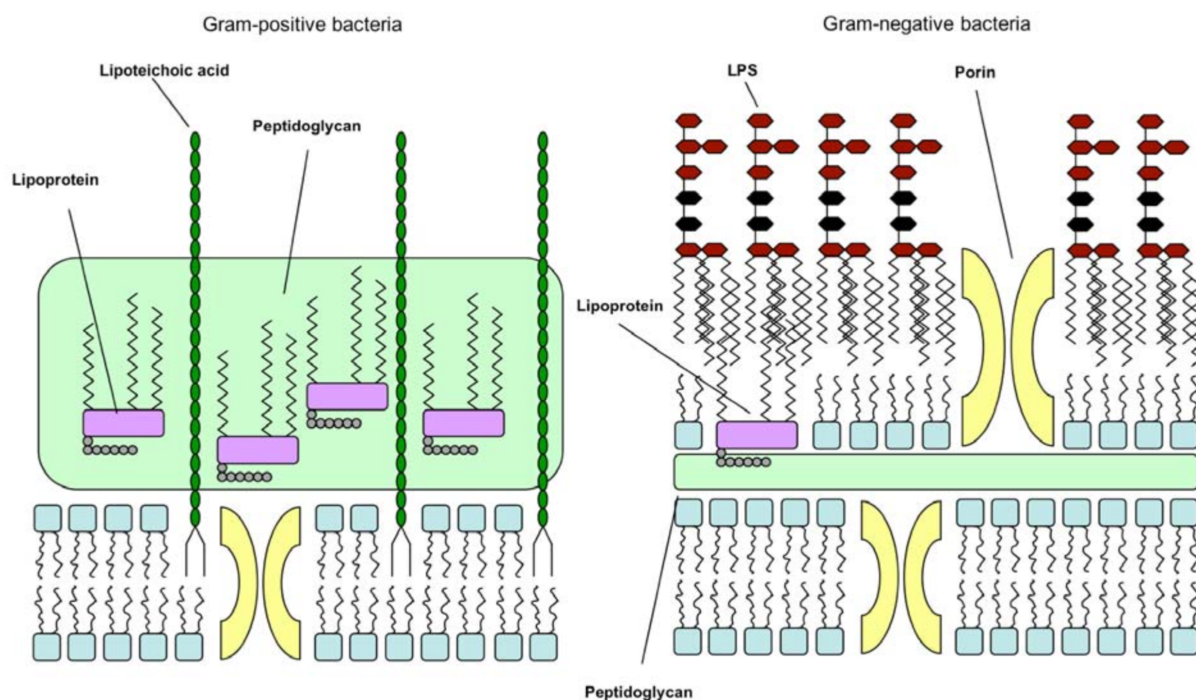


FIG.3: Schematic illustration of Gram-positive and Gram-negative bacterial cell wall

Gram-positive bacterial cell wall contains a thick layer of Peptidoglycan (PGN) and is embedded with Lipoteichoic acids (LTA) and various lipoproteins. The presence of LPS and a thin layer of PGN characterize the cell wall of Gram-negative bacteria. Lipoproteins are common for various types of bacteria.

TLR2

Like most members of the TLR family TLR2 was also found by database researches and was identified as a protein homologous to *Drosophila* Toll and able to induce NF-κB. After further analysis activation of macrophages upon challenge with bacterial lipopeptide in terms of IL-12 secretion as well as respiratory burst induction and initiation of apoptosis was demonstrated to be dependent of TLR2 (Aliprantis, Yang et al. 1999; Brightbill, Libraty et al. 1999). Thus, TLR2 was considered to initiate immune responses towards Gram-positive bacterial infections, which carry substantial fractions of lipoproteins within their cell walls. Upcoming reports confirmed these assumptions and introduced other microbial products like LTA and PGN as TLR2 agonists (Takeuchi, Hoshino et al. 2000). Also the yeast cell wall component zymosan and specific types of LPS were

described as TLR2 ligands whereupon reports demonstrating LPS recognition via TLR2 are yet under discussion (Werts, Tapping et al. 2001; Janeway and Medzhitov 2002). TLR2 recognizes some of its ligands independently but in contrast to other TLRs it was shown that TLR2 cooperates with TLR1 or TLR6. TLR2 mediated cell activation was enhanced if TLR1 or TLR6 were coexpressed and it was concluded that TLR2 dimerizes with TLR1 or TLR6 to recognize different PAMPs (Ozinsky, Underhill et al. 2000; Takeuchi, Kawai et al. 2001). Furthermore TLR2 mediated signalling was shown to be facilitated by CD14 (Hirschfeld, Kirschning et al. 1999). Notably, a monoclonal antibody antagonising the function of TLR2 was demonstrated to protect wild-type mice from fatal shock induced by tri-acylated lipoprotein or *B. subtilis* underlining the importance of TLR2 in responsiveness to Gram-positive bacterial infection (Meng, Rutz et al. 2004).

TLR4

LPS (endotoxin) the major cell wall constituent of Gram-negative bacteria is a strong inducer of proinflammatory immune responses in mice and human and can eventually cause fatal septic shock-like syndromes (Gormus and Shands 1975; Doe, Yang et al. 1978). However mechanisms how LPS is recognized by the immune system and how signalling occurs were mysterious until the Toll-like receptors were identified. Before TLR4 was described other serum-proteins and also receptors were found to be involved in LPS signalling. One important serum protein with LPS-binding capacity is the LPS binding protein (LBP). LBP is an acute phase protein, which is produced in the liver during Gram-negative bacterial infections. Human cDNAs encoding LBP were isolated upon screening a human liver cDNA library with an oligonucleotide derived from the rabbit LBP protein sequence. The predicted human LBP protein consists of a 25-amino acid signal sequence followed by a 452-amino acid mature protein containing 4 cysteine residues and 5 potential glycosylation sites. In rabbits it was found that LBP functions as a carrier protein for LPS in the plasma and controls LPS-dependent monocytic responses (Schumann, Leong et al. 1990). Later experiments with KO-mice deficient for LBP confirmed previous findings and showed that LBP is not required for LPS clearance but rapidly induces LPS mediated inflammatory

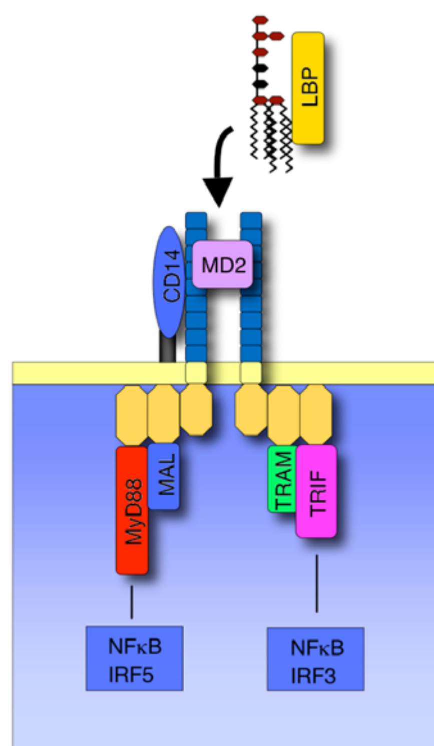


FIG.4: TLR4, the LPS receptor complex
LPS binding protein (LBP) detects LPS and transfers it to surface-TLR4 that interacts with MD-2. The GPI-anchored coreceptor CD14 supports LPS recognition. Signalling is mediated by the intracellular TIR-domain of activated TLR4 and induces MyD88 and TRIF. Both pathways result in NF-κB activation whereas MyD88 further activates IRF5 and TRIF activates IRF3. Notably IRF3 promotes production of type I interferon whereas IRF5 does not.

processes (Jack, Fan et al. 1997). Meanwhile the myeloid-cell-specific-leucine-rich glycoprotein CD14 was identified. CD14 is a single-copy gene encoding 2 protein forms: a 50- to 55-kD glycosylphosphatidylinositol (GPI)-anchored membrane protein (mCD14) and a monocyte or liver-derived soluble serum protein (sCD14) that lacks the anchor. Both molecules were found to be critical for LPS-dependent signal transduction, and sCD14 confers LPS sensitivity to cells lacking mCD14 (Goyert, Ferrero et al. 1988; LeVan, Bloom et al. 2001). Notably as a GPI-anchored receptor CD14 lacks transmembrane and intracellular domains and consequently is unable to transduce signalling into the cell. However, mice lacking CD14 were described as resistant to endotoxin shock and increased sCD14 levels are associated with inflammatory infectious diseases and high mortality in Gram-negative shock (Haziot, Ferrero et al. 1996). Furthermore LBP was shown to rapidly catalyze the transfer of LPS to membrane bound or soluble CD14 but the receptor responsible for signalling was further elusive (Wright, Ramos et al. 1990). Finally, by gene analysis of the mouse strains C3H/HeJ and C57BL10/ScCR, which both fail to induce proinflammatory responses upon LPS challenge the *LPS* gene was identified to encode murine TLR4. Thereby it was shown that C3H/HeJ mice reveal a point mutation (P712H) in the TIR domain whereas C57BL10/ScCR mice lack a genomic region including the TLR4 gene (Poltorak, He et al. 1998; Qureshi, Lariviere et al. 1999). Further experiments with TLR4$^{-/-}$ mice demonstrated the importance of TLR4 in LPS signalling (Hoshino, Takeuchi et al. 1999). However, it was demonstrated that an additional molecule named MD-2, which forms a complex with TLR4, is required for effective LPS recognition. Although TLR4 is indispensable for LPS signalling, overexpression of TLR4 did not constitute LPS responsiveness in non sensitive cells such as human embryonic kidney (HEK)-293 or mouse IL-3 dependent pro B cell line Ba/F3 cells (Kirschning, Wesche et al. 1998; Shimazu, Akashi et al. 1999). However, co-expression of MD-2 or culture in soluble MD-2 conditioned medium conferred full LPS responsiveness (Visintin, Mazzoni et al. 2001). KO-studies showed that MD-2 is indispensable for LPS responses *in vivo* and enhances expression of TLR4 and contributes to its surface expression (Akashi, Shimazu et al. 2000; da Silva Correia, Soldau et al. 2001; Miyake, Nagai et al. 2002; Nagai, Akashi et al. 2002). MD-2 is a secreted glycoprotein with an apparent size in SDS-PAGE of approximately 25 kilo Dalton (kDa) and shorter mono- and unglycosylated forms (da Silva Correia and Ulevitch 2002). Whether MD-2 itself binds to LPS or mediates signalling is yet under discussion. An LPS-binding peptide fragment of MD-2 implicates an amphipatic cluster composed of basic and hydrophobic residues as binding site (Mancek, Pristovsek et al. 2002). Taken together LPS recognition is mediated by a receptor complex consisting of LBP, which opsonizes LPS and transfers it to CD14 (**Figure 4**). CD14 supports LPS recognition and activation of TLR4/MD-2 complex results in signalling transduction (Aderem and Ulevitch 2000).

1.2.1.3 Regulation of TLR signalling

TLR activation upon recognition of invading pathogens is essential for the initiation of an effective immune response. On the other hand it is reported that specific members of the TLR family are involved in pathogenesis of autoimmune diseases, chronic inflammation diseases as well as infectious diseases. The most severe disease mediated by TLR activation is sepsis, which frequently results in lethal shock syndromes. Furthermore TLRs are postulated to play a role in diabetes susceptibility and TLR9 for instance is demonstrated to mediate self-DNA recognition within lupus erythematosus. Consequently if out of control innate immune responses can lead to hyper-inflammation, chronic inflammatory diseases and autoimmune disorders. Therefore proinflammatory processes are controlled by several mechanisms to inhibit above-mentioned dysregulations and maintain immunological balance (Liew, Xu et al. 2005).

Over the last years several regulatory mechanisms to modulate TLR-signalling have been identified. For TLR2 and TLR4 soluble receptors (sTLRs) are identified and represent negative regulators directly interacting with the respective ligand to terminate TLR activation and further signalling. Therefore production of sTLRs is a first line negative regulatory mechanism preventing overreaction of the host against microbial products. Other negative regulators interfere intracellularly with the TLR signal cascades. For example, IRAK-M is up-regulated upon stimulation of TLRs in monocytes and macrophages, indicating a negative feedback loop (Wesche, Gao et al. 1999). Although IRAK-M lacks the typical kinase activity of other family members, it prevents the formation of the IRAK1-TRAF6 complex by inhibiting dissociation of the IRAK1-IRAK4 complex from MyD88, which results in the interruption of signalling to NF-κB and MAPK (Kobayashi, Hernandez et al. 2002). Suppressor of cytokine signalling (SOCS) 1 is a negative regulatory molecule of cytokine signalling (Alexander and Hilton 2004). However, whether SOCS1 exerts a direct effect on TLR signalling is still controversial, but one potential mechanism might be the inhibition of IRAK1 activation (Kinjyo, Hanada et al. 2002; Nakagawa, Naka et al. 2002; Baetz, Frey et al. 2004; Gingras, Parganas et al. 2004). Other negative regulators are the adaptor molecule Toll-interacting protein (TOLLIP), which also inhibits IRAK1 activation, probably by decreasing its autophosphorylation (Zhang and Ghosh 2002) and MyD88s a splice variant of MyD88 that is unable to bind to IRAK4 and to promote phosphorylation of IRAK1 (Burns, Janssens et al. 2003). Furthermore the protein A20 was identified as a negative regulator for activation of all TLRs. A20 is a cysteine protease deubiquitylating enzyme that blocks TLR signalling by deubiquitylation of TRAF6 inhibiting downstream signalling and translocation of NF-κB into the nucleus (Boone, Turer et al. 2004; Wertz, O'Rourke et al. 2004). Also transmembrane proteins were implicated in modulating TLR signalling. Examples are the orphan receptors single immunoglobulin IL-1R-related protein (SIGIRR) and suppressor of tumorgenicity (ST2). Amongst others, they are recruited to TLR4 where SIGIRR interferes with IRAK1 and TRAF6, whereas ST2 addresses MyD88 and TIRAP, thereby inhibiting proper signal transduction (Wald, Qin et al. 2003; Brint, Xu et al.

2004). Finally in contrast to the other TIR-domain containing proteins, which positively regulate immune responses the TIR domain containing adaptor protein SARM was identified to negatively regulate TRIF-mediated proinflammatory responses upon direct interaction (Carty, Goodbody et al. 2006).

Conclusively all TLR pathways are somehow regulated by cutting off the signalling events either extracellular or intracellular. Not all TLRs are affected in the same way and are regulated by specific mechanisms by the different molecules. But the interplay of these regulators provides a potent mechanism to balance the TLR-mediated immune activation in order to avoid detrimental and inappropriate inflammatory responses.

1.2.2 Non-Toll-like pattern recognition receptors

Although TLRs seem to play a dominant role they are not the sole receptor family that mediates innate immunity and pattern recognition. The TLRs were the first family of PRRs that were studied in detail but recently two other families of PRRs were described: the NOD-like receptors (NLRs) and the RIG-like helicases (RLHs). In contrast to TLRs these receptor families lack transmembrane domains and consequently are located and active in the cytosolic fraction of the cell.

1.2.2.1 The NOD-like receptors (NLRs)

The NOD-like receptors (NLRs) also known as NOD-LRRs or CATERPILLAR are cytosolic proteins containing three distinct domains: an N-terminal protein-protein interacting domain composed of a CARD (caspase activation and recruitment domain) or a pyrin domain, a central nucleotide binding domain called NACHT and a C-terminal domain containing several LRRs (Takeuchi and Akira 2007). The NLRs include the two major subfamilies the NODs and the NALPs and a few further proteins like IPAF and NAIP. However the NALPs consist of 14 members of whom only for NALP1 and NALP3 specific ligands are identified yet (Petrilli, Dostert et al. 2007). The first discovered intracellular PRRs are NOD1 and NOD2. Both are described as sensors for bacterial products whereas NOD1 was identified to recognize a molecule called *meso*-DAP (*meso*-diaminopimelic acid), that is a peptidoglycan constituent of only Gram-negative bacteria and NOD2 was reported to recognize intracellular MDP (muramyl dipeptide), which is a peptidoglycan constituent of both Gram positive and Gram negative bacteria. Upon ligand recognition by the LRRs of NOD1 and NOD2 CARD-CARD domain interaction leads to the recruitment of the serine/threonine kinase RIP-2 resulting in NF-κB and MAP kinase activation (Fritz, Ferrero et al. 2006).

In contrast to the NODs the NALPs carry a pyrin effector domain N-terminally instead of a CARD domain. Till now only a few NALPs are characterized in detail and have been shown to activate proinflammatory processes via activation of caspases through formation of a protein complex called the inflammasome. The two inflammasomes identified are the NALP1 and the NALP3 inflammasome.

Whereas both inflammasomes recruit the adaptor molecule Apoptosis-Associated Speck-Like Protein Containing a CARD (ASC) the adaptor molecule CARDINAL is a constituent only in the NALP3 inflammasome. Furthermore the NALP3 inflammasome contains and finally activates caspase 1 alone whereas the NALP1 inflammasome consists of and further activates caspase 1 together with caspase 5 (Meylan, Tschopp et al. 2006). Both inflammasomes control the processing of the proinflammatory cytokines IL-1β and IL-18 by cleaving pro-IL-1β and pro-IL-18 leading to secretion of their active form. In contrast to NALP1 for which no ligand is identified yet many signals, which activate NALP3 (also known as cryopyrin), are described and consist not only microbial PAMPs but also host danger signals (danger-associated molecular patterns, DAMPs). It was described that high concentrations of ATP upon acute cellular stress, uric acid or bacterial toxins such as *Staphylococcus aureus* α-toxin activate the inflammasome by induction of massive potassium ion efflux (Perregaux and Gabel 1994; Walev, Reske et al. 1995; Shi, Evans et al. 2003; Martinon, Petrilli et al. 2006). Furthermore NALP3 was reported to sense TLR-ligands like bacterial DNA, the imidazoquinoline components R837 and R848. Additionally NALP3-dependent activation of caspase1 was observed upon LPS, lipopeptide and LTA challenge in the presence of ATP (Kanneganti, Ozoren et al. 2006; Mariathasan, Weiss et al. 2006). Upon observation of a large diversity of ligands it is therefore likely that NALP3 acts as the central adaptor molecule activating proinflammatory caspase1 downstream of more specific cytosolic receptors, which could be the remainder of NALPs. Conclusively due to its potentially dangerous proinflammatory activity IL-1β release is tightly controlled and therefore requires two parallel signalling pathways initiated by different specific receptors. During microbial infections TLR activation initiates NF-κB induced production of pro IL-1β and pro IL-18, which accumulates within the cell. As a second stimulus DAMPs like ATP, uric acid or bacterial pore-forming toxins entering the cytosol activate NLRs and induce formation of the caspase1-activating inflammasome resulting in processing and finally secretion of active IL-1β and IL-18 (Meylan, Tschopp et al. 2006; Kanneganti, Lamkanfi et al. 2007). The further members of the NLR-family NAIP and IPAF were both reported to sense *Legionella pneumophila* flagellin by activating caspase1 upon direct interaction with caspase1 independent of ASC. Notably it was observed that NAIP and IPAF physically interact (Franchi, Amer et al. 2006; Zamboni, Kobayashi et al. 2006).

1.2.2.2 The RIG-like helicases (RLHs)

Type-I interferons include a single IFN-β and several IFN-α molecules and are considered as cytokines bearing strongest antiviral capacity. Their expression is regulated by distinct transcription factors, mainly IRF3, IRF7, NF-κB and AP-1. As type-I interferons inducing receptors TLR3, TLR7, TLR8 and TLR9 are described. These TLRs are located intracellularly within endosomes and sense viral and bacterial nucleic acids. Aside of these TLRs three specific independent homologous DexD/H

box RNA helicases were identified to promote antiviral immune responses upon recognizing dsRNA, which is known to present a molecular intermediate during viral replication of many viruses within infected cells. The retinoic acid inducible gene-I (RIG-I) was found to specifically recognize dsRNA and recruits by CARD-CARD domain interaction the adaptor molecule IFN-β promoter stimulator (IPS-1). Activation of IPS-1 activates crucial signalling molecules resulting in activation of NF-κB and IRF3. Another RIG-like helicases, recruiting IPS-1 as an adaptor is the melanoma-differentiation-associated gene 5 (Mda5), which has also been implicated in viral dsRNA recognition (Kawai, Takahashi et al. 2005; Meylan and Tschopp 2006; Takeuchi and Akira 2007). Both helicases RIG-I and Mda5 were reported to signal cell-type specific. Plasmacytoid dendritic cells (pDC's), which are the main IFN-α producers were found to initiate antiviral responses independent of RIG-I and Mda5. IFN-α production in pDC's was found to be mediated by TLR7 and TLR9, which respond to ssRNA and unmethylated DNA with CpG motifs respectively. pDC's from mice lacking MyD88 challenged with RNA-viruses did not produce type-I interferons and other proinflammatory cytokines, whereas absence of IPS-1 did not impair type-I interferon production (Kato, Sato et al. 2005; Kato, Takeuchi et al. 2006). However, RIG-1 and Mda5 were demonstrated to defend the host against viral infection in the cytoplasm of mouse embryonic fibroblast (MEFs) and conventional dendritic cells (cDCs). Although both receptors are described to sense dsRNA *in vivo* experiments showed different ligand specificity in regard to infections with specific viruses. Experiments with knock-out mice showed that RIG-I mediates antiviral responses upon infection with the positive-stranded single-stranded (ss)RNA virus Japanese encephalitis virus and a set of negative-strand ssRNA viruses such as Newcastle disease virus, Vesicular Stomatitis virus (VSV) , Sendai and influenza virus. In contrast Mda5-deficient mice were almost unresponsive towards infection with positive-strand ssRNA picornavirus encephalomyocarditis virus (Kato, Sato et al. 2005; Kato, Takeuchi et al. 2006). Consequently potential cooperation of RIG-I and Mda5 in response to specific viral infections as well as further analysis regarding ligand specificity on the molecular level have to be determined in the future.

1.3 Sepsis

Sepsis describes a clinical condition including the spread of a local infection to a systemic infection and the therefore induced immune response. It represents a substantial burden of modern health care and 750.000 cases of sepsis in the US are described annually. This number composes 2% of all hospitalizations. The mortality ranges between 18 to 30% but could dramatically increase according to series of pathogenesis. 215.000 deaths, representing 9.3% of all deaths occur in the US each year and care for septic patients results in hospital costs, which exceed $16 billion (O'Brien, Ali et al. 2007). In Germany at least 65.000 deaths are counted annually and therefore sepsis is the third prevalent cause

of death after myocardial disorders and is the most frequent cause of death in non-cardiological intensive care units. Development of sepsis is independent of gender and patients diagnosed for sepsis are at an average age of 61. However half of relevant patients are already hospitalized due to another disease before developing septic conditions (www.sepsis.bbraun.de). Despite frequency, high mortality rate and enormous costs an explicit patient phenotype for sepsis is lacking. Due to the heterogeneity of the patients diagnosis and therapy is still difficult and must be improved.

1.3.1 Pathogenesis of sepsis

The most common mediators of sepsis are bacteria. Thereby pathogenic bacteria as well as commensal bacteria could be involved in sepsis development. The most common sites of infection are primary infections of the blood stream and infections of the respiratory tract, the abdominal cavity and the urinary tract. If a microbiological diagnosis could have been preformed Gram-negative bacteria are responsible for about 60% of all cases whereas Gram-positive bacteria account for the remainder. Interestingly the spectrum of responsible bacteria shifts and Gram-positive bacteria induced sepsis increases whereas Gram-negative bacteria induced sepsis decreases (Riedemann, Guo et al. 2003). Most cases of Gram-negative sepsis are caused by *Pseudomonas aeruginosa* and Enterobacteriacae such as *Escherichia coli* and *Klebsiella* species. The commonest causes of Gram-positive sepsis are Staphylococci (mainly *Staphylococcus aureus* and coagulase-negative staphylococci) and Streptococci (*Streptococcus pyogenes* and *Streptococcus pneumoniae*) that usually infect the skin, the respiratory tract and the primary blood stream. Notably, aside of bacteria any microorganisms including fungi, parasites and also viruses can cause sepsis (Bochud and Calandra 2003).

Sepsis is described as a condition of an ongoing, uncontrolled and therefore dangerous immune response induced upon systemic hyperinfection. Interaction of microbial compounds and the host that is susceptible due to specific factors induces different proinflammatory cascades that include procoagulant and antifibrinolytic pathways as well as several innate proinflammatory processes. The general symptoms and accepted criteria to diagnose sepsis are hyper- (>38°C) or hypothermia (<36°C), tachypnea (high respiratory rate, >20/min), increased heart rate (>90/min) and a white blood cell count lower than 4000/mm^3 or higher than 12 000/mm^3. Conclusively sepsis conditions are classified in four different stages according to the clinical severity and symptoms (Wiedemann 2007).

1. SIRS (Systemic Inflammatory Response Syndrome):

A general immune response towards specific disease patterns like trauma, burns, hypoxia or pancreatitis without an acute infection. At least two of the symptoms mentioned above exist.

2. Sepsis:

Sepsis describes the systemic reaction towards a specific infection without organic dysregulations. At least two of the symptoms mentioned above exist.

3. Severe Sepsis:

Parallel to sepsis syndromes functionality of organs like lung, kidney, liver or heart is dysregulated.

4. Septic Shock:

Symptoms of organic dysfunction persist and continuous hypotension despite of adequate fluid resuscitation is observed. Finally decreased blood pressure leads to hypoxemia resulting in cell death and multiple organ failure (MOF). Mortality rate of patients suffering septic shock is above 70%.

These potentially life threatening symptoms within sepsis pathogenesis are mediated by proinflammatory cytokines and other innate immune processes. High levels of cytokines and complement proteins like C5a are detected in sepsis patients and embrace the so called “cytokine storm”, which is characteristic for the disease and is related to its severity. Early proinflammatory mediators like TNFα and IL-1 within the first two hours as well as subsequent acute phase proteins like IL-6 are significantly increased in sepsis patients and promote inflammation in terms of vessel relaxation, production of reactive oxygen species and increase of body temperature. Reactive oxygen species (ROS) like H_2O_2 for instance are capable to cause tissue damage leading to increased vascular permeability and organ injury (Riedemann, Guo et al. 2003). During a systemic infection treatment with antibiotics is indispensable to stop further spread of bacteria. Unfortunately antibiotic treatment and according killing of the bacteria sometimes has an adverse effect and supports ongoing inflammation and significantly worsens the condition of the patient. This effect is called Jarisch-Herxheimer reaction and is induced when immune-stimulatory microbial components are released rapidly from bacteria exposed to antibiotics (Pound and May 2005).

1.3.2 The role of TLRs in sepsis pathology

As detectors for microbial components and mediators of inflammation pattern recognition receptors like TLRs were reported to play a crucial role in sepsis pathology. It was shown that application of LPS, which is the major immune-stimulatory agent in Gram-negative bacteria, induces septic-shock like syndromes. The endotoxin shock model shows that in absence of an infection shock can be induced and must be mediated by an overwhelming immune response. Furthermore mice lacking TLR4 are more susceptible to infection with Gram-negative bacteria but are resistant to LPS-induced shock strengthening the important role of TLRs in septic shock induction (Hoshino, Takeuchi et al.

1999). Several other bacterial TLR agonists, like flagellin, lipopeptide, unmethylated CpG sequences as well as naked bacterial DNA have been shown to be able to induce shock in experimental systems mediated by the respective TLR (Sparwasser, Miethke et al. 1997; Eaves-Pyles, Murthy et al. 2001; Meng, Rutz et al. 2004).

1.4 Aims of the study

As detectors of microbial challenge and inducers of initial immune responses TLRs play an essential role within innate immunity. Although TLRs meanwhile represent a well characterized receptor family reports regarding their ligand specificities are still contradictory and require further analysis. To expand present sound knowledge we focused on bacterial compounds like specific bacterial lipopeptides and various LPS-species and analyzed their capacity to activate TLR2 and/or TLR4, which both are PRRs mainly involved in bacterial recognition. Furthermore we aimed to elucidate the role of TLRs during bacterial hyperinfection and sepsis. Therefore we planned to establish mouse models for Gram-negative as well as Gram-positive bacterial infections. By using specific TLR knock-out mice our intention was to define which TLRs are essential in recognition of particular bacteria *in vivo*. Upon identification of specific TLRs mediating bacteria induced fatal hyperinflammation our purpose was to specifically inhibit TLR activity during ongoing sepsis in order to avert fatal shock-like syndromes which are induced by overwhelming proinflammatory processes. Therefore we planned to treat mice suffering fatal bacterial hyperinfection with a combination of antagonistic monoclonal antibodies towards TLR2 or TLR4. While an antagonistic monoclonal antibody towards TLR2 was already established we aimed to screen and characterize another monoclonal antagonistic antibody towards TLR4. Taken together aim of the study was to demonstrate that blockade of TLRs by application of specific monoclonal antibodies might provide a potentially clinically relevant therapeutic approach to inhibit bacteria induced hyperinflammation.

2 MATERIAL AND METHODS

2.1 Material

2.1.1 Equipment

BioFuge fresco	Heraeus
Centrifugal Filter Units (100kDa)	Centricon
CyAn ADP LX9 analyzer	DakoCytomation
Electrophoresis Mighty Small SE250 Vertikal	Hoefer
Electroporation device Gene Pulser	Bio-Rad
ELISA reader Sunrise	Tecan
ELISA washing machine	Skatron
Freezer -20°C	Siemens
Freezer -80°C	Thermo Scientific
Fridge	Liebherr
FUJI Medical X-Ray Film	FujiFilm
Gel documentation Eagle Eye II	Stratagene
Heatblock Dri-Block DB 2D	Techne
Incubator Hera Cell 240	Heraeus
MaxiSorp 96-well ELISA plates	Nunc
Microcentifuge tubes 1.5 ml	Eppendorf
Microscope Zeiss IDO3	Zeiss
MegaFuge 1.RS	Heraeus
Multichannel pipettes	ThermoLabsystems
Multipipette plus	Eppendorf
NanoDrop®ND-1000 Spectrophotometer	Peqlab
Neubauer counting chamber	Roth
Nitrogen freezing tank Espace 300	Air Liquide
Orion Microplate Luminometer	Berthold
Parafilm®	Pechiney, Plastic Packaging
pH-meter Multical	WTW
Pipettes	Gilson
Pipetboy acu	Integra Biosciences
Plastic ware	Nunc; Falcon

Power Supply Power Pac 200	Amersham/Bio-Rad
Rotator OV3	Biometra
SDS-PAGE gel chamber	Amersham
Semi-dry blotting apparatus	Amersham
Shaker	Peqlab
Sonicater UW 60	Bandelin Electronic
Sterile bench	Heraeus
Thermocycler Trio Thermoblock	Biometra
Thermomixer Comfort	Eppendorf
Ultra-Turrax T25	IKA Labortechnik
Vortexer Genie 2	Scientific Industries
Waterbath	Memmert
Whatman Paper	Schleicher & Schuell

2.1.2 Kits and enzymes

AccuTaq LA DNA polymerase	Sigma
BCA™ Protein Assay Kit	Pierce
Cytofix / Cytoperm Kit	BD
EndoFree® Plasmid Maxi Kit	Qiagen
EndoFree® Plasmid Mini Kit	Qiagen
ELISA-Kits	R&D Systems
Pfu DNA polymerase	Fermentas
QIAEX II Gel Extraction Kit	Qiagen
QuickChange Multi Site-Directed Mutagenesis Kit	Stratagene
Restriction enzymes	Fermentas
Shrimps Alkaline Phosphatase	USB
Taq DNA polymerase	Clontech
T4 DNA Ligase Kit	Fermentas
Topo-TA-Cloning-kit	Invitrogen
Western Lightning Chemiluminescence	PerkinElmer Life Sciences

2.1.3 Reagents

Acrylamide/Bis-Acryamidesolution 30%	Biorad
Accutase	PAA
Agarose	Invitrogen
Ammouniumperoxysulfate (APS)	Sigma

Ampicillin	Sigma
β-Mercaptoethanol	Gibco
BSA	Sigma
CpG1668	TIB MolBiol
D-Galactosamine	Sigma
D-Luciferin	PJK
Desoxytrinucleotides (dNTPs)	Fermentas
DMSO	Sigma
DNA-Ladder 1 Kb	Invitrogen
DTT	Sigma
Dulbecco PBS	Biochrom
EDTA	Sigma
Erythrocyte lysis buffer (hybri Max)	Sigma
Ethidiumbromide	Roth
FCS	PAA
G418 (Neomycin)	PAA
Golgi-Plug	Pharmingen
HAT Media Supplement (50x) Hybri-Max	Sigma
Hybridoma Fusion and Cloning Supplement (HFCS)	Roche
Hygromycin B	Roche
L-cell-conditioned medium	Own production
Loading Dye Solution 6x (Western)	Fermentas
LPS *E. coli* O111:B4	Sigma
LPS *S.minnesota* Re595 (rough strain)	Sigma
Magnesium chloride ($MgCl_2$)	Roth
Methanol	Roth
Milk powder	Roth
murine IFN-γ recombinant	tebu-bio
Myristoyl coenzyme A Lithium salt (MyrCoA)	Sigma
Myr_1GSK_4 (Myristoyl-glcyl-seryl-tetralysine)	EMC Microcollections
Myr_3CSK_4 (Tri-myristoyl-cysteinyl-seryl-tetralysine)	EMC Microcollections
Natrium chloride (NaCl)	Roth
Natrium fluoride (NaF)	Merck
Normal goat serum (NGS)	Invitrogen
Nonident P-40 (IGEPAL CA-630)	Sigma
Ofloxacin	Apotheke Klinikum r. d. Isar
Pam_2CSK_4 (Di-palmitoyl-cysteinyl-seryl-tetralysine)	EMC Microcollections

Pam_3CSK_4 (Tri-palmitoyl-cysteinyl-seryl-tetralysine)	EMC Microcollections
Pancoll (human)	PAN (Biotech)
Penicillin/Streptomycin	Biochrom
Poly I:C	Invivogen
Protease Inhibitor Cocktail	Sigma
Proteinase K	Roche
Resiquimod R848	Coley pharmaceuticals
Reporter Lysis Buffer 5x	Promega
Rocephin	Sigma
Natriumdodecyle sulfate (SDS)	Merck
SCGK(Myr)RTEK	EMC Microcollections
SCGK(Dpr)RTEK	EMC Microcollections
SCGK(Pam)RTEK	EMC Microcollections
SCGKRTEK	EMC Microcollections
Streptavidin-HRP	R&D Systems
TEMED	Sigma
TMB tablets	Sigma
TRIS	Roth
TRIS hydro chloride	Roth
Tween20	Sigma

2.1.4 Primers and probes

Name (Position relative to TSS)	**5'-3' sequence (inserted restriction sites underlined)**
Exchange **LRR7-12** from hTLR2 in hTLR4	F-Primer vector-hTLR4-insert-hTLR2 GACTTGCGGGTTCTACATCAAATG–ATTCAGAATGTAAGTCA TCTGATCC R-Primer insert-hTLR2-vector-hTLR4 GATCTAGAAACTCAAGGCTTGGTAGA-TGTTGTGAAAGTAAA CAAGGAACC
Exchange **LRR7-12** from hTLR4 in hTLR2	F-Primer vector-hTLR2-insert-hTLR4 CTATGAGCCAAAAAGTTTGAAGTC-CCCCTACTCAATCTCTCT TTAGACCTG R-Primer insert-hTLR4-vector-hTLR2 CTGAGATCCAAGTATTCTAATGATTTTAAA-TCAACTTCTGAA AAAGCATTCCCAC
Exchange **LRR6-13** from	

hTLR2 in hTLR4	F-Primer vector-hTLR4-insert-hTLR2 CTTTCAAATTACCTGAGTATTTTTCTAAT-CTTACCTTCCTTGA GGAACTTGAG R-Primer insert-hTLR2-vector-hTLR4 CAGATCTAAATACTTTAGGCTGGTTGT-GGCATCCTCACAGG CTGAATTTTTC
Exchange **LRR6-13** from hTLR4 in hTLR2	F-Primer vector-hTLR2-insert-hTLR4 GATTCAAAGAAAAGATTTTGCTGGA-CTGACCAATCTAGAGC ACTTGGAC R-Primer insert-hTLR4-vector-hTLR2 CTTAAAATTAAAGTTTGTAGAGAGGGCCA-CAAAATCACTTT GAGAACAGCAACC
Exchange **LRR7-9** from hTLR2 in hTLR4	F-Primer vector-hTLR4-insert-hTLR2 CTTGCGGGTTCTACATCAAATG–ATTCAGAATGTAAGTCATC TGATCC R-Primer insert-hTLR2-vector-hTLR4 CAAATTGCACAGGCCCTCTAG–CTGATTCAAAAGTTTCATAA CCAG
Exchange **LRR7-11** from hTLR2 in hTLR4	F-Primer vector-hTLR4-insert-hTLR2 CTTGCGGGTTCTACATCAAATG–ATTCAGAATGTAAGTCATC TGATCC R-Primer insert-hTLR2-vector-hTLR4 CTTGCGGGTTCTACATCAAATG–ATTCAGAATGTAAGTCATC TGATCC
Exchange **LRR8-12** from hTLR2 in hTLR4	F-Primer vector-hTLR4-insert-hTLR2 CTTTATCCAACCAGGTGCATTTAAA–ACAAGTTCCGTGGAAT GTTTG R-Primer insert-hTLR2-vector-hTLR4 CTAGAAACTCAAGGCTTGGTAG–ATGTTGTGAAAGTAAACA AGGAAC
Exchange **LRR7** from hTLR2 in hTLR4	F-Primer vector-hTLR4-insert-hTLR2 CTTGCGGGTTCTACATCAAATG-ATTCAGAATGTAAGTCATC TGATCC R-Primer insert-hTLR2-vector-hTLR4 GTCAGCTTATGAAGCCTAATTTC-AACATCTACAAAAATCTC CAGCAG
Exchange **LRR8** from hTLR2 in hTLR4	F-Primer vector-hTLR4-insert-hTLR2 CTTTATCCAACCAGGTGCATTTAAA-ACAAGTTCCGTGGAAT GTTTG

	R-Primer insert-hTLR2-vector-hTLR4 CTAAACCAGCCAGACCTTGAAT−TTCACCAGTGGATAGTTCTG
Exchange **LRR9** from hTLR2 in hTLR4	F-Primer vector-hTLR4-insert-hTLR2 GATAGTTTAAATGTAATGAAAACTTGT−ACAAATTCATTGATTAAAAAG R-Primer insert-hTLR2-vector-hTLR4 CAAATTGCACAGGCCCTCTAG-CTGATTCAAAAGTTTCATAACCTG

Table 1. Oligonucleotide primer for splice/mutagenesis PCRs.

2.1.5 Vectors

Vector	Marker	Descprition	Reference
pCMV-Tag2A	P CMV, P bla, P SV40, SV40 pA, TK, pA, f1 ori, pUC ori, neo/kanR, FLAG	Expressionvector, flag-tagged at 5’ of MCS	Stratagene
pCMV-Tag3A	P CMV, P bla, P SV40, SV40 pA, TK, pA, f1 ori, pUC ori, neo/kanR, MYC	Expressionvector, myc-tagged 5’ at of MCS	Stratagene
pCMV-Tag4A	P CMV, P bla, P SV40, SV40 pA, TK, pA, f1 ori, pUC ori, neo/kanR, FLAG	Expressionvector, flag-tagged 3’ at of MCS	Stratagene
pMycCMV-1	P CMV, P SV40, hHG pA, pretrypsine leader, f1 ori, ampR, MYC	Expressionvector, pretrypsine leader and myc-tagged 5’ at of MCS	Stratagene
pFlagCMV-1	P CMV, P SV40, hHG pA, pretrypsine leader, f1 ori, ampR, FLAG	Expressionvector, pretrypsine leader and flag-tagged 5’ at of MCS	Stratagene
pRK5	P CMV, P SV40, P SP6, SV40 pA, pUC ori, ampR, lacZ, lacI, FLAG	Expressionvector, flag-tagged 3’ at of MCS	U. Schindler
pEFBOS	P SV40, P hEF1 pA, M13 ori, ampR, lacI, FLAG	Expressionvector, pretrypsine leader and flag-tagged 5’ at of MCS	Stratagene

Plasmids

Promoter	Insert	Vector	Donor
P_{CMV}	humanTLR 4 (Flag)	pFlag-CMV-1	C.Kirschning H.Wesche
P_{CMV}	mouseTLR 4 (Flag)	pFlag-CMV-1	H. Heine
P_{CMV}	mouseTLR 4 (Myc)	pMyc-CMV-1	H. Heine
P_{CMV}	humanTLR 2 (Flag)	pFlag-CMV-1	C.Kirschning H.Wesche
P_{CMV}	mouseTLR 2 (Flag)	pFlag-CMV-1	H. Heine
P_{CMV}	hmutH TLR2 (Flag)	pFlag-CMV-1	GM
P_{CMV}	human CD14 (Flag)	pRK5	C.Kirschning H.Wesche
P_{EF1}	human MD-2 (Flag)	pEFBOS	Stratagene
P_{EF1}	mouse MD-2 (Flag)	pEFBOS	Stratagene
P_{CMV}	human TLR2-4(LRR6-13)-2 (Flag)	pFlag-CMV-1	S. Spiller
P_{CMV}	human TLR2-4(LRR7-12)-2 (Flag)	pFlag-CMV-1	S. Spiller
P_{CMV}	human TLR4-2(LRR6-13)-4 (Flag)	pFlag-CMV-1	S. Spiller
P_{CMV}	human TLR4-2(LRR7-12)-4 (Flag)	pFlag-CMV-1	S. Spiller
P_{CMV}	human TLR4-2(LRR7-9)-4 (Flag)	pFlag-CMV-1	S. Spiller
P_{CMV}	human TLR4-2(LRR7-11)-4 (Flag)	pFlag-CMV-1	S. Spiller
P_{CMV}	human TLR4-2(LRR8-12)-4 (Flag)	pFlag-CMV-1	S. Spiller
P_{CMV}	human TLR4-2(LRR7)-4 (Flag)	pFlag-CMV-1	S. Spiller
P_{CMV}	human TLR4-2(LRR8)-4 (Flag)	pFlag-CMV-1	S. Spiller
P_{CMV}	human TLR4-2(LRR9)-4 (Flag)	pFlag-CMV-1	S. Spiller
P_{ELAM-1}	Luc	pELAM-1	U. Schindler
P_{T7}	Renilla	pHRL	?
P_{CMV}	-	pRK5	U. Schindler

Table 2 Plasmids and expression constructs used

Mammalian expression vectors pRK5 and pCMV contain the early promotor of human cytomegalovirus (CMV) mediating high expression of recombinant proteins. The promoter of pELAM-1 is NF-κB dependent. The plasmids pFLAG-CMV-1 is a derivate of pCMV. A heterologous preprotrypsin leader precedes a FLAG epitope tag, N-terminally fused to the overexpressed protein. GM – Guangxun Meng.

2.1.6 Antibodies used for Western blot

Primary antibodies

Antigen	Isotype	Dilution	Blocking	Company
ß-actine	mouse, IgG1	1:5000	5 % milk	Sigma-Aldrich
p38	rabbit	1:1000	5 % milk	Cell Signaling
phospho-p38 (Thr180/Tyr182)	rabbit	1:1000	5 % milk	Cell Signaling
phospho-ERK (Thr202/Tyr204)	rabbit	1:1000	5 % milk	Cell Signaling
α-Flag (polyclonal)	rabbit	1:1000	5 % milk	Sigma
α-Myc (polyclonal)	rabbit	1:1000	5 % milk	Sigma
α-TLR2 (polyclonal)	rabbit	1:500	5 % milk	Sigma

Secondary antibodies

Antigen	Conjugate	Source	Dilution	Company
α-mouse IgG F(ab')2-Fragment	HRP	goat	1:5000	Dianova
α-rabbit IgG F(ab')2-Fragment	HRP	donkey	1:5000	Dianova

Table 3. Antibodies for Western blot analysis.

2.1.7 Antibodies used for flow cytometry

Antibodies

Antigen	Isotype	Conjugate	Dilution	Company
mouse CD16/CD32	rat IgG2b	-	1:200	BD Bioscience
human CD11b	rat IgG1	PE	1:100	BD Bioscience
mouse CD11b	rat IgG2a	FITC/PE/APC	1:300	BD Bioscience
mouse CD3	rat IgG2b	FITC/APC-Cy7	1:200	BD Bioscience
mouse CD4	rat IgG2b	FITC/PE	1:200	BD Bioscience
mouse CD8	rat IgG2a	FITC/Alexa564	1:200	BD Bioscience
mouse F4/80	rat IgG2b	PE-Cy5	1:300	BD Bioscience

mouse NK1.1	rat IgG2b	PE	1:100	BD Bioscience
mouse IFNγ	rat IgG2b	APC	1:100	BD Bioscience
mouse TNFα	rat IgG2b	APC	1:100	BD Bioscience
mouse/human TLR2 (Cl. 2.5)	mouse IgG1	FITC	1:50	HBT
mouse TLR2 (Cl. 2.7)	mouse IgG1	FITC	1:50	HBT
mouse TLR4/MD-2 (Cl. 1A6)	rat IgG2b	-	1:50	NovImmune
mouse TLR4/MD-2 (Cl. 5E3)	rat IgG2b	-	1:50	NovImmune
mouse TLR4/MD-2 (MTS510)	rat IgG2a	-	1:50	Abcam
rat IgG	goat IgG1	FITC	1:400	Caltag
mouse IgG	rat IgG1	PE	1:1000	BD Bioscience
rabbit IgG	goat IgG1	FITC	1:300	BD Bioscience

Conjugates

Antigen	Isotype	Conjugate	Dilution	Company
Biotin	Streptavidin	PE	1:100	Molecular Probes

Table 4. Antibodies and conjugates for flow cytometry.

2.1.8 Antagonistic antibodies used for *in vitro* and *in vivo experiments*

Antibodies

Antigen	Name	Isotype	Company
murine / human TLR2	T2.5	mouse IgG1	G. Meng / HBT
Non-specific isotype control	T2.13	mouse IgG1	G. Meng
mouse TLR4/MD-2	1A6	rat IgG2b	NovImmune
mouse TLR4	5E3	rat IgG2b	NovImmune
Non-specific isotype control	11G8	Rat IgG2b	NovImmune

human TLR4	15C1	mouse IgG1	NovImmune

Table 5: Antagonistic antibodies used for *in vitro* and *in vivo* application

2.2 Methods

2.2.1 Cell biology

2.2.1.1 Culture of murine RAW 264.7 and human THP-1 macrophages

RAW264.7 medium:	500 ml	RPMI 1640
	50 ml	FCS
	5 ml	Penicillin/Streptomycin
	5 ml	Antibiotic/Antimycotic
	500 µl	β-mercaptoethanol 50 mM

THP-1 medium:	500 ml	RPMI 1640
	50 ml	FCS
	5 ml	Penicillin/Streptomycin
	5 ml	Antibiotic/Antimycotic

RAW 264.7 and THP-1 macrophages were cultured in cRPMI medium at 37°C, 6 % CO_2 and 95 % humidity. Cells were cultured to 60-70 % confluency. To split the cells, they were rinsed from the plate and centrifuged at 1200 rpm for 5 min. The cells were gently resuspended and 1/5th to 1/10th of this solution were transferred to a new cell culture dish containing fresh medium. This procedure was carried out every two to three days.

2.2.1.2 Culture of HEK 293 cells

HEK293 medium:	500 ml	DMEM
	50 ml	FCS
	5 ml	Penicillin/Streptomycin
	5 ml	Antibiotic/Antimycotic

The human embryonic kidney cell line (HEK) 293 was applied for protein overexpression and functional analysis. HEK 293 cells were cultured as adherent monolayer at 37°C, 6% CO_2 and 95% humidity. The cells were grown to confluence and split. Therefore the medium was removed and cells

detached in 5 ml (per 10 cm dish) of 1% (w/v) trypsin-EDTA (Gibco) for 5 min. Trypsin was inhibited by addition of 1 volume of medium and the cells were thoroughly resuspended. 1/10 of this solution was transferred to a new plate and fresh medium was added.

2.2.1.3 Cryopreservation of cells

Cryopreservation medium:	10%	DMSO
	90%	FCS

To cryopreserve eukaryotic cells, FCS substituted with 10 % DMSO was prepared. After detaching the cells, they were counted and centrifuged at 1200 rpm for 5 min. The cells were resuspended in 1 ml per $5x10^6$ cells, transferred to cryotubes and deep frozen at -20°C for 120 min before they were stored at -80°C overnight. Finally the frozen cells were transferred for long-term storage in liquid nitrogen. Cells were thawed very quickly at 37°C and then transferred in 10 ml of pre-warmed medium. After two washes with 1x PBS and spins at 1200 rpm cells were then resuspended in 5 to 10 ml of pre-warmed medium and transferred to a cell culture flask

2.2.1.4 Generation/cultivation of human peripheral mononuclear cells (PBMCs)

PBMC cultivation medium:	500 ml	RPMI 1640
	50 ml	FCS
	5 ml	Penicillin/Streptomycin
	5 ml	Antibiotic/Antimycotic

To analyze inflammatory processes occurring in primary human cells, peripheral mononuclear cells were isolated from human blood. Therefore 50 ml of human blood was sampled from a selected donor. Blood was heparinized to avoid coagulation and was mixed with 50 ml of naive RPMI 1640 at RT. Within a 50 ml Falcon tube, 15 ml of human Pancoll were poured and were gently covered very slow with 33 ml of blood without intermixing. To separate mononuclear cells from erythrocytes and plasma Falcon tubes were centrifuged at 2200 rpm for 25 min at RT. Importantly, centrifugation must be stopped with using the breaking function. Upon centrifugation plasma-containing medium was soaked off till the white distinct monolayer of PBMCs. Next PBMCs were collected carefully with a 10ml pipette, were pooled and washed with RPMI by centrifugation. Pellet was resuspended in 10 ml PBMC cultivation medium; cells were counted and finally plated on a 96-well plate with 300.000 cells per well. After incubation for 3 hours cells were challenged for experimentation.

2.2.1.5 Transient and stable transfection of HEK293 cells

For transient overexpression of proteins, HEK 293 cells were transfected by application of the calciumphosphate precipitation method. Cells were seeded for 96-well plates 10^4 cells/well, whereas for 10-cm dishes 2-4 $x10^6$ cells/dish. Dilutions were prepared, distributed carefully and incubated for 6-8 h. For transfection, the following compounds were mixed under sterile conditions:

96-well-plate (per well):	350 ng	DNA
	0.98 µl	FCS
	∑ 7.8 µl	ddH_2O

10-cm dish:	10-50 µg	DNA
	62.5 µl	FCS
	∑ 500 µl	ddH_2O

This DNA mix was added to 1 volume of 2 x HBS on a vortex and the resulting mixture was added drop-wise to the cells. The dish was tilted to ensure homogenous distribution of the precipitates and cultured o.n. In the following morning, medium was exchanged, either by medium containing 2% FCS or 10% FCS for transfection. High serum concentrations (10% FCS) might interfere with ligand binding by TLRs such as through LBP binding. Protein was overexpressed for 48 h up to 72 h.
For preparation of stable HEK293 clones, the plasmid pTK-neo, encoding the neomycin (G418) resistance gene, was co-transfected in a ratio of 1:20. Transfection was performed on 10-cm dishes and transfected clones were positive selected in G418 supplemented medium. G418 inhibits growth of untransfected cells. In the course of selection, specifically transfected cells were able to grow and formed dense island after several days. These clones were picked carefully and were expanded under constant selection stepwise on 24- well plates and then 6-well plates. Finally potential stable clones were screened by recombinant protein detection via immuno blot analysis or FACS. Positive clones were expanded, cryopreserved and used for further experimentation.

2.2.1.6 Generation of murine bone marrow-derived macrophages (BMDMos)

BMDMo medium:	500 ml	RPMI 1640
	50 ml	FCS
	5 ml	Penicillin/Streptomycin
	5 ml	Antibiotic/Antimycotic
	500 µl	β-mercaptoethanol 50 mM

BMDMo differentiation medium:	500 ml	RPMI 1640
	50 ml	FCS
	50 ml	L-cell supernatant (LCCM)
	5 ml	Penicillin/Streptomycin
	5 ml	Antibiotic/Antimycotic
	500 µl	β-mercaptoethanol 50 mM

The mice were sacrificed, the hind legs were removed and the bones were separated. Femora and tibiae were flushed with PBS using a syringe (best needle 27G) into a 10 ml petri dish. Bone marrow cells were taken up in RPMI supplemented with 10 % L-cell-conditioned medium (LCCM) as a source of M-CSF and incubated overnight in 10-cm bacteriological plastic dishes. Non-adherent cells were counted and re-plated at a density of 0.5×10^6 cells/ml in cDMEM with 10 % LCCM in 10-cm standard plastic dishes. At day four, 5 ml of fresh BMDMo differentiation medium was added. After 6-7 days of differentiation, cultures were nearly confluent. Non-adherent cells were removed, the remaining cells were washed once with 1x PBS and then incubated with 5 ml Accutase at 37°C for 5 min to detach the cells. Accutase was then inhibited by the addition of 5 ml cDMEM. Cells were counted, re-plated and used for further experimentation.

2.2.1.7 Generation of hybridoma, producing TLR4 specific antibodies

A TLR4$^{-/-}$ or TLR4$^{-/-}$MD-2$^{-/-}$ mouse was immunized three times within eight weeks by intraperitoneal (i.p.) injection of either 50µg of recombinant protein (extracellular domain of TLR4) or 1×10^6 macrophages isolated from a wild-type or MD-2 deficient mouse, carrying TLR4 protein, together with adjuvantive 10 nmol of a thiolated DNA oligonucleotide (5'-TCCATGACGTTCCTGA-3', Tib Molbiol, Berlin, Germany). Two days after third immunization its splenocytes were fused with murine P3X cells and hybridomas were selected.

Culture and preparation of P3X cells

P3X cells medium:	500 ml	RPMI 1640
	50 ml	FCS
	5 ml	Penicillin/Streptomycin
	5 ml	Antibiotic/Antimycotic
	500 µl	β-mercaptoethanol 50 mM

P3X cells are mouse myeloma cell line used for fusion experiments with murine B-cells for creating monoclonal antibody producing hybridoma cells. For B-cell fusion procedure cells were harvested,

counted and washed 3 times in serum free RPMI, and use about 3.5 x10^7 cells in 50 ml for mouse mAb generation.

Preparation of Spleen cells

Immunized mouse was sacrificed by cervical dislocation and spleen was carefully removed after the site of operation was disinfected with 70 % EtOH. Isolated spleen was put into 50ml RPMI for washing and was finally pressed through a sterile cell filter (0.22µm) with a syringe plug. The push-through was collected from the dish and was transferred into a 15 ml tube. Splenocytes were pulled down by centrifugation (1200 rpm, RT, 5 min.), resuspended in 5ml of Red cell lysing buffer to remove erythrocytes and were incubated for 4 minutes on RT. To neutralize lysing buffer 10ml RPMI was added, cells were washed twice afterwards and were resuspended in 5ml RPMI. Splenocytes were counted via Neubauer cell-counting chamber and 8x10^7 cells were used for α-mouse TLR4 mAb generation.

Fusion experimental procedures

To generate antibody-producing hybridoma cells, splenocytes and P3X cells were mixed by a 2:1 ratio in a glass tube with round bottom. Cell mixture was centrifuged and media was sucked of completely. For the following steps cooperation of two persons was necessary. One person kept the tube constantly turning while the other person added 1 ml PEG 50% drop by drop on the wall of the tube within ~1 minute. Afterwards, in a similar way, 10 ml serum-free RPMI were added within approximately 4 minutes. Fused cells were gently centrifuged (400g, 10min, RT) and cells were transferred into 60ml of hybridoma selection media. 10ml were used for plating one 96-well plate.

Culture of hybridoma cells

Hybridoma selection medium:	500 ml	RPMI 1640
	50 ml	FCS
	1 x	HAT
	1 x	HFCS
	5 ml	Penicillin/Streptomycin
	5 ml	Antibiotic/Antimycotic
	500 µl	β-mercaptoethanol 50 mM
Hybridoma culture medium:	500 ml	RPMI 1640
	50 ml	FCS
	5 ml	Penicillin/Streptomycin

5 ml	Antibiotic/Antimycotic
500 µl	β-mercaptoethanol 50 mM

Hybridoma were cultured in 96-well plates incubated in 5% CO2 and 37°C. During the first 14 days after fusion half of the medium was changed every 24h and wells were monitored for growing hybridoma. Large cells are fused cells that can survive in contrast to splenocytes and P3X cells which will die in hybridoma selection medium. Since the unfused normal B cells cannot survive long in an *in vitro* culture, they derive immortality by fusion to a partner tumor cell line. The tumor line is resistant to the purine analogue 6-thioguanine because of deficiency of hypoxanthine-guanine phosphoribosyI transferase (HGPRT). This deficiency results in lethal sensitivity to aminopterin, which blocks de novo synthesis of purines. The normal B cell is not sensitive to aminopterin when hypoxanthine and thymidine are supplied, salvage pathways utilizing HGPRT are necessary for survival. Thus, only hybridoma (normal B cells fused to tumor cells) will survive in HAT (hypoxanthine, aminopterin and thymidine) selection medium.

Wells containing colonies of hybridoma cells were marked. To isolate single clones, hybridomas from one well were subjected to 10ml of hybridoma selection medium and were plated on another 96-well plate. Growing hybridoma were monitored daily and supernatants were analyzed for antibody content and possible antigen specificity. Positive clones were once again diluted on another 96-well plate (to ensure that one single clone is isolated) and again supernatants were analyzed. Positive clones were upscaled to cell culture flasks and hybridomas were adjusted to hybridoma culture medium by removing HAT and HFCS content within multiple steps of titration. Furthermore supernatants were collected and antibodies were isolated via Protein G solid phase chromatography.

2.2.2 Molecular biology

2.2.2.1 Basic tools

Minipreps and Maxipreps were performed using the respective kits from QIAGEN following the manufacturer’s guidelines. Kits from QIAGEN were also used for Gel extraction and DNA-purification. Ligations and restriction digests were done with the Quick Ligation Kit and restriction enzymes, respectively, from Fermentas following their instructions. All plasmids were transformed into chemically competent *E. coli* strains, DH5α (Invitrogen) or XL-10 (Stratagene), according to the manufacturer’s guidelines. For *in vitro* site-directed mutagenesis appropriate kit from Stratagene was used.

2.2.2.2 *Agarose gel electrophoresis*

Agarose gels (1 %) were used to separate DNA fragments variable in size. TAE-buffer (1x) with EtBr (100 ng/ml) was used as electrophoresis and gel buffer. Samples were mixed 4:1 with sample buffer and separated at 120 V. For size determination of the fragments, 5 µl of a 1 kb ladder were used. DNA bands were visualized with UV light (254 nm).

50x TAE:	242 g	Tris Base
	500 ml	ddH_2O
	57.1 ml	Acetic acid
	100 ml	0.5 M EDTA
	∑ 1000 ml	ddH_2O and adjust to pH8.5
6x DNA running buffer:	50 mg	Orange G
	15 ml	Glycerol
	0.5 ml	1 M Tris-HCl
	∑ 50ml	ddH_2O

2.2.2.3 *Polymerase chain reaction (PCR)*

If not stated otherwise, all PCRs were performed using the AccuTaq Polymerase (Sigma). AccuTaq is polymerase providing proof-reading activity, which minimizes mutation rates significantly in comparison to conventional Taq polymerases.

A typical PCR protocol starts with an initial denaturation of the DNA at 95°C. The following steps are repeated 35 to 45 times and contain a denaturation phase at 95°C, an annealing phase (enables specific attachment of specific primers to the DNA) at 46°C to 58°C and an elongation phase at 72°C to synthesize DNA as long as required. The temperature used during annealing phase is set 2°C below to the fusion temperature of selected primers, which should be between 46°C to 58°C and should not differ more than 2°C between forward and reverse primer. The elongation is performed at 72°C for all Taq-polymerases and time of elongation phase depends on the rate of synthesis of used polymerase and lengths of the DNA fragment to amplify. For the AccuTaq polymerase a rate of synthesis of 1kb per minute is described by the manufacturer's guidelines.

Typical PCR program:	94°C	5 min	
	94°C	30 s	35-40 x
	46°C-58°C	45 s	35-40 x
	72°C	1-5 min	35-40 x

	72°C	10 min
	4°C	pause

PCR mix for 50µl:	5 µl	10x buffer
	2 µl	10 mM dNTPs
	2 µl	10 µM forward primer
	2 µl	10 µM reverse primer
	1 µl	DNA (equals 500 ng)
	1 µl	AccuTaq Polymerase (1 U/µl)
	1 µl	$MgSO_4$
	∑ 50 µl	ddH_2O

2.2.2.4 Restriction digestion, dephosphorylation and ligation of DNA fragments

Restriction Digestion

To manufacture gene-expression vectors, specific DNA fragments are cleaved and remerged enzymatically. Gene expression vectors could be cleaved by application of specific endonucleases. These restriction enzymes detect specific recognition sites, cut DNA highly specific and generate DNA fragments with defined overlaps, which enable specific remerging afterwards. All restriction enzymes were used according to the manufacturer's guidelines with current reaction buffers for about 2h at a temperature of 37°C.

Mix for enzymatic restriction digestion:	2 µl	specific 10x reaction buffer
	1 µg	DNA
	0.5 µl	restriction enzyme (10 U/µl)
	∑ 20 µl	ddH_2O

Dephosphorylation

To avoid spontaneous religation of cleaved DNA fragments the 5'-phosphate residues were removed by *shrimp alkaline phosphatase* (SAP). 1µl of SAP was added directly into the digestion mix and was incubated for 2h at 37°C before proceeding with specific ligation. Notably dephosphorylation was performed only with vector-DNA. DNA fragments to insert (Insert-DNA) must be applied in phosphorylated form.

Ligation of DNA fragments

To insert specific DNA fragments into DNA-vectors to finally express required genes within an overexpression system, ester-binding of the phosphatase groups of appropriate ends is cloned by T4 DNA Ligase activity. Ligation mix was prepared according to the manufacturer's guidelines and incubated for 10 min at RT. 30 to 100ng of vector DNA was applied whereas three to ten times higher amounts of insert-DNA were used for ligation processes.

Mix for ligation:	4 µl	5x Rapid Ligation Buffer
	y µg	Vector-DNA
	x µg	Insert-DNA
	1µl	T4 DNA-Ligase (1 U/ml)
	∑ 20 µl	ddH_2O

2.2.2.5 Site-Directed Mutagenesis

In vitro site-directed mutagenesis is an invaluable technique for studying gene and protein structure-function relationships and for modifying vector sequences to facilitate cloning and expression strategies. Usage of primers, which are not identical to the region they anneal, performs mutagenesis. The primers encode mutagenic regions and are used for PCR to amplify fragments containing mutations from template DNA. In order to insert a single point mutation one PCR performance, containing mutagenic primer and plasmid-DNA encoding "wild-type" - gene was sufficient. If larger fragments were exchanged mutagenic ologonucleotide was amplified first with primers encoding the overlapping region of the fragment, which should be exchanged, and fragments of the target gene. Created single mutagenic oligonucleotides are applied together with target vector DNA and function as primers within a further PCR performance. Notably programs for Mutagenesis-PCRs imply very long elongation times enabling synthesis of whole vectors, which were in our case up to 10kBs large. Accordingly, PCR products are treated with the endonulease *Dpn 1*. The *Dpn 1* endonuclease is specific for methylated DNA and is used to digest the parental DNA template. DNA isolated from almost all *E. coli* strains is methylated and therefore suitable to digestion. Finally, DNA was precipitated by adding 190µl of ddH_2O, 24µl of Sodium-acetate (3M pH5.2) and 520µl EtOH (100%). Precipitation mix was incubated at -20°C for 1h and was centrifuged at 13.000 rpm at 4°C afterwards. Pellet was washed once with EtOH 70%, was pulled down by centrifugation again and finally dried at 50°C in the heat-block. Dried DNA was resolved in 5µl of 1x TE-buffer and was transformed into supercompetent XL-10 bacteria. After selection of positive clones mutagenic plasmids were isolated and used for further experimentation.

Mutagenesis PCR mix for 50µl:		
	5 µl	10x Pfu / AccuTaq buffer
	2 µl	10 mM dNTPs
	15 µl	mutagenic PCR product
	x µg	vector DNA
	1 µl	Pfu / AccuTaq Polymerase (1 U/µl)
	1 µl	$MgSO_4$
	∑ 50 µl	ddH_2O

2.2.2.6 *Heat-shock transformation of E. coli*

Chemically competent *E. coli* is able to take up plasmid DNA. Therefore 25µl of E. coli (DH5α or XL10-strain) were mixed wit 10ng-100ng of plasmid-DNA and were incubated on ice for 30 min. Afterwards cells were heat-shocked for 45 sec at 42°C, cooled down on ice for 2 min and were further incubated and shaked in 1ml LB_o-medium for 1h. Upon centrifugation of bacteria at 13000 rpm for 1 min, 800µl of supernatant were rejected and 100µl to 200µl were plated on and an agar-plate containing specific selective antibiotics. Plates were incubated over night and colonies were picked next day.

2.2.2.7 *DNA sequencing*

Sequencing of sub-cloned DNA fragments was performed by MWG-Biotech, Ebersberg.

2.2.3 Protein biochemistry

2.2.3.1 *Cell lysis*

Cell lysis was carried out on ice. After stimulation cells were washed twice with ice-cold 1x PBS and then lysed with 100 µl lysis buffer per $1x10^6$ cells for 30 min. The lysate was then collected and centrifuged at 13000 rpm for 15 min in a tabletop centrifuge to remove nuclei and debris. The supernatants were collected in a 1.5 ml tube, stored at -80°C or were directly subjected to electrophoresis. Protein concentration was determined using the BCA Protein Assay Reagent Kit according to manufacturer's guidelines.

Lysis buffer:		
	50 mM	HEPES pH7.6
	50-150 mM	NaCl
	1 mM	DTT

1 mM	EDTA
1 mM	EGTA
0.5-1.5% (v/v)	Nonident P-40
10% (v/v)	Glycerol
20 mM	β-Glycerophosphate
1 mM	Na_3VO_4
0.4 mM	PMSF
1 Tab/ml	Protease Inhibitor Cocktail Tablet (Roche)

2.2.3.2 Immunoprecipitation

To analyze protein-protein interaction or specificity of antibodies, protein complexes consisting tagged proteins are specifically pulled down by application of tag-specific beads. By centrifugation, protein complexes attached to the relatively heavy beads are isolated from remaining cell lysate and can be further analyzed.

Constructs and controls applied were overexpressed as hybrid proteins carrying different tags. For our purpose two constructs encoding potentially interacting proteins (one protein carrying a flag-tag and one myc-tagged protein), were used for transfection. For immunoprecipitation of transiently overexpressed proteins, 10 µg of total expression plasmid DNA for expression of the regarding two proteins was transfected into 3 x 10^6 HEK293 cells seeded on 10 cm dishes by the calcium phosphate precipitation method. After cultivation of transfected cell for 3 days, cells were lysed as described and 20µl of Flag mAb M2 beads were added for precipitation (Sigma). When non-tagged protein was precipitated, specific antibody was applied in the cell lysate together with 20µl of Protein A/G, which strongly bind to Fc-parts of antibodies. Precipitation was performed in 1.5 ml tubes overnight under constant circulation on a turning wheel. Next, beads, bound to specific protein complexes, were washed by centrifugation (13.000 rpm, 20sec. and 4°C) five times and were incubated with 500 µl IP washing buffer for 5min after each washing step. IP washing buffer contains higher salt concentrations compared to lysis buffer in order to disrupt unspecific and low affine protein interactions. After washing procedure pellet was resuspended in 50µl of IP washing buffer and samples were mixed with 6x sample buffer and heated for 5 min at 95°C to disrupt protein - bead interaction. Upon centrifugation (13.000 rpm, 20sec., 4°C) supernatants were subjected to SDS gel electrophoresis and proteins were specifically visualized by immuno western blotting.

IP washing buffer:	50 mM	HEPES pH7.6
	50-200 mM	NaCl
	1 mM	DTT
	1 mM	EDTA

1 mM	EGTA
0.5% (v/v)	Nonident P-40
10% (v/v)	Glyerole

2.2.3.3 SDS-polyacrylamid gel electrophoresis (PAGE) of proteins

SDS-PAGE was carried out using gels of 1.5 mm thickness. First the resolving gel (10 or 12 %) was poured according SDS-PAGE gel recipes and gels were covered with isopropanol. After resolving gel was polymerized the stacking gel (4 %) was poured and the comb was inserted. After complete polymerisation the gel was installed, overlayed with Laemmli buffer and the combs were removed. Then the wells were flushed with Laemmli buffer to remove residual acrylamid. The cell lysates were mixed 1:6 with 6x sample buffer and incubated for 5 min at 95°C. Usually 20 µl of the lysate were loaded on each well and for the determination of protein size, 5 µl of a protein standard (Fermentas) were loaded on the gel. Gel electrophoresis took place at 80 V until probes reached the resolving gel. The voltage was increased to 120V to separate subjected proteins.

Buffers for SDS-polyacrylamide gel electrophoresis

Resolving gel buffer:	1.5M	Tris-HCl pH 8.8
Stacking gel buffer:	0.5M	Tris-HCl pH 6.8
10 % SDS:	10%	SDS in ddH_2O
10 % APS:	10%	Ammonium persulfate in ddH_2O
6x sample buffer:	7 ml	stacking gel buffer
	1 g	SDS
	3 ml	Glycerol
	0.9 g	DTT
	0.06%	Bromphenol blue
Laemmli buffer:	30.28g	Tris
	208.2g	Glycine
	50ml	10 % SDS
	∑ 10 l	ddH_2O

	Resolving Gel		Stacking Gel
	10 %	12 %	4 %
Acrylamide solution	3.3 ml	4 ml	0.66 ml
4x Resolving gel buffer	2.5 ml	2.5 ml	-
4x Stacking gel buffer	-	-	0.3 ml
10 % SDS	0.1 ml	0.1 ml	0.2 µl
ddH_2O	4 ml	3.3 ml	4 ml
TEMED	5 µl	5 µl	5 µl
10 % APS	50 µl	50 µl	25 µl

Table 5: SDS-PAGE gel recipes

2.2.3.4 Western blot analysis

For the transfer of the proteins from the polyacrylamide gels to a nitrocellulose membrane the semi-dry blot method was carried out. After SDS gel electrophoresis the gel was carefully transferred from the gel-chamber and the stacking gel was removed. Four Whatman papers in the size of the gel, the nitrocellulose membrane and the gel were equilibrated in blotting buffer for 5 min. Afterwards the gel was placed on the membrane and both were sandwiched between four Whatman papers. Whatman papers including gel and membrane were transferred into the semi-dry blotting apparatus. After removing potential air bubbles apparatus was closed and blotting procedure was performed wait 1mA / cm^2 for 60 min. During blotting process proteins migrate from the gel, which is placed on the cathode side, to the membrane located at the anode side. On the membrane, proteins are bound due to interactions with hydrophobic membrane surface. To avoid unspecific binding the membrane was blocked for 1 h in 5 ml of blocking buffer. Incubation with the primary antibody (diluted in 1x blocking buffer) took place at 4°C overnight. On the next day the membrane was washed three times for 5 min in PBT and then incubated with the secondary HRP-conjugated antibody (diluted in PBT) for 2 h at RT on the shaker. After three washing steps with PBT for 10 min each, proteins were detected using Western Lightning Chemiluminescence Reagent.

If the membrane was subject to incubation with a different antibody, a stripping protocol was carried out to remove previous antibodies from the membrane. Briefly, the membrane was incubated in H_2O for 5 min, followed by incubation with pre-warmed 0.2 N NaOH for 20 to 40 min and a final wash in H_2O for 5 min. All steps were performed on a shaker. The membrane was then blocked again in blocking buffer for 1 h.

Buffers for Western Blot

Blotting buffer:	1.94 g	Tris
	8,656 g	Gylcine
	200 ml	Methanol
	∑ 1000 ml	ddH_2O
PBT:	1x	PBS
	0.05%	Tween20
Blocking buffer:	1x	PBT
	3%	NGS
	50 g/l	Milk powder

2.2.3.5 Luciferase reporter gene assay

The luciferase reporter assay was used to measure NF-κB-dependent activation of a luciferase gene. Therefore, HEK 293 cells, which largely lack TLR expression but express downstream molecules essential for signaling, were transfected with cDNAs coding for TLRs and the reporter. As internal control for transfection efficiency, a Renilla-assay was performed. Luciferase as well as Renilla activities were determined by chemiluminoscence assays. All assays were prepared in 96-well scale and duplicate values were determined.

1.5 x 10^4 HEK293 cells in 200µl of culture medium were plated on single wells of 96-well plates in the morning. HEK293 cells were cotransfected by the calcium phosphate precipitation method with an NF-κB recruiting endothelial-leukocyte adhesion molecule (ELAM)-1 (CD62E) promoter luciferase construct, a phRL-null plasmid, encoding the co-reporter Renilla controlled by the constitutive active T7 promoter, as well as a cytomegalovirus (CMV)-promoter regulated expression plasmid for human/murine TLRs. Transfection was performed in the evening and next morning medium was soaked off carefully and was replaced by fresh HEK293 culture medium containing only 2% FCS. 7 h after medium change preparations of bacterial products or immune stimulatory analogues were added to transfected cells for 16 h. Afterwards medium was soaked off and cells were lysed by adding 40µl 1 x lysis buffer (Promega) to each well. The mixture was incubated and covered for 1 h at RT. For measurement cell lysates was transferred to a photo-resist plate and plate was inserted into the luminometer, which automatically injected 50 µl/well of luciferase or Renilla substrate. The emitted

light was measured and luciferase activity was accounted to Renilla activity to normalize NF-κB fold induction upon Toll-like receptor challenge.

The transfection mix contained the following compounds:

96-well-plate (per well):	34 ng	pELAM-1-Luc
	64 ng	phRL-null
	1-50 ng	expression vector
	5 ng	pRK5 (empty vector)
	∑ 7.5-µl	ddH_2O
Renilla substrate:	1.1 M	NaCl
	2.2 mM	Na_2EDTA
	220 mM	$KHPO_4$
	1.3 mM	NaN_3
	440 µg/ml	BSA
	1,43 µM	Coelenteracine
	pH 5.0	
Luciferase substrate:	470 µM	D-Luciferine
	270 µM	Coenzyme A
	33.3 mM	DTT
	530 µM	ATP
	1.07 mM	$(MgCO_3)_4Mg(OH)_2$
	2.67 mM	$MgSO_4$
	20 mM	Tricine
	0.1 mM	Na_2EDTA
	pH 7.8	

2.2.4 Immunology

2.2.4.1 Enzyme linked immunosorbent assay (ELISA)

Cytokine concentrations in cell supernatants or murine sera (see below) were analyzed by enzyme linked immunosorbent assay with enzyme-mediated colorimetry (Magellan, Tecan, Crailsheim, Germany) according to supplier protocols.

All proteins were detected by DuoSet ELISA Development System (R&D Systems) following the manufacturer's protocol. Briefly, plates were coated with 100 µl per well of capture antibody (720 ng/ml) in 1x PBS and incubated overnight at 4°C. The next day the plate was washed by ELISA washer (3 washing steps with 250µl washing buffer), tapped dry and 250 µl per well of blocking buffer were added. The following incubation took place for 1 h at room temperature. Afterwards, the plate was washed by ELISA washer, tapped dry and 100 µl per well of the samples in reagent diluent and standards were added. Incubation took place at room temperature for 2 h or at 4°C overnight and was followed by washing. The detection antibody (36 µg/ml) was diluted in reagent diluent and added in a volume of 50 µl per well, the plate was incubated for 2 h and washed. This was followed by incubation with 100 µl per well of Streptavidin-HRP (50 µl in 10 ml reagent diluent). Finally the plate was washed and fresh substrate reagent was added (100 µl/well). The plate was incubated in the dark because the substrate reagent contains H_2O_2, which is known to be light sensitive. The incubation time ranged from 10-60 min, depending on the protein detected. To stop the reaction 50 µl per well of stop solution were added and the plate was analyzed in the ELISA reader at 450 nm (reference at 570 nm).

ELISA buffers and solutions

Blocking buffer:	1x	PBS
	1%	BSA
	5%	Sucrose
Reagent Diluent:	1x	PBS
	1%	BSA
Washing buffer:	1x	PBS
	0.05%	Tween 20
Phosphate-Citrate buffer:	25.7 ml	0.2 M Na_2HPO_4
	24.3 ml	0.1 M Citric Acid 1-hydrate (pH 5.0)
	50 ml	ddH_2O
	adjust to pH 5.0	with HCl
Substrate reagent:	1	tablet Tetramethylbezine (TMB)
	10 ml	0.05 M Phosphate-Citrat buffer
	2 µl	H_2O_2 30 %
Stop solution:	2N	H_2SO_4

2.2.4.2 NO-Assay

The concentration of nitric oxide in the supernatant of the cultured cells was measured using the Griess-Reagent (Green, Wagner et al. 1982).

Griess Reagent A:	0.4 g	N-(1-Naphtyl) Ethlyendiamin Dihydrochlorid
	∑ 200 ml	ddH_2O
Griess Reagent B:	4 g	Sulphanilamid
	10 g	H_3PO_4
	∑ 200 ml	ddH_2O

The supernatant (50 µl) was mixed with 50 µl of a 1:1 mixture of Reagent A and B in an ELISA plate (Nunc). After incubation for 10 min at RT, the plate was measured in the ELISA reader at 540 nm. The NO concentration of the samples was calculated by comparing their absorbance to that of a range of standard concentrations of $NaNO_2$ on the same plate.

2.2.4.3 Flow cytometry

Analysis of cell surface antigens

Up to 1x10^6 cells per staining were centrifuged at 1500 rpm at 4°C for 2 min in a well of a round bottom 96-well plate. The supernatant was discarded and the cells were washed twice with 50µl FACS buffer (1 % BSA in 1 x PBS*)* by centrifugation at 1500 rpm at 4°C for 2 min and resuspended in 50µl FACS buffer. To stain dead cells, Ethidium monoazide (0.5 mg/ml) was added in a ratio 1:1000 and cells were incubated 10 min on ice in darkness, followed by 10 min in direct light. After washing the cells once, cell surface Fc-receptors were blocked by incubating the cells with unlabeled α-CD16/CD32 antibody for 10 min at 4°C. Meanwhile the appropriate staining antibodies were prepared. After blocking Fc-receptors the cells were centrifuged one more time. The cells were resuspended in 50 µl of the ready made staining solutions including specific labelled antibody combination. Incubation lasted for 20 min in the dark at 4°C and was followed by two washing steps (50 µl/sample, centrifugation at 1500 rpm, 4°C, 2 min). In the case of Biotin-conjugated or non-conjugated antibodies, a second staining step with Streptavidin-PE conjugates or a species-specific conjugated secondary antibody respectively was attached. After the two final washing steps, the cells were resuspended in 50 µl of the staining solutions containing the Streptavidin-PE or the secondary antibody-conjugates for 20 min at 4°C in darkness. Finally, the supernatant was discarded; the cells were washed twice and resuspended in 200 µl FACS buffer. Stained samples were analyzed by flow cytometry using a CyAn ADP LX9 analyzer (DakoCytomation) and the data were analysed using the

software FlowJo. If peripheral blood samples were analysed, they were treated with Erythrocyte lysis buffer according to manufacturer's guidelines prior to starting general staining procedures.

Staining of intracellular antigens

If intracellular proteins were stained, cells were fixated and permeabilized prior to staining with Cytofix/Cytoperm Kit, enabling antibodies to reach specific antigens inside of the cell. For intracellular staining EMA application was dispensable and instead of EMA, cells were incubated for 20 min in Cytofix/Cytoperm solution. Notably, after permeabilization and fixation, for further procedure, cells were held in 1x Perm/Wash buffer instead of FACS buffer without exception. Also staining antibodies were diluted in 1x Perm/Wash buffer. Otherwise intracellular staining protocol accords to protocol described for staining of cell surface molecules.

Specific Cytokine staining

For analysis of cytokine expression by flow cytometry, cells were treated with Golgi-plug (1:100, Pharmingen) for 4h prior to staining procedure. Golgi-plug inhibits the secretion of cytokines by preventing their transportation via the Golgi apparatus, resulting in accumulation of specific cytokines within the cell. By application of fluoresecent labelled antibodies, cytokines were specifically detected accordingly to the protocol for intracellular antigen staining and were analyzed via flow cytometry.

2.2.5 Bacteria

For infection experiments bacteria were grown within adjusted media. For our analysis we used clinical isolate clones of *Salmonella. enterica* (subspecies enterica serovar enteriditis) and *Echerichia coli* as representers of the Gram-negative bacteria, whereas the attenuated strain D39 of *Streptococcus pneumoniae* was used for experimentation with Gram-positive bacteria. To get reproducible numbers of bacteria for each experiment growth conditions for bacteria were standardized and therefore never changed. Bacteria were stored in –80°C and were grown, while shaking at 37°C, in 200 ml of specific media for exact 16 hours. Afterwards suspensions were centrifuged (4500 rpm, 20min, 4°C), and washed with PBS twice. Upon one further centrifugation step pellet of bacteria was resuspended in 5 ml of PBS and bacterial suspension was directly used for *in vitro* or *in vivo* studies. Side by side an aliquot of bacterial suspension was titrated within PBS and plated on blood agar plates to determine concentration. Next day plates were analysed and bacterial colonies were counted and calculated to concentration of bacteria.

Growth media (preparation according to standardized protocols):

- **LB** (Lysogeny Broth)-medium (*E.coli*)
- **BHI** (Brain-Heart-Infusion)-medium (*S. enterica*, *S. pneumoniae*)

2.2.6 Mice

All mice were kept under pathogen-free conditions at the animal facility of the Institute of Medical Microbiology, Immunology and Hygiene (Technical University, Munich, Germany). Animal experiments were approved and authorized by local government. Experiments were performed with 10-12 week old mice, unless otherwise stated. Prior to the experiment all the mice were genotyped according to standard laboratory protocol.

2.2.7 Animal experiments

2.2.7.1 Organ withdrawal

Mice were sacrificed by cervical dislocation and the respective organs were carefully removed after the site of operation was disinfected with 70 % EtOH.
To analyze bacterial counts within different organs upon infection, organs were collected from mice and were homogenized in 1 ml of sterile PBS using an Ultra-Turrax device. Serial dilutions of organ homogenates in PBS were plated on blood agar plates. CFU were counted after incubation at 37°C for 24 h and calculated per organ.

2.2.7.2 Blood withdrawal

Blood was collected either from the retrobulbar plexus upon anesthetization, the tail vein or, upon scarification, directly from the heart. To isolate serum from blood, samples were centrifuged once (13.000 rpm, 20min., 4°C) and serum was collected and frozen at -20°C if not used directly for experimentation.

2.2.7.3 OVA-Immunization using Myr_3CSK_4 as an adjuvant

Mice were challenged by injection of 200 µg Myr_3CSK_4 and 50 µg OVA solubilized in 400 µl of PBS i. p. for three times consecutively. First injection was followed by second injection after 3 weeks and third injection upon additional 4 weeks. 3 days after third injection serum aliquots were collected. Concentrations of OVA-specific-immunoglobulins in the serum samples were monitored by ELISA using a murine Ig-specific antibody.

2.2.7.4 Sterile, PAMP-induced shock

Mice were used for shock induction at an age of 5-10 weeks. Experimental groups were matched for age and sex. In case of the survival experiments, the mice were under observation twice daily for 7 days upon challenge. For shock experiments, TLR agonist preparations were applied alone or upon sensitization using D-GalN alone or in combination with IFNγ. For IFNγ priming, 50 µg/kg IFNγ was injected i. v. 45 min prior to application of TLR agonists. Lethality was monitored within 72 h in respect to high dose application (no sensitization) and within 16 h upon low dose application (sensitization with 800 mg/kg D-GalN alone or additional IFNγ priming).

2.2.7.5 Model for bacteria induced septic shock

In order to establish a clinical relevant model for septic shock, mice, after infection with a specific dose of bacteria, were treated with antibiotics to stop spread of infection. Antibiotics as well as bacteria were applied intraperitoneally. To combat Gram-negative bacteria, 68mg/kg Ampicillin together with 2.8 mg/kg Ofloxacine, were applied after infection 4 times hourly. In terms of Gram-positive infection 25µg/ml of Rocephin was added. To establish effective antibiotic treatment sterility of blood samples from control mice, isolated 24 hours upon infection, were analyzed and were plated on blood agar. To analyze protective effect of TLR-blockade upon hyper-inflammation mice were infected with a dose of bacteria, which was lethal despite of antibiotic treatment. The dose of bacteria applied was investigated by monitoring survival of wild-type mice upon infection with titrated amounts of bacteria followed antibiotic treatment. Depending on sort of bacteria and time point of start of antibiotic treatment after infection titre of injected bacteria was investigated a newly to sustain a reproducible model.

3 RESULTS

3.1 Overlap in ligand specificity of TLR2 and TLR4

3.1.1 Myr_3CSK_4 and LPS activate both overexpressed TLR2 and TLR4

Most TLRs have been shown to specifically bind distinct microbial products. TLR2 has been reported to be a pattern recognition receptor for bacterial lipopeptides and TLR4 is regarded as the LPS receptor. The structural constitution of lipoproteins and LPS is illustrated in **Figure 5**. However, controversial results have been published. TLR2 for instance has been described to mediate LPS-signalling upon its interaction with LBP or CD14 (Yang, Mark et al. 1998). Furthermore, Leptospiral LPS was shown to induce cell activation in a TLR2 dependent manner (Yang, Mark et al. 1998; Werts, Tapping et al. 2001). On the other hand, TLR4 has been demonstrated to recognize pneumolysin, which is a secreted lipoprotein from *Streptococcus pneumoniae* (Malley, Henneke et al. 2003). Accordingly we analyzed TLR4-mediated cell activation upon challenge with specific lipopeptides. Thereby we observed Myr_3CSK_4-induced TLR4 activation. In contrast challenge of TLR4 with Lau_3CSK_4 and Pam_3CSK_4 was ineffective (data not shown). Moreover we analyzed whether TLR4-induced recognition of Myr_3CSK_4 is species-specific (**Figure 6**). HEK293 cells were transfected with murine or human TLR4 alone or were cotransfected either with murine or human MD-2. Whereas cells expressing TLRs alone were unresponsive both human and murine TLR4/MD-2 receptor complexes responded to Myr_3CSK_4-challenge by NF-κB-mediated reportergene activation. Notably, a mixed species TLR4/MD-2 complex did not transduce cell activation if challenged with Myr_3CSK_4, whereas LPS used as positive control was recognized readily via the indicated complex. Myr_3CSK_4 represents an

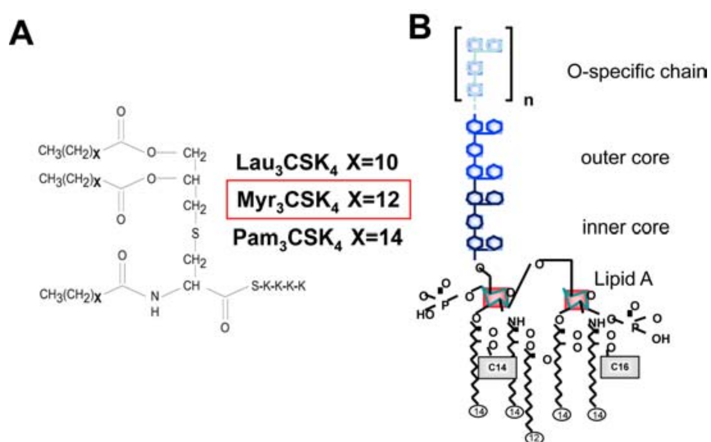


FIG.5: Bacterial lipoprotein analogues and LPS

(A) Bacterial **lipoproteins** carry three acylations that are bound to a central moiety consisting of a cysteine to which a glycerol rest is coupled via a thioester bond. The cysteine constitutes the N-terminal amino acid to which the C-terminal peptide chain is bound. (B) **LPS** carry up to 6 acylations bound to their central diglucosamine-di-phospho moiety. A polysaccharide chain of variable length is bound to the di-glucosamine. The acylated di-glucosamine constitutes the lipidA moiety of LPS.

The size of the fatty acids in both molecules varies and is characterized typically by between 12 and 16 linearly ordered C atom residues but can be more numerous.

entire subpopulation of the *E. coli* outer membrane protein (OMP), which is tri-acylated at the N-terminal cysteine. Thereof 53% of acyl chains were found to contain a line up of 16 carbon atoms (palmitoylation) and about 43% of lipoproteins carried longer fatty acids with up to 19 carbon atoms. Notably, 3.1% of all fatty acids were acylated with myristic acid and thus contained only 14 carbon atoms.

Furthermore we analyzed TLR2 mediated LPS responsiveness. HEK293 cells transiently overexpressing TLR2 or TLR4/MD-2 were challenged with increasing amounts of LPS (**Figure 7**). We applied regular smooth O111:B4 *E. coli* LPS, a repurified O111:B4 *E. coli* LPS preparation and a highly purified rough *Salmonella minnesota* LPS, which carries very short polysaccharide-chains. Luciferase activity was significantly induced in cells expressing TLR4 upon challenge with all LPS preparations independent of the bacterial origin. Notably, aside of TLR4 all LPS preparations used significantly activated TLR2 if applied at higher doses (**Figure 7**).

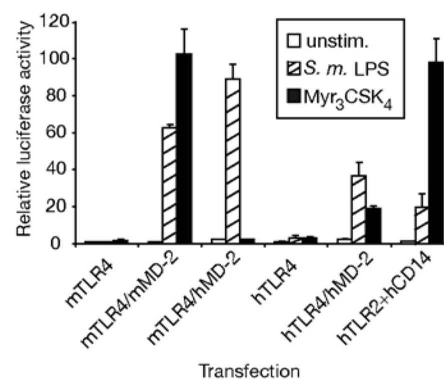


FIG.6: TLR4-recognition of Myr_3CSK_4 is not species-specific
Reportergene assay of HEK293 cells transiently expressing reporter constructs, encoding murine (m) or human (h) TLR4 or MD-2. TLR2/CD14 was used as a control. Cells were challenged wit 10µg/ml of *S. m.* LPS (*S. enterica* serovar *minnesota* strain R595) as a positive control or synthetic Myr_3CSK_4 (unstim., untreated). Upon 16h cells were lysed for analysis of intracellular NF-κB-induced luciferase activity.

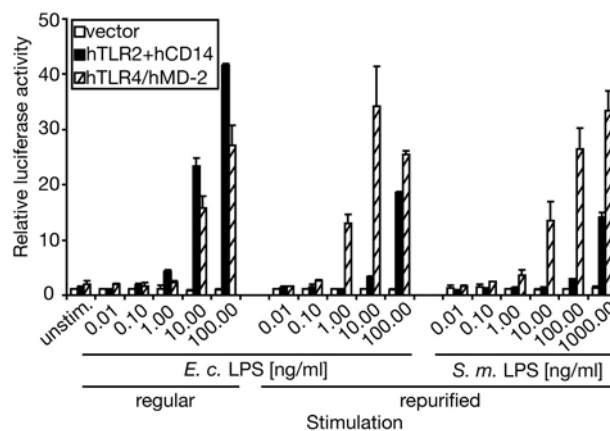


FIG.7: Overexpressed TLR2 and TLR4 recognize LPS
Reporter gene assay of HEK293 cells transiently expressing reporter constructs, as well as receptor molecules indicated (h, human). Two different preparations of LPS from *E. coli* strain O111:B4 named regular (prepared according to standard protocol) or repurified, as well as repurified rough *Salmonella enterica* serovar Minnesota strain R595 (*S. m.*) were applied to cells for 16 h at concentrations indicated (unstim., untreated). Luciferase activity was analyzed to determine NF-κB driven cell activation.

3.1.2 Analysis of endogenous TLR2 and TLR4 in terms of LPS and Myr_3CSK_4 recognition

In order to analyze endogenous TLR2 and TLR4 in respect to ligand specificity BMDMo's isolated from wild-type mice, $TLR2^{-/-}$, $TLR4^{-/-}$ and $TLR2^{-/-}/TLR4^{-/-}$ mice were challenged with Myr_3CSK_4 and were analyzed in terms of Nitric Oxyde (NO)-production (**Figure 8A**). While wild-type macrophages as well as macrophages lacking TLR2 or TLR4 responded to Myr_3CSK_4 challenge, cells isolated from $TLR2^{-/-}/TLR4^{-/-}$ mice were unresponsive in respect of NO-release. Furthermore Myr_3CSK_4 induced phosphorylation of the MAP kinases p38 and p42/44 was comparably dependent on TLR2 and TLR4 and was undetectable only if expression of both receptors were lacking (**Figure 8B**). PolyI:C as a TLR3 ligand was used as a positive control to show cell viability for all genotypes. Additionally we examined TLR2 and TLR4 dependent responsiveness of BMDMo's upon IFNγ priming. Priming with IFNγ enhanced cell activation in terms of cytokine release upon challenge with specific TLR ligands (**Figure 9A**). Accordingly with NO-release data, we found Myr_3CSK_4 induced TNFα production in the absence of TLR2 (**Figure 9B**). BMDMo's primed with IFNγ and challenged with Myr_3CSK_4 enhanced cytokine production while macrophages from $TLR2^{-/-}TLR4^{-/-}$ were unresponsive independently of IFNγ priming. Furthermore, TNFα-release upon LPS challenge was observed independent of TLR4 (**Figure 9C**). While macrophages lacking TLR4 did not respond to LPS challenge, priming with IFNγ rendered them responsive. Only macrophages lacking TLR2 and TLR4 failed to produce TNFα upon LPS challenge independent of IFNγ priming.

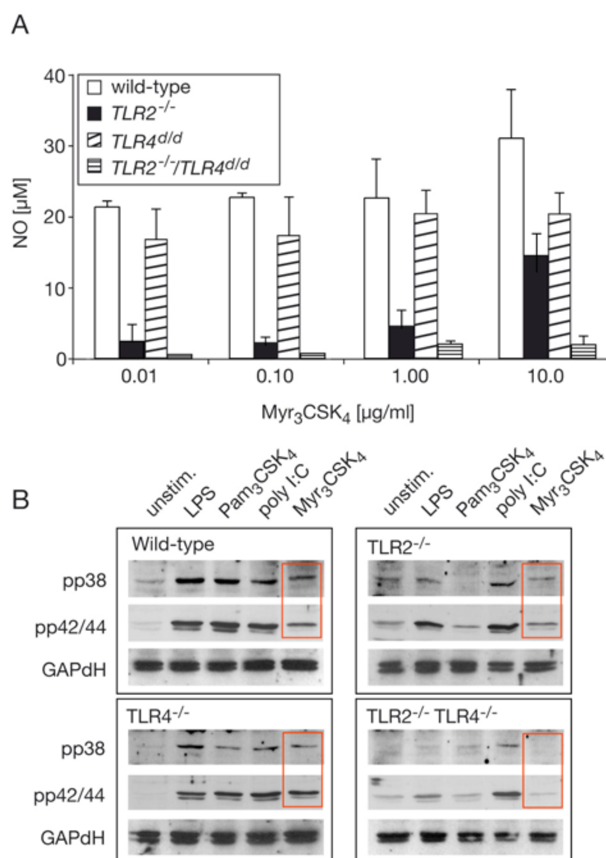


FIG.8: NO-release and activation of MAP kinases upon Myr_3CSK_4 challenge in the absence of TLR2

(A) Bone marrow derived macrophages from mice indicated were challenged with increasing amounts of Myr_3CSK_4 for 24h upon which NO-release was quantified within the supernatant. (B) Macrophages from the genotypes indicated were challenged for 20 minutes. Afterwards cells were lysed and phosphorylation (p) of p38 and p42/44 was analyzed by immuno blotting. GAPdH was used as loading control. ($^{-/-}$, knock-out; *$TLR4^{d/d}$*, C3H/Hej TLR4-defective mice)

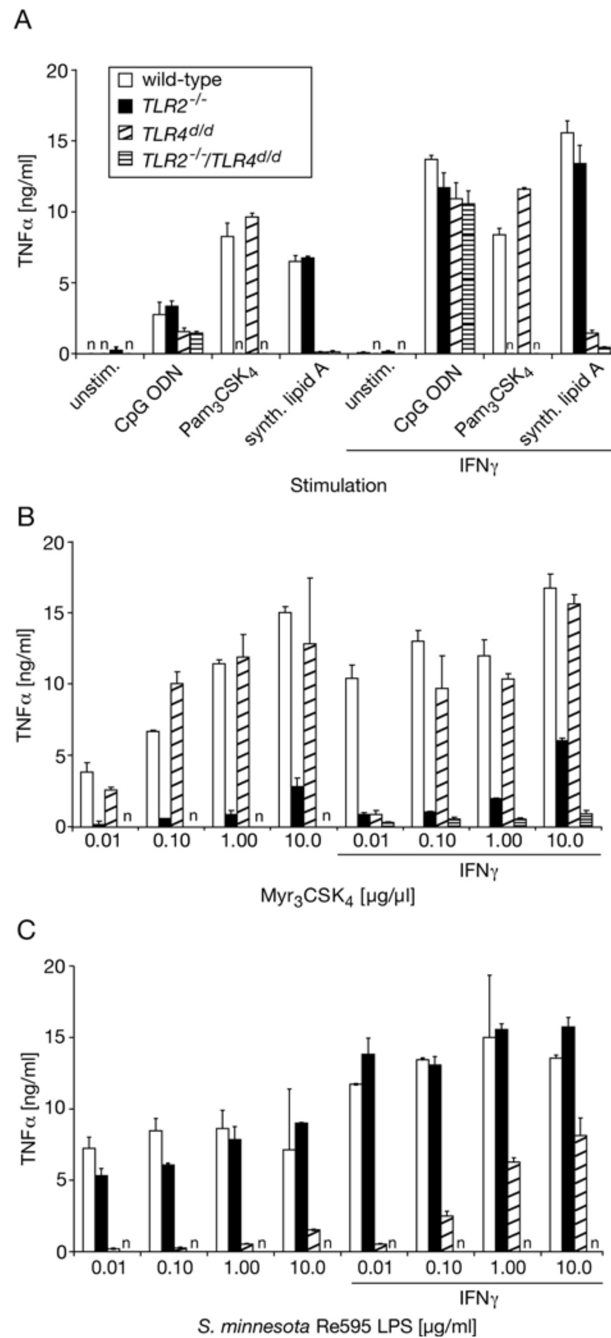


FIG.9: Myr$_3$CSK$_4$ and LPS-induced TNFα-release of primary macrophages primed with IFNγ or not

(A-C) Legend in A applies to B and C also. Challenge of BMDMo's without or upon IFNγ priming with microbial products or analogues as indicated. Stimuli were applied for 16 h at concentrations indicated or of 10 µg/ml except for oligodeoxynucleotide 1668 (CpG ODN, 2 µM). Subsequently supernatants were subjected to ELISA (n, not detectable; synth., synthetic; unstim., untreated; $^{-/-}$, knock-out; *TLR4$^{d/d}$*, C3H/Hej TLR4-defective mice)

3.1.3 Blockade of murine and human TLR2 does not inhibit responsiveness to Myr_3CSK_4

Murine RAW264.7 macrophages or human peripheral blood mononuclear cells (PBMCs) were challenged with increasing amounts of Myr_3CSK_4. Prior to challenge cells were preincubated with a monoclonal antibody (T2.5) antagonizing murine and human TLR2. T2.5 application specifically inhibited NF-κB mediated reportergene expression upon activation of overexpressed TLR2 challenged with Pam_3CSK_4 and Myr_3CSK_4 (**Figure 10A**).

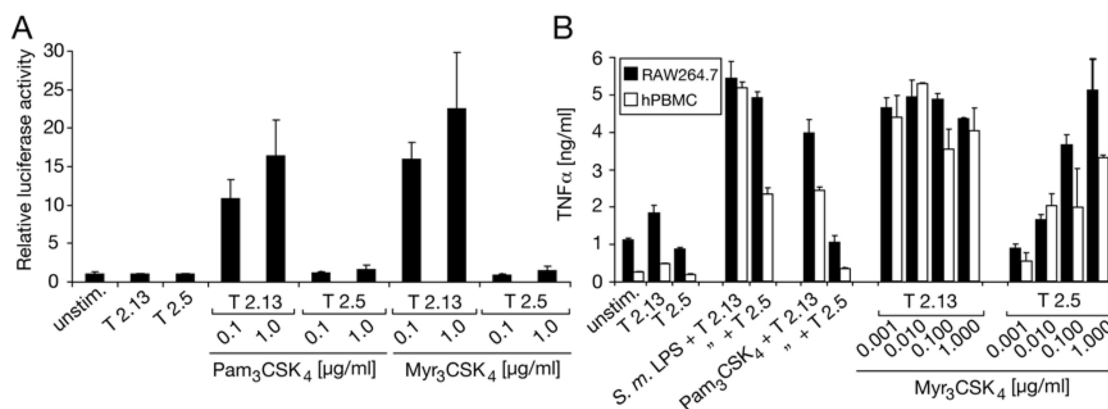


FIG. 10: Lipopeptide induced cell activation in spite of specific TLR2 blockade.
Cells were left untreated (unstim.), pretreated with α-TLR2 mAb (T2.5) or isotype control mTLR2-specific antibody (T2.13) at a concentration of 10 µg/ml for 30 min. (A) HEK293 cells transfected with NF-κB reporter and control reporter plasmids, as well as plasmids mediating expression of human TLR2 and CD14 were challenged with different lipopeptides as indicated. Lysates were lysed 18 h thereafter for analysis of luciferase activity. (B) Murine RAW264.7 marcophages or human PBMC were challenged with compounds at a concentration of 1 µg/ml or as indicated. Supernatants were recovered for subjection to ELISA after 24 h. (*S. m., Salmonella Minnesota*)

Accordingly, Pam_3CSK_4 induced cell activation was decreased by T2.5 application whereas LPS-mediated TNFα release by RAW macrophages and PBMCs was not. Control antibody (T2.13) did not interfere with Myr_3CSK_4 induced cell activation irrespective of the dose applied. However, inhibition of TLR2 activity was overcome by large amounts of myristoylated lipopeptide indicating partial Myr_3CSK_4 recognition independent of TLR2 (**Figure 10B**).

3.1.4 Systemic responsiveness towards Myr_3CSK_4 and LPS

In order to analyze the capacity of Myr_3CSK_4 to activate TLR4 aside of TLR2 *in vivo* I immunized mice with Ovalbumin (OVA) using Myr_3CSK_4 as adjuvant. After immunization according to standard immunization protocols described in Material & Methods, serum of mice was monitored for OVA specific IgGs (**Figure 11**). As expected wild-type as well as TLR4-deficient mice produced highest amounts of OVA-specific Ig whereas Ig levels from mice lacking TLR2 were reduced but still

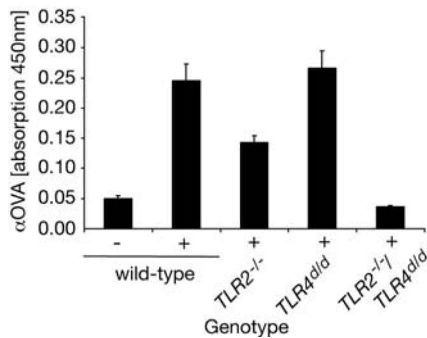


FIG. 11: Myr$_3$CSK$_4$ as an adjuvant induces OVA specific immune response dependent on TLR2 and TLR4

Mice of the genotypes indicated (n = 2 for each group) were challenged by intraperitoneal injection of Myr$_3$CSK$_4$ and ovalbumin (OVA, +) for three times consecutively or left untreated (-). Content of OVA-specific Ig in serum was monitored by ELISA (α, specific Ig; $^{-/-}$, knock-out; *TLR4$^{d/d}$*, TLR4-defectiv mice).

significant. Notably, only serum of mice lacking both TLRs did not contain OVA-specific Ig upon immunization , which indicates a Myr$_3$CSK$_4$ signal transducer role of TLR4. Next I analyzed TLR2 and TLR4 dependent susceptibility of mice to Myr$_3$CSK$_4$ as well as LPS challenge. While wild-type mice succumbed to Myr$_3$CSK$_4$ induced shock TLR2$^{-/-}$, TLR4$^{-/-}$ and TLR2$^{-/-}$TLR4$^{-/-}$ mice were resistant implicating both TLRs involved in Myr$_3$CSK$_4$ sensing (**Figure 12A**). Upon challenge with a lethal dose of LPS wild-type mice as well as mice lacking either TLR2 or TLR4 alone were susceptible and succumbed to fatal hyper inflammation. Only TLR2$^{-/-}$TLR4$^{-/-}$ mice were resistant to fatal endotoxemia indicating an important role of TLR2 in LPS recognition aside of TLR4 (**Figure 12B**).

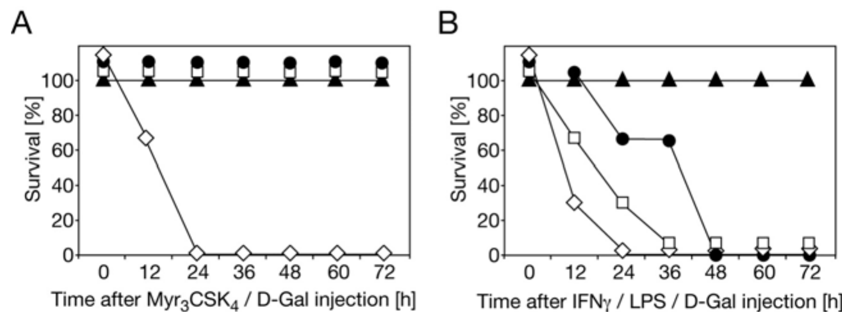


FIG. 12: Systemic Myr$_3$CSK$_4$ and LPS challenge induced lethality dependent on both TLR2 and TLR4

Mice of genotypes wild-type (◇), *TLR4$^{-/-}$* (●), *TLR2$^{-/-}$* (□), or *TLR2$^{-/-}$/TLR4$^{-/-}$* (▲) were challenged by intraperitoneal injection of (A) Myr$_3$CSK$_4$ and D-galactosamine (D-GalN) or (B) LPS and D-GalN with additional IFNy priming and viability was monitored for 72 hours (n = 3 for each group; $^{-/-}$, knock-out).

3.1.5 TLR2 and TLR4 activation is not due respective contamination

To show absence of LPS in our Myr_3CSK_4 fraction HEK293 cells overexpressing TLR2 were challenged with LPS, Pam_3CSK_4 or Myr_3CSK_4 preincubated with endotoxin inactivating polymyxin-B (**Figure 13A**). Whereas stimulatory capacity of LPS was completely abolished in the presence of polymyxin-B, cell activation upon Pam_3CSK_4 and Myr_3CSK_4 challenge was not influenced by polymyxin-B treatment. Furthermore, Myr_3CSK_4 mediated activation of TLR2 and TLR4/MD-2 was independent of serum components while stimulation with LPS requires presence of serum components like LBP to activate TLR2 and TLR4/MD-2 (**Figure 13B**). Notably by using TNFα as a TLR-independent inducer of NF-κB we showed that cell viability was not diminished by absence of serum components (data not shown). Conclusively, TLR4-stimulatory capacity of Myr_3CSK_4 was not caused by LPS contamination within the lipopeptide preparation.

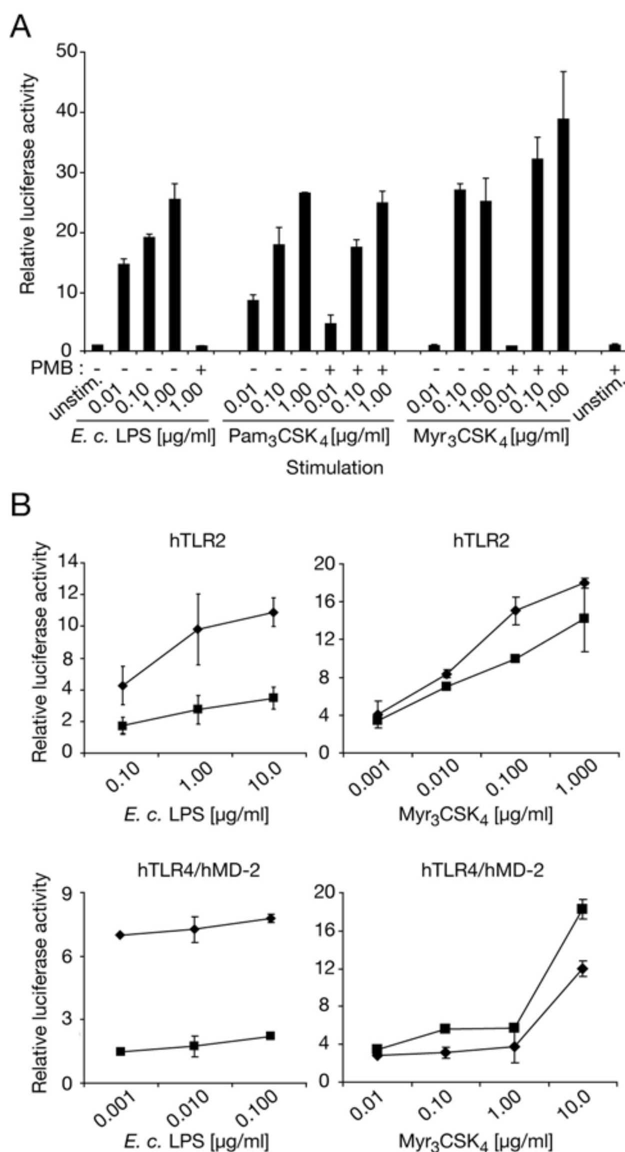


FIG.13: Myr_3CSK_4 activates TLR4 independent of Polymyxin-B and serum components

NF-κB dependent reporter gene assay of HEK293 cells expressing reporter constructs, as well as hTLR2 and hCD14 or hTLR4/hMD-2 transiently (h, human). (A) *E. coli* strain O111:B4 (*E. c.*) LPS, Pam_3CSK_4 or Myr_3CSK_4 were preincubated in sterile PBS (-) or with polymyxin B (PMB) (+). Subsequently, preincubated TLR agonists were added to TLR2/CD14 expressing cells at the concentrations indicated (unstim., untreated) and cellular lysates were analyzed for luciferase activity. (B) Cells were challenged in the absence (filled space) or presence (filled diamond) of serum with LPS or Myr_3CSK_4 at the concentrations indicated for 16 h after which cell lysates were sampled for reporter gene assay.

Furthermore contamination with nucleic acid (detection limit: 0.3 %) in *Salmonella enterica* serovar Minnesota strain R595 (Re mutant) LPS was not detectable by gas chromatography of alditol acetates. Protein content was determined by Bradford microassay (Biomol, Hamburg, Germany) using 160 µl of an LPS solution (50 mg/ml) and 40 µl of dye reagent. The amount of protein detected (2.83 µg)

corresponds to 0.035 % of LPS by weight. These results were in accordance with a UV spectrum in 0.1 M NaOH, which exhibited a single maximum at a wavelength of 220 nm.

3.1.6 Tri-myristoylation is required for TLR4 stimulation

Upon identification of Myr_3CSK_4 as a ligand for TLR4 we wondered whether tri-myristoylation is required for rendering lipopeptides detectable for TLRs. Thus we initiated synthesis of lipopeptide analogues carrying only a single myristoylation. Both Myr_1ASK_4 and Myr_1SSK_4 were applied to TLR2 or TLR4/MD-2 expressing HEK293 cells aside of Myr_3CSK_4 used as a positive control and myristic acid as negative control (**Figure 14**). In terms of TLR-induced NF-κB mediated reportergene activation single myristoylated lipopeptides activated TLR2 but failed to induce TLR4 mediated luciferase production.

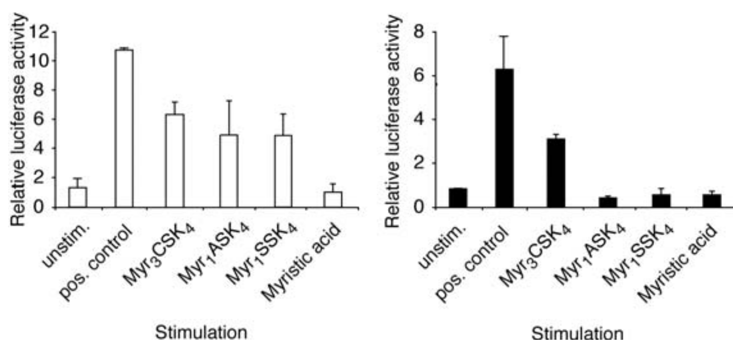


FIG.14: Single-myristoylated lipopeptides fail to activate TLR4.
HEK293 cells overexpressing hTLR2/CD14 (white bars) or hTLR4/hMD-2 (black bars) were challenged with 1µg/ml of lipopeptide indicated (h, human). 100ng/ml O111:B4 LPS was used as positive control. 16 h after stimulation cells were lysed and analyzed for luciferase activity.

3.1.7 Synthetic pneumolysin analogues are TLR2-specific stimuli

The lipoprotein pneumolysin is an exotoxin from *Streptococcus pneumoniae* and has been reported to activate TLR4 (Yoshimura, Lien et al. 1999; Malley, Henneke et al. 2003). Furthermore pneumolysin is acylated intramolecularly at specific positions. The peptide sequence SCGKRTEK represents a short part of pneumolysin and carries an arginine, which has been reported to be acylated. To analyze stimulatory capacity of pneumolysin we used different synthetic pneumolysin analogues synthesized by EMC Microcollections that consist of the SCGKRTEK-peptide carrying either a myristoylation (Myr) a palmitoylation (Pam) or carry a 2, 3-Diaminopropionic (Dpr) acid chain at the internal arginine. The non-acylated peptide was used as a negative control. Surprisingly, cells

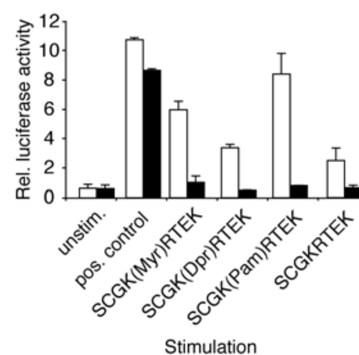


FIG.15: Synthetic lipopeptides representing pneumolysin are sensed only through TLR2.
Human (h) TLR2 (white bars) and hTLR4/hMD-2 (black bars) expressing HEK293 cells were challenged over night with 1µg/ml of pneumolysin analogues. NF-κB-mediated reportergene activation was analyzed by luciferase assay.

overexpressing TLR2 were responsive towards all lipopeptides used (**Figure 15**) while overexpression of TLR4/MD-2 was unable to confer pneumolysin analogue responsiveness. Next supernatant from *S. pneumoniae* was analyzed for pneumolysin content and potential immune stimulatory capacity. *S. pneumoniae* were grown in standard Brain Heart (BH) medium. Bacteria were centrifuged, supernatant was collected and protein content within the supernatant was accumulated by using Centricon® Centrifugal Filter Units. Pneumolysin was specifically detectable by western blotting and concentration of pneumolysin was enhanced upon accumulation of supernatant (**Figure 16A**). Application of *S. pneumoniae* supernatants induced NF-κB activation in terms of Luciferase production in cells overexpressing TLR2 but not in cell overexpressing TLR4/MD-2 (**Figure 16B**). Challenge with bacteria supernatant induced TNFα secretion in RAW macrophages. However, blockade of TLR2 by preincubation of cells with an antagonistic monoclonal TLR2-specific antibody (T2.5) did not inhibit cell activation upon administration of *S. pneumoniae* supernatant (**Figure 16C**).

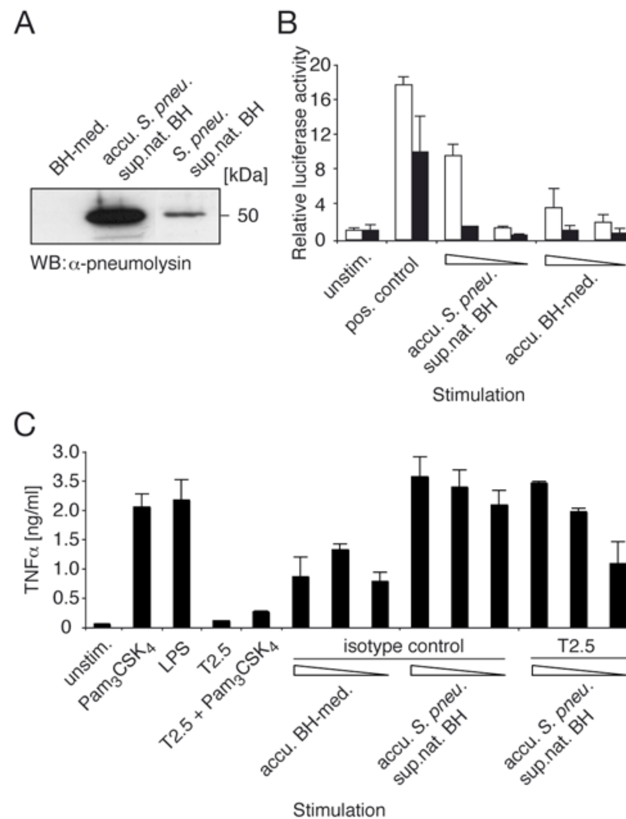


FIG.16: *S. pneumoniae* supernatant contains pneumolysin and activates TLR2.

(A) BH-medium and naive supernatants from *S. pneumoniae* and supernatant accumulated by Centricon® Centrifugal Filter Units were analyzed for pneumolysin content by western blotting (BH, Brain Heart; med., medium; sup.nat., supernatant; accu., accumulated). (B) hTLR2 (white bars) and hTLR4/hMD-2 (black bars) expressing HEK293 cells were challenged with 1µl or 0.1µl of *S. pneumoniae* supernatant or standard BH-medium. 100ng/ml of Pam_3CSK_4 and LPS were used as positive control for hTLR and hTLR4/MD-2 respectively. NF-κB-mediated reportergene activation was analyzed by luciferase assay (h, Human). (C) RAW264.7 macrophages either pre-treated with 25µg/ml of T2.5 or isotype control were subjected to decreasing amounts (10µl, 1µl, 0.1µl) of BH-medium or supernatant. 16 h upon stimulation TNFα levels were determined in the cell supernatant.

3.1.8 Exchange of relevant LRRs to analyze structural requirements determining ligand specificity

Our finding of overlapping specificity of TLR2 and TLR4 for LPS and tri-myristoylated lipopeptide questions how structural homology and diversity within both receptors enable distinct ligand specificity. In order to identify specific LRRs that determine optimal LPS responsiveness of TLR4 and lipopeptide responsiveness of TLR2, I generated chimeric expression constructs by exchange of specific LRRs between both receptors. First I analyzed protein sequences of TLR2 and TLR4 and identified all LRRs within their extracellular portion according to the established LRR consensus motifs LxxLxxLxLxxN and LxxLxxLxLxxNxLxxL (**Figure 17**).

Altogether I identified 21 LRRs for hTLR4 and 20 LRRs for hTLR2. Next, LRRs of both receptors

hTLR4 LRRs

1. PFSTKNLDLSFNPLRHLGSYSFFS
2. FPELQVLDLSRCEIQTIEDGAYQS
3. LSHLSTLILTGNPIQSLALGAFSG
4. LSSLQKLVAVETNLASLENFPIGH
5. LKTLKELNVAHNLIQSFKLPEYFSN
6. LTNLEHLDLSSNKIQSIYCTDLRVLHQM
7. PLLNLSLDLSLNPMNFIQPGAFK
8. EIRLHKLTLRNNFDSLNVMKTC
9. IQGLAGLEVHRLVLGEFRNEGNLEKFDKSA
10. LEGLCNLTIEEFRLAYLDYYLDDIIDLFNC
11. LTNVSSFSLVSVTIERVKDFSYNFGWQHLELVNCKFGQFPTLK
12. LKSLKRLTFTSNKGGNAFSEVD
13. LPSLEFLDLSRNGLSFKGCCSQSDFG
14. TTSLKYLDLSFNGVITMSSNFLG
15. LEQLEHLDFQHSNLKQMSEFSVFLS
16. LRNLIYLDISHTHTRVAFNGIFNG
17. LSSLEVLKMAGNSFQENFLPDIFTE
18. LRNLTFLDLSQCQLEQLSPTAFNS
19. LSSLQVLNMSHNNFFSLDTFPYKC
20. LNSLQVLDYSLNHIMTSKKQELQHF
21. PSSLAFLNLTQN

hTLR2 LRRs

1. TEAVKSLDLSNNRITYISNSDLQR
2. CVNLQALVLTSNGINTIEEDSFSS
3. LGSLEHLDLSYNYLSNLSSSWFKP
4. LSSLTFLNLLGNPYKTLGETSLFSH
5. LTKLQILRVGNMDTFTKIQRKDFAG
6. LTFLEELEIDASDLQSYEPKSLKS
7. IQNVSHLILHMKQHILLLEIFVDV
8. TSSVECLELRDTDLDTFHFSELSTGE
9. TNSLIKKFTFRNVKITDESLFQVKLLNQ
10. ISGLLELEFDDCTLNGVGNFRASDNDRVID
11. PGKVETLTIRRLHIPRFYLFYDLSTLYSL
12. TERVKRITVENSKVFLVPCLLSQH
13. LKSLEYLDLSENLMVEEYLKNSACEDA
14. WPSLQTLILRQNHLASLEKTGETLLT
15. LKNLTNIDISKNSFHSMPETCQW
16. PEKMKYLNLSSTRIHSVTGCI
17. PKTLEILDVSNNNLNLFSLN
18. LPQLKELYISRNKLMTLPDASL
19. LPMLLVLKISRNAITTFSKEQLDS
20. FHTLKTLEAGGNNFICSCEFLSFT

FIG.17: Identifiction of LRRs within the extracellular domain of human (h) TLR4 and hTLR2 according to the typical LRR consensus motifs LxxLxxLxLxxN and LxxLxxLxLxxNxLxxL.

were systematically aligned and analyzed for homology towards each other. Whereas LRRs 1-6 and LRRs 13-20 showed homology of more than 38%, diversity of LRRs 7-12 was specifically high (**Figure 18**). Assuming that observed diversity within this specific region of the extracellular domain is responsible for ligand specificity, LRR6-13 and LRR7-12 were exchanged between both receptors (**Figure 18**).

Upon controlling expression levels of flag-tagged chimeras by western blot (data not shown) expression-constructs encoding chimeric hTLR4 or hTLR2 were overexpressed together with hMD-2

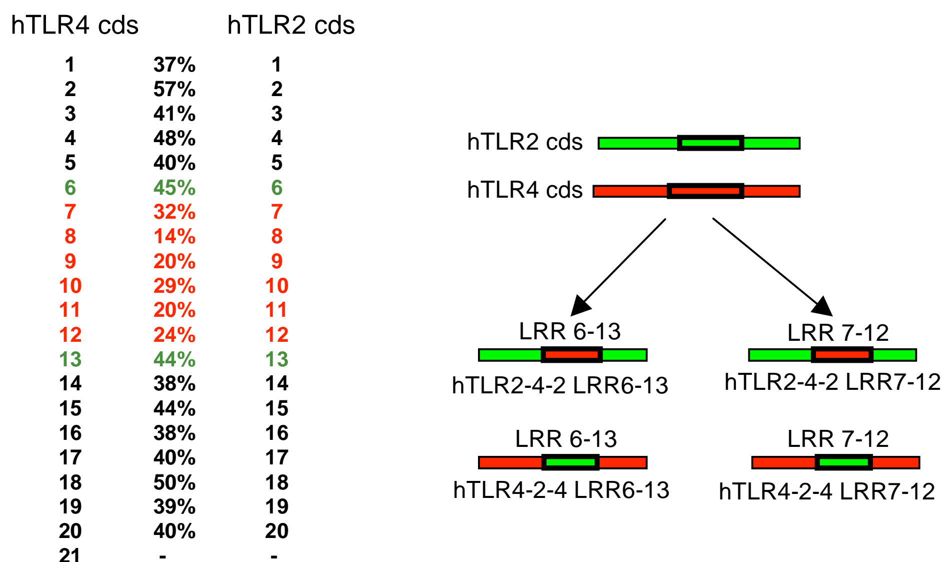


FIG.18: Conserved homology of LRRs between human (h) TLR2 and hTLR4 extracellular domain as well as strategy to exchange specific LRR fragments bearing highest diversity between both receptors to analyze which LRRs are responsible for characteristic ligand specificity of respective TLRs.

and were analyzed for ligand specificity by Luciferase assay. The chimeric constructs encoding a mutated form of human TLR2 bearing the LRR6-13 or LRR7-12 from TLR4 were functionally inactive and did not induce NF-κB mediated reportergene expression (**Figure 19A**). In contrast the chimeric constructs hTLR4-2-4 LRR6-13 and hTLR4-2-4 LRR7-12 constitutively induced NF-κB activation independently of challenge with specific TLR4 or TLR2 ligands (**Figure 19B**). Notably constitutive activity of the chimeric constructs was not due to exceeding overexpression (data not shown). Due to the observation of inactivity of mutated TLR2 constructs and constitutive activity of TLR4 mutants I decided to focus on further mutating TLR4. In order to abolish ligand independent activity and increase potential ligand specificity I decreased number of LRRs and exchanged shorter LRR-fragments. Consequently I exchanged LRR7-9, LRR7-11 and LRR8-12 of TLR4 by respective LRR fragments of TLR2. While TLR4 constructs carrying LRR7-11 and LRR8-12 from TLR2 despite of normal expression levels (data not shown) were non-functional the mutant hTLR4-2-4 LRR7-9 was constitutively active (**Figure 19C**). Accordingly I focused at the LRRs 7-9 and exchanged each LRR separately. Chimeric constructs of human TLR4 including LRR7, LRR8 or LRR9 of hTLR2 were active in terms of NF-κB activation independent of ligand application (**Figure 19D**). Challenge with different forms of LPS as well as Pam_3CSK_4 did not induce Luciferase activity in comparison to the non-stimulated control indicating lack of ligand specificity of generated chimeric constructs.

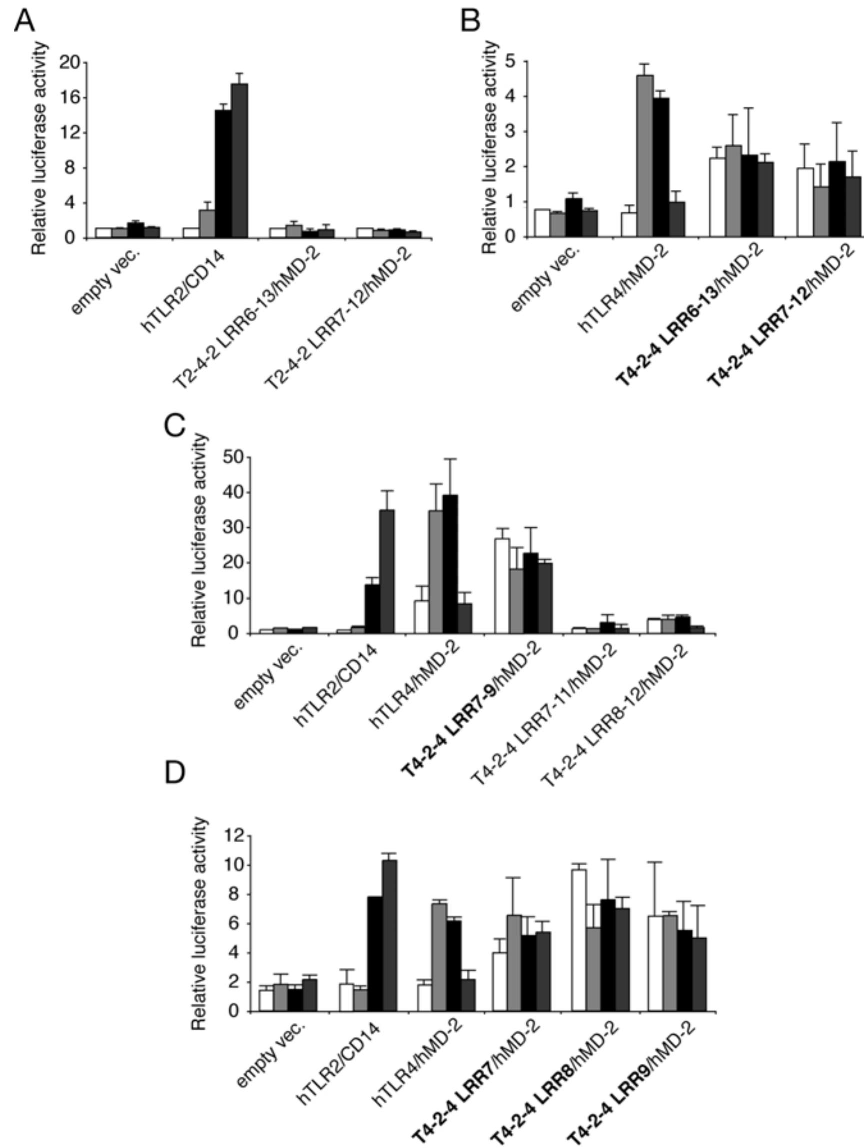


FIG.19: Functional analysis of chimeric TLR2 and TLR4 constructs in terms of potentially modified ligand specifictiy

Generated mutants of human (h) TLR2 (A) and hTLR4 (B) carrying exchanged fragments of LRR6-13 and LRR7-12 were transiently overexpressed together with a NF-κB promotor carrying Luciferase gene construct and hMD-2 in HEK293 cells. Cells were either unchallenged (white bars) or challenged with 100ng/ml of *Salmonella enterica* serovar Minnesota strain LPS R595 (bright grey bars), O111:B4 *E. coli* LPS (black bars) or Pam_3CSK_4 (dark grey bars) for 16 h and cell lysates were afterwards analyzed for Luciferase activity. Constructs bearing NF-κB mediating activity (bold lettering) were further mutated by exchanging shorter LRR regions (C) and finally by exchanging single LRRs 7, 8 and 9 (D).

3.2 Antagonism of TLR2 and TLR4 to prevent septic shock

According to our finding, reporting that only mice lacking TLR2 and TLR4 are unresponsive towards LPS as well as specific lipopeptides, we asked whether antagonizing both TLRs diminishes inflammation upon recognition of bacterial infections. Therefore in contrast to application of synthetic bacterial compounds we analyzed the role of TLR2 and TLR4 during infection with Gram-negative as well as Gram-positive bacteria. Furthermore we planned to impede TLR2- and TLR4-signalling by usage of monoclonal antagonistic antibodies towards both TLRs. Preliminarily blockade of TLR-activity provides a therapeutic tool for termination of hyper inflammation during sepsis pathogenesis but offers as well an effective instrument to experimentally analyze the role of TLRs in allergy or induction of autoimmunity.

3.2.1 Contribution of TLR2 and TLR4 to bacteria recognition and sepsis

3.2.1.1 Responsiveness to Gram-negative bacteria depends on TLR2 and TLR4

To analyze the role of TLR2 and TLR4 in bacterial infection we used BMDMo's isolated from wild-type, TLR2$^{-/-}$, TLR4$^{-/-}$ and TLR2$^{-/-}$TLR4$^{-/-}$ mice and infected them with Gram-positive as well as Gram-negative bacteria. Specifically, macrophages were challenged with clinical isolates of *Salmonella enterica* or *Escherichia coli* representing Gram-negative bacteria and with the Gram-positive *Streptococcus pneumoniae* or *Streptococcus epidermidis* (**Figure 20**). To terminate bacterial proliferation antibiotic cocktails (100µg/ml Ampicillin / 10µg/ml Ofloxacin, Gram-negative bacteria; 100µg/ml Ampicillin / 10µg/ml Ofloxacin / 25µg/ml Rocephin, Gram-positive bacteria) were applied 1 h after bacterial challenge. Cells were incubated for additional 16 h upon which supernatants were analyzed for TNFα concentration. While Gram-positive bacteria were sensed independently of TLR2 and TLR4, recognition of Gram-negative bacteria at lower concentrations was dependent on TLR2 and TLR4. Macrophages lacking both, TLR2 and TLR4 showed decreased responsiveness to *S. enterica* and *E. coli* challenge.

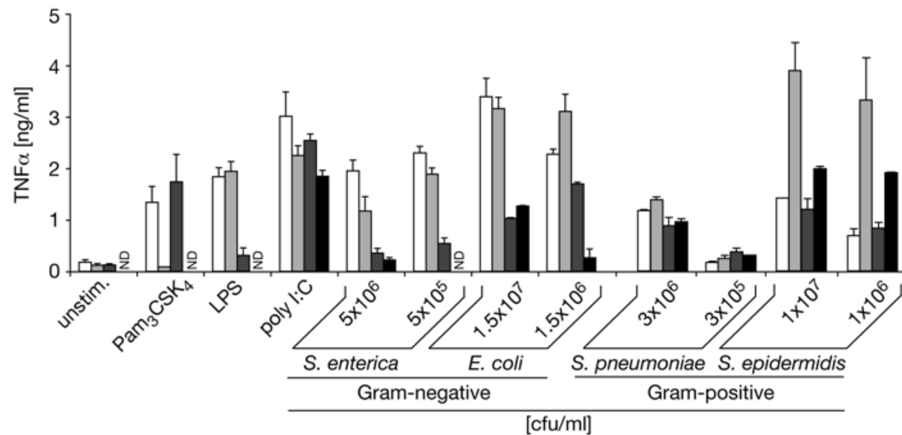
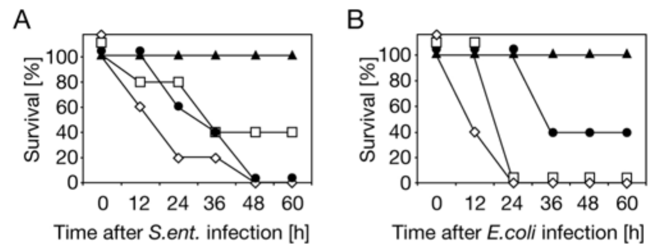


FIG.20: TLR2 and TLR4 dependent recognition of Gram-negative bacteria

BMDMo's from wild-type (white bars), $TLR2^{-/-}$ (light grey bars), $TLR4^{-/-}$ (dark grey bars) and $TLR2^{-/-}TLR4^{-/-}$ (black bars) mice were infected with Gram-negative and Gram-positive bacteria and subjected to antibiotic therapy after 1h. Cell culture supernatants were analyzed for TNFα content by ELISA 16 h after challenge or infection as indicated. Pam_3CSK_4, LPS and poly I:C were used as positive controls to verify genotype of macrophages (ND, not detected).

3.2.1.2 *$TLR2^{-/-}TLR4^{-/-}$ mice are resistant to Gram-negative bacteria induced septic shock*

Next I analyzed role of TLR2 and TLR4 during bacterial infection *in vivo*. Therefore I established a clinically relevant septic shock model including infection with viable bacteria and subsequent antibiotic therapy to terminate spread of infection (**Figure 21**). Notably, the dose of bacteria applied



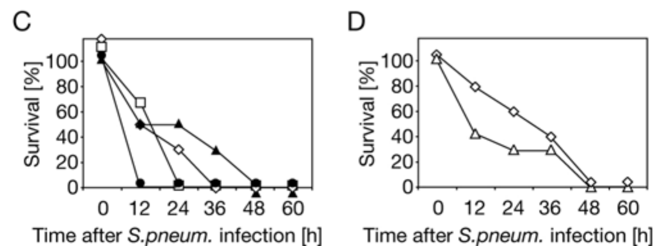


FIG.21: Impaired responsiveness due to absence of both TLR2 and TLR4 protects mice from fatal hyper-inflammation induced by Gram-negative bacteria but not Gram-positive bacteria if infection is terminated by antibiotic therapy.

(A-D; ◇, wild-type; ■ $TLR2^{-/-}$; ●, $TLR4^{-/-}$; ▲, $TLR2^{-/-}TLR4^{-/-}$; △, 5xKO - mice). Mice were infected i.p. with $1x10^9$ CFU *S. enterica* (*ent.*; *n* = 5 for each group; A), $5x10^9$ CFU *E. coli* (*n* = 5 for each group; B) or $2.5x10^9$ CFU *S. pneumoniae* (*pneum.*; ◇, *n* = 6; ■, *n* = 6; ●, *n* = 3; ▲, *n* = 6, C; ◇, *n* = 6, △, n = 7; D). After 1 h mice were treated four times hourly with 68mg/kg Ampicillin together with 2.8mg/kg Ofloxacin (A, B) or 68mg/kg Ampicillin together with 2.8mg/kg Ofloxacin and 8mg/kg Rocephin (C, D). Survival was monitored for 1 week.

was adjusted to be lethal despite of antibiotic therapy mimicking fatal Jarisch-Herxheimer reaction during sepsis pathogenesis. Effectiveness of antibiotic treatment was thoroughly checked by analysis of blood by plating. In terms of Gram-negative bacteria induced septic shock we observed resistance to fatal *S. enterica* (**Figure 21A**) and *E. coli* (**Figure 21B**) challenge only of TLR2$^{-/-}$TLR4$^{-/-}$ mice. All wild-type mice as well as most TLR2$^{-/-}$ and TLR4$^{-/-}$ mice succumbed to lethal shock. In case of Gram-positive bacteria mice succumbed to septic shock independent of TLR2 and TLR4 (**Figure 21C**). Even 5x-knockout mice lacking TLR2, 3, 4, 5 and 9 comparable to wild-type mice succumbed to *S. pneumoniae* infection that was barely lethal although subsequent antibiotic treatment was performed (**Figure 21D**).

3.2.2 Identification of hybridoma producing TLR4-specific monoclonal antibody

After identifying TLR2$^{-/-}$TLR4$^{-/-}$ mice as unresponsive to Gram-negative bacteria and thus resistant to Gram-negative bacteria induced lethal septic shock we aimed to inhibit activity of both TLRs by application of specific antagonistic antibodies. While a monoclonal antagonistic antibody towards murine TLR2 was already available (Meng, Rutz et al. 2004), a specific antibody inhibiting TLR4-

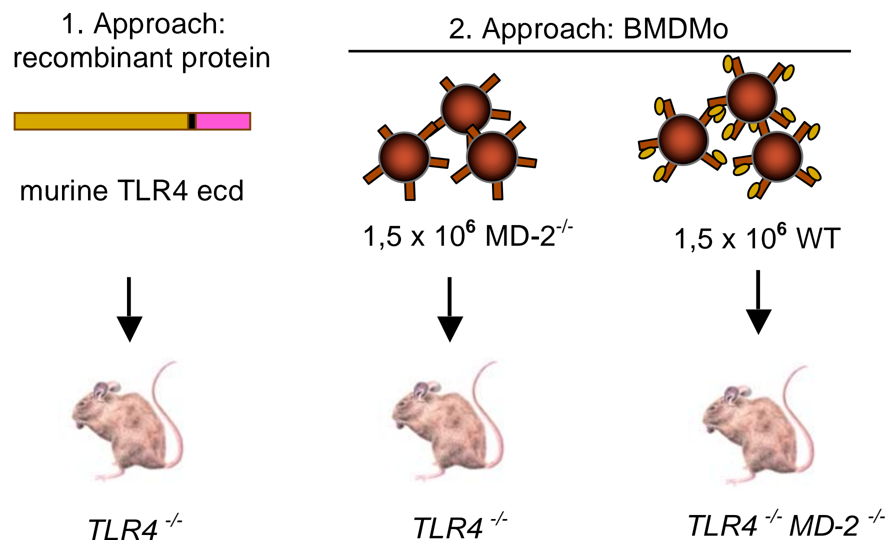


FIG.22: Generation of TLR4-specific antibodies within by different approaches

Mice lacking TLR4 were immunized with recombinant extracellular portion of murine TLR4 (1. Approach). Mice lacking TLR4 or TLR4/MD-2 complex were immunized with $1.5x10^6$ primary macrophages isolated from MD-2-deficient or wild-type mice respectively.

induced signalling had not yet been described. Therefore I followed two different strategies of immunizing mice to generate TLR4 specific antibodies (**Figure 22**). Mice lacking TLR4 or TLR4/MD-2-complex were immunized either with recombinant protein of murine TLR4 extracellular domain or BMDMo's isolated from wild-type or MD-2-deficient mice according to the established procedure described in M&M (3.2.1.7). Afterwards isolated splenocytes were fused to hybridoma cells and were selectively cultured. By limiting dilutions single clones were isolated for further analysis. Altogether 142 different hybridoma clones immunization with recombinant protein and 380 clones derived from the second approach were considered for further analysis. To identify

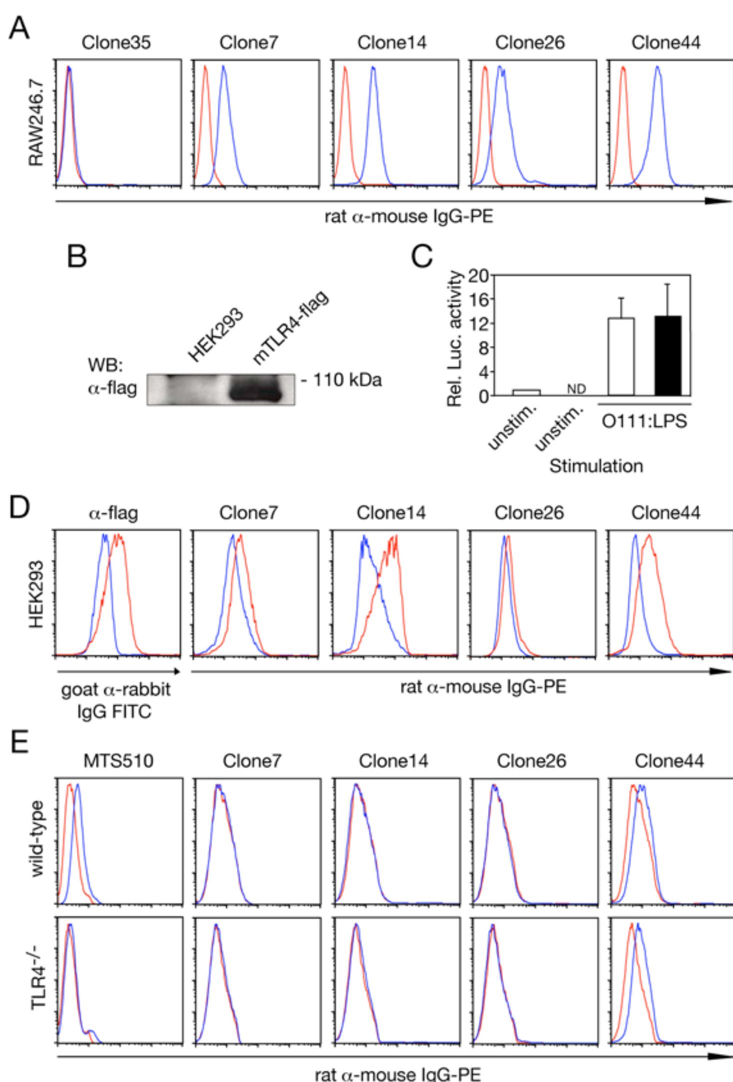


FIG.23: Screening for a monclonal antibody towards TLR4 by FACS
Hybridoma supernatants were applied on RAW264.7 macrophages (A, clone 35 represents antibody-negative supernatant), HEK293 cells expressing flag-tagged mTLR4/hMD-2 (D) or primary macrophages from wild-type or TLR4$^{-/-}$ mice (E) to screen for α-TLR4. Bound antibody was detected by rat α-mouse IgG-PE and visualized by flow cytometry. (B) Flag-tagged mTLR4 stably expressed by HEK293 cells was detected by immuno blotting with flag-specific antibody (α-flag). (C) HEK293 cells stably expressing mTLR4/mMD-2 (white bars) or mTLR4/hMD-2 (black bars) were transiently transfected with NF-κB promotor carrying Luciferase gene construct and were either left untreated (unstim.) or were challenged with *E. coli* O111:B4 LPS for 16h. Afterwards cell lysates were analyzed for Luciferase activity. (D) α-flag antibody was used as a positive control showing TLR4 surface expression. Flag-specific antibody was detected by goat α-rabbit IgG-FITC. (E) MTS510 (rat α-mTLR4/MD-2) was used as positive control and was detected by goat α-rat IgG-FITC.

clones producing specific αTLR4-antibody, supernatants from all hybridoma clones were subjected to RAW macrophages and analyzed by flow cytometry (**Figure 23A**). Potential TLR4-specific antibodies bound to surface of RAW-macrophages were detected by PE-labelled rat α-mouse IgG antibody and specific clones were selected for further characterizations. Therefore I generated HEK293 lines stably overexpressing either mTLR4/mMD-2 or mTLR4/hMD-2. Flag-tagged TLR4 was highly expressed while MD-2-flag was not detectable by western blotting (**Figure 23B**). Notably stable overexpression

of both mTLR4/mMD-2 and mTLR4/hMD-2 rendered HEK293 cells responsive for LPS challenge indicating expression of functional TLR4/MD-2 complex (**Figure 23C**). Using these stably TLR4-expressing cells I further screened hybridoma supernatants for TLR-specific antibodies (**Figure 23D**). Finally supernatants of four clones specifically stained TLR4 complex on the surface of HEK293 cells. Only clone 7, 14, 26 and 44 detected RAW cells as well as specifically mTLR4/MD-2 expressed on HEK293 cells and were further characterized. However, while the established α-TLR4 antibody named MTS510 specifically detected endogenous TLR4 on the cell surface of primary macrophages supernatants from hybridoma cells were unspecific for TLR4 (**Figure 23E**). Independent of applied amounts (data not shown) of hybridoma supernatants from clones 7, 14 and 26, no specific antibody was found to specifically bind to primary macrophages whereas antibodies within the clone 44 supernatant bound to wild-type macrophages likewise to cells lacking TLR4. Furthermore we analyzed hybridoma supernatants for TLR4-antagonistic capacity. None of the selected clones produced TLR4-antagonistic antibody (data not shown).

3.2.3 Characterisation of TLR4-specific antibody

In the course of a collaborative effort we received two novel monoclonal antibody named 1A6 (rat IgG2b) and 5E3 (rat IgG2b), which were identified upon immunizing male wistar rats three times subcutaneously with $1x10^6$ mTLR4/MD-2-expressing CHO-cells. Monophosphoryl-lipid A/trehalose dicorynomycolate (Sigma) was used as an adjuvant. Immunized rats were boosted once by subcutaneous injection of 10μg of recombinant mTLR4/mMD-2 fusion protein. After three days lymph nodes were fused with Sp2/0-myeloma cells and hybridoma supernatants were analyzed for TLR4-binding capacity by FACS. I further characterized both 1A6 and 5E3 and examined TLR4-specificity and antagonistic capacity *in vitro* and *in vivo* for 1A6 whereas 5E3 was analyzed only *in vivo* and used for other purpose (Daubeuf, Mathison et al. 2007). Notably, further experimentations shown in my thesis were all performed with 1A6.

3.2.3.1 1A6 specifically detects murine and human TLR4

1A6 specifically bound overexpressed flag-tagged human and murine TLR4/MD-2 complex and also detected a mixed species complex of TLR4/MD-2 (**Figure 24A**). Furthermore FACS analysis with a fluorescent-labelled antibody towards rat-Fcγ showed 1A6 binding on wild-type macrophages but not on macrophages from $TLR4^{-/-}$ mice (**Figure 24B**). Confocal microscopy confirmed TLR4-specificity of 1A6 and illustrates primarily intracellular TLR4 localization (**Figure 24C**). Additionally mTLR4

and mMD-2 were co-precipitated by 1A6 from HEK293 cells overexpressing both proteins (**Figure 24D**).

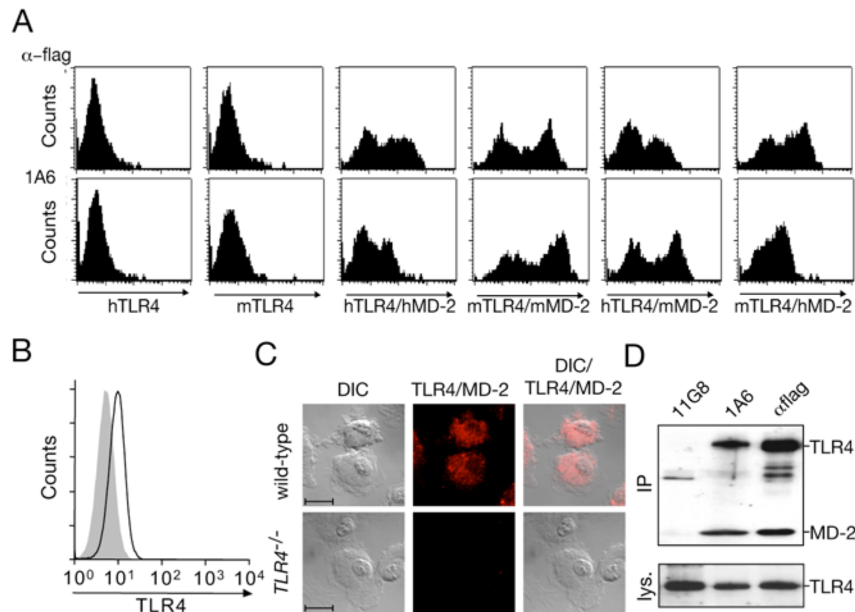


FIG.24: Specific binding of 1A6 to murine and human TLR4/MD-2

(A) HEK293 cells overexpressing mTLR4 or hTLR4 alone or together with m/hMD-2 were stained with 1A6 or α-flag as a positive control. (B) BMDMo's from WT (black line) or TLR4$^{-/-}$ (grey field) were subjected to 1A6 and analyzed by FACS. (C) Confocal microscopy of BMDMo' from genotypes indicated were incubated with 1A6 and were shown by Nomarski differential interferences conrast microscopy (DIC; bar represents 10µm), fluorescenc exposure (middle panel) and superimposition of both. (D) Overexpressed flag-tagged mTLR4/mMD-2 were precipitated by 1A6 or α-flag as a positive control. Analysis was performed by immuno blotting and detection using flag-specific antiserum.

3.2.3.2 Antagonism of murine TLR4 upon 1A6 administration

In order to analyze TLR4-inhibitory potential of 1A6 HEK293 cells overexpressing mTLR2 or mTLR4/mMD-2 were incubated with 11G8 (isotype control) or 1A6 for 30 min. prior to challenge with specific TLR agonist (**Figure 25**). 11G8 and 1A6 treatment alone did not induce activation of

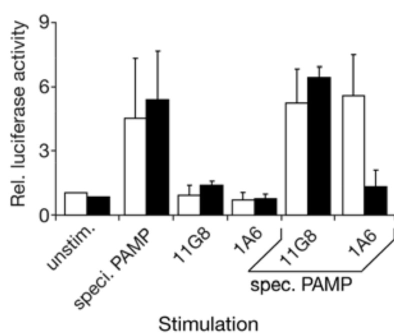


FIG.25: TLR4-anatagonizing potential of 1A6

HEK293 cells overexpressing NF-κB induced reportergene and murine (m) TLR2 (white bars) or mTLR4/mMD-2 (black bars) were either pretreated with 25µg/ml of isotype control (11G8) or α-TLR4 (1A6) or were left untreated. 30 min later cells were challenged with 1µg/ml of specific PAMP (Pam_3CSK_4, white bars; or LPS, black bars) for 16 h and were further analyzed for luciferase activity.

TLR2 and TLR4/MD-2. Preincubation with 1A6 specifically inhibited luciferase induction of cells expressing TLR4/MD-2 upon LPS challenge but did not influence Pam_3CSK_4 mediated TLR2-activation. Application of 11G8 did not alter responsiveness of TLR2 and TLR4/MD-2 upon Pam_3CSK_4 or LPS challenge respectively.

3.2.3.3 Systemic application of 1A6 protects mice from LPS induced lethal shock

Consequently TLR4-antagonistic activity of 1A6 was analyzed *in vivo*. First we analyzed whether systemic 1A6 administration inhibits secretion of proinflammatory cytokines upon LPS challenge. Therefore mice were pretreated with increasing amounts of 1A6 or isotype control. 1h after antibody injection mice were challenged with a non-lethal dose of LPS and serum was drawn 4 h later (**Figure 26A**). 1A6 pre-treatment dose-dependently inhibited LPS induced IL-6 production in contrast to 11G8 application. Furthermore we applied 1A6 within a high-dose LPS-shock model and injected antibody at several time points either prior (**Figure 26B**) or after (**Figure 26C**) LPS injection. Mice pretreated with 750µg of 1A6 were fully protected from fatal endotoxemia even if antibody was administered 5h prior to LPS injection. Injection of 1A6 6h before LPS challenge reduced survival rate while mice treated with equal amounts of isotype control succumbed independent of administration time point. Mice were even protected from LPS induced fatal shock when 1A6 was applied up to 4h upon LPS injection. Protective effect of therapeutic 1A6

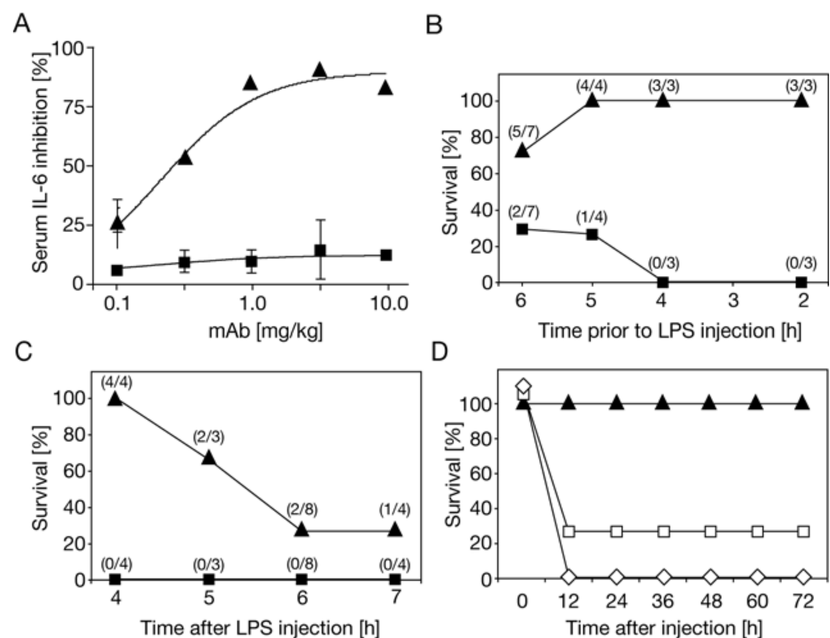


FIG.26: Systemic 1A6 administration prevents hyperinflammation and protects mice from lethal endotoxemia.

(A) C57BL/6 mice were pretreated with indicated amounts of 11G8 (■, isotype control) or 1A6 (▲) by i.p.injection and were challenged i.p. with 100µg of LPS 1 h later. 4 h thereafter serum of mice was drawn and analyzed by ELISA. IL-6 concentrations of mice treated with 11G8 were related to 1A6 treated mice (*n* = 2 per experimental group). (B-D) Mice were challenged by i.p. injection of 1.2 mg *E. coli* O111:B4 LPS. Application of 750µg of 11G8 (■) or 1A6 (▲) was performed at specific indicated time points either prior (B) or after (C, D) LPS challenge. Survival of mice was monitored for 1 week (survivors/ *n* applied at each time point indicated). (D) 4 hours after injection of 1.2 mg LPS mice were treated with 750µg (▲), 375µg (□) or 187µg (◇) of 1A6 by i.p. injection (*n* = 4 per experimental group).

administration was reduced time dependently if 1A6 was applied later. Notably injection of 750µg of 1A6 was necessary to sustain its protective activity if administered 4 h after LPS challenge (**Figure 26D**). Reduction of 1A6 dose was accompanied by reduced survival rate.

3.2.4 Blockade of TLR2 and TLR4 *in vitro* and *in vivo*

3.2.4.1 Inhibition of inflammatory response upon application of antagonistic antibodies in vitro and in vivo

Upon confirming the crucial roles of TLR2 and TLR4 in sensing of Gram-negative bacteria, we applied antagonistic antibodies to inhibit Gram-negative bacteria induced inflammatory responses. Aside of an α-TLR4 antibody (1A6) we applied an α-TLR2 antibody named T2.5 on RAW264.7 macrophages. 1A6 specifically inhibited LPS-mediated cell activation whereas Pam_3CSK_4 mediated TNFα release was effectively blocked by T2.5 (**Figure 27A**). Next we challenged antibody-treated RAW macrophages with variable doses of *S. enterica* or *E. coli,* which were subsequently subjected to antibiotics exposure 1h upon infection (**Figure 27B**). Cells pretreated with isotype controls fully responded to bacterial challenge and secreted high amounts of TNFα. While antagonizing TLR2 or

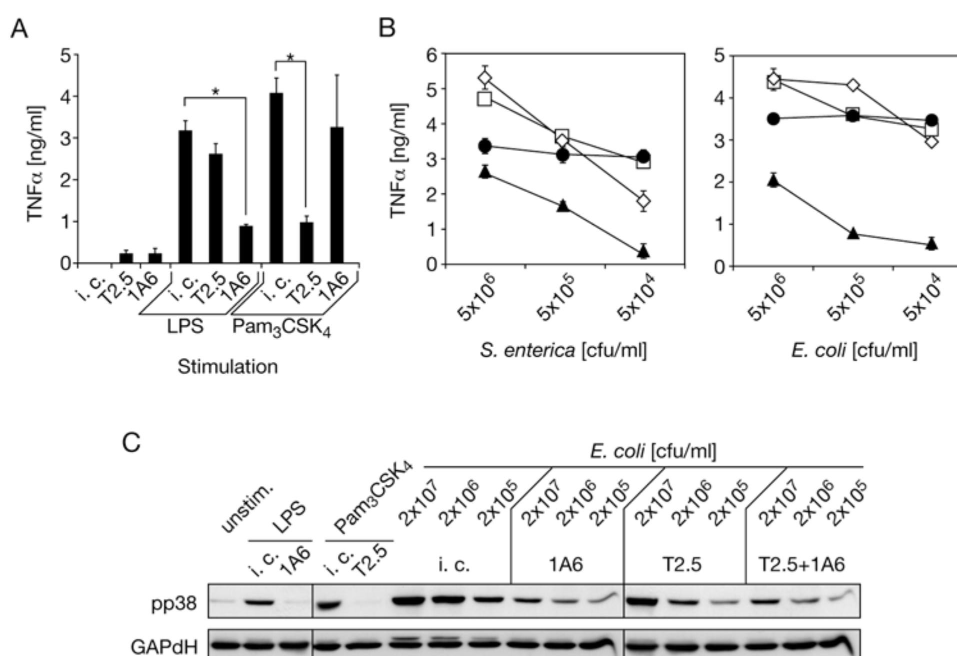


FIG.27: Effective blockade of TLR2 and TLR4 on macrophages infected with Gram-negative bacteria

(A) RAW264.7 macrophages were incubated with α-TLR2 (T2.5), α-TLR4 (1A6) or respective isotype control (i. c.; 11G8 as control for 1A6 and T2.13 as control for T2.5) for 30 min and were further challenged with 1µg/ml LPS or Pam_3CSK_4 (*$P < 0.003$). (B) RAW264.7 macrophages were infected with *S. enterica* and *E. coli* as indicated, 30 min after incubation with 1A6 (●), T2.5 (□), 1A6 and T2.5 (▲) or isotype control (◇) mAb. Antibiotics were applied 1h after infection and supernatants were analyzed by ELISA 6h later. (C) Upon preincubation with antibodies as indicated macrophages were infected for 20 min with *E. coli*. Thereafter cells were lysed and analyzed by immuno blotting in respect to phosphorylation of p38. GAPdH was used as loading control. (A-C) Antibodies including isotype controls were applied at 25µg/ml.

TLR4 alone did not downregulate *S. enterica* and *E. coli* induced cell activation, preincubation with α-TLR2 and α-TLR4 together effectively inhibited bacterial recognition and consequently diminished TNFα release. Furthermore phosphorylation of the MAP kinase p38 upon *E. coli* infection was effectively inhibited by co-application of T2.5 and 1A6 (**Figure 27C**). Consequently antibodies were administrated systemically in wild-type mice prior to challenge with antibiotic-killed bacteria. Upon specific timepoints serum was drawn and analyzed for cytokine content by ELISA (**Figure 28**). Parallel blockade of TLR2 and TLR4 in wild-type mice effectively inhibited inflammatory processes. Serum levels of TNFα, IL-6 and IL-10 in mice pretreated with T2.5 and 1A6 were significantly decreased in comparison to mice treated with isotype controls and were equally low as in $TLR2^{-/-}$ $TLR4^{-/-}$ mice used as a negative control.

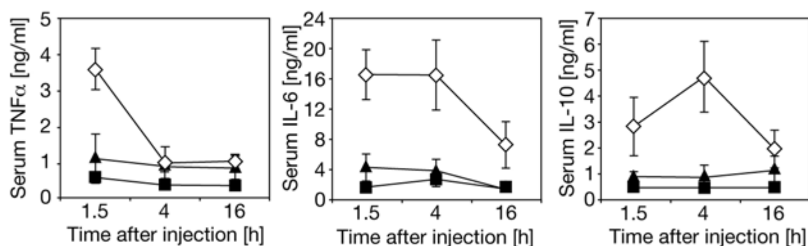


FIG.28: Systemic TLR2 and TLR4 blockade leads to inhibition of cytokine production

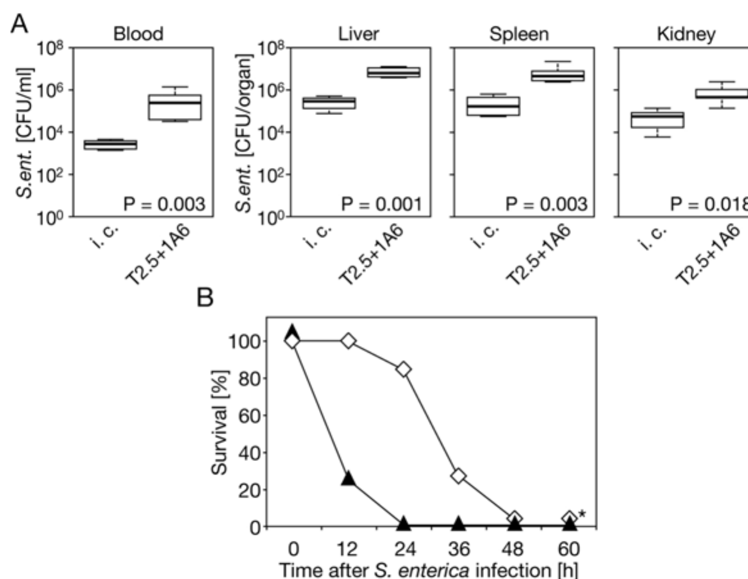
C57Bl/6 mice received 750µg of 1A6 and T2.5 (▲, *n* = 6) or 1.5mg of isotype control (◇, *n* = 6) 1h prior to i.p. injection of $5x10^7$ CFU *E. coli* that had been killed by antibiotic treatment *in vitro* 2h prior to administration. $TLR2^{-/-}TLR4^{-/-}$mice were used as negative controls (■, *n* = 3). Serum was sampled at indicated time points and was analyzed by ELISA.

3.2.4.2 TLR2- and TLR4-blockade during Gram-negative infection without subsequent antibiotic therapy promotes bacterial spread and accelerates fatality

To analyze effect of systemic TLR2- and TLR4-blockade during acute Gram-negative infection we applied antagonistic antibodies and monitored progression of infection in wild-type mice as well as survival. C57BL/6 mice were pretreated with isotype controls or α-TLR2 and α-TLR4 mAb 1h prior to infection with a low dose of *S. enterica*. We analyzed bacterial loads of different compartments from infected mice, which received either isotype controls or 1A6 and T2.5. 24h upon infection mice were sacrificed and blood, liver, spleen and kidneys were isolated, homogenised and analyzed for bacterial load by plating on blood agar over night. Bacterial counting illustrated that dual TLR-blockade significantly promotes systemic bacterial spreading in mice upon low-dose infection

(**Figure 29A**). Consequently accelerated pathogenesis due to TLR2- and TLR4-blockade prior to acute infection without antibiotic treatment increased fatality of mice (**Figure 29B**).

FIG.29: Increased susceptibility to Gram-negative infection upon blockade of TLR2 and TLR4
(A) Wild-type mice received mAbs (i.c., isotype control; T2.5, α-TLR2; 1A6, α-TLR4) 1 h prior to infection with 1 x 10^6 CFU *S. enterica* (*ent.*) by i.p. injection. 24 h later mice were sacrificed and bacterial loads of compartments indicated were determined (*n* = 6 for each experimental group). (B) Wild-type mice received mAb (◇, isotype control; ▲, 1A6 and T2.5; *P < 0.004 for comparison to specific dual-TLR-blockade groups) 1 h prior to infection with 1 x 10^8 CFU *S. enterica* to be left untreated thereafter and monitored for survival (*n* = 7 per group).



3.2.4.3 *Antibiotic therapy requires additional blockade of TLR2 and TLR4 to protect mice from fatal Gram-negative induced septic shock*

In contrast to ongoing infection we next applied 1A6 and/or T2.5 within a clinical relevant mouse model for Gram-negative septic shock including infection with a lethal dose of bacteria followed by subsequent antibiotic therapy. Notably, dose of bacteria applied was lethal in spite of effective antibiotic treatment. 1h (**Figure 30A**) or 4h (**Figure 30B**) after infection of mice treatment with 1.7mg Ampicillin and 70µg Ofloxacin was performed and was accompanied by administration of isotype controls or antagonizing antibodies. Afterwards antibiotic administration was repeated for three times hourly to ensure termination of bacterial growth. Mice which received isotype control, α-TLR2 or α-TLR4 antibody alone 1h upon infection succumbed to both *E. coli* as well as *S. enterica* induced fatal septic shock despite of antibiotic therapy. Only mice treated with antibiotics together with TLR2- and TLR4-antagonizing antibodies were protected from fatal septic shock (**Figure 30A**). Application of both α-TLR2 and α-TLR4 antibodies accompanied by start of antibiotic therapy was protective even if administrated 4h upon infection (**Figure 30B**). Notably in comparison to the 1h model dose of bacteria injected had to be reduced within the 4h model to compensate systemic bacterial spreading during the additional time upon infection. Nevertheless quantity of *S. enterica* and *E. coli* applied

within the 4h model was lethal although antibiotic therapy was performed. Surprisingly, while blockade of TLR2 in addition to that of TLR4 after infection was indispensable for survival, application of α-TLR4 antibody alone prior to bacterial challenge and start of subsequent antibiotic treatment 2h later was sufficient to protect mice (**Figure 30C**).

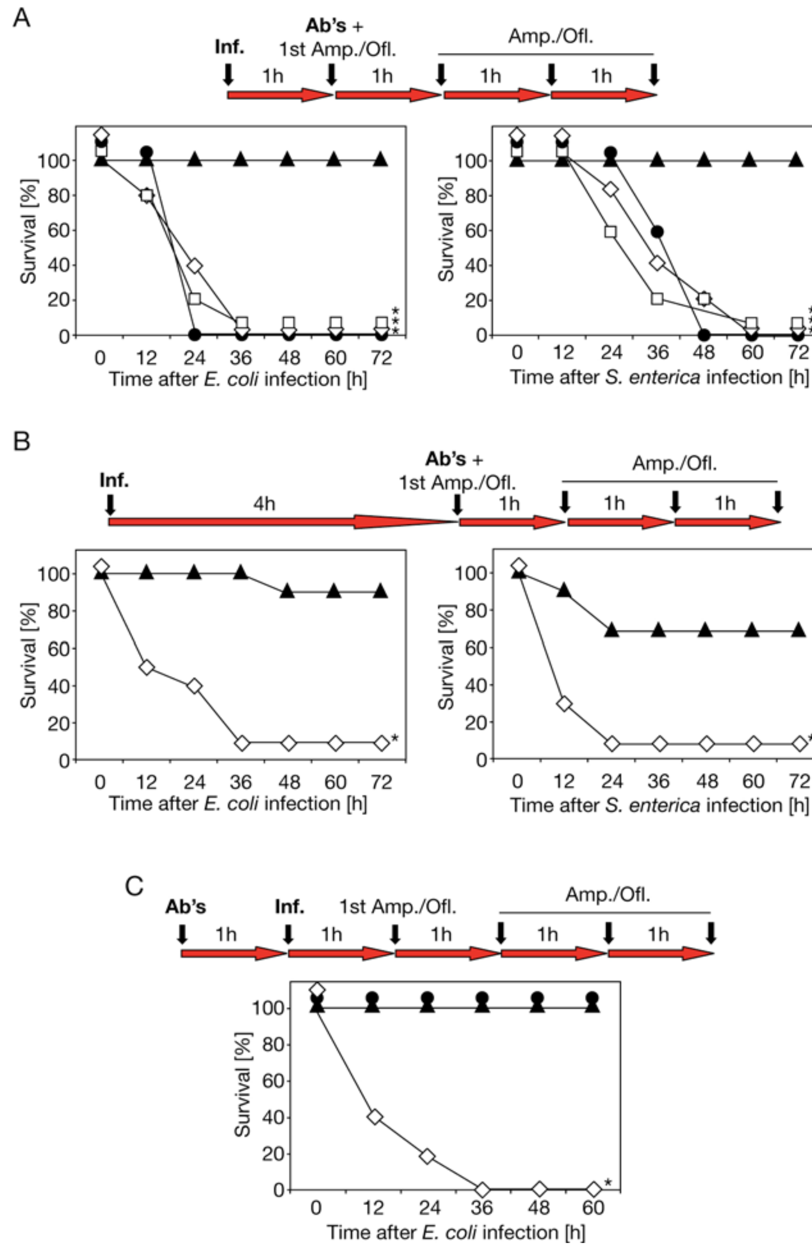


FIG.30: Application of 1A6 together with T2.5 in combination with subsequent antibiotic therapy protects mice from fatal septic shock

(A-C) Wild-type mice received 750µg of mAb (◇, isotype control; ●, 1A6; ▽, T2.5; ▲, 1A6 and T2.5): (A, B) at the start of antibiotic therapy (A) 1 h after infection with 5 x 10^9 CFU *E. coli* or 1 x 10^9 CFU *S. enterica* (*n* = 5 per group), or (B) at the start of antibiotic therapy 4 h after infection with 5 x 10^8 CFU *E. coli* (*n* = 9 for both experimental groups) or 1 x 10^8 CFU *S. enterica* (*n* = 10 for both experimental groups). (C) Antibodies were administrated 1 h prior to infection with 5 x 10^9 CFU *E. coli* and 2 h prior to antibiotic therapy (*n* = 5 per group). (A-C) Antibiotic therapy consisted of four i.p. injections of 1.7mg Ampicillin (Amp.) and 70µg Ofloxacin (Ofl.) hourly. (Ab's, antibodies; Inf., infection; *P < 0.004 for comparison to specific dual-TLR-blockade groups)

3.3 TLR4-driven activity of TLR2 during Gram-negative infection is mediated by IFNγ

3.3.1 TLR4-activation rapidly enhances lipopeptide-sensitivity *in vivo*

Due to our observation of a protective effect of α-TLR4 antibody application prior to infection but requirement of additional TLR2-blockade if antibodies were administrated after infection we assumed a trigger function of TLR4 that enhances functionality of TLR2 during Gram-negative infection. To address a potential mechanism we analyzed serum concentration of TNFα upon single or double injection of LPS or Pam_2CSK_4 within a time interval of 3 hours. TNFα concentration in the serum peaks at 90 min after challenge and is no more detectable 3h after challenge (**Figure 31A**). To analyze whether the chronology of activation of TLR2 or TLR4 determines the extent of the second TNFα induction 90 min after re-challenge mice were double-challenged alternately and consecutively with TLR2- or TLR4-ligands or were challenged only once. Serum was drawn 90 min after last challenge and was analyzed for TNFα content. Pre-activation of TLR4 in contrast to pre-activation of TLR2 significantly enhanced TNFα content upon re-challenge with TLR2-ligand in the serum (**Figure 31B**). Notably TNFα concentration in the second peak upon chronologic TLR4-TLR2 stimulation was higher as compared to TNFα concentration 90 min after single TLR4 or TLR2 activation, indicating that TLR4 signalling induces enhanced responsiveness of TLR2 (**Figure 31A,B**). Furthermore we observed persistence of an increased TNFα level 180 min after second challenge and fatal outcome in mice upon TLR4-TLR2 challenge in contrast to other TLR stimulation progressions (data not shown).

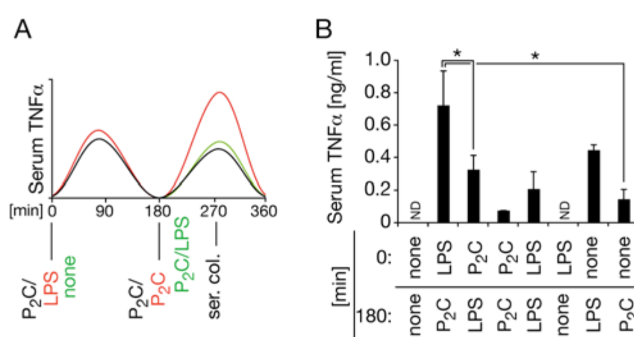


FIG.31: Pre-activation of TLR4 specifically induces enhanced TLR2-responsiveness.

(A) Schematic overview of performed sequential TLR-activation and observed process of TNFα-secretion (P_2C, Dipalmytoylated hexapeptide; ser col., serum collection). (B) Mice were challenged with 50µg of LPS or P_2C or were left untreated and were challenged a second time (or not) at 180 min. 90 min after last challenge serum was drawn and analyzed by ELISA ($n = 6$ per group; *$P < 0.004$; ND, not detected)

3.3.2 Early IFNγ-production upon challenge with Gram-negative bacteria is mediated TLR4-dependently and is secreted by NK and NKT cells

Upon reporting that IFNγ drives TLR2 sensitivity *in vitro* and *in vivo* we analyzed potential TLR4 mediated IFNγ induction. Therefore we infected wild-type mice and $TLR4^{-/-}$ mice with *E. coli* or *S. enterica* and analyzed serum for IFNγ content 2h after infection (**Figure 32A**). In contrast to $TLR4^{-/-}$ mice, wild-type mice produced significant amounts of IFNγ upon Gram-negative bacterial challenge. Furthermore LPS induced IFNγ-release of splenocytes was dependent on MyD88 but independent of TRIF (**Figure 32B**). Finally we identified NK-cells ($CD3^-NK1.1^+$) as well as NKT-cells $CD3^+NK1.1^+$) as early IFNγ producers dependent on TLR4. Wild-type mice as well as TLR4 deficient mice were infected with *S. enterica* for 2h. Thereafter mice were sacrificed and splenocytes were incubated with GolgiPlug upon which cells were stained and analyzed for intracellular IFNγ by flow cytometry. Number of IFNγ-producing NK-cell and NKT-cells were significantly higher within splenocytes from wild-type mice in comparison to cells isolated from TLR4-deficient mice (**Figure 32C**).

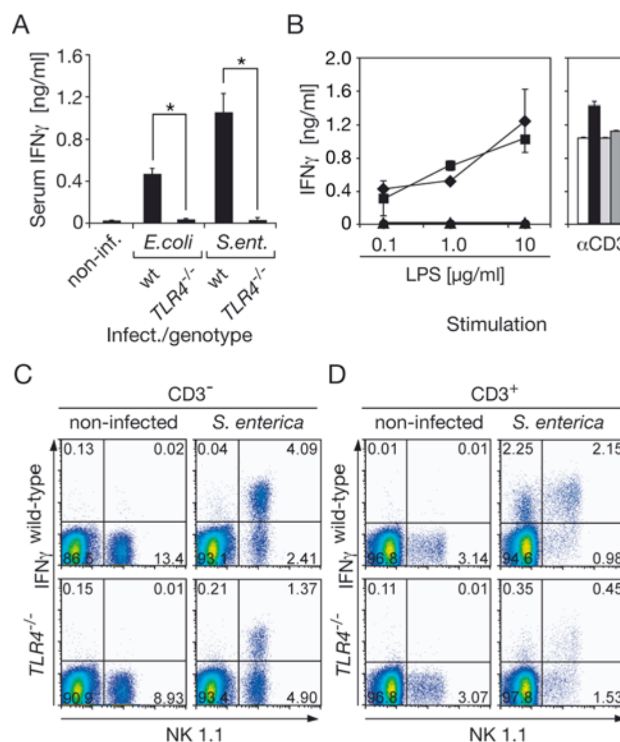


FIG.32: Gram-negative bacteria induced IFNγ is released by NK- and NKT-cell in a TLR4- and MyD88-dependent fashion.

(A) Mice were infected with $5x10^8$ CFU *E. coli* or $1x10^8$ CFU *S. enterica* for 3h upon which serum was analyzed by ELISA ($n = 3$ for each experimental group; $*P < 0.004$). (B) Splenocytes from wild-type (◆, white bar), $MyD88^{-/-}$ (●, light grey bar), $TRIF^{-/-}$ (■, black bar) and $MyD88^{-/-}TRIF^{-/-}$ (▲, dark grey bar) mice were challenged for 24h ex vivo with indicated amounts of LPS or α-CD3 antibody as positive control. Supernatants were analyzed for IFNγ-concentration by ELISA. (C, D) Murine splenocytes were prepared 2h upon injection of $1x10^8$ CFU *S. enterica*. Upon *ex vivo* incubation with GolgiPlug for additional 3h cells were divided by CD3 and NK1.1 staining and analyzed for intracellular IFNγ production by FACS.

3.3.3 TLR4-induced IFNγ increases TLR2-sensitivity and accompanies with enhanced surface TLR2-expression on $CD11b^+$ cells

Upon observing enhanced TLR2 sensitivity induced by LPS and TLR4 dependent IFNγ secretion we analyzed direct effect of IFNγ on macrophages in terms of TLR2 mediated responses. BMDMo's from C57Bl/6 mice were primed with IFNγ for 3h upon which cells were washed and challenged with TLR2 ligand. Cells pre-treated with IFNγ secreted higher amounts of TNFα compared to non-primed cells upon challenge with different amounts of Pam_3CSK_4 (**Figure 33A**). In addition we analyzed regulation of surface TLR2-expression on monocytes in dependence of TLR4 as well as IFNγ. Splenocytes from wild-type, $TLR4^{-/-}$, $IFN\gamma R^{-/-}$ and $TLR2^{-/-}$ mice were isolated 3h upon infection with *S. enterica* and $CD11b^+$ cells were analyzed for TLR2 surface expression by FACS. Only cells isolated from wild-type mice showed enhanced surface TLR2-expression upon infection while TLR2-upregulation on cells isolated from mice lacking either TLR4 or IFNγR was not detectable (**Figure 33B**). $TLR2^{-/-}$ mice were used as a negative control.

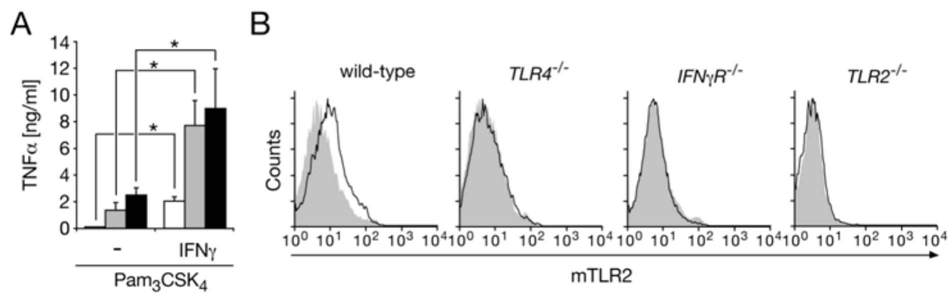


FIG.33: IFNγ-induces enhanced TLR2 sensitivity and TLR2-upregulation upon Gram-negative infection is mediated by TLR4-induced IFNγ.

(A) BMDMo's were primed with IFNγ (20ng/ml) for 3h or were left untreated. Cells were washed twice with PBS and were challenged with Pam_3CSK_4 (1ng/ml, white bars; 10ng/ml grey bars; 100ng/ml, black bars; *$P < 0.006$) for 6 h upon which supernatants were analyzed by ELISA. (B) Mice with indicated genotypes were infected with $1x10^8$ CFU *S. enterica* for 3h. Splenocytes were isolated, stained and $CD11b^+$-fraction was analyzed for surface TLR2-expression by FACS.

4 DISCUSSION

4.1 TLR2 and TLR4 share ligand-specificity for LPS and Myr_3CSK_4

The identification of TLRs more than a decade ago and their further characterization implicated their specificity for single ligands and their role of PRRs. However, some TLRs had been reported to bind more than one microbial compound. For instance, TLR2 has been shown to sense bacterial lipopeptide whereas TLR4 was regarded as a sole LPS-receptor. My findings extend this view and describe an overlap in ligand specificity of TLR2 and TLR4 regarding recognition of LPS and a tri-myristoylated lipopeptide, namely Myr_3CSK_4. We found that Myr_3CSK_4, which is a synthetic lipopeptide analogue representing a subpopulation of *E. coli* OMP activates both TLR2 and overexpressed human as well as murine TLR4/MD-2 complexes. Contrariwise we observed capacity of overexpressed TLR2 to detect *E. coli* LPS as well as *S. minnesota* LPS independent of TLR4 in the presence of CD14. Analyses of responsiveness of primary macrophages to Myr_3CSK_4 as well as LPS confirmed involvement of TLR2 and TLR4 in such pattern recognition.

Myr_3CSK_4 is recognized by TLR4 aside of TLR2

Myr_3CSK_4 induced NO-release, phosphorylation of MAP-kinases as well as TNFα in macrophages from wild-type, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice. In contrast cells isolated from TLR2$^{-/-}$TLR4$^{-/-}$ mice were unresponsive to Myr_3CSK_4 challenge demonstrating involvement of TLR2 and TLR4 in Myr_3CSK_4 recognition. Furthermore, blockade of murine as well as human TLR2 in RAW-macrophages or primary human PBMC's respectively inhibited Pam_3CSK_4 mediated cell activation but failed to inhibit TNFα-release upon high dose Myr_3CSK_4 challenge implicating an additional Myr_3CSK_4 receptor aside of TLR2 (**Figure 8-10**). Additionally, we demonstrated TLR4-dependent Myr_3CSK_4 responsiveness *in vivo*. While wild-type mice succumbed to otherwise lethal Myr_3CSK_4 challenge, TLR2$^{-/-}$, TLR4$^{-/-}$ and TLR2$^{-/-}$TLR4$^{-/-}$ mice were resistant. Accordingly, in contrast to mice lacking both TLR2 and TLR4 immunization using Myr_3CSK_4 as adjuvant induced OVA-specific Ig in TLR2$^{-/-}$ mice and also implicates TLR4 as a signal transducer for Myr_3CSK_4 (**Figure 11,12**).

Notably, we thoroughly analyzed the Myr_3CSK_4 preparation to exclude relevant LPS contamination potentially falsifying our observed TLR4-specificity regarding Myr_3CSK_4. First, observing that an overexpressed mixed species complex of mTLR4 and hMD-2 responds to LPS but not to Myr_3CSK_4 in contrast to the regular human and murine TLR4/MD-2 complexes indicated absence of LPS within our Myr_3CSK_4 preparation (**Figure 6**). Furthermore we could show that polymyxin B specifically inhibited LPS-mediated cell activation but had no influence in Myr_3CSK_4 induced activation of

overexpressed TLR2 as well as TLR4/MD-2 complex (data not shown). Finally we analyzed whether immune-stimulatory capacity of Myr_3CSK_4 is dependent of serum components (Schumann, Leong et al. 1990). While LPS recognition was diminished under serum-free conditions validating the requirement of LBP for LPS signalling, Myr_3CSK_4 signalling in case of TLR2 as well as TLR4 was independent of serum components and further indicated absence of LPS in our synthetic Myr_3CSK_4 preparation (**Figure 13**).

In order to analyze TLR4 mediated lipopeptide recognition we used further synthetic lipopeptide analogues carrying only a single myristoylation in contrast to the tri-myristoylation of Myr_3CSK_4. Single myristoylated Myr_1ASK_4 and Myr_1SSK_4 activated TLR2 likewise to Myr_3CSK_4 but failed to induce cell activation via TLR4 indicating requirement of poly-myristoylation to render lipopeptides detectable by TLR4/MD-2 complex (**Figure 14**).

Previous reports implicated TLR4 as the receptor for the bacterial lipoprotein pneumolysin secreted from *Streptococcus pneumoniae*. Pneumolysin is an exotoxin and is described to carry different intramolecular acylations within specific amino-acid motifs (Malley, Henneke et al. 2003). In order to confirm these data we applied specific pneumolysin analogues carrying different acylation and observed no TLR4/MD-2 dependent recognition of pneumolysin. In contrast all applied preparations of pneumolysin analogues activated overexpressed TLR2 but not TLR4/MD-2 (**Figure 15**). We reasoned that synthetic pneumolysin analogue display only a part of the natural protein and therefore might lack capacity to activate TLR4. Furthermore the pneumolysin analogues carry only one acylation, which possibly makes them according to our results discussed above undetectable for TLR4. Consequently we used supernatants of *S. pneumoniae* containing secreted pneumolysin to challenge TLR2 or TLR4/MD-2 expressing cells. Although pneumolysin was specifically detected and was enriched in the supernatants it failed to induce TLR4 activation but potently activated TLR2 confirming our data with the pneumolysin analogues. It remains to be analyzed whether higher concentrations of purified or recombinant pneumolysin would induce cell activation in a TLR4 dependent fashion.

LPS is recognized by TLR2 aside of TLR4

Aside of characterising TLR specificity of Myr_3CSK_4 we further addressed LPS mediated TLR2 activation *in vitro*, *ex vivo* and *in vivo*. BMDMo's gained responsive for LPS independently of TLR4 when pre-treated with IFNγ while macrophages from $TLR2^{-/-}TLR4^{-/-}$ mice were unresponsive to LPS despite of IFNγ priming. IFNγ was shown to induce protein upregulation of TLR2 independent of MyD88 and it was reported recently that otherwise resistant $TLR4^{-/-}$ mice gain responsiveness to LPS upon IFNγ priming (Sun and Ding 2006). In line with this, alike wild-type mice, $TLR4^{-/-}$ mice

succumbed to LPS induced septic shock upon IFNγ priming while mice lacking TLR2 and TLR4 were resistant implicating TLR2 as an additional signal transducer for LPS *in vivo* (**Figure 7, 9, 12**).

In order to exclude interference of undefined PAMPs to activation we analyzed LPS preparations for contamination. Nucleic acids were not detectable within *Salmonella enterica* serovar Minnesota strain R595 LPS preparation thus excluding TLR9-recruitment within our experimental settings. Furthermore protein content was below 0.035% and thus considered as irrelevant within the amounts we used for experimentations (data not shown).

In general, observed TLR2 sensitivity for rough LPS was lower compared to preparation of smooth LPS indicating a prominent role of saccharide chains bound to Lipid A in TLR2-dependent LPS recognition. For both agonists substantially higher amounts were needed to induce cross-activation as compared to amounts applied for sensing lipopeptide via TLR2 and LPS via TLR4 (**Figure 9**). This could be explained by lower affinity of TLR2 for LPS and TLR4 for Myr_3CSK_4 maybe dependent on numbers and types of acylations or length of polysaccharide chains within lipopeptides or LPS respectively. Notably, LPS recognition independent of TLR4 in primary cells is detectable only upon IFNγ induced TLR2 upregulation (**Figure 9**). But also IFNγ induced increase of LBP, CD14 or CD36 might play a role in TLR2 mediated LPS recognition.

In order to analyze structural requirements that are responsible within both receptors for specific LPS and lipopeptide recognition I exchanged specific LRR fragments between both receptors. Chimeric TLR4 constructs carrying LRRs 7, 8 or 9 were active independent of LPS or lipopeptide challenge (**Figure 19**). However, constitutive activity upon exchange of LRR7-9 displays importance of these LRR fragments in terms of receptor activation. Whether constitutive activity accompanies with persistent TIR-domain phosphorylation or is dependent on MD-2 or serum components remains to be analyzed in the future.

In summary we describe here new aspects in TLR2 and TLR4 function by demonstrating formely unknown overlap in ligand specificity in terms of LPS and a specific tri-myristoylated lipopeptide. We found specificity of both receptors for both agonists and consequently argue that interference with both TLRs is required to inhibit systemic responses upon infection with Gram-positive as well as Gram-negative bacteria.

4.2 Protection from lethal Gram-negative septic shock requires blockade of TLR2 aside of TLR4 in combination with antibiotic therapy

The crucial role of TLRs as triggers of sepsis pathogenesis is well established. Activation of TLRs by specific PAMPs leads to sterile inflammation and in higher doses induces shock-like syndromes with fatal outcome. For instance mice lacking TLR4 fail to respond to high doses of LPS and are resistant to LPS-challenge induced shock. Consequently the reduced immune-responsiveness of mice lacking TLRs leads to higher susceptibility towards infections. Conclusively while TLR signalling induces controlled inflammation to terminate local infections or to fight recently invaded bacteria, systemic activation of innate immunity in case of sepsis development leads to fatal hyper inflammation and finally shock induction. Treatment of septic patients with antibiotics is indispensable to combat ongoing infection by bacterial clearance. However, as a side effect, antibiotic therapy drives hazardous Harisch-Herxheimer reaction. Harisch-Herxheimer reaction is the sudden induction of shock-syndrome upon rapid PAMPs release from bacteria exposed to antibiotics. Accordingly, blockade of pattern recognition during antibiotic therapy might prevent overwhelming inflammation and fatal hyperactivation of the innate immune system.

Several strategies to inhibit bacterial recognition have been put into practice and include application of LBP, antagonistic Lipid A, antagonism of CD14 and blockade of complement system components (Lamping, Dettmer et al. 1998; Schimke, Mathison et al. 1998; Rittirsch, Flierl et al. 2008). However, numerous efforts such as TNFα inhibition failed to establish practical applications (Grau and Maennel 1997). In contrast to target mediators like TNFα or other proinflammatory cytokines interference with pathogen recognition directly might be a more promising strategy to impede dysregulated hyper response to microbial challenge. Thus administration of TLR-specific small molecules or monoclonal antibodies to circumvent the encounter of invading pathogens and the innate immune system provide potential clinically relevant tools.

Accordingly, we analyzed importance of TLR2 and TLR4 as cellular sensors of pathogenic Gram-negative and Gram-positive bacteria and aimed at interference with pattern recognition through both TLRs to potentially inhibit excess of inflammation during sepsis pathogenesis. Therefore we established a clinically orientated mouse model for septic shock. Mice were infected with a lethal dose of bacteria and treated with antibiotics afterwards. The dose of bacteria was adjusted to be high enough to induce lethal septic shock despite of effective antibiotic therapy after infection. In this experimental model we observed that only mice deficient for both TLR2 and TLR4 were resistant to Gram-negative bacteria induced septic shock (**Figure 21A, B**). Mice lacking either TLR2 or TLR4

alone showed an intermediate phenotype but predominately succumbed to *E. coli* and *S. enterica* induced septic shock. However, in terms of Gram-positive bacteria even 5x KO-mice lacking TLR2, 3, 4, 5, 7 and 9 succumbed to *S. pneumoniae* induced shock indicating involvement of further PRR receptor in sensing of Gram-positive bacteria (**Figure 21C, D**). Nevertheless in terms of Gram-negative infection we observed a crucial role of TLR2 and TLR4 in bacteria sensing.

Based on unresponsiveness of $TLR2^{-/-}TLR4^{-/-}$ mice to LPS and also Gram-negative bacteria (**Figure 20**) we focussed on specific blockade of TLR2 and TLR4 under Gram-negative septic conditions. While a cross-reactive (α-mouse, α-human) antagonistic TLR2 monoclonal antibody named T2.5 that protects mice from *Bacillus subtilis* induced shock was already available (Meng, Rutz et al. 2004) we characterized a novel α-mTLR4 mAb named 1A6. 1A6 specifically detected overexpressed as well as endogenous murine TLR4 and co-precipitates MD-2 aside of TLR4 (**Figure 24**). Application of 1A6 *in vitro* and *in vivo* inhibited LPS-induced cytokine release and protected mice from lethal endotoxin shock even if applied 4h after LPS injection. Further pharmacological analyses revealed that 1A6 persists on the surface of macrophages for at least 4h upon *in vitro* administration and that 750µg of 1A6 are required to protect mice from endotoxemia (**Figure 25, 26**). Notably, protective TLR4-antagonism by 1A6 is not due to depletion of $TLR4^{+}$ cells upon 1A6 binding (data not shown).

Consequently we analyzed both antibodies T2.5 and 1A6 for their potential to inhibit responsiveness to Gram-negative bacteria. We observed that administration only of both antibodies was effective and reduced cell activation *in vitro* as well as release of pro- and anti-inflammatory cytokines *in vivo* upon challenge with *E. coli* and *S. enterica* and subsequent antibiotic treatment (**Figure 27, 28**). In line with previous reports demonstrating enhanced susceptibility towards bacterial infection of mice lacking specific TLRs we observed higher systemic bacterial load in mice that received T2.5 together with 1A6 in comparison to mice receiving isotype control (Takeuchi, Hoshino et al. 2000). Increased bacterial burden upon *S. enterica* infection due to TLR2 and TLR4 inhibition correlated with accelerated fatal outcome if mice were not treated with antibiotics subsequently to terminate infection (**Figure 29B**).

In contrast blockade of TLR2/4 function by antibody administration was beneficial in our Gram-negative septic shock model including antibiotic therapy. While wild-type mice infected with Gram-negative bacteria and thereafter treated with isotype control, α-TLR2 mAb or α-TLR4 mAb alone succumbed to septic shock despite of subsequent antibiotic therapy, mice that received T2.5 together with 1A6 survived even if both antibodies and antibiotics were administrated four hours after infection (**Figure 30A, B**). Conclusively, antibiotic therapy concurrently with blockade of both TLR2 and TLR4 provides a therapeutic approach to protect from Gram-negative bacteria induced septic shock. Notably, effective antibiotic therapy is indispensable aside of TLR2/4-blockade to compensate dampening of

the antimicrobial immune response. Conclusively, combination of immunosuppressive TLR-blockade with antimicrobial therapy unifies inhibition of fatal cytokine-storm on the one side and termination of bacterial infection on the other side.

Based on the finding that administration of α-TLR4 mAb alone prior to infection is protective (**Figure 30 C**) but contrariwise application of antibodies after infection requires additional TLR2-blockade (**Figure 30A, B**) aside of antibiotic therapy we asked for a potential TLR2 trigger function of TLR4. In order to evaluate this hypothesis we analyzed cytokine release upon sequential and alternate TLR2-TLR4 challenge. We observed significant increase of TNFα-production upon TLR2 activation in mice previously challenged with low doses of LPS 3h before re-challenge (**Figure 31**). As shown by consecutive TLR2-TLR2 or TLR4-TLR4 stimulation we did not induce tolerance within a time window of 3h between injection of TLR2-ligand or TLR4-ligand. Instead of induction of cross-tolerance we observed an increased TNFα-release in mice that received TLR4-TLR2 agonists sequentially compared to mice challenged in reversed order or mice without LPS pre-injection indicating a TLR4-mediated enhanced sensitivity for lipopeptides via TLR2. Notably, TNFα was no more detectable in the serum drawn 3h upon single lipopeptide or LPS injection of mice.

Our reports demonstrated that IFNγ increases cell sensitivity towards several TLR-ligands. We observed that mice lacking TLR4 were unresponsive to infection with Gram-negative bacteria in respect to IFNγ-release and found that TLR4-induced IFNγ-release depends on MyD88 but not on the adaptor TRIF (**Figure 32A, B**). IFNγ release upon Gram-negative infection was detected in wild-type mice already 2h after infection.

As source of early IFNγ-release we identified NK as well as NKT-cells, which produced IFNγ already 3h after infection with *S. enterica* in a TLR4-dependent manner. Notably human PBMCs as well produced IFNγ TLR4-dependently (**Figure 32C**). Antibody-mediated blockade of hTLR4 diminished release of IFNγ by human PBMCs upon challenge with LPS but not upon challenge with lipopeptide (data not shown). Along this line we demonstrated that murine macrophages as well as human PBMCs primed with IFNγ for 3h were more sensitive towards lipopeptide challenge as indicated by production of higher amounts of TNFα in comparison to respective non-primed cells (**Figure 33A**).

Enhanced sensitivity towards lipopeptides induced by IFNγ priming correlated with increased surface expression of TLR2 on $CD11b^{+}$ cells. 3h upon infecting mice with *S. enterica* TLR2 surface expression on monocytes isolated from wild-type mice was increased (**Figure 33B**). TLR2 surface expression was not altered by infection on cells derived from mice lacking TLR4 or IFNγR. We thus provided striking evidence for a cross-talk between TLR4 and TLR2 during Gram-negative infection. Notably, LPS-induced upregulation of TLR2 mRNA as well as TLR2 upregulation on human

leukocytes have been already published and are in line with my data (Wittebole, Coyle et al. 2005; Maris, Dessing et al. 2006). Conclusively TLR4 activation induced IFNγ-release by NK and NKT-cells that mediated upregulation of TLR2 on monocytes (**Figure 34**).

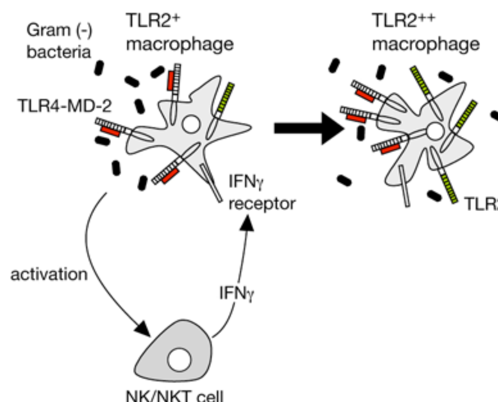


FIG.34: TLR4-TLR2 cross-talk upon Gram-negative bacterial challenge
Upon Gram-negative (-) bacterial infection bacteria were sensed through TLR4, which led to rapid activation of NK- and NKT cells to produce IFNγ. IFNγ increased macrophage surface TLR2 expression and TLR2 ligand sensitivity, which correlated with lack of substantial TLR2 activity contributing to toxemia in case of TLR4 blockade prior to Gram-negative bacterial infection.

By demonstrating protective effects of antagonizing TLR2 and TLR4 in combination with antibiotic therapy we provide a therapeutic approach to treat Gram-negative bacteria induced severe septic shock. In addition the identification of a mechanism including TLR4 mediated TLR2 upregulation underlines the necessity of additional TLR2-blockade aside of TLR4-blockade. Notably, upon observing that administration of mAbs together with antibiotics was still protective 4h upon infection (**Figure 30B**) we conclude that TLR activation must not be initiated by only one TLR-PAMP contact. Blockade of TLR2 and TLR4 might be effective even when infection pathology is advanced. In contrast to our model in which extremely high doses of bacteria are injected once into mice development of infection and accumulation of a high bacterial load in patients presumably occurs over a substantially longer period of time; thus, a time window wider than 4 h might be appropriate for antibody-mediated TLR-blockade during antibiotic therapy of sepsis patients. Although we observed resistance of mice that received 1A6 5h prior to otherwise lethal LPS injection (**Figure 26B**) it remains to be shown how long antibody-mediated inhibition of TLR-functionality persists *in vivo* during infection. Furthermore, one has to address whether TLR2/TLR4-blockade influences later immune-paralysis that is often fatal for sepsis patients by promoting bacterial proliferation during the advanced phase of sepsis pathogenesis. Accordingly, inhibition of anti-inflammatory IL-10 release by TLR2/TLR4-blockade (**Figure 28**) is a promising observation indicating potential ablation of later immune-paralysis. Whether TLR2/TLR4-blockade diminishes secretion of the late-induced sepsis relevant alarming protein high-mobility-group box 1 (HMGB1) should also be analyzed in the future.

Additionally it has to be analyzed whether combined TLR2/TLR4-blockade is effective in terms of other Gram-negative bacterial species aside of *E. coli* and *S. enterica*. However, both *E. coli* and *S. enterica* differ in terms of occurrence, habit and pathogenesis. Whereas Salmonella are versatile highly pathogenic bacteria, which use a type-III secretion system to enter host cells and are absorbed mostly orally in relatively low numbers, *E. coli* as a low-pathogenic component of the gut flora infiltrates the host mostly via the urinary tract or upon gut lesions in higher amounts (Haraga, Ohlson et al. 2008). However, *E. coli* displays the most frequent sepsis bacteria aside of the Gram-positive *S. aureus* (http://www.uniklinik-ulm.de/struktur/institute/medizinische-mikrobiologie-und-hygiene). Although TLR2/TLR4-blockade effectively reduces septic shock syndromes upon infections with both bacteria species, it has to be shown whether provided therapeutic approach protects from otherwise lethal shock induced by other species of sepsis relevant Gram-negative bacteria such as Klebsiella or Pseudomonas.

Aside of Gram-negative infections application of α-TLR2 and α-TLR4 mAb might also mediate diminished hyperinflammation upon challenge with Gram-positive bacteria, which carry ligands for TLR2 and also TLR4. Although mice lacking TLR2 and TLR4 subjected to antibiotic therapy were not resistant to lethal doses of *S. pneumoniae* (**Figure 21C**) inhibition of both receptors might partially reduce Gram-positive bacteria induced proinflammatory processes and might be beneficial during specific phases of Gram-positive bacteria induced sepsis pathogenesis.

In addition TLR2/TLR4-blockade might also be relevant in polymicrobial sepsis for the reasons mentioned above and might be combined with blockade of further TLRs or other PRRs. TLR9-blockade for instance was recently demonstrated to be beneficial during polymicrobial sepsis. However, effect of TLR9-blockade was not inhibition of cytokine storm but was reduced apoptosis of peritoneal granulocytes resulting in improved bacterial clearance and increased survival (Plitas, Burt et al. 2008). Also TLR2 and TLR4 activation have been reported to induce apoptosis (Oliveira, Ochoa et al. 2003). Whether application of T2.5 or 1A6 influences sepsis-relevant apoptosis has to be analyzed in the future. Notably, I did not observe reduced numbers of $CD11b^+$ cells in the spleen 4 hours upon administration of T2.5 together with 1A6 (data not shown). But combination of TLR9-blockade and subsequent TLR2/TLR4-blockade with antibiotic therapy might improve conditions of patients suffering sepsis and might provide a clinical approach to treat polymicrobial infections. Antagonism of TLR9 and induced inhibition of apoptosis might conserve numbers of phagocytes thus supporting bacterial clearance during early sepsis pathogenesis without antibiotic treatment. If adopted TLR9 inhibition fails to terminate infection antibiotic therapy paralleled by TLR2/TLR4-blockade might follow as a measure to inhibit development of septic shock.

Aside of TLR2, TLR4 and TLR9, immune-factors such as several complement proteins or the late-induced cytokine HMGB1 have been described to be involved in mediation of sepsis pathogenesis and provide potential targets for intervention. Also IFNγ or IFNγR antagonists have been described to beneficially interfere with inflammatory processes (Yin, Gribbin et al. 2005). Although we identified IFNγ as a mediator of TLR4-TLR2 cross-talk and illustrated its striking role in our model of Gram-negative septic shock, IFNγ is a central mediator of NO-production and also promotes other proinflammatory mechanisms aside of activating T-bet to drive Th1 responses. Finally, a suitable combination of antagonists towards several receptors, cytokines or factors paralleled by antibacterial and anti-coagulating treatment might provide most effective protection of the host in microbial-induced hyper-inflammation.

4.3 TLR interference: Not only relevant in sepsis

Although TLRs were found as crucial mediators of inflammation several reports during the last years arise and expanded the role of TLRs within immunity. Predominantly upon analyses of specific TLR knockout mice and analyses of TLR-polymorphisms involvement of TLRs in apoptosis induction, tumour suppression, allergic disease and arthrosclerosis have been postulated. Consequently, TLR agonists and antagonists may not only provide a useful tool for experimental research or clinical agent in the field of inflammation and infection but interference with TLR function also displays a potential strategy to analyse or treat cancer, allergy, auto-immunity or cardiovascular diseases.

In terms of chronic inflammation occurring for example in bowel disease TLRs have been described to influence the composition of the commensal microbial flora in the gut. TLR2 has been shown to mediate specific a specific polysaccharide (PSA) secreted from the commensal bacteria *Bacteroides fragilis*. PSA has been shown to activate TLR2 but finally mediates downregulation of inflammatory processes in the intestine and protects from bowel disease (Wang, McLoughlin et al. 2006). Activation of TLR2 by PSA was shown to induce TNFα but also upregulates MHCII and thus promotes in turn activation of Th1 orientated $CD4^{+}CD45Rb^{low}$ cells, which constitutively produce high amounts of anti-inflammatory IL-10 (Mazmanian, Round et al. 2008). Consequently, TLR2 activation links innate and adaptive immunity and might be participated in modulation of gut homeostasis. Specifically TLR2 agonists might induce increase in IL-10 production to inhibit inflammatory processes during bowel disease or other inflammatory disorders.

TLR2 as well as TLR4 have also been described to polarize T cell differentiation in the respiratory tract either toward the Th1 type or the pro-allergenic Th2 type (Re and Strominger 2001; Redecke, Hacker et al. 2004). Although previous reports rather dispute above-mentioned publications since they implicate TLR2 and TLR4 in Th1 polarization repressing susceptibility for allergic airway disease and suggests activation rather than neutralization of both receptors inhibit development of allergies. For instance, lipopeptide administration had been reported to impair airway inflammation in mice suffering acute OVA-induced asthma (Patel, Xu et al. 2005).

Furthermore analyses of TLR deficient mice implicate a striking role of TLR2 in cardiovascular diseases. For instance, TLR2 was described to regulate ventricular remodelling after myocardial infarction resulting in longer survival of TLR2-deficient mice compared to wildtype mice after induced myocardial infarction (Shishido, Nozaki et al. 2003). In addition, absence of TLR2 was found to significantly reduce atherosclerosis in atherosclerosis-susceptible LdlR-null mice whereas injection of TLR2 agonists drives progression of atherosclerosis. Within that report it was suggested that chronic bacterial infections but also stress-mediated endogenous TLR2 ligands derived from the host contribute to atherosclerosis development (Mullick, Tobias et al. 2005).

Another striking observation implicated TLR4 in elimination of tumour cells. TLR4 was described to mediate activation of tumour-specific T-cells upon recognition of HMGB1, which is released from dying tumour cells subjected to radiotherapy or chemotherapy (Apetoh, Ghiringhelli et al. 2007). Activation of TLR4 by an endogenous ligand, serving as an adjuvant promotes a tumour-specific DC-mediated T-lymphoycte (CTL) response.

By means of illustrated examples it is shown that TLRs fulfil various important functions aside of mere pathogen recognition. Therefore antibodies or substances that specifically interfere with TLR-function might be of great value for a broad field of application potentially in the clinic to improve human health in the future.

5 SUMMARY

Toll-like receptors (TLR) are a well-studied family of pattern recognition receptors, which recognize broad classes of molecular structures common to groups of microorganisms. TLR-activation initially induces proinflammatory processes and leads to production of a wide spectrum of immune stimulatory factors and therefore induces not only innate but also adaptive immunity. Lipopolysaccharide (LPS), a cell wall component from Gram-negative bacteria, is described as the agonist for TLR4 whereas lipopeptides from Gram-positive bacteria are known to activate TLR2. We identified a specific lipopeptide (Myr_3CSK_4) carrying three myristoylations at the N-terminal cysteine as compared to classical tri-palmitoylated lipopeptide (Pam_3CSK_4) as a TLR agonist, activating not only TLR2, but also TLR4. Furthermore we found that overexpressed TLR2 is responsive to highly purified rough enterobacterial LPS and that macrophages lacking TLR4 sense LPS in a TLR2-dependent fashion. Taken together, upon analyses *in vitro, ex vivo* and *in vivo* we found an overlap in pattern recognition for both of the two TLRs for both of the two agonists (LPS and Myr_3CSK_4). Consequently, we observed that recognition of Gram-negative bacteria depended on both, TLR4 and TLR2. Along this line we established a clinical orientated septic shock model and infected mice with a dose of *E. coli* or *S. enterica*, which was lethal despite of effective subsequent antibiotic treatment. In order to protect mice from fatal cytokine storm, antagonistic monoclonal antibodies towards TLR2 and TLR4 were applied prior to infection or after infection. Only application of both antibodies synchronously, prevented lethal septic shock and was protective even if applied 4h after infection in combination with antibiotic therapy. Surprisingly we observed that application of α-TLR4 mAb alone, prior to infection, was sufficient to protect mice from fatal septic shock contrary to the finding that its application after infection was not. So we assumed that TLR4 functionally triggers TLR2 and identified IFNγ as a TLR4-dependent inducer of TLR2-upregulation and enhancer of its responsiveness. Collectively, here we report on timely graduated TLR-activation and offer a potentially clinically relevant therapeutic approach to inhibit Gram-negative bacteria induced hyper-inflammation.

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