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Cellular recognition of microbial patterns through toll-like receptor (TLR) 2: Analysis of molecular requirements and monoclonal antibody mediated blockage

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App. II Meng G, Rutz M, Schiemann M, Metzger J, Grabiec A, Schwandner R, Luppa PB, Ebel F,Busch DH, Bauer S, Wagner H, Kirschning CJ. Antagonistic antibody prevents Toll-like receptor 2 driven lethal shock-like syndromes. J Clin Invest. 2004 May 15; 113:1473-81.

Abbrevation list

A

AA	Amino acid
Ab	Antibody
Ag	Antigen
AP-1	Activating protein-1
APC	Antigen presenting cell
APC	Allophycocyanin
APS	Ammonium peroxidedi sulfate
ARDS	Adult respiratory distress syndrome

B

β-gal	β-galactosidase
BH	Brain heart
β–ME	β–Mercaptoethanol
BSA	Bovine serum albumin

C

C5αR	C5α Receptor
CD	Cluster of differentiation
CHO	Chinese hamster ovary
CLP	Cecal Ligation and Puncture
CMV	Cytomegalovirus
CREB	cAMP response element binding (protein)

D

DC	Dendritic cell
D-gal	D-galactosamine
DID	Death inducing domain
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Desoxynucleotidetriphosphate
DTT	Dithiothreitol

E

EB	Ethdium bromide

ECD	Extracellular domain
ECL	Enhanced chemoluminescence
E. coli	*Escherichia coli*
EDTA	Ethylendiamintetraacetate
EGTA	Ethylenglycoltetraacetate
ELAM-1	Endothel cell-leucocyte-adhesion molecule-1
ELISA	Enzyme linked immunosorbent assay
EMA	Photoactivated ethidium monozide
EMSA	Electro mobility shift assay
ER	Endoplasmic reticulum
ERK1/2	Extracellular-signal-regulated kinase 1/2
EtOH	Ethanol

F

FACS	Fluorescence activated cell sorting
FADD	Fas associated death domain
FC	Flow cell
FCS	Fetal calf serum
FITC	Fluorescein Isothiocyanate

G

GBS	Grop B Strptococci
GIPL	Glycoinositol phospholipid
GPI	Glycosyl-phosphatidyl-inositol (anchor)

H

H	Hour
HAT	Hypoxanthine, aminopterin, thymidine (hybridoma selection supplement)
HBS	Hepes buffered saline
HEK 293	Human embryonic kidney 293
HFCS	Hybridoma fusion and cloning supplement
HGPRT	Hypoxanthine-guanine phospho-ribosy I transferase
h.i.B.s.	heat inactivated *Bacillus subtilis*
h.i.E.c.	heat inactivated *Escherichia coli*
HMGB1	High Mobility Group Box 1 (protein)
HRP	Horse radish peroxidase
HSP60	Heat shock protein 60

I

ICU	Intensive Care Unit

ICD	Intracellular domain
IFN-β	Interferon-β
I-κB	Inhibitor of NF-κB
IKKβ	I-κB kinase β
IL	Interleukin
IL-1RI	IL-1 receptor I
Imd	Immune deficiency
IP	Immunoprecipitation
i.p.	intra-peritoneal (injection)
IRAK	IL-1-receptor-associated kinase
IRF3	Interferon regulatory factor 3

J

JNK	c-Jun NH_2-terminal kinase

K

kDa	Kilodalton

L

LAM	Lipoarabinomannan
LB	Luria Bertani
LBP	LPS binding protein
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
LRRCT	LRR C-terminal
LTA	Lipoteichoic acid
LSM	Laser scanning microscope

M

M	mol/l
MAb	Monoclonal antibody
Mal (TIRAP)	MyD88 adaptor-like
MALP-2	Macrophage-activating lipopeptide, 2 kDa
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MEF	Mouse embryonic fibroblast
MIF	Macrophage migration Inhibitory Factor
Min	Minute
MKK	MAP kinase kinase
MOF	Multiple organ failure

Mut	Mutant
MyD88	Myeloid differentiation marker 88
M/SAPK	Mitogen/stress activated protein kinase

N

Na Ac	Sodium acetate
NF-κB	Nuclear factor κB
NGS	Normal goat serum
NO	Nitric Oxide
NP40	Nonidet P-40

O

OD	Optical density
Omp (Osp)	Outer membrane protein
o.n.	Over night
Osp (Omp)	Outer surface protein

P

p	Plasmid
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen-activator inhibitor type-1
PAMP	Pathogen associated molecular pattern
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood monocyte
PBS	Phosphate buffered saline
PBT	Phosphate buffered Tween 20
PCR	Polymerase chain reaction
P_3CSK_4	tripalmitoylcysteinyl-seryl-(lysyl)3- lysine
P_2CSK_4	bispalmitoylcysteinyl-seryl-(lysyl)3- lysine
$PCSK_4$	palmitoylcysteinyl-seryl-(lysyl)3- lysine
$PHCSK_4$	N-palmitoyl-S-(1,2-bishexadecyloxy-carbonyl-ethyl-(R)-cysteinyl-seryl-(lysyl)3-lysine
PE	Phycoerythrin
PEG	Polyethylene glycol
PFA	Paraformaldehyde
PGRP	Peptidoglycan recognition protein
PI3K	Phosphatidyl-inositol-3-kinase
PMA	phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear (leukocyte)
PMSF	Phenylmethylsulfonylfluoride

PRR	Pattern recognition receptor

R

RAGE	Receptor for advanced glycation end product
RI	Ribonuclease inhibitor
RNA	Ribonucleic acid
rpm	Rounds per minute
RSC786	Randomly sequenced cDNA 786
RSV	Rous-Sarkoma-Virus
RT	Room temperature
RU	Resonance unit

S

s. B.b.	sonicated *Borrelia burgdorferi*
SDS	Sodium dodecyl sulfate
Sec	Second
SMART	Simple modular architecture research tool
SP-1	Stimulating factor 1
sPGN	Soluble peptidoglycan (from *Staphylococcus aureus)*
SPR	Surface plasmon resonance
STAT	Signal transducer and activator of transcription

T

TAE	Tris-acetate-EDTA
TAK1	TGF-β-activated kinase 1
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TEMED	N' N' N' N'-Tetramethylethylendiamine
TGF-β	Transforming growth factor β
Th	T-helper cell
TICAM1 (TRIF)	TIR containing adaptor molecule 1
TIL	Toll/interleukin-1 receptor like
TIR	Toll/IL-1-receptor
TIRAP (Mal)	TIR domain containing adapter protein
TIRP (TRAM)	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TMB	3' 3' 5' 5'-Tetramethylbenzidine
TNF	Tumor necrosis factor
TRAF6	TNF-receptor-associated factor 6
TRAM	TRIF related adaptor molecule

TRIF (TICAM1)	TIR domain containing adaptor inducing IFN-β
Tris	Tris-(Hydroxylmethyl-) Aminomethan

U

U	Units

V

v/v	Volume per volume

W

Wt	Wild-type
w/v	Weight per volume

Y

YPD	Yeast peptone dextrose

1 Introduction

The members of the Toll-like receptor (TLR) family are thought to recognize conserved microbial structures and initiate an eventually fatal signaling cascade resulting in life threatening septic shock. The initial identification of TLRs based on their homologies to *Drosophila* Toll that is a trans-membrane receptor involved in both embryonic development and host defense towards microbial challenge. Type I receptor Toll-like receptor 2 is a member of the TLR family that has been studied in substantial detail over the recent years. TLR2 carries an ectodomain containing 20 leucine rich repeat (LRR) and LRR like motifs, a transmembrane domain and a cytodomain sharing high homology with that of Toll and IL-1 receptor named Toll/IL-1 receptor/plant Resistance protein (TIR) domain. TLR2 has been proven to be directly involved in recognition of various pathogen associated molecular patterns (PAMPs) representing species such as Gram-positive and Gram-negative bacteria, mycobacteria, spirochetes, and mycoplasm. Host material such as heat shock protein (HSP) 60 has been demonstrated to activate cells through TLR2 as well. This receptor has been proven to fulfill its pattern recognition function in association with other molecules such as CD14, MD2, TLR1, and TLR6. Thus, TLR2 is a sensor of PAMPs and mediates specific defense processes. Detailed understanding of TLR2 biology will most likely contribute to further development of therapeutic immune modulation.

1.1 Septic shock and Innate immunity

Shock is a clinical condition of patients whose underlying causes and outcomes vary widely. A substantial number of patients develop septic shock, the most lethal of the infection-triggered physiological disturbances. Mortality rate of septic shock is 30 to 50%, and it is the 13th leading reversible cause of death in the United States. This syndrome affects approximately 700,000 people annually and accounts for about 210,000 deaths per year in the US. According to recent reports, the incidence is rising at rates between 1.5% and 8% per year, despite technical developments in intensive care units (ICUs) and advanced supportive treatment. As a consequence large societal and financial costs are associated with septic shock (http://www.septicshock.org/research/septic_shock.htm).

Progressive multiple-organ failure (MOF) rather than the primary infection causes lethality during shock pathogenesis. In the early phase of a localized infection, release of endotoxins or exotoxins by bacterial cells induces release of inflammatory cytokines from immune cells. Although these early response cytokines play an important role in host defense such as by attracting activated neutrophils to the site of infection, the entry of these cytokines and bacterial products into the systemic circulation triggers widespread microvascular injury (Fig. 1.1) (1).

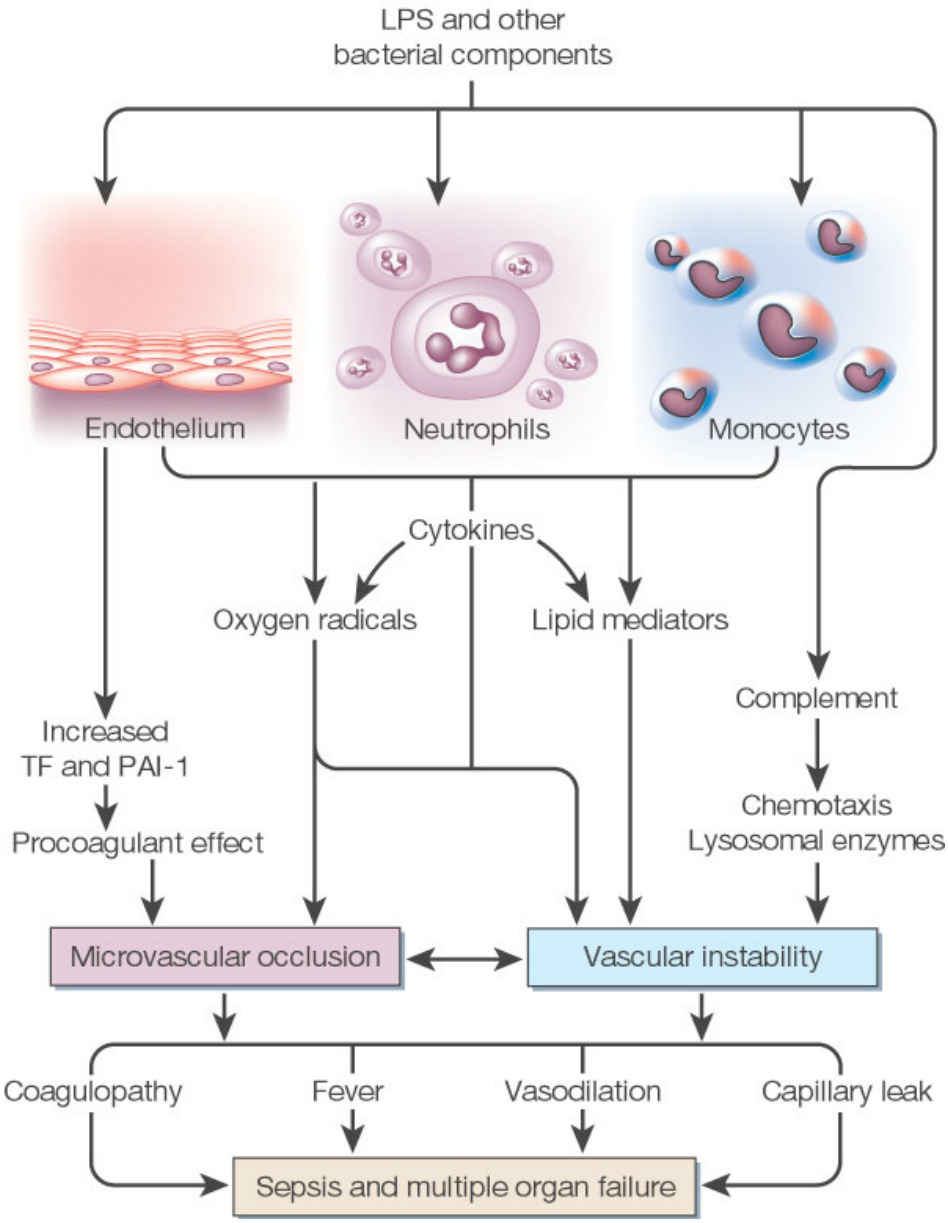

Fig. 1.1 **Pathogenetic networks in shock.** Lipopolysaccharide (LPS) and other microbial components simultaneously activate multiple parallel cascades that contribute to the pathophysiology of adult respiratory distress syndrome (ARDS) and shock. The combination of poor myocardial contractility, impaired peripheral vascular tone and microvascular occlusion leads to tissue hypoperfusion and inadequate oxygenation, and thus to organ failure.

Nature 2002 Dec 19-26; 420 (6917): 885-91. The immunopathogenesis of sepsis. Cohen J.

The immune system defends the host against infection. Traditionally, the immune system has been divided into innate and adaptive components, each of which have different roles in defending the host against infectious agents. The innate immune response is a preprogrammed first line of defense that is primarily responsible for elimination of pathogens at the site of entrance into the host. Therefore, innate immunity provides a first line of defense, but its ability to specifically recognize pathogens and to provide specific protective immunity is somewhat limited. Innate pathogen recognition is mediated through germ-line encoded receptors that bind to classes of conserved molecules produced by infectious organisms. Primary effector cells of innate immune response are macrophages, neutrophils, and dendritic cells. Effective long-term defense, however, requires development of an adaptive immune response mediated by B cells and T cells. Innate immune cells instruct the adaptive immune response. For instance, dendritic cells and macrophages ingest and degrade microbes and present microbe-derived antigens to T cells in the lymph nodes, which induces clonal expansion of antigen-specific T cells. T cell-derived cytokines and chemokines in turn regulate B cell proliferation and antibody production, as well as the activity of cells of the innate immune system. These communication loops facilitate a flexible and robust immune response. In the early phases of infection, inflammatory responses arise solely from the innate immune system; later during infection, cooperative effects of both the innate and adaptive arms of the immune system causes coordinated inflammatory responses (2).

Innate immunity protects the body from infection by microbes, including those found among the microbial flora that normally inhabit the surfaces of human skin and mucosae. Failure of innate immune mechanisms renders the body unable to contain commensal microbes when they invade, often through a break in an epithelial barrier, and allows them to multiply within tissues. The local inflammatory response intensifies, and, for poorly understood reasons, severe sepsis/shock oftenly ensues (http://www.septicshock.org/research/septic_shock.htm).

In 1989, Janeway pointed out that the “non-specific” immune defenses of the host are “hard-wired” in the genome, rapidly mobilized, and able to recognize microbes that express conserved molecular patterns (3). Some of the elements of this innate immunity are pre-formed and ready to act without modification (natural antibodies, the alternative complement system). Others (phagocytes) require activation by non-self signals; sentinel host molecules (pattern receptors) recognize conserved microbial molecular patterns and trigger signal-transducing pathways within host cells. When stimulated in this way, tissue macrophages activate their own antimicrobial killing mechanisms and release mediators that increase local blood flow, attract neutrophils to the site of infection, and provoke fluid extravasation. In

other words, the sensory or recognition arm of innate immunity is tightly coupled to the antimicrobial effector functions provided by local inflammation.

Our understanding of essential features of the innate immune system has increased dramatically during the past decade, beginning with the discovery of the LPS binding protein (LBP)/CD14 pathway and continuing now to the recent findings for the importance of the Toll-like receptor (TLR) family of proteins. TLR-mediated signaling is known to activate the transcription factor nuclear factor (NF) κB and to upregulate tumor necrosis factor (TNF) expression. The accepted paradigm is that activation of innate immunity occurs when products of infectious organisms bind to specific plasma membrane receptors on host cells. This response is characterized by the synthesis and release of multiple pro- and anti-inflammatory mediators such as lipid mediators, cell surface proteins, and a myriad of cytokines and other bioactive proteins. At issue are the signaling pathways that elicit production of these mediators, the temporal sequence of mediator production, the specific actions of mediators throughout the body, and the counter-regulatory responses that they set in motion (4, 5).

In the past, basic and clinical researches have focused on Gram-negative (G-) sepsis, mainly through studies using LPS. It is generally acknowledged that LPS plays an important role in septic shock, and a large body of important data have emerged from studies of the effects of LPS on the innate immune system. However, a broader approach is required for studying inflammatory reactions to invading bacteria. One reason for this contention is found in investigations that implicate Gram-positive (G+) bacteria in septic shock (6). Until recently, little was known about the cellular mechanisms involved in recognition and responsiveness to G+ bacteria. Now it is clear that membrane constituents from G+ bacteria use a Toll-like receptor distinct from that used by the LPS; i.e. TLR2 rather than TLR4. The distinct and overlapping features of signaling via the individual TLRs have not yet been fully elucidated.

1.2 *Drosophila* Toll receptor and Mammalian TLRs

The innate immune system has developed a series of phylogenetically conserved receptors, termed pattern recognition receptors (PRRs) to impede the entrance of infectious microorganisms. These PRRs have the ability to recognize specific PAMPs such as LPS of Gram-negative organisms, the lipoteichoic acids of Gram-positive organisms, and the glycolipids of mycobacteria. Recognition of PAMPs by PRRs results in the activation of different intracellular cascades leading to the expression of effector molecules such as cytokines, chemokines, and adhesion molecules that are involved in inflammation. Specific members of the innate immune system include PRRs such as CD14, and the newly described

TLRs (7). Given that CD14 lacks a transmembrane domain, the mechanism by which LPS binding to CD14 induces cell activation leading to proinflammatory cytokine production had remained an enigma. One of the major advances in the understanding of early events in microbial recognition and subsequent development of sepsis has been the identification of TLRs, the human homologs of *Drosophila* Toll (2).

1.2.1 *Drosophila* Toll

The first mutants in the *Drosophila* Toll signaling pathway were discovered in the genetic screens performed by Nuesslein-Volhard and Wieschaus, which led to identification of this membrane bound Toll receptor (8). While searching for zygotic lethal mutations that affected embryonic patterning, Wieschaus discovered a line in which none of the embryos laid by heterozygous femals hatched. When Wieschaus showed the cuticle pattern of the unhatched embryos to Nuesslein-Volhard, she exclaimed, "Toll!" (a German slang term comparable to "crazy" or "far-out"), and the gene became known by that name (9).

An intracellular signaling pathway from Toll ligand Spaetzle to the transcription factor and Rel protein family member Dorsal, as well as further signaling pathways were identified and elucidated to a large extent by genetic screens in *Drosophila* (8, 10). Involvement of Toll signaling components in antifungal response of the adult fruitfly, as well as of one out of eight *Drosophila* Toll homologs, 18 wheeler, in antibacterial response has been demonstrated subsequently (7).

The induction of genes for the antimicrobial peptides has provided a good starting point from which to study the mechanisms of the immune response in *Drosophila*. At least two signaling pathways are involved, which were originally defined genetically by mutants in the Toll and imd genes. Recent genetic and molecular work has led to a detailed characterization of these pathways (Fig. 1.2), both of which involve members of the Rel family of transcription factors, which belong to the Rel-protein family such as human transcription factor NF-κB (11).

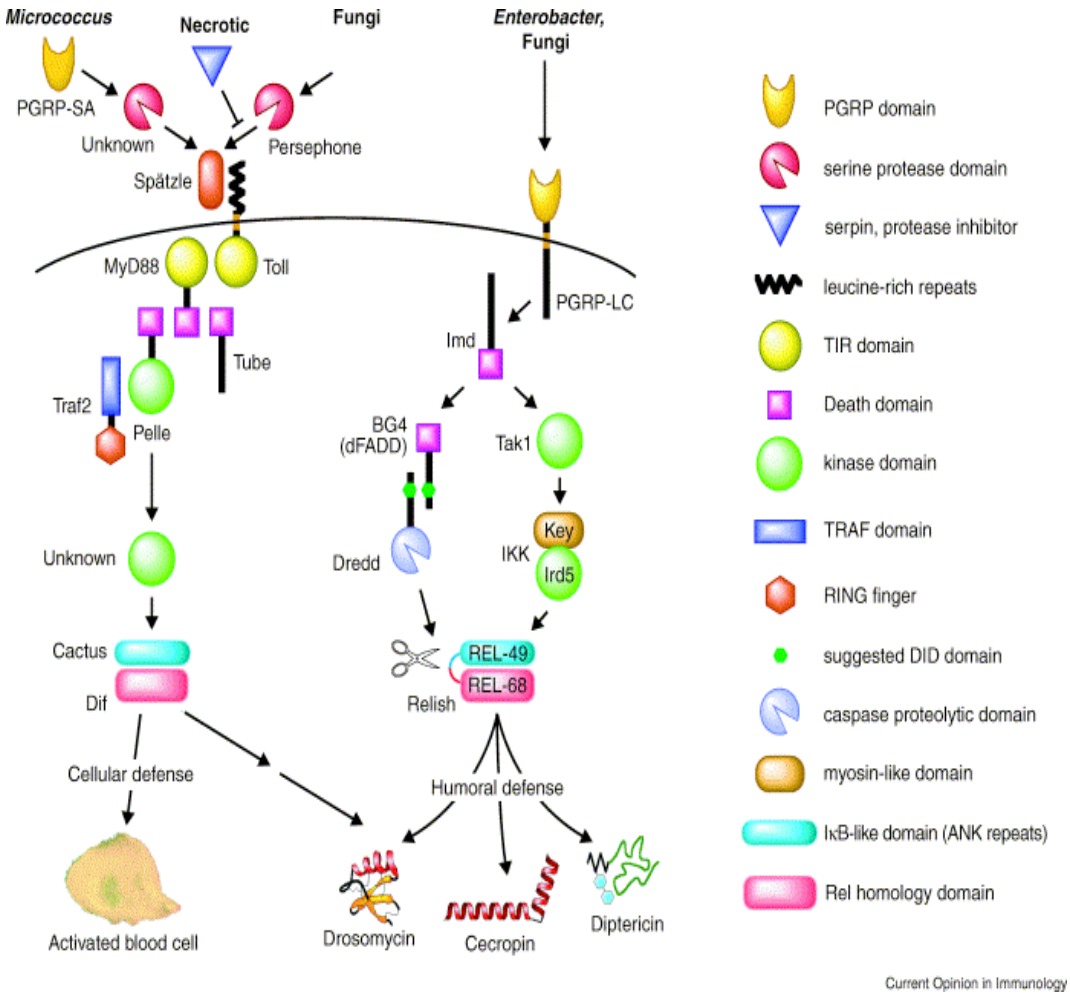

Fig. 1.2 Two different NF-κB-like signaling pathways mediate cellular and humoral immune reactions in *Drosophila*. The imd/Relish pathway is probably complex, with two parallel branches. Several variants of this scheme are compatible with published studies. Through genetic techniques, most of the indicated factors have been shown to be required *in vivo* and their relative positions in the pathways have been determined. But the actual physical interactions between the factors are only partially known. The serine proteases that activate Spaetzle in response to stimulation with Micrococcus are not known, nor is the kinase that targets Cactus for degradation. ANK, ankyrin; DID, death-inducing domain; FADD, Fas-associated death domain protein; TIR, Toll/IL-1 receptor homology; TRAF, TNF receptor-associated factor.

Curr Opin Immunol. 2003 15(1):12-9. Drosophila immunity: paths and patterns. Hultmark D.

In one pathway, signaling from the membrane Toll receptor activates two Rel factors, Dif and Dorsal. At the resting stage, Dif and Dorsal form inactive complexes with Cactus, a *Drosophila* member of the IκB family of NF-κB inhibitors. Toll signaling results in the phosphorylation of Cactus, followed by the ubiquitination and proteasome-dependent degradation of this inhibitor. Dif and Dorsal then translocate to the nucleus where they participate in the transcriptional activation of target genes. Specific gene products mediate the signaling between Toll and Dif (Fig. 1.2), and most of them are homologues to factors in the human IL-1 and TLR pathways. No mammalian homolog of Tube has been identified, but the other genes all have counterparts in human IL-1 receptor signaling. The kinase Pelle corresponds to human IL-1 receptor associated kinase (IRAK); *Drosophila* Traf2 to human TNFR associated factor (TRAF) 6; and myeloid differentiation marker (MyD) 88 to the human homolog with the same name. The crucial phosphorylation of Cactus is carried out by a kinase that remains unknown.

A second pathway leads to the activation of the Rel factor Relish. Within seconds after an infection, Relish is cleaved into two parts. The amino (N)-terminal REL-68 fragment, which contains the DNA-binding Rel homology domain, translocates to the nucleus where it binds to κB-like enhancer elements in the promoters of antimicrobial genes. The other fragment, REL-49, is IκB-like and remains in the cytoplasm, surprisingly without inhibiting the activity of REL-68. Activation of Relish also requires the *Drosophila* homolog of inhibitor of NF-κB kinase (IKK), for which Relish is a substrate. Activation is blocked in mutations in the ird5 or kenny (key) genes, which encode kinase and accessory subunits of the enzyme, respectively. The catalytically active ird5 gene product is closely related to both the α and β subunits of human IKK. The key gene encodes a protein that is distantly related to the γ subunit of human IKK (NEMO), although the two proteins are probably not orthologous. Mutant analysis has also identified a second kinase, Tak1 (transforming growth factor activated kinase 1), which is required for activation of Relish. Tak1 acts upstream of ird5 and key, and downstream of imd. The human homolog, TAK1, can mediate the phosphorylation and activation of IKK, possibly as part of an alternative pathway for NF-κB activation (11).

1.2.2 Mammalian Toll-like receptors

Application of the sequences of the intracellular domain of the interleukin (IL)-1 receptor (R)-I and *Drosophila* Toll in database analyses resulted in identification of human Toll-like receptors. Randomly sequenced cDNA (RSC) 786/TIL/TLR1 and human Toll/TLR4 were the first two of currently ten human TLRs to be identified (12, 13). Induction of nuclear factor (NF)-κB through the TLR4 TIR domain proved conservation of the Toll signaling pathway in TLR function. TLR4-mediated signaling activated genes encoding proteins with roles in inflammatory processes such as IL-6 and costimulatory molecule B7.1. The abundance of TLR4 mRNA in the spleen and in peripheral blood lymphocytes (PBL) further implicated a role of TLR4 in immunity. Primary sequences, expression patterns, genomic localization, and functional data were presented for the first five human TLRs (14, 15). Additional human TLRs were discovered as well, to date, 10 TLRs have been extensively characterized (Fig. 1.3) (16). Of those, solely TLR10 remains an orphan receptor. The other TLRs mediate recognition of exogenous agonists through binding, which is in contrast to *Drosophila* Toll and IL-1 receptor both binding endogenous ligands. The pattern recognition receptor thus is positioned upstream of Toll while TLRs are pattern recognition receptors by themselves.

Genomic analysis of two resistant mouse strains that are both hyporesponsive to LPS identified TLR4 as the *lps* (LPS gene) product (17, 18). In the C3H/HeJ mouse, a single missense mutation within the TLR4 coding sequence was identified. C3H/HeJ mice are homozygous for a mutation that substitutes histidine for proline at position 712. In contrast, C57BL/10ScCr strain mice were found to lack the Toll like receptor 4 gene. Implication of TLR4 as the LPS receptor has been continued by targeted disruption of the TLR4 gene which renders mice resistant to LPS as well (19). These studies provided a direct link between TLR4 and physiologic responses to LPS. They further provided critical information about LPS binding to CD14 and transmembrane signaling via TLR4. Although TLR2 was initially linked to LPS signaling, genetic evidence now supports that TLR4 is the primary signal transducer for LPS. Studies in TLR2- deficient mice have confirmed the minimal role of this receptor in LPS signaling, as these mice are susceptible to the toxic effect of LPS. However, studies indicated that TLR2, but not TLR4, is a major receptor for Gram-positive bacteria lacking LPS (*ie*, *Staphylococcus aureus* and *Streptococcus pneumoniae*) and their cell wall components, as well as for bacterial lipoproteins produced by both Gram-positive and Gram-negative bacteria (7).

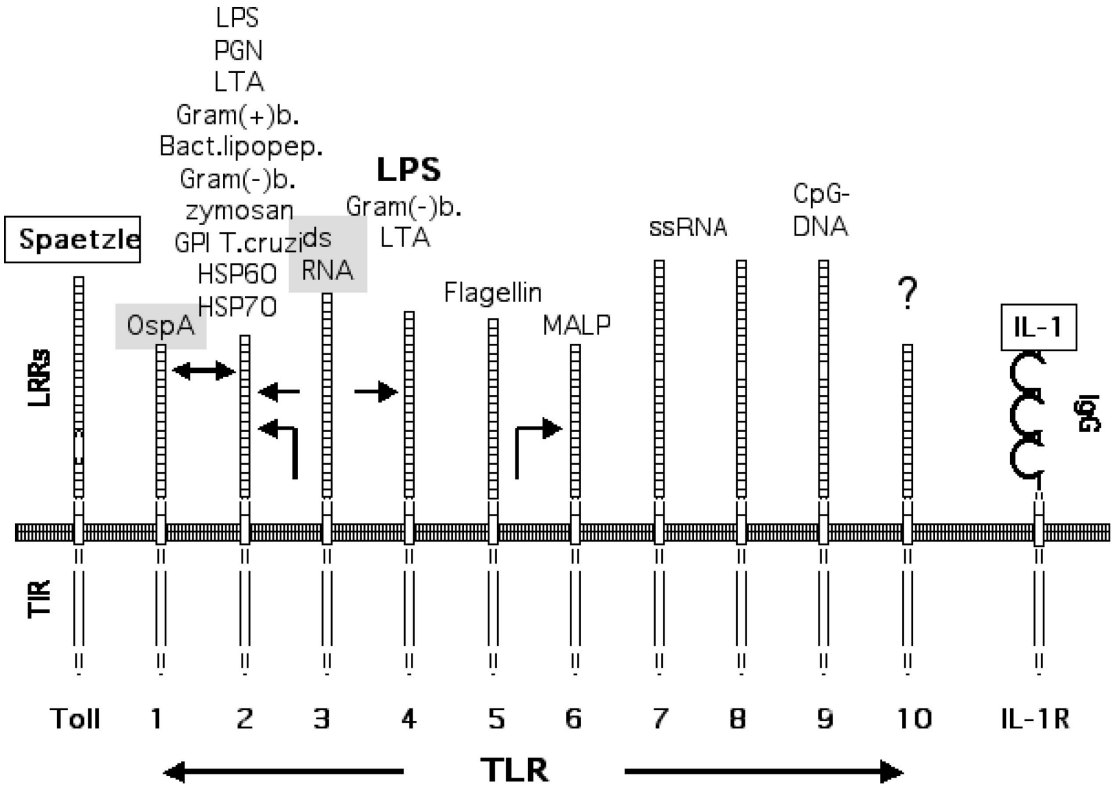

Fig. 1.3 Schematic comparison of Drosophila Toll, human TLRs 1 to 10, and vertebrate IL-1RI. Arrayed are TLRs 1 to 10 positioned between *Drosophila* Toll and vertebrate IL-1RI. LRRs (Leucine rich repeats) are indicated as boxes and Cys-rich clusters are drawn as half ovals in the extracellular domain; intracellular TIR domains are depicted as ovals. Examples for molecular patterns reported to utilize the regarding TLR are given. Arrows depict functional cooperation in terms of transmission of crosstolerance or pattern recognition. TIR, intracellular domain of Toll, TLR, and IL-1 receptors; CpG-DNA, unmethylated DNA fragments containing CG-motives flanked by particular DNA sequences; Bact.lipopep., bacterial lipopeptide; MALP, mycoplasmal monocyte-activating lipopeptide; Gram(-)b., Gram-negative bacteria; Gram(+)b., Gram-positive bacteria; LPS, lipopolysaccharide from different bacterial species; PGN, peptidoglycan; LTA, lipoteichoic acid.

Int. J.Med.Microbiol. 2001 291: 251-260. Toll-like receptors: cellular signal transducers for exogenous molecular patterns causing immune responses. Kirschning CJ, Bauer S.

The engagement of TLRs by microbial products leads to the activation of multiple intracellular signal transduction pathways. Among the best-characterized pathways is the one leading to NF-κB activation. TLRs most likely form dimers, leading to conformational change in the cytoplasmic Toll/IL-1R/Resistance (TIR) module (20). Conformational change in TIRs results in a subsequent recruitment of the adapter protein MyD88, which consists of a C-terminal TIR domain that binds TLR via the cytoplasmic TIR module and an N-terminal portion that is a death-domain. When associated with a TLR, MyD88 recruits members of the IRAK family through death domain-death domain homophilic interactions. IRAK1 and IRAK4 are serine/threonine kinases involved in the phosphorylation and activation of TRAF6. In contrast, IRAK2 and IRAK-M lack kinase activity and play different roles. IRAK-M negatively regulates TLR signaling by preventing dissociation of phosphorylated IRAK1 and IRAK4 from MyD88, a necessary step for signal transduction. The function of IRAK-2 remains unclear. After phosyphorylation by IRAKs, the RING-finger containing factor TRAF6 activates a heterodimer composed of two ubiquitination proteins called Ubc13 and Uev1A (21). These proteins trigger nonclassical polyubiquitination of TRAF6 that leads to its association with the MAP3 kinase TAK1. Once activated, TAK1 can directly phosphorylate and activate the IκBα kinase complex (IKK), which consists of the kinases IKKα and IKKβ and the scaffolding protein IKKγ/NEMO. The activation of the IKK complex by TAK-1 leads to the phosphorylation of the NF-κB cytoplasmic inhibitor IκBα that leads to its degradation via classical ubiquitination, thus releasing the transcription factor into the nucleus where it activates target genes. Activated TAK-1 can also activate mitogen/stress activated protein kinases (M/SAPK) p38, c-Jun NH_2-terminal kinase (JNK), and extracellular signal-regulated kinases (ERK; p42/p44) 1/2 via phosphorylation of their respective MKKs (Fig. 1.4)(22).

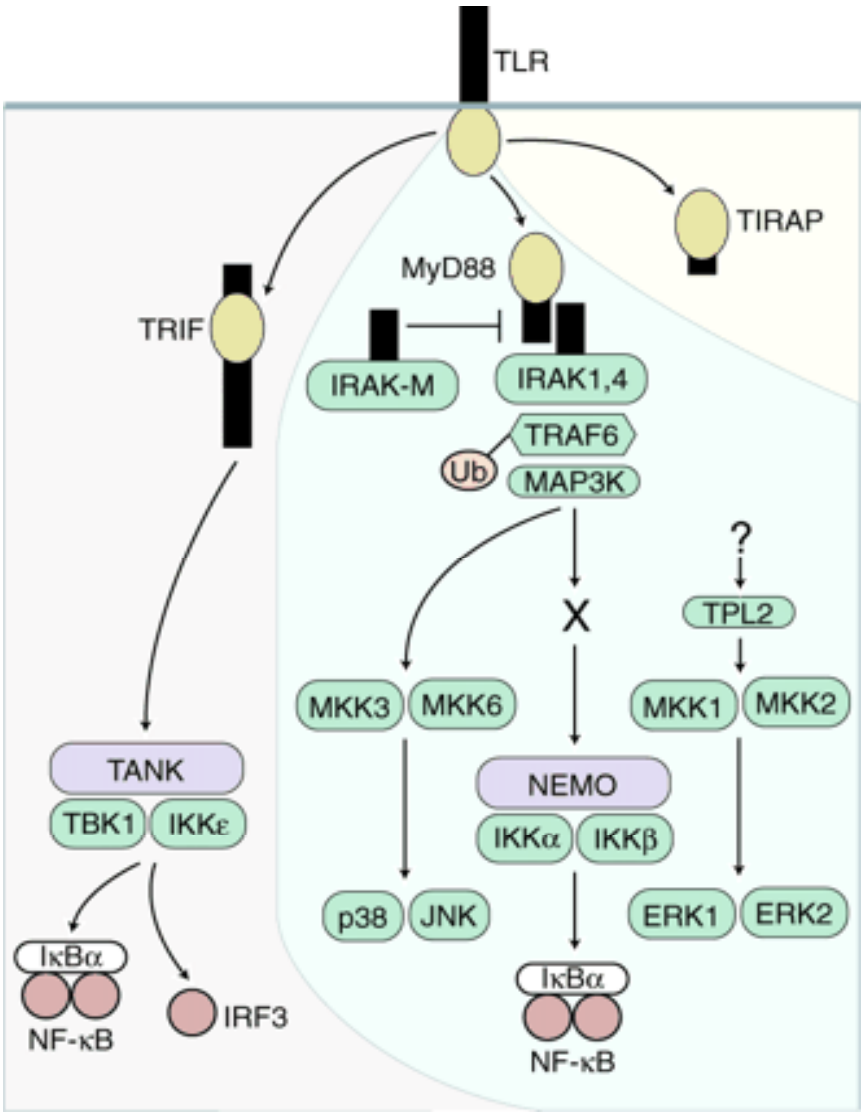

Fig. 1.4 TLR signal transduction pathways. Individual TLR family members induce different signaling pathways and can be grouped based on their usage of the known TLR adaptors. All TLRs signal through the MyD88-dependent pathway (shaded in blue). TLR3, and TLR4, can induce IFN α/β expression through the TRIF (also known as TICAM-1) pathway (shaded in red). TIRAP (also known as Mal) functions downstream of TLR2 and TLR4 (shaded in yellow). Where appropriate, the modular organization of individual signaling components is represented schematically. The role played by TAK1 in IKK activation remains unclear, so a generic MAP3K is shown downstreamof TRAF6. TRAF6 is a RING domain-containing ubiquitin ligase and is ubiquitinated upon activation.

Science. 2003 Jun 6;300(5625):1524-5. Toll-like receptor signaling pathways. Barton GM, Medzhitov R.

The MyD88-dependent signaling pathway described above is shared by all members of the TLR family and results in the induction of a core set of responses. However, analysis of cells from mice lacking MyD88 has demonstrated that TLR3 and TLR4 are capable of inducing certain signaling pathways independent of this adaptor. Two additional TIR-containing adaptors have been identified. TIR domain-containing adaptor protein (TIRAP, also known as Mal, MyD88 adaptor like) functions downstream of TLR2 and TLR4 but is not involved in signaling by other TLRs (23, 24). TIR domain-containing adaptor-inducing IFN-β (TRIF, also known as TICAM-1/LPS2, TIR containing adaptor molecule) appears to function downstream of TLR3 and TLR4, being responsible for the induction of interferon (IFN)-α and IFN-β (IFN α/β) genes by these TLRs (25-27). The induction of IFN α/β expression by TLR3 and TLR4 occurs through a MyD88-independent pathway that leads to the activation of interferon regulatory factor (IRF) 3, a key transcription factor responsible for the induction of IFN genes. Recent identification of a new TIR domain-containing adaptor, TRIF-related adaptor molecule (TRAM, also known as TIRP, TIR domain containing adaptor protein) showed that it is a TLR4 specific adaptor positioning downstream of this receptor and upstream of TRIF while is not involved in TLR3 iniated signaling (28). In addition, two noncanonical IKKs, IKKε and TBK-1 have been shown recently to function downstream of TRIF and upstream of IRF3 (22, 29). These kinases are likely to be responsible for the MyD88-independent induction of NF-κB by TLR3 and TLR4 as well (Fig. 1.4). The known TLR signaling components still do not explain all of the known differences in signaling between individual TLRs, indicating that additional gene products and signaling mechanisms have yet to be discovered.

1.3 TLR2 Structure

The N-terminal extracellular domain of vertebrate TLRs is characterized by a sequence of leucine rich repeat (LRR) motifs flanked by a membrane proximal LRR C-terminal cysteine-rich domain (LRRCT), which is followed by a transmembrane domain and a C-terminal TIR domain (exemplified by TLR2, Fig. 1.5 and Fig. 1.6) (7). The complete human and murine TLR2 cDNAs have been identified recently (14, 30) (accession numbers U88878 and AF124741, respectively). The premature human TLR2 molecule is a polypeptide encompassing 785 amino acid residues, of which 15.4% is leucine. The extracellular domain, the transmembrane domain, and the intracellular domain of TLR2 contain 13, 3, and 4 cysteine residues, respectively. The size of the mature protein is 97 kDa as revealed from immunoblot analysis.

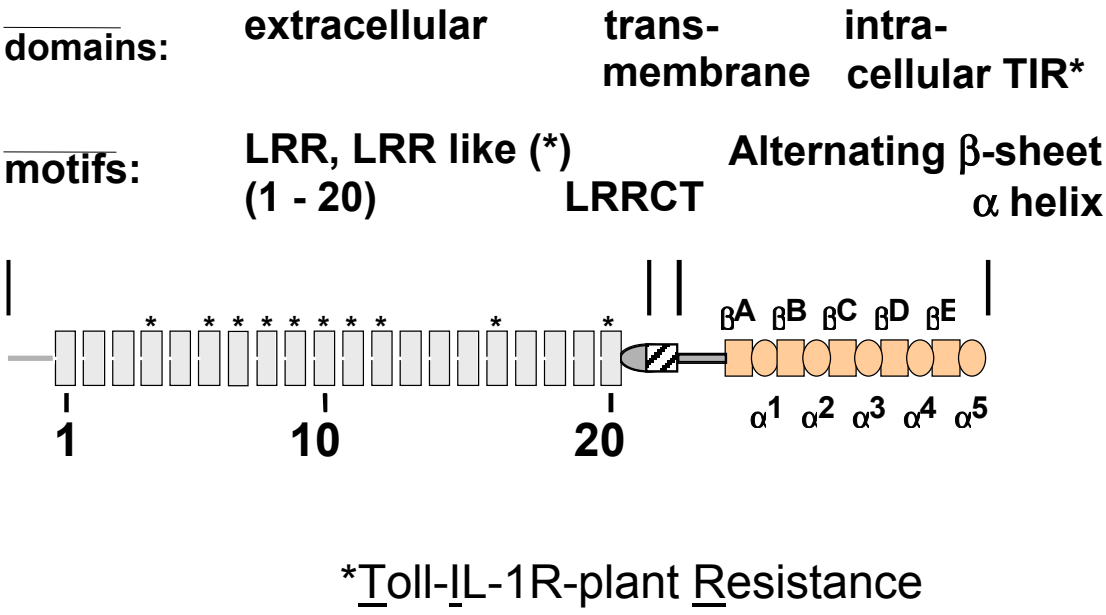

Fig. 1.5 **TLR2 protein structure.** Cartoon of the type I receptor molecule TLR2 extending from its extracellular amino terminus (left) to its intracellular C-terminus (right). An amino terminally located portion of TLR2 (dark Grey box) is followed by 20 LRR and LRR like motifs proposed (light Grey boxes). Unmarked LRR motifs have been defined by application of the SMART program. LRR motifs marked with an asterisk have been defined based on their similarity to a consensus motif (see text and Fig. 1.6 for details). The transmembrane domain (lined box) is preceded by the membrane proximal LRR C-terminal motif (LRRCT, dark gray half oval) and is followed by a structurally undefined stretch (second dark gray box) and the Toll/IL-1R/Resistance (TIR) domain, respectively. The TIR domain forms a specific three-dimensional structure encompassing five alternating β sheet (open boxes)-α helix motif (open ovals) pairs and provides the C-terminus of the TLR2 molecule.

Current topics in microbiology and immunology, 2002, Vol.270, Toll-like receptor family members and their ligands. Springer, Pp121-144. Kirschning CJ, Schumann RR.

TLR2 contains twenty leucine rich repeat (LRR)/ LRR-like motifs within its extracellular domain as revealed by application of the SMART program (31) for identification of motifs number 1, 2, 3, and 5, as well as motifs number 13 through 19 with the exception of #16 (http://smart.embl-heidelberg.de/) (7, 32). The other motifs were localized by definition of at least two matches within a LRR core motif (LxxLxxLxLxxN) as minimal requirement for a LRR like motif (marked by asterisk in Fig. 1.5 and Fig. 1.6).

The TLR2 extracellular domain sequence shares particularly high similarity with the regarding sequences of TLR1, TLR6, and TLR10 (33). The similarity of TLR2 and the subgroup formed by TLR7, TLR8, and TLR9 is particularly low due to differences in the numbers and irregularities of LRRs, as well as non-LRR-like motif insertions. Comparison of the sequence spanning the region of LRR motifs numbers 13 to 16 in the extracellular domain of TLR2 (Fig. 1.6) and the membrane-proximal region of CD14 revealed considerable similarity implying functional resemblance of both proteins (16). The crystal structure of one LRR protein, ribonuclease inhibitor (RI), has been determined providing a model for TLRs. RI LRRs represent units of one β-sheet and one α-helix, which are arranged in parallel to one axis. The β-sheets form an inner surface and the α-helices provide the interconnecting outer structure resulting in formation of a horseshoe shaped molecule (34).

The transmembrane domain of TLR2 spans from T588 to H610 of the premature protein. The Tyrosine 617 located in close proximity, as well as the “intra TIR” Y761, have been demonstrated to be phosphorylated upon activation of TLR2. They furthermore have been shown to be involved in NF-κB activation (35). Thus the N-terminus of the intracellular domain of TLR2 not part of the TIR domain is actively involved in signal transduction through TLR2. The TIR domain starts 30 AA C-terminally from the transmembrane domain and extends to the C-terminal residue S785 of TLR2. The intracellular TIR forms a cassette of ten motifs of alternating β-sheets and α-helices (14, 36). Except for the inter connection of the third β-sheet and the third α-helix these ten alternating motifs are connected by eight loops. The structure of the loop between the second β-sheet and α-helix containing a sequence motif characteristic for IL-1R/Toll type receptors has been a major focus of structure-function analysis (36). It includes the amino acid (AA) residue P681 equivalent to mouse TLR4 P712 crucial for LPS responses in mice (18, 37). This so called BB loop strongly contributes to a surface patch potentially important for interaction with the adapter molecule MyD88 by forming a protrusion. It was proposed that although the structural changes caused by a P681H mutation in TLR2 are not significant, the residue P681 located at the tip of the loop may be important for interaction with other molecules such as those carrying TIR domains including MyD88 (36).

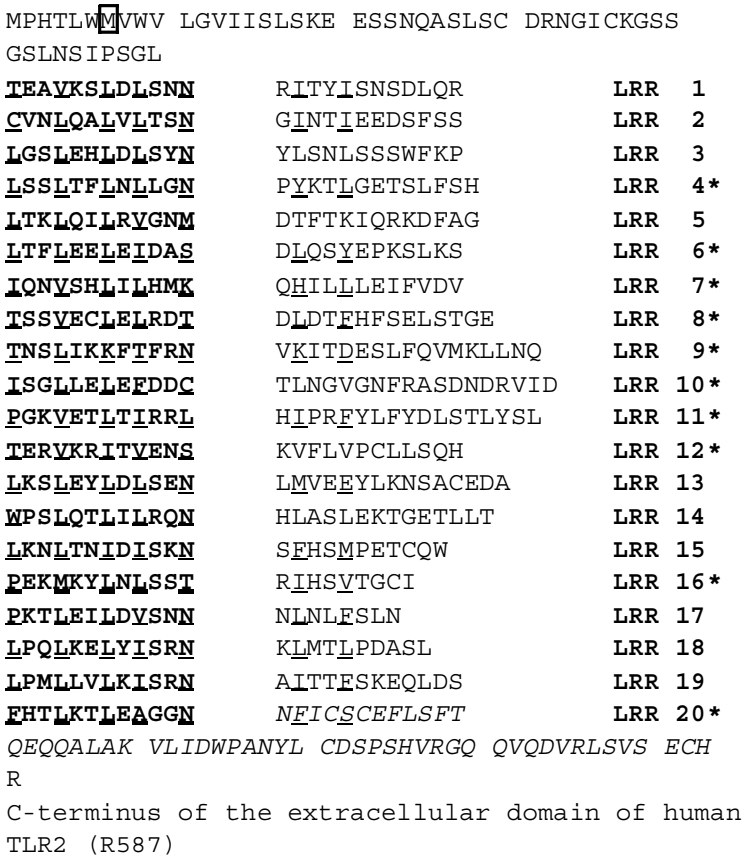

Fig. 1.6 Sequences of the extracellular domain of human TLR2 ordered by structural properties. Depicted are the amino acid sequences of the extracellular domain of human TLR2 (immature protein). Core motifs of the extracellular domain of TLR2 proposed hereby as leucine rich repeat (LRR) or LRR like (marked with an asterisk) motifs are separated from the flanking sequences and aligned, as well as printed in bold. The motifs are numbered from 1 to 20, successively. Most LRR motifs and a LRR C-terminal domain (printed in italic) have been identified by application of the SMART program (see text and Fig. 1.5). Amino acid residues principally conserved according to the minimal LRR consensus motif (LxxLxxLxLxxN; L, leucin; x, amino acid) are underlined. The C-terminally flanking two residues that are occupied by conserved leucines according to an extended LRR consensus motif (LxxLxxLxLxxNxLxxL) are underlined and fit the consensus sequence in three cases (LRR motif 3, 14, and 18). A potential translation start at amino acid (aa) residue M7 results in calculation of a signal peptide encompassing the C-terminally flanking 20 aa and is boxed.

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1.4 TLR2 Function

1.4.1 Recognition of microbial agonists

Gram-positive bacteria are of major clinical relevance and cause up to 50% of all cases of bacterial sepsis (38). Main immuno-stimulatory cell wall components of Gram-positive bacteria are lipoteichoic acid (LTA) and peptidoglycan (PGN) (39). Like LPS, PGN and LTA elicit the release of pro-inflammatory cytokines such as TNFα, IL-1β, and IL-6 from immune cells (40).

Initial implication of TLR2 in recognition of Gram-positive bacteria based on application of whole bacteria such as *Staphylococcus aureus* or bacterial cell wall preparations to cells ectopically overexpressing TLR family members including TLR2. Application of highly purified soluble *S. aureus* PGN and commercially available *Bacillus subtilis* LTA identified both bacterial products as TLR2 agonists and further implied their role as PAMPs representing Gram-positive bacteria to the immune system (41, 42). Application of highly purified LTA preparations from various bacterial species such as *Treponema pallidium* and *S. aureus* to cell lines or primary mouse cells supported the earlier notion that TLR2 is the exquisite LTA signal transducer (43-45).

Bacterial and mycoplasmal lipopeptides are further TLR2 specific agonists. Modifications such as tripalmytilation and diacylation are typical and appearently important for this interaction (46, 47). For a varierty of different microbial lipopeptides including the soluble bacterial lipopeptide analog, synthetic tripalmitoyl-cysteinyl-seryl-$(lysyl)_3$-lysine (P_3CSK_4), OspA from *Borrelia burgdorferi*, and *Mycoplasma fermentans* macrophage activating lipopeptide (MALP)-2, a clear TLR2-dependent cell stimulation pattern has been observed (48-50). A cooperation of TLR2 with TLR6 or TLR1 in recognition of lipopeptides and other TLR2 agonists has been demonstrated (51, 52).

LPS unresponsiveness despite CD14 overexpression paralleled by susceptibility to TNFα and IL-1β qualified the HEK293 fibroblast cell line for complementation experiments with TLR protein family members. Responsiveness to commercially available LPS and Lipid A preparations was gained by overexpression of human TLR2 in HEK293 cells. Cell activation through TLR2 required the presence of the serum components LBP or soluble CD14 when low concentrations of LPS were applied (53, 54). Further studies showed that TLR4 is the major LPS signal transducing receptor for classical LPS, but variants such as LPS from enterobacterial species signal through TLR2 (55). LPS from *Porphyromonas gingivalis* has been shown to not only specifically recruit TLR2, but also elicit partially different cellular responses as compared to *E. coli* LPS induced effects (56). Furthermore,

LPS of the spirochete *Leptospira interrogans* has been demonstrated to require TLR2 for activation of immune cells *in vitro* and *in vivo* (57).

The first report of a role for TLR2 in infection *in vivo* showed that gene targeted *TLR2*$^{-/-}$ mice displayed increased susceptibility to *S. aureus* infection as compared to wild-type mice (58). It has also been shown that TLR2 plays a major role for recognition of Group B streptococci (GBS), which are of major clinical importance in infection of newborn children (59). A role of TLR2 in recognition of *Chlamydia pneumoniae*, which currently is discussed as being involved in arteriosclerosis pathogenesis, has been implicated as well (60). Finally, Neisserial porins have been demonstrated to employ TLR2 for cell activation (61).

TLR2 appeared to be the major molecular sensor for Spirochetes (62). Spirochetes including the genera *Treponema*, *Borrelia,* and the family *Leptospira* are causative agents of a number of severe and frequently occurring chronic inflammatory diseases in humans, with Syphilis caused by *T. pallidum*, and Borreliosis or Lyme disease caused by *B. burgdorferi* being the most prominent ones (63, 64). Impaired bacterial clearance of *TLR2*$^{-/-}$ mice in *B. burgdorferi* infection as compared to wild-type mice has been demonstrated (65). In search for spirochetal compounds responsible for inflammatory reactions of the host, the presence of LPS in the outer membrane was reported (7). The search for biologically active products of these bacteria has focused on lipoproteins, of those outer surface proteins (Osps) A-F were found to be strong inducers of pro-inflammatory cytokines in mononuclear cells via TLR2, so were spirochetal lipoproteins (50).

TLR2 involvement in mycobacterial host-interaction has been confirmed by many publications. A prime focus has been placed on mycobacterial lipoarabinomannan (LAM) which stimulates host cells via TLR2 (66, 67).

It has also been shown that yeast zymosan induced cell activation but not uptake is TLR2 dependent (68). Glycosylphosphatidylinositol (GPI) anchors and glycoinositol phospholipids (GIPLs) from parasitic protozoa have been shown to trigger NF-κB activation in Chinese hamster ovary-K1 (CHO) cells ectopically overexpressing CD14 and TLR2, but not wild-type CHO cells. Analytical comparison of wild-type and TLR2 knockout mouse macrophages confirmed that TLR2 expression appears to be essential for induction of IL-12, TNFα, and NO by GPI anchors derived from Trypanosoma cruzi trypomastigotes (69). Fig. 1.7 tries to summarize some of the microbial PAMPs recognized through TLR2.

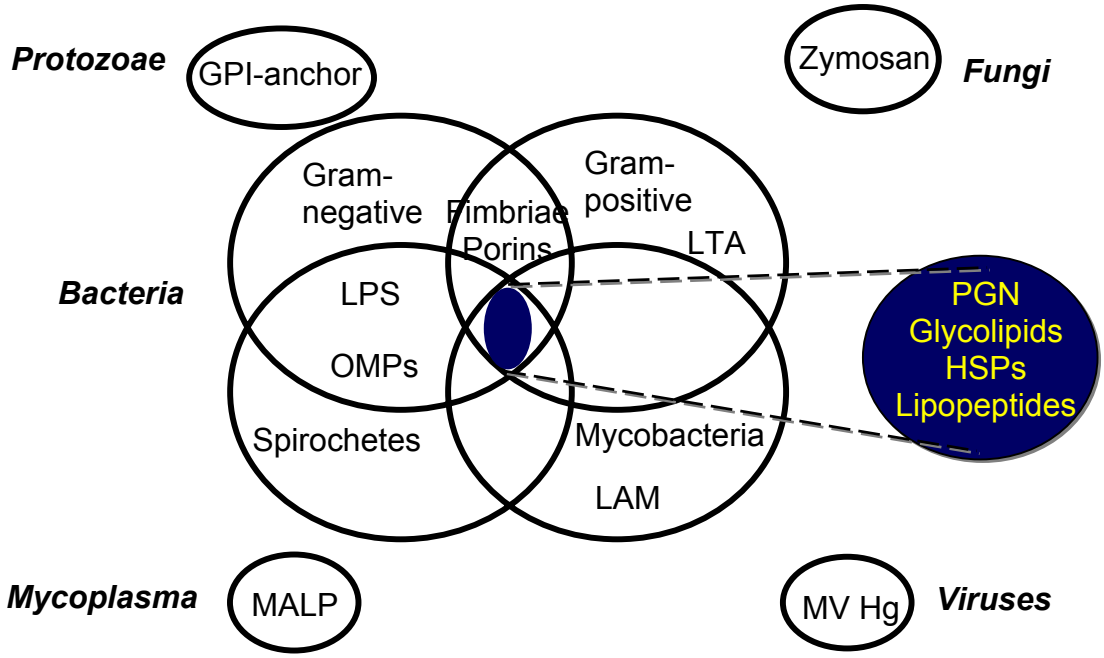

Fig. 1.7 Schematic overview of subgroups of bacterial species, mycoplasma, protozoae, and fungi, as well as of PAMPs recognized through TLR2. Gram-positive, Gram-negative, spirochetes, mycobacteria as major bacterial groups, mycoplasma and eukaryotic microbes such as protozoae and fungi are depicted (circles or ovals). Some PAMPs are present in all bacterial species while others are present in subgroups only. OMPs, outer membrane proteins; LPS, lipopolysaccharide; PGN, soluble peptidoglycan; LTA, lipoteichoic acid; HSPs, bacterial heat shock proteins; Glycolipids encompass different subgroups of PAMPs such as mycobacterial lipoarbinomannans (LAM); zymosan is a lyophilized lysate of yeast cells from which a TLR2 agonist has not been identified yet.

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1.4.2 Recognition of endogenous ligands

The first report implicating TLR2 in recognition of endogenous but unidentified molecular patterns suggested a protective role against cell destruction mediated by TLR2. It was shown that oxidative stress induced activation of NF-κB and AP-1 as well as p38 MAP kinase in primary rat myocytes. Application of an anti-TLR2 antiserum blocked activation of NF-κB and AP-1 but not of p38 MAP kinase. The authors concluded that hydrogen peroxide induces activation of NF-κB and AP-1 but not of p38 in a TLR2 dependent fashion. TLR2 dependent cell activation thus may be brought about indirectly by prior induction of release of a TLR2 agonist. This agonist could be released from cells driven to necrosis or apoptosis by oxidative stress and activate TLR2 dependent anti-apoptotic pathways protecting further myocytes from death (70). By usage of lysed mouse embryonic fibroblasts (MEFs) and irradiated MEFs as necrotic and apoptotic cells, respectively, for stimulation, a study employing DCs, MEFs, macrophages, and transiently transfected HEK293 cells showed that necrotic but not apoptotic cells induced expression of genes involved in inflammatory reponses through TLR2 (71).

Members of the heat shock protein family HSP60 of both human and bacterial origin were identified as potent stimuli of mouse macrophages in terms of TNFα and nitrogen monoxide (NO) release (72). Two lines of evidence suggested the involvement of TLR4 and TLR2 in cellular HSP60 recognition as proposed earlier: TNFα release upon stimulation with chlamydial HSP60 of mouse immune cells of the C3H/HeJ strain lacking functional TLR4, as well as of *TLR2*$^{-/-}$ mouse DCs was profoundly decreased as compared to control cells (72, 73). Secondly, overexpression of TLR4/MD2 or TLR2 in human epithelial cells was shown to confer responsiveness to both, human and chlamydial HSP60 (73). Further studies demonstrated that HSP70 induced proinflammatory cytokine production from human monocytes was mediated via the MyD88-NF-κB pathway utilizing both TLR2 and TLR4 (74, 75). Another member of the heat shock protein family, Gp96 (HSP90) was also shown to carry the capacity to activate dendritic cells via TLR2 and TLR4 resulting in activation of NF-κB and MAP kinases (76). From a recent study using dominant negative constructs of molecules involved in TLR-NF-κB pathway, it was demonstrated that receptors such as TLR2, TLR4 and receptor for advanced glycation end product (RAGE) were all shown to be involved in high mobility group box (HMGB) 1 induced activation of NF-κB in neutrophiles, monocytes or macrophages (77).

The above findings about endougenous ligands of TLRs seemed very exciting, however, many recent researches challenged them strongly by raising the concern that the cellular activation induced by heat shock proteins in previous studies might due to the contamination of LPS and/or LPS associated molecule(s) (78). Two years ago, it was shown

that in the presence of LPS antagonist polymyxin B, commercial recombinant human HSP (rhHSP) 70 failed to induce the maturation of human monocyte derived DC (79). Further studies suggested that LPS and other contaminats were responsible for both rhHSP70 and rhHSP60 induced macrophage activation (80-82). Evidences to this direction include a recent sutdy showing that highly purified Gp96 retaining its native conformation and ligand binding activity did not activate macrophage (83) and mycobacterial HSP65 enhanced antigen cross-presentation in DCs independent from TLR4 signaling (84). Further efforts should be directed to conclusively determine whether the endougenous ligands of TLR2 are results of contamination before exploring further the implication and therapeutic potential of these endougenous agonists (85).

1.4.3 Cellular activation and Phagocytosis

The recognition of molecular patterns ininates signal transduction through the intracellular domains of cellular receptors such as TLRs. Biological parameters such as release of TNFα, IL-1β, IL-6, and IL-12 have been analyzed for characterization of TLR functions. Also, induction of direct anti-microbial activity through TLR2 such as release of NO has been demonstrated (86). Whether TLR2 activates distinct signaling pathways as compared to those triggered by other TLR family members and whether immune responses towards particular pathogens differ has been addressed to a wide extent (56, 87-89). Whether different TLR2 ligands elicit distinct effects or whether different affinities cause activation to specific degrees remains to be analyzed (7).

Subcellular translocation of TLR2 from the cell surface to phagosomes within five minutes upon microbial challenge has been shown (68). Another study showed that the phagocytosis was TLR2 independent, while another receptor, Dectin-1, mediates the pathogen internalization and collaborates with TLR2 in induction of inflammatory responses (90). Related work showed that Dectin-1 colocalized with TLR2 on the surface of macrophage cells upon fungal pathogen challenge which was followed by phagocytosis. Cellular immune responses, however, occurred on the cell surface and were independent from engulfment (91). TLR2 was also found to be involved in bacteria induced phagosome maturation in a recent study conducted by Medzhitov and colleagues. In *TLR2*$^{-/-}$ macrophages, *S. aureus* were found to be docked in the phagosomes, but defected in fusions with lysosomes, whereas *TLR4*$^{-/-}$ macrophages looked similar to wild type cells (92). In addition, recent study showed that in airway epithelial cells, upon bacteria exposure, TLR2 mobilized into lipid rafts in association with asialo-glycolipids and recruited its downstream signaling molecules such as MyD88, IRAK1 and TRAF6 (93).

1.5 TLR2 Polymorphism and Expression

1.5.1 Genomic organization and Genetic polymorphism

The human TLR2 gene is localized at 4q32 while the mouse TLR2 gene is located on chromosome 3 (14, 15, 94). Among others, two genetic polymorphisms both locating in the intracellular portion of TLR2 have been identified. The first one, R753Q, has been shown to be associated with *Staphylococcus aureus* infection, tuberculosis and atopic dermatitis (95-97). The second mutation, R677W, has been found to alter intracellular mycobacterial signaling and was associated with lepromatous leprosy as well as tuberculosis (98-100). Studies on R753Q polymorphism of TLR2 as well as D299G and T399I mutations of TLR4 have shown that one functional allele for any of these sites suffices for full function of the respective receptors (101, 102). A microsatellite polymorphism in human TLR2 has been identified recently (103). Previous analysis of TNF gene mutation showed association with increased susceptibility to ultraviolet-B radiation stress (104). And a recent finding showed that a TLR5 stop codon polymorphism is associated with increased susceptibility to legionnaires' disease (*legionella pneumophila* infection) (105). These investigations raised the concern that genetic variations in innate immune receptors could be risk factors for infectious diseases and provided important information for potential targeted therapy of acute infections.

1.5.2 Expression and Regulation

Transcriptional regulation of the TLR2 gene has been analyzed by reporter gene assays. Two NF-κB and two stimulating factor (SP) 1 recognition elements, as well as a signal transducer and activator of transcription (STAT) binding consensus sequences have been identified in the promoter sequence of TLR2. And they all were found to be functionally involved in the gene regulation of this receptor upon either Lipid A and IL-15 stimulation or *Mycobacterium avium* infection in various cell systems (106-108).

Initial analysis of human TLR2 expression by Northern Blot analysis revealed abundant TLR2 mRNA accumulation in the lung, spleen, and PBL (14, 15), while mouse TLR2 mRNA expression was found to be most pronounced in spleen, lung, and thymus (109).

Human polymorphnuclear (PMN) leukocytes, as well as DCs were demonstrated to express TLR2 mRNA. Upon LPS stimulation TLR2 mRNA levels were upregulated in PMNs only, but not in monocytes. TLR2 mRNA expression furthermore was downregulated during differentiation of monocytes to DCs (110). Stimulation with LPS increased TLR2 mRNA accumulation also in endothelial cells and IFNγ further enhanced this increase (111). And in monocytes from sepsis patients, TLR2 mRNA was upregulated (112).

Analysis of TLR2 protein expression in human cells has been performed by several groups. It was reported that the human myeloid cell lines THP1, U937, and Mono Mac 6 as well as human PBMCs and memory T cells expressed TLR2 while many other types of cells did not (113-116). The CD16+ and/or CD14+ monocytes express high levels of TLR2 (113, 117). In human dendritic cells, TLR2 was found inside of the cell and associated with microtubules and the Golgi apparatus but not detectable on the cell surface (118). TLR2 was also found expressed in human keratinocytes and upregulated upon differention (119, 120). The expression level of TLR2 in human PBMCs was decreased upon surgical operation stress (121). TLR2 expression in primary intestinal epithelial cells was low while patients with inflammatory bowel disease displayed significantly increased expression of TLR2 in the ileal or colonic epithelium (122). Furthmore, it was shown that glucocorticoids upregulated TLR2 expression in human epithelial cells from different studies (123, 124).

Treatment of primary mouse T cells from spleen and thymus with anti-CD3ε mAb resulted in upregulation of TLR2 mRNA accumulation as did stimulation of mouse T cell lines with IL-2, IL-15, or PMA (109). Stimulation of RAW264.7 mouse macrophage like cells with IL-2, IL-1β, IFNγ, as well as TNFα strongly induced TLR2 mRNA expression resulting in proposal of a model in which upregulation of TLR2 leads to sensitization of immune cells to TLR2 agonists (125). Infection of mouse macrophages with *Mycobacterium avium* resulted in a rapid increase of TLR2 mRNA levels in a TLR4 independent manner while LPS induced TLR2 protein synthesis without inducing its mRNA upregulation (107). Either IL-1α itself or TGF-β alone upregulated TLR2 expression at both mRNA and protein levels, but TGF-β inhibited the upregulation of TLR2 by IL-1α in murine hepatocytes (126). *In vivo* Gram-positive bacterium infection as well as LPS challenge upregulated TLR2 protein expression in various murine primary cells such as macrophages and granulocytes (116).

TLR2 expression and regulation was also investigated in specises other than human and mouse. In dairy calves, it was found that TLR2 mRNA expression in blood leukocytes, lung lavage cells as well as spleen and thymus cells was enhanced by treatment with dexamethasone and growth hormone (127).

1.6 Perspectives

Binding of LPS, as well as lipopeptide and TLR2 have been demonstrated (116, 128). PGN, as well as MD2 and surfactant protein A have been shown to also bind to TLR2, leading to enhancement or inhibition of function, respectively (129-131). Results from analysis of species-specific PAMP recognition further support the "TLR=PRR" model (116, 132). Since numerous TLR2 agonists have been implicated, different PAMPs might interact with different sub-domains of TLR2 ectodomain or they may share a common recognition motif of this receptor (Fig. 1.8).

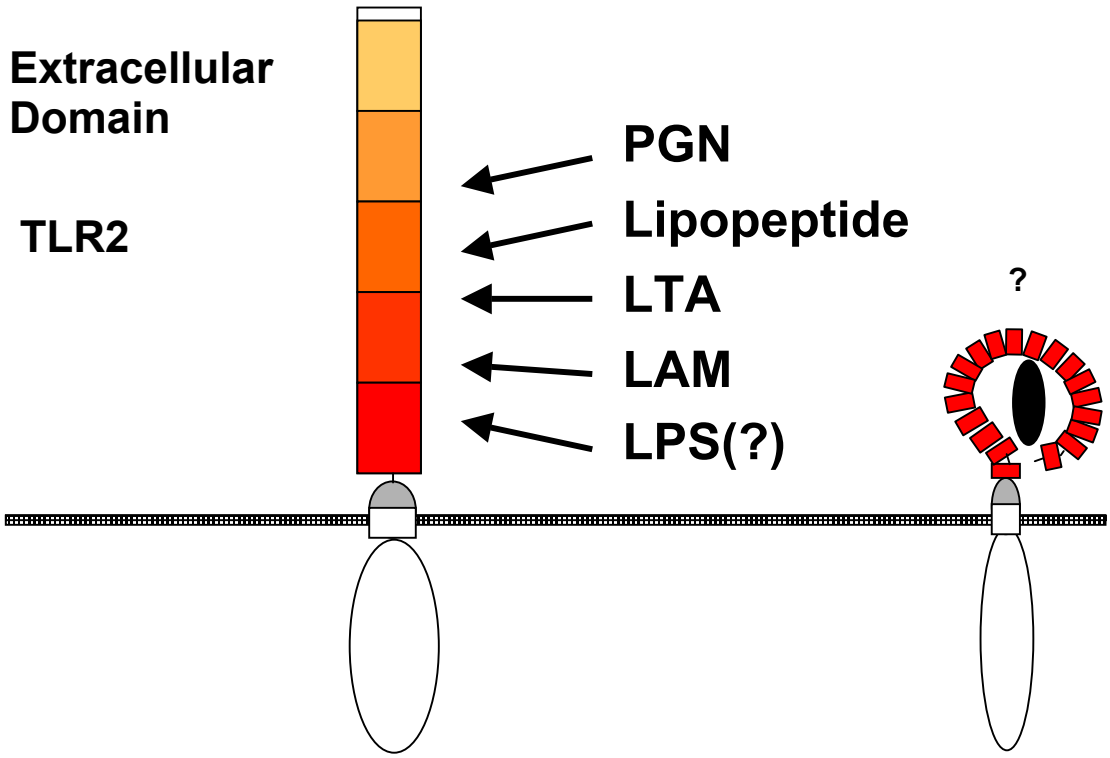


Fig. 1.8 Models for PAMPs recognition through TLR2. Possible models for cellular recognition of agonists via TLR2 (left and right respectively) are proposed. According to the first model, TLR is a pattern recognition receptor (PRR) that binds different pathogen associated molecular patterns (PAMPs) with respective sub domains leading to TLR dimerization and cell activation (left). Alternatively, TLR2 specifc agonists may share a common binding motif of the recepor for recognition by TLR2 perior to downstream cell activation signaling (right).

As has been described above, it is the recognition of PAMPs released from invading microorganisms through TLRs that is causative for overactivation of the host immune system eventually leading to septic shock syndrome. If this process can be inhibited at an early stage such as at the level of PAMP recognition by TLRs at the cell surface, the arising overwhelming host reactions may become controllable, resulting in protection of shock, such as exemplified in Fig. 1.9.

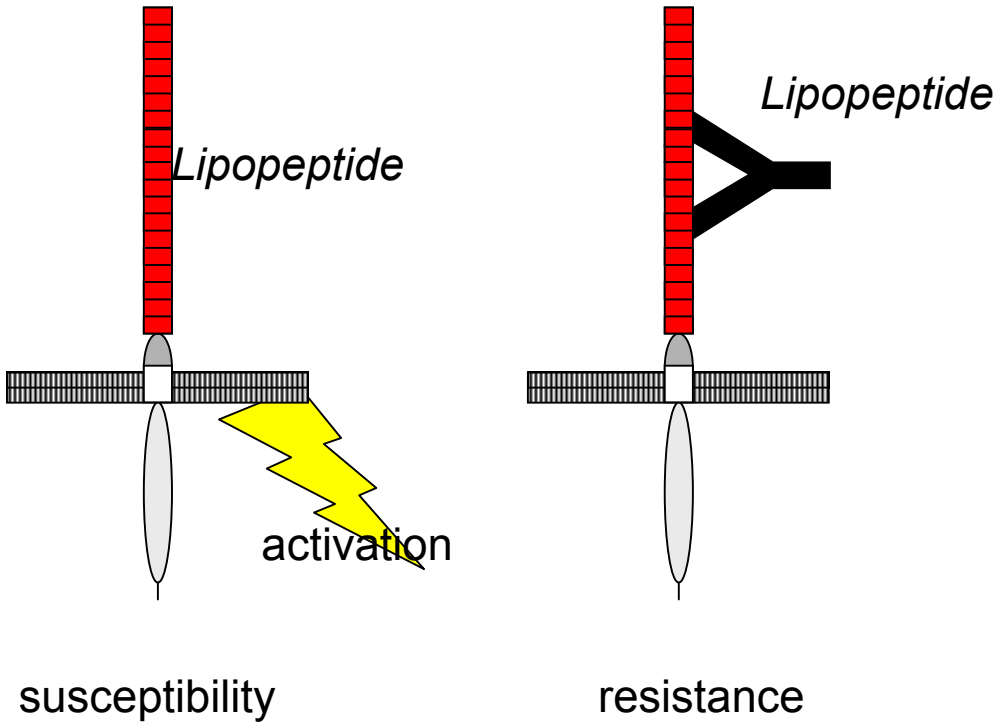


Fig. 1.9 A model for modulation of PAMP recognition via TLR2. Cellular recognition of PAMPs via TLR2 iniates anti inflammatory responses which might result in septic shock. If this process can be modulated at the stage of PAMP-TLR interaction, such as the level of TLR binding, the immune reaction of the host might be controllable and therefore shock preventable. Lipopeptide is illustrated examplifing all TLR2 agonists.

1.7 Objectives

1.7.1 Analysis of structural requirements for the TLR2 extra-cellular domain in specific pattern recognition

Although the intracellular domain of TLR2 has been crystallized resulting in basic understanding of the structure-function relationship of the TIR domain, the three dimensional structure of the leucin-rich extracellular TLR domain has only been modeled (7, 14, 133). Physical interaction of TLR agonists with the extracellular domain has been demonstrated in specific cases (116, 128, 129, 134).

Based on our own protein sequence analysis and published data on LRR-rich protein structures a set of deletion mutants covering motifs throughout the entire TLR2 molecule were generated. Mutant TLR2 constructs were ectopically overexpressed and functionally analyzed. Specific cell lines and primary cells of mice were applied for overexpression experiments. Our results contributed substantially to understanding of the molecular mechanisms underlying TLR2 function in PAMP recognition and cell activation.

1.7.2 Identification and characterization of TLR2 specific monoclonal antibody

As an important tool for analysis of the molecular basis of TLR2 dependent pattern recognition and further functional analysis, monoclonal antibodies were raised against the extracellular domain of mouse (m) TLR2. Recombinant TLR2 extracellular domain protein was overexpressed and used for immunization of gene targeted TLR2 deficient (*TLR2*$^{-/-}$) mice. Spleen cells were fused with immortal tumor cells in order to generate monoclonal antibody-producing hybridoma cell clones. A panel of 12 independent clones was identified. Antibodies were applied to analysis of cross-reactivity, epitope mapping, as well as of functional properties such as agonistic, antagonistic, or neutral potentials.

One antagonistic antibody clone was both cross-reactive and inhibitory for TLR2 specific pattern recognition. It was further applied in animal models for intervention in the fatal signaling cascade leading to shock in an experimental TLR2-specific shock system. This antibody opens a novel avenue for medical intervention in acute infection.

2 Materials and Methods

2.1 Materials

2.1.1 Buffers and solutions

Buffers and solutions were prepared using Milipore Q-destilled water. Chemicals were purchased from Sigma (Deisenhofen) or Roth (Karlsruhe), unless indicated otherwise.

PBS:	10 g/l	Dulbecco PBS (Biochrom)
	pH 7.4	
PBT:	1 x	PBS
	0.05% (v/v)	Tween 20
TAE buffer:	40 mM	Tris-acetate
(Invitrogen)	1 mM	EDTA
	pH 8.3	
TBE buffer:	10.8 g/l	Tris base
	5.5 g/l	Boric acid
	1mM	EDTA
	pH 8.3	
6 x Loading buffer:	1 g/l	Orange G
(agarose gel)	20 mM	Tris
	15% (v/v)	Glycerol
	pH 8.5	
2 x HBS:	16 g/l	NaCl
	0.74 g/l	KCl
	0.21 g/l	Na_2HPO_4
	10 g/l	Hepes
	pH 7.1	sterile filtrated
Lysis buffer:	50 mM	Hepes pH 7.6

	100-300 mM	NaCl
	1 mM	DTT
	1 mM	EDTA
	1 mM	EGTA
	0.5% (v/v)	Nonidet P-40
	10% (v/v)	Glycerol
	20 mM	β-Glycerolphosphate
	1 mM	Na_3VO_4
	0.4 mM	PMSF
	1 Tab/ml	Protease inhibitor cocktail tablets (Roche)
	1 mM	NaF
Washing buffer:	50 mM	Hepes pH 7.6
	150-350 mM	NaCl
	1 mM	DTT
	0.5% (v/v)	Nonidet P-40
	10% (v/v)	Glycerol
4 x SDS Sample buffer:	200 mM	Tris-HCl pH 6.8
(polyacrylamid gel)	400 mM	DTT
	10% (w/v)	SDS
	16% (v/v)	Glycerol
	2 g/l	Bromphenolblue
Laemmli buffer:	2.9 g/l	Tris
	14.4 g/l	Glycine
	1 g/l	SDS
	pH 8.3	
Blotting buffer:	5.8 g/l	Tris
	2.9 g/l	Glycine
	20% (v/v)	Methanol
Blocking buffer:	1 x	PBT
	3% (v/v)	NGS
	50 g/l	Milkpowder

Stripping solution:	100 mM	Glycine
	10 mM	β-Mercaptoethanol
	pH 2.75	

FACS buffer:	1 x	PBS
	2% (v/v)	FCS
	0.01% (v/v)	NaN_3
	5 mM	$CaCl_2$
	10 mM	$MgCl_2$

10 x Tris-Glycine:	29 g/l	Glycine
	58 g/l	Tris

Citrate buffer:

A	0.1M citric acid	21.01 g/l	Citric acid
B	0.1M sodium citrate	29.41 g/l	$C_6H_5O_7Na_3 \cdot 2H_2O$

X ml of A puls Y ml of B and diluted to a total of 100 ml with distilled water:

X	Y	pH	X	Y	pH
46.5	3.5	3.0	23.0	27.0	4.13
43.7	6.3	3.2	20.5	29.5	5.0
40.0	10.0	3.4	18.0	32.0	5.2
37.0	13.0	3.6	16.0	34.0	5.4
35.0	15.0	3.8	13.7	36.3	5.6
33.0	17.0	4.0	11.8	38.2	5.8
31.5	18.5	4.2	9.5	41.5	6.0
28.0	22.0	4.4	7.2	42.8	6.2
25.5	24.5	4.6			

Citrate Phosphate buffer:	50 mM	Na_2HPO_4
	25 mM	Citric acid
	pH 5.0	

EMSA buffer:

A: Cell lysis buffer:	10 mM	HEPES pH 7.9

	10 mM	KCl
	0.5 %	NP-40
	0.1 mM	EDTA
	0.2 mg/ml	leupeptin and aprotinin
	0.5 mM	PMSF
	1 mM	DTT
B: Nuclei lysis buffer:	20 mM	HEPES pH 7.9
	0.4 M	NaCl
	1 mM	EDTA
	0.2 mg/ml	leupeptin and aprotinin
	1 mM	PMSF
	1 mM	DTT
Saponin buffer:	0.2%	saponin
	0.5%	BSA
	in 1 x PBS	
Saponin block:	0.2%	saponin
	3%	BSA
	in 1 x PBS	
mAb purification buffer:	50mM	Tris
	150 mM	NaCl
	pH8.5	

2.1.2 Kit systems

PCR QuickChange Site directed Mutagenesis PCR Kit (Stratagene)
Liga Fast Rapid DNA Ligation system (Promega)
QIAquick Gel extraction kit (250) (QIAgen)
QIAprep spin Miniprep kit (250) (QIAgen)
QIAfilter Plasmid Maxi kit (25) (QIAgen)
Effectance Transfection Reagent (QIAgen)
Wizard SV gel and PCR clean up system (Promega)
Luciferase assay system (Promega)
Tropix-Galacto-light-plus (PerkinElmer)

WesternBlot Chemoluminescence Reagent Plus (PerkinElmer)

Centriprep/ Centrifugal filter devices (Millipore)

Human IL-8 ELISA (R&D)

Mouse TNF alpha ELISA (R&D)

Mouse IL-12p40 ELISA (R&D)

Mouse IL-6 ELISA (R&D)

Mouse GRO alpha / KC ELISA (R&D)

Cytofix/cytoperm Kit (BD)

2.1.3 Media

HEK 293 medium:	1 x	DMEM (Gibco)
	10% (v/v)	FCS (PAA)
	1% (v/v)	Penicilin-Streptomycine (Gibco)
	1% (v/v)	Antibiotic-antimycotic (Gibco)
THP1 medium:	1 x	RPMI (Gibco)
	10% (v/v)	FCS (PAA)
	1% (v/v)	Penicilin-Streptomycine (Gibco)
	1% (v/v)	L-glutamine (Gibco)
	1% (v/v)	Antibiotic-antimycotic (Gibco)
RAW264.7 medium:	1 x	RPMI (Gibco)
(Peritoneal macrophage)	10% (v/v)	FCS (PAA)
	1% (v/v)	Penicilin-Streptomycine (Gibco)
	1% (v/v)	Antibiotic-antimycotic (Gibco)
	1% (v/v)	L-glutamine (Gibco)
	50 µM	β-Mercaptoethanol (PAN)
mEF cell medium:	1 x	DMEM (Gibco)
	10% (v/v)	FCS (PAA)
	1% (v/v)	Penicilin-Streptomycine (Gibco)
	1% (v/v)	Antibiotic-antimycotic (Gibco)
	1% (v/v)	L-glutamine (Gibco)
	3.5 µg/ml	Glucose (additional, Sigma)
	50 µM	β-Mercaptoethanol (PAN)

Hybridoma selection medium:	1 x	RPMI (Gibco)
	1 x	HAT (Sigma)
	1 x	HFCS (Roche)
	5% (v/v)	FCS (PAA)
	1% (v/v)	Penicilin-Streptomycine (Gibco)
	1% (v/v)	Antibiotic-antimycotic (Gibco)
	1% (v/v)	L-glutamine (Gibco)
	50 µM	β-Mercaptoethanol (PAN)
Hybridoma culture medium:	1 x	RPMI (Gibco)
	8% (v/v)	FCS (PAA)
	1% (v/v)	Penicilin-Streptomycine (Gibco)
	1% (v/v)	Antibiotic-antimycotic (Gibco)
	1% (v/v)	L-glutamine (Gibco)
	50 µM	β-Mercaptoethanol (PAN)
Human PBMC medium:	1 x	RPMI (Gibco)
	20%	Autologous serum
	1% (v/v)	Penicilin-Streptomycine (Gibco)
	1% (v/v)	L-glutamine (Gibco)
	1% (v/v)	Antibiotic-antimycotic (Gibco)
Freezing medium:	10% (v/v)	DMSO (Sigma)
	90% (v/v)	FCS (PAA)
LB-medium:	10 g/l	Bacto-Trypton
	5 g/l	Yeast-extract
	10 g/l	NaCl
BH-medium:	27.5 g/l	Brain/Heart extract and peptones
	2.0 g/l	D (+) glucose
	5.0 g/l	NaCl
	2.5 g/l	Na_2HPO_4

2.1.4 Antibodies and antibody conjugates

Name	Antigene	Conjugate	Source	Conc. (mg/ml)	Company/Donor
Poly-αFlag	Flag-Tag	-	Rab	0.8	Sigma
Poly-αMyc	c-Myc-Tag	-	Rab	0.4	Sigma
Poly-αMsTLR2	MsTLR2 ECD	-	Rab	0.4	BioRad
Poly-αMs-HRP	Ms IgG	HRP	Goat	0.5	BD Pharmingen
Poly-αRab-HRP	Rab IgG	HRP	Goat	0.5	BioRad
Poly-αRab-PE	Rab IgG	PE	Don	0.5	JacksImmuRes
Mono-TL2.1	Hu TLR2	-	Ms	0.3	Dr. Lien
Poly-αNFκB/p65	NFκB/p65	-	Rab	0.2	Santa Cruz
Mono-αCD19	Ms CD19	APC	Rat	0.2	BD Pharmingen
Mono-αCD11c	Ms CD11c	FITC	Rat	0.2	BD Pharmingen
Mono-αCD11b	Ms CD11b	APC	Rat	0.2	BD Pharmingen
Mono-αGr1	Ms Gr1	FITC	Rat	0.2	BD Pharmingen
Mono-αMsIgG1	Ms IgG1	PE	Rat	0.5	BD Pharmingen
Mono-αMsIgG1	Ms IgG1	FITC	Rat	0.5	BD Pharmingen
Mono-αMsIgG_k	Ms IgG_k	PE	Rat	0.5	BD Pharmingen
Mono-αMsIgG_λ	Ms IgG_λ	FITC	Rat	0.5	BD Pharmingen
Poly-αPhop38	Phospho P38	-	Rab	0.04	Cell signaling
Poly-αPhoErk	Phospho Erk	-	Rab	0.05	Cell signaling
Poly-αPhoAkt	Phospho Akt	-	Rab	0.04	Cell signaling
Poly-αPhoJNK	Phospho JNK	-	Rab	0.05	Cell signaling
Poly-αp38	P38	-	Rab	0.01	Cell signaling
Poly-αErk	Erk	-	Rab	0.01	Cell signaling
Poly-αJNK	JNK	-	Rab	0.01	Cell signaling
Poly-αRab-546	Rab IgG	AleFlo546	Goat	2	Molecular Probes
Poly-αMs-546	Ms IgG	AleFlo546	Goat	2	Molecular Probes
Concanavalin A	-	AleFlo488	-	5	Molecular Probes
Mono-αMsFab	MsIgGFab	FITC	Goat	0.7	Caltag
Mono-αMsIgG	Ms IgG	AleFlo488	Rat	0.5	BD Pharmingen
Poly-αHu-IgG	Hu IgG	-	Goat	1.8	JacksImmuRes
Mono-αFlag M2	Flag-Tag	Agarose beads	Ms	-	Sigma

Table 2.1 Antibodies and conjugates used. Poly = Polyclonal, Mono = Monoclonal, ECD = extra cellular domain, HRP = Horse Radish Peroxidase, PE = Phycoerythrin, FITC = Fluorescein Isothiocyanate, APC = Allophycocyanin, Hu = human, Rab =rabbit, Ms =mouse, Don = Donkey, AleFlo = AlexaFlour, Pho = Phosph = Phosphorylated, JacksImmuRes = Jackson Immuno Research laboratories INC (hamburg).

2.1.5 Plasmids

Promotor	Insert	Vector	Donor
P_{CMV}	-	pRK5	U. Schindler
P_{CMV}	TLR1, 2, 3, 4	pFlag-CMV-1	C. Kirschning H. Wesche
P_{CMV}	TLR1, 2, 3, 4	pMyc-CMV-1	C. Kirschning H. Wesche
P_{CMV}	mTLR2 ECD	pCDNA3.1 (-)	S. Bauer
P_{ELAM-1}	Luc	pELAM-1	U. Schindler
P_{RSV}	β-Gal	pRSV	M. Rothe
P_{TK}	neo R	PTK-neo	Z. Cao

Table 2.2 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 and pMyc-CMV-1 are derivates of pCMV. A heterologous preprotrypsin leader precedes a FLAG or c-Myc epitope tag, N-terminally fused to the overexpressed protein.

2.1.6 Oligonucleotides

Oligonucleotides were purchased from MWG (Ebersberg) and applied as primers for following PCR or Sequencing reactions:

Primer name Sequence (5-prime to 3-prime)

Primers for mutagenesis PCR

Mut1_F
ATCTTTAAACTCC ATT CCC // GCTGGACTTACCTTCCTT (37bp)
Mut1_R
AAGGAAGGTAAGTCCAGC // GGGAATGGAGTTTAAAGAT

Mut2_F
ACTAAGATTCAAAGAAAAGAT // AGAGTTATAGATCCAGGTA (40bp)
Mut2_R
TACCTGGATCTATAACTCT // ATCTTTTCTTTGAATCTTAGT

Mut3_F

TTAGAGCATCTGATAATGAC // TGTCAGTGGCCAGAAAAG (38bp)
Mut3_R
CTTTTCTGGCCACTGACA // GTCATTATCAGATGCTCTAA

Mut4_F
AACATTGATATCAGTAAGAAT // AACTTCATTTGCTCCTGTGAAT (43bp)
Mut4_R
ATTCACAGGAGCAAATGAAGTT // ATTCTTACTGATATCAATGTT

MutA_F
CACTAAGATTCAAAGAAAAGATTTTGCTGGA//ATTCAGAACGTAAGTCATCTGATCCT(57bp)
MutA_R
AGG ATC AGA TGA CTT ACG TTC TGA AT//TCCAGCAAAATCTTTTCTTTGAATCTTAGTG

MutB_F
CACTAAGATTCAAAGAAAAGATTTTGCTGGA//ACAAGTTCCGTGGAATGTTTGGAAC (56bp)
MutB_R
GTTCCAAACATTCCACGGAACTTGT//TCCAGCAAAATCTTTTCTTTGAATCTTAGTG

MutC_F
GTC TTC CTG GTT CAA GCC C//GC TGG ACT TAC CTT CCT TGA G (40bp)
MutC_R
C TCA AGG AAG GTA AGT CCA GC//G GGC TTG AAC CAG GAA GAC

MutD_F
GTC TTC CTG GTT CAA GCC C//AC AAG TTC CGT GGA ATG TTT GG (41bp)
MutD_R
CC AAA CAT TCC ACG GAA CTT GT//G GGC TTG AAC CAG GAA GAC

MutE_F
GTC TTC CTG GTT CAA GCC C//CA GAT TTC TGG ATT GTT AGA ATT AG AG (46bp)
MutE_R
CT CT AAT TCT AAC AAT CCA GAA ATC TG//G GGC TTG AAC CAG GAA GAC

MutF_F
CTCAGGATCTTTAAACTCCATTCCC//CCCCTTTCTTCTTTAACATTCTTAAACTTAC (56bp)
MutF _R
GTAAGTTTAAGAATGTTAAAGAAGAAAGGGG//GGGAATGGAGTTTAAAGATCCTGAG

MutG_F

CTCAGG ATC TTT AAA CTC CAT TCC C//AT TCA GAA CGT AAG TCA TCT GAT CCT (51bp)
MutG _R
AGG ATC AGA TGA CTT ACG TTC TGA AT//G GGA ATG GAG TTT AAA GAT CCT GAG

MutH _F
CTC AGG ATC TTT AAA CTC CAT TCC C//AC AAG TTC CGT GGA ATG TTT GGA AC (50bp)
MutH_R
GTT CCA AAC ATT CCA CGG AAC TTG T//GG GAA TGG AGT TTA AAG ATC CTG AG

MutI _F
CTCAGGATCTTTAAACTCCATTCCC//GATGAAAGTTTGTTTCAGGTTATGAAAC (53bp)
MutI _R
GTTTCATAACCTGAAACAAACTTTCATC//GGGAATGGAGTTTAAAGATCCTGAG

MutJ _F
CTCAGGATCTTTAAACTCCATTCCC//ACCCTTAATGGAGTTGGTAATTTTAGAG (53bp)
MutJ _R
CTCTAAAATTACCAACTCCATTAAGGGT//GGGAATGGAGTTTA AAG ATC CTG AG

MutCK_F
C AAG CTT GCG GCC GCG AAC TTC ATT TGC TCC TGT GAA TTC C (41bp)
MutCK_R
G GAA TTC ACA GGA GCA AAT GAA GTT CGC GGC CGC AAG CTT G

MutCD_F
C AAG CTT GCG GCC GCG CGC CTC TCG GTG TCG G (32bp)
MutCD_R
C CGA CAC CGA GAG GCG CGC GGC CGC AAG CTT G

Mut_del_ICD_F
CGT TTC CAT GGC CTG TGG TAG GGA TCC CGG GTG GC (35bp)
Mut_del_ICD_R
GC CAC CCG GGA TCC CTA CCA CAG GCC ATG GAA ACG

Primers for cloning of human TLR2 intracellular domain in pRK5/SN-Myc-Vector

Forward: ATCTTGGTCGACTCGTTTCCATGGCCTGTGG (31bp) Sal I
Reverse: GTACATGCGGCCGCCTAGGACTTTATCGCAGCTC (34bp) Not I

Primers for sequencing

F1-hT2
ATG CCA CAT ACT TTG TGG ATG G (22bp)

F2-hT2
CTA ATT TAT CGT CTT CCT GGT TC (23bp)

F3-hT2
CAG AAC TAT CCA CTG GTG AAA C (22bp)

F4-hT2
CCT GGC CCT CTC TAC AAA CTT (21bp)

F5-hT2
CAC ACT GAA GAC TTT GGA AGC TG (23bp)

F6-hT2
CAG GAG CTG GAG AAC TTC AAT C (22bp)

R1-hT2
CTA GGA CTT TAT CGC AGC TCT C (22bp)

R2-hT2
CCG CTT ATG AAG ACA CAA CTT G (22bp)

R3-hT2
GAG GAA TTC ACA GGA GCA AAT G (22bp)

R4-hT2
CAG AGT GAG CAA AGT CTC TCC (21bp)

R5-hT2
CCT GAA ACA AAC TTT CAT CGG TG (23bp)

R6-hT2
GAA GAA AGG GGC TTG AAC CAG (21bp)

CMV30 (pFLAG-CMV 5 prime)
AATGTCGTAATAACCCCGCCCCGTTGACGC (30bp)

CMV24 (pFLAG-CMV 3 prime)
TAGGACAAGGCTGGTGGGCAC (24bp)

F1-mT2
ATG CTA CGA GCT CTT TGG CTC (21bp)

F2-mT2
T TTG TCT GAT AAT CAC CTA TC (21bp)

F3-mT2
CGA TGA AGA AGC TGG CAT TC (20bp)

F4-mT2
C CTG GCC TTC TCT ACA AAC C (20bp)

F5-mT2
CAA ACT GGA GAC TCT GGA AG (20bp)

F6-mT2
TGG TCC AGC AGC TGG AGA AC (20bp)

R1-mT2
CTA GGA CTT TAT TGC AGT TCT C (22bp)

R2-mT2
CCC GCT TGT GGA GAC ACA G (19bp)

R3-mT2
AAG GAT AGG AGT TCG CAG GAG (21bp)

R4-mT2
TT GCA TTG ATC TCA AAT GAT TC (22bp)

R5-mT2
C AGG AGC TCG TTA AAG CTT TC (21bp)

R6-mT2
AA GAG GAA AGG GGC CCG AAC (20bp)

3F-mT2
CG CAA GAT AAT GAA CAC CAA G (21bp)

5R-mT2
C AGA AGC ATC ACA TGA CAG AG (21bp)

T7 primer (pCDNA3.1(-) 5 prime)
TAATACGACTCACTATAGGG (20bp)

BGH primer (pCDNA3.1(-) 3 prime)
TAGAAGGCACAGTCGAG (17bp)

Primers for construction of mT2ECD in pCDNA3.1 (-) for overexpression as antigen for mAb generation

Forward: TATAT GCGGCCGC *CCACC* ATGCTACGAGCTCTTTGGCTC (40bp) Not I
Reverse: TATAT GCGGCCGC CCTGGTGACATTCCAAGACGGAG (36bp) Not I

2.1.7 Reagents

B. subtilis (DSMZ.1087) and *E. coli* (DH5α, Gibco BRL, Gaithersburg, MD) were cultured in standard brain-heart medium over night at 37°C. Bacterial cells were adjusted to a concentration of 1 x 10^{12} cfu/ml. Bacterial suspensions were heat inactivated at 56°C for 45 min and adjusted to a concentration of 1 x 10^{9} cfu/ml in cell culture experiments (h.i.B.s., heat inactivated *B. subtilis*; h.i.E.c., heat inactivated *E. coli*). *B. burgdorferi* inactivated through sonication (s.B.b.) was kindly provided by Janis Weis (University of Utah, Salt Lake City, UT) and applied at a protein concentration of 1.9 µg/ml. LPS from *E. coli* 0111:B4 (Sigma, Deisenhofen, Germany) was generally applied at a concentration of 0.1 µg/ml. Soluble peptidoglycan (sPGN) was prepared from *S. aureus* Rb by vancomycin affinity chromatography and applied at a concentration of 10 µg/ml or as indicated. Highly purified LTA from *B. subtilis* (DSMZ 1087) was applied at a concentration of 5 µg/ml. Synthetic mycoplasmal macrophage activating lipoprotein (R-MALP)-2 was from Dr. Muehlradt (GBF Braunschweig, Germany) and applied at a concentration of 1.3 ng/ml or as indicated. Synthetic N-palmitoyl-S-(bis (palmitoyloxy) propyl) cysteinyl-seryl-(lysyl) 3-lysine (P_3CSK_4), S-bis (palmitoyloxy) propyl-CSK_4 (P_2CSK_4), and N-palmitoyl-CSK_4 ($PCSK_4$) were purchased from ECHAZ microcollections (Tuebingen, Germany) and applied at a concentration of 0.1 µg/ml if not indicated otherwise. Lipidated OspA, a tripalmitylated

lipoprotein from *B. burgdorferi*, was from Dr. Dunn (Brookhaven National Laboratory, Upton, NY) and applied at 4.5 μg/ml. Highly purified recombinant chlamydial HSP60 was applied at a concentration of 8 μg/ml (135). Zymosan and phorbol 12-myristate 13-acetate (PMA) were from Sigma and applied at concentrations of 50 μg/ml and 0.1 μg/ml, respectively. Ultra pure LPS from *Salmonella minnesota Re595* was from List Laboratory (Campbell, California, USA), recombinant murine IFNγ and IL-1β from Peprotech (London, England), and D-galactosamine from Sigma (Deisenhofen, Germany).

2.1.8 Cell lines

Human Embryonic Kidney (HEK) 293 cells (ATCC-Nr. CRL-1573) are fibroblast cell like and provide a well characterized experimental system for investigation of TLR function.

RAW 264.7 cell (ATCC-Nr. TIB-71) was established from a tumor induced by Abelson murine leukemia virus. It is mouse monocyte/macrophage like and served as a popular experimental system for functional investigation of various innate immune molecules such as TLRs.

THP-1 cells (ATCC-Nr. TIB 202) are human macrophage like cells originating from a leukemic cell line cultured from the blood of a boy with acute monocytic leukemia.

2.1.9 Mice

Matched groups of wild-type (*TLR2*$^{+/+}$) C57BL/6 and *TLR2*$^{-/-}$ mice generated by Deltagen (Redmond City, California, USA) were kindly provided by Tularik (South San Francisco, California, USA; nine-fold crossed towards B57BL/6 background).

2.2 Methods

2.2.1 Site directed mutagenesis

A wild-type human TLR2 expression plasmid lacking the original leader sequence in favor of a 5'-terminally fused trypsin leader followed by a Flag-tag coding sequence (pFLAG-CMV, Sigma) was employed as template in overlap-PCR based mutagenesis (Quick change kit, Stratagene, Amsterdam, Netherlands). Deletion mutants lacking the following internal peptides as determined from the the TLR2 cDNA sequence (gene bank accession number HSU88878) were generated: Mut1 (ΔS48-F170), Mut2 (ΔF170-D301), Mut3 (ΔR302-T431), Mut4 (ΔS424-N533), MutA (ΔL173-L196), MutB (ΔL173-V220), MutC (ΔL123-F170), MutD (ΔL123-V220), MutE (ΔL123-N274), MutF (ΔS48-K121), MutG (ΔS48-G196), MutH (ΔS48-V220), MutI (ΔS48-T262), and MutJ (ΔS48-C287), as well as MutCK (ΔK19-N533), and MutCD (ΔK19-N578). Positioning of deletion termini was performed by application of the psipred software program (http://bioinf.cs.ucl.ac.uk/psipred/). Minimal changes of the secondary structure and line up of LRR β sheet sub domains as revealed from computer based calculation served as main criterion.

Fig. 2.1 illustrates the basic mechanism for the site directed mutagenesis. The primers for mutagenesis PCR were calculated T_m of ≥78°C (T_m = 81.5 + 0.41 x (% G/C)-675 / size - % mismatch) and designed to provide an approximately 15 bp flanking region of complementary basepairs at both sides of the mutations introduced. Both sides (on the left and right arm of the mutations) of the primer had a similar T_m and carried 3'-terminal C or G to improve accurate annealing and polymerization. The reaction was carried out in a T3 Thermocycler (Biometra).

The parental DNA template is methylated and therefore sensitive to Dpn I restrict digestion. After digestion with 10 units Dpn I specifically newly amplified plasmid (mutant) DNA was not degraded. Subsequently, DNA was precipitated in EtOH (70%), NaAc (300 mM) at -20°C for 1 h and pelleted at 13000 rpm (Biofuge fresco, Haraeus) for 15 min. The pellet was washed in 70% EtOH, air-dried and finally resolved in 10 µl ddH_2O for transformation in bacteria. The complete sequence of human TLR2 mutants was confirmed by sequencing.

Reaction mix (1x):		5 µl	10x Reaction buffer	
		50 ng	Template DNA	
		0.4 µM	Primer (each)	
		1 µl	dNTP-Mix (2.5 mM each)	
		1 µl	PfuTurbo DNA-Polymerase	
		ddH_2O		
		50 µl		
PCR-cycling:	1.	95°C	30 sec	
	2.	95°C	30 sec	12-18x
	3.	55°C	1 min	
	4.	68°C	2 min/kb	
	5.	68°C	step 4 + 1 min	
	6.	4°C	-	

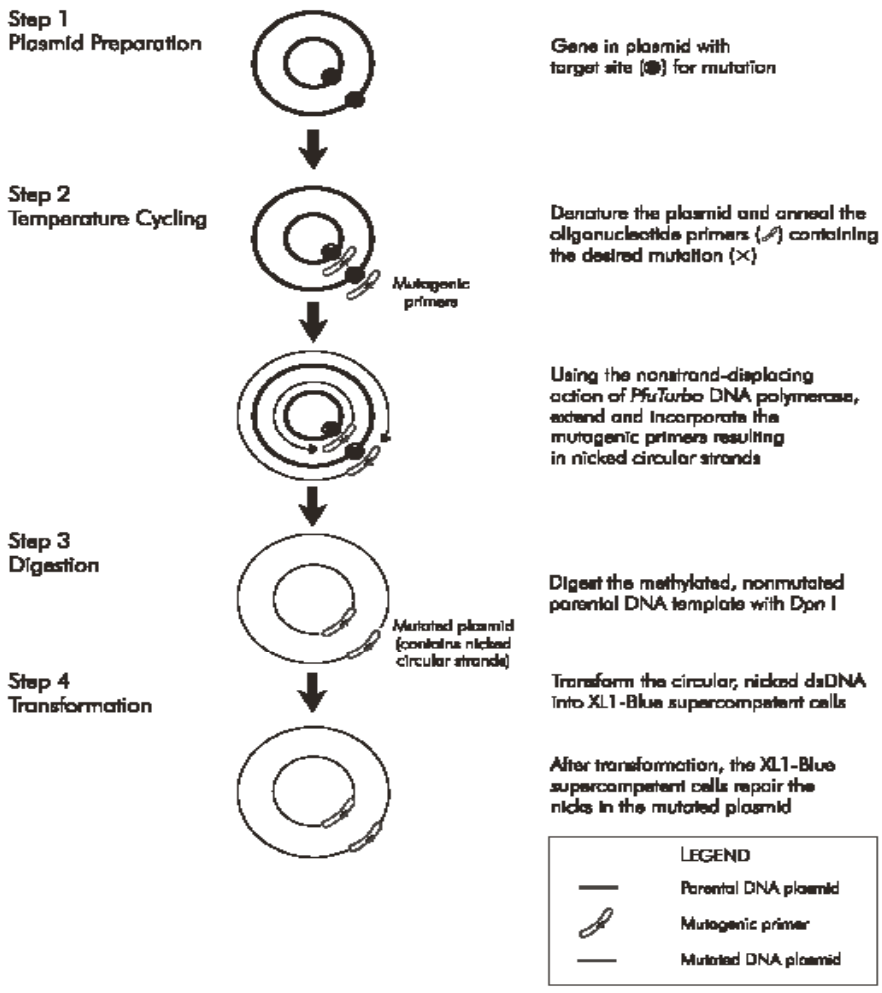


Fig. 2.1 Cartoon illustrating site directed mutagenesis.

2.2.2 Restrict digestion and ligation of DNA

DNA was digested for analytical or preparative purposes. Reactions were carried out with excess of restriction enzymes (purchased from Invitrogen and New England Biolabs) in a total volume of 20 µl. Digestion was performed for a maximum of 4 h and DNA was resolved on an agarose gel. For preparation, bands of interest were cut out under mild UV-conditions and DNA was purified using a gel extraction Kit (QIAgen). Vector and insert DNA were ligated and transformed in *E. coli*. Miniprep plasmid DNA using a Miniprep Kit (QIAagen) and analytic restriction digestion, confirming proper ligation of the insert, was performed.

DNA fragments (vector and insert) with compatible ends were ligated using T4-DNA ligase according to a ligation protocol (Promega). Vector and insert were mixed in a molar ratio of 1:2, the reaction mixture was prepared and incubated for 5-10 min at RT.

Reaction mix (1x):	2 µl	10x Ligase buffer
	50-100 ng	DNA (vector-insert mix)
	1 µl	T4-DNA-Ligase
		ddH_2O
	10 µl	

2.2.3 Agarose gel electrophoresis

DNA fragments were resolved on agarose gels. Agarose was dissolved in 100 ml of TAE buffer by heating for approximately 1-2 min in the microwave. After cooling to 50°C, ethidium bromide (EB) was added to a final concentration of 300 µg/l and mixed properly. The gel was poured and combs were attached. Polymerization at room temperature (RT) lasted around 15-20 min. The gel was transferred into the TAE buffer filled chamber and covered with buffer. DNA samples were mixed with a 6 x DNA loading buffer and loaded into the slots (maximum volume 25-30 µl). As size marker, a 1kb-ladder (Gibco) was used. The gel was run at 10 V/cm until intended resolution was achieved.

2.2.4 Transformation of *E. coli*

For transformation of ligated DNA, 5 µl of prechilled reaction and 50 µl chemically competent *E. coli* DH5α-cells (Clontech) were incubated on ice for 30 min. For retransformation of plasmids, 50-100 ng plasmid-DNA (max. 2 µl) and 20 µl competent *E. coli* DH5α-cells were used. After 30 min, a heat shock was performed for 30 sec at 37°C, followed by incubation on ice for 2 min. For regeneration, transformed cells were incubated

under constant agitation for 1 h in 1 ml of LB-medium. 100-200 µl of bacterial suspension were plated on LB-amp-plates and incubated o.n. at 37°C.

For transformation of mutagenesis products, 50 µl of competent *E. coli* XL10-Gold cells and 2 µl β-Mercaptoethanol (Stratagene) were incubated on ice for 10 min, 5 µl of prechilled PCR product was added and incubation was prolonged for 30 min. A heat shock for 30 sec at 42°C was performed, followed by incubation on ice for 2 min. Regeneration and plating was performed as described above.

2.2.5 DNA Plasmid preparation from *E. coli*

Plasmid preparation in mini- and maxi- scale was performed using Kit-systems purchased from QIAgen.

For mini preparation, a single clone was picked from a plate and inoculated in 3 ml of LB-amp medium. The culture was grown o.n. at 37°C under constant agitation. 2 ml of cell suspension was pelleted (1 min, 13000 rpm, Biofuge fresco) and plasmid was prepared according to manufactures protocol. Plasmid DNA was eluted in 30 µl of ddH_2O.

For maxi preparation, clones from a plate or a glycerol stock were inoculated in 200 ml of LB-amp medium and grown o.n. at 37°C under constant agitation. At the following day, cells were pelleted by centrifugation for 15 min at 6000 rpm (Sorvall RC26 plus, rotor SLA1500) and plasmid preparation according to the protocol was performed. DNA was eluted in 100-200 µl of ddH_2O and concentration was determined photo metrically at a wavelength of 260 nm. An optical density of OD_{260} = 1.0 corresponded to a concentration of 50 ng/µl of double stranded DNA.

2.2.6 Silver and coomassie brilliant blue staining of protein resolved on gel

A. Silver staining

1. Soultions

a. Fix soultion:
   500 ml/l Methanol
   120 ml/l Acidic Acid

b. Ethanol solution:
   500 ml/l Ethanol

c. Sodium-Thiosulfat solution: ($Na_2S_2O_3.5H_2O$), fresh
   10 ml/l Na-Thiosulfat from Na-Thiosulfat Stock solution (10 x, 2 g/l in ddH_2O)

d. SilverNitrate solution: fresh
   100 ml ddH_2O
   0.4 g SilverNitrate ($AgNO_3$)

76 µl Formaldehyd 37%

e. Developing solution: fresh

100 ml Na_2CO_3 from Na_2CO_3 Stock solution (60 g/l, water-free, in ddH_2O)

200 µl Na-Thiosulfat from Na-Thiosulfat Stock solution (10 x, 2 g/l in ddH_2O)

50 µl Formaldehyd 37%

2. Procedures

(Always handel the gel with gloves bearing hands!)

a. 30 min incubation in Fix soultion;

b. 3 times wash with Ethanol solution, 15 min each time;

c. Incubate gel in Sodium-Thiosulfat solution for 1 min;

d. 3 times wash with ddH_2O, 20 sec each time;

e. 20 min in SilverNitrate solution incubation;

f. 2 times wash with ddH_2O, 30 sec each time;

g. Incubate in developing solution;

Crucial! One has to stand by to stop reaction when the protein band is clear!!

h. Stop developing with Fix soultion;

i. Scan gel or dry it for record.

B. Coomassie Brilliant Blue staining

a. Staining with 0.025% Coomassie Brilliant Blue R-250 in (50% methanol, 12% Acidic acid) at room temperature for $\geq$ 1 hour, until protein bands are visible.

b. Destaining with 50% methanol, 12% Acidic acid until protein bands get clear (background decrease).

c. Dry gel in drying buffer (12% ethanol, 5% glycerol) covered with drying buffer pre-weted Cellophane (Novex, San diego, CA, USA) fixed in a proper frame.

Notes:

a. The concentration of methanol in the buffers used above is for staining of 12-15% gels, for low percentage gels, it can be as low as 40% or 30%.

b. The stained gels can be kept in water for 1 or 2 days before drying.

2.2.7 Cytochemical staining

A. Cytochemical staining of TLR2

Macrophage cells or Pools of transfected HEK293 cell clones were grown on polylysine-coated glass-carriers each with 8 culture dishes with removable walls (Becton Dickinson, Le Point de Claix, France). Cells were washed with PBS and incubated with 50

μg/ml Alexa Fluor 488-conjugated concanavalin A (Molecular Probes, Amsterdam, Netherlands) in serum free DMEM medium at 4°C for 15 min. The medium was removed and the cells were washed with PBS and fixed with 2 % formalin for 20 min at room temperature. Cells were washed and blocked with PBS containing 0.2 % saponin and 3 % BSA for 30 min at room temperature. A first antibody, either anti Flag polyclonal rabbit antisera (3 μg/ml) from Sigma, mouse monoclonal anti human TLR2 TL2.1 (5 μg/ml) provided by Egil Lien or mouse monoclonal anti mouse TLR2 antibody such as mT2.5 (2 μg/ml) was applied prior to washing after 30 min of incubation. As a second antibody, Alexa Fluor 546-conjugated Goat anti rabbit/mouse IgG (4 μg/ml) was applied for 30 min (Molecular Probes, Amsterdam, Netherlands) and washed. Cells were sealed in the presence of mounting fluid (*C. pneumoniae* micro-IF, Labsystems Oy, Helsinki, Finnland) for analysis with a laser scanning microscope with documentation unit (LSM510, Carl Zeiss, Oberkochen, Germany).

B. Cytochemical staining of NF-κB

THP1 cells were grown on coverslips (Eppendorf). Primary human macrophages were isolated as $CD14^+$ peripheral blood leukocytes by centrifuagtion of heparinized blood in Ficoll (Seromed, Munich). Followed by isolation with magnetic anti-CD14 antibody beads and MS^+ Separation Column (Miltenyi Biotec, Auburn, CA), and seeded onto Cellocate ccoverslips (Eppendorf) at a density of 5×10^4 cells and cultured in RPMI containing 20% of autologous serum (136). Cells were washed with PBS, permeabilized and fixed with Methanol at -20°C for 8 minutes. Then, cells were washed 3 times with PBS and blocked with 2% goat serum containing PBS at room temperature for 20 minutes and incubated with 4 μg/ml anti NF-κB/p65 (polyclonal rabbit, Santa Cruz) at 37 °C for 1 hour in a humid chamber. After 3 times wash with PBS, a specific secondary α rabbit IgG antibody labeled with Alexa-Flour-546 (4 μg/ml) was applied and cells were incubated at 37 °C for 30 minutes in a humid chamber. After wash with PBS, cells were sealed in the presence of mounting fluid (*C. pneumoniae* micro-IF, Labsystems Oy, Helsinki, Finnland) for analysis with a laser scanning microscope with documentation unit (LSM510, Carl Zeiss, Oberkochen, Germany).

2.2.8 Cell culture

The human embryonic kidney cell line (HEK) 293, as well as *TLR2*$^{-/-}$ embryonic fibroblasts (MEFs) were applied for protein overexpression and functional analysis. *TLR2*$^{-/-}$ mice were kindly provided by Tularik Inc. (San Francisco, CA). *TLR2*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were generated from embryos isolated at day 12 post fertilization. Cells were grown under regular mammalian cell culture conditions in Dulbecco's modified eagle medium (DMEM, Invitrogen, Auckland, Scotland) supplemented with 10 % FCS (Roche), standard antibiotics (Invitrogen, Auckland, Scotland), and 50 μM Thioglycerol (Sigma). Cells

were passaged and expanded for 5 times. Frozen stocks were thawed and cultured for experiments.

HEK 293 cells and RAW264.7 cells were cultured as adherent monolayer at 37°C, 8% 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 resuspend. 1/10 of this solution was transferred to a new plate and fresh medium was added.

For preparation of frozen stocks, cells were grown on 15 cm plates to high density, detached by incubation with trypsin-EDTA solution and spun down for 7 min at 1200 rpm (Megafuge 1.0RS, Haraeus). The cell pellet was resuspend in 1 ml of ice cold freezing medium and kept for 2 h at -20°C before the tube was finally transferred to -80°C. For prolonged storage, cells were transferred to liquid nitrogen tanks. To reculture the cells, cells were thawed rapidly at 37°C and washed immediately with 10 ml of pre-warmed medium. The cells were spun down, resuspend in medium and transferred to a 15 cm plate. After o.n. culture, cells were used for experiments.

Hybridoma and THP1 cells were grown in suspension culture. For culture, 10-50 fold dilutions in fresh medium were prepared and grown in tissue culture flasks for three days.

2.2.9 Transient and stable transfection of HEK 293 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 1-2 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:	150 ng	DNA
(per well)	0.98 µl	$CaCl_2$ (2 M)
		ddH_2O
	7.8 µl	total volume
10-cm dish:	10-50 µg	DNA
(per dish)	62.5 µl	$CaCl_2$ (2 M)
		ddH_2O
	500 µl	total volume

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 HEK 293 clones, the plasmid pTK-neo, encoding the neomycin resistance gene, was co-transfected in a ratio of 1:20. Transfected clones were positively selected in G418 supplemented medium. G418 inhibits growth of untransfected cells. Cells were transfected in a 10 cm dish as described above. In the morning after transfection, fresh medium containing 10% FCS and G418 at a concentration of 600 µg/ml was added. In the course of selection, specifically transfected cells were able to grow and formed dense islands. These clonal cell aggregates were picked and expanded stepwise (24-well to 6-well-plates) under constant selection. Screening was performed by recombinant protein detection through immuno blot analysis. Stocks were prepared for positive clones.

2.2.10 Electroporation

A. Prepare:

a. 400µl electroporation medium (25% FCS in RPMI or DMEM, depending on the culture conditions of regarding cells, eg, RWA264.7 cell---RPMI, HEK 293 cell----DMEM) for each sample.
b. Electroporation machine at 960 µFD and X Volt (RAW264.7, 280V; HEK293, 220V; mEF, 260-300V; ES cells, 340V).
c. BioRad cuvet.
d. Cuvet holder.

B. Procedures:

a. Count and prepare cells according to the calculation of 5 x 10^6 cells / electroporation / 410 µl electroporation medium.
b. Prepare 20 µg DNA / electroporation / 410 µl electroporation medium in BioRad cuvet.
c. Electroporation

   Power on,

   Back connection to get extender to 960 µFD,

   Set voltage,

   Put DNA/Cell mixture in cuvet at position for electroporation,

   Press both bottons for electroporation,

   Stop press when machine alarms and remove cells back to clean bench.

d. Put electroporated cells into 10 ml normal culture medium, let stay at room temperature for 10 minutes.
e. Pellet cells down (1200 rpm, 4°C, 7 min).
f. Resuspend cells with culture medium, incubate under normal condition for cell culture.

2.2.11 Protein isolation

HEK 293 cells were detached from the plate with 5 ml of chilled PBS and harvested by spinning for 7 min, 4°C, 1200 rpm (Megafuge 1.0RS, Haraeus). The cell pellet was mixed in 40-100 µl of lysis buffer, transferred to eppendorf tubes and incubated on ice for 20 min. Cell debris was removed from the suspension by spinning twice for 20 min, 4°C, 13000 rpm (Biofuge fresco).

2.2.12 Electro mobility shift assay (EMSA)

1 x 10^6 HEK293 cells or double amount of RAW264.7 cells were stimulated for 2 h in DMEM or RPMI medium containing 2 % FCS. Briefly, cells were washed with ice cold PBS and lysed (10 mM HEPES pH 7.9, 10 mM KCl, 0.5 % NP 40, 0,1 mM EDTA, 0.2 mg/ml leupeptin and aprotinin, 0.5 mM PMSF, and 1 mM DTT). Nuclei were pelleted with high speed (13000rpm) for 15 min at 4°C and lysed (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 0.2 mg/ml leupeptin and aprotinin, 1 mM PMSF, and 1 mM DTT) followed by a sonication for 10 seconds. Debris was pelleted at 13000rpm for 15 min at 4°C and supernatant recovered. 5 µg of protein was applied to electro mobility shift assay (EMSA) with a radioactively labeled double stranded DNA oligonucleotide (5'-GATGCC ATTGGG GATTTC CTCTTT ACTG-3') representing a NF-κB recognition element of the ELAM-1 promoter sequence. Results were visualized by phospo-imager (Storm 840, Molecular Dynamics, Amersham Biosciences, Freiburg, Germany) aided analysis.

2.2.13 Immunoprecipitation

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. Mutant constructs and controls applied were overexpressed as Flag-tagged hybrid proteins while the coexpressed protein was Myc-tagged. Flag mAb M2 beads were used for precipitation (Sigma) (137, 138). Immune complexes were analyzed by application of polyclonal anti Myc-tag antiserum for immuno blot analysis (Santa Cruz, CA).

For characterization of mouse TLR2 specific monoclonal antibodies, lysates of Flag-TLR2 transfected HEK293 cells or macrophages, as well as 1 µg of antibody and protein G

beads (Santa Cruz, California, USA) were mixed for o.n. precipitation. Immune complexes or cell lysates were analyzed by immunoblot analysis as described (135). Precipitations were controlled by application of Flag specific (mAb M2, Sigma) or protein G beads only. Flag (HEK293) or mTLR2 (RAW264.7) specific antisera were applied for immunoblot analyses. In contrast, total lysates of macrophages (see inhibition experiments) were analyzed for phosphorylation of kinases indicated.

2.2.14 SDS-Polyacrylamide-gel electrophoresis (SDS-PAGE)

Proteins were seperated due to their size by SDS-PAGE described by Laemmli (1970). The lengh of the stacking gel was 1 cm, of the separating gel 5 cm, while the thickness was 1 mm. The gels were prepared as follows:

	Separating gel			**Stacking gel**
	8%	10%	12%	5%
Acrylamide-bisacrylamide[1]	2.6 ml	3.3 ml	4 ml	0.66 ml
1.5 M Tris-HCl pH 8.8	2.5 ml	2.5 ml	2.5 ml	0,3 ml[2]
10% (w/v) SDS	100 µl	100 µl	100 µl	200 µl
ddH_2O	4.4 ml	4 ml	3.3 ml	3.9 ml
TEMED	5 µl	5 µl	5 µl	5 µl
10% (w/v) APS	50 µl	50 µl	50 µl	25 µl

[1] 29:1 (Biorad)

[2] 2.5 M Tris-HCl pH 6.8

The separating gel was poured and overlaid with isopropanol to ensure homogenous polymerization. After incubation for around 20-30 min at RT, the gel was washed with ddH_2O, the stacking gel was poured, and the combs were attached carefully avoiding trapping of air bubbles. The gels were kept wrapped in wet sheets at 4°C for up to 1 week. For electrophoresis, gels were attached to chamber reservoirs, filled with Laemmli buffer, and air bubbles were removed. Protein samples were denatured for 5 min at 95°C and spun down for 1 min at 13000 rpm (Biofuge fresco) before supernatants were loaded to the gels. Electrophoresis was performed at 12 V/cm for the stacking gel and then increased to maximum speed of 20 V/cm until the control dye ran out completly.

Separated protein samples were transferred to nitrocellulose-membranes by semi-dry electroblotting. The membranes and filter paper were pre-wet in blotting buffer. The gel was carefully placed on the membrane and positioned between two layers of paper. Protein was

blotted from the gel (cathode side) towards the membrane (anode side) for 1 h 10 min at 1 mA/cm^2.

2.2.15 Immunoblot detection of protein

HEK293 cells were lysed upon protein overexpression and stimulation for 30 min. Lysates from 2.5 x 10^5 cells or immune complexes prepared from 3 x 10^6 cells for each sample were prepared and analyzed by immunoblot analysis. For analysis of JNK phosphorylation three fold amounts of total lysates (approx. 7.5 x 10^5 cells) were applied. Rabbit polyclonal anti sera specific for phosphorylated p38, ERK 1/2, JNK, or Akt/PKB were used (Cell signaling, Frankfurt, Germany). Specific epitopes were visualized by enhanced chemoluminescence (ECL) (Western lightning, Perkin Elmer, Boston, MA).

The following is the brief procedure of immuno bloting experiment. The membrane (blot) was briefly washed in PBT prior to blocking for at least 1 h at RT. Incubation with primary antibody was performed o.n. at 4°C. Primary antibody was used at dilutions 1:1000 (α Flag) or 1:300 (poly α mouse TLR2) in blocking buffer. The blot was washed 3 times for 5 min in PBT and incubated with secondary antibody (1:5000 dilution of anti rabbit/mouse-HRP in blocking buffer), washed twice with PBT and once with PBS for 5 min each.

Washing with PBS was necessary to remove Tween which interferes with HRP activity. All washing steps and the incubation with secondary antibody was done in a small tray on the shaker, whereas incubation with primary antibody was done in a 50 ml tube on the roler. For detection of bound antibody, the blot was overlied with 2 ml ECL Reagent Plus (Perkin Elmer) and incubated for 1 min. Excess of substrate was removed and the blot placed between a layer of plastik wrap in a film cassette. Exposure to a photographic film was performed for 1 min up to 1 h.

2.2.16 Biosensor based binding analysis (kindly provided by Jochen Metzger and Mark Rutz)

Real-time binding analysis was performed using surface plasmon resonance (SPR) detection on a Biacore X device (Biacore AB, Uppsala, Sweden). The two flow cells (FC) of a streptavidin precoated chip were loaded with biotinylated $PHCSK_4$ (FC1) and P_3CSK_4 (FC2), respectively. Specific binding of a recombinant T2EC protein was controlled by application of a human mAb carrying the same Fcγ domain. This antibody did not bind in either FC1 or FC2 (data not shown). After prior incubation in 45 µl of running buffer (50 mM morpholino ethane sulfonic acid, 150 mM $MgCl_2$, pH 6.5) at 25°C for 15 min, 200 nmol of purified T2EC alone (maximum control) or in combination with mAbs (T2.5 or an isotype matched irrelevant mAb at molar excesses indicated) were injected over FC1 and FC2 at a flow rate of 10 µl/min. For negative control, mAbs alone were administered at the highest

amounts also used for blocking analysis of TLR2 ligand-binding. The values obtained upon continuous resonance monitoring at 25°C over 570 s (delay time 300 s) from the control FC1 were subtracted from the respective values resulting from simultaneously performed analysis of FC2. Generally, biomolecular interaction between receptor and its respective ligands immobilized on the sensor chip is optically monitored as a function of time and expressed in resonace units (RUs). Regeneration of the chip was achieved by washes with 50 mM NaOH, 1 M NaCl and extensive re-equilibration with running buffer.

2.2.17 Generation, identification and purification of TLR2 specific antibodies

A cDNA fragment encoding the N-terminal 587 amino acids of mTLR2 was amplified from a RAW264.7 cell cDNA library (advantage kit, BD Clontech, Heidelberg, Germany). The murine TLR2ECD was fused to a C-terminal thrombin cleavage site followed by a human IgGFcγ moiety. The murine TLR2ECD protein was purified upon overexpression in HEK293 cells and thrombin digestion. A *TLR2*$^{-/-}$ mouse was immunized three times within eight weeks by intraperitoneal (i.p.) injection of 50 µg of TLR2ECD and 10 nmol of a thioated DNA oligonucleotide (5'-TCCATGACGTTCCTGA-3', Tib Molbiol, Berlin, Germany). Its splenocytes were fused with murine P3X cells and hybridomas were selected (116).

Protocol of fusion experiment

1. Preparation of P3X cells

Harvest and count cells, wash 3 times in serum free RPMI, about 1x10^8 cells in 50 ml were sufficient for my experiment with mouse TLR2 mAb generation.

2. Preparation of Spleen cells

A. Prepare paper towers on bench, 70% ethanol in a beaker, sterilize surgical tools in 70% ethanol.

B. Kill the mouse by neck fracture, take off skin, sterilize with 70% ethanol throughly, open peritoneum, carefully get the spleen out and transfer it into 50 ml RPMI for washing, do not hurt the outer membrane of the spleen, no spleen cells should leak out if the capsule is intact.

C. Transfer the spleen from the 1st 50 ml RPMI to the second one for washing (the blood can be collected for polyclonal anti serum collection).

D. Press the spleen through a cell filter (steril, 100µm) with a syringe plug. Collect the push-through from the dish and transfer it to a 15 ml tube, wash twice with serum

free RPMI and resuspend in 5 ml serum free RPMI. Count cells. In my case for anti mouse TLR2 mAb generation, we got $5x10^7$ cells.

3. Fusion experimental procedures

A. Mix spleen cells and P3X myeloma cells by 1:1 ratio in a glass tube with round bottom, pellet and suck off media completely.

B. The following steps need cooperation of two persons: One person keep the tube constantly turning, another one add 1 ml PEG 50% drop by drop in 1 minute, with pipette tip touching the wall of the tube. In the similar way, add 2 ml serum free RPMI in 1 minute (go on side of the tube wall, relatively close to cells to creat a stream). In the same way, add 4 ml serum free RPMI in 1 minute. Then, 8 ml serum free RPMI in 1 minute.

C. Pellet down, RT, 400g, 10 min. Soak off media and release pellet gently, add hybridoma selection media (RPMI, 5% FCS, 1 x HAT, P/S, 1x HFCS or 5 x 10^4/ml macrophage cells as feeder cells) to 40 ml for plating.

D, Plate the first 3 96-well plates, 10 ml per plate, 2 drops (100-125 μl) each well, 37°C, 5% CO_2 incubate.

E. Add 10 ml medium to the rest 10 ml of mixed cells, plate another two 96-well plates.One day later, add another half (100-125 μl) selection medium and change half of the medium every 24 hours. Large cells are fused cells that can survive, spleen cells and P3X cells without fusion will die. Since the unfused normal B cells can not 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 defieiency of hypoxanthine-guanine phosphoribosyI transferase (HGPRT). This defieiency 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.

Protocol of ELISA for mAb clone identification

1. plate coating, 2.7 μl/96 well plate (1.8mg/ml) goat anti huIgG1-Fc-γ in PBS. 4 °C over night, wash with 1 x PBT.
2. blocking, RT, 30-90 min, in blocking buffer (5 g/l sucrose; 1 g/l BSA; 50 mg/l NaN_3 in PBS), wash with 1 x PBT.
3. binding, mT2ECD/ mT9ECD/Vector overexpressing HEK293 cells, 15 cm dish, about 75% confluence, lysate in 2 ml lysis buffer (1% triton, 150 mM NaCl, 1mM EDTA, pH 7.5), take 2 μl for each well of the 96 well plate for binding. RT, 90 min, wash with 1 x PBT.

4. detection, apply hybridoma clone supernatants with positive controls, RT, 90 min, wash with 1 x PBT.
5. apply HRP conjugated anti mouse/rabbit IgG antibody, 1:5000-10000, RT, 90 min, wash with 1 x PBT.
6. substrte application, for each 96 well plate, apply 10 ml phophate citrate buffer (pH 5.0) with 2 µl H_2O_2 and 1 piece of peroxidase substrate, when the positive clones have clear blue signal, add 2N H_2SO_4 to stop the reaction, measure plates with ELISA reader.

Antibody purification from hybridoma cell supernatants

1. Adjust pH of the supernatant according to Table Puri (***table on next page***), referring the isotype of certain antibody clone.
2. Filtrate the supernatant with 0.22 µm filter.
3. Wash the Hi-trap protein A HP column (Amersham Biosciences, Freiburg, Germany) loaded on AKTA prime (Amersham Pharmacia Biotech, Freiburg, Germany) with 20 ml 20% ethanol first, then, wash with PBS, at least 10 column volume.
4. Set program for flow through (binding) of the supernatant, 1 ml/min. Make sure that the supernatant bottle can not be totally empty so that the air can not run into the column.
5. Wash column with the following buffers after binding.
   IgG2a, IgG2b, 50 ml **PBS**
   IgG1, 50ml **50mM Tris, 150 mM NaCl, pH8,5**. 2 ml/min
6. Elute antibody with **Citrate buffer, pH 3,6.** (set the UV lamp for auto zero)
   when OD280 goes high, stop elution program and start Manual runing, set tubes for fractioning (**115 ul** of **1,5M Tris, pH 8,5**), elution fraction, **400 ul**, final pH 7.2-7.4.
7. Wash column with elution buffer further after collection for at least another 2 ml.
8. Wash column with 25 ml PBS first, then with 20 ml 20% ethanol, store column in 20% ethanol at 4 °C.
9. Pool collected protein, dialysis in PBS with Snakeskin pleated dialysis tubing (PIERCE, Rockford, Illinois, USA), 12 hours later, change PBS once, dialysis for another 3 hours.
10. Measure the concentration of antibody with BCA method according to manufactures instructions (BCA protein assay reagent, PIERCE, Rockford, Illinois, USA).
11. Apply silver gel staining, Coomassie Brilliant Blue or PoncauS staining for analysis.
12. Filtrate antibodies with 0.22 µm filter, store at -80 °C.

Table Puri, Affinity of Protein A for selected classes of monoclonal antibodies, approximately.

Antibody	Affinity	Binding pH	Elution pH
Human			
IgG1	Very high	6.0-7.0	3.5-4.5
IgG2	Very high	6.0-7.0	3.5-4.5
IgG3	Low-none	8.0-9.0	<7.0
IgG4	Low-high	7.0-8.0	3.0-6.0
Mouse			
IgG1	Low	8.0-9.0	4.5-6.0
IgG2a	Moderate	7.0-8.0	3.5-5.5
IgG2b	High	about 7.0	3.0-4.0
IgG3	Low-high	about 7.0	3.5-5.5

2.2.18 FACS analysis

Stably transfected HEK293 cell clones, as well as uninduced peritoneal wash-out macrophages were cultured o.n. as described (135). Flow cytometry was performed upon staining with either T2.5, affinity purified polyclonal rabbit antiserum specific for the murine TLR2ECD (139), or the Flag tag (Sigma), as well as rabbit or mouse IgG-specific secondary antibodies (phycoerythrin or Fluorescein Isothiocyanate labelled, BD Pharmingen, Heidelberg, Germany), respectively.

For establishment of mTLR2 expression analysis in primary cells, surface and intracellular T2.5 dependent staining of $CD11b^+$ splenocytes (140) from wild-type and *TLR2*$^{-/-}$ mice challenged with LPS (0.5 mg, i.p., 24h) were compared by flow cytometry (CyAn, Dako Cytomation, Fort Collins, Colorado, USA). Cells were stained with photoactivated ethidium monoazide (Molecular Probes, Amsterdam, Netherlands) immediately upon isolation, followed by TLR2 specific surface staining, or intracellular staining (cytofix/cytoperm, BD Pharmingen). In order to analyze TLR2 expression in non- or *B. subtilis*-infected (5×10^8 cfu, i.p., 24h) mice, peritoneal washout cells and splenocytes (116, 140) from five wild-type or *TLR2*$^{-/-}$ mice were pooled, respectively. Fluorescence labeled cell surface marker antibodies (BD Pharmingen) and T2.5 counter-stained with secondary anti mIgG1 were used as indicated.

Briefly, for surface staining, cells were blocked in 5% normal goat serum (NGS) and 2 µg/ml α-mouse CD16/CD23 antibody (cross reactive for human cells) for 15 min on ice before spun down for 6 min at 4°C, 1000 rpm (Megafuge 1.0RS, Haraeus). Liquid was removed and primary antibody was applied at a concentration of 8 µg/ml and incubated for 20 min on ice. Cells were spun down and unbound antibody was removed by washing twice with 200 µl/well of FACS buffer. Secondary antibody was added to a final concentration of 2 µg/ml. Samples were incubated for 20 min on ice before they were washed twice additionally. Cells were finally resuspended in 300 µl FACS buffer and subjected to FACS analysis. Measurement was carried out in a FACS detector and data were processed applying the Cellquest software.

For intracellular staining, cells were blocked with 5% NGS and Fc Block (α mouse CD16/CD32 (FCγ III/II receptor), 0.5 mg/ml), 1:300 in FACS buffer (10mM $MgCl_2$ and 5mM $CaCl_2$, 2% FCS in 1 x PBS), on ice, 15 min before centrifugation at 1000 rpm, 4°C for 6 min. Then, supernatant was discarded and cells were resuspended in 100 µl of BD Cytofix/Cytoperm intracellular staining buffer per well. Next, samples were incubated for 30 minutes at room temperature and washed twice with 200 µl of 1 x Perm/Wash buffer (BD) per well. Cells were then centrifuged at 250 g for 5 minutes and supernatants between washes were aspirated. Intracellular antigen was stained with specific antibody in 50 µl of 1 x Perm/Wash buffer/well and incubated for 30 minutes at room temperature before the next wash (2 x with 200 µl of 1 x Perm/Wash buffer (BD) per well). Centrifugation at 250 g for 5 minutes was followed and supernatants between washes were aspirated. Then, samples were resuspended and transfered in 400 µl of staining buffer (PBS+2%FCS+0.1% sodium azide) to tubes appropriate for analysis with a flow cytometer. Samples were analyzed on a flow cytometer. If the analysis can not be done immediately upon finish of staining, cells can be fixed in 1 % PFA (PARAFORMALDEHYDE) in 1 x PBS at 4°C and cytometer measurement can be done later.

20 % PFA stock: 10 g PFA in 40ml PBS+8ml NaOH (3M), solve at 56°C, adjust PH to 7.4, fill in with PBS to 50 ml.

2.2.19 Luciferase reporter assay

3 x 10^4 HEK293 cells or *TLR2*$^{-/-}$ MEFs were cultured on single wells of 96-well plates. HEK293 cells were cotransfected with an NF-κB recruiting endothelial-leukocyte adhesion molecule (ELAM)-1 (CD62E) promoter luciferase construct and a Rous sarcoma virus (RSV) promoter β-galactosidase reporter plasmid (141), as well as a cytomegalovirus (CMV)-promoter regulated expression plasmid for human TLR2 by the calcium phosphate

precipitation method (30, 142). For equilibration of expression levels, DNA amounts used were adjusted and expression levels analyzed by immuno blot analysis. *TLR2*$^{-/-}$ MEFs were transfected by electroporation at 960 µF and 260 V (Gene Pulser II system, Biorad, Munich, Germany). 7 h after medium change preparations of bacterial products or analogues were added to transfected cells for 16 h. Cells were lysed for measurement of luciferase and β-galactosidase activities using reagents from Promega (Madison, WI) and PE Biosystems (Bedford, MA). Luciferase activities were related to β-galactosidase activities for normalization.

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 β-galactosidase-assay was performed. Luciferase as well as β-galactosidase activities were determined by chemiluminoscence assays. All assays were prepared in 96-well scale and duplicate values were determined. The transfection mix contained the following compounds:

96-well-plate:	30 ng	pELAM-1-Luc
(per well)	30 ng	pRSV-β-Gal
	1-2.5 ng	expression vector
	80 ng	pRK5 (empty vector)

Cells were transfected as described in 2.2.9. 32 h later, cells were stimulated with TLR agonists for 16 h.

For β-galactosidase-assay, 5 µl/well of each lysate were transferred to light impervious plates and 40 µl/well of substrate (Tropix, PerkinElmer) was added. The mixture was incubated and covered for 1 h at RT. For measurement, the plate was inserted into the luminometer, which automatically injected 30 µl/well of Accelerator solution (PerkinElmer). The emitted light was measured and normalized luciferase activity calculated according to the formula:

Normalized luciferase activity = βgal_{max} x luc / βgal. Diagrams illustrate fold induction of normalized luciferase activity compared to unstimulated vector control.

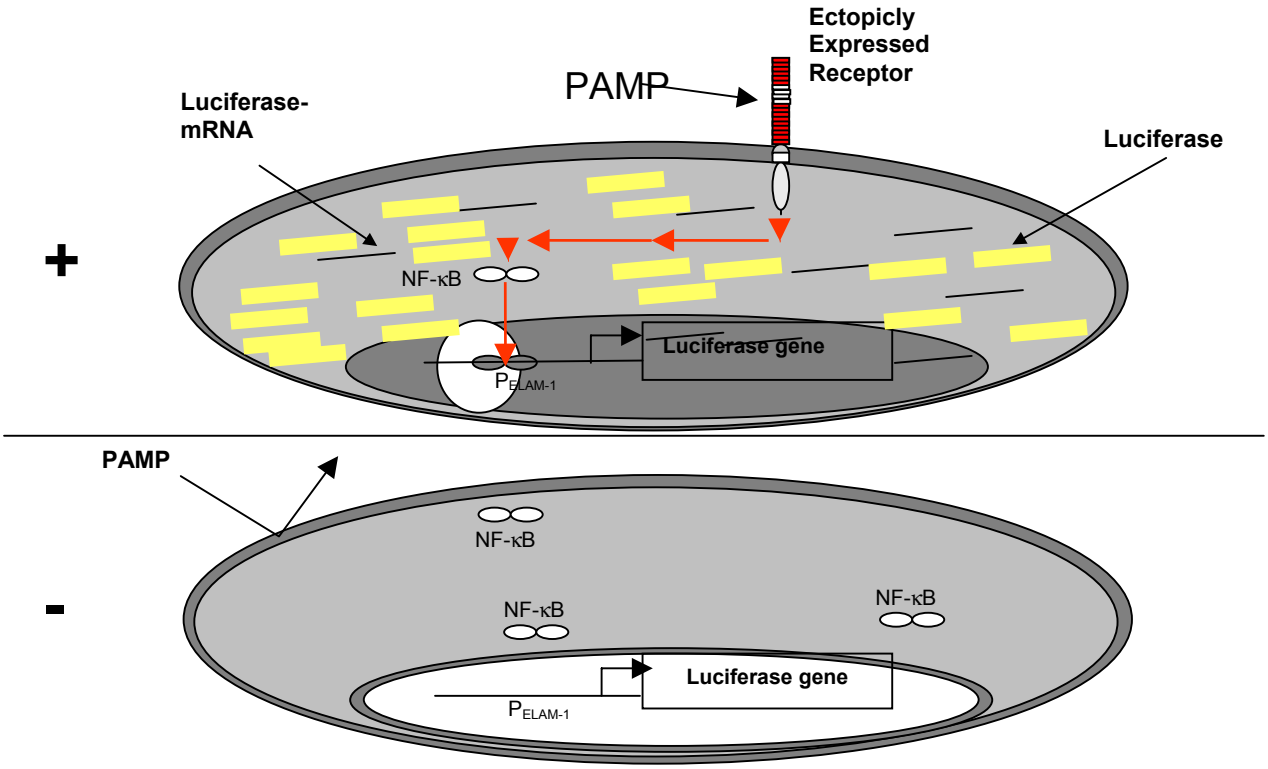


Fig. 2.2 NF-κB dependent luciferase reporter assay. Cartoon illustrating HEK 239 cells which express signaling molecules mediate NF-κB activation but do not express endogenous TLRs to sufficient extents. Therefore, these cells do not react to PAMP challenge without ectopic overexpression of TLRs (lower panel of the figure (-)), however, ectopic expression of TLR2 conferred NF-κB activation upon specific PAMP challenge (uper panel of the figure (+)). In this experimental system, NF-κB activity was correlated with luciferase activity since cells were contransfected with TLR2 and a NF-κB-recruiting endothelial-leukocyte adhesion molecule-1 (ELAM-1) promoter luciferase construct, the later serving as reporter. Upon TLR2 recognition of PAMP, NF-κB is activated and translocated into the nucleus where it binds to the ELAM-1 promoter and leads to expression of the luciferase gene. Accumulated luciferase protein in cytoplasm reflecting NF-κB activity can be then released upon cell lysis and visulized as chemiluminoscence upon reaction with substrate reagent in a luminometer.

2.2.20 Enzyme linked immunosorbent assay (ELISA)

MAb specificities for TLR2ECD, as well as cyto- and chemokine concentrations in cell supernatants or murine sera (see below) were analyzed by enzyme linked immuno sorbent assay (ELISA, R&D systems, Minneapolis, Minnesota, USA) with enzyme mediated colorimetry (Magellan, Tecan, Crailsheim, Germany) according to supplier protocols. Significance of serum concentration differences were determined by application of the student's *t*-test for unconnected samples.

The following is the experimental procedures for the human IL-8 ELISA as an example for all the cyto- and chemokine ELISAs. Interleukin 8 (IL-8) is a member of the neutrophil specific CXC subfamily of chemokines and a potent chemotactic and activating factor. It is produced by many cells in response to proinflammatory stimuli such as IL-1, TNF or PAMPs. HEK 293 cells transfected with TLRs secrete IL-8 in response to stimulation with specific TLR agonists. Measurement of the IL-8 concentrations in the supernatants provides therefore information about TLR dependent cell activation. IL-8 amount was determined by application of ELISA kit purchased from R&D.

96-well plates were coated o.n. at RT with monoclonal αIL-8 capture antibody (2 µg/ml in PBS). The working volume was 100 µl/well, antibody-, conjugate- and standard-dilutions were carried out in reagent diluent. At the following day, plates were washed with PBT in an ELISA washer and blocked for 1 h in ELISA blocking buffer. Liquid was removed and plates were again washed with PBT. A standard curve was calculated upon stepwise dilution of a recombinant human IL-8 standard. The supernatant samples were thawed carefully and administered onto the plate. The plates were incubated for 2 h at RT. After incubation, unbound antibody was removed by washing with PBT. Polyclonal αIL-8-biotin conjugate (detection antibody) was applied at a concentration of 20 ng/ml and plates were incubated for 2 h at RT. Unbound conjugate was removed by washing before HRP conjugated streptavidin (1:200 dilution) was added. Plates were incubated for 20 min at RT, unbound conjugate was removed and the substrate was added. As substrate, 1 tab per plate of 3'3'5'5'-tetramethylbenzidine was resolved in a phosphate-citrate buffer in the presence of 0.006% H_2O_2. Substrate was added and plates incubated in the dark until staining for positive samples was detected. The reaction was stopped by addition of 2 N H_2SO_4. Amounts of catalyzed substrate were determined by measurement of the $OD_{480\ nm}$. Final IL-8 amounts were automatically calculated according to the standard curve. Duplicate values were prepared for all samples.

2.2.21 Inhibition of TLR2 dependent cell activation *in vitro* and *in vivo*

Transiently transfected HEK293 cells, murine RAW264.7, as well as primary macrophages were used for *in vitro* analysis of TLR2 inhibition by monoclonal antibodies generated by us. 50 μg/ml of antibodies were applied 30 min prior to challenge with 100 ng/ml of LPS, IL-1β, P_3CSK_4, or 1 x 10^6 cfu/ml of h. i. *B. subtilis*. HEK293 cells were cotransfected with reporter (141), human CD14, human or mTLR2, and MD2 (provided by Tularik, Drs. Golenbock and Heine, as well as Miyake, respectively) expression plasmids, and NF-κB dependent reportergene activity as well as IL-8 were assayed after 6 h of stimulation (135). TNFα and IL-6 concentrations in supernatants of RAW264.7 and primary murine macrophages, as well as NF-κB translocation in THP1 cells and human macrophages (136) were analyzed 24 h and 90 min after challenge, respectively. RAW264.7 macrophages were used for analysis of challenge and antibody dose dependent NF-κB- and MAP kinase activation. NF-κB specific electro mobiliy shift assay (EMSA), as well as p38, Erk1/2, and Akt phosphorylation specific immunoblot analysis (Cell signaling, Frankfurt, Germany) were carried out in order to analyze cell activation. 1 x 10^6 cells were pretreated with antibodies as described above at various concentrations and stimulated for 90 min (EMSA) or 30 min (kinase phosphorylation analysis) (116).

For analysis of TLR2 inhibition *in vivo*, mice were injected i.p. with 1 mg of T2.5 or left untreated. 1 h later, 100 μg of P_3CSK_4 and 20 mg of D-galactosamine were injected i.p. Serum concentrations of TNFα, GROα/KC, a murine IL-8 homologue, IL-6, and IL-12p40 in five unchallenged control mice were 0.05 ng/ml, 0.43 ng/ml, not detectable, and 0.44 ng/ml, respectively. Significance of results was determined by performance of the student's *t*-test for unconnected samples.

2.2.22 Systemic shock induction

In an experimental sensitization dependent model (57), mice were injected intravenously with 1.25 μg of murine IFNγ. 20 min later, mice were injected i.p. with doses of mAb as indicated. 50 min after IFNγ injection, 100 μg of synthetic P_3CSK_4 and 20 mg of D-galactosamine were injected i.p. as well.

The experimental high dose shock model encompassed a single i.p. injection of 1 x 10^{10} cfu of h.i.*B. subtilis* with prior (1 h) or subsequent (1 h, 2 h, or 3 h) i. p. injection of 1 mg of mAb or as indicated.

3 Results and discussion (I)

Since the extracellular domain (ECD) of TLR2 was considered to interact with various PAMP (143), we hypothesized that different parts of the ECD interact with these ligands. To address the question, TLR2 ECD deletion mutants were generated and the resulting protein constructs were compared in respect to their ability to mediate recognition of a variety of TLR2 specific PAMP. We have found that cell activation by distinct TLR2 specific PAMP requires different subdomains of the TLR2 ECD. Data presented here supplements original publications attached in appendix I.

3.1 Mutagenesis

Single or groups of LRRs from the TLR2 ECD were deleted with the assumption that removal of entire LRR subdomains would not alter overall protein structure (App. I, Fig. 1). All TLR2 constructs were expressed at similar levels as revealed by anti-Flag tag immunoblot analysis of total lysates of HEK293 cells after transfection of equal amounts of specific expression plasmid DNA preparations, either transiently or stably (Fig. 3.1).

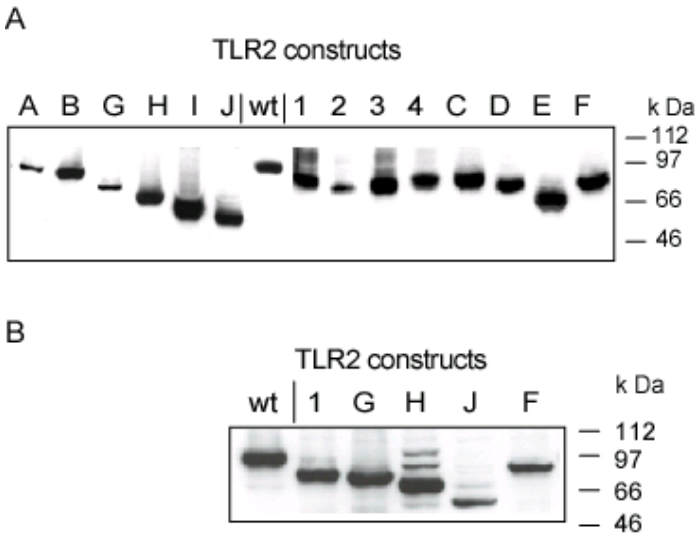


Fig. 3.1 (Suppl. App. I) Expression of wild-type and mutant TLR2 constructs upon transfection in HEK293 cells. HEK293 cells were transfected with Flag-tagged wild-type (wt) and mutant TLR2 expression constructs (Mut 1 to 4, A to J). 48 h after transfection start cells were lysed and subjected to Flag-specific immuno bloting analysis (A). Cell lysates from HEK293 cell clones stably expressing indicated constructs were also analyzed as described above (B).

3.2 Functional analysis of TLR2 ecd mutant constructs

Wild-type TLR2 conferred NF-κB dependent reporter gene activation and release of IL-8 in HEK293 cells upon challenge with all preparations of bacterial products (App. I, Tables 1 and 2). Interestingly, of all mutant constructs analyzed Mut1 mediated a weak signal upon application of P_3CSK_4 while MutG and MutH mediated successively increasing cell activation upon application also of P_3CSK_4, as well as OspA and inactivated *B. subtilis* (App. I, Fig. 1, Tables 1 and 2). Results from analysis of stably and transiently transfected HEK293 cells were similar (Table 3.1, see highlighted numbers in red color; App. I, Tables 1 and 2). Notably, transfection of fifty fold amounts of expression plasmid for MutH as compared to wild-type TLR2 expression plasmid conferred partial cellular activation by the diacylated peptide R-MALP-2. However, activation by application of further TLR2 agonists such as sPGN was barely detectable even upon application of very high amounts of stimulants (App. I, Fig. 2A).

Wild-type TLR2 conferred NF-κB activation upon application of two tripalmitylated peptide derivatives, P_2CSK_4 (S-bis (palmitoyloxy) propyl cysteinyl-seryl-(lysyl) 3-lysine) and $PCSK_4$ (N-palmitoyl-CSK_4) aside from P_3CSK_4 (N-palmitoyl-S-(bis (palmitoyloxy) propyl)-CSK_4). In contrast, the constructs MutG and MutH mediated response to P_3CSK_4, as well as P_2CSK_4 to specify degrees but not to $PCSK_4$ (App. I, Fig. 2B). Similar results were obtained upon transfection of 50 fold amounts of mutant expression plasmid as compared to wild-type TLR2 plasmid, as well as application of very high amounts of stimulants (App. I, Fig. 2C). Cotransfection of both wild-type TLR2 and each of the mutant DNA constructs in a ratio of 1:50 was performed for analysis of potential dominant negative/positive mutant effects on wild-type TLR2 mediated cell activation. TLR2 deletion mutants inhibited wild-type TLR2 mediated cell activation when transfected cells were stimulated with heat inactivated *B. subtilis* (h.i.B.s) or P_3CSK_4 except for MutH (Table 3.2, see highlighted numbers in red color). Consistent with results from analysis of transfection of HEK293 cells, overexpression of wild-type TLR2 restored responsiveness towards LTA, as well as P_3CSK_4 in *TLR2*$^{-/-}$ mouse embryonic fibroblasts (MEFs) as indicated by NF-κB dependent reporter gene activation and release of IL-6. In contrast, MutH mediated cell activation was restricted to P_3CSK_4 stimulation and further mutants such as MutJ were inactive (App. I, Fig. 3).

Table 3.1 (Suppl. App. I)
TLR2 ecd mutant mediated IL-8 release from
HEK293 cells stably expressing regarding constructs

IL-8 (ng/ml)	vect.	wild-type	Mut 1	F	G	H	J
unstim.	-	0,03	-	0,01	-	-	-
s. B.b.	-	0,69*	-	0,01	-	0,01	-
h.i.E.c.	0,01	0,56*	-	0,01	-	0,01	0,01
h.i.B.s.	0,01	0,71*	-	0,01	0,01	0,03	0,01
Zymos.	0,01	0,59*	-	0,01	-	-	-
LPS	0,01	0,61*	-	0,01	-	0,02	-
sPGN	-	0,48*	-	0,01	-	-	-
LTA	0,01	0,59*	-	0,01	-	0,01	0,01
HSP60	-	0,21*	-	-	-	-	-
OspA	-	0,59*	-	0,01	-	0,08*	0,01
P_3CSK_4	-	0,72*	-	0,01	-	0,13*	0,02
MALP-2	-	0,42*	-	0,01	-	-	-
PMA	0,18*	0,21*	0,19*	0,18*	0,16*	0,17*	0,16*

(-) </= 0,005; * P<0,05; significance as revealed from t-test for unconnected samples by relation to vector control for the regarding stimulant (PMA induction values were related to the vect.-unstim.-value).

Table 3.2 (Suppl. App. I)
Effect of TLR2 ecd mutant on the receptor mediated
NF-κB dependent reporter gene activation in HEK293 cells

Rel. luc. act.	vect.	wild-type	Mut 1	2	3	4	A	B	C	D	E	F	G	H	I	J
unstim.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
s. B.b.	11,2	9,4	-	-	-	5,5*	5,5*	10,9	6,3	13,5	14,9	6,6	6,1	7,4	11,7	10,0
h.i.E.c.	7,1	7,4	5,3	-	-	4,4	-	5,4	4,7	6,6	12,5	4,6	-	7,5	5,3	6,4
h.i.B.s.	45,0	52,9	12,8*	10,4*	17,8	22,1	14,7*	25,5	12,9*	10,7*	27,4	8,0*	23,7	27,4	13,5*	5,4*
Zymos.	18,6	17,8	-	-	-	-	4,5*	7,2	-	-	5,8*	-	5,6*	7,3	-	4,5*
LPS	22,2	23,6	-	-	-	-	5,6*	11,7	-	4,8*	6,5*	4,5*	8,7	17,1	7,3	4,4*
sPGN	10,7	15,2	-	-	-	-	-	-	-	-	4,3*	-	-	6,4	-	-
LTA	22,3	22,0	-	-	-	8,4	4,7*	6,9*	5,5*	6,5*	11,5	7,2	-	11,1	5,8*	7,3
HSP60	6,9	9,1	-	-	-	-	-	5,6	-	-	-	4,3	-	4,8	-	4,4
OspA	30,3	36,1	5,5*	6,8*	-	7,2	5,5*	7,4	7,3	10,4	15,7	8,3	6,5*	7,7	5,0*	7,5
P_3CSK_4	32,8	37,6	6,9*	7,0*	-	9,6	11,6	12,3	8,4	11,8	13,7	8,3	17,8	24,9	13,3	5,4*
MALP-2	6,8	6,7	-	-	-	4,8	-	7,5	4,2	7,3	13,8	4,4	4,7	7,5	8,2	7,2
PMA	8,1	8,9	6,7	5,2	5,9	8,8	11,6	13,2	8,8	13,4	27,5	6,2	5,6	9,2	13,3	13,3

(-) </= 4,0; * P<0,05; significance as revealed from t-test for unconnected samples by relation to vector control for the regarding stimulant (PMA induction values were related to the vect.-sPGN-value).

3.3 Cellular localization of wild-type and mutant TLR2

In order to figure out whether the functional differences between mutant TLR2 constructs were due to their potentially different cellular localization upon overexpression, HEK293 cell clones overexpressing wild-type TLR2, Mut1, MutF, MutG, MutH, or MutJ were analyzed immunocytochemically. While control HEK293 cells did not express a Flag epitope, overexpression of wild-type Flag-TLR2 as well as the mutant constructs analyzed as represented by MutH and MutJ revealed the localization of the tagged proteins specifically at the cell membrane (App. I, Fig. 4).

3.4 DNA binding of NF–κB and phosporylation of cellular kinase Akt, as well as of MAP kinases p38, ERK1/2, and JNK mediated by TLR2 and mutant receptors

Control cells and HEK293 cells stably expressing wild-type TLR2, MutH, or MutJ were subjected to molecular analyses of cell activation. Nuclear extracts, as well as total lysates of cells were prepared 2 h or 30 min respectively after start of stimulation with sPGN, P_3CSK_4, or PMA. Nuclear extracts were applied to NF-κB specific electro mobility shift assay (EMSA) and total lysates were subjected to immuno blot analysis for comparison of cellular kinase Akt phosphorylation, as well as MAP kinases p38, ERK1/2, and JNK phosphorylations. EMSA revealed nuclear translocation and binding of NF-κB to a canonical NF-κB DNA recognition element, as well as immunoblot analysis revealed kinase phosphorylation in all clones upon PMA stimulation as compared to unstimulated cells (App. I, Fig. 5).

NF-κB activation and phosporylation of kinases analyzed upon stimulation with sPGN depended on expression of wild-type TLR2 but was absent in all other clones applied. P_3CSK_4 induced activation of NF-κB and phosphorylation of cellular kinases was not restricted to HEK293 cells overexpressing wild-type TLR2, but was also observed in cells overexpressing MutH lacking the N-terminal seven LRRs. Control HEK293 cells as well as cells overexpressing MutJ did not respond to challenge with P_3CSK_4 as revealed from NF-κB EMSA and immuno blot analysis of kinases phosphorylation (App. I, Fig. 5).

3.5 Interaction of mutant TLR2 constructs with wild-type TLR2 or MyD88

Since TLR2 dimerization was considered necessary to iniate down stream signaling (7), immunoprecipitation experiments were performed for analysis of the role of the TLR2 extra- or intracellular domain in homo- or heterologous interaction of TLR2 and TLR1. Flag-tagged constructs indicated, or vector as negative control were cotransfected with Myc-tagged wild-type TLR2 or TLR1. Flag-tagged mutants, as well as wild-type proteins all coprecipitated with the Myc tagged wild-type TLR2 or TLR1 (App. I, Fig. 6 and data not shown).

TLR2 signaling is initiated by interaction of its TIR domain with that of the adaptor molecule MyD88 (144). Mutated TLR2 might have an abnormal conformation resulting in failure of MyD88 recruitment. In order to analyze MyD88-TLR2 ecd mutant interaction, immunoprecipitation experiments were perfomed. Flag-tagged wild type TLR2, MutH, MutG, MutF, MutJ, or vector as negative control were cotransfected with Myc-tagged MyD88. Flag-tagged mutant, as well as wild-type proteins as indicated all coprecipitated Myc-tagged MyD88 (Fig. 3.2). This indicated that the TIR domains of these TLR2 mutant constructs were not abrogated in respect of their abilities of MyD88 recruitment, the functional difference between them most likely due to the conformal/structural change in the extracellular domain caused by different deletion.

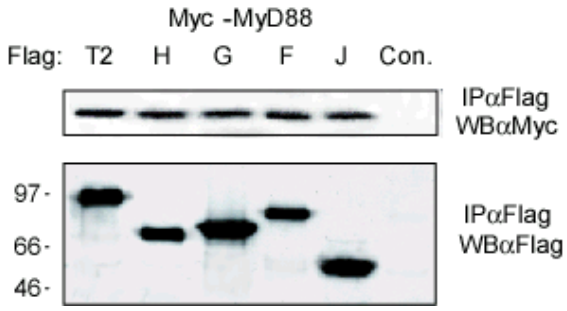


Fig. 3.2 (Suppl. App. I) Immunoprecipitation of mutant constructs and MyD88. For interaction analysis of TLR2 mutant constructs with downstream signaling molecules, Myc-tagged MyD88 and Flag-tagged TLR2 mutant constructs (Mut H, G, F and J) as well as wild-type TLR2 (T2) expression plasmids were transfected into HEK293 cells as indicated. 48 h after transfection start cells were lysed and subjected to Flag-specific immuno precipitation (IP, immuno precipitation; WB, immuno blot analysis with antibodies indicated)

3.6 Epitope difference between TLR2 ecd mutants

A monoclonal antibody against human TLR2 named TL2.1 (provided by Dr. Lien) was applied for analysis of cellular localization of and potential epitope difference between wild-type TLR2 and mutant constructs upon overexpression. The presence of wild-type TLR2 and notably MutH, at the cell membrane was confirmed. In contrast, none of the other mutants used were recognized by TL2.1 to a detectable degree although most of them inevitably carried the domain forming the respective epitope in wild-type TLR2 and MutH (Fig. 3.3), indicating that its tertiary structure rather than linear integrity of the TLR2 ecd is important for PAMP recognition through this receptor (Fig. 3.4).

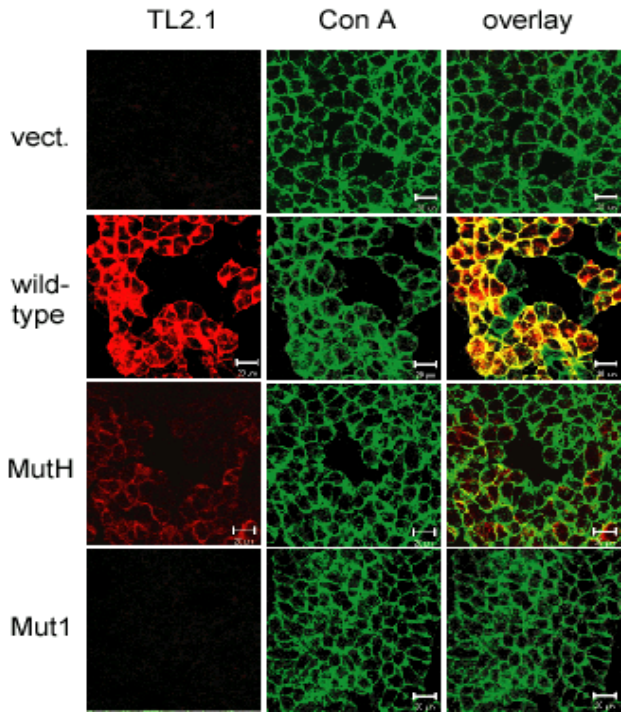


Fig. 3.3 (Suppl. App. I) Epitope difference between overexpressed TLR2 ecd mutants. Upon application of a monoclonal antibody recognizing human TLR2, only wild-type receptor and mutant H expressing HEK293 cells were detectable while expression of other mutant constructs such as Mut1 was not recognized by the same antibody. Although MutH was only weakly detected by the antibody, this construct mediated cellular recognition of tri-/di-palmitoylated peptide upon expression in HEK293 cells. The experimental procedure was the same like that for App. I, Fig. 4 except that a monoclonal antibody named TL2.1 instead of polyclonal α Flag antibody was applied to detect TLR2 / mutants.

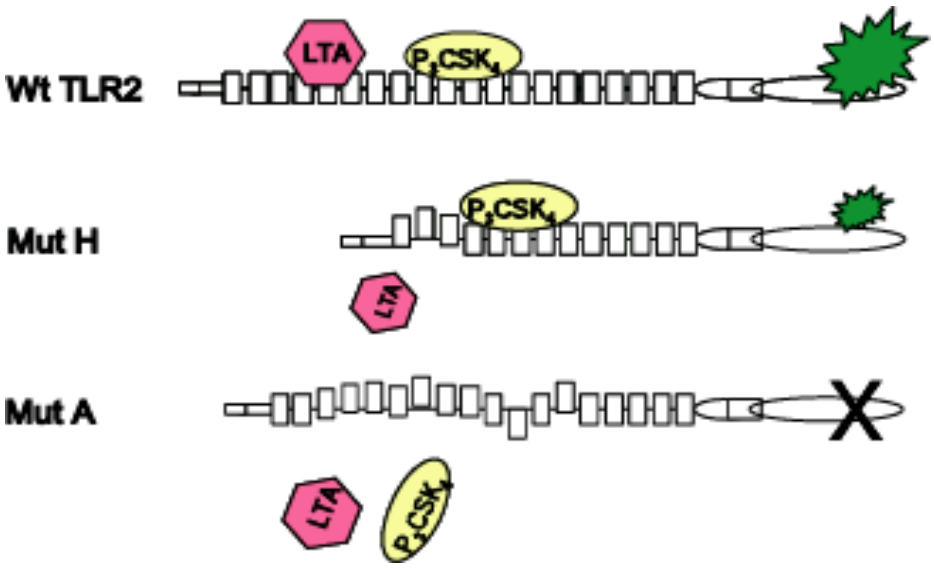


Fig. 3.4 (Suppl. App. I) Model implicating PAMP binding to TLR2. Different PAMPs might interact with different subdomains of the TLR2 extracellular domain and the subdomains mediating binding of specific PAMP may be constituted by spatial motifs instead of primary sequences. Therefore, in the case of MutH (N-terminal seven LRRs deleted), the tri-/di-palmitoylated peptide recognition subdomain was largely intact. In contrast, in other mutant constructs such as MutA, albeit lacking a smaller part of the TLR2 ECD, in some cases the ligand recognition domain has been disrupted. Tertiary structure rather than linear integrity is important for TLR2-PAMP recognition.

Results from this study indicated that the N-terminal seven LRRs of TLR2 are not involved in cellular recognition of tri- and diacylated microbial lipopeptides. A large functionally important subdomain might be formed by the N-terminal third of the TLR2 ecd. It might be structurally independent from the rest of the TLR2 ecd and possibly either consist of the subgroup of the 7 LRRs or just coincidentally represented by the respective region. Deletions within and beyond this proposed N-terminal seven-LRR subdomain might have caused severe structural changes biasing TLR2 function. In contrast, removal of the whole domain might have rather preserved the structure and function of the rest part of the receptor thus retaining perceptibility of agonists such as P_3CSK_4 and P_2CSK_4 that do not require its presence (Fig. 3.4). PAMP-TLR binding and structural analysis in the future will further contribute to understanding of TLR-PAMP recognition/interaction mechanisms.

4 Results and discussion (II)

Overactivation of innate immune cells by bacterial TLR agonists during infection is thought to be a main cause of septic shock (1). We raised antibodies against the extracellular domain (ECD) of TLR2 and found that monoclonal antibody T2.5 recognized both the murine and human receptor. Notably, T2.5 inhibited TLR2 specific activation of murine and human macrophages and protected pretreated mice against lethal shock upon challenge with synthetic lipopeptide or a Gram-positive bacterium. These results indicated that TLR2 is capable of mediating cellular signaling leading to lethal shock, as well as the potential therapeutic application of neutralizing anti TLR2 antibodies in sepsis. Figures shown supplement original publications as attached in appendix II.

4.1 Identification and characterization of mTLR2 specific mAbs

ELISA was used for analysis of supernatants of hybridomas generated. Therefore, recombinant IgG Fc fused TLR2 extracellular domain protein was immobilized on anti-Fc antibody coated plates. Potential positiveness of hybridoma clones was visualized via HRP conjugated streptavidin application (Fig. 4.1A). As control for mTLR2 ecd overexpressed mTLR9 or irrelevant protein was applied. If the ELISA signal was positive for all three samples, tested clone was considered as false positive. If signals were all negative clones were considered negative. Only specific clones like Nr.2 which produced a supernatant which confered a TLR2 specific signal and were clearly negative for the controls were taken as positive clones (Fig. 4.1B). Finally, 12 different clones were identified and characterized (Table 4.1).

4.2 Application of mAb T2.5 for expression analysis *in vitro*

We have selected an IgG1κ anti-TLR2 mAb named T2.5 which was cross reactive with human TLR2 and specifically recognized both overexpressed and endogenous antigen as revealed from surface immunostaining based flow cytometry analysis and immuno-precipitation experiments (App. II, Fig. 1). This specific immuno-detection of TLR2 also held true on the subcellular level. Upon permeabilization, overexpressed and endogenous murine as well as human TLR2 were all detectable in primary murine and human macrophages by T2.5 (App. II, Fig. 2).

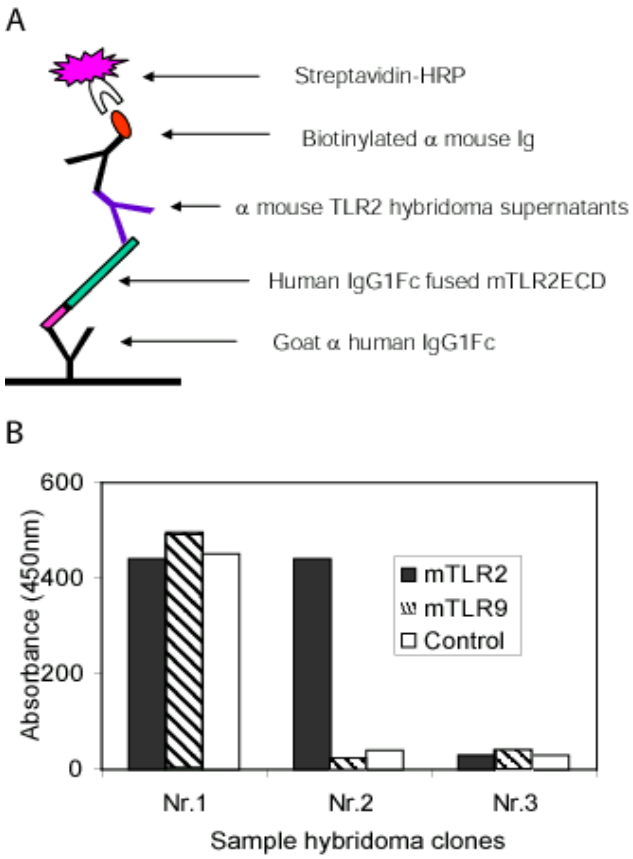


Fig. 4.1 (Suppl. App. II) Schematic overview of TLR2 specific mAb identification ELISA design and results. For hybridoma cell clone identification, IgG1 Fc fused TLR2 extracellular domain protein was immobilized on anti Fc antibody coated plates, and potential positive hybridoma clone was visualized via HRP conjugated streptavidin application (A). As control for mTLR2, overexpressed mTLR9 or irrelevant protein was applied, only those clones like Nr.2, which were specifically positive for TLR2 and clearly negative for the controls were taken as positive clones (B).

Nr.	Name1	Name2	Isotype	ELISA	western	FACS-293-mT2	FACS-293-hT2	FACS MoT2$^{-/-}$	FACS MoT2$^{+/+}$	IP-mT2	IP-hT2
Nr.4	F1A4	MOA5-1-E1-C5-A4	IgG2b$_k$	+	-	+	-	-	+	-	-
Nr.5	F1B1	HFD8-1-H4-E4-B1	IgG1$_k$	+	-	+	+	-	+	+	++
Nr.7	F1B3	HFC4-1-C4-A7-B3	IgG2a$_k$	+	-	+	-	-	+	++	-
Nr.10	F1C2	MOA4-1-G4-B12C2	IgG2a$_k$	+	-	+	-	-	+	+++	-
Nr.12	F1C4	MOA4-1-F10-C6-C4	IgG2a$_k$	+	-	+	-	-	+	-	-
Nr.13	F2A1	HFA11-2-B4-A8-A1	IgG1$_k$	+	-	+	-	-	+	-	-
Nr.15	F2A3	ALTP4G2-1-G7B2A3	IgG2a$_k$	+	-	-	-	-	-	-	-
Nr.16	F2A4	HFB11-1-A11B12A4	IgG2a$_k$	+	-	-	-	-	-	-	-
Nr.17	F2B1	ALTP4G2-1-A4C3B1	IgG2a$_k$	+	-	-	-	-	-	-	-
Nr.18	F3A1	ALT-P4-1-18-A1	IgG2a$_k$	+	-	-	-	-	-	++	-
Nr.26	F3C1	RMOG7-2-D4-C4-C1	IgG2a$_k$	+	-	-	-	-	-	+	-
Nr.28	F3C3	RHFB6-2-D10-B7C3	IgG2a$_k$	+	-	-	-	-	-	++	-

Nr.	IHC 293-mT2	IHC 293-hT2	IHC MoT2$^{-/-}$	IHC MoT2$^{+/+}$	neutra. 293-mT2	neutra. 293-hT2	neutra. Raw	neutra. PeriMo	neutra. THP1	neutra. huPBMC	neutra. in vivo	IHC hT2mutH
Nr.4	+	-	-	+	+	-	+	+	-	-	+	-
Nr.5	++	++	-	+++	++	++	++	++	++	++	++	+
Nr.7	+	-	-	+++	-	-	-	-	-	-	-	-
Nr.10	+	-	-	+++	-	-	-	-	-	-	-	-
Nr.12	+	-	-	-	-	-	-	-	-	-	-	-
Nr.13	+	-	-	++	-	-	-	-	-	-	-	-
Nr.15	-	-	-	-	-	-	-	-	-	-	-	-
Nr.16	-	-	-	-	-	-	-	-	-	-	-	-
Nr.17	-	-	-	-	-	-	-	-	-	-	-	-
Nr.18	+	-	-	+++	-	-	-	-	-	-	-	-
Nr.26	+	-	-	+++	-	-	-	-	-	-	-	-
Nr.28	+	-	-	++	-	-	-	-	-	-	-	-

Table 4.1 (Suppl. App. II) Summary of murine TLR2 specific mAb clones including characterization for them. Mo = Macrophage; mT2 = mouse TLR2; hT2 = human TLR2; IHC = immunohistochemistry; neutra = neurtalization; Mut = mutant; Peri = peritoneal.

4.3 Inhibitory effects of T2.5 on TLR2 specific cell activation

T2.5 inhibited murine TLR2 mediated cell activation by TLR2 specific agonists applied to murine macrophage like RAW264.7 cells and primary peritoneal macrophages as determined by analysis of TNFα as well as IL-6 release, respectively (App. II, Fig. 3 C and D; Fig. 4.2 A and B). The inhibitory effect from T2.5 was effective not only for mouse TLR2, but also for its human homologe. T2.5 inhibited both murine and human TLR2 mediated cell activation by P_3CSK_4 or *B. subtilis* applied to HEK293 cells overexpressing TLR2 as determined by analysis of NF-κB activation as well as IL-8 release, respectively (App. II, Fig. 3A and B; Fig. 4.2 C and D). The samples analyzed for NF-κB dependent luciferase activity and IL-8 concentration were from same experiments.

A second newly generated IgG1κ anti TLR2 mAb, conT2, was used as control. This mAb binds native murine (m) TLR2 like T2.5 but does not bind human TLR2 (Table 4.1, conT2 = mAb Nr.13) and failed to inhibit TLR2 dependent cell activation *in vitro* and *ex vivo*. T2.5 did not inhibit IL-1 receptor or TLR4 signaling which indicated that TLR2 independent signaling pathways in T2.5 treated cells remained intact. Moreover, TLR2 mediated nuclear translocation of NF-κB was specifically inhibited by T2.5 in human macrophage like THP1 cells and primary macrophages (Fig. 4.2E; App. II, Fig 3E).

Fig. 4.2 (Suppl. App. II) Inhibitory effect of mAb T2.5 on cell activation *in vitro*. IL-6 concentrations in supernatants of RAW264.7 (A) or primary murine macrophages (B) and IL-8 release in HEK293 cells overexpressing either murine (C) or human TLR2 (D), challenged with inflammatory stimulants are shown (ND, not detectable). Cells were incubated either with T2.5 or conT2 only (empty bars), or additionally challenged with ultra pure LPS (A and B, bold upward hatched bars), or IL-1β (C and D, horizontally hatched bars), P_3CSK_4 (filled bars), or h. i. *B.subtilis* (downward hatched bars). (E) shows inhibitory effect of T2.5 on challenge (P_3CSK_4, LPS) dependent NF-κB/p65 nuclear translocation in THP1 cells analyzed by cytochemical staining (Unstim., unstimulated), arrow shows NF-κB activated and translocated into the nucleus upon stimulation, arrow head indicates NF-κB cytoplasm location (inactivation) either without PAMP challenge or inhibited by T2.5 upon TLR2 specific, in this case P_3CSK_4 stimulation.

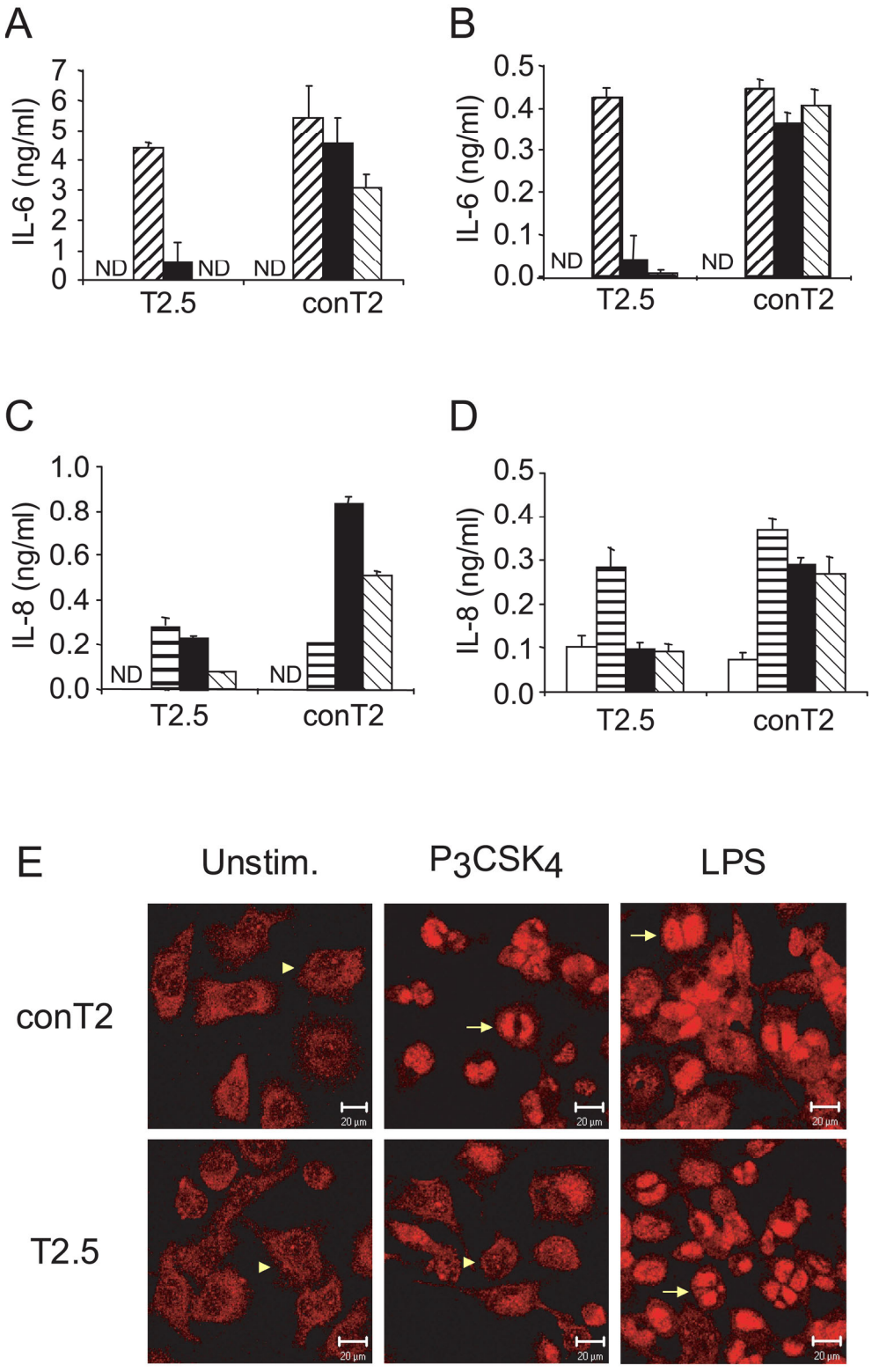


Fig. 4.2 (Suppl. App. II)

NF-κB specific EMSA, as well as anti phospho p38, Erk1/2, and Akt immunoblot analysis revealed T2.5 dose dependent inhibition of TLR2 agonists induced NF-κB-DNA binding and kinase phosphorylation (App. II, Fig. 3 F and G and data not shown). In addition, application of T2.5 inhibited TLR2 mediated NF-κB activation in HEK 293 cells upon challenge with various TLR2 agonists, indicating that the inhibition of the function of this receptor was universal but not specific to certain PAMP binding motif (Fig. 4.3A).

The N-terminal third of the LRR-rich domain of human TLR2 is not involved in lipopeptide recognition (135) and T2.5 cross-reacts with human TLR2. Thus, we applied T2.5 to HEK293 cells overexpressing a mutant construct of human TLR2 lacking the respective portion of the wild-type ECD, namely MutH (135). Immunochemical detection of MutH and specific abrogation of NF-κB dependent reporter gene activation upon P_3CSK_4 challenge after administration of T2.5 strongly suggests localization of the epitope recognized by T2.5 within the C-terminal portion of the TLR2ECD (Fig. 4.3B; App. II, Fig. 4C).

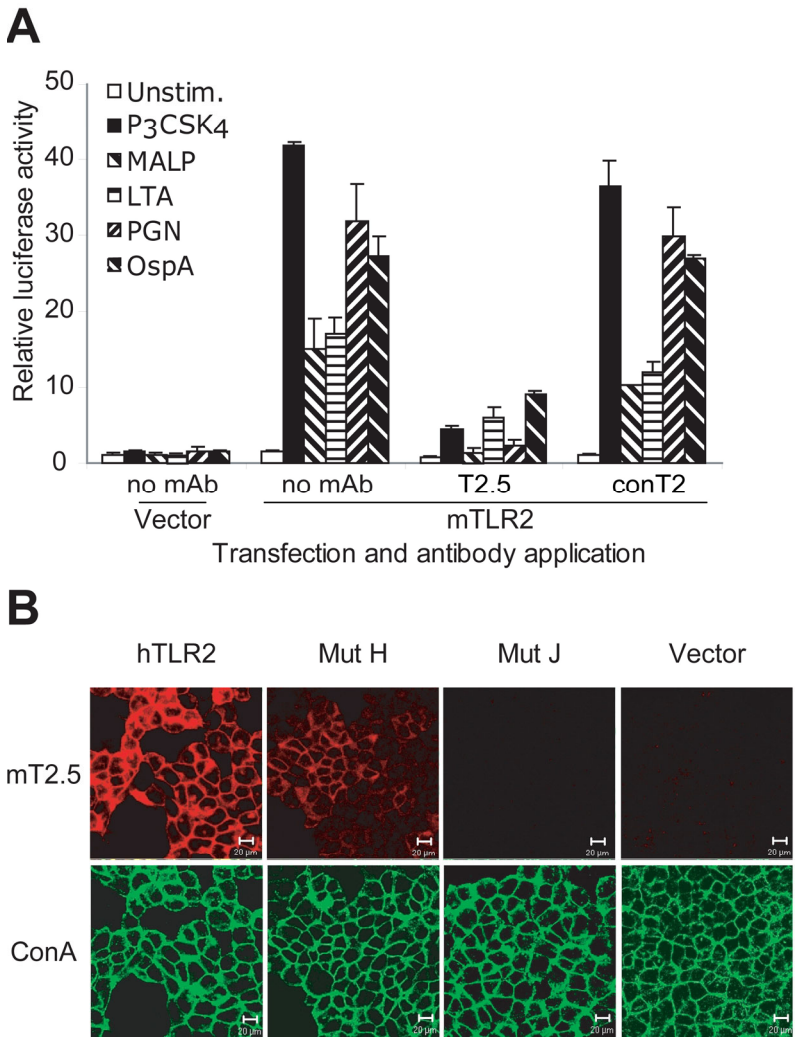
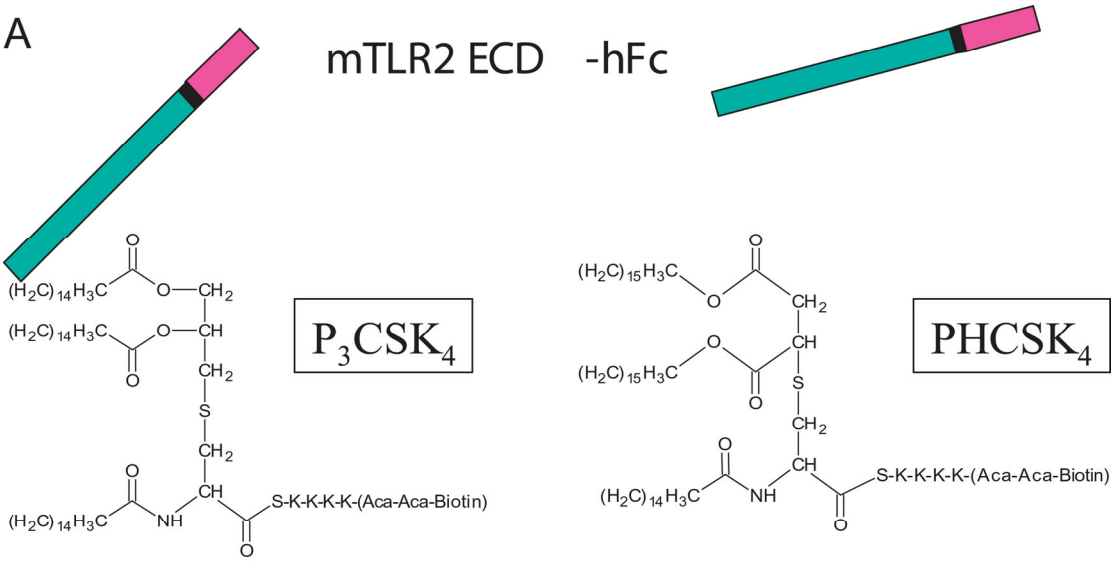


Fig. 4.3 (Suppl. App. II) Inhibitory effect of T2.5 on cell activation upon various TLR2 agonist challenges and epitope localization analysis. NF-κB dependent luciferase activities in HEK293 cells overexpressing murine TLR2 was inhibited significantly upon pre-incubation with T2.5 and subsequent TLR2 specific challenge with MALP (synthetic analogue of mycoplasmal diacylated peptide), P_3CSK_4, LTA, PGN, or OspA representing TLR2 agonists of bacterial origin (A). These results demonstrate a general antogonistic property of T2.5 for chemically diverse agonists and not restriction to only a single TLR2 agonist. For analysis of approximate localization of T2.5 epitope within the TLR2ECD, a mutant human TLR2 construct lacking the N-terminal third of the LRR-rich ECD domain (hTLR2-mutH) was used for immuno detection (B). Concanavalin A (ConA) was used for staining of cell membranes (B).

4.4 Abrogation of TLR2ECD ligand-binding by T2.5 in a SPR biosensor system and analysis of T2.5 epitope localization

To investigate whether T2.5 blocked binding of TLR2 to its synthetic agonist P_3CSK_4 we established a surface plasmon resonance (SPR) biosensor based binding assay. With $PHCSK_4$, a nonactive analogue of P_3CSK_4 as control, biotinylated P_3CSK_4 was immobolized on the surface of a streptavidin-precoated chip and binding of T2EC was tested under various conditions (Fig. 4.4A). By application of a human protein fused with the same IgG1-Fc domain like in T2EC protein as control, it turned out that the binding of T2EC to P_3CSK_4 but not $PHCSK_4$ was highly specific (Fig. 4.4A and data not shown). T2.5 as well as another antagonistic mAb named mT2.4 dose dependently inhibited this binding (App. II, Fig. 4A; Fig. 4.4B). In contrast, application of the isotype control antibody for T2.5 (con T2) and another nonneutralizing mAb named mT2.7 did not intervene the binding between T2EC and P_3CSK_4 (App. II, Fig. 4B; Fig. 4.4C).

Fig. 4.4 (Suppl. App. II) Molecular base of the SPR biosensor based T2EC-α mTLR2 mAb binding assay and representative results. $PHCSK_4$ is an inactive analogue of P_3CSK_4, in this experiment system it served as negative control (A). The flow cells (FC) of a streptavidin-precoated chip were loaded with biotinylated $PHCSK_4$ (FC1) and P_3CSK_4 (FC2), respectively (A). Binding of T2EC to immobilized P_3CSK_4 but not $PHCSK_4$ upon preincubation with a neutralizing mAb mT2.4 (mT2ECD +mT2.4) at different molar excesses (B, x1, x3.3, x10) or with a nonantagonistic mAb mT2.7 (mT2ECD +mT2.7) at 10 fold molar excesses (C, x10). MT2ECD alone (C) and mAbs alone at high amounts (B, mT2.4, x10; C, mT2.7, x10) were applied as control for binding. Response units at 300 seconds are a measure for P_3CSK_4-binding capacities of mT2ECD and mT2ECD + mAbs.



N-palmitoyl-S -(2,3 -bis (palmitoyloxy)-(2R,S) -propyl)-(R) -cysteinyl -seryl -(lysyl)3 -lysine

N-palmitoyl-S -(1,2 -bishexadecyloxy -carbonyl) -ethyl-(R) -cysteinyl -seryl -(lysyl)3 -lysine

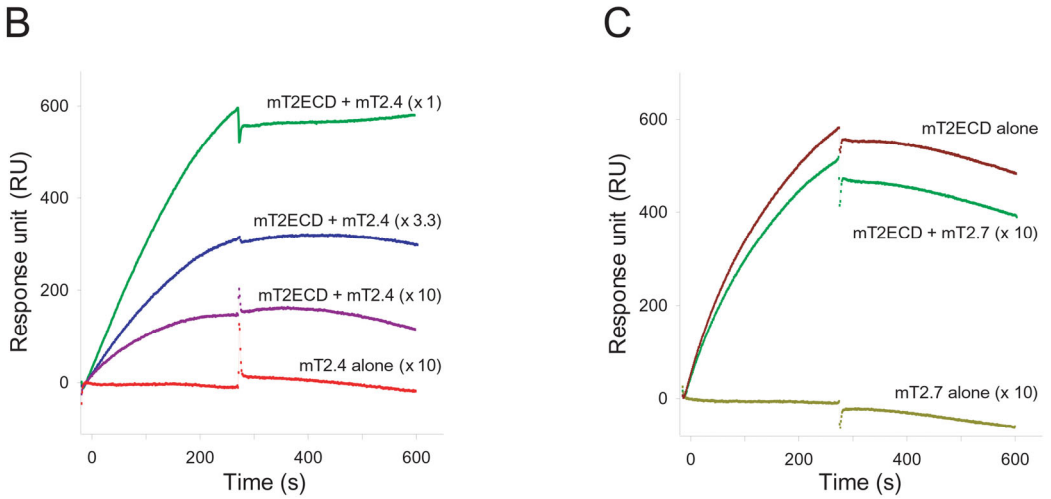


Fig. 4.4 (Suppl. App.II)

4.5 Surface and intracellular TLR2 expression *ex vivo* as analyzed immediately upon primary cell preparation

Since LPS induces TLR2 expression in primary macrophages *in vitro*, we first compared T2.5 specific staining of $CD11b^+$ (macrophage) splenocytes from LPS challenged wild-type and *TLR2*$^{-/-}$ mice by flow-cytometry. Weak surface staining and more pronounced intracellular staining were evident (App. II, Fig. 5A). In subsequent experiments, peritoneal washout cells and splenocytes from mice infected with Gram-positive bacteria *B. subtilis* were analyzed. While surface expression of TLR2 in primary murine macrophages was relatively strong upon *in vitro* culture (App. II, Fig. 1D), surface expression was weak or not detectable in unchallenged $CD11b^+$, $CD11c^+$ (dendritic cell), $CD19^+$ (B cell), and $GR1^+$ (granulocyte) subpopulations of splenocytes and peritoneal washout cells (App. II, Fig. 5B; Fig. 4.5A and data not shown). Upon microbial challenge, however, TLR2 surface expression increased in $CD11b^+$ and $GR1^+$ but not $CD19^+$ and $GR1^+$ cells (App. II, Fig. 5C; Fig. 4.5 B, C, D and data not shown). Enhancement of intracellular signals through prior challenge, however, was more pronounced as compared to enhancement of extracellular staining (App. II, Fig. 5 B and C; Fig. 4.5 A and B) and signals detected were largely TLR2-specific.

Fig. 4.5 (Suppl. App. II) TLR2 expression *in vivo*. Flow-cytometry of splenocytes and peritoneal washout cells from wild-type and *TLR2*$^{-/-}$ mice *ex vivo* immediately upon isolation (n = 5, cells pooled for each sample). For analysis of TLR2 regulation upon infection (A to D), mice were either left uninfected (-) or infected with *B. subtilis* and sacrificed after 24 h (+). Upon staining of populations of cells as indicated, cells were stained with T2.5 (TLR2) either without (A) or upon permeabilization (B, C and D). Numbers in quadrants represent the proportion of single or double stained cells, respectively, as compared to the total number of viable cells analyzed (%).

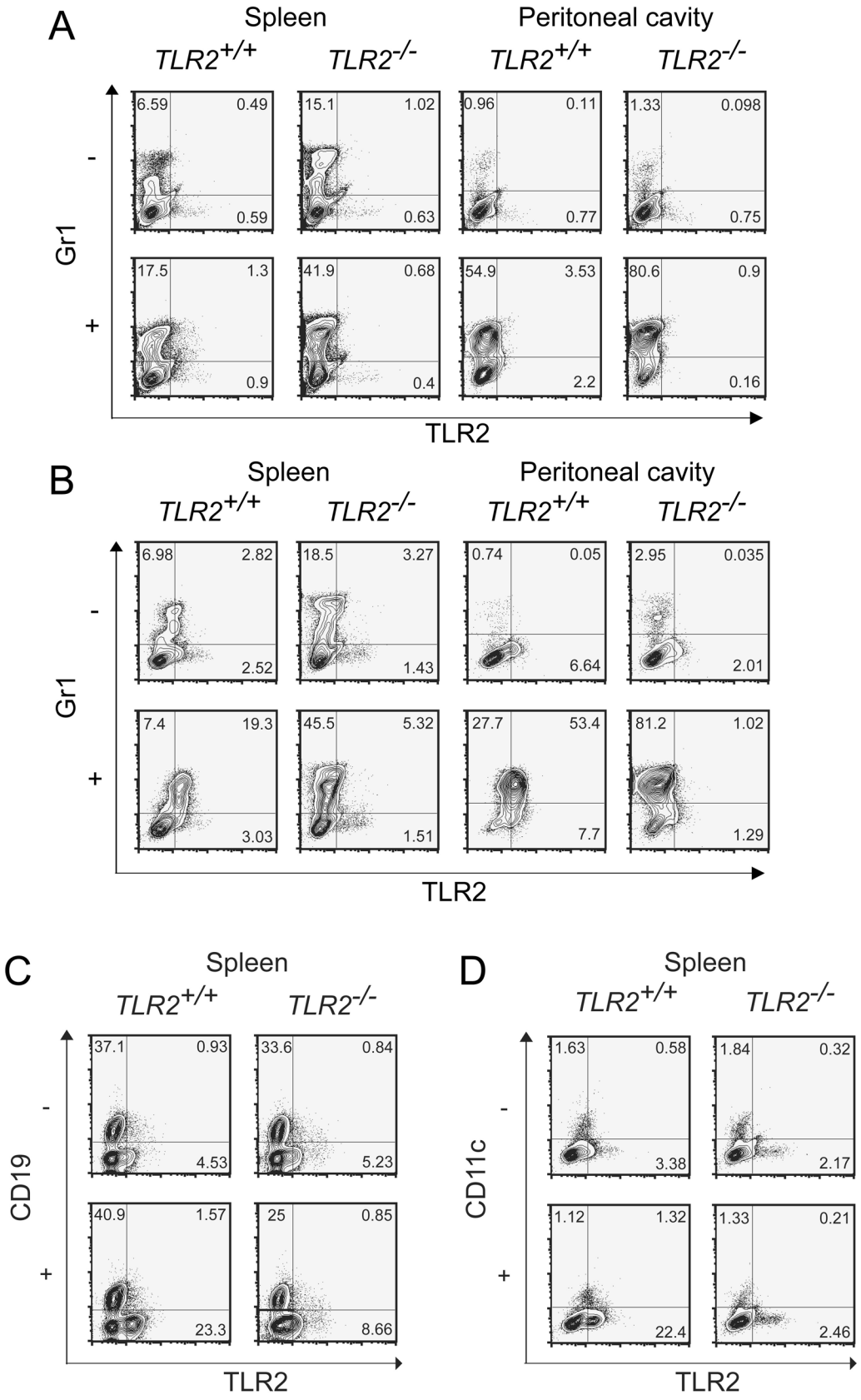


Fig. 4.5 (Suppl. App. II)

4.6 Antibody mediated interference with TLR2 specific immune responses towards systemic challenge

Next, we asked whether our *in vitro* and *ex vivo* evidence could be expanded to a systemic situation. Thus, cytokine and chemokine serum concentrations in mice, either pretreated, or not pretreated with T2.5 upon challenge with a lipopeptide analogue P_3CSK_4 were determined. While cytokine and chemokine concentrations were low in sera of untreated mice (App. II, materials and methods), serum levels of TNFα, GROα/KC (murine IL-8 homologue), IL-6, and IL-12p40 were significantly lower in mice preinjected with T2.5 as compared to controls upon challenge with P_3CSK_4 (App. II, Fig. 6). These results indicated that under *in vivo* situation, T2.5 was still effective in blockage of TLR2 mediated inflammatory responses towards microbial challenge.

Both a high dose (microbial product only) and a low dose model (additional sensitization with D-galactosamine) have been established for bacterial product-induced shock-like syndromes in mice (145). In order to interfere in a TLR2 specific model of septic shock, we applied the bacterial lipopeptide analogue and TLR2 agonist P_3CSK_4 upon sensitization of mice with IFNγ and D-galactosamine (57, 146). Sensitization was used to prime host defense and mimic an underlying primary infection. While mice that had received no mAb or conT2 30 min prior to injection died from lethal toxemia within 24 h, mice treated with T2.5 survived (Fig. 4.6A).

In a distinct shock model, we took advantage of the finding that induction of shock-like syndrome by viable or inactivated *B. subtilis* bacteria was TLR2-dependent (A. G. and C. J. K., unpublished observation) and applied heat inactivated *B. subtilis* (h. i. *B. subtilis*) as a more complex challenge. Thus, mice were pretreated with different doses of T2.5 prior to administration of h. i. *B. subtilis*. While pretreatment with 1 mg and 0.5 mg of T2.5 protected mice from lethal toxemia (protective protocol), lower amounts were ineffective (Fig. 4.6B).

Fig. 4.6 A+B (Suppl. App. II) Effects of mAb T2.5 administration on viability upon TLR2 specific systemic challenging.

A, Lipopeptide blockage *in vivo*. IFNγ and D-galactosamine sensitized mice received either no mAb, 1 mg of mAb T2.5, or 1 mg of conT.2 i.p. 30 min prior to microbial challenge with bacterial lipopeptide analogue P_3CSK_4 (O, no mAb, n = 4; Δ, mAb conT2, n=3; ■, mAb T2.5, n=4).

B, Dose kinetic in blockage of bacteria induced experimental shock. Mice challenged with a high dose of h.i. *B. subtilis* were left untreated or treated 1 h later with dosages of mAb T2.5 indicated (◆, 1 mg, n=3; □, 0.5mg, n=3; Δ, 0.25mg, n=4; ✕, 0.13mg, n=4; O, no mAb, n=4).

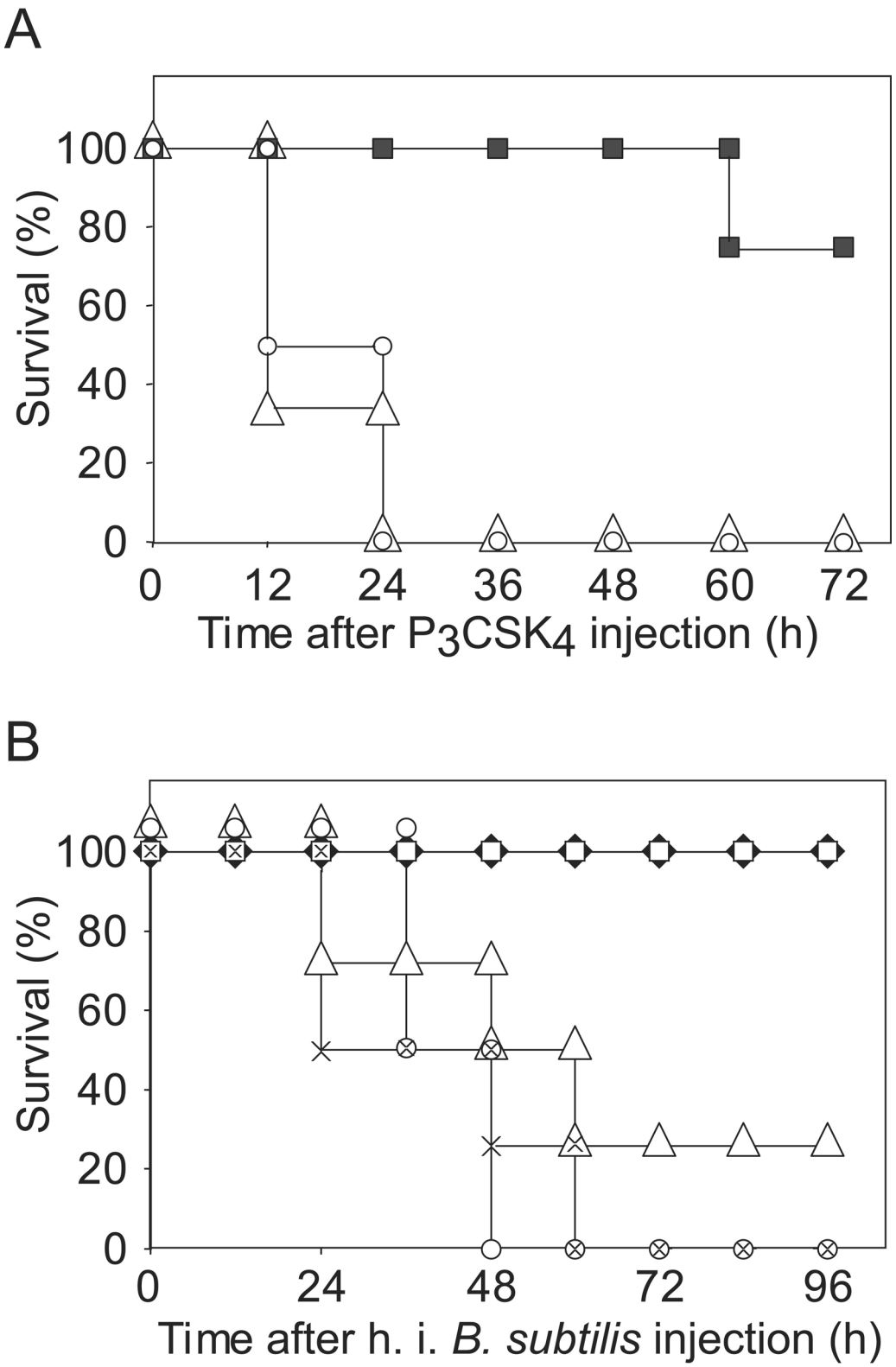


Fig. 4.6 A+B (Suppl. App.II)

Next, T2.5 or conT2 followed by challenge with a lethal dose of h. i. *B. subtilis* were applied simultaneously. In one group of mice, we first administered h. i. *B. subtilis* and applied T2.5 up to 3 h later (therapeutic protocol). Without sufficient amount of T2.5 the high dose h. i. *B. subtilis* challenge was lethal for all mice tested (Fig. 4.6 B and C). However, when T2.5 was given either prior (1 h), or up to 2 h after bacterial challenge, all h. i. *B. subtilis* injected mice survived (Fig. 4.6C). Most notably, treatment with T2.5 even 3 h after principally lethal challenge saved 75% of mice injected (Fig. 4.6C).

When we reversed the order of experimental T2.5 and h. i. *B. subtilis* application, a completely protective effect of T2.5 administration was evident if the bacterial challenge was started 3 h later (Fig. 4.6D). While T2.5 treatment was protective for 2 out of 3 mice applied even for 4 h followed by microbial challenge, it was not effective in protecting at the 5 h and 6 h time points in the respective experimental settings (Fig. 4.6D).

Fig. 4.6 C+D (Suppl. App. II) Effects of mAb T2.5 administration on viability upon TLR2-specific systemic challenging.

C, Antibody treatment after bacterial challenge. Mice challenged with a high dose of h.i. *B. subtilis* were left untreated or treated with 1mg of mAbs as indicated at different time points. Administration of TLR2-specific mAbs prior to (-), as well as after (+) bacterial challenge (▼, no mAb, *n*=8; ❍, mAb conT2, -1h, *n*=3; ◆, mAb T2.5, -1h, *n*=4; ❑, mAb T2.5, +1h, *n*=3; ✕, mAb T2.5, +2h, *n*=3; ◊, mAb T2.5, +3h, *n*=4; Δ, mAb T2.5, +4h, *n*=3).

D, mAb treatment prior to bacterial challenge. Mice challenged with a high dose of h.i. *B. subtilis* were left untreated or treated with 1mg of T2.5 prior (-) to bacterial challenge as indicated at different time points. Administration of TLR2-specific mAb T2.5 (*n*=3 for experimental groups: Δ, no mAb; ■, mAb T2.5, -3h; ◊, mAb T2.5, -4h; ❍, mAb T2.5, -5h; ▼, mAb T2.5, -6h).

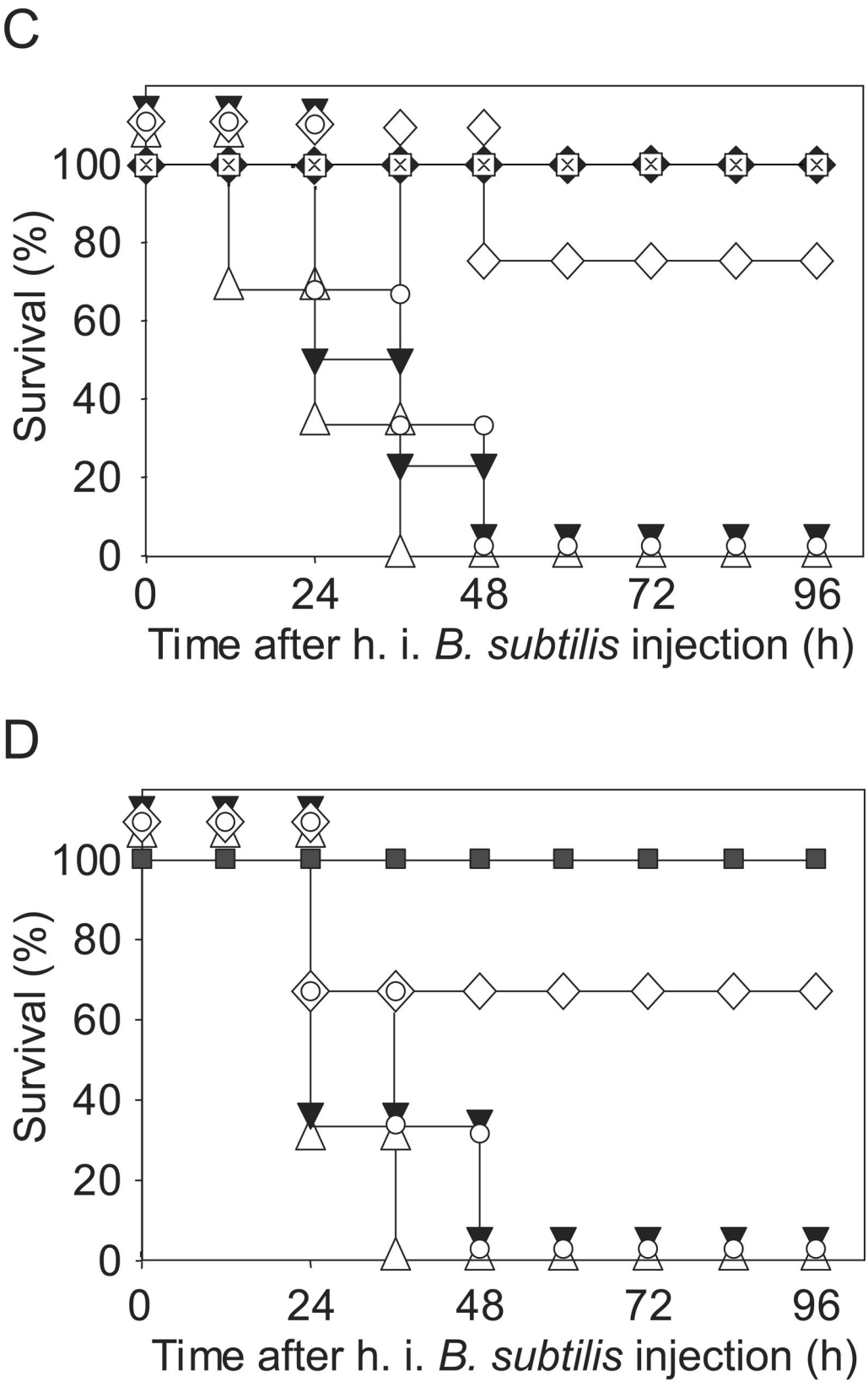


Fig. 4.6 C+D (Suppl. App.II)

Our data indicated a therapeutically useful function of a neutralizing mAb raised against murine TLR2 in Gram-positive bacteria driven-toxemia. In both a sensitization dependent low dose and a sensitization independent high dose TLR2 specific experimental model, the beneficial and specific effects of mAb T2.5 on the host, in this case mice were evident. Suprisingly, *B. subtilis* induced shock-like syndrome was prevented by application of T2.5 two hours or even three hours after shock induction (100% and 75% of mice survived, respectively). However, it was not protective after 4 hours (Fig. 4.6C). When the order of mAb T2.5 and *B. subtilis* application was reversed, a protective effect of the antibody was evident if the bacterial challenge was started 3 h or even 4 h later (100% and 67% survival, respectively). In contrast, a 5 h time period was too long for T2.5 to be still protective (Fig. 4.6D). In fact, upon acute infection in the clinical situation, the onset of septic shock may be postponed in contrast to sudden induction of toxemia in experimental models, therefore, a larger time window might be allowed for interference. T2.5 was cross reactive with and antagonistic for human TLR2. This may support transferability of out results to elimination of the TLR2 dependent share in septic shock induction. The surprisingly low level of TLR2 expression in host immune cells might account for the notably effective antagonistic effect from T2.5 in TLR2-mediated cell activation. This mAb antagonized TLR2 function through masking of the ligand-binding domain of TLR2 thus inhibited ligand-TLR2 interaction as revealed from SPR binding experiments. Upon antibiotic therapy, TLR2 blockage may contribute to prevention of an excessive host immune reaction upon sudden release of large amounts of microbial products from disintegrated microbial cells. It may have to be complemented by blockage of further surface receptors such as TLR4, in order to facilitate further inhinition of host immune cell activation. In conclusion, our study indicated systemic cell surface TLR specific antibody application as potentially usefully strategy for therapeutic blocking of TLR-mediated cell activation in the course of acute infection.

5 Summary

Various agonists have been attributed to Toll-like receptor (TLR) 2, a member of the TLR family that has been shown to bind microbial products such as lipopeptide. Distinct agonists might interact with different subdomains of the TLR2 extracellular domain (ecd) which carries 10 leucin rich repeat (LRR) motifs and 10 LRR-like motifs. Therefore, transfection of TLR2 LRR/LRR-like motif deletion constructs in HEK293 cells and primary TLR2 deficient mouse fibroblasts was performed for analysis of structural requirements for TLR2 ecd in specific pattern recognition. Preparations applied as agonists were highly purified PGN, LTA, OspA, MALP-2, tripalmitoyl-cysteinyl-seryl-(lysyl)3-lysine (P_3CSK_4), dipalmitoyl-/ P_2-CSK_4, and palmitoyl-/ P-CSK_4, as well as LPS and inactivated bacteria. We found that a block of the N-terminal 7 LRR/LRR-like motifs was not involved in TLR2 mediated cell activation by P_3CSK_4 and P_2CSK_4 ligands mimicking triacylated and diacylated bacterial lipopeptides, respectively. In contrast, the integrity of the TLR2 holoprotein was compulsory for effective cellular recognition of all other TLR2 agonists applied, including $PCSK_4$. Formation of a functionally relevant subdomain by a region including the N-terminal seven LRR/LRR-like motifs rather than by single LRR is suggested by our data. This study further indicated that TLR2 contains multiple binding domains for ligands, which may contribute to the characterization of its promiscuous pattern recognition.

Over-activation of immune cells by microbial products through TLRs was considered as causative mechanism underlying septic shock pathology. Infection with bacteria providing TLR2-specific agonists is one of the major causes of severe sepsis. In order to intervene in TLR2-driven toxemia, we raised mAbs against the murine TLR2 ecd. Application of mAb T2.5 inhibited cell activation in experimental mice models of infection. This mAb also antagonized TLR2-specific activation of primary human macrophages. Surface plasmon resonance analysis demonstrated direct and specific interaction of TLR2 and immuno-stimulatory lipopeptide which was blocked by T2.5 in a specific and dose dependent manner. In contrast to TLR2-specific intracellular staining, surface staining of murine macrophages and granulocytes was surprisingly weak and increased only slightly upon microbial challenge. Upon lipopeptide challenge, systemic application of T2.5 inhibited release of inflammatory mediators such as TNFα and prevented lethal shock-like syndrome in mice. i.p. application of 20 mg/kg of T2.5 was sufficient to protect mice and its administration with 40 mg/kg was protective even 3 h after start of lethal-dose *Bacillus subtilis* challenge. These results implied potential therapeutic application of a neutralizing anti TLR2 antibody in acute infection.

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Cellular Recognition of Tri-/Di-palmitoylated Peptides Is Independent from a Domain Encompassing the N-terminal Seven Leucine-rich Repeat (LRR)/LRR-like Motifs of TLR2*

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Toll-like receptors (TLRs) mediate microbial pattern recognition in vertebrates. A broad variety of agonists has been attributed to TLR2 and three TLRs, TLR4, TLR2, and TLR5, have been demonstrated to bind microbial products. Distinct agonists might interact with different subdomains of the TLR2 extracellular domain. The TLR2 extracellular domain sequence includes 10 canonical leucine-rich repeat (LRR) motifs and 8–10 additional and potentially functionally relevant LRR-like motifs. Thus, the transfection of TLR2 LRR/LRR-like motif deletion constructs in human embryonic kidney 293 cells and primary TLR2-deficient mouse fibroblasts was performed for analysis of the role of the regarding domains in specific pattern recognition. Preparations applied as agonists were highly purified soluble peptidoglycan, lipoteichoic acid, outer surface protein A from *Borrelia burgdorferi*, synthetic mycoplasmal macrophage-activating lipoprotein-2, tripalmitoyl-cysteinyl-seryl-(lysyl)3-lysine (P_3CSK_4), dipalmitoyl-CSK_4 (P_2-CSK_4), and monopalmitoyl-CSK_4 ($PCSK_4$) as well as lipopolysaccharide and inactivated bacteria. We found that a block of the N-terminal seven LRR/LRR-like motifs was not involved in TLR2-mediated cell activation by P_3CSK_4 and P_2CSK_4 ligands mimicking triacylated and diacylated bacterial polypeptides, respectively. In contrast, the integrity of the TLR2 holoprotein was compulsory for effective cellular recognition of other TLR2 agonists applied, including $PCSK_4$. The formation of a functionally relevant subdomain by a region including the N-terminal seven LRR/LRR-like motifs rather than by single LRRs is suggested by our results. They further imply that TLR2 contains multiple binding domains for ligands that may contribute to the characterization of its promiscuous molecular pattern recognition.

Immune responses toward microbes are preceded by their recognition. Pathogen-associated molecular patterns (PAMPs)[1] are cell constituents representing groups of microbes or parasites rather than single species. For instance, lipopolysaccharide (LPS) is a key PAMP of Gram-negative bacteria (1–3). Mannose- and LPS-binding protein, scavenger receptors, and CD14 as well as members of the toll-like receptor (TLR) family are examples of pattern recognition receptors mediating recognition of microbial products prior to the early phase of host defense (4). Many receptors of the innate immune system are expressed constitutively, thus enabling immediate-early responses (2) including the release of proinflammatory cytokines.

The human protein family of TLRs encompasses 10 members from which TLR4 was the first to be implicated in vertebrate immunity (2, 5–8). Although *in vitro* evidence suggested involvement of TLR2 in LPS recognition, an analysis of specific rodent strains proved a role for TLR4 as the prime LPS signal transducer (3, 4, 9, 10). More specifically, the identifications of an inactivating TLR4 point mutation and a TLR4 null allele in C3H/HeJ and B57BL/10ScCr mice, respectively, provided the initial evidence. The phenotype of consequently LPS-resistant gene targeted $TLR4^{-/-}$ mice as well as identification of an inactivating point mutation in TLR2 in Chinese hamsters displaying normal LPS responsiveness ($TLR2^{d/d}$/$TLR4^{+/+}$; d = defect) further validate these findings. Peptidoglycan (sPGN) and lipoteichoic acid (LTA) from Gram-positive bacteria, lipoarabinomannan from mycobacteria, neisserial porins, bacterial tripalmitoylated, and mycoplasmal diacylated lipoproteins, as well as yeast products and glycosylphosphatidylinositol-anchored proteins of the protozoa *Trypanosoma cruzi* are examples of microbial PAMPs eliciting host responses via TLR2 (4). Principal differences in pattern recognition through TLRs such as the distinct necessity for intracellular PAMP uptake in the case of TLR9 have been demonstrated previously (11). In addition, for sPGN as well as a substructure thereof, muramyl dipeptide, the cytoplasmic nucleotide-binding oligomerization domain 2 protein has been identified as a signaling pattern recognition receptor (12, 13).

The cellular reactions induced by TLR2 agonists involve activation of nuclear factor (NF)-κB and kinases such as p38, c-Jun N-terminal kinase (JNK), extracellular signal regulated kinase (ERK) 1/2, and Akt/protein kinase B. In this regard,

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[1] The abbreviations used are: PAMP, pathogen-associated molecular pattern; TLR, toll-like receptor; LRR, leucine-rich repeat; sPGN, soluble peptidoglycan from *S. aureus*; LPS, lipopolysaccharide; LTA, lipoteichoic acid; OspA, outer surface protein A; MALP-2, mycoplasmal monocyte-activating lipoprotein 2; P_3CSK_4, tripalmitoyl-cysteinyl-seryl-(lysyl)3-lysine; PMA, phorbol 12-myristate 13-acetate; HSP60, heat shock protein 60; NF, nuclear factor; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; ECD, extracellular domain; ICD, intracellular domain; EMSA, electromobility shift assay; HEK, human embryonic kidney; MEF, $TLR2^{-/-}$ embryonic fibroblasts; IL, interleukin; mAb, monoclonal antibody.

TLR2 agonist largely resemble TLR4 agonist effects but differ in some aspects (4, 14). In addition, cooperation between TLR1 and TLR2 in recognition of triacylated peptides, as well as cooperation between TLRs 6 and 2 for diacylated mycoplasmal peptides, has been reported previously (15–17). Whether heteromerization is obligatory for cellular recognition of specific PAMPs such as acylated proteins or whether it is required for all TLR2-mediated effects remains unknown. In addition, specific TLR homodimers/heterodimers might associate with further receptor chains such as CD14, MD-2, and/or MD-1/Rp105 as has been demonstrated for TLR4 (18, 19).

Because the extracellular domain of TLR2 is considered to interact with various PAMPs (see above) (15), the question arises whether different parts of the ECD interact with these various ligands. TLR ECD sequences include arrays of leucine-rich repeat (LRR) motifs. The LRR consensus sequence encompasses 24–29 amino acid residues containing a highly conserved core region (L*XXLXLXX*(N/L)*XLXXLXX*L) and is implicated in protein-protein interaction (5, 20). Crystal structures of multi-LRR domains of proteins such as ribonuclease inhibitor and internalin potentially provide a model of TLR ECD structure (20, 21). The ribonuclease inhibitor and internalin crystal structures revealed that the LRR motifs are composed of β strand-helix modules with the β strands being oriented in parallel and positioned in close proximity. Based on these structural considerations, it might be expected that mutations of the extracellular domain of TLR2 could influence susceptibilities to infections. This has been implicated for a polymorphism of the TLR2 intracellular domain (ICD), which correlates with altered functionality of the receptor (22).

Accordingly, we generated TLR2 ECD deletion mutants and compared the ability of the resulting protein constructs to mediate recognition of a variety of TLR2-specific PAMPs. We have found that cell activation by distinct TLR2-specific PAMPs requires different subdomains of the TLR2 ECD.

EXPERIMENTAL PROCEDURES

Reagents—Bacillus subtilis (DSMZ.1087) and *Escherichia coli* (DH5α, Invitrogen) were cultured in standard brain-heart medium overnight at 37 °C. Bacterial cells were adjusted to a concentration of 1×10^{11} colony-forming units/ml. Bacterial suspensions were heat-inactivated at 56 °C for 45 min and adjusted to a concentration of 1×10^8 colony-forming units/ml in cell culture experiments (heat-inactivated *B. subtilis* and heat-inactivated *E. coli*). *Borrelia burgdorferi* inactivated through sonication was kindly provided by Dr. Weis (University of Utah, Salt Lake City, UT) and applied at a protein concentration of 1.9 μg/ml. LPS from *E. coli* 0111:B4 (Sigma) was generally applied at a concentration of 0.1 μg/ml. sPGN was prepared from *Staphylococcus aureus* by vancomycin affinity chromatography (23) and applied at a concentration of 10 μg/ml or as indicated. Highly purified LTA from *B. subtilis* (DSMZ 1087) was applied at a concentration of 5 μg/ml (24). Synthetic mycoplasmal macrophage-activating lipoprotein (R-MALP)-2 was from Dr. Mühlradt (GBF Braunschweig, Germany) and applied at a concentration of 1.3 ng/ml or as indicated (17). Synthetic *N*-palmitoyl-*S*-(bis (palmitoyloxy)propyl)cysteinyl-seryl-(lysyl)-3-lysine (P_3CSK_4), *S*-bis(palmitoyloxy)propyl-CSK_4 (P_2CSK_4), and *N*-palmitoyl-CSK_4 ($PCSK_4$) were purchased from ECHAZ microcollections (Tübingen, Germany) (25) and applied at a concentration of 0.1 μg/ml if not indicated otherwise. Lipidated OspA, a tripalmitoylated lipoprotein from *B. burgdorferi*, was from Dr. Dunn (Brookhaven National Laboratory, Upton, NY) and applied at 4.5 μg/ml (26). Highly purified recombinant chlamydial HSP60 was as described previously (27) and applied at a concentration of 8 μg/ml. Zymosan and phorbol 12-myristate 13-acetate (PMA) were from Sigma and applied at concentrations of 50 μg/ml and 0.1 μg/ml, respectively.

Mutagenesis—A wild-type human TLR2 expression plasmid lacking the original leader sequence in favor of a 5′-terminally fused trypsin leader followed by a FLAG tag coding sequence (pFLAG-CMV, Sigma) was employed as template in overlap-PCR based mutagenesis (QuikChange kit, Stratagene, Amsterdam, Netherlands). Deletion mutants lacking the following internal peptides as determined from the TLR2 cDNA sequence (GenBank™ accession number HSU88878) were generated as follows: Mut1-(ΔS48-F170); Mut2-(ΔF170-D301); Mut3-(ΔR302-T431); Mut4-(ΔS424-N533); MutA-(ΔL173-S196); MutB-(ΔL173-V220); MutC-(ΔL123-F170); MutD-(ΔL123-V220); MutE-(ΔL123-N274); MutF-(ΔS48-K121); MutG-(ΔS48-S196); MutH-(ΔS48-V220); MutI-(ΔS48-T262); and MutJ-(ΔS48-C287) as well as MutCK-(ΔK19-N533); MutCD-(ΔK19-V578); TLR2ΔICD-(ΔY617-S784); and TLR2ICD-(ΔM1-H610). Positioning of deletion termini was performed by application of the Psipred software program (bioinf.cs.ucl.ac.uk/psipred/). Minimal changes of the secondary structure and line up of LRR β sheet subdomains as revealed from computer based calculation served as main criterion.

Cell Culture—The human embryonic kidney cell line (HEK) 293 as well as $TLR2^{-/-}$ embryonic fibroblasts (MEFs) were applied for protein overexpression and functional analysis. $TLR2^{-/-}$ mice were kindly provided by Tularik Inc. (South San Francisco, CA) (28). $TLR2^{-/-}$ mouse embryonic fibroblasts (MEFs) were generated from embryos isolated at day 12 post-fertilization. Cells were grown under regular mammalian cell culture conditions in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (Roche Applied Science), standard antibiotics (Invitrogen, Auckland, Scotland), and 50 μM thioglycerol (Sigma). Cells were passaged and expanded for five times. Frozen stocks were thawed and cultured for experiments.

Reporter Gene Assay—3×10^4 HEK293 cells or $TLR2^{-/-}$ MEFs were cultured on single wells of 96-well plates. HEK293 cells were cotransfected with an NF-κB-recruiting endothelial-leukocyte adhesion molecule-1 (CD62E) promoter luciferase construct and a Rous sarcoma virus promoter β-galactosidase reporter plasmid (29) as well as a cytomegalovirus promoter-regulated expression plasmid for human TLR2 by the calcium phosphate precipitation method (10, 30). For equilibration of expression levels, DNA amounts used were adjusted and expression levels were analyzed by immunoblot analysis (data not shown). $TLR2^{-/-}$ MEFs were transfected by electroporation at 960 microfarads and 260 mV (Gene Pulser II system, Bio-Rad). 7 h after medium change, preparations of bacterial products or analogues were added to transfected cells for 16 h. Cells were lysed for measurement of luciferase and β-galactosidase activities using reagents from Promega (Madison, WI) and PE Biosystems (Bedford, MA). Luciferase activities were related to β-galactosidase activities for normalization.

Enzyme-linked Immunosorbent Assay—$TLR2^{-/-}$ MEFs and HEK293 cells were cultured on 96-well plates (2×10^5 cells/well) with bacterial components for 16 h as indicated. Culture supernatants were applied to enzyme-linked immunosorbent assay (R&D systems, Minneapolis, MN) for measurement of murine IL-6 as well as human IL-8 concentrations, respectively, by enzyme-mediated colorimetry (Magellan, Tecan, Crailsheim, Germany) according to supplier protocols.

Immunoblot Analysis—HEK293 cells were lysed upon protein overexpression and stimulation for 30 min. Lysates from 2.5×10^5 cells or immune complexes prepared from 3×10^6 cells for each sample were prepared and analyzed by immunoblot analysis as described previously (31). For analysis of JNK phosphorylation, 3-fold amounts of total lysates (approximately 7.5×10^5 cells) were applied. Rabbit polyclonal antisera specific for phosphorylated p38, ERK1/2, JNK, or Akt/protein kinase B were used (Cell Signaling). Specific epitopes were visualized by enhanced chemiluminescence (Western lightning, PerkinElmer Life Sciences).

Electromobility Shift Assay (EMSA)—1×10^6 HEK293 cells were stimulated for 2 h in Dulbecco's modified Eagle's medium serum containing 2% fetal calf serum. Cells were washed with ice-cold phosphate-buffered saline and lysed (10 mM HEPES, pH 7.9, 10 mM KCl, 0.5% Nonidet P-40, 0,1 mM EDTA, 0.2 mg/ml leupeptin and aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). Nuclei were pelleted and lysed (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 0.2 mg/ml leupeptin and aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). Debris was pelleted, and supernatant recovered (32). 5 μg of protein was applied to EMSA with a radioactively labeled double-stranded DNA oligonucleotide (5′-GATGCC ATTGGG GATTTC CTCTTT ACTG-3′) representing an NF-κB recognition element of the endothelial-leukocyte adhesion molecule-1 promoter sequence (29). Results were visualized by PhosphorImager (Storm 840, Amersham Biosciences) aided signal detection.

Intracellular Staining—Pools of transfected HEK293 cell clones were grown on polylysine-coated glass carriers each with eight culture dishes with removable walls (BD Biosciences). Cells were washed with phosphate-buffered saline and incubated with 50 μg/ml Alexa Fluor 488-conjugated concanavalin A (Molecular Probes) in serum-free Dulbecco's modified Eagle's medium at 4 °C for 15 min. The medium was removed, and the cells were washed with phosphate-buffered saline and fixed with 2% formalin for 20 min at room temperature. Cells were washed

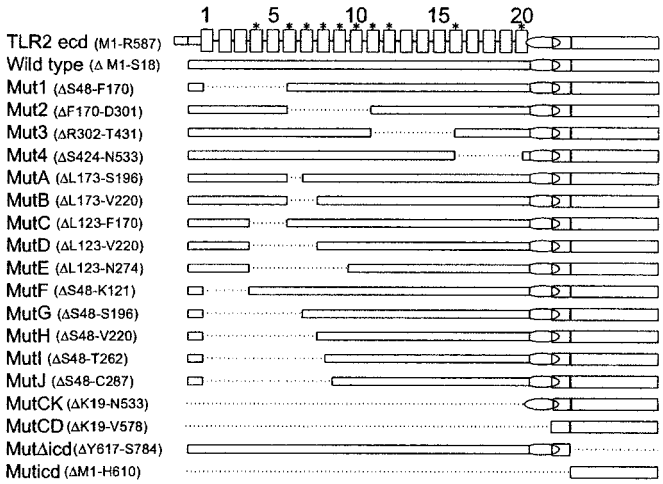

FIG. 1. **Illustration of deletion mutants.** Scheme of TLR2 with its ECD containing 20 LRRs (*boxes*)/LRR-like motifs (*boxes* with *asterisk*), the LRR C-terminal (*oval*), and transmembrane (*small rectangle*) as well its intracellular domain (*large rectangle*) from its N terminus (*left*) to its C terminus (*right*) in relation to 18 deletion mutants generated thereof is shown. Deleted regions are depicted as *dotted lines* (Δ amino acid residues). In wild-type and mutant constructs (with the exception of Muticd lacking a signal peptide), the original signal peptide was replaced by a heterologous signal and an N-terminal FLAG tag peptide.

and blocked with phosphate-buffered saline containing 0.2% saponin and 3% bovine serum albumin for 30 min at room temperature. A first antibody, either anti-FLAG polyclonal rabbit antiserum (3 μg/ml) from Sigma or mouse monoclonal anti-human TLR2 2.1 (5 μg/ml) provided by Dr. Lien was applied prior to washing after 30 min of incubation. As a second antibody, Alexa Fluor 546-conjugated goat anti-rabbit/mouse IgG (4 μg/ml) was applied for 30 min (Molecular Probes) and washed. Cells were sealed in the presence of mounting fluid (*Chlamydia pneumoniae* micro-IF, Labsystems Oy, Helsinki, Finland) for analysis with a laser-scanning microscope with documentation unit (LSM510, Carl Zeiss, Oberkochen, Germany).

Immunoprecipitation—For immunoprecipitation of transiently overexpressed proteins, 10 μg of total expression plasmid DNA for the expression of the respective two proteins was transfected into 3×10^6 HEK293 cells seeded on 100-mm dishes by the calcium phosphate precipitation method (30). Mutant constructs and controls applied were overexpressed as FLAG-tagged hybrid proteins while the co-expressed protein was Myc-tagged. FLAG mAb M2 beads were used for precipitation (Sigma) (31). Immunocomplexes were analyzed by application of polyclonal anti-Myc tag antiserum for immunoblot analysis (Santa Cruz Biotechnology, Santa Cruz, CA).

RESULTS

Mutagenesis—Structural information about TLR ECDs is restricted to sequence-based domain assignment at this stage. Using this method, the presence of 19 LRRs in TLR2 has been described previously (5). In addition to the 10 canonical LRR motifs, we have assigned 10 LRR-like motifs to the TLR2 ECD previously (9). We deleted single or groups of LRR/LRR-like motifs from the TLR2 ECD with the assumption that the removal of entire LRR subdomains would not alter overall protein structure (Fig. 1). We used the resulting constructs for potential identification of domains distinctively involved in cellular PAMP recognition. All of the TLR2 constructs were expressed at similar levels as revealed by anti-FLAG tag immunoblot analysis of total lysates of HEK293 cells following transfection of equal amounts of specific expression plasmid DNA preparations. The sizes observed were in agreement with expected mutant protein sizes. DNA amounts were adjusted for transfection, and expression levels were controlled by immunoblot analysis (data not shown).

Functional Analysis of TLR2 ECD Mutant Constructs—For all of the preparations of bacterial products used, wild-type TLR2 conferred NF-κB dependent reporter gene activation and release of IL-8 in HEK293 cells (Tables 1 and 2). The mean values displayed in both tables represent the results of at least three independent experiments. The data in Table 1 were normalized by calculation of the ratio of NF-κB-dependent and constitutive reporter gene activity. The significance of all of the values listed in the tables was analyzed through application of the Student's *t* test for unconnected samples upon relation to vector controls. sPGN and all of the additional non-tripalmitoylated TLR2 agonists used did not induce cellular activation through any of the TLR2 mutant constructs overexpressed at equal levels. Examples are constructs Mut2 to Mut4, which cover three-fourths of the entire LRR-rich region (Fig. 1 and Tables 1 and 2). Mut1, however, mediated a weak signal upon application of P_3CSK_4 (Tables 1 and 2). In contrast, MutA and MutB lacking the LRRs adjacent to the Mut1 deletion, single LRR6, or LRRs 6 and 7, respectively, were not functional. This was also true for MutC carrying a deletion that was limited to the C terminus of Mut1 (LRRs 4 and 5) and two constructs carrying C-terminally extended deletions, namely MutD (LRRs 4–7) and MutE (LRRs 4–9) (Fig. 1 and Tables 1 and 2). Deletion of the N-terminal three LRRs abrogated cell activation as well (MutF). Notably, C-terminal extension of a deletion represented by Mut1 resulted in a successively increasing cell activation upon application of P_3CSK_4, OspA, and inactivated *B. subtilis* through MutG (LRRs 1–6) and MutH (LRRs 1–7), respectively (Fig. 1 and Tables 1 and 2). However, further C-terminal extension of the deletion abrogated cell activation as revealed from overexpression and analysis of the constructs MutI and MutJ, which lack the eight and nine N-terminal LRRs, respectively (Fig. 1 and Tables 1 and 2). Results from analysis of transiently and stably transfected HEK293 cells were similar (data not shown).

We further analyzed whether increased expression levels of MutH or other mutants would enable cellular recognition of a wider variety of PAMPs. Transfection of 50-fold amounts of expression plasmid for MutH as compared with wild-type TLR2 partially conferred cellular activation by the diacylated peptide R-MALP 2 in a dose-dependent manner. However, activation by application of other TLR2 agonists such as sPGN was barely detectable even upon application of very high amounts of stimulants (Fig. 2*A*). None of the other mutants mediated responsiveness following either increased expression or through application of ligands at high concentrations (Fig. 2*A*).

To assess the role of single palmitoylations for recognition of tripalmitoylated peptides by TLR2, two P_3CSK_4 derivatives, P_2CSK_4 and $PCSK_4$, were used to challenge transiently transfected HEK293 cells expressing each of the TLR2 mutants that were generated (Fig. 2*B*). Wild-type TLR2 conferred NF-κB activation upon application of all three derivatives. The constructs MutG and MutH mediated response to P_3CSK_4 as well as P_2CSK_4 to different degrees. In the case of MutH, P_2CSK_4 induced a more robust NF-κB activation compared with P_3CSK_4 (Fig. 2*B*). $PCSK_4$, albeit clearly activating cells expressing wild-type TLR2, did not elicit a significant signal through any of the mutant constructs analyzed (Fig. 2*B*). Similar results were obtained upon transfection of 50-fold amounts of mutant expression plasmid as compared with wild-type TLR2 plasmid as well as application of very high amounts of stimulants (Fig. 2*C*). Cotransfection of both wild-type TLR2 and each of the mutant DNA constructs in a ratio of 1:50 was performed for analysis of mutant effects on wild-type TLR2-mediated cell activation. TLR2 deletion mutants inhibited wild-type TLR2-mediated cell activation when transfected cells were stimulated with heat-inactivated *B. subtilis* or P_3CSK_4 with the exception of MutH (data not shown). Consistent with results from analysis of transfection of HEK293 cells, overex-

TABLE I

TLR2 ECD mutant-mediated NF-κB dependent reporter gene activation in HEK293 cells

The values indicated by minus signs are <1.0.

Rel. Luc. activity	Vector	Wild-type	Mutant													
			1	2	3	4	A	B	C	D	E	F	G	H	I	J
Unstim.	–	2.1	1.6	–	–	–	–	–	–	–	–	–	–	–	–	–
s. B.b.	–	21.2[a]	2.1[a]	–	1.4	–	–	–	–	–	–	1.3	1.7	4.3[a]	–	–
h.i.E.c.	2.1	11.1[a]	2.9	2.2	2.3	1.6	2.3	1.5	1.6	1.6	1.7	2.3	2.5	2.2	1.8	1.9
h.i.B.s.	5.3	32.5[a]	7.5	6.3	3.1	2.8	4.0	3.7	4.8	2.9	3.7	5.2	12.9[a]	31.6[a]	4.1	4.2
Zymos.	–	9.1[a]	1.4	–	–	–	1.5	–	–	–	–	–	1.2	–	1.2	–
LPS	1.5	16.0[a]	2.6	1.1	1.1	–	–	–	–	–	–	–	1.4	2.5	1.3	–
sPGN	1.2	20.2[a]	2.3	0.8	1.2	–	1.4	1.3	–	–	–	–	1.1	1.2	1.1	–
LTA	1.3	22.0[a]	1.6	1.4	1.3	1.1	1.4	–	–	–	–	1.2	–	1.3	1.2	–
HSP60	1.5	10.7[a]	1.6	1.8	1.7	1.5	1.4	1.2	–	–	–	1.1	1.1	2.1	1.2	1.9
OspA	1.9	27.1[a]	1.9	1.7	1.6	1.7	1.4	1.1	–	–	–	1.1	2.5	3.4	1.2	–
P_3CSK_4	1.7	34.9[a]	4.3[a]	1.4	–	1.2	1.5	1.5	–	–	–	2.0	4.8[a]	16.2[a]	–	–
MALP-2	–	22.0[a]	1.3	–	1.4	–	–	–	–	1.4	1.1	–	–	1.2	–	1.1
PMA	8.5[a]	8.2[a]	12.8[a]	17.5[a]	16.9[a]	10.7[a]	18.7[a]	18.9[a]	14.8[a]	13.3[a]	12.5[a]	7.1[a]	13.0[a]	9.5[a]	14.2[a]	5.0[a]

[a] $p < 0.001$; significance as revealed from Student's *t* test for unconnected samples by relation to vector control for the regarding stimulant (PMA induction values were related to the vector-unstimulated value).

TABLE II

TLR2 ECD mutant mediated IL-8 release from HEK293 cells upon PAMP application

The values indicated by minus signs are <0.005.

IL-8 ng/ml	Vect.	Wild-type	Mutant													
			1	2	3	4	A	B	C	D	E	F	G	H	I	J
Unstim.	–	–	–	–	–	–	–	0.01	0.01	–	–	0.01	0.01	0.01	0.01	0.01
s.B.b.	0.01	0.26	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.02	0.01	0.02
h.I.E.c.	0.01	0.74[a]	0.01	0.01	–	0.01	–	–	–	0.01	0.01	0.03	0.01	0.02	0.01	0.02
h.i.B.s.	0.26	1.30[a]	0.27	0.23	0.10	0.14	0.10	0.14	0.31	0.31	0.29	0.38	1.18[a]	3.53[a]	0.63	0.43
Zymos.	–	0.36[a]	0.06	–	0.07	–	–	–	–	–	–	–	–	–	–	–
LPS	–	0.55[a]	–	–	–	–	0.01	0.01	0.01	0.00	0.01	0.03	0.03	0.02	0.02	0.02
sPGN	–	0.45[a]	–	–	–	–	–	–	–	–	–	0.01	–	0.01	–	0.01
LTA	–	1.15[a]	–	–	–	–	–	–	–	0.02	–	0.02	–	0.01	–	0.01
HSP60	–	0.30[a]	–	–	–	–	–	–	–	–	–	–	–	0.01	–	–
OspA	–	1.05[a]	–	–	0.10	–	0.01	0.02	–	–	–	0.04	0.02	0.20	0.02	0.03
P_3CSK_4	0.03	1.39[a]	0.08	0.05	0.05	0.05	0.05	0.05	0.05	0.03	0.04	0.04	0.08	1.32[a]	0.15	0.03
MALP-2	–	0.28[a]	–	–	–	–	–	–	–	–	–	0.02	–	–	–	0.01
PMA	0.24[a]	0.29[a]	0.24[a]	0.28[a]	0.30[a]	0.29[a]	0.21[a]	0.30[a]	0.32[a]	0.23[a]	0.18[a]	0.31[a]	0.21[a]	0.22[a]	0.21[a]	0.35[a]

[a] $p < 0.05$; significance as revealed from Student's *t* test for unconnected samples by relation to vector control for the regarding stimulant (PMA induction values were related to the vector-unstimulated value).

pression of wild-type human TLR2 restored responsiveness toward LTA as well as P_3CSK_4 in $TLR2^{-/-}$ MEFs as indicated by NF-κB-dependent reporter gene activation (Fig. 3*A*) and release of IL-6 (Fig. 3*B*). In contrast, MutH-mediated cell activation was restricted to P_3CSK_4 stimulation and further mutants such as MutJ were inactive (Fig. 3, *A* and *B*).

Cellular Localization of Wild-type and Mutant TLR2—Pools of six cell clones overexpressing wild-type TLR2, Mut1, MutF, MutG, MutH, or MutJ were analyzed immunocytochemically. Concavalin A was used for staining of the cell membrane. Although control HEK293 cells did not express a FLAG epitope, overexpression of wild-type FLAG-TLR2 revealed the localization of the tagged protein specifically at the cell membrane (Fig. 4). Overexpressed wild-type TLR2 and all of the mutant proteins analyzed were located at the cellular membrane and not within the cell as revealed by comparison with overexpressed FLAG-tagged IL-1 receptor-associated kinase 1, which represents a cytoplasmically located protein (Fig. 4 and data not shown). We further applied an anti-human TLR2 monoclonal antibody (mAb 2.1) for analysis. The presence of wild-type TLR2, MutH, and MutG at the cell membrane was confirmed. None of the other mutants used was recognized by mAb 2.1 to a detectable degree, although most of them inevitably carried the domain forming the respective epitope in wild-type TLR2 and MutH (data not shown).

DNA Binding of NF-kB and Phosphorylation of Cellular Kinase Akt as Well as That of Mitogen-activated Protein Kinases p38, ERK1/2, and JNK Mediated by TLR2 and Mutant Receptors—Controls as well as HEK293 cell clone pools stably expressing wild-type TLR2, MutH, or MutJ were subjected to molecular analyses of cell activation. Nuclear extracts as well as total lysates of cells were prepared 2 h or 30 min after the start of stimulation, respectively, with sPGN, P_3CSK_4, or PMA. Nuclear extracts were applied to EMSA and total lysates for analyses of cellular kinase Akt as well as mitogen-activated protein kinases p38, ERK1/2, and JNK phosphorylations by immunoblot analysis. EMSA revealed nuclear translocation and binding of NF-κB to a canonical NF-κB DNA recognition element as well as kinase phosphorylation in all of the clones upon PMA stimulation as compared to unstimulated cells (Fig. 5, *A* and *B*). NF-κB activation and phosphorylation of kinases analyzed upon stimulation with sPGN depended on the expression of wild-type TLR2 but were absent in all of the other clones tested. P_3CSK_4 induced activation of NF-κB, and phosphorylation of cellular kinases was not restricted to HEK293 cells overexpressing wild-type TLR2 but was also observed in cells overexpressing MutH lacking the N-terminal seven LRRs. Control HEK293 cells as well as cells overexpressing mutant J did not respond to challenge with P_3CSK_4 as revealed from NF-κB EMSA and analysis of kinase phosphorylation (Fig. 5, *A* and *B*).

Interaction of Wild-type TLR2 with Mutant TLR2 Constructs—To analyze the role of the TLR2 ECD in homologous or heterologous interaction of TLR2, we performed immunoprecipitation experiments. FLAG-tagged wild-type TLR2, Mut1, Mut2, Mut3, Mut4, MutH, MutJ, MutCK, MutCD, or vector as negative control were cotransfected with Myc-tagged wild-type TLR2 or TLR1. FLAG-tagged mutant as well as wild-type proteins as indicated all coprecipitated with the Myc-tagged wild-

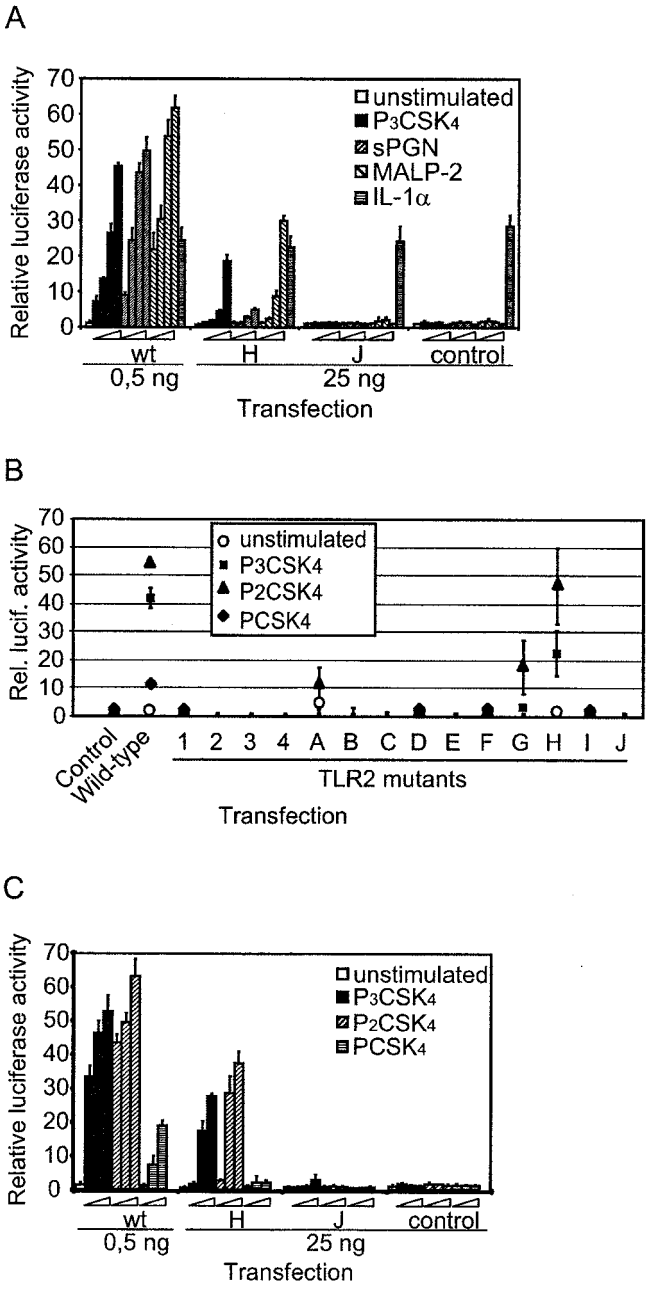

FIG. 2. **TLR2 mutant-mediated stimulus and NF-κB-dependent reporter gene activation in HEK293 cells.** Cells were cotransfected with reporter gene constructs as well as CD14 and wild-type or mutant TLR2 expression plasmids. 24 h after transfection started, cells were stimulated with the microbial products or synthetic derivatives as indicated for 16 h and lysed. Cell activation was measured as luciferase reporter activity in the lysates. Experiments were repeated at least twice. (*A*) dose kinetics of agonists applied to cells transfected with 50-fold amounts of expression plasmids for TLR2 mutants MutH and MutJ as compared to wild-type TLR2 (IL-1α as positive control). Amounts of agonists applied increased successively: P_3CSK_4, 1 ng/ml, 10 ng/ml, 100 ng/ml, and 1 μg/ml; sPGN, 10 ng/ml, 100 ng/ml, 1 μg/ml, and 10 μg/ml; MALP-2, 10 pg/ml, 0.1 ng/ml, 1 ng/ml, and 10 ng/ml; and IL-1α, 20 ng/ml. *B*, P_3CSK_4 in comparison with P_2CSK_4 and $PCSK_4$ induced cell activation through TLR2 and mutants as indicated. *C*, dose kinetics of agonists applied to cells transfected with 50-fold amounts of expression plasmids for TLR2 mutants MutH and MutJ as compared to wild-type TLR2. Amounts of agonists applied increased successively: P_3CSK_4 and P_2CSK_4, 1 ng/ml, 10 ng/ml, 100 ng/ml, and 1 μg/ml; $PCSK_4$, 10 ng/ml, 100 ng/ml, 1 μg/ml, and 10 μg/ml.

type TLR2 or TLR1 (Fig. 6 and data not shown). Even TLR2 mutant constructs either lacking both the entire LRR-rich domain and the LRR C-terminal C-rich (LRRCT, MutCD) domain

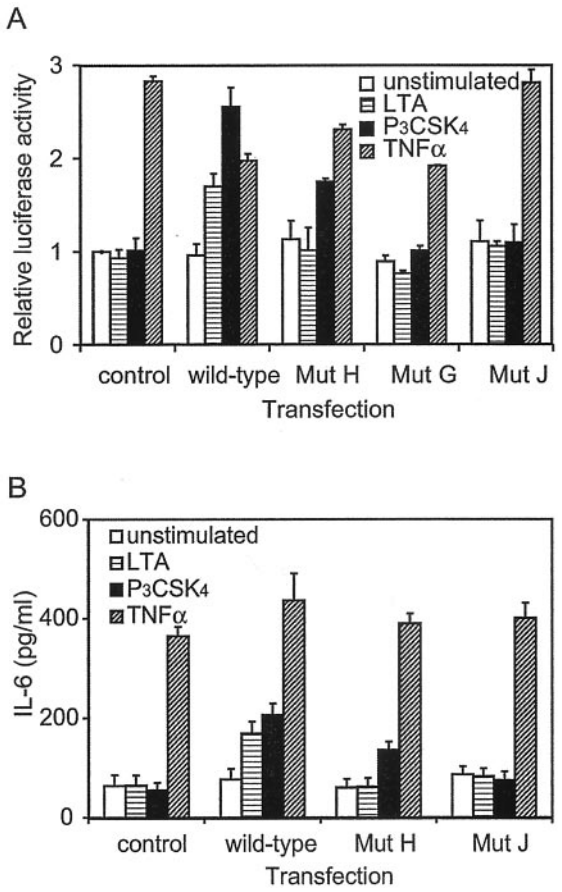

FIG. 3. **TLR2 wild-type/mutant-mediated stimulus-dependent reporter gene activation in $TLR2^{-/-}$ MEFs and IL-6 release from $TLR2^{-/-}$ MEFs.** Results upon overexpression of wild-type human TLR2 or TLR2 mutant constructs in $TLR2^{-/-}$ MEFs in terms of NF-κB-dependent reporter gene activation (*A*) and IL-6 release (*B*) are illustrated. tumor necrosis factor α was applied for stimulation as positive control.

or the LRR-rich domain only (MutCK) coprecipitated with the wild-type TLRs 2 and 1 (Fig. 6 and data not shown).

DISCUSSION

Comparative mutational analysis of mouse and human TLR4 and analysis of TLR5 implicated particular ECD domains in species-specific recognition of LPS modifications and binding of flagellin, respectively (33, 34). TLR2 and/or TLR4 binding of glucuronoxylomannan capsules of *Cryptococcus neoformans* as well as of LPS and sPGN have been reported previously (35–37). To date, evidence for direct binding of PAMPs to TLRs as well as recognition of a relatively large variety of PAMPs particularly through TLR2 is compelling and may imply the existence of different binding sites of various specific ligands. Here we used mutagenesis of the TLR2 ECD for its functional analysis.

We speculated that in addition to the 10 canonical LRR, 8–10 LRR-like motifs present in the TLR2 ECD sequence might represent functionally relevant subdomains (5, 9). The LRRs are evenly distributed throughout the TLR2 ECD, and we deleted them in four blocks, each containing five motifs (Fig. 1). We then focused on the 10 N-terminal LRRs by successive deletion of internal regions. In total, 14 mutant ECD TLR2 constructs were generated (Fig. 1). Specifically, we asked which of the ligands within a representative group of known agonists were able to induce cell activation through mutant constructs. We identified one class of agonists inducing signaling in the absence of the seven N-terminal LRRs. As such, our results

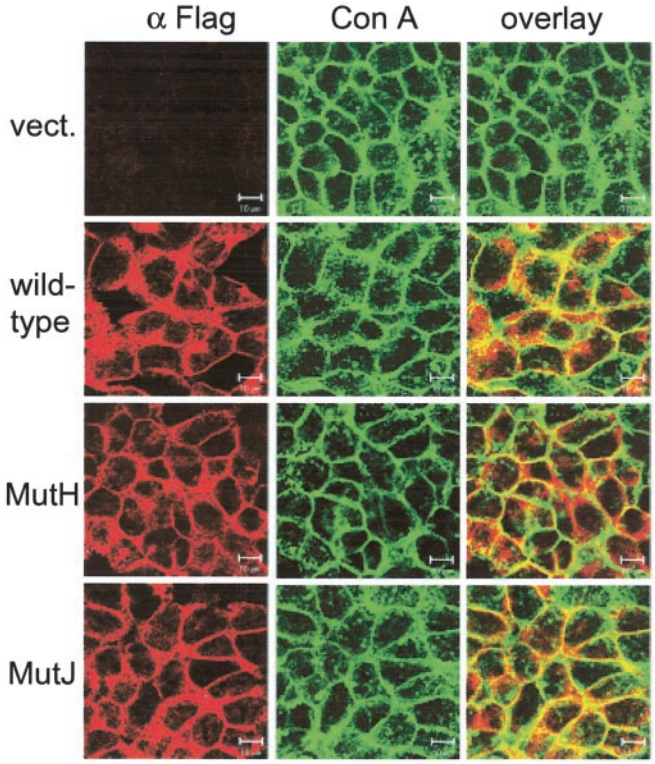

FIG. 4. **Cellular localization of TLR2 mutant constructs upon overexpression in HEK293 cells.** Pools of HEK293 cell clones stably overexpressing wild-type TLR2 or mutants MutH or MutJ as well as negative controls were analyzed for cellular localization of FLAG-tagged proteins. Concavalin a (*Con A*) was applied for staining of cellular membranes (*green*), whereas FLAG epitope (*α-Flag*) localization was analyzed by immunostaining (*red*). Overlay of both signals was performed (*yellow*). *Bar* in each picture represents distance of 10 μm on the original slide.

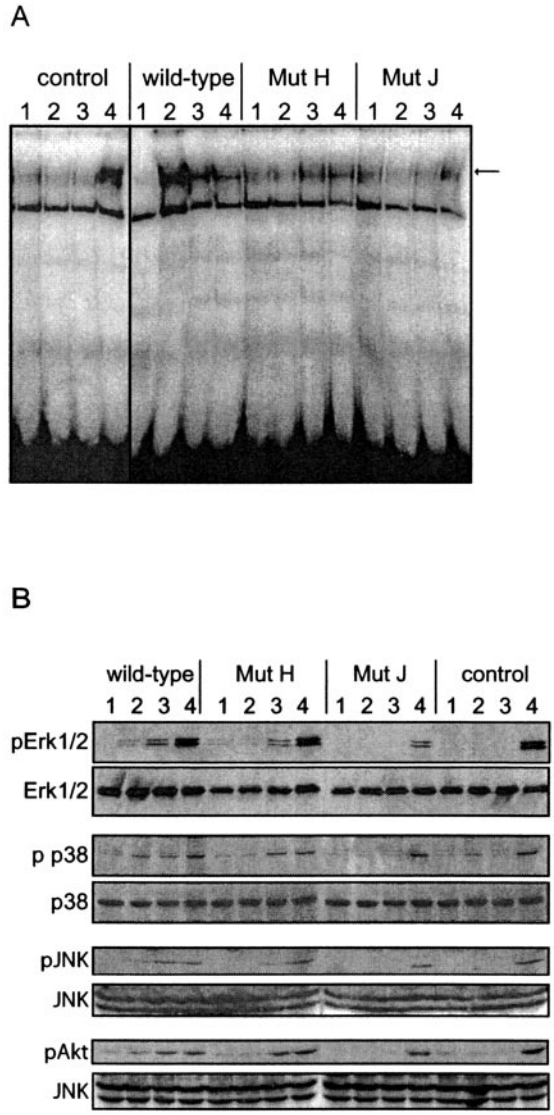

FIG. 5. **Stimulus-dependent cellular activation of TLR2 mutant overexpressing HEK293 clone pools as revealed by NF-κB specific EMSA and phospho-ERK1/2, p38, JNK, and Akt-specific immunoblot analysis.** Pools of HEK293 cell clones stably overexpressing wild-type TLR2 or mutants MutH or MutJ as well as negative controls were analyzed for NF-κB DNA binding activity 2 h upon stimulation by EMSA (*A*), as well as after 30 min of stimulation by phospho- (*p*) Erk1/2, p38, JNK, and Akt-specific immunoblot analysis of total cell lysates (*B*): *1*, unstimulated; *2*, sPGN (10 μg/ml); *3*, P_3CSK_4 (0.1 μg/ml); and *4*, PMA (1 μg/ml). The NF-κB-specific signal is *marked* by an *arrow*, whereas a signal beneath was nonspecific. Equal protein loading was controlled by application of antibodies specific for the regarding kinases independent from activation as indicated. Only pAkt analysis was controlled by application of an antibody specific for a distinct kinase (*B*, *JNK*).

imply interaction of dipalmitoylated/tripalmitoylated peptides with the C-terminal region of the TLR2 ECD (38).

A trend of mutant activity became evident at equal expression levels of each TLR2-derived construct. Only MutG and MutH conferred cell activation to significant degrees upon challenge with P_3CSK_4, P_2CSK_4, or OspA but not upon application of any of the other PAMPs and analogues as indicated (Fig. 2 and Tables 1 and 2). Tripalmitoylation is a typical characteristic of bacterial proteins eliciting host responses through TLR2 (4, 39). Additionally, increased overexpression in combination with application of increased amounts of synthetic R-MALP-2 rendered MutH-expressing cells responsive, whereas sPGN-induced cell activation was barely detectable (Fig. 2*A*). None of the mutants encompassing Mut2 to Mut4, MutA to MutF, as well as MutI and MutJ (Fig. 1) mediated activation of the signaling pathways analyzed upon application of any TLR2-specific agonist (Tables 1 and 2).

TLR2 dependence of cell activation upon challenge with P_3CSK_4, P_2CSK_4 lacking the amide-linked fatty acid, and $PCSK_4$ missing the two ester-bound fatty acids was in line with a recent report describing primary immune cell responses to two of these ligands *ex vivo* as TLR4-independent (39). Although stimulating activities of P_2CSK_4 and P_3CSK_4 were almost equal, those of $PCSK_4$ were only 30–50% (Fig. 2*B*). On the other hand, palmitoylation of a peptide at the amino group of a terminal cysteine was sufficient for recognition through TLR2, yet dipalmitoylation increased the stimulatory potential of the peptide considerably. These results suggest that triacylation is not obligatory for TLR2-dependent stimulatory activity. It also raises questions for further aspects of structural properties, which recently have been addressed in the case of a diacylated peptide such as R-MALP-2 (17). Notably, the levels of P_2CSK_4-induced cell activation were similar when mediated through MutH or wild-type TLR2. However, $PCSK_4$ did not induce cell activation through any of the mutant TLR2 constructs (Fig. 2*B*). Thus, the additional palmitoylation of $PCSK_4$ confers independence of cellular recognition from the N-terminal third of the TLR2 LRR-rich domain. This was confirmed upon increased TLR2 mutant expression and amounts of stimulants applied (Fig. 2*C*). Interestingly, recognition of diacylated R-MALP-2 through MutH was detectable only at increased expression levels (Fig. 2, *A* and *B*).

Overexpression of mouse TLR2 (15) as well as that of human TLR2 (Fig. 3, *A* and *B*) complemented cellular responsiveness

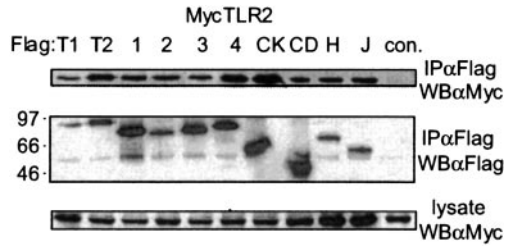

FIG. 6. **Immunoprecipitation of wild-type TLR2 and single mutant constructs.** HEK293 cells were transfected with Myc-tagged wild-type TLR2 and FLAG-tagged TLR2 mutant constructs (Mut1–4, Muts H and J), wild-type TLR1 (T1), or TLR2 (T2) expression plasmids as indicated. Additionally, two deletion mutants lacking the entire LRR-rich domain (*CK*) or the LRR-rich and the LRRCT domain (*CD*) of TLR2 were analyzed. 48 h after transfection started, cells were lysed and subjected to FLAG-specific immunoprecipitation (*IP*). *WB*, immunoblot analysis with antibodies indicated *con*., Myc-TLR2 only (protein marker size in kDa).

to specific agonists of primary TLR2$^{-/-}$ MEFs. Consistent with the above described results, overexpression of construct MutH in TLR2$^{-/-}$ MEFs mediated P_3CSK_4 but not LTA or sPGN-induced NF-κB-dependent reporter gene activation and release of IL-6 (Fig. 3, *A* and *B*). Our results further demonstrated comprehensive effects of specific LRR deletions on signal transduction in terms of nuclear translocation and DNA binding of NF-κB as well as Akt, p38, ERK1/2, and JNK phosphorylation upon challenge with TLR2 agonist P_3CSK_4 or sPGN (Fig. 5, *A* and *B*).

Localization of the overexpressed TLR2 mutant proteins at the cell membrane was observed (Fig. 4 and data not shown), while no evidence for cytoplasmic localization was revealed that might have indicated non-functionality (40, 41). Although we intended to minimize disruptions of protein structure, the malfunction of mutants such as MutA to MutD might result from disruption of a complex tertiary protein structure. Thus, a larger functionally important subdomain might be formed by the N-terminal third of the TLR2 ECD. This proposed subdomain might be structurally independent from the rest of the TLR2 ECD and possibly either consists of a LRR subgroup or is just coincidentally represented by the respective seven sequence motifs. Deletions within (Mut1 to Mut4, MutA to MutG) and deletions extending beyond this proposed N-terminal seven LRR subdomain (MutI, MutJ) might have caused severe structural changes biasing TLR2 mutant function. In contrast, the removal of the whole domain might have rather preserved the structure and function of the rest of the protein, thus retaining recognition of agonists that do not require its presence. These notions are further supported by results obtained from application of anti-FLAG antibodies for FACS analysis and of a human TLR2-specific monoclonal antibody (mAb T2.1) for cytochemical analysis, suggesting deletion-dependent disruption of wild-type TLR2 structure in most constructs, to a limited extent only in MutG but not in MutH (data not shown).

We found no evidence for involvement of the TLR2 ECD in receptor homodimerization or heteromerization. As revealed by immunoprecipitation upon overexpression, none of the deletions in the TLR2 ECD caused abrogation of interaction with wild-type TLR2 and TLR1 (Fig. 6 and data not shown). This finding might explain dominant negative effects of mutants (data not shown) through their binding to the wild-type receptors. Because the TLR4 ECD mediates ligand specificity of receptor activation (42), TLR2 ECD mutants may interfere with homodimerization or heteromerization of wild-type TLR2 or TLR1 and prevent appropriate receptor complex activation upon specific extracellular challenge. Interaction between a mutant TLR2 construct lacking the entire ICD (TLR2ΔICD) with wild-type or mutant TLR2 lacking the entire ECD (MutCD) was evident, whereas only a TLR2ΔICD construct did not coprecipitate with the tagged cytoplasmic TLR2 domain (TLR2ICD, signal sequence deleted, data not shown). These results further indicate a role of the transmembrane domain rather than that of the TLR ECD in receptor dimerization/oligomerization as has been proposed also by others previously (33, 34) and that might be mediated by unknown proteins within a receptor complex.

Our data imply that the N-terminal 7 of 18–20 LRRs are not involved in cellular recognition of triacylated and diacylated microbial polypeptides through TLR2. The activity of a respective TLR2 mutant (MutH) was only slightly diminished when compared with wild-type TLR2 (Tables 1 and 2 and Fig. 2*B*). In accordance with the findings of Mitsuzawa *et al.* (38) who demonstrated the involvement of the domain Ser-40 to Ile-64 as an sPGN-binding domain, our results suggest involvement of the N-terminal third of the TLR2 ECD in cellular recognition of sPGN and other TLR2 agonists applied (38). Thus, potential binding domains most probably differ for tripalmitoylated and diacylated polypeptides as compared with those of the other TLR2 ligands tested. These conclusions might contribute to elucidation of the molecular basis of TLR-mediated PAMP recognition including possible differences in cell activation triggered by distinct ligands or different doses of one ligand via one receptor (43, 44). One possible explanation of our findings could be that there exist yet unknown recognition proteins for LTA and other TLR2 agonists, which differ from a potential P_3CSK_4 recognition protein. If so, both types of endogenous proteins might function serum independently (39) and mediate cell activation by interacting with distinct regions of the TLR2 ECD. Future PAMP-TLR-binding and structural analyses will further clarify the perspective on pattern recognition receptor function of TLRs.

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Antagonistic antibody prevents toll-like receptor 2–driven lethal shock-like syndromes

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Hyperactivation of immune cells by bacterial products through toll-like receptors (TLRs) is thought of as a causative mechanism of septic shock pathology. Infections with Gram-negative or Gram-positive bacteria provide TLR2-specific agonists and are the major cause of severe sepsis. In order to intervene in TLR2-driven toxemia, we raised mAb's against the extracellular domain of TLR2. Surface plasmon resonance analysis showed direct and specific interaction of TLR2 and immunostimulatory lipopeptide, which was blocked by T2.5 in a dose-dependent manner. Application of mAb T2.5 inhibited cell activation in experimental murine models of infection. T2.5 also antagonized TLR2-specific activation of primary human macrophages. TLR2 surface expression by murine macrophages was surprisingly weak, while both intra- and extracellular expression increased upon systemic microbial challenge. Systemic application of T2.5 upon lipopeptide challenge inhibited release of inflammatory mediators such as TNF-α and prevented lethal shock-like syndrome in mice. Twenty milligrams per kilogram of T2.5 was sufficient to protect mice, and administration of 40 mg/kg of T2.5 was protective even 3 hours after the start of otherwise lethal challenge with *Bacillus subtilis*. These results indicate that epitope-specific binding of exogenous ligands precedes specific TLR signaling and suggest therapeutic application of a neutralizing anti-TLR2 antibody in acute infection.

Introduction

Host cells recognize specific microbial components through pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) that mediate immune responses (1, 2). LPS from the outer membrane of Gram-negative bacteria is a potent agonist for TLR4, whose effects on host organisms of different species have been studied extensively in experimental models of infection and septic shock (3–6). Hyperstimulation of host immune cells by microbial products causes the release of large amounts of inflammatory mediators such as the cytokine TNF-α (7). Its systemic presence at high concentrations is recognized as a major cause of septic shock, characterized by clinical parameters such as abnormal coagulation, profound hypotension, and organ failure (8–10). Also, further inflammatory cytokines such as macrophage inhibitory factor have been shown to directly bias host responsiveness to microbial challenge through modulation of TLR expression (11).

The concept of PRR-dependent induction of hyperinflammation by microbial products has been validated using both gene-targeted mice lacking the expression of respective receptors, and receptor-specific inhibition of microbial product–induced host cell activation. For example, application of CD14-specific antibodies inhibited LPS-induced cell activation, protected rabbits against LPS-induced pathology, and is being evaluated in clinical trials (12, 13). Blockage of further LPS receptors or extracellular effector proteins such as high-mobility group 1 protein has been shown to be preventive as well (14). Another approach of therapeutic intervention in inflammation has been interference with the functions of proinflammatory cytokines such as TNF-α or IL-1β. For instance, competitive inhibition of the binding of a cytokine to its signaling receptors by application of recombinant extracellular domain (ECD) or naturalizing receptor antagonist proteins has been shown to be protective in LPS-induced shock-like syndrome (15). Alternatively, antagonistic antibodies targeting cytokines or ECDs of cytokine receptors have been applied for inhibition of inflammatory immune reactions (16). Therapeutic blockage of cytokines such as TNF-α and IL-6 is used already for treatment of chronic inflammations (17, 18).

Besides Gram-negative bacteria, Gram-positive bacteria lacking LPS play an equally important role in the clinical manifestation of shock (10). Cell wall components from these bacteria, such as peptidoglycan (PGN) and lipoteichoic acid (LTA), are considered major causative agents of Gram-positive shock (19, 20). PGN is a main component of Gram-positive and is also present in Gram-negative bacterial cell walls, and it consists of an alternating β(1,4)-linked *N*-acetylmuramyl and *N*-acetylglucosaminyl glycan cross-linked by small peptides (21). In contrast, the macroamphiphile LTA, a saccharide chain molecule consisting of repetitive oligosaccharides connected by alcohols such as ribitol and carrying acyl chains through which it is anchored to the bacterial cell

Nonstandard abbreviations used: electrophoretic mobility shift assay (EMSA); extracellular domain (ECD); flow cell (FC); heat inactivated (h.i.); human embryonic kidney 293 [cell line] (HEK293); intraperitoneal(ly) (i.p.); leucine-rich repeat (LRR); lipoteichoic acid (LTA); murine toll-like receptor 2 (mTLR2); murine TLR2ECD–human IgGFcγ fusion protein (T2EC); *N*-palmitoyl-S-(1,2-bishexadecyloxy-carbonyl)-ethyl-(R)-cysteinyl-seryl-(lysyl)3-lysine ($PHCSK_4$); *N*-palmitoyl-S-(2,3-bis(palmitoyloxy)-(2R,S)-propyl)-(R)-cysteinyl-seryl-(lysyl)3-lysine (P_3CSK_4); pattern recognition receptor (PRR); peptidoglycan (PGN); surface plasmon resonance (SPR); toll-like receptor (TLR).

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membrane, is specific for Gram-positive bacteria (22). For example, LTA has been described to carry the major stimulatory activity of *Bacillus subtilis* (23). Further, tripalmitoylated proteins, which have been identified in Gram-negative bacteria initially, are mimicked by the synthetic compound *N*-palmitoyl-S-(2,3-bis(palmitoyloxy)-(2R,S)-propyl)-(R)-cysteinyl-seryl-(lysyl)3-lysine (P_3CSK_4) (24).

The bacteria and bacterial products named above are known to trigger the TLR2 signaling cascade (2). For example, the bacterial species *Listeria monocytogenes* and *Staphylococcus aureus*, recognized as clinically more important, are phylogenetically closely related to *B. subtilis*, and the Gram-positive bacteria of all three species produce TLR2 agonists (25–28). However, recent reports indicate that *L. monocytogenes* and *S. aureus* generate additional molecular patterns that elicit immune responses in a TLR2-independent manner in vivo. Susceptibilities of *TLR2*$^{-/-}$ as compared with wild-type mice to respective bacterial challenges differed to a limited degree or did not differ (29–31), implicating further PRRs in their cellular recognition. Of note, triacylated P_3CSK_4 has been demonstrated to use TLR2 in combination with TLR1, while a diacylated mycoplasmal protein uses TLR6 in addition to TLR2 or cell activation (32–34). The TLR2ECD, whose N-terminal portion has been implicated in direct PGN recognition (35), contains an array of distinct leucine-rich repeat (LRR) motifs. The LRR-rich domain is followed by an LRR C-terminal, a transmembrane, and an intracellular C-terminal toll–IL-1 receptor typical signaling domain (TIR) (36).

Here, we show by application of surface plasmon resonance (SPR) biosensor technology that the TLR2-specific mAb T2.5 abrogated TLR2ECD binding to P_3CSK_4. Consequently, TLR2-mediated activation of murine and human cells was inhibited in the presence of T2.5, demonstrating ligand binding to a specific epitope within the TLR2ECD to cause signaling-receptor complex formation. Using two different TLR2-dependent shock models, we demonstrate the protective potential of neutralization of TLR2 function with this antibody in vivo. We propose that antagonism of extracellular TLR2ECD function might provide a therapeutic option for prevention of septic shock.

Results

Application of murine mAb T2.5 for TLR2 expression analysis in vitro. We have selected an IgG1κ anti-TLR2 mAb named T2.5, which recognized TLR2. Human embryonic kidney 293 (HEK293) cells stably expressing murine or human TLR2 were stained specifically on their surface by T2.5 (Figure 1, A and B). Furthermore, T2.5 did not bind to primary murine *TLR2*$^{-/-}$ but bound to wild-type macrophages cultured in vitro (Figure 1, C and D). T2.5 immunoprecipitated native murine and human TLR2 from lysates of HEK293 cells overexpressing one or the other of the two receptors (Figure 1E). Most importantly, T2.5 precipitated endogenous TLR2 from lysates of RAW264.7 macrophages (Figure 1E). We further analyzed T2.5 for its capacity to specifically detect TLR2 on the subcellular level. Detection of overexpressed murine and human TLR2 was specific (Figure 2A). Further, endogenous TLR2 was detectable on the surface of primary murine human macrophages, as well as within the cytoplasmic space (Figure 2B).

Inhibitory effects of T2.5 on TLR2-specific cell activation in vitro and in vivo. T2.5 inhibited murine and human TLR2-mediated cell activation by the TLR2-specific stimuli P_3CSK_4 or *B. subtilis* applied to HEK293 cells overexpressing TLR2, as well as murine RAW264.7 and primary macrophages. NF-κB activation and IL-8 release, as well as TNF-α and IL-6 release, respectively, were analyzed upon cellular challenge (Figure 3, A–D, and data not shown). A second newly generated IgG1κ anti-TLR2 mAb, conT2, was used as a control. This mAb bound to native murine TLR2 (mTLR2), as T2.5 did, but it did not bind to human TLR2 (data not shown) and failed to inhibit TLR2-dependent cell activation in vitro and ex vivo (Figure 3). Also, no inhibition of IL-1 receptor or TLR4 signaling by T2.5 was evident, which indicates that TLR2-independent signaling pathways in T2.5-treated cells remain intact (Figure 3, A–D). Moreover, TLR2-mediated nuclear translocation of NF-κB was specifically inhibited by T2.5 in human macrophages (Figure 3E). NF-κB–specific electrophoretic mobility shift assay (EMSA), as well as anti–phospho-p38, anti–phospho-Erk1/2, and anti–phospho-Akt immunoblot analysis, revealed T2.5 but not conT2 dose-dependent inhibition of P_3CSK_4-induced NF-κB–DNA binding and cellular kinase phosphorylation (Figure 3, F and G).

Abrogation of TLR2ECD ligand binding by T2.5 and analysis of T2.5 epitope localization. To investigate whether T2.5 blocked binding of TLR2 to its synthetic agonist P_3CSK_4, we established an SPR biosensor–based binding assay. P_3CSK_4 was immobilized on a chip surface, and binding of murine TLR2ECD–human IgGFcγ

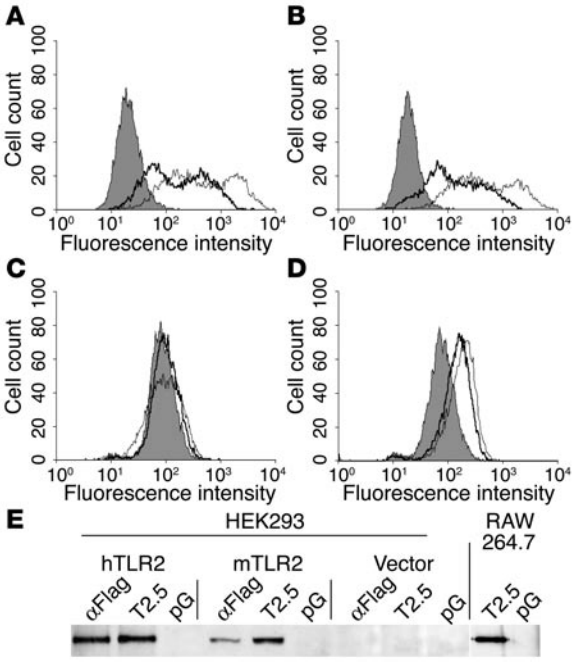

Figure 1
Application of mAb T2.5 for specific detection of TLR2. (**A**–**D**) Results of flow cytometry of HEK293 cells stably overexpressing Flag-tagged mTLR2 (**A**) or human TLR2 (**B**), as well as primary *TLR2*$^{-/-}$ (**C**) and wild-type murine macrophages (**D**), by staining with mAb T2.5 (bold line). Negative controls represent cells incubated with a mouse IgG-specific secondary antibody only (filled areas). For positive controls, Flag-specific (**A** and **B**) and mTLR2-specific (**C** and **D**) polyclonal antisera were used (thin line). (**E**) For immunoprecipitation with T2.5, lysates of HEK293 cells overexpressing murine or human TLR2, as well as of murine RAW264.7 macrophages, were applied as indicated. TLR2 precipitates were visualized by application of Flag-specific (HEK293) or mTLR2-specific (RAW264.7) polyclonal antisera. Flag-specific beads (αFlag) and protein G beads in the absence of antibodies (pG), as well as vector-transfected HEK293 cells, were used as controls. The size of TLR2 was 97 kDa.

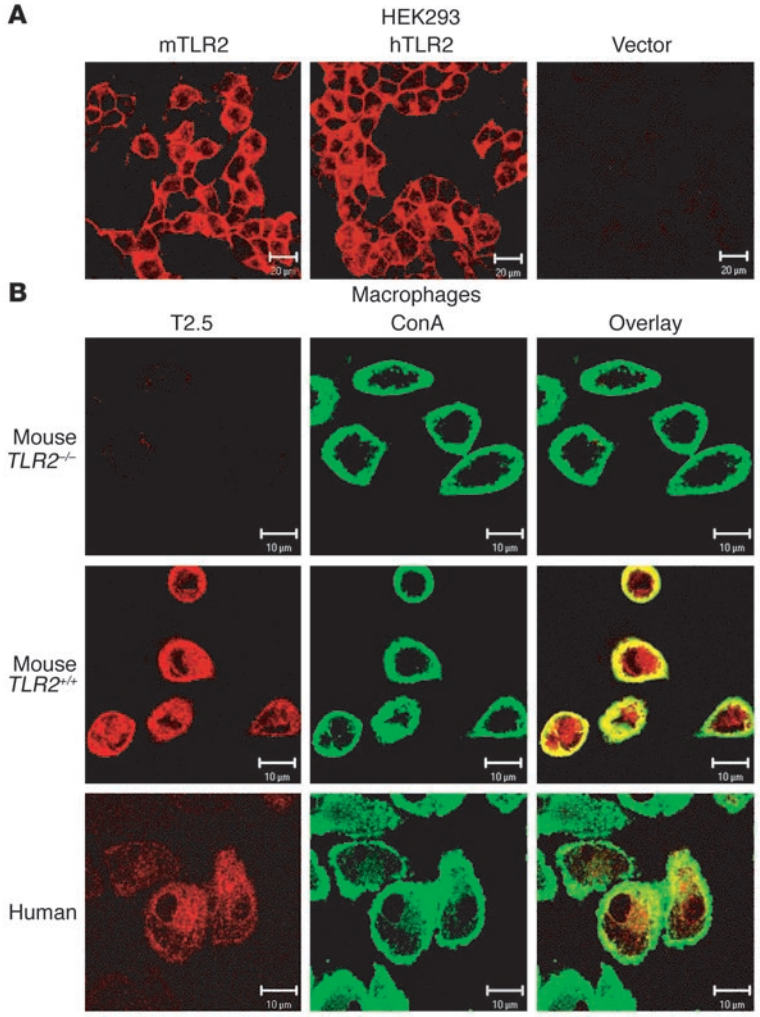

Figure 2
Subcellular localization of TLR2 in vitro. Monoclonal antibody T2.5 was used for cytochemical detection of overexpressed mTLR2 and human TLR2 (hTLR2) (**A**), as well as endogenous murine (*TLR2*$^{+/+}$, wild-type) or human TLR2 in primary macrophages (**B**). Vector-transfected HEK293 cells as well as *TLR2*$^{-/-}$ primary macrophages were analyzed as controls. Concanavalin A (ConA) was used for staining of cellular membranes. The bar in the lower right corner of each field represents a distance of 20 μm (**A**) or 10 μm (**B**) on the slides analyzed.

fusion protein (T2EC) was tested under various conditions. *N*-palmitoyl-S-(1,2-bishexadecyloxy-carbonyl)-ethyl-(R)-cysteinyl-seryl-(lysyl)3-lysine (PHCSK$_4$), a nonactive analogue of P$_3$CSK$_4$, was used as a control, and sensorgrams are displayed as subtracted binding curves. Binding of T2EC to P$_3$CSK$_4$ was specific (Figure 4A). When T2EC was preincubated with T2.5, the antibody dose-dependently inhibited T2EC-P$_3$CSK$_4$ binding (Figure 4A). A molar ratio of 3.3 (T2.5/T2EC) was required to reduce binding to 50%. Preincubation of T2EC with T2.5 at tenfold molar excess abrogated T2EC-P$_3$CSK$_4$ interaction (Figure 4A). In contrast, an isotype-matched control antibody did not block binding of T2EC to P$_3$CSK$_4$ even when applied at tenfold molar excess (Figure 4B). When applied alone, both mAb's did not interact with the sensor-chip surface (Figure 4, A and B). The N-terminal third of the LRR-rich domain of human TLR2 is not involved in lipopeptide recognition (37), and T2.5 cross-reacts with human TLR2 (Figures 2B and 3E). Thus, we applied T2.5 to HEK293 cells overexpressing a mutant construct of human TLR2 that lacks the respective portion of the wild-type ECD (37). Specific abrogation of NF-κB–dependent reporter gene activation upon P$_3$CSK$_4$ challenge after administration of T2.5 strongly suggests localization of the epitope recognized by T2.5 within the C-terminal portion of the TLR2ECD (Figure 4C).

Surface and intracellular TLR2 expression ex vivo as analyzed immediately after primary cell preparation. Since LPS induces TLR2 expression in primary macrophages in vitro, we first compared T2.5-specific staining of CD11b$^+$ splenocytes from LPS-challenged wild-type and *TLR2*$^{-/-}$ mice by flow cytometry. Weak surface staining and more pronounced intracellular staining were evident (Figure 5A). In subsequent experiments, peritoneal cells and splenocytes from mice infected with Gram-positive *B. subtilis* bacteria were analyzed. While surface expression of TLR2 in primary murine macrophages was relatively strong upon in vitro culture (Figure 1D), surface expression was weak or not detectable in unchallenged CD11b$^+$, CD11c$^+$, CD19$^+$, and peripheral neutrophil marker GR1$^+$ subpopulations of splenocytes and peritoneal washout cells (Figure 5, A and B, and data not shown). Upon microbial challenge, however, TLR2$^+$ cell numbers and TLR2 surface expression increased in CD11b$^+$ and GR1$^+$ cells (Figure 5B and data not shown). The increase in the numbers of cells expressing intracellular TLR2 because of prior challenge, however, was more pronounced than the propagation of extracellular TLR2$^+$ cells (Figure 5, A–C), and the signals detected were largely TLR2-specific (Figure 5, B and C).

Antibody-mediated interference with TLR2-dependent cell activation in vivo leading to cytokine and chemokine release into the serum. Next, we determined cytokine and chemokine serum concentrations in mice either pretreated or not pretreated with T2.5 and challenged with P$_3$CSK$_4$. While cytokine and chemokine concentrations were low in sera of untreated mice (see Methods), serum levels of TNF-α, GROα/KC (a murine homolog of human IL-8), IL-6, and IL-12p40 were significantly lower in mice preinjected with T2.5 than in controls upon challenge with P$_3$CSK$_4$ (Figure 6, A–D).

Antibody-mediated interference with systemic induction of shock-like syndromes through TLR2-specific challenge. Both a high-dose (microbial product only) and a low-dose model (additional sensitization with D-galactosamine) have been established for bacterial product–induced shock-like syndromes in mice (38). In order to interfere in a specific model of septic shock, we applied the bacterial lipopeptide analogue P$_3$CSK$_4$ (a TLR2 agonist) upon sensitization of mice with IFN-γ and D-galactosamine (39, 40). Sensitization was used to mimic an underlying primary infection priming host defense. While mice that had received no mAb or conT2 30 minutes prior to injection succumbed to lethal toxemia within 24 hours, mice treated with T2.5 survived (Figure 7A). Intending to use a more complex challenge for a distinct shock model, we took advantage of the finding that shock-like syndrome induction by viable or heat-inactivated (h.i.) *B. subtilis* bacteria was TLR2-

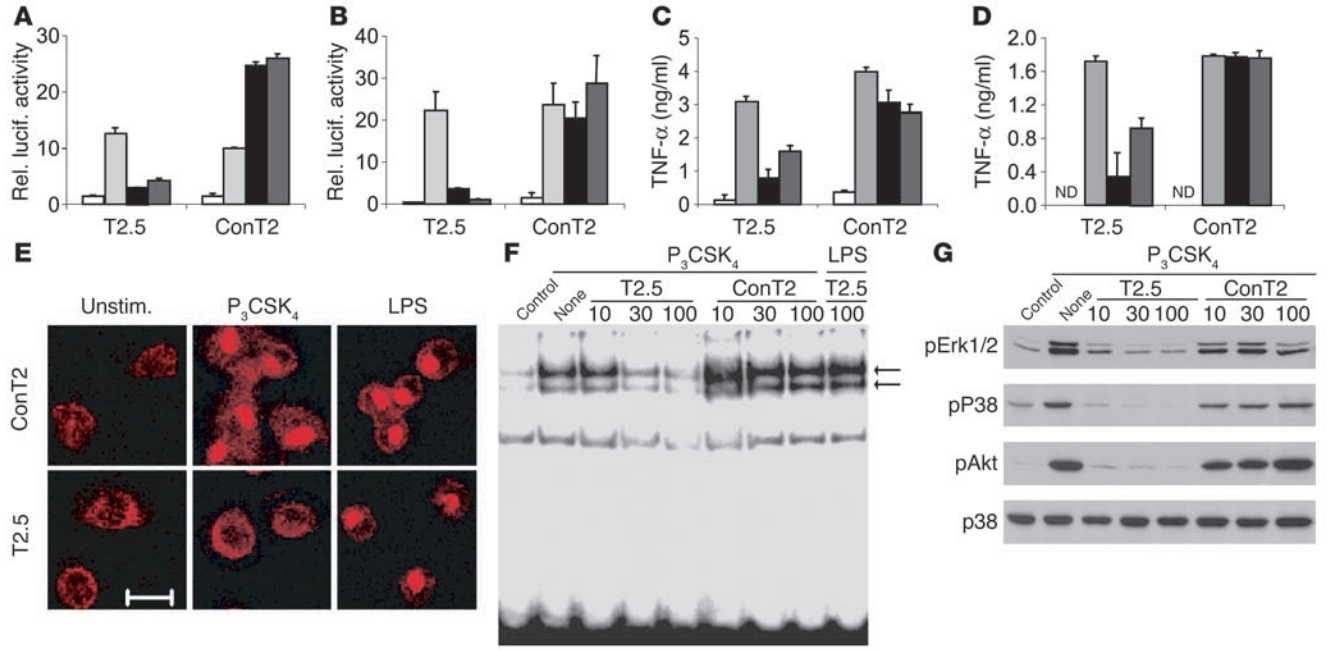

Figure 3

Inhibitory effect of mAb T2.5 on cell activation in vitro. (**A**–**D**) NF-κB–dependent luciferase activities in HEK293 cells overexpressing either murine (**A**) or human TLR2 (**B**), as well as TNF-α concentrations in supernatants of RAW264.7 (**C**) or primary murine macrophages (**D**) challenged with inflammatory agonists. Rel. lucif. activity, relative luciferase activity; ND, not detectable. Cells were incubated with T2.5 or conT2 only (white bars), or additionally challenged with IL-1β (**A** and **B**, light gray bars), ultrapure LPS (**C** and **D**, medium gray bars), P_3CSK_4 (black bars), or h.i. *B. subtilis* (**A**–**D**, dark gray bars). (**E**) NF-κB/p65 nuclear translocation dependent on mAb, P_3CSK_4 challenge, or LPS challenge in human macrophages was analyzed by cytochemical staining. Unstim., unstimulated. Scale bar: 20 μm; magnification was equal for all recordings. (**F** and **G**) NF-κB–dependent EMSA was analyzed by application of nuclear extracts from RAW264.7 macrophages, and phosphorylation of MAPKs Erk1/2 (pErk1/2), p38 (pP38), and Akt (pAkt) was analyzed by application of total extracts from RAW264.7 macrophages. Cells were preincubated with the indicated amounts of mAb T2.5 or conT2 (μg/ml) and challenged with P_3CSK_4 or LPS subsequently for 90 minutes (**F**; arrows indicate specific NF-κB–DNA complexes) or 30 minutes (**G**; phosphorylation-independent p38-specific immunoblot analysis as positive control). Untreated cells were analyzed as controls (Control).

dependent. Clearance of *B. subtilis*, notably, was complete within 48 hours in *TLR2*$^{-/-}$ mice challenged intraperitoneally (i.p.) with viable *B. subtilis* at dosages lethal for wild-type mice. These findings encompassing TLR2 dependence in vivo indicated toxemia as the major pathophysiologic cause of wild-type mouse lethality in both experimental models (P_3CSK_4-induced and *B. subtilis*–induced experimental shock-like syndrome; A. Grabiec and C.J. Kirschning, unpublished observations). Thus, mice were pretreated with different doses of T2.5 prior to administration of h.i. *B. subtilis*. While pretreatment with 1 mg and 0.5 mg of T2.5 protected mice from lethal toxemia (protective protocol), lower amounts were ineffective (Figure 7B). Next, aside from T2.5, conT2 was also applied prior to administration of a principally lethal dose of h.i. *B. subtilis*. In a separate group of mice, we first administered h.i. *B. subtilis* and applied T2.5 up to 4 hours later (therapeutic protocol). In the absence of sufficient dosage of T2.5, the high-dose h.i. *B. subtilis* challenge was lethal for all mice tested (Figure 7, B and C). However, when given T2.5 either 1 hour before or up to 2 hours after microbial challenge, all mice challenged with h.i. *B. subtilis* survived. Most notably, treatment with T2.5 even 3 hours after otherwise lethal injection saved 75% of mice challenged (Figure 7C). When the order of experimental T2.5 and h.i. *B. subtilis* application was reversed, a completely protective effect of T2.5 administration was evident if the bacterial challenge was started 3 hours later (Figure 7D). While T2.5 treatment was effective for two out of three mice even when applied for 4 hours, a protective effect was not detectable at the 5-hour and 6-hour time points in the respective experimental setting (Figure 7D).

Discussion

Our results suggest a therapeutically useful function of an antagonistic TLR2 mAb in TLR2-driven toxemia. We found that application of TLR2 agonists was lethal in two experimental models of septic shock and aimed to identify antibodies that recognize TLR2. One mAb, named T2.5, blocked mTLR2-dependent cell activation. T2.5 also blocked human TLR2 function, since subcellular NF-κB translocation upon TLR2-specific challenge of primary human macrophages was inhibited upon its application. The neutralizing effect of T2.5 application is based on abrogation of TLR2ECD-agonist binding as revealed by SPR analysis upon immobilization of P_3CSK_4. Here we show that T2.5 prevents lethal shock-like syndromes induced by P_3CSK_4 or Gram-positive bacteria (*B. subtilis*) in mice.

The lack of TLR functions negatively affects humans, at least upon acute infection (41, 42). However, in a systemic model of polymicrobial sepsis encompassing standardized influx of the gut flora into the peritoneal cavity, mice benefit from the lack of TLR functions (43), which indicates TLR-dependent mediation of harmful effects in acute infection. Accordingly, blockage of LPS-binding protein (LBP) (44), as well as application of LBP, of peptides rep-

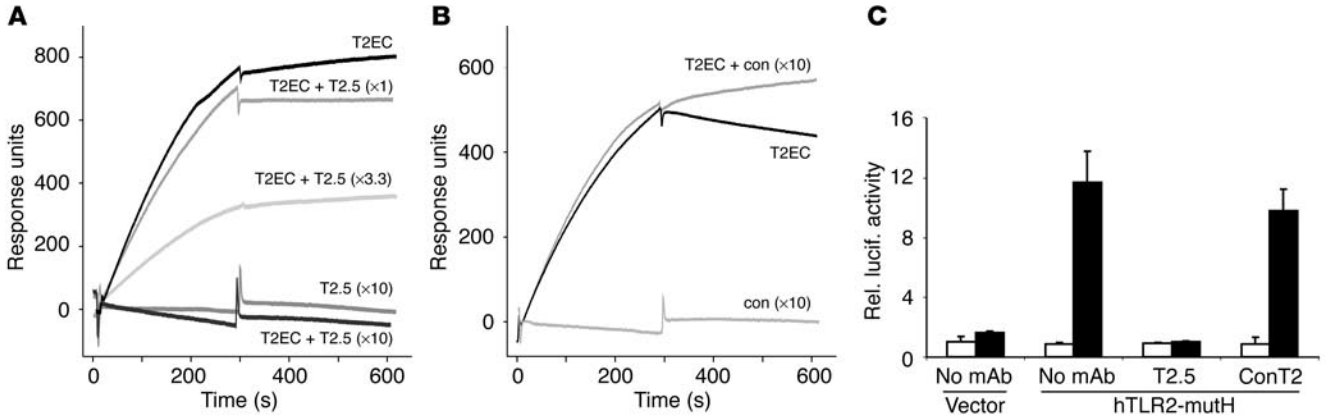

Figure 4

Molecular analysis of the effects of mAb T2.5 on TLR2ECD-P_3CSK_4 interaction. (**A** and **B**) Binding of recombinant TLR2ECD-Fc fusion protein (T2EC, positive controls) to immobilized P_3CSK_4 upon preincubation with T2.5 (T2EC + T2.5) at different molar excesses (**A**, ×1, ×3.3, ×10) or with an isotype-matched control mAb (T2EC + con) at tenfold molar excess only (**B**, ×10). Binding was continuously monitored in an SPR biosensor device, and amounts of antibodies used to gain high molecular excess over T2EC (coincubation) were applied alone as negative controls (**A**, T2.5; **B**, Con). Response units at 300 seconds are a measure for P_3CSK_4-binding capacities of T2EC and T2EC plus mAb. (**C**) For analysis of approximate localization of T2.5 epitope within the TLR2ECD, a mutant human TLR2 construct lacking the N-terminal third of the LRR-rich ECD (hTLR2-mutH) was used for NF-κB–dependent luciferase assay upon transient transfection, preincubation with mAb (T2.5, conT2), and P_3CSK_4 challenge (black bars). Absence of mAb treatment (No mAb) and/or of P_3CSK_4 challenge (white bars), and empty vector (Vector), represent respective controls.

resenting its subdomains, or of bactericidal/permeability-increasing protein (BPI), has been effective in inhibiting LPS-induced pathology (45–49). Attempting to inhibit a TLR-specific immune activation as has been exemplified by systemic tolerance induction through TLR2-specific challenge prior to principally fatal microbial challenge (40), we applied an antagonistic mAb T2.5 raised against the murine TLR2ECD. Its application enabled analysis of murine and human TLR2 localization on the surface and inside of immune cells (Figures 1 and 2). Direct interaction between TLR2 and P_3CSK_4 was demonstrated and allowed comparison of the affinities of TLR2 and of the TLR2-T2.5 complex to this ligand. SPR analysis showed the direct and specific interaction between TLR2ECD and P_3CSK_4, as well as a specific and dose-dependent inhibition of this interaction by T2.5 (Figure 4, A and B), indicating that binding of T2.5 masked the ligand-binding domain in TLR2. Accordingly, T2.5 antagonized not specifically P_3CSK_4, but also h.i. *B. subtilis*, PGN, LTA, and TLR2-dependent cell activation induced by mycoplasmal macrophage-activating protein (Figure 3 and data not shown). Blockage was specific and dose-dependent (Figure 3). Taken together, these findings show that specific binding of ligands to a discrete site within the TLR2ECD is a prerequisite for TLR2-mediated signaling.

Surface expression of TLR2 in vivo was a precondition of systemic effects of T2.5 application. Relatively weak surface expression of TLR2 even upon LPS or bacterial challenge ex vivo (Figure 5, A and B), however, was in contrast with relatively high surface expression on unchallenged primary murine (Figures 1D and 2B) as well as human myeloid cells upon in vitro culture (50). However, comparative TLR2 staining of nonpermeabilized and permeabilized cells indicated localization of a major portion of TLR2 in the intracellular compartment of murine $CD11b^+$ and $GR1^+$ cells, as well as human macrophages (Figure 5, Figure 2B, and data not shown). In fact, we noted increased surface and, to a larger extent, intracellular TLR2 expression in specific cell populations 24 hours after bacterial infection, which was similar upon LPS challenge (Figure 5 and data not shown). Weak unspecificity of intracellular staining with T2.5, detected mostly in permeabilized spleen cells, had to be taken into account (Figure 5C). The time course of TLR2 regulation in distinct immune cells upon microbial contact needs to be investigated in more detail, because it might determine the time frame within which intervention based on TLR2 blockage can be effective.

Perhaps it is the surprisingly low constitutive surface expression of TLR2 in host cells such as $CD11b^+$ (macrophage) cells, $GR1^+$ (granulocyte) cells, $CD19^+$ (B) cells, and $CD11c^+$ (dendritic) cells in vivo (Figure 5 and data not shown) that explains the high efficacy of T2.5-mediated prevention of TLR2-driven hyperinflammation (Figures 6 and 7). Application of T2.5 30 minutes prior to application of a principally lethal dose of P_3CSK_4 or 1 hour prior to administration of a principally lethal dose of h.i. *B. subtilis* protected mice against the otherwise lethal effects of both stimulants (Figure 7, A–C), but not against the lethal effects of LPS (data not shown). In fact, *B. subtilis*–induced toxemia was prevented upon application of T2.5 2 hours or even 3 hours after shock-like syndrome induction (100% or 75% of survival, respectively). In contrast, application of T2.5 was not effective after 4 hours (Figure 7C). However, the onset of septic shock upon acute infection in the clinical situation may be delayed as compared with sudden induction of toxemia by experimental injection of large amounts of stimulant and may allow interference within a larger time window. Our results indicate that complement-mediated depletion of $TLR2^+$ cells is unlikely to be a mechanism of prevention of T2.5-dependent prevention of TLR2-driven shock-like syndrome, since application of the mTLR2-specific isotype-matched mAb conT2 in vivo did not result in protection (Figure 7A). This is in line with reversibility of mAb-mediated TLR2 blockage within 5 hours (Figure 7D), which may be important for timely recovery of TLR2-dependent cellular responsiveness

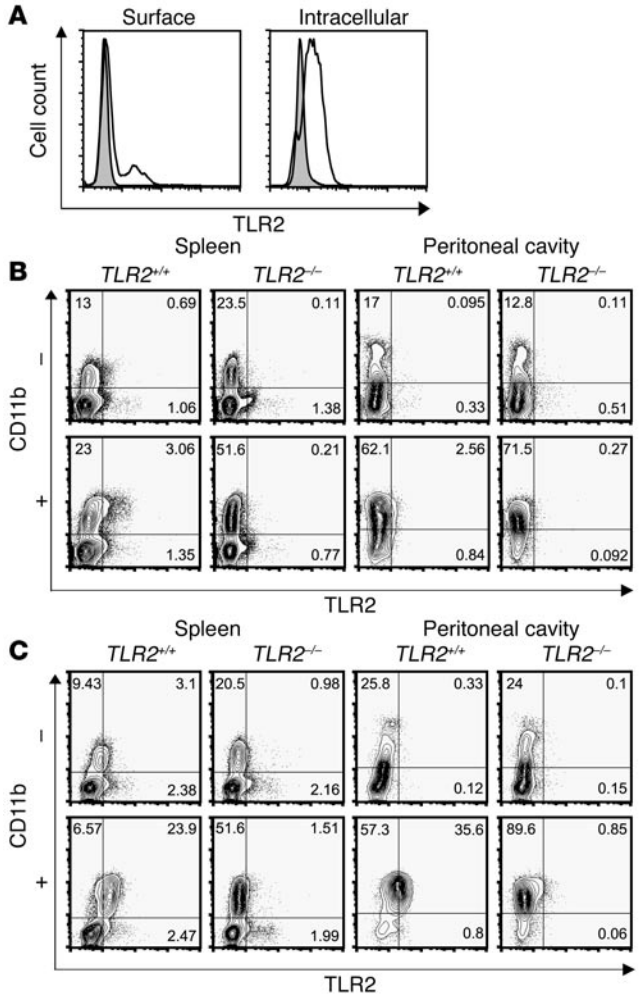

Figure 5
TLR2 expression ex vivo immediately after primary cell isolation. Flow cytometry of splenocytes and peritoneal washout cells from wild-type (*TLR2*$^{+/+}$) and *TLR2*$^{-/-}$ mice ex vivo immediately after isolation (n = 5, cells pooled for each sample). (**A**) CD11b^{+} splenocytes from mice challenged with LPS for 24 hours were analyzed for surface and intracellular TLR2 expression by staining with T2.5 (bold line, *TLR2*$^{+/+}$; filled area, *TLR2*$^{-/-}$). (**B** and **C**) For analysis of TLR2 regulation upon infection, mice were either left uninfected (–) or infected with *B. subtilis* and sacrificed after 24 hours (+). Upon staining of CD11b, cells were stained with T2.5 (TLR2) either without permeabilization (**B**) or after permeabilization (**C**). Numbers in quadrants represent the percentage of single- or double-stained cells with respect to the total number of viable cells analyzed.

in later phases of sepsis at which diminished immune function is fatal (9). Systemic presence of T2.5 1 hour prior to challenge did not interfere with resistance of a *TLR2*$^{-/-}$ mouse challenged with h.i. *B. subtilis* at a dose that was lethal for wild-type mice in the absence of T2.5 application (data not shown). The demonstration of beneficial and specific effects of T2.5 in both a sensitization-dependent and a high-dose TLR2-specific experimental model may support transferability of our results to elimination of the TLR2-dependent share in septic shock induction (9). Specifically, TLR2 blockage upon antibiotic therapy may substantially contribute to prevention of an excessive host immune reaction upon sudden release of large amounts of microbial products from disintegrating microbial cells. It may have to be complemented by blockage of further surface receptors, for which TLR4 is a prime candidate, in order to facilitate inhibition of cell activation. Conversely, failure of therapy to compensate for a decrease in biocidal immune cell activity upon TLR blockage by antibiotic treatment might compromise a beneficial outcome (13).

We have identified exclusively antagonistic or neutral TLR2-specific mAb's, and antagonistic properties have recently been demonstrated in vitro also for two different human TLR2-specific mAb's (28, 51). Active complex formation of TLRs as compared with receptors for which agonistic antibodies have been identified might differ. However, T2.5 antagonized TLR2 function through inhibition of ligand-TLR2-complex formation (Figure 4A), which is a prerequisite of TLR2-driven cell activation. T2.5 may therefore recognize the possibly single ligand-binding site within the C-terminal portion of the TLR2ECD. We expect that identification of the epitope will show its conservation between mice and humans. In conclusion, our results implicate antibody-mediated TLR blockage on immune cells as a promising strategy for attenuation of potentially fatal host-response amplification in the course of acute infection.

Methods

Material. Overnight *B. subtilis* (DSMZ.1087) cultures in brain-heart medium containing approximately 1 × 10^{9} CFUs/ml were used immediately or heat-inactivated at 56°C for 50 minutes. Synthetic P_3CSK_4 and, as a negative control, $PHCSK_4$, a nonstimulatory derivative thereof (52), were purchased from EMC microcol-

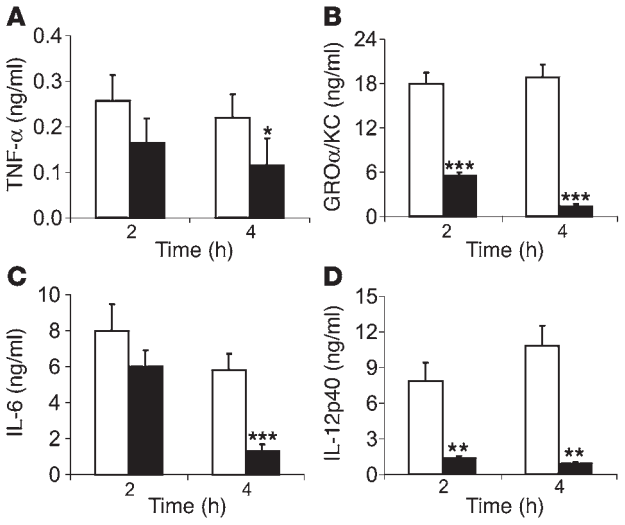

Figure 6
Inhibitory effect of mAb T2.5 on host activation by microbial challenge in vivo. Mice were pretreated i.p. with 1 mg mAb T2.5 (black bars) or left untreated (white bars). Mice were challenged i.p. with P_3CSK_4 and D-galactosamine after 1 hour and sacrificed 2 or 4 hours later (n = 4 for each group at each time point). Serum concentrations of TNF-α (**A**), GROα/KC (human IL-8 homolog) (**B**), IL-6 (**C**), and IL-12p40 (**D**) were analyzed by ELISA. *P < 0.05, **P < 0.005, ***P < 0.001, Student's t test for unconnected samples.

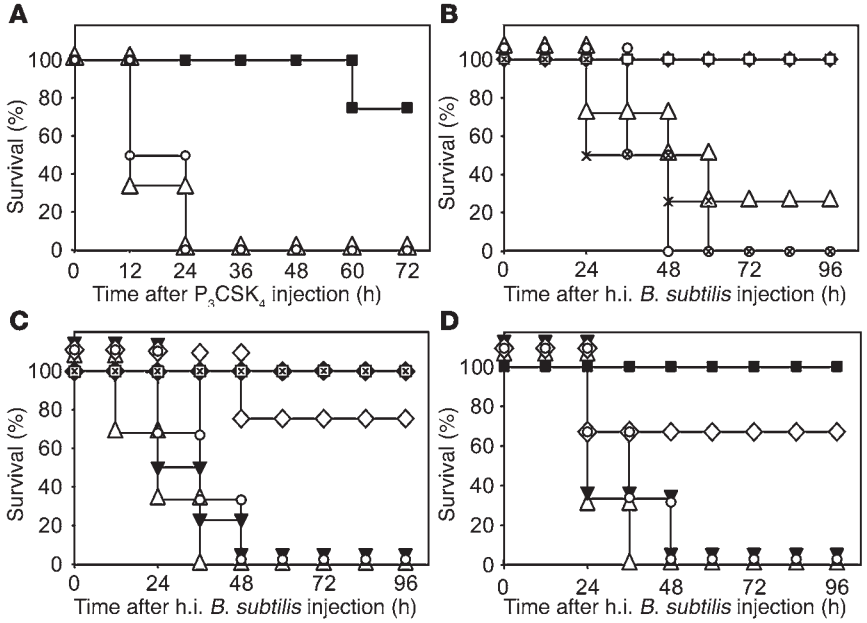

Figure 7
Effects of mAb T2.5 administration on viability after TLR2-specific systemic challenge. (**A**) IFN-γ– and D-galactosamine–sensitized mice received no mAb, 1 mg of mAb T2.5, or 1 mg of conT2 i.p. 30 minutes prior to microbial challenge with bacterial lipopeptide analogue P_3CSK_4 (open circles, no mAb, $n = 4$; open triangles, mAb conT2, $n = 3$; filled squares, mAb T2.5, $n = 4$). (**B**–**D**) Mice challenged with a high dose of h.i. *B. subtilis* were left untreated, treated 1 hour later with the indicated dosages of mAb T2.5 (**B**; filled diamonds, 1 mg, $n = 3$; open squares, 0.5 mg, $n = 3$; open triangles, 0.25 mg, $n = 4$; ×'s, 0.13 mg, $n = 4$; open circles, no mAb T2.5, $n = 4$), or treated with 1 mg of mAb's at the different time points indicated below (**C** and **D**). (**C**) TLR2-specific mAb was administered before (–) or after (+) bacterial challenge (filled inverted triangles, no mAb, $n = 8$; open circles, mAb conT2, –1 hour, $n = 3$; filled diamonds, mAb T2.5, –1 hour, $n = 4$; open squares, mAb T2.5, +1 hour, $n = 3$; ×'s, mAb T2.5, +2 hours, $n = 3$; open diamonds, mAb T2.5, +3 hours, $n = 4$; open triangles, mAb T2.5, +4 hours, $n = 3$). (**D**) TLR2-specific mAb T2.5 was administered before (–) bacterial challenge (open triangles, no mAb; filled squares, mAb T2.5, –3 hours; open diamonds, mAb T2.5, –4 hours; open circles, mAb T2.5, –5 hours; filled inverted triangles, mAb T2.5, –6 hours; $n = 3$ for all groups).

lections GmbH (Tuebingen, Germany); both carried biotin tags. Ultrapure LPS from *Salmonella minnesota Re595* was from List Laboratory (Campbell, California, USA), recombinant murine IFN-γ and IL-1β were from PeproTech EC Ltd. (London, United Kingdom), and D-galactosamine was from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany).

Mice. Matched groups of wild-type (*TLR2*$^{+/+}$) C57BL/6 and *TLR2*$^{-/-}$ (39) mice generated by Deltagen Inc. (Redmond City, California, USA) were kindly provided by Tularik Inc. (South San Francisco, California, USA) and crossed ninefold from a mixed Sv129×C57BL/6 toward a C57BL/6 genetic background. Experiments were approved by the government of Upper Bavaria, Germany.

Generation of TLR2ECD-specific antibodies and ELISA. A cDNA fragment encoding the N-terminal 587 amino acids of mTLR2 (53) was amplified from an RAW264.7 cell cDNA library (Advantage kit; BD Biosciences Clontech, Heidelberg, Germany). The murine TLR2ECD was fused to a C-terminal thrombin cleavage site followed by a human IgGFcγ moiety (T2EC). The murine TLR2ECD protein was purified upon overexpression in HEK293 cells and thrombin digestion. A *TLR2*$^{-/-}$ mouse was immunized three times within 8 weeks by i.p. injection of 50 μg of TLR2ECD and 10 nmol of a thioated DNA oligonucleotide (5′-TCCATGACGTTCCTGA-3′; TIB MOLBIOL, Berlin, Germany). Its splenocytes were fused with murine P3X cells, and hybridomas were selected (54). Monoclonal antibody specificities for TLR2ECD, as well as cytokine and chemokine concentrations, in cell supernatants or murine sera were analyzed by ELISA (R&D Systems Inc., Minneapolis, Minnesota, USA). Significance of serum-concentration differences was determined by application of the Student's *t* test for unconnected samples.

Flow cytometry. Stably transfected HEK293 cell clones, as well as uninduced peritoneal washout macrophages, were cultured overnight as described previously (37). Flow cytometry was performed upon staining with T2.5 and a secondary mouse IgG-specific mAb, as well as affinity-purified polyclonal antisera specific for the murine TLR2ECD (55) or the Flag-tag (Sigma-Aldrich Chemie GmbH) and a rabbit IgG-specific secondary mAb. Secondary mAb's were phycoerythrin-labeled (BD Biosciences Pharmingen, Heidelberg, Germany). For establishment of mTLR2 expression analysis in primary cells, surface and intracellular T2.5-dependent staining of CD11b$^+$ splenocytes (54) from wild-type versus *TLR2*$^{-/-}$ mice challenged with LPS (0.5 mg, i.p., 24 hours) was compared by flow cytometry (CyAn; DakoCytomation, Fort Collins, Colorado, USA). Cells were stained with photoactivated ethidium monoazide (Molecular Probes Europe BV, Amsterdam, The Netherlands) immediately upon isolation, followed by TLR2-specific surface staining, or intracellular staining (Cytofix/Cytoperm; BD Pharmingen). In order to analyze TLR2 expression in uninfected or *B. subtilis*–infected mice (5×10^8 CFUs, i.p., 24 hours), peritoneal washout cells and splenocytes (54) from five uninfected or infected wild-type or *TLR2*$^{-/-}$ mice were pooled. Fluorescence-labeled cell surface marker antibodies (BD Pharmingen) and T2.5 counterstained with secondary anti-mIgG1 were used as indicated.

Immunoprecipitation and immunoblot analysis. Lysates of Flag-TLR2–transfected HEK293 cells or macrophages were mixed with 1 μg of antibody and protein G beads (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) for overnight precipitation. Immune complexes or cell lysates were analyzed by immunoblot analysis as described previously (37). Precipitations were controlled by application of Flag-specific (mAb M2; Sigma-Aldrich Chemie GmbH) or protein G beads only. Flag- or mTLR2-specific antisera were applied for immunoblot analyses of HEK293 or RAW264.7 cell lysates, respectively. In contrast, total lysates of macrophages were analyzed for phosphorylation of kinases as indicated.

Cytochemical staining of TLR2 or NF-κB. Transfected HEK293 cell clones, as well as primary murine or human macrophages, the latter isolated as CD14$^+$ peripheral blood leukocytes and cultured in 20% of autologous serum (56), were grown on slides. Cells were washed with PBS, permeabilized, and incubated with 5 μg/ml TLR2-specific mAb or anti–NF-κB/p65 (polyclonal

rabbit; Santa Cruz Biotechnology Inc.) (37). Specific secondary anti-IgG antibodies labeled with Alexa Fluor 546 (anti-TLR2) or Cy5 (anti–NF-κB; both from BD Biosciences Pharmingen) were applied. Cell membranes were stained with labeled concanavalin A (Molecular Probes Europe BV).

Inhibition of TLR2-dependent cell activation in vitro and in vivo. Transiently transfected HEK293 cells as well as murine RAW264.7 and primary macrophages were used. Fifty micrograms per milliliter of antibodies were applied 30 minutes prior to challenge with 100 ng/ml of LPS, IL-1β, or P_3CSK_4 or 1×10^6 CFUs/ml of h.i. *B. subtilis*. HEK293 cells were cotransfected with reporter (57), human wild-type TLR2, human mutant TLR2 (lacking the N-terminal third of the LRR-rich domain; ref. 37), or mTLR2, as well as MD2 and CD14 (provided by Tularik Inc., South San Francisco, California, USA; D. Golenbock, University of Massachusetts Medical School, Worcester, Massachusetts, USA; H. Heine, Research Center Borstel, Borstel, Germany; and K. Miyake, University of Tokyo, Tokyo, Japan) expression plasmids, and NF-κB–dependent reporter gene activity was assayed after 6 hours of stimulation. TNF-α concentrations in supernatants of RAW264.7 and primary murine macrophages were analyzed 24 hours after challenge, and NF-κB translocation in human macrophages (56) was analyzed 90 minutes after challenge. RAW264.7 macrophages were used for analysis of challenge and of antibody-dose-dependent activation of NF-κB and MAPK. NF-κB–specific EMSA and p38, Erk1/2, and Akt phosphorylation–specific immunoblot analysis (Cell Signaling Technology, Frankfurt, Germany) were carried out. Prior to TLR-specific challenge of 1×10^6 cells, as described above, for 90 minutes (EMSA) or 30 minutes (kinase-phosphorylation analysis) (37), antibody was administered at various concentrations. For analysis of TLR2 inhibition in vivo, mice were injected i.p. with 1 mg of T2.5 or left untreated. One hour later, 100 μg of P_3CSK_4 and 20 mg of D-galactosamine were injected i.p. Serum concentrations of TNF-α, GROα/KC (murine homolog of human IL-8), IL-6, and IL-12p40 in five unchallenged control mice were 0.05 ng/ml, 0.43 ng/ml, not detectable, and 0.44 ng/ml, respectively. Significance of results was determined by the Student's *t* test for unconnected samples.

SPR biosensor measurements. Real-time binding analysis was performed using SPR detection on a Biacore X device (Biacore AB, Uppsala, Sweden). The two flow cells (FCs) of a streptavidin-precoated chip were loaded with biotinylated $PHCSK_4$ (FC1) and P_3CSK_4 (FC2), respectively. Specific binding of a recombinant T2EC protein was controlled by application of a human mAb carrying the same Fcγ domain. This antibody did not bind in either FC1 or FC2 (data not shown). After prior incubation in 45 μl of running buffer (50 mM morpholino ethane sulfonic acid, 150 mM $MgCl_2$, pH 6.5) at 25°C for 15 minutes, 200 nmol of purified T2EC alone (maximum control) or in combination with mAb's (T2.5 or an isotype-matched irrelevant mAb at the molar excesses indicated) was injected over FC1 and FC2 at a flow rate of 10 μl/min. As negative control, mAb's alone were administered at the highest amounts also used for blocking analysis of TLR2 ligand binding. The values obtained upon continuous resonance monitoring at 25°C over 570 seconds (delay time 300 seconds) from the control FC1 were subtracted from the respective values resulting from simultaneously performed analysis of FC2. Generally, biomolecular interaction between receptor and its respective ligands immobilized on the sensor chip is optically monitored as a function of time and expressed in response units. Regeneration of the chip was achieved by washes with 50 mM NaOH and 1 M NaCl and extensive re-equilibration with running buffer.

Systemic induction of shock-like syndrome. In an experimental sensitization-dependent model (39), mice were injected i.v. with 1.25 μg of murine IFN-γ. Twenty minutes later, mice were injected i.p. with doses of mAb as indicated. Fifty minutes after IFN-γ injection, 100 μg of synthetic P_3CSK_4 and 20 mg of D-galactosamine were injected i.p. as well. The experimental high-dose shock model encompassed a single i.p. injection of 1×10^{10} CFUs of h.i. *B. subtilis*, with i.p. injection of 1 mg of mAb 1 hour to 6 hours earlier or 1 hour to 4 hours later as indicated. Survival was monitored and did not change within 7 days after injection after the latest time points indicated in Figure 7.

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