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Cellular detection of infections:

Analysis of systemic toll-like receptor 2 function and species specificity

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Abbreviation list

A

AP-1	activating protein-1
APS	ammoniumperoxidedisulfate
AA	aminoacid
APC	antigen presenting cells

B

BSA	bovine serum albumin

C

CARD	caspase-activating and recruitment domain
CD	cluster of differentiation
CMV	cytomegalovirus
CREB	cAMP response element binding protein
CRP	C-reactive protein

D

DCs	dendritic cells
DD	death domain
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	desoxynucleotidetriphosphate

E

ECD	extracellular domain
ECL	enhanced chemoluminescence
EDTA	ethylendiamintetraacetate
EGTA	ethylenglycoltetraacetate
ELAM-1	endothelcell-leucocyte-adhesion molecule-1
ELISA	enzyme linked immunosorbent assay
EMSA	electro mobility shift assay
ER	endoplasmic reticulum
ERK1/2	extracellular-signal-regulated kinase 1/2

F

FACS	fluorescence activated cell sorting
FCS	fetal calf serum

G

GARG 16	glucocorticoid attenuated response gene 16

H

h	hour
HBS	hepes buffered saline
HEK 293	human embryonic kidney 293 cells
HSP	heat shock protein
h. i.	heat inactivated

I

ICD	intracellular domain
I-κB	inhibitor of NF-κB
IFN-β, γ	interferon-β, γ
Ig	immunoglobulin
IL	interleukin
IKKβ	I-κB kinase β
i.p.	intraperitoneal
IRAK	IL-1-receptor-associated kinase
IRF3	interferon regulatory factor 3
IRG1	immunoresponse gene 1

J

JNK	c-Jun N-terminal kinase

K

kDa	kilodalton

L

Lau_3CSK_4	tri-lauroylated-lipopeptide analogue
LB	Luria Bertani medium
LDL	low density protein
LM	*Listeria monocytogenes*

LPS	lipopolysaccharide
LRR	leucine rich repeat
LRRCT	LRR C-terminal rich domain
LTA	lipoteichoic acid

M

M	mol/l
Mal (TIRAP)	MyD88 adaptor-like
MALP-2	macrophage-activating lipopeptide, 2 kDa
MAPK	mitogen-activated protein kinase
MEFs	mouse embrionic fibroblasts
min	minute
mut	mutant
MyD88	myeloid differentiation marker 88
Myr_3CSK_4	tri- myristoylated -lipopeptide analogue

N

Na Ac	sodium acetate
NF-κB	nuclear factor κB
NGS	normal goat serum
NOD	nucleotide-binding-oligomerisation-domain

O

OD	optical density
ON	over night
OspA	outer surface protein A

P

PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PBT	phosphate buffered Tween 20
P_3CSK_4	tri-palmitoylated -lipopeptide analogue
P_2CSK_4	di-palmitoylated -lipopeptide analogue
PCR	polymerase chain reaction
PGN	peptidoglycan
PGRP	peptidoglycan recognition protein
PI3K	phosphatidyl-inositol-3-kinase
PMA	phorbolmyristatacetate

PMSF	phenylmethylsulfonylfluoride
PRR	pattern recognition receptor

R

ROS	reactive oxygen species
RSV	respiratory syncitial virus
RT	room temperature
rpm	rounds per minute

S

SAP	serum amyloid protein
SARM	sterile α and HEAT-Armadillo motifs
SDS	sodium dodecyl sulfate
sec	second
SIRS	systemic inflammatory response syndrome
SRE-1	serum response element binding factor
SMN	survival motor neuron protein

T

TAB-2	TAK binding protein 2
TAE	Tris-acetate-EDTA
TAK1	TGF-β-associating kinase 1
TBE	Tris-borate-EDTA
TBK-1	TANK-binding kinase 1
TE	Tris-EDTA
TEMED	N, N, N‘, N‘-Tetramethylethylendiamine
TGF-β	transforming growth factor β
Th	T-helper cell
TIR	Toll/IL-1-receptor
TIRAP (Mal)	TIR domain containing adaptor protein
TLR	toll-like receptor
TMB	3, 3‘, 5, 5‘-Tetramethylbenzidine
TNFα	tumor necrosis factor α
TRAF6	TNF-receptor-associated factor 6
TRAM	TRIF related adaptor molecule
TRIF(TICAM)	TIR domain containing adaptor inducing IFN-β
Tris	Tris-(Hydroxylmethyl-)Aminomethan

U

U	units

V

v/v	volume per volume

W

w/v	weight per volume
WT	wild type

Y

YPD	yeast peptone dextrose

1. Summary

Toll-like receptors (TLRs) represent a family of membrane proteins mediating recognition of pathogen derived ligands, endogenous ligands liberated from disintegrating cells, or metabolic intermediates. In mammalians cells, activation of TLRs results in stimulation of the innate immune system through signaling pathways some of which are well characterized (*Dunne et al. 2003*).

The thesis is divided into three chapters. In the first chapter, we describe analysis of species–specific TLR2 response to different pathogen derived ligands. Comparative analysis of TLR2-driven cell activation by various bacteria and pure TLR2 agonists showed that the tri-lauroylated-lipopeptide analogue (Lau_3CSK_4) is recognized efficiently through murine (m) but not through human (h) TLR2. Genetic complementation of human embryonic kidney (HEK) 293 cells and *TLR2*$^{-/-}$ murine embryonic fibroblasts, as well as comparative analysis of murine RAW264.7 and human MonoMac6 and THP1 macrophages were performed. In contrast to activation, cellular uptake of Lau_3CSK_4 and tri-palmitoylated peptide (P_3CSK_4) was not only species independent, but also TLR2 independent. A low-conserved region spanning from leucine rich repeat (LRR) / LRR-like motif 7 to 10 was found to control species-specific Lau_3CSK_4 recognition. Specifically, exchange of mouse LRR8 by human LRR8 within wild type mTLR2 abrogated mTLR2 typical cell activation upon cellular challenge with Lau_3CSK_4 but not P_3CSK_4, implicating mLRR8 as a main element in cellular Lau_3CSK_4 recognition. Furthermore, the LRR consensus-motif point mutation L112P abrogated human TLR2-dependent recognition of lipopeptides, but only attenuated murine TLR2 function, while deletion of the N-terminal third of the respective LRR-rich domains had reverse effects. Human TLR2 possess 4 putative N-glycosylation sites whereas the murine TLR2 sequence carries three putative N-glycosylation sites. Functional analysis of N-glycosylation mutants showed that the glycosylation site N 442 plays a central role in function of both molecules.

In the second chapter of the thesis, we present the analysis of TLR2 function in *Listeria monocytogenes* (LM) infection. *L. monocytogenes* is a facultative intracellular Gram-positive bacterium causing potentially lethal infection in newborns. The control of *L.monocytogenes* infection depends on the rapid activation of the

innate immune system, of which TLRs are important elements. At the beginning of our work a role of TLR2 in *L. monocytogenes* infection *in vivo* had not yet been described. We analysed *TLR2*$^{-/-}$ mice upon infection with *L. monocytogenes*. Susceptibility of *TLR2*$^{-/-}$ mice to infection was analyzed by determination of bacterial burden in liver, spleen and blood, as well as proinflammatory cytokine concentrations in the serum upon infection. Although TNFα and IL-6 serum concentrations were decreased in *TLR2*$^{-/-}$ mice as compared to wild type mice in the early phase of infection, lethality of *TLR2*$^{-/-}$ mice and bacterial burden in organs was undistinguishable from the burden in organs of wild type mice.

In the third part of the thesis, we describe the analysis of potential interactions of TLR2 with cytoplasmic proteins. The intracellular domain of TLR2 was applied as a bait in yeast two-hybrid studies to identify new proteins involved in TLR-mediated signaling (Lamping N.; Kirschning C. unpublished data). Five proteins were found to interact with the intracellular domain of TLR2 or TLR1, namely: filamine, α-actinin, Hsp40, a new protein with high sequence similarity to human SSA/Ro protein and SMN. Of these proteins only α-actinin and Hsp40 was found to co-immunoprecipitate with intracellular domain of TLR2. Further biochemical analysis, however, did not implicate these proteins in TLR2 signaling.

2. Introduction

2.1 Immune system

2.1.1 Innate Immunity

All organisms are challenged constantly by infectious agents such as bacteria, viruses or fungi which threaten to invade their body. The ability of the organism to discriminate between infectious non-self and self is essential for identification of invading pathogens and defence against them (*Medzhitov et al. 2002*). Higher vertebrates carry two main systems that act in cooperation against infection: the adaptive and the innate immune system (*Abbas 2000*). Adaptive immunity is mediated by T and B lymphocytes and is characterised by high specificity and memory; the adaptive immune system is critically dependent on somatic gene rearrangement and diversification processes that generate millions of antigen receptors with random specificities. The innate immune system, in contrast, is phylogenetically ancient and most likely present in all multicellular organisms. It relies on a set of germline-encoded receptors that are expressed on a wide variety of cells, especially effector cells like macrophages or dendritic cells (*Medzhitov et al. 2000*).

The innate immunity uses a series of phylogenetically conserved receptors termed pattern recognition receptors (PRRs) for sensing invading pathogens. They can be expressed on the cell surface, in intracellular compartments, or secreted into the bloodstream. This group of receptors recognizes pathogen-associated molecular patterns (PAMPs), that is, molecular patterns alien to the host cell that are specifically produced by microorganisms. The principal functions of PRRs include opsonisation and activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory signaling pathways, and induction of apoptosis (*Medzhitov et al. 2000*). Examples of PRRs are C-reactive protein (CRP) and serum amyloid protein (SAP), both of which are secreted during the acute phase response. CRP and SAP function as opsonins upon binding to bacterial surfaces. Another group of PRRs represent scavenger receptors. These are cell surface glycoproteins which have the ability to bind a broad range of ligands including low density

lipoprotein (LDL) and lipopolisacharid (LPS). Activated scavenger receptors trigger pathogen removal through phagocytosis (*Gough et al. 2000*). C-type lectins are member of scavenger receptors family and they are known for their ability to recognise specific pathogen associated carbohydrate structures. Dectin-1 is an example of a C-type lectin involved in phagocytic uptake and killing of yeast cells (*McGreal 2004*).

A relatively new family of proteins involved in intracellular pattern recognition are nucleotide-binding-oligomerisation-domain (NOD) proteins also known as CARDs (caspase-activating and recruitment domain). Their structure is tripartite and characterised by a C-terminal peptide recognition (LRR) domain, a central NOD domain, and a N-terminal CARD domain (NOD2 carries 2 CARD domains) (*Athman et al. 2004*). The expression of NOD1 is ubiquitous in adult tissues whereas NOD2 is expressed preferentially in antigen presenting cells (APCs) (*Athman et al. 2004*); (*Watanabe et al. 2004*). NODs recognise bacterial components through a C-terminal LRR domain: NOD1 recognises Gram-negative bacteria derived peptidoglycan, containing a GlcNAc-MurNAc tripeptide motif where the terminal amino acid is diaminopimelic acid; NOD2 recognises a component of bacterial peptidoglycan called muramyl dipeptide (*Athman et al. 2004*). A recent study shows that NOD2 signaling inhibits Toll like receptor (TLR) 2 driven Th1 responses by decreasing NF-κB activation. Notably, TLR2 mediated NF-κB activation and IL-12 production are increased in the absence or mutation of NOD2 (*Watanabe et al. 2004*).

2.1.2 Sepsis

A major function of the immune system is to protect the host organism against microbial infection. Immunity is necessary for survival - but also has the potential for causing injury to the organism for instance during sepsis.

It is known that sepsis and the systemic inflammatory response syndrome (SIRS) are accompanied by the inability to regulate the inflammatory response (*Riedemann et al. 2003*). The diagnosis of sepsis and SIRS requires confirmation of bacterial growth in blood cultures, as well as presence of at least three of the following symptoms: hypothermia or hyperthermia, tachycardia, tachypnea, leukocytopenia or leukocytosis. The immune system becomes hyperactive upon

onset of sepsis (Figure 1): Lymphocytes, macrophages, as well as endothelial and epithelial cells produce proinflammatory mediators such as TNFα, IL-6, IL-1, and IL-8. Also production of acute phase proteins is increased in the liver and humoral defence mechanisms are active. Phagocytic cells produce reactive oxygen species (ROS) such as H_2O_2 in response to respective cytokines. In contrast to this overactive state of the inflammatory response at the early phase of the sepsis, various functions of the immune system are suppressed at a later stage. The result is hyporeactivity of the host immune system and subsequent immunoparalysis (*Riedemann et al. 2003*). Basic and clinical research has focused on sepsis caused by Gram-negative bacteria. However, an increasing number of studies implicate an equally important role of Gram-positive bacteria as a causative agent of severe sepsis and septic shock (*www.septicshock.org/research*).

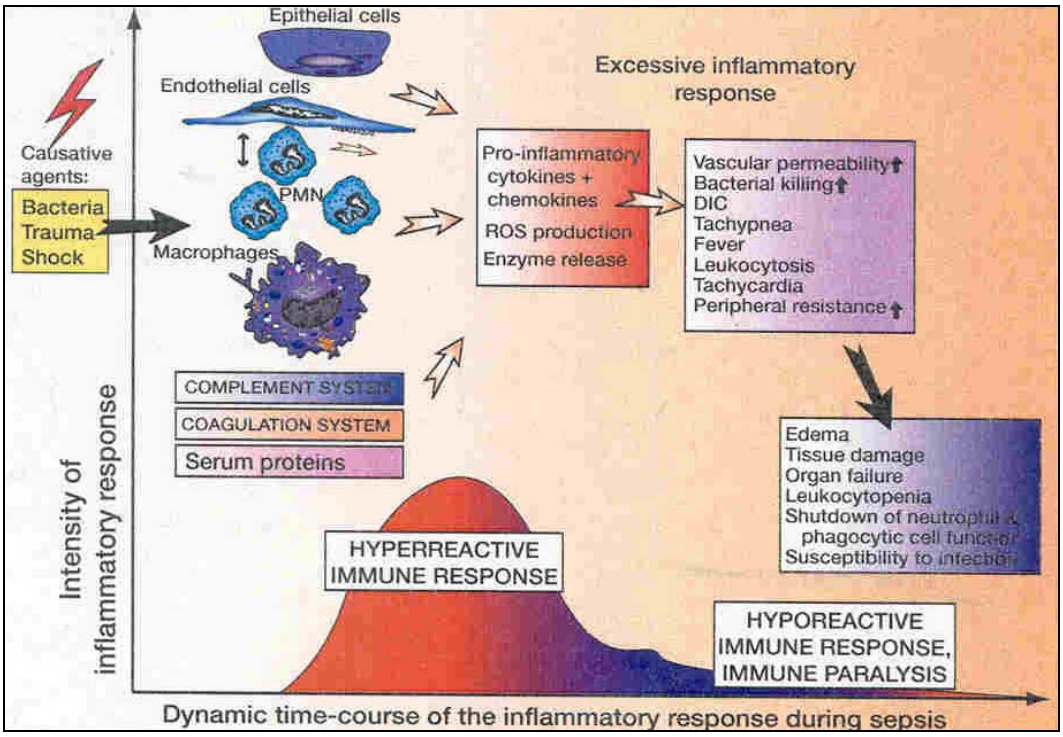


Figure 1 The inflammatory response during sepsis

Infection or trauma can cause sepsis through signaling cascades leading to inflammation and disseminated coagulation. The result is a life-threatening septic shock syndrome, characterized by multi organ failure. Source: (*Riedemann et al. 2003*).

2.2 Toll like receptors are pattern recognition receptors

2.2.1 Drosophila Toll receptor and mammalian Toll Like Receptors (TLRs)

Toll like receptors (TLRs) are pattern recognition receptors (PRRs). The discovery of TLRs was based on identification of *Drosophila* Toll: Toll drives the establishment of dorso-ventral polarity in the developing *Drosophila* embryo and is involved in anti-fungal and Gram-positive bacterial responses of the adult fruit fly (*Lemaitre et al. 1996*); (*Meister et al. 1997*); (*Anderson et al. 1985*). The ligand that activates Toll in both cases is Spätzle (*Morisato et al. 1994*). Spätzle is a cleavage product of the Pro-Spätzle protein generated upon activation of a protease cascade upon challenge with specific pathogens. Toll activates a protein kinase termed Pelle upon interaction with adaptor proteins dMyd88 and Tube. Pelle leads to the phophorylation of Cactus, which dissociates from the transcription factor Dorsal in order to translocate to the nucleus and increase expression of target genes involved in dorsoventral polarity. In the adult fruit fly, Toll activates another NF-κB/Rel family member, Dif, which regulates the expression of antifungal peptides such as Drosomycin (*Dunne et al. 2003*).

Database analysis applying the sequence of *Drosophila* Toll resulted in identification of mammalians TLRs. Humans express at least ten TLRs, all of which except TLR10 are also expressed by mice. Instead, mice express the two paralogs TLR11 and TLR12 that are not present in humans (*Beutler 2004*). TLRs are characterised by the presence of leucine-rich repeats (LRR) in the extracellular domain (ecd) and the TIR domain in the intracellular domain (icd). A comparison of the sequences of the TLRs members reveals five subfamilies among TLRs: TLR2, TLR3, TLR4, TLR5, and TLR9. The TLR2 subfamily is composed of TLR1, 2, 6 and 10 while the TLR9 subfamily is composed of TLR7, 8, and 9 (*Takeda et al. 2003*). TLRs sense microbial products (PAMPs), as well as endogenous ligands (*Tsan et al. 2004*) (Figure 2).

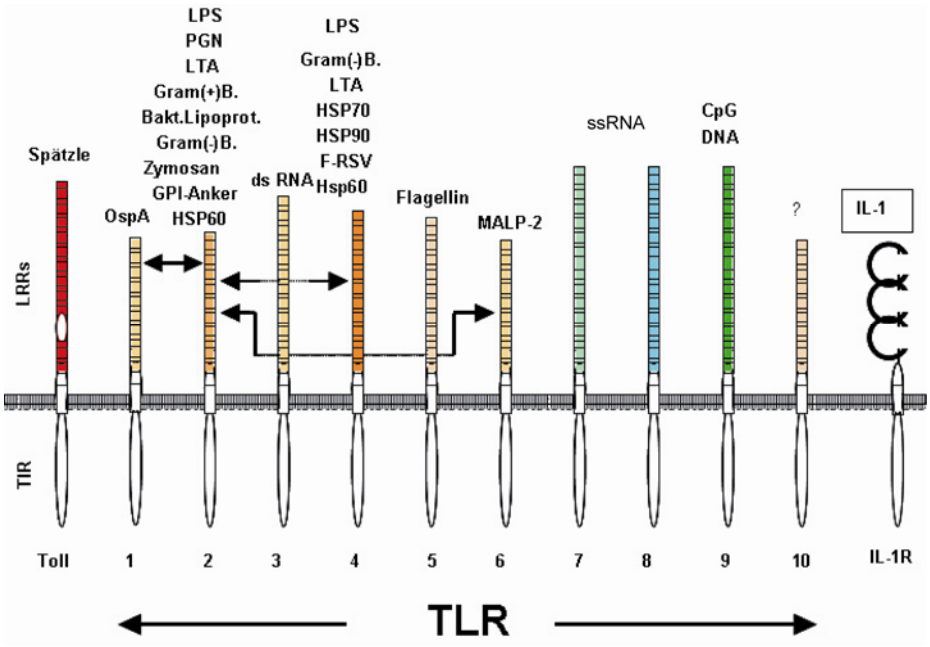


Figure 2 Family of human TLRs and some exemplary ligands

Comparison between 10TLRs, Toll receptor from *Drosophila* and interleukin 1 receptor (IL-1R). Legend: LRR - leucin rich repeat, TIR - Toll/Interleukin-1 receptor, OspA - outer surface protein A, LPS - lipopolysaccharide, PGN - peptidoglycan, LTA - lipoteichoic acid, HSP60 - heat shock protein 60 kDa, RSV - respiratory syncitial virus, MALP-2 - macrophage activating lipoprotein. Source: (*Kirschning et al. 2001*).

TLRs are expressed mainly at the surface and within specific organelles of immune cells. Human monocyte/macrophages express mRNA for most TLRs except TLR3 (*Muzio et al. 2000*). Expression of TLRs on dendritic cells depends on the subset and maturation status of the cells. Human myeloid dendritic cells express TLR1, 2, 4, 5 and 8 and plasmacytoid dendritic cells express TLR7 and 9 (*Takeda et al. 2003*). TLR3 is expressed in mature dendritic cells preferentially (*Muzio et al. 2000*). Mast cells, which have the capacity to phagocytose pathogens, process antigens, and produce inflammatory cytokines, express TLR2, 4, 6 and 8 but not TLR5 (*Supajatura et al. 2001*); (*McCurdy et al. 2001*). In addition to innate immune cells, TLRs are expressed in several other types of cells that contribute to inflammatory responses. The mucosal surface of the respiratory and intestinal tract contains a layer of epithelial cells forming a protective barrier against pathogens. The inflammatory response occurs only when the pathogenic bacteria invade into the basolateral compartment from the apical side. TLR5 is expressed exclusively on the basolateral surface of the intestinal epithelial cells and TLR4 is expressed at relatively low levels in intestinal epithelial cells (*Naik et al. 2001*); (*Abreu et al. 2001*).

TLR expression is strictly regulated in epithelial cells possibly explaining why pathogenic Gram-negative bacteria, but not commensal bacteria, induce inflammatory response in the intestine. Expression of TLR2 and 4 in renal epithelial cells is induced by IFNγ and TNFα (*Wolfs et al. 2002*). Epidermal keratinocytes in normal human skin constitutively express TLR1, 2 and 5, while TLR3 and 4 are expressed at very low level (*Baker et al. 2003*).

2.2.2 TLR signaling

2.2.2.1 Myd88 dependent pathway

Upon binding of their ligands, TLRs induce expression of a set of defence gens such as those encoding inflammatory cytokines and chemokines, costimulatory molecules, or antimicrobial peptides. TLRs activate an intracellular signaling pathway that is conserved from *Drosophila* to mammals. Upon recognition of PAMPs through TLRs, the adaptor molecule myeloid differentiation marker 88 (Myd88) is recruited to the receptor complex. It contains a COOH-terminal TIR domain and a NH_2-terminal death domain (DD). Myd88 dimerizes through both the TIR and DD domain. The N-terminal death domain of Myd88 engages the death domain of IRAK-4 (IL1R associated kinase 4), a serine threonine kinase. IRAK-4 mediates phophorylation of a crucial residue in the kinase-activation loop of IRAK-1. IRAK-1 is bound to Tollip where it is prevented from undergoing phosphorylation in resting cells. Upon activation, IRAK-1 is phosphorylated, weakening its affinity for Tollip and making it more accessible for further phosphorylation. The secondarily phosphorylated form of IRAK-1 is then released and can interact with TRAF6 (tumor-necrosis factor receptor-associated factor 6). TRAF6 interacts with TAB-2 (TAK1 binding protein 2), and this complex activates TAK-1 (TGF β activated kinase) (*Janeway et al. 2002*); (*Dunne et al. 2003*); (*Akira et al. 2004*). TAK-1 serves as a branch point, mediating activation of the IκB kinase complex and NF-κB, and the upstream kinases that activate p38 and JNK.

Activated IKK (I-kappa B kinase) phosphorylates and targets the NFκB inhibitor IκB for degradation, and thereby liberates NFκB for translocation into the nucleus and finally activate transcription of proinflammatory genes (Figure 3).

2.2.2.2 Myd88 independent pathway

Cellular responses downstream of TLR activation involve the expression and release of numerous cytokines, chemokines and co-stimulatory molecules required for the activation of adaptive immunity. Stimulation with different PAMPs can result in overlapping responses but specific response appears to be attributed to a particular TLR. The type of the downstream signaling of the individual TLR is dependent on the signaling pathway that is activated; which pathway is activated, in turn, appears to be dependent on the adaptor molecules that interact with the different TLRs (*Athman et al. 2004*). The first indication of such a mechanism resulted from analysis of Myd-88 deficient mice (*Kawai et al. 1999*). In these mice activation of NF-κB and MAP kinases, as well as upregulation of surface expression of MHC and costimulatory molecules in response to IL-1 and many TLRs ligands was diminished. However, in the absence of Myd88, the TLR4 ligand LPS still induced NF-κB and MAP kinases, although according to altered time kinetics. The Myd88-independent pathway was further characterized by determining the genes expressed in Myd88 deficient macrophages that had been exposed to LPS: A number of genes known to be interferon (IFN) inducible genes were identified, such as immunoresponsive gene 1 (IRG1) and glucocorticoid-attenuated response gene 16 (GARG16). Additionally, the Myd88-independent pathway mediates LPS-induced maturation of dendritic cells (DCs): upon stimulation with LPS, bone-marrow derived DCs upregulate the cell surface expression of co-stimulatory molecules such as CD40, CD80, and CD86 and induces proliferation of T cells (*Akira et al. 2004*).

The discovery of the Myd88 independent pathway led researches to the analysis of signaling pathways of the various TLRs and the discovery of other adaptor molecules. Until now, there are four known adaptor proteins besides Myd88: (1) MAL (Myd88 adaptor-like) or TIRAP (TIR domain containing adaptor protein), (2) TRIF (TIR domain containing adaptor inducing interferon β) or TICAM-1 (TIR containing adaptor molecule-1), (3) TRAM (TRIF related adaptor molecule), and (4) SARM (sterile α and HEAT-Armadillo motifs) (*Beutler 2004*); (*O'Neill et al. 2003*).

MAL/TIRAP contains a C-terminal TIR domain but does not contain a death domain. MAL/TIRAP deficient mice are impaired in their response not only to TLR4 ligands, but also to TLR2 ligands. LPS induction of interferon-inducible genes is

normal in these mice, suggesting the existence of another adaptor molecule involved in the Myd88 independent pathway (*Yamamoto et al. 2002*).

Database screening resulted in identification of TRIF representing a third class of adaptor proteins. TRIF functions as an adaptor mediating TLR3 signaling. TRIF was found to associate with TLR3 and IRF-3 in co-immunoprecipitation experiments and in overexpression studies TRIF activated the IFNβ promoter (*Yamamoto et al. 2004*). IRF-3 is a IRF-transcription factor that binds to the interferon stimulated response element (ISRE) and leads to induction of type 1 interferons (*Au et al. 1995*) (Figure 3):

The most recently described adaptor proteins are TRAM and SARM (*O'Neill et al. 2003*). TRAM deficient mice showed defects in cytokine production in response to TLR4 ligand, but not to the other TLRs ligands. TLR4 but not TLR3 mediated Myd88 independent IFNβ production and activation of signaling cascade was abolished in TRAM deficient cells. Thus, TRAM is an adaptor molecule that provides specificity for the Myd88 independent pathway of TLR4 signaling (*Yamamoto et al. 2003*).

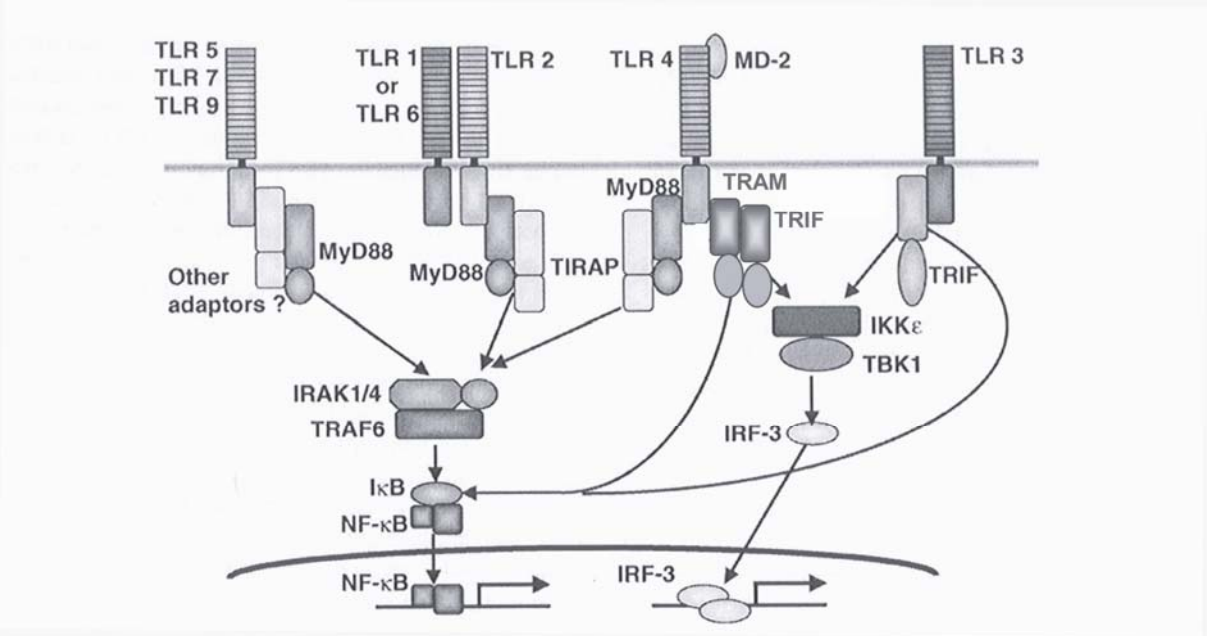


Figure 3 Myd88 dependent and independent signaling pathway

The Myd88 adaptor molecule mediates TLRs signaling pathway that activates IRAKs and TRAF6 and leads to the activation of NF-κB complex. This pathway is used by TLR1, 2, 4, 5, 6, 7, and 9. The released NF-κB from its inhibitor translocates to the nucleus and induces expression of inflammatory cytokines. Another TIR-domain containing adaptor molecule, TIRAP, is involved in the signaling pathway through TLR2 and TLR4. By contrast, TLR3 and TLR4 mediate activation of interferon (IFN)-regulatory factor 3 (IRF3)

and induction of IFNβ in Myd88-independent manner, using a third TIR-domain-containing adaptor TRIF (*Akira et al. 2004*). Source: (*Yamamoto et al. 2004*), modified.

2.2.2.3 TLRs and adaptive immunity

Dendritic cells are placed at the border between innate and adaptive immunity (*Banchereau et al. 1998*). Immature DCs reside in the peripheral tissues; upon encountering the pathogen, they undergo a maturation process which includes increased MHC molecule expression, and subsequent antigen processing, induction of costimulatory activity, and migration to the lymph node where they can prime naïve antigen-specific T cells. In this way, activation of the adaptive immune system occurs only after recognition of the pathogen. Expression of TLRs on the surface of dendritic cells and specificity for microbial ligands allows them to mediate control of adaptive responses (*Janeway et al. 2002*). TLR signaling can induce production of proinflammatory cytokines and lead to upregulation of expression of co-stimulatory molecules such as CD86 and CD80. Together with antigen-MHCII complex presentation, this leads to instruction of naïve T cells to differentiate into a Th1 or Th2 subpopulation, finally resulting in the establishment of an adaptive immunity (Figure 4) (*Kaisho et al. 2002*). Analysis of Myd88-deficient mice demonstrated the importance of TLRs in generation of adaptive immunity responses. Stimulation with different PAMPs (for example peptidoglycan or CpG motifs) except LPS does not result in DC maturation, so the DCs cannot activate naïve T cells in vitro nor produce IL-12. However, B cells from Myd88-deficient mice produce antigen specific IgG1 and IgE antibodies. *Myd88*$^{-/-}$ mice are impaired in Th1 type responses but not in Th2 responses (*Janeway et al. 2002*).

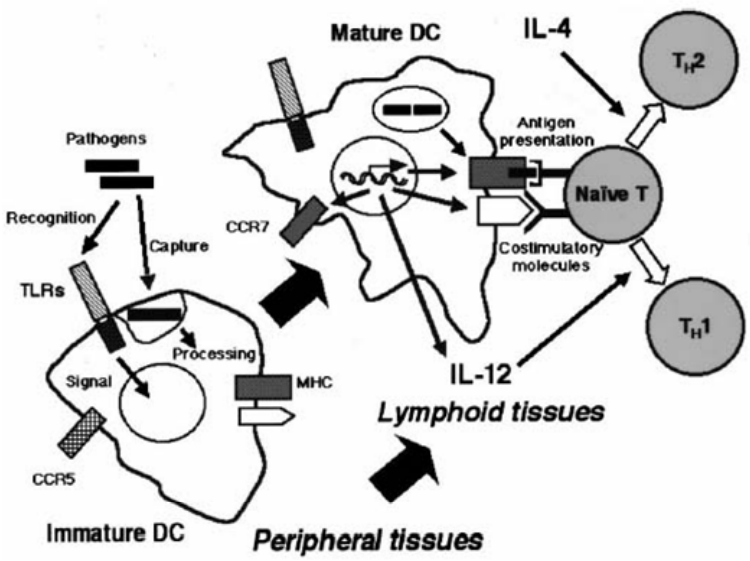


Figure 4 Activation of adaptive immunity through TLRs

Immature DCs in the peripheral tissues sense invading pathogen through TLRs or capture by endocytosis and processing. Processed products are then presented to naïve T cells as antigen-MHC complexes together with proinflammatory cytokines inducing differentiation of naïve T cells. Source: (*Kaisho et al. 2002*)

2.2.3 TLRs mediate species specific PAMPs recognition

The mediation of species specific PAMPs recognition has been reported for TLR4, MD2, TLR 7, TLR 8 and TLR9.

Species-specific differences in cellular pattern recognition have been revealed for particular LPS variants and taxol, retrospectively implicating TLR4 and MD2. The extracellular domain of human TLR4 discriminates between *Pseudomonas aeruginosa* LPS structures. The opportunistic bacterium *Pseudomonas aeruginosa* synthesizes more highly acylated (hexa-acylated) LPS structures during adaptation to the cystic fibrosis airway; human TLR4 recognizes this modification and induces proinflammatory signals (*Hajjar et al. 2002*). Human TLR4 can also discriminate between different acylation states of lipid A: A tetra-acyl lipid A (lacking secondary acyl chains) does not effectively induce signaling via human TLR4, whereas lipid A does. In contrast, mTLR4 transduces signal initiated by both lipid A and tetra-acyl lipid A (*Poltorak et al. 2000*). Also the lipid A analogues lipid IVa and *Rhodobacter sphaeroides* lipid A (RSLA) exhibit species specific pharmacology: both are potent LPS antagonists in LPS-responsive human cells but in hamster macrophages they

are LPS mimetic (*Lien et al. 2000*). Additionally, in mice RSLA acts as an LPS antagonist, whereas lipid IVa acts as LPS mimetic (*Lien et al. 2000*).

MD-2 is associated with TLR4 and imparts LPS responsiveness to it. Both TLR4 and MD-2 are implicated in species specific recognition of *Salmonella* lipid A. *Salmonella* lipid A has been reported to have a little stimulatory effect on human macrophages while being highly active in respect to murine macrophages. Muroi and colleagues showed that unresponsive human monocytic THP-1 cells upon overexpression of mCD14, mTLR4 and mMD-2 are capable of responding to *Salmonella* lipid A (*Muroi et al. 2002*). It has been demonstrated that MD-2 is able to directly regulate the recognition of LPS by TLR4. Humans but not mice recognize a lipid A analogue, lipid IVA. Human cells discriminate between these molecules and respond only to lipid A, whereas mouse cells respond to both lipid A and lipid IVA (*Akashi et al. 2001*). Akashi et al. showed that human MD-2 confers on mTLR4 responsiveness to lipid A but not to lipid IVa. Moreover, lipid IVa acts as a lipid A antagonist on mTLR4 associated with hMD-2.

Mouse MD-2 but not human MD-2 is involved in Taxol signaling (*Kawasaki et al. 2001*). Taxol was suggested to share a receptor and/or signaling molecules with LPS. The LPS mimetic activities of Taxol were species specific; human cells were unresponsive to Taxol whereas mouse cells mediated Taxol signaling. The Gln^{22} of mouse MD2 was found to be essential for LPS mimetic Taxol signaling but not for LPS signaling (*Kawasaki et al. 2001*).

TLR7 and TLR8 recognize synthetic ligand R-848 in species specific manner (*Heil et al. 2003*). Human and mouse TLR7 recognize R-848, whereas only human TLR8 confers response to R-848. Recently, Heil et al. showed that murine TLR7 and human TLR8 mediate species-specific recognition of GU-rich ssRNA (*Heil et al. 2004*). Also TLR9 has been implicated as the mediator of species-specific DNA sequence recognition. Human and mouse TLR9 required distinct CpG-motives for signal initiation: mouse TLR9 prefers motif containing ACG, whereas human TLR9 prefers the sequence TCG, implying that TLR9 directly engage immunostimulatory CpG-DNA in a species specific manner (*Bauer et al. 2001*).

2.3 TLR2

2.3.1 TLR2 structure

TLR2 is a type I transmembrane receptor. The N-terminal extracellular domain consists of leucine rich repeats (LRRs) motifs, followed by a membrane proximal LRR C terminal cystein rich domain (LRRCT), transmembrane domain, and a C-terminal TIR domain (Figure 5) (*Kirschning et al. 2002*). The premature human TLR2 is a polypeptide encompassing 785aa residues with 15.4% leucine residue in the total protein. The size of the mature protein is 95 kDa, the difference from the calculated size of unmodified protein (approximately 10kDa) could be due to post-translational modification such as glycosylation. Analysis by program (*http://www.cbs.dtu.dk/ services/NetNGlyc/*) reveal 4 putative N-glycosylation sites for human TLR2 and 3 sites for mouse TLR2.

The extracellular domain of TLR2 contains 20 LRR motifs as revealed by application of the SMART program (*Schultz et al. 1998*) for identification of motifs 1-3, 5, 13-15, and 17-19. The other motifs were localized by definition of at least two matches within a LRR main motif – LxxLxxLxLxxN – as a minimal requirement for an LRR-like motif. The LRR12 motif lacks identity with consensus sequence, although it displays similarities with it.

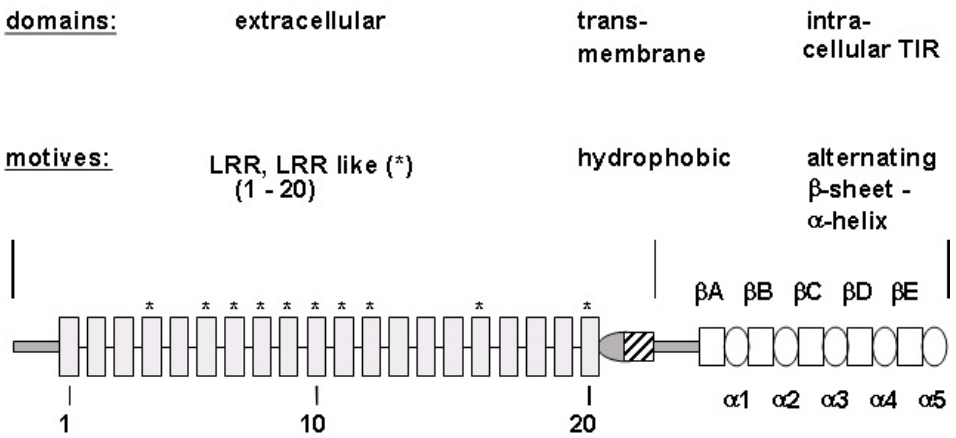


Figure 5 TLR2 protein structure

The schematic representation of TLR2 protein extending from extracellular domain (left) to its intracellular domain (right). The N-terminally located boxes represent LRRs or LRR-like motifs (box with an asterisk). The transmembrane domain (upward hatched box) is preceded by an LRRCT motive. The TIR domain consists of five alternating β-sheet (box) and α-helix motifs (oval) pairs. Source: (*Kirschning et al. 2002*)

TLR extracellular domains have not been crystallised to date. A model of the TLR2-ecd is based on the 3D structure of several other LRR containing proteins like ribonuclease inhibitor (*Kobe et al. 2001*) or internalin from *Listeria monocytogenes* (*Schubert et al. 2002*). In this model each individual LRR forms a loop and several LRR loops form a coil or solenoid structure. The conserved hydrophobic residues of the LRR consensus motif point inward and form the core of the solenoid. In all LRR-ligand complexes solved up to date, the ligand binding site is located on the concave surface of the solenoid (*Bell et al. 2003*). Recently, Weber et al. indicated that the ectodomain of human TLR2 has a similar structure to that of glycoprotein Ib and the Nogo receptor. In their model the convex surface is not α-helical but of variable, extended secondary structure (*Weber et al. 2004*).

The TIR domain starts 30aa C-terminally from the transmembrane domain and extends to last C-terminal residue S785 of TLR2. The TIR domain forms a cassette of ten motifs of alternating β-sheets α-helices (*Xu et al. 2000*) The so called BB loop, which is formed between the second β-sheet and α-helices, contains a sequence motif characteristic for IL-1/TLRs. This loop contributes to a surface patch potentially important for interaction with the adaptor molecule Myd88 (*Xu et al. 2000*) (Figure 6).

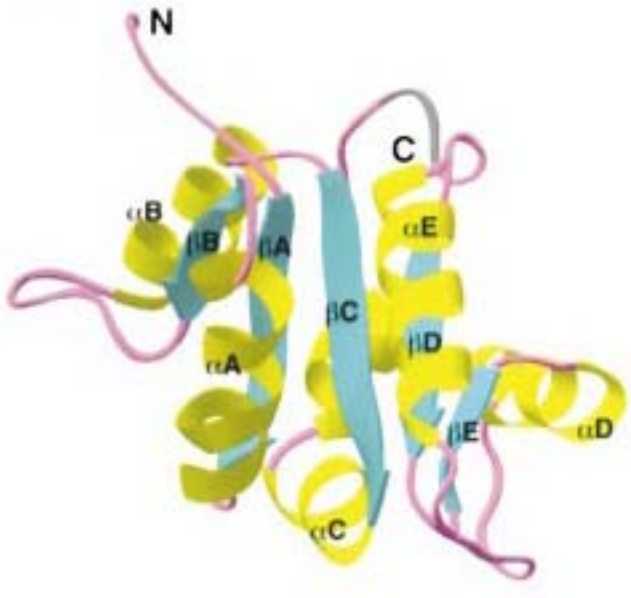


Figure 6 The 3D structure of TIR domain of human TLR2

The structure contains the central five stranded parallel β-sheet βA-βE (blue) that is surrounded by a total of five α-helix αA-αE (yellow) on both sides. Source: (*Xu et al. 2000*).

2.3.2 TLR2 function

Among all TLRs, TLR2 has been described to mediate response to a most diverse set of molecular structures to date. The initial implication of TLR2 in recognition of Gram-positive bacteria was based on the application of the whole bacteria such as *Staphylococcus aureus*, or bacterial cell wall preparations (*Schwandner et al. 1999*); (*Yoshimura et al. 1999*). Application of highly purified soluble peptidoglycan (PGN) from *S.aureus* and commercially available lipoteichoic acid (LTA) from *B.subtilis* implicated both bacterial products as TLR2 agonists. LTA and PGN are the main immunostimulatory cell wall components of Gram-positive bacteria (*Heumann et al. 1994*) inducing the release of proinflammatory cytokines such as TNFα or IL-6. *TLR2*$^{-/-}$ mice displayed unresponsiveness to PGN, whereas *S.aureus* LTA has been implicated as TLR4 agonist (*Takeuchi et al. 1999*). However, *in vitro* analysis including over-expression of MD-2 in HEK293 cells implicated TLR2 and TLR4 as LTA recognition receptors (*Dziarski et al. 2001*). In contrast, high purification of LTA from *S.aureus* or *Treponema* implied LTA as an exquisite TLR2 agonist (*Morath et al. 2001*).

Another group of specific TLR2 agonists consists of bacterial and mycoplasmal lipopeptides. Specific TLR2-dependent cell stimulation has been observed for a variety of different microbial lipopeptides including the synthetic lipopeptide analogue tripalmitoyl-cysteinyl-seryl-(lysyl)3- lysine (P_3CSK_4), OspA of *Borrelia burgdoferi*, and *Mycoplasma fermentans* macrophage activating lipopeptide (MALP-2) (*Aliprantis et al. 1999*); (*Brightbill et al. 1999*); (*Hirschfeld et al. 1999*). Additionally, the stereoisomeric orientation of this lipopeptide appears to be of major importance for TLR2 interaction (*Takeuchi et al. 2001*).

Aderem and co-workers first suggested the possibility that TLR2 ligands are recognized by heterodimers formed between TLR2 and other TLRs (*Ozinsky et al. 2000*). This was confirmed in *in vivo* experiments: neither *TLR2*$^{-/-}$ nor *TLR6*$^{-/-}$ responded to synthetic MALP-2 whereas *TLR6*$^{-/-}$ but not *TLR2*$^{-/-}$ responded to P_3CSK_4 (*Takeuchi et al. 2001; Takeuchi et al. 2002*). Recent studies showed that 19kDa lipoprotein from *M. tuberculosis* and Osp A from *B. burgdoferi* are recognized by a heterodimer formed between TLR2 and TLR1(*Akira 2003*).

TLR4 is a major LPS receptor (*Beutler 2002*). However, TLR4-independent recognition of LPS such as *Porphyrymonas gingivalis* and the spirochete *Leptospira interrogans* LPS has been demonstrated and is TLR2 dependent (*Hirschfeld et al. 2001*); (*Werts et al. 2001*). TLR2 and TLR4 are also implicated in recognition of heat shock proteins (HSP) of microbial or endogenous origin (*Vabulas et al. 2001*).

2.3.3 TLR2 signaling

TLR2 shares signaling pathways with other TLRs (see 2.2.2) that lead to activation of NF-κB and MAPKs. However, TLR2 also induces specific pathways not employed by other TLRs (*Hirschfeld et al. 2001*). Application of lipid A or LPS variants from two different bacterial species *E.coli* and *Porphyrymonas gingivalis* revealed striking differences in the degree, time course and quality of gene induction (*Hirschfeld et al. 2001*); (*Martin et al. 2001*). While *E.coli* was a stronger inducer of T helper (Th)1 cytokines IFNγ and IL-12, *P.gingivalis* LPS preferentially induced Th2 cytokines such as IL-10 in immune cells (*Pulendran et al. 2001*).

It has been shown that phosphorylated TLR2 - intracellular domain recruits the p85 subunit of phosphatidylinositol-3 kinase (PI3K) (*Arbibe et al. 2000*), which leads to activation of Akt and p65 phosphorylation. However, NF-κB p65 kinase downstream from Akt is not yet defined (*Arbibe et al. 2000*).

Aliprantis et al. have suggested that TLR2 not only mediated cellular activation, but also apoptotic effects. Fas associated death domain protein and caspase 8 were implicated in a TLR2-dependent apoptotic pathway (*Aliprantis et al. 1999*); (*Aliprantis et al. 2000*). Transcription factors such as NF-κB, AP-1, CREB (cAMP response element binding factor) and SRE-1 (serum response element binding factor) have been implicated in TLR-mediated gene activation (*Xu et al. 2001*).

2.3.4 TLR2 and *in vivo* studies

The first report of a TLR2 role in *in vivo* infection showed that gene targeted *TLR2*$^{-/-}$ mice displayed increased susceptibility to *S.aureus* infection as compared to wild-type mice (*Takeuchi et al. 2000*). Genetic analysis revealed that a

polymorphism in TLR2 might be associated with increased susceptibility to *S.aureus* infection in humans (*Lorenz et al. 2000*).

Group B streptococci (GBS), which are of major clinical importance for newborn children, display unique immuno-stimulatory activity. It has been shown, that TLR2 plays a major role for recognition of extracellular GBS products, but not to whole bacteria using peritoneal macrophages from TLR2-deficient mice (*Henneke et al. 2001*). *In vivo* studies of invasive GBS disease provide evidence of the dual role of TLR2 and Myd88 as important components of the host immune system during initial infection and as mediators of lethality in the presence of overwhelming sepsis (*Mancuso et al. 2004*).

TLR2-deficient mice infected with *Candida albicans* displayed significant impaired survival and decreased recruitment of neutrophiles to the peritoneal cavity. Production of reactive oxygen intermediates, however, was not affected in macrophages from *TLR2*$^{-/-}$ mice (*Villamon et al. 2004*). Additionally, *TLR2*$^{-/-}$ produce Th1 cytokines (IFNγ, IL-12, TNFα) at lower levels than wild types, but both *TLR2*$^{-/-}$ and wild types are equally capable of inducing specific humoral response to the fungus and developing a vaccine-induced resistance (*Villamon et al. 2004*). A similar observation has been made by Wooten et al. studying *B. burgdorferi* infection (*Wooten et al. 2002*). Despite the fact that TLR2 is reported to be involved in innate host defence against *B. burgdorferi* infection, development of acquired humoral response and clearance from *B. burgdorferi* infection is TLR2 independent (*Wang et al. 2004*); (*Wooten et al. 2002*).

Mycobacterial cell wall components activated macrophages through TLR2. Consequently, *TLR2*$^{-/-}$ mice show reduced bacterial clearance, a defective granulomatous response and develop chronic pneumonia after aerosol infection with live mycobacteria (*Drennan et al. 2004*). Interestingly, *TLR2*$^{-/-}$ mice develop chronic pneumonia despite enhanced cell mediated immunity. Analysis of $CD11c^+$ and $Ly\text{-}6G^+$ cells isolated from the lungs of infected mice showed increased expression levels of MHC class II, accompanied by increased numbers of $CD4^+$ and $CD8^+$ cells in the lungs. Additionally, pulmonary levels of IFNγ, IL-12, TNFα were elevated in *TLR2*$^{-/-}$ mice (*Drennan et al. 2004*).

Edelson and colleagues showed equivalent resistance of wild type and *TLR2*$^{-/-}$ mice against LM infection. However, Myd88 is required for full activation of

innate immunity response in LM infection. In contrast, direct *Listeria* killing by activated macrophages occurs through not only a TLR2 independent but also Myd88 independent mechanism (*Edelson et al. 2002*). A second study by Seki (*Seki et al. 2002*) showed only partial involvement of TLR2 in *Listeria* infection. *TLR2*$^{-/-}$ mice displayed only partial impairment in their capacity to produce IL-12, IFNγ, and TNFα upon infection (*Seki et al. 2002*). In contrast, Torres et al. showed that TLR2 is required for optimal control of LM infection. TLR2 deficient mice were more susceptible to the infection as compare to wild type mice. They had reduced survival rates and released lower cytokine amounts. Additionally, *TLR2*$^{-/-}$ mice expressed costimulatory molecules at lower levels and an increased bacterial burden was observed in the liver (*Torres et al. 2004*).

3. Aims

3.1 Identification of species-specific TLR2 agonist, as well as analysis of molecular requirements

Species-specific differences in cellular pattern recognition have been observed for specific LPS variants and taxol (*Lien et al. 2000*); (*Poltorak et al. 2000*); (*Akashi et al. 2001*). Similarly, TLR9 and TLR7, 8 have been recognized as mediators of species-specific DNA and ssRNA sequence recognition, respectively (*Bauer et al. 2001*); (*Chuang et al. 2002*); (*Heil et al. 2004*). Here, we used complementation of human embryonic kidney (HEK) 293 cells or murine embryonic *TLR2*$^{-/-}$ fibroblasts with murine TLR2, human TLR2 or TLR2 chimera constructs to identify and analyze species-specificity of pattern recognition through TLR2 orthologue products. In addition, we analyzed the murine RAW264.7 cell line, as well as human MonoMac6 and THP1 macrophage cell lines.

The results showed species-specific recognition of a tri-lauroylated peptide through murine TLR2 and involvement of a relatively low conserved LRR-rich sub-domain of the TLR2ECD in this process.

3.2 Analysis of role of TLR2 in the host response to *Listeria monocytogenes* infection *in vivo*

TLR2 has been described to mediate cellular recognition of *Listeria monocytogenes* (*Flo et al. 2000*). Here, we analysed immunoresponsiveness of *TLR2*$^{-/-}$ mice to *L.monocytogenes* infection. Cytokine levels, bacterial burden in organs, as well as lethality were measured. Results indicate a minor phenotype of *TLR2*$^{-/-}$ mice in *L.monocytogenes* infection.

3.3 Analysis of potential interaction between TLR2 and specific cytoplasmic proteins

Knowledge of intracellular signaling pathway of TLRs is restricted to NF-κB and MAP kinases, as well as IRF-activation. In *Drosophila*, filamin has been reported as a potential adaptor linking Tube to the transmembrane receptor Toll (*Edwards et al. 1997*), which was one of the 5 proteins identified as TLR2 interactors by yeast two hybrid assay.

Further proteins identified in the yeast two hybrid system were: α-actinin, Hsp40, a new protein with high sequence similarity to human SSA/Ro protein, and SMN (Lamping N., Kirschning C., unpublished data). We focused on further biochemical analysis of potential interaction between these proteins and intracellular domain of TLR2.

By co-immunoprecipitation analysis upon overexpression in HEK293 cells, we showed interaction between: (1) TLR2 intracellular domain (icd) and α-actinin (2) TLR2icd and Hsp40, and (3) TLR1icd and Hsp40.

4. Material and Methods

In the following, we present the materials that were used and the methods that were applied.

4.1 Materials

4.1.1 Reagents

As purified bacterial components were applied: LPS from *E. coli* 0111:B4, LPS from *K. pneumoniae*, LPS from *S. flexneri* (Sigma, Deisenhofen, Germany), LPS D31m4 and LipA31m4 from *E.coli* (List Chemicals, California, USA). Soluble peptidoglycan (PGN) from *S. aureus* Rb prepared by vancomycin affinity chromatography (*Dziarski et al. 1998*), and highly purified LTA from *S. aureus* prepared by propanol extraction (*Morath et al. 2001*). Synthetic mycoplasmal macrophage-activating lipoprotein (MALP)-2 was supplied from Dr. Mühlradt (GBF Braunschweig, Germany). Tri-/di-/monopalmitoyl-cysteinyl-seryl-(lysyl)3-lysine (P_3CSK_4, P_2CSK_4, $PCSK_4$) and tri-lauroyl/trimyristoyl-cysteinyl-seryl-(lysyl)3-lysine (Lau_3/Myr_3CSK_4), as well as biotinoylated analogues were purchased from ECHAZ microcollections (Tübingen, Germany) (*Bessler et al. 1992*). Lipidated OspA, a tripalmitoylated lipoprotein from *B. burgdorferi*, was supplied by Dr. Dunn (Brookhaven National Laboratory, Upton, NY). Highly purified recombinant chlamydial HSP60 was supplied by Drs. Prazeres da Costa and Miethke (*Costa et al. 2002*). Yeast lyophilisate zymosan and phorbol 12-myristate13-acetate (PMA) were purchased from Sigma.

4.1.2 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.

Buffer	Composition
PBS:	10 g/l Dulbecco PBS (Biochrom) pH 7.4
PBT:	1xPBS 0.05% (v/v) Tween 20
TAE-buffer:	40 mM Tris-acetate pH 8,3 1 mM EDTA
6x Loading buffer: (Agarose gel)	1 g/l Orange G 20 mM Tris pH 8.5 15% (v/v) Glycerol
2x 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 Protease inhibitor cocktail 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
4xSDS-Sample buffer:	200 mM Tris-HCl pH 6,8 400 mM DTT 10% (w/v)SDS 16% (v/v) Glycerol 2g/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:	1x PBT 3% (v/v) NGS 50 g/l Milk powder
Stripping solution	100 mM Glycine 10 mMβ-Mercaptoethanol pH 2.75
FACS buffer:	1x PBS 2% (v/v) FCS
Citrate-Phosphate buffer:	50 mM Na_2HPO_4 25 mM Citric acid
Renilla buffer	64,28 g/lNaCl 6,42 g/l Na_2EDTA 5,44 g/l K_4PO_4 (pH 5,1) 440 mg/l BSA 0,084 g/l NaN_3 1 mg/l Coelenterazin pH 5,0

Luciferase substrate	132 mg/l D-luciferin 210 mg/l Coenzym A 5140 mg/l DTT 292 mg/l ATP 520 mg/l$(MgCO_3)_4Mg(OH)_2$ x5H_2O 322 mg/l $MgSO_4$ 3584 mg/l Tricine 37,2 mg/l EDTA pH 7,8
FACS blocking buffer	2%FCS 5% NGS Fcγ block or Endoglobulin (Baxter)
Immunocytochemistry blocking buffer	2% NGS in PBS
Totex buffer	20 mM HEPES (pH7,9) 0,35 M NaCl 20% Glycerol 1% NP-40 1 mM $MgCl_2$ 0,5 mM EDTA (pH8,0) 0,1 mM EGTA 10 mg/ml Leupeptin Aprotinin 100 mM PMSF 100 mM DTT
10 x TBE	108 g/l TRIS 55 g/l Boric acid 0,5 M EDTA
5x Buffer F	20% Ficoll 400 100 mM Hepes pH 7.2 300 mM KCl 10 mM DTT 0.1 mM PMSF

Buffer D	20 mM Hepes pH 7.2 20% glycerin 100 mM KCl 0.5 mM EDTA 0.25% NP-40 2 mM DTT 0.1 mM PMS
10x Buffer L	0.1 M Tris pH 7.5 0.1 M $MgCl_2$ 0.01 M DTT
1 x STE	100 mM NaCl 20 mM Tris-Hcl 10 mM EDTA

4.1.3 KIT-systems

The following KIT-systems were used:

KIT-system
Chemoluminescence Reagent Plus – ECL (PerkinElmer)
Human IL-8, IL-6, TNFα ELISA (R&D)
Mouse IL-6, TNFα ELISA (R&D)
Cytofix/Cytoperm (BD)
PNGase F (New England Biolabs)
Advanatge cDNA polymerase Mix (Clontech)
BCA® protein assay kit (Pierce)
PCR QuickChange Site directed Mutagenesis PCR Kit (Stratagene)
LigaFast Rapid DNA Ligation system (Promega)
QIAquick Gel extraction kit (250) (QIAgen)
QIAprep spin Miniprep kit (250) (QIAgen)
QIAfilter Plasmid Maxi kit (25) (QIAgen)

4.1.4 Media

The following media were applied:

Media	Composition
HEK 293	1xDMEM (Gibco) 10% (v/v) FCS (PAA) 1% (v/v) Anti-mycoticum (Gibco) 1%(v/v) Penicilin-Streptomycine (Gibco)
Electroporations medium	25% (v/v) FCS in DMEM
Freezing medium:	10% (v/v) DMSO 90% (v/v) FCS
THP-1	1xRPMI (Gibco) 10% (v/v) FCS 1% (v/v) Anti-mycoticum (Gibco) 1% (v/v) Penicilin-Streptomycine (Gibco) 1% (v/v) L-glutamine (Gibco)
RAW 264.7	1xRPMI (Gibco) 10% (v/v) FCS 1% (v/v) Anti-mycoticum (Gibco) 1% (v/v) Penicilin-Streptomycine (Gibco) 1% (v/v) L-glutamine (Gibco) β mercapohoethanol
MonoMac6	1xRPMI (Gibco) 10% (v/v) FCS 1% (v/v) Anti-mycoticum (Gibco) 1% (v/v) Penicilin-Streptomycine (Gibco) 1% (v/v) L-glutamine (Gibco) non canonical amino acids OPImedia supplement (Sigma)
MEF *TLR2*$^{-/-}$	1xDMEM (Gibco) 10% (v/v) FCS (PAA) 1% (v/v) Anti-mycoticum (Gibco) 1% (v/v) Penicilin-Streptomycine (Gibco) 10 μM monothioglycerol

LB-medium:	10 g/l Bacto-Trypton 5 g/l Yeast-extract 10 g/l NaCl
Brain/Heart (BH) medium	27.5g/l Nutrient substarte (brain, heart extract and peptones) 2g/l D+glucose 5g/l sodium chloride 2.5g/l di-sodium hydrogen phosphate

4.1.5 Antibodies and antibody conjugates

The following antibodies were applied:

Name	Conjugate	Source	Con mg/ml	Company	Application
poly-αHA	-	rabbit	0.2	Santa Cruz	1:1000 WB
poly-αFlag	-	rabbit	0.8	Sigma	1:1000 WB
poly-αrabbit-HRP	HRP	goat	0.5	BioRad	1:5000 WB
αFlag-M2 affinity gel	agarose beads	mouse	-	Sigma	IP
α-mouse CD16/CD23		rat	0.5	BD	1:100 FACS
mono α-mouse/humanT2.5	-	mouse	0.9	GM	1:100 FACS, IH
mono α-mouse T2.13	-	mouse	0.6	GM	1:100 FACS, IH
αpp38	-	rabbit	0.04	Cell Signaling	1:1000 WB
αp38	-	rabbit	0.01	Cell Signaling	1:1000 WB
αpERK	-	rabbit	0.05	Cell Signaling	1:1000 WB
αERK	-	rabbit	0.01	Cell Signaling	1:1000 WB
mono α mouse IgG	FITC	goat	0.7	Caltag lab	1:200 FACS
α mouse IgG	AleFlo546	goat	0,02	Cell Signaling	1:250 IH
streptavidin	AleFlo647	-	1	Mol. Probes	1:100 IH
concavalin A	AleFlo488	-	5	Mol. Probes	1:200 IH

Table 1 Antibodies and conjugates used.

Poly - Polyclonal, Mono - Monoclonal, HRP - Horse Radish Peroxidase, PE - Phycoerythrin; WB-Western blot; FACS - fluorescence-activated cell sorting; IH - immunochemistry; AleFlo – AlexaFlour, Mol. Probes – Molecular Probes.

4.1.6 Plasmids

Table 2 lists the plasmids that were used/generated.

Promotor	Insert	Vektor	Donor
P_{CMV}	humanTLR 2	pFlag-CMV-1	C.Kirschning; H.Wesche
P_{CMV}	mouseTLR 2	pFlag-CMV-1	H. Heine
P_{CMV}	h/m TLR2	pFlag-CMV-1	AG
P_{CMV}	m/h TLR2	pFlag-CMV-1	AG
P_{CMV}	h_{1-10}m/m TLR2	pFlag-CMV-1	AG
P_{CMV}	m_{1-10}h/h TLR2	pFlag-CMV-1	AG
P_{CMV}	hm_{7-10}h/h TLR2	pFlag-CMV-1	AG
P_{CMV}	mh_{7-10}m/m TLR2	pFlag-CMV-1	AG
P_{CMV}	$hm_{7-10}hm_{19-20}$/h TLR2	pFlag-CMV-1	AG
P_{CMV}	$mh_{7-10}mh_{19-20}$/m TLR2	pFlag-CMV-1	AG
P_{CMV}	hm_{8-10}h/h TLR2	pFlag-CMV-1	AG
P_{CMV}	hm_{9-10}h/h TLR2	pFlag-CMV-1	AG
P_{CMV}	hm_{10}h/h TLR2	pFlag-CMV-1	AG
P_{CMV}	hm_{8-9}h/h TLR2	pFlag-CMV-1	AG
P_{CMV}	hm_{9}h/h TLR2	pFlag-CMV-1	AG
P_{CMV}	hm_{8}h/h TLR2	pFlag-CMV-1	AG
P_{CMV}	mh_{8}m/m TLR2	pFlag-CMV-1	AG
P_{CMV}	hm_{14-18}h/h TLR2	pFlag-CMV-1	AG
P_{CMV}	mh_{14-18}m/m TLR2	pFlag-CMV-1	AG
P_{CMV}	hm_{19-20}h/h TLR2	pFlag-CMV-1	AG
P_{CMV}	mmutH TLR2	pFlag-CMV-1	GM
P_{CMV}	hmutH TLR2	pFlag-CMV-1	GM
P_{CMV}	hmutL112P TLR2	pFlag-CMV-1	AG
P_{CMV}	mmutL112P TLR2	pFlag-CMV-1	AG
P_{CMV}	human dG N114Q TLR2	pFlag-CMV-1	AG
P_{CMV}	human dG N199Q TLR2	pFlag-CMV-1	AG
P_{CMV}	human dG N414Q TLR2	pFlag-CMV-1	AG
P_{CMV}	human dG N442Q TLR2	pFlag-CMV-1	AG
P_{CMV}	mouse dG N147Q TLR2	pFlag-CMV-1	GM
P_{CMV}	mouse dG N414Q TLR2	pFlag-CMV-1	GM
P_{CMV}	mouse dG N442Q TLR2	pFlag-CMV-1	GM
P_{CMV}	mouse dG3 TLR2	pFlag-CMV-1	GM
P_{CMV}	hTLR2 icd	pFlag-pRK SN	AG
P_{CMV}	hTLR1 icd	pFlag-pRK SN	AG
P_{CMV}	Myd88	pFlag-pRK SN	AG

Promotor	Insert	Vektor	Donor
P_{CMV}	hTLR1	pFlag-CMV-1	C.Kirschning
P_{CMV}	Hsp40	HA - pcDNA	N.Lamping
P_{CMV}	α-actinin	HA - pcDNA	N.Lamping
P_{CMV}	-	pRK5	U. Schindler
P_{ELAM-1}	Luc	pELAM-1	U. Schindler
P_{renila}	renilla	pHRL	R. Hasse

Table 2 Plasmids and expression constructs used

Mammalian expression vectors pcDNA, pRK-SN, pRK5 and pCMV contain the early promoter of human cytomegalovirus (CMV) that allows the transcription in eukaryotic cells. The promoter of pELAM-1 is NF-κB dependent. The plasmid pFLAG-CMV-1 is a derivate of pCMV, a heterologous preprotrypsin leader precedes a FLAG epitope tag, N-terminally fused to the overexpressed protein, pcDNA vector has an HA epitope tag N-terminally fused to the overexpressed protein. AG – Alina Grabiec, GM – Guangxun Meng.

4.1.7 Oligonucleotides

Oligonucleotides were purchased from MWG (Ebersberg, Germany) and applied as primers for following PCR reactions. The numbers in the name of primers for site directed mutagenesis correspond to the number of exchanged LRRs.

Primers for NF-κB labelling:

Primer name	Sequence (5' to 3')
Sense	AGT TGA GGG GAC TTT CCC AGG C
Antisense	GCC TGG GAA AGT CCC CTC AAC T

Primers for mTLR2 sequencing:

Primer name	Sequence (5' to 3')
1. F1-m2	ATG CTA CGA GCT CTT TGG CTC
2. 5R-m2	C AGA AGC ATC ACA TGA CAG AG
3. F2-m2	T TTG TCT GAT AAT CAC CTA TC
4. R6-m2	AA GAG GAA AGG GGC CCG AAC
5. F3-m2	CGA TGA AGA AGC TGG CAT TC
6. R5-m2	C AGG AGC TCG TTA AAG CTT TC
7. F4-m2	C CTG GCC TTC TCT ACA AAC C
8. R4-m2	TT GCA TTG ATC TCA AAT GAT TC
9. F5-m2	CAA ACT GGA GAC TCT GGA AG

10. R3-m2	AAG GAT AGG AGT TCG CAG GAG
11. F6-m2	TGG TCC AGC AGC TGG AGA AC
12. R2-m2	CCC GCT TGT GGA GAC ACA G
13. 3F-m2	CG CAA GAT AAT GAA CAC CAA G
14. R1-m2	CTA GGA CTT TAT TGC AGT TCT C

Primers for hTLR2 sequencing:

Primer name	Sequence (5' to 3')
1. F1-hT2	ATG CCA CAT ACT TTG TGG ATG G
2. F2-hT2	CTA ATT TAT CGT CTT CCT GGT TC
3. F3-hT2	CAG AAC TAT CCA CTG GTG AAA C
4. F4-hT2	CCT GGC CCT CTC TAC AAA CTT
5. F5-hT2	CAC ACT GAA GAC TTT GGA AGC TG
6. F6-hT2	CAG GAG CTG GAG AAC TTC AAT C
7. R1-hT2	CTA GGA CTT TAT CGC AGC TCT C
8. R2-hT2	CCG CTT ATG AAG ACA CAA CTT G
9. R3-hT2	GAG GAA TTC ACA GGA GCA AAT G
10. R4-hT2	CAG AGT GAG CAA AGT CTC TCC
11. R5-hT2	CCT GAA ACA AAC TTT CAT CGG TG
12. R6-hT2	GAA GAA AGG GGC TTG AAC CAG

Primers for cloning:

Primer name	Sequence (5' to 3')
1. F h/m	ACG ATG ACG ACA AGC TTG CGG CCG CGC AGG CTT CTC TGT CTT GTG ACC G
2. R h/m	CAC AGC AGA CTC CAG ACA CCA GTG CAG CCC TGT GAC ATT CCG ACA CCG AG
3. F m/h	GAT GAC GAC AAG CTT GCG GCC GCG CAG GAG TCT CTG TCA TGT GAT GCT TCT G
4. R m/h	CAC AGC ACA TGC CAG ACA CCA GTG CTG TCT GGT GAC ATT CCA AGA CGG AGG G
5. R $h_{1\text{-}10}$m/m	CAA CCT CCG GAT AGT GAC TGT TTC TAC TTT ACC CAG ATC TAT AAC TCT GTC ATT ATC AGA TGC TC
6. R $m_{1\text{-}10}$h/h	CCT CCG GAT TGT TAA CGT TTC CAC TTT ACC TGG CTC GCT CAC TAC GTC TGA CTC CGA

7. F $hm_{7\text{-}10}h$	CAG ATC TAC AGA GCT ATG AGC CAA AAA GTT TGA AGT CAA TCC GCG ACA TCC ATC ACC TGA CTC
8. F $mh_{7\text{-}10}m$	GTC TCC GGA ATT ATC AGT CCC AAA GTC TAA AGT CGA TTC AGA ACG TAA GTC ATC TGA TCC TTC
9. F $hm_{7\text{-}10}hm_{19\text{-}20}$	GTT GAT GAC TCT ACC AGA TGC CTC CCT CTT CCC TGT GTT GCT GGT CAT GAA AAT C
10. R $hm_{7\text{-}10}hm_{19\text{-}20}$	CTT TGG CCA GTG CTT GCT GCT CCT GAG TAA AGG ATA GGA GTT CGC AGG AG
11. F $mh_{7\text{-}10}mh_{19\text{-}20}$	CTG AAA ACA CTC CCA GAT GCT TCG TTG TTA CCC ATG TTA CTA GTA TTG AAA ATC AG
12. F $h_{14\text{-}18}$	GAA GAA CTC AGC CTG TAA GGG AGC CTG GCC CTC TCT ACA AAC TTT AAT TTT AAG GC
13. R $h_{14\text{-}18}$	CTG ATT TTC ATG ACC AGC AAC ACA GGG AAG AGG GAG GCA TCT GGT AGA GTC
14. F $m_{14\text{-}18}$	GAA AAA TTC AGC CTG TGA GGA TGC CTG GCC TTC TCT ACA AAC CTT AGT TTT GAG C
15. R $m_{14\text{-}18}$	CCT ACT GAT TTT CAA TAC TAG TAA CAT GGG TAA CAA CGA AGC ATC TGG GAG TGT TTT CAG
16. Fh_8	CTG CTT TCC TGC TGG AGA TTT TTG CAG ATA TTA CAA GTT CCG TGG AAT GTT TGG AAC TGC
17. Rh_8	CCA GCT TCT TCA TCG GTG AGC TGA CTT CAC CAG TGG ATA GTT CTG AAA AAT GGA AAG
18. $Fm_{8\text{-}10}$	GCA GCA TAT TTT ACT GCT GGA GAT TTT TGT AGA TGT TCT GAG TTC TGT GAG ATA TTT AGA ACT AAG AGA TAC
19. $Fm_{9\text{-}10}$	GGA CAC TTT CCA TTT TTC AGA ACT ATC CAC TGG TGA AGT CAG CTC ACC GAT GAA GAA GCT GG
20. Fm_{10}	CCG ATG AAA GTT TGT TTC AGG TTA TGA AAC TTT TGA ATC AGA TCT TGG AAC TGT CGG AGG TAG AGT TCG
21. Fm_8	GGT GAT TTT CAC ATT TCT AAA TGT AAA CTT TTT AAT CAA TGA ATT TGT TTC ATC TAC GGG CAG TGG TGA AAA CTG
22. $Fm_{8\text{-}9}$	GTA CAG TCA TCA AAC TCT AAT TCT AAC AAT CCA GAA ATG TAA CGC AAC AGC TTC AGG AGC TCG
23. F dG N442Q	GAA AAG ATG AAA TAT TTG CAG TTA TCC AGC ACA CGA ATA C

24. F dG N414Q	GAC TTT GCT CAC TCT GAA ACA GTT GAC TAA CAT TGA TAT C
25. F dG N199Q	GTT TGA AGT CAA TTC AGC AGG TAA GTC ATC TGA TCC TTC
26. F dG N114Q	CCT ATA ATT ACT TAT CTC AGT TAT CGT CTT CCT GGT TC
27. R dG N442Q	GTA TTC GTG TGC TGG ATA ACT GCA AAT ATT TCA TCT TTT C
28. R dG N414Q	GAT ATC AAT GTT AGT CAA CTG TTT CAG AGT GAG CAA AGT C
29. R dG N199Q	GAA GGA TCA GAT GAC TTA CCT GCT GAA TTG ACT TCA AAC
30. R dG N114Q	GAA CCA GGA AGA CGA TAA CTG AGA TAA GTA ATT ATA GG
31. FhL112P	CAT TTA GAC TTA TCC TAT AAT TAC CCA TCT AAT TTA TCG TCT TCC TGG TTC AAG CCC
32. RhL112P	GGG CTT GAA CCA GGA AGA CGA TAA ATT AGA TGG GTA ATT ATA GGA TAA GTC TAA ATG
33. FmL112P	CAT TTG GAT TTG TCT GAT AAT CAC CCA TCT AGT TTA TCC TCC TCC TGG
34. RmL112P	CCA GGA GGA GGA TAA ACT AGA TGG GTG ATT ATC AGA CAA ATC CAA ATG

4.1.8 Bacterial strains

The following bacterial strains were used:

Bacterial strains	
E. coli DH5α (Clontech)	deoR, endA1, gyrA96, hsdR17 ($r_k^-m_k^-$), recA1, relA1, supE44, thi-1, Δ(lacZYA-argFV169), f80lacZΔM15, F'
E. coli XL10-Gold (Stratagene)	Tet^R, Δ(mcrA)183, Δ(mcrCB-hsdSMR-mrr)173, endA1, supE44, thi-1, recA1, gyrA96, relA1, lac, Hte [F' proAB $lacI^qZ\Delta M15$, Tn10 (Tet^R) Amy Cam^R]
L. monocytogenes	ATCC 43251
S. aureus	DSMZ 20231
Legionella pneumophila	Nr. 2064

B. subtilis	DSMZ 1087
E. faecalis	DSMZ 20478
S.pyogenes	DSMZ 20565
S. epidermidis	DSMZ 20044

4.1.9 Cell lines

The following cell lines were used:

Cell lines	
HEK 293	(ATCC Nr. 305) human embryonic kidney fibroblasts
THP-1	(ATCC Nr. 16) human monocytic cell line
MonoMac6	(ACC Nr. 124) human monocytic cell line
RAW264.7	(ATCC Nr. TIB71) mouse monocytic cells line
MEF	mouse embryonic fibroblasts were prepared from *TLR2*$^{-/-}$mice embryos isolated at day 12 post fertilization

4.1.10 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). They were nine-fold crossed towards C57BL/6 background. All mice were kept under pathogen-free conditions at the animal facility of the Institute of Medical Microbiology, Immunology and Hygiene (Technical University, Munich, Germany). Animal experiments were approved and authorized by local government under the permission nr. 209.1/211-253-95/99. Experiments were performed with 10-12 week old mice both sexes, unless otherwise stated. Prior to the experiment all the mice were genotyped according to standard laboratory protocol.

4.2 Methods

4.2.1 Site directed mutagenesis

A human and a murine TLR2 expression plasmid (pFlag-CMV, Sigma) (*Heine et al. 1999*) were employed as templates in splice PCR based mutagenesis (Quick Change kit, Stratagene, Amsterdam, Netherlands). Chimera constructs were generated as deduced from the primary sequences of both immature proteins.

Name	TLR2 chimera amino acid sequence
h/m or m/h	h or m K19/Q25-R/Q587 fused to m or h A/T588-N785
h_{1-10}m/m or m_{1-10}h/h	h or m K19/Q25-D/E305 fused to m or h L/P306-N785
hm_{7-10}h/h or mh_{7-10}m/m	h or m K19/Q25-S196 fused to m or h I197-D/E305 and to h or m L/P306-N785
$hm_{7-10}hm_{19-20}$/h or $mh_{7-10}mh_{19-20}$/m	h or m K19/Q25-S196 fused to m or h I197- D/E305, h or m L/P306-L497, m or h F/L498-T545, and h or m Q/M546-N785
hm_{8-10}h/h	hK19-V220 fused to m L221-E305 to h P306-N785
hm_{9-10}h/h	hK19-E246 fused to m V247-E305 to h P306-N785
hm_{10}h/h	hK19-Q275 fused to m I276-E305 to h P306-N785
hm_{8-9}h/h	hK19-V220 fused to m L221-Y275 to h I276-N785
hm_{9}h/h	hK19-E246 fused to m V247-Y275 to h I276-N785
hm_{8}h/h or mh_{8}m/m	h or m K19/Q25-V/I220 fused to m or h L/T221-E246 to h or m T/V247-N785
hm_{14-18}h/h or mh_{14-18}m/m	h or m K19/Q25-A385 fused to m or h W386-L497 to h or m L/F498-N785
hm_{19-20}/h	h K19-L497 fused to m F498-T545 to h Q546-N785

Table 3 Amino acid sequence of chimera constructs

h-humanTLR2; m-mouse TLR2

Based on hMutH lacking the N-terminal seven LRRs as has been described by Meng et al. 2003 (*Meng et al. 2003*) an analogous murine construct denoted mMutH lacking the respective subdomain (ΔS48-I220) through deletion has been generated. One point mutation of a leucine residue within the LRR consensus sequence of the third LRR motif (L112P) was introduced into both wild-type constructs. A three-fold point mutated (N147Q, N414Q, and N442Q) mTLR2 construct named ΔG3 as well as single point mutants of human TLR2 (N114Q;

N199Q; N414Q; N442Q) and mouse TLR2 (N147Q; N414Q; N442Q) were generated in order to impede h- and mTLR2-typical N-glycosylation.

4.2.2 Splice PCR and point mutagenesis

Using a site directed mutagenesis kit (Stratagen), all chimera constructs as well as specific point mutations were generated. All reactions were performed according to the manufacture protocol. The primers were 50-80 bp in length, GC% content was more than 50% and the primers carried on 5' and 3'- terminal C or G bp to improve accurate annealing and polymerization. The reaction was carried out in a T3 Thermocycler (Biometra). For chimera constructs, amplification of the desired region in human or mouse TLR2 was performed as first PCR reaction, followed by splice PCR reaction.

Reaction mix (1x)	
5 µl	10x Reaction buffer
4 µl	dNTP-Mix (2.5 mM each)
2 µl	Primer (each 10 µM)
50 ng	Template DNA
1 µl	Klenov DNA-Polymerase (Advanatge cDNA polymerase Mix)
Up to 50 µl	ddH_2O

PCR cycle		
1. 94°C	30 sec	
2. 94°C	30 sec	step2-3 20x
3. 68°C	3 min 30 sec	
4. 68°C	3 min	
5. 4°C		

The PCR product was mixed with the 6x DNA loading buffer and loaded into the slots (maximum volume 25-30 µl) of the 1% agarose gel prepared in TAE buffer with ethidium bromide (final con. of 300 µg/l). As size marker, a 1kb-ladder (Gibco) was used. The gel was run at 10 V/cm until the intended resolution was achieved. The PCR fragment was cut out from the gel and purified using the QIAquick Gel extraction kit (QIAgen). The amount and quality of the PCR-DNA was checked again on the agarose gel. The PCR product was next used as a primer in the splice PCR.

Splice PCR mix (1x)	
5 µl	10x Reaction buffer
1 µl	dNTP-Mix (2.5 mM each)
	PCR product in ratio to vector - 20:1
50 ng	Template DNA – vector
1 µl	Pfu DNA-Polymerase
up to 50 µl	ddH_2O

PCR-cycling		
1. 94°C	30 sec	
2. 94°C	30 sec	step2-3 18x
3. 68°C	1kb/2min	
4. 68°C	5 min	
5. 4°C		

In contrast to the newly amplified chimera DNA, the parental DNA template is methylated and therefore sensitive to Dpn I restriction digest. Thus, after digest with 10 units Dpn I, the newly amplified chimera DNA was not degraded. Subsequently, DNA was precipitated in EtOH (100%), 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 resuspended in 10 µl ddH_2O for transformation in bacteria. The PCR reaction for the point mutants was performed as follows:

Reaction mix (1x)	
5 µl	10x Reaction buffer
1 µl	dNTP-Mix (2.5 mM each)
2 µl	Primer (each 10 µM)
50 ng	Template DNA
1 µl	Pfu DNA-Polymerase
Up to 50 µl	ddH_2O

PCR-cycling		
1. 94°C	30 sec	
2. 94°C	30 sec	steps 2 -4 16x
3. 55°C	1 min	
4. 68°C	1kb/2min	
5. 68°C	3 min	
6. 4°C		

After Dpn I digest, 1 µl of the PCR product was used for transformation in bacteria. The complete sequence of mutants was confirmed by restriction digest of DNA and sequencing.

4.2.3 Restriction digest of DNA and ligation

DNA was digested for analytical or preparative purposes. Reactions were carried out with restriction enzymes (purchased from Fermentas) in a total volume of 23 µl.

Digest mix	
2,5µl	Buffer10x
600 ng	DNA
1µl	restriction enzyme
20 µl	ddH_2O

The digest was performed for 2 h and DNA was loaded on an agarose gel. Before ligation, vector and insert cut with restriction enzymes were purified from agarose gel using a gel extraction kit (QIAgen). DNA fragments (vector and insert) were ligated using T4-DNA ligase according to the protocol (Promega). After ligation DNA was transformed into *E. coli* competent cells.

4.2.4 Transformation of *E. coli*

For transformation of purified DNA, 10 µl (1 µl for point mutation or 5 µl of ligation mix) of pre-chilled reaction and 50 µl chemically competent *E. coli* XL10-Gold (Stratagen) (for mutagenesis) or DH5α-cells (Clontech) (for ligation) 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 42°C, followed by incubation on ice for 2 min. As a next step, the 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 ON at 37°C.

4.2.5 DNA Plasmid preparation in *E. coli*

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

For mini preparations, a single clone was picked from a plate and inoculated in 3 ml of LB-amp medium. The culture was grown ON at 37°C under constant agitation. 2 ml of cell suspension was pelleted for 1 min, 13000 rpm (Biofuge fresco) and plasmid prepared according to manufactures protocol. Plasmid DNA was eluted in 50 µl of ddH_2O.

For maxi preparation, clones were grown in 250 ml LB-amp medium ON at 37°C under constant agitation. At the next day, the cells were pelleted by centrifugation for 15 min at 6000 rpm (Sorvall RC26 plus, rotor SLA 1500). DNA preparation was performed according to the manufacture protocol. DNA was eluted in 100-200 µl of ddH_2O.

4.2.6 Preparation of glycerol stocks

E. coli- suspension cultures were stored in glycerol at –80°C. 0.9 ml of glycerol (50% v/v) and 0.5 ml of an overnight culture were mixed, kept for 2 h at -20°C, and transferred to a –80°C refrigerator.

4.2.7 Cell culture

All the cell lines were grown at 37°C, 8% CO_2 and 95% humidity. HEK 293 cells, *TLR2*$^{-/-}$ embryonic fibroblasts (MEFs) and RAW264.7 cells were cultured as adherent monolayer to confluence and split. For HEK 293 cells, the medium was removed and the cells detached in 5 ml (per 15 cm dish) of 1% (v/v) trypsin-EDTA (Gibco) for 4 min. Trypsin was inhibited by addition of 1 volume of medium and the cells were thoroughly resuspended: 1/10 of this solution was transferred to a new plate and fresh medium was added. RAW 264.7 cells were washed from the plate and a drop of the cell suspension was added to the fresh medium. THP-1 and MonoMac6 cells were grown in suspension. For culture, 10-50 fold dilutions in fresh medium were prepared and grown in tissue culture flasks for three days.

For preparation of frozen stocks, the cells were grown on 15 cm plates to high density, detached by incubation with trypsin-EDTA solution (or washing) and spun down for 5 min at 1200 rpm (Megafuge 1.0RS, Haraeus). The cell pellet was resuspended 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, the cells were transferred to liquid nitrogen tanks. To reculture the cells, they were thawed rapidly at 37°C and washed immediately with 10 ml of pre-warmed medium. The cells were spun down, resuspended in medium and transferred to a 15 cm plate. After ON culture, the cells were used for experiments.

4.2.8 Transfection of HEK 293 cells

HEK 293 cells were transfected by the calciumphosphate precipitation method. In 96-well plates and 6-well plates, 10^4 cells/well and $3x10^5$ cells/well were plated, respectively. For transfection, the following compounds were mixed under sterile conditions:

Transfection mix	
96-well-plate (per well):	150 ng DNA
	0.98 µl $CaCl_2$ (2 M)
	ddH_2O up to total volume of 7.8 µl
6 well plate (per well):	2.5 µg DNA
	15.6 µl $CaCl_2$ (2 M)
	ddH_2O up to total volume of 110 µl

This DNA mix was added to 1 volume of 2x 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 ON. At the following morning, the 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 PAMP binding such as through LBP. Proteins were overexpressed for 48 h up to 72 h.

For preparation of stable HEK 293 clones, the plasmid pTK-neo, which encodes the neomycin resistance gene, was co-transfected in a ratio of 1:20.

Transfected clones were positively selected in G418 supplemented medium. In the following morning after transfection, fresh medium containing G418 (600 µg/ml) was added. Specifically transfected cells were able to grow and formed dense islands. These clonal aggregates were picked and expanded stepwise under constant selection. Screening of the positive clones was performed through immunoblot analysis.

4.2.9 Luciferase reporter assay

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

Transfection mix	
96-well-plate (per well):	30 ng pELAM-1-Luc
	30 ng pHRL renilla
	1-2.5 ng expression vector
	80 ng pRK5 (empty vector)

Cells were transfected as described in Section 4.2.8. After 32 h the cells were stimulated with TLR agonists for 16 h. After stimulation, the supernatants were kept at –20°C for further analysis by IL-8 ELISA. The cells were lysed in 35 µl/well reporter lysis buffer (Promega). Lysates were incubated for 1 h at room temperature under constant agitation. 20 µl/well of each lysate were transferred to light impervious 96-well-plates and luciferase activity was measured in a luminometer. 50 µl/well of luciferase substrate were injected automatically and chemoluminescence was determined. After the measurement of luciferase activity, 50 µl/well of the substrate for renilla activity was injected and chemiluminescence

was determined. The emitted light was measured and normalized luciferase activity calculated according to the formula:

Normalized luciferase activity = ren_{max} * luc / ren.

For analysis of the potential blockage of P_3CSK_4 -induced hTLR2-dependent cell activation by Lau_3CSK_4, HEK293 cells overexpressing the receptor were preincubated with Lau_3CSK_4 at a concentration of 100 ng/ml for 30 minutes. Subsequently, increasing amounts of P_3CSK_4 were added to distinct wells for 6 h. Similarly, a mAb T2.5 which has been demonstrated to neutralize TLR2 by blockage of ligand binding (*Meng et al. 2004*) was added at a concentration 50 µg/ml 30 min prior to cellular challenge with P_3CSK_4 or Lau_3CSK_4.

4.2.10 Electroporation of MEF *TLR2*$^{-/-}$

MEF *TLR2*$^{-/-}$ cells ($5x10^6$) were centrifuged 5 min, 1200 rpm (Megafuge 1.0RS, Haraeus), 4°C. Cells pellets were resuspended in 400 µl electroporation medium and 20 µg DNA was added to each probe. The cell-DNA solution was mixed and placed in the electroporation tubes. The electroporation was done at 300V, 960 µFD. After 10 min incubation at room temperature 10 ml of medium was added and the cells were centrifuged 5 min, 1200 rpm, 4°C and finally cultured over night before stimulation.

4.2.11 Protein isolation and Western blot analysis

HEK 293 and RAW264.7 cells were detached from the plate with 1 ml of chilled PBS, while MonoMac6 cells were removed from the plate and harvested by spinning for 1 min, 1200 rpm (Megafuge 1.0RS, Haraeus), 4°C. The cell pellet was washed with cold PBS and mixed in 20-100 µl of lysis buffer (150mM NaCl for expression analysis and 250 mM NaCl for MAPKs analysis), transferred to tubes and incubated on ice for 30 min. Cell debris was removed from the suspension by spinning down for 30 min, 13000 rpm (Biofuge Fresco), 4°C. Protein samples were then denatured for 5 min at 95°C and spun for 1 min at 13000 rpm before supernatants were loaded to the gels and subjected to the SDS-polyacrylamide-gel electrophoresis (SDS-PAGE). 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 completely.

Proteins were separated due to their size by SDS-PAGE described by Laemmli (1970). The length 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%	3%
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

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

4.2.12 Immunodetection of protein

The membrane (blot) was briefly washed in PBT prior to blocking for at least 1 h at RT. Incubation with primary antibody was performed ON at 4°C. The blot was washed 3 times for 5 min in PBT and incubated with secondary antibody, washed twice with PBT and once with PBS for 5 min each. Washing in PBS was necessary to remove Tween which interferes with HRP activity. All washing steps and the incubation with secondary antibody were done in a small tray on the shaker, whereas incubation with primary antibody was done in a 50 ml tube on the roller. For detection of bound antibody, the blot was overlaid with 2 ml Chemoluminescence Reagent Plus (PerkinElmer) and incubated for 1 min. Excess of substrate was

removed and the blot placed between a layer of plastic wrap in a film cassette. Exposure to an X-ray film was performed for 1 min up to 1 h.

4.2.13 FACS analysis

By means of fluorescence-activated cell sorting (FACS) surface and intracellular staining of proteins can be performed. For this purpose, cells are stained with specific antibodies and antibody-fluorochrome-conjugates. Apart from distinguishing surface and intracellular location, this technique can also be used to characterize or separate distinct sub-populations of a cell pool.

4.2.13.1 Surface staining

RAW264.7, THP1, and MonoMac6 0.5 x10^6 cells were washed in 5 ml of cold PBS. To prevent unspecific binding of antibody to the Fc-receptor, the cells were blocked in α-mouse CD16/CD23 antibody (for RAW264.7) or Endoglobin (THP1 and MonoMac6) for 15 min on ice. Cells were spun down and washed twice for 2 min, 800 rpm (Megafuge 1.0RS, Haraeus), 4°C. The primary antibody α-mouse TLR2.5 (cross-reactive to human TLR2) (*Meng et al. 2004*) was applied 30 min on ice. Cells were spun down and unbound antibody was removed by washing twice in the FACS buffer followed by incubation in secondary antibody α-mouse IgG FITC conjugated. Samples were incubated for 30 min on ice before they were washed again twice. Finally, the cells were resuspended in 300 µl PBS and subjected to FACS analysis. Measurement was carried out in a FACS detector and data were processed applying the Cell-Quest software.

4.2.13.2 Intracellular staining

For intracellular staining, the cells were fixed and permeabilized by incubation with BD Cytofix/Cytoperm reagent (BD Bioscience) according to the manufacture protocol prior to the incubation with antibodies (see surface staining protocol).

4.2.14 ELISA - Enzyme Linked Immune Sorbent Assay

The supernatant from transfected HEK293 ($1x10^4$ per 96 well) cells was collected and applied for hIL-8 ELISA. The ELISA was performed according to the supplier protocol (R&D Systems). The *TLR2*$^{-/-}$ MEFs ($3x10^5$), RAW264.7 ($1x10^5$) as well as MonoMac6 ($1x10^5$) were cultured in 96 well plates and challenged as indicated in Section 5 and the appendix for 24h in the 2%FCS medium. Culture supernatants were collected and applied to ELISA for measurement of murine and human IL-6 and TNFα. ELISA was performed according to the supplier protocol.

4.2.15 Immunocytochemistry

THP-1 cells differentiated with PMA, RAW264.7 cells or peritoneal macrophages from *TLR2*$^{-/-}$ mice were grown on glass carriers in 24-wells culture plate and incubated with biotinoylated P_3CSK_4 or Lau_3CSK_4 for time periods indicated in Section 5. The cells were washed with PBS and incubated with 50 µg/ml Alexa Fluor 488-conjugated concanavalin A in serum free RPMI1640 medium at 4°C for 15 min. The medium was removed and the cells were washed with PBS and fixed with cold methanol for 8 min at -20°C. The cells were blocked with 2% normal goat serum in PBS for 30 min at 37°C. After washing with PBS – a first antibody: a TLR2-specific mAb cross-reacting with murine (*Meng et al. 2004*) and human TLR2 – was applied for 30 min of incubation. As second antibody, Alexa Fluor 546-conjugated goat anti murine IgG (4 µg/ml), as well as Alexa Fluor 647-conjugated Streptavidin (10 µg/ml) for detection of labelled lipopeptides were applied for 30 min and following another washing with PBS. The cells were sealed by incubation in mounting fluid (Chlamydia pneumoniae micro-IF) for analysis with a laser-scanning microscope with documentation unit (LSM510, Carl Zeiss, Oberkochen, Germany).

4.2.16 EMSA - Electro Mobility Shift Assay

RAW264.7 and MonoMac6 cells ($1x10^6$) were culture in 6-wells plates in 2% FCS medium following stimulation for 2h. The cells were washed with PBS and centrifuged for 15 min, 13000 rpm (Biofuge fresco), 4°C. The supernatant was

removed and the pellet was resuspended in 50 µl of Totex buffer, left on ice for 30 min and finally centrifuged for 5 min, 13000 rpm, 4°C. The supernatant was put into a new tube and frozen immediately in ethanol-dry ice bath. The protein concentration was estimated by application of BCA® protein kit and following the supplier protocol.

The NF-κB oligonucleotides were labelled as follows:

Annealing mix:	
1 µl	NF-κB sense oligo
1 µl	NF-κB antisense oligo
5 µl	10x buffer L
14,5µl	H_2O_{dd}

The annealing mixture was incubated for 15 min at 55°C followed by incubation at room temperature for another 15 min.

Labelling mix:	
8µl	annealing mix
2µl	10x T4 PNK buffer
5µl	γ ^{32}P-ATP
1µl	T4 PNK
4µl	H_2O_{dd}

The mixture was incubated 30 min at 37°C, refilled with water to the volume of 50 µl and purified on the BioRad column at 2000 rpm for 4 min. The probe was kept at -20°C.

Components	6%
Acrylamide-bisacrylamide[1]	12.5 ml
10xTBE	5 ml
ddH_2O	82.5 ml
TEMED	75 µl
10% (w/v) APS	750 µl

[1] 39:1 (Biorad)

EMSA mix	
40µl	BSA 10 µg/ µl
40µl	dIdC
80µl	buffer F
40µl	buffer D
100µl	H_2O_{dd}
4 - 12µl	radioactive probe (depending of the radioactivity)

10-15 µg of the nuclear extracts were used for EMSA. If the volume of the extracts was lower than 5 µl, the probe was refilled with water to the volume of 5 µl. 15 µl of EMSA mix was added to the nuclear extracts probe and after vortexing the probes were incubated for 25 min at room temperature. In this time 6% EMSA gel was pre-run at 100V for 20-25 min with the 6 µl of marker in the last slot. After incubation time, the probes were loaded on the gel and run at 220V for ca. 1.5 h, which should correspond to the distance of 10 cm measured from the slot end.

When the run was finished, the gel was put on the Whatman paper, covered with plastic foil and dried 1 h at 80°C. Finally, the dried blot was exposed on the phospho-screen over night followed by exposure on the X-ray film.

4.2.17 Immunoprecipitation

Immunoprecipitation was performed to detect protein-protein interactions. Proteins were overexpressed in HEK 293 cells and detected with antibodies specific for fused epitope tags (Flag or HA). The cells were transiently transfected in 10 cm dishes with 10 µg DNA as described in section 4.2.8. Fresh medium 10% FCS was added at the morning following transfection. After 48 h, the cells were harvested in chilled PBS and lysed as described in section 4.2.11. An aliquot of 20 µl was removed for expression control and the remaining lysate was adjusted to a final volume of 500 µl. 15 µl of αFlag beads (Sigma) were added to the suspension and the tubes rotated overnight in cold room. The beads were then spun down for 15 sec, 13000 rpm (Biofuge Fresco), 4°C and the supernatant was removed quantitatively. 500 µl of washing buffer was added and rotation prolonged for 15 min. These steps were repeated three times before the recovered material was resuspended in 30 µl of lysis buffer and 10 µl 4x SDS-sample buffer.

4.2.18 Deglycosylation assay

The deglycolysation assay was done according to the supplier protocol. In brief, humanTLR2, mouse TLR2, human mutH, mouse mutH, and an N-glycosylation site deficient mouse TLR2 construct (ΔG3) were subjected to N-specific deglycosylation. Flag-tag specifically immunoprecipitated constructs were incubated at 100°C for 10 min in denaturing buffer following the addition of NP40 and PNGase F within a total volume of 25 µl. In parallel, the same amount of precipitated protein was incubated in the absence of PNGase F. After incubation at 37°C for 1 h, the samples were loaded on 8% SDS-PAGE gel and subjected to electrophoresis, as well as immunoblot analysis subsequently.

4.2.19 Bacterial preparation

Bacteria (see section 4.1.8) were cultured at 37°C in standard brain-heart (BH) medium over night. Bacterial cells were washed twice with PBS and the resulting suspensions were heat inactivated (h. i.) through incubation at 56°C or 100°C for 50 min. Additionally, bacteria culture were plated out on the blood agar plates for determination of the CFU/ml and purity of the culture. *Legionella pneumophila* (*Garduno et al. 1998*) was seeded on coal agar plates, incubated at 37°C under 8% of CO_2 for 5 days, and scraped from the plates for suspension in PBS.

4.2.20 *In vivo* experiments and preparation of organs

The *Listeria monocytogenes* infection experiments were done according to the protocol from Prof. K. Pfeffer's laboratory. In brief, a single pearl from the frozen stock of bacteria was picked and inoculated in the 3 ml BH following by incubation overnight at 37°C. The next day the $O.D._{600nm}$ was measured and the culture was diluted to the $O.D._{600nm}$ = 0.7. This was used as the first point for the serial dilution of the culture (10^{-1} - 10^{-8}) in the BH medium. The dilutions 10^{-3} and 10^{-4} were used for the intraperitoneal injection of the mouse (350 µl per mouse), which correspond to 1x LD_{50} and 0.1 LD_{50}, respectively. The 20 µl from each dilution was plated out on

the blood plate (5% Columbia sheep blood) and grown overnight at 37°C in order to estimate CFU/ml and purity of the bacterial culture.

The mice were killed at the day 1 or day 3 by cervical dislocation. The peritoneal region was disinfected with 70% ethanol and the organs (spleen, liver) and blood were removed under aseptic conditions. The organs were homogenised and serial dilutions were plated on blood plate for estimating CFU/ml. The blood was collected to the tubes with heparin, centrifuged for 10 min at 13000 rpm (Biofuge fresco) and serum was used for plating out as well as for cytokine analysis by ELISA (IL-6 and TNFα).

5. Results and discussion – part I

Part I and the appendix describe identification and analysis of a species-specific TLR2 agonist and molecular requirements. We used complementation of human embryonic kidney (HEK) 293 cells or murine embryonic *TLR2*$^{-/-}$ fibroblasts with murine TLR2, human TLR2, as well as TLR2 chimera constructs to identify and analyze species-specificity of pattern recognition through TLR2 orthologue products. In addition, we analyzed responsiveness of murine RAW264.7 macrophages, as well as human MonoMac6 and THP1 monocyte cell lines. The results reveal species-specific recognition of a tri-lauroylated peptide through murine TLR2 and involvement of a relatively low conserved sub-domain encompassing LRR 7-10 of the TLR2ECD in this process.

This work resulted in a publication (*Grabiec et al. 2004*), which is presented in the appendix. The results within the publication are ordered as follows:

1. Comparative mutagenesis of wild-type human and murine TLR2
2. Comparative analysis of TLR2-construct activities by genetic complementation of HEK293 cells
3. Comparative analysis of TLR2-construct activities by genetic complementation *TLR2*$^{-/-}$ MEFs
4. Effect of cellular preincubation with Lau_3CSK_4 or TLR2-specific neutralizing mAb and subsequent challenge with specific lipopeptide analogues
5. TLR2 expression of murine and human monocyte/macrophage cell lines and analysis of species-specific responsiveness to distinct TLR ligands confirming species specificity
6. Immunocytochemical analysis of lipopeptide uptake by human macrophage cell lines
7. Deletion- and point-mutagenesis based analysis of structural requirements for species-specific TLR2 function

Supplementary results not presented within the publication are shown and present additional data to the results shown in parts 1, 2, 3, 6 and 7 of the paper.

5.1 Results

5.1.1 Comparative mutagenesis of wild-type h- and m TLR2

The extracellular (ecd)-sequences of human and murine TLR2 contain 18-20 LRR/LRR-like motifs (*Kirschning et al. 2002*) referred to as “LRRs”. Alignment of both TLR2 sequences showed similar localization of these motifs, which enabled pairwise comparison of LRRs motives. Comparative amino acid sequence analysis of human and murine TLR2 revealed that extracellular and intracellular domain sequences display 65% and 84% of identity, respectively. The LRRs 7-10 are low conserved with identities between 57 and 44%. This group is followed by a highly conserved region comprised of LRRs 10-13 with identity between 83 and 88% (Appendix, Figure 1A and Figure 7).

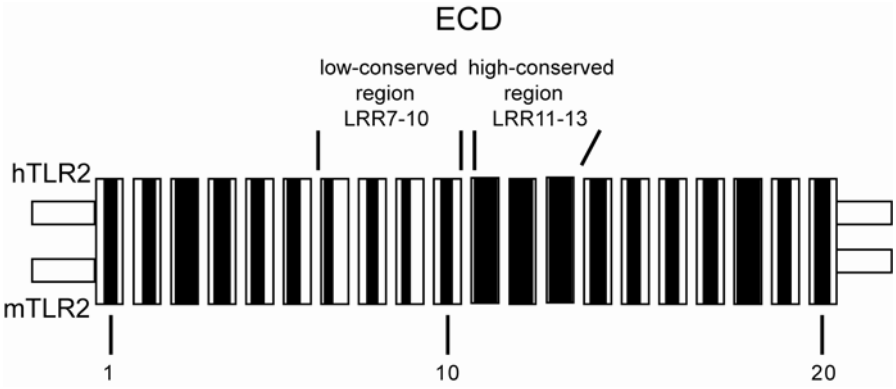


Figure 7 Illustration of human and murine TLR2 extracellular domain homology

The cartoon represents the alignment of human and murine extracellular TLR2 primary sequences. Each box corresponds to one of 20 LRR/LRR-like motifs (boxes numbered according to order from N-terminus towards C-terminus). Black colour corresponds to identical amino acids; low-conserved region and high-conserved region are indicated.

In order to search for species specificity, we generated a set of human-mouse TLR2 chimera constructs by exchanging LRRs. Further point and deletion mutants of human and mouse TLR2 were analysed as well (Appendix, Figure 1B). Expression of all constructs generated was analysed by immunoblot (see Sections 4.2.11 and 4.2.12) and was similar to expression of wild type receptors. Lysate of untransfected HEK 293 cells was applied as a control (Figure 8).

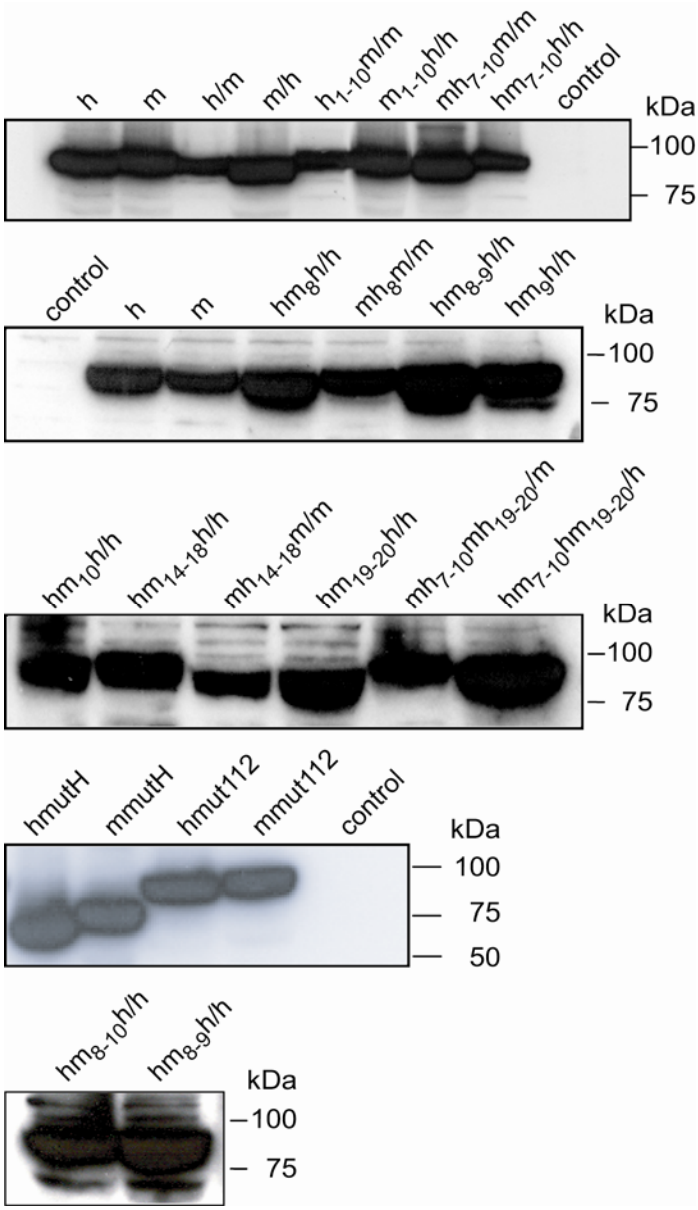


Figure 8 Expression of TLR2 constructs generated

All constructs (h – wild type human TLR2; m – wild type mouse TLR2; and for further constructs see Appendix Figure 1B) were flag tagged, overexpressed in HEK 293 cells and comperatively analysed by immunoblot analysis upon PAGE. Immunoblot analysis was performed by using anti-flag serum. Lysate of untransfected HEK 293 cells was applied as a control.

5.1.2 Comparative analysis of TLR2-construct activities by genetic complementation of HEK293 cells

In order to compare responsiveness to whole bacterial cells or specific bacterial products, HEK293 cells expressing either human or murine TLR2 were challenged with suspensions of heat inactivated (h. i.) bacteria at increasing

concentrations or single bacterial products at one concentration. Wild-type receptors mediated NF-κB dependent reporter gene activation to similar degrees. Cell activation by h. i. bacteria through TLR2 was species-independent (Appendix, Figure 2A and Figure 9).

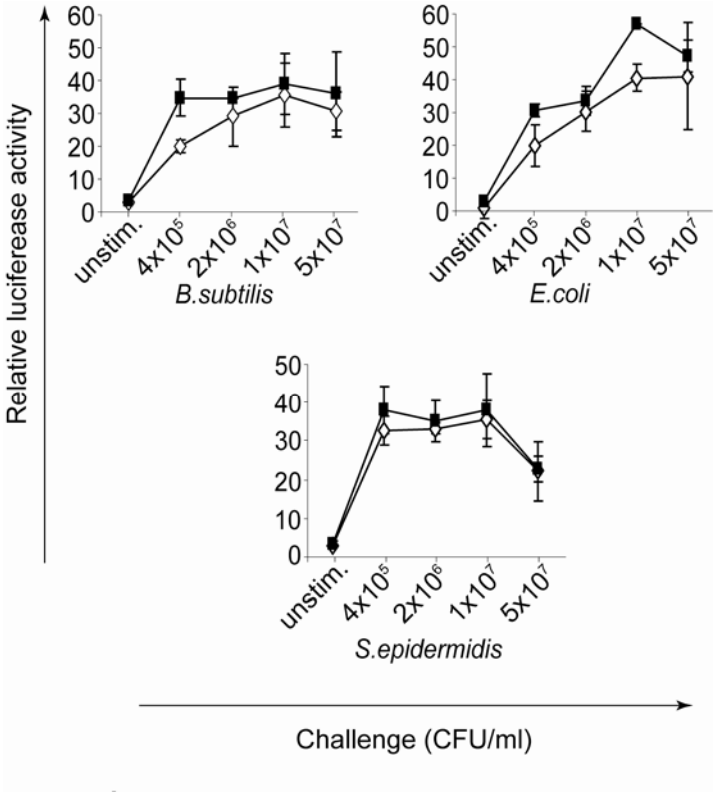


Figure 9 Human and murine TLR2 specific responsiveness to bacterial challenge

HEK293 cells overexpressing human (unfilled diamond) or murine (filled square) TLR2 were challenged with suspensions of heat inactivated bacteria at concentrations indicated prior to measurement of NF-κB dependent reporter gene activation.

Also isolated microbial products (for example LTA or PGN) did not elicit species - specific responses (Appendix, Figure 2B and Figure 10). Exchange of the extracellular domains between human and murine TLR2 did not alter cellular responsiveness, indicating full function of the murine TLR2ICD in human embryonic fibroblasts (Appendix, Figure 2B and Figure 10).

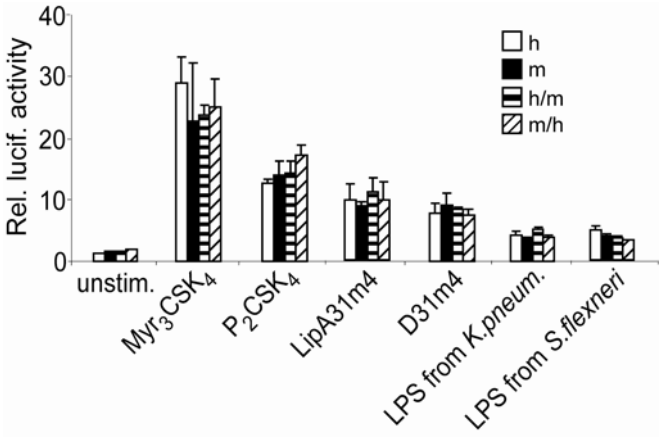


Figure 10 Human and murine TLR2 specific responsivness to defined microbial products

hTLR2- (white bars), mTLR2- (black bars), h/m-construct (horizontally hatched bars), or m/h-construct (upwards hatched bars) mediated reporter gene activation upon challenge with defined microbial products indicated (Myr_3CSK_4, 1 μg/ml; P_2CSK_4, 10 μg/ml; LipA31m4, 10 μg/ml; LPS - D31m4 from *E. coli*, 1 μg/ml; LPS from *K. pneum.*, 10 μg/ml; LPS from *S.flexneri*; 1 μg/ml; Rel. lucif. activity, relative luciferase activity).

However, application of lipopeptide analogues (Figure 11) carrying acyl chains of reduced length (as compared to the classical P_3CSK_4, which contains 16 C-atoms in its palmitoyl-chain) revealed TLR2 species-specificity (Appendix, Figure 3B-C and Figure 12A-B).

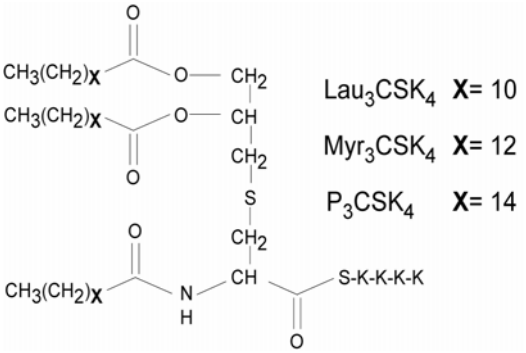


Figure 11 Chemical structure of lipopeptide analogues

Structure of lipopeptide analogues (-CSK_4, -cysteinyl-seryl-(lysyl)3-lysine) carrying triacylations of distinct lengths (x, CH_2-group number as indicated; Lau, lauroyl; Myr, myristoyl; P, palmitoyl).

While P_3CSK_4 (tri-palmitoylated peptide) and Myr_3CSK_4 (tri-myristoylated) induced cell activation to similar degrees, Lau_3CSK_4 (tri-lauroylated) was recognized preferentially through mTLR2ECD but not hTLR2ECD unless more than hundred-fold increased ligand-concentrations were used (Appendix, Figure 3B-C, and Figure 12A-B).

Next, we tested cross-species subdomain containing constructs in order to identify the region responsible for this species-specificity (Appendix, Figure 3B-C, and Figure 12A-B). Replacement of the mTLR2ECD domain containing the first ten LRRs with the corresponding hTLR2 domain did not abrogate signal transduction. The reverse change (introducing mTLR2 LRR 1 to 10 into hTLR2) conferred Lau_3CSK_4-responsiveness through this otherwise human construct. Responsiveness mediated by both constructs was intermediate as compared to both wild-type receptors (Figure 12A-B). Exchange of a sub-domain encompassing LRRs 7 to 10 had a similar effect (Appnedix, Figure 3B-C and Figure 12A-B). Analysis of a mh_8m/m construct indicated a central role of LRR8 in recognition of Lau_3CSK_4. The activation level mediated through this construct upon Lau_3CSK_4 challenge was nearly as low as wild-type hTLR2 dependent cell activation (Appendix, Figure 3B-C). In conclusion, these results indicate that the murine LRR block from motif 7 to 10 is involved in mouse specific Lau_3CSK_4 recognition and LRR8 plays a prominent role within this block.

Additionally, we analysed IL-8 release upon Lau_3CSK_4 challenge of HEK293 cells transfected with chimera constructs (Appendix, Figure 3D and Figure 12C). The results confirmed species – specificity of Lau_3CSK_4 recognition.

Further fusion constructs were generated and analyzed (Appendix, Figure 1B; further supportive data not shown) in order to analyze additional sub-domains for involvement in species-specific pattern recognition. Neither of the regions encompassing LRRs 14 to 18, nor LRR 19 and 20 within the C-terminal portion of TLR2 was found to contributed to species specific pattern recognition (Figure 13 and data not shown).

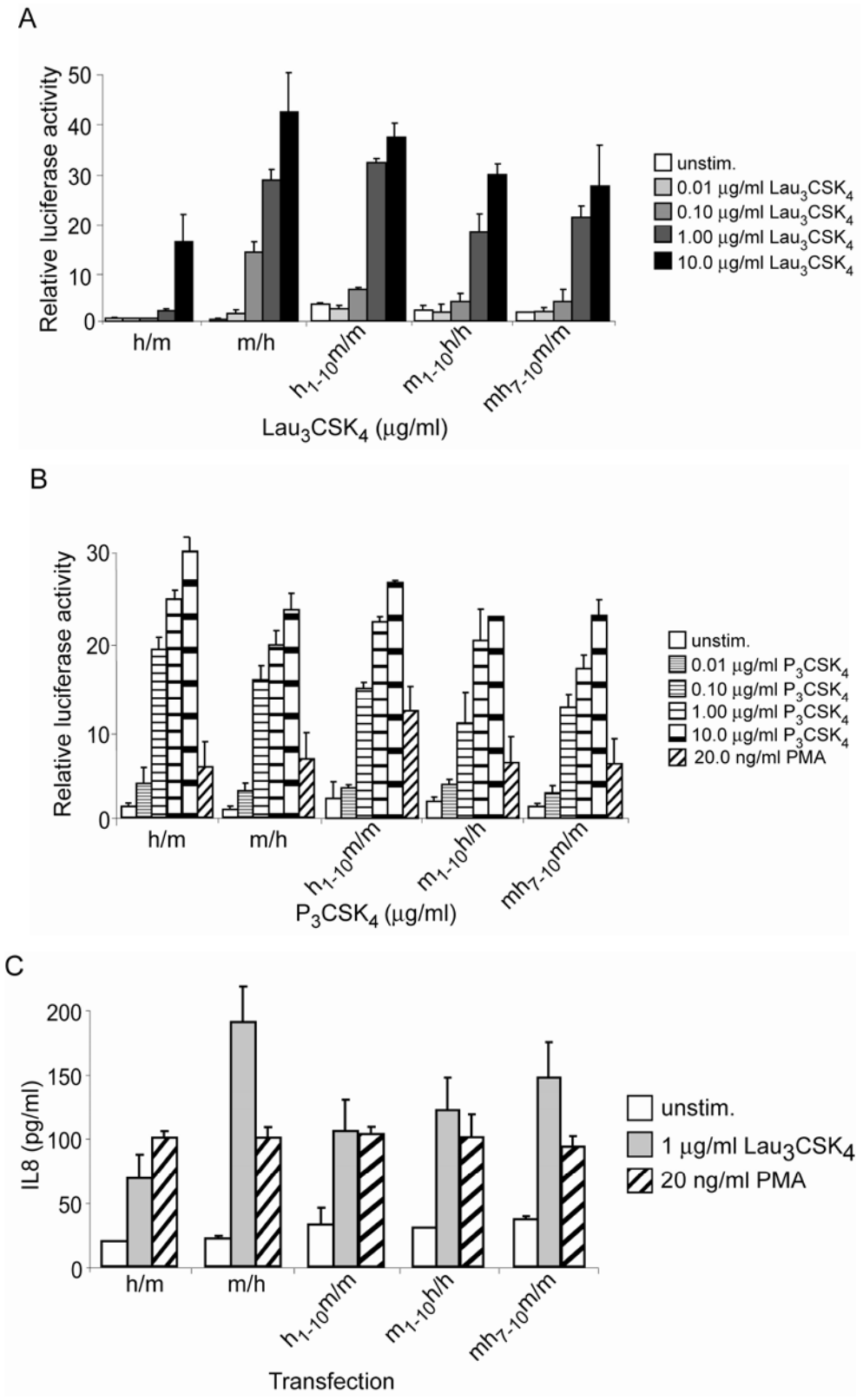


Figure 12 Human and murine TLR2 specific responsiveness to lipopeptide challenge

NF-κB–dependent reporter gene activation as compared to vector control upon challenge with lipopeptides at increasing concentrations (unfilled bars, unstimulated; light grey bars, 10 ng/ml; grey, 100 ng/ml, dark grey bars, 1 µg/ml; black bars, 10 µg/ml) for Lau_3CSK_4 (A) and (unfilled bars, unstimulated; horizontaly hatched bars of decreasing density for

concentartion 10 ng/ml, 100 ng/ml, 1 μg/ml, 10 μg/ml) for P_3CSK_4 (B) through TLR2-constructs indicated. IL-8 release upon stimulation of transfected HEK293 cells with lipopeptides (C, respectively; unstimulated, unfilled bars; grey bars,1 μg/ml Lau_3CSK_4; upward hatched bars, 20 ng/ml PMA, phorbol 12-myristate 13-acetate; e.vec., empty vector).

Notably, exchange of human motifs 19 and 20 for its murine counterpart abrogated recognition of TLR2 agonists in both constructs: $hm_{19\text{-}20}$/h and $hm_{7\text{-}10}hm_{19\text{-}20}$/h (Appendix, Figure 1B, and Figure 13). In contrast, the respective "human-in-murine"-exchange had no detectable effect ($mh_{7\text{-}10}mh_{19\text{-}20}$/m versus $mh_{7\text{-}10}$m/m; Appendix, Figure 1B and Figure 13, Figure 12A-B).

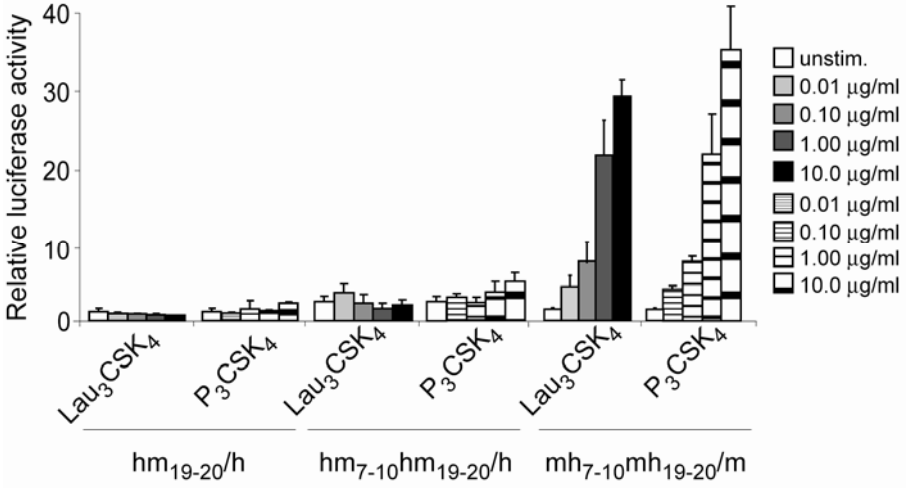


Figure 13 $hm_{7\text{-}10}hm_{19\text{-}20}$/h, $mh_{7\text{-}10}mh_{19\text{-}20}$/m and $hm_{19\text{-}20}$/h TLR2 constructs specific responsiveness to lipopeptide challenge

Reporter gene activation as compared to vector control upon challenge with lipopeptides at increasing concentrations for Lau_3CSK_4 and P_3CSK_4 through TLR2-constructs indicated (unfilled bars, unstimulated; light grey bars, 10 ng/ml; grey, 100 ng/ml, dark grey bars, 1 μg/ml; black bars, 10 μg/ml for Lau_3CSK_4 and unfilled bars, unstimulated; horizontaly hatched bars of decreasing density for concentartion 10 ng/ml, 100 ng/ml, 1 μg/ml, 10 μg/ml for P_3CSK_4).

Expression analysis by FACS showed that $hm_{7\text{-}10}hm_{19\text{-}20}$/h construct is not expressed on the surface but intracellulary, whereas the construct $mh_{7\text{-}10}mh_{19\text{-}20}$/m is expressed on the surface and within cells (Figure 14A-B).

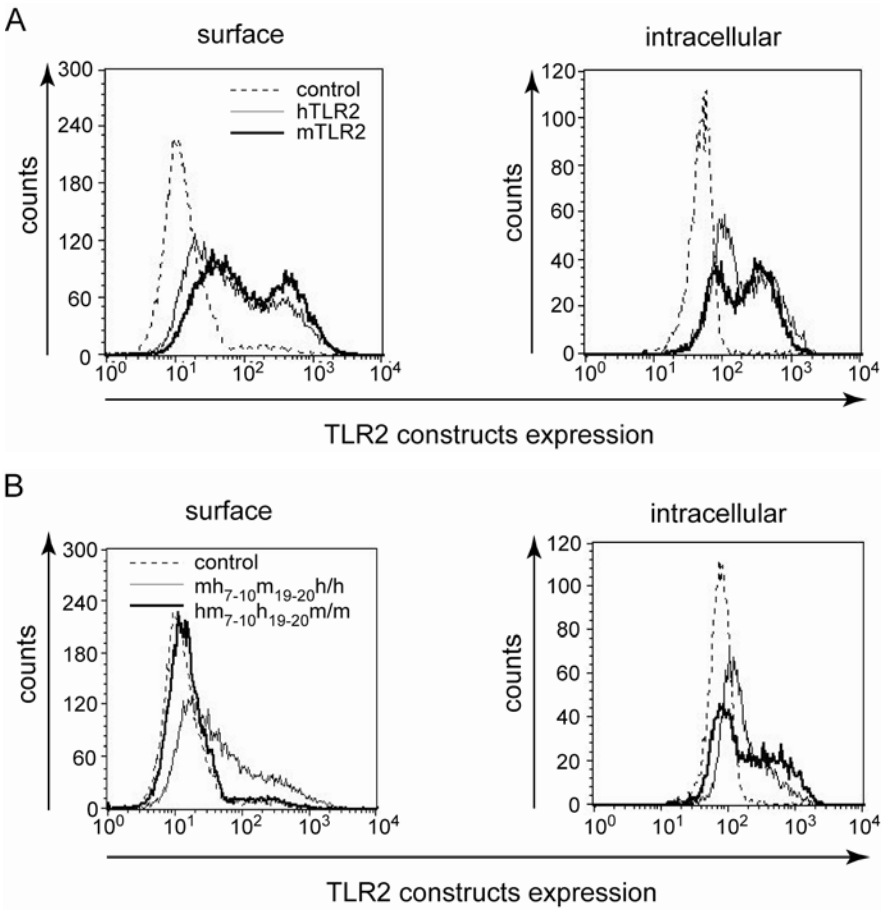


Figure 14 Subcellular expression of $hm_{7\text{-}10}hm_{19\text{-}20}$/h and $mh_{7\text{-}10}mh_{19\text{-}20}$/m TLR2 constructs

Transiently transfected HEK293 cells were analyzed for cell surface and intracellular TLR2 construct expression (A, bold line - murine TLR2, thin line – human TLR2; B, bold line - $hm_{7\text{-}10}hm_{19\text{-}20}$/h, thin line - $mh_{7\text{-}10}mh_{19\text{-}20}$/m) by flow cytometry. (A-B: dotted line, control; bold and thin line, flag tag-specific antibody).

5.1.3 Comparative analysis of TLR2-construct activities by genetic complementation of *TLR2*$^{-/-}$ MEFs

Next, we analyzed TLR2 deficient mouse fibroblasts genetically complemented with TLR2-constructs. *TLR2*$^{-/-}$ fibroblasts gained responsiveness to both lipopeptide analogues upon transfection of mTLR2; in contrast, hTLR2 did confer detectable responsiveness to P_3CSK_4 but not to Lau_3CSK_4 (Appendix, Figure 3E). The finding that Lau_3CSK_4 was recognized exclusively through murine TLR2ECD was confirmed through analysis of ECD-ICD exchange constructs (Figure 15). Results obtained by transient over-expression of TLR2 murine-human fusion constructs in *TLR2*$^{-/-}$ MEFs corroborated the results from complementation analysis

of HEK293 cells over-expressing the same constructs (Appendix, Figure 3E and Figure 15).

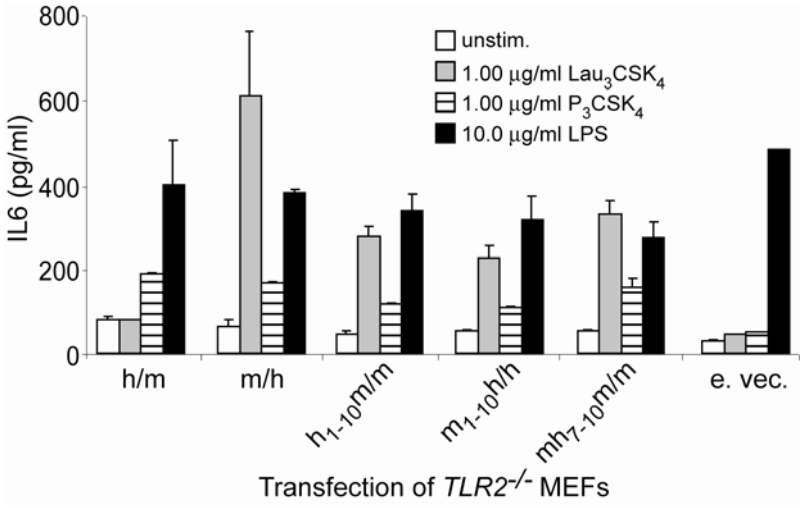


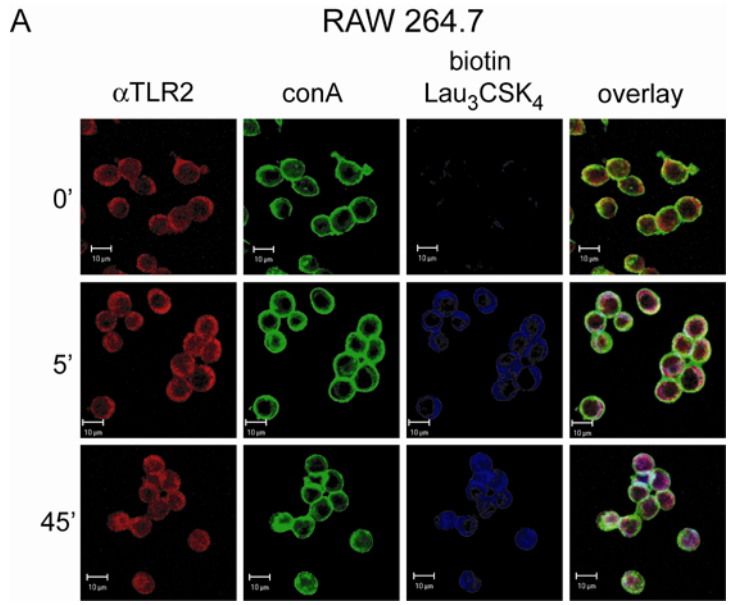
Figure 15 IL-6 release from transfected (as indicated) *TLR2*$^{-/-}$ MEFs

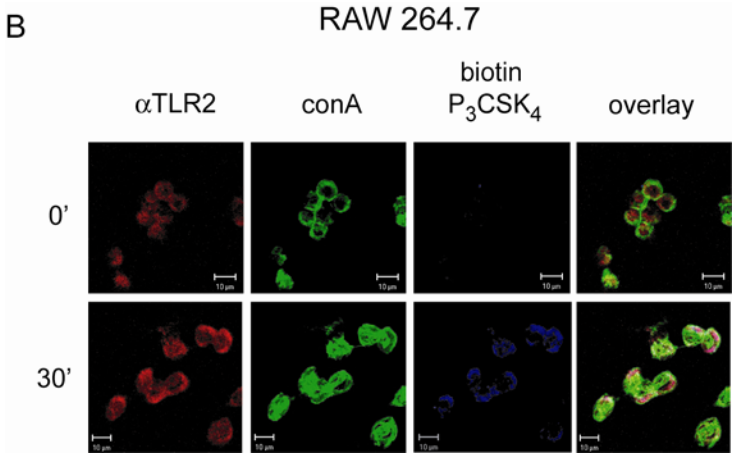
TLR2$^{-/-}$ MEFs were transfected and stimulated as indicated. IL-6 release was measured (unstim.-unstimulated; unfilled bars; grey bars, 1 µg/ml Lau_3CSK_4; horizontally hatched bars, 1 µg/ml P_3CSK_4; black bars, 10 µg/ml LPS; e.vec., empty vector).

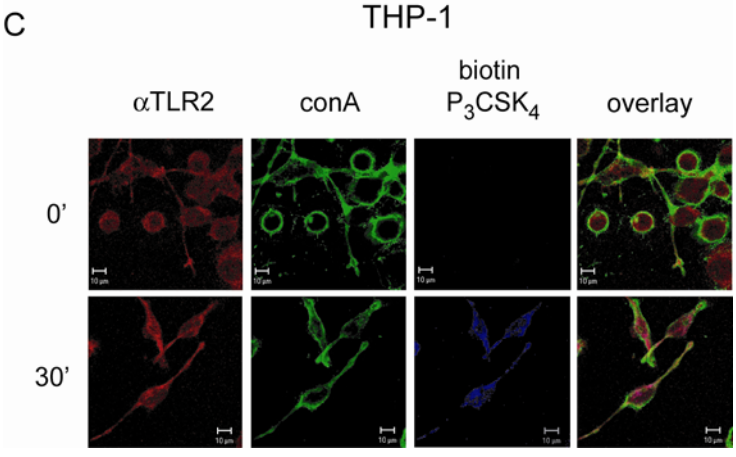
5.1.4 Immunocytochemical analysis of lipopeptide uptake by RAW 264.7 (mouse) cell line, THP-1 (human) cell line and macrophages from *TLR2*$^{-/-}$ mice

We compared uptake of P_3CSK_4 and Lau_3CSK_4 in human and murine macrophages in order to determine whether cellular uptake was also species specific. Weak lipopeptide specific cell surface staining after 5 min of incubation but significant intracellular staining after 30 or 45 min was observed in THP-1 cells and RAW 264.7 cells (Appendix, Figure 7 and Figure 16A-C). This finding indicated time-dependent cellular uptake by human wild-type (Appendix, Figure 7 and Figure 16C) and mouse wild-type (Figure 16A-B) macrophages. Similar kinetics of internalization and similar uptake of Lau_3CSK_4 by murine *TLR2*$^{-/-}$ macrophages (Figure 16D) indicated TLR2-independent binding of the lipopeptide analogues P_3CSK_4 and Lau_3CSK_4 to both human and murine macrophages. Free biotin was not detectable within cells after application under the same conditions used for biotinylated

lipopeptides (data not shown), indicating lipopeptide specificity of cellular uptake observed.







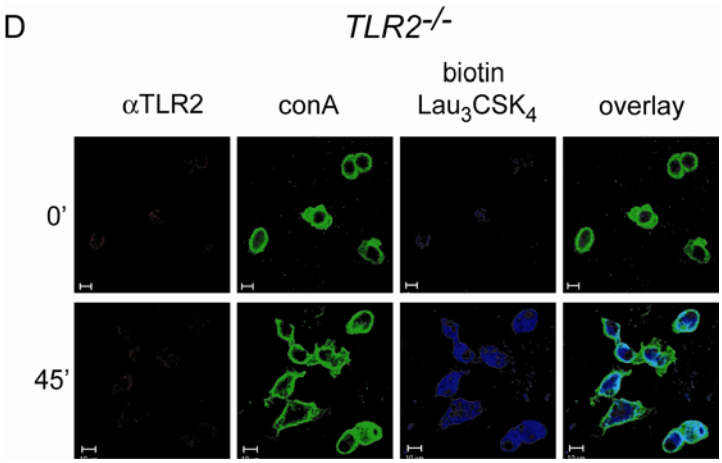


Figure 16 Time dependent uptake of Lau_3CSK_4 or P_3CSK_4 to RAW264.7 cells, THP-1 cells and macrophages from *TLR2*$^{-/-}$ mice

RAW264.7 (mouse) macrophages (A and B), THP-1 (human) macrophages (C) and primary peritoneal macrophages from *TLR2*$^{-/-}$ mice (D) were either left untreated (0') or challenged with biotinoylated Lau_3CSK_4 (A and D) or biotinoylated P_3CSK_4 (B and C) for time periods indicated prior to washing and fixation. Subsequently, TLR2 (αTLR2), cell surfaces (conA, fluoresceine labeled concanavalin A), and biotinoylated Lau_3CSK_4 (biotin Lau_3CSK_4) were stained. All three signals were superimposed (overlay). Bar corresponds to 10µm distance.

5.1.5 Specific N-glycosylation of human and mouse TLR2

N-glycosylation is critical for TLR4 function (*da Silva Correia et al. 2002*) and for TLR2 surface expression (*Weber et al. 2004*), suggesting, that distinctive N-glycosylation might lead to species-specificity of pattern recognition through TLR2. Application of the NetNGlyc 1.0 algorithm (*http://www.cbs.dtu.dk/services/NetNGlyc/*) revealed presence of four N-residues that are putatively glycosylated in hTLR2ECD (amino acid residues 114, 199, 414, and 442, respectively), while in mTLR2ECD only three putative glycosylation sites are localized within ECD (amino acid residues 147, 414, and 442). We considered glycosylation of a putative fourth site as unlikely (residue 296) due to conformational constraints induced by a proline following the respective asparagin (*http://www.cbs.dtu.dk/services/NetNGlyc/*). Glutamine (Q) -residues were introduced into a wild-type mTLR2 expression construct to replace: single (147; 414; 442), pairs of (147&414; 147&442; 414&442) or all three (147&414&442 - ΔG3) central asparagines within the canonical N-glycosylation sites (constructs were

generated by GM). Glutamine (Q) residues were introduced to replace single asparagines within the canonical N-glycosylation sites (114, 199, 414, and 442) in the human wild-type TLR2 construct. All TLR2 constructs were expressed at similar levels upon transfection in HEK293 cells as analyzed by immunoblot analysis (Figure 17).

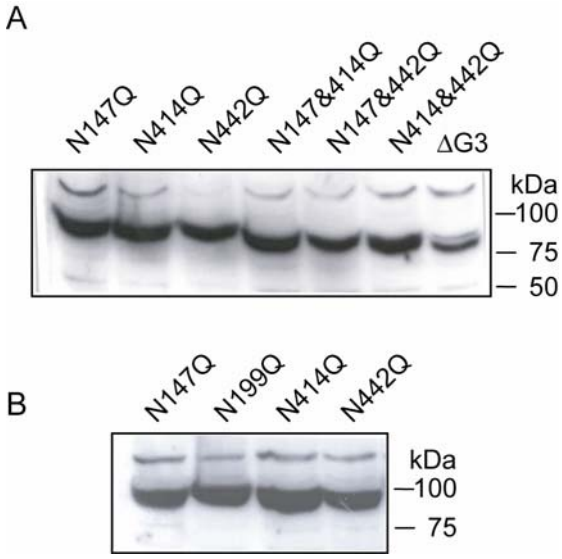


Figure 17 Expression of human and mouse deglycosylation mutants

Mouse (A) and human (B) TLR2 constructs were flag tagged, overexpressed in HEK 293 cells and visualised upon lysis of cells, SDS-PAGE and blotting. Immunoblot analysis was performed by application of anti-flag antibody.

In order to compare responsiveness of human and mouse deglycosylation mutants to lipopeptides, HEK293 cells expressing either human or murine TLR2 mutant were challenged with Lau_3CSK_4 or P_3CSK_4. Results showed that for both human and mouse TLR2 receptor glycosylation integrity of residue 442 is critical for function. Mutants N442Q did not mediate Lau_3CSK_4 or P_3CSK_4 signals (Figure 18).

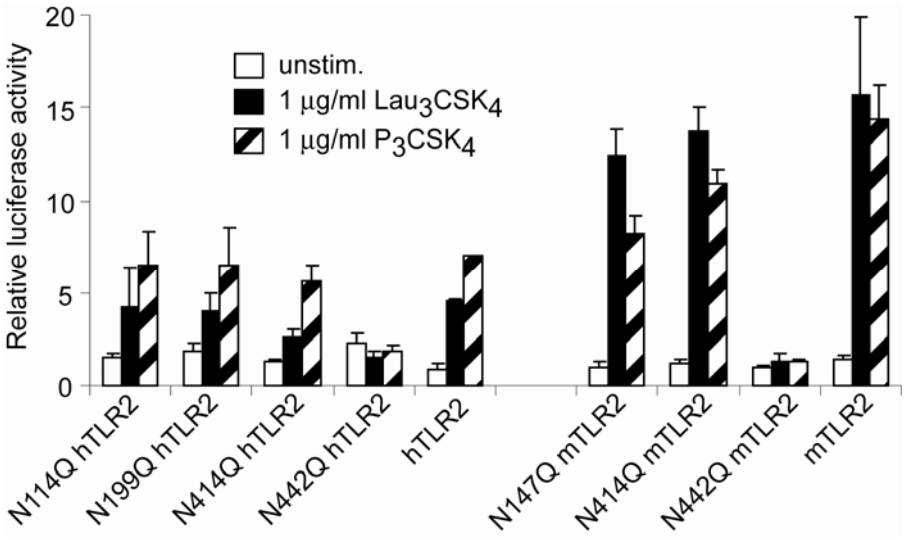


Figure 18 Effect of human and mouse TLR2 specific deglycosylation

HEK293 cells overexpressing human and murine TLR2 as well as deglycosylation mutants were challenged with 1 μg/ml Lau_3CSK_4 or P_3CSK_4 prior to measurement of NF-κB dependent reporter gene activation (unstimulated- unfilled bars; Lau_3CSK_4- filled bars, P_3CSK_4- horizontally upwards hatched bars).

5.2 Discussion

We (see the appendix for details) aimed at the identification of species-specific ligands for TLR2 and analysis of a respective structure-function relationship. Through our analysis lauroylated lipopeptides were identified as species-specific TLR2-agonists. Furthermore, LRR8 and the surrounding region spanning from LRR7 to LRR10 control species-specific ligand recognition. Comparative analysis of wild-type mTLR2 and mTLR2ECD-hTLR2ICD construct demonstrated equal potential of both human and murine ICDs to mediate intracellular signaling such as NF-κB-signaling.

It has been reported that TLR4 requires gylcosylation for receptor function (*da Silva Correia et al. 2002*). Nine glycosylation sites which are present in TLR4 are highly conserved and functionally important. Two of them: Asn^{526} and Asn^{575} play a critical role in a surface transport of TLR4. A recent report by Weber et al. shows involvement of specific N-glycosylation in surface expression of TLR2. Secretion of all N-glycosylation mutants generated was reduced as compared to wild-type receptor suggesting requirement of all glycosylation sites for secretion (*Weber et al. 2004*). Among 4 glycosyaltion sites, site 4 (442aa) (located on the inner LRR

solenoid surface) is one of the major determinants for proper TLR2 biosynthesis and subcellular localisation. The authors speculate that this site could be functionally important (*Weber et al. 2004*). Our data (Figure 18) confirm this observation. Both human and murine TLR2 mutant N442Q does not mediate NF-κB dependent reporter gene activation upon challange with lipopeptides. Additionally, mTLR2 N442Q mutant does not mediate stimulation with different TLR2 agonists (Guangxun Meng, unpublished results).

Uptake of P_3CSK_4 and Lau_3CSK_4 by human macrophages, as well as murine wild-type and *TLR2*$^{-/-}$ macrophages was undistinguishable (Figure 16 and see Appendix Fig. 7). However, efficient uptake and processing of a TLR2-specific antibody have been demonstrated (*Schjetne et al. 2003*). A human TLR2 specific mAb (TL2.1) bound to TLR2 was internalized and subjected to the endocytic pathway suggesting that TLR2 ligands bound to TLR2 are internalized in the similar manner (*Schjetne et al. 2003*). A recent report by West et al. shows that TLR ligands stimulate antigen macropinocytosis, leading to enhanced presentation on class I and class II MHC (*West et al. 2004*). However, our results indicate TLR2 independent uptake of lipopeptides suggesting involvement of other cellular receptors in this process for which CD14 is a candidate (*Wright et al. 2000*).

Our results demonstrate species-specific characteristics of TLR2 and strongly suggest that these differences provide a molecular basis for distinct susceptibilities of humans and mice to specific infections. Most notably, they provide strong evidence for binding of lipopeptides to TLR2 supporting direct TLR-interaction as the mechanism underlying TLR-dependent cell activation. Specifically, they complement recently reported evidence for physical interactions of specific agonists to TLR4 and TLR9 (*Akashi et al. 2003*); (*Rutz et al. 2004*).

6. Results and discussion - part II

Listeria monocytogenes (LM) is a gram-positive, facultatively intracellular bacterium. Its virulence is due to its ability to invade host cells, to exploit their cellular machinery for their purposes, and to evolve highly sophisticated strategies to evade the host immune response (*Torres et al. 2004*). The activation of the host's innate immune system is critical for LM clearance in the early phase of infection. LM-infected macrophages produce IL-12 and IL-18 that synergistically induce NK cells and dendritic cells to produce IFNγ. IFNγ leads to activation of macrophages and neutrophiles to kill LM via production of listericidal molecules such as NO (*Seki et al. 2002*). Furthermore, activated macrophages secrete IL-6, IL-1 and chemokines which control lymphocyte and neutrophil recruitment and activation. *In vitro* studies applying human monocytes revealed that TLR2 is required for macrophages activation in response to LM (*Flo et al. 2000*). In order to investigate the role of TLR2 in control of LM infection, we used TLR2–deficient mice and examined their response to LM infection *in vivo*.

6.1 Results

6.1.1 The survival of TLR2-deficient mice after infection with *Listeria monocytogenes* (LM)

To evaluate the role of TLR2 in LM infection *in vivo*, wild-type and TLR2-deficient mice were infected intra-peritoneal (i.p.) with $1xLD_{50}$ corresponding to a dose of $1{,}65x10^6$ CFU/ml of LM. Their survival was monitored for 14 days. As shown in Figure 19 after infection with $1xLD_{50}$ all TLR2-deficient mice succumbed to LM infection and died within 5 days whereas less than 25% wild-type mice survived on day 14. The observed difference in the survival of wild type mice versus TLR2-deficient mice, however, is not statistically significant.

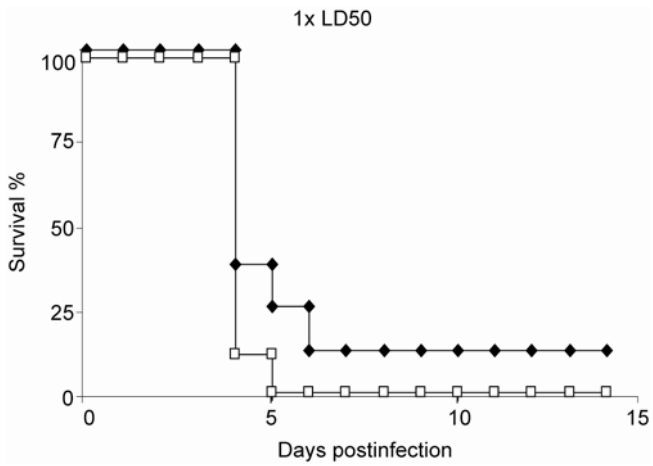


Figure 19 Survival of TLR2-deficient mice upon *L. monocytogenes* infection

TLR2$^{-/-}$ mice (n=8) and wild type mice (n=8) were infected i.p. with 1,65x10^6 CFU/ml of *L. monocytogenes* (filled rhombus - wild type; unfilled squares - *TLR2*$^{-/-}$ mice). Survival was monitored up to 14 days.

6.1.2 Bacteria load in TLR2-deficient mice upon infection with *Listeria monocytogenes*

In order to comparatively analyze bacterial dissemination in wild type and *TLR2*$^{-/-}$ mice, bacterial loads in blood, spleen and liver were determined at day 3 post infection.

As shown in Figure 20 organs of *TLR2*$^{-/-}$ mice carried normal bacterial loads upon LM infection, with *Listeria* titres in blood, spleen and liver being equivalent to those of wild type mice. Thus, the absence of TLR2 did not affect control of *Listeria* growth within the host organism.

6.1.3 Cytokines production by *TLR2*$^{-/-}$ mice in response to *Listeria monocytogenes* infection

The innate immune response to *Listeria* requires production of numerous proinflammatory cytokines. To evaluate the role of TLR2 in production of proinflammatory cytokines we analysed TNFα and IL-6 serum levels upon infection with *Listeria*. Serum samples were collected from wild type and *TLR2*$^{-/-}$ mice and analysed by ELISA at day 1 and day 3 postinfection (Figure 21).

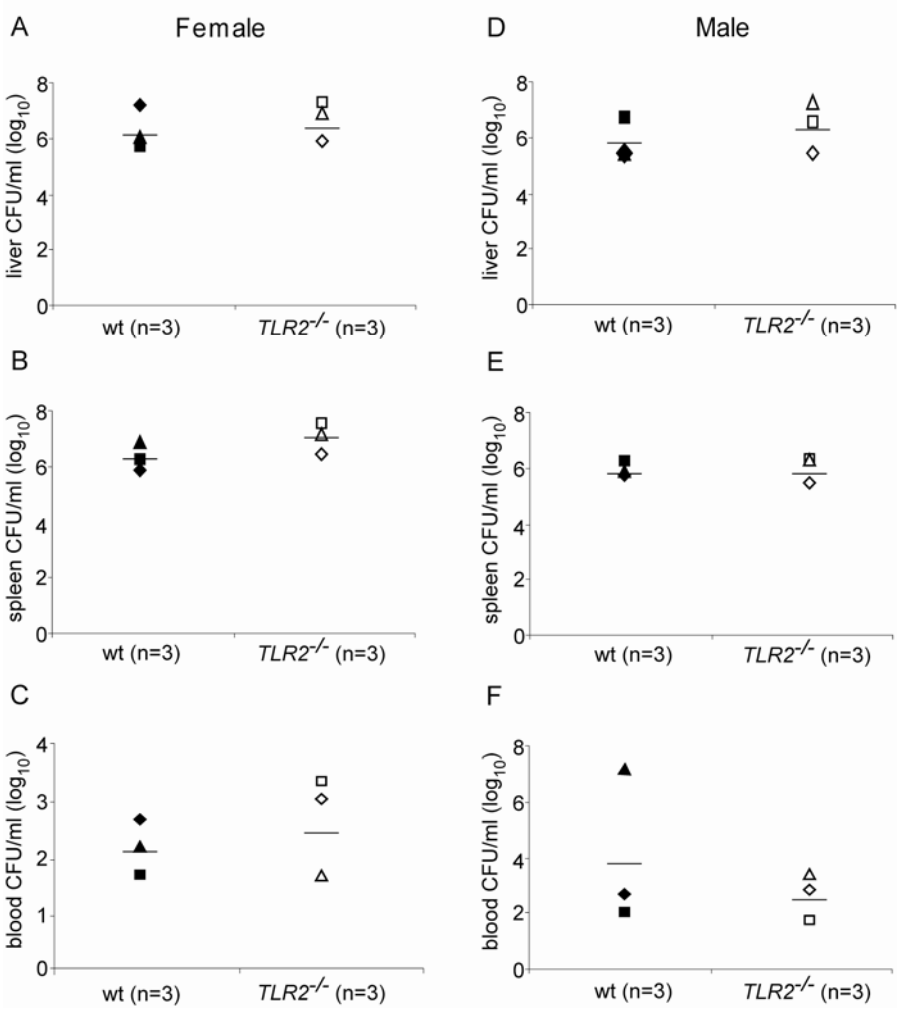


Figure 20 Bacterial loads in mouse organs upon *L. monocytogenes* infection

Wilde type and *TLR2*$^{-/-}$ mice were infected with 1,65x10^5 *Listeria* i.p. and killed at day 3 post-infection (A-C females, D-F males). Symbols represent single mice and bars represent geometric mean CFU/organ or CFU/ml of blood. Filled symbols represent wild type mice, unfilled symbols represent *TLR2*$^{-/-}$ mice.

A decreased TNFα and IL-6 release to the serum in *TLR2*$^{-/-}$ as compared to wild type sera was observed at day 1 after *Listeria* infection (Figure 21 A left and right panel). At day 3, TNFα levels in sera of wild type and *TLR2*$^{-/-}$ mice were similar (Figure 21 B left panel) whereas still more IL-6 was released into sera of wild-type mice as compared to *TLR2*$^{-/-}$ mice (Figure 21 B right panel). This suggests the involvement of TLR2 in a very early phase of *Listeria* infection not affecting bacterial load at later time points (Figure 20).

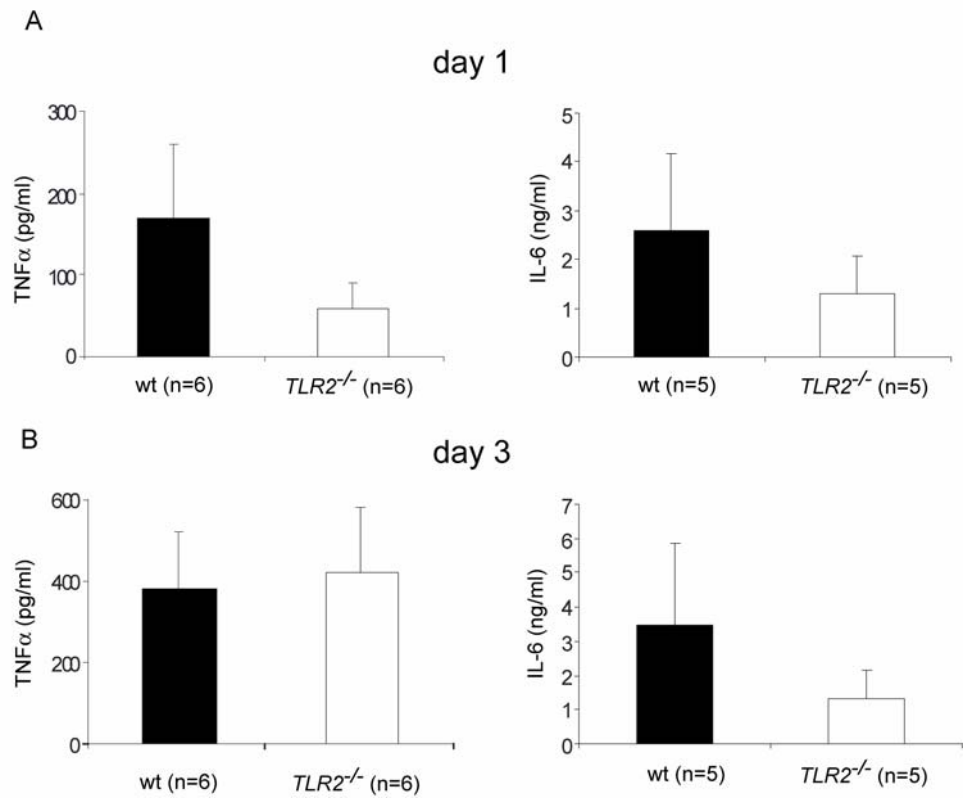


Figure 21 TNFα and IL-6 serum levels upon *L. monocytogenes* infection

Wilde type (black bars) and *TLR2*$^{-/-}$ (white bars) mice were infected with 1,65x10^6 *Listeria* i.p. and killed at day 1 (A) and day 3 (B) postinfection. Blood was collected and subjected to TNFα and IL-6 ELISA.

6.2 Discussion

The recognition of invading bacteria by the immune system mediates host response aiming on eradication of the pathogen. Toll-like receptors expressed by cells of the innate immune system are involved in recognition of infection. *Listeria monocytogenes* is an intracellular, parasitic bacterium responsible for severe systemic infections in immunocompromised individuals. Here, we investigated the role of TLR2 in *L. monocytogenes* infection *in vivo*.

The innate immune response to *Listeria* involves a coordinated interaction between many cell types and the production of numerous cytokines (*Edelson et al. 2000*). Neutrophiles play a key role in the early control of *Listeria* growth, appearing at the sites of infection within the first 24h. Infection of macrophages with *Listeria* results in production of TNFα and IL-12, which synergistically cause secretion of

IFNγ by NK cells. IFNγ induced signaling together with TNFα leads to full macrophage activation. Activated macrophages display increased levels of MHC class II and produce free radicals (*Edelson et al. 2000*); (*Unanue 1997*). Additionally, cytokines IL-1 and IL-6 play an important role during the early immune response to *Listeria* infection. IL-1 is required for neutrophile infiltration during infection and it induces increase of MHC II by peritoneal macrophages, in response to *Listeria* infection (*Rogers et al. 1994*); (*Unanue 1997*). Similarly to IL-1, IL-6 induces recruitment of neutrophile during listeriosis (*Dalrymple et al. 1995*) and promotes activation of T cells to produce IFNγ (*Liu et al. 1994*).

TLR2 has been reported to recognise heat inactivated *Listeria monocytogenes* (HKLM) (*Flo et al. 2000*). The authors demonstrated TLR2 mediated cellular activation in response to HKLM which was blocked by application of a TLR2 specific antibody. Our studies showed minor requirement of TLR2 in the protective immune response to *L. monocytogenes* infection *in vivo*. No difference in susceptibility or bacterial burden in organs analysed between wild type and *TLR2*$^{-/-}$ mice was evident (Figure 19, Figure 20). This observation is in agreement with recent reports (*Seki et al. 2002*); (*Edelson et al. 2002*). Edelson et al. demonstrated that *TLR2*$^{-/-}$ mice are normally resistant to *Listeria* infection, while Myd88-deficient mice were significantly higher susceptible to infection. Bacterial growth in spleen and liver was uncontrolled, while IL-12p40, IFNγ and TNFα levels were decreased in *Myd88*$^{-/-}$ mice as compared to wild type mice. Thus, Myd88 is an adaptor molecule essential for *in vivo* resistance to *Listeria* infection. However, impaired IL-1 and IL-18 signaling in *Myd88*$^{-/-}$ mice has to be considered as an additional factor contributing to the increased susceptibility observed.

Another report showed high importance of Myd88 for proinflammatory cytokine release in an early phase of *Listeria* infection affecting clearance (*Seki et al. 2002*). The authors compared IL-12, IL-18, IL-12/IL-18, IFNγ and Myd88 knock-out mice in terms of susceptibility to *Listeria* infection. Bacterial burden in liver, as well cytokine release in blood were determined. Specifically, IL-12, TNFα and IFNγ release in TLR2, TLR4, TLR2/TLR4 and Myd88 knock-out mice were analysed. The results underline the importance of IL-12 and IL-18 in the early phase for clearance of *Listeria* infection. Myd88 is essential for induction of IL-12 and IL-18 dependent IFNγ production, as well as TNFα release. Seki et al. observed partial impairment in

cytokine production (IL-12, TNFα and IFNγ) in *TLR2*$^{-/-}$ mice. The cytokine measurement was carried out 24h post infection. These data corroborate our results presented in Figure 21. We also observed decreased TNFα release and additionally reduced IL-6 release 24 h upon infection with *Listeria*. However, 72 h after infection the TNFα level was equal in sera of wild type and *TLR2*$^{-/-}$ mice whereas the level of IL-6 was still decreased in the *TLR2*$^{-/-}$ mice as compared to its level in wild type mice. These results suggest involvement of TLR2 in the early phase of *Listeria* infection. Thus, more than one TLR is involved in recognition of live *Listeria in vivo*, for example TLR5 recognizing flagellin (*Hayashi et al. 2001*) or TLR9 recognizing bacterial DNA (*Hemmi et al. 2000*).

In contrast, a recent publication by Torres et al. shows TLR2 requirement for NO, TNFα, and IL-12p40 production, CD40 and CD86 expression on macrophages and dendritic cells upon *Listeria* infection (*Torres et al. 2004*). Additionally, *TLR2*$^{-/-}$ mice showed increased susceptibility to *Listeria* infection (*Torres et al. 2004*). The discrepancy in the results may be due to the different route of the *Listeria* injection. Torres applied *Listeria* intravenously whereas we and other authors used intraperitoneal injection. Another reason could be different characteristics of different *Listeria* strains used in the different studies.

Innate and adaptive immunity are closely connected to each other. *Listeria* specific cellular immune responses are generated in the absence of Myd88 and they are protective (*Way et al. 2003*). IL-12 secreted from infected macrophages participate in the development of T lymphocytes expressing Th1-type cytokines such as IFNγ, TNFα or IL-2 (*Edelson et al. 2000*). A modest reduction in the *Listeria* specific Th1 CD4 T cells response in Myd88 deficient mice has been reported. However, CD8 T cell generation was unaffected. The *Listeria*-specific CD8 T cells generated from Myd88 deficient mice conferred protective immunity to subsequent lethal challenge upon transfer into naïve immunocompetent mice (*Way et al. 2003*); (*Kursar et al. 2004*).

It can be concluded that either TLRs involved in recognition of *Listeria* PAMPs do not use Myd88 as adaptor molecule but rather signal through Myd88 independent pathways. Alternatively, Ag-specific immunity can develop in the absence of TLR mediated signaling. However, it should be kept in mind that in *Borrelia burgdoferi* infection in *TLR2*$^{-/-}$ mice spirochetes persisted at elevated levels despite normal

antibody levels (*Wooten et al. 2002*). This suggests that development of the acquired humoral response can occur in the absence of TLR2 but the cellular response is TLR2 dependent (*Wooten et al. 2002*).

In summery, our results attribute only a minor role to TLR2 in clearance of *Listeria monocytogenes* infection *in vivo*. The role of the adaptor molecule Myd88 is more prominent for *in vivo* resistance to the infection. However, even Myd88 deficient mice are able to generate *Listeria* specific CD8 and CD4 T cells.

7. Results and discussion - part III

Five proteins: filamine, α-actinin, Hsp40, a new protein with high sequence similarity to human SSA/Ro protein, and SMN (Lamping N., Kirschning C unpublished data) were identified by yeast two hybrid screening with TLR2 intracellular domain as a bait. We were able to confirm (1) interaction of TLR2icd with Hsp40 and α-actinin, and (2) TLR1icd with Hsp40 in co-immunoprecipitation. Extensive further biochemical studies did not confirm interaction between TLR2 and above mentioned proteins.

7.1 Results: Co-immunoprecipitation of α-actinin and Hsp40

α-actinin or Hsp40 and the flag tagged constructs TLR2icd, TLR1icd, Myd88, TLR2, and TLR1 were overexpressed in 293 HEK cells for three days. Cellular lysates were subjected to co-immunoprecipitation with anti flag antibody and subsequently to immunoblot analysis with anti HA antibody. Results are presented in Figure 22

α-actinin interacted with TLR2 intracellular domain constructs whereas Hsp40 interacted with both TLR2 and TLR1 intracellular domains. No interaction with Myd88 or full length Toll like receptors 1 and 2 was evident. Empty vector was used as a control for specificity.

7.2 Discussion

Our studies focused on further biochemical analysis of potential TLR2 icd-interactors identified as such by yeast two hybrid screening. We were able to establish co-immunoprecipitation between α-actinin and TLR2 intracellular domain (icd).

α-actinin is an actin crosslinking protein. The α-actinin construct used in these studies represents the C-terminal fragment of the full length α-actinin and contains an in frame insertion of 43 aa. This variant of α-actinin has not been described, but the insert is present within published sequence of chromosome 14 (human genome project). α-actinin has been reported to directly bind to phosphatidylinositol 3-kinase (PI 3-K) through its p85 subunit.

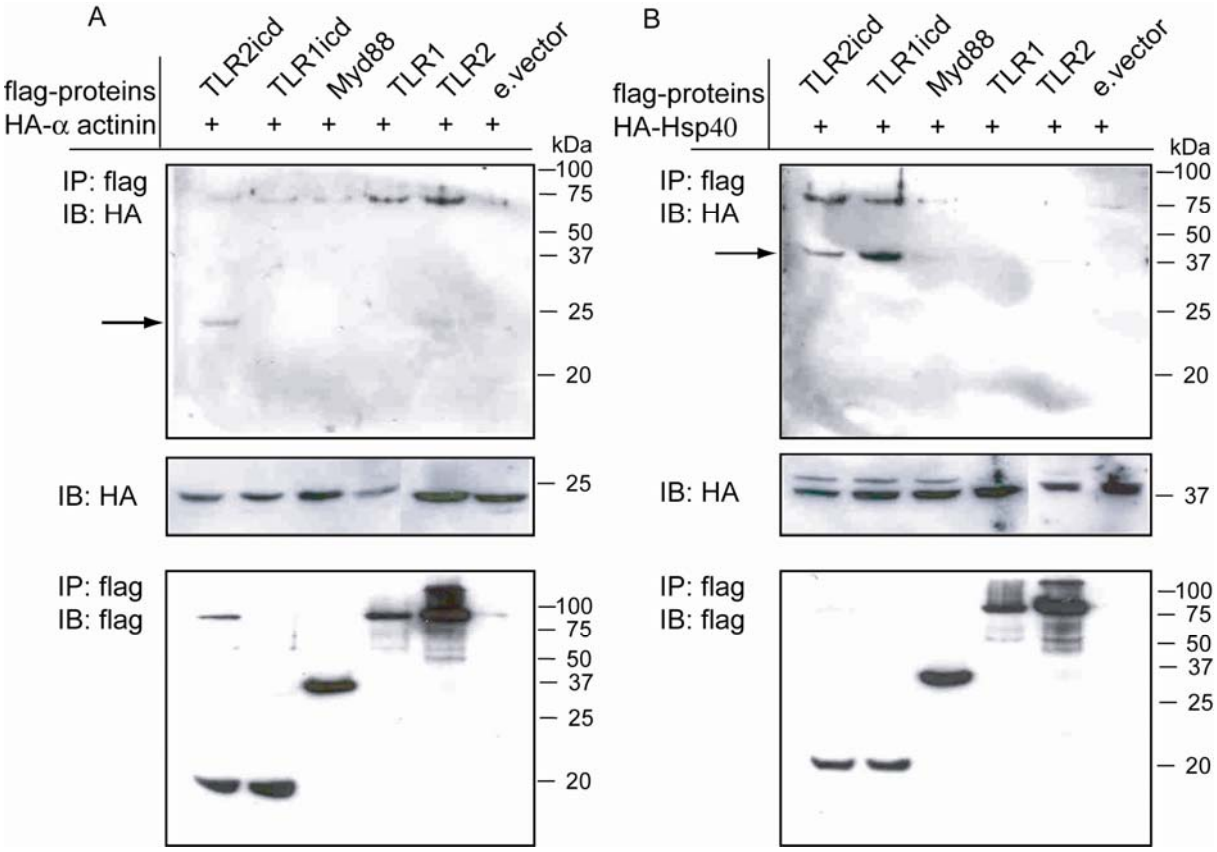


Figure 22 Co-immunoprecipitation of α-actinin and Hsp40

293 cells were transfected with α-actinin construct (A) and Hsp40 construct (B) and flag tag proteins as indicated above. Lysates from 293 cells were incubated with agarose beads coupled with anti flag antibodies overnight. Co-precipitated (IP) α-actinin (A) and Hsp40 (B) were detected by immunoblotting (IB) with anti HA antibodies. The expression of HA (middel panel A and B) and flag (lower panel) proteins was detected by immunoblotting (IB) with anti HA antibodies and anti flag antibodies, respectively. Positions of molecular mass standard (kDa kilodaltons) are indicated. Arrows indicate α-actinin (A) and Hsp40 (B) (e. vector – empty vector).

This binding was observed also after depolarization of actin fibres, suggesting that PI 3-kinase directly binds to α-actinin and can regulate cytoskeleton reorganisation (*Shibasaki et al. 1994*). On the other hand, it has been reported that intracellular domain of TLR2 can bind to p85 and form a stimulus-dependent signaling complex (*Arbibe et al. 2000*). It could be speculated that α-actinin is linking the TLR2 and PI 3-kinase to the cytoskeleton and membrane. A very recent report by West et al. shows that the dendritic cell actin cytoskeleton can be rapidly mobilized in response to Toll-like receptors stimuli to enhance antigen capture and presentation (*West et al. 2004*). Thus, it could be envisioned that, again α-actinin could link TLR2icd with

cytoskeleton via actin and in this way serve as a connection between TLR2 and cellular cytoskeleton.

We were able to show co-immunoprecopitation between α-actinin construct and intracellular domain of TLR2 (Figure 22). However, our biochemical analysis did not indicate interaction between α-actinin construct and full length human TLR2 (data not shown).

Filamin, another actin crosslinking protein, was also analysed for interaction with TLR2. Filamin is known to interact with a number of receptors in mammalian systems, both signal dependent and signal independent (*Fox 1985*); (*Ohta et al. 1991*). The cytoplasmic domain of immunoglobulin G Fc receptor I (FcγRI) in leukocytes interacts with filamin. Binding of ligand disrupts this highly specific interaction with filamin (*Ohta et al. 1991*). Unlike FcγRI, the glycoprotein Ib-IX complex (GPIb-IX), the platelet von Willebrand factor receptor, engages in the signal-independent interaction with filamin (*Fox 1985*). The *Drosophila* Toll receptor was also reported to interact with filamin: Edwards et al. identified the C-terminal part of filamin to interact with Toll (*Edwards et al. 1997*). They proposed a model in which filamin associates with Tube, Dorsal and Cactus as well as Toll receptor. In this way, filamnin is bringing the receptor and intracellular signaling complex into close proximity at the cell surface. The construct used in our studies contains only a C-terminal fragment of filamin. However, we were not able to co-immunoprecipitate filamin with neither full length TLR2 nor the intracellular domain of human TLR2.

Heat shock proteins are not only involved in the control of integrity of cellular proteins but also they can mediate induction of immune responses. Hsp60, hsp70 or gp96 can induce production of proinflammatory cytokines, release of nitric oxide and maturation of DCs. Hsp60 of both bacterial and human origin recruit TLR2 and TLR4 for their recognition (*Vabulas et al. 2001*). Furthermore, endogenous Hsp70 was reported to induce proinflammatory cytokine production from human monocytes via the Myd88 - NF-κB pathway utilizing both TLR2 and TLR4 (*Vabulas et al. 2002*). Another member of the heat shock family, gp96 (Hsp90) was shown to activate dendritic cells via TLR2 and TLR4 (*Vabulas et al. 2002*). The discovery of endogenous ligands for TLRs capable to induce not only the innate immune response but also adaptive immune system shows that immune system primarily recognize the danger signals rather than the nonself signals (*Matzinger 2002*). For

example, the presence of Hsps potentially signals tissue damage or cellular stress to the immune system (*Chen et al. 1999*).

Interestingly, the cytosolic heat shock protein Hsp27 has been shown to be phosphorylated upon IL-1 signaling (*Freshney et al. 1994*). Additionally, a recent report shows that Hsp27 plays a negative role in down-regulating IKK signaling by reducing its activity following TNFα stimulation (*Park et al. 2003*). TNFα increases p38-MK2 (MAPK-activated protein kinase kinase2) phosphorylation of Hsp27 and in this way enhances Hsp27 association with IKKβ which results in decreased IKK activity (*Park et al. 2003*). Another group identified Hsp90 protein in the IKK complex interacting with IKKα (*Chen et al. 2002*). Based on these findings one could imply possible involvement of Hsp40 in TLR signaling.

In summery, we were able to show co-immunoprecipitation between Hsp40 and intracellular domains of TLR2 and TLR1 (Figure 22). Hsp40 did also interact with the TLR4 intracelullar domain (Lamping N., unpublished data). However, our biochemical analysis did not indicate interaction between Hsp40 and full length human TLR2 (data not shown).

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Appendix

The following appendix contains our publication *Grabiec et al. 2004*, to which Section 5 presents supplementary results and discussion.

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Human but Not Murine Toll-like Receptor 2 Discriminates between Tri-palmitoylated and Tri-lauroylated Peptides*

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Toll-like receptors (TLRs) mediate activation of the immune system upon challenge with microbial agonists, components of disintegrating cells of the body, or metabolic intermediates of lipidic nature. Comparison of murine (m) and human (h) TLR2 primary sequences revealed 65% of identical residues within the extracellular domains in contrast to 84% in the intracellular domains. Comparative analysis of TLR2-driven cell activation by various TLR2 agonists showed that the tri-lauroylated lipopeptide analog (Lau_3CSK_4) is recognized efficiently through mTLR2 but not hTLR2. Genetically complemented human embryonic kidney 293 cells and murine *TLR2*$^{-/-}$ embryonic fibroblasts, as well as human and murine macrophage cells, were used for this analysis. In contrast to cellular activation, which depended on blockable access of the TLR2-ligand to TLR2, cellular uptake of Lau_3CSK_4 and tri-palmitoylated peptide (P_3CSK_4) was independent of TLR2. A low-conserved region spanning from leucine-rich repeat (LRR) motif 7 to 10 was found to control TLR2 species-specific cell activation. Exchange of mLRR8 for hLRR8 in mTLR2 abrogated mTLR2-typical cell activation upon cellular challenge with Lau_3CSK_4 but not P_3CSK_4, implicating mLRR8 as a central element of Lau_3CSK_4 recognition. The point mutation L112P within LRR3 abrogated hTLR2-dependent recognition of lipopeptides but merely attenuated mTLR2 function, whereas deletion of the N-terminal third of each LRR-rich domain (LRRs 1 to 7) had the opposite effect on P_3CSK_4 recognition. Despite similar domain structure of both TLR2 molecules, species-specific properties thus exist. Our results imply distinct susceptibilities of humans and mice to challenge with specific TLR2 ligands.

Immediate-early host responses to potentially harmful microbial challenges depend on innate immunity, whereas adaptive immune responses come into play later. Innate immune receptors with specificity for pathogen-derived ligands are expressed constitutively and allow sensing of pathogens when they appear in the host. LPS,[1] peptidoglycan, lipoproteins, flagellin, and nucleic acids are examples of microbial and viral products eliciting host responses (1–3). Both Gram-negative and Gram-positive bacterial products induce overactivation of the host immune system and are a major cause of severe sepsis and septic shock (4).

The toll-like receptor (TLR) family includes 10 members in humans. Mice have no TLR10 but carry TLR11 (5). TLRs and other pattern-recognition receptors such as dectin 1 and complement receptor 3 mediate specific host cell activation by components of microorganisms (6, 7) as well as by endogenous cellular products liberated upon disruption (8). In contrast to *Drosophila* toll and cytokine receptors, which exclusively bind endogenous ligands, TLRs directly interact with exogenous pathogen-derived ligands (9–11). They contain leucine-rich repeat (LRR) line-ups in the TLR-extracellular domains (ECDs), which resemble known structures of LRR-rich domains (12). In addition, the structures of other microbial ligand receptors such as CD14 may be organized similarly. Structurally unrelated proteins can exert similar receptor functions, as exemplified by the LPS binding capacity of LPS-binding protein (13, 14).

Bacterial species, such as Gram-positive *Listeria monocytogenes* and *Staphylococcus aureus*, and Gram-negative *Chlamydia pneumoniae* elicit host cell activation through TLR2 (3, 15). Bacterial products, for instance lipoteichoic acid, bacterial lipoproteins or their analogs such as di-/tri-palmitoyl-cysteinyl-seryl-(lysyl)3-lysine ($P_{2/3}CSK_4$), and mycoplasmal macrophage activating lipoprotein are agonists of TLR2 (3). Although further cellular detection mechanisms exist that participate in innate host defense such as TLR2-independent, but dectin-dependent, immune reactions, TLRs seem to be essential for induction of a comprehensive innate immune response (6, 16, 17).

Species-specific differences in cellular pattern recognition have been found for certain LPS variants and Taxol; this may reflect the variant use of TLR4 and MD2 (18–20). Similarly, TLR9 has been recognized as the mediator of species-specific DNA sequence recognition (21, 22). Here, we used complementation of human embryonic kidney (HEK) 293 cells or murine embryonic *TLR2*$^{-/-}$ fibroblasts with murine and human TLR2 to identify and analyze species specificity of pattern recognition through *TLR2* ortholog products. In addition, we analyzed the murine RAW264.7 cell line, as well as human MonoMac6 and THP1 macrophage cell lines. Our results reveal species-specific recognition of a tri-lauroylated peptide through murine TLR2

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[1] The abbreviations used are: LPS, lipopolysaccharide; TLR, toll-like receptor; LRR, leucine-rich repeat; ECD, extracellular domain; ICD, intracellular domain; P_3CSK_4, tri-palmitoyl-cysteinyl-seryl-(lysyl)3-lysine; Lau, lauroyl; Myr, myristoyl; MEF, murine embryonic fibroblast; PBS, phosphate-buffered saline; NF-κB, nuclear factor-κB; mAb, monoclonal antibody; IL, interleukin.

and involvement of a relatively low conserved LRR-rich subdomain of the TLR2ECD in this process.

MATERIALS AND METHODS

Reagents—Purified bacterial components applied were LPS from *Escherichia coli* 0111:B4 (Sigma), soluble peptidoglycan from *Staphylococcus aureus* retinoblastoma prepared by vancomycin affinity chromatography (23), and highly purified lipoteichoic acid from *S. aureus* prepared by propanol extraction (24). Synthetic mycoplasmal macrophage-activating lipoprotein-2 was from Dr. Mühlradt (GBF Braunschweig, Germany), whereas tri-/di-/mono-palmitoyl-cysteinyl-seryl-(lysyl)3-lysine (P_3CSK_4, P_2CSK_4, $PCSK_4$) and tri-lauroyl/tri-myristoyl-cysteinyl-seryl-(lysyl)3-lysine (Lau_3/Myr_3CSK_4), as well as biotinoylated analogs, were purchased from ECHAZ microcollections (Tübingen, Germany) (25). Lipidated OspA, a tri-palmitoylated lipoprotein from *Borrelia burgdorferi*, was from Dr. Dunn (Brookhaven National Laboratory, Upton, NY), and highly purified recombinant chlamydial heat shock protein 60 was from Drs. Prazeres da Costa and Miethke (15). Yeast liophylisate zymosan and phorbol 12-myristate 13-acetate were from Sigma. Rabbit polyclonal antisera against phosphorylated and non-phosphorylated mitogen-activated protein kinases p38 and extracellular signal-regulated kinase 1/2 were from New England Biolabs (Frankfurt, Germany).

Cell Culture—HEK293 cells (ATCC305) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics (Invitrogen, Auckland, Scotland), whereas for culture of primary murine embryonic fibroblasts (MEFs), 10 μM monothioglycerol (Sigma) was added to these medium components. MEFs were prepared from *TLR2*$^{-/-}$ mice (Tularik, South San Francisco, CA) as described (26). Murine RAW264.7 (ATCC No.TIB71) cells, as well as human MonoMac6 (ATCC124) and THP1 (ATCC16) cells, were cultured in RPMI 1640 medium supplemented as described for Dulbecco's modified Eagle's medium (Invitrogen), whereas nonessential amino acids and media supplement (OPI, Sigma) were added for culture of MonoMac6 cells specifically.

Mutagenesis—A human and a murine TLR2 expression plasmid (pFlag-CMV, Sigma) (27) were used as templates in splice polymerase chain reaction-based mutagenesis (Quick Change kit, Stratagene, Amsterdam, Netherlands). Chimera constructs were generated as deduced from the primary sequences of both immature proteins. Human (h) or murine (m) K19/Q25-R/Q587 was fused to m or h A/T588-N785, and the resulting constructs were designated h/m or m/h, respectively; a fusion construct carrying h or m K19/Q25-D/E305, and m or h to L/P306-N785 was designated h_{1-10}m/m or m_{1-10}h/h, respectively; h or m K19/Q25-S196 fused to m or h I197-D/E305 and to h or m L/P306-N785 was designated hm_{7-10}h/h or mh_{7-10}m/m, respectively; m or h Q25/K19-S196 fused to h or m I197-E/D305, m or h P/L306-L497, h or m L/F498-T545, and m or h M/Q546-N785 was named $mh_{7-10}mh_{19-20}$/m or $hm_{7-10}hm_{19-20}$/h; hK19-V220 fused to m L221-E305 to h P306-N785 was named hm_{8-10}h/h; hK19-E246 fused to m V247-E305 to h P306-N785 was named hm_{9-10}h/h; hK19-Q275 fused to m I276-E305 to h P306-N785 was named hm_{10}h/h; hK19-V220 fused to m L221-Y275 to h I276-N785 was named hm_{8-9}h/h; hK19-E246 fused to m V247-Y275 to h I276-N785 was named hm_9h/h; h or m K19/Q25-V/I220 fused to m or h L/T221-E246 to h or m T/V247-N785 was named hm_8h/h or mh_8m/m, respectively; h or m K19/Q25-A385 fused to m or h W386-L497 to h or m L/F498-N785 was named hm_{14-18}h/h or mh_{14-18}m/m, respectively; and h K19-L497 fused to m F498-T545 to h Q546-N785 was named hm_{19-20}h/h.

hMutH lacking the N-terminal seven LRRs has been described (26), and an analogous murine construct denoted mMutH lacked the respective subdomain (ΔS48-I220) through deletion. One point mutation of a leucine residue within the LRR consensus sequence of the third LRR motif (L112P) was introduced into both wild-type constructs. A threefold point mutated (N147Q, N414Q, and N442Q) mTLR2 construct with named ΔG3 was generated to impede mTLR2-typical N-glycosylation.

Preparation of Inactivated Bacteria Suspensions—Bacteria of the species *S. aureus* (DSMZ 20231), *S. pyogenes* (DSMZ 20565), as well as of *Enterococcus faecalis* (DSMZ 20478) were cultured at 37 °C in standard brain-heart medium overnight and plated on standard blood agar plates for determination of culture purity and colony forming unit titers. *Legionella pneumophila* (28) was seeded on coal agar plates, incubated at 37 °C under 8% of CO_2 for 5 days, and scraped from the plates for suspension in PBS. Bacterial cells were washed twice with PBS, and the resulting suspensions were heat inactivated through incubation at 56 °C for 50 min.

Reporter Gene Assay—HEK293 cells were plated on 96-well plates and cotransfected with an NF-κB-dependent promoter firefly luciferase construct (29), a reporter plasmid mediating constitutive expression of Renilla luciferase (30), as well as cytomegalovirus-promoter-dependent expression plasmids for human and murine TLR2 fusion constructs by the calcium phosphate precipitation method. Preparations of TLR agonists were added to the transfected cells for 16 h. Cells were lysed for measurement of firefly- and Renilla-luciferase activities using reagents from Promega (Madison, WI) and PJK GmbH (Kleinblittersdorf, Germany). Firefly-luciferase activities were related to Renilla-luciferase activities for normalization. For analysis of potential blockage of P_3CSK_4-induced hTLR2-dependent cell activation by Lau_3CSK_4, HEK293 cells overexpressing the receptor were preincubated with Lau_3CSK_4 at a concentration of 100 μg/ml for 30 min. Subsequently, increasing amounts of P_3CSK_4 were added to distinct wells for 6 h. Similarly, mAb T2.5, which has been demonstrated to neutralize TLR2 by blockage of ligand binding (31), was added at a concentration of 50 μg/ml 30 min before cellular challenge with P_3CSK_4 or Lau_3CSK_4. NF-κB-dependent luciferase activity and IL-8 release were analyzed.

Analysis of Supernatants by Enzyme-linked Immunosorbent Assay—Transfected HEK293 cells and *TLR2*$^{-/-}$ MEFs, as well as RAW264.7 and MonoMac6 cells, were cultured on 96-well plates (1×10^4, 3×10^5, 1×10^5, and 1×10^5 cells per well, respectively) and challenged as indicated for 24 h. Culture supernatants were applied to enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) for measurement of human IL-8, as well as human and murine IL-6 and tumor necrosis factor-α concentrations by enzyme-mediated colorimetry (Magellan, Tecan, Crailsheim, Germany), according to enzyme-linked immunosorbent assay material supplier protocols.

Immunoblot Analysis—Flag-tagged and overexpressed proteins were visualized upon lysis of overexpressing cells, optional immunoprecipitation, SDS-PAGE, and blotting as described (26). RAW 264.7 and MonoMac6 cells were challenged for 30 min and lysed. Lysates were applied to each lane of an SDS-PAGE gel. Polyclonal rabbit antisera specific for Flag (Sigma) or phosphorylated p38 and extracellular signal-regulated kinase 1/2 (Cell Signaling, Frankfurt, Germany) were used. Specific epitopes were visualized by enhanced chemiluminescence (Western Lightning, Perkin-Elmer). Whereas lysate of 3×10^5 cells/lane was sufficient to gain significant signals in general, lysate of 1×10^6 MonoMac6 cells had to be fractionated per lane to gain clear signals representing conditional kinase phosphorylation.

Flow Cytometry—RAW 264.7 and MonoMac6 cells (0.5×10^6 cells/ml) were rinsed from the culture plates, whereas THP1 cells differentiated by incubation with 50 ng/ml phorbol 12-myristate 13-acetate overnight were detached through incubation with PBS on ice for 5 min. Cells were incubated first with 2% fetal calf serum, 5% normal goat serum, and anti-murine Fcγ III/II Receptor mAb (BD PharMingen, Heidelberg, Germany) or Endobulin S/D (Baxter, Unterschleissheim, Germany) for murine or human cells, respectively.

After washing, cells were incubated with a murine and human TLR2-specific (31) and a secondary murine IgG-specific mAb (FITC-labeled, Caltag Laboratories, Burlingame, California) subsequently. For intracellular staining, cells were fixed and permeabilized by incubation with BD Cytofix/Cytoperm reagent (BD Bioscience, Heidelberg, Germany) according to the supplier protocol before incubation with antibodies. Samples were analyzed on a FACSCalibur flow cytometer (BD Bioscience).

Electrophoretic Mobility Shift Analysis—RAW264.7 or MonoMac6 cells were challenged in RPMI 1640 serum containing 2% fetal calf serum for 2 h, and nuclear extracts were prepared. Cells were lysed, and nuclear proteins analyzed as described previously (26).

Immunocytochemical Staining and Uptake of Biotinoylated Lipopeptide—THP1 cells, differentiated with phorbol 12-myristate 13-acetate or murine macrophages, were grown on glass carriers in a 24-well culture plate and incubated with biotinoylated P_3CSK_4 or Lau_3CSK_4 for the time periods indicated. Cells were washed with PBS and incubated with 50 μg/ml Alexa Fluor 488-conjugated concanavalin A (Molecular Probes, Amsterdam, Netherlands) in serum-free RPMI 1640 at 4 °C for 15 min. The medium was removed, and the cells were washed with PBS and fixed with cold methanol for 8 min at −20 °C. Cells were blocked with 2% normal goat serum in PBS for 30 min at 37 °C. As first antibody a TLR2-specific mAb cross-reacting with murine and human TLR2 (31) was applied before washing after 30 min of incubation. As second antibody, Alexa Fluor 546-conjugated goat anti-murine IgG (4 μg/ml), as well as Alexa Fluor 647-conjugated Streptavidin (10 μg/ml) for detection of labeled lipopeptides, was applied for 30 min (Molecular Probes). Cells were washed and sealed by incubation in 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).

Deglycosylation—Human and murine wild-type TLR2 constructs, as well as designated MutH constructs and an *N*-glycosylation site-deficient mTLR2 construct (ΔG3), were subjected to *N*-specific deglycosylation assay according to the protocol provided by the supplier of peptide:*N*-glycosidase F (New England Biolabs, Frankfurt, Germany), which cleaves complex oligosaccharides from *N*-linked glycoproteins. In brief, Flag-tag specifically immunoprecipitated (Flag-beads, Sigma) (26) constructs were incubated at 100 °C for 10 min in denaturing buffer to which Nonidet P-40 and peptide:*N*-Glycosidase F were added within a total volume of 25 μl thereafter. In parallel, the same amount of precipitated protein was incubated in the absence of peptide:*N*-glycosidase F. After incubation at 37 °C for 1 h, samples were loaded to an 8% SDS-polyacrylamide gel and subjected to PAGE, as well as immunoblot analysis subsequently.

RESULTS

Comparative Mutagenesis of Human and Murine TLR2—Comparative amino acid sequence analysis of human and murine TLR2 revealed 70% overall identity. In contrast, extracellular and intracellular domain sequences show 65 and 84% identity, respectively. We subdivided the ECD sequences of human and murine TLR2 into 20 LRR/LRR-like motifs (32) and refer to these as LRRs. Alignment of both TLR2 sequences showed identical localization of these motifs and thus enabled the individual comparison of each of the LRRs (Fig. 1*A*). The intracellular domain was exchanged between human and murine TLR2 to control for their potential species-specific activity. No evidence for species-specific properties was apparent from analysis in human (HEK293) or murine (*TLR2* $^{-/-}$ MEF) cells (Fig. 1*B*, constructs h/m and m/h, and data not shown). To search for species specificity through non-conserved regions of the two TLR2ECD sequences, we swapped the first half of the LRR-rich domains because LRR motifs 1 to 10 displayed lower interspecies similarity than motifs 11 to 20 (Fig. 1*B*, $h_{1-10}m/m$ and $m_{1-10}h/h$). We also exchanged a region encompassing the four LRR motifs 7 to 10 because conservation of their sequences is particularly low (Fig. 1, *A* and *B*, $mh_{7-10}m/m$ and $hm_{7-10}h/h$). For fine-mapping of this block of LRRs, LRRs 8 to 10, 9 to 10, 8 to 9, as well as single motifs 8, 9, and 10 were swapped (constructs $hm_{8-10}h/h$, $hm_{9-10}h/h$, $hm_{8-9}h/h$, hm_8h/h, mh_8m/m, hm_9h/h, and $hm_{10}h/h$). To analyze a potential role of another low-conserved region within the TLR2ECD, we additionally exchanged the LRR motifs 19 and 20 (Fig. 1*B*, constructs $mh_{7-10}mh_{19-20}/m$ and $hm_{7-10}hm_{19-20}/h$) and exchanged LRRs 19 and 20 only (hm_{19-20}/h). Another relatively small conserved region is the LRR block encompassing motifs 14 to 18, which was analyzed similarly ($mh_{14-18}m/m$ and $hm_{14-18}h/h$,). A mutant "H" lacking the N-terminal third of the LRR-rich domain of human TLR2 has been described previously (26), and an analogous murine TLR2 construct was generated (Fig. 1*B*, mMutH). Two constructs of human and murine TLR2 carrying the point mutation L112P (this affects a consensus leucine residue within the respective third LRR motifs) were also prepared.

N-Glycosylation is critical for TLR4 function (33) and for TLR2 surface expression (34), suggesting that distinctive *N*-glycosylation might lead to species specificity of pattern recognition through TLR2. Application of the NetNGlyc 1.0 algorithm[2] revealed presence of four *N*-residues that are putatively glycosylated in hTLR2ECD (amino acid residues 114, 199, 414, and 442, respectively), whereas in mTLR2ECD only three sites have a significant potential for being *N*-glycosylated (amino acid residues 147, 414, and 442). We considered glycosylation of a putative fourth site as unlikely (residue 296) because of conformational constraints induced by a proline following the respective asparagine. Glutamine residues were introduced into a wild-type mTLR2 expression construct to replace the central asparagines within the three canonical *N*-glycosylation

A

	Identities (%)		Identities (%)
LRR1	62	*LRR11*	*86*
LRR2	62	*LRR12*	*83*
LRR3	83	*LRR13*	*88*
LRR4	72	LRR14	76
LRR5	64	LRR15	69
LRR6	58	LRR16	66
LRR7	**45**	LRR17	75
LRR8	**57**	LRR18	86
LRR9	**44**	LRR19	62
LRR10	**56**	LRR20	70

B

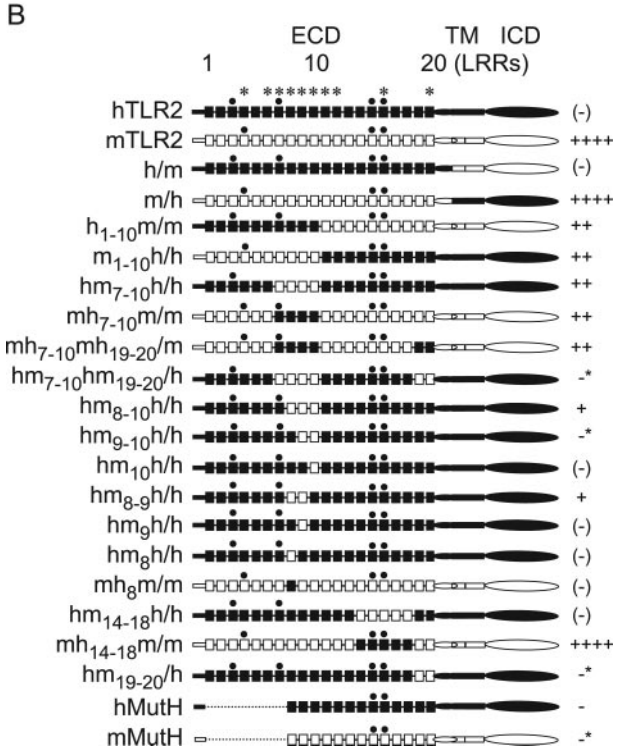

FIG. 1. **Illustration of human and murine TLR2ECD homology and the TLR2 constructs generated.** *A*, alignment and comparative analysis of human and murine extracellular TLR2 primary sequences and definition of 20 LRR/LRR-like motifs (*boxes* numbered according to the order from the N terminus) revealed similarities illustrated as identities within each of the sequence motifs; low-conserved regions (*bold* and *italics*) and high-conserved regions (*italics*) are highlighted within the N-terminal and the C-terminal half of the LRR-rich domain. *B*, fusion constructs of human (*h*, *black*) and murine (*m*, *white*) TLR2 are aligned schematically with wild-type proteins, as well as deletion constructs (MutH, ΔS48-V220) to illustrate relative localization of LRR (unmarked) and LRR-like (*asterisks*) motifs (*rectangles*). ●, localizations of putative *N*-glycosylation sites; *ECD*, ECD encompasses LRRs symbolized as *boxes*, N-terminal domain as *rectangles*, and C-terminal domain as *small half-oval*; *TM*, *trans*-membrane domain depicted as *small rectangles*; N terminus pictured as *large rectangles* and Toll-IL-1 receptor/TIR-domain as *large ovals*. Nomenclature in combination with the graphic code illustrates the order (N toward C terminus) of subdomains from both receptors within the fusion constructs; numbers correspond to the LRR motifs exchanged. "*f*" symbolizes the *trans*-membrane domain; symbols beside each construct cartoon represent the relative cellular activity upon challenge with Lau_3CSK_4 mediated through the respective TLR2 construct: ++++, high; ++, intermediate; +, low; (−), very low; −, no detectable signal upon Lau_3CSK_4 challenge; −*, no detectable signal upon TLR2-specific challenge.

[2] Internet address: www.cbs.dtu.dk/ services/NetNGlyc/.

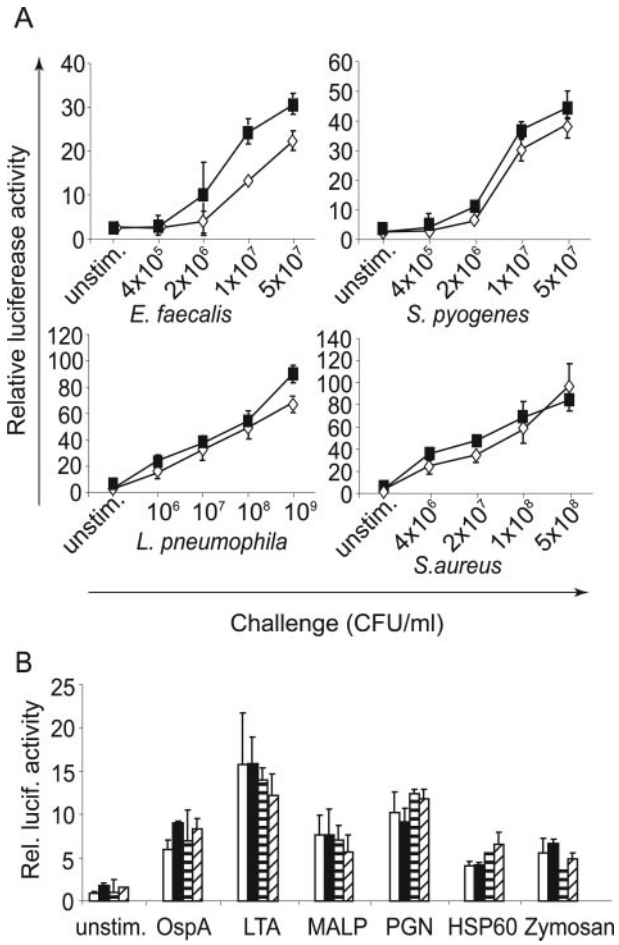

FIG. 2. **Human and murine TLR2-specific responsiveness of transfected HEK293 cells to bacterial challenge.** *A*, HEK293 cells overexpressing human (◇) or murine (■) TLR2 were challenged with suspensions of heat-inactivated bacteria at the concentrations indicated before measurement of NF-κB-dependent reporter gene activation. *unstim.*, unstimulated; *CFU*, colony-forming unit. *B*, human wild-type TLR2 (□), murine wild-type TLR2 (■), human TLR2 ECD fused to complete C-terminal portion of murine TLR2 (▤), or murine TLR2 ECD fused to complete C-terminal portion of human TLR2 construct (▨) mediated reporter gene activation upon challenge with the defined microbial products indicated. *OspA*, 4.5 μg/ml of outer surface protein A of *B. burgdorferi*; *LTA*, 5 μg/ml of lipoteichoic acid of *S. aureus*; *MALP*, 100 ng/ml of mycoplasmal monocyte activating lipoprotein; *PGN*, 5 μg/ml of peptidoglycan of *S. aureus*; *HSP60*, 8 μg/ml of recombinant heat shock protein 60 of *C. pneumoniae*; 50 μg/ml of *Zymosan*, resuspended liophylisate of yeast. *Rel. lucif. activity*, relative luciferase activity.

sites (ΔG3). All TLR2 constructs were expressed at similar levels upon transfection in HEK293 cells as analyzed by immunoblot analysis (data not shown).

Comparative Analysis of TLR2 Construct Activities by Genetic Complementation of HEK293 Cells—To compare responsiveness to whole bacterial cells, HEK293 cells expressing either human or murine TLR2 were challenged with suspensions of heat inactivated bacteria at increasing concentrations. Results revealed a similar trend in respect to NF-κB-dependent reporter gene activation and IL-8 release through both wild-type receptors. Cell activation by heat-inactivated bacteria (Fig. 2*A*) or defined microbial products such as lipoteichoic acid and peptidoglycan through TLR2 was species-independent (Fig. 2*B*). Exchange of the extracellular domains between human and murine TLR2 did not alter cellular responsiveness, indicating full function of the murine TLR2ICD in human embryonic fibroblasts (Fig. 2*B* and data not shown). However, application of lipopeptide analogs (Fig. 3*A*) carrying acyl chains of reduced length (as compared with the classical P_3CSK_4, which contains 16 C-atoms in its palmitoyl chain) revealed TLR2 species specificity. Although P_3CSK_4 (tri-palmitoylated peptide) and Myr_3CSK_4 (tri-myristoylated) induced cell activation to similar degrees, Lau_3CSK_4 (tri-lauroylated) was recognized specifically through mTLR2ECD but not hTLR2-ECD unless more than one hundred-fold-increased ligand-concentrations were used (Fig. 3, *B* to *D* and data not shown).

Constructs containing cross-species subdomains were next tested to identify the subdomain responsible for this species specificity (Fig. 1*B*). Replacement of the mTLR2ECD domain containing the first 10 LRRs with the corresponding hTLR2 domain did not abrogate signal transduction. The reverse change (introducing mTLR2 LRR 1 to 10 into hTLR2) conferred Lau_3CSK_4 responsiveness through this otherwise human construct. Responsiveness mediated by both constructs was intermediate as compared with both wild-type receptors (data not shown). Exchange of a subdomain encompassing LRRs 7 to 10 had a similar effect, not only in HEK293 cells but also in *TLR2*$^{-/-}$ MEFs (Figs. 1*B* and 3, *B* to *E*, and data not shown) mapping species specificity to this narrowed subdomain. For detailed analysis of the LRR 7 to 10-block constructs carrying blocks of three, two, or only single murine LRRs within the otherwise human receptor were prepared. Although not enhancing cellular Lau_3CSK_4 recognition to a degree similar to wild-type mTLR2, all constructs containing blocks of three or two cross-species LRRs conferred enhanced activity to hTLR2. The only exception was construct $hm_{9-10}h/h$, which did not confer detectable responsiveness. Exchange of single LRRs within the LRR 7 to 10 subdomain in hTLR2 did not promote increased cell activation as compared with wild-type hTLR2 activity. The most active construct was $hm_{7-10}h/h$, the activity of which was intermediate as compared with mTLR2 (high) and hTLR2 (low) (Figs. 1*B* and 3, *B* to *E*, and data not shown). Analysis of a cross-specific LRR8 construct (mh_8m/m, carrying the human motif in the otherwise murine receptor) indicated a central role of LRR8 in recognition of Lau_3CSK_4. The activation level mediated through this construct upon Lau_3CSK_4 challenge was nearly as low as wild-type hTLR2-dependent cell activation (Fig. 3*B* to *D*, and data not shown). In conclusion, these results indicate that the murine LRR block from motif 7 to 10 is involved in mouse-specific Lau_3CSK_4 recognition, and LRR8 plays a prominent role within this block.

To analyze additional subdomains for involvement in species-specific pattern recognition, further fusion constructs were generated and analyzed (Fig. 1*B* and data not shown). We found that neither the region encompassing LRRs 14 to 18, nor LRR 19 and 20 within the C-terminal portion of TLR2, contributed to species-specific pattern recognition because their cross-species exchange did not alter characteristics of the respective constructs. Notably, exchange of human motifs 19 and 20 for its murine counterpart abrogated recognition of known TLR2 agonists (Fig. 1*B*, hm_{19-20}/h). In contrast, the respective "human-in-murine" exchange had no detectable effect (Fig. 1*B*, $mh_{7-10}mh_{19-20}/m$ *versus* $mh_{7-10}m/m$ and data not shown).

Comparative Analysis of TLR2 Construct Activities by Genetic Complementation of TLR2$^{-/-}$ *MEFs*—Next, TLR2-deficient mouse fibroblasts genetically complemented with TLR2 constructs were analyzed. Although *TLR2*$^{-/-}$ fibroblasts gained responsiveness to both lipopeptide analogs upon transfection of mTLR2, hTLR2 did confer detectable responsiveness to P_3CSK_4 but not to Lau_3CSK_4 (Fig. 3*E*). The finding that Lau_3CSK_4 was recognized exclusively through murine TLR2ECD was confirmed through analysis of ECD-ICD exchange constructs (data not shown). Results obtained by transient overexpression of TLR2 murine-human fusion constructs

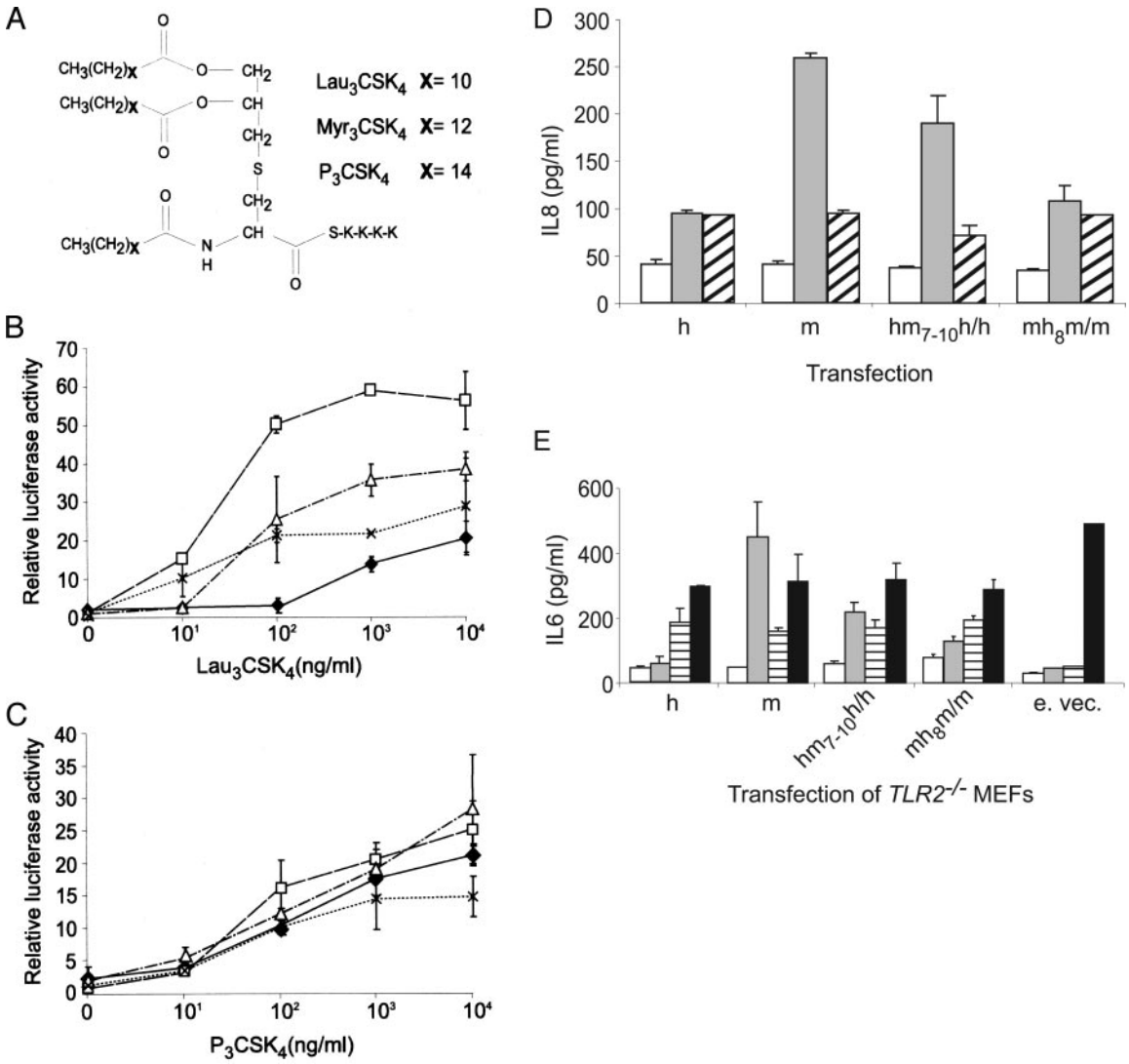

FIG. 3. **Human and murine TLR2-specific responsiveness to lipopeptide challenge.** *A*, structure of lipopeptide analogs (CSK_4, cysteinyl-seryl-(lysyl)3-lysine) carrying triacylations of distinct lengths (*x*, CH_2-group number as indicated; *Lau*, lauroyl; *Myr*, myristoyl; *P*, palmitoyl). *B* and *C*, reporter gene activation as compared with vector control upon challenge with lipopeptides at increasing concentrations as indicated (♦, hTLR2; □, mTLR2; *x*, mh_8m/m; △, $hm_{7-10}h/h$) for Lau_3CSK_4 (*B*) and P_3CSK_4 (*C*). *D* and *E*, IL-8 release upon stimulation of transfected HEK293 cells (*D*) and IL-6 release from transfected (as indicated) *TLR2*$^{-/-}$ MEFs with lipopeptides (*E*). □, unstimulated; ▨, 1 μg/ml Lau_3CSK_4; ▤, 1 μg/ml P_3CSK_4; ▨, 20 ng/ml phorbol 12-myristate 13-acetate; ■, 100 ng/ml LPS; *e. vec.*, empty vector.

in *TLR2*$^{-/-}$ MEFs confirmed our results from analysis of HEK293 cells overexpressing the same constructs (Fig. 3*E* and data not shown).

Effect of Cellular Preincubation with Lau$_3$CSK$_4$ or TLR2-specific Neutralizing mAb and Subsequent Challenge with Specific Lipopeptide Analogs—We then asked whether Lau_3CSK_4, although it fails to activate at regular concentrations, would bind to TLR2 like P_3CSK_4. We therefore preincubated hTLR2-transfected HEK293 cells either with Lau_3CSK_4 or with neutralizing TLR2-specific mAb before addition of increasing amounts of lipopeptides Lau_3CSK_4 or P_3CSK_4, respectively. Preincubation with Lau_3CSK_4 did not significantly alter cellular responsiveness to P_3CSK_4. This indicates that either Lau_3CSK_4 binds to a distinct site in TLR2 or a high on-off rate of lipopeptide-TLR2 binding prevents detectable competition at the concentration of Lau_3CSK_4 applied within the time periods (4 to 24 h) analyzed (Fig. 4*A* and data not shown). In contrast, preincubation with a TLR2-specific antagonizing mAb and subsequent application of increasing amounts of Lau_3CSK_4 blocked cellular activation with Lau_3CSK_4, not only through mTLR2 (Fig. 4*C*) but also through hTLR2 (the latter was detectable only after application of high amounts of the lipopeptide analog; Fig. 4*B*).

TLR2 Expression of Murine and Human Monocyte/Macrophage Cell Lines and Species-specific Responsiveness to Distinct TLR Ligands—We extended our analysis on species-specific cell activation through TLR2 to macrophage cell lines of human or murine origin. Using a cross-reactive TLR2 mAb recognizing both human and murine TLR2, we analyzed TLR2 expression of RAW264.7, MonoMac6, and THP1 cells. Intracellular TLR2 expression was higher than cell surface expression, but all three cell lines expressed detectable amounts of TLR2 on the cell surface (Fig. 5, *A* and *B*). Both human and murine macrophages responded to P_3CSK_4 or P_2CSK_4 and LPS. In contrast, Lau_3CSK_4 activated murine RAW264.7 but not human macrophage-like cells unless the ligand was applied at one hundred- to thousand-fold higher concentrations (Fig. 5*C*). In line with these results, nuclear translocation and DNA binding of NF-κB, as well as the phosphorylation of mitogen-activated protein kinases p38 and extracellular signal-regulated kinase 1/2 (all of which play central roles in TLR-mediated signal transduction (3)), were different. The measured events of signal

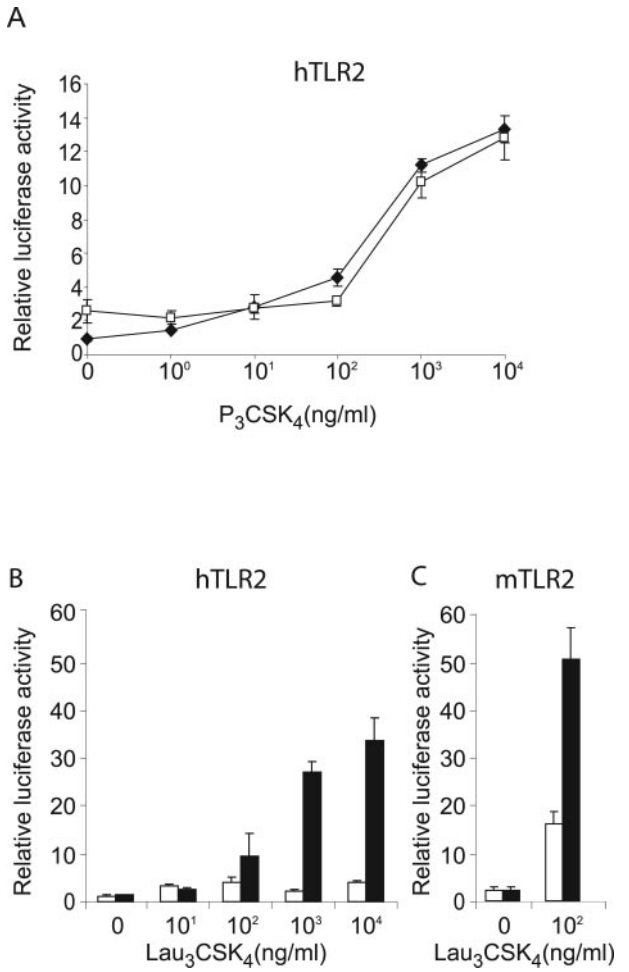

FIG. 4. **Analysis of potentially direct interaction between TLR2 and Lau_3CSK_4.** *A*, NF-κB-dependent reporter gene activation in HEK293 cells overexpressing hTLR2 without (♦) or upon preincubation with (□) Lau_3CSK_4; preincubation for 30 min at a concentration of 100 ng/ml and subsequent challenge with P_3CSK_4 at concentrations indicated for 6 h. *B* and *C*, NF-κB-dependent luciferase activities in HEK293 cells overexpressing human TLR2 (*hTLR2*, *B*) or murine TLR2 (*mTLR2*, *C*) challenged with Lau_3CSK_4 at the concentrations indicated. Cells were preincubated before Lau_3CSK_4 challenge either with mAb T2.5 (□, 50 μg/ml) or isotype control mAb (*B* and *C*, ■, 50 μg/ml).

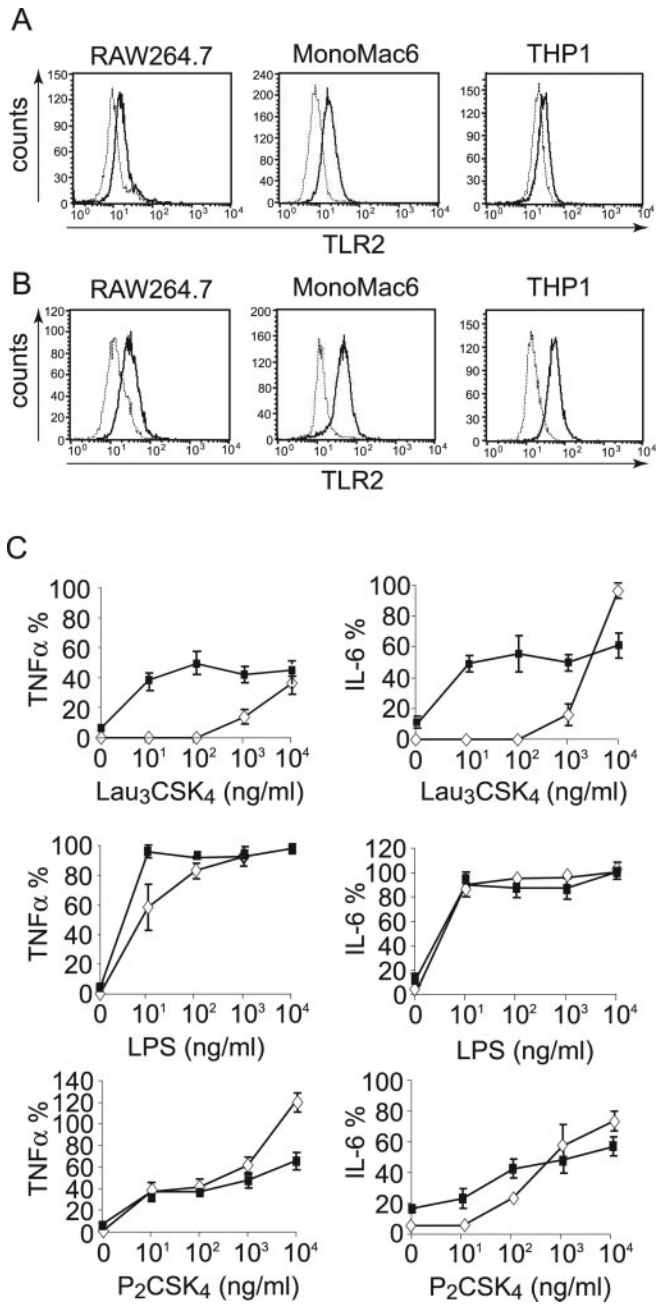

FIG. 5. **TLR2 expression by and cytokine release from human and murine macrophages upon TLR2-specific challenge.** Human MonoMac6 and THP1 cells, as well as murine RAW264.7 macrophages, were analyzed for cell surface (*A*) and intracellular (*B*) TLR2 expression by flow cytometry (*thin line*, isotype control; *bold line*, TLR2-specific antibody). Human MonoMac6 (◇) and RAW264.7 (■) macrophages were challenged with lipopeptides or LPS (as positive control) at different concentrations for 24 h before application of supernatants to ELISA as indicated (*C*). In general, RAW264.7 released ~10-fold amounts of cytokines analyzed as compared with MonoMac6 cells. For comparative analysis, LPS (10 μg/ml)-induced cytokine release was fixed as 100%, and respective sample values were related to it in a cell line- and cytokine-specific manner (see *ordinates*).

transduction upon Lau_3CSK_4 challenge were not detectable or strongly decreased in human as compared with murine macrophages (Fig. 6, *A* and *B*).

Immunocytochemical Analysis of Lipopeptide Uptake by Human Macrophage Cell Lines—Uptake of P_3CSK_4 and Lau_3CSK_4 in human and murine macrophages was compared to determine whether cellular uptake was also species specific. Weak lipopeptide-specific cell surface staining after 5 min of incubation but significant intracellular staining after 30 or 45 min indicated time-dependent cellular uptake by human wild-type macrophages (Fig. 7) and also by murine *TLR2*$^{-/-}$ macrophages (data not shown). Similar kinetics of internalization indicated TLR2-independent binding of the lipopeptide analogs P_3CSK_4 and Lau_3CSK_4 to both human and murine macrophages (Fig. 7 and data not shown). Free biotin was not detectable within cells after application under the same conditions used for biotinylated lipopeptides (data not shown), indicating lipopeptide specificity of the cellular uptake observed.

Deletion- and Point Mutagenesis-based Analysis of Structural Requirements for Species-specific TLR2 Function—A hTLR2 construct, lacking the N-terminal third of its LRR-rich domain (mutant H), mediated di- or tri-palmitoylated peptide-induced cell activation but not to other TLR2 agonists (26). We generated an analogous mTLR2 construct. This mutant failed to mediate P_3CSK_4-induced cell activation (Fig. 8*A*, *right panel*), indicating a species-specific difference of TLR2 structure. Neither "H"-mutant mediated a Lau_3CSK_4 signal (Fig. 8*A*). Although diminished, a mTLR2 construct containing a point mutation in its ECD mediated a P_3CSK_4 signal, whereas the analogous human construct did not (Fig. 8*B*). All mutant constructs were expressed at similar levels, and molecular

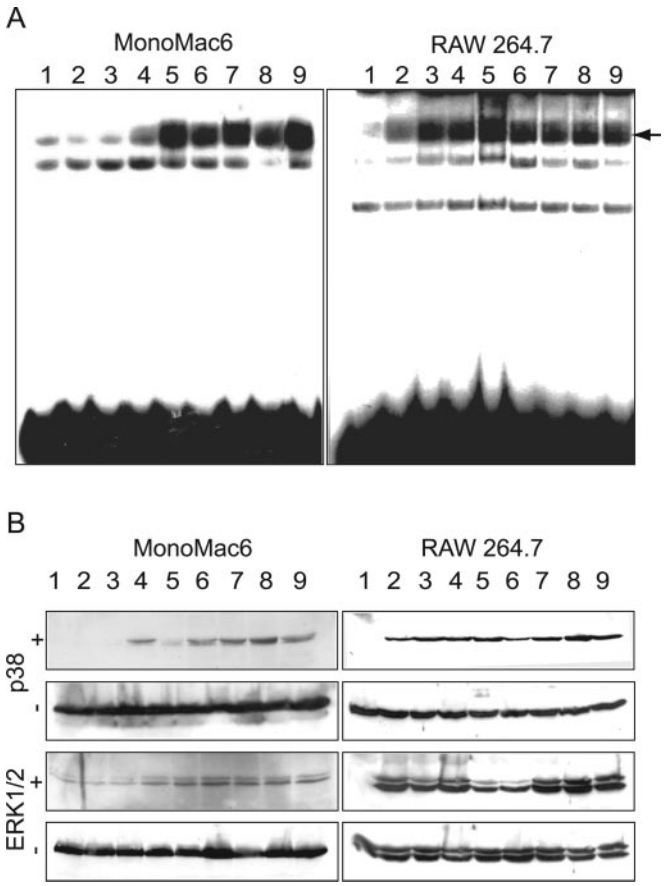

FIG. 6. **Species-specific signal transduction upon lipopeptide challenge of human and murine macrophages.** MonoMac6 (human) and RAW264.7 (murine) macrophages were challenged with stimulants at increasing concentrations. *Lane 1*, unstimulated; *Lanes 2–4*, Lau_3CSK_4 (10 ng/ml, 100 ng/ml, and 1 μg/ml); *Lanes 5–7*, P_2CSK_4 (10 ng/ml, 100 ng/ml, and 1 μg/ml); *Lanes 8* and *9*, LPS and P_3CSK_4 (10 μg/ml), respectively, for 2 h for analysis of NF-κB activation by electrophoretic mobility shift analysis (*A*, *arrow*, specific NF-κB-DNA complexes) or for 30 min for analysis of mitogen-activated protein kinase phosphorylation by immunoblot analysis as indicated (*B*, +, phosphorylated kinase; −, non-phosphorylated kinase as control), respectively.

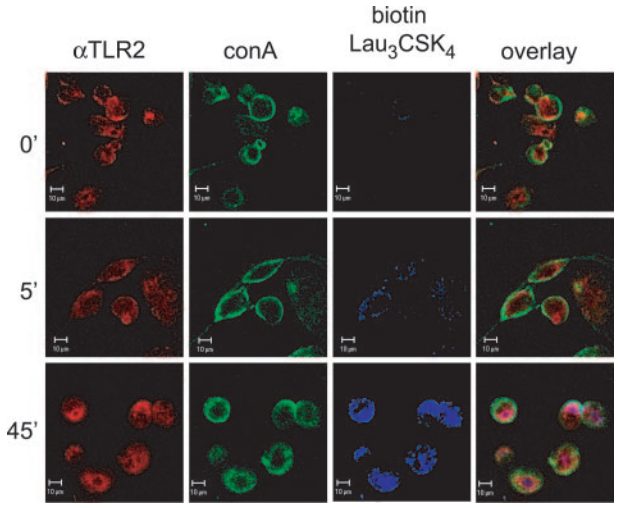

FIG. 7. **Time-dependent uptake of Lau_3CSK_4 to THP1 cells.** THP1 (human) macrophages were either left untreated (0′) or challenged with biotinoylated Lau_3CSK_4 for the time periods indicated before washing and fixation. Subsequently, TLR2 (*αTLR2*), cell surfaces (*conA*, fluorescein-labeled concanavalin A), and biotinoylated Lau_3CSK_4 (*biotin Lau_3CSK_4*) were stained. All three signals were superimposed (*overlay*). *Bar* corresponds to a distance of 10 μm on original slide.

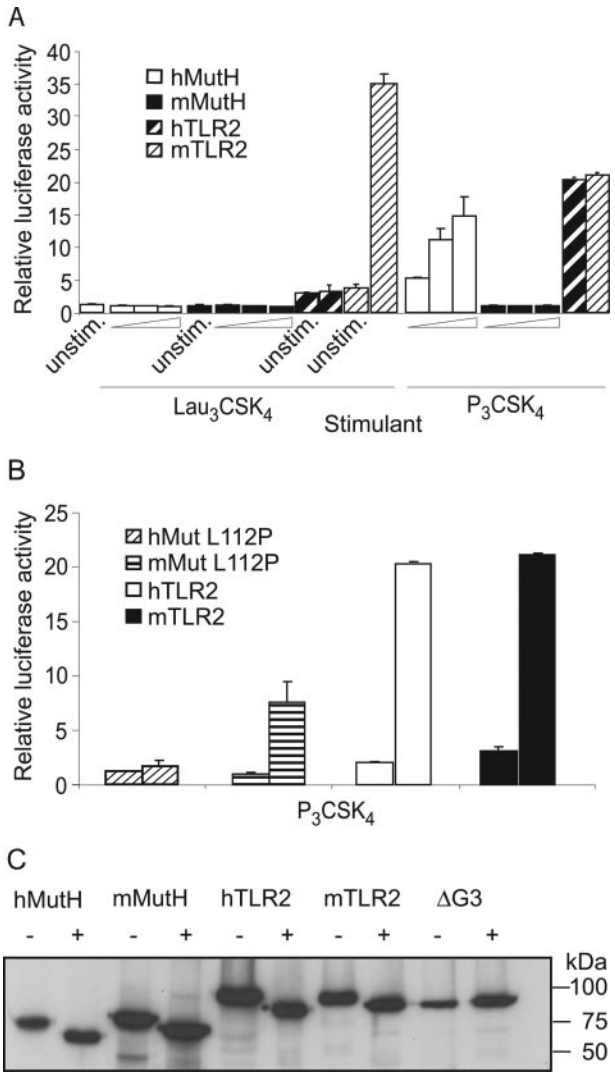

FIG. 8. **Comparative analysis of human and murine TLR2 mutant construct activity and *N*-glycosylation.** Human and murine mutant TLR2 constructs, either lacking the first seven LRRs (*A*, *mutH*; *unstim.*, unstimulated) or carrying a point mutation in the consensus core sequence of LRR3 (*B*, *L112P*), were analyzed by NF-κB-dependent reporter gene assay, whereas wild-type receptors were used as controls. Lipopeptides, as indicated, were applied at concentrations of 100 ng/ml, 1 μg/ml, and 10 μg/ml or 1 μg/ml only (*A*), as well as 1 μg/ml (*B*, each *first column* represents *unstim.* control). Both human and murine overexpressed and immunoprecipitated MutH constructs, as well as a murine construct lacking all three putative *N*-glycosylation sites through point mutation (ΔG3) and wild-type constructs, were treated with peptide:*N*-glycosidase F and comparatively analyzed by immunoblot analysis upon PAGE (*C*; −, untreated; +, *N*-specific amidase treated).

weights were as expected (Fig. 8*C* and data not shown). As mentioned above, both h and mTLR2 primary sequences contain putative *N*-glycosylation sites, the localization of which within h and mTLR2ECD sequences differs. *N*-specific deglycosylation of both wild-type TLR2 proteins attenuated their apparent molecular weights to that of the mutant TLR2, in which all three glycosylation sites had been mutated (Fig. 8*C*). Significant size reduction through *N*-deglycosylation was also evident for h and mTLR2 constructs lacking the N-terminal third of their LRR-rich domains (Fig. 8*C*).

DISCUSSION

The structural requirements for the known species-specific LPS and Taxol recognition through murine and human TLR4-MD2 complexes have been analyzed previously by domain-exchange between the respective TLR4 proteins. These data

implicated a non-conserved region in species-specific pattern recognition through TLR4-MD2 (35). We aimed at the identification of species-specific ligands for TLR2 and analysis of the respective structure-function relationship. Our results identify lauroylated lipopeptides as a species-specific TLR2-agonist. They further indicate that LRR8 and the surrounding region spanning from LRR7 to LRR10 control species-specific ligand recognition.

Comparative analysis of a variety of distinct microbes for their potential to activate hTLR2 or mTLR2 did not reveal significant differences. This indicates that expression of a set of TLR2 agonists by a given microorganism overcomes species specificity of individual TLR2 ligands. This assumption, however, does not exclude differences in the potential of individual TLR2 ligands. Furthermore, incubation with heat-inactivated bacterial cells is not the same as confrontation of host cells with bacterial products that occurs upon lysis, upon treatment with antibiotics, or inside phagosomes/lysosomes in cells of infected host organisms. TLR2 agonists integrated into the cell wall or residing in the bacterial cytoplasm might reach TLR2 only under specific conditions. Additionally, digestive processes catalyzed either by enzymes released from disintegrating microbial cells themselves or provided by the host organism might change the chemical properties of microbial products and TLR specificity. For instance, lipases might degrade acyl moieties of immunostimulatory lipopeptides as exemplified by neutralization of LPS through deacylation (36). Furthermore, individual bacterial species might produce distinct molecular variants of one microbial product, such as lipopeptide, under specific conditions. For instance, distinct LPS species are synthesized by bacteria under specific growth conditions (35).

Considering such chemical modification of immunostimulatory bacterial products during infection, we analyzed distinct synthetic analogs of lipopeptides. The lipopeptides used contained distinct acylations in respect to palmitoyl-moiety numbers (37) or acyl chain lengths (Fig. 3*A*). Although analysis of the first did not show species specificity of TLR2 (data not shown), analysis of the latter set of lipopeptides indicated that the analog Lau_3CSK_4 (Fig. 3*A*) lacking 4 CH_2-groups (12 C-atoms, lauroyl) in each acyl chain as compared with P_3CSK_4 carrying 16 C-atoms per acyl chain was recognized specifically through mTLR2. This finding was corroborated by use of HEK293 cells and *TLR2* $^{-/-}$ MEFs transfected with hTLR2 or mTLR2 variants (Fig. 3, *B* to *E*).

Comparative analysis of wild-type mTLR2 and a mTLR2ECD-hTLR2ICD construct demonstrated equal potential of both human and murine ICDs to mediate intracellular signaling, such as NF-κB-signaling (Figs. 2 and 3). Exchange of a region encompassing the N-terminal half of the LRR-rich domain conferred Lau_3CSK_4 responsiveness to hTLR2 and diminished but did not abrogate mTLR2 activity. Exchange of LRRs 7 to 10 only was sufficient to mediate activation to a similar degree, whereas exchange of mLRR8 by its human counterpart resulted in "loss of function" of mTLR2. These results attribute a central role to murine LRR8 and its surrounding LRRs in cellular recognition of lauroylated peptides. In contrast, exchange of low-conserved LRRs 14 to 18, or of 19 and 20, did not affect activity to a detectable degree. The exchange of LRR19 and 20 in hTLR2 unexpectedly resulted in complete loss of function. When overexpressed, the construct hm_{19-20}/h was detectable by surface staining and flow cytometry at similar levels as active TLR2 fusion constructs (data not shown), indicating that disruption of its regular integrity was causative for malfunction of this hTLR2 variant.

Specific binding of P_3CSK_4 and TLR2 (31) suggests direct interaction also of TLR2 and Lau_3CSK_4. In this respect, high exchange rates of ligand molecules at binding sites of the receptor or differences in affinities could explain lack of detectable competition between Lau_3CSK_4 and P_3CSK_4 for binding to hTLR2 (Fig. 4*A* and data not shown). However, mAb-mediated inhibition, not only of Lau_3CSK_4-dependent activation of $mTLR2^+$ cells but also of high-dose Lau_3CSK_4, induced activation of cells expressing hTLR2, which suggests that both analogs bind to a single site in the TLR2ECD (Fig. 4, *C* and *B*, respectively). Alternatively, the mAb used might block several distinct sites in TLR2 involved in lipopeptide recognition either directly or indirectly.

Both murine RAW264.7 and human MonoMac6 macrophages expressed cell surface and intracellular TLR2 and responded equally well to P_3CSK_4 and LPS. In line with the results discussed above, murine macrophages responded to Lau_3CSK_4 challenge. Only when Lau_3CSK_4 was applied at one hundred- or thousand-fold increased concentrations did human macrophages respond, as indicated by detectable cytokine release and intracellular signal transduction (Figs. 5 and 6). These results confirm species specificity of Lau_3CSK_4 recognition.

Time-limited proximity of TLR2 to zymosan particles bearing TLR2 agonist β-glycan, as well as efficient uptake and processing of a TLR2-specific antibody, have been demonstrated recently (38, 39). However, uptake of P_3CSK_4 and Lau_3CSK_4 by human macrophages, as well as murine wild-type and *TLR2* $^{-/-}$ macrophages, was indistinguishable (Fig. 7 and data not shown), implicating a TLR2-independent lipopeptide uptake receptor. Cellular lipid receptors, such as CD14, are candidates for this function (40). Recently, saturated and free lauric acid has been shown to activate TLR2. We propose that free lauric acid activates cells specifically through mTLR2 used in that study (41). Lauric acid might represent a metabolic intermediate resulting from degradation of microbial lipoproteins in the host organism.

Comparison of two different types of mutant TLR2 constructs based on both wild-type hTLR2 and mTLR2 showed functional details of TLR2 species specificity. A hTLR2 mutant lacking an N-terminal portion of the LRR-rich domain conferred responsiveness specifically to P_3CSK_4, whereas the analogous murine construct did not. Notably, Lau_3CSK_4 signals were mediated through neither construct, even when this lipopeptide was applied at high concentrations. This suggests a distinctive mechanism of TLR2-dependent P_3CSK_4 and Lau_3CSK_4 recognition. In contrast, a point mutation identified by random mutagenesis and localized within the consensus sequence of the third LRR did not abrogate P_3CSK_4 signaling through mTLR2, whereas cells overexpressing the analogous human construct were unresponsive to P_3CSK_4 challenge. Results of *N*-glycosylation analysis indicate similar *N*-glycosylation of both TLR2 proteins because a constitutively non-*N*-glycosylated construct resembled the size of the *N*-deglycosylated wild-type proteins. More pronounced size reduction of wild-type and mutant hTLR2 as compared with mTLR2 through *N*-deglycosylation might be explained by the potential presence of an additional *N*-glycosylation site within the seventh LRR in the human TLR2ECD but not in mTLR2. Specific *N*-glycosylation is involved in TLR2 surface expression (34). Furthermore, *N*-glycosylation is important for signaling, and sequence-specific differences of TLR2ECDs were also evident from analysis of a variety of monoclonal antibodies raised against mTLR2ECD protein.[3] Only one of the mAbs analyzed recognized TLR2 of both species (31). Our results demonstrate species-specific characteristics of TLR2 and strongly suggest that these differences provide a molecular basis for distinct susceptibilities of humans and mice to specific infections.

[3] G. Meng, A. Grabiec, and C. J. Kirschning, unpublished results.

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