Nadra Jesmine Nilsen

TOLL-LIKE RECEPTOR 2 EXPRESSION, REGULATION AND SIGNALING

Thesis for the degree of doctor philosophiae

Trondheim, June 2008

Norwegian University of
Science and Technology
Faculty of Medicine
Department of Cancer Research and Molecular Medicine

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TOLL-LIGNENDE RESEPTOR 2
UTTRYKK, REGULERING OG SIGNALISERING

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TOLL-LIGNENDE RESEPTOR 2 UTTRYKK, REGULERING OG SIGNALISERING

Den første responsen mot infeksiøse mikroorganismer er koordinert av det medfødte immunforsvaret. Cellene i dette systemet er ansvarlig for det første steget i fjerning av mikroorganismer, og modulering av den adaptive immunresponsen. Toll- lignende reseptorene (TLR) er en familie på 13 signalreseptorer (TLR1-13) som uttrykkes på immunceller som monocytter/makrofager, granulocytter og dendritiske celler. TLRene er essensielle i gjenkjenningen av en rekke komponenter fra invaderende mikroorganismer og for indusering av inflammatorisk respons mot disse organismene.

Den best karakteriserte TLR er TLR4 som er signalreseptoren for endotoksin (også kalt lipopolysakkarid (LPS)). LPS finnes i ytterveggen til Gram- negative bakterier, og er en viktig virulensfaktor for Gram- negative bakterieinfeksjoner. TLR2 gjenkjenner Gram-positive bakterier, lipoproteiner og lipoteikoinsyre (LTA) som også er uttrykt i celleveggen til bakterier. Gjenkjennelse av disse komponentene via TLRene er viktig for at kroppen skal oppdage begynnende infeksjoner og sette i gang responsene som er viktig for fjerning av infeksjon.

I dette studiet har vi studert TLR2 uttrykk, regulering og signalisering. Vi fant TLR2 uttrykt på plasma membranen til immunceller og at reseptoren er oppregulert i respons på svært lave konsentrasjoner av stimuli. Vi har også studert uttrykket av TLR2 inni humane monocytter, der vi fant TLR2 uttrykt i tidlige og resirkulerende endosomer og samt i lysosomer. Reseptorene CD14 og CD36 er vist å fungere som ko-reseptorer for TLR2 og vi viser at disse er viktige for at humane monocytter skal kunne internalisere TLR2 liganden LTA, samt forsterke TLR2 signalisering. TLR2 og ko-reseptorene CD14 og CD36 er høyt uttrykt på plasmamembranen til monocytter. Vi viser videre at signalisering initieres hovedsakelig ved binding av ligand til plasmamembranen og er i stor grad uavhengig av internaliseringen av LTA. Videre viser vi at TLR2 har en rolle i antigen-presentasjon og initiering av adaptiv immunitet.

Vi fant at TLR2 på celleoverflaten oppreguleres i respons på en rekke stimuli. Oppreguleringen av TLR2 i respons på lave konsentrasjoner av lipoprotein forsterket responsen av sekundære stimuli, noe som samsvarte med oppregulering av TLR2. Oppregulering av TLR2 kan derfor være viktig for å forsterke responsen mot lave konsentrasjoner av stimuli og for bekjempelse av begynnende infeksjon. I respons på høyere konsentrasjoner av prestimuli, ble imidlertid toleranse indusert i respons på sekundær stimuli, til tross for oppregulert TLR2. Signalveiene som induserer oppregulering av TLR2 ble derfor videre studert. TLRene signaliserer via fire intracellulære signal adaptor molekyler; MAL, MyD88, TRIF og TRAM. TLR4 kan signalisere via et MAL/MyD88 avhengig spor, som fører til aktivering av transkripsjonsfaktoren NF-κB og induksjon av proinflammatoriske cytokiner. TLR4 kan også signalisere via et TRAM/TRIF avhengig spor, som aktiverer transkripsjonsfaktoren IRF3 og induserer interferon (IFN)-β. TLR2 har hittil vært kjent å kun signalisere via MAL/MyD88 sporet. Vi viser imidlertid at begge sporene regulerer TLR2 overflateuttrykk på makrofager i respons på TLR4 liganden LPS. Overraskende nok fant vi en ny rolle for TRAM/TRIF signalveien i TLR2 signalisering. Denne signalveien var viktig for induksjon av chemokinet RANTES (CCL5), men ikke for induksjon av det proinflammatoriske cytokinet TNF.

Resultatene i denne avhandlingen gir ny forståelse og innsyn i TLR2 uttrykk, regulering og signalisering, som vi mener kan være viktig for utviklingen av vaksiner og immunmodulerende medisiner for behandling av akutt- og kronisk inflammasjon. Resultatene bidrar også til å øke vår forståelse av hvordan det medfødte immunforsvarets bekjemper infeksjoner.

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EXPRESSION, REGULATION AND SIGNALING

Dr. Philos. Thesis

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CONTENTS

ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AP-1	Activator protein 1
APC	Antigen presenting cell
ATP	Adenosine triphosphate
B DNA	Bacterial DNA
CBP	CREB-binding protein
CCL5	CC-chemokine ligand 5
CCR	Chemokine receptor
CKII	Casein kinase II
CpG	deoxycytidyl-deoxyguanosine
CREB	Cyclic-AMP-responsive-element binding protein
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ds	Double-stranded
ER	Endoplasmatic reticulum
FRAP	Fluorescence recovery after bleaching
FRET	Fluorescence resonance energy transfer
FSL-1	Fibroblast-stimulating lipopeptide 1
GPI	Glycoylphosphatidylinositol
HCMV	Human cytomegalovirus
HEK	Human epithelial kidney
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSP	Heat shock protein
HSV	Herpes simplex virus
hToll	Human Toll (also known as TLR4)
IFN	Interferon
IFNRI	Interferon receptor I
IKK	IκBα kinase complex
IL	Interleukin
IP-10	Interferon-inducible protein-10
Ipaf	IL-1β -converting enzyme-protease-activating factor
IPS	IFN-β promoter stimulator (also known as MAVS/Cardiff/VISA)
IRAK	Interleukin-1 receptor associated kinase
IRF	IFN regulatory factor
ISG15	IFN-stimulated gene 15
ISGF3	IFN-stimulated gene factor 3
ISRE	Interferon stimulating response element
JNK	Jun N-terminal kinase
LBP	LPS-binding protein
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LTA	Lipoteichoic acid
mAb	Monoclonal antibody
MAL	Myeloid adapter-like protein (also known as TIRAP)
MALP-2	Macrophage activating lipopeptide 2
MAPK	Mitogen-activated protein kinase
MAPKKK	MAPK kinase kinase

MBL	Mannan-binding lectin
mCD14	Membrane bound CD14
MDA5	Melanoma differentiation-associated gene 5
mDC	Myeloid DC
MEF	Mouse embryonic fibroblast
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MR	Macrophage-mannose receptor
MyD88	Myeloid differentiation factor 88/Myeloid differentiation primary response gene 88
NALP	Nacht domain-, leucine-rich repeat- and PYD-containing protein
NAP1	NF-kappaB-activating kinase (NAK)-associated protein 1
NDV	Newcastle disease virus
NF-κB	Nuclear Factor–κB
NK	Natural killer cell
NLR	NOD-like receptor
NOD	Nucleotide oligomerization domain
Pam_3CysSK_4	S-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys_4-OH
PAMP	Pathogen-associated molecular pattern
pDC	Plasmacytoid dendritic cell
PGN	Peptidoglycan
PI3K	Phosphoinositide-3 kinase
PMN	Polymorphonuclear
polyIC	polyinosine-polycytidylic acid
PRR	Pathogen-recognition receptor
PYD	pyrin N-terminal homology domain
R848	Resiquimod
Rac1	Ras-related C3 botulinum toxin substrate 1
RANTES	Regulated upon activation, normal T cell expressed and secreted/ CCL-5
RHD	Rel homology domain
RIG-1	Retinoic acid Inducible gene-1
RIP	Receptor interacting protein
RNA	Ribo nucleic acid
RSK1	Ribosomal S6 kinase 1
SARM	Sterile alpha and HEAT-Armadillo motifs containing protein
sCD14	Soluble CD14
ss	Single-stranded
STAT	Signal transduced and activator of transcription
SV	Sendai virus
TAB	TAK1 binding protein
TAK1	TGFß–activated kinase 1
TANK	TRAF family member associated NF-κB activator
TBK1	TANK binding kinase 1 (also known as NAK or T2K)
TGF	Transforming growth factor
T_H	T helper
TICAM	TIR-containing adaptor molecule
TIR	Toll-IL-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	TNF receptor
TRAF	TNFR-associated factor
TRAM	TRIF-related adapter molecule/TICAM-2
TRIF	TIR-domain containing adaptor inducing protein inducing IFN-β/ TICAM-1
VRE	Virus-responsive element

1. INTRODUCTION

The immune system is a collection of cellular and humoral components which serve to discriminate between self- and non-self and protect the host against invading organisms, as well as eradicate malignant and foreign cells. The immune system can be divided into the innate and adaptive system. The innate immune system provides the host with an immediate, broad, first-line defense against a range of foreign components. Adaptive immunity provides a slower, but more specific response, involving clonal expansion of antigen-specific effector cells which combat infection and provide immunological memory. Although innate and adaptive immunity evolved separately, the efficiency of the overall immune response depends on complex interplay and regulation between the two systems.

1.1 PATTERN RECOGNITION RECEPTORS

The initial response to an invading organism is coordinated by the innate immune system. Innate immune signals then play a critical role in initiating and instructing the adaptive immunity. The main players in innate immunity are phagocytes, such as neutrophils, macrophages and dendritic cells (DCs). These cells discriminate between pathogens and self via pathogen-recognition receptors (PRRs); germ-line encoded receptors that play a crucial role in the innate host defense. PRRs recognize evolutionary conserved pathogen-associated molecular patterns (PAMPs), rare or absent in vertebrates. These patterns have alternatively been denoted microbe-associated molecular patterns (MAMPs), since these patterns neither define, nor are exclusively derived from pathogens. PRR-mediated recognition of pathogens by phagocytes triggers engulfment, killing and digestion of invading microbes, and initiation of a cascade of inflammatory responses. The pattern of responses induced by PRRs depends on the origin of the PAMP and which PRRs are activated. PRRs are also activated by endogenous self-molecules associated with cellular stress and damage of host cells. These endogenous stress signals, often termed damage-associated molecular patterns (DAMPs) include nucleic acids released from damaged cells, heat shock proteins (HSPs), interferon (IFN)-α (an important mediator in host-response to viral infection), CD40-L (a surface molecule on activated platelets and activated T cells), and decomposition products of hyaluron[3].

Secreted PRRs function as opsonins by binding microbes, and promoting activation of the complement system and recognition by phagocytes. Secreted PRRs include complement receptors, collectins, pentraxin proteins (i.e. serum amyloid) and C-reactive protein. One of

the best characterized secreted PRRs is mannan-binding lectin (MBL), a member of the calcium-dependent lectin family, which binds to a wide range of bacteria, viruses, fungi and protozoa. The MBL predominantly recognizes certain sugar groups on the surface of microorganisms, but also binds phospholipids, nucleic acids and non-glycosylated proteins[4].

Endocytic PRRs are expressed on the surface of phagocytes. These receptors efficiently bind PAMPs and are an essential part of the clearance of bacteria from circulation by triggering internalization of pathogens and targeting these for lysosomal destruction. Examples of endocytic PRRs include the macrophage-mannose receptor (MR) and scavenger receptors. The MR is a member of the calcium-dependent lectin family which specifically recognizes carbohydrates with large numbers of mannoses. The receptor binds both microbial glycans and self-glycoproteins and initiates phagocytosis by macrophages.

Signaling PRRs activate signal transduction pathways and induce the expression of a variety of immune response genes following PAMP recognition. Signaling PRRs include transmembrane and cytoplasmic signaling PRRs. The cytoplasmic signaling PRRs consist of nucleotide oligomerization domain (NOD) -like receptors (NLRs) and the RNA helicases retinoic acid inducible gene-1 (RIG-1) and melanoma differentiation-associated gene 5 (MDA5), while the Toll-like receptor (TLR) family (See **Chapter 1.2**) are transmembrane signaling PRRs.

The best studied NLRs are NOD1 and NOD2. These specifically recognize subcomponents of peptidoglycan (PGN)[5-7], and induce nuclear factor-κB (NF-κB) activation through recruitment and oligomerization of receptor-interacting protein (RIP) 2[8, 9]. Other NLRs include nacht domain-, leucine-rich repeat- and PYD-containing protein (NALP)1, NALP3 and interleukin (IL) -1β-converting enzyme-protease-activating factor (Ipaf), which all have been implicated in the activation of the inflammasomes[10-12]. Inflammasomes are cytoplasmic multiprotein complexes that mediate the activation of the inflammatory caspases-1 and -5 required for the cleavage of pro-IL-1β and pro-IL-18 and formation of mature IL-1β and IL-18 for release [11]. NALP3 is activated by stimuli such as bacterial RNA, bacterial toxins, ATP and uric acid crystals[13]. Ipaf is involved in inflammasome assembly following infection with *Salmonella typhimurium* and is required for cytosolic flagellin to activate caspase-1 and induce IL-1β[11, 14-16].

The RNA helicases RIG-1 and MDA5 are cytoplasmic signaling sensors of double-stranded RNA (dsRNA)[17, 18]. RIG-1 recognizes viruses, such as Newcastle disease virus (NDV),

vesicular stomatitis virus (VSV) and Sendai Virus (SV)[19, 20], while MDA5 is required for picornavirus detection and dsRNA-induced IFN-α production in mouse embryonic fibroblasts (MEF)s and myeloid DCs (mDC)s[21]. Activation of RIG-1 and MDA5 induces recruitment of the adapter protein IFN-β promoter stimulator (IPS)-1, activation of the transcription factors interferon regulatory factor (IRF) -3 and NF-κB, and subsequent induction of type I IFN[22].

1.2 TOLL-LIKE RECEPTORS

TLRs are transmembrane signaling PRRs which are crucial for immune recognition of a number of PAMPs and DAMPs. TLR activation results in the induction of pro-inflammatory cytokines (i.e. chemokines and type I IFNs) and upregulation of co-stimulatory molecules; important mediators in both innate immunity and in initiation of adaptive responses.

1.2.1 Discovery

The Toll gene was first discovered in the fruit fly *Drosophila melanogaster* by Christiane Nüsslein-Volhard and co-workers[23, 24]. During a screening for lethal zygotic mutations that affected embryonic patterning, they came across an extraordinary dominant and ventralized phenotype in fly embryos. Upon this discovery Nüsslein-Volhard is said to have exclaimed "Toll!", which means "fantastic" in German slang. The mutated gene thus became known by this name. Together with Eric Wieschaus and Edward B. Lewis, she received the Nobel Prize in Physiology or Medicine in 1995 for their research on genetic control of embryonic development. Jules Hoffman and colleagues later showed that the Toll gene was important for the flies' resistance to fungal infection[25].

The first human homolog of the Toll receptor was described by Nomura and colleagues in 1994[26], and mapped to chromosome 9q32-33 by Taguchi and colleagues in 1996[27]. In 1997, Charles Janeway and Ruslan Medzhitov suggested that the human homolog hToll (now known as TLR4) induced activation of NF-κB and induction of proinflammatory cytokines and costimulatory molecules[28]. This was done by constructing a constitutively active CD4/hToll chimera, since the Toll ligand was unknown at the time. In 1998, Bruce Beutler and colleagues discovered the function of the hToll gene[29]. They showed that C3H/HeJ mice were unresponsive to lipopolysaccharide (LPS) due to a proline to histidine point-mutation at proline 712 in the TIR domain of TLR4, identifying TLR4 as a key receptor for LPS, and suggesting that other TLRs might detect microbe signature molecules. Shizuo Akira and colleagues have later contributed considerably to revealing the functions of the other TLRs through the generation of an extensive collection of mice with targeted deletions of TLRs and TLR signaling proteins[30].

1.2.2 Introduction to the TLR family

Thirteen mammalian TLRs, TLR1-13, have been identified to date[31]. Different microbial structures are recognized by different TLRs (**Figure 1**). LPS from Gram-negative bacteria is recognized by TLR4[29, 32] (described in Chapter 1.2.5). Gram-positive bacteria activate TLR2, while bacterial components, such as lipopeptides and lipoteichoic acid (LTA) are recognized by TLR2 in cooperation with TLR1 or TLR6[33-39] (described in Chapter 1.2.3). Viral and/or bacterial nucleic acids are recognized by TLR3, TLR7, TLR8 and TLR9 (described in Chapters 1.2.4 and 1.2.7). TLR3 recognizes viral dsRNA and synthetic polyinosine-polycytidylic acid (polyIC), while viral single-stranded RNA (ssRNA) and the antiviral compounds imiquimod and resiquimod (R848) are ligands for TLR7 and TLR8[40-43]. Bacterial DNA (B DNA) and the synthetic unmethylated oligonucleotides containing CpG-dinucleotides (CpG) are recognized by TLR9[44]. TLR5 is activated by flagellin from bacterial flagella[45]. TLR11 recognizes uropathogenic bacteria and a protozoan-derived profilin-like protein[46,47] (Chapter 1.2.8). The ligands for TLR10, TLR12 and TLR13 are currently unknown[30] (Chapter 1.2.3 and 1.2.8).

The TLRs are transmembrane proteins that traverse cellular membranes; either the plasma membrane or intracellular vacuolar membranes. Their common structure consists of an extracellular leucine-rich repeat (LRR) domain and a cytoplasmic domain, sharing homology with the mammalian IL-1 receptor[48, 49]. The LRR domain binds ligands, while the cytoplasmic Toll-IL-1 receptor (TIR) domain initiates intracellular signaling pathways through homotypic protein-protein interaction with TIR-adapter molecules[50]. Four TIR-adapter molecules have been shown to mediate TLR signaling; myeloid differentiation factor 88 (MyD88), myeloid adapter-like protein (MAL), TIR-domain containing adaptor inducing protein inducing IFN-β (TRIF) and TRIF-related adapter molecule (TRAM) (**Figure 1**). In TLR4 signaling MyD88 and MAL form one pathway, resulting NF-κB activation and production of inflammatory cytokines[51-56]. TRIF and TRAM form the second pathway leading to activation of IRF-3 and induction of Type I IFNs[57-59]. TLR2 signaling utilizes both MAL and MyD88 in a manner similar to TLR4[55, 56], while TLR7/8 and TLR9 require only MyD88 for signaling[30]. TLR3 signaling requires TRIF alone, and is the only TLR known to signal in a MyD88-independent manner[60]. A fifth TIR-adapter SARM (sterile alpha and HEAT-Armadillo motifs containing protein) may interact with TRIF and inhibit its function[61]. However, cells from animals deficient in SARM do not display altered TLR signaling[62]. In contrast, SARM is expressed primarily in neurons, where it is shown to mediate stress-induced neuronal toxicity.

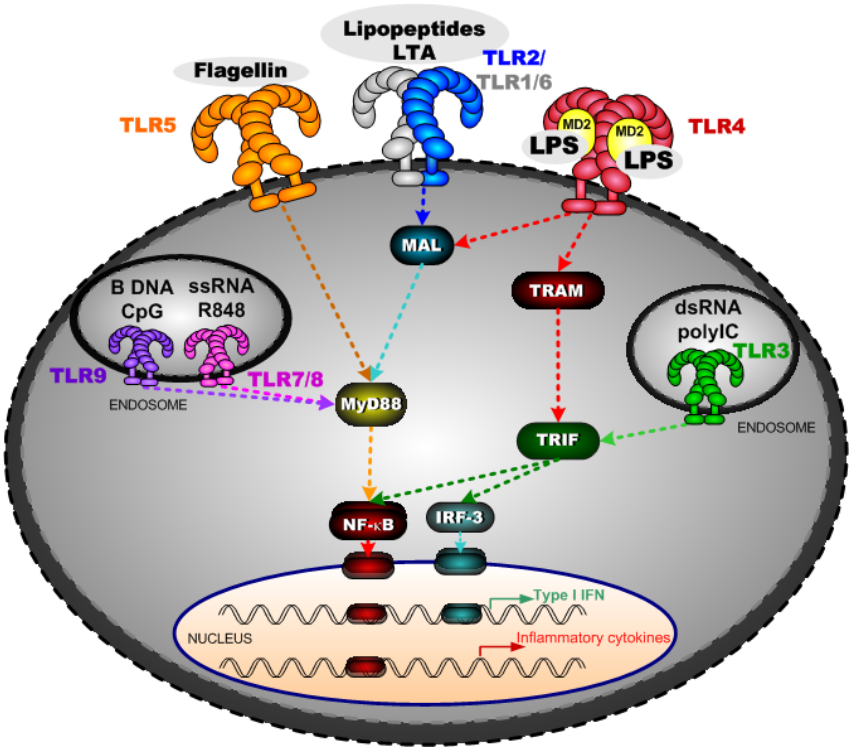

FIGURE 1: Overview of selected members of the TLR family, their ligands and the TIR-adapters they utilize upon activation.

1.2.3 The TLR2 subfamily

TLR1, TLR2, TLR6 and TLR10 are commonly categorized into the TLR2 subfamily, based on chromosomal localization, genomic structure and amino acid sequences. The genes encoding TLR1, TLR6 and TLR10 genes are located closely on chromosome 4p14, while tlr2 maps to 4q32[26, 48, 63-65].

TLR2 recognizes Gram-positive bacteria, such as *Staphylococcus aureus* (*S. aureus*) and *Mycobacteria,* as well as cell wall components such as LTA, peptidoglycan and lipoproteins[33-35, 66-68]. Additional TLR2 ligands may include zymosan, glycolipids from spirochetes, lipoarabinomannan and porins from *Neisseria*, among others[30, 69]. TLR2-deficient mice fail to respond to purified lipoproteins from Gram-positive bacteria, but are more susceptible than wild-type mice to septicemia due to *S. aureus*, meningitis due to *S. pneumoniae* and *L. monocytogenes* and infection with *M. tuberculosis*[70-72]. An Arg753Gln polymorphism in human TLR2 also confers significantly lower responsiveness to bacterial lipoproteins derived from *M. tuberculosis, B. burgdorferi*, and *T. pallidum*[73, 74], and may predispose to *Staphylococcus* infection and tuberculosis susceptibility[73-75]. The Arg677Trp polymorphism in TLR2 is shown to impair activation of NF-κB in response to

Mycobacterium leprae and *Mycobacterium tuberculosis* and may enhance susceptibility to leprosy and tuberculosis[76]. TLR2 has also been implicated in diseases such as rheumatoid arthritis and atherosclerosis[50, 75].

The ability of TLR2 to recognize a wide repertoire of ligands is partially explained by heterodimerization with TLR1 or TLR6. TLR2/TLR1 heterodimerization occurs in response to triacylated lipopeptides, such as Pam_3CysSK_4[77, 78], as well as in response to *Borrelia burgdorferi* outer surface protein A[79]. On the other hand, optimal response towards diacylated lipopeptides is attained by heterodimerization of TLR2 with TLR6[37, 80]. LTA is also recognized by the TLR2/TLR6 heterodimer, supposedly due to the two diacyl chains in the molecule[39]. TLR2 is furthermore shown to mediate cytokine responses towards viral components such as the envelope proteins of human cytomegalovirus (HCMV) and Herpes Simplex virus (HSV)-1, as well as measles virus hemagglutinin[81-83].

The crystal structure of the ectodomain of both the human TLR2/TLR1-lipopeptide complex and the mouse TLR2-lipopeptide complex have recently been published[1] (**Figure 2**). The ectodomain of TLR2 has a three-domain architecture consisting of an N-terminal, a central, and a C-terminal subdomain with unusual β-sheet conformations. The lipopeptide-binding site of TLR2 consists of a large internal pocket found on the convex region of TLR2, at the border between the central and C-terminal domdain. The lipid chains of a single Pam_3CysSK_4 molecule mediates the heterodimerization of TLR2 and TLR1 through insertion of its two esterbound lipid chains into the pocket in TLR2, while the amide-bound lipid chain inserts into a hydrophobic channel in TLR1. The lipid-binding channel of TLR1 is located in the same unusual region as in TLR2. The glycerol

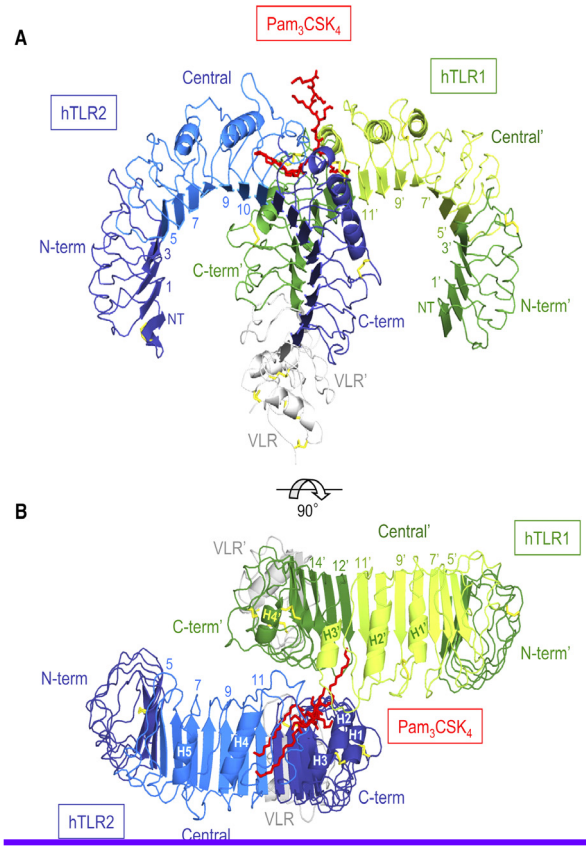

FIGURE 2: Crystal structure of TLR2/TLR1 in complex with Pam_3CySK_4.

Proposed structure of the ectodomains of TLR2 (blue) and TLR1 (green) in complex with the lipopeptide Pam_3CysSK_4 (red). The central domains are shown in light green or light blue. Disulfide bridges are represented as yellow lines. Domains belonging to the TLR1 hybrid proteins are labeled with apostrophes. **(A)** side view; **(B)** top view (from Kim *et al*, 2007)[1]. TLR2 and TLR1 were crystallized using the Hybrid LRR Technique involving fragments of TLR and hagfish were fused a clone of the hagfish Variable Lymphocyte Receptors (VLRs)[1].

backbone of the lipopeptide is placed in a narrow opening formed where the TLR1 and TLR2 pockets join. An extensive network of hydrogen-bonds, as well as hydrophobic interactions between TLR1 and TLR2 further stabilize the heterodimer[1]. Notably, no lipopeptide was observed in complex with only TLR2 or TLR1 alone.

TLR1 and TLR6 share 56% sequence identity. Using the structure of TLR1 as a template Jin *et al* modeled the structure of TLR6[1]. Their analysis suggests that two bulky phenylalanines block the potential lipid-binding channel in TLR6. Consequently, tri-acylated lipopeptides should not be able to interact with TLR6.

Early studies on leukocytes revealed that TLR2 mRNA is expressed in monocytes, polymorphonuclear (PMN) cells and dendritic cells (DC), while TLR1 mRNA is additionally expressed in T lymphocytes and NK cells[84]. Additional studies showed that TLR2 is expressed on the cell surface of monocytes and granulocytes, while lymphocytes expressed low levels of TLR2[84, 85]. TLR1 is also expressed on the cell surface in monocytes, DC and neutrophils[78, 86, 87]. TLR6 is expressed on the cell surface of monocytes, myeloid DCs (mDC), and neutrophils, but not on B, T, or NK cells[88]. Human TLR10 is expressed in B cells and pDC and is shown to homodimerize, as well as heterodimerize with TLR2 and TLR1[89]. Ligands for TLR10 remain unknown. TLR10 in mice is truncated and non-functional, however, the full gene is presumably expressed in rats[89].

1.2.4 TLR3

The human *tlr3* gene is located at 4q32. TLR3 recognizes dsRNA produced by RNA viruses during replication. TLR3 also recognizes the viral dsRNA mimic polyIC. Activation of TLR3 results in the production of anti-viral cytokines such as IFN-β[40]. TLR3 is expressed in immune cells such as conventional DCs[84, 86, 90], natural killer (NK) cells[91-93] and mast cells[94, 95]. TLR3 expression differs between species, since murine macrophages express TLR3[40, 96], while human macrophages do not[84]. Other cell types, i.e. fibroblasts and epithelial cells express TLR3 as well[30, 97]. In contrast to TLR2 which is expressed at the cell surface, TLR3 is expressed intracellularly in most cells. In dendritic cells TLR3 is expressed in the endoplasmatic reticulum (ER) and is recruited to endosomes upon stimulation with dsRNA[98].

1.2.5 TLR4

The *tlr4* gene maps to chromosome 4 in mice[99] and to chromosome 9q32-33 in humans[48]. TLR4 is expressed on immune cells, including monocytes, DCs and PMNs[84, 100], but is also

expressed on a number of other cell types, such as osteoblasts[101], endothelial cells[102, 103], adipocytes[104], Kupffer cells[105], keratinocytes[106], and epithelial cells[107, 108].

TLR4, in complex with the small secreted glycoprotein MD-2, recognizes LPS from the outer membrane of Gram-negative bacteria[29, 32]. Soluble CD14 (sCD14) and LPS-binding protein (LBP) initially bind LPS and transfer the ligand to membrane bound CD14 (mCD14). mCD14 further presents LPS to the TLR4/MD-2 signaling complex[109-113]. MD-2 is responsible for binding LPS and is required for TLR4-signaling in response to LPS [114-116]. Signaling by the TLR4/MD2 complex is believed to predominantly occur at the plasma membrane[117, 118]. The LPS/TLR4/MD-2 complex has further been shown to shuttle between the plasma membrane and the Golgi, independent of signaling[119]. In addition to LPS, TLR4 has been implicated in the recognition of taxol and endogenous ligands such as heat shock protein (HSP)60, HSP70, the extra domain A of fibronectins, oligosaccharides of hyaluronic acid, heparan sulfate and fibrinogen. High concentrations of all these endogenous ligands are, however, required to activate TLR4, in contrast to LPS which stimulates TLR4 at nanogram to picogram per milliliter concentrations[120].

The structure of the murine TLR4-MD-2 complex in association with the TLR4 antagonist Eritoran has recently been crystallized[2]. Eritoran, (also called B1287 and E5564), is a synthetic molecule developed from the lipid A structure of the non-pathogenic LPS of *Rhodobacter spharoides*, and acts as a strong antagonist of LPS signaling[121-123]. The crystal structure of the TLR4 ectodomain revealed that it has a horse-shoe-shape similar to that of TLR2. MD-2 was first crystallized by Ohto *et al* 2007, both in its native form and in complex with the TLR4 antagonist lipid IVa[124]. The crystal of MD-2 consists of two separable anti-parallel β-sheets, permitting the formation of a narrow and deep internal pocket, lined with hydrophobic residues. The hydrophobic acyl chains of lipid IVa and Eritoran fit neatly into

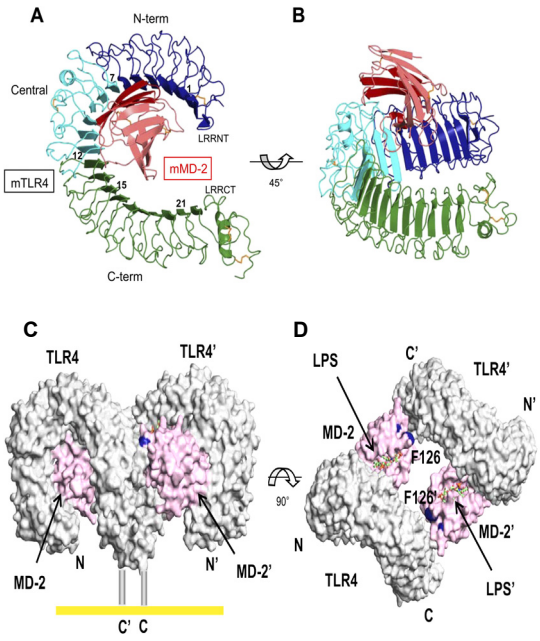

FIGURE 3: Crystal structure of TLR4-MD2 complex.

(A) and **(B)** Two views of mouse TLR4-MD-2 complex. The N-terminal, central, and C-terminal domains of TLR4 are colored in blue, cyan, and green, respectively. The beta strands of MD-2 are shown in pink and red, and the LRR modules of TLR4 are numbered. **(C)** and **(D)** Two views of the proposed three-dimensional TLR4-MD-2 complex. Surfaces of TLR4 and MD-2 are gray and magenta, respectively. The Phe126 and His155 residues important for dimerization are blue. The labels that belong to the second TLR4-MD-2 complex are marked with apostrophes (from Kim *et al*, 2007 [2]).

the hydrophobic pocket of MD-2, while the di-glucosamine backbone of the molecules are exposed to solvent[2, 124]. MD-2 binds to the concave surface of the N-terminal and central domains of TLR4 in a stable 1:1 complex[2]. There is no direct interaction between Eritoran and TLR4. The interaction between TLR4 and MD-2 is mediated by an extensive network of charged-enhanced hydrogen bonds in two patches on TLR4, termed the A patch and B patch, which interact with two patches on MD-2, termed the A' and B' patch, respectively. Gel filtration chromatography and SDS gel electrophoresis suggest that hexa-acylated LPS bound TLR4-MD-2 forms a heterotetramer, whereas tetra-acetylated lipids does not[2].

An Asp299Gly polymorphism in the ectodomain of TLR4 has been implicated in susceptibility to Gram-negative bacterial infections[125]. A Thr399Ile polymorphism in TLR4, as well as the Asp299Gly, have also been associated with increased frequency and severity of Gram-negative bacterial infections in septic shock patients[126].

1.2.6 TLR5

The human *tlr5* gene is located at 1q33.3[48, 64]. TLR5 initiates responses to flagellin, a monomeric constituent of bacterial flagella[45]. TLR5 is expressed on monocytes, DCs and PMNs[84]. TLR5 is suggested to play an important role in microbial recognition at mucosal surfaces, since TLR5 expression is observed on the basolateral, but not on the apical side of both intestinal epithelial cells and lung epithelial cells[127, 128]. A common stop codon polymorphism in the ligand-binding domain of TLR5 has been shown to be associated with susceptibility to legionnaires' disease[128].

1.2.7 The TLR9 subfamily

TLR7, TLR8 and TLR9 belong to the TLR9-subfamily[129]. The genes encoding TLR7 and TLR8 are located as a tandem on Xp22, while the gene encoding TLR9 maps to 3p21.3[130]. TLR7 and TLR9 are expressed in pDC and B cells, but not in mDC or macrophages in humans[131-136]. In contrast, human TLR8 is expressed on monocytes and mDC, but not on pDC[135, 137]. Murine macrophages, as well as mDC, express both TLR7 and TLR9, while TLR8 is shown to be non-functional in mice[42, 43, 137]. Human TLR7 and TLR8 have also been shown to be expressed on regulatory CD25+ T cells[138].

TLR7, TLR8 and TLR9 are all expressed intracellularly where they trigger signaling in endosomal compartments in response to nucleic acids and synthetic analogues. TLR7 recognizes single-stranded RNA (ssRNA) of both viral origin (e.g. influenza virus, vesicular stomatitis virus and HIV-1 genomic RNA) and non-viral origin[41, 137, 139]. TLR9 recognizes

unmethylated bacterial DNA, synthetic CpG[44, 140] and dsDNA of viral origin (e.g. from HSV1, HSV2 and MCMV)[141-144]. TLR9 additionally recognizes non-DNA components such as hydrophobic heam polymer homozoin which is produced when malaria parasites digest hemoglobin[145].

Activation of TLR7 and TLR9 in human pDC induces the production of IFN-α and IFN-regulated cytokines, while activation of TLR8 in monocytes and mDC induces proinflammatory cytokines[135]. Signaling mediated by all members of the TLR9 subfamily is completely abrogated in the absence of the TIR-adapter MyD88[146].

1.2.8 TLR11, 12 and 13

Murine TLR11 has been shown to be expressed in bladder epithelial cells and implicated in defense against uropathogenic bacteria[46]. TLR11 has also been shown to recognize a protozoan-derived profilin-like protein[47]. Humans do not express functional TLR11 and human orthologs of TLR12 and TLR13 have not been identified. TLR12 and TLR13 have been identified in mice[144], however, the ligands of these TLRs are currently unknown.

1.3 TLR SIGNALING PATHWAYS

TLR dimers presumably exist in a low-affinity complex before ligand binding. Ligand binding is suggested to induce a conformational change that brings the two TIR-domains on the cytosolic face of each receptor into closer proximity, thereby creating the signaling platform necessary for recruitment of adaptor molecules[50]. TLR signaling is initiated upon interaction between intracellular TIR signaling domains on TLRs and the corresponding TIR domains on the cytoplasmic adapter molecules; MyD88, MAL, TRIF and TRAM. The TLR signaling pathways are commonly divided into MyD88- and the TRIF-dependent signaling[146].

1.3.1 MyD88-dependent signaling

TLR2/TLR4 signaling

TLR2 and TLR4 signaling require the bridging adaptor MAL, in addition to MyD88 (**Figure 4**). MyD88 contains a C-terminal TIR-domain and an N-terminal death domain. The death domain recruits the serine/threonine kinases of the IL-1 receptor associated kinase (IRAK) family[147, 148]. Of these, IRAK1 and IRAK4, have been shown to be involved in TLR–induced activation of NF-κB[148,149-152]. Upon phosphorylation the IRAKs dissociate from MyD88 and interact with the E3 ligase TNF receptor-associated factor (TRAF)6. TRAF6 forms a complex

with E2 ubiquitin-conjugating enzymes Ubc13 and Uev1A promoting the synthesis of lysine 63-linked polyubiquitin chains, which in turn activate the MAPK kinase kinase (MAPKKK) transforming growth factor ß–activated kinase 1 (TAK1)[153] (**Figure 4, pathway 1**). TAK1 activates the IκBα kinase complex (IKK) which phosphorylates IκB in complex with NF-κB subunits. Phoshorylation of IκB leads to its proteolytic degradation, allowing nuclear translocation of NF-κB subunits, which bind the promoters of NF-κB target genes. Activated TAK1, in combination with the TAK1 binding proteins (TAB) TAB1, TAB2 and TAB3, can additionally phosphorylate the MAP kinase kinases (MKK)3 and MKK6, upstream of p38 mitogen-activated protein kinase (MAPK) and Jun N-terminal Protein Kinase (JNK)[153].

The transcription factor IRF-5 also interacts directly with MyD88 and TRAF6, mediating the induction of pro-inflammatory cytokine genes, such as those encoding TNF, IL-6 and IL-12p40 in response to ligands of TLR4[146] (**Figure 4, pathway 2**). Ligands of TLR5, TLR7 and TLR9 also activate this pathway.

TLR7/9 signaling

TLR7 and TLR9 ligands induce type I IFNs, in addition to proinflammatory cytokines, in a MyD88-dependent manner[137, 154]. Downstream of MyD88, signaling pathways are split into NF-κB, IRF-5- and IRF-7-dependent pathways. NF-κB is required for pro-inflammatory gene expression and is activated by the same pathway as described above for TLR2/4 (**Figure 4, pathway 1**). It is currently unclear how TLR-mediated signaling diverges from MyD88 and engages the IRF-5, IRF-7 and NF-κB pathways.

IRF-5 is also required for the induction of proinflammatory cytokines in response to TLR9 and TLR7 ligands, but not for type I IFN production[155, 156] (**Figure 4, pathway 2**). IRF-5 interacts directly with MyD88 and TRAF6. Signaling through TLR7 or TLR9 induces nuclear translocation of IRF-5 where it is shown to bind the interferon stimulating response element (ISRE) in the promoter of the gene encoding IL12p40 in response to CpG[155]. IRF-4 negatively regulates TLR7/9-mediated induction of proinflammatory cytokines induced by the IRF5-dependent pathway by competing with IRF5 in binding MyD88[157].

IRF-7 is essential for type I IFN gene induction in response to TLR7 and TLR9 ligands in plasmacytoid dendritic cells (pDC) (**Figure 4, pathway 3**)[41, 137, 158, 159]. IRF-7 activation, as well as NF-κB activation, is defective in the absence of MyD88, IRAK4 and TRAF6 in response to CpG, suggesting that these molecules are involved in both these pathways[160-163]. In contrast, IRAK1 deficiency results in the loss of IRF-7 activation, without affecting NF-κB

activity[164]. TRAF3 and IKKα are also required for IRF-7 activation and IFN-α induction in response to TLR7 and TLR9 ligands[165, 166,160] (**Figure 4, pathway 3**).

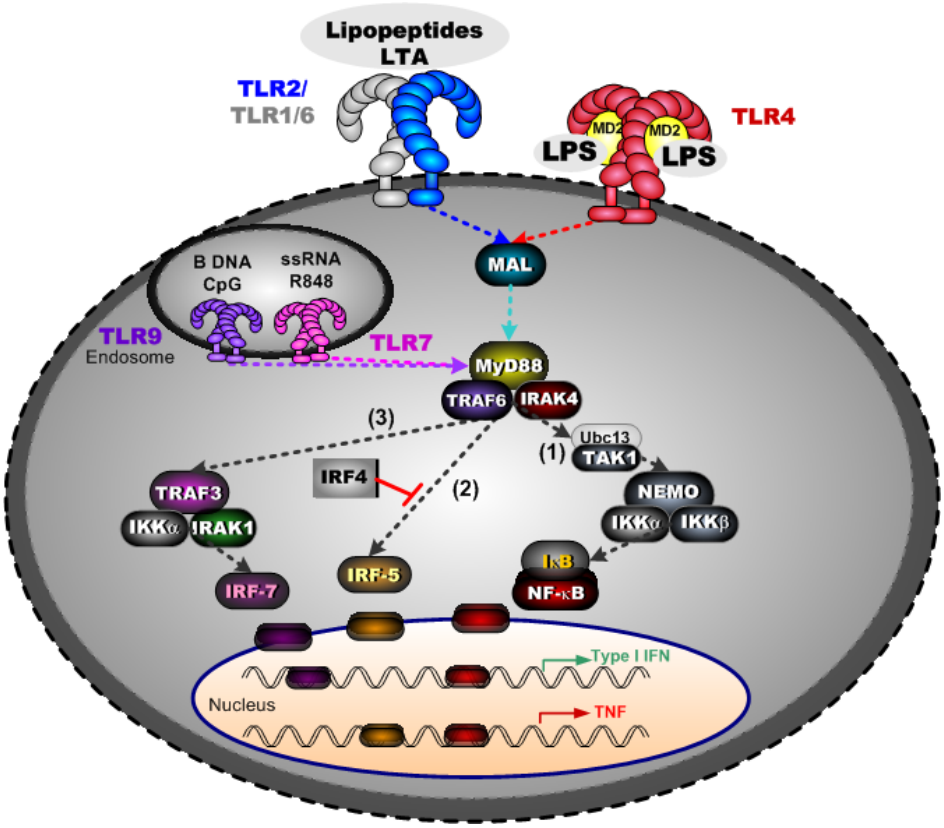

FIGURE 4: MyD88-dependent TLR signaling.

An outline of MyD88-dependent signaling pathways utilized by TLR2/1/6, TLR4, and TLR 7/8/9. TLR2 and TLR4 require MAL as a bridging adapter to recruit MyD88, while TLR7/8/9 recruit MyD88 directly. MyD88 subsequently recruits TRAF6 and IRAK4. Pathway (1) is utilized by all TLRs shown. Pathway (2) is activated in response to all TLRs shown, except TLR2/6/1. Only TLR7/9 ligands are shown to activate pathway (3) leading to IRF-7 activation. (Adapted from Kawai and Akira, 2007[146]).

1.3.2 TRIF-dependent signaling

TLR3 and TLR4 signaling

TLR3 and TLR4 signal via MyD88-independent pathways, involving the adapter molecule TRIF (**Figure 5**)[60]. TLR3 recruits TRIF directly to its TIR domain, while TLR4 requires TRAM as a bridging adaptor for the recruitment of TRIF[57, 58, 167]. Activation of the TRIF-pathway by TLR3 and TLR4 ligands results in activation of the transcription factor IRF-3 and subsequent induction of IFN-β and IFN-β-inducible genes and late activation of NF-κB. TRIF-dependent activation of IRF-3 involves the noncanonical IκB kinase homologues IKKε and TANK binding kinase 1 (TBK1)[30, 162, 168-171]. TRAF3 has been shown to form a complex with TRIF, as well as TBK1, and to positively regulate TRIF-dependent IFN-β gene induction[165, 166]. It is also been reported that NAK-associated protein 1 (NAP1) forms a

complex with TRIF and is required for activation of TBK1[172]. Thus, TRAF3 and NAP1 form a complex with TRIF and cooperate to activate TBK1 and, consequently induce IRF-3 activation[156].

TLR3 and TLR4-mediated signaling via TRIF also leads to NF-κB activation through recruitment of TRAF6 and RIP1 which further activate the IKK-complex (**Figure 5**). TRIF-dependent NF-κB activation additionally depends on TAK1 and IRAK4 in response to LPS and polyIC[152, 165, 173].

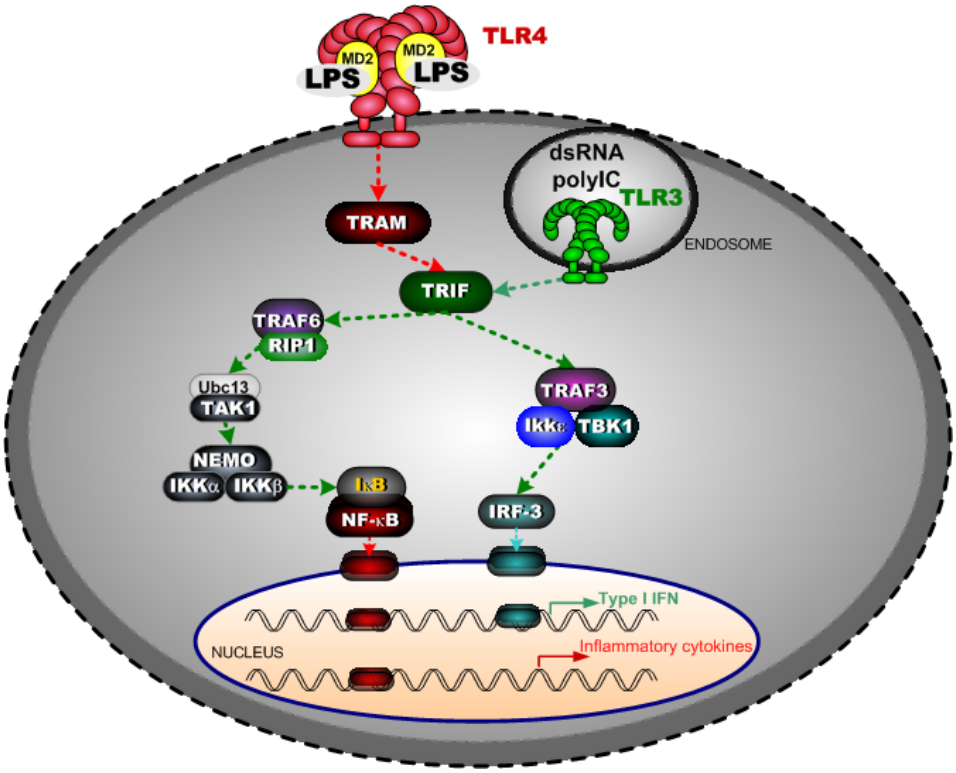

FIGURE 5: TRIF-dependent TLR signaling.

Outline of TRIF-dependent signaling pathways utilized by TLR3 and TLR4. TLR4 requires TRAM as a bridging adapter for recruitment of TRIF, while TLR3 recruits TRIF directly. TRIF subsequently activates NF-κB and IRF-3 by separate pathways (Adapted from Kawai and Akira, 2007[146]).

1.3.3 Transcription factors

Transcription factors control gene expression by binding the promoter of target genes and allowing RNA polymerase to bind and initiate transcription of the gene. Transcription of TLR-mediated induction of proinflammatory cytokines such as TNF requires cooperative binding of activating protein 1 (AP-1) and NF-κB to the promoter region of the gene encoding TNF. Induction of TLR-mediated induction of Type I interferons such as IFN-β requires the additional activation and promoter binding of IRFs.

Nuclear Factor -κB

NF-κB comprises a family of dimeric transcription factors which regulate the expression of a vast number of genes involved in immune, stress and antiapoptotic processes. The core components of NF-κB signaling are the IKK complex, the IκB inhibitory proteins and the NF-κB subunits themselves[174]. In the uninduced state, IκB inhibitory proteins retain the NF-κB subunits in the cytoplasm. Upon activation, the IKK complex phosphorylates IκB, leading to its proteolytic degredation. Following degradation of IκB, nuclear translocation signals on the NF-κB subunits are exposed, resulting in nuclear translocation, where they bind the promoters of NF-κB activated genes[153].

IκBα is the most extensively studied inhibitory protein. Other IκB proteins include IκBβ, IκBγ, IκBε, IκBδ, IκBNS and Bcl-3[153, 175]. The IKK-complex that phosphorylates IκB consists of four essential elements; IKKα, IKKα, NEMO/IKKγ and the recently identified ELKS. IKKα and IKKβ are serine/threonine kinases which share high sequence homology and are both important for TLR-induced NF-κB activation[176]. NEMO's function is unclear, but it is suggested to serve as a platform for interaction between IKKα and IKKβ, as well as other modulators of NF-κB[148, 177]. Two IKK-related kinases also play an important role in TLR signaling; IKKε (also called IKK-ι) and TBK1 (also called NAK or T2K). IKKε and TBK1 exhibit structural similarity to IKKα and IKKβ, but are not part of the classical IKK-complex[178-180]. Although several studies have failed to show a direct role of TBK1 in NF-κB activation, TBK1-deficient mice display a lethal phenotype that is very similar to that of NEMO$^{-/-}$, IKKβ$^{-/-}$ and RelA$^{-/-}$deficient mice[148, 181-185]. The lethality associated with TBK1 deficiency is furthermore prevented by disruption of TNF receptor I (TNFRI), suggesting that TBK1 is required for TNF-dependent activation of NF-κB[181]. Detailed analysis of TBK1-deficient MEFs did not reveal an appreciable decrease in TNF-induced NF-κB DNA binding activity, however, although TBK1 MEFs did display impaired NF-κB transcriptional activity[181].

Five NF-κB genes, NF-κB1, NF-κB2, RELA, c-REL, and RELB, encode for the seven NF-κB proteins p105, p50, p100, p52, p65 (RelA), c-Rel and RelB. p50 and p52 are generated by limited proteosomal processing of p105 and p100. All of the NF-κB proteins contain a Rel homology domain (RHD) which is approximately 300 amino acids of the N-terminal region. The RHD is responsible for NF-κB dimerization, DNA binding and interaction with IκB. All NF-κB proteins, except p50 and p52, also contain a transactivational domain found in the C-

terminal region. p50 and p52 are considered repressors of transcription since they lack this domain[153].

The seven NF-κB proteins form hetero- and homodimers giving 15 possible dimers. These dimers bind a NF-κB DNA binding site with the consensus sequence 5'-GGGRNWYYCC-3', where R is a purine, N is any base, W is an adenine or thymine and Y is a pyrimidine. NF-κB target genes are numerous and include a large number of immunomodulatory factors such as cytokines and chemokines[153].

The NF-κB pathway is further regulated by multiple posttranslational modifications of the core components of NF-κB signaling. The most extensively studied NF-κB component is p65 (RelA) which is the target for a number of modifications which affect NF-κB translocation and transcription[174]. One well studied phosphorylation site is Ser-536 found within the C-terminal transactivation domain. This site is a target of multiple kinases, including IKKβ, IKKα, IKKε and TBK1[186-192]. Phosphorylation of Ser-536 by the serine/threonine kinase RSK1 (ribosomal S6 kinase 1) can also induce IκB-independent NF-κB activation[193, 194]. Ser-529, another phosphorylation site in the transactivation domain, is to date only shown to be phosphorylated by the kinase Casein Kinase II (CKII)[195]. Simultaneous phosphorylation of multiple serines on p65 may be necessary for optimal activation, but details and level of redundancy for phosphorylations are unclear.

Interferon regulatory factors

The mammalian IRF family comprises nine members; IRF-1, IRF-2, IRF-3, IRF-4 (also known as LSIRF, PIP or ISCAT), IRF-5, IRF-6, IRF-7, IRF-8 (also known as ICSBP) and IRF-9[156]. IRF-1, IRF-3, IRF-5 and IRF-7 are positive regulators of the transcription of type I IFN genes[156, 196, 197]. Each IRF contains a well-conserved DNA-binding domain which recognizes the consensus ISRE DNA sequence[196, 198-200]. ISREs are found in the promoters of the genes that encode the type I IFNs, as well as in the promoters of many other genes that are involved in immunity and oncogenisis[156]. IFN genes are induced in virus-infected cells, mainly as a consequence of transcriptional activation of virus-responsive elements (VREs) which are located in the upstream region of the transcription-initiation site of type I IFN genes. The VRE of the IFN-β gene contains ISREs, as well as binding sites for NF-κB and AP-1[156, 197, 201].

IRF-3 and IRF-7, which are highly homologous, are considered key regulators of type I IFN gene expression elicited by viruses. Both IRF-3 and IRF-7 reside in the cytosol and are

phosphorylated and translocated to the nucleus upon activation. IRF-3 is a potent activator of the IFN-β gene, whereas IRF-7 efficiently activates both IFN-α and IFN-β genes[202-206]. Following viral infection IRF-3 is phosphorylated and forms a dimer (either homodimer or a heterodimer with IRF-7), enabling it to interact with the co-activators CBP or p300 to form a holocomplex[202, 203, 207-210]. The holocomplex subsequently binds its target sequences in the promoter of type I IFN genes and certain chemokine genes, and initiates transcription of these genes[156]. IRF-7 is expressed at low levels in most cells and is strongly induced by type I-IFN-mediated signaling. The binding of type I IFNs to the type I IFN receptor I (IFNRI) results in the activation of a heterotrimeric transcriptional activator known as IFN-stimulated gene factor 3 (ISGF3). ISGF3 consists of IRF-9 and signal transduced and activator of transcription (STAT) 1 and STAT2, and is responsible for the induction of the IRF-7 gene[162, 163, 211]. TBK1 and IKKε have been implicated in the activation of both IRF-3 and IRF-7 in response viral infection[58, 212]. TBK1 functions downstream of TLR3 and TLR4 to activate IRF-3, whereas IRF-7 is activated by other protein kinases during TLR7 and TLR9 signaling[164, 168, 169, 213].

NF-κB and IRFs cooperatively control the transcription of several cytokine genes. Transcription of the IFN-β gene requires the coordinated binding of the transcription factors AP-1, NF-κB and homodimers or heterodimers of IRF-3 and IRF-7[156, 214, 215]. The promoters of the RANTES, IP-10 also contain transcription factor binding elements for NF-κB and IRF3 [216, 217]. The resultant DNA-bound complex, known as the enhanceosome, is more stable and effective at inducing transcription than any of the individual transcription factors on their own.

1.4 TLR RESPONSES

Ligand interactions with TLRs induce production of proinflammatory cytokines such as TNF, IL-6 and IL-1β and IL-12. Proinflammatory cytokines induced by TLR ligands are predominantly mediated by MyD88-dependent signaling pathways. The induction of cytokines activates surrounding cells to produce chemokines and adhesion molecules which serve to facilitate the passage of leukocytes from circulation into the tissues. IL-8 is a typical chemokine which functions as a neutrophil chemoattractant, and also activates neutrophils to degranulate and cause tissue damage. Other chemokines induced by TLRs include the CC-chemokine RANTES (CCL5) and the macrophage inflammatory protein 1 (MIP) MIP-1α and MIP-1β. These chemokines have also been implicated in inhibiting viral infection[218]. Activation of TLR7 and TLR9 induces robust IFN-α production in pDCs, while activation of TLR3 and TLR4 in mDC induce robust IFN-β production, and induction of IFN-inducible genes. Type I IFNs possess antiviral activities, as well as important immune regulatory functions, and also serve to link innate and adaptive immune responses[219].

TLRs expressed on DC aid the initation of adaptive immune responses. Adaptive immunity is triggered when immature dendritic cells residing in local tissue phagocytose exogenous antigen (Ag) and TLR ligands, initiating the maturation of DCs. During maturation DC produce IFNs, cytokines and chemokines, upregulate co-stimulatory molecules, NK-activating ligands and MHC, and activate a variety of lymphocytes. The immune response is determined by which TLRs are activated[219, 220]. DCs activated by TLR9 and TLR7/8 ligands yield IL-12, IFNα and induce strong T-helper (T_H) 1 and CTL responses in a MyD88-dependent manner. DCs triggered via TLR3 yield mostly IFNα and also induce T_H 1 and CTL responses. TLR4 ligands predominantly induce IL-12, some IFNα and yield T_H1 responses. LPS and polyIC additionally induce upregulation of costimulatory molecules such as CD40, CD80 and CD86 in a IFN-β dependent manner, via IFNRI, on macrophages and DC[171]. TLR2 ligands in contrast are shown to induce IL-10 production in DCs, suppressing the production of proinflammatory cytokines and yielding a T_H2/T regulatory response[219].

2. AIMS OF STUDY

The overall aim of this study was to increase basic understanding of TLR2 signaling, regulation and trafficking and to unveil new functions of TLR2 in controlling adaptive immune responses. Understanding TLR biology is fundamental in order to apply these receptors in the development of anti-inflammatory and immune modulating compounds, since this requires finding the balance between immune activation and th toxic effects of inflammation.

Our initial aim was to study the role of CD14 as a co-receptor for TLR2 and the role of CD14, CD36 and other TLRs in response to different TLR2 ligands (**Paper I** and **II**). Our next objective was to study the subcellular expression of TLR2 and determine the subcellular compartments where signaling occurs (**Paper II**). The finding that TLR2 is highly expressed on the plasma membrane and in endocytic structures (**Paper II**) prompted us to investigate whether TLR2 is involved in antigen presentation and in the induction of adaptive immune responses (**Paper III**). An important goal in the following study was to develop and characterize a monoclonal antibody against murine TLR2 in order to study TLR2 expression and regulation in murine models (**Paper IV**). The finding that surface expression of TLR2 is a sensitive marker for a range of microbial products, and is upregulated independent of MyD88 in response to LPS and polyIC, prompted us to further investigate TLR signaling pathways involved in TLR2 regulation. The regulation of TLR2 protein expression in macrophages deficient in important signaling adapter molecules was compared to the regulation of the co-stimulatory molecule CD86 and the release of TNF and RANTES in response to different TLR-ligands, in order to unveil similarities and differences in the signaling pathways regulating these different responses (**Paper V**). In particular our objectives were as follows;

1) Study subcellular expression and trafficking of TLR2 and colocalization and association of TLR2 with co-receptors and other TLRs (**Paper I** and **II**).
2) Study LTA internalization in relation to TLR2 and co-receptors and determine whether ligand internalization is required for TLR2-mediated signaling in response to LTA (**Paper II**).
3) Determine whether TLR2 is involved in antigen presentation and in the induction of adaptive immune responses (**Paper III**).
4) Develop a monoclonal antibody against murine TLR2 in order to study expression and regulation of the receptor in murine models (**Paper IV**).
5) Investigate the contribution of different signaling pathways in the regulation of TLR2, as well as in the regulation of CD86, TNF and RANTES (**Paper V**).

3. SUMMARY OF PAPERS

PAPER I: Binding of Lipopeptide to CD14 induces physical proximity of CD14, TLR2 and TLR1

TLR2 signaling in response to triacylated lipopeptide such as Pam_3CysSK_4 is mediated by the TLR2/TLR1 heterodimer. In this paper we show that pretreatment of monocytes with monoclonal antibodies (mAbs) against TLR2 and CD14 inhibited TNF release in response to Pam_3CysSK_4, illustrating that CD14 acts as a co-receptor for TLR2 in response Pam_3CysSK_4. Using FLAG-tagged functional Pam_3CysSK_4 we observed that CD14, but not TLR2, markedly enhanced the binding of Pam_3CysSK_4 both in human monocytes and transfected human epithelial kidney (HEK) cells. Confocal microscopy revealed colocalization between Pam_3CysSK_4 and CD14 and between Pam_3CysSK_4 and TLR2. Association between TLR2 and Pam_3CysSK_4, as well as between CD14 and Pam_3CysSK_4, was furthermore observed by fluorescence resonance energy transfer (FRET). Importantly, FRET was further observed between CD14 and TLR2 upon stimulation with Pam_3CysSK_4, but not in the absence of stimuli. This association could further be inhibited by both an anti-TLR2 mAb and unlabeled Pam_3CysSK_4. Combined these results suggest that Pam_3CysSK_4 induces physical proximity between CD14 and TLR2. Fluorescence recovery after bleaching (FRAP) studies further showed a decrease in TLR2 mobility upon stimulation with Pam_3CysSK_4, suggesting that TLR2 is target to a low-mobility signaling complex in the plasma membrane upon activation, presumably in order to facilitate the association with signaling TIR-adapter molecules.

PAPER II: Internalization and Cellular Trafficking of Lipoteichoic acid and Toll- like Receptor 2 in Relation to Signaling; Involvement of CD14 and CD36.

In this study we initially explored the signaling, uptake and trafficking pattern of the TLR2/TLR6 ligand LTA in relation to expression of TLR2 and its co-receptors CD36 and CD14 in human monocytes. We found TLR2 expressed in the plasma membrane, early endosomes and in late endosomes/lysosomes, proposing that signaling may occur at the plasma membrane, or along the endocytic pathway. We further observed rapid internalization of fluorescently labeled LTA in human monocytes, colocalizing with markers for early and late endosomes and lysosomes, where we also observed TLR2 expression. We also observed LTA in the ER and Golgi network, however, we did not observe TLR2 in these compartments. Expression of CD14 markedly enhanced LTA binding to the plasma membrane and also enhanced NF-κB activation. Blocking either CD14 or CD36 with antibodies inhibited LTA binding, as well as LTA-induced TNF release from monocytes,

emphasizing an important role for both molecules in the binding of LTA and in aiding TLR2 signaling. LTA internalization, but not NF-κB activation, was inhibited in Dynamin-I K44A dominant negative transfectants, suggesting that LTA is internalized by receptor-mediated endocytosis, but that internalization is not required for signaling. Indeed, immobilizing LTA, and thereby inhibiting internalization, enhanced TNF release in monocytes. In summary, these results support that LTA signaling preferentially occurs at the plasma membrane, and that both CD36 and CD14 are required as co-receptors for TLR2 for optimal signaling to occur.

PAPER III: Link between Innate and Adaptive Immunity: Toll- like receptor 2 Internalizes Antigen for Presentation to $CD4^+$ T Cells and could be an Efficient Vaccine Target.

In this paper we further investigated the role of TLR2 as an endocytic receptor and addressed whether TLR2 may be involved in the induction of adaptive immune responses. An antagonistic TLR2-specific mAb (TL2.1) was used to investigate whether peptides bound to TLR2 are presented on major histocompatibility complex class II (MHCII) molecules. We found that the TL2.1 mAb was efficiently presented to cloned mouse Cκ-specific HLA-DR4-restricted human $CD4^+$ T cells by PBMC, monocytes and immature DCs, triggering both proliferation and IFNγ release in T cells. TL2.1 induced T cell proliferation 100-1000 times higher than that induced by an isotype control or CD62L, showing that only a subset of cell surface receptors channel Ag into the MHCII presentation pathway. The presentation of the Cκ-epitope derived from the TLR2 specific Ab appeared to be dependent on conventional Ag processing since the inhibitors chloroquine, leupeptin and Brefeldin A all intervened with T-cell proliferation induced by TL2.1. Combined, these results show that antibodies bound to TLR2 are processed by the conventional MHCII-pathway, presented to T-cells and induce adaptive immune responses. Targeting antigens to TLR2 could consequently be an effective strategy for the development of Ab-based vaccines.

PAPER IV: Lipopolysaccharide and Double-stranded RNA Up-regulate Toll- like Receptor 2 Independently of Myeloid Differentiation Factor- 88

In this paper we sought to investigate the mechanisms regulating TLR2 expression. In order to study the expression, signaling and regulation of TLR2 in mouse cells, we developed a monoclonal antibody (mAb) against mouse TLR2 (named 6C2). Pretreatment of macrophages with the TLR2 antibody diminished TNF release in response to lipopeptides and down-regulated membrane TLR2, suggesting that the mAb has antagonistic properties and that crossbinding TLR2 induces down-regulation of the receptor and signal abrogation. Using this antibody, we detected TLR2 protein expression on macrophages, neutrophils and dendritic cells. Endogenous TLR2 in macrophages localized mostly to the cell membrane, with

particular accumulation around phagosomes containing zymosan. We also observed marked upregulation of surface TLR2 on macrophages in response to whole bacteria, lipoproteins, LPS, polyI:C, R848 and CpG DNA. This upregulation appeared to be a sensitive marker for the presence of microbial products since it was induced by very low concentrations of ligand. Upregulation of TLR2 in response to stimuli furthermore correlated with increased responsiveness towards secondary exposure of lipoprotein, following low concentrations of primary lipoprotein challenge. Upregulation of TLR2 may consequently be an important mechanism by which the immune system boosts its response to beginning infection. However, exposure to larger doses of primary challenge induced a hyporeactive state, despite upregulated surface TLR2, suggesting that excessive signaling is blunted down-stream of the receptor. Interestingly, we found that LPS- and polyIC-induced upregulation of surface TLR2 in macrophages was MyD88-independent, while upregulation in response to lipoproteins, R848 and CpG DNA was entirely MyD88-dependent.

PAPER V: TIR adapters TRIF and TRAM mediate TLR2-induced Release of the Chemokine CCL5

In this paper we further investigated the signaling pathways involved in the regulation of TLR2 expression. In light of Paper IV, we initially investigated whether the TRIF-pathway may be responsible for the upregulation of TLR2 in response to LPS, since upregulation of TLR2 in response to LPS was normal in MyD88$^{-/-}$ macrophages (**Paper IV**). We found that LPS-triggered upregulation of surface TLR2 was only partially reduced in macrophages from TRIF$^{-/-}$, TRAM$^{-/-}$ or TRIF$^{-/-}$TRAM$^{-/-}$ mice. This was in contrast to LPS-induced CD86 surface expression and RANTES release, which was absent in all these cells. TLR2 upregulation was, however, completely abrogated in MyD88$^{-/-}$TRIF$^{-/-}$ macrophages treated with LPS, showing that MyD88 can participate in a response that initially appeared MyD88-independent. Surprisingly, we also found that RANTES release was markedly reduced in TRIF$^{-/-}$, TRAM$^{-/-}$ and TRIF$^{-/-}$TRAM$^{-/-}$ macrophages in response to several TLR2 ligands, despite normal TNF release and TLR2 expression in these cells, suggesting a new role of TRIF and TRAM in TLR2 signaling. We also found that macrophages from TRIF$^{-/-}$TRAM$^{-/-}$ mice failed to phosphorylate Serine-536 on NF-κB p65 (RelA) normally in response to MALP-2, supporting a role for the TRIF/TRAM pathway in TLR2 signaling. We propose that TRIF/TRAM signaling may be important for RANTES induction in response to TLR2 ligands, possibly by a mechanism involving phosphorylation of Ser-536 on NF-κB p65/RelA. Combined these results provide further insight on the contribution of the MyD88- and TRIF- pathways in response to different TLR ligands and cross-talk between these pathways during TLR-mediated signaling.

4. DISCUSSION

4.1 TLR2 EXPRESSION

4.1.1 Cell types that express TLR2

In **Paper I** and **III**, we found TLR2 protein expressed on human macrophages, neutrophils and dendritic cells. This is in line with previous reports [85]. In Paper **IV** we found that TLR2 is expressed on the cell surface of mouse macrophages, neutrophils and dendritic cells, whereas CD19+ B-cells stained only weakly for TLR2. Our results indicate that surface expression pattern of TLR2 is similar in mice and humans, with macrophages displaying the highest expression of TLR2, as shown by others [84, 85]. TLR2 ligands have later been shown to activate B cells and induce IL-6 and upregulation of MHCII in a T-cell-dependent, and -independent manner[221, 222]. Murine follicular B cells and marginal zone B cells have also been shown to respond to TLR2 ligands[223]. Thus, several types of B cells appear to express functional TLR2, although at low levels, compared to macrophages.

TLR2 expression also has been reported in thymic T cells at the mRNA level[224]. Although we did not observe TLR2 expression on the surface of thymic T cells (**Paper IV**), recent studies have shown that TLR2 is expressed on T_H1 and T_H2 effector cells, as well as on T memory cells and regulatory T cells. Murine T_H1 have furthermore been shown to respond directly to TLR2 ligands in the absence of TCR signaling[225]. Memory T cells and regulatory T cells also respond to stimulation by TLR2 ligands, supporting that these cells express functional TLR2[226, 227]. TLR2 expression is low in resting T cells and is upregulated upon stimulation. Upregulation of TLR2 in response to stimuli, which we observed in **Paper IV** and **V**, could therefore be particularly important in these cells. Low expression of surface TLR2 in resting cells may also be more important for restricting inappropriate activation of these cells. Whether TLR2 requires the same co-receptors and utilizes the same signaling pathways in T cells, as in other immune cell such as macrophages, is unclear. Although T cells do not express CD14, bovine $\delta\gamma$ T cells have been shown to express functional CD36 which participates LTA-induced MIP-1α induction[228]. In light of your findings that CD36 and CD14 play similar roles in the response to LTA in human macrophages (**Paper II**), we speculate that CD36 may perhaps substitute CD14 in cells that do not express CD14. Our results in human monocytes suggest, however, that blocking either one of these receptors diminishes TNF release in response to LTA.

4.1.2 Subcellular expression of TLR2

In **Paper II** we further investigated the subcellular expression of TLR2 in human monocytes and found TLR2 expressed on the plasma membrane, in endosomes, lysosomes and Rab-11-positive compartments, but not in the Golgi, in contrast to previous reports[229]. TLR2 is highly expressed at the plasma membrane, in likeness with TLR4, but in contrast to TLR3, TLR7/8 and TLR9. The differences in subcellular TLR expression are presumably due to the nature of the components different TLRs recognize. While TLR2 and TLR4, predominantly recognize extracellular bacterial components, TLR3, TLR7/8 and TLR9 recognize nucleic acids that are released during endosomal degradation of microbes, or released by damaged host cells. The expression of TLR2 on the plasma membrane appears to be important for recognition of different ligands, indicated by the upregulation of the receptor in response to low concentrations of stimuli (**Paper II, III, IV** and **V**). Sensitive upregulation in response to low concentrations of lipopeptide may also aid in priming macrophages towards secondary stimuli (**Paper IV**). Tolerance is, however, induced in response to high concentrations of primary challenge by lipopeptides, despite upregulated TLR2, suggesting that excessive signaling is terminated down-stream of the receptor (**Paper IV**).

In **Paper II** we observed TLR2 expressed intracellularly in endosomes and lysosomes. We also observed that TLR2 mAb bound to surface TLR2 was targeted to the endocytic pathway and presented on MHCII molecules (**Paper II** and **III**), suggesting a role for TLR2 in the endocytic pathway.

4.2 DOES TLR2 PLAY A ROLE IN PHAGOCYTOSIS?

Phagocytosis triggers degradation of pathogens and presentation of pathogen-derived peptide antigen. The expression pattern of TLR2 suggests that the receptor is involved in endocytosis and phagocytosis. In **Paper IV** we found that TLR2 was recruited to the phagocytic cup containing the yeast particle zymosan, confirming previous reports on transfected TLR2[230]. Phagocytosis and cytokine production occurs as seperate processes[230, 231], however, implying that TLR2 does not primarily function as a phagocytic receptor. TLR2 is instead recruited to phagosomes independent of contents, where the receptor aids in the recognition of microbial components present in the phagosome[80]. The cytosolic domain of TLR2 has, however, also been shown to complex with molecules implicated in cytoskeletal rearrangement, such as the Rho GTPase Rac1 and the p85 subunit of Phosphoinositide-3 kinase (PI3K) in response to *S.aureus*, implying an indirect role for TLR2 in the phagocytic process[232, 233]. Bone-marrow derived dendritic cells have also been shown to increase their endocytic ability upon stimulation with a range of TLR ligands, and stimulation also triggers cytokskelatal

rearrangement and Ag-presentation in these cells[234], further supporting a role for TLRs in phagocytosis. Whether TLR stimulation affects phagosome and lysosome fusion remains unclear. Although some studies suggest that TLR stimulation has an enhancing effect[235], other studies showed no effect[236, 237]. LPS stimulated macrophages have, however, been shown to upregulate a large number of genes involved in all stages of phagocytosis[238-240], suggesting a role for TLRs in phagocytosis.

A role for TLRs in phagosome maturation and antigen presentation has been emphasized by the finding that only phagosome cargo containing TLR4 ligands could trigger efficient antigen processing and MHCII presentation and activate CD4+ cells, whereas phagosome cargo containing apoptotic cells retained MHCII intracellularly[241, 242]. We have shown that TLR2-bound Ab is internalized in endosomes, processed by the MHCII pathway and induces proliferation of CD4+ T cells (**Paper III**). Ab bound to TLR4 is processed and presented in a similar manner[243]. TLR2 may therefore play a similar role as shown for TLR4 in sampling phagosome content and triggering effecient processing and MHCII presentation. However, we note important differences in TLR2 and TLR4 regulation, trafficking and signaling, suggesting that the two receptors also differ in many ways (see Chapter 4.5).

In line with our observations in **Paper I**, the crystal structure of the ectodomain of TLR2 suggests that Pam_3CysSK_4 induces the association of TLR2 and TLR1 and that TLR2 and TLR1/TLR6 bind their ligands directly[1]. We observed, however, that TLR2 bound both Pam_3CysSK_4 and LTA poorly in comparison to CD14 (**Paper I** and **II**), although TLR2 was completely necessary for signaling in response to these ligands. Our current understanding suggests that CD36/CD14 may concentrate TLR2 ligands on the cell surface and transfer the compounds to the signaling receptor complex.

4.3 THE ROLE OF CD36 AND CD14 IN TLR2 ACTIVATION?

We showed in **Paper I** that CD14 binds the TLR2 ligand Pam_3CysSK_4. We furthermore show FRET between TLR2 and CD14, and TLR1 and CD14 upon stimulation with Pam_3CysSK_4, suggesting that CD14 initially binds Pam_3CysSK_4 and presents it to the TLR2/TLR1 complex. The crystal structure of TLR2 and TLR1 suggests that Pam_3CysSK_4 binds both TLR2 and TLR1, which induces the dimerization of the receptors [1]. It remains unclear how CD14 participates in this TLR2/TLR1 complex and in enhancing the response towards Pam_3CysSK_4, in light of the proposed crystal structure of TLR2/TLR1 ectodomain[1]. CD14

may perhaps bind Pam_3CysSK_4 in a manner that allows the presentation of the acyl chains to TLR2 and TLR1, and thereby facilitate TLR2/TLR1 complex formation.

Although we generally observed upregulation of TLR2 in response to a range of ligands (**Paper II-IV**), we also observed internalization and trafficking of the receptor upon stimulation (**Paper II**, **III** and **IV**). The findings in **Paper II** suggest that CD14 may aid in binding LTA to the membrane, where we propose that signaling may occur. CD14 binds a range of ligands, including LPS, lipopeptides and LTA[111, 112, 244, 245]. We and others have observed that CD14 also enhances signaling in response to LPS, lipopeptides and LTA (**Paper I, II** and [244,246]). CD14 is however a GPI-linked membrane protein, devoid of an intracellular portion, and is incapable of inducing signaling on its own[247]. Nevertheless, CD14 may enhance signaling by immobilizing ligands on the cell surface, where both TLR2 and TLR4, are highly expressed, as suggested in **Paper I** and **II**. Alternatively, CD14 may target ligands to early endosomes and endocytic pathway where some signaling may occur, as suggested for TLR4[243].

CD14 is clearly involved in enhancing TLR2 signaling in response to both TLR2/TLR1 and TLR2/TLR6 ligands (**Paper I** and **II**), but does not appear to discriminate between different TLR ligands. The scavanger receptor CD36 has, in contrast, been shown to recognize TLR2/TLR6 ligands, but not other TLR ligands[171]. In **Paper II** we showed that CD36 is involved in both the binding of LTA and in TNF release in human monocytes, in response to LTA, but not in response to the TLR2/TLR1 ligand Pam_3CysSK_4, or LPS. It is unclear how TLR2/TLR6 ligands bind both CD36 and CD14, in light of the proposed model of how TLR2/TLR6 bind their ligands. It is also puzzling how CD36 recognizes structurally different molecules such as MALP-2 and LTA, but discriminates between structurally similar molecules like MALP-2 and Pam_3CysSK_4. CD36 has been suggested to discriminate between these molecules by recognizing the diacyl chains present in TLR2/TLR6 ligands. However, the proposed model of how TLR2/TLR6 ligands recognize their ligands suggests that these acyl chains are inserted into binding pockets in TLR2 and TLR6[1]. How TLR2 and TLR6 recognize larger ligands such as LTA remains unclear.

The finding that CD36 specifically discriminates between TLR2/6 ligands and TLR2/1 ligands has somewhat overshadowed the role of CD14 as a co-receptor for TLR2. In **Paper II** we showed, however, that both co-receptors are required for optimal response to LTA, and that inhibition of either CD14 or CD36 had a profound effect on TNF release in human monocytes in response to LTA. We speculate that CD36 may be required for correct complex formation at the plasma membrane or for targeting to lipid rafts, as suggested by Triantafilou

et al[229]. Alternatively, CD36 may be required for targeting LTA to endosomes during internalization, as suggested by Stuart *et al*[248]. Since CD36 appears to act as a co-receptor for TLR2/TLR6 ligands, we speculate that an unknown receptor may play an equivalent role in response to TLR2/TLR1 ligands.

TLR2 recognizes a broad range of ligands. Discrimination between TLR2/1 ligands and TLR2/6 ligands and other TLR2 ligands, appears to occur at the plasma membrane, since TLR2, as well as co-receptors of TLR2, such as CD14, CD36, TLR1, TLR6 and Dectin-1, all are expressed at the plasma membrane, or are recruited to the plasma membrane, upon stimulation. The number of surface receptors that have been shown to be involved in TLR2-signaling in response to different TLR2 ligands is puzzling, since both TLR2/TLR1 ligands and TLR2/TLR6 ligands recruit the same TIR-adapters, apparently leading to initiation of the same MAL/MyD88 signaling pathway and induction of the same responses. The need to discriminate between TLR2 ligands may be required for correct internalization and for correct targeting of different TLR2 ligands to different endocytic routes. In **Paper III** we show that peptides bound to TLR2 are processed by the MHCII pathway and induce T cell proliferation. Only TLR2 ligands containing peptide moieties, such as lipopeptide and peptidoglycan are, however, expected to be presented on MHCII molecules, while TLR2 ligands devoid of peptide units, (e.g. LTA) are likely to be processed differently. Indeed, we observed in **Paper II** that the internalization pattern og LTA differs from the internalization of other TLR ligands, (e.g. Pam_3CysSK_4 in **Paper I**), in that it appears to be targeted to the Golgi and ER, presumably by a retrograde pathway.

4.4 WHERE DOES TLR2 SIGNALING OCCUR?

In **Paper III** we argue that signaling in response to LTA predominantly occurs at the plasma membrane where TLR2 and its co-receptors CD14 and CD36 are highly expressed. Upregulation of TLR2 in response to LTA furthermore suggests that signaling may occur at the plasma membrane. Expression of the Dynamin I mutant K44A inhibited LTA internalization, but did not affect NF-κB activation. Immobilization of LTA on a plastic surface, furthermore, enhanced TNF release from monocytes, suggesting that LTA internalization is not required for signaling to occur, and that signaling predominantly occurs at the plasma membrane. This finding is supported by the findings Triantafilou *et al*[229] and Sandor *et al*[78] that show that NF-κB activation can be induced by cross-linking TLR2 and TLR1 with TLR2- and TLR1-specific mAb. Stuart *et al*[248], however, claim that LTA internalization is required for signaling to occur. Although we cannot exclude that some

signaling occurs in early endosomes, we argue on the basis of our findings in **Paper II**, that signaling in response to LTA at least predominantly occurs at the plasma membrane, independent of ligand internalization. Notably, TIR-adapter MAL is constitutively expressed at the plasma membrane[249]. Upon stimulation of TLR2 transfected cells with LTA, we also observed translocation of MyD88 to the plasma membrane by confocal microscopy (Nilsen, Unpublished data), further supporting that TLR2 signaling in response to LTA occurs at the plasma membrane.

4.5 DIFFERENCES AND SIMILARITIES BETWEEN TLR2 AND TLR4

Both TLR2 and TLR4 are highly expressed on the plasma membrane, supposedly in order to sense extracellular pathogens. TLR4 and TLR2 are, however, also expressed in endocytic compartments (**Paper II**, **III** and [243]), and peptides bound to both TLR2 and TLR4 are processed by the MHCII pathway and induce T cell proliferation (**Paper III** and [243]). TLR2 and TLR4 ligands furthermore induce upregulation of costimulatory molecules on human DCs (**Paper III**). Although both TLR2 and TLR4 ligands have been reported to upregulate CD86 on murine DCs[250, 251], we did not observe upregulation of CD86 on macrophages in response to TLR2 ligands, although these cells efficiently upregulated co-stimulatory molecules in response to LPS (**Paper V**). These results suggest that both TLR2 and TLR4 are involved in Ag-presentation and induction of adaptive immune responses, however, certain responses appear to be cell-type specific.

In contrast to surface TLR2, which is rapidly upregulated in response to a range of TLR ligands, TLR4 is down-regulated in response to LPS, ubiquitylated and targeted for degradation[243, 252]. TLR2 is not ubiquitylated in the same manner[253]. In **Paper IV** we showed that TLR2 was rapidly upregulated in response to low doses of Pam_3CysSK_4, which prime macrophages towards secondary stimuli, suggesting that TLR2 upregulation may function as a sensitive sensor for infection. Tolerance was, however, induced upon prestimulation with higher doses of Pam_3CysSK_4, despite upregulation of TLR2 (**Paper IV**), suggesting that excessive signaling may be controlled by inhibition of down- stream signaling pathways, rather than by degradation of the signaling receptor. In contrast to TLR2 upregulation, Miyake *et al* showed that TLR4/MD-2 on mouse macrophages is down-regulated in a MyD88-dependent manner in response to LPS, using the anti mouse MD-2/TLR4 mAb MTS510[254]. Using a similar mAb (Sa15-21), we observed relatively small changes in MD-2/TLR4 expression following a similar challenge (Nilsen, Lien, Unpublished data). We

conclude that the surface expression of TLR2 on macrophages in general is upregulated upon challenge, whereas TLR4 expression is not.

TLR4 and TLR2 ligands, such as LPS and LTA, respectively, share certain similarities with regard to structure. CD14 appears to play a similar role in response to both these ligands by markedly enhancing the binding of these ligands to the plasma membrane (**Paper II** and [119, 255]). CD14 may further aid the targeting of the ligands to endocytic pathways. Both LPS and LTA were internalized by Dynamin-dependent mechanisms, and both TLR4 and TLR2 have been shown to signal from the plasma membrane (Paper II and [243]). The internalization patterns of the two ligands appear to differ though. While LPS is internalized in vesicular structures colocalizing neatly with transferrin[243], LTA is rapidly targeted to the Golgi and ER, as well as endocytic compartments (**Paper II**), suggesting that LTA may utilize several internalization pathways.

TLR2 heterodimerizes with TLR1 and TLR6 in response to different ligands, while TLR4 forms heteromers (2x MD-2 and 2x TLR4) in response to LPS. The ability of TLR2 to heterodimerize may partially explain the receptors ability to recognize an array of different ligands. Both TLR2 and TLR4 require coreceptors for optimal signaling in response to their respective ligands. The small glycoprotein MD-2 is a crucial coreceptor for TLR4 in response to LPS. However, neither MD-2, nor MD-1 are involved in TLR2 signaling[61]. The crystal structure of TLR2 ectodomain suggests that TLR2 binds Pam_3CysSK_4 directly[1], while the crystal structure of TLR4 suggests that there is no direct binding between TLR4 and its ligand. In the latter case the ligand binds MD-2, and this complex in turn binds TLR4[2].

TLR2 recognizes a broad range of structurally different ligands through cooperation with other TLRs and co-receptors. Ligation of TLR2, however, results in induction of the same MAL/MyD88-dependent signaling pathway. TLR4, in cooperation with MD-2 and CD14 on the other hand induces signaling by both the MAL/MyD88 pathway and the TRIF/TRAM pathway in response to LPS. The role of CD14 may differ in triggering MyD88 and TRIF-dependent pathways[256]. TLR4 consequently induces a broader spectrum of responses in macrophages, including proinflammatory cytokines, chemokines, interferons and upregulation of costimulatory molecules. Thus, the complexity of TLR2 responses appears to be in ligand recognition, while the complexity of TLR4 signaling is reflected by its ability to activate both signaling pathways and induce an array of responses upon recognition of the same ligand. Although we unravel a new role for the TRIF/TRAM pathway in TLR2 signaling in **Paper V**, this pathway mediated responses by all TLR2 ligands tested (**Paper V**). An apparent contrast lies in the MyD88 utilization for RANTES release following cell stimulation with TLR2 or

TLR4 ligands. Lipoprotein stimulation inducing RANTES is completely dependent on both TLR2 and MyD88, whereas LPS induction of RANTES is independent of TLR2 and only partially reduced in the absence of MyD88. This emphasizes the fact that for TLR2 signaling, all responses are MyD88 dependent.

4.6 TLR2 REGULATION AND SIGNALING PATHWAYS

In **Paper IV** we hypothesized that TLR2 upregulation primes macrophages in response to low levels of prestimuli. Higher amounts of prestimuli induced tolerance towards secondary stimulation, despite TLR2 upregulation, suggesting that signal-termination occurs down-stream of TLR2. In **Paper IV** and **V** we found that surface TLR2 was differentially regulated by both the MyD88- and TRIF/TRAM-dependent pathway in response to LPS, since upregulation was only abolished in the absence of both TRIF and MyD88 (**Figure 6**). These results are supported by microarray studies showing that TLR2 gene expression is differentially regulated by TRIF and MyD88 in response to LPS[257]. Notably, the TRIF/TRAM signaling branch appeared to be more crucial for correct regulation of TLR2 in response to LPS, since the effect of the MAL/MyD88 pathway only became apparent in the absence of TRIF and/or TRAM. Upregulation TLR2 may be particularly important in other cell types, such as B cells and T cells, which express low levels of TLR2 in resting cells and lack CD14 and CD36.

In **Paper V** we found that upregulation of CD86 and RANTES induction were crucially dependent on the TRIF/TRAM pathway in response to LPS. CD86 is upregulated in response to LPS in a TRIF-IFNβ-IFNRI dependent manner[171] (**Figure 6**). Surface expressed TLR2 was, in contrast, only modestly upregulated upon treatment with recombinant IFN-β (Nilsen, Unpublished data) compared to the level induced by LPS and MALP-2. These observations indicate that mechanisms leading to regulation of CD80/86, TLR4, and TLR2 differ. Although CD86 and RANTES are both IFN-inducible proteins, which were regulated similarly in response to LPS, we found important differences in the regulation of these genes in response to different TLR ligands. CD86 upregulation was only observed in response to LPS, polyIC and Sendai virus, but not in response to TLR2 ligands, R848 or CpG in macrophages (**Paper V** and Unpublished data). RANTES was in contrast induced by all these ligands in macrophages, suggesting that RANTES and CD86 surface expression are not regulated by the same mechanisms. We hypothesize that differences ma may be explained in terms of differential dependence on IRF/IFN induction.

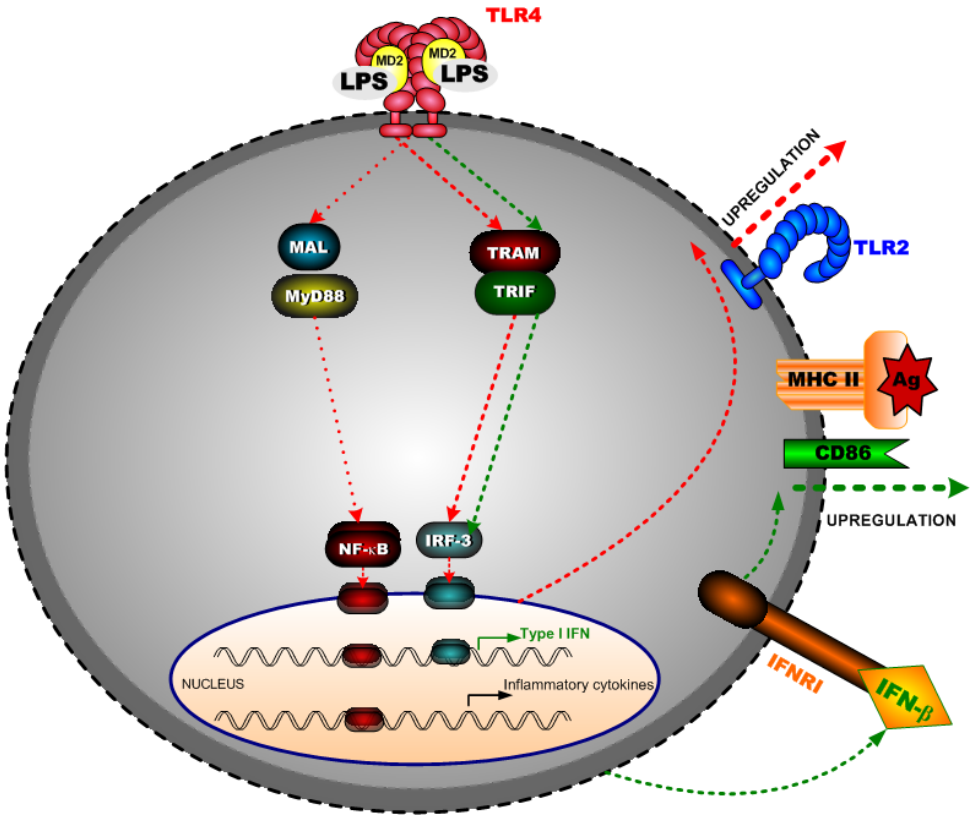

FIGURE 6: TLR2 regulation and CD86 regulation in response to LPS.

An outline of signaling pathways leading to upregulation of TLR2 in response to LPS. Both MAL/MyD88 and the TRIF/TRAM pathway control the upregulation of surface TLR2 (red arrows), while upregulation of CD86 in response to LPS occurs in a TRIF-IFN-β-IFNRI –dependent manner (green arrows). The TRIF/TRAM pathway appeared more important for TLR2 upregulation in response to LPS than the MyD88/MAL pathway (thin dotted red lines).

Interestingly, we found that TLR2-mediated RANTES induction was dependent on the TRIF/TRAM pathway, suggesting a new role for these adapter molecules in TLR2 signaling. Notably, we found that TRIF and TRAM are specifically involved in RANTES release, but not TNF release. The promoter region of RANTES, as well as IFN-β, contain transcription factor binding sites for NF-κB and IRF-3[216, 217]. LPS induces RANTES and IFN-β by activating both NF-κB and IRF-3 in a TRIF/TRAM-dependent manner[58]. TLR2 ligands fail, however, to induce IRF-3 binding to the ISRE of interferon stimulated gene 15 (ISG15) in macrophages[58, 258] and are poor inducers of type I IFN[191], something which may be related to lack of TRAF3 recruitment[259]. In light of the generally lower levels of RANTES induced by TLR2 ligands, compared to LPS, we reason that TRIF/TRAM-mediated RANTES induction only induces NF-κB binding to the RANTES promoter in response to TLR2 ligands. We further speculate that the TRIF/TRAM-mediated induction of RANTES in response to TLR2 ligands may be dependent on the death-domain kinase RIP-1, which is shown to be activated

by LPS and polyIC in a TRIF-dependent manner, activating NF-κB, but not IRF-3[260, 261]. The TRIF/TRAM-dependent RANTES induction we observed in response to TLR2 ligands could therefore possibly utilize the TRIF-RIP-1-NF-κB-pathway (**Figure 7**).

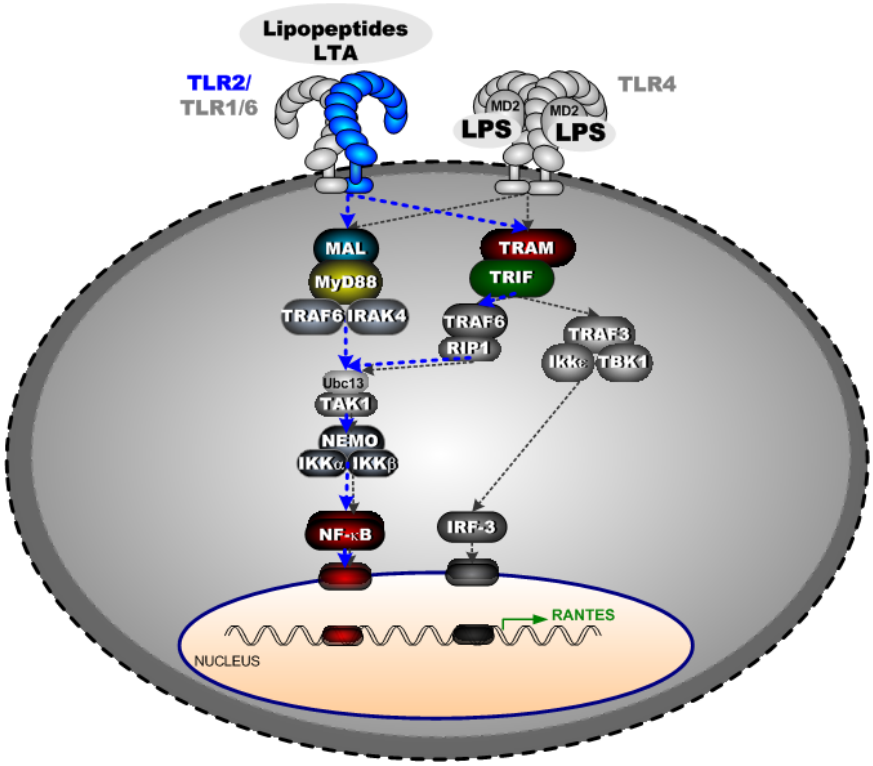

FIGURE 7: TRAM and TRIF are involved in TLR2 signaling.

Proposed signaling pathways leading to RANTES induction in response to TLR2 ligands (blue arrows). The TRIF/TRAM pathway, as well as the MAL/MyD88 pathway mediate RANTES induction in response to TLR2 ligands. TLR4 signaling leading to RANTES induction is also shown (grey arrows).

In **Paper V**, we also found that phosphorylation of nuclear p65$^{Ser\text{-}536}$ was impaired in response to MALP-2 in TRIF$^{-/-}$TRAM$^{-/-}$ macrophages, supporting a role for TRIF and TRAM in TLR2-signaling. This phosphorylation site may possibly be involved in TLR2-mediated RANTES-induction, although this has yet to be shown. A number of kinases have been reported to phosphorlyate p65$^{Ser\text{-}536}$ in addition to IKKβ, including IKKα, IKKε, TBK1 and RSK1. The details regarding the kinases involved in TLR2-mediated, TRIF-dependent induction of RANTES have yet to be resolved. A number of posttranslational modifications at different sites of p65 may likely play a role in this response. In this respect it is highly possible that different serine residues may be phosphorylated simultaneously, however, it is unclear if there is a redundancy in serine phosphorylations at different sites.

Both the TRIF and the MyD88 pathway mediate TLR2-induced RANTES release in macrophages (**Paper V**), in contrast to TNF induction, which is tightly controlled by the MyD88-dependent pathway alone. The reason for this redundancy is unclear. Other cell types may, however, rely more heavily on either the one pathway or the other. Our results emphasize, however, that a role for TRIF and TRAM in TLR2 signaling should not be ignored. The TRIF-pathway may moreover be more prominent in other cell types, such as dendritic cells, which are efficient producers of IFNs, or T_H1 effector cells, which have recently been shown to be directly activated by TLR2 ligands to produce IFN-γ. The contribution of TRIF in TLR2 signaling *in vivo* is currently unknown. Induction of genes by both MyD88 and TRIF pathways may be necessary for optimal responses towards certain infections.

4.7 TLR2 IN DISEASE PATHOGENISIS AND INFLAMMATION

TLRs normally sense small amounts of microbial ligands, induce limited local inflammation and eliminate and infection, before it becomes systemic. A number of TLR polymorphisms have been implicated in infection and sepsis[75, 76]. Polymorphisms in TLR2 may predispose to *Staphylococcus* infection and tuberculosis susceptibility[73-75], as well as enhance susceptibility to leprosy and tuberculosis[76]. Upregulation of TLR2 in these could consequently serve as a means as to boost detection of an initial infection.

Antagonists of TLRs are applicable in the regulation of TLR-mediated inflammation or autoimmune status[262]. Alternative strategies to inhibiting these responses include designing TLR interfering molecules or by positively or negatively regulating TIR adapter molecules or other TLR signaling molecules[220]. In **Paper IV** we developed an antagonistic mAb against murine TLR2 which indirectly inhibited TNF release in macrophages in response to Pam_3CysSK_4. An antagonistic antibody against TLR2 could potentially inhibit the initial step of systemic inflammation by disrupting the activation of TLR2 by Gram-positive bacteria. Another murine TLR2 mAb; T2.5, similar to our 6C2 TLR2 mAb was developed by Meng *et al*[263] which presumably directly affects ligand-receptor interactions. This antibody was shown to suppress lethality due to septic shock syndrome provoked by Pam_3CysSK_4 and heat-inactivated *Bacillus subtilis* when administered within three hours after infection. The 6C2 anti-TLR2 mAb we developed in **Paper IV** also inhibited macrophage stimulation *in vitro*. We did not assess the effect of our 6C2 mAb *in vivo*, however, we did find that preatreatment of macrophages with the antibody down-regulated surface TLR2 *in vitro*. This mechanism, and not direct blocking of ligand binding epitopes, likely explains why the antibody dimished

responses to subsequent lipoprotein challenge. Expression, downregulation or blocking TLRs could consequently be an approach to inhibiting septic syndrome. However, we note that neither antibodies towards LPS, TNF or IL-1 have proven effective in human trials, despite their ability to protect mice from septic shock syndrome. Whole bacteria and viruses also contain a number of TLR ligands, as well as ligands for other PPRs, suggesting that a number of receptors must be blocked in order to inhibit signaling. The rapid response of TLRs towards ligands could furthermore limit the therapeutic window for intervention. It has therefore been suggested that targeting late mediators of septic shock syndrome may be more successful, since this strategy should allow a wider time span for intervention[264]. TLR signaling pathways could alternatively be targeted in order to terminate excessive signaling. Our observations in **Paper IV** and **V** regarding compensation and redundancy between different signaling pathways should however be taken into account when employing such a strategy.

4.8 TLR2 AS A BRIDGE TO ADAPTIVE IMMUNITY

While inhibiting TLR activation is potentially useful in suppressing inflammation and autoimmunity, enhancing TLR activation is applicable in immunotherapy in order to enhance anti-pathogenic responses during vaccination. TLR2 is broadly expressed on immune cells (**Paper IV**) and activation of TLR2 induces a variety of diverse immune responses depending on the cell type in question, making TLR2 an interesting target in drug development.

4.8.1 Initiation of adaptive immune responses via TLR2

TLRs influence the differentiation of T_H1 and T_H2 cells through activation of APC, resulting in the generation of lineage specific cytokines. T_H1 responses are important in protecting against many microbial infections, while T_H2 response are implicated in defense against parasitic infections and in the pathologies of allergy and asthma[265]. TLR3, TLR4, TLR7 and TLR9 ligands are all important forces in driving TLR-mediated Th1 responses through stimulation of IL-12p70 and IFN-γ in DCs[266-268]. TLR2 stimulation by Pam_3CysSK_4, in contrast, induces IL-10 release in DCs resulting in a T_H2 profile[268, 269]. The TLR2 ligand FSL-1 has also been shown to induce IL-10 and T_H2 type responses *in vivo*[267].

The expression of co-stimulatory molecules is also required to sustain an adaptive immune response. The induction of type I IFN-α/β promotes CD8+ T cell proliferation and survival, as well as B cell isotype switching and differentiation[270]. We observed upregulation of CD86 on DC in response to TLR2 ligands on human DC (**Paper III**), however, we did not observe

upregulation of surface CD86 on macrophages in response to TLR2 ligands (**Paper V**), suggesting that DC and macrophages respond differently to TLR2 ligands. Notably, both LPS and polyIC markedly upregulates surface CD86 in macrophages in a TRIF-dependent manner.

Murine Th1 effector cells have recently been shown to respond directly to TLR2 ligands[225], inducing IFN-γ production, as well as proliferation and survival of T_H1, directly, in the absence of TCR signaling. TLR2 stimulation of MyD88 or IRAK4 deficient mice still induced p38 and JNK phosphorylation, but did not induce ERK phosphorylation or NF-κB nuclear translocation, suggesting the TLR2-mediated signaling pathways in T_H1 cells and APC differ. In light of our finding that TRIF plays a role in TLR2-induced RANTES (**Paper V**), it is intriguing to speculate that the direct stimulation of T_H1 cells may be mediated by TRIF.

The upregulation of surface TLR2 observed in **Paper IV** and **V** may be particularly important on T cells, which normally express low levels of TLR2. Naïve human T cells have been shown to upregulate surface TLR2 in response to anti-T cell receptor antibody and IFN-α. These cells also produce cytokines in response to TLR2 ligands. Memory T cells from peripheral blood also express TLR2 and produce IFN-α in response to bacterial lipopeptide. Proliferation and IFN-γ production was also enhanced upon costimulation with TLR2 ligands in combination with IL-2 or IL-15. TLR2 therefore appears to function as a costimulatory receptor for antigen-specific T cell development and may participate in the maintenance of T cell memory [224].

4.8.2 TLR2 as a target for vaccine development

The expression of TLR2 and the role of TLR2 in inducing and shaping adaptive immune responses is becoming appreciated with regard to vaccine development. In **Paper III** we showed that targeting Ag by to TLR2 using recombinant Ab, called troy bodies, could be an effective strategy for development of Ab based vaccines. Troy bodies are recombinant Ab that are specific for APC surface molecules and which carry T cell epitopes as an integral part of their constant region[271]. Targeting TLR2 using Troy bodies induced a specific CD4+ T cell response and could allow efficient delivery of antigen to dendritic cells, as well as macrophages and B-cells (**Paper III**). Recent studies have furthermore suggested that both TLR2 and CD14 are suitable as targets for delivery of large antigens using vaccibodies. Vaccibodies are recombinant antibody-like vaccination vehicles consisting of homodimers of an antibody V-region specific for surface molecules on antigen-presenting cells (APC), a

linker, and an antigenic unit. TLR2 and CD14-specific vaccibodies have been shown to target and deliver large antigens to APC and efficiently induce CD4+T cell responses[272].

Chimeras composed of TLR2 ligands such as Pam_3CysSK_4, MALP-2 and FSL-1 fused to tumor antigens have been been suggested as an alternative immune therapy for cancer and other diseases [220]. These chimera are proposed to enhance Ag-presentation by specifically targeting antigens to APC, as well as stimulate cytokine production and upregulation of costimulatory molecules in DC. Fused proteins, consisting of TLR5 and TLR7/8 agonists and targeted antigens effiently induce anti-pathogenic CTL responses *in vivo* in animal studies[273, 274]. Vaccines consisting of a TLR2 ligand, a T_H epitope and a target antigen epitopes conferred protection in a *Listeria monocytogenes* model, as well as in a lung tumor cell challenge model[275]. These chimeras of TLR ligands fused to antigens may therefore be suitable for boosting immune responses against infectious diseases and cancer. A specific immune response could furthermore potentially be tailored by applying different TLR ligands in combination.

The unique ability of TLR2 agonists to stimulate IFN-γ production and proliferation and survival of T_H1 cells in the absence of TCR signaling could possibly be manipulated in immunotherapy to directly activate T_H1 cells[225]. Memory T cells from peripheral blood have also been shown to express TLR2 and produce IFN-γ in response to bacterial lipopeptide. TLR2 ligands could therefore possibly be applied to enhance maintenance of T cell memory[224]. TLR2 has also been implicated in the regulation of regulatory CD4+CD25+ T cells[227]. Stimulation of these cells with TLR2 ligands enhanced the proliferation of these cells and reversed their suppressive activity. Although this effect was transient, TLR2 ligands may aid in enhancing immune responses in this manner.

TLR2 ligands could potentially be applied to induce a humoral response directly, since TLR2 ligands such as Pam_3CysSK_4 and MALP-2 have been shown to directly activate B cells and induce IL-6 and upregulation of MHCII in a T-cell independent manner[221, 222]. TLR2 ligands, as well as TLR4, TLR7 and TLR9 also induce robust proliferation and antibody secretion in murine follicular B cells and marginal zone B cells[223]. TLR2 ligands also induce the differentiation and proliferation of B cells[276].

TLR2 ligands appear to affect a number of cell-types, and induce a number of different responses. Although TLR2 and other TLR ligands appear to hold valuable potential in immunotherapy strategies, further research is needed to map the precise mechanisms of TLR biology in order to fully apply TLRs therapeutically and avoid the potentially adverse effects these ligands may cause.

5. CONCLUSION

In this study we show that TLR2 is highly expressed on immune cells and that surface TLR2 is upregulated in response to low concentrations of stimuli. We provide further insight into the subcellular expression and trafficking of TLR2, and a role for the co-receptors CD14 and CD36 in enhancing TLR2 signaling. We also reveal a role for TLR2 in antigen-presentation and in the induction of adaptive immunity. We further provide insight into TLR2 signaling and signaling pathways controlling the regulation of TLR2 expression, and describe a new role for the TIR-adapters TRIF and TRAM in TLR2 signaling. Combined, these results provide further insight into TLR2 expression, regulation and signaling, which we propose could be helpful in the development of vaccines and immunomodulatory drugs. Our findings also add significantly to our understanding of innate immune responses to infection.

6. REFERENCES

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Dissertations at the Faculty of Medicine, NTNU

1977
1. Knut Joachim Berg: EFFECT OF ACETYLSALICYLIC ACID ON RENAL FUNCTION
2. Karl Erik Viken and Arne Ødegaard: STUDIES ON HUMAN MONOCYTES CULTURED *IN VITRO*

1978
3. Karel Bjørn Cyvin: CONGENITAL DISLOCATION OF THE HIP JOINT.
4. Alf O. Brubakk: METHODS FOR STUDYING FLOW DYNAMICS IN THE LEFT VENTRICLE AND THE AORTA IN MAN.

1979
5. Geirmund Unsgaard: CYTOSTATIC AND IMMUNOREGULATORY ABILITIES OF HUMAN BLOOD MONOCYTES CULTURED IN VITRO

1980
6. Størker Jørstad: URAEMIC TOXINS
7. Arne Olav Jenssen: SOME RHEOLOGICAL, CHEMICAL AND STRUCTURAL PROPERTIES OF MUCOID SPUTUM FROM PATIENTS WITH CHRONIC OBSTRUCTIVE BRONCHITIS

1981
8. Jens Hammerstrøm: CYTOSTATIC AND CYTOLYTIC ACTIVITY OF HUMAN MONOCYTES AND EFFUSION MACROPHAGES AGAINST TUMOR CELLS *IN VITRO*

1983
9. Tore Syversen: EFFECTS OF METHYLMERCURY ON RAT BRAIN PROTEIN.
10. Torbjørn Iversen: SQUAMOUS CELL CARCINOMA OF THE VULVA.

1984
11. Tor-Erik Widerøe: ASPECTS OF CONTINUOUS AMBULATORY PERITONEAL DIALYSIS.
12. Anton Hole: ALTERATIONS OF MONOCYTE AND LYMPHOCYTE FUNCTIONS IN REALTION TO SURGERY UNDER EPIDURAL OR GENERAL ANAESTHESIA.
13. Terje Terjesen: FRACTURE HEALING AN STRESS-PROTECTION AFTER METAL PLATE FIXATION AND EXTERNAL FIXATION.
14. Carsten Saunte: CLUSTER HEADACHE SYNDROME.
15. Inggard Lereim: TRAFFIC ACCIDENTS AND THEIR CONSEQUENCES.
16. Bjørn Magne Eggen: STUDIES IN CYTOTOXICITY IN HUMAN ADHERENT MONONUCLEAR BLOOD CELLS.
17. Trond Haug: FACTORS REGULATING BEHAVIORAL EFFECTS OG DRUGS.

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1987
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1988
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1997

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1998

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137. Juan A. Pareja: SUNCT SYNDROME. ON THE CLINICAL PICTURE. ITS DISTINCTION FROM OTHER, SIMILAR HEADACHES.
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1999

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142. Harm-Gerd Karl Blaas: THE EMBRYONIC EXAMINATION. Ultrasound studies on the development of the human embryo.
143. Noèmi Becser Andersen:THE CEPHALIC SENSORY NERVES IN UNILATERAL HEADACHES. Anatomical background and neurophysiological evaluation.
144. Eli-Janne Fiskerstrand: LASER TREATMENT OF PORT WINE STAINS. A study of the efficacy and limitations of the pulsed dye laser. Clinical and morfological analyses aimed at improving the therapeutic outcome.
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146. Terje Haug: STRUCTURE AND REGULATION OF THE HUMAN UNG GENE ENCODING URACIL-DNA GLYCOSYLASE.
147. Heidi Brurok: MANGANESE AND THE HEART. A Magic Metal with Diagnostic and Therapeutic Possibilites.
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152. Katarina Tunòn: ULTRASOUND AND PREDICTION OF GESTATIONAL AGE.
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2000

158. Ola Dalsegg Sæther: PATHOPHYSIOLOGY DURING PROXIMAL AORTIC CROSS-CLAMPING CLINICAL AND EXPERIMENTAL STUDIES
159. xxxxxxxxx (blind number)
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2001

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CELLULAR TRAFFICKING OF LIPOTEICHOIC ACID AND TOLL-LIKE RECEPTOR 2 IN RELATION TO SIGNALING; ROLE OF CD14 AND CD36

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Running Title: LTA and TLR2 Trafficking in relation to Signaling

ABSTRACT

Lipoteichoic acid (LTA) is a central inducer of inflammatory responses caused by Gram- positive bacteria, such as *Staphylococcus aureus* (*S. aureus*), via activation of Toll-like receptor 2 (TLR2). Localization of TLR2 in relation to its co-receptors may be important for function. This study explores the signaling, uptake and trafficking pattern of LTA in relation to expression of TLR2 and its co-receptors CD36 and CD14 in human monocytes. We found TLR2 expressed in early endosomes, late endosomes/lysosomes and in Rab-11-positive compartments, but not in the Golgi apparatus or endoplasmic reticulum (ER). Rapid internalization of fluorescently labeled LTA was observed in human monocytes, colocalizing with markers for early and late endosomes, lysosomes, ER and Golgi network. Blocking CD14 and CD36 with antibodies inhibited LTA-induced TNF release from monocytes and LTA uptake, emphasizing an important role for both molecules as co-receptors for TLR2. Importantly, blocking CD36 did not affect TNF release induced by Pam_3CysSK_4 or LPS. Expression of CD14 markedly enhanced LTA binding to the plasma membrane and also enhanced NF-κB activation. LTA internalization, but not NF-κB activation, was inhibited in Dynamin-I K44A dominant negative transfectants, suggesting that LTA is internalized by receptor-mediated endocytosis, but that internalization is not required for signaling. In fact, immobilizing LTA, and thereby inhibiting internalization, resulted in high TNF release from monocytes. Our results suggest that LTA signaling preferentially occurs at the plasma membrane, is independent of internalization, and is facilitated by both CD36 and CD14 as co-receptors for TLR2.

INTRODUCTION

Both Gram-negative and Gram-positive bacteria, as well as viruses and fungi, induce proinflammatory responses that can cause fatal sepsis syndrome [1, 2]. *Staphylococcus aureus* (*S. aureus*) is the most commonly isolated infectious Gram-positive pathogen, and strains are rapidly becoming resistant to nearly all current antibiotics [3]. While lipopolysaccharide (LPS) from Gram-negative bacteria is suggested as a principal inducer of Gram-negative septic shock [4, 5], lipoteichoic acid (LTA) may be an equivalent component responsible for septic shock provoked by Gram-positive bacteria [6].

Toll-like receptors (TLRs) recognize a range of pathogen-associated molecular patterns (PAMPS), such as LPS and LTA. TLRs further initiate proinflammatory responses required for clearance of infection, by the same mechanisms that potentially cause sepsis [7]. Thirteen mammalian TLRs (TLR1-13), which recognize different PAMPs have been identified to date [7]. These are germ-line encoded, transmembrane proteins, consisting of an extracellular leucine-rich repeat (LRR) domain, and a cytoplasmic domain sharing homology with the mammalian interleukin-1 (IL-1) receptor [8, 9]. A signaling cascade initiated by activation of the TLRs results in translocation of the transcription factor nuclear factor kappa-B (NF-κB), which subsequently induces the expression of TNF, IL-1β, IL-6 and IL-8 and maturation of antigen presenting cells [10]. While TLR4, in complex with the small secreted glycoprotein MD2, recognizes LPS from Gram-negative bacteria [11, 12], TLR2 recognizes a particularly broad range of ligands, including Gram-positive bacteria and cell wall components such as LTA, as well as peptidoglycan and lipoproteins [13-18]. Additional TLR2 ligands may include zymosan, glycolipids from spirochetes, lipoarabinomannan, porins from *Neisseria*, among others [19]. The ability of TLR2 to recognize such a wide repertoire of ligands is partially explained by heterodimerization of TLR2 with TLR1 and TLR6. TLR2/TLR1 heterodimeriztion occurs in response to triacetylated lipopeptides, such as Pam_3CysSK_4 [20, 21], while optimal response towards diacetylated lipopeptides is attained by heterodimerization of TLR2 with TLR6 [22, 23]. LTA is recognized by the TLR2/TLR6 heterodimer, supposedly due to the two diacyl chains in the molecule [24].

The monocyte differentiation antigen CD14 is a glycosylphosphatidylinositol (GPI) -linked receptor expressed by cells of the monocytic lineage [25, 26]. The receptor is shown to be highly concentrated in lipid raft microdomains of these cells [27]. Soluble CD14 (sCD14) and LPS-binding protein (LBP) in serum transfer LPS to membrane bound CD14, which further presents LPS to the TLR4/MD2 signaling complex [28-31]. The entire complex has further been shown to shuttle between the plasma membrane and the Golgi, independent of signaling, which is believed to

predominantly occur at the plasma membrane [32, 33]. CD14 has further been shown to bind lipopeptides and LTA in a similar manner [34-36]. The multifunctional B class scavanger receptor CD36 has, however, also been found to be involved in immune responses to TLR2/TLR6 ligands such as LTA, in a manner analogous to CD14 [37]. Whether LTA binding to the plasma membrane is sufficient to induce signaling through TLR2, or whether internalization of the ligand is required is still under debate. Although some reports support that signaling occurs in lipid rafts, independent of ligand internalization [36, 38], other reports show that reduced internalization of both *S. aureus* and its component LTA, correlated with diminished inflammatory response [39]. The relative role of CD14 and CD36 in response to LTA is furthermore in question with regard to whether the co-receptors participate in the same TLR2/TLR6 signaling complex, or whether they enhance TLR2-mediated responses independent of one another.

In this study we explored the uptake and trafficking pattern of LTA from *S. aureus*, in relation to subcellular expression of TLR2 and its co-receptors CD36 and CD14, in human monocytes. We found TLR2 expressed in the plasma membrane, endosomes, lysosomes and in Rab11-positive compartments, but not in the Golgi apparatus or the ER. LTA rapidly accumulated in early and late endosomes, lysosomes, as well as in the ER and Golgi. Both CD14 and CD36 were required for optimal LTA-binding/internalization and TNF release in monocytes. We further found that LTA internalization, but not NF-κB activation, was inhibited in Dynamin-I K44A dominant negative transfectants, showing that LTA is internalized by receptor-mediated endocytosis, but that internalization is not required for signaling. These results support the hypothesis that the main signaling in response to LTA occurs preferentially at the plasma membrane, is independent of internalization, and requires both CD36 and CD14 as co-receptors for TLR2.

EXPERIMENTAL PROCEDURES

Reagents

Tissue culture medium, trypsin/EDTA, penicillin, streptomycin and PBS were obtained from BioWhittaker (Walkersville, MD). Culture medium was supplemented with 2mM L-glutamine and 10 μg/ml ciprofloxacin (Cellgro/Mediatech, Herndon, VA or from BioWhittaker). G418 was purchased from Calbiochem (San Diego, CA) and Life Technologies (Gaithersburg, MD). Low endotoxin fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT) and Integro (Zaandam, The Netherlands). Lipoteichoic acid from *S. aureus* was prepared by butanol extraction as described [16]. The purity of LTA was over 99%, measured by nuclear magnetic resonance and mass spectrometry [16]. Endotoxin contamination was minimal (<0.1 pg/μg), measured by negative Limulus amoebocyte

lysate assay, QCL-1000, (Charles River Endosafe, Charleston, WV, USA). Fluorescein- (FITC) and Rhodamine-conjugated LTA was prepared by sonifying LTA from *S. aureus* (3 mg) and fluorescein 5-isothiocyanate or sulforhodamine Q 5-acid fluoride (4.5 mg) (Fluka, Buchs, Switzerland), dimethyl sulfoxide (2.5 ml) (Wak-Chemie-Medical GmbH, Steinbach, Germany) and trimethylamine (25 µl) (Acros Organics, Leicestershire, UK) for 10 min, and then shaked overnight at 37°C. The mixture was further spun at 7000 g for 90 min at room temperature four times in a pyrogen-free centrifugal ultrafilter unit (cut-off 3 kDa, Microsep 3K Centricons, Pall, USA) and additionally filtered through a PD-10 desalting column, (Amersham Biosciences, Freiburg, Germany). The yield of labeled LTA was determined by phosphate content, measured by the molybdenum blue method; LTA solution (50 µl) was mixed with ashing solution [H_2SO_4 : $HClO_4$: H_2O (556:105: 3339, v:v:v)] (200 µl) and incubated at 145 °C for 2 h. Reducing solution [ascorbic acid: ammoniumheptamolybdenum sodium acetate (1: 9, v: v)] (1 ml) was subseqently added prior to incubation at 50°C for 2 h. Absorption was measured at 700 nm. Labeling efficiency, calculated as fluorescence (560 nm/620 nm) per phosphate content, was ~ 1 molecule rhodamine or fluorescein per LTA. The labeled LTA was negative in the Limulus test for Gram-negative endotoxin (<0.1 pg/µg). Lipopolysaccharide (LPS) was from Escherichia coli strain O111:B4 and purchased from Invivogen (San Diego, CA). Synthetic Pam_3Cys-Ser-Lys_4 (Pam_3CysSK_4) was purchased from EMC microcollections (Tübingen, Germany). Antibodies used were; anti-TLR2 (TL2.1) [18], anti-TLR4 (HTA125) purified from hybridoma cells, kindly provided by Dr Kensuke Miyake (Saga Medical School, Japan) [40], unconjugated and FITC conjugated anti-CD36 (FA6-152) (Immunotech, France), anti-CD14 mAbs 3C10 [41], 5C5 [42] and MEM-18 (HyCult Biotechnology, Uden, The Netherlands). Additional antibodies used include Mouse IgG and Tricolor (PE-Cy5)-conjugated Goat-anti-Mouse secondary Ab (Caltag/Invitrogen, CA), anti-LAMP-1 (R&D Systems, MN), anti-Golgin-97 (CDF4) (Invitrogen, CA), anti-GM130, anti-Eea-1 and unconjugated and FITC-conjugated anti-Calnexin and MouseIgG (BD Biosciences, NJ). Alexa-conjugated antibodies were generated by protein labeling with Alexa 488 (A488), 546 (A546) or 647 (A647) according to manufacturer's instructions (Invitrogen, CA). Alexa 633 (A633) labeled transferrin was purchased from Invitrogen. The following expression vectors were used; pcDNA3 (Invitrogen, CA), human CD14 and TLR2-YFP in pcDNA3 [43], and early endosomal antigen-1 (Eea-1) tagged with green fluorescent protein (GFP) (Eea-1^{GFP}) [44]. pORF9 and human CD36 in pORF9 were purchased from Invivogen (San Diego, CA). MD-2 in pEF-BOS was kindly provided by Dr Miyake [45]. ER^{CFP} encoding the ER targeting sequence of calreticulin fused to cyan fluorescent protein (CFP) (Clontech, CA). Dynamin-I wild-type and Dynamin-I K44A in pcDNA3 were kindly provided by Dr Sandy Schmid

(Scripps, USA). Transient transfections were performed using GeneJuice^{TM} transfection reagent (Novagen, Darmstadt, Germany) according to manufacturer's instructions.

Cells and Cell lines

Human monocytes were isolated from peripheral blood mononuclear cells (PBMC) by adherence. PBMC were seperated from A^+ buffycoats (Blood bank, St Olav's Hospital Trondheim, Norway) using Lymphoprep, as described by manufacturer (Axis- Shield, Norway). Monocytes were allowed to adhere in RPMI supplemented with 5 or 10% pooled A^+ serum (St Olav's Hospital Trondheim, Norway) for 1 h at 37 °C, 5% CO_2, before cells were washed 3 times and added fresh medium. Human epithelial kidney 293 (HEK293) cell lines expressing TLR2 or TLR2 in combination with CD14 [43] were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and the selection antibiotic G418 (0.5 mg/ml). Untransfected HEK293 cells were cultured in 10%FBS/DMEM. For confocal imaging, cells were seeded on 35 mm glass bottom γ-irradiated tissue cell dishes (MatTek Corporation, Ashland, MA). Madine Darby Canine Kidney (MDCK) cells stably expressing Eea-1^{GFP} were grown in DMEM supplemented with 9% FBS, 2 mM glutamine, 25 U/ml penicillin and 25 μg/ml streptomycin at 37 ^{o}C, 6% CO_2.

Live Microscopy

Madine Darby Canine Kidney (MDCK) cells stably expressing Eea-1^{GFP} were transiently transfected with the TLR2 over night in microwell dishes for microscopy (Mattek, MA), using Lipofectamin according to protocol (Invitrogen, CA). Preceding imaging the cells were added microscopy medium, DMEM without phenol red and sodium carbonate, supplemented with 3.5 g/L D-glucose to a final concentration of 4.5 g/L; 25 mM HEPES; with 10% FBS. Cells were kept on ice for 45 min and then incubated with Alexa 546 conjugated anti-TLR2 mAb TL2.1 on ice for an additional 45 min. Image acquisition was performed on an Olympus Fluoview 1000 at 37°C with an Olympus PlanApo 60X/1.42 Oil objective. Internalization analysis was carried out with ImageJ software, measuring the intensity of conjugated TLR2 as a function of time.

Confocal microscopy of subcellular expression of TLR2 and LTA internalization

Freshly isolated live monocytes were added fresh RPMI medium supplemented with 0.1% A^+ serum incubated with LTA^{Rhodamine} (20 μg/ml) and internalization of the ligand was followed by confocal microscopy at 37 °C for 1 h.

Freshly isolated monocytes were either left unstimulated or stimulated with $LTA^{Rhodamine}$ for 1 h, 37 °C, 8% CO_2, prior to fixating with 4% para- formaldehyde, 10 min on ice, and then with PEM buffer 10 min at room-temperature (RT). Cells were then permeabilized with 50 mM NH_4Cl/0.05% BSA/0.05% Saponin, 20 min, RT. Cells were subsequently stained in 50 mM NH_4Cl/0.05% BSA/0.05% Saponin, 20 min, RT. Cells were also stained with A488 or A647 conjugated anti-TLR2 and antibodies against early endosome marker $Eea\text{-}1^{FITC}$, lysosome marker $anti\text{-}LAMP\text{-}1^{A647}$, trans-Golgi marker $anti\text{-}Golgin\text{-}97^{A647}$, *cis*-Golgi marker anti-GM130, $anti\text{-}CD36^{FITC}$, $anti\text{-}CD14^{A647}$ (3C10) or $anti\text{-}CD14^{A488}$ (5C5). Freshly isolated monocytes were additionally stained intracellularly, as described, with anti-Rab-11, and subsequently with secondary antibody $Goat\text{-}anti\text{-}Rabbit^{A647}$, prior to staining with $TL2.1^{A488}$. HEK293 cells were stained intracellularly after fixing cells with 4% para-formaldehyde for 10 min on ice and permeabilization with 20%A^+/0.1% saponin/PBS for 20 min at RT. Cells were stained in 2%A^+/0.1% saponin/PBS for 45 min at RT using 2-10 μg/ml Ab before cells were washed and added PBS. Cells were observed by confocal microscopy using an Axiovert 100-M inverted microscope (Zeiss), equipped with an LSM 510 laser scanning unit and a 63X 1.4-NA plan Apochromat oil-immersion objective (Zeiss). Appropriate filters were selected for the individual stainings.

LTA binding/internalization studies

A^+ buffycoat from healthy donors (St Olav's Hospital Trondheim, Norway) was incubated with $LTA^{Rhodamine\ Green}$ (10 μg/ml) for 45 minutes at 4 °C or at 37 °C, 8% CO_2. Incubation at 4 °C should permit LTA binding to receptors on the plasma membrane, but delay LTA internalization, while binding and internalization of LTA in monocytes was expected to proceed normally at 37 °C. Erythrocytes were lysed with formic acid- based lysis buffer for 1 min, neutralized and fixed using the Coulter Immunoprep Epics Leukocyte preparation system (Coulter, FL). Samples of cells were stained with Fluoroscein (FITC) or Phycoerytrin (PE) conjugated monoclonal antibodies (mAb) against CD14, CD3 or CD19. Samples were analyzed by flow cytometry and populations were gated by CD14 high expression (monocytes), CD14 low expression (granulocytes), and CD3 or CD19 expression (lymphocytes), as well as by size and granularity. Gates were subsequently applied to determine $LTA^{Rhodamine\ Green}$ internalization in the samples by determining median fluorescence of each population. Monocytes were also stimulated by plating cells in sterile 96-plate wells coated overnight at 4 °C with titrations of LTA or PBS, or stimulated by adding titrations of LTA in solution, or medium. Wells were washed 4 times with PBS after coating and prior to addition of cells to remove excess

unbound LTA. Cells were stimulated overnight in 1%A^+/RPMI at 37 °C, 5% CO_2 before supernatant was harvested and TNF levels were assessed by ELISA.

Cell staining for flow cytometry

Freshly islolated monocytes were detached with 0.02% EDTA, fixed with 4% para-formaldehyde and stained extracellularly in 1% FBS/ PBS with anti- $TLR2^{A488}$, $CD36^{FITC}$, $CD14^{A488}$ (5C5) or $MouseIgG^{A488/FITC}$ and analyzed by flow cytometry. Freshly isolated monocytes were furthermore left unstimulated or stimulated for 16 h with LTA (0.1, 1, 10, 100 or 1000 ng/ml), prior to staining with anti-TLR2 or MouseIgG and subsequently with secondary Ab Goat-anti-$Mouse^{Tricolor}$, or $CD36^{FITC}$ or $MouseIgG^{FITC}$, as described.

Luciferase reporter assay

NF-κB activation was determined by an NF-κB luciferase reporter assay as previously described [43]. Briefly, HEK293 cells were transiently transfected with reporter plasmid ELAM-luciferase reporter gene (ELAM-Luc), containing a NF-κB dependent portion of the ELAM promoter driving luciferase. Cells were additionally transfected with control plasmids pcDNA3 and pORF9 and/or CD36, CD14, TLR2; CD36, CD14 and TLR2 in combination, or TLR4, CD14 and MD2 for 24 h. The total amount of each vector was kept constant, by filling up with the appropriate control plasmids. Cells were subsequently stimulated with LTA (5 μg/ml) or LPS (100 ng/ml) for 5 h before cells were lysed and assayed for luciferase activity as a measure for NF-κB activation.

Blocking studies

Freshly isolated monocytes were preincubated with MouseIgG, anti-TLR2, anti-CD36, anti-CD14 (MEM-18) or anti-TLR4 (10 ug/ml) in RPMI at RT for 30 min and subsequently added $LTA^{Rhodamine\ Green}$ (2 μg/ml) for 45 min at 37 °C. Cells were detached with 0.02% EDTA/PBS, washed and analyzed for LTA binding and internalization by flow cytometry. Monocytes were pretreated with optimized concentrations of anti-CD36 (0.5 μg/ml), anti-CD14 (3C10) (10 μg/ml), a mixture of TL2.1 and TL2.3 (10 μg/ml) or MouseIgG (10 μg/ml) for 45 min, RT, prior to stimulation with medium, LTA (10 μg/ml), LPS (20 ng/ml) or Pam_3CysSK_4 (50 ng/ml) in 1%A^+/RPMI for 5 h at 37 °C, 5% CO_2 before supernatant was harvested and analyzed for TNF by ELISA (R&D Systems, MN).

Dynamin-I expression studies

HEK293-TLR2 cells were transiently transfected with wild- type Dynamin-I or the mutant Dynamin-I K44A, in the presence or absence of transfected CD14 for 72 h before cells were incubated with $LTA^{Rhodamine}$ (2 μg/ml) and $Transferrin^{A633}$ (2 μg/ml) for 30 min at 37 °C and observed by confocal microscopy. HEK-TLR2 cells were transiently transfected as described with the ELAM- Luc, and combinations of CD14, and Dynamin-I WT or the mutant Dynamin-I K44A and/or control pcDNA3 for 72 h. Cells were subsequently stimulated with medium or LTA (5 μg/ml) for 5 h, before cells were lysed and analyzed for NF-κB activation.

RESULTS

Surface TLR2 is rapidly internalized into endosomes and lysosomes

In order to study TLR2 trafficking, MDCK cells expressing TLR2 and a GFP-tagged early endosomal antigen-1 ($Eea\text{-}1^{GFP}$), were incubated with the Alexa 546 labeled TLR2 mAb TL2.1 ($TL2.1^{A546}$). We observed that $TL2.1^{A546}$ bound to the plasma membrane of TLR2 transfected cells (Figure 1 A). We further observed gradual internalization of the $TL2.1^{A546}$ mAb into Eea-1-positive early endocytic compartments (Figure 1 A and B, Supplementary Video 1), followed by maturation as the Eea-1 coat detached (Figure 1 A, Arrow). No binding or internalization was observed upon incubation with an isotype control (not shown), demonstrating the specificity of the staining.

Figure 1 B shows an increase in colocalization of $TL2.1^{A546}$ and $Eea\text{-}1^{GFP}$ within the initial 4 min of incubation, while colocalization between $TL2.1^{A546}$ and $lysotracker^{Green}$ began to increase after 10 min of incubation. Calculations from images after 180 min showed that 20-30% of the TLR2 mAb colocalized with the lysosome marker LysotrackerGreen after 180 min (images not shown). These results suggest that surface TLR2 traffics along the conventional endosomal pathway.

TLR2 is expressed in the plasma membrane, endosomes, lysosomes and Rab-11- positive compartments in monocytes

We further applied confocal microscopy to investigate the intracellular expression of TLR2 in freshly isolated monocytes in order to assess the subcellular compartments where LTA- induced signaling could occur. In line with previous studies [46], TLR2 was found to be highly expressed in the plasma membrane of monocytes (Figure 2), as

well as in a population of early endosomes (Figure 2 A) and lysosomes (Figure 2 B). Furthermore, we found that TLR2 was not expressed in the Golgi network, using antibodies against Golgin-97 (Figure 2 C) and GM130 (not shown), which are specific markers for the *trans*- and *cis*-Golgi, respectively [47, 48]. TLR2 expression was, however, often observed in close proximity to the trans-Golgi network, which led us to examine whether TLR2 was expressed in Rab-11 positive compartments. The GTPase Rab-11 localizes to pericentriolar recycling endosomes and *trans*-Golgi and is essential for development of multivesicular body (MVB) endosomal compartments [49-52]. TLR2 colocalized with Rab-11 in the perinuclear area (Figure 2 D). These results suggest that TLR2 may also be expressed in recycling endosomes and possibly in multivesicular endosomes. Minimal colocalization was observed between TLR2 and the ER-marker Calnexin (not shown), showing that TLR2 is not retained in the ER, which is in contrast to other TLRs such as TLR3 and TLR9 [53, 54].

Monocytes efficiently bind and internalize fluorescently labeled LTA and upregulate TLR2

The ability of leukocyte populations to bind and internalize LTA was assessed by incubating white blood cells from healthy human donors with fluorescently labeled $LTA^{Rhodamine\ Green}$ at 4 °C or 37 °C. We found that monocytes, as well as granulocytes, bound LTA efficiently at 4 °C, while only marginal binding was observed to lymphocytes (Figure 3 A). Additional fluorescence was observed at 37 °C, suggesting both binding and internalization of LTA in monocytes and granulocytes. Though both monocytes and granulocytes express TLR2, CD36 and CD14 [46, 55, 56], monocytes express particularly high levels of all three receptors on the surface (Figure 3 B). We have previously shown that murine macrophages rapidly upregulate TLR2 in response to heat- killed *S. aureus* [57]. Here we show that LTA from *S. aureus* upregulated surface TLR2 on human monocytes in a dose- dependent manner (Figure 3 C). The expression of surface CD36 on monocytes was, however, less affected upon stimulation with LTA and was found to be slightly down-regulated at the highest LTA concentration, in contrast to TLR2 (Figure 3 D).

LTA is rapidly internalized in tubular endocytic structures and targeted to the trans- Golgi network and the ER

We next investigated whether LTA localized to the same compartments as TLR2 in human monocytes. Using fluorescently labeled LTA we observed LTA internalization in live monocytes by confocal microscopy. LTA initially bound to the plasma membrane of monocytes and was rapidly internalized (not shown). Figure 4 A shows LTA in extensive tubular structures and concentrated in the perinuclear area in monocytes after 20 min of incubation. Confocal images and fluorescence intensity profiles of the fluorescently conjugated Ab and LTA showed that LTA was localized in the Golgi network (Figure 4 B), in early endosomes (Figure 4 C), as well as in lysosomes (Figure 4 D), after 1 h of incubation. LTA localization to LAMP-1 positive vesicles was more apparent at later time points, (2- 6 h), (data not shown). Extensive overlay was observed between fluorescent LTA and calnexin staining, particularly in close proximity to the nucleus (Figure 4 E), suggesting that LTA localizes to the ER. These results were confirmed in HEK293-TLR2 cells transiently expressing the targeting sequence of calreticulin fused to CFP which localizes the protein to the ER (ER^{CFP}). Confocal images of these cells with $LTA^{Rhodamine}$ revealed overlap between LTA and the ER marker (Figure 4 F), suggesting that LTA is targeted to the ER upon internalization. Though LTA clearly localized to the *trans*-Golgi network and the ER of monocytes, TLR2 was not expressed in these compartments (Figure 2 C and data not shown). Colocalization between LTA and TLR2 was, however, observed in early endosomes and lysosomes (not shown). Since LTA requires TLR2 for signaling, the results suggest that signaling may occur at the plasma membrane and along the endocytic pathway.

Both CD36 and CD14 enhance TLR2-mediated NF-κB activation in response to LTA

Both CD36 and CD14 are shown to act as coreceptors for TLR2 in response to LTA [34, 37]. In this experiment the role of CD36 and CD14 in response to LTA was compared. HEK293 cells were transiently transfected with CD36, CD14, TLR2 or in combination. HEK293 cells were also transiently transfected with TLR4, MD2 and CD14 as a control. NFκB-activation in response to LTA was only observed upon expression of TLR2 (Figure 5). Coexpression of TLR2 with either CD36 or CD14 enhanced LTA induced NFκB-activation approximately three-fold compared to cells expressing TLR2 alone (Figure 5). These results indicate that both CD36 and CD14 function as co-receptors for TLR2 in response to LTA, and that expression of either co-receptor enhances LTA-induced NFκB-activation markedly. A small additive effect on NFκB-activation was observed upon co-expression of both CD36 and CD14 with TLR2 in

response to LTA (Figure 5). NFκB- activation was not observed in HEK293 cells transfected with TLR4, CD14 and MD2 in response to LTA, though these cells responded normally to LPS (Figure 5), illustrating the purity of the LTA.

CD36 and CD14 colocalize with TLR2 at the plasma membrane in monocytes

Since both CD36 and CD14 function as coreceptors for TLR2 in response to LTA, we studied the localization of these receptors in unstimulated and LTA- stimulated monocytes. Freshly isolated human monocytes were either left unstimulated, or incubated with $LTA^{Rhodamine}$ for 1 h, and then stained for TLR2, CD36 or CD14. Confocal microscopy showed that all three receptors were highly expressed in the plasma membrane, as well as in intracellular vesicles (Figure 6). TLR2 was also found to colocalize with CD36 (Figure 6 A) and CD14 (Figure 6 C) both at the plasma membrane and in internal vesicular structures, that may represent early endosomes and lysomes where TLR2 is expressed (Figure 2 A and B). Upon stimulation of monocytes with fluorescent LTA, particularly CD36 (Figure 6 B), but also CD14 (Figure 6 D), redistributed to the plasma membrane (Figure 6, profile graphs). LTA was predominantly localized in the perinuclear area, and surprisingly little LTA was observed bound to the plasma membrane. However, profile graphs of fluorescence intensity in a cross section of the cells show that LTA was often localized in close proximity to the plasma membrane (Figure 6 B and D), often overlapping partially with TLR2, CD14 and CD36 at the plasma membrane. Thus, it is likely that LTA signaling occurs at the plasma membrane, as well as along the endocytic pathway.

Blocking CD14 or CD36 impairs LTA cell association and subsequent TNF release in monocytes

We further investigated the contribution of TLR2 and its co-receptors CD36 and CD14 in the cell association (binding plus internalization) of LTA in freshly isolated human monocytes. Monocytes were pre-treated with antibodies against TLR2, CD36 and CD14 prior to incubation with fluorescently labeled LTA. Interestingly, we found that cell association of LTA was markedly reduced in cells pretreated with antibodies against either CD36 or CD14, while antibodies against TLR2 and TLR4 alone did not affect LTA cell association (Figure 7 A). Thus, both CD14 and CD36 play an important role in the cell association of LTA in human monocytes.

Given the reduction in cell associated LTA in the presence of blocking CD36 and CD14 antibodies, we next determined whether inhibiting CD36 and CD14 also had an effect on LTA-induced signaling in monocytes. Indeed, inhibition of CD36 was found to markedly reduce TNF release in monocytes in response to LTA, but had no effect on

the response to the TLR4 ligand LPS, or the TLR2/TLR1 ligand Pam_3CysSK_4 (Figure 7 B). Though inhibition of TLR2 had no effect on cell association of LTA, inhibition of TLR2 significantly reduced TNF release from monocytes in response to LTA, as well as in response to Pam_3CysSK_4, but not in response to LPS (Figure 7 B). Pretreatment of monocytes with anti-CD14 inhibited TNF release in response to LTA, as well as LPS and Pam_3CysSK_4 (Figure 7 B). These results suggest though TLR2 is required for signaling in response to LTA, CD14 and CD36 play a prominent role in the LTA cell association, and in LTA-induced signaling in human monocytes.

TLR2 signaling in response to LTA occurs mainly at the plasma membrane, independently of receptor-mediated endocytosis via Dynamin- I

Since LTA colocalized with TLR2 both at the plasma membrane and along the endocytic pathway, we further examined whether internalization of LTA was necessary for signaling. Monocytes were incubated on LTA- coated wells or with LTA added in solution. Immobilization of LTA on a plastic surface should allow binding, but restrict internalization of the ligand. Interestingly, we found that immobilizing LTA greatly enhanced TNF release from monocytes, compared to cells that received LTA in solution (Figure 8 A). We argue that the prominent effect on TNF release from monocytes observed upon immobilizing LTA, shows that signaling predominantly occurs at the plasma membrane and does not require internalization of LTA.

The significance of LTA internalization with regard to signaling was further studied by inhibiting LTA internalization. A dominant negative mutant of Dynamin-I (Dynamin-I K44A) inhibits receptor-mediated endocytosis by interfering with the function of endogenous Dynamin-I by blocking vesicle internalization before membrane scission occurs [58]. The internalization route of LTA was studied in HEK-TLR2 cells, transiently expressing wild-type Dynamin-I, or the mutant Dynamin-I K44A, in the presence or absence of CD14 expression. Transfected cells were incubated with $LTA^{Rhodamine}$, as well as with $Transferrin^{Alexa633}$ as a control for receptor- mediated endocytosis, prior to imaging by confocal microscopy (Figure 8 B). Confocal images show that internalization of both LTA and transferrin occured in cells expressing wild-type Dynamin-I, but not in cells expressing the mutant Dynamin-I K44A. As expected, transferrin bound to the plasma membrane, but was not internalized in the presence of Dynamin-I K44A. The mutant Dynamin-I K44A also inhibited LTA uptake in CD14 expressing cells. LTA binding to the plasma membrane was, however, only observed in the presence of CD14, where LTA colocalized with transferrin (Figure 8 B). The results suggest that LTA is internalized by a receptor-mediated mechanism.

To assess whether internalization of LTA was necessary for signaling, NF-κB activation was assessed in HEK-TLR2 cells in the absence or presence of CD14 and in the presence of wild- type Dynamin-I or the mutant K44A Dynamin-I. Figure 8 C shows that introduction of CD14 strongly enhanced LTA-induced NF-κB activation, but that the Dynamin-I K44A mutant did not affect NF-κB activation, neither in the presence, nor absence of CD14. These results show that CD14 enhances signaling by binding LTA to the plasma membrane and that LTA internalization is not required for signaling.

DISCUSSION

In this study we have investigated the mechanistical details of LTA internalization in monocytes. It has been suggested that LTA uptake is required for signaling [39], however, this is in contrast with previous reports suggesting that TLR2 activation by LTA occurs in lipid rafts in the plasma membrane, independent of LTA internalization [36, 38]. In this study we examined the intracellular trafficking of LTA in human monocytes using directly labeled LTA. We found that monocytes, which express high levels of surface TLR2 and its co-receptors CD14 and CD36 bind and internalize LTA efficiently.

Using a TLR2 specific mAb, we found that TLR2 rapidly traffics from the plasma membrane to early endosomes in live cells that over-expressed the receptor. Early endosomes containing TLR2 subsequently matured (Figure 1 A). We further observed TLR2 in lysosomes. Though only 20-30% of the TLR2 Ab was observed colocalizing with lysotracker^{Green} after 3 h, this observation could be due to several factors. Incubation for longer time periods, or during stimulation, may have shown more TLR2 in the lysosomes. The fluorochrome bound to the TL2.1 Ab may have possibly lost fluorescence in the acidic environment of lysosomes of live cells, or the Ab may have been degraded, resulting in the low percentage of TLR2 mAb observed in lysosomes. Though TLR2 has been reported to not be ubiquitinylated by the ubiquitin- protein ligase TRIAD3A, in contrast to TLR4 and TLR9 [59], other ligases, for instance TRIAD3B, may ubiquitinylate TLR2 and target it for degradation. The TL2.1 mAb used in this study has been shown to be presented on MHC class II and induce proliferation of a mouse C[illegible]-specific human $CD4^+$ T cell clone [60], supporting that TLR2 traffics along the classical MHCII- pathway.

TLR2 was further found to be highly expressed in a population of Eea-1 positive early endosomes and LAMP-1 positive lysosomes in human monocytes. In contrast to previous reports [36], we did not observe TLR2 in the Golgi network in human monocytes, however, we did observe TLR2 in the Golgi network of HEK293 cells overexpressing

the receptor (data not shown). We interpret these results as characteristic of epithelial cells, or an effect of over-expression of TLR2. In monocytes, TLR2 colocalized with Rab-11-positive structures localized in close proximity to the trans-Golgi network, suggesting that TLR2 is expressed in endosomal recycling compartments. Whether Rab-11 is required for LTA internalization and signaling remains to be investigated.

TLR2 is essential for inflammatory responses towards highly purified LTA [34]. The high amount of TLR2 at the plasma membrane, and upregulation of the receptor in response to LTA, supports the notion that TLR2 signaling occurs at the plasma membrane. Localization of TLR2 in endosomes and lysosomes, however, suggests that signaling may occur in these compartments as well, as shown for TLR4 [61]. Signaling from endosomes/lysosomes also occurs for TLR3 [53] and TLR9 [54], though these receptors are recruited to endosomes from the ER. Using directly labeled functional LTA we found that LTA was rapidly internalized in characteristic tubular structures, which colocalized with markers for ER- and Golgi network, showing that LTA has a uptake and trafficking pattern in phagocytic cells which is different from other TLR ligands such as LPS, CpG and polyIC [32, 53, 54, 61]. The colocalization of LTA with markers of the ER and the Golgi suggests that it follows a retrograde pathway, possibly resembling the trafficking pattern of the plant toxin Ricin and the bacterial toxin Shiga toxin [62]; which follow a retrograde transport to the ER [63-66]. While Shiga toxin is shown to be internalized in clathrin-coated pits, Ricin is internalized by Dynamin-, clathrin- and caveolea- independent mechanisms [62]. In light of the finding that LTA appears to be targeted to the Golgi and ER it would be interesting to compare LTA internalization with Ricin and Shiga toxin.

Our results demonstrate that LTA was endocytosed in a Dynamin-I dependent manner. Previous reports have suggested that LTA is internalized by a lipid raft-dependent pathway [67-69]. Colocalization between LTA and transferrin was observed during the initial minutes of endocytosis in HEK293 cells expressing TLR2 (Nilsen, Unpublished data). In addition, some colocalization between LTA and Choleratoxin B was seen, predominantly in the Golgi network (Nilsen, Unpublished data). These results suggest that LTA may be internalized by both clathrin- and caveolea-dependent pathways. Colocalization between LTA and transferrin and Choleratoxin B was, however, only partial and the internalization pattern of LTA did not mimic the uptake of LPS, FSL-1 or Pam_3CysSK_4. These ligands are internalized slower than LTA and are seen in endocytic vesicles that clearly colocalize with transferrin [35, 61], (Nilsen, Unpublished data). Our findings suggest that LTA may utilize several endocytic pathways, which also has been described for CD14-mediated LPS uptake [61, 70].

The role of CD36 as a co-receptor for TLR2 in response to LTA has predominantly been studied in the presence of CD14 [37, 39], and the contribution of each of these co-receptors has previously not been compared. In accordance with previous reports [34, 39], we found that both CD36 and CD14 enhanced LTA-induced TLR2-mediated NF-κB activation in transfected cells. Only a minor additive effect was observed upon coexpression of both receptors in HEK-TLR2 cells. In monocytes CD36 and CD14 mAbs inhibited both cell association of LTA and TNF release to a similar extent (Figure 7). The results suggest that inhibiting either CD14 or CD36 down-regulates binding or internalization of LTA, which in turn reduces signaling and the induction of TNF. Consequently, both CD36 and CD14 appear to be important in the TLR2 signaling complex in response to LTA. CD36 is, however, specifically involved in TLR2/TLR6-mediated responses, but not in TLR2/TLR1-mediated responses, while CD14 is involved in signaling in response to LPS and Pam_3CysSK_4 as well in signaling in response to LTA.

Expression of CD14 in HEK-TLR2 cells profoundly enhanced both binding of LTA to the plasma membrane and the NF-κB activation, suggesting that this co-receptor may upregulate signaling by accumulating LTA at the plasma membrane. Furthermore, immobilizing LTA on a plastic surface induced a high level of TNF release in monocytes, independent of internalization of the ligand. This appears to be a specific property of LTA, but not LPS or Pam_3CysSK_4 (Deininger, Unpublished data). Expression of Dynamin-I K44A inhibited LTA internalization, both in the absence and presence of CD14, suggesting that LTA is internalized by a receptor- mediated mechanism. Expression of Dynamin-I K44A had, however, no significant effect on LTA-induced NF-κB activation, showing that binding of LTA to the plasma membrane is sufficient to induce signaling. This finding is in contrast to previous reports describing that CD36-mediated internalization of LTA is required for signaling [39]. Though CD36 is shown to be necessary for internalization, the presence of CD36 may also be required for formation of the correct receptor clustering in response to LTA, or for recruitment of LTA to lipid rafts, as suggested in recent reports [38]. In summary, our results show that signaling in response to LTA occurs independent of internalization of the ligand and provide further insight into the mechanisms of LTA internalization, trafficking and signaling through TLR2 and its coreceptors CD14 and CD36.

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ABBREVIATIONS

LTA, lipoteichoic acid; S. aureus, Staphylococcus aureus; LPS, lipopolysaccharide; TLR, Toll-like receptor; LRR, Leucine-rich repeats; GPI, glycosylphosphatidylinositol; LBP, Lipopolysaccharide binding protein; sCD14, soluble CD14; ER, endoplasmic reticulum; MyD88, myeloid differentiation antigen-88; IL, interleukin; TNF, tumor necrosis factor-α; mAb, monoclonal antibody; HEK Human epithelial kidney; FITC, fluorescein; NF-κB, nuclear factor-kappa B; FBS, Fetal Bovine Serum; DMEM, Dulbecco's Modified Eagle's Medium; GFP; Green Fluorescent Protein, Eea-1, Early Endosomal Antigen-1, LAMP-1, Lysosome-associated membrane protein-1.

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FIGURE LEGENDS

Figure 1: Surface TLR2 is internalized into endosomes. A) Confocal images of internalization of TLR2 mAb (red) in MDCK cells expressing Eea-1-GFP (green) and TLR2. Images show enlargements of a portion of a representative cell at 4 s interval time-points (two top panels). Arrows denote an EEA-1^{GFP} (green) containing TLR2 mAb (red) that matures and looses the early endosome tag. The full picture of the cell (bottom picture) shows TLR2 mAb internalization after 612 s. Cells were transiently transfected with TLR2 24 h prior to the experiment using Oligofectamine transfection reagent. Cells were kept on ice for 45 min and then incubated with $TLR2^{A546}$ mAb on ice for 45 minutes. Image acquiring was initiated 15 min post incubation. Image acquisition was performed on an Olympus Fluoview 1000 at 37°C with an Olympus PlanApo 60X/1.42 Oil objective. **B)** Plot of relative intensity of total $TLR2^{A546}$ florescence (black) and colocalization of $TLR2^{A546}$ mAb with Eea-1^{GFP} (green) and $lysotracker^{Green}$ (red), as a function of time. Internalization analysis was carried out with ImageJ software. Dotted lines denote polynomial trendlines.

Figure 2: TLR2 is expressed in the plasma membrane, endosomes, lysosomes and Rab-11- positive compartments, but not in the Golgi of monocytes.

Confocal images of freshly isolated monocytes stained intracellularly with the TLR2 monoclonal antibody TL2.1 (red) and antibodies against **A)** early endosome marker Eea-1 (green), **B)** lysosome marker LAMP-1 (green), **C)** *trans*-Golgi marker Golgin-97 (green) or **D)** Rab-11 (green). Overlay of each staining is shown in addition to overlay and single tracks of enlargements of two sections, denoted by squares in each image. Profile graphs show fluorescence intensity of each color in a cross-section denoted by an arrow in each image (**A-D**). Images of cells shown are representative of the cells observed in each dish, and are representative of three experiments.

Figure 3: Monocytes efficiently bind and internalize LTA and upregulate TLR2. A) Monocytes efficiently bind and internalize LTA. A^+ buffy coat from healthy donors was incubated with $LTA^{Rhodamine\ Green}$ for 45 min at 4 °C or at 37 °C, 8% CO_2. Red blood cells were subsequently lysed and remaining cells were analyzed by flow cytometry to determine LTA binding and uptake. Populations were gated by size and granularity and CD14 high expression (monocytes), CD14 low expression (granulocytes) and CD3 or CD19 expression (lymphocytes). **B)** Monocytes were

fixed and stained extracellularly with Ab against TLR2, CD14 and CD36 for 45 min on ice and analyzed by flow cytometry. Monocytes were stimulated with LTA (0, 0.1, 1, 10, 100 or 1000 ng/ml) for 16 h and were subsequently stained for surface expression of **C)** TLR2 or **D)** CD36, prior to determination of median fluorescence by flow cytometry. Results shown are representative of three independent experiments.

Figure 4: LTA is rapidly internalized in tubular structures and targeted to the *trans*-Golgi network and the ER.

A) Internalization of $LTA^{Rhodamine}$ (20 μg/ml) (red) in live monocytes after 20 min of incubation at 37 °C. **C-F)** Monocytes incubated with $LTA^{Rhodamine}$ (red) (20 μg/ml) for 1 h at 37 °C, 8% CO_2, and subsequently fixed and stained intracellularly with antibodies against **B)** Eea-1 (green), **C)** Golgin97 (green), **D)** LAMP-1 (green) or **E)** ER marker Calnexin and secondary antibody Goat-anti-$mouse^{A647}$ (green). Cells were visualized by confocal microscopy. Overlay images are shown to the left with enlargements of sections denoted by a square in the overlay image shown to the right along with separate tracks of each color. Profile graphs are included showing fluorescence intensity of each color in a cross-section denoted by an arrow in each image (**B-E)**. **F)** Confocal images of live HEK293-TLR2 cells transiently expressing CFP fused to the targeting sequence of calreticulin (ER^{CFP}), which localizes to the endoplasmic reticulum (green), incubated with $LTA^{Rhodamine}$ (red) (20 μg/ml) for 1hr at 37 °C. Overlay (Over) and separate tracks of a new image of the same cell are shown to the right. Images of cells shown are representative of the cells observed in each dish, and are representative of three independent experiments.

Figure 5: CD14 and CD36 enhance LTA- induced NF-κB activation mediated by TLR2. A) HEK 293 cells transfected with an NF-κB luciferase reporter plasmid and TLR2, or TLR2 in combination with CD36 or/and CD14 for 24 h were stimulated with LTA (5 μg/ml) or LPS (100 ng/ml) for 5 h at 37 °C, 8% CO_2. Cells were subsequently lysed and assayed for NF-κB activation. Results shown are representative of three independent experiments.

Figure 6: CD36 and CD14 are expressed at the plasma membrane where they colocalize with TLR2.

Freshly isolated monocytes incubated with medium **(A** and **C)** or $LTA^{Rhodamine}$ for 1 h **(B** and **D)** at 37 °C, 5% CO_2, and subsequently fixed and stained intracellularly with TLR2 mAb $TL2.1^{A647}$ (red) and anti-$CD36^{FITC}$ (green) **(A** and **B)**, or $TL2.1^{A647}$ (red) and anti-$CD14^{A488}$ (green) (**C** and **D)**. Staining was observed by confocal microscopy. Overlay

images are shown to the left, with enlargements of sections denoted by a square in the overlay image shown to the right, along with separate tracks of each color. Profile graphs show fluorescence intensity of cross-sections denoted by an arrow in (**A-D)**.

Figure 7: Blocking CD14 or CD36 impairs LTA cell association and subsequent TNF release in monocytes

A) Monocytes were pretreated with mAb against TLR2, CD36, CD14 or control Ab (10 μg/ml) for 45 min, RT, before addition of $LTA^{Rhodamine\ Green}$ (2 μg/ml) for 45 min at 37 °C, 5% CO_2. Cells were subsequently washed and analyzed by flow cytometry to assess LTA cell association (binding and internalization). **B)** Monocytes were pretreated with Control Ab or mAb against CD36, CD14 or TLR2 or CD36, CD14 and TLR2 in combination for 45 min, before cells were stimulated with Medium, LTA (10 μg/ml), Pam_3CysSK_4 (50 ng/ml) or LPS (20 ng/ml) for 5 h at 37 °C, 5% CO_2. Supernatant was harvested and analyzed for TNF by ELISA. Results shown are representative of three independent experiments.

Figure 8: TLR2 signaling in response to LTA occurs at the plasma membrane, and is not dependent on Dynamin-I.

A) Immobilizing LTA on a plastic surface enhances TNF release in monocytes. Monocytes were stimulated by plating cells in wells coated with LTA or PBS, or stimulated by adding LTA or medium in solution. Supernatant was harvested after overnight incubation and TNF levels were analyzed by ELISA. Results show average TNF release of duplets and are representative of three independent experiments. **B)** LTA is internalized by a receptor-mediated mechanism is inhibited by the Dynamin-I mutant Dynamin-I K44A. Confocal images of HEKTLR2 cells transiently expressing wild-type Dynamin-I or the mutant Dynamin-I K44A in the presence and absence of CD14, incubated with $LTA^{Rhodamine}$ (red) or $Transferrin^{A633}$ (green) for 30 min at 37 °C, 8% CO_2 prior to imaging. The nucleuses of cells are outlined in Dynamin-I K44A expressing cells. **C)** LTA-induced NF-κB activation occurs at the plasma membrane, independent of LTA-uptake. HEK293-TLR2 cells were transfected with an NF-κB luciferase reporter plasmid and wild- type Dynamin-I or the mutant Dynamin-I K44A, in the presence of control pcDNA3 or CD14. Cells were subsequently stimulated with LTA (5 μg/ml) or medium for 6 h, 37 °C, 8% CO_2, before cells were lysed and assayed for NF-κB activation. Results shown are representative of three experiments.

Figure 1

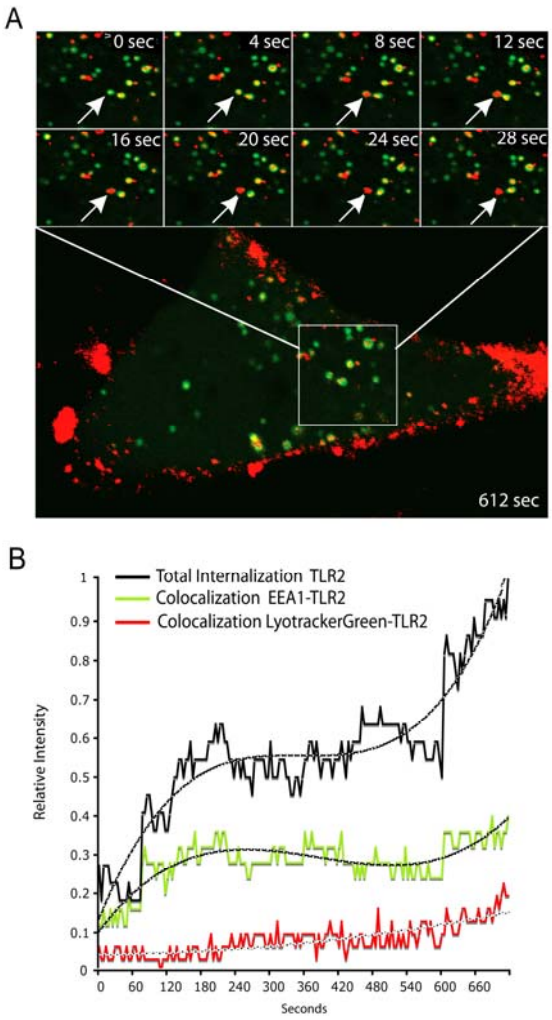

A
0 sec
4 sec
8 sec
12 sec
16 sec
20 sec
24 sec
28 sec
612 sec
B
Total Internalization TLR2
Colocalization EEA1-TLR2
Colocalization LyotrackerGreen-TLR2
Relative Intensity
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
60
120
180
240
300
360
420
480
540
600
660
Seconds

Figure 2

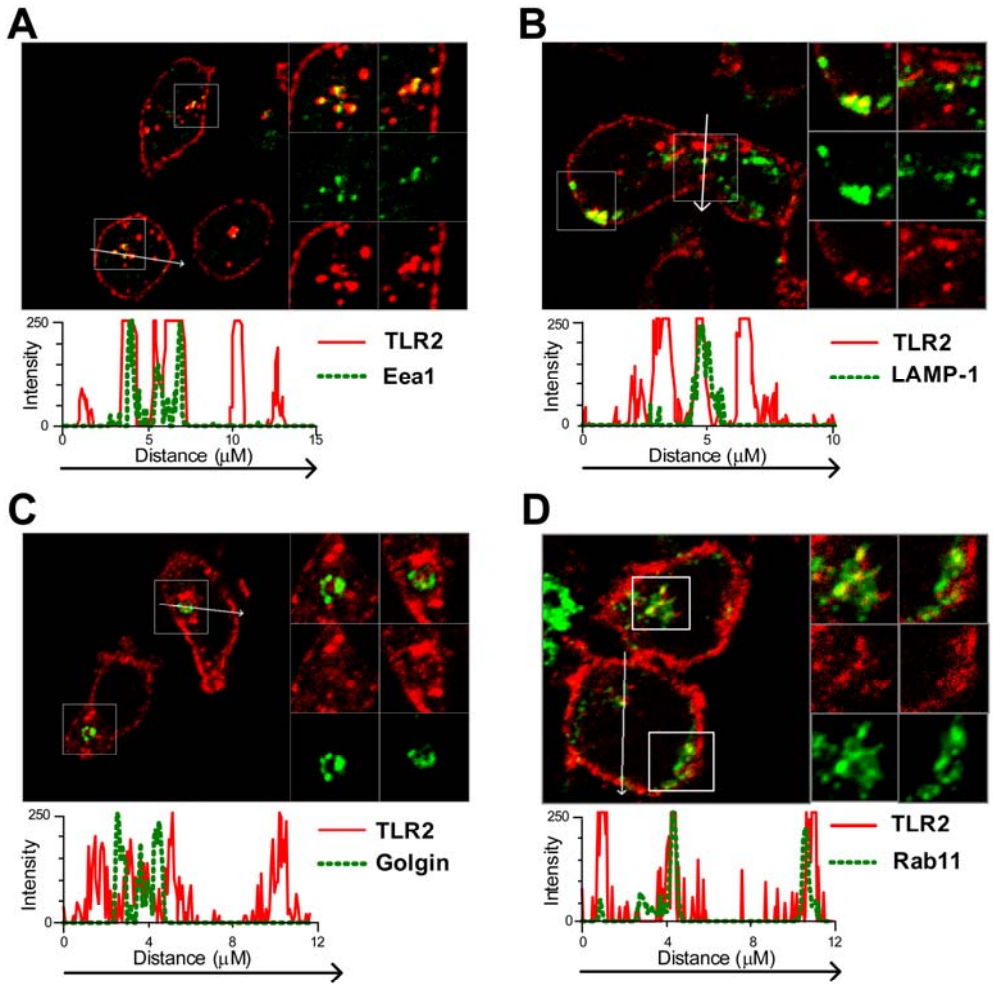

A
B
C
D
TLR2
Eea1
LAMP-1
Golgin
Rab11
Intensity
Distance (μM)
250
0
5
10
15
4
8
12

Figure 3

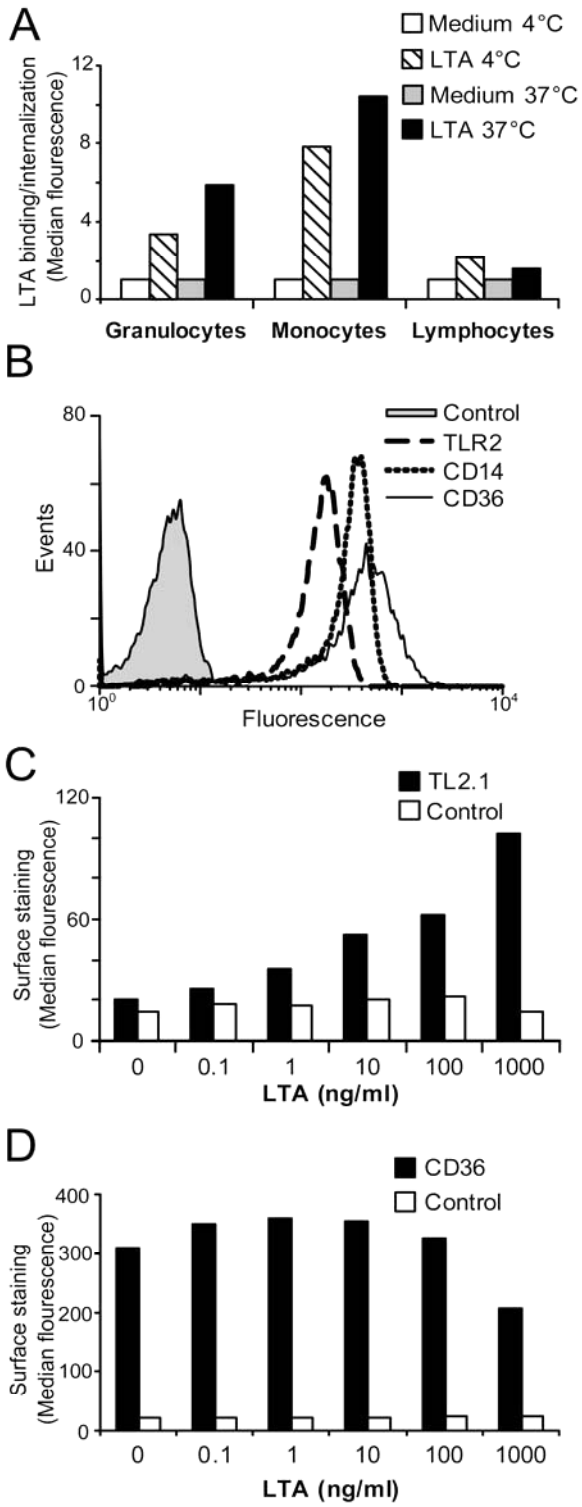

A
Medium 4°C
LTA 4°C
Medium 37°C
LTA 37°C
LTA binding/internalization (Median flourescence)
12
8
4
0
Granulocytes
Monocytes
Lymphocytes
B
Control
TLR2
CD14
CD36
Events
80
40
0
10^0
10^4
Fluorescence
C
TL2.1
Control
Surface staining (Median flourescence)
120
60
0
0
0.1
1
10
100
1000
LTA (ng/ml)
D
CD36
Control
Surface staining (Median flourescence)
400
300
200
100
0
0
0.1
1
10
100
1000
LTA (ng/ml)

Figure 4

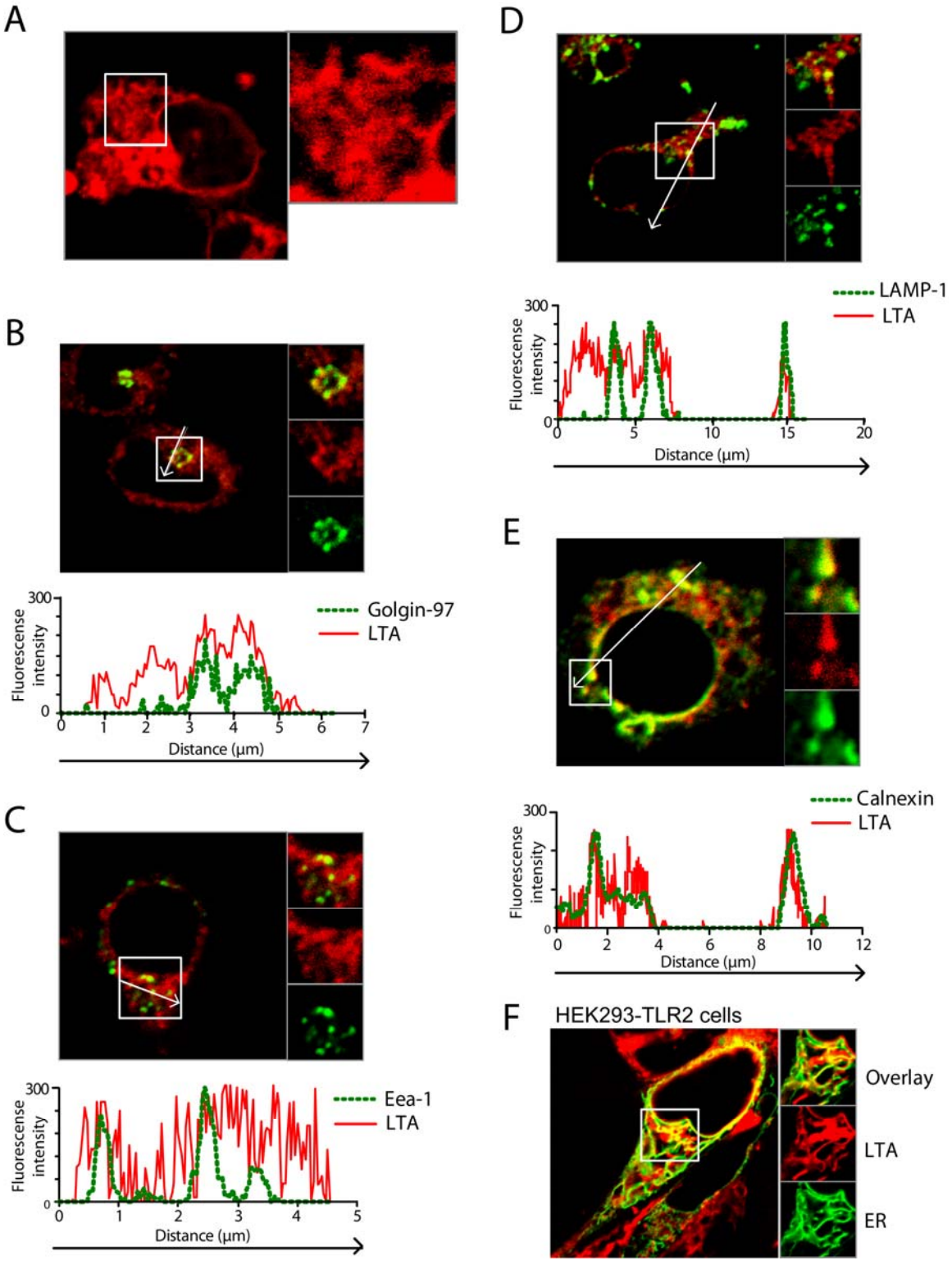

A
B
Golgin-97
LTA
Fluorescense intensity
Distance (µm)
C
Eea-1
LTA
D
LAMP-1
LTA
E
Calnexin
LTA
F
HEK293-TLR2 cells
Overlay
LTA
ER

Figure 5

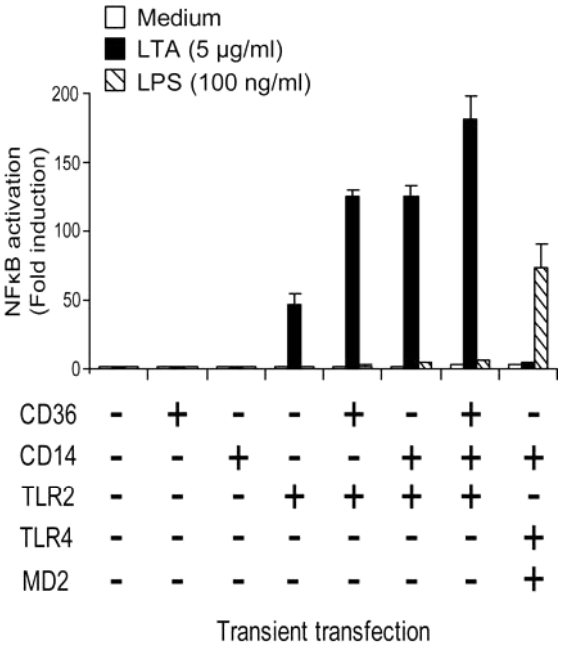

Medium
LTA (5 µg/ml)
LPS (100 ng/ml)
NFκB activation (Fold induction)
200
150
100
50
0
CD36 - + - - + - + -
CD14 - - + - - + + +
TLR2 - - - + + + + -
TLR4 - - - - - - - +
MD2 - - - - - - - +
Transient transfection

Figure 6

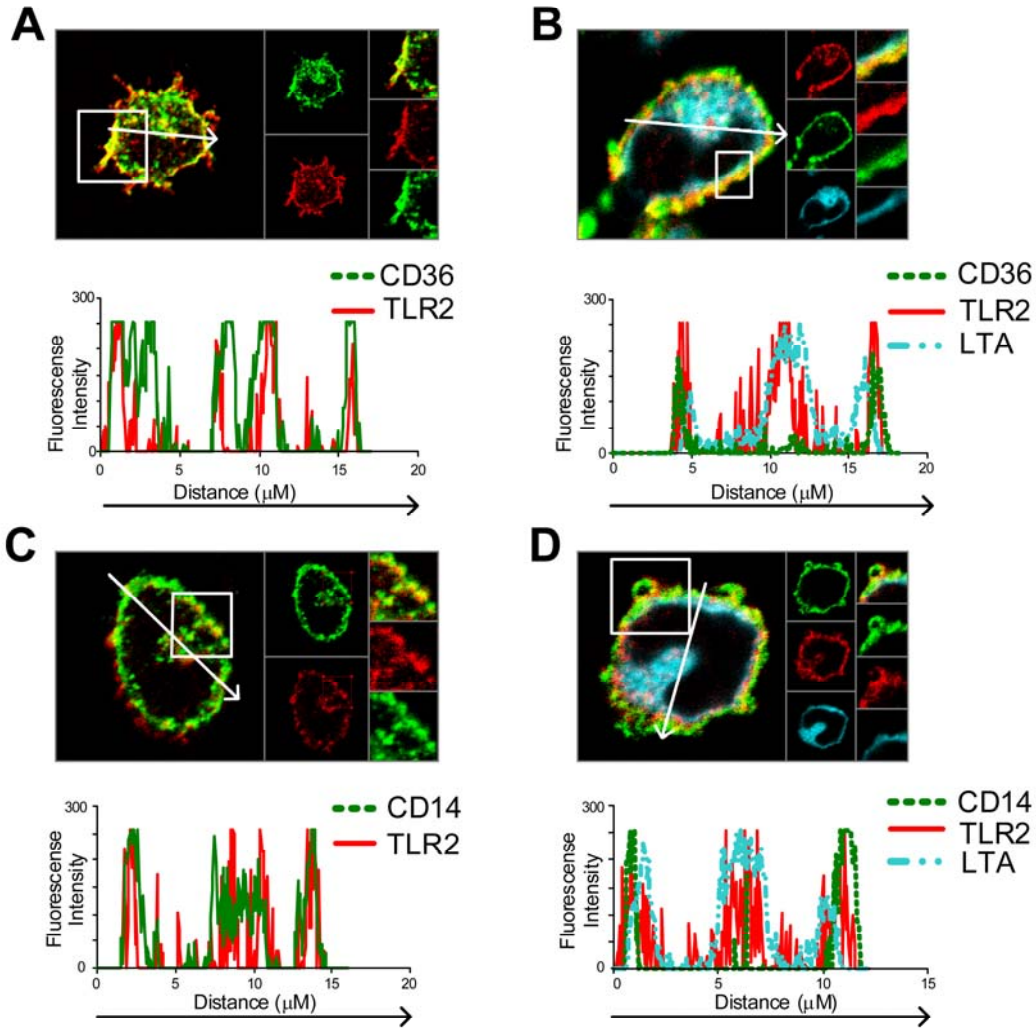

A
B
C
D
CD36
TLR2
LTA
CD14
Fluorescense Intensity
Distance (μM)
300
0
5
10
15
20

Figure 7

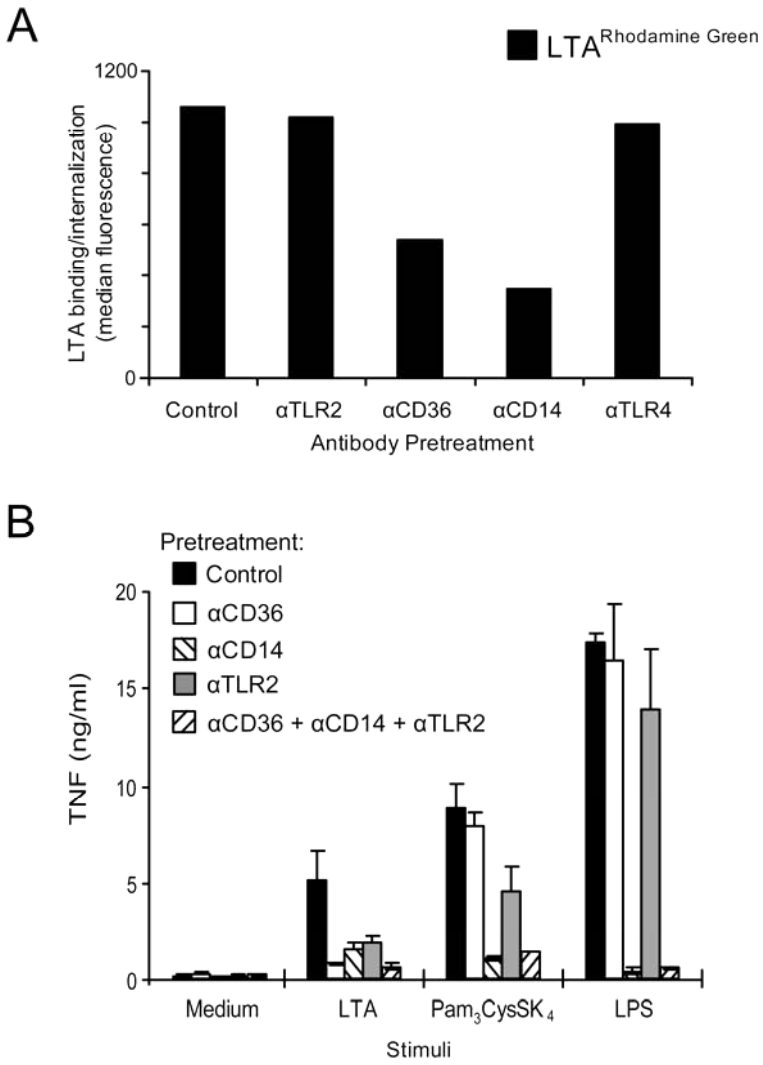
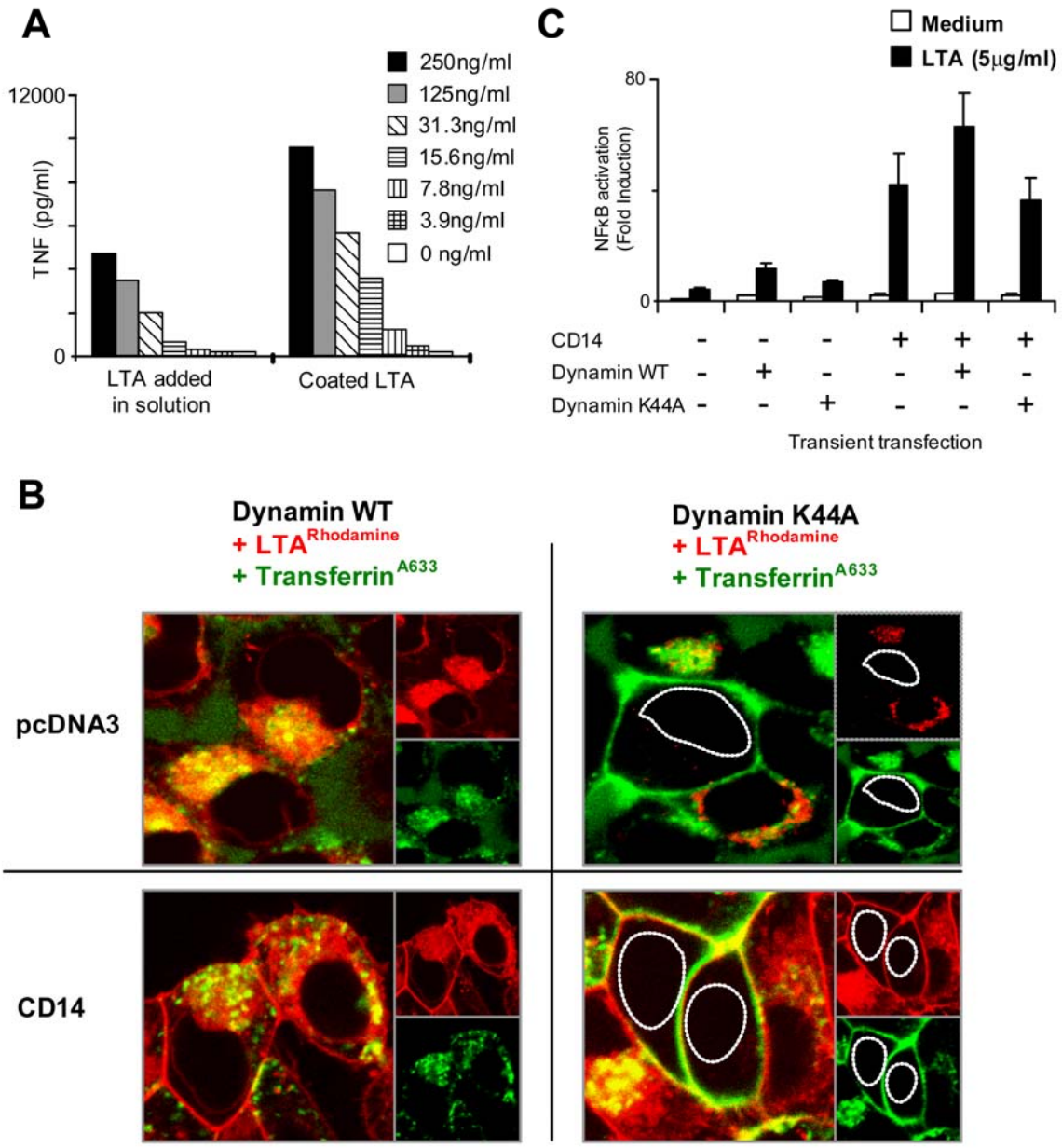

A
LTA Rhodamine Green
1200
0
LTA binding/internalization (median fluorescence)
Control
αTLR2
αCD36
αCD14
αTLR4
Antibody Pretreatment
B
Pretreatment:
Control
αCD36
αCD14
αTLR2
αCD36 + αCD14 + αTLR2
20
15
10
5
0
TNF (ng/ml)
Medium
LTA
Pam3CysSK4
LPS
Stimuli

Figure 8


A
12000
0
TNF (pg/ml)
250ng/ml
125ng/ml
31.3ng/ml
15.6ng/ml
7.8ng/ml
3.9ng/ml
0 ng/ml
LTA added in solution
Coated LTA
C
Medium
LTA (5μg/ml)
80
0
NFκB activation (Fold Induction)
CD14 - - - + + +
Dynamin WT - + - - + -
Dynamin K44A - - + - - +
Transient transfection
B
Dynamin WT
+ LTA Rhodamine
+ Transferrin A633
Dynamin K44A
+ LTA Rhodamine
+ Transferrin A633
pcDNA3
CD14

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