TOLL-LIKE RECEPTORS (TLR) 4 AND 2 REGULATE THE INNATE IMMUNE RESPONSE

Study of endotoxin influence in mice

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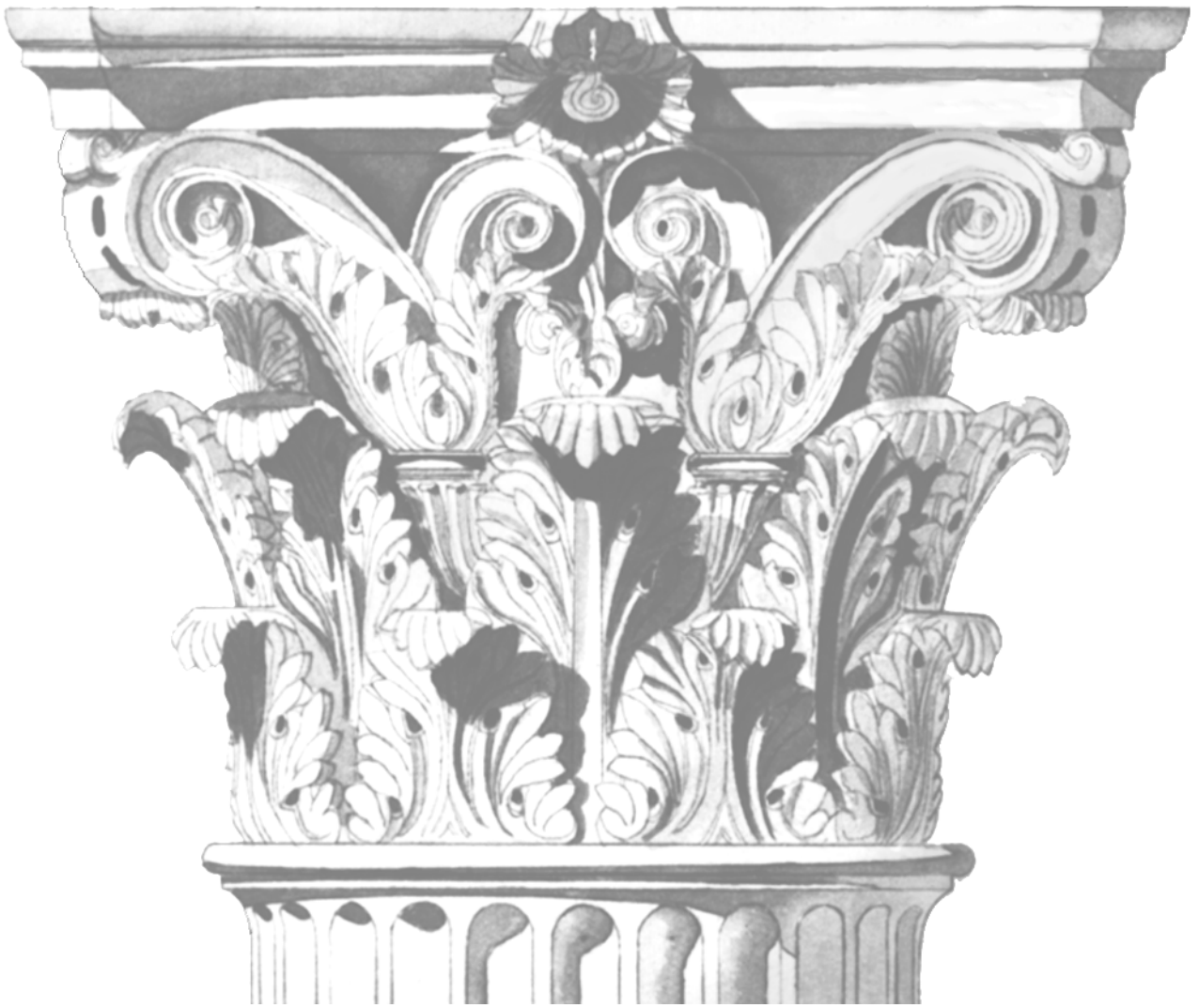
Abstract in Finnish



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Abstract

The response of the innate immune system is triggered through Toll-like transmembrane receptors (TLR) that recognize a variety of microbial products. TLR4 is the principal mediator for Gram negative bacterial endotoxin (LPS), whereas TLR2 mediates the response to Gram positive bacteria, mycobacteria, and yeast. Stimulation of TLR activates complex cascades leading first to the production of inflammatory mediators, such as proinflammatory cytokines IL-1 α/β and TNF-α.

Overproduction of inflammatory cytokines as well as failure in the activation of innate immunity are detrimental to the host. Excess inflammatory stimulation leads to a septic shock, which may cause multi-organ failure and even death. The lack of any innate response exposes the host to overwhelming bacterial infections. Appropriate regulation of the innate immune response could be a target for attempts to find therapeutics to septic shock. This experimental study focuses on functional activation of the signaling receptors TLR4 and TLR2 upon a LPS challenge.

An acute inflammation model was used for both *in vivo* and *in vitro* experiments. LPS was used to stimulate a mouse macrophage cell line. It was administred intraperitoneally or intra-amniotically to non-pregnant or time-mated mice. The basal and induced mRNA expression levels and the protein production of TLRs as well as the mRNA expression of several inflammatory mediators were studied.

The present study showed that the expression of TLR4 and TLR2 is strain and tissue-specific. At the mRNA level, the levels of TLR4 expression limited the extent of the acute cytokine response. The quality of the cytokine response was modulated by protein aggregates formed by TLR4 on the cell surface. The LPS challenge caused a marked increase in the expression of TLR2 mRNA but not the protein; the significance of this remains to be studied. The study further showed that the expression of TLRs is regulated during the perinatal period, and that the acute cytokine response to LPS in the lung develops during antenatal differentiation.

The present study provides information about how the activation of TLR regulates the acute inflammatory response and further helps to elucidate new targets for the anti-inflammatory strategies in controlling inflammatory events.

Keywords: cytokines, immunity; natural, infant; premature, inflammation, lipopolysaccharides, receptors; cell surface, signal transduction

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Tiivistelmä

"Toll-like"-reseptorit (TLR) ovat solukalvon proteiineja, jotka spesifisesti tunnistavat erilaisia bakteerirakenteita. Infektiossa tällainen bakteerirakenne sitoutuu reseptoriin ja seurauksena solussa käynnistyy synnynnäinen immuunivaste eli tulehdusvälittäjäaineiden tuotto. Liiallinen tulehdusvälittäjäaineiden tuotto voi johtaa septiseen shokkiin eli verenmyrkytykseen, elinvaurioihin ja jopa kuolemaan. Septisen shokin synty voisi olla estettävissä immuunivasteen voimakkuuden tarkoituksenmukaisella säätelyllä. Väitöskirjassa on tutkittu, miten TLR4 ja TLR2 aktivoituvat bakteeri-infektiossa, tarkoituksena selvittää, säätelevätkö reseptorit immuunivasteen käynnistystä ja voimakkuutta solussa.

Tutkimuksessa todettiin, että TLR4:n ja TLR2:n geenien ilmentymistä säädellään eri tavoin eri hiirikannoilla ja eri kudoksissa. TLR4-tason nousu aiheutti voimakkaamman immuunivasteen, kun taas reseptorin matala esiintymistaso laski immuunivasteen voimakkuutta. Lisäksi TLR4:aan solukalvolla sitoutuvat muut proteiinit vaikuttivat immuunivasteen laatuun. Tutkimuksessa todettiin myös, että TLR:n määrä sikiön keuhkoissa rajoittaa keuhkojen immuunivasteen kehittymistä.

Tutkimus antaa tietoa siitä, miten TL-reseptorien aktivaatio säätelee synnynnäistä immuunipuolustusta ja selventää mahdollisuutta kontrolloida immuunivasteen voimakkuutta vaikuttamalla TL-reseptoriin.

Asiasanat: ennenaikainen syntymä, lipopolysakkaridi, solukalvoreseptorit, solusignalointi, synnynnäinen immuniteetti, sytokiinit, tulehdus

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Oulu, April 2004

Kirsi Harju

Abbreviations

AP	activation protein
Arg	arginine
Asp	asparagine
BPD	broncho-pulmonary dysplasia
Btk	Bruton's tyrosine kinase
CD	cluster of differentiation
DC	dendritic cell
(d)NTP	(deoxy) nucleotide triphosphate
EMSA	electrophoretic mobility shift assay
Gln	glutamine
Gly	glycine
GM-CSF	granulocyte macrophage colony stimulating factor
Hsp	heat shock protein
i.a.	intra-amnion
IFN	interferon
IKK	NF-κB inhibitor kinase
IL	interleukin
IL-1R	interleukin-1 receptor
Ile	isoleucine
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
IRAK	IL-1R associated kinase
I-κB	NF-κB inhibitor
JNK/SAPK	c-Jun NH_2-terminal kinase/ stress activated protein kinase
LBP	LPS binding protein
LPS	lipopolysaccharide
LRR	leucine rich repeat
LTA	lipoteichoic acid
Mal	MyD88 adapter-like
MAP	mitogen activated protein
MD	myeloid differentiation

MHC	major histocompatibility complex
MIF	macrophage migration inhibitory factor
MIP	macrophage inflammatory protein
MyD	myeloid differentiation factor
NF-κB	transcription factor-κB
NIK	NF-κB inducing kinase
NO	nitric oxide
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PDTC	pyrrolidinedithiocarbamate
PI3K	phosphatidylinositol 3-kinase
PRR	pattern recognition receptor
RPA	ribonuclease protection assay
RT-PCR	reverse transcriptase polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate-polyacryl amide gel electrophoresis
Ser	serine
Sp	stimulating factor
SP	surfactant protein
TGF	transforming growth factor
Th	T-helper cell
Thr	threonine
TIR	Toll/IL-1R
TIRAP	Toll/IL-1R domain containing adapter protein
TLR	Toll-like receptor
TNF	tumour necrosis factor
TRAF	TNF receptor associated factor

List of original publications

This thesis is based on the following articles, which are referred to in the text by Roman numerals:

I Harju K, Glumoff V & Hallman M (2001) Ontogeny of Toll-like receptors Tlr2 and Tlr4 in mice. Pediatr Res 49: 81-83

II Ojaniemi M, Harju K, Glumoff V & Hallman M (2004) Rapid Toll-like receptor-2 induction and high hepatic cytokine response during endotoxemia in mice. Submitted.

III Ojaniemi M, Glumoff V, Harju K, Liljeroos M, Vuori K & Hallman M (2003) Phosphatidylinositol 3-kinase is involved in Toll-like receptor 4-mediated cytokine expression in mouse macrophages. Eur J Immunol 33: 597-605.

IV Harju K, Ojaniemi M, Rounioja S, Glumoff V, Paananen R, Vuolteenaho R & Hallman M (2004) Expression of Toll-like receptor 4 and endotoxin responsiveness in mice during perinatal period. Submitted.

Additionally, some unpublished data are presented.

Contents

1 Introduction

Nobel prize winners Christiane Nüsslein-Volhard and Eric Wieschaus made their careers studying the fruit fly *Drosophila melanogaster*. When they were screening for lethal zygotic mutations that affected embryonic patterning, Wieschaus discovered a line in which none of the embryos from heterozygous females hatched. They had no lateral or ventral cell types developed and no nervous system. When Wieschaus showed the unhatched embryos to Nüsslein-Volhard, she exclaimed: "Toll!", which means 'fantastic' in German slang. The mutated gene thus became known by that name. The phenotype was extraordinary because it was dominant and ventralized, opposite to the two previously known recessive dorsalizing mutations. With two opposing mutant phenotypes, the scientists started to build a genetic pathway. (Anderson 2000).

In addition to being crucial in embryonic development, the Toll gene also proved to be very important in another way. Evidence of Toll involvement in host defence came from an analysis of immune responses of *Drosophila* mutants carrying loss-of-function mutations in various components of the Toll signaling pathway. Flies deficient in Toll or some other signaling component of the pathway were unable to recognize fungal infection or to produce the antifungal peptide drosomycin. It also turned out that an individual peptide from the group of antimicrobial peptides produced by rapid induction against microbial infection has selective activity against a particular class of micro-organisms. Also, an infection with a given class of pathogens results in preferential induction of only the appropriate peptides. The Toll receptors of *Drosophila* appeared not only to detect the presence of infection, but also to discriminate between classes of pathogens. (Janeway, Jr. & Medzhitov 1999).

That led to the discovery of mammalian Toll-like receptors, TLRs. TLRs do not seem to have any developmental function, but they have been shown to play a central role in the mammalian innate immune system. They are involved in infectious diseases by recognizing various microbial products and by activating appropriate signaling cascades. Inappropriate activation may lead to septic multi-organ failures, including lung damage, cardiac failure, and brain damage (Fenton & Golenbock 1998, Knuefermann *et al.* 2002, Hagberg *et al.* 2002). In addition, TLRs are capable of reacting with environmental antigens and even self-antigens and may thus contribute to the generation of several non-infectious diseases, such as allergies or auto-immune diseases. TLRs are also considered

to be “surveillance” receptors, which indicates that they are capable of monitoring tissues for disease states (Johnson *et al.* 2003). Dysregulation of TLR function is thus critical for the innate immune system. Especially premature infants are vulnerable to any disturbance in innate immunity because of the inadequate development of the other parts of the host defence system. An excessive cytokine response has been proposed to be a major cause of spontaneous premature deliveries and life-threatening diseases in premature infants (Romero *et al.* 1989).

This experimental study focuses on the functional activation of two signaling receptors, TLR2 and TLR4, which result in the induction of the cytokine cascade. According to the current hypothesis, cytokines play a major role in the pathogenesis of serious diseases.

2 Review of the literature

2.1 Innate and adaptive immunity

The mammalian immune system consists of two types of immunity: innate and adaptive. Innate immunity constitutes the first-line host defence system, the components of which are encoded by DNA rather than expressed by clonal cells after antigenic exposure, as in the case of adaptive immunity. The innate immune system destroys many pathogens, determines the localization and extent of the challenge, and facilitates the adaptive immune response. Innate immunity is mediated by genes that remain in the germ line and encode for proteins that recognize conserved structural patterns on micro-organisms. Defensins and other antimicrobial peptides, complement, opsonins as well as signal-transducing and endocytic receptors and soluble proteins that bind and agglutinate microbes are components of the innate immune system. (For a rewiew, see Janeway, Jr. & Medzhitov 1998, Zhang & Ghosh 2001, Hallman *et al.* 2001).

Specific components of microbial cell walls are strong activators of innate immune responses. These molecules are called pathogen-associated molecular patterns, PAMPs. They are all essential, conserved microbial components that are recognized as foreign by specific pattern recognition receptors, PRRs. PRRs are preferentially expressed in monocytes and macrophages and in other cell types as well. The receptors can be structurally divided into those containing a LRR (leucine-rich repeat) domain, a calcium-dependent lectin domain, or a scavenger receptor protein domain. Functionally, they are divided into secreted or endocytotic proteins or signaling molecules (Medzhitov & Janeway, Jr. 1997). Secreted receptors usually activate the complement cascade, endocytotic receptors move the pathogen from the surface of the phagocyte into its intracellular lysosomes for destruction, and signaling receptors induce the expression of a variety of acute-phase reaction products. In mammals, the PAMPs activate the production of, for example, bioactive lipids (e.g. platelet-activating factor), reduced oxygen species (NO), and proteins, such as the cytokines interleukin (IL)-1, IL-6, and tumor necrosis factor alpha (TNF-α), which all are important in the response to infection (rewiewed by, for example, Janeway, Jr. & Medzhitov 1998, Zhang & Ghosh 2001, Hallman *et al.* 2001). A simplified presentation of action of the innate immunity system is shown in figure 1.

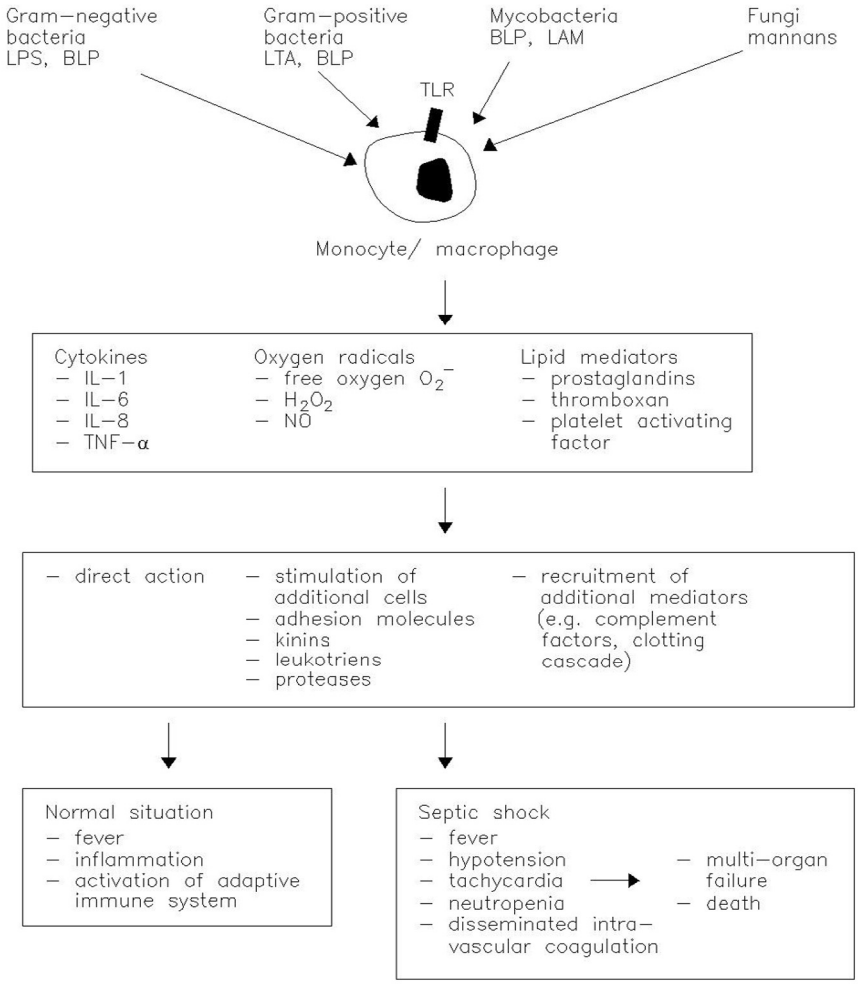

Fig. 1. Schematic representation of the elements of innate immunity (modified from Schletter *et al.* 1995b, Beutler 2000). Abbreviations: TLR, Toll-like receptor; LPS, lipopolysaccharide; LTA, lipoteichoic acid; BLP, bacterial lipoprotein; LAM, lipoarabinomannan.

Separate from, but linked with, innate immunity is adaptive immunity. The key features of adaptive immunity are the clonal expansion of lymphocytes in response to a particular antigen and the ability to evoke an immunologic memory. The response to a specific antigen rises through specific B- and T-cell receptors for each clone of cells. These receptors are structurally unique, not predestined to recognize any particular antigen and not encoded by the germ line. Therefore, they must be established by every generation. (For references, see Janeway, Jr. & Medzhitov 1998, Hallman *et al.* 2001).

Adaptive immunity in vertebrates uses the innate immune system in the selection and presentation of antigens to clonotypic recognition systems. Innate immunity also serves to restrict the infectious challenge during the lag period required for adaptive immunity to develop. The role of innate immunity is especially critical during early childhood, when the clonal development of antibodies and other host defences is inadequate. Especially premature infants are particularly vulnerable to dysregulation of innate immunity. (Hallman *et al.* 2001).

2.2 Pathogen-associated molecular patterns (PAMP)

2.2.1 Lipopolysaccharide (LPS)

In the late 19th century, Richard Pfeiffer used heat-inactivated lysates of *Vibrio cholerae* to induce a range of pathophysiological reactions in guinea pigs. He named the toxic substance endotoxin. Since then, the substance has become the most widely studied activator of the innate immune system. The term 'lipopolysaccharide' (LPS), which is descriptive of the structure of endotoxin, is now used as a synonym for endotoxin. LPS is characteristic of Gram negative bacteria and present inside and outside the outer lipid layer of the bacterial cell wall. Innermost in the cell wall of Gram negative bacteria is a double lipid layer, and between the inner and outer layers there is a peptidoglycan layer, which is a network containing carbohydrate and peptide chains. (Schletter *et al.* 1995b, Järveläinen & Miettinen 2001).

LPS consists of a lipid part and a polysaccharide part (Schletter *et al.* 1995b). The polysaccharide part includes a highly variable sugar chain and an intermediate variable core. The sugar chain may consist of up to 50 repeating oligosaccharide units, and it is unique for each bacterial strain and highly antigenic. It determines the bacterial serotype. The lipid part is highly conserved among the bacterial strains, and it is responsible for the endotoxic activity of LPS (Galanos *et al.* 1985). Regardless of the type of the Gram negative bacterium, the lipid A is composed of a diglucosamine backbone containing ester-linked and amide-linked long-chain fatty acids. Any alteration in the structure causes partial or total loss of endotoxic activity (Rietschel *et al.* 1994).

Mammals are in permanent contact with Gram negative bacteria and LPS. Low doses may be beneficial for enhancing resistance to infections, but larger doses lead indirectly to dramatic pathophysiological reactions, which are caused by excessive amounts of inflammatory endogenous mediators, such as cytokines (Schletter *et al.* 1995b). Subsequent exposure to LPS causes a phenomenon called LPS tolerance or LPS desensitization. Macrophages show a reduced response to second LPS exposure in a time- and dose-dependent manner determined by the reduced production of inflammatory cytokines. To induce any cellular response in the host, LPS must have the capacity for cell activation and must bind specifically to a receptor. It has been shown that both of these criteria require the breakdown of LPS aggregates into monomers (Takayama *et al.* 1994). These cell surface events leading to a host cell response have been mostly elucidated during the past 10 years and will be reviewed in the subsequent chapters.

It was suggested earlier that LPS, because of its amphipathic character, interacts non-specifically with a responsive host cell by hydrophobic insertion into the cell membrane. Nowadays, several cell surface receptors and other proteins specifically binding LPS have been described, including the LPS-binding protein (LBP), the glycerolphosphatidylinositol (GPI)-anchored protein CD14, β2 integrins, the pulmonary surfactant proteins A and D, and the macrophage scavenger receptor (Wright *et al.* 1990, Wright 1991, McIntosh *et al.* 1996, Fenton & Golenbock 1998). Binding of LPS to β2 integrins and macrophage scavenger receptors leads to clearance and degradation of LPS, but, most likely, not to the activation of any signaling cascade. The pulmonary surfactant proteins, SP-A and SP-D, in addition to phagocytosis and intracellular killing (for a rewiew, see Holmskov *et al.* 2003), also modulate the response to pulmonary infection triggered by LPS (Kuan *et al.* 1992, Kalina *et al.* 1995). SPs further inhibit the growth of Gram negative bacteria (Wu *et al.* 2003).

2.2.2 Other PAMPs

Gram positive bacteria exhibit their own PAMPs, peptidoglycans, and lipoteichoic acid (LTA). The cell wall of Gram positive bacteria is structurally simpler than the cell wall of Gram negative bacteria. No outer lipid layer exists, and as much as 90 % of the cell wall of Gram positive bacteria may consist of peptidoglycan. Carbohydrate structures, i.e. teichoic acids, are connected to the peptidoglycan layer, and the teichoic acid connected to the cell membrane is called lipoteichoic acid. LTA is a biologically active molecule characteristic of Gram positive bacteria (Järveläinen & Miettinen 2001). It has been studied much less than LPS, but is known to have many biologically similar impacts. It induces the production of cytokines and causes septic shock (Bhakdi *et al.* 1991, De Kimpe *et al.* 1995). Lipoteichoic acid has also been shown to induce preterm delivery in mice (Kajikawa *et al.* 1998).

Other examples of common PAMPs are the non-methylated CpG repeats in bacterial DNA, the lipoarabinomannan of mycobacteria, and the mannans of fungi (Janeway, Jr. & Medzhitov 1999, Järveläinen & Miettinen 2001).

2.3 Toll-like receptors

The Toll protein was first characterized in the fruit fly *Drosophila melanogaster* in investigations of the molecular basis of embryonic specification of body axes, i.e. dorsoventral patterning (Anderson *et al.* 1985, Morisato & Anderson 1995). The patterning results from a signaling cascade that centers on the transmembrane receptor Toll. The Toll pathway also controls the antimicrobial response in the adult fly.

The first clue of a mammalian 80 kDa cell membrane receptor that binds LPS in the presence of certain serum proteins came from Jens Schletter and his co-workers in 1995 (Schletter *et al.* 1995a). However, the first characterized mammalian Toll-like receptor (first named hToll and later re-named TLR4) was found in 1997 by Charles Janeway Jr. and his co-workers (Medzhitov *et al.* 1997). Since then, at least ten TLRs have been

identified (Rock *et al.* 1998, Chaudhary *et al.* 1998, Takeuchi *et al.* 1999b, Sebastiani *et al.* 2000, Chuang & Ulevitch 2000, Du *et al.* 2000, Chuang & Ulevitch 2001). Mammalian TLRs are pattern recognition receptors that function as a cluster of differentiation (CD)-14 associated signal transducers, help cells to recognize and distinguish between pathogens, and initiate appropriate signaling cascades. They also help to bridge innate and adaptive immunity by inducing various co-stimulatory and effector molecules (Zhang & Ghosh 2001). However, it is unlikely that TLR has a developmental role in mammals, because the myeloid differentiation factor (MyD)-88, a close structural and functional partner of mammalian TLR, shows no function in embryonic development.

All TLRs share the same structure: a large (550 to 980 amino acids) extracellular domain consisting of leucine-rich repeats, a transmembrane domain, and a TIR (Toll/IL-1R-like) cytoplasmic domain approximately 200 amino acids long. The extracellular domain has ligand-binding capacity, and the TIR domain mediates the signal. TIR regions have also been found in vaccinia virus, and they are used by the virus to suppress host IL-1 and TLR signaling (Bowie *et al.* 2000). Despite these similarities, TLRs are differentially expressed and regulated in many tissues and cell types (Muzio *et al.* 2000b), (Zarember & Godowski 2002, Bottcher *et al.* 2003). Different TLRs are also capable of activating distinct cellular responses in spite of their shared capacities to signal through the activation of NF-κB, AP-1, and MAP kinases (Jones *et al.* 2001b). This may be explained by differential use of adapter proteins (O'Neill *et al.* 2003).

Ligands for many TLRs have been characterized: TLR4 mediates the innate immune response to LPS, TLR2 has been shown to mediate the response to yeast and Gram-positive bacteria (Lien *et al.* 1999, Takeuchi *et al.* 1999a), TLR1 functions as an accessory protein (Wyllie *et al.* 2000), TLR3 recognizes viral double-stranded RNA (Alexopoulou *et al.* 2001, Doyle *et al.* 2003), TLR5 is activated by bacterial flagellin (Hayashi *et al.* 2001, Szabo 2003), TLR6 functions to assist TLR2 (Takeuchi *et al.* 2001, Bulut *et al.* 2001), and TLR9 responds to unmethylated CpG dinucleotide motifs (Hemmi *et al.* 2000). There is some lack of distinction between the ligands of TLR4 and TLR2, and it thus seems quite possible that some of the TLRs can replace each other under certain circumstances.

TLRs have the capacity to oligomerize in their cytoplasmic domains. It seems that their cytoplasmic tails are not functionally equivalent, but certain TLRs require assembly into heteromeric complexes, whereas others are active as homomeric (Ozinsky *et al.* 2000). TLR4 acts as a homodimer, and a recent study has also implicated the formation of a TLR5/TLR4 heterodimer in the signaling of bacterial flagellin (Mizel *et al.* 2003). TLR2 can form functional pairs with TLR6 and TLR1, but also functions alone (Ozinsky *et al.* 2000).

2.3.1 Toll-like receptor 4 (TLR4)

2.3.1.1 Structure

TLR4 has been mapped to chromosome 4 in mouse (Poltorak *et al.* 1998b) and to chromosome 9q32-33 in human (Rock *et al.* 1998). Recently, Pereira and his co-workers showed that the expression is monoallelic in bone marrow granulocytes and splenic B-cells (Pereira *et al.* 2003). The TLR4 gene from mouse and human has been cloned and sequenced (Smirnova *et al.* 2000). The human gene is 19 kb in length and consists of three exons. Promoter analysis has shown that location approximately 75 bp upstream from the transcriptional start site is sufficient to direct the gene expression. This promoter region is highly conserved between human and mouse. The size of the mouse gene is 91.7 kb, its length being due to the longer intronic sequences. Otherwise, it is structurally similar to the human gene (Smirnova *et al.* 2000).

The extracellular domain of the TLR4 protein contains 22 copies of LRRs (Rock *et al.* 1998). Most likely, however, LRRs do not present the LPS-binding property of TLR4, because mapping studies with its close binding partner CD14 revealed that most LRRs can be deleted without affecting LPS binding (Juan *et al.* 1995). The extracellular part of TLR4 contains 9 N-linked glycosylation sites. Glycosylation is important for the transportation of the protein to the cell surface and the maintenance of the functional integrity of the LPS receptor complex (da Silva & Ulevitch 2002). The signal-mediating part of TLR4 is the intracellular TIR domain (Rock *et al.* 1998).

2.3.1.2 Gene expression

TLR4 expression has been found in heart, lung, fetal skin, fetal brain, placenta, fetal ileum, and many other tissues (Medzhitov *et al.* 1997, Laflamme & Rivest 2001, Fusunyan *et al.* 2001, Holmlund *et al.* 2002). In addition to immunocompetent cells (Muzio *et al.* 2000a), TLR4-expressing cells include fetal small-intestinal enterocytes (Fusunyan *et al.* 2001), gastric pit cells (Kawahara *et al.* 2001), osteoblasts (Kikuchi *et al.* 2001), endothelial cells (Frantz *et al.* 1999, Faure *et al.* 2000), adipocytes (Lin *et al.* 2000), gingival fibroblasts (Wang *et al.* 2000), smooth muscle cells (Watari *et al.* 2000), Kupffer cells (Su *et al.* 2000), hepatic stellate cells (Paik *et al.* 2003), keratinocytes (Pivarcsi *et al.* 2003), and epithelial cells (Cario *et al.* 2000, Song *et al.* 2001).

The regulation of TLR4 expression is complex, involving tissue and cell-specific differences. Proper regulation, however, is crucial for the innate immune system. The amount of TLR4 receptors in the cell is small (only up to 1000), and over-expression of TLR4 not only enhances the sensitivity to LPS, but may also contribute to heart failure (Frantz *et al.* 1999) and genetic susceptibility to ozone-induced lung hyperpermeability (Kleeberger *et al.* 2000, Kleeberger *et al.* 2001). Downregulation of TLR4 gene expression, on the other hand, is involved in LPS tolerance (Nomura *et al.* 2000, Medvedev *et al.* 2000).

Only a few mechanisms for regulating TLR4 gene expression have been characterized. First, there is an alternatively spliced form of mouse TLR4, in which an additional exon

between the second and third exons of the reported gene encodes for 36 additional amino acids and a stop codon. This alternatively spliced form is expressed as a partially secretory 20 kDa soluble protein. It significantly inhibits LPS-mediated TNF-α production and NF-κB activation in mouse macrophages and may thus function to inhibit an excessive LPS response (Iwami *et al.* 2000). Second, the TLR4 promoter region has a myeloid cell-specific transcription factor PU.1 and interferon consensus sequence-binding protein (ICSBP) binding sites. TLR4 promoter activity in myeloid cells is dependent on them. However, PU.1 and ICSBP in a non-myeloid cell line do not induce promoter activity, suggesting a need for additional transcription factors or some inhibitory regulation in some other cells (Rehli *et al.* 2000). Third, alteration of mRNA stability serves another transcriptional and posttranscriptional regulatory mechanism. Fan *et al*. showed, in a rodent model *in vivo* and *in vitro*, that the reduction of lung TLR4 mRNA after intratracheal LPS was due to a lowering of mRNA stability, and that the prevention of mRNA reduction after an antecedent shock was due to prevention of mRNA destabilization (Fan *et al.* 2002).

Table 1. Stimulators of TLR4. (For references, see Johnson et al. 2003)

Stimulant	Source (example)
Exogenous	
Lipopolysaccharide	Gram negative bacteria
Flavolipin	Gram negative bacteria
P fimbriae	Gram negative bacteria
Mannuronic acid polymers	Gram negative bacteria
Lipoteichoic acid	Spirochete
Heat-labile component	Mycobacteria
Glucuronoxylomannan	Yeast
Envelope glycoprotein	Retrovirus
Fusion glycoprotein	Paramyxovirus
Paclitaxel (taxol)[a]	Synthesized from English yew
Heat-shock protein (Hsp) 60	Gram negative bacteria
Endogenous	
Heparan sulfate	Cell surface, extracellular matrix
Hyaluronan	Extracellular matrix, synovium
Hsp60	Mitochondria
Hsp70	Cytoplasm
Gp96	Endoplasmic reticulum
Fibronectin extra domain A	Extracellular matrix, serum
Fibrinogen	Serum
Surfactant protein A	Lung endothelium

[a] Agonist for murine TLR4, not human

2.3.1.3 Ligands and function

In addition to mediating cytokine production in response to LPS and some other bacterial and viral products, TLR4 has been suggested to be the receptor for some endogenous ligands and, therefore, to participate in other central phenomena in the immune response. For example, fibrinogen stimulates macrophage chemokine secretion through TLR4 (Smiley *et al.* 2001). Fibronectin, which is produced in cells in response to tissue injury, activates TLR4 (Okamura *et al.* 2001). Heat shock protein 60 (hsp60) induces a proinflammatory response via TLR4 as a danger signal of stressed and damaged cells (Ohashi *et al.* 2000). Dendritic cell maturation by LPS and murine β-D2-defencin is TLR4-mediated (Tsuji *et al.* 2000, Kaisho *et al.* 2001, Biragyn *et al.* 2002). According to a recent finding, TLR4 controls the migration of polymorphonuclear leucocytes by regulating cell surface chemokine receptors (Fan & Malik 2003). Pulmonary surfactant protein A has also recently been found to stimulate TLR4 (Guillot *et al.* 2002).

The known stimulators of TLR4 are listed in table 1, but not all of them have been shown to directly interact with it.

2.3.1.4 Deficiency

TLR4 was discovered to be the principal mediator of the LPS response by characterizing the LPS-hyporesponsive mouse strains. A few decades ago, the mouse strains C3H/HeJ and C57BL/10ScCr were found to show a deficient response to bacterial endotoxin (Sultzer 1968, Coutinho *et al.* 1977). They could tolerate enormous amounts of LPS without any lethal effects, but were highly susceptible to Gram negative bacterial infection. Genetic studies revealed a single *Lps* locus in chromosome 4, which was responsible for this LPS hyporesponsiveness. In the late 90's, this locus was mapped as the TLR4 gene (Poltorak *et al.* 1998a, Poltorak *et al.* 1998b, Qureshi *et al.* 1999). The C3H/HeJ mouse strain has a single co-dominant missense mutation in the third exon of the TLR4 gene, which causes a replacement of proline with histidine at position 712 of the polypeptide. The strain C57BL/10ScCr is homozygous for a null mutation of TLR4, which is caused by a deletion of about 75 kb on chromosome 4. The TLR4 knock-out mouse generated by Hoshino and his co-workers had the same phenotype as naturally occurring TLR4-mutant mice (Hoshino *et al.* 1999). The animals developed otherwise normally, but showed no response to LPS or synthetic lipid A to a certain dose level. The hyporesponsiveness was later suggested to be due to disruption of the TLR4-mediated signaling pathway resulting from the inability of mutant TLR4 to interact with the second messenger MyD88 (Rhee & Hwang 2000). In addition to interrupted LPS signaling, the loss-of-function mutations in TLR4 have been shown to be beneficial in preventing, for instance, neurodegeneration and the generation of atherogenesis (Kiechl *et al.* 2003, Lehnardt *et al.* 2003).

TLR4 polymorphism has been reported (Arbour *et al.* 2000). Two common missense mutations (Asp299Gly and Thr399Ile) affect the extracellular domain of TLR4 in human. These mutations have been shown to be associated with an increased risk of Gram

negative bacterial infections and impaired LPS signaling (Agnese *et al.* 2002), and the Asp299Gly mutation is associated with premature birth in a Finnish population (Lorenz *et al.* 2002).

2.3.1.5 Signaling

The known mammalian TLR-signaling cascade is analogous to the *Drosophila* Toll-signaling pathway. In *Drosophila*, a proteolytically processed product of a Spätzle gene activates the Toll receptor. Toll signals the activation through Tube (corresponds to MyD88 in mammals) and the serine-threonine kinase Pelle (IRAK in mammals) to Cactus, which is the analog of the mammalian NF-κB inhibitor. Degradation of Cactus releases Dorsal, i.e. the NF-κB-like protein, from the complex, and Dorsal translocates into the nucleus, where it activates appropriate specific target genes, such as the antifungal peptide Drosomycin and the antibacterial peptide Attacin (Anderson 2000). The Spätzle gene needs to be processed by serine protease to activate the Toll receptor, and it has been suggested that LPS may first activate the mammalian serine protease, which generates a product required for further signaling, but there is no direct evidence for that argument (Mansell *et al.* 2001).

LPS signaling through TLR4 is shown schematically in figure 2. According to current knowledge, LPS is first recognized near the cell surface by circulating LBP, which breaks down LPS aggregates and moves LPS monomers to the membrane protein CD14. LBP has a high-affinity binding site for LPS, and it functions as a catalytic lipid transfer protein (Schumann *et al.* 1990, Tobias *et al.* 1995, Su *et al.* 2000). The extracellular domain of CD14 is very similar to the extracellular domain of TLR4, but as it lacks the cytoplasmic domain, it is not capable of inducing cellular signaling (Medzhitov *et al.* 1997). TLR4 has been shown to be the signaling part of the receptor complex, which involves a fourth member as well, namely myeloid differentiation (MD)-2. MD-2 is physically associated with TLR4 and essential for TLR4 to translocate on the cell surface (Ohnishi *et al.* 2003) and also for an efficient response to LPS (Shimazu *et al.* 1999, Akashi *et al.* 2000, Akashi *et al.* 2001, Visintin *et al.* 2001, Schromm *et al.* 2001, Mancek *et al.* 2002). LPS has been shown to be in direct contact with each of the other three members of the receptor complex (da Silva *et al.* 2001).

The downstream effector from TLR4 is an adapter protein MyD88, which interacts with the transmembrane receptor through the C-terminal TIR domain (Lord *et al.* 1990, Hultmark 1994, Medzhitov *et al.* 1998). MyD88 recruits Ser/Thr kinase IRAK (IL-1R associated kinase) to the receptor complex (Wesche *et al.* 1997). IRAK associates with an adapter molecule TNF receptor associated factor (TRAF6) (Cao *et al.* 1996a, Cao *et al.* 1996b, Muzio *et al.* 1997, Kanakaraj *et al.* 1998). TRAF6, in turn, activates the MAP3K family member NIK (NF-κB-inducing kinase) (Malinin *et al.* 1997), which activates the NF-κB inhibitor kinases (IKKs) (Ling *et al.* 1998, Takeda *et al.* 1999, Hu *et al.* 1999, Li *et al.* 1999). Degradation of the NF-κB inhibitor I-κB releases NF-κB to subsequently translocate to the nucleus and to activate to induce appropriate gene expression (Mercurio *et al.* 1997, Woronicz *et al.* 1997, Zandi *et al.* 1997).

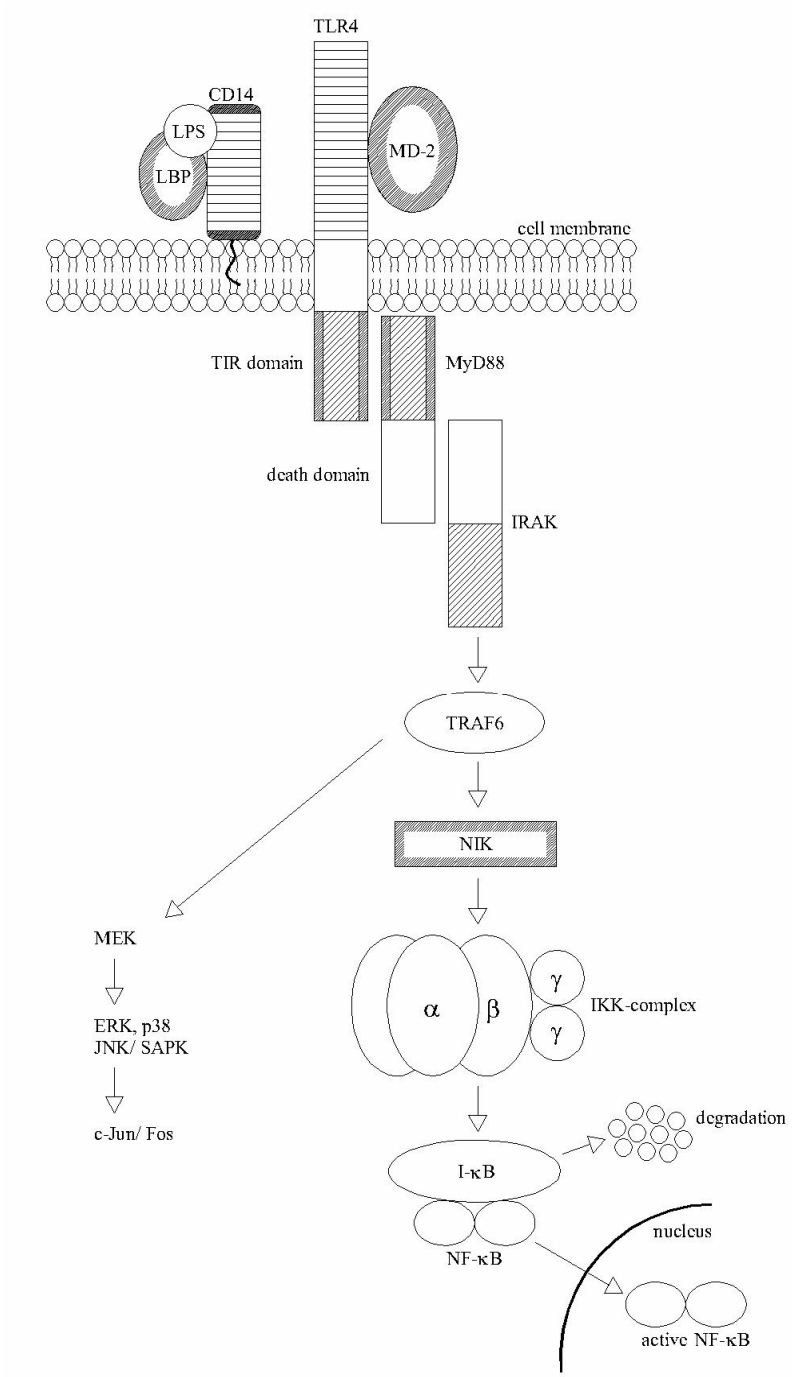

Fig. 2. TLR4-signaling cascade according to the current opinion.

Recently, at least two novel proteins, MyD88 adapter-like Mal/TIRAP (Fitzgerald *et al.* 2001, Yamamoto *et al.* 2002, Schilling *et al.* 2002, O'Neill *et al.* 2003) and Bruton's tyrosine kinase (Btk) (Jefferies *et al.* 2003), have been reported to participate in the pathway. Mal/TIRAP is an adapter protein, which forms heterodimers with MyD88, activates NF-kB in association with IRAK-2, and also associates with TLR4. Btk binds to the TIR domain and is important for NF-κB activation by TLR4. In B-cells, a TIR family protein RP105 regulates the LPS response by cooperating with TLR4 (Ogata *et al.* 2000, Miyake *et al.* 2000).

Other pathways downstream from TLR4 have been suggested. There is some evidence of a MyD88-independent TLR4-signaling pathway, which does not lead to the activation of NF-κB and the production of cytokines, but responds to LPS by activating the IFN-regulatory factor 3 as well as by inducing the genes containing IFN-stimulated regulatory elements, such as IP-10 (Kawai *et al.* 2001, Akira & Hoshino 2003). An adapter protein Mal/TIRAP has been suggested to replace MyD88 in the MyD88-independent signaling pathway (Horng *et al.* 2001) in addition to the possibility to function along with MyD88 (Horng *et al.* 2002). Many other alternatives for members of the TLR4-signaling pathway have been suggested. LPS is known to activate a number of signaling molecules involved in inflammatory phenomena, such as phosphatidylinositol 3-kinase (PI3K), Akt (a downstream mediator from PI3K), and many others able to activate NF-κB (Herrera-Velit & Reiner 1996, Ninomiya-Tsuji *et al.* 1999, Irie *et al.* 2000, Valledor *et al.* 2000, Hippenstiel *et al.* 2000, Jones *et al.* 2001a). LPS also activates the pulmonary surfactant proteins SP-A and SP-D, which directly bind to CD14 (Sano *et al.* 1999, Sano *et al.* 2000).

2.3.2 Toll-like receptor 2 (TLR2)

2.3.2.1 Structure

The TLR2 gene has been mapped on chromosome 4q32 in human (Rock *et al.* 1998). Similarly to its murine homolog, the human TLR2 gene is composed of three exons, of which the first and second are non-coding, and the complete open reading frame is located on exon three. Alternative spliced forms also exist (Haehnel *et al.* 2002).The human, as well as the mouse, 5'-flanking regions have been cloned (Matsuguchi *et al.* 2000b, Musikacharoen *et al.* 2001, Wang *et al.* 2001b, Haehnel *et al.* 2002), and interestingly, no sequence homology has been detected between the human and mouse promoter regions. The mouse 5' untranslated region contains two NF-κB positions, which play a role in regulating TLR2 gene expression.

The extracellular domain of the receptor molecule TLR2 contains 18 to 20 LRRs and LRR-like motives. Like other TLRs, the intracellular domain of TLR2 contains the Toll/IL-1 receptor-like TIR domain (Kirschning & Schumann 2002).

2.3.2.2 Gene expression

TLR2 expression has been found in lymphoid tissues, such as spleen, lymph node, thymus, and bone marrow (Yang *et al.* 1998, Kirschning *et al.* 1998), and in lung, heart, muscle, and brain (Rock *et al.* 1998). The expressing cells include adipocytes (Lin *et al.* 2000), gingival fibroblasts (Mori *et al.* 2003), epithelial cells (Cario *et al.* 2000), keratinocytes (Pivarcsi *et al.* 2003), type II alveolar cells (Droemann *et al.* 2003), hepatocytes (Matsumura *et al.* 2003), and smooth muscle cells (Watari *et al.* 2000).

Unlike TLR4, TLR2 gene expression is upregulated by LPS and synthetic lipid A in various cells, including murine hepatocytes (Matsuguchi *et al.* 2000a, Matsumura *et al.* 2003). Circulating Gram negative bacteria cell wall components also cause a marked increase in TLR2 transcription, at least in cerebral tissue (Laflamme *et al.* 2001). The upregulation of TLR2 gene expression by LPS could indicate that, although TLR2 is dispensable for the initial host response against LPS, it may contribute to the accelerated macrophage response seen at subsequent stages of infection. Various cytokines, such as IL-2, IL-15, IL-1α, IL-1β, and TNF-α, also induce TLR2 expression either directly or indirectly by active NF-κB. The NF-κB sites in the TLR2 promoter bind the NF-κB protein in macrophages following LPS and TNF-α stimulation, suggesting an important role of these sites in TLR2 regulation (Liu *et al.* 2000, Matsuguchi *et al.* 2000a, Liu *et al.* 2001, Wang *et al.* 2001b, Matsumura *et al.* 2003). IFN-γ and macrophage colony-stimulating factor (M-CSF) also upregulate TLR2 expression in macrophages and peripheral blood monocytes (Mita *et al.* 2001, Miettinen *et al.* 2001).

Downregulation of TLR2 gene expression is involved in LTA tolerance, in which LTA causes the stage of hyporesponsiveness to second stimulation (LTA tolerance or desensitization). LTA tolerance does not exist in TLR2-deficient mice, suggesting that TLR2 regulation or an impaired signaling pathway is the reason for tolerance (Lehner *et al.* 2001).

2.3.2.3 Ligands and function

TLR2 was first considered the principal mediator of the LPS response (Yang *et al.* 1998, Kirschning *et al.* 1998). This consideration was based on the facts that TLR2 mRNA was upregulated upon stimulation of isolated macrophages with LPS, that the treatment of TLR2-expressing culture cells with LPS resulted in significant NF-κB activation dose-dependently, which effect was not seen with TLR4, and that mutations in the TLR2 cytoplasmic portion inhibited NF-κB activation, indicating that the cytoplasmic domain starts the LPS-signaling cascade. In addition, it was shown that efficient LPS signal transmission by TLR2 requires the serum components CD14 and LBP, which are known to be part of the LPS-signaling cascade. However, discovery of LPS-hyporesponsive mice with TLR4 deficiency and a study where TLR2-deficient cells responded normally to LPS (Heine *et al.* 1999) suggested an alternative role for TLR2. A study with TLR2 knock-out mice provided verification of the assumption (Takeuchi *et al.* 1999a). Mice with TLR2 deficiency responded to LPS equally to wild-type mice, and TLR2-deficient macrophages were hyporesponsive to several Gram positive bacterial cell wall products.

In addition, it has been proposed that the signaling via TLR2 is caused by the highly bioactive contaminants in LPS preparations, known as endotoxin proteins, rather than by pure LPS itself (Hirschfeld *et al.* 2000, Ingalls *et al.* 2001). Although the role of TLR2 in LPS signaling is still somewhat unclear (see, for example, (Mokuno *et al.* 2000, Matsuguchi *et al.* 2000b, Muta & Takeshige 2001), it is basically accepted that TLR2 confers the responsiveness to Gram positive bacteria.

In 1999, Schwandner and his co-workers showed that peptidoglycan and lipoteichoic acid-induced cell activation is mediated by TLR2 (Schwandner *et al.* 1999). On the basis of structural and functional homology as well as the result of signaling, it is most likely that the signaling takes place through the same machinery as that with IL-1R and TLR4 (Yang *et al.* 1999, Muta & Takeshige 2001), though alternative and TLR2-specific signaling pathways (Arbibe *et al.* 2000, Rhee *et al.* 2003) have also been suggested. Since then, several studies have come to the same conclusion that TLR2 recognizes Gram positive bacteria (Lien *et al.* 1999, Yoshimura *et al.* 1999, Hirschfeld *et al.* 1999, Underhill *et al.* 1999a, Opitz *et al.* 2001, Wang *et al.* 2001a, Schroder *et al.* 2003), mycobacteria (Underhill *et al.* 1999b), yeast (Underhill *et al.* 1999a), and parasitic protozoa (Campos *et al.* 2001).

Analogous to TLR4, some endogenous stimuli can activate TLR2. Necrotic cells induce inflammatory and tissue repair responses, including chemokine MIP-2, by activating NF-κB. This activation is dependent on TLR2 and requires the TLR2-signaling cascade (Li *et al.* 2001). The lung surfactant protein SP-A significantly reduces peptidoglycan-induced TNF-α secretion by directly binding to TLR2 (Murakami *et al.* 2002, Sato *et al.* 2003). In addition to mediating cytokine production, TLR2 induces dendritic cell maturation in the same way as does TLR4 (Tsuji *et al.* 2000, Hertz *et al.* 2001). Recently, TLR2 has been shown to internalize the antigen for presentation to adaptive immune cells, and it could thus be an efficient vaccine target (Schjetne *et al.* 2003).

Activation of TLR2 also has harmful effects. TLR2 has been suggested to be a “death receptor” mediating bacterial lipoprotein-caused apoptosis (Aliprantis *et al.* 1999, Aliprantis *et al.* 2000). TLR2 has also been shown to help *Mycobacterium tuberculosis* to survive in host cells and to maintain chronic infection (Noss *et al.* 2001). *Mycobacterium tuberculosis* bacilli and lysate inhibit macrophage expression of class II MHC molecules and antigen processing and thus decrease recognition by T-cells despite the innate immune responses in early infection, and that inhibition is dependent on TLR2.

2.3.2.4 Deficiency

TLR2 deficiency has been studied with the human polymorphisms Arg753Gln and Arg677Trp and with TLR2-deficient mice. Deficiency in TLR2 function may predispose to life-threatening bacterial infections because of impaired NF-κB activation and cytokine production (Lorenz *et al.* 2000, Bochud *et al.* 2003). In addition, studies with TLR2(-/-) mice showed that the lack of TLR2 was associated with earlier death from meningitis, which was not due to sepsis but to reduced brain bacterial clearing followed by increased intrathecal inflammation (Echchannaoui *et al.* 2002)

2.4 Nuclear transcription factors

2.4.1 General

There are a number of transcription factors that control the expression of genes. All transcription factors function by recognizing specific promoter response elements in the gene to be regulated, binding to DNA and allowing RNA polymerase to initiate the transcription. The activation of a transcription factor is controlled in several ways: by synthesis of the protein (e.g. homeoproteins), by phosphorylation (heat shock protein responsible element recognizing transcription factors, HSTF) or dephosphorylation (AP-1) of the protein, by ligand binding (steroid receptors), by changing the partner bound to the protein (helix-loop-helix proteins), or by release by inhibitor (NF-κB) (Lewin 1994). The focus in this study was on the genes mainly controlled by the transcription factor NF-κB.

2.4.2 Nuclear factor-κB (NF-κB)

On a molecular level, the innate immune system centers on the activation of NF-κB, which has the ability to induce gene transcription of several proinflammatory cytokines, chemokines, adhesion molecules, and inducible nitric oxide in response to stimulation by signals related to pathogens or stress. In addition, NF-κB controls the expression of many evolutionarily conserved antimicrobial peptides, adaptive immunity genes, such as MHC proteins, and genes critical for regulating apoptotic processes. (Zhang & Ghosh 2001).

Cloning of NF-κB subunits has revealed a family of proteins exhibiting a conserved central region known as the Rel homology domain, which is involved in DNA binding. Five members of the mammalian family have been defined: NF-κB1 (p50/p105), NF-κB2 (p52/p100), p65 (RelA), RelB, and c-Rel. The classical form is a heterodimer of p50 and p65 subunits. NF-κB exists in cytoplasm in an inactive form associated with the regulatory proteins I-κB. The most important of these inhibitors are α, β, ε, and the recently found nuclear ζ, which have distinct but overlapping specificities in the regulation of distinct genes in various tissues (Muta *et al.* 2003). The nuclear translocation and activation of NF-κB is usually a result of phosphorylation and degradation of inhibitors. NF-κB can also function in concert with other transcription factors, such as AP-1. (Ghosh *et al.* 1998, May & Ghosh 1998).

2.5 Cytokines

In general, cytokines are defined as secreted regulatory proteins that control the survival, growth, differentiation, and effector function of tissues and cells. Their production and secretion by host cells is stimulated by specific signals from micro-organisms. For example, interleukins, interferons, lymphokines, chemokines, and growth factors are considered as cytokines. They share some common features. They are often undetectable

in circulation, but they are produced by cells that are widespread in the body, such as macrophages. They are secreted into the extracellular medium, where they interact with specific target cells, resulting in an appropriate biological response. They contribute to developmental regulation, tissue repair, hemopoiesis, inflammation, and specific and non-specific immune responses. These responses are mostly mediated by several cytokines. Thus, it is infrequent that a loss or neutralization of one cytokine will markedly interfere with the function. Cytokines act as a complex network where one cytokine can influence the production of, and response to, many other cytokines. It is believed that a constitutive level of cytokine production is necessary to maintain steady-state levels of tissue renewal, and that constitutive production of cytokines may be required for continued cell survival and selection. Mostly, cytokine production represents an emergency response to tissue damage, infection, or other insults. (For references, see Haddad 2002).

Based on their function, cytokines can be divided into proinflammatory and anti-inflammatory ones. Proinflammatory cytokines, such as IL-1 and TNF-α, are produced soon after infection, and they are responsible for the acute inflammatory reaction. Anti-inflammatory cytokines, such as IL-10, balance and inhibit the response. (Haddad 2002).

The molecular processes responsible for the regulation of cytokines are poorly understood. The regulation may take place via the expression or activity of cytokine receptors or via competition for the same receptor. Many cells observe a hierarchy in cytokine action, where some early cytokines activate cells to produce late-acting cytokines. In addition, there are a variety of microbial response modifiers that function as cytokine antagonists. (Haddad 2002).

A more detailed description is given of a few common cytokines that were under investigation in this study. Selective aspects of the cytokines involved in inflammation are shown summarized in table 2.

2.5.1 Interleukin (IL)-1 and IL-1 receptor

Three IL-1 cytokines, receptor agonists IL-1α and IL-1β and receptor antagonist IL-1ra, are central mediators in the activation and maintenance of the inflammatory response. Their production and release from activated macrophages is induced by several proinflammatory stimuli, such as the bacterial cell wall products LPS and LTA, IL-1 itself, zymosan, activated complement components, TNF, and adhesion to surfaces. IL-1 production is downregulated by steroids, transforming growth factor-(TGF)β, and retinoic acid. (Dinarello 1991).

IL-1 has a wide range of target cells, such as fibroblasts, smooth muscle cells, keratinocytes, endothelial cells, hepatocytes, islet cells, and immune system cells (B-cells, T-cells, monocytes, and neutrophils), in which it causes proliferation and induces secretion of other cytokines and many other substances, such as adhesion molecules and insulin. In connective tissue, IL-1 has a central role in the recovery of tissue after injury. In osteoclasts and chondrocytes, IL-1 mediates bone resorption and cartilage breakdown. IL-1 also mediates many of the toxic reactions observed in acute septicaemia and inflammation. (Dinarello 1989, Civitelli *et al.* 1989, Raines *et al.* 1989).

The receptor that mediates all the known biological activities of IL-1 is IL-1 type I receptor (IL-1RI). It binds both IL-1α and IL-1β in the same activity. IL-1R1-deficient mice have a reduced inflammatory response (Labow *et al.* 1997). IL-1R1 belongs to the TIR family because of the homologous cytoplasmic domain shared with the other family members, such as Toll-like receptors, IL-18R, and MyD88 (O'Neill 2000).

There are two ways of antagonizing IL-1 activity. One is the binding of IL-1ra to the IL-1 receptor, since IL-1ra is not capable of inducing signaling. The only biological role of IL-1ra seems to be the suppression of the IL-1-mediated inflammatory response, and mice lacking IL-1ra show an exaggerated and overwhelming response (Hirsch *et al.* 1996). The other way of antagonizing IL-1 is the expression of IL-1R2. It is a receptor that binds IL-1α and IL-1β, but not IL-1ra, and it has a short cytoplasmic domain incapable of mediating the signal (Slack *et al.* 1993).

2.5.2 IL-6

IL-6 is constitutively active in a number of tumor cells, but it is not produced in normal cells, unless appropriately stimulated by, for example, LPS. Other cytokines, such as IL-1 and TNF, induce IL-6 production, as do also various viruses and adenylate cyclase activators (Akira *et al.* 1993). IL-6 has many biological functions: it promotes immunoglobulin secretion in B-cells, activates osteoclasts, matures megakaryocytes, and promotes the growth of T-cells, keratinocytes, and myeloma cells (Kishimoto 1989, Kishimoto *et al.* 1992). It is also suggested to be involved in several diseases, including uterine cervical carcinoma, rheumatoid arthritis, and AIDS (Kishimoto 1989, Akira *et al.* 1993).

2.5.3 IL-18

IL-18 (previously called IFN-γ-inducing factor) is a pro-inflammatory cytokine that belongs to the IL-1 cytokine family, since its receptor contains the Toll/IL-1R-like cytoplasmic domain (Bazan *et al.* 1996). IL-18 mRNA is expressed in a wide range of cells, including Kupffer cells, macrophages, T-cells, B-cells, dendritic cells, osteoblasts, keratinocytes, astrocytes, and microglia. In unstimulated cells, IL-18 is primarily present in an inactive precursor form called pro-IL-18 (Puren *et al.* 1999). Alone, it stimulates the production of inducible nitric oxide synthase (iNOS), TNF-α, Th2 cytokine production, and allergic inflammation (Olee *et al.* 1999), (Puren *et al.* 1998). The combination of IL-18 and IL-12 stimulates Th1-mediated immune responses through the induction of IFN-γ (Okamura *et al.* 1995) and enhances the activity of cytotoxic T-lymphocytes (Dao *et al.* 1998).

Table 2. Central proinflammatory and anti-inflammatory cytokines in bacterial infection (modified from Järveläinen & Miettinen 2001).

Cytokine	Primary source	Effect
Proinflammatory		
IL-1α, IL-1β	monocytes, macrophages, T- and B-cells, endothelial cells	activation of inflammatory cells, induction of the expression of adhesion molecules, activation of thrombocytes
IL-2	T- cells	growth and proliferation of T- cells
MIP-2 (IL-8)	monocytes, macrophages	neutrophil infiltration
IL-6	monocytes, macrophages	acute phase protein synthesis
IL-12	monocytes, macrophages, dendritic cells	proliferation and differentiation of Th1-cells, production of IFN-γ together with IL-18
IL-18	monocytes, macrophages	induction of proinflammatory and catabolic responses
TNF-α	monocytes, macrophages, T- and B-cells, endothelial cells	activation of inflammatory cells, induction of the expression of adhesion molecules, activation of thrombocytes
MIF	macrophages, epithelial cells	stimulation of immune cells, modulation of specific receptors
IFN-γ	natural killer cells, T-cells	activation of macrophages, antigen presentation
Anti-inflammatory		
IL-4	Th2 cells	growth and proliferation of B and T-cells, inhibition of IL-12 and IFN-γ
IL-10	macrophages, T cells	inhibition of other cytokines, inhibition of antigen presentation
TGF-β	fibroblasts	inhibition of cell growth

2.5.4 Tumor necrosis factor (TNF)-α

TNF-α expression has been found in macrophages, lymphocytes, fibroblasts, smooth muscle cells, tumor cells, Kupffer cells, and many others. It exists as a trimer under native conditions. TNF-α is produced in response to Gram negative and Gram positive bacteria, viruses, mycoplasma, cytokines, such as IL-1, tumor cells, reactive oxygen species, platelet-activating factor, etc. Its production is inhibited by dexamethasone, TGFβ, thalidomide, and IL-6. (Nicola 1994).

TNF-α has many biological effects. *In vitro*, it induces fibroblast proliferation, mediates antiviral effects, induces endothelial cell adhesion molecules and other cytokines, and precedes myeloid cell differentiation. *In vivo*, it induces tumorigenesis and metastasis, induces septic shock, and promotes cerebral malaria. (Nicola 1994).

TNF-α has been used as an anti-tumor agent since it inhibits the proliferation of some tumor cells *in vitro*. However, its systemic administration has major side effects, such as

hepatotoxicity, neurotoxicity, cyanosis, and fever (Aggarwal & Reddy 1994). TNF receptor antagonist and antibody have been successfully used as drugs for rheumatoid arthritis (Baumgartner 2000).

2.5.5 Macrophage migration inhibitory factor (MIF)

The pituitary hormone and macrophage/T-cell cytokine macrophage migration inhibitory factor (MIF) has recently emerged as an important mediator of inflammation and innate immunity. Microbial products and other pro-inflammatory cytokines rapidly induce the release of MIF from macrophages, and MIF, in turn, stimulates the production of other pro-inflammatory cytokines. There is evidence suggesting that MIF is an enzyme and may thus function, at least in part, via non-receptor-mediated pathways. No membrane receptor for MIF has been identified. (Bernhagen *et al.* 1998, Roger *et al.* 2001a)

Glucocorticoids as anti-inflammatory agents inhibit cytokine production, but induce rather than suppress MIF production. MIF influences the magnitude of the inflammatory response by functioning as a physiological counterregulator of glucocorticoid action (Bernhagen *et al.* 1998). The absence of MIF protects animals from lethal endotoxemia, staphylococcal toxic shock, and septic shock. MIF-deficient macrophages are hyporesponsive to LPS and Gram negative bacteria (Roger *et al.* 2001a, Roger *et al.* 2001b, Roger *et al.* 2003).

2.5.6 IL-10

IL-10 is an anti-inflammatory cytokine that reduces the lethality of septic shock and is expressed late in the activation process of T-cells, B-cells, monocytes, macrophages, mast cells, and keratinocytes. Its physiological function is the limitation and final shut-off of the inflammatory reaction in immune defence once the pathogen has been eliminated. IL-10 expression is activated by anti-CD3 antibodies and phorbol esters and inhibited by IL-4 and IFNγ (de Waal *et al.* 1991a, de Waal *et al.* 1992). IL-10 inhibits class II MHC and cytokine expression and can act as a co-stimulator of the growth of several hematopoietic cells, including T-, B-, and mast cells, and of the proliferation of megakaryocytes and erythroid and primitive hematopoietic cells (Fiorentino *et al.* 1991, de Waal *et al.* 1991b). It also inhibits the production of reactive oxygen species and NO by macrophages (Bogdan *et al.* 1991, Moore *et al.* 1993).

2.5.7 Macrophage inflammatory protein 2 (MIP-2)

MIP-2 is a mouse protein, a member of the α-chemokine family, and a homolog of human IL-8. It is produced primarily by monocytes and macrophages in response to LPS. MIP-2 causes neutrophil infiltration, chemotaxis, and neutrophil degranulation, and it suppresses the colony formation of immature myeloid progenitors. Similarly to other α-

chemokines, it is implicated as a major participant in both acute and prolonged inflammatory reactions, modulation of angiogenesis, and fibroplasia. (Taub & Oppenheim 1994).

2.6 Current and future therapies of inflammatory diseases

Severe sepsis and septic shock are life-threatening complications of infections and major causes of death despite the availability of antibiotics. Gram negative bacterial infections (LPS) have earlier been predominant, and they still comprise about half of all cases of septic syndromes (Bochud *et al.* 2001). Susceptibility to sepsis may be due to inherited or acquired mutations of innate immune genes (Bochud & Calandra 2003).

Most of the symptoms of septic syndrome are caused by cytokines. Normally, cytokines act in balance to overcome the infection, but a too massive or too long-lasting stimulus leads to excessive activation and overproduction of cytokines, resulting in dramatic pathophysiological conditions. These include fever, tachycardia, hypotension, disseminated intravascular coagulation, and finally, multi-organ failure and even death (Schletter *et al.* 1995b). On the other hand, failure to activate innate immunity may also have deleterious outcomes, such as overwhelming bacterial infection.

Numerous adjunctive treatments for sepsis have been tested (table 3), such as immunosuppressive drugs and neutralization of microbial toxins, none of which have proven to be very successful (Bochud & Calandra 2003, Riedemann *et al.* 2003). Problems are caused by the redundancy of cytokine action, by the fact that Gram positive and Gram negative sepsis cannot be distinguished based on clinical symptoms, and by the inherent need for the innate immune system: it cannot be totally eliminated. Antibodies only neutralize existing cytokines, but do not inhibit their production. Corticosteroids inhibit the production of nearly all cytokines, unfortunately also anti-inflammatory ones, and their influence is often too unspecific. (Chernoff *et al.* 1995, Nathan & Ding 2001, Järveläinen & Miettinen 2001).

Some possible new treatment strategies have been suggested, which may prove useful in the future (Bochud & Calandra 2003, Riedemann *et al.* 2003). First, genetic studies may help to identify people who are at an increased risk of sepsis. Second, IL-10 as an effective anti-inflammatory cytokine is a promising candidate for clinical application, and as a treatment, it does not seem to have any remarkable disadvantages (Chernoff *et al.* 1995). Third, MIF has been shown to regulate the innate immune response and to cause LPS hyporesponsiveness by modulating the expression of TLR4 (Roger *et al.* 2001a). Finally, modulation of the expression of the receptors for bacterial agents or blocking of the interaction of microbial products and the receptor may be some other therapeutic targets.

Table 3. Selected therapies for inflammatory diseases (modified from Bochud & Calandra 2003).

Type of therapy	Target(s)	Agents
Neutralization of microbial toxins	LPS	Anti-LPS antibodies, anti-lipid A antibodies, lipopolysaccharide removal, lipopolysaccharide analogues
Non-specific anti-inflammatory and immunomodulating drugs	Multiple inflammatory and immune mediators	Corticosteroids, immunoglobulins, interferon-γ
Inhibition of specific mediators	*Pro-inflammatory cytokines:*	
	TNF-α	Anti-TNF-α antibodies, soluble TNF-α receptors
	IL-1	IL-1 receptor antagonist
	Phospholipid components:	
	Phospholipase A2	Phospholipase A2 inhibitor
	Cyclo-oxygenase (Cox)	Ibuprofen, other inhibitors of Cox1 and/or Cox2
	Platelet-activating factor	Platelet-activating factor antagonist
	Oxygen free radicals	N-acetylcysteine, allopurinol
	Nitric oxide	N-methyl-L-arginine
Correction of coagulopathy	Coagulation cascade	Antithrombin III, tissue pathway inhibitor, activated protein C
Agonists of the acute innate response	Rescuing on essential missing function	Prostaglandin E1, granulocyte colony-stimulating factor, nitric oxide

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3 Outlines of the present study

Despite antiseptic techniques, immunizations against specific infections, and antibiotics, serious generalized infections and inflammatory diseases remain major causes of morbidity and mortality, particularly during the perinatal period. The efforts invested in the investigation of the molecular mechanisms leading to septic shock syndrome and to serious inflammatory diseases may eventually provide new ancillary methods of prevention and treatment. The attempts to find ancillary therapies have so far focused on neutralizing the bacterial agent, blocking the activity of transcription factors that causes the expression of cytokines, and manipulating directly the expression or function of cytokines. None of these have proven very successful. A new approach to the clinical treatment of sepsis is the manipulation of the machinery between the bacterial agent and the activation of the transcription of cytokines (see, for example, Nathan & Ding 2001). Discovery of the mammalian Toll-like receptors in 1997 as the signal-mediating part of the receptor complexes recognizing the bacterial agents was a major milestone in that investigation.

Elucidation of the TLR function and signaling will have important implications for the anti-inflammatory strategies to control inflammatory events. The focus of this study was on how the functional activation of two signaling receptors, TLR4 and TLR2, regulates the acute inflammatory response. The following goals were set for this work:

1. To determine the developmental and the tissue and species-specific expression patterns of TLR4 and TLR2 in mice.
2. To determine the patterns of TLR4 and TLR2 activation and the acute cytokine response in adult tissues to LPS.
3. To study whether there is an additional component associated with TLR4 in the signaling pathway for LPS, and whether it may further explain the cellular responses to LPS.
4. To determine the expression of TLR4 and TLR2 in fetal tissues during the perinatal period and to study the pattern of TLR4 activation and the acute cytokine response in the feto-placental compartment to LPS.

4 Materials and methods

Detailed descriptions of the materials and methods have been given in the original articles I-IV.

4.1 Experimental animals (I, II, IV)

All experiments were approved by the Animal Experimentation Board of the University of Oulu and the Regional Civil Board Authority.

The DBA/2 mouse strain was used for the adult experiments (II). Female mice aged 3-4 months were used without any treatment, or they were injected intraperitoneally (ip) with 0.2 ml of PBS or LPS (*Escherichia coli* lipopolysaccharide, serotype 055:B5, Sigma Chemical Co, St. Louis) of different concentrations (0.6 mg/kg, 1.2 mg/kg and 2.4 mg/kg). At two, five, or twenty-four hours after any injection, the mice were killed with cervical dislocation, the tissues were harvested and frozen in liquid nitrogen for RNA and protein isolation, and pieces of all tissues were formalin-fixed for immunohistochemistry

The developmental experiments were done with two different mouse strains, FVB (I) and DBA/2 (IV). Female mice aged 3-4 months were time-mated, and the day of the vaginal plug was designated as the day 0. The mice were used at different dates of pregnancy, known within ±12 h. The DBA/2 mice were injected in the same way as non-pregnant mice with PBS or LPS or left untreated. Other DBA/2 mice received an injection of PBS or 0.25 μg LPS into the amniotic sac. These dams were anesthetized on day 17 of pregnancy, the uterine horns were exposed, and PBS or LPS was given intra-amniotically (i.a.) (Rounioja *et al.* 2003). The newborn mice (age 12-24 h) were injected subcutaneously with PBS or an equivalent amount of LPS. The mice were exposed to LPS for five hours. The FVB pregnant and newborn mice were left untreated. All animals were killed with cervical dislocation, and the adult, newborn, and fetal tissues were harvested into liquid nitrogen for RNA and protein isolation..

4.2 Experimental cells (III)

The mouse macrophage cell line RAW 264.7 was used for *in vitro* experiments. The cells were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum, 50 IU/ml penicillin and 50 μg/ml streptomycin. The cells were or were not treated with 1 μg/ml LPS in the presence or absence of inhibitors as indicated.

4.3 RNA analyses

Total RNA of the murine tissues and cultured cells was isolated with Trizol reagent (GibcoBRL, Life Technologies Inc., Grand Island) according to the manufacturer's instructions. The tissue samples used in mRNA expression studies *in vivo* are shown in table 4.

Table 4. The samples used in mRNA expression studies in vivo. The abbreviation 'gd' refers to the gestational days of a fetus.

Mouse strain	Age	Tissue	Analysis method	Factors studied
FVB	13-19 gd	lung	RT-PCR	TLR2/4 basal
DBA/2	14, 17 gd	lung	RPA	TLR4 basal + induced + cytokines
FVB	13-19 gd	liver	RT-PCR	TLR2/4 basal
FVB	10-19 gd	placenta	RT-PCR	TLR2/4 basal
DBA/2	14, 17 gd	placenta	RPA	TLR4 basal + induced + cytokines
DBA/2	14, 17 gd	fetal membranes	RPA	TLR4 basal + induced + cytokines
DBA/2	newborn	lung	RPA	TLR4 basal + induced + cytokines
FVB	newborn	lung	RT-PCR	TLR2/4 basal
FVB	newborn	liver	RT-PCR	TLR2/4 basal
FVB	adult	lung	RT-PCR	TLR2/4 basal
DBA/2	adult	lung	RPA	TLR2/4 basal + induced + cytokines
FVB	adult	liver	RT-PCR	TLR2/4 basal
DBA/2	adult	liver	RPA	TLR2/4 basal + induced + cytokines

4.3.1 Quantitative reverse transcriptase-PCR (I)

Quantitative reverse transcriptase-PCR was carried out at the Department of Physiology in the University of Oulu.

Half a microgram of total RNA was used in each reverse transcriptase reaction. The quantitative PCRs were driven by the ABI PRISM 7700 Sequence Detection System (Perkin Elmer, Norwalk, CT). The primer sequences used for TLR2 were 5'-GCCA-CCATTTCCACGGACT-3' and 5'-GGCTTCCTCTTGGCCTGG-3', and the TaqMan probe sequence was 5'(FAM)-TGGTACCTGAGAATGATGTGGGCGTG-(TAMRA)3'.

The primer sequences used for TLR4 were 5'-CCTCTGCCTTCACTACAGAGA-CTTT-3' and 5'-TGTGGAAGCCTTCCTGGATG-3', and the TaqMan probe sequence was 5'(FAM)-CCTGGTGTAGCCATTGCTGCCAACA-(TAMRA)3'. All results were normalized to 18S rRNA.

4.3.2 Northern analysis (II)

A 10 μg sample of total RNA was resolved on 1.5% agarose-formamide gel and transferred on to Biodyne B Membrane (Pall Corporation, Ann Arbor) by capillary transfer. After overnight prehybridization, the membranes were hybridized with α^{32}P-dCTP-labelled probe overnight at 42°C. After hybridization, the filters were washed twice with 2x SSC-0.1% SDS at room temperature and once with 0.1x SSC-0.1% SDS at 60°C. The bands were quantitated with a PhosphorImager scanner. The results were normalized with 28S rRNA.

The 300 bp TLR4 probe was generated by reverse transcriptase-PCR from adult mouse lung. The primers were chosen from the extracellular domain of mouse TLR4 (GenBank AF110133). For sense primer the sequence was 5'-GCAAAGTCCC-TGATGACATT-3', and for antisense primer the sequence was 5'-CCAATAGGGAAG-CTTTCTA-3', corresponding to the nucleotides 155 to 174 and 440 to 458, respectively. PCR amplification was performed under conditions of initial 94°C denaturation for 5 minutes, 35 cycles of 15-second denaturation at 94°C, 45-second annealing at 50°C, and 45-second chain synthesis at 72°C, and final elongation at 72°C for 10 minutes. The synthesized DNA was ligated into pGEM-T easy vector (Promega, Madison) to create pmTLR4-1 for easy preparation of DNA and RNA probes. Radioactive labeling was performed with random priming.

4.3.3 Ribonuclease protection assay (II,III, IV)

10 μg of total RNA was dried in a vacuum dryer for ribonuclease protection assay (RPA), which was performed with the RiboQuant-RPA kit (Pharmingen, San Diego).

The radiolabeled antisense RNA probe was synthesized of a custom template set (Pharmingen) of various cytokines according to the manufacturer's instructions. The 303 bp TLR4 probe was synthesized from linearized plasmid pmTLR4-1. The probe synthesis reaction contained the linearized template, each NTP and α-^{32}P-UTP (Amersham Biosciences, UK), DDT, transcription buffer, RNase inhibitor, and T7 RNA polymerase (Promega). DNA was digested from the mixture after 1 h synthesis at 37°C with DNase-free RNase. The TLR4 probe was purified with PAGE-urea gel followed by further purification with phenol-chloroform extraction and ethanol precipitation. The custom template mix and the TLR4 probe were dissolved and mixed together. The precipitate was dissolved in hybridization buffer, and the probe was added to 10 μg of total RNA to be analyzed. After overnight hybridization at 56°C, the unprotected RNA was digested with RNase A and RNase T1 mixture, and the protected RNA fragments were purified with phenol extraction and ethanol precipitation. The electrophoresis was carried out on a 5 % denaturing polyacrylamide gel. The bands were quantitated with a PhosphorImager

scanner after exposure to the imaging plate. The results were normalized to the housekeeping gene L32.

4.4 Protein analyses

4.4.1 Antibodies and enzyme inhibitors

The antibodies used in these experiments are listed in table 5. The PI3-kinase inhibitors LY294002 and wortmannin (Vlahos *et al.* 1994, Nakanishi *et al.* 1995) were from Calbiochem, and the NF-κB inhibitor pyrrolidinedithiocarbamate (PDTC) was from Sigma Chemical Co.

Table 5. Antibodies used in protein analyses. IHC refers to immunohistochemistry.

Antibody	Source	Usage
Primary		
Goat anti-mouse TLR4 (L-14)	Santa Cruz Biotechnology, Inc.	IHC (II)
Goat anti-human TLR4 (C-18)	Santa Cruz Biotechnology, Inc.	Western analysis (IV)
Rabbit anti-human TLR4 (H-80)	Santa Cruz Biotechnology, Inc.	Western analysis (II)
Rabbit anti-human TLR2 (S-16)	Santa Cruz Biotechnology, Inc.	Western analysis, IHC (II)
Mouse anti-human PI3K	Transduction Laboratories	Western analysis (III)
Rabbit anti-mouse Akt	Cell signalling Technology, Inc.	Western analysis (III)
Rabbit anti-mouse phospho-Akt (Ser473)	Cell signalling Technology, Inc.	Western analysis (III)
Goat anti-mouse NF-κB p50 (C-19)	Santa Cruz Biotechnology, Inc.	EMSA (III)
Goat anti-mouse NF-κB p65 (C-20)	Santa Cruz Biotechnology, Inc.	IHC (II, III), EMSA (III)
Rabbit anti-human MyD88	Santa Cruz Biotechnology, Inc.	Immunoprecipitation, Western analysis (III)
Secondary		
HRP-goat anti-rabbit IgG	Santa Cruz Biotechnology, Inc.	Western analysis (II)
HRP-donkey anti-goat IgG	Santa Cruz Biotechnology, Inc.	Western analysis (IV)
HRP-rabbit anti-mouse IgG	Biorad	Western analysis (III)
Biotin-donkey anti-goat IgG-B	Santa Cruz Biotechnology, Inc.	IHC (II, III)

4.4.2 Protein isolation and preparation of cell lysates (II, III, IV)

To isolate the total protein from the tissues, the samples were lysed in extraction buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS, 0.1 % Na-deoxycholate, 50 mM NaF, 1 mM PMSF containing 0.1 units/ml aprotinin and 10 μg/ml leupeptin) followed by centrifugation at low speed. For cell lysates, the extraction buffer contained 1 mM Na_3VO_4 and 5 mM EDTA, and the centrifugation was performed at high speed at 4º C. The protein contents were quantitated by Bio-Rad DC Protein Assay.

4.4.3 Immunoprecipitation (III)

The supernatant from the cell lysate was incubated with antibody on ice for 1 hour. The antigen-antibody complex was precipitated with 50 % Gammabind-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) at constant agitation for 2 hours at +4°C. The precipitates were washed with wash buffer (lysis buffer without detergents) and collected by centrifugation.

4.4.4 Affinity association using GST fusion proteins or glutathione-Sepharose (III)

The GST fusion proteins containing the Src homology (SH)-2 or SH3 domains of PI3-kinase were expressed in *E. coli* and purified. SH2 binds proteins at the phosphotyrosine site, and SH3 binds proline-rich domains. Cell lysates were incubated with 10 μg of GST alone or GST fusion proteins, which had been immobilized on glutathione-Sepharose beads. Protein complexes were allowed to associate for 4 h at 4 ºC, followed by centrifugation and washes with lysis buffer. Complexes were analyzed by SDS-polyacrylamide gel (SDS-PAGE) and immunoblotting.

4.4.5 Western analysis (II, III, IV)

A 10 or 50 μg sample of tissue protein and a 20 μg sample of cell protein were boiled in SDS sample buffer for 5 min and resolved in SDS-PAGE. The proteins were elecrotransferred onto a Protran BA85 nitrocellulose filter (Schleicher & Schuell, Dassel, Germany). The membranes were blocked at 4°C overnight in 5% milk powder-TBST (or TBS containing 5 % BSA for PI3K antibody) and incubated with primary antibody in TBST for one hour at room temperature followed by washes with TBST and incubation with HRP-conjugated secondary antibody. The blot was developed with the ECL system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

4.4.6 Immunohistochemistry (II, III)

The formalin-fixed and paraffin-embedded tissues were cut into 5 μm sections. The sections were deparaffinized and rehydrated. Target retrieval was carried out with the citrate method. After blocking of endogenous peroxidase activity with 3 % H_2O_2, the samples were blocked with appropriate serum in 0.1% BSA/PBS followed by incubation with the primary antibody diluted in BSA/PBS for one hour. After rinses in PBS, the samples were incubated with biotin-conjugated secondary antibody for 30 minutes. Detection was carried out by the avidin-biotin method and AEC staining (detection kit by Dako, Glostrup, Denmark) according to the manufacturer's instructions. The detection of NF-κB in the liver (II) was carried out in the presence of 0.4 % Triton X-100.

4.5 Nuclear extraction and electrophoretic mobility shift assay (EMSA) (II, III)

Nuclear extracts were prepared by harvesting cells in PBS and centrifuging and resuspending them in ice-cold hypotonic buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DDT, 0.5 mM PMSF containing 10 μg/ml pepstatin, aprotinin, and leupeptin). The cells were incubated on ice for 15 min, Nonidet P-40 was added, and the sample was centrifuged at 8000x g for 15 min after vigorous vortexing. The nuclear pellet was resuspended in nuclear extraction buffer (20 mM Hepes, pH 7.9, 0,4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DDT, 0.5 mM PMSF containing 10 μg/ml pepstatin, aprotinin, and leupeptin), incubated on ice for 30 min, and centrifuged at 12000x g for 15 min. The protein content of the supernatants was determined as before.

The NF-κB binding site containing oligonucleotide probe for EMSA (5'-AGTTGAGGGGACTTTCCCAGGC-3', Promega) was end-labeled with γ^{32}P-ATP. The probe was incubated with nuclear protein in binding buffer (10 mM Tris, pH 7.5, 1 mM EDTA, % mM $MgCl_2$, 5 % glycerol 5 % sucrose, 0.1 mM DDT, and 0.01 % Nonidet P-40) for specific binding of DNA and protein. Specificity was determined by competition with 50-fold excess of the unlabeled NF-κB oligonucleotide. The sample was electrophoresed in 7 % polyacrylamide gel. The supershift analysis was done by incubating the nuclear extracts with antibodies specific for p50 or p65 subunits of NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA). The intensities of the bands were quantitated by PhosphorImager.

4.6 PI3-kinase assay (III)

The cells were stimulated with LPS in the presence or absence of PI3K inhibitors and lysed in Nonidet P-40/Triton X-100 buffer (20 mM Tris pH 7.5, 145 mM NaCl, 10 % glycerol, 0.5 % NP-40, 1 % Triton X-100, 5 mM EDTA and phosphatase and protease inhibitors). Immunoprecipitations were carried out with anti-MyD88 antibody or anti-PI3K antibodies, as indicated. PI3K activity of the precipitated complexes was measured according to (Whitman *et al.* 1988).

4.7 Transfection and reporter gene analysis (III)

Transient transfections were performed using the FuGENE 6 reagent (Roche Molecular Biochemicals). pNF-κB-luciferase plasmid was co-transfected with pSV-β-galactosidase control plasmid. After 24 h, the cells were treated with LPS in the presence or absence of inhibitors. Non-transfected cells and transfected but untreated cells were used as controls. Cell lysates were prepared and assayed for luciferase and β-galactosidase activity, and luciferase activity was normalized to β-galactosidase activity.

5 Results

5.1 Preliminary studies

5.1.1 In vivo studies (I, II, IV)

To evaluate the LPS responsiveness of experimental animals, PBS or LPS-treated DBA/2 strain mice were monitored for clinical signs of inflammation. Four hours after the LPS challenge, the mice developed symptoms of fever, ruffled hair, and lethargy. Their serum IL-6 levels rose after the LPS challenge. However, there were no deaths, spontaneous abortions, or fetal deaths within six hours. In adult experiments, the acute responsiveness to different doses of LPS was evaluated at 2, 5 and 24 hours. A LPS dose of 2.4 mg/kg and a time of five hours were chosen for fetal experiments to establish a primary inflammatory response, minimizing the secondary maternal response likely to interfere with the intrauterine response. The results were obtained with the DBA/2 mouse strain. The ontogeny of TLR2 and TLR4 expression during the perinatal period and in newborn and adult FVB mice was also studied.

5.1.2 In vitro studies (III)

To evaluate the LPS responsiveness of experimental cells, the TLR4-expressing mouse macrophage cell line RAW 246.7 was studied for acute production of proinflammatory cytokines and other mediators. LPS treatment for 4 hours at a dose of 1 μg/ml resulted in increased mRNAs of IL-1α, IL-1β, TNF-α, and MIP-2. The activation and involvement of PI3-kinase in the LPS-induced inflammatory response was confirmed by evaluating the capacity of LPS to activate a PI3K-dependent downstream mediator Akt. A rapid increase in Akt serine 473 phosphorylation was detected as a sign of the activation of Akt in RAW 246.7 cells. Phosphorylation was blocked by the PI3K inhibitors LY294002 and wortmannin.

5.2 TLR4 and LPS responsiveness in adult

5.2.1 Animal model

5.2.1.1 Basal TLR4 and TLR2 mRNA expression and protein production (I, II)

In DBA/2 adult mice, as detected by Northern analysis, TLR4 mRNA was more abundant in lung but barely detectable in liver. The TLR4 protein was detected both in lung and in liver by Western blotting. Immunohistochemical staining located the pulmonary TLR4 protein basolaterally in bronchiolar epithelium. Hepatic staining was detected in the cytoplasm of perisinusoidal cells and hepatocytes.

RT-PCR studies with FVB adult mice showed an opposite pattern in the expression of TLR4 mRNA, with higher expression in liver and lower in lung.

The expression of TLR2 was also studied in DBA/2 adult lung and liver by Northern analysis. Basal TLR2 mRNA expression was low in both tissues. However, the TLR2 protein was clearly detectable in Western blot.

The expression level of TLR2 mRNA in FVB mouse lung and liver was similar to that of TLR4, being high in liver and lower in lung.

5.2.1.2 Responsiveness to LPS (II)

Detected by Northern blot in the DBA/2 mouse strain, LPS upregulated TLR4 mRNA dose-dependently in mouse lung, the induction being 1.3 to 2.1-fold at 1.2 and 2.4 mg/kg LPS, respectively. The protein levels after the LPS challenge reflected the RNA levels. The liver TLR4 mRNA showed no induction by LPS challenge, but the protein production showed a slight increase against LPS.

Interestingly, the expression of TLR2 mRNA in both lung and liver was robustly and dose-dependently increased during the LPS challenge.

To study the association between TLR expression and the tissue-specific response of cytokines/chemokines previously reported to be involved in LPS/TLR4 signaling, the mRNA expression of the proinflammatory cytokines IL-1α, IL-1β, IL-6, IL-18, TNF-α, and MIF, the chemokine MIP-2, the anti-inflammatory cytokine IL-10, and the cytokine receptor IL-1RI were studied by RPA. The response was studied at 2, 5, and 24 hours after the challenge.

In adult lung, a strong cytokine response developed after the LPS challenge. The mRNA expressions of IL-1α, IL-1β, TNF-α, IL-6, IL-18, MIF, and MIP-2 were upregulated, but the response peaked first at five hours after the LPS challenge. The cytokines were barely detectable at two hours, and within 24 hours the expression levels had nearly reached the baseline. The expression levels of IL-10 and IL-1RI were not affected, however, and these mediators also showed a slight increase in the lungs of pregnant mice. In contrast, the hepatic response was remarkable at two hours, when the expression levels of IL-1α, IL-1β, TNF-α, IL-18, and MIP-2 were increased, the

induction remaining after five hours. IL-10, MIF, and IL-1R1 were upregulated at five hours after the LPS challenge. The hepatic response also nearly reached the baseline after 24 hours of challenge. A summary of the basal and LPS-induced expressions of TLR4 mRNA in the DBA/2 mouse strain and the association with the acute cytokine response to LPS are shown in table 6a.

NF-κB nuclear translocation and DNA binding during the LPS challenge were associated with an induction of the cytokine response both in lung and in liver, as measured by gel mobility shift assays and immunohistochemistry. Following PBS treatment, only cytoplasmic NF-κB was evident. NF-κB turned out to be translocated to the nuclei of hepatocytes and Kupffer cells after the challenge with LPS. The nuclear extracts from PBS-treated mice showed only low levels of NF-κB DNA-binding activity, and after the LPS challenge, the marked enhancement in NF-κB DNA-binding activity was measured both in lung and in liver.

Table 6a. A simplified summary of the basal and LPS-induced expression of TLR4 mRNA and the correlation with the acute cytokine response to LPS in adut and newborn tissues of the DBA/2 mouse strain measured by RPA.[1]

	Adult and dam			Newborn		
	TLR4 basal	TLR4 induced	Cytokine response	TLR4 basal	TLR4 induced	Cytokine response
Lung	+	++	++	+	+	+(+)
Liver	–	–	+			

Table 6b. A simplified summary of the basal and LPS-induced expression of TLR4 mRNA and the correlation with the acute cytokine response to LPS in fetal tissues of the DBA/2 mouse strain measured by RPA.[1]

	TLR4 basal		TLR4 induced		Cytokine response	
	14 days	17 days	14 days	17 days	14 days	17 days
Lung	–	+	–	+	–	+
Liver	–	–	–	–	–	–
Placenta (i.a.)		+		+		++
Fetal membranes (i.a.)		++		++(+)		+++

[1] Marks: – indicates no detectable TLR4 expression or cytokine response, + indicates weak or moderate TLR4 expression or cytokine response, and ++ indicates intense TLR4 expression or cytokine response. Blank means 'not tested'.

5.2.2 Cell model (III)

5.2.2.1 Role of PI3-kinase in LPS-induced cytokine production

The proximal signaling mechanisms leading to gene expression of inflammatory mediators are incompletely defined. The possible role of PI3K in the LPS-induced production of the inflammatory mediators IL-1β, TNF-α, and MIP-2 was studied using the PI3K inhibitors LY294002 and wortmannin. Only the mRNA expression and protein production of IL-1β was blocked, whereas no inhibitory effect was detected in LPS-induced TNF-α and MIP-2 production.

5.2.2.2 TLR4 aggregate during LPS challenge

MyD88 is considered obligatory for LPS-induced cytokine production, and it possesses a putative PI3K-binding site YKAM (amino acids 257-260). MyD88 precipitated with PI3K, and the amount of this change was rapidly but transiently increased by the LPS challenge. When immunoprecipitating TLR4, transient co-precipitation of PI3K could be detected during the LPS challenge. Further studies showed that the SH2 domain of PI3 kinase bound to MyD88 in lysates of RAW 264.7 cells, the amount of bound MyD88 being increased by the LPS challenge. The enzymatic activity of PI3K was low in MyD88 immunoprecipitates from untreated cells, but markedly increased following the LPS challenge.

5.2.2.3 Role of NF-κB in PI3-kinase-mediated cytokine production

The studies on the role of NF-κB activation in LPS-induced expression of IL-1β, TNF-α, and MIP-2 by RPA revealed that the NF-κB inhibitor PDTC blocked IL-1β and TNF-α mRNA production, but had no effect on MIP-2 production.

Untreated cells showed low levels of NF-κB DNA-binding activity. LPS robustly enhanced NF-κB nuclear translocation and DNA binding, but neither of the PI3K inhibitors were able to block the response measured by gel mobility shift assay. Treatment of cells with the NF-κB inhibitor PDTC reduced LPS-induced DNA binding to the level of untreated cells. Repoter studies on PI3K involvement in regulating the transactivation of NF-κB revealed that the PI3K inhibitors LY294002 and wortmannin resulted in 51 % and 14 % reductions of NF-κB promoter activity, respectively, whilst without these inhibitors, the induction of NF-κB promoter activity was over threefold after the LPS challenge.

5.3 TLR4 and LPS responsiveness during the perinatal period

5.3.1 Basal TLR4 and TLR2 mRNA expression and protein production (I, IV)

The two different mouse strains studied, DBA/2 (studied with RPA) and FVB (studied with RT-PCR), showed different TLR4 mRNA basal expression patterns. In FVB mice, pulmonary TLR4 mRNA was low at the fetal stage and increased toward birth. Hepatic TLR4 mRNA expression showed no developmental trends and was high compared to pulmonary expression. Placental expression was extremely high at early pregnancy (days 11-12), decreasing toward term.

In DBA/2 mice, fetal lung and liver did not show any basal expression of TLR4 mRNA at 14 days of gestation. At 17 days of gestation, fetal lung showed clear basal TLR4 mRNA expression, as did also neonatal lung. The mRNA and protein expression levels of TLR4 in liver remained barely detectable at 17 days of gestation.

In placental tissue and fetal membranes, basal expression of TLR4 mRNA and the protein were evident at 17 days of gestation.

The basal TLR2 mRNA expression in fetal life was studied with the FVB mouse strain. The expression in lung and liver was very similar to the expression of TLR4, being low in fetal lung and increasing toward birth, while remaining strong and constant in liver. The placental TLR2 mRNA expression was low compared to TLR4 expression, and no significant trends were evident during gestation.

5.3.2 Responsiveness to LPS (IV)

At 14 and 17 days of gestation and after birth, no induction in the expression of TLR4 by LPS was evident in the lung or liver of DBA/2 mice. The LPS responsiveness of placenta and fetal membranes was dependent on the site of LPS administration. LPS exposure did not increase the placental expression of TLR4 in either i.a. or i.p. challenged mice, but the expression of TLR4 in fetal membranes was increased during the i.a. LPS challenge, although no acute increase was detectable in the protein content. The i.p. LPS challenge had no detectable effect on the expression of TLR4.

To study the association between TLR expression and the tissue-specific response of the cytokines/chemokines previously reported to be involved in LPS/TLR4 signaling, the mRNA expressions of the proinflammatory cytokines IL-1α, IL-1β, TNF-α, and IL-6, the chemokine MIP-2, the anti-inflammatory cytokine IL-10, and the cytokine receptor IL-1RI were studied five hours after the LPS challenge by RPA. In very premature, 14-day-old fetal lung, neither any expression nor any change in the expression of the inflammatory mediators were detected after maternal LPS. 14-day-old fetal liver expressed low, but non-inducible levels of IL-1α, TNF-α, and IL-1RI. In contrast to the 14-day-old fetus, 17-day-old fetal lung showed occasional responses to maternal LPS, as demonstrated by the induction of the expression of the proinflammatory cytokines and chemokines IL-1α, IL-1β, IL-6, TNF-α, and MIP-2. However, i.a. LPS failed to activate

the fetal innate immune response. Neonatal lung expressed a similar pattern as adult lung. 17-day-old fetal liver showed no significant increase of inflammatory mediators consistent with the lack of the signaling receptor to LPS.

Placental tissue and fetal membranes induced or increased the expression of several inflammatory mediators in response to LPS. Placental tissue expressed basal levels of IL-1α, TNF-α, and IL-1RI. Intraperitoneal LPS increased the expression of IL-1α, IL-1β, MIP-2, and TNF-α. No IL-6 or IL-10 was evident. Intra-amniotic LPS increased more robustly the expression of IL-1β and MIP-2, and the levels of IL-6 and IL-10 expression were also induced. In fetal membranes, i.p. administration had a minor effect on the cytokine expression levels. On the contrary, the intra-amniotic LPS challenge resulted in strong upregulation of IL-1α, IL-1β, TNF-α, IL-6, and MIP-2, and IL-10 was also slightly increased. Only IL-1RI was constitutional and non-inducible.

The basal and LPS-induced expression of TLR4 mRNA of the DBA/2 mouse strain and the association with the acute cytokine response to LPS are shown in table 6b.

6 Discussion

Multiple bacterial infections lead to inflammation and activation of the innate immune system. Inappropriate activation and function of the innate immune system may cause deleterious outcomes. A lack of response leads to overwhelming bacterial infection (Janeway, Jr. & Medzhitov 1998, Zhang & Ghosh 2001, Hallman *et al.* 2001). Excessive activation leads to overproduction of diverse inflammatory mediators, which may be followed by septic shock and is a likely cause of chronic diseases in various organs (Schletter *et al.* 1995b, Hallman *et al.* 2001). For example, respiratory failure remains a common cause of sepsis-related deaths. Septic multi-organ failures include cardiac failure and brain damage in addition to lung damage (Fenton & Golenbock 1998, Knuefermann *et al.* 2002, Hagberg *et al.* 2002). In addition, an excessive cytokine response has been proposed to be a major cause of spontaneous premature deliveries and life-threatening diseases in premature infants (Romero *et al.* 1989).

It would be beneficial to eliminate bacteria without the activation of a pattern recognition receptor, since the activation unavoidably causes the host to feel sick. However, this is far from being always possible. Regulation of the machinery starting from the bacterial agent that leads to cytokine production would give an enormous opportunity to avoid overwhelming bacterial infections and problems associated with inappropriate cytokine responses. Total blockade of the inflammatory response would not be appropriate, since some response by the individual is needed for survival through bacterial invasion. Uncontrolled function of the innate immune system is also very harmful. To manipulate the innate immune system from outside the host requires good candidates involved in the signaling machinery. In this study, the principal signaling receptor for Gram negative bacterial LPS, TLR4, and another signaling receptor, TLR2, were studied for their functional activation in the presence of LPS, as possible rate-limiting factors involved in normal and pathological regulation of the cytokine cascade.

6.1 Basal expression of TLR4 and TLR2

We studied the basal mRNA expression of TLR4 and TLR2 with two different mouse strains, FVB and DBA/2. We found strain-specific differences in all tissues studied.

TLR4 expression was lower in FVB lung than in liver both at fetal age and in adults. In contrast, TLR4 expression in DBA/2 lung was high compared to the almost non-detectable expression in liver. However, the developmental trends in both strains were similar. Pulmonary TLR4 expression was low during the perinatal period, increased toward term, and increased further toward adulthood. Hepatic TLR4 expression showed no developmental trends. The expression of TLR4 in the placenta of FVB mice was extremely high during the early stages of pregnancy, decreasing toward term. The placental expression of TLR4 of DBA/2 mice showed no developmental trends.

The expression of TLR2 in FVB adult mice was similar to that of TLR4. The level of expression was higher in liver than in lung. In contrast, the pulmonary expression of TLR2 in DBA/2 mice was higher than hepatic expression, although both expressions were very low.

These results indicate that the regulation of the expression of TLR4 and TLR2 is, besides tissue-specific, also strain-specific. Differential expression of TLRs is likely to affect the susceptibility to infections and also to non-infectious diseases that involve TLRs (Wells *et al.* 2003, Lehnardt *et al.* 2003). Studies of the LPS responsiveness of different mouse strains have been reported (Lorenz *et al.* 2001, Wells *et al.* 2003). LPS responsiveness measured by the number, amplitude, and rate of induction of genes expressed varied greatly between the strains studied. This implies that there are differences in the regulation of TLR4 expression. Although the TLR4 locus is clearly important in determining LPS sensitivity, many additional genetic loci are likely to control the extent and nature of the LPS response. The meaning and relevance of different TLR expression profiles and the differences in LPS responsiveness remain to be studied.

6.2 Association between TLR4 expression and acute inflammatory response

Naturally occurring downregulative mechanisms of the innate immune response have been reported (for references, see (Fukao & Koyasu 2003). IL-1-associated kinase-M (IRAK-M) is inducible upon TLR activation and regulates TLR signaling. The suppressor of cytokine signaling-1 (SOCS-1) is also a negative regulator of TLR signaling, althoug its induction occurs only through TLR. Endotoxin tolerance, including downregulation of the TLR4-MD-2 receptor complex, and the limited activation of NF-kB are another means to avoid overexpression of inflammatory mediators. TLR4 has been shown to be a limiting factor of the LPS responsiveness of mouse macrophages (Du *et al.* 1999). According to our studies, there are three patterns of TLR4 expression and the acute cytokine response.

1. TLR4 is basally expressed and upregulated during a LPS challenge. This results in activation of the signaling cascade and acute production of inflammatory mediators. Both adult lung and fetal membranes showed this pattern. The finding of LPS-inducible TLR4 in lung is consistent with the previous results (Matsumura *et al.* 2000). Although this pattern is the most obvious and likely to function in a controlled and appropriate way, inducible TLR4 expression may increase the susceptibility to severe toxicity during LPS exposure. A recent study shows that an early increase in TLR4 (and also TLR2) mRNA

and protein expression correlated with mortality, whereas blunting of the expression correlated with improved long-term survival (Williams *et al.* 2003).

The lung possesses a unique innate immune system. The surfactant proteins SP-A and SP-D participate in pulmonary host defence as phagocytosis stimulants, agglutinins, opsonins, and immunomodulators, and they directly bind LPS (Kuan *et al.* 1992, Kalina *et al.* 1995). SP-A has been shown to have both up- and downregulative effects on cytokine secretion, which both involve TLR4. SP-A-deficient mice produce more proinflammatory cytokines than wild-type mice after treatment with LPS (Borron *et al.* 2000). This downregulation involves complex formation of SP-A with CD14, TLR4, and MD-2 followed by inhibition of LPS binding and cellular responses (Sano *et al.* 1999, Sano *et al.* 2000). On the other hand, other *in vitro* data show that SP-A directly enhances the secretion of proinflammatory cytokines in a TLR4-dependent manner (Guillot *et al.* 2002). This suggests that SPs and TLRs may act together to regulate the cellular responses to microbial pathogens in lung. Alternatively, SPs regulate the activation or functioning of TLRs by binding directly to TLR or by binding LPS, CD14, or other members of the TLR-signaling complex.

2. TLR4 is basally expressed, but the expression levels do not increase during a LPS challenge. However, an acute cytokine response is evident. This situation was seen in adult liver, in placenta, and in newborn and 17-day fetal lung. The non-inducibility of TLR4 may serve as a regulator to moderate the inflammatory response to LPS, since the levels of TLR4 are likely to limit the acute response (Kalis *et al.* 2003).

Our finding of non-inducible hepatic TLR4 is consistent with the previous reports on murine hepatocytes (Matsumura *et al.* 2000), showing that LPS did not induce TLR4 *in vivo* or *in vitro*, and of cultured human hepatic stellate cells (Paik *et al.* 2003), showing that LPS stimulation for 6 hours did not affect the expression of CD14 and TLR4. The regulation of the acute inflammatory response in liver appears to be appropriate. The liver is considered a key organ in the host defence against pathogens. Various liver cells are involved in antigen processing and in the induction of the immune response (Blomhoff *et al.* 1984, Magnusson & Berg 1989). The liver has an important protective role in the defence against gut-derived Gram negative bacteria. It receives blood from the portal vein, which drains the intestinal tract, containing LPS from intestinal bacteria. The inflammatory reactions of the liver need to be well controlled, as otherwise the continuous flow of bacteria and LPS would cause an overwhelming and continuous cytokine response. Hepatic TLR4 expression and cytokine production may be diminished due to the massive LPS exposure. Vice versa, paucity of intestinal LPS may lead to hepatic proinflammatory activation during an acute LPS challenge.

3. TLR4 is not detectably expressed basally, the expression is not acutely induced during an LPS challenge, and no acute cytokine response is evident. In our experiments, this situation was found only in very immature fetal lung and in fetal liver. The non-responsiveness may protect the tissues against toxic inflammatory mediators. However, the paucity of the beneficial components of the inflammatory response is likely to result in serious consequences, including susceptibility to infections.

According to the present studies, there was a rough correlation between the expression and inducibility of TLR4 and the extent of cytokine production. The evidence shown is not strictly quantitative, however. The tissues with basally expressed and inducible TLR4 (adult lung and the fetal membranes) generally showed the most drastic increase and

inducibility of cytokine production. Cytokine production in adult liver and in placenta with basally but not inducibly expressed TLR4 was clearly detectable, but lower than in the first case. In fetal liver and very immature lung, where no TLR4 was evident even after a LPS challenge, no cytokine response remained detectable. These findings are in accordance with the hypothesis that TLR4 is a potent modulator of the LPS response.

Although TLR4 is expressed in cells of many kinds, the abundance of TLR4 relates to the amount of responsive cells (e.g. monocytes and macrophages) in tissue, which is naturally also related to the power of the innate immune response involving proinflammatory cytokines (Hoshino *et al.* 1999, Nomura *et al.* 2000). Kupffer cells, which are located mainly in the periportal area of the liver, comprise approximately 70% of the total macrophage content of the body effectively removing Gram negative bacteria and LPS from the systemic circulation (Mathison & Ulevitch 1979, Katz *et al.* 1991). Isolated Kupffer cells have also been shown to be able to produce inflammatory cytokines and to modulate the host reaction against LPS, and recent *in vitro* studies suggest that TLR4 is necessary for the response (Feder *et al.* 1993, Su *et al.* 2000, Seki *et al.* 2001). However, our studies showed that this response may be restricted, as the expression of TLR4 was not induced by LPS. The macrophage content of the placenta is also very high and increases during inflammation (Hunt 1989). Our studies show a limiting factor in the placental cytokine response, too. TLR4 is constantly expressed in placenta, and the expression levels did not increase during inflammation despite the increased amount of macrophages. This may serve as a factor limiting excessive cytokine production during endotoxemia. The lung macrophage content is less abundant, but increases during inflammation from the bloodstream. In these tissues, the upregulated level of TLR4 expression correlates with the amount of macrophages and the acute inflammatory response. The present finding further supports the role of TLR4 as a limiting factor for the cytokine response. The most drastic negative feedback appears to be evident in the tissues with the highest macrophage contents.

6.3 Modulation of the LPS-signaling pathway

6.3.1 Role of PI3-kinase

We examined the possibility of an alternative signaling cascade for LPS. Phosphatidylinositol 3-kinase (PI3K) is an enzyme implicated in host defence and inflammation in macrophages (Leevers *et al.* 1999). LPS has been shown to induce activation of PI3K and its downstream mediator Akt, and PI3K has also been shown to participate in the signaling pathway that leads to NF-κB-dependent gene transcription in macrophages (Herrera-Velit & Reiner 1996, Diaz-Guerra *et al.* 1999). TLR2 and IL-1R have been shown to potentially use PI3K in their signaling, and TLR2 binds directly to PI3K (Reddy *et al.* 1997, Arbibe *et al.* 2000). PI3K activation is triggered by TLR2, TLR4, and TLR9 in various cell lines, in which PI3K expresses pro- or anti-inflammatory actions (for references, see Fukao & Koyasu 2003). Using mouse macrophage RAW 264.7 cells *in vitro*, we detected an association between PI3K, MyD88, and TLR4 suggesting that TLR4 utilizes PI3K for downstream signaling. TLR4 lacks any PI3K-

binding motif in the intracellular domain, but the TLR adapter MyD88 possesses a putative p85-binding site. As MyD88 associates with TLR4 on the cell surface, it may also serve as an adapter to recruit PI3-kinase to activated TLR4 (figure 3).

Our study showed that PI3K inhibition selectively removed the induction of IL-1β, whereas the induction of TNF-α and MIP-2 remained unaffected. PI3K inhibition reduced the NF-κB promoter activity, and the nuclear translocation and DNA binding of NF-κB were not sufficient for effective IL-1β production. Further, the inhibition of the nuclear translocation of NF-κB blocked the induction of IL-1β as well as TNF-α, whereas MIP-2 was unaffected. These observations suggest that the induction of different cytokines may result in differential transcriptional regulation mediated by PI3K, and that regulation of the promoter activity of NF-κB is more probable than regulation of nuclear translocation or DNA binding. Based on these results, the quality of the protein aggregate formed by TLR4 on the cell surface controls the quality of the acute cytokine response.

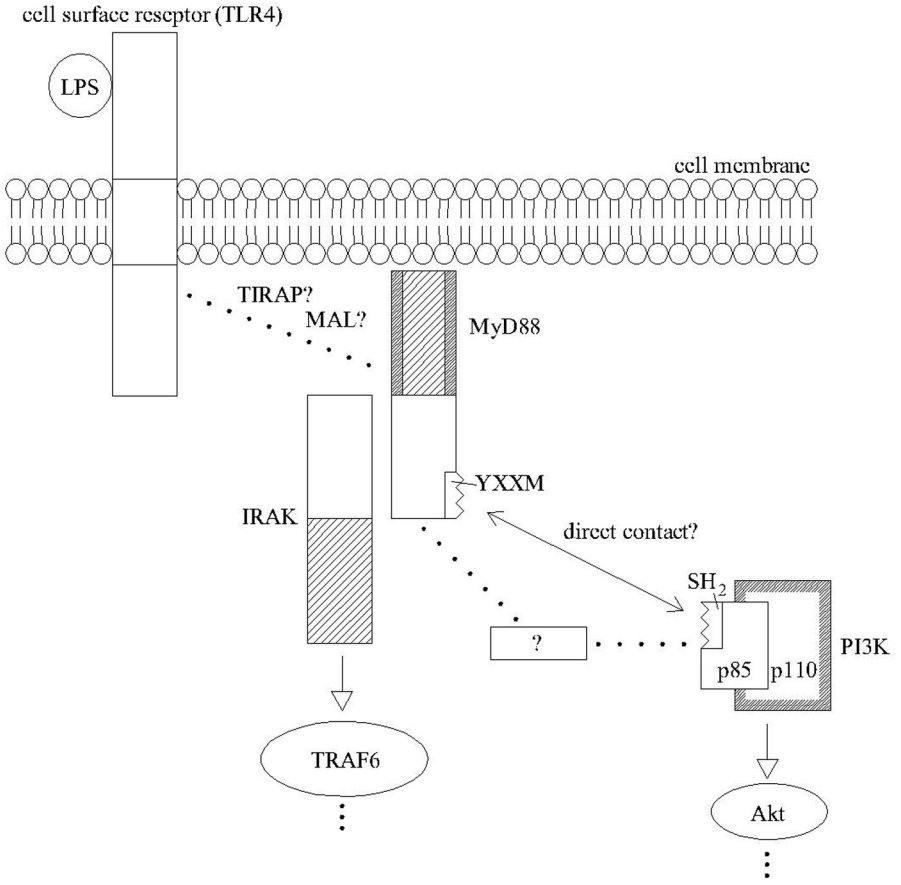

Fig. 3. A schematic presentation of the LPS-signaling cascade involving PI3K.

6.3.2 Role of TLR2

TLR2 is considered to signal the presence of Gram positive bacteria components, fungi, yeast, mycobacteria, and parasitic protozoa (Yoshimura *et al.* 1999, Underhill *et al.* 1999a, Underhill *et al.* 1999b, Campos *et al.* 2001). The signaling mechanism of TLR2 is unclear, although evidence of a Rac-1-dependent pathway has been published (Arbibe *et al.* 2000). We studied TLR2 expression as a response to LPS in the lung and liver of an adult mouse and found a robust and dose-dependent increase in TLR2 mRNA. This phenomenon has been observed earlier in lung, liver, and skeletal muscle (Matsumura *et al.* 2000, Lang *et al.* 2003), which suggests that the LPS-induced up-regulation of TLR2 is mediated via TLR4-dependent activation of NF-κB. A recent study hypothesized that TLR2 expression upregulated in alveolar macrophages following bacterial respiratory infections (LPS) may render the lung responsive to TLR2 ligands or may contribute to the accelerated macrophage response seen at subsequent stages of infection, both of which may enhance innate immunity against pathogens in lung (Oshikawa & Sugiyama 2003). In liver, we detected *in vivo* activation of NK-κB in hepatocytes and Kupffer cells, which is consistent with the strong upregulation of TLR2. It is possible that, after its own activation, TLR4 promotes the activation of other TLRs and thus improves the host's resistance against pathogens. In fact, it has been shown in adipocytes that activation of TLR4 with LPS also results in induction of TLR2 synthesis (Lin *et al.* 2000).

6.4 Perinatal development of LPS responsiveness

Diseases in infants often involve infection and inflammation. Part of the events leading to susceptibility to damage often take place in the perinatal period already. Premature infants have a high risk of developing chronic disease, particularly chronic lung disease or brain damage, before full development of their adaptive immunity (Jobe & Ikegami 1998, Speer 1999, Hallman *et al.* 2001). Prematurity is strongly associated with intrauterine infections or the intrauterine inflammatory syndrome. Among the predisposing conditions, urinary tract infections and infections of the abdominal organs are often caused by Gram negative bacteria. In addition, the endotoxin levels in amniotic fluid are associated with the onset of premature birth (Romero *et al.* 1989). High levels of cytokines in amniotic fluid and in cord blood at premature birth have also been associated with serious chronic diseases involving the lung (bronchopulmonary dysplasia, BPD) (Jobe & Ikegami 2001), the central nervous system (periventricular leukomalasia and cerebral palsy) (Yoon *et al.* 2003), and the intestine (necrotizing enterocolitis) (Hitti *et al.* 2001).

We administrated LPS i.p. to the dam or directly into amniotic fluid. We detected inflammatory signaling across the feto-placental compartment in both cases. The fetal membranes possessed a much stronger cytokine response against i.a. compared to i.p. LPS. In theory, the placenta and the fetal membranes could serve as barriers eliminating LPS from the maternal source. This could protect the fetus from the harmful effects of the activation of the innate immune system and the production of inflammatory mediators. The cytokines generated by the fetal membranes and the placenta in intrauterine infection

have been implicated in the induction of prostaglandin synthesis and other mediators required for the onset and progression of labor, and thus as likely causes of premature labor and birth (Hunt 1989, Romero *et al.* 1998).

17-day-old fetal lung responded poorly to maternal LPS, and very immature lung from day 14 showed no acute response. However, we often failed to elicit any pulmonary response against i.a. LPS even on day 17. This may be due to the lack of fetal breathing movements during anesthesia, or the time window required for the activation of the immune response is longer than that used here. The fact that very premature ovine and human fetuses develop a prominent cytokine response to toxins from microbes (Watterberg *et al.* 1996, Kramer *et al.* 2001) may also indicate species-specific differences in the ontogeny of the innate response.

According to the present study, TLR4 is an important modulator of the degree of LPS responsiveness during the perinatal period. The basal expression of TLR4 appeared in fetal lung after day 14 of gestation (measured on day 17), but LPS did not influence the level of expression. In accordance with undetectable TLR4 expression in very immature fetal lung, no induced cytokine production was detected, whereas 17-day-old lung responded slightly to the LPS challenge by producing proinflammatory cytokines. Newborn lung showed non-inducible TLR4 expression and LPS responsiveness similar to that seen in adult lung. It seems that the acute responsiveness of the innate immune system develops during the antenatal differentiation of the lung.

In premature newborn infants developing BPD, the cytokine response is abnormally high, suggesting dysregulation of the innate immune response (Jobe & Bancalari 2001). Development of BPD also involves a lack of anti-inflammatory cytokine IL-10 in lung (Jones *et al.* 1996). We detected the paucity of IL-10 in fetal and placental tissues in contrast to the induction of IL-10 in adult lung as a response to LPS. This may be due to inducible TLR.

As in adult liver, the basal TLR4 expression in fetal liver was unaffected by the LPS challenge or by gestational age. There was no detectable increase in the production of inflammatory mediators in fetal liver after the LPS. According to previous evidence, LPS downregulates TLR4 expression in fetus. Human fetal intestine has been shown to express LPS-downregulative TLR4 at mid-pregnancy, whereas IL-1β upregulated this expression (Fusunyan *et al.* 2001). In our study, no IL-1β production after LPS stimulation was detected. Thus, a negative feedback system may prevent the upregulation of TLR4 and the response to LPS.

The regulation of the development of TLR4 expression and LPS responsiveness in the perinatal period remains to be studied. The number of growth factors, including GM-CSF, increase strongly during the perinatal period and are involved in the functional regulation of alveolar macrophages (Bry *et al.* 1997, Trapnell & Whitsett 2002). They may be potent regulators of TLR (Mita *et al.* 2001). It is also possible that the lack of other factors result in TLR non-activation. TLR4 fails to be properly processed and does not bind to the cell membrane without MD-2. First, the development of functional MD-2 may activate TLR4 (Nagai *et al.* 2002, Ohnishi *et al.* 2003).

6.5 Future prospects

According to the present study, the levels of TLR4 expression influence the intensity of the innate immune response. Thus, TLR4 may prove to be a possible target for new therapies that modulate the generation and strength of the inflammatory response and thereby influence the pathogenesis of several sepsis-related diseases. Neurodegeneration has been shown to follow the activation of TLR4, and animals bearing a loss-of-function mutation on the TLR4 gene are resistant to neuronal injury in the same model (Lehnardt *et al.* 2003). Cardiac failure as a result of endotoxin shock is likely to be mediated by TLR. Again, this may be prevented by appropriate regulation of TLR (Knuefermann *et al.* 2002). However, there are a number of issues that remain challenging:

1) A recent study of mice shows that overexpression of TLR4 at an early stage of infection gives a survival advantage, but this advantage was not maintained later during the infection, because mice overexpressing TLR4 developed an excessive inflammatory response detrimental to the host (Bihl *et al.* 2003). It requires much research to understand and control the balance between non- and overresponsiveness, which may both be harmful to the host.
2) Natural variation in the number, amplitude, and rate of induction of genes expressed in response to LPS as seen in several different mouse strains (Wells *et al.* 2003) and the species differences in the TLR4 gene (Smirnova *et al.* 2000) make it difficult to interpret the results and to develop applications. In addition to TLR4, many other genetic loci appear to control the nature and extent of the response promoted by a single bacterial agent, such as LPS. Our data show that there are strain-specific differences in TLR4 expression in mouse, a phenomenon that relates to the variation in the inflammatory response. That, together with the tissue-specific differences in TLR expression, requires targeted methods for TLR4 manipulation in the regulation of the immune response.
3) We showed evidence that the quality of the protein aggregate associated with TLR4 influences the quality of the cytokine response. Regulation of the activated protein aggregate containing the TLR4 dimer could be another target of potential therapies.
4) In our experiments, the expression of TLR2 was robustly increased after a LPS challenge. The functional implications of this phenomenon remain to be studied.

In the future, active efforts should be made to better understand the function and regulation of TLR4 in an attempt to develop TLR4-based means to regulate the innate immune response and to control the inappropriate cytokine response.

7 Summary and conclusion

According to the present findings, there were three basically different patterns of TLR4 and acute inflammatory responses *in vivo*.

1) TLR4 is basally expressed, and the expression is upregulated during a LPS challenge. A strong acute cytokine response is detected.
2) TLR4 is basally expressed, but the expression does not increase during an acute LPS challenge, although a clear acute cytokine response is evident.
3) There is little if any detectable basal TLR4 expression, the expression is not induced during an acute LPS challenge, and no acute cytokine response can be detected.

The expression and inducibility of TLR4 and the strength of cytokine production appeared to be mutually associated. In tissues with inducible TLR4 expression, the cytokine response to LPS was strongest. The response to basal and non-inducible TLR4 expression was lower, and no cytokine response was evident during paucity of TLR4. In the tissues where the major responsive cells, i.e. macrophages, are most abundant and their number increases most during inflammation, the TLR4 expression levels were not inducible, and the cytokine production remained low or moderate (liver). In tissues with rather lower macrophage contents, levels of TLR4 expression were inducible, and acute induction of cytokines was most remarkable after LPS (lung).

In fetal life, the expression of TLR4 and the capability to induce an acute response seem to develop along with functional maturation (lung). The lack of cytokine response in an immature fetus may be partly a matter of protection against the toxic effects of inflammatory stimuli, while partly it could explain the susceptibility to infections in association with premature birth.

Based on the present results, TLR4 may be considered a therapeutical target, since it modulates and regulates the acute inflammatory cytokine response, and the levels of TLR4 expression limit the cytokine response. However, the present study reveals some complexities related to this approach: First, the species and tissue-specific differences in TLR4 expression and regulation require targeting. Second, by binding PI3-kinase, the activated TLR4-MyD88 complex modulates the quality of the acute inflammatory

response and may result in differential regulation of TLR. Third, LPS causes a robust increase in TLR2 expression, which may affect or compensate for the function of TLR4. The future aim would be to better understand the function and regulation of TLR4 and to develop TLR4-based means to regulate the innate immune response and thus to control the inappropriate cytokine response.

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