Biochemical and functional study of the immunomodulatory capacity of the soluble form of human Toll-like receptor 2

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ABSTRACT

The Toll-like receptor (TLR) family plays a crucial role in innate immunity by mediating the recognition of, and response to a variety of microorganisms. Dysregulation of TLR-mediated inflammatory responses may, however, lead to a variety of acute and chronic inflammatory conditions. The description of a naturally occurring soluble form of TLR2 (sTLR2), and the observation that sTLR2-depleted serum renders leukocytes hypersensitive to TLR2-mediated stimulation, demanded a full assessment of sTLR2's regulatory capacity and an investigation of the underlying mechanism and therapeutic potential. The present study addressed these issues, and reports that cells overexpressing sTLR2, or stimulated in the presence of the sTLR2 protein, are TLR2 hyposensitive. Regulation was TLR2-specific, affected NF-κB activation, phagocytosis and superoxide production. Mice administered sTLR2 with Gram-positive bacteria-derived components showed lower levels of the neutrophil (PMN) chemoattractant, keratinocyte-derived chemokine; lower PMN numbers and late apoptotic PMN. Mononuclear cell recruitment was not affected, and endogenous peritoneal sTLR2 levels increased. Notably, the anti-inflammatory effect of sTLR2 did not compromise the capacity of mice to clear a live infection. sTLR2 interfered with the mobilisation of TLR2 to lipid rafts, acted as a decoy receptor, and disrupted the receptor (TLR2)-co-receptor (CD14) interaction by associating with CD14. In order to identify the region(s) of sTLR2 involved in the interaction with CD14, the leucine-rich repeats (LRRs) of TLR2 were mutated, and the ability of these mutants to affect CD14-dependent signalling was evaluated. Peptides representing the LRR6 and LRR20 were found to specifically inhibit CD14-dependent TLR2 triggering, indicating that these LRRs are directly involved in the TLR2-CD14 interaction. This study defines sTLR2 as an efficient regulator of TLR2-mediated inflammatory responses. The capacity of sTLR2 and TLR2-derived peptides to exert regulatory effects by targeting CD14 may inform the design of therapeutics against inflammatory conditions that will aim at disrupting the co-receptor's activity, thus blunting, but not abrogating, microbial recognition and host responses.

ABBREVIATIONS

Ab:	Antibody
Ag:	Antigen
AP-1:	Activating protein-1
APCs:	Antigen presenting cells
BIR:	Baculovirus inhibitor of apoptosis protein repeat domain
BLP:	Bacterial lipopepides
BSA :	Bovine serum albumin
CARD:	Caspase activation and recruitment domain
CFU:	Colony-forming unit
CL:	Collectin
CRD :	Carbohydrate recognition domain
CRP:	C-reactive protein
DCs:	Dentritic cells
Dif:	Dorsal-related immunity factor
dsRNA:	Double-stranded RNA
ECD:	Extracellular domain
FADD:	Fas-associated protein with dead domain
FCS:	Foetal calf serum
FRET:	Fluorescence resonance energy transfer
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
GNBP:	Gram-negative binding protein
His-rhsTLR2:	6x histidine-tagged recombinant human soluble TLR2
HKLM:	Heat-killed *Listeria monocytogenes*
HMGB:	High-mobility group B
HRP:	Horseradish peroxidase
IE-DAP:	γ-D-glutamyl-meso-diaminopimelic acid
IFN:	Interferon
Igs:	Immunoglobulins
IKK:	IκB kinase
IL:	Interleukin

IL-1R:	IL-1 receptor
Imd:	Immune deficiency
IPAF:	ICE protease-activating factor
IRAK:	Interleukin-receptor-associated kinase
IRF:	Interferon response factor
ISRE:	Interferon-stimulated response element motifs
JKN:	c-Jun N-terminal kinase
KC:	Keratinocyte-derived chemokine
LBP:	LPS-binding protein
LDL:	Low-density lipoproteins
LAM:	Lipoarabinomannan
LM:	Lipomannan
LPS:	Lipopolysaccharide
LRR:	Leucine-rich repeat
LTA:	Lipoteichoic acid
m:	Membrane-bound
MAC:	Membrane attack complex
Mal:	MyD88 adaptor-like
MALP:	Macrophage-activating lipopeptide
ManLAM:	Mannosyl-capped lipoarabinomannan
MAPK:	Mitogen-activated protein kinase
MARCO:	Macrophage receptor with collagenous structure
MASP:	Mannose-binding-lectin-associated serine protease
MBL:	Mannose-binding lectin
MCMV:	Murine cytomegalovirus
MCP-1 :	Monocyte chemoattractant protein-1
MDA:	Melanoma differentiation associated gene
MDP:	Muramyl dipepdtide
MFI:	Mean of fluorescence intensity
MHC:	Major histocompatibility complex
MIP:	Macrophage inflammatory protein
MKK:	MAPK kinase

MMR:	Macrophage mannose receptor
MNC:	Mononuclear cells
MUC:	Mucin-like glycoprotein
MyD88:	Myeloid differentiation primary response protein 88
NAIPs:	Neuronal apoptosis inhibitor proteins
NEMO:	NF-κB essential modulator
NF-κB:	Nuclear factor-κB
NLR:	Nucleotide-binding oligomerisation domain–like receptor
NO:	Nitric oxide
NOD:	Nucleotide-binding oligomerisation domain
NS:	Non-structural protein
Ni-NTA:	Nitrilotriacetic acid
OAS:	Oligoadenylate synthase
ODN:	Oligodeoxynucleotide
Pam$_3$Cys:	Trypalmitoyl-cysteinyl-seryl-(lysyl)$_3$-lysine (Pam$_3$-Cys-Ser-Lys$_4$)
PAMPs:	Pathogen-associated molecular patterns
PBMC:	Peripheral blood mononuclear cells
PFU:	Plaque-forming unit
PGN:	Peptidoglycan
PIAS:	Protein inhibitor of the activated signal transducer and activator of transcription
PILAM:	Phosphoinositol-capped lipoarabinomannan
PI3K:	Phosphoinositide 3-kinase
PMN:	Polymorphonuclear cells
poly(I:C):	Polyinosine-deoxicytidylic acid
PRGP:	Peptidoglycan recognition protein
PRRs:	Pattern recognition receptors
PSM:	Phenol soluble modulin
PTX:	Pentraxin
RANTES:	Regulated upon activation, normal T-cell expressed, and secreted
RIG:	Retinoid-inducible gene protein
RIP:	Receptor-interacting protein

ROS:	Reactive oxygen species
s:	Soluble
SAP:	Serum amyloid protein
SARM:	Sterile alpha and HEAT-Armadillo motifs-containing protein
SES:	*Staphylococcus epidermidis* cell-free culture supernatant
SHP:	Src homology-2 (SH-2)- containing tyrosine phosphatase
SIGIRR:	Single immunoglobulin IL-1R-related molecule
SIKE:	Supressor of IκB kinase ε
SOCS:	Suppressor of cytokine signaling
SP:	Surfactant protein
SR:	Scavenger receptor
SRCL:	Scavenger receptor with C type lectin
srMD-2:	Soluble recombinant MD-2
TAB:	Transforming growth factor-β-activated kinase -1 binding proteins
TAK:	Transforming growth factor-β-activated kinase
TBK:	TRAF-family-member-associated-NF-κB activator -binding kinase
TGF-β:	Transforming growth factor-β
Th:	T helper
TICAM:	TIR-containing adapter molecule
TIR:	Toll/interleukin-1 receptor
TIRAP:	TIR domain-containing adapter
TLR:	Toll-like receptor
TNF:	Tumour necrosis factor
TOLLIP:	Toll-interacting protein
TRAF:	TNF-α-receptor-associated factor
TRAIL:	TNF-related apoptosis-inducing ligand
TRAILR:	TNF-related apoptosis-inducing ligand receptor
TRAM:	TRIF-related adaptor molecule
TRIF:	TIR domain-containing adaptor inducing interferon-β
Ubc13:	Ubiquitin-conjugating enzyme 13
Uev1A:	Ubiquitin-conjugating enzyme E2 variant 1

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1. INTRODUCTION

1.1 Overview of the innate and adaptive immune response

Resolution of a microbial infection requires an efficient as well as controlled immune response, which is based on the activity of the innate immune system – a universal and ancient form of host defence – and the more specific, adaptive immune system (Janeway and Medzhitov, 2002).

The fine specificity of adaptive immune responses is ensured by the work of two types of specialised cells, B-lymphocytes and T-lymphocytes, through their somatically-generated receptors for antigens (Ags), whose expressions depend upon gene arrangement (Medzhitov and Janeway, 2000). This mechanism involves a clonal distribution of receptors, each cell displaying a unique receptor capable of recognising a specific Ag.

Ag recognition by a receptor leads to activation of the specific lymphocyte (B or T) and subsequent cell proliferation (clonal expansion), thus ensuring a strong and highly specific immune response. Furthermore, the clonal expression of Ag receptors in lymphocytes provides the basis of immunological memory, which allows for a faster, stronger and more specific response to re-encountered Ags.

In spite of being a very specific and powerful defence mechanism, the process of building up an adaptive immune response is relatively long, taking between 2 to 5 days after the initial contact with the Ag. This time constitutes a considerable delay when combating fast replicating microbial pathogens.

In addition to pathogen-derived Ags, the adaptive immune system also recognises and responds to elements from the environment or from self. Uncontrolled response to such Ags may lead to allergies and autoimmune diseases, respectively.

Although lacking the specificity of the adaptive immune system, the innate immune system provides an immediate, yet efficient, answer to a microbial challenge. The cells of the innate immune system express germ-line-encoded receptors specific for highly conserved molecular structures present in a variety of microorganisms, termed pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002). This is a misnomer, as these molecular patterns are not exclusive of pathogens. Importantly, the innate immune response targets mainly microbial pathogens, as mostly they, and not the host, produce these patterns. The fact that a given PAMP is expressed by a range of microorganisms allows a single innate immune receptor to sense different microbes. Moreover, given that PAMPs are essential either to microbial survival or pathogenicity it makes it more difficult for the pathogen to escape innate immune recognition by mutation.

The innate immune receptors recognising PAMPs are called pattern recognition receptors (PRRs). In general, PRR triggering by PAMPs induces, either directly or indirectly, immediate cell activation. The activated cell performs its effector function instantly, thus providing the host organism with a first line of defence against the pathogen.

Although innate and adaptive immunity can be considered two distinct systems, as they rely on different receptors and effector cells, the immune response is often presented as an organisational continuum (Fearon and Locksley, 1996). Indeed, dentritic cells (DCs) act as antigen presenting cells (APCs): they recognise and phagocyte pathogens through their PRRs, degrade them, and subsequently, display pathogen-derived Ags at their surface. APCs then interact with T-lymphocytes to initiate an Ag-specific adaptive immune response, and such cells form a crucial link between the innate and adaptive

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Table 1.1. Overview of human antimicrobial peptides

Antimicrobial peptide	Activities	Origin
α-defensins: Human neutrophil defensins precursor 1 to 3 (HNP-1 to −3)	Antibacterial, antifungal, antiviral activity. Chemotactic effect on monocytes, T cells, dendritic cells.	Neutrophils, macrophages
Human defensin 5 and 6 (HD-5 and −6)	Antibacterial activity, activates mast cells.	Paneth cells
β-defensins Human β-defensins 1 to 4 (HBD-1 to −4)	Antibacterial activity. Chemotactic effect on monocytes, T cells, dendritic cells.	Epithelial cells
Bacterial Permeability Increasing protein (BPI)	Binding to lipopolysaccharides. Gram negative antibacterial activity.	Neutrophils
Cathelicidins LL-37	Binding to lipopolysaccharides. Antibacterial activity. Chemotactic effect on monocytes, T cells, mast cells.	Neutrophils, epithelial cells
Histatins Histatin 1 to 3	Metal-binding properties. Antibacterial and antifungal activity.	Parotid gland
Lactoferrin	Iron sequester. Antibacterial and antifungal activity.	Neutrophils, mammary gland
Lysozyme	Hydrolysis of peptidoglycan. Antibacterial activity (mainly Gram postive). Non-specific opsonin.	Neutrophils, macrophages, monocytes epithelial cells
Phospholipase A-2 (PLA-2)	Antibacterial activity. Cleavage of bacterial cell wall phospholipids.	Neutrophils, macrophages, epithelial cells, paneth cells

immunity (Banchereau and Steinman, 1998). Cells of the innate immune system may also express co-stimulatory molecules and produce cytokines and chemokines, which are essential for the activation of the adaptive immune system (Janeway and Medzhitov, 2002).

1.2 Mediators of innate immune responses

The capacity of the innate immune system to respond to a microbial challenge in a prompt and efficient manner depends, in the first place, on the production of small protein mediators with diverse structures and functions, which are able to interact directly with and inactivate pathogens. The innate immune mediators include: antimicrobial peptides, antimicrobial chemokines, angiogenins and the complement system.

1.2.1 Antimicrobial peptides

Antimicrobial peptides are phylogenetically ancient 'weapons' present in both the vegetal and animal kingdom (Zasloff, 2002). The ability of these peptides to destroy pathogens lies in their cationic and amphiphilic nature. Their overall positive charge allows the peptides to interact with the negative outer leaflet of the microbial bilayer, which consists of lipids with negatively charged phospholipid headgroups. After insertion into the membrane, antimicrobial peptides either disrupt the integrity of the microbial bilayer – affecting its barrier function – or diffuse from the cell membrane to intracellular membranes (Hancock and Sahl, 2006). Because most microbial membranes have a negatively charged outer leaflet, antimicrobial peptides have a very broad spectrum of action. Susceptibility to antimicrobial peptides, however, varies among

Table 1.2. Human chemokines with antimicrobial properties

Chemokine	Microorganism reported to be susceptible
CXC family	
CXCL-4 or platelet factor-4 (PF-4)	*Esherichia coli, Staphylococcus aureus, Cryptococcus neoformans, Candida albicans.*
CXCL-7 or transporter-associated with antigen processing-2 (TAP-2)	*Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Lactococcus, Cryptococcus neoformans.*
CXCL-9 or mitogen inducible gene (MIG)	*Esherichia coli, Listeria monocytogenes.*
CXCL-10 or interferon-γ - induced protein of 10 kDa (IP-10)	*Esherichia coli, Listeria monocytogenes.*
CXCL-11 or interferon-inducible T-cell α-chemoattractant (I-TAC)	*Esherichia coli, Listeria monocytogenes.*
Connective tissue activating peptide 3 (CTAP-3)	*Esherichia coli, Staphylococcus aureus, Cryptococcus neoformans.*
CC family	
CCL-5 or 'Regulated upon activation, normal T-cell expressed, and secreted' (RANTES)	*Esherichia coli, Staphylococcus aureus, Cryptococcus neoformans, Candida albicans.*
CCL-28	*Pseudomonas aeruginosa, Klebsiella pneumoniae, Streptococcus mutans, Staphylococcus aureus, Candida albicans.*

different bacterial species (Zasloff, 2002), and it is believed that any mammalian organism may have a considerable number of different peptides to ensure the best possible response to any microbial challenge (Hancock and Sahl, 2006). By contrast, antimicrobial peptides have very little action on mammalian membranes, because their outer leaflet usually presents no net charge and the presence of cholesterol stabilises their structure. Thus, cationic peptides combine the wide range of action and the specificity required for an efficient first line of antimicrobial defence. It is now accepted, however, that their action is not limited to pathogen destruction, and that they possess important immunomodulatory properties (Durr and Peschel, 2002; Hancock and Sahl, 2006), including direct chemoattraction of immune cells, induction of chemokine release and modulation of monocyte gene expression. Table 1.1 presents an overview of the human antimicrobial peptides, their activities and origins.

1.2.2   Antimicrobial chemokines

Chemokines are small (8-14 kDa), mostly cationic, polypeptides known to play pivotal roles in immunity by regulating migration and activation of leukocytes (Hieshima et al., 2003). In addition, direct antimicrobial properties have lately been attributed to several chemokines: CXCL-9, CXCL-10, CXCL-11 (Cole et al., 2001), the platelet chemokines CXCL-4, CATP-3 and CCL-5 (Tang et al., 2002), as well as CXCL-7 – also stored in platelets (Durr and Peschel, 2002) – and CCL-28 (Hieshima et al., 2003), produced at high levels in mucosal tissues such as exocrine glands, the respiratory tract and colon. These chemokines act, as most antimicrobial peptides, by perturbing the integrity of the microbial membrane (Table 1.2).
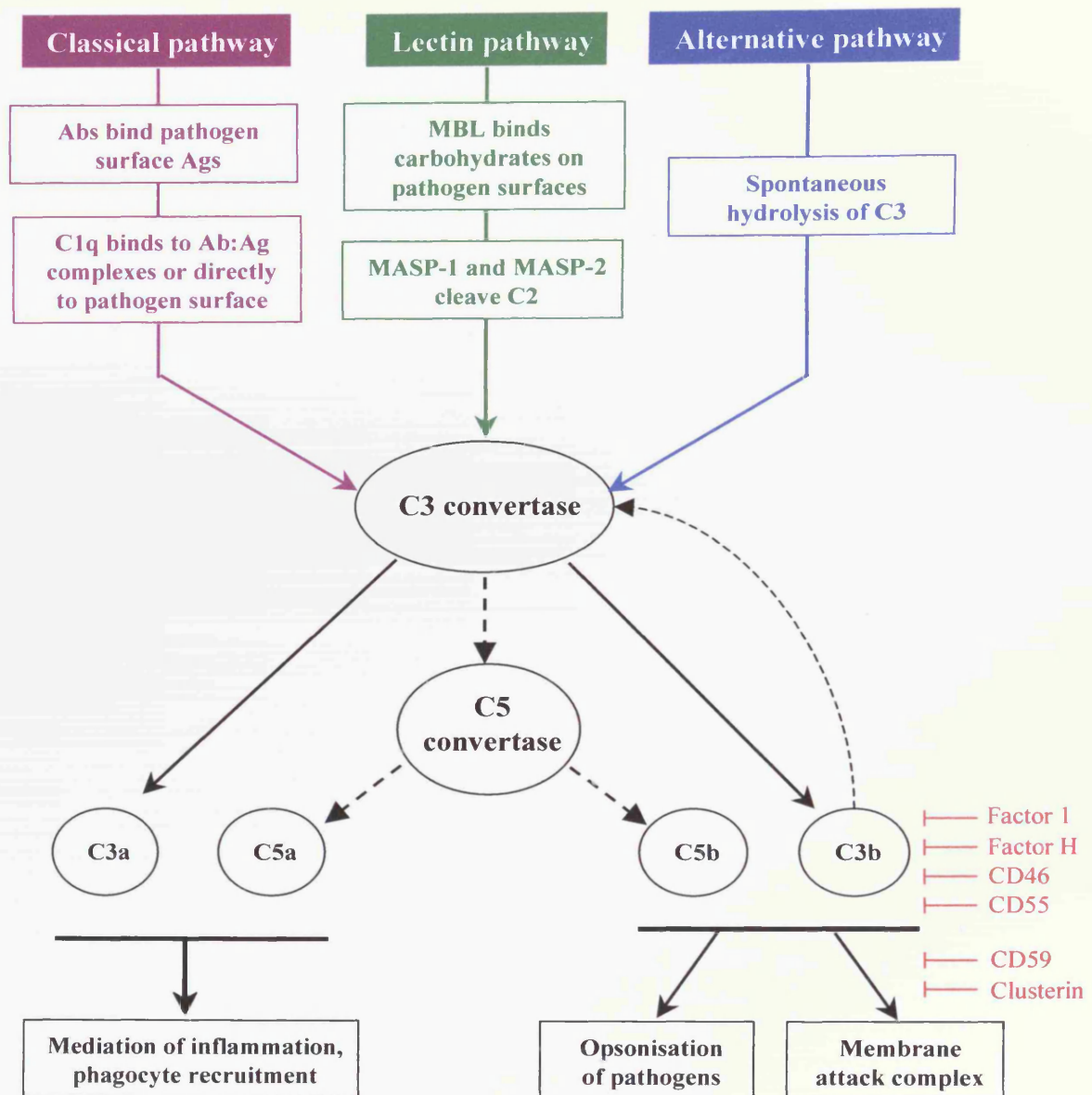
Figure 1.1. Schematic overview of the complement cascade

In the three pathways of complement activation, a series of protein cleavage reactions converge in the formation of the C3 convertase, which cleaves C3 into C3a and C3b (plain black arrows). C3b binds to the C3 convertase to form the C5 convertase, which cleaves C5 into C5a and C5b (dotted black arrows). C3a and C5a are powerful peptides mediators of inflammation. C3b binds to and opsonises pathogens and, together with C5b, initiates the formation of the membrane attack complex (MAC) that can damage pathogen membrane. MAC's formation can be inhibited by a number of complement regulators (red). Ab, antibody; Ag, antigen; MBL, mannose-binding lectin; MASP, mannose-binding-lectin-associated serine protease.

It has become apparent that antimicrobial peptides and chemokines have overlapping functions, as some members of the two groups contribute to recruitment of immune cells and direct neutralisation of pathogens (Durr and Peschel, 2002).

1.2.3 Angiogenins

Angiogenins are members of the ribonuclease superfamily that have long been known to be involved in vasculogenesis and tumour angiogenesis. Both human and mouse angiogenins have later been found to exert a microbicidal activity against bacterial and fungal pathogens *in vitro* (Hooper et al., 2003). However, this antimicrobial effect of angiogenins has lately been challenged, as it appeared to be limited, and nonspecific (Avdeeva et al., 2006). Thus, whether or not angiogenins constitute a class of microbicidal proteins is still unclear.

1.2.4 The complement system

The complement system is made up of many distinct plasma proteins that react with one another to destroy pathogens directly and also promote their opsonisation to help clear the infection. Many complement proteins are zymogens – proteases that can themselves be activated by proteolytic cleavage – and their precursors are widely distributed in body fluids and tissues without adverse effects for the host (Janeway's immunobiology, 2008)

There are three established pathways of complement activation: the direct, the mannose-binding lectin (MBL) and the alternative pathways (Fig. 1.1). The direct pathway is triggered when C1q, the first protein in the complement cascade, binds to the surface of a pathogen, either directly or to antibody:antigen complexes. The MBL pathway is

initiated by binding of MBL to mannose-containing carbohydrates present on bacteria and viruses. MBL forms a complex with the mannose-binding-lectin-associated serine proteases (MASPs)-1 and -2, which in turn activate the complement cascade by cleaving the component C2. The alternative pathway is stimulated by the spontaneous hydrolysis of the complement component C3 (Lambris et al., 2008).

After activation, each pathway follows a different sequence of enzymatic cleavages, all leading to the generation of the same complement protease called C3 convertase (Fig. 1.1). This protease cleaves C3 to generate C3a, a mediator of inflammation, and C3b, a major effector molecule of the complement system. C3b acts in two ways : 1) as an opsonin, it binds to the pathogen and so targets it for destruction by phagocytes expressing the C3b receptor. 2) C3b binds to the C3 convertase to create a C5 convertase complex, which cleaves C5 into C5a and C5b. C5b interacts with the other terminal complement components, C6, C7, C8 and C9, to form a membrane attack complex (MAC), which is capable of creating pores in the membrane of certain pathogens, leading to their destruction (Janeway's immunobiology 2008 and Fig. 1.1). Notably, the activity of a number of complement regulators (Clusterin, CD46, CD55, CD59, Factor 1, Factor H) prevents MAC from damaging the host cells (McDonald and Nelsestuen, 1997; Gelderman et al., 2002; Janeway's immunobiology, 2008).

The anaphylatoxins C3a and C5a, released during complement activation, bind to immune cells and trigger a range of chemotactic and pro-inflammatory responses. This results in the recruitment of inflammatory cells and the activation of downstream immune mechanisms (Lambris et al., 2008).

1.3   Receptors of the innate immune system

The innate immune system uses a variety of PRRs. Recognition of a PAMP by these receptors induces an immediate response aimed at the destruction of the pathogen in many different ways, including: opsonisation, phagocytosis, triggering of complement, activation of pro-inflammatory signalling pathways and induction of apoptosis. PRRs are expressed either as soluble receptors (secreted proteins), in intracellular compartments or at the cell surface.

1.3.1   Soluble PRRs

Typical soluble PRRs include members of the collectin, ficolin and pentraxin families.

Collectins are an important group of PRRs present in plasma and on mucosal membranes. Seven soluble members of this family have been identified: MBL, surfactant protein (SP)-A and -D, conglutin, collectin (CL)-43, CL-46 and CL-K1. They belong to a family of mammalian lectins which contain collagen-like regions and a C-type lectin carbohydrate recognition domain (CRD) (Janeway and Medzhitov, 2002) . Collectins bind to a wide range of glycoconjugates on microbial surfaces, thanks to an open and flexible binding pocket in their CRD (Gupta and Surolia, 2007). Upon recognition of the infectious agents, collectins put into action different effector mechanisms to ensure the destruction of the pathogen. Some mechanisms are common to the seven secreted collectins (Gupta and Surolia, 2007), like agglutination or opsonisation of pathogens, both leading to an enhanced phagocytosis. More specific ways to ensure microbial destruction are however used by specific collectins: MBL, a

plasma protein, can initiate the activation of the complement system and enhance chemokine production by macrophages. The pulmonary collectins SP-A and SP-D have a more direct antimicrobial action, as they can kill Gram-negative bacteria by disrupting their membrane integrity. They can also act as chemoattractants for phagocytes at the infection site. In addition to their antimicrobial activities, MBL, SP-A and SP-D also exert a host protective role. They ensure an enhanced clearance of apoptotic cells by acting as opsonins for free DNA and RNA, inhibiting pro-inflammatory cytokine production (MBL), or sequestering C1q to block complement activation (SP-A) (Gupta and Surolia, 2007). Furthermore, SP-A and SP-D can affect the adaptive immune response by inhibiting T-cell proliferation (Borron et al., 1996) or modulating the differentiation of immature DCs. Indeed, SP-D was found to associate with immature DCs, facilitate their interaction with bacterial pathogen, and enhance their cell-surface Ag presentation capacity (Brinker et al., 2001), whereas SP-A was found to reduce the expression of major histocompatibility complex (MHC) class II on DCs, and thus inhibiting the allostimulation of T cells by DCs (Brinker et al., 2003). CL-K1's cDNA was detected by screening human liver databases, and RT-PCR analysis showed that most human tissues express CL-K1 mRNA – with the exception of skeletal muscles and bone marrow (Keshi et al., 2006). CL-K1 was found capable of binding to LPS from several Gram-negative bacterial strains and mannan from *Saccharomyces cerevisiae*, but the relevance of this binding in terms of antimicrobial activity remains to be evaluated.

Conglutin, CL-43 and CL-46 are bovine collectins, which provide a first line of defence against rumen microbes by using the common effector mechanisms, i.e. agglutination and opsonisation.

Members of the ficolin family are related to MBL (Endo et al., 2007), and have been shown to be involved in non-self clearance. Depending on the species of mammals, two or three kinds of ficolin have been identified. They are produced by different cell types, and have different affinity for different bacterial carbohydrates. In humans, the first member of the ficolin family, termed L-ficolin, was isolated from plasma and shown to recognise N-acetylglucosamine residues in sugar chains (Matsushita et al., 1996). The second human ficolin, M-ficolin, was identified by genome comparison with L-ficolin. M-ficolin is 76% identical to L-ficolin, is not expressed in plasma, but mainly in peripheral monocytes, lung and spleen (Endo et al., 1996). The latest human ficolin to be identified was isolated as a serum Ag recognised by an autoantibody in patients with systemic lupus erythematosus (Sugimoto et al., 1998). It was first termed Hakata antigen, and later renamed H-ficolin. H-ficolin is produced mainly by the liver and lungs, and is secreted in the blood and bile duct. Upon binding to the ligand, ficolins can activate complement through the lectin pathway by binding to MBL-associated serine proteases (MASPs), or induce opsonophagocytosis (Endo et al., 2007).

C-reactive protein (CRP), serum amyloid protein (SAP) and pentraxin 3 (PTX-3) are members of the pentraxin family. They function as opsonins upon binding to phosphorylcholine on bacterial membranes (Janeway and Medzhitov, 2002; Lee and Kim, 2007). CRP and SAP can also bind to C1q and, thus, activate the classical pathway of complement activation.

1.3.2   Intracellular PRRs

Viruses and some bacteria can gain access to intracellular compartments, and several PRRs expressed in the cytosol can detect these intracellular pathogens: the 2'-5'-oligoadenylate synthase (OAS), the protein kinase R (PKR), the melanoma differentiation associated gene-5 (MDA-5), the retinoid-inducible gene protein-1 (RIG-1) and members of the the nucleotide-binding oligomerisation domain (NOD)–like receptors (NLRs).

OAS is activated by binding to viral double-stranded RNA (dsRNA), and produces a nucleotide messenger, 2'-5' oligoadenylate, which in turn activates the ribonuclease (RNase) L. This RNase digests both viral and cellular RNAs, leading to viral destruction and apoptosis of the infected cells (Janeway and Medzhitov, 2002).

PKR is a TNF-α-induced dsRNA-dependent serine threonine kinase (Garcia et al., 2007). Upon binding to dsRNA, PKR phosphorylates and inactivates the translation initiation factor eIF2α. This blocks both viral and cellular synthesis, leading to the destruction of the virus and the infected cell. In addition to its translational regulatory function, PKR can also activate NF-κB, leading to the induction of various pro-inflammatory genes (Garcia et al., 2007).

MDA-5 and RIG-1 are cytoplasmic caspase activation and recruiting domain (CARD) helicases, containing a RNA helicase domain involved in RNA recognition and two CARDs responsible for initiating dowstream signalling (Lee and Kim, 2007) leading to viral RNA destruction. *In vivo* experiments have demonstrated that MDA-5 is the main receptor for the viral double-stranded RNA (dsRNA) mimic polyinosinic-polycytidylic acid (poly I:C, also a TLR3 ligand, see section 1.3.3.B.2.5), whereas RIG-

1 recognises single-stranded (ss) 5′-triphosphorylated and uncapped RNA (Takeuchi and Akira, 2008). As most of the 5′ ends of host mRNAs are capped, RIG-1 is able to discriminate viral and host RNAs.

Contrary to the specific recognition of viruses by OAS, MDA-5 and RIG-1, NLRs are mainly involved in antibacterial defence. The NLRs constitute a large family of receptors, more than 20 in mammals. They consist of three distinct domains: a C-terminal leucine-rich repeat (LRR) domain – mediating ligand binding – , a NACHT central domain – involved in self oligomerisation – (NACHT stands for domain present in: neuronal apoptosis inhibitor proteins (NAIPs), class II transactivator (CIITA), the plant het product involved in vegetative incompatibility HET-E and telomerase associated protein-1 (TP-1)), and an N-terminal effector domain for protein-protein interaction, which initiates downstream signalling. NLRs have been grouped into three subfamilies, depending on their effector and NACHT domains: 1) NOD-1, NOD-2 and the ICE protease-activating factor (IPAF) contain a CARD domain, 2) NAIPs have three baculovirus inhibitor of apoptosis protein repeat (BIR) domains (Lee and Kim, 2007), and 3) NALPs (NATCH, LRR, PYD) possess a pyrin effector domain (PYD) (Lee and Kim, 2007).

Human NOD-1 and -2 recognise the different peptidoglycan (PGN) fragments γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), respectively. NOD-1 acts mainly as a sensor for Gram-negative bacteria, as most Gram-positive PGNs do not contain iE-DAP. On the contrary, because PGNs from both Gram-positive and Gram-negative bacteria contain MDP, NOD-2 has a broader spectrum of action (Strober et al., 2006). Ligand recognition by NODs can activate both NF-κB and MAP kinase pathways (Underhill, 2007). NOD-1 or -2 deficiency increases murine

sensitivity to gastrointestinal bacterial infections (Strober et al., 2006), and LRR mutations in NODs have been linked with inflammatory disorders such as Crohn's disease in humans.

Recently, IPAF and NAIP-5 (murine paralogue of NAIP) have been shown to recognise cytosolic flagellin (Miao et al., 2006), the principal protein of bacterial flagella – known to be a ligand for TLR5 (see section 1.3.3.B.2.1). Activation of IPAF or NAIP-5 leads to Caspase-1-mediated secretion of IL-1β and macrophage cell death. It is believed that NAIP-5 collaborates with IPAF to achieve macrophage cell death (Miao et al., 2006).

Fourteen members of the NALP family have been identified, and the function of most of them remains to be investigated. Various stimuli activating human NALP-3 were recently identified. These include various exogenous stimuli such as bacterial RNA, antiviral compounds (R848 and R837), and bacteria (*Staphylococcus aureus* and *Listeria monocytogenes*, but not *Salmonella typhimurium* or *Francisella tularensis*), as well as endogenous ligands that act as 'danger' signals such as uric acid crystals formed after the release of uric acid from dying cells, and extracellular ATP released by dying cells. Upon ligand recognition, NALP-3 recruits Caspase-1 via interaction with the CARD-containing adaptors Cardinal and Asc, forming a so-called inflammasome (Lee and Kim, 2007). The close proximity of the caspases in the inflammasome is thought to promote maturation of pro-inflammatory cytokines such as IL-1β and IL-18, and subsequent cell apoptosis (Lee and Kim, 2007).

Several members of the Toll-like receptor (TLR) family capable of recognising viral and bacterial RNA and DNA are also intracellularly located. They will be described, as the rest of the TLR family, in the next section.

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1.3.3 Cell membrane PRRs

Cell-membrane-expressed PRRs include: Dectin-1, the macrophage mannose receptor (MMR), scavenger receptors (SRs), and the Toll-like receptors (TLRs).

Dectin-1, a C-type lectin, is able to induce intracellular signalling by itself (Lee and Kim, 2007). It is expressed on DCs, macrophages and neutrophils. Dectin-1 recognises β-1,3-glucan in the core of the fungal cell wall, which is exposed upon separation of budding yeast cells. Macrophages from Dectin $^{-/-}$ animals have been used to demonstrate that this receptor is essential for the phagocytosis of zymosan (a glucan from the yeast cell wall) and live fungi as well as for the production of antimicrobial reactive oxygen species (ROS). It has also been shown that Dectin-1 co-operates with TLR2 for the zymosan-induced production of TNF-α and IL-12p40 by macrophages, and IL-2 and IL-10 by DCs (Rogers et al., 2005).

MMR is a C-type lectin which binds carbohydrates on the surface of pathogens. Its structure and recognition properties are very similar to those of MBL (section 1.3.1), but because MMR has a transmembrane domain, it can act as a direct phagocytic receptor (Fraser et al., 1998).

SRs are capable of binding to a variety of microbial ligands as well as to host-derived molecules, such as modified low-density lipoproteins (LDL), promoting their phagocytosis (Mukhopadhyay and Gordon, 2004). SRs are expressed mainly at the cell surface of macrophages, DCs and some selected endothelial cells. SR-A, one of the best described SR, has been shown to recognise different bacterial ligands, such as lipopolysaccharide (LPS) – a component of the outer membrane of Gram-negative bacteria, lipoteichoic acid (LTA) from Gram-positive bacteria, and bacterial CpG DNA.

The SR termed macrophage receptor with collagenous structure (MARCO) also binds a wide range of Gram-positive and Gram-negative ligands, but it is only expressed in subpopulations of macrophages, such as resident peritoneal macrophages. Other SRs are involved in viral recognition and phagocytosis: gp-340, identified in both saliva and lung surfactant (Mukhopadhyay and Gordon, 2004), has been shown to neutralise different strains of the influenza-A virus, and the human immunodeficiency virus-1. SRs are not only involved in pathogen destruction. For example, MARCO and the SR with C-type lectin-1 (SRCL-1) ensure the removal of modified host molecules such as oxidised-LDL (Ox-LDL), a metabolite generated by host cells and trapped in the subendothelial spaces. Failure to remove oxidised-LDL from the subendothelial spaces typically leads to atherosclerosis (Mukhopadhyay and Gordon, 2004).

TLRs are also membrane-bound PRRs. They are of critical importance to host defence and the subject matter of the present study. Therefore, they will be described in more detail in the next section.

1.3.3.A   The Toll receptors

Toll-like receptors take their name from the Toll family of receptors. The Toll receptors are evolutionary conserved, and homologous receptors are found in plants, insects and vertebrates (Lemaitre et al., 1996). The first member of this family was identified in the fruit fly *Drosophila melanogaster* in 1984 and named Toll, the german word for 'strange, bizarre', because fly larvas bearing a mutation in the *toll* gene were very strange looking, as the ventral portion of their body was underdeveloped. Indeed, Toll was first discovered as part of a pathway required for
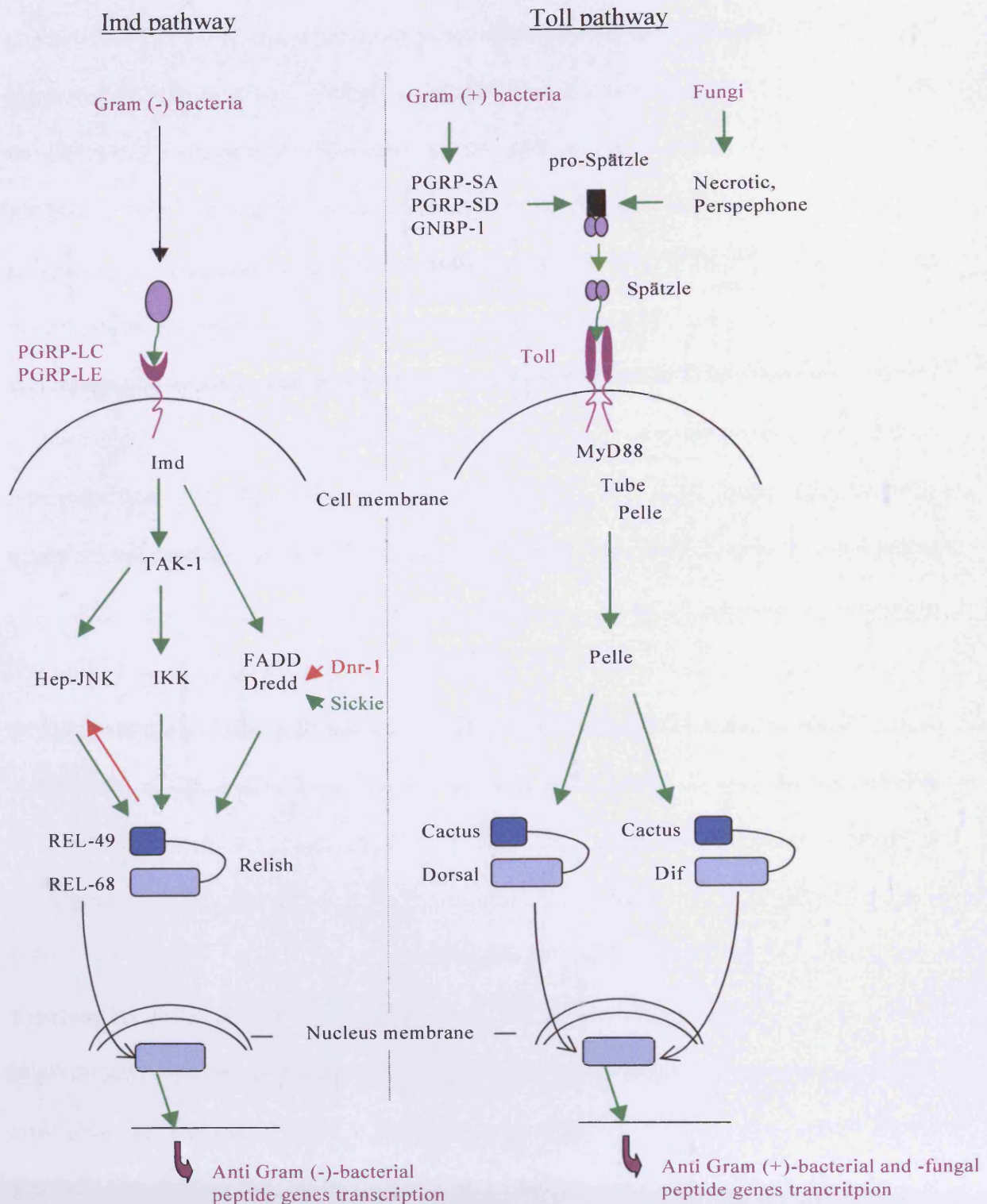
Figure 1.2. The *Drosophila* Imd and Toll pathways
Comparison of the mechanims involved in ligand recognition and subsequent signalling by the Imd pathway and the Toll pathway in *Drosophila* immunity. Dif, Dorsal-related immunity factor; Dnr-1, defence repressor 1; FADD; Fas-associated protein with dead domain; GNBP-1, Gram-negative binding protein-1; IKK, IκB kinase complex; Imd, Immuno-deficiency; JNK, c-Jun N-terminal kinase; MyD88, myeloid differentiation primary response protein 88; PGRP, peptidoglycan recognition protein; TAK-1, transforming growth factor-β-activated kinase-1. Green and red arrows indicate activation and inhibition processes, respectively.

dorso-ventral axis differentiation in the fly embryo (Anderson and Nusslein-Volhard, 1984). The observation that the intracellular (C-terminal) domain of Toll was closely related to that of the vertebrate IL-1 receptor (IL-1R), which is involved in the signalling cascade leading to NF-κB activation and the release of immune defence proteins during infection (Gay and Keith, 1991), was critical. Indeed, it suggested that Toll may be involved in signalling processes related not only to insect development, but also to immune defence mechanisms. These findings therefore prompted Lemaitre and co-workers to investigate the role of Toll in insect immunity.

Multiple self defence mechanisms contribute to the insect innate immunity, including entrapment of pathogens by deposition of melanine, engulfment by blood cells, and killing by ROS and antimicrobial peptides (Tanji and Ip, 2005). The expression of these peptides is induced by the presence of pathogens, and Lemaitre and co-workers showed that two different pathways control their production: the Immune deficiency (Imd) and Toll pathways (Lemaitre et al., 1996 and Fig. 1.2).

The Imd pathway governs the expression of antimicrobial peptide genes in response to Gram-negative bacterial infections. Imd is not a PRR, but an adaptor protein homologous to the mammalian tumour necrosis factor (TNF) receptor interacting protein. There are two identified PRRs activating the Imd pathway: the peptidoglycan recognition proteins (PRGP)-LC and -LE, which co-operate to recognise and respond to various PGNs and possibly LPS from Gram-negative bacteria (Tanji and Ip, 2005). Imd triggering recruits transforming growth factor-β-activated kinase-1 (TAK-1), the most upstream kinase of the cascade identified so far. TAK-1 activates the *Drosophila* IκB kinase (IKK) complex, which in turn directs the site-specific proteolytic cleavage and activation of the inducible transactivactor Relish. This releases the REL-68 subunit of

Relish, which translocates to the nucleus and binds to κB-like enhancer elements in the promoter of antimicrobial genes (Fig. 1.2). This mechanism is considered homologous to the processing of NF-κB by the IKK complex in mammals (Tanji and Ip, 2005).

The homologue of the mammalian Fas-associated protein with dead domain (FADD) and the *Drosophila* caspase homologue Dredd, are also involved in the Imd-mediated antimicrobial response in *Drosophila*. They act downstream of Imd, but upstream of Relish, and not through TAK-1 and IKK, thus constituting a separate Imd pathway. Activation of the FADD-Dredd pathway is controlled by at least two regulators: Sickie – which induces Dredd-mediated activation of Relish – , and defence repressor-1 (Dnr-1) – which negatively regulates this process. A third pathway of Imd-mediated signalling involves the Hemipterous (Hep)-c-Jun N-terminal kinase (JKN) cascade, which acts downstream of TAK-1 (Silverman et al., 2003). Although this pathway is not required for the production of antimicrobial peptides, it regulates other immune genes, such as *Relish*, and activates immediate early genes after septic injury before being shut off by a Relish-dependent mechanism (Tanji and Ip, 2005).

Upon infection by Gram-positive bacteria or fungi, the Toll pathway is activated, leading to antimicrobial peptide synthesis (Fig. 1.2). Gram-positive bacteria-derived peptidoglycan is recognised by multiple host proteins, PRGP-SA, -SD, and Gram-negative binding protein-1 (GNBP-1). Like for the Imd pathway, it is believed that these molecules co-operate to recognise a wide range of microorganisms. In the case of a fungal infection, pathogen recognition involves a serine protease, Persephone, and a protease inhibitor, Necrotic. Notably, the upstream regulatory cascades in dorso-ventral differentiation, and the response to Gram-positive bacteria and the fungi all lead to the processing of the Toll ligand Spätzle. The Spätzle protein is secreted as a precursor,

pro-Spätzle, which is cleaved as a result of a serine-protease activation cascade induced

following ligand recognition by PRGP-SA, -SD and GNBP-1 (Weber et al., 2003). The

truncated form of Spätzle has been shown to bind to Toll and activate the Toll pathway,

indicating that Spätzle as acts a unique Toll ligand in *Drosophila* immunity (Tanji and

Ip, 2005).

Toll triggering involves multimerisation of the receptor, and *in vitro* studies have

demonstrated that Spätzle binds to Toll with a stoichiometry of one Spätzle dimer to

two Toll monomers (Weber et al., 2003), suggesting that Spätzle may induce Toll

dimerisation for signalling. Activated Toll associates with the myeloid differentiation

primary response protein 88 (MyD88), a cytoplasmic adaptor protein with a

Toll/interleukin-1 receptor (TIR) domain that interacts with the TIR domain of Toll.

Subsequently, Tube, another adaptor protein, and Pelle, a serine-threonine kinase, are

recruited to form the signalling complex. Activation of Pelle leads to the processing of

the cytoplasmic Dorsal-Cactus and Dorsal-related immunity factor (Dif)-Cactus

complexes. Dif and Dorsal are NF-κB homologues and are retained in the cytoplasm by

the IκB-related inhibitor Cactus. After the signal-induced degradation of Cactus, Dif

and Dorsal translocate to the nucleus and activate the transcription of antimicrobial

genes, leading to the production of anti-Gram-positive bacterial and anti-fungal peptides

(Tanji and Ip, 2005).

1.3.3.B   The mammalian Toll-like receptors

A year after *Drosophila* Toll (dToll) was demonstrated to play an

important role in insect innate immunity, the first mammalian Toll was identified in

humans, and called human Toll (hToll) (Medzhitov et al., 1997). Like dToll, hToll is a

Toll receptor

Toll-like receptor

Ectodomain

Cytoplasmic domain

N terminus

LRR

C terminus

Juxtamembrane region

Transmembrane region

TIR domain

BB loop
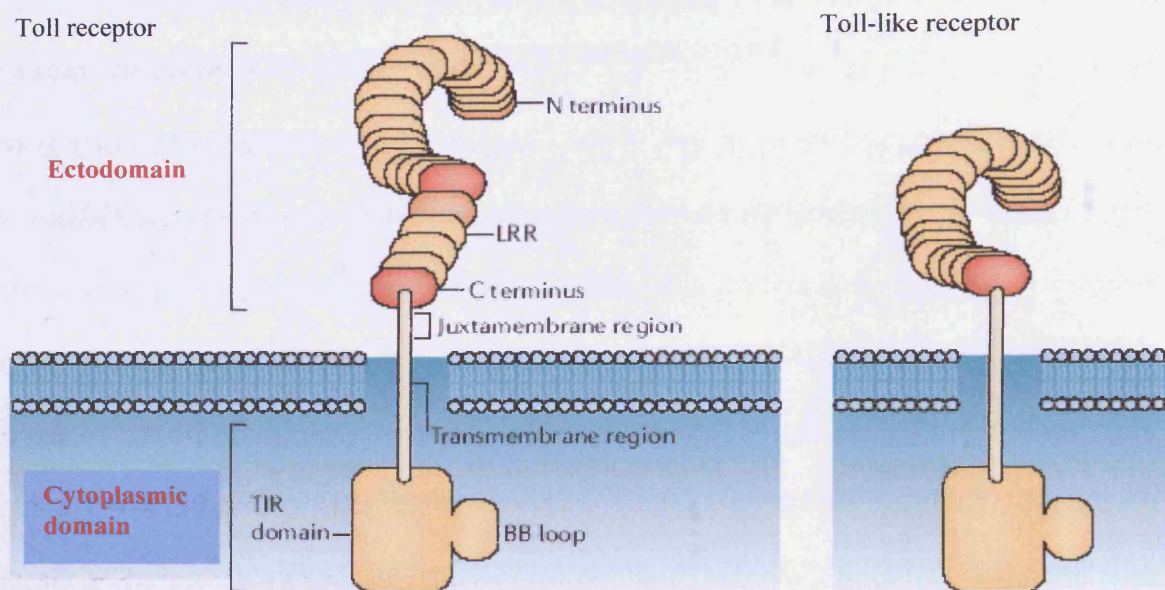
(Adapted from *Gay et al., 2006*)

Figure 1.3. Schematic structure of Toll and Toll-like receptors
Comparative diagram of Toll and Toll-like receptor structure showing their extracellular domain (ectodomain), consisting of leucine-rich repeat (LRR) domains, a short transmembrane domain, and the intracellular domain containing the Toll/Interleukin 1 receptor (TIR) critical for signalling. LRR, leucine-rich repeat.

type I transmembrane protein, with an extracellular domain consisting of a series of leucine-rich repeat (LRR) domains, and a cytoplasmic domain homologous to that of IL-1R, hence its name Toll/interleukin-1 receptor (TIR) domain (Fig. 1.3). No ligands for hToll were yet known, but using a constitutively active mutant of hToll, Medzhitov and co-workers showed that activation of hToll leads to NF-κB activation and NF-κB-controlled gene expression of the pro-inflammatory cytokines IL-1, IL-6 and IL-8. Within a year, five human Toll-like receptors (TLRs) were identified by genomic sequence comparison, and named TLR1 to TLR5, hToll corresponding to TLR4 (Rock et al., 1998), human TLR6 to TLR10 were characterised later (Takeda et al., 2003).

1.3.3.B.1   *TLR tissue distribution*

Consistent with their function in early pathogen recognition, TLRs are widely expressed in immune cells and tissues in constant contact with microorganisms. Notably, TLR1, 2, 4, 5 and 6 are mainly expressed at the cell surface, whereas TLR3, 7, 8 and 9 are mainly present intracellularly (Watts, 2008).

Within peripheral blood leukocytes, professional phagocytes express the greatest variety of TLRs. Neutrophils, monocytes and macrophages were indeed found to express mRNA for all TLRs but TLR3 (Muzio et al., 2000; Sandor and Buc, 2005). TLR3 mRNA is mainly expressed by DCs (Muzio et al., 2000; Hornung et al., 2002). Interestingly, immature DCs do not express TLR3, they acquire it during their maturation following stimulation with various microbial components, while the expression of TLR1, 2, 4 and 5 starts decreasing (Muzio et al., 2000; Hornung et al., 2002; Sandor and Buc, 2005). Myeloid DCs have been shown to express TLR1-6, 8 and 10, whereas plasmacytoid DCs were reported to express high amounts of TLR7 and

TLR9, and lower levels of TLR1 and TLR10 (Iwasaki and Medzhitov, 2004). Eosinophils express TLR1, 2, 4, 6, 7, 9 and 10 mRNAs constitutively, whereas basophils express TLR2 and TLR4, and mast cells TLR1, 2 and 6 mRNAs.

Lymphocytes have also been shown to express TLRs. NK cells – major players in the antiviral response – express TLR3 together with TLR2, 5 and 9. B-lymphocytes show high expression levels of TLR1, 6, 9 and 10, and lower levels of TLR2, 4 and 7. Differential TLR expression has been detected on T cell subsets. TLR3, 5, 6, 7 and 9 mRNAs have been detected in $CD4^+$ T cells, and TLR2 and TLR4 proteins can be detected at the surface of activated and memory T cells, however, naïve $CD4^+$ do not show significant levels of expression. There is also evidence that regulatory T cell (Treg) activity can be directly modulated by TLRs. TLR2 activation on Tregs has been shown to induce proliferation of otherwise anergic Tregs and transiently reverts their suppressive activity (Sutmuller et al., 2006). However, opposite effects were observed by Zanin-Zhorov and co-workers, who reported that TLR2 triggering enhanced Tregs' suppressive activity (Zanin-Zhorov et al., 2006). Similarly, depending on the dose of agonist used, TLR5 was shown to supress (low dose) or enhance (high dose) Tregs' regulatory activity (Crellin et al., 2005). TLR8 triggering was also shown to revert the suppressive function of Tregs *in vivo* (Peng et al., 2005).

In addition to leukocytes, TLRs are expressed in epithelial tissues including the skin, respiratory, intestinal and genitourinary tracts. These are potential routes of entry of pathogens into the host organism.

Keratinocytes express TLR1-5 constitutively, and mRNAs for TLR2 and TLR4 were detected in nasal mucosa, adenoids and salivary glands (Sandor and Buc, 2005; Kuroishi et al., 2007). Immunohistochemical staining of human airways revealed

expression of TLR2 throughout the epithelium, and TLR4 has been shown to be expressed in pulmonary epithelial cells as well as corneal epithelium.

Intestinal epithelial cells are in constant contact with bacteria on their apical surface, and yet, only pathogens invading the basolateral compartment of the epithelium elicit an inflammatory response. Consistent with these observations, these cells express very low levels of TLR2 and TLR4, and TLR5 is only expressed on the basolateral surface.

With regards to the genitourinary tract, TLR1, 2, 3, 5 and 6 mRNAs were found in the lower female genital tract. Endothelial cells may also have a role in detecting pathogens, as they have been shown to express TLR2, 4 and 5 (Sandor and Buc, 2005). TLR2 on endothelial cells is thought to promote the development of atherosclerosis, as TLR2 deficiency results in decreased lesion burden. Notably, atherosclerosis-associated lesions do not develop uniformly throughout the arterial tree, but at sites of disturbed flow – i.e. vessel bifurcations and the lesser curvature of the aortic arch. It has been shown that endothelial TLR2 expression is only found in these regions of disturbed flow and that this local TLR2 expression increases with the development of atherosclerosis (Dunzendorfer et al., 2004). By recreating the flow conditions observed in the aortic arch *in vitro*, the authors found that laminar flow, not disturbed/low flow, induced phosporylation of the transcription factor signal protein-1 (Sp-1) and thereby blocked its binding to the TLR2 promoter, which is required for TLR2 expression (Dunzendorfer et al., 2004). It was demonstrated that TLR2 on epithelial cells can be activated by endogenous ligands, such as high-mobility group B1 (HMGB-1), hyaluronic acid fragments and serum amyloid A, but not by exogenous TLR2 agonist, which specifically trigger TLR2-mediated activation of bone-marrow-derived cells (Mullick et al., 2005). It is possible that TLR2-mediated activation of endothelial cells

1

MPHTLWMVWV *LGVIISLSKE* ESSNQASLSC DRNGICKGSS GSLNSIPSGL <u>TEAVKSLDLS</u>   60

<u>NNRITYISNS</u> DLQR<u>CVNLQA</u> LVLTSNGINT IEEDSFSSLG <u>SLEHLDLSYN</u> YLS**N**LSSSWF   120
                                                                -CHO-

KPL<u>SSLTFLN</u> <u>LLGN</u>PYKTLG ETSLFS<u>HLTK</u> <u>LQILRVGNMD</u> TFTKIQRKDF AG<u>LTFLEELE</u>   180

<u>IDASDLQSYE</u> PKSLKSIQ**N**V <u>SHLILHMKQH</u> ILLLEIFVDV <u>TSSVECLELR</u> <u>DTDLDTFHFS</u>   240
              -CHO-

ELSTGE<u>TNSL</u> <u>IKKFTFRNVK</u> ITDESLFQVM KLLNQ<u>ISGLL</u> <u>ELEFDDCTLN</u> GVGNFRASDN   300

DRVID<u>PGKVE</u> <u>TLTIRRLHIP</u> RFYLFYDLST LYSLTERVKR <u>ITVENSKVFL</u> VPCLLSQ<u>HLK</u>   360

<u>SLEYLDLSEN</u> LMVEEYLKNS ACEDA<u>WPSLQ</u> <u>TLILRQNHLA</u> SLEKTGETLL TLK**N**<u>LTNIDI</u>   420
                                                                -CHO-

SK<u>NSFHSMPE</u> TCQ<u>WPEKMKY</u> L**N**LSSTRIHS VTGCI<u>PKTLE</u> <u>ILDVSNNNLN</u> LFSLNL<u>PQLK</u>   480
                               -CHO-

<u>ELYISRNKLM</u> TLPDAS<u>LLPM</u> <u>LLVLKISRNA</u> ITTFSKEQLD <u>SFHTLKTLEA</u> <u>GGNN</u>FICSCE   540

FLSFTQEQQA LAKVLIDWPA NYLCDSPSHV RGQQVQDVRL SVSECHRTAL VSGMCCALFL   600

LILLTGVLCH RFHGLWYMKM MWAWLQAKRK PRKAPSRNIC YDAFVSYSER DAYWVENLMV   660

QELENFNPPF KLCLHKRDFI PGKWIIDNII DSIEKSHKTV FVLSENFVKS EWCKYELDFS   720

HFRLFDENND AAILILLEPI EKKAIPQRFC KLRKIMNTKT YLEWPMDEAQ REGFWVNLRA   780

AIKS 784

Figure 1.4. Amino acid sequence of TLR2
The first twenty amino acids (*italics*) correspond to the putative leader peptide of TLR2. The twenty LRR domains are underlined. The four N-glycosylation sites are indicated by the symbol –CHO- and the acceptor N in red. The putative transmembrane domain is shown in green, and the TIR cytoplasmic domain in blue.

leads to the recruitment of bone-marrow-derived cells – such as neutrophils, monocytes and macrophages – which could in turn be activated in response to potential exogenous TLR2 agonists. Thus, it is thought that TLR2 promotes atherosclerosis by exacerbating the local inflammation process in response to endogenous ligands and/or exogenous microbial ligands.

1.3.3.B.2   *TLR structure and ligand specificity*

The extracellular domain (ECD) of TLRs is responsible for ligand recognition. It is composed of 19 to 25 leucine-rich repeat (LRR) domains which are 24 to 29 amino acids long and contain a consensus motif: LXXLXXLXLXXN (X, any amino acid) or a LRR-like motif – characterised by a minimal requirement of two matches within an LRR core motif (Kirschning and Schumann, 2002). As an example, the human TLR2 ECD contains 20 LRR/LRR-like domains (Fig. 1.4). Each LRR consists of a β-strand and a α-helix connected by loops. Molecular modelling predicted a horseshoe-like structure for the LRR domain of several TLRs (LeBouder et al., 2003; Akira and Takeda, 2004). This predicted structure has lately been confirmed by the description of the crystal structure of TLR3 (Choe et al., 2005), TLR4 (Kim et al., 2007), and TLR2/TLR1 complexes (Jin et al., 2007). Although it has long been thought that the TLR ligand would interact with the concave part of the horseshoe, resolution of the crystal srtucture of the TLR2/ligand complex showed that the lipopeptidic ligand was bound to the convex surface of TLR2 (Jin et al., 2007). Similarly, TLR3 crystal structure revealed a glycosylation-free, positively charged, domain on the convex surface of TLR3 which was predicted to be the binding
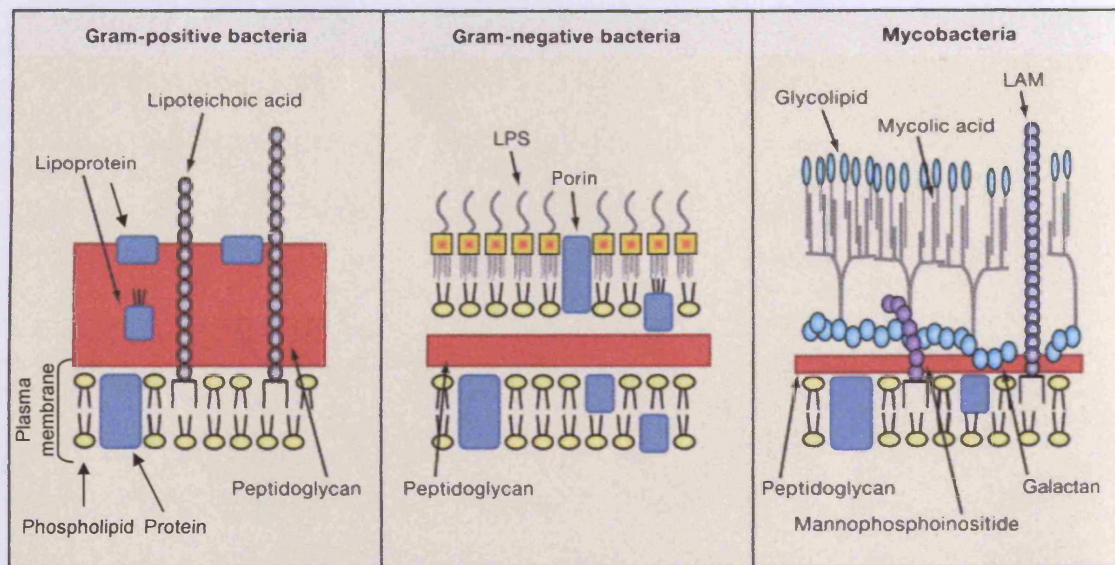
(Adapted from *Akira et al., 2006*)

Figure 1.5. Schematic representation of bacterial cell walls
The lipid bilayer cell membrane of Gram-positive bacteria is covered by a poreous peptidoglycan layer, containing lipoproteins and lipoteichoic acids. In Gram-negative bacteria, the peptidoglycan layer is thiner, but an additional outer membrane containing lipopolysaccharides (LPS) and porins is present. Mycobacteria produce a thick mycolate-rich outer covering that ensures a very efficient barrier function. LAM, lipoarabinomannan.

site of RNA to TLR3 (Choe et al., 2005). The question of how general this model is can

only be answered by the crystallographic analysis of other TLRs.

Through their LRR domains, TLRs specifically recognise a wide range of microbial and

host components.

1) Bacterial ligands

Cell wall components

Bacteria can be classified into two major groups, Gram-positive and Gram-

negative depending on the composition and, thus, Gram staining characteristics of their

cell wall. Some of the cell wall components are unique to one group of bacteria and

recognised specifically by individual TLRs. The Gram-positive cell wall is

characterised by the presence of a very thick peptidoglycan (PGN) layer, also

containing lipoproteins and lipoteichoic acids. In contrast, the peptidoglycan layer of the

Gram-negative cell wall is very thin, but Gram-negative bacteria are surrounded by an

additional outer membrane composed mainly of phospholipids and lipopolysaccharides

(LPS). Mycobacterial species share a characteristic mycolate-rich, thick and

hydrophobic cell wall (Fig. 1.5).

LPS, also known as endotoxin, is the major component of the outer wall of

Gram-negative bacteria. It is made up of a lipid A portion, a core oligosaccharide and an

O-antigen (Brade and Galanos, 1983). Studies using TLR4 mutated (C3H/HeJ) or

deficient mice have shown that TLR4 is crucial for LPS responsiveness (Poltorak et al.,

1998; Hoshino et al., 1999). The use of highly purified natural or synthetic lipid A

helped to conclude that lipid A is indeed the part of LPS responsible for TLR4

activation (Ogawa et al., 2002). Different strains of bacteria produce structurally

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different LPS, varying in their phosphate patterns, number of acylations and fatty acid composition. These variations account for the varied biological activities of lipid A from different origins (Akira et al., 2006). Notably, some bacteria can escape the immune system by modulating the structure of their lipid A upon invasion (Hajjar et al., 2002). It is important to note that, for an efficient recognition by TLR4, LPS has to be in monomeric form. The acute phase response serum protein, LPS binding protein (LBP), is responsible for converting oligomeric micelles of LPS to LPS monomers, thus facilitating its transfer to the TLR4 co-receptor, CD14, and subsequent recognition by the TLR4 signalling complex (Pugin et al., 1993; Hailman et al., 1994). The possibility of the existence of another LPS transfer molecule was suggested as LBP-deficient mice showed an almost normal inflammatory response after LPS injection (Wurfel et al., 1997). This possibility was confirmed by the observation that high mobility group-1 protein (HMGB-1) – a nuclear protein involved in nucleosome stabilisation and gene transcription – works in concert with LPS to increase the production of TNF-α by monocytes (Youn et al., 2008). Youn and co-workers also demonstrated that HMGB-1 binds to and catalytically disaggregates LPS, thus, facilitating its transfer to CD14 (Youn et al., 2008).

Interestingly, the non-enteric bacteria *Leptospira interrogans, Helicobacter pylori* and *Porphyromonas gingivalis* produce atypic LPS, which have been shown to induce inflammation through TLR2 and not TLR4 (Takeda and Akira, 2005). However, the difficulty of removing TLR2 contaminants from LPS preparations makes these results controversial.

Components of Gram-positive bacteria cell wall can also trigger TLRs. Lipoteichoic acids (LTA) is a negatively-charged glycolipid believed to be the

counterpart of LPS of Gram-negative bacteria. The biological activity of LTA has long been controversial, due to the high levels of endotoxin present in the commercial LTA preparations that have been purified from *Staphylococcus aureus* or *Enterococcus hirae*. However, the use of a synthetic analogue of LTA from *S. aureus* showed that LTA on its own is biologically active and induces cell activation via TLR2 (Morath et al., 2002), in collaboration with TLR6 (Alexopoulou et al., 2002).

Peptidoglycan (PGN) is composed of long linear sugar chains of alternating N-acetyl glucosamine and N-acetyl muramic acid. Although present in Gram-negative bacteria, PGN is most abundant in the cell wall of Gram-positive bacteria and has been shown to induce cell activation via TLR2, in association with TLR1 (Takeuchi et al., 2001; Iwaki et al., 2002). Like in the case of LTA, this observation remains controversial (Uematsu and Akira, 2008), due to the possible presence of contaminants in the PGN preparations purified from bacteria. PGN from *S. aureus* has been proven to bind directly to the extracellular domain of TLR2, both using a plate-based assay and by sedimentation experiments (Iwaki et al., 2002).

Phenol soluble modulin (PSM), an inflammatory polypeptide complex secreted by *Staphylococcus epidermidis* has also been shown to trigger cell activation via TLR2 (Mehlin et al., 1999; Hajjar et al., 2001; Liles et al., 2001). The *S. epidermidis* PSM is constituted of three strongly hydrophobic active polypeptides: PSM-α, PSM-β and PSM-γ, each of them with a molecular mass of < 5 kDa.

Lipoproteins and lipopeptides, present on both Gram-negative and Gram-positive bacteria, are potent immunostimulants. TLR2 responds to lipopeptides from a wide range of pathogens (Carpenter and O'Neill, 2007). Lipid modification seems to be critical for TLR2 activation, since the unlipidated outer-surface protein A (OspA)

lipoprotein purified from *Borrelia burgdorfer* cannot activate TLR2 cell transfectants (Hirschfeld et al., 1999). To confirm the importance of the lipid modification, a trypalmitoylated (Pam$_3$), lipopeptide (Pam$_3$)-Cys-Ser-Lys$_4$ (Pam$_3$CSK$_4$ or Pam$_3$Cys) was synthesised and demonstrated to be a potent TLR2 agonist. Pam$_3$Cys is now commonly used as a prototypic TLR2 ligand, and has been shown to bind directly to the TLR2 ECD (Vasselon et al., 2004). Importantly, discrimination of subtle changes in the lipid portion of lipoproteins is ensured by the collaboration of TLR2 with TLR1 or TLR6. Indeed, macrophages from TLR6-deficient animals proved to be insensitive to the presence of the diacylated mycobacterial macrophage activating lipopeptide-2kDa (MALP-2), whereas they produced TNF-α at similar levels to wild-type cells in response to triacylated lipopeptides, such as Pam$_3$Cys (Takeuchi et al., 2001), indicating that TLR6 is involved in diacylated lipopeptide recognition. A year later, Takeuchi and co-workers confirmed the involvement of TLR1 in triacylated lipopeptides recognition by TLR2 (Takeuchi et al., 2002).

Porins, the major outer membrane proteins of the pathogenic neisserial species, have been shown to trigger B-lymphocyte activation, as indicated by the upregulation of MHC class II molecules (Massari et al., 2002). However, B-lymphocytes from TLR2-deficient mice were found unresponsive to porins from *Neisseria meningitidis*, indicating that B-cell activation by porins is TLR2-mediated (Massari et al., 2002).

Flagellin

Flagellin, the major constituent of bacteria flagella, is a potent activator of innate immune responses. Analysis of the crystal structure of the *Salmonella*'s flagellin shows different domains: N- and C-terminal α helix chains (D0), the central α helix chain (D1)

and a hypervariable central region with β sheets (Akira et al., 2006). TLR5 is responsible for the detection of flagellin, through recognition of the D1 constant domain – highly conserved among different species (Hayashi et al., 2001). Some bacteria, such as *Helicobacter pylori* and *Campylobacter jejuni* produce flagellin lacking pro-inflammatory properties – because of mutations within a specific region of the D1 domain (amino acids 89 to 96) – and, therefore, escape the flagellin-specific host immune response (Andersen-Nissen et al., 2005). Notably, these 8 amino acids (89-96) of the D1 domain were also found critical to flagellar filament formation and bacterial mobility. To preserve mobility, *H. pylori* and *C. jejuni* possess compensatory amino acid changes in another region of the flagellin molecule (Andersen-Nissen et al., 2005).

Bacterial DNA

Bacterial genomic DNA is also a stimulator of the host immune response, and is recognised by TLR9 (Hemmi et al., 2000; Carpenter and O'Neill, 2007). TLR9 recognises unmethylated CpG dinucleotides, which are very abundant in bacterial genomes. By contrast, mammalian CpG motifs are highly methylated and are not recognised by TLR9 (Akira et al., 2006). This discrimination ensures that the host DNA does not induce TLR9 activation.

Since TLR9 resides in the endosome, bacterial DNA must be delivered to this intracellular compartment where the acidic and reducing conditions lead to the degradation of double-stranded sDNA into multiple single-stranded CpG-motif-containing regions that will interact with TLR9 (Akira et al., 2006). This results in the multiplication of the inflammatory signal from a single molecule of bacterial DNA. The use of compounds blocking endosomal acidification, such as chloroquine or

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bafilomycin, has shown that an acidic pH is required for DNA digestion and optimal recognition by TLR9. However, this model has recently been challenged by the observation that a chimeric TLR9 localised at the cell surface is still capable of recognising non methylated CpG-DNA, suggesting that DNA degradation in the endosome may not be critical for TLR9 triggering (Barton et al., 2006).

Miscellaneous ligands

The pathogenic *Yersinia* species (Gram-negative bacteria) is recognised by the immune system via TLR4, thus triggering a pro-inflammatory response. However, *Yersinia* releases a virulence factor,V antigen (LcrV), which stimulates the production of the anti-inflammatory cytokine IL-10 via TLR2 and suppresses production of TNF-α and IFN-γ, thus exploiting TLR2 activation as a way to evade the host's immune system. As a consequence mechanism, $TLR2^{-/-}$ mice are actually less susceptible to oral infection with *Yersinia enterocolitica* (Sing et al., 2002).

It is worth noting that an unknown component of uropathogenic *E. coli* activates murine TLR11 (Zhang et al., 2004a). It is suggested that susceptibility to uropathogenic *E. coli* in humans may result from the fact that human TLR11 is not functional.

Malaria parasites within red blood cells digest host hemoglobin into a hydrophobic heme polymer, known as hemozoin, which was found to induce pro-inflammatory responses *in vivo* in a TLR9-dependent manner (Coban et al., 2005). A more recent study, however, reported that highly purified Malaria hemozoin is immunologically inert, but dramatically enhances innate responses by presenting malaria DNA to TLR9 (Parroche et al., 2007).

2) Mycobacterial ligands

The mycobacterial cell wall is composed of a thick mixture of lipids and polysaccharides (Fig. 1.5) and a high content of mycolic acid. Lipomannan (LM), and its arabinosylated form, lipoarabinomannan (LAM), are potent immunomodulatory lipoglycans. The arabinan domain is capped by either a mannosyl (ManLAM) or a phosphoinositol residue (PILAM). LM – found on both pathogenic and non-pathogenic species – and PILAM – present on fast-growing non-pathogenic species – are potent TLR2 activators, whereas ManLAM, characteristic of slow-growing virulent microorganisms, such as *Mycobacterium tuberculosis,* is a strong anti-inflammatory molecule. It is thought that pathogenic and non-pathogenic mycobacteria are discriminated according to the ManLAM/LM ratio in their cell wall (Akira et al., 2006). The mycobacterial diacylated lipopeptide, MALP-2, is also recognised by TLR2 – in collaboration with TLR6. Indeed, as described previously in this section, the TLR2/TLR6 complex detects diacylated lipopeptides, often associated with mycobacteria. In addition, the TLR2/TLR1 heterodimer recognises a 19-kDa cell-wall associated and secreted lipoprotein initially purified from *M. tuberculosis,* and also present in *M. leprae,* that induces cell activation. It is reported that a mutation in the intracellular domain of TLR2 (Arg677Trp) is associated with lepromatous leprosy in the Korean population, confirming the involvement of TLR2 in *M. leprae* recognition (Takeuchi et al., 2002; Malhotra et al., 2005).

Unmethylated CpG mycobacterial DNA is recognised by TLR9 (Akira et al., 2006).

3)   <u>Fungal ligands</u>

The observation that Toll-deficient *Drosophila* are highly susceptible to fungal infection suggested that mammalian TLRs may also be involved in fungal recognition. This was confirmed by the demonstration that TLR4 recognises mannans from *Saccharomyces cerevisiae* and *Candica albicans* (Shoham et al., 2001), as well as glucoronoxylomannan from *Cryptococcus neoformans* (Netea et al., 2004). It was also shown that zymosan, a *S. cerevisiae* cell-wall component, is phagocytosed by macrophages via the mannose receptor and then activates TLR2 present on phagosomes (Underhill et al., 1999). In addition, direct binding of zymosan to the ^{125}I-labelled TLR2 ectodomain was demonstrated (Sato et al., 2003). TLR2 also collaborates with another PRR, Dectin-1, to optimise the recognition of fungal β-glucans (Brown et al., 2003 and section 1.3.3.B.3, page 43).

T-cell helper 1 (Th1) responses are critical to the protection against fungi, and it has been shown that most TLR-mediated signals induce Th1-directed responses. TLR2 activation – including that induced by fungal ligands – appears to favour Th2 responses through the induction of IL-10 production (Agrawal et al., 2003). In fact, *in vivo* studies using TLR-deficient mice suggest differential roles of TLR2 and TLR4 in fungal infections. While TLR4$^{-/-}$ animals showed an increased susceptibility to *C. albicans*, TLR2 deficiency increased mice resistance. TNF-α and IL-1-β levels were found unaffected in TLR2$^{-/-}$ animals, but the release of IL-10 was strongly impaired, indicating that *C. albicans* induces immunosuppression through the anti-inflammatory cytokine IL-10. A similar escape mechanism is also observed with the hyphae form of *Aspergillus fumigatus*, which specifically activates TLR2 and not TLR4, as opposed to the conidia form (Netea et al., 2004).

4)   Protozoan parasite ligands

Trypanosoma-derived molecules, such as glycosylphosphatidylinositol-mucin, glycoinositolphospholipids and genomic DNA have been reported to activate TLR2, TLR4 and TLR9, respectively.

Profillins – small ubiquitous proteins thought to be involved in parasite mobility and/or invasion – appear to activate TLRs. Indeed, a profillin-like molecule from *Toxoplasma gondii* was identified as the first ligand for murine TLR11 (Yarovinsky et al., 2005). In humans, however, TLR11 is not functional due to the presence of an additional stop codon in its gene.

Notably, mice deficient in individual TLRs do not show impaired responses to live protozoan parasites, as opposed to those deficient in MyD88 – a signal adaptor common to most TLRs (Adachi et al., 2001). This suggests that several TLRs are involved in the response against protozoan parasites.

5)   Viral ligands

Viruses contain genetic material, either DNA or RNA, encoding viral structural components and enzymes for replication and protein synthesis. TLRs are involved in the recognition of various viral PAMPs, including viral DNA, ssRNA, dsRNA and surface glycoproteins.

Viral DNA

The genome of DNA viruses, like that of herpes simplex virus (HSV)-1, HSV-2 and murine cytomegalovirus (MCMV), is rich in CpG-DNA motifs which are recognised by TLR9 and lead to the production of type 1 IFNs (Akira et al., 2006).

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The obsevation that mice lacking TLR9 or MyD88 can still control HSV-1 infection indicates that an effective viral response can be ensured in a TLR9- and MyD88-independent manner (Krug et al., 2004).

Single-stranded RNA

Uridin-rich and uridin/guanosine-rich ssRNA from the host or of viral origin is recognised by mouse TLR7 and human TLR7 and TLR8 – mouse TLR8 is non functional – (Hemmi et al., 2002; Heil et al., 2004). Notably, although human TLR7 and human TLR8 are structurally related and both recognise ssRNA, their functions are not redundant. Indeed, ssRNA recognition by human TLR7 or human TLR8 seems to depend on the number of uridine (U) residues in the ssRNA sequence: TLR7 recognising U-low sequences and TLR8 U-rich sequences (Sioud, 2006; Gantier et al., 2008). Furthermore, it has been shown that human TLR7 triggering stimulates DCs to produce mainly IFN-α, whereas activation of TLR8 leads to pro-inflammatory cytokines production by monocytes (Gorden et al., 2005). However, a more recent study suggested that ssRNA sequence-dependent ligand recognition can be modulated by the relative expression of human TLR7 and TLR8, and that these two receptors collaborate for an effective ssRNA recognition (Gantier et al., 2008).

Human/mouse TLR7 and human TLR8 are also activated by synthetic antiviral components, e.g. R848, Imiquimod. In addition, human TLR7 can be activated by guanine nucleotide analogs e.g. loxoribine (Hemmi et al., 2002; Gantier et al., 2008).

TLR7 and *TLR8* genes show high homology, and both receptors are expressed within the endosome membrane. This location ensures high ligand accessibility, since many enveloped viruses traffic into the cytosol through the endosomal compartment. Like in

the case of TLR9, an acidic environment is of critical importance for TLR7 and TLR8 activity, as it ensures – together with degradation enzymes – the destruction of viral particles, the release of ssRNA and subsequent recognition by these receptors (Akira et al., 2006).

Importantly, unlike viral RNAs which are protected by a capside, host RNAs are degraded by extracellular RNases and rarely reach the endocytic compartment, thus, allowing for self/non-self discrimination.

Double-stranded RNA

During viral infection, dsRNA can be generated as a replication intermediate of ssRNA viruses, or as a by product of symmetrical transcription in DNA viruses. This makes dsRNA a universal viral PAMP. Alexopoulou and co-workers have shown that dsRNA and its synthetic analog, polyinosine-deoxicytidylic acid (poly I:C), are recognised by TLR3 and induce secretion of IFN-α by DCs (Alexopoulou et al., 2001). Reported evidence indicates, however, that TLR3 is not required for the initial recognition of viral infection that induces the first wave of IFN-α (Lopez et al., 2004). Consistent with this finding, mice lacking TLR3 do not show increased sensitivity to infection by MCMV, VSV, lymphocytic choriomeningitis virus (LCMV) and reoviruses (Akira et al., 2006). Furthermore, TLR3$^{-/-}$ animals are more resistant than wild-type to West Nile virus infection, suggesting that this virus benefits from its interaction with TLR3 (Akira et al., 2006). Thus, the exact role of TLR3 in viral infection remains to be clarified.

Different components of the viral envelope have been identified as TLR ligands. For instance, TLR4-deficient mice showed lower levels of infiltrating mononuclear cells and reduced production of IL-12 following infection with the respiratory syncytial virus (RSV). Indeed, the fusion protein from RSV was found to activate TLR4 (Kurt-Jones et al., 2000). Similarly, TLR2 is activated by viral components such as Measles virus hemagglutinin protein, and non-identified components from human CMV and HSV-1 (Bieback et al., 2002; Akira et al., 2006).

TLR4 is also thought to recognise the envelope protein (Env) of mouse mammary tumour virus (MMTV). Notably, Env appears to benefit from this interaction, as it results in the overexpression of the MMTV entry receptor, CD71 on DCs (Burzyn et al., 2004).

Triggering of TLR2 and TLR4 by viral glycoproteins does not lead to the production of IFN-α, but to that of pro-inflammatory cytokines which direct the response towards a general inflammation rather than to a specific antiviral action.

6)   Host derived ligands

In addition to the recognition of pathogens, TLRs also detect endogenous ligands, thus signalling other types of 'danger' conditions.

One of the most well established endogenous TLR ligands are host nucleic acids, which can be recognised by TLR9 (DNA), TLR3 (dsRNA), TLR7 and TLR8 (ssRNA). As described previously, host RNAs are normally not accessible to TLRs, but under certain condition, like tissue/cell necrosis or injury, they may become available for recognition by these receptors. Recognition of host nucleic acids leads to pro-

inflammatory cytokine production, recruitment of phagocytes and a rapid clearance of the necrotic cells (Tsan and Gao, 2004).

Patients suffering from systemic lupus erythematosus (SLE) and other systemic autoimmune diseases produce a wide range of autoantibodies. Very frequently these autoantibodies bind to chromatin or other subcellular nucleic acid-protein particles. B cells expressing an antigen receptor specific for self-immunoglobulin-γ (IgG) make a type of autoantibody known as rheumatoid factor (RF). IgG2a–chromatin immune complexes have been shown to mediate RF$^+$ B cell activation, which could be blocked by inhibitors of TLR9 sigalling (Leadbetter et al., 2002). This data suggests a potent synergistic functional interaction between the B-cell receptor and TLR9 mediated by chromatin-containing immune complexes. It is postulated that B-cell receptor engagement by an autoantibody–chromatin immune complex triggers the endocytosis of the immune complex-associated antigen, and that this results in the highly efficient delivery of chromatin fragments to endosome-associated TLR9 (Leadbetter et al., 2002).

The use of animals deficient in or mutated for individual TLRs have lead to the indentification of other endogenous TLR ligands, which mainly signal through TLR2 and/or TLR4.

Hyaluronan, one of the major structural components of the extracellular matrix, is a high-molecular-weight polymer that undergoes rapid degradation at sites of tissue injury. Low-molecular weight products of hyaluronan have been shown to initiate inflammatory responses through TLR2 and TLR4. In addition, by using *in vivo* models of lung injury, it was demonstrated that activation of TLR2 and TLR4 by hyaluronan fragments promotes wound defence and repair by enhancing transepithelial migration of

Table 1.3. Human Toll-like receptors and their ligands

TLR	Ligand	Source
TLR2 (in association with TLR1 or TLR6)	Lipoprotein/lipopeptides	Bacteria, mycobacteria
	Triacyl lipopeptides (Pam$_3$CSK$_4$)	Bacteria, mycobacteria
	Diacyl lipopeptides	Mycoplasma
	Diacyl lipopeptides (Pam$_2$CSK$_4$ and MALP2SK$_4$)	Synthetic compound
	Peptidoglycan	Gram-positive bacteria (not accessible in gram negative)
	Lipoteichoic acid (LTA)	Gram-positive bacteria
	Lipoarabinomannan (LAM)	Mycobacteria
	A phenol-soluble modulin	*Staphylococcus epidermidis*
	Glycoinositolphospholipids	*Trypanosoma cruzi*
	Glycolipids	*Treponema maltophilum*
	Porins	*Neisseria meningitidis*
	Virulence factor LcrV	*Yersinia* species
	Zymosan	Fungi
	Atypical LPS	*Leptospira interrogans*
	Atypical LPS	*Porphyromonas gingivalis*
	Hsp70	Host
	Hyaluronan	Host
	Hemagglutinin	Measles virus
TLR3	dsRNA	Viruses
	Poly I:C	Synthetic compound
TLR4	LPS	Gram-negative bacteria
	Fusion protein	Respiratory syncytial virus
	Envelope proteins	Mouse mammary tumor virus
	Hsp60	*Chlamydia pneumoniae*
	Hsp60	Host
	Hsp70	Host
	Type III repeat extra domain A of fibronectin	Host
	Oligosaccharides of hyaluronic acid	Host
	Polysaccharide fragments of heparan sulfate	Host
	Fibrinogen	Host
TLR5	Flagellin	Bacteria
TLR7	ssRNA	Viruses
	Imidazoquinolines (imiquimod, R-848)	Synthetic compounds
	Guanosine analogs	Synthetic compounds
TLR8	ssRNA	Viruses
	R-848	Synthetic compounds
TLR9	Unmethylated CpG DNA	Bacteria, virus, yeast, insects
	Chromatin-IgG complexes	Host

neutrophils and inhibiting epithelial cell apoptosis (Jiang et al., 2005). The involvement of TLRs in tissue repair has been supported by the observation that LPS helped tissue regeneration following ethidium-bromide induced brain damage (Glezer et al., 2006).

Other extracellular matrix breakdown products, i.e. heparan sulfate, the extra domain A of fibronectin, fibrinogen, SP-A and HMGB-1, have been shown to activate TLR4. HMGB-1 is also recognised by TLR2. These endogenous ligands are exposed during cellular injury and matrix remodelling, thus constituting 'danger' signals for the innate immune system.

The cellular chaperones heat shock proteins 60 and 70 (Hsp 60 and 70) are released by mammalian cells or bacteria following cellular stress. The use of both cell extracts and recombinant Hsp has shown that they are ligand for both TLR2 and TLR4, and thus may signal cellular stress or damage to the immune system (Tsan and Gao, 2004).

Recognition of endogenous ligands is, however, not always beneficial to the host, as it may promote autoimmunity. Indeed, it is thought that the induction of pro-inflammatory cytokines by Hsp60 and Hsp70 may contribute to the development of a number of autoimmune diseases, such as type I diabetes, Crohn's disease and atherosclerosis. Furthermore, the reported recognition of SP-A and fibrinogen by TLR4 may be deleterious to the host, as these TLR ligands are constitutively present at significant concentrations in the circulation (fibrinogen) and the lung (SP-A) (Zhang and Schluesener, 2006).

Table 1.3 presents a summary of the ligands described here for each human TLR.

1) TLR triggering

TLR oligomerisation

The basic mechanism of triggering used by TLRs and Toll appears to involve ligand induced (hetero/homo) dimerisation or oligomerisation. The first evidence of this mechanism comes from the observation that Späztle crosslinks two *Drosophila* Toll to induce signalling. Chimaeric TLR4 molecules in which the ECD is replaced by that of Toll can also be activated by Späztle, suggesting that the TIR domains of two TLR4 molecules can establish downstream signalling if the receptors are arranged in a symmetrical ligand-crosslinked complex manner (Gay et al., 2006). Furthermore, TLRs have often been reported to form homo-, or heterodimers. Cross-linking experiments have led to the conclusion that TLR4 dimerises in the presence of the ligand LPS (Kim et al., 2007), whereas co-immunoprecipitation experiments have shown that TLR2 associates with TLR6 (Ozinsky et al., 2000) and TLR1 (Nakata et al., 2006), even in the absence of ligand. Fluorescence resonance energy transfer (FRET) experiments seem to indicate that the TLR2/TLR1 or TLR2/TLR6 dimers form bigger oligomers in response to their ligands (Triantafilou et al., 2006).

Although receptor crosslinking is crucial, it was shown that the process of activation induces conformational changes in the receptor ectodomains that are required for signal triggering (Gay and Gangloff, 2007). Truncations in the Toll N-terminus sequence allow the formation of a stable dimeric complex and induces constitutive cellular activation, suggesting that the N-terminal region of the Toll ectodomain exerts a steric constraint and prevents self association of the receptor. Therefore, binding of
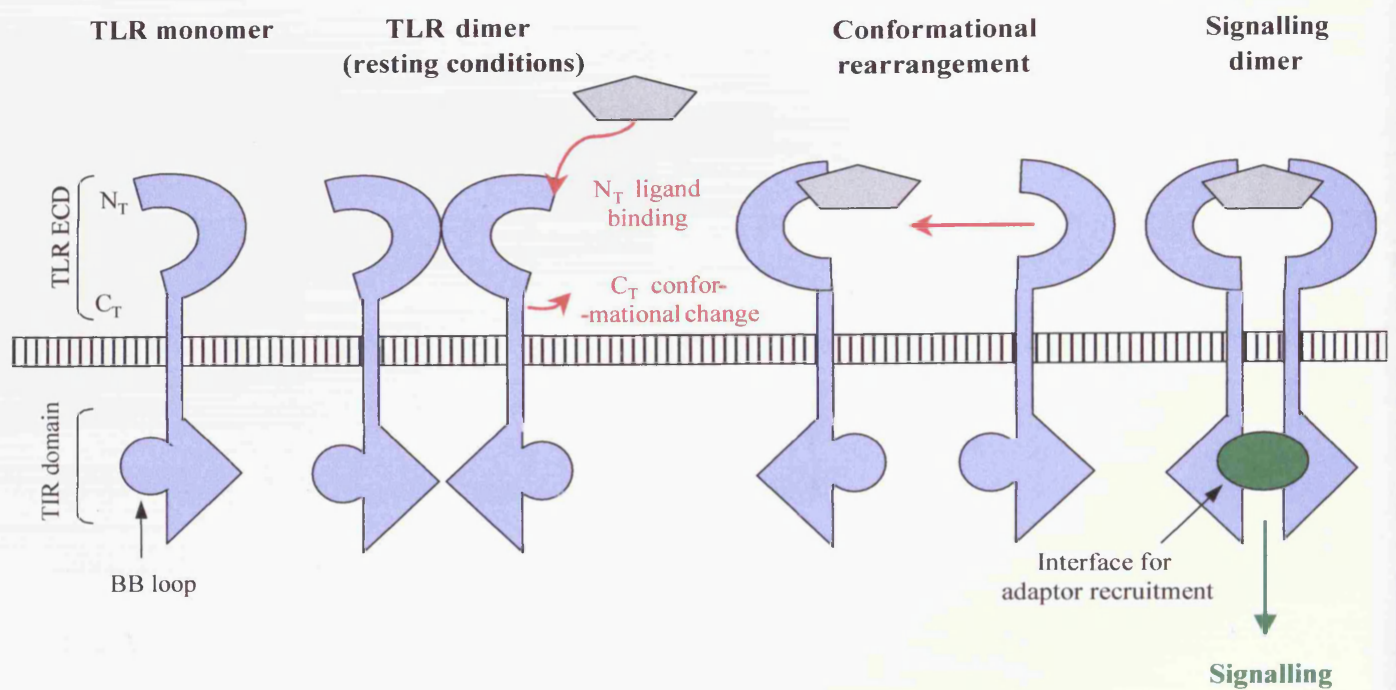
Figure 1.6. Model of the ligand-induced TLR conformational rearrangement
Binding of the ligand to the N-terminus domain of a TLR is thought to induce a conformational change in the C-terminus part of the ectodomain that allows for a successful contact between the two TLRs TIR domains. The BB loop of the signalling TIR domain is likely to be involved in receptor–receptor contacts. Formation of the signalling complex is proposed to generate a new interface for the recruitment of signal adaptors. ECD, ectodomain; C_T/N_T, C/N-terminus; TIR domain, Toll/interleukin-1 receptor domain; TLR, Toll-like receptor.

Spätzle must alter the receptor's conformation in order to relieve this constraint (Gay et al., 2006). Notably, point mutations in the C-terminal capping structure of the juxtamembrane region of Toll (Fig. 1.3) result in constitutive activation, showing that the integrity of this region is essential to control autodimerisation and signalling. Furthermore, while chimaeric Toll-TLR4 receptors containing the Toll juxtamembrane region are only activated in response to Spätzle, Toll-TLR4 receptors containing the TLR4 juxtamembrane region, although still activated by Spätzle, show a substantial constitutive activation (Weber et al., 2005). This suggests that there is an important role for the Toll juxtamembrane region in suppressing signalling activity and that there are significant mechanistic differences in the activation of the *Drosophila* Toll and TLR4 pathways.

Indication that a triggering mechanism similar to that used by Toll may be involved in TLR triggering comes from the fact that many TLR-specific antibodies can crosslink two TLR molecules together without inducing signalling (Shimazu et al., 1999; Matsumoto et al., 2002). This indicated that dimerisation is not sufficient to ensure signal transduction, and that correct orientation of the receptors with respect to each other may be required.

By considering these observations, Gay and co-workers (Gay et al., 2006), propose a model of sequential activation of TLRs in which ligand binding at the N-terminus induces a conformational change at the C-terminal region of the TLR ECD, and this change allows for a stable receptor-receptor interaction. This in turn promotes the rearrangement of the transmembrane helices of the receptor dimer so that downstream signalling is initiated (Fig. 1.6).

Fully efficient microbial recognition by some TLRs requires the activity of accessory molecules, also termed co-receptors. Sensitive cell response to most ligands activating via TLR2, 3 and 4 requires the activity of the co-receptor CD14, and TLR4 additionally requires the accessory molecule MD-2 for ligand recognition and responses.

- **CD14**

CD14 is a 55-kDa glycosyl-phosphatydil-inositol (GPI)-anchored cell-surface glycoprotein found on the surface of myeloid cells and, to a lesser extent, on B-lymphocytes and non-immune cells such as mammary cells, placental trophoblasts and gingival fibroblasts (Antal-Szalmas, 2000). The mature CD14 protein is 356 amino acid long, possesses seven LRR domains, nine cysteine residues and four potential N-glycosylation sites (Goyert et al., 1988).

In addition to the membrane bound form (mCD14), a soluble form of CD14 (sCD14) that lacks the GPI anchor has been detected in plasma – at μg/ml concentrations – and in human milk – at levels up to 100 times higher than in plasma (Durieux et al., 1994; Labéta et al., 2000). CD14 (mCD14 or sCD14) has been shown to increase sensitivity to stimulation via TLR2, 3 and 4 substantially (Frey et al., 1992; Schroder et al., 2003; Lee et al., 2006; Nakata et al., 2006) and mCD14 and sCD14 were found to bind to Gram-negative and Gram-positive cell-wall components (Akashi et al., 2003; Nakata et al., 2006). For example, LPS-induced monocyte activation can be blocked by some anti-CD14 antibodies in such a dramatic way that CD14 was first believed to be the main receptor for LPS, before the discovery of TLR4 (Wright et al., 1990). Later, the use of

CD14$^{-/-}$ animals helped to confirm the importance of CD14 for ligand recognition via TLR2, TLR3 and TLR4 *in vivo*. Indeed, animals lacking CD14 were found to be less sensitive to LPS (Haziot et al., 1996; Brass et al., 2007), or to live Gram-negative bacteria (Yang et al., 2002). Similarly, susceptibility to live *Listeria monocytogenes*, a pathogen recognised by TLR2, was found greatly increased in CD14-deficient mice (Janot et al., 2008).

dsRNA-induced IL-12p40 production and IFN-β promoter activity were also found dramatically reduced in CD14$^{-/-}$ animals (Lee et al., 2006), indicating that CD14 is also required for efficient TLR3 triggering.

The precise mechanism by which CD14 enhances TLR activation is still unclear, although several mechanisms have been proposed. It was first thought that CD14 may induce conformational changes in the TLR molecule leading to signal triggering (Schromm et al., 2001). A later study proposed a model in which CD14 helps loading LPS onto the TLR4/MD2 complex (Akashi et al., 2003). This is consistent with the model proposed by Nakata and co-workers to explain the enhancing effect of CD14 on TLR2 triggering (Nakata et al., 2006). Indeed, these authors concluded that CD14 helps the TLR2/TLR1 dimer to recognise triacylated lipopetides without binding to the complex. However, a direct interaction of sCD14 with TLR2 has been reported by our laboratory (LeBouder et al., 2003) and Iwaki and co-workers (Iwaki et al., 2005), and similar observations will be described in the present study. Similarly, the co-immunoprecipitation of sCD14 and TLR3 has been reported (Lee et al., 2006), thus demonstrating a direct interaction between the co-receptor and the signalling receptor.

In addition to its role in TLR activation, CD14 was also reported to modulate T and B cell responses (Rey Nores et al., 1999; Arias et al., 2000). The mechanism underlying such modulatory activity remains to be clarified.

- **MD-2**

Even in the presence of CD14, TLR4 transfected cells have still been found unable to respond to LPS (Shimazu et al., 1999). By sequence homology comparison with MD-1 – an accessory molecule of radioprotective 105 (RP105) involved in B-lymphocyte responses to LPS – a small protein, MD-2, was isolated and found to be required for TLR4 function (Shimazu et al., 1999). MD-2 is a secreted protein of about 18 kDa that contains two N-glycosylation sites and seven cystein residues. The interaction of MD-2 with TLR4 was first shown by co-immunoprecipitation (Shimazu et al., 1999), and has lately been confirmed by co-purification (Kim et al., 2007). According to this later study, the stoichiometry of the TLR4/MD-2 complex is 1:1, but a dimer of dimers is formed in the presence of LPS. In addition to binding to TLR4 with an apparent K_D of 12 nM, free MD-2 also binds to LPS, although with a weaker affinity (Visintin et al., 2005).

A number of *in vitro* studies have now demonstrated LPS hyporesponsiveness in cells expressing TLR4 alone or TLR4 and mutated MD-2. The importance of MD-2 has been confirmed *in vivo*, as mice lacking MD-2 did not respond to LPS, and were resistant to endotoxin shock (Miyake, 2003). Futhermore, inhibition of responses to LPS by targeting MD-2 has been proposed as a therapeutic strategy against septic shock and severe inflammation (Visintin et al., 2005). Like in the case of CD14, the mechanism by which MD-2 collaborates with TLR4 for signalling is still unclear. Kim and co-workers

A

LPS

N_T

N_T *

TLR4

TLR4 *

F126
H155

MD-2

C_T

C_T *

B

TLR4

TLR4*

LPS

C_T*

TLR4*

MD-2

N_T*

MD-2

N_T

N_T*

MD-2*

F126

F126*

N_T

MD-2*

TLR4

LPS*

C_T* C_T

C_T

90°

(Adapted from *Kim et al. 2007*)

Figure 1.7. Model of the TLR4/MD-2 tetramer after binding of LPS to MD-2
Grey and magenta surfaces represent TLR4 and MD-2, respectively. Elements of the second TLR4-MD-2 dimer of the complex are marked with an asterix. **A,** Schematic presentation of the MD-2/TLR4/LPS complex. Binding of LPS to MD-2 induces a conformational change in the F126-H155 region of MD-2 which results in the interaction of MD-2 with a second TLR4 molecule. **B,** Three dimensional representation of the model presented in A. N_T/C_T, N/C terminus.

proposed that LPS binding to MD-2, in a 1:1 complex with TLR4, induces a structural change in the Phe126 – His155 edge of MD-2, and that this change promotes the interaction of MD-2 with a second TLR4 (Kim et al., 2007 and Fig. 1.7).

Other than LPS, Taxol (a plant-derived anti-mitotic compound), hsp60 and fibronectin domain A have also been shown to require MD-2 to induce signalling via TLR4 (Hajjar et al., 2002; Miyake, 2003).

TLR heterodimerisation and collaboration with other PRRs

- **TLR heterodimerisation**

In addition to the heterodimerisation of TLR2 with TLR1 and TLR6 for ligand discrimination and signalling, there is evidence that other TLRs may also collaborate, although the exact conditions for collaboration are still unknown.

The observation that TLR4-negative/ TLR5-positive cells do not produce NO in response to flagellin, a TLR5 ligand, suggested a collaboration between TLR4 and TLR5 (Mizel et al., 2003). Consistent with this possibility, Mizel and co-workers also demonstrated the direct interaction between TLR4 and TLR5 by co-immunoprecipitation (Mizel et al., 2003).

A subsequent study showed that stimulation of mouse macrophages with both poly I:C, a TLR3 ligand, and CpG DNA, a TLR9 ligand, induced more than additive levels of TNFα, IL-6 and IL12p40, thus supporting the concept that co-operation between certain TLRs may occur (Whitmore et al., 2004).

- **Collaboration with other receptors**

There is growing evidence that TLRs may collaborate with other PRRs for PAMP recognition.

Overexpression of the β-glucan receptor, Dectin-1, was found to enhance zymosan- and live fungi-mediated TNF-α production in macrophages, while TLR2$^{-/-}$ macrophages were found unable to induce TNF-α production in response to zymosan (Brown et al., 2003). These findings, together with the fact that TLR2 and Dectin-1 co-localise at the cell surface, suggest that TLR2 and Dectin-1 collaborate for an efficient recognition of fungal pathogens or their components (Brown et al., 2003). Notably, Gantner and co-workers reported the collaboration between TLR2 and Dectin-1 for the zymosan-induced production of cytokines by macrophages, but also showed that TLR2 is not required for phagocytosis of zymosan and zymosan-induced ROS production (Gantner et al., 2003). These findings indicated that Dectin-1 can directly trigger phagocytosis and ROS production, and that different pathways are involved in pro-inflammatory responses to, and killing of fungal pathogens.

In addition to its role in anti-fungal response, Dectin-1 has also been shown to collaborate with TLR2 for mycoplasmal recognition (Yadav and Schorey, 2006; Shin et al., 2008). There is indication that collaboration of TLR2 and Dectin-1 may require physical interaction, as these two PRRs could be co-immunoprecipitated from mycobacterial-activated macrophages (Shin et al., 2008).

The observation that macrophages from CD36$^{-/-}$ animals produced less TNF-α than their wild-type counterparts in response to MALP-2 and LTA, but not to zymosan or the triacylated lipopeptide Pam$_3$Cys, or ligands for TLRs other than TLR2 (Hoebe et al., 2005), suggested that CD36 plays a role in cell activation mediated by the

TLR2/TLR6 complex (MALP-2- or LTA-induced). Notably, the LTA-induced secretion of TNF-α by CD36$^{-/-}$ macrophages was higher than that by TLR2$^{-/-}$ macrophages.. Furthermore, CD36$^{-/-}$ mice injected with live *S. aureus* are more susceptible and die faster than wild-type animals, but not as fast as TLR2$^{-/-}$ animals (Hoebe et al., 2005). Thus, CD36 appears to act as a facilitator for diacylglyceride recognition by the TLR2/TLR6 complex, but is not absolutely required for LTA/MALP-2 sensing by TLR2.

A recent study suggested that vitronectin and integrin-αvβ3 are also required for the recognition of bacterial lipopeptides (BLP) by TLR2. Vitronectin is a glycoprotein present in the extracellular matrix and in blood. It has been known to be involved in platelet aggregation, coagulation, and fibrinolysis (Preissner, 1991). It is also known to regulate mechanisms of the immune response such as cell migration and complement activation, and to opsonise bacteria (Chhatwal et al., 1987). The main receptor for vitronectin is integrin-αvβ3, which is expressed on many cell types, including monocytes. Integrin-αvβ3 participates in diverse immune processes such as cell migration, opsonisation of bacteria and clearance of apoptotic cells by phagocytes (Hynes, 2002; Scibelli et al., 2007).

It was found that vitronectin promotes BLP-induced TNF-α production by monocytes, and that this activity can be abrogated by blocking integrin-αvβ3 with a specific Ab (Gerold et al., 2008). These *in vitro* observations were consistent with the fact that monocytes from patients with Glanzmann thrombasthenia – who lack integrin-αvβ3 – were largely insensitive to BLP activation. Mechanistically, Gerold and co-workers demonstrated that vitronectin can interact with BLP, and that TLR2 and integrin-αvβ3 form a complex that dissociates after BLP stimulation. The authors propose a model in

which vitronectin concentrates TLR2 agonists at the signalling complex composed of TLR2 and integrin-$\alpha_V\beta_3$. BLP is then transferred to TLR2, and integrin-$\alpha_V\beta_3$ dissociates and is degraded.

2) TLR signalling pathways

Following ligand binding, the formation of TLR oligomers results in the recruitment of specific signalling adaptors to the TLRs' cytoplamic TIR domain (O'Neill, 2006). The TIR domain is between 135 and 160 amino acids long, and its molecular structure – resolved for the TIR of TLR1, TLR2 and TLR10 – shows a central, five-stranded, parallel β-sheet which is surrounded by a total of 5 α-helices on both sides (Xu et al., 2000). In terms of sequence, each TIR domain contains three conserved regions named Box 1, Box 2 and Box 3. The Box 1 sequence is common to all TLRs, Box 2 contains a large and conserved surface with consensus sequences ((F/Y)-(V/L/I)-(P/G)) present in all TLRs as well as in the adaptor MyD88 – the BB loop – , and Box 3 contains amino acids that have been identified as important for IL-1R signalling. Notably, a proline to histidine natural point mutation of the conserved proline of the TLR4 BB loop in C3H/HeJ mice was shown to be responsible for the absence of signal transduction via TLR4 in these animals (Poltorak et al., 1998). This was the first indication of the importance of the BB loop in TLR signal transduction. The BB loop has emerged as key to the TIR domain-mediated homotypic protein–protein interaction (Bartfai et al., 2003). However, a computer-predicted docking model (Fig. 1.8, A) suggested that the BB loop of TLR1 interacts with the DD loop of TLR2 – another loop located at the opposite side of the BB loop – , therefore leading to heterodimerisation and subsequent signalling initiation (Gautam et al., 2006). The first

A

TLR1

TLR2

C_T

N_T

N_T

C_T

BB loop

DD loop
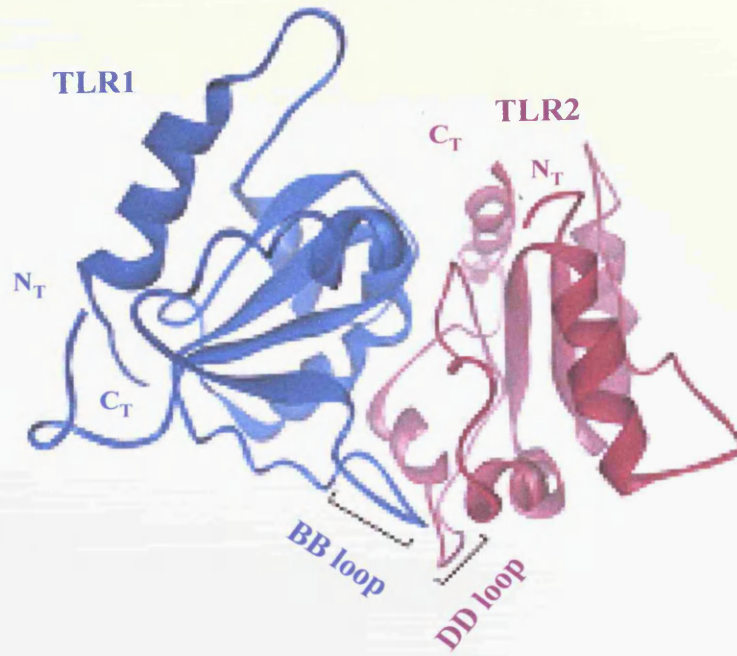
(Adapted from *Gautam et al. 2006*)

B

TLR10

TLR10

DD loop

C_T

BB loop   BB loop

N_T
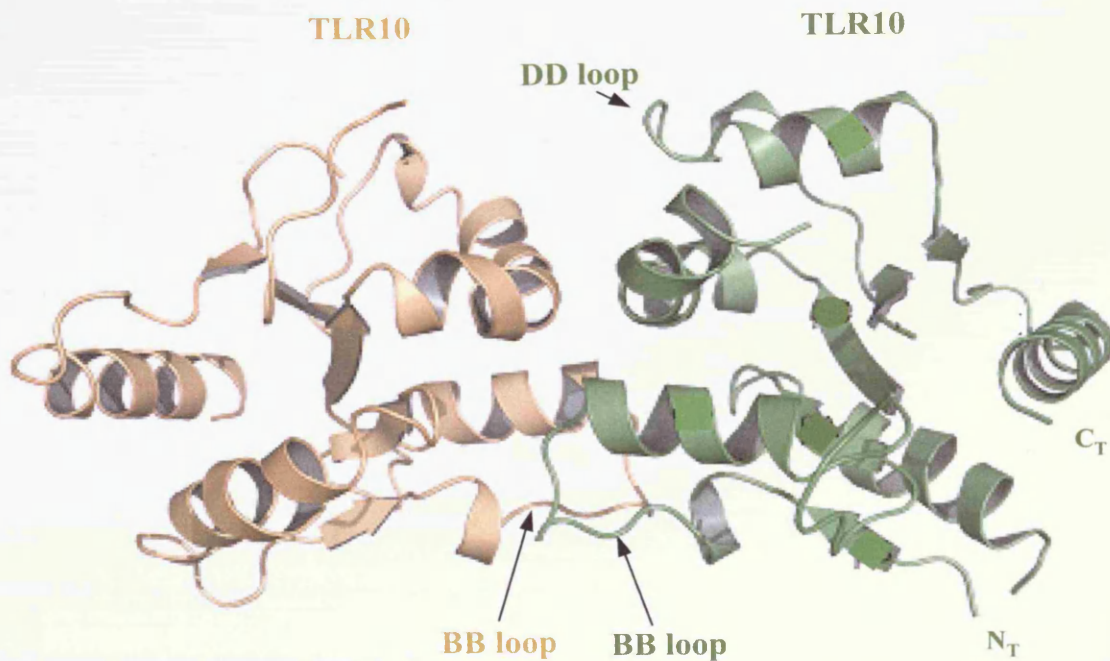
(Adapted from *Nyman et al. 2008*)

Figure 1.8. Computer-predicted models of asymmetrical and symmetrical interactions of TLR TIR domains **A,** Model of asymmetrical heterodimerisation of the TIR domain of TLR1 (blue) with the TIR domain of TLR2 (purple). The ribbon diagram shows structural features, including the proximity of the TLR1 BB to the TLR2 DD loop. **B,** Model of symmetrical homodimerisation of the TIR domains of two TLR10 molecules (beige and green). The ribbon diagram shows structural features, including the proximity of the two TLR10 BB loops. Part of the DD-loop of the left TLR10 monomer is disordered and not resolved, thus, it is not shown. N_T/C_T, N/C-terminus.

direct structural data regarding the formation of a symetrical TIR-TIR domain upon TLR homodimerisation was obtained following the resolution of the human TLR10 cytoplasmic domain crystal structure. Based on this structure (Fig. 1.8, B), two TLR10 TIR domains are predicted to interact through their BB loops (Nyman et al., 2008). Thus, it is possible that TLR homodimerisation involves only TLR BB loops, while TLR asymmetrical heterodimerisation involves the BB loop of one receptor and the DD loop of the other receptor (Fig. 1.8).

It is now well established that the TIR-TIR platform formed by the dimerisation of two TLRs promotes homotypic protein-protein interactions with additional cytoplasmic adapter molecules, resulting in an active signalling complex. These adaptors initiate a chain of phosphorylation and ubiquitination events that lead to activation of the transcription factor NF-κB, mitogen-activated protein kinases (MAPKs), and interferon-response factors (IRFs) (Watts, 2008).

To date, five TLRs adaptor proteins have been identified: 1) MyD88, 2) MyD88 adaptor-like (Mal; also known as TIR domain-containing adaptor protein (TIRAP)), 3) TIR domain-containing adaptor inducing IFNβ (TRIF; also known as TIR-containing adapter molecule-1 (TICAM-1)), 4) TRIF-related adaptor molecule (TRAM; also known as TICAM-2), and 5) sterile-alpha and HEAT-Armadillo motifs-containing protein (SARM). The differential recruitment of these adaptor proteins to the TLRs forms the basis of the specificity in the signalling processes activated by TLRs.
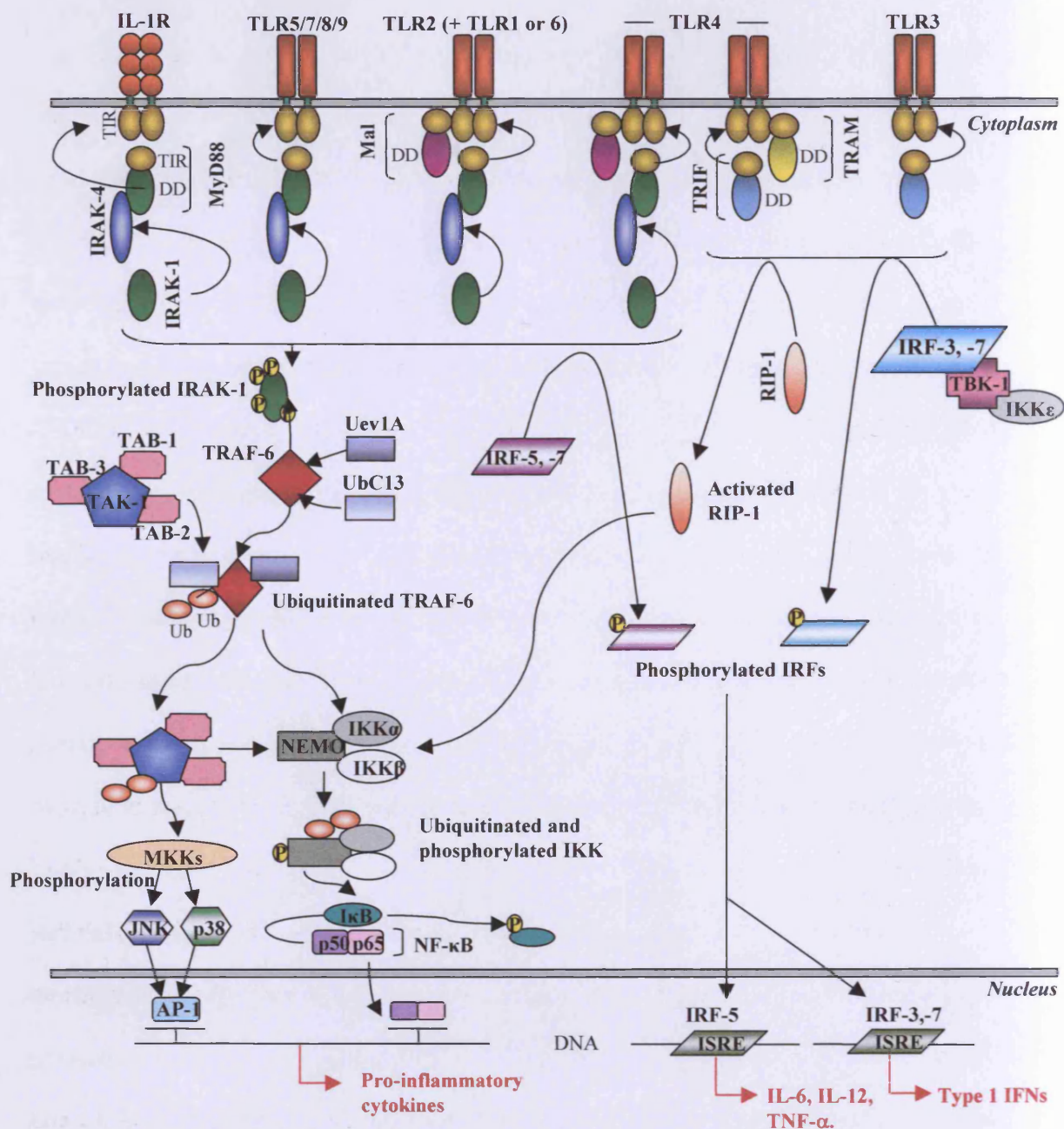
Fig 1.9. TLR signalling pathways
Schematic representation of the MyD88- and TRIF-dependent pathways for TLR signalling *(see description in the text)*. AP-1, activating protein-1; DD, death domain; IκB, inhibitor of NF-κB; IKK, IκB kinases; IL, interleukin; IL-1R, interleukin-1 receptor; INF, interferon; IRAK, interleukin-receptor-associated kinase; IRF, interferon-response factor; ISRE, IFN-stimulated response element motifs JNK, c-jun-N-terminal kinase; Mal, MyD88 adaptor-like; MKKs, mitogen-activated protein-kinase kinases; MyD88, myeloid differentiation primary response protein 88; NEMO, NF-κB essential modulator; NF-κB, nuclear factor-kappa B; RIP-1, receptor interacting protein-1; TAB, TAK-1 binding protein; TAK-1, TGF-β-activated kinase-1; TBK-1, TRAF-family member-associated-NF-κB activator (TANK)-binding kinase-1; TIR, Toll/interleukin-1 receptor domain; TLR , Toll-like receptor; TNF-α, tumour necrosis factor-α; TRAF, TNF-α-receptor-associated factor-6 TRAM, TRIF-related adaptor molecule; TRIF, TIR domain containing adaptor inducing INF-β; Ub C13, ubiquitin-conjugating enzyme 13; Uev 1A, ubiquitin-conjugating enzyme E2 variant 1.

The MyD88-dependent signalling pathway

The TLR adaptor MyD88 was first found expressed in myeloid tissues, and its RNA levels were used as a marker for differentiation, hence its name, as MyD stands for Myeloid Differentiation, and 88 is the number of the gene expressing the MyD88 protein. MyD88 was the first adaptor described as a member of the Toll/IL-1 receptor family (Hultmark, 1994), and its function was found to be crucial for signalling induced by several TLRs (Bonnert et al., 1997; Medzhitov et al., 1998). By using MyD88-deficient mice, it was demonstrated that MyD88 is involved in TLR signalling in response to different microbial components: LPS and PGN from different strains of bacteria, cell wall preparations from the Gram-positive bacteria *S. aureus,* whole extract from *M. tuberculosis* (Takeuchi et al., 2000b), and live *S. aureus* (Takeuchi et al., 2000a). This confirmed the involvement of MyD88 in TLR2- and TLR4-induced signalling. It is now known that MyD88 mediates signalling for all TLRs, except for TLR3 (Doyle and O'Neill, 2006 and Fig. 1.9).

MyD88 contains a death domain (DD) at its N-terminus and a TIR domain at the C-terminus. The DD domain of MyD88 interacts with the BB loop of TLRs cytoplasmic domain (Gautam et al., 2006). Following TLR ligand-induced binding of MyD88 to the TLR dimer, MyD88 recruits the first kinase of the signalling cascade, interleukin-receptor-associated kinase-4 (IRAK-4). The presence of IRAK-4 is required for the recruitment of IRAK-1, and mediates its phosphorylation (Fig. 1.9). Phosphorylated IRAK-1 then dissociates from the TLR/MyD88/IRAK-4 complex, and associates with TNF-α-receptor-associated factor-6 (TRAF-6). It was demonstrated using deletion mutants that efficient LPS signalling requires the activity of another member of the IRAK family, IRAK-2 (Zhang et al., 1999). Recently, the use of IRAK-2 loss-of-

function mutants revealed that IRAK-2 was involved in NF-κB activation via TLR2, 5, 7, 8 and 9. IRAK-2 was shown to interact with TRAF-6 directly, and initiate its polyubiquitination (Keating et al., 2007). Unlike IRAK-1, however, IRAK-2 was found unable to activate interferon response factors (IRFs).

TRAF-6, the next signalling intermediate after IRAK, links the IL-1R/IRAKs or TLR/IRAKs complexes with the activation of the NF-κB and the MAPK cascade. It interacts with the ubiquitin-conjugating enzyme E2 variant 1 (Uev1A) and enzyme 13 (Ubc13), becomes polyubiquitinated, oligomerises, and consequently becomes activated (Chen, 2005). Subsequently, activated TRAF-6 associates with four downstream proteins: TGF-β-activated kinase-1 (TAK-1), and the TAK-1 binding proteins-1, -2 and -3 (TAB-1, TAB-2, TAB-3). The formation of this complex leads to the ubiquitination of TAK-1, and ubiquitinated TAK-1 in turn phosporylates the inhibitor of NF-κB (I-κB) kinases (IKK) complex. IKK is also ubiquitinated by the Uev1/Ubc13/TRAF-6 complex (Doyle and O'Neill, 2006). The IKK complex consists of three subunits, IKKα, IKKβ and IKKγ (Brikos and O'Neill, 2008). IKKγ (also called NF-κB essential modulator, NEMO), is a scaffold protein for the other subunits, and links the complex to I-κB. IKKα and IKKβ are the kinases of the complex, and their role is to phosphorylate I-κB proteins that are associated with NF-κB in the cytoplasm of resting cells. NF-κB consists of a dimer of the subunit p50 − which links NF-κB to I-κB − and the subunit p65 − that is needed for transactivation of gene expression. Phosphorylation of I-κB by IKKα and IKKβ liberates NF-κB, which then translocates to the nucleus where it binds to and activate the promoters of genes responsible for inflammatory responses (Fig. 1.9 and Doyle and O'Neill, 2006).

Notably, the cytoplasmic adaptor IRAK-1 can also be found in the nucleus, and a recent study suggests that nuclear IRAK-1 binds to the promoter of the NF-κB-regulated gene, *IκB-α*, and enhances binding of the NF-κB p65 subunit to NF-κB responsive elements within the *IκB-α* promoter (Liu et al., 2008).

TAK-1 also activates the MAPKs cascade. TAK-1 phosphorylates MAPK kinases (MKKs), which in turn phosphorylate and activate the MAPKs p38 and c-jun-N-terminal kinase (JNK). Activated p38 and JNK then activate the transcription factor, activating protein-1 (AP-1) that, like NF-κB, initiates the transcription of genes coding for pro-inflammatory cytokines (Fig. 1.9).

In addition to this general signalling pathway, the MyD88-dependent pathway also activates members of the interferon response factor (IRF) family (Fig. 1.9). Indeed, after TLR7 or TLR9 triggering, IRF-7 has been found to associate with the MyD88/IRAK-4/IRAK-1 complex. Most likely upon phosphorylation by IRAK-1, IRF-7 translocates to the nucleus where it binds IFN-stimulated response element motifs (ISRE), thus activating type-1 IFNs production (Uematsu et al., 2005). Similarly, IRF-5 was shown to associate with MyD88 and TRAF-6, after TLR4 and TLR9-induced cell activation. Subsequently, it moves to the nucleus, binds to ISRE motifs, and induces the production of IL-6, IL-12 and TNF-α.

The observation that NF-κB activation in response to TLR4 agonists in mice lacking MyD88 was only delayed initiated the search for other adaptors involved in TLR signalling (Doyle and O'Neill, 2006).

Mal (also known as TIRAP) was identified based on its sequence similarity with MyD88, and was initially shown to have a role in TLR4 signalling, because a mutation

in the BB loop of Mal could inhibit TLR4-, but not IL-1R-induced signalling. In addition, Mal-deficient animals behaved similarly to those MyD88-deficient, as they showed a delayed response to TLR4 agonists. Notably, Mal $^{-/-}$ mice responded similarly to wild-type animals when challenged with IL-1 or any other TLR ligand except for those for TLR2. In fact, the lack of Mal was found to cause a more pronounced effect on signalling via TLR2 than via TLR4 (Brikos and O'Neill, 2008). Recent studies have shown that Mal possesses a binding domain for a major component of the plasma membrane, phosphatidylinositol-4,5-biphosphate (PIP-2) (Kagan and Medzhitov, 2006). The interaction of Mal with PIP-2 facilitates the recruitment of MyD88 to the TLR4 complex (Kagan and Medzhitov, 2006). In addition, and unlike MyD88, Mal can associate with TRAF-6, and thus appears to be responsible for the recruitment of TRAF-6 to the TLR2 and TLR4 signalling complexes.

Although Mal was shown to be part of the TLR4 MyD88-dependent activation pathway, animals lacking both MyD88 and Mal were still capable of responding, although with some delay, to a challenge via TLR4 (Yamamoto et al., 2002). This suggested that, in addition to TLR3, TLR4 may also signal through an MyD88-independent pathway.

MyD88-independent signalling: the TRIF pathway

Following the observation that TLR3 and TLR4 can signal independently of MyD88, Yamamoto and co-workers searched databases for TIR-containing proteins, and identified TRIF as the essential adaptor for MyD88-independent TLR signalling (Yamamoto et al., 2002). Overexpression of TRIF resulted in a similar activation of NF-κB to that via MyD88/Mal. However, unlike MyD88/Mal, activation of TRIF also leads to the activation of the IFN-β promoter. Recently, cytokine production in animals

lacking TRIF was found reduced in response to TLR4 activation, but unaffected following TLR2, TLR7 or TLR9 triggering (Brikos and O'Neill, 2008). In addition, TRIF/MyD88 double mutant mice were unable to activate NF-κB, showing that TRIF was responsible for the delayed activation of NF-κB in MyD88$^{-/-}$ animals.

In order to signal downstream the TLR3 and TLR4 pathways, TRIF forms a complex with the IKK-like kinase termed TRAF-family-member-associated-NF-κB activator (TANK)-binding kinase 1 (TBK-1), the IKK homologue IKKε, and IRF-3 or IRF-7 (Fig. 1.9). The formation of this complex results in the phosphorylation of IRF-3 or IRF-7 by TBK-1 and IKKε, and the activated IRF then binds to INF-stimulated response element motifs (ISRE) on its target genes, inducing the production of class I IFNs. Similarly, TBK-1 and IKKε phosphorylate IRF-5, which then bind to ISREs and initiates IL-6, IL-12 and TNF-α.

In TLR3 signalling, TRIF has also been shown to activate NF-κB through its binding to the TRAF-6/TAK-1/TAB-2 complex which activates IKK. However, the exact role of TRAF-6 in NF-κB activation through TRIF is still unclear. To signal to NF-κB, TRIF also interacts with the receptor interacting protein-1 (RIP-1), which then binds to and activates the IKK complex (Fig. 1.9).

A major difference between the activation of the TRIF pathway by TLR3 and TLR4 is that TLR3 interacts with TRIF directly, whereas TLR4 requires the presence of the fourth adaptor protein, TRAM. Thus, TRAM is also a bridging adaptor, like Mal, but it is only utilised by TLR4 (Doyle and O'Neill, 2006; Brikos and O'Neill, 2008). Thus, unlike TLR3, which signals exclusively through TRIF, TLR4 is the only TLR to activate both the MyD88 and TRIF pathways. There is now evidence that TLR4 does induce the MyD88/Mal and TRIF/TRAM pathways sequentially, rather than

MyD88 pathway

TRIF pathway

soluble TLRs, soluble MD-2

TLR

TLR

Triad-3, TGF-β, GM-CSF (?),
IL-4, IFN-γ, TNF-α

SIGIRR, A46R

Cytoplasm

sMyD88, TGF-β,
NS5A, ST-2, A46R

SARM, TRAF-1,-4, RIP-3
PIASy, NS3/4A, A46R

MyD88

TRIF

ST-2, A46R

Mal

A46R

TRAM

IRAK-4

RIP-3

RIP-1

TKB-1

SIKE,
SHP-2

IRAK-M, TOLLIP,
SOCS-1, SHP-1

IRAK-1

IKKε

SIKE

SIGIRR, TRAF-4
A-20, A52R

TRAF-6

IRF-3

TAK-1

PI3K

MKKs

IKK

IRF-4

IRF-5

IRF-7

IRF-3

PIASy

JNK   p38

IκB-NF-κB

TRAILR

Nucleus

AP-1

NF-κB

Pro-inflammatory cytokines

IL-6, IL-12, TNF-α

Type-1 INFs

Fig 1.10. Negative regulation of TLR activation

Schematic representation of the TLR signalling pathways (green) and their regulators whose targets are known (red; *see description in the text*). AP-1, activating protein-1; DD, death domain; GM-CSF, granulocyte-macrophage colony-stimulating factor; IκB, inhibitor of NF-κB; IKK, IκB kinases; IL, interleukin; INF, interferon; IRAK, interleukin-receptor-associated kinase; IRF, interferon-response factor; ISRE, IFN-stimulated response element motifs JNK, c-jun-N-terminal kinase; Mal, MyD88 adaptor-like; MKKs, mitogen-activated protein-kinase kinases; MyD88, myeloid differentiation primary response protein 88; NEMO, NF-κB essential modulator; NF-κB, nuclear factor-kappa B; NS, HCV non-structural proteins; PIASy, protein inhibitor of the activated signal transducer and activator of transcription y; PI3K, Phosphoinositide 3-kinase; RIP, receptor interacting protein; SARM, sterile-alpha and HEAT-Armadillo motifs-containing protein; SHP, Src homology containing tyrosine phosphatase; SIGIRR, single immunoglobulin IL-1R-related molecule; SIKE, supressor of IKKε; SOCS-1, suppressor of cytokine signaling-1; TAK-1, TGF-β-activated kinase-1; TBK-1, TRAF-family member-associated-NF-κB acivator (TANK)-binding kinase 1; TGF-β, transforming growth factor-β; TIR, Toll-interleunkin-1 receptor domain; TLR , Toll-like receptor; TNF-α, tumour necrosis factor-α; TOLLIP, Toll-interacting protein; TRAF, TNF-α-receptor-associated factor-6; TRAILR, TNF-related apoptosis-inducing ligand (TRAIL) receptor; TRAM, TRIF-related adaptor molecule; TRIF, TIR domain containing adaptor inducing INF-β.

simultaneously. Kagan and co-workers proposed that following an initial phase of MyD88-dependent signalling, TLR4 is internalised and starts off a second phase, TRIF-dependent, signalling via TRIF molecules located in the endosomes (Kagan et al., 2008).

SARM – the fifth and latest TLR adaptor to be identified – acts as a negative regulator of TLR signalling (Carty et al., 2006). Therefore, it will be described in the next section, dedicated to the regulators of TLR activation.

3) Regulation of TLR activation

Activation of the immune system has to be tightly regulated to avoid excessive and inappropriate responses, which could be detrimental to the host. In the case of TLRs, excessive TLR-mediated responses resulting from overactivation of the receptor or dysregulation of intracellular signalling pathways may lead to severe, acute and chronic inflammatory conditions, such as myocardial dysfunction, respiratory, renal and multiorgan failure, septic shock, arthritis, asthma and autoimmunity (Zuany-Amorim et al., 2002; O'Neill, 2003). Therefore, TLR-mediated responses are controlled by a number of mechanisms, including: apoptosis of the activated cells, reduction of TLR expression, prevention of ligand binding to its TLR, and inhibition of TLR signalling (Fig. 1.10).

TLR regulation by apoptosis

TLRs can also function as death receptors, and TLR-induced apoptosis plays a role in the control of TLR response. Indeed, TLR4 was the first TLR to be reported as a

death receptor, as it was demonstrated that LPS can induce macrophage apoptosis (Choi et al., 1998). Similarly, the interaction of TLR2 with microbial agonists triggers apoptosis in macrophages and epithelial cells (Aliprantis et al., 1999). A loss-of-function mutation of MyD88 – but not TRAF-6 or IKK-β – was found to inhibit TLR2-mediated cell apoptosis, indicating that the TLR signalling pathway to NF-κB and the TLR apoptotic pathway bifurcate at the level of MyD88. (Aliprantis et al., 2000). The authors provided further insight into the mechanism involved in the TLR apoptotic pathway by showing that MyD88 can interact with and recruit FADD (Aliprantis et al., 2000). FADD then binds to pro-caspase 8 and induces enzyme maturation and subsequent cell death, as previously described (Muzio et al., 1998). A more recent study suggested that NF-κB activation and induction of cell apoptosis by TLRs were not simultaneous, but sequential phenomenons. Indeed, TLR2 ligand recognition was found to induce MyD88-mediated NF-κB activation as an early event, and MyD88/FADD-mediated cell apoptosis as a later event (Into et al., 2004).

Activation of apoptosis via TLRs may serve different purposes: the clearance of activated cells can help to reduce the level of inflammation, and may help in host defence by destroying the host-infected cells.

Reduction of TLR expression

Reduction of TLR expression can be achieved by protein degradation, mainly through ubiquitination. Triad-3A – the most abundant member of the Triad-3 family of RING-finger E3 ligases – was found to bind to the cytoplasmic domain of TLR4 and TLR9, but not TLR2 (Chuang and Ulevitch, 2004). Triad-3A promotes ubiquitination of TLR4 and TLR9 and their subsequent proteolytic cleavage. Consistent with these

findings, it was demonstrated that overexpression of Triad-3A significantly reduces TLR activation in response to LPS (TLR4 ligand), CpG DNA (TLR9 ligand), but not to bacterial lipopeptide (TLR2 ligand). Thus, Triad-3A selectively modulates activation of certain TLRs by promoting their degradation through ubiquitination.

Reduction of TLR levels can also be achieved by inhibiting their expression. Indeed, the anti-inflammatory cytokine transforming growth factor-β (TGF-β) has been shown to suppress LPS-mediated function by down-regulating TLR4 expression (McCartney-Francis et al., 2004). Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been found to influence TLR2 expression. Treatment with GM-CSF was found to reduce the expression of TLR2 on granulocytes (Flo et al., 2001). However, Kurt and co-workers reported an increase in TLR2 expression on neutrophils upon treatment with GM-CSF (Kurt-Jones et al., 2002). By contrast, these authors and Flo and co-workers found increased TLR2 expression on macrophages and monocytes following GM-CSF treatment (Flo et al., 2001; Kurt-Jones et al., 2002). IL-4, IFN-γ and TNF-α were found to reduce TLR2 expression on monocytes (Flo et al., 2001).

Very low levels of TLR expression are observed in intestinal epithelial cells, this is believed to ensure tolerance, or a low-level response to commensal bacteria in the gut. However, the mechanism by which commensal bacteria (or their products) inhibit TLR expression remains to be clarified.

Soluble TLRs as decoy microbial receptors

A powerful way of negatively regulating TLR-induced pro-inflammatory responses is to reduce TLR triggering by preventing the interaction of the receptor with

its ligand. Soluble forms of TLR (sTLR) 2, 3, and 4, have been shown to inhibit activation of their repective membrane-bound counterparts.

- **Soluble TLR4**

The first observation on the existence and activity of a soluble form of a TLR was made by Iwami and co-workers (Iwami et al., 2000). Although there is only one copy of the *TLR4* gene, several TLR4 mRNA isoforms can be detected. In an attempt to identify these different isoforms, Iwami and co-workers screened a mouse macrophage cDNA library. In addition to the published mouse TLR4 cDNA sequence, a cDNA containing a 144-bp insertion between the reported second and third exon sequences was detected. This 144-bp sequence was found in the reported genomic DNA, and matched the AG/GT rule, suggesting that it was produced by alternative splicing. This novel exon contained an in-frame stop codon at 110 pb, and the predicted protein sequence indicated that the alternatively spliced cDNA encodes a 122-amino acid protein, whose first 86 amino acids were indentical to those of the TLR4 ECD. In order to study the activity of this putative soluble protein, the alternatively spliced cDNA was cloned, Flag-tagged, and transfected into CHO cells. A soluble 20-kDa protein was detected in culture supernatants using an anti-Flag mAb. This sTLR4-encoding cDNA was also introduced in a mouse macrophage cell line, and proved to be capable of inhibiting LPS-induced NF-κB activation and TNF-α production by these cells. It remains to be established, however, whether this putative soluble TLR4 protein fragment is naturally expressed and released by normal mouse cells.

- **Soluble TLR2**

A soluble form of human TLR2 (sTLR2) was first detected and characterised by our laboratory (LeBouder et al., 2003; Heggelund et al., 2004; Ueland et al., 2006). Unlike sTLR4, sTLR2 occurs naturally, as it is constitutively released by normal monocytes, and present in normal human plasma, breast milk (LeBouder et al., 2003), saliva (Kuroishi et al., 2007), mouse peritoneal lavage fluids (this study), and plasma, as well as bovine and porcine plasma (our unpublished observations). Other than sTLR2, no naturally occurring soluble form of a mammalian TLR has so far been identified. Western blot analysis showed six sTLR2 isoforms (25 to 83 kDa) present in human plasma, with a major polypeptide band of ~66 kDa. Because blood monocytes express the highest levels of membrane-bound TLR2 (mTLR2), it was speculated that they are the main source of sTLR2 in plasma. Consistent with this possibility, Western blot analysis of culture supernatants from freshly-isolated human monocytes showed a sTLR2 pattern similar to that of plasma sTLR2. Similar experiments failed to demonstrate the release of detectable amounts of sTLR2 by tonsillar B cells, B cell lines and peripheral T cells (LeBouder et al., 2003).

RT-PCR and Southern blot analyses of human monocyte RNA did not show the presence of splice variants, deletions or mutations of the *TLR2* gene, indicating that sTLR2 originates from a post-translational modification of mTLR2. Indeed, following cell-surface monocyte biotinylation, biotinylated sTLR2 was detected in the cell culture supernatant, and increased levels of sTLR2 correlated with lower levels of mTLR2 over the time following cell activation. Furthermore, monensin – an antibiotic that blocks processing events taking place in internal acidic compartments – was found to affect the release of sTLR2, and an intracellular pool of sTLR2 was found in monocytes

(LeBouder et al., 2003). Together, these findings indicated that sTLR2 results from post-translational modification of the TLR2 protein, most likely by proteolytic cleavage of endocytosed mTLR2.

Notably, although non-activated monocytes constitutively produce sTLR2, *in vitro* activation was found to increase the kinetics of sTLR2 release. Thus, cell activation seems to modulate the production of sTLR2 (LeBouder et al., 2003). Modulation of sTLR2 levels *in vivo* during infection was also observed (LeBouder et al., 2003; Heggelund et al., 2004; Kuroishi et al., 2007).

The biological activity and physiological relevance of naturally occurring sTLR2 was evaluated by depleting sTLR2 from plasma using a TLR2-specific mAb. It was found that sTLR2-depleted plasma confers higher sensitivity to TLR2-mediated PBMC stimulation (LeBouder et al., 2003), indicating that plasma sTLR2 may act as a natural regulator of cell activation via TLR2.

Given that a direct interaction of the TLR2 ECD with many TLR2 agonists has been demonstrated (see section 1.3.3.B.2, pages 25, 26 and 30), it is possible that sTLR2 inhibits mTLR2-mediated responses by competing for the ligand, thus acting as a decoy microbial receptor. However, the fact that sTLR2 and sCD14 could be co-immunoprecipitated from breast milk and plasma (LeBouder et al., 2003), indicated that they may naturally associate, and suggested that sTLR2 may also modulate TLR2 triggering by binding to CD14 and, thus, disrupting its co-receptor function. The mechanism underlying sTLR2's activity was addressed in more details in the present study.

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- **Soluble TLR3**

Cloning of the cDNA coding for the human TLR3 ECD and subsequent transfection in HEK293 cells led to the production of a 110-kDa soluble protein termed hTLR3 ECD (Sun et al., 2006). Consistent with the demonstrated activity of sTLR4 and sTLR2, hTLR3 ECD was also found to inhibit mTLR3-mediated NF-κB activation in response to the synthetic ligand poly(I:C). However, the biological relevance of this finding is not evident, as there is no indication that a soluble form of TLR3 naturally occurs.

- **Soluble TLR5**

Like the mammalian sTLR2, the existence of a TLR5-like soluble protein naturally present in the rainbow trout and a variety of other fish species was reported (Tsujita et al., 2006). By using a recombinant form of sTLR5, Tsujita and co-workers showed that sTLR5 can directly bind to the TLR5 ligand, flagellin. Interestingly, sTLR5 was not found to inhibit, but to enhance flagellin-induced TLR5-mediated cell stimulation. The authors proposed that sTLR5 binds to flagellin and then helps its recognition by mTLR5. sTLR5 has not been found in humans. The presence of sTLR5 in the rainbow trout may reflect a prototype of humoral immunity in the fish, which may be required for a very sensitive recognition of flagellated microorganisms (Tsujita et al., 2006).

- **Soluble MD-2**

As MD-2 is essential for LPS recognition via TLR4, and has been found to directly interact with LPS, Kuroki and co-workers sought the possibility of dampening TLR4 triggering using a soluble recombinant form of human MD-2 (srMD-2) together

with sTLR4 (Mitsuzawa et al., 2006). They showed that sTLR4 does not interact with the lipid A fraction of LPS, whereas srMD-2 does, and that the binding of lipid A to srMD-2 increases when srMD-2 is in a complex with sTLR4. Consistent with this observation, they found that sTLR4 did not inhibit LPS-induced NF-κB activation and IL-8 release by a mouse macrophage cell line. srMD-2, however, had a slight inhibitory effect on its own, and this inhibitory effect was enhanced in the presence of sTLR4. *In vivo*, the administration of the complex srMD-2/sTLR4 reduced LPS-induced neutrophil recruitment and TNF-α production in a mouse model of pulmonary inflammation (Mitsuzawa et al., 2006). Whether natural sMD-2 regulates mTLR4-mediated responses *in vivo* remains to be evaluated.

Inhibition of TLR signalling

TLR-mediated responses can also be controlled through several intracellular negative regulators of TLR signalling.

- **SARM**

SARM is a TIR domain-containing protein, and is the latest TLR adaptor so far identified. Unlike other TIR-containing adaptors, SARM acts as an inhibitor of NF-κB and IRF activation in response to TLR triggering. Indeed, the knock-down of SARM expression using small interfering RNA (siRNA), leads to enhanced production of pro-inflammatory cytokines upon stimulation with TLR3 and TLR4 agonists (Carty et al., 2006). In addition, cell stimulation via TLR3 or TLR4 results in an enhanced expression of SARM. Thus, it is likely that SARM is involved in a negative feedback mechanism preventing prolonged and excessive pro-inflammatory responses via TLRs.

It is known that SARM can interact directly with the signalling adaptor TRIF, but whether SARM inhibits TRIF activation directly, or by favouring the interaction of TRIF with another inhibitory molecule is still unclear (Brikos and O'Neill, 2008; Carty et al., 2006).

- **SIGIRR**

The single immunoglobulin IL-1R-related molecule (SIGIRR), is a cell membrane-bound, TIR domain-containing protein, which has been shown to inhibit IL-1R and TLR4 signalling. Indeed, initial studies showed that overexpression of SIGIRR inhibited NF-κB activation in response to IL-1, suggesting that SIGIRR could also act as an inhibitor of IL-1R-related receptors, like TLRs. This was confirmed *in vivo*, as SIGIRR-deficient mice showed enhanced inflammatory responses to LPS and IL-1 (Wald et al., 2003). Furthemore, NF-κB and JNK activation were enhanced in splenocytes from SIGIRR$^{-/-}$ animals in response to both LPS (TLR4 agonist) and CpG DNA (TLR9 agonist), indicating that the SIGIRR inhibitory effect is not limited to one TLR (Wald et al., 2003). SIGIRR was found to co-immunoprecipitate with TLR4, 5, 9 and IL-1R, suggesting a TIR-TIR domain interaction. SIGIRR was also shown to interact with the TLR signalling intermediate, TRAF-6. Thus, it is thought that SIGIRR inhibits TLR activation by preventing the recruitment of signalling adaptors to the TLR TIR domain (Wald et al., 2003). Alternatively, SIGIRR may inhibit the dissociation of the activated signalling components from the receptor, thus reducing the activation of downstream signalling events.

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- **ST-2**

ST-2 is another member of the TIR domain-containing family of receptors. It was first identified as crucial to Th2-cell activity (Townsend et al., 2000). Regarding TLR signalling, overexpression of ST-2 leads to inhibition of MyD88-dependent signalling induced via IL-1R and TLR4, but not TLR3 (Brint et al., 2004). Consistent with this finding, IL-1, LPS, lipopeptides and CpG DNA, but not poly I:C, were found to induce higher levels of pro-inflammatory cytokines by macrophages from ST-2-deficient animals (Brint et al., 2004). A recombinant form of ST-2 has been shown to interact with Mal and MyD88, but not TRIF, thus it is likely that ST-2 inhibits MyD88-dependent TLR signalling by sequestering its adaptors (Brint et al., 2004).

- **TRAILR**

TNF-related apoptosis-inducing ligand (TRAIL) receptor (TRAILR) is a membrane-bound receptor for TRAIL – a member of the TNF family of cytokines that promotes apoptosis. It does not contain a TIR domain, as it belongs to the TNF-α receptors superfamily. Both TRAIL- and TRAILR-deficient animals show increased levels of IL-12, IFN-β and IFN-γ upon infection with mouse CMV (TLR2 ligand), and TRAILR-deficient macrophages were found to produce higher levels of pro-inflammatory cytokines in response to activation via TLR2, 3 or 4 (Diehl et al., 2004). TRAILR seems to inhibit TLR signalling by stabilising IκB, thus reducing the nuclear translocation of NF- κB (Diehl et al., 2004; Liew et al., 2005).

- **Soluble MyD88**

Soluble MyD88 (sMyD88) is a naturally-occurring alternatively-spliced variant of MyD88 that contains its death domain, but lacks the domain involved in binding to IRAK-4. Overexpression of sMyD88 inhibits IL-1 and LPS induced NF-κB activation, and promotes the formation of MyD88-sMyD88 complexes. As sMyD88-complexed MyD88 cannot bind to IRAK-4, this kinase is not recruited to the signalling complex, thus IRAK-1 cannot be phosphorylated and activate downstream processes (Liew et al., 2005).

- **TRAF-1 and TRAF-4**

Another adaptor-mediated inhibition of TLR signalling involves two members of the TRAF family, TRAF-1 and -4. Unlike TRAF-6, which is essential for TLR signalling, TRAF-1 and -4 play inhibitory roles (Brikos and O'Neill, 2008). Overexpression of TRAF-1 inhibited TRIF- and TLR3-mediated activation of NF-κB, ISRE and IFN-β promoter (Su et al., 2006). TRAF-1 has been shown to bind to TRIF, which then induces cleavage of TRAF-1. Consequently, TRAF-1 becomes activated and inhibits TRIF-dependent signalling (Su et al., 2006).

TRAF-4 can interact not only with TRIF, but also with TRAF-6, and it has been found to prevent NF-κB activation induced via a number of TLRs, including TLR2, 3, 4 and 9, as well as TLR3 and TLR4-induced IFN-β promoter activation (Takeshita et al., 2005).

- **IRAK-M, IRAK-2c and IRAK-2d**

In humans, four members of the IRAK family have been identified: IRAK-1, IRAK-2, IRAK-M and IRAK-4. IRAK-1, IRAK-2 and IRAK-4 have been shown to

participate in the MyD88-dependent pathway of TLR signalling. IRAK-M – whose expression is restricted to monocytes and macrophages (Wesche et al., 1999) – is an inactive kinase, because it lacks a critical aspartate residue in the catatalytic site. IRAK-M knock-out mice produced markedly higher levels of pro-inflammatory cytokines than the wild-type after a challenge with LPS or CpG DNA (Kobayashi et al., 2002). This indicated a negative role of IRAK-M in, at least, TLR4 and TLR9 signalling. In addition, expression of IRAK-M was found increased by LPS, suggesting that IRAK-M is involved in a negative regulatory feedback in TLR4 signalling (Kobayashi et al., 2002). Although the exact mechanism of IRAK-M activity has not been determined, it is known that it does not interfere with the binding of IRAK-1 to the TLR/MyD88/IRAK-4 signalling complex, but inhibits the formation of the IRAK-1/TRAF-6 complex. It is believed that IRAK-M blocks the dissociation of IRAK-1 from MyD88/IRAK-4, thus preventing the interaction of IRAK-1 with TRAF-6 (Kobayashi et al., 2002).

Four splice variants of the mouse (but not human) *Irak2* gene have been reported: *Irak2a, Irak2b, Irak2c* and *Irak2d* (Hardy and O'Neill, 2004). Overexpression of IRAK-2a and b have been shown to enhance LPS-induced NF-κB activation, whereas IRAK-2c and d had an inhibitory effect (Hardy and O'Neill, 2004). LPS-activation enhanced the expression of IRAK-2c, but not IRAK-2a, by a mouse macrophage-like cell line, suggesting a negative regulatory feedback of IRAK-2c in TLR4 signalling. However, as no animal models for IRAK-2 have been generated, the physiological relevance of IRAK-2's regulatory activity has not been fully assessed.

- **TOLLIP**

Overexpression of the Toll-interacting protein (TOLLIP) results in reduction of IL-1R- (Burns et al., 2000), TLR2- and TLR4-mediated NF-κB activation (Bulut et al., 2001; Zhang and Ghosh, 2002). TOLLIP has been found to naturally associate with IRAK-1 in resting cells (Burns et al., 2000). This association results in TOLLIP phosphorylation by IRAK-1, which leads to the release of TOLLIP from the TOLLIP/IRAK-1 complex, thus, allowing subsequent signalling and ubiquitination of IRAK-1 (Zhang and Ghosh, 2002). However, IRAK-1 ubiquitination promotes its degradation and thereby may also inhibit TLR-signalling. In view if these findings, the physiological effect of TOLLIP was addressed using TOLLIP-deficient mice. *Ex vivo* experiments showed that cell activation in response to TLR3, 4, 5, and 9 agonists was normal in DCs and lymphocytes from TOLLIP$^{-/-}$ animals, suggesting that TLR-mediated activation is TOLLIP-independant (Didierlaurent et al., 2006). *In vivo* experiments, however, suggested that TOLLIP controls the magnitude of IL-1R- and TLR4-mediated cytokine production. Indeed, Didierlaurent and co-workers showed that TOLLIP$^{-/-}$ animals produce less IL-6 and TNF-α in response to relatively low doses of IL-1β or LPS. However, TOLLIP regulatory activity was not observed when a lethal concentration of LPS was used. Given the apparent lack of consistency of the *in vitro*, *ex vivo* and *in vivo* results, the authors suggest that TOLLIP 'fine tunes' optimal signalling through IL-1R and TLRs, and predict that the concentration of TOLLIP may be critical to its capacity to regulate signalling (Didierlaurent et al., 2006).

- **PI3K**

Phosphoinositide 3-kinase (PI3K) is a heterodimer consisting of a p85 regulatory subunit and a p110 catalytic chain. IL-12 production in response to TLR2, 4 and 9 ligands was found markedly increased in DCs from animals lacking PI3K, and p85-deficient mice showed enhanced TLR signalling (Fukao et al., 2002). Although the exact mechanism of PI3K activity is unclear, it is believed that PI3K blocks the MAPK pathway. PI3K positively regulates the activation of protein kinase B, a kinase that phosphorylates MAPK kinase kinase (MAPKK-K) apoptosis signal-regulating kinase-1 (ASK-1). Phosphorylation of ASK-1 suppresses its activity, which is required for the activation of upstream regulators of p38, MAPK kinases-3 (MKK-3) and MKK-6. Therefore, inhibition or lack of PI3K was found to upregulate p38 activity in DCs (Fukao and Koyasu, 2003). *In vivo*, inhibition of PI3K resulted in increased serum levels of IL1-β, IL-2, IL-6, IL-10, IL-12, and TNF-α in septic mice, and was associated with enhanced disease severity, and decreased survival outcome (Williams et al., 2004). Thus, PI3K may play a pivotal role in the maintenance of homeostasis and the integrity of the immune response via TLRs.

- **SOCS-1**

Several members of the suppressor of cytokine signalling (SOCS) family have been shown to suppress cytokine signalling. The macrophages of SOCS-1^{-/-} animals produce higher levels of NO and pro-inflammatory cytokines upon stimulation with the TLR4 and TLR9 agonists LPS and CpG DNA, respectively. In addition, both LPS and CpG DNA enhanced the expression of SOCS-1 in macrophages, suggesting a negative feedback mechanism (Kinjyo et al., 2002; Liew et al., 2005). The crucial role

of SOCS-1 in the negative regulation of inflammatory processes is highlighted by the fact that mice lacking SOCS-1 die within three weeks of birth due to multi-organ inflammation (Starr et al., 1998). It has been demonstrated that SOCS-1 reduces phosphorylation of IκB, p38 and JNK, thus blocking downstream signalling most likely by targeting IRAK-1 (Kinjyo et al., 2002).

■ **RIP-3**

RIP-3, like RIP-1, is a member of the receptor interacting proteins, and can also associate with TRIF. Unlike RIP-1, RIP-3 cannot induce downstream signalling, on the contrary, RIP-3 was shown to inhibit TLR signalling in two ways. Firstly, RIP-3 can phosphorylate RIP-1, thus inhibiting NF-κB activation (Meylan et al., 2004). Secondly, the authors also showed that RIP-3 acts as an inhibitor of TRIF-induced NF-κB activation by competing with the binding of TRIF to RIP-1 (Meylan et al., 2004).

■ **IRF-4**

As mentioned previously (section 1.3.3.B.3.2), IRF-5 and -7 associate with MyD88, leading to the production of type I IFNs and pro-inflammatory cytokines. IRF-4 can also bind to MyD88, but this interaction does not result in downstream signalling, on the contrary, it prevents the formation of the MyD88/IRF-5 complex. However, IRF-4 does not impair the binding of IRF-7 to MyD88. Thus it blocks specifically MyD88/IRF-5-mediated signalling, most likely by impairing the translocation of IRF-5 to the nucleus, as it was shown for TLR9 induced signalling (Negishi et al., 2005). The inhibitory activity of IRF-4 in TLR signalling was confirmed *in vivo* by the observation that IRF-4-deficient mice are hypersensitive to DNA-induced shock and show elevated

serum levels of pro-inflammatory cytokines (Negishi et al., 2005). IRF-4 appears to be involved in a negative feedback mechanism, as its mRNA levels increase upon cell stimulation with agonists of TLR4, 7 and 9 (Negishi et al., 2005).

- **TGF-β**

TGF-β has been shown to down-regulate TLR4 expression (section 1.3.3.B.3.3). It is also known that TGF-β induces ubiquitination of MyD88 and its subsequent proteosomal degradation. Consistent with this observation, TGF-β was found to act as an intracellular signalling regulator, as it inhibits the MyD88-dependent pathway in response to ligands for TLR2, 4 and 5, whereas the TRIF-dependent pathway of TLR4-mediated activation is not affected (Naiki et al., 2005).

- **PIASy**

A member of the protein inhibitor of the activated signal transducer and activator of transcription (PIAS) family, PIASy, has been shown to suppress TRIF-induced NF-κB activation, as well as TRIF-, IRF-3- and IRF-7 induced ISRE activation (Zhang et al., 2004b). PIASy was found to interact with TRIF, IRF-3 and IRF-7 (Zhang et al., 2004b), suggesting that PIASy may regulate TLR activation by seggregating these three signalling intermediates (Zhang et al., 2004b; Brikos and O'Neill, 2008).

- **SIKE**

The supressor of IKKε (SIKE) interacts with both TBK-1 and IKKε, and blocks their respective association with TRIF and IRF-3. As a consequence, SIKE

inhibits ISRE and IFN-β promoter activation via TLR3, but not the TLR3-induced NF-κB activation (Huang et al., 2005).

- **SHP-1 and SHP-2**

Src homology-2 (SH-2)-containing tyrosine phosphatase (SHP)-1 and -2 have been shown to modulate TLR activation. SHP-2 was first found to negatively regulate TLR4- and TLR3-mediated IFN-β production, but without affecting the activation of TLR2, 7 and 9 (An et al., 2006). SHP-2 was also found to inhibit poly(I:C)-induced activation of MAP kinase pathways (An et al., 2006). In addition, SHP-2 was shown to interact directly with the kinase domain of TBK-1, suggesting that SHP-2 specifically regulates TRIF-mediated gene expression in TLR signalling, at least in part, by inhibiting TBK1-activated signal transduction (An et al., 2006). SHP-1 was found to inhibit the production of pro-inflammatory cytokines in response to agonists of TLR2, 3, 4 and 9 *in vitro*. Furthermore, SHP-1 deficiency resulted in inhibition of TLR3-mediated production of IFN-β (An et al., 2008). Mechanistically, SHP-1 was found to bind to and block the activity of the TLR signalling intermediate IRAK-1, thus inhibiting the activation of NF-κB and MAPKs. An and co-workers also demonstrated that inactivation of IRAK-1 promotes the activation of MyD88- and TRIF-dependent production of IFN-β, which is consistent with the fact that SHP-1 deficiency impaired IFN-β production. The authors also provided evidence of an opposite regulatory effect of SHP-1 and SHP-2 in TLR signal transduction through a cross-regulation mechanism by which endogenous SHP-2 'antagonises' SHP-1-mediated positive regulation of TLR-activated IFN-β expression by reducing the expression of mRNA encoding SHP-1 (An et al., 2008).

- **MUC-1**

The transmembrane mucin-like glycoprotein-1 (MUC-1 in humans, muc-1 in other species) is expressed in epithelial cells lining various mucosal surfaces as well as in hematopoietic cells. *Ex vivo* experiments have revealed that peritoneal macrophages, alveolar macrophages and tracheal surface epithelial cells from muc-1$^{-/-}$ animals were more sensitive than their wild-type counterparts to ligands for TLR2, 3, 4, 5, 7 and 9 (Ueno et al., 2008). The cytoplasmic tail of MUC-1 – but not its extracellular domain – was found critical to MUC-1 regulatory activity, suggesting that MUC-1 targets TLR intracellular signalling, rather than extracellular ligand recognition. Although the exact mechanism involved in MUC-1 inhibitory ability remains to be determined, Ueno and co-workers propose that MUC-1 interacts with and sequesters TLR signalling intermediates, thus inhibiting TLR-mediated NF-κB activation (Ueno et al., 2008).

- **A-20**

A-20 is a cysteine protease deubiquitinating cytoplasmic enzyme that inhibits TLR signalling. Macrophages from the A-20-deficient mice were found to produce higher levels of pro-inflammatory cytokines in response to TLR2, 3, 4 and 9 agonists. A-20 expression is up-regulated by LPS. These observations indicate that A-20 may be involved in a negative feedback mechanism preventing TLR overactivation. A-20 was demonstrated to cleave the ubiquitin chain of TRAF-6, which is essential for its activity. Because TRAF-6 is utilised by all members of the TLR family, A-20 inhibits NF-κB activation via both the MyD88 and TRIF-dependent pathways (Liew et al., 2005).

- **Virus-derived inhibitors of TLR signalling**

A number of viral proteins have been shown to be used by the invading pathogens to prevent TLR signalling. They include, A46R, A52R and the HCV non-structural proteins (NS)-3, -3/4A, -4B and -5A.

A46R and A52R are two viral proteins expressed by the vaccinia virus that contribute to virulence and suppresion of host defence (Harte et al., 2003; Stack et al., 2005). A46R contains a TIR domain and can associate with MyD88, Mal, TRAM, TRIF and TLR4 through its TIR domain and, thus, is capable of impairing signalling through TLR2, 4, 5, 7 and 9, as well as of inhibiting IRF-3 activation via TLR3 (Stack et al., 2005).

A52R, which does not contain a TIR domain, can interact with IRAK-2 and TRAF-6, disrupting signalling complexes containing these proteins and, consequently, suppressing NF-κB activation by TLR2, 3, 4 and 5 (Harte et al., 2003).

Overexpression of NS3, NS3/4A, NS4B and NS5A in macrophages was found to inhibit MyD88-dependent TLR signalling in response to PGN (TLR2), LPS (TLR4), R837 (TLR7) and CpG DNA (TLR9) (Abe et al., 2007). In addition, expression of NS5A inhibited the TLR-mediated MAPK cascade. NS5A was found to interact with the death domain of MyD88, and inhibit recruitment of IRAK-1 to MyD88, thus, blocking subsequent signalling (Abe et al., 2007). Notably, NS3/4A was also found to inhibit dsRNA-induced IFN production, by cleaving the adaptor molecule TRIF, thereby disrupting signalling via TLR3 (Li et al., 2005).

The different levels at which TLR activity can be regulated (summarised in Fig. 1.10) highlight the importance of such regulation to the maintenance of immune homeostasis

4) TLR-based therapeutic strategies

Given that TLRs are central to innate immunity and also control, directly or indirectly, the extent and quality of the adaptive immune response, they have become a major target for the development of novel therapeutics which, depending on the pathological condition, aim at strengthening or dampening the immune response.

Therapeutic applications of TLR agonists

TLR agonists are mainly used to strengthen or re-establish an immune response.

- **Vaccine adjuvants**

The use of TLR agonists as vaccine adjuvants has been extensively evaluated. Indeed, linkage of a TLR agonist to an antigen can increase antigen uptake by DCs and facilitate antigen processing, antigen presentation, and the subsequent development of the adaptive immune response. At present, a number of prophylactic and therapeutic TLR-based vaccines against viral infections (hepaptitis B virus, human papilloma virus, human immunodeficiency virus, influenza virus), cancer (melanoma, lung cancer) and general vaccine adjuvants are being developed and evaluated by pharmaceutical companies (Kanzler et al., 2007).

- **Cancer therapy**

TLR stimulation may be beneficial to cancer therapies by enhancing: innate immunity, T-cell activity, cytotoxic antibody function, and apoptosis of TLR positive tumours.

Triggering of the innate immune response induces innate antitumour mechanisms, such as activation of NK cells, monocytes and macrophages, and production of antitumoural

Table 1.4. Principal TLR targeting therapeutics under development

Company	Compound	Target	Indication	Status
3M	Aldara (imiquimod) cream	TLR7/8 agonist	Actinic keratosis; superficial basal cell carcinoma; external genital warts	Marketed
	Follow on compounds	TLR7/8 agonist	Human papilloma virus; cancer	Phase I
Coley	ProMune (CPG 7909)	TLR9 agonist	Melanoma;other cancers	Entering Phase III
	Actilon (CPG 10101)	TLR9 agonist	Hepatitis C virus; infectious diseases	Entering Phase III
	AVE7279 and AVE 0675	TLR9 agonist	Asthma; other respiratory diseases	Phase I
	Vaximmune (vaccine adjuvant)	TLR9 agonist	Cancer; anthrax; biowarfare	Phase I
Dynavax	AIC (1018 ISS + ragweed allergen)	TLR9 agonist	Ragweed allergy	Phase II/III
	1018 ISS + Hepatitis B virus antigen	TLR9 agonist	Hepatitis B virus vaccine	Entering Phase III
Anadys	ANA245 (isatoribine)	TLR7 agonist	Hepatitis B virus; Hepatitis C virus; cancer	Phase II
	ANA975 (prodrug of ANA245)	TLR7 agonist	Hepatitis B virus; Hepatitis C virus	Phase I
Hybridon	IMOxine (HYB2055) vaccine adjuvant	TLR9 agonist	Renal cell carcinoma; cancer	Phase II
	Amplivax (HYB2903) Vaccine adjuvant	TLR9 agonist	Human immunodeficiency virus; vaccine adjuvant	Phase I
Eisai	Eritoran (E5564)	TLR4 antagonist	Severe sepsis	Phase III
Takeda Pharmaceutical Company	TAK-242	TLR4 antagonist	Severe sepsis	Phase III
Mologen	dSLIM	TLR9 agonist	Cancer	Phase I/II
Corixa	CRX-675	TLR4 agonist	Seasonal allergic rhinitis	Phase I
	CRX-567	TLR4 agonist	Infectious diseases	Phase I
Vaxinnate	Preclinical candidates	TLR5 agonists; others	N/A	Preclinical

cytokines such as IFN-α, IFN-γ and TNF-α. Activation of innate antitumoural mechanisms may also promote, indirectly, T-cell activity by increasing the release of antigens by the dying tumor cells. Efficient induction of tumour cell apoptosis via TLRs has been demonstrated *in vitro* for TLR9$^+$ chronic lymphocytic leukemia cells (Jahrsdorfer et al., 2005) and TLR3$^+$ breast carcinoma cells (Salaun et al., 2006).

To date, several TLR ligands have shown significant promise for the treatment of cancer, and the TLR7 agonist Imiquimod – developed by 3M Pharma – is now approved for the treatment of basal cell carcinoma (Hoffman et al., 2005 and Table 1.4).

▪ Antimicrobial therapy

The use of TLR3, 7, 8 and 9 agonists has shown promise as therapeutic strategies against infectious – in particular viral – diseases. TLR activation by agonists leads to the production of antiviral type I IFNs. The TLR-induced enhancement of NK cell cytotoxicity and virus-specific T-cell responses play an equally important role in the resolution of the infection. Small agonists of TLR7 are the most advanced, and Imiquimod has been approved for the treatment of genital warts caused by the human papilloma virus. ANA-975, an oral prodrug of isatoribine from Anadys, is being tested for the treatment of chronic hepatitis C virus, although trials were suspended when toxicity developed in long-term animal studies (Fletcher et al., 2006 and Table 1.4). Ligands for TLR3 are also being developed, mainly for targeting the human immunodeficiency virus. The use of TLR agonists as prophylactic treatments to increase resistance to and protect from microbial infections is also being considered, e.g. the TLR9 ligand, CpG-oligodeoxynucleotide (ODN) (Kanzler et al., 2007).

■ **Allergic diseases**

Although inadequate TLR activation is thought to be involved in different forms of allergies (Hoffman et al., 2005), the use of TLR agonists can, paradoxically, be beneficial to allergic patients. A general feature of allergies is an inappropriate Th2 cell response to harmless environmental antigens. It is known that Th2 cell development and activity can be inhibited by Th1 cytokines, and that TLRs can induce strong Th1 responses (section 1.3.3.B.2.3). Thus, TLR agonists could, indirectly, inhibit inadequate Th2 responses causing allergies. Ligands for TLR4 and TLR9 are being evaluated for the treatment of allergic rhinitis (Table 1.4). The administration of CpG-ODN has been shown to shift the Th2/Th1 ratio, and attenuate allergic reactions in patients allergic to ragweed. CpG-ODN has also been proven to be efficient against asthma in both rodent and primate models (Serebrisky et al., 2000; Fanucchi et al., 2004).

Therapeutic applications of TLR antagonists

Most therapeutic strategies to dampen TLR responses use TLR antagonists to inhibit TLR ligand recognition, and are based on the use of structural analogs of agonists that bind to the TLR but fail to signal, or of TLR blocking Abs, or small molecules antagonists selected from compound libraries. Alternative strategies use small molecules to block intracellular TLR signalling.

■ **Microbial inflammation**

Bacterial-induced inflammation may lead to sepsis and septic shock when TLR activation gets out of control. Two lipid A analogs acting as potent TLR4

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antagonists are being tested for the treatment of Gram-negative sepsis, Eritoran and TAK-242 (Table 1.4).

- **Autoimmunity**

One of the challenges of the innate immune system is to differentiate self from non-self. To date, endogenous ligands have been described for most TLRs. They originate mainly from damaged or infected cells and tissues, and their recognition is normally beneficial to the host. However, activation of TLRs by damaged host tissue may lead to a cycle of chronic activation and tissue damage by TLR-activated effector mechanisms (Kanzler et al., 2007). In such cases, it is reasonable to believe that the use of TLR antagonists would reduce self-induced TLR activation. For example, systemic lupus erythematosus is an autoimmune disease driven by TLR3, 7 and 9, and bifunctional ODN – blocking both TLR7 and TLR9 – have proven to be an efficient strategy to control autoimmunity in animal models that remains to be tested in humans (Barrat et al., 2005).

Sterile inflammation (inflammation not caused by pathogens) occuring during bleomycin-induced lung inflammation and ischaemia-reperfusion have also been shown to be driven by TLRs recognition of ligands from dying cells. Bleomycin-induced lung inflammation is mediated, at least in part, by chemokines and cytokines generated by TLR2 and TLR4 recognition of hyaluronan fragments derived from the extracellular matrix (Jiang et al., 2005). Similarly, TLR2 and TLR4 appear to contribute to ischemia-reperfusion injury, although the specific ligand has not been identified (Mollen et al., 2006). Thus, appropriate TLR2 and TLR4 antagonists may represent a new class of therapeutics for this type of autoimmune driven inflammation (Kanzler et al., 2007).

Thus, the TLR family of receptors is currently being considered as a major therapeutic target for the treatment of a variety of pathological conditions. In particular, novel TLR-based therapeutic approaches against acute and chronic microbial-induced inflammatory conditions aim at reducing TLR-mediated pro-inflammatory responses without eliminating the capacity of TLRs to recognise the pathogen and clear the infection.

1.4 Hypotheses and aims of the project

Following the description of a naturally occurring soluble form of TLR2 by our laboratory, the assessment of sTLR2's activity as a negative regulator of TLR responses and the evaluation of sTLR2's potential as a therapeutic against inflammatory conditions have become the focus of intense research in our laboratory.

1.4.1 Hypotheses

Given that sTLR2 consists of most of TLR2's extracellular domain, which is involved in ligand binding, it was hypothesised that sTLR2 may play a critical role in the control of TLR2-mediated responses by acting as a decoy bacterial receptor, thus negatively regulating mTLR2 triggering (LeBouder et al., 2003; Liew et al., 2005; Kuroishi et al., 2007). Furthermore, given that sTLR2 and sCD14 naturally associate − as they co-immunoprecipitate from plasma and milk − , it is possible that sTLR2 regulates mTLR2 signalling not only by acting as a decoy receptor, but also by interacting with CD14 (mCD14 or sCD14) and disrupting its co-receptor activity. If this possibility were confirmed, identifying the region(s) within the TLR2 ECD involved in

the interaction with CD14 would make possible the design of soluble peptides incorporating such region(s). It was hypothesised that, by binding to CD14, these peptides may disrupt CD14's co-receptor activity (reducing pro-inflammatory responses) while preserving TLR2's capacity to respond to microbial ligands.

1.4.2   Aims and objectives

This study thus sought to fully assess the negative regulatory capacity of sTLR2, shed light on the underlying mechanism, and evaluate the possibility of targeting the TLR co-receptor, CD14, to reduce pro-inflammatory responses. Specifically it was planned to:

I.   Use the baculovirus expression system to generate sufficient amounts of human recombinant sTLR2 to allow for a full assessment of sTLR2's biological activity.

II. Evaluate the extent of sTLR2's regulatory capacity *in vitro*, and *in vivo* by using two established mouse models of peritoneal inflammation.

III. Use biochemical and cell imaging approaches to study the mechanism underlying sTLR2's regulatory activity.

IV. Identify the region(s) of the TLR2 ECD that are involved in the interaction with CD14, design peptides incorporating such region(s) and evaluate their capacity to disrupt CD14's co-receptor activity.

2. RESULTS

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ESSNQASLSC DRNGICKGSS GSLNSIPSGL TEAVKSLDLS NNRITYISNS DLQRCVNLQA LVLTSNGINT IEEDSFSSLG
SLEHLDLSYN YLSNLSSSWF KPLSSLTFLN LLGNPYKTLG ETSLFSHLTK LQILRVGNMD TFTKIQRKDF AGLTFLEELE
IDASDLQSYE PKSLKSIQNV SHLILHMKQH ILLLEIFVDV TSSVECLELR DTDLDTFHFS ELSTGETNSL IKKFTFRNVK
ITDESLFQVM KLLNQISGLL ELEFDDCTLN GVGNFRASDN DRVIDPGKVE TLTIRRLHIP RFYLFYDLST LYSLTERVKR
ITVENSKVFL VPCLLSQHLK SLEYLDLSEN LMVEEYLKNS ACEDAWPSLQ TLILRQNHLA SLEKTGETLL TLKNLTNIDI
SKNSFHSMPE TCQWPEKMKY LNLSSTRIHS VTGCIPKTLE ILDVSNNNLN LFSLNLPQLK ELYISRNKLM TLPDASLLPM
LLVLKISRNA ITTFSKEQLD SFHTLKTLEA GGNNFICSCE FLSFTQEQQA LAKVLIDWPA NYLCDSPSHV RGQQVQDVRL
SVSECHRLEV LFQGPLHHHH HH
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587

B
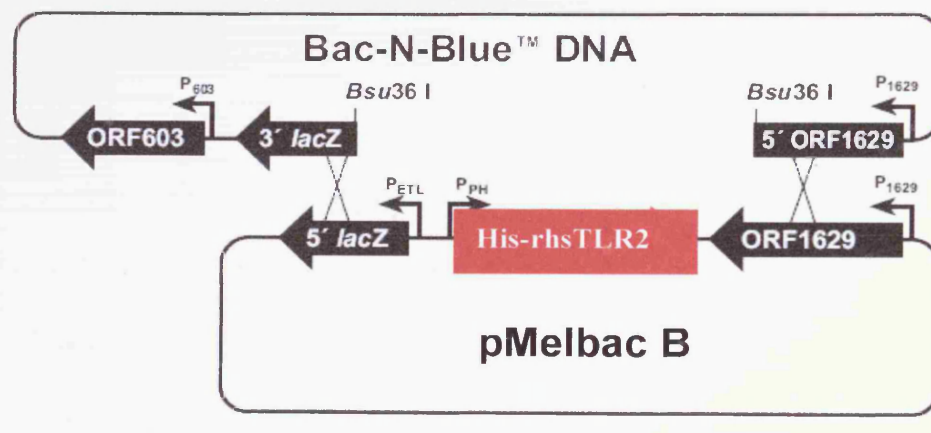
Figure 2.1.1. Generation of viral DNA coding for His-rhsTLR2
A, Amino acid sequence of 6x histidine-tagged recombinant human soluble TLR2 (His-rhsTLR2). The sequence encompasses amino acids Glu[21] to Arg[587] of the human TLR2 protein (black), a cleavage sequence (green), and a 6x histidine tag (red). **B,** Phenomenon of homologous recombination: transfer of the His-rhsTLR2 coding cDNA sequence from the pMelBac B vector to the viral Bac-N-Blue ™ DNA.

2.1 Production and purification of recombinant human soluble TLR2

The assessment of the modulatory capacity of sTLR2 required a considerable amount of purified and biologically active soluble protein. Therefore, the first phase of this work consisted in the generation of sufficient amounts of recombinant human soluble TLR2 (rhsTLR2). It consisted of the full TLR2 ECD, thus resembling the main naturally occurring sTLR2 forms found in plasma and milk (LeBouder et al., 2003).

2.1.1 Generation of recombinant human sTLR2

The full-length human TLR2 cDNA – previously prepared in our laboratory (LeBouder et al., 2003) – was used as a template to prepare a construct coding for the putative human TLR2 ECD (Glu^{21}-Arg^{587}) and incorporating a 6x histidine tag at the N-terminus (Fig. 2.1.1, A and *Materials and Methods*, section 4.8.1, page 129). The His-rhsTLR2 cDNA construct was introduced in the pMelBac B plasmid, and co-transfected with the viral DNA (Bac-N-Blue™ DNA) into *Spodoptera frugiperda* (Sf9) insect cells (*Materials and Methods*, section 4.8.2, page 131). A sequence encompassing the polyhedrin promoter (to ensure high transcription levels), the honeybee-melitin signal (for high secretion levels) and the in-frame His-rhsTLR2 cDNA was incorporated into the viral DNA by homologous recombination (Fig. 2.1.1, B). Following a series of low-multiplicity infections to increase the viral titre, High Five insect cells were infected with Sf9 culture supernatants of the optimal viral titre (typically, between 5 x 10^7 and 2 x 10^8 PFU/ml) and cultured for three days before the culture supernatant was collected and processed in preparation for His-rhsTLR2 purification, as described under

Generation of a viral vector containing His-rhsTLR2 cDNA

Transfection of Sf9 cells with the His-rhsTLR2 cDNA-containing plasmid and the viral DNA
Homologous recombination (Fig. 2.1.1)

Collection of supernatant from Sf9 transfectants

Viral titration and selection of the cells infected with the His-rhsTLR2 cDNA-containing virus,
(plaque assays)

Increase of viral titre in culture supernatants by low-multiplicity infections of Sf9 cells

Production of His-rhsTLR2

High-multiplicity infection of High Five insect cells with Sf9 supernatant containing
the recombinant virus

Harvest of High Five culture supernatant

Purification of His-rhsTLR2

Concentration and dialysis (binding buffer) of High Five culture supernatant

Metal-affinity chromatography (Ni-NTA)

Resin washing (10 mM Imidazole)

Elution of His-rhsTLR2 (250 mM Imidazole)

Dialysis (PBS) of pooled fractions

Final preparation

Protein concentration assay/ SDS-PAGE and Coomassie blue staining/
anti-TLR2 and anti-His immunoblotting, cell activation test

Figure 2.1.2. Summary of the strategy used for the production of His-rhsTLR2
Diagram showing the main steps for the production of His-rhsTLR2 (see detailed description under *Materials and Methods*, section 4.8 and 4.9, pages 129-133).

A

Last wash   Eluted fractions

W16   E1   E2   E3   E4

kDa

100 —

75 —   ← ~ 72 kDa rhsTLR2

50 —

37 —

25 —

B

Last wash   Eluted fractions

W16   E1   E2   E3   E4   E5   F.P.

kDa

100 —   ~ 90 kDa

75 —   ~ 83 kDa

50 —   ~ 72 kDa

37 —

25 —
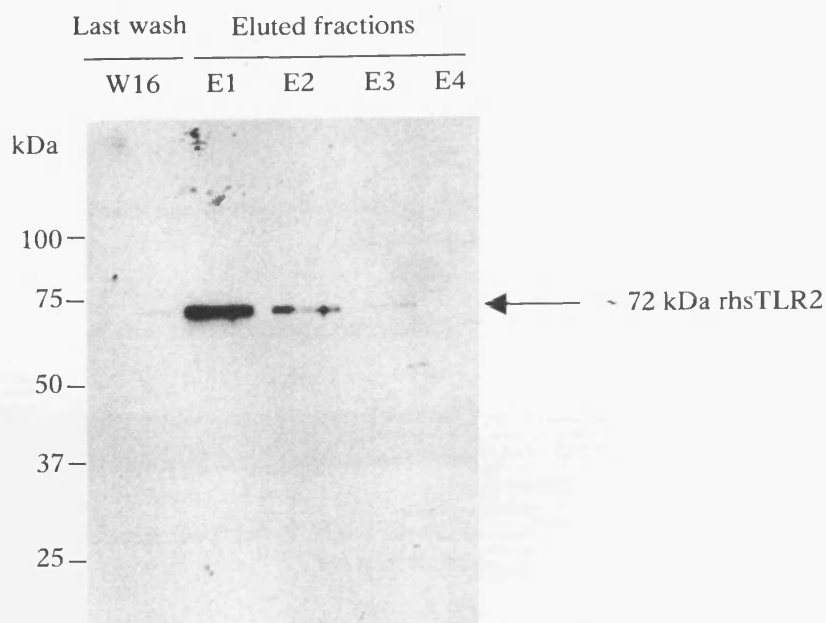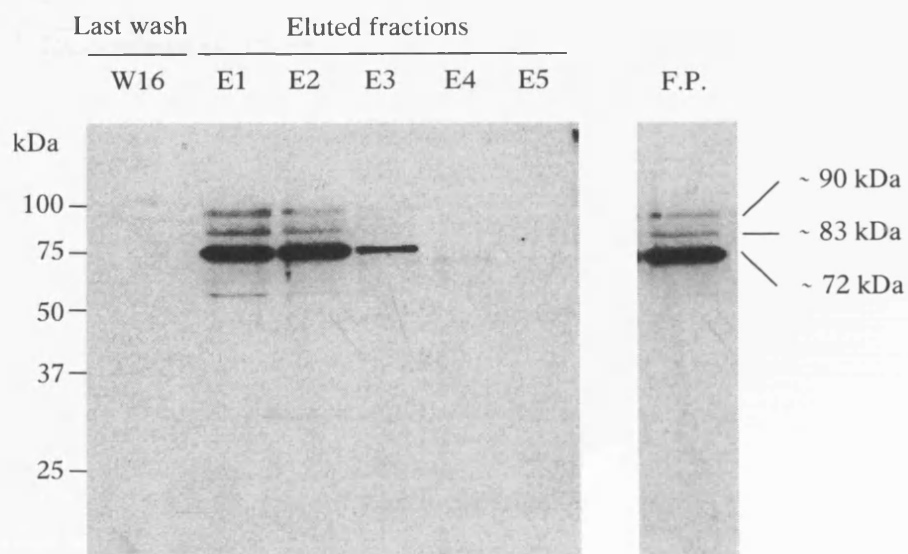
Figure 2.1.3. Detection of His- rhsTLR2 eluted from Ni-NTA-coated beads
A and B, Elution profile of His-rhsTLR2 following metal (Ni)-affinity chromatography. Ni-NTA-coated beads (200 μl) were washed 16 times with 250 μl of 10 mM imidazole/ binding buffer before elution of the bound protein by successive incubations of the beads with 200 μl of 250 mM imidazole/ binding buffer. The elution of His-rhsTLR2 was monitored by Western blotting using an anti-His5 mAb **(A)** or an anti-TLR2 polyclonal Ab **(B)**. W16, 16th wash; F.P., final preparation.
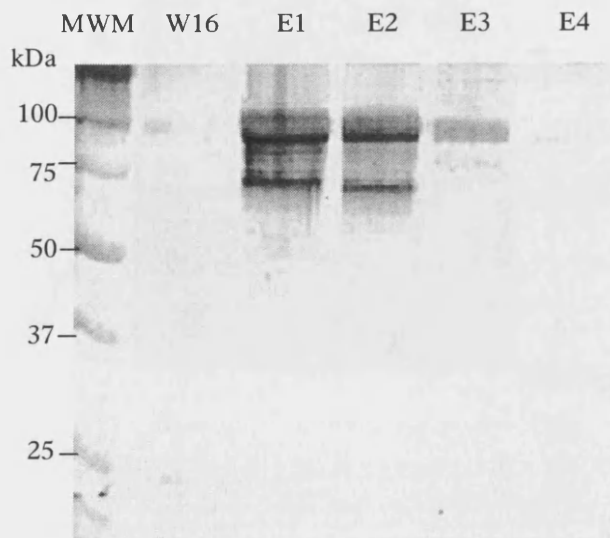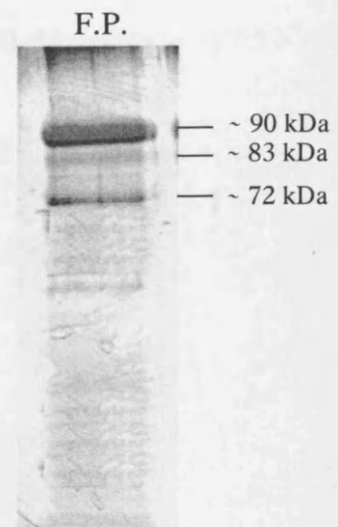
Figure 2.1.4. His-rhsTLR2 purification assessed by Coomassie blue staining
A, Coomassie blue staining of the Ni-NTA-coated beads 16th wash (W16) and first 4 eluted fractions (E1-E4). Washing and protein elution were as described in Figure 2.1.3. The mobility of molecular weight markers (MWM) is shown. **B,** Coomassie blue staining pattern of rhsTLR2 final preparation (F.P.), corresponding to the pooled and dialysed fractions E1 to E4.

Materials and Methods (section 4.9, page 133). Figure 2.1.2 shows a schematic

representation of the strategy used to generate His-rhsTLR2.

2.1.2 Purification of recombinant human sTLR2

Histidine-tagged recombinant human sTLR2 was purified from High Five cell

culture supernatants by metal (Ni)-affinity chromatography, as described under

Materials and Methods (section 4.9, page 133). Supernatants from High Five cells were

concentrated, dialysed (binding buffer), and incubated with Ni-NTA coated beads.

Following extensive washing with binding buffer, the elution of His-rhsTLR2 was

achieved by increasing the concentration of imidazole in the buffer to 250 mM. The

elution of His-rhsTLR2 was monitored by Western blotting using anti-His and anti-

TLR2 specific Abs (Fig. 2.1.3, A and B). Both Abs detected a major ~72-kDa

polypeptide band, whose elution was completed after three or four washes (depending

on the preparation) of the nickel-coated beads. The anti-TLR2 Ab (TLR2p) detected

additional sTLR2 polypeptides of ~83 and ~90 kDa (Fig. 2.1.3, B). The three sTLR2

isoforms have previously been described, and most likely correspond to partially (~72

kDa, Iwaki et al., 2002), fully (~83 kDa, LeBouder et al., 2003), and hyperglycosylated

(~90 kDa, Weber et al., 2004) glycoforms of the TLR2 ECD. The 90-kDa form

originates by using the baculovirus expression system.

SDS-PAGE followed by Coomassie blue staining was used to monitor the elution of

His-rhsTLR2 and evaluate the purity of the preparations (Fig. 2.1.4). Consistent with

the results of the immunoblotting with the anti-TLR2 Ab (Fig. 2.1.3.B), Coomassie blue

staining of the eluted fractions also showed ~90-, ~83-, and ~72-kDa sTLR2

polypeptides (Fig. 2.1.4, A), the 90-kDa glycoform was the most prominent. The final
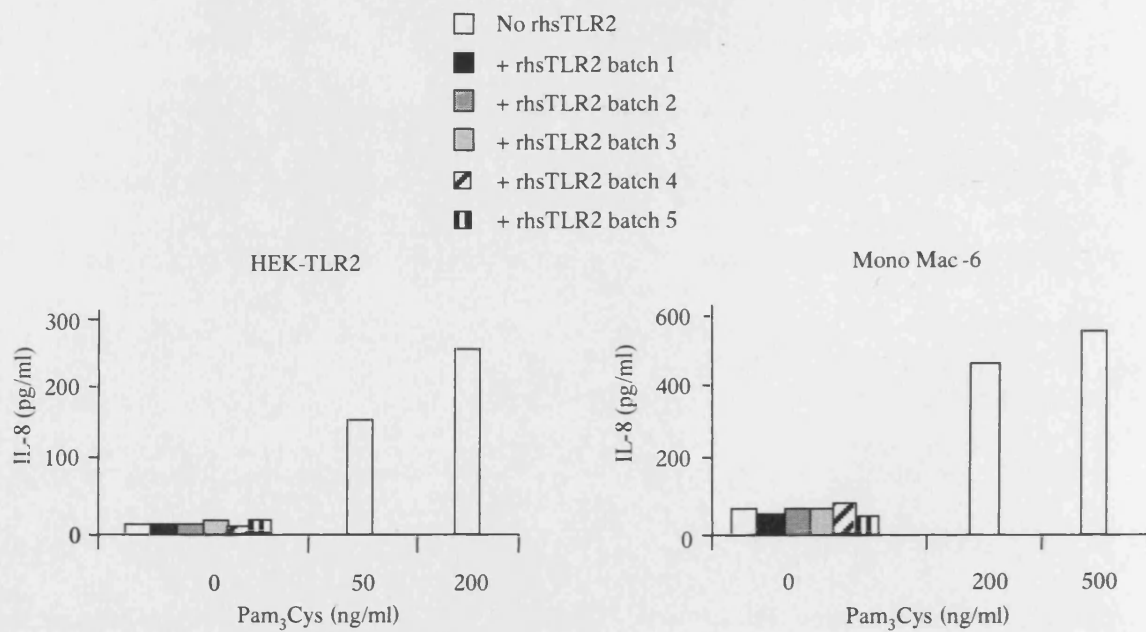
Figure 2.1.5. Purified sTLR2 preparations do not induce cell activation.
Cells (1 x 10⁵/well) were cultured in the presence of 10 μg/ml of purified rhsTLR2 or the indicated concentrations of the synthetic bacterial lipopeptide. The cultures were supplemented with 500 ng/ml (HEK-TLR2) or 200 ng/ml (Mono Mac-6) of sCD14. After incubation (16 h), cell supernatants were collected and tested for IL-8 by ELISA. Results are the mean of triplicate cultures (±SD included in the bars) of one experiment representative of three.

(pooled and dialysed) preparation (F.P.) showed a staining pattern similar to that of the eluted fractions (Fig. 2.1.4, B).

The purity of the rhsTLR2 preparations was tested further. The possibility that undetected contaminants in the sTLR2 preparations induce cell activation, thus affecting the results of biological assays, was investigated. Human embryonic kidney (HEK) 293 cell transfectants expressing cell-membrane bound TLR2 (HEK-TLR2, previously prepared in our laboratory), and Mono Mac-6 human monocytes – which express a number of TLRs constitutively – were cultured in the presence of a relatively high concentration of rhsTLR2 (10 μg/ml) or the TLR2 agonist synthetic bacterial lipopeptide $Pam_3CysK(Lys)_4$ (Pam_3Cys). As shown in Figure 2.1.5, none of the different rhsTLR2 batches tested induced cell activation, as measured by the release of IL-8. Cell sensitivity to activation was confirmed by the relatively strong stimulation induced by Pam_3Cys. This result indicated that the sTLR2 preparations were free of contaminants that could trigger cell activation and thus interfere with the *in vitro* or *in vivo* functional assays. Figure 2.1.2 shows a schematic representation of the strategy used to purify His-rhsTLR2.

2.1.3 Discussion

In the first phase of this project, the preparation of recombinant human sTLR2 was successfully achieved using the baculovirus expression system.

It has been demonstrated that glycosylation is essential for TLR2 and TLR3 biological activity (Kataoka et al., 2006; Sun et al., 2006). Furthermore, it has been shown that glycosylation is required for cell surface expression of TLR4 (Ohnishi et al., 2003) and secretion of the TLR2 ECD (Weber et al., 2004). Therefore, a strategy

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ensuring not only substantial amounts of protein, but also glycosylation of the mature sTLR2 protein had to be used. Given these requirements, mammalian or bacterial (*E. Coli*) expression systems were not considered for the expression of sTLR2, as preliminary work in our laboratory indicated a poor expression of sTLR2 in mammalian cell systems and *E. Coli* expression of sTLR2 would result in a non-glycosylated protein. The baculovirus expression system was therefore selected to generate rhsTLR2, as it ensures production of relatively high levels of a glycosylated protein (Ailor and Betenbaugh, 1999; Kost et al., 2005).

The routine production of a considerable, but not substantial, amount of sTLR2 protein described here – i.e. 200 µg per 200 ml of High Five cell culture supernatant – reflects the difficulties encountered by laboratories that have sought to produce large quantities of TLR proteins for *in vivo* or crystallographic studies. Poor processing, aggregation, and/or insufficient levels of ER chaperons seem to affect the production of TLR proteins in particular (Ailor and Betenbaugh, 1999).

The purification of His-rhsTLR2 was efficiently achieved using metal (Ni)-affinity chromatography. This technique has been widely used for the purification of His-tagged proteins, and is based on the high affinity of the interaction between the nickel ions immobilised by the NTA groups of the resin and the imidazole rings of the histidine residues present in the protein's tag (Petty, 2001). A relatively high concentration of imidazole (here, 250 mM) is then used to dissociate and elute the tagged protein from the resin, as imidazole competes with the histidine tag for binding to the Ni^{2+} ions in the resin. A batch, rather than a column procedure was used for purification, since batch purification promotes a more efficient binding of the His-tagged protein to the resin (Qiagen protein purification handbook, 2003). This was

critical for the purification of sTLR2, since sTLR2 was present at a low concentration in the insect cell culture supernatants. Given the high protein binding capacity of the Ni-NTA resin used, only 200 µl of Ni-NTA agarose beads were routinely required to purify sTLR2 from 200 ml of cell culture supernatant. By using a small amount of resin, the non-specific binding to the beads of irrelevant proteins present in the cell supernatant was reduced to a minimum. The use of the column format in purification would have required larger amounts of resin, which would have increased non-specific protein binding.

The elution profile of sTLR2 was monitored using anti-His and anti-TLR2 Abs, and showed a ~72-kDa band as the most prominent sTLR2 polypeptide (Fig. 2.1.3, A and B). Additional, minor, bands of ~83 and ~90 kDa were detected by the anti-TLR2 polyclonal Ab (TLR2p). A lower sensitivity of the anti-His mAb than that of the anti-TLR2 polyclonal Ab most likely explains the different rhsTLR2 pattern obtained by using the two Abs. By contrast, the Coomassie blue staining of the eluted fractions and final preparations showed the ~90-kDa sTLR2 glycoform as the main sTLR2 polypeptide (Fig. 2.1.4, A and B). It is possible that the hyperglycosylation of the 90-kDa sTLR2 isoform affected its staining pattern, as well as its detection by the anti-TLR2 and -His Abs.

Having achieved the generation and preparation of sTLR2, the issue of its biological activity *in vitro* and *in vivo* was addressed next.

A

HEK-TLR2

+EV + sTLR2-Myc   +EV + sTLR2-Myc

kDa

100 —

75 —   ← sTLR2-Myc

50 —

W.B.: — anti-TLR2 —   — anti-Myc —
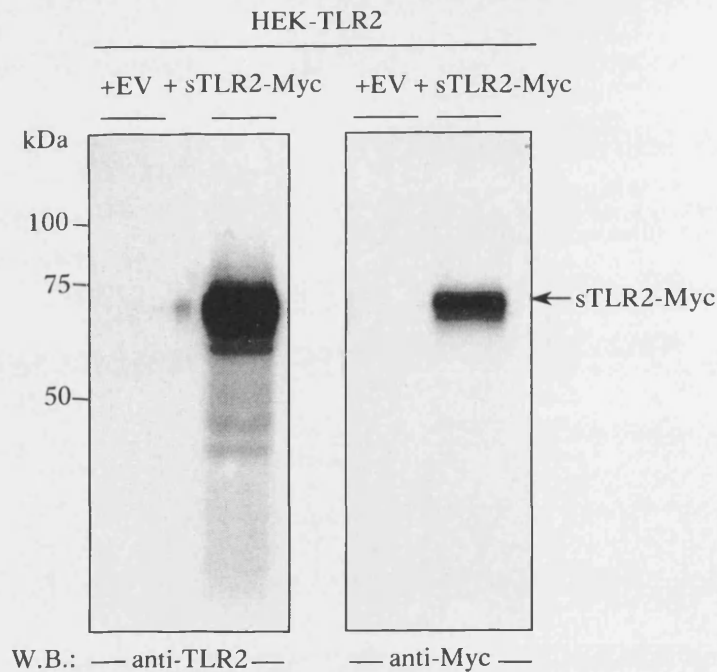
B
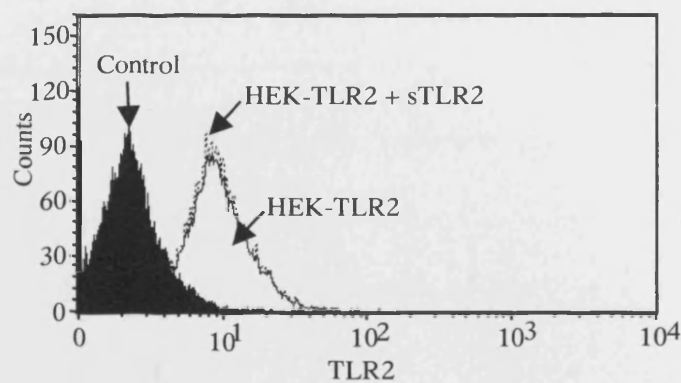
Figure 2.2.1. HEK-TLR2 + sTLR2 stable cell transfectants release sTLR2, and express mTLR2 levels similar to those of HEK-TLR2 cells. **A,** Detection of sTLR2 in 10x concentrated HEK-TLR2 + sTLR2 culture supernatants (2×10^6 cells) by Western blot (W.B.) using the anti-TLR2 polyclonal Ab, TLR2p, or the anti-cMyc epitope mAb, 9E10 (HEK-TLR2 + sTLR2 cells express an N-terminus c-Myc-tagged sTLR2 protein). For control experiments, culture supernatants from HEK-TLR2 + empty expression vector (EV) stable cell transfectants were tested. **B,** Fluorescence profiles of mTLR2 expression in HEK-TLR2 and HEK-TLR2 + sTLR2 cell transfectants stained with the PE-conjugated anti-TLR2 mAb, TL2.1, or an isotype-matched control IgG.

2.2 Biological activity of sTLR2 *in vitro*

Following the reported detection by our laboratory of a naturally occuring soluble form of human TLR2 in biological fluids (LeBouder et al., 2003), the observation that depletion of sTLR2 from plasma renders PMBC more sensitive to TLR2-mediated stimulation (LeBouder et al., 2003) indicated that sTLR2 might play an important modulatory role *in vivo*. This prompted us to fully assess the modulatory capacity of sTLR2 *in vitro* and *in vivo* by making use of the engineered recombinant sTLR2

2.2.1 Effect of overexpressing sTLR2 on cell activation

To test for a possible modulatory activity of sTLR2 *in vitro*, the effect of overexpressing sTLR2 on TLR2-mediated cell stimulation was tested first.

Previously generated HEK293 cell transfectants stably expressing either the membrane-bound TLR2 (mTLR2) receptor (HEK-TLR2) or both mTLR2 and sTLR2 – the latter tagged at the N-terminus with a Myc epitope (HEK-TLR2 + sTLR2) – were analysed by Western blotting with anti-Myc and anti-TLR2 specific Abs and by flow cytometry to confirm that the engineered sTLR2 protein was secreted into the medium by HEK-TLR2 + sTLR2 cells (Fig. 2.2.1, A) and that the HEK-TLR2 and HEK-TLR2 + sTLR2 cells expressed similar levels of mTLR2 (Fig. 2.2.1, B).

Following this initial confirmatory analysis, a comparative study of the sensitivity of HEK-TLR2 and HEK-TLR2 + sTLR2 to TLR2-mediated stimulation was performed. HEK-TLR2 + sTLR2 cells were found markedly insensitive to stimulation with relatively low and high doses of the TLR2 agonist synthetic bacterial lipopeptide Pam_3Cys, as judged by the release of the pro-inflammatory chemokine IL-8 (CXCL-8;
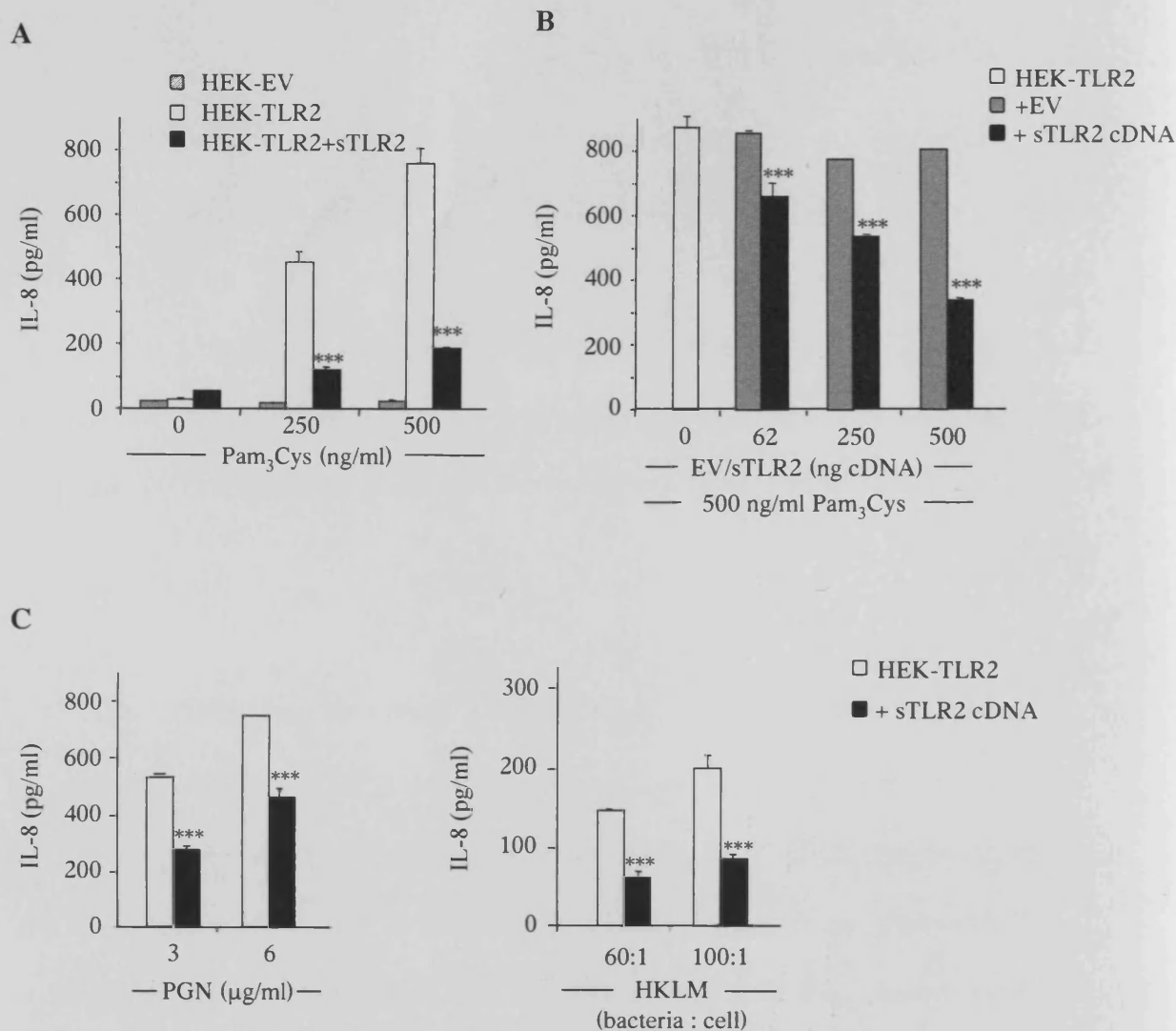
Figure 2.2.2. Overexpression of sTLR2 renders cells hyposensitive to TLR2-mediated stimulation.
Triplicate cultures (1.5×10^5 cells/well) of: **A,** HEK293 cells stably expressing mTLR2 (HEK-TLR2), mTLR2 and sTLR2 (HEK-TLR2 + sTLR2), or the empty vector (HEK-EV), were stimulated (16h) with the indicated concentrations of the TLR2 agonists Pam₃Cys. **B,** HEK-TLR2 cells transiently transfected with the indicated amounts of EV or sTLR2 cDNA were stimulated (16h) with 500 ng/ml of Pam₃Cys. **C,** HEK-TLR2 cells transiently transfected with 250 ng of sTLR2 cDNA were stimulated (16h) with the indicated concentrations of the TLR2 agonists peptidoglycan (PGN) or heat-killed *Listeria monocytogenes* (HKLM). All cultures were supplemented with 500 ng/ml of sCD14. Culture supernatants were tested for IL-8 by ELISA. Results are means ±SD of one experiment representative of four (A) or three (B and C). The differences in IL-8 release between sTLR2 expressing cells and HEK-TLR2, or HEK-TLR2 + EV (B) were compared using the Student's *t* test: ***, $p < 0.0001$.
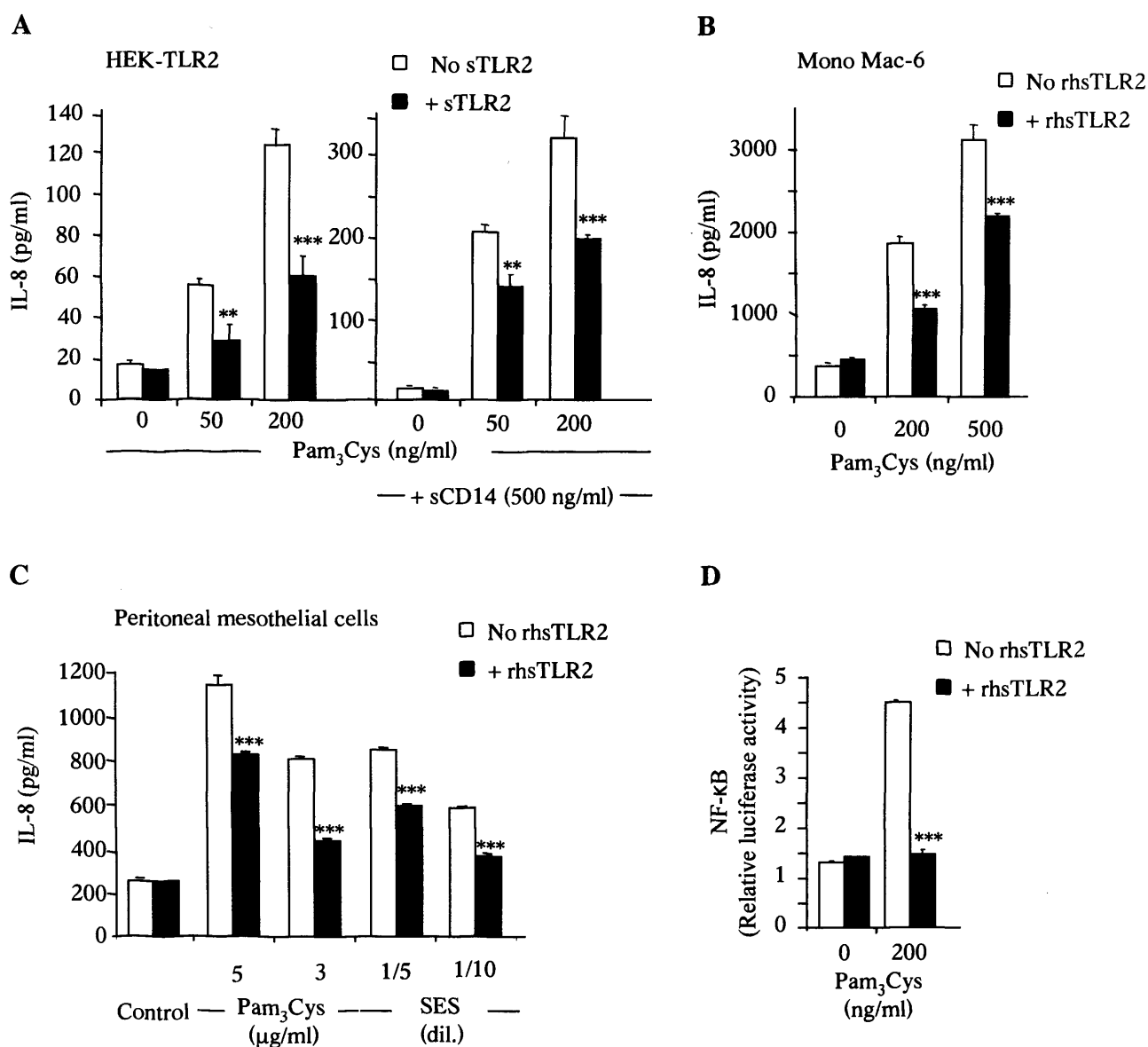
Figure 2.2.3. sTLR2 renders cells hyposensitive to TLR2-mediated stimulation.

The effect of 5 μg/ml rhsTLR2 was tested in triplicate cultures (1.5 x 10⁵ cells/well) of cells stimulated for 16h or 24h (mesothelial cells) with the indicated concentrations of Pam₃Cys or dilutions of a cell-free supernatant from *S. epidermidis*, termed SES. Cultures were supplemented or not (Mono Mac-6 cells) with 500 ng/ml sCD14. For NF-κB reporter assays **(D)**, HEK-TLR2 cells (3 x 10⁵ cells/well) were transiently transfected with firefly and *Renilla* luciferase reporter plasmids. Forty-eight hours post-transfection, the cells were stimulated with Pam₃Cys, followed by luciferase activity measurements (as described under *Materials and Methods*, section 4.16, page 141). Culture supernatants were tested for IL-8 by ELISA. Results shown are of one experiment (±SD) representative of at least three. Differences in IL-8 release or NF-κB activity between sTLR2-treated and non-treated cultures were significant: **, $p < 0.01$, ***, $p < 0.0001$

Fig. 2.2.2, A). To test the sTLR2 cDNA dose-dependency of the inhibitory effect, HEK-TLR2 cells were transiently transfected with increasing amounts of sTLR2 cDNA and activated with Pam_3Cys. As shown in Figure 2.2.2, B, increasing the amount of sTLR2 cDNA transfected lead to a concomitant progressive reduction in IL-8 production, indicating that sTLR2 inhibitory activity was concentration-dependent.

The hyporesponsiveness of the sTLR2-overexpressing cells was not limited to stimulation with Pam_3Cys lipopeptide, since the HEK-TLR2 + sTLR2 cells also showed reduced responses to the TLR2 agonists peptydoglycan (PGN) and the whole Gram-positive bacterium heat-killed *Listeria monocytogenes* (HKLM; Fig. 2.2.2, C).

2.2.2 The effect of rhsTLR2

Having observed hyporesponsiveness in cells overexpressing sTLR2, the modulatory capacity of the purified recombinant sTLR2 protein was evaluated next.

The effect of rhsTLR2 was tested in cell lines and primary cultures of cells that exhibit different levels of expression of TLR2 and the co-receptor, CD14, and thus show different sensitivity to activation via TLR2. HEK-TLR2 cell transfectants do not express the co-receptor CD14, but their sensitivity to TLR2 activation can be increased by addition of soluble CD14 (sCD14; Fig. 2.2.3, A). The TLR2-mediated release of IL-8 by HEK-TLR2 cells was found reduced in the presence of sTLR2, both in the presence and absence of sCD14 (Fig. 2.2.3, A).

The inhibitory effect of sTLR2 was also observed in the human monocyte-like cell line, Mono Mac-6, which constitutively expresses high and moderate levels of CD14 and TLR2, respectively (Fig. 2.2.3, B).
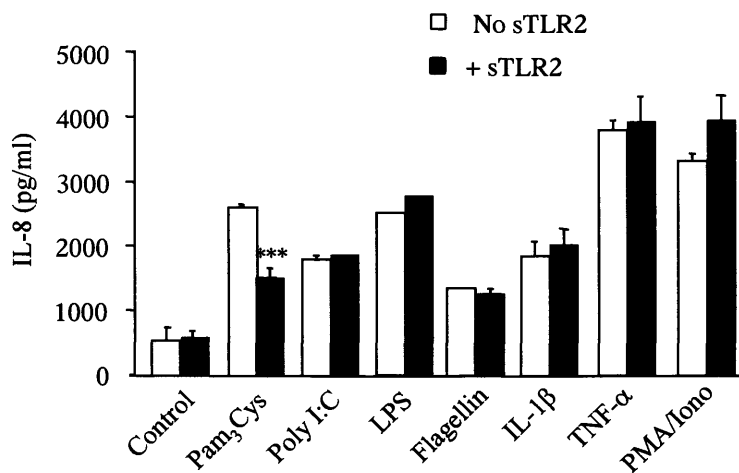
Figure 2.2.4. sTLR2 inhibits TLR2-mediated monocyte activation specifically.
Triplicate cultures (1.5 x 10^5 cells/well) of Mono Mac-6 cells were activated with 200 ng/ml Pam$_3$Cys, 80 µg/ml poly I:C, 10 ng/ml LPS, 5 µg/ml flagellin, 5 ng/ml IL-1β, 10 ng/ml TNF-α or 50 ng/ml PMA + 500 ng/ml inonomycin in the absence or presence of 5 µg/ml of sTLR2. Results shown are of one experiment (±SD) representative of three. Differences in IL-8 release between sTLR2-treated and non-treated cultures were significant: ***, $p < 0.0001$.

Peritoneal mesothelial cells were also tested. These CD14⁻ cells – which express low

levels of mTLR2 – play a pivotal role during the course of a peritoneal infection – like

the one studied here (section 2.3) – by secreting chemokines that regulate leukocyte

infiltration into the peritoneal cavity and by expressing adhesion molecules (Topley et

al., 1996; Park et al., 2007). It was therefore relevant to evaluate the capacity of sTLR2

to modulate the TLR2-mediated response of these cells. Mesothelial cells were

stimulated in the presence of sCD14 with Pam₃Cys or dilutions of a cell-free

supernatant prepared from the Gram-positive bacterium, *Staphylococcus epidermidis*

(termed SES, see *Materials and Methods*, section 4.19.1, page 143). In the presence of

sTLR2, the release of IL-8 – a typical human neutrophil chemoattractant – was found

reduced (Fig. 2.2.3, C).

TLR activation leads to the activation and nuclear translocation of the

transcription factor NF-κB, which in turn induces the transcription of genes that code

for a wide variety of pro-inflammatory cytokines (Doyle and O'Neill, 2006). An NF-κB

reporter assay was, thus, used to test whether sTLR2 affects NF-κB activation. The

activity of the luciferase reporter was found markedly reduced when HEK-TLR2 cells

were stimulated with Pam₃Cys lipopeptide in the presence of sTLR2 (Fig. 2.2.3, D),

indicating a profound modulatory effect of sTLR2 on NF-κB activation.

2.2.3 Specificity of the sTLR2 modulatory activity

The specificity of the sTLR2 inhibitory effect was evaluated next. It was tested

whether sTLR2 affects human monocyte (Mono Mac-6) activation induced by

suboptimal doses of the TLR agonists, viral dsRNA mimic polyinosinic-polycytidylic

acid (poly I:C), LPS and flagellin, which activate cells via TLR3, TLR4 and TLR5,
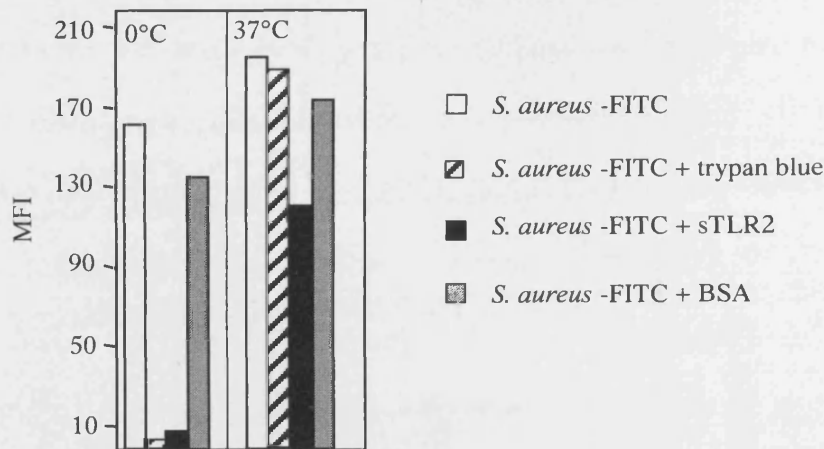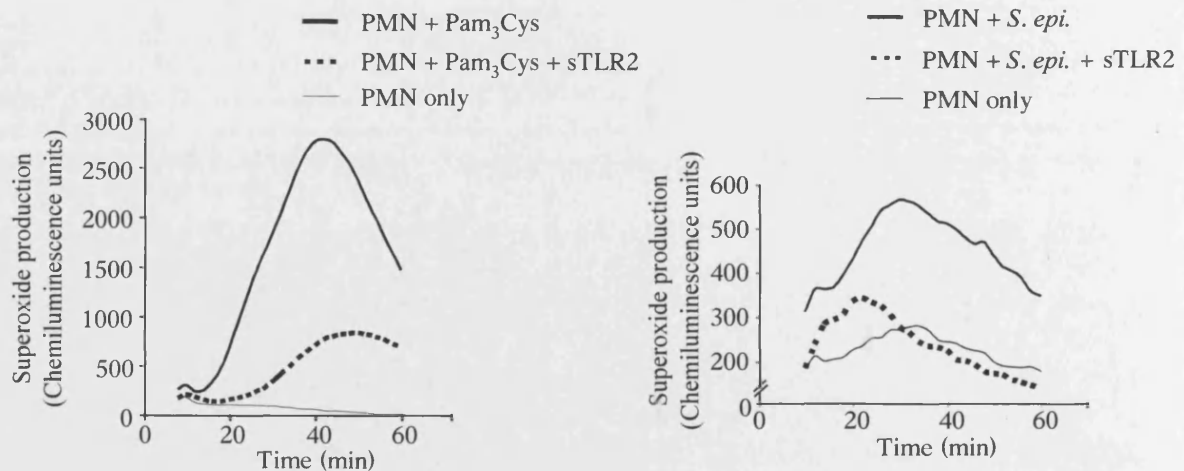
Figure 2.2.5. Bacterial clearance mechanisms can be affected by sTLR2 *in vitro*.
A, Extent of FITC-labelled bacteria bound (0°C) or phagocytosed (37°C) by RAW264 macrophages (4 x 10^5 cells) preincubated or not with 5 μg/ml sTLR2 or an irrelevant protein (BSA, 2 x sTLR2 molarity), as determined by flow cytometry. Macrophages were incubated with bacteria (bacteria:cell ratio, 10:1) for 30 min at the indicated temperature. To distinguish between cell-surface bound and phagocytosed bacteria, the cell-surface fluorescence was quenched with trypan blue before flow cytometric analysis. Results are of one representative out of three independent experiments. **B,** Luminol-dependent chemiluminescence generated by superoxide produced over the time by triplicate cultures of human neutrophils (PMN, 2 x 10^5/well) stimulated with 5 μg/ml Pam$_3$Cys or 5 x 10^6 heat-killed *S. epidermidis* in the absence or presence of 5 μg/ml sTLR2. Results are from one representative experiment out of four.

respectively. In addition, the effect of sTLR2 on signalling via the IL-1R (that shares with TLRs the MyD88-dependent signalling pathway), the TLR non-related receptor TNFα-R, and on non-receptor-mediated cell stimulation (PMA + ionomycin) was tested. Figure 2.2.4 shows that only the TLR2-mediated production of IL-8 by monocytes was inhibited by sTLR2, indicating that sTLR2 targets monocyte TLR2 signalling specifically.

2.2.4 The effect of sTLR2 on bacterial clearance mechanisms

In order to extend the assessment of the negative regulatory potential of sTLR2, the capacity of sTLR2 to affect typical mechanisms associated with bacterial clearance, namely phagocytosis and superoxide production was tested.

2.2.4.A   Effect of sTLR2 on phagocytosis

RAW264 macrophages were used to test macrophage phagocytic capacity in the absence and presence of sTLR2. To differentiate binding from phagocytic uptake of bacteria by the macrophages, the RAW264 cells were incubated with FITC-labelled bacteria at 0°C and 37°C in the presence and absence of trypan blue to quench cell-surface fluorescence. In this way, the amount of bacteria bound to (0°C, trypan blue-sensitive fluorescence) and phagocytosed by (37°C, trypan blue-resistant fluorescence) macrophages was evaluated separately. Figure 2.2.5, A shows that sTLR2 interfered strongly with the macrophage binding (0°C) of a typical Gram-positive bacterium, *Staphylococcus aureus*. The effect of sTLR2 on phagocytosis (37°C) was however comparatively modest.

2.2.4.B Effect of sTLR2 on superoxide production

Freshly-isolated human neutrophils were used to test the effect of sTLR2 on microbial-induced superoxide production (Fig. 2.2.5, B). Upon stimulation with Pam₃Cys or whole heat-killed *S. epidermidis*, the production of superoxide by neutrophils showed a rapid increase for approximately 40 min (Pam₃Cys) or 30 min (*S. epidermidis*). In the presence of sTLR2, however, the capacity of neutrophils to generate superoxide over time in response to either Pam₃Cys or *S. epidermidis* was substantially reduced.

2.2.5 Discussion

Collectively, the *in vitro* data described in this section demonstrated the potential of sTLR2 to negatively regulate TLR2-mediated cell signalling and effector functions that are critical during microbial infection.

sTLR2 was found to modulate the TLR2-mediated responses induced by microbial components and whole Gram-positive bacteria in a variety of cells, including peritoneal mesothelial cells. These cells are known to play a pivotal role during a peritoneal infection – like the one studied in this project (section 2.3) – by regulating leukocyte infiltration into the peritoneum. Thus, these results suggest that during peritoneal infections sTLR2 may also target mesothelial cells for negative regulation.

sTLR2's inhibitory effect was found to be stronger when sTLR2 was overexpressed in cell transfectants than when it was exogenously added as a purified protein. This may reflect a difference in sTLR2 local concentration in the two model systems. Exogenous rhsTLR2 may be degraded over time, whereas overexpression of

sTLR2 may ensure that the local concentration of sTLR2 remains relatively high throughout the activation period, hence the more efficient inhibitory effect. The modulatory effect was not limited to the release of IL-8, since NF-κB activation was markedly affected by sTLR2, indicating that sTLR2 has a wide spectrum of effects. Furthermore, sTLR2 also demonstrated the capacity to modulate phagocytosis and superoxide production – two effector functions that are critical for microbial clearance. The effect on the phagocytic uptake of bacteria (37°C) was modest compared to that on binding (0°C) and, most probably, a consequence of the marked effect on bacterial binding. It is likely that, at 37°C, the activity of typical phagocytic receptors (e.g. scavenger receptors, C-type lectins) compensated for the interfering effect of sTLR2, hence the relatively low effect of sTLR2 on phagocytosis. It should be noted that to assess sTLR2's full potential as a regulator of the phagocytic process, the experiments were performed in serum-free medium, thereby excluding the contribution of Fc and/or complement receptors. It is likely that *in vivo* these mediators of phagocytosis compensate for the inhibitory capacity of sTLR2. Consistent with this possibility, we observed that the negative effect of sTLR2 on phagocytosis is significantly reduced in the presence of serum (data not shown).

The modulatory capacity of sTLR2 observed *in vitro* posed the question of its activity *in vivo*. This question was addressed in the next phase of the project.
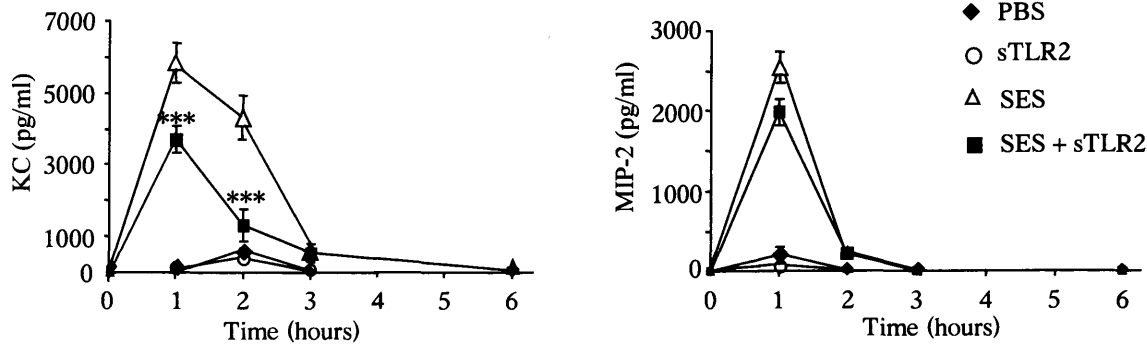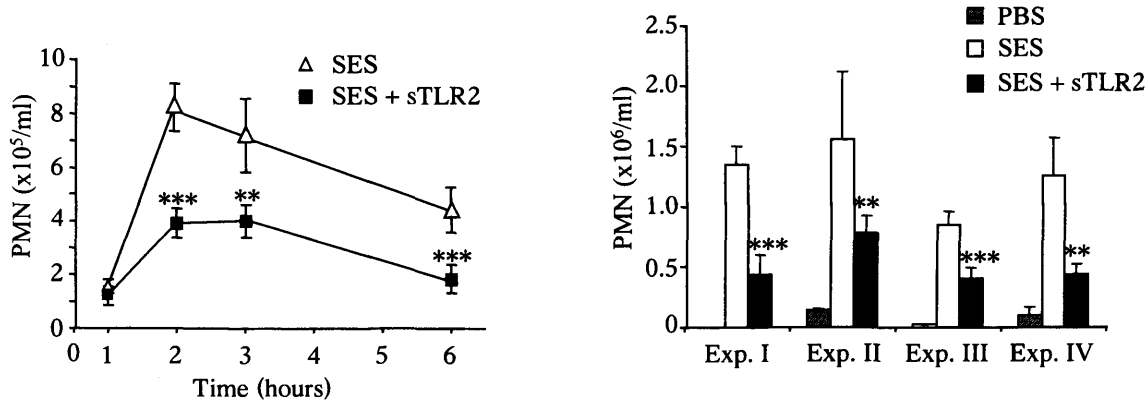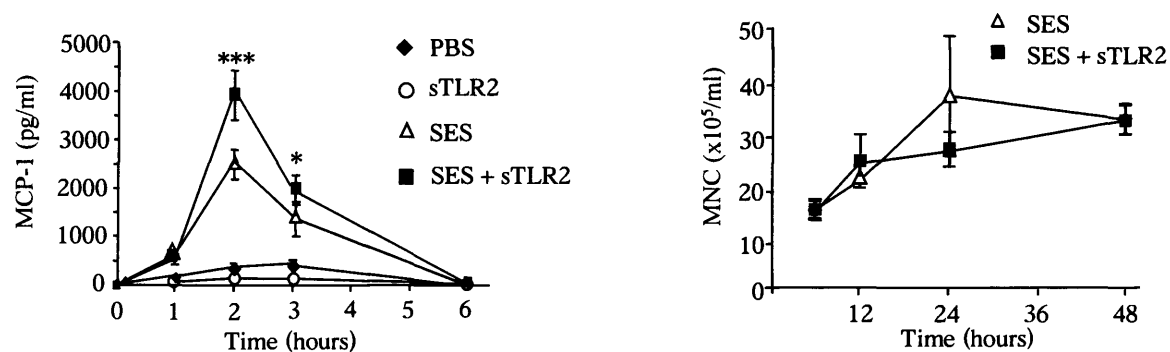
Figure 2.3.1. sTLR2 affects PMN recruitment in a mouse peritoneal inflammation model.

Mice were injected i.p. with a defined dose of a cell-free supernatant from *S. epidermidis* (SES), SES + 100 ng sTLR2 or the indicated controls. At the indicated time points, the peritoneal cavity was lavaged and profiles of chemokine expression in the lavages were determined by ELISA. To determine cell numbers, differential cell counts on cytospin preparations were performed (B, right; results from four independent experiments are shown) or leukocytes were double stained with anti-F4/80-FITC and -CD11b-APC mAbs and analysed by flow cytometry (B and C, time courses). Values in A-C are expressed as the mean ±SEM (n=5/condition; $*, p < 0.05$, $**, p < 0.01$; $***, p < 0.0001$ SES + sTLR2 versus SES).

2.3 Biological activity of sTLR2 *in vivo*

2.3.1 Effect of sTLR2 on leukocyte recruitment

In order to evaluate the biological activity of sTLR2 and assess its potential as modulator of inflammation *in vivo*, the effect of sTLR2 on a well-established mouse model of acute peritoneal inflammation (Hurst et al., 2001; McLoughlin et al., 2003) was tested. This model is based on the intraperitoneal (i.p.) administration of a defined dose of a cell-free supernatant from *S. epidermidis*, termed SES. In this way, the progression of a *S. epidermidis*-induced peritonitis episode – typically seen in end-stage renal failure patients on continuous ambulatory peritoneal dialysis – is mimicked (Topley et al., 1996).

The SES-induced acute peritoneal inflammation was monitored over the time by determining the leukocyte count and the chemokine levels in the peritoneal lavages. As shown in Figure 2.3.1, A, intraperitoneal administration of SES to mice resulted in a fast and transient increase in the peritoneal levels of the neutrophil (PMN) chemoattractants, keratinocyte-derived chemokine (KC) and macrophage inflammatory protein-2 (MIP-2) – murine functional counterparts of human IL-8 and GRO-α – with peak levels occurring at 1 h post-injection. Corresponding determinations of PMN numbers recruited to the peritoneal cavity showed peak levels at 2-3 h (depending on the experiment) after SES administration (Fig. 2.3.1, B, left). The simultaneous administration of SES and sTLR2 (100 ng/mouse) resulted in reduced levels of KC and MIP-2. These levels were significantly reduced in the case of KC, but not MIP-2 (Fig. 2.3.1, A). Consistent with the inhibitory effect on PMN chemoattractants, sTLR2 administration resulted in a marked reduction in PMN numbers recruited to the
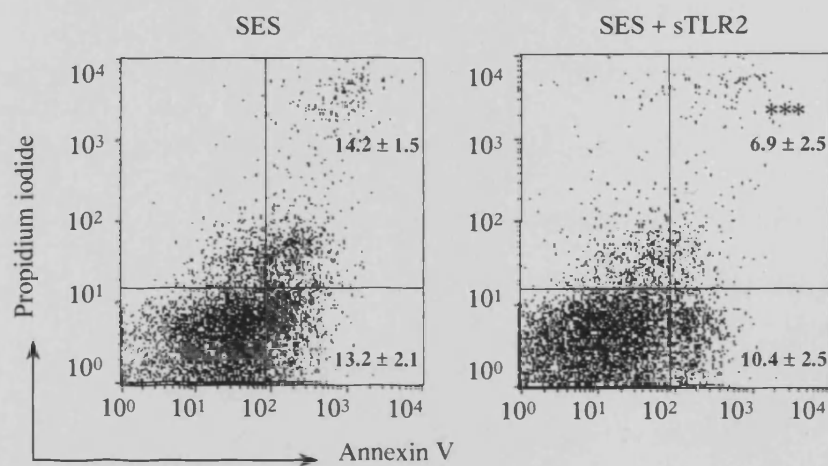
Figure 2.3.2. sTLR2 affects late apoptotic PMN numbers in a mouse peritoneal inflammation model.
Acute peritoneal inflammation in mice was induced with SES as described in Fig. 2.3.1. At the peak time of
PMN influx (shown, 3 h), leukocytes present in the peritoneal lavages were annexin V-FITC/ propidium iodide
(PI) stained and analysed by flow cytometry. The representative scatter plots shown are from analyses of gated
PMN. Apoptotic cells were identified according to the annexin V^+/PI^- (lower right quadrant, early apoptosis) and
annexin V^+/PI^+ (upper right quadrant, late apoptosis/necrosis) staining. The percentage of cells in the apoptotic
quadrants is shown (mean ±SEM, n=5/condition; ***, $p < 0.0001$, significant reduction versus SES).

peritoneum either over the time course (Fig. 2.3.1, B, left) or at the peak time of their

influx (Fig. 2.3.1, B, right).

The effect of sTLR2 on the SES-induced release of the mononuclear cell (MNC)

chemoattractant, monocyte chemoattractant protein-1 (MCP-1), and on the subsequent

recruitment of monocytes/macrophages was also tested. Figure 2.3.1, C shows that

induction of MCP-1 reached a maximum level 2 h after injection of SES (left panel),

and that the consequent recruitment of monocytes/macrophages reached peak levels

approximately twenty two hours later (right panel). Notably, in this case, sTLR2 was

found to exert a positive and significant effect on MCP-1 levels over the time period

post-SES injection. The relatively late recruitment of MNC, however, was not found to

be affected.

2.3.2 Effect of sTLR2 on PMN apoptosis

As macrophages are responsible for the removal of the dying PMN, the

suppressive effect of sTLR2 on early (PMN), but not late (MNC) leukocyte recruitment

posed the question of whether such a disproportionate leukocyte influx influences PMN

survival and thus inflammatory resolution. Therefore, the apoptotic status of PMN at the

peak of their peritoneal influx in SES-challenged mice was compared with that in mice

challenged with SES + sTLR2. One of the earliest indications of apoptosis is the

translocation of the membrane phospholipid phosphatidylserine from the inner to the

outer leaflet of the plasma membrane, thus rendering binding sites on

phosphatidylserine available to the protein annexin V, which binds to

phosphatidylserine with high affinity. Thus, positive staining with annexin V indicates

early or late cell apoptosis. During late apoptosis or early necrosis, propidium iodide
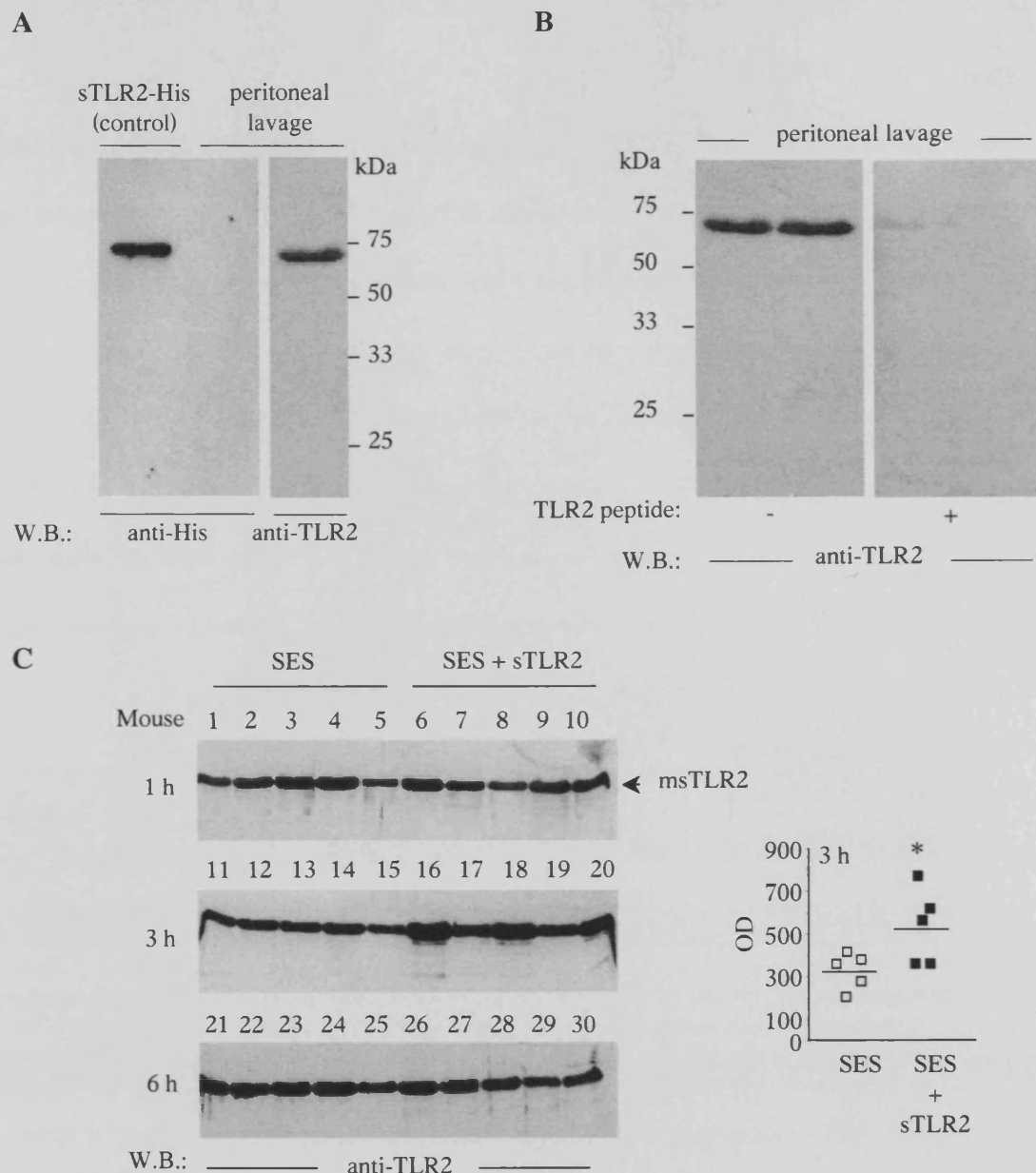
Figure 2.3.3. Detection of mouse sTLR2 in mice peritoneal lavages.
Acute peritoneal inflammation in mice was induced with SES as described in Fig 2.3.1. In **A,** mouse sTLR2, but not His-rhsTLR2, was detected in the peritoneal lavages of mice i.p. inoculated with SES + 100 ng sTLR2. Western blot analysis was performed with an anti-His$_5$ mAb or the anti-TLR2 polyclonal Ab, TLR2p. Purified His-rhsTLR2 (10 ng) was included as positive control (left track). Representative mouse sTLR2 detection from 3h post-inoculation lavages is shown. **B,** The specificity of sTLR2 detection in the peritoneal lavages was confirmed by performing peptide competition by immunoblotting. The TLR2p Ab was preincubated (+) or not (-) with 10x mass excess of the peptide used for immunisation, and the samples were analysed by Western blotting with the TLR2p Ab. **C,** Western blot analysis of aliquots of peritoneal lavages taken at the indicated time points and tested for mouse sTLR2 release by blotting with the anti-TLR2 polyclonal Ab, TLR2p. Densitometric scanning of sTLR2 levels at the peak of PMN influx (3 h) is shown (right; n=5/condition,; *, $p < 0.05$, SES + sTLR2 versus SES).

(PI) – a fluorescent DNA intercalating agent – can stain DNA, as it becomes accessible due to the loss of integrity of the plasma membrane.

Cells from lavages obtained at the peak time point of PMN recruitment (3 h) were double-stained with annexin V-FITC and PI, and gated PMN were analysed by flow cytometry (Fig. 2.3.2). Profile comparison of the annexin V/PI scatter plots showed no significant difference in the proportion of early apoptotic PMN (annexin V positive/PI negative, lower right quadrant) between SES- and SES + sTLR2-treated mice. Examination of the proportion of late apoptotic/early necrotic PMN (annexin V positive/PI positive, upper right quadrant), however, showed a marked reduction (~ 50%) of their numbers in the SES + sTLR2-treated mice.

2.3.3 Effect of sTLR2 on the release of endogenous sTLR2

The effect of administering sTLR2 together with SES to mice on the levels of endogenous (mouse) sTLR2 in the peritoneal lavages was also tested, as our laboratory and others have demonstrated that sTLR2 release is affected by cell activation and infection (LeBouder et al., 2003; Heggelund et al., 2004; Kuroishi et al., 2007; Srinivasan et al., 2008). The detection of endogenous sTLR2 was facilitated by the absence of exogenous sTLR2 (His-rhsTLR2) in the peritoneal lavages (Fig. 2.3.3, A). The specificity of the detection was confirmed by the negative results of immunoblottings in the presence of the TLR2 peptide used to immunise the mice and generate the polyclonal anti-TLR2 Ab (TLR2p) used in this study (Fig. 2.3.3, B).

At 1 h post-injection, no differences in the levels of sTLR2 between SES- and SES + sTLR2-challenged mice were observed (Fig. 2.3.3, C). At 3 h, i.e. when PMN influx was high, mouse sTLR2 levels in the peritoneal lavages of the sTLR2-treated mice were

A
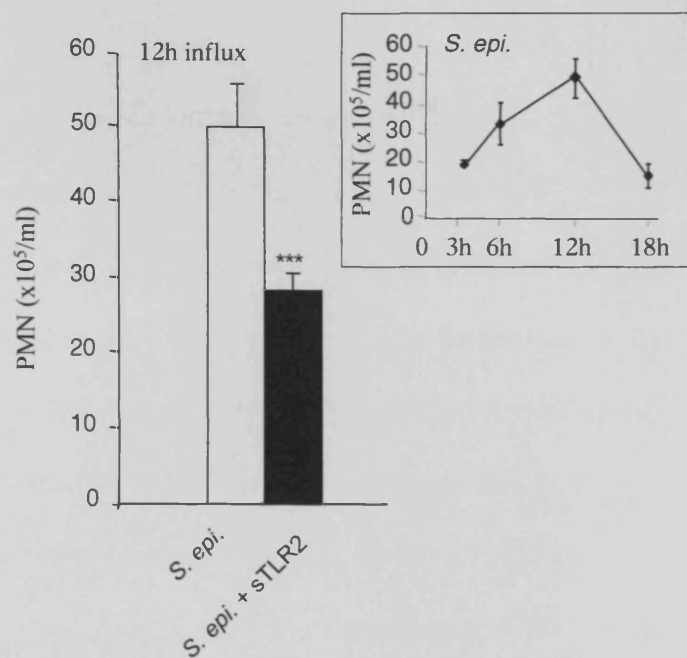
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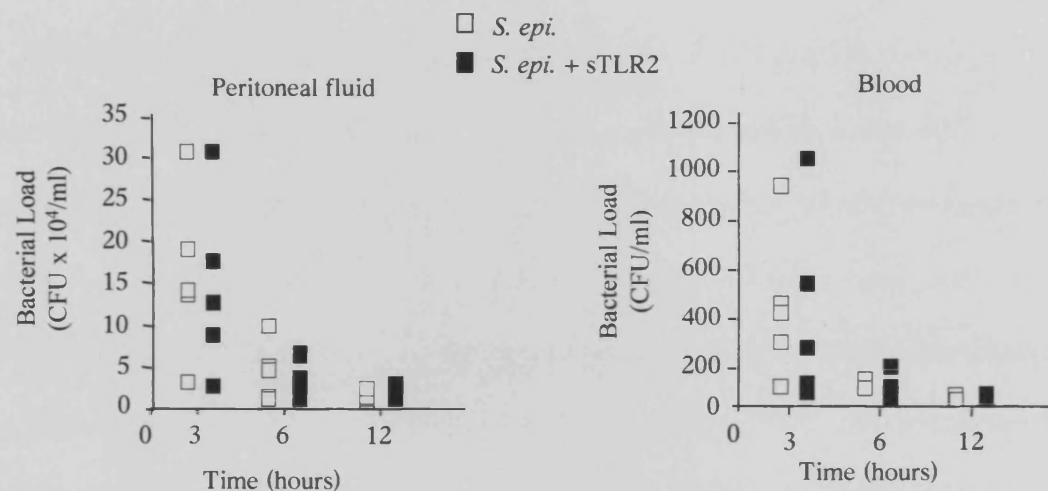
Figure 2.3.4. sTLR2 reduces peritoneal PMN infiltration without compromising bacterial clearance.
Mice (n=5/condition) were i.p. inoculated with 5 x 10^7 CFU *S. epidermidis* alone or together with 100 ng sTLR2. At the indicated times, mice were sacrificed, blood samples were obtained by cardiac puncture and the peritoneal cavity was lavaged. PMN numbers in the lavages **(A)** were determined by differential cell counts on cytospin preparations. Bacterial titres in the peritoneal fluid and blood **(B)** were determined as described under *Material & Method*s (section 4.19.3 page 145). Values in A are expressed as the mean ±SEM (n=5/condition; ***, $p < 0.0001$, *S epi.* + sTLR2 versus *S. epi.*).

found increased. By 6 h post-injection, sTLR2 levels between sTLR2-treated and non-treated mice were comparable and similar to those at the 1 h time point.

2.3.4 Effect of sTLR2 in a live infection model

The anti-inflammatory effects of sTLR2 observed *in vivo* using the SES model of mouse peritoneal inflammation raised the question of whether such negative effects would be detrimental to bacterial clearance during infection. To address this issue, an experimental model of acute peritoneal inflammation consisting of an i.p. challenge with live *S. epidermidis* in the absence or presence of sTLR2 was used. An innoculum of 5×10^7 CFU of *S. epidermidis* per mouse was administered. This dose allowed the mice to clear the infection almost completely by 12 h. PMN numbers in the peritoneum of mice injected with *S. epidermidis* showed peak levels at 12 h post-injection (Fig. 2.3.4, A, inset). In the presence of sTLR2, peritoneal neutrophil accumulation at the peak time of their influx was found significantly reduced (Fig. 2.3.4, A).

Having confirmed that sTLR2 exerts a negative regulatory effect on peritoneal inflammation during a live infection, a possible effect of sTLR2 on bacterial clearance was evaluated next. The bacterial load in the peritoneal cavity and blood over the 12 h period post-infection in the mice injected with *S. epidermidis* and *S. epidermidis* + sTLR2 was compared. No difference in bacterial count either in the peritoneal cavity or blood between sTLR2-treated and -non-treated mice was observed (Fig. 2.3.4, B).

2.3.5 Discussion

By using two mouse models of acute peritoneal inflammation, it was established that administration of sTLR2 reduced the level of PMN recruitment to the peritoneal

cavity in animals challenged with either Gram-positive bacteria-derived microbial components or live Gram-positive bacteria.

In spite of sTLR2's ability to control the inflammatory response, and its *in vitro* capacity to interfere with the phagocytic uptake of bacteria and bacteria-induced superoxide production, sTLR2 administration did not have a negative effect on the clearance of bacteria. This is likely to be due in part to the fact that TLR2 does not seem to play a critical role in bacterial clearance. Indeed, initial studies with TLR2- and MyD88-deficient mice indicated that TLR2 plays a role in the inflammatory response and bacterial clearance only at a high bacterial dose, and that other receptors might be involved (Takeuchi et al., 2000a). Subsequent studies with TLR2-deficient mice challenged with *S. pneumoniae* (Knapp et al., 2004) or *S. aureus* (Mullaly and Kubes, 2006) clearly demonstrated that TLR2 plays a critical role in the inflammatory response, but not in clearing the infection. Notably, a pivotal role for C5aR in bacterial clearance was demonstrated instead (Mullaly and Kubes, 2006). Additional recent work on the activity of the mycoplasma-derived FSL-1 lipopeptide in phagocytosis of bacteria also supported the conclusion that TLR2 does not function as a phagocytic receptor and is not involved in bacterial clearance (Mae et al., 2007). Furthermore, a number of humoral mediators that contribute to efficient bacterial clearance and killing, including complement components, mannose-binding lectin and immunoglobulins (Igs) as well as cell-surface Fc and scavenger receptors may counteract the activity of sTLR2. Consistent with this possibility is the observation that the negative effect of sTLR2 on phagocytosis *in vitro* described in this study was significantly reduced in the presence of serum. The possibility that sTLR2 affects bacterial clearance in certain pathologies (e.g.

complement deficiency) or when higher bacterial doses are used, however, remains to be investigated. Notably, preliminary experiments conducted very recently did not show the modulatory effect of sTLR2 on PMN recruitment at early time points – i.e 3h and 6h – post-infection with live *S. epidermidis*. Confirmation of this finding would indicate that sTLR2 affects PMN recruitment only at the peak (12 h) of their influx, when the animals had cleared the infection almost completely (see Fig. 2.3.4).

Consistent with its negative effect on PMN recruitment, sTLR2 exerted a negative effect on the two major murine neutrophil chemoattractants, KC and MIP-2 (Kobayashi, 2006). The effect on KC was marked and significant, whereas that on MIP-2 was very modest and statistically not significant. In this model of peritoneal infection, the marked effect on KC appeared to be sufficient to impact on PMN recruitment. There is conflicting evidence as to the individual importance of KC and MIP-2 in neutrophil recruitment. While studies on the effect of administering recombinant KC or MIP-2 in models of inflammation showed that each chemokine can cause substantial PMN influx, but that MIP-2 is the most efficient (McColl and Clark-Lewis, 1999; Zwijnenburg et al., 2003), other studies showed that KC is the most important PMN chemoattractant in the response to *Klebsiella* and *Aspergillus* lung infections (Tsai et al., 1998; Mehrad et al., 1999), fibrosis (Keane and Strieter, 1999), and atherosclerosis (Huo et al., 2001).

The reason for the very modest effect of sTLR2 on MIP-2 compared with that on KC is not known at present. It may be related to the recently reported finding that KC and MIP-2 synthesis is induced via TLRs through the MyD88-dependent pathway, but that MIP-2, not KC, is also synthetised as a direct product of the alternative TLR signalling pathway that uses TRIF as the adaptor protein (De Filippo et al., 2008). This pathway is

selectively triggered by TLR3 and TLR4 – but not TLR2 – ligands, and thus cannot be affected by sTLR2.

By contrast to its inhibitory effect on PMN mobilisation to the site of injury, sTLR2 did not influence the recruitment of macrophages to the peritoneal cavity, despite causing increased production of MCP-1. Additional effects of sTLR2 on the complex chemokine network controlling MNC recruitment most likely account for these observations. Indeed, recent work using mouse models of pulmonary pneumococcal infections (Fillion et al., 2001; Dessing et al., 2006) and haemolitic-uremic syndrome (Keepers et al., 2007) demonstrated that the chemokines regulated upon activation, normal T-cell expressed, and secreted (RANTES/CCL-5) and macrophage inflammatory protein-1α (MIP-1α/CCL-3) also play a direct and substantial role in inducing monocyte/macrophage infiltration to the site of infection. It is thus possible that the positive effect of sTLR2 on MCP-1 is compensated for by these other mediators, whose production may have been negatively affected by sTLR2. Alternatively, sTLR2 may have negatively affected the expression of the MCP-1 receptor, CCR-2. Further investigation will thus be required to test whether sTLR2 affects RANTES and/or MIP-1α production and CCR-2 expression, as well as to define the mechanism underlying the positive effect of sTLR2 on MCP-1 production.

Nevertheless, the differential effect of sTLR2 on early (PMN) and late (macrophages) leukocyte recruitment, and the consequent skewing of the leukocyte influx in favour of the macrophages, appears to promote the more efficient removal of senescent PMN, as indicated by the substantially reduced proportion of late apoptotic/early necrotic PMN found in the peritoneal cavity of sTLR2-treated mice. This effect might ultimately favour more rapid resolution of inflammation.

Notably, at the peak of PMN influx, mouse sTLR2 levels in the peritoneal lavages of the sTLR2-treated mice were found to have increased. The mechanism responsible for this positive effect of sTLR2 remains to be investigated. Nevertheless, this finding suggests that the administration of sTLR2 together with SES induced a positive feedback for the release of sTLR2, resulting in transiently higher local concentrations of endogenous sTLR2, which may well contribute to maintaining its regulatory effect on inflammation.

The modulatory capacity of sTLR2 observed *in vivo* and *in vitro* demanded further investigation of the underlying mechanisms. This issue was addressed in the next phase of this project.

2.4 Study of the mechanism underlying sTLR2 activity

The fact that sTLR2 did not affect signalling via other TLRs, the IL-1R and non-TLR related receptors, or non-receptor-mediated signalling, suggested that the primary effect of sTLR2 is exerted upstream of signalling, proximal to TLR2 ligand recognition. To test this possibility, the capacity of sTLR2 to: 1) affect the mobilisation of TLR2 to lipid rafts, 2) act as a decoy receptor and, 3) disrupt the interaction between mTLR2 and mCD14 was tested.

2.4.1 Effect of sTLR2 on the mobilisation of mTLR2 to lipid rafts

It has been demonstrated that the TLR co-receptor CD14 resides mainly in cholesterol and sphingolipid-rich detergent-resistant membrane microdomains, termed lipid rafts (Triantafilou et al., 2002). It has also been shown that, in resting conditions, TLR2 and TLR4 are localised mainly outside the lipid rafts in the detergent-soluble membrane fractions. Upon TLR ligand-induced cell stimulation, the specific TLR is recruited to lipid rafts where it is found in close proximity to CD14 and other cell-surface molecules, thus forming a receptor cluster. The ligand-induced mobilisation of TLR2 and TLR4 to lipid rafts and their close proximity to CD14 is believed to be critical for signalling (Pfeiffer et al., 2001; Triantafilou et al., 2004; Triantafilou et al., 2006). It was therefore tested whether sTLR2 inhibited TLR2 triggering by affecting the ligand-induced clustering of mCD14 and mTLR2 in lipid rafts. Freshly-isolated human monocytes were Pam_3Cys-stimulated in the presence or absence of sTLR2, and the cell lysates subjected to sucrose density gradient centrifugation. By this procedure, the high-
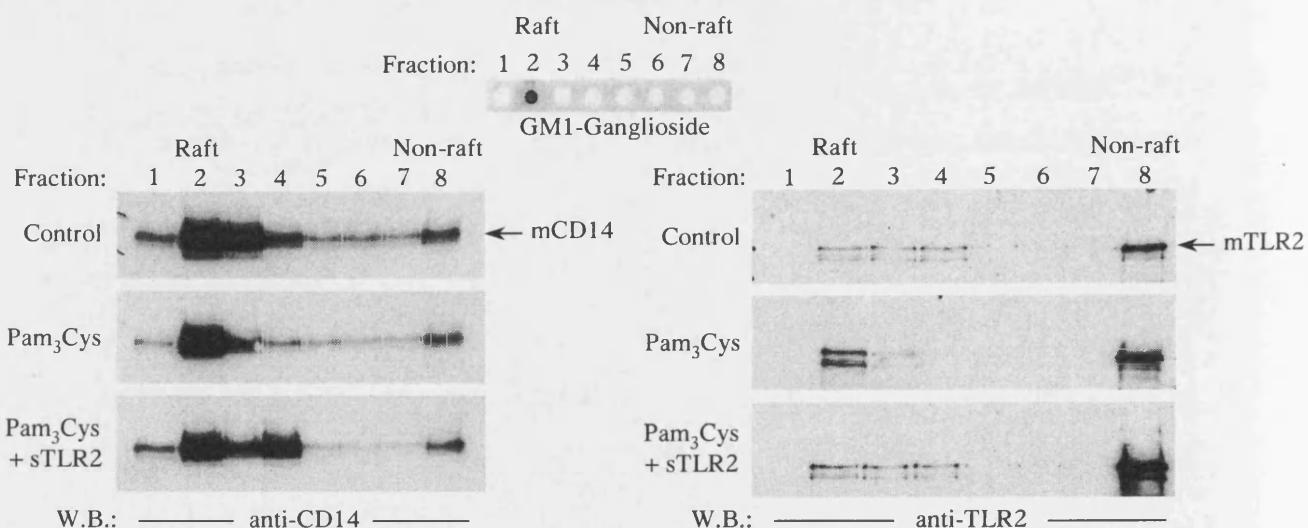
Figure 2.4.1. sTLR2 prevents the ligand-induced mobilisation of mTLR2 to lipid rafts.

mCD14 and mTLR2 partitioning into lipid raft and non-raft fractions following stimulation of freshly-isolated monocytes (1×10^8 cells) with 5 μg/ml Pam$_3$Cys in the absence or presence of 5 μg/ml sTLR2. Triton X-100 cell lysates were subjected to sucrose density gradient centrifugation. Fractions were then collected from the top of the gradient and analysed by Western blotting with anti-CD14 (MY4) or anti-TLR2 (IMG319) mAbs. Dot blots (top) were used to determine the position of the raft marker - GM1 ganglioside - in the gradient, as detected by the binding of HRP-conjugated cholera toxin B. Similar results to those shown were obtained from four independent experiments.

density, detergent-soluble, subcellular fractions move to the lower fractions of the gradient, whereas the low-density, detergent-insoluble fractions – which include lipid rafts – move to the top of the gradient. To define the lipid raft-containing fractions in the sucrose gradient more precisely, aliquots of each fraction were analysed by dot blotting to determine the presence of the raft-associated ganglioside, GM1. As shown in Figure 2.4.1 (top), GM1 was detected in the fraction 2 of the gradient, thus indicating that lipid rafts were mainly located in this fraction. However, due to the heterogeneity of the lipid rafts, they may locate not in a single but in neighbouring fractions of the gradient.

Analysis of lipid raft preparations from non-stimulated (control) monocytes confirmed the preferential association of mCD14 with lipid rafts and mTLR2 with detergent-soluble (non-raft) fractions (Fig. 2.4.1, upper panels). Pam_3Cys stimulation resulted in an enrichment of mTLR2 in lipid rafts and reduced levels of mCD14 (Fig. 2.4.1, middle panels), most likely as a consequence of the activation-induced shedding of soluble CD14 (Durieux et al., 1994). However, when cells were stimulated in the presence of sTLR2, the pattern of mCD14 and mTLR2 partition into membrane domains resembled that in non-stimulated cells (Fig. 2.4.1, lower panels), indicating that sTLR2 interferes with the ligand-induced mTLR2 mobilisation to lipid rafts for signalling, and consequently with the approximation of mTLR2 to mCD14 in the rafts.

2.4.2 Decoy bacterial receptor activity of sTLR2

A decoy activity would explain, at least in part, the interfering effect of sTLR2 on the mTLR2 mobilisation to lipid rafts described previously. This possibility was tested, and it was found that sTLR2, but not an irrelevant control protein (CD46), binds

A
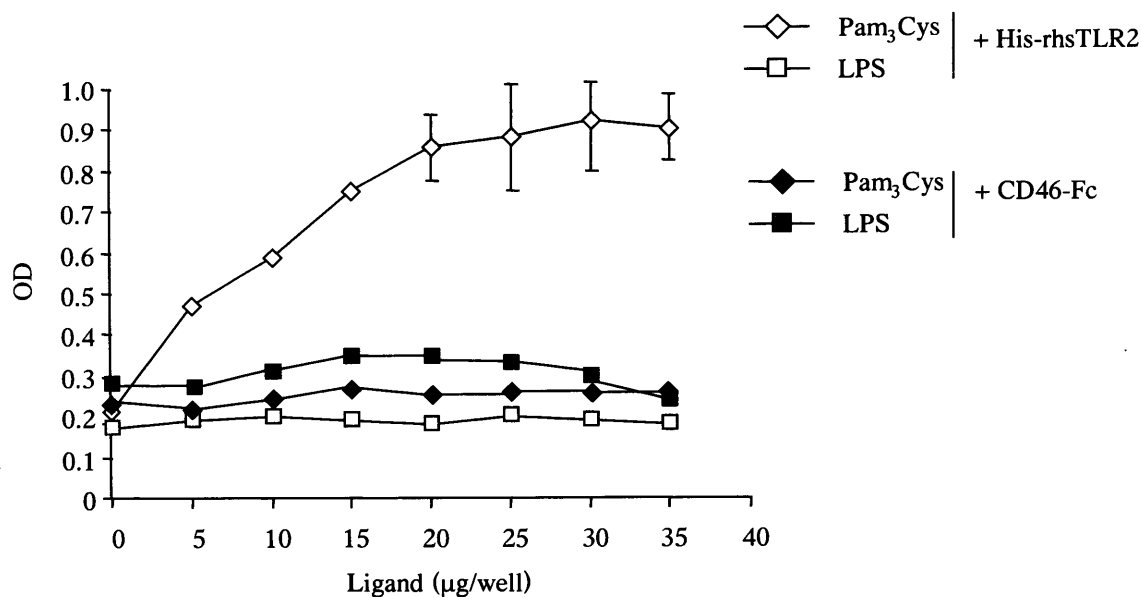
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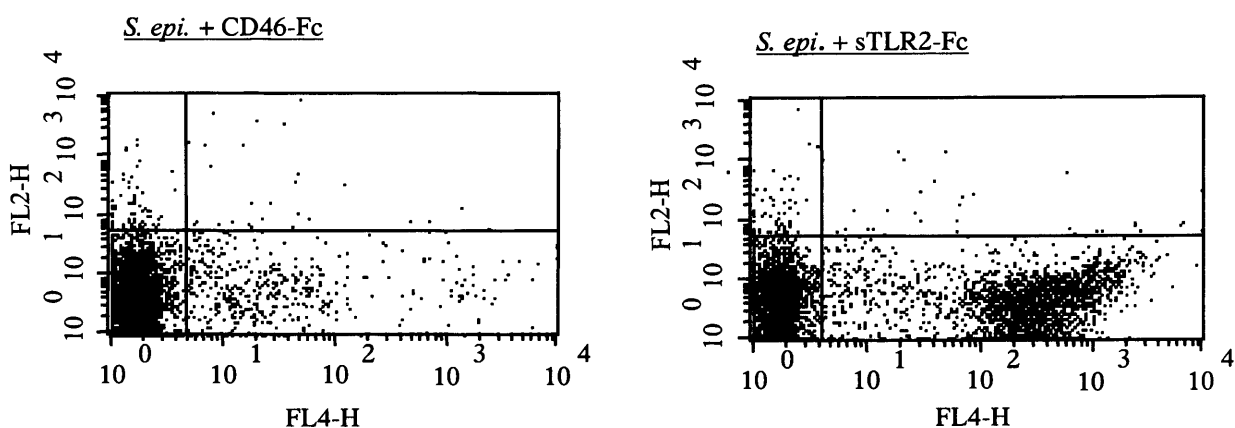
Figure 2.4.2. sTLR2 can act as a decoy receptor.

A, Triplicate microtiter wells were coated with the indicated amounts of Pam₃Cys or LPS and incubated (4h, 37°C) with 5 µg/ml of His-rhsTLR2 or irrelevant control protein (CD46-Fc). His-rhsTLR2 bound to the wells was detected by incubation with an anti-His₅ mAb followed by a biotin-conjugated anti-mouse IgG Ab, and CD46-Fc was detected with a biotin-conjugated anti-human IgG Ab, as described under *Materials & Methods*. All wells were incubated with streptavidin-HRP and then substrate. Colour development was measured at 450 nm. **B,** Binding of sTLR2-Fc or irrelevant (CD46-Fc) fusion proteins (1 µg) to *S. epidermidis* (5 x 10⁴) was tested for 30 min at room temperature. Bound protein was detected with a biotin-conjugated anti-IgG Ab followed by streptavidin-APC and analysis by flow cytometry, as described under *Materials & Methods*. Binding results are from one experiment representative of three (Pam3Cys) or five (*S. epidermidis*) experiments.

Pam₃Cys lipopeptide in a ligand-concentration-dependent and saturable manner (Fig. 2.4.2, A), confirming previous reports (Iwaki et al., 2002; Vasselon et al., 2004). The interaction of sTLR2 with whole bacteria was also tested. A sTLR2-Fc fusion protein, but not an irrelevant control, specifically bound heat-killed *S. epidermidis* (Fig. 2.4.2, B).

2.4.3 Effect of sTLR2 on the natural association of mTLR2 with mCD14

The ectodomain of TLR2 has been shown to interact with CD14 (Iwaki et al., 2005) and sTLR2 was found to co-immunoprecipitate with sCD14 from milk and plasma (LeBouder et al., 2003). It was therefore speculated that sTLR2 may also disrupt the close proximity of mCD14 to mTLR2 directly – i.e. in the absence of ligand – by interacting with mCD14. Alternatively, sTLR2 may interact with mTLR2 (homodimerisation). To test these possibilities, co-immunoprecipitation experiments, FRET analysis and chemical crosslinking strategies were performed.

2.4.3.A Effect of sTLR2 on the co-immunoprecipitation of mCD14 and mTLR2

First, the effect of sTLR2 on the ligand-independent natural association of mTLR2 with mCD14 in the detergent-soluble fractions of normal human monocytes lysates was examined. As shown in Figure 2.4.3, A, the typical ~110-kDa mTLR2 polypeptide band (LeBouder et al., 2003) was consistently detected by Western blotting in mCD14 immunoprecipitates from monocyte cell lysates (left track). In addition, faint TLR2 polypeptide bands most likely corresponding to an intracellular

Figure 2.4.4. sTLR2 associates with the co-receptor mCD14, but not with mTLR2.
The indicated cells were incubated with 5 µg His-sTLR2 or irrelevant control protein His-sCD55. Crosslinking was carried out with the membrane-impermeable, non-cleavable crosslinker, BS^3, as described under *Materials and Methods* (section 4.11 page 135). Crosslinked His-tagged proteins in the cell lysates were pulled-down by Ni-NTA precipitation, and the precipitates analysed by Western blotting with anti-CD14 (69.4) or -TLR2 (sc8689) polyclonal Abs. Head arrows point at Ni-NTA-precipitated, CD14-crosslinked, polypeptide bands. Upper panels shows the mobility of sTLR2, mCD14 and mTLR2 monomers pulled-down (sTLR2) or immunoprecipitated (mCD14, mTLR2) from purified stock or CHO-CD14 and CHO-TLR2 transfectants, respectively. Results are representative of three crosslinking experiments.

A

B

Figure 2.4.3. sTLR2 disrupts the natural association of mTLR2 with the co-receptor CD14.
A, Anti-TLR2 (IMG319 mAb) Western blots (W.B.) of CD14 immunoprecipitates (MY4 mAb) from cell lysates of freshly-isolated human monocytes incubated with 5 μg/ml sTLR2, 10 μg/ml BSA or left untreated. Monocyte preparations from four donors gave identical results to those shown. H, mouse Ig heavy chain. **B,** FRET analysis were carried out on freshly-isolated monocytes incubated for 1h in the absence or presence of 5 μg/ml sTLR2 or BSA before labelling with the anti-CD14 mAb MY4-Cy3 (acceptor) and anti-TLR2 mAb TL2.5-Alexa 488 (donor). FRET between TLR2 and CD14 was determined by the increase in donor fluorescence after acceptor photobleaching, as detailed under *Materials & Methods*. The threshold for significant FRET was determined with the acceptor isotype-matched control IgG2b-Cy3. Results are from five independent experiments.

(~95 kDa) glycoform of the mature protein and to fully glycosylated (~83 kDa) and intracellularly located sTLR2 (LeBouder et al., 2003) were detected. The ~110-kDa mTLR2 polypeptide band was not detected when the co-immunoprecipitation experiments were performed following preincubation of monocytes with sTLR2 (Fig. 2.4.3, A, middle track). This disrupting effect was specific of sTLR2, as it could not be reproduced using an irrelevant protein, BSA (Fig. 2.4.3, A, right track). These findings indicated that sTLR2 interfered with the natural association of mCD14 with mTLR2. The fact that sTLR2 did not prevent the co-immunoprecipitation of the intracellular forms of TLR2 with CD14 suggested that such interference takes place at the cell surface.

2.4.3.B   Effect of sTLR2 on the interaction between mCD14 and mTLR2

Confirming evidence of sTLR2's interfering effect on the mCD14-mTLR2 interaction was obtained by fluorescence resonance energy transfer (FRET) studies. FRET was used as it allows for the evaluation of interactions between neighbouring molecules by determining their proximity within ≤ 10 nm range. FRET results from the (non-radioactive) transfer of energy from an excited donor to an acceptor fluorophore (Kenworthy, 2001). Here, FRET efficiency was measured between the anti-TLR2-Alexa488 (donor) and anti-CD14-Cy3 (acceptor) mAbs, used to label mTLR2 and mCD14, following dequenching of donor fluorescence after photobleaching of the acceptor fluorophore. An increase in mTLR2 (donor, green) fluorescence after mCD14 (acceptor, red) photobleaching was detected in monocytes, indicating energy transfer, and thus close proximity between the two molecules (Fig.
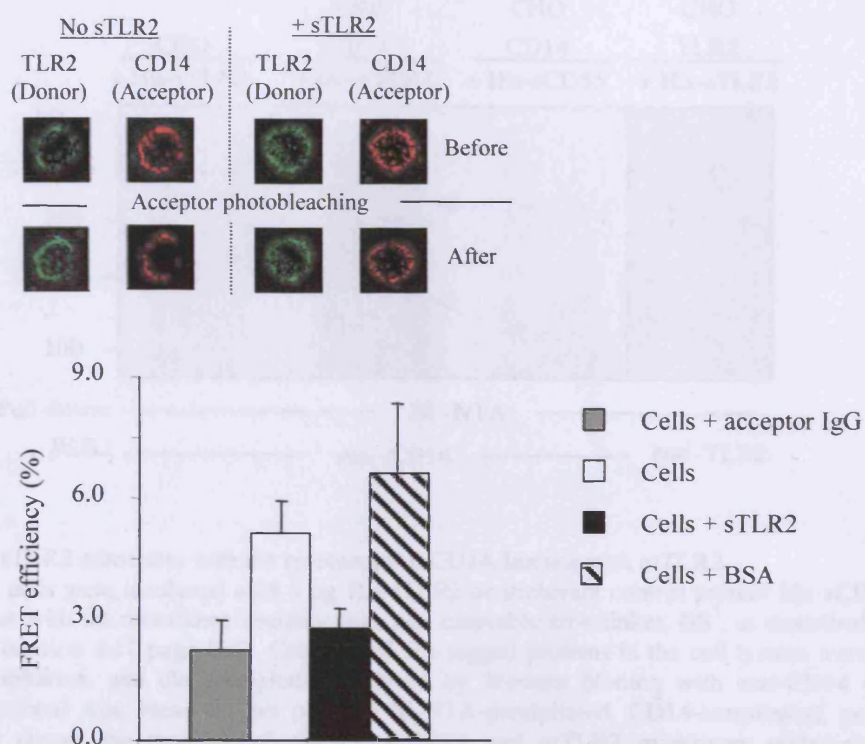
Figure 2.4.4. sTLR2 associates with the co-receptor mCD14, but not with mTLR2.
The indicated cells were incubated with 5 μg His-sTLR2 or irrelevant control protein His-sCD55. Crosslinking was carried out with the membrane-impermeable, non-cleavable crosslinker, BS³, as described under *Materials and Methods* (section 4.11 page 135). Crosslinked His-tagged proteins in the cell lysates were pulled-down by Ni-NTA precipitation, and the precipitates analysed by Western blotting with anti-CD14 (69.4) or -TLR2 (sc8689) polyclonal Abs. Head arrows point at Ni-NTA-precipitated, CD14-crosslinked, polypeptide bands. Upper panels shows the mobility of sTLR2, mCD14 and mTLR2 monomers pulled-down (sTLR2) or immunoprecipitated (mCD14, mTLR2) from purified stock or CHO-CD14 and CHO-TLR2 transfectants, respectively. Results are representative of three crosslinking experiments.
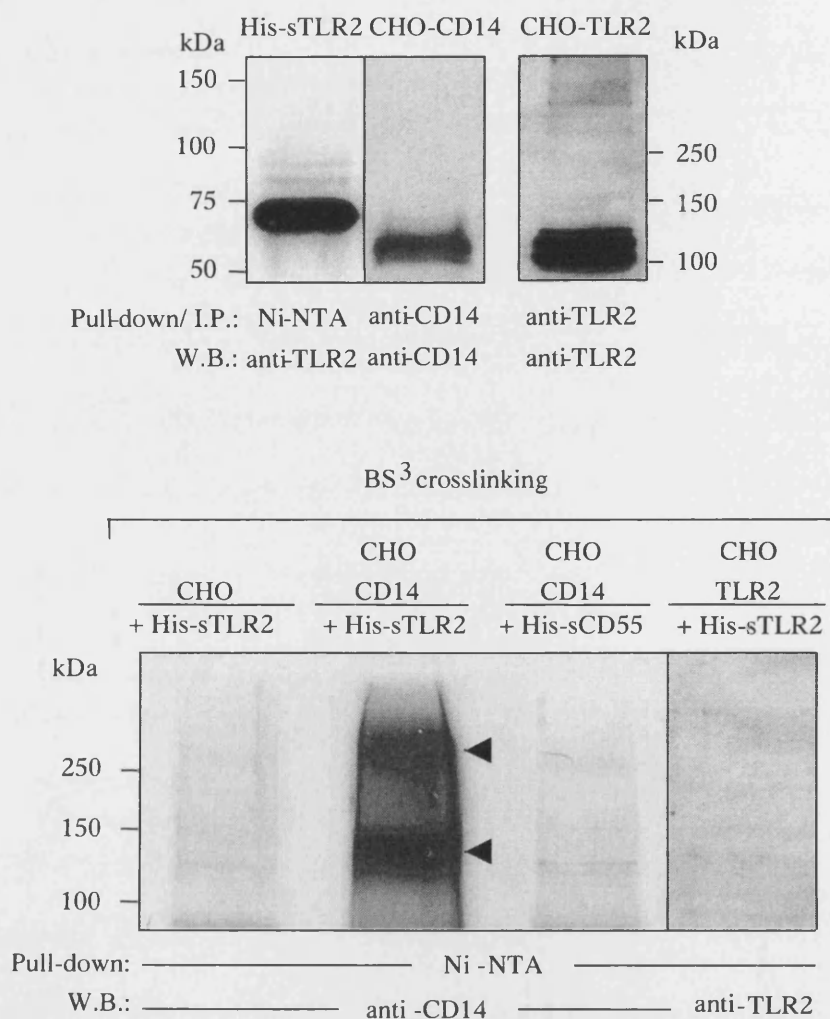
2.4.3, B, upper panel). This was in agreement with the results of the co-immunoprecipitation experiments. In the presence of sTLR2, however, no increase in TLR2 fluorescence after mCD14 photobleaching was observed, and FRET efficiency between mTLR2 and mCD14 was reduced to almost background levels, i.e. 2% FRET, threshold for significant energy transfer defined with the acceptor isotype-matched control IgG2b-Cy3 (Fig. 2.4.3, B, lower panel). These findings thus confirmed that sTLR2 perturbs the mCD14-mTLR2 interaction.

2.4.3.C   Association of sTLR2 with the co-receptor CD14

The interfering effect exerted by sTLR2 in the absence of ligand raised the question of whether this effect results from an interaction of sTLR2 with mCD14 and/or mTLR2. To address this issue, chemical crosslinking experiments were performed. CHO, CHO-CD14 and CHO-TLR2 transfectant cells were incubated with His-sTLR2 or an irrelevant His-tagged soluble protein. Subsequently, a non-cleavable, membrane-impermeable, crosslinking reagent, BS^3, was used to stabilise potential interactions between cell-surface molecules. The cells were then washed, lysed, and crosslinked His-sTLR2 in the cell lysates was pulled-down using Ni-NTA-coated beads. The precipitates were tested by Western blotting for the presence of CD14 (CHO and CHO-CD14 cells) or TLR2 (CHO-TLR2 cells). Western blot analysis of control precipitates of His-sTLR2 (from purified stock), mCD14 and mTLR2 (from CHO-CD14 and CHO-TLR2 cell transfectants, respectively) showed bands of the expected sizes, ~72kDa (His-sTLR2), ~55 kDa (mCD14) and ~110 kDa (mTLR2; Fig. 2.4.4, upper panels). Incubation of CHO-CD14 transfectants with His-sTLR2 followed by chemical crosslinking, Ni-NTA-bead pull-down from the CHO-CD14 cell lysates, and

anti-CD14 Western blotting revealed bands of ~125-130 kDa and ~250 kDa, i.e. of

lower mobility than that of mCD14 (Fig. 2.4.4, lower panels). The size of these bands

was consistent with that estimated for CD14/sTLR2 heterodimers (~72+~55=~127 kDa)

and CD14/sTLR2 dimer of dimers (~254 kDa). To test for an interaction of sTLR2 with

mTLR2, the same crosslinking strategy was applied to CHO-TLR2 cell transfectants

preincubated with His-sTLR2. Here, however, Ni-NTA-bead pull-down followed by

anti-TLR2 immunoblotting did not show any crosslinked TLR2 polypeptide band (Fig.

2.4.4, right track).

2.4.4 Discussion

The results presented in this section indicate that sTLR2 can act as a decoy

bacterial receptor and also disrupt the close proximity between the co-receptor, CD14,

and the receptor, TLR2 – which is critical to efficient signalling. Such disruption is

likely to result from the capacity of sTLR2 to interact with CD14.

The results of the *in vitro* studies presented in section 2.2 suggested that the

primary effect of sTLR2 is exerted upstream of signalling, proximal to ligand

recognition. It was therefore tested whether sTLR2 affects the ligand-induced clustering

of mCD14 and mTLR2 in lipid rafts, which is believed to be critical to signalling. It was

found that sTLR2 indeed interferes with the ligand-induced mobilisation of mTLR2 to

lipid rafts. Such interference would be explained, at least in part, by the sTLR2's

capacity to act as a decoy microbial receptor, as demonstrated in this study. However, it

was also found that sTLR2 disrupts the close proximity of mCD14 to mTLR2 in the

absence of ligand by associating with mCD14, as indicated by the co-
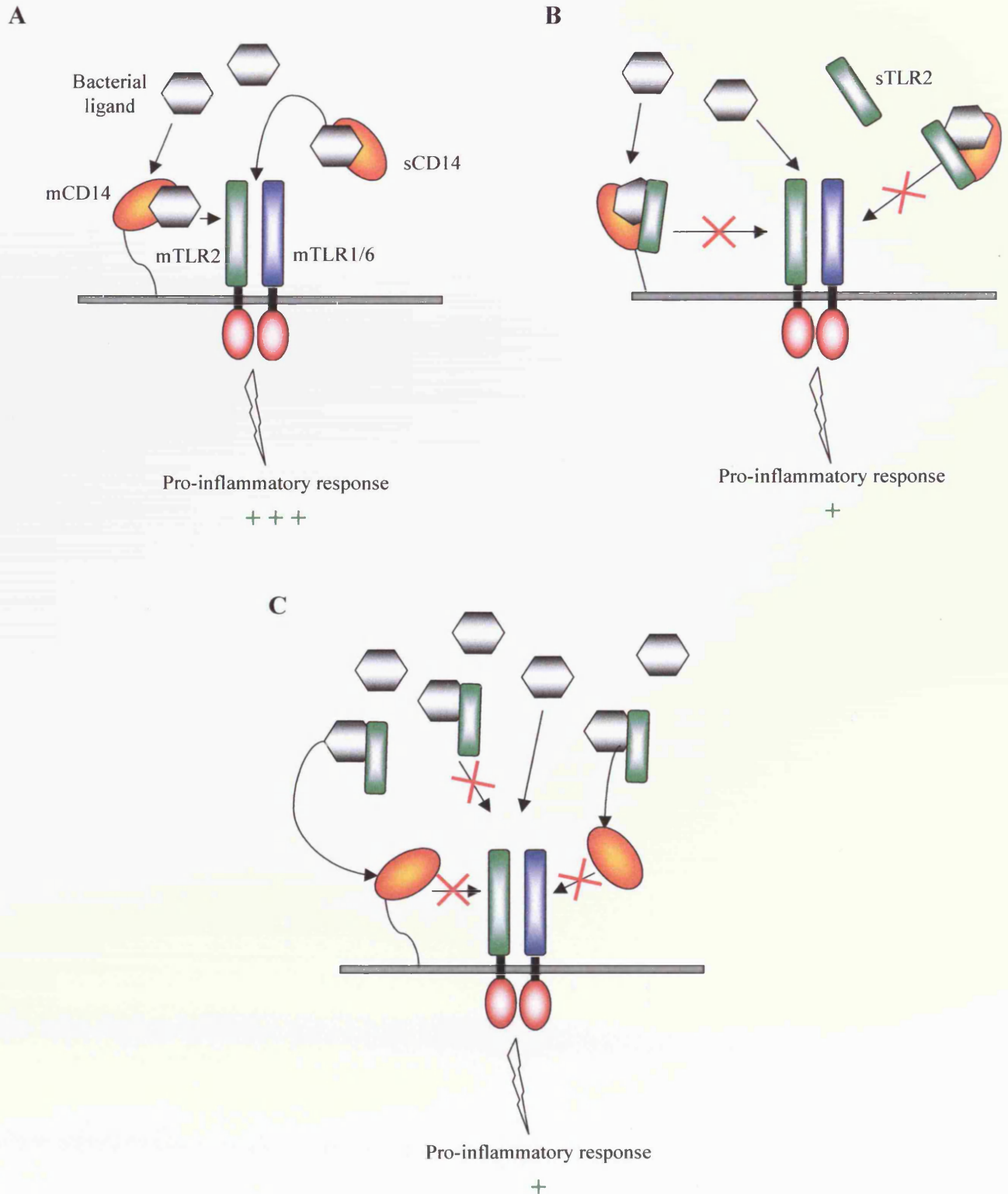
Figure 2.4.5. Postulated mechanisms underlying sTLR2 inhibitory activity.
A, CD14 (mCD14 or sCD14) presents the bacterial ligand to TLR2/TLR1 or TLR2/TLR6 heterodimer. This results in the efficient triggering of TLR2, which in turn leads to the induction of pro-inflammatory responses.
B, sTLR2 may modulate TLR2 triggering by associating with mCD14 and/or sCD14 and disrupting the close proximity between CD14 and TLR2, thus interfering with CD14 co-receptor activity. This results in a reduced pro-inflammatory response. **C,** sTLR2 may interfere with and reduce TLR2 triggering by binding to bacterial ligands, thus acting as a decoy receptor. Through these mechanisms, sTLR2 may affect the mobilisation of TLR2 to lipid rafts and its approximation to CD14 in the rafts for efficient signalling.

immunoprecipitation, FRET and chemical crosslinking experiments. Such close proximity is crucial to CD14's co-receptor function and highly efficient TLR2 signalling. Thus, sTLR2's capacity to interfere with the mCD14-mTLR2 interaction and disrupt the co-receptor function by associating with CD14, together with sTLR2's decoy receptor activity may affect the mobilisation of mTLR2 to lipid rafts for signalling upon cell stimulation, and lead to reduced pro-inflammatory responses, which in turn result in the observed reduction in PMN recruitment to the site of infection. A schematic representation of the mechanisms underlying sTLR2's modulatory capacity discussed here is shown in Figure 2.4.5.

The ability of sTLR2 to affect the co-receptor activity of CD14 raises the question of why TLR4- and TLR3-mediated monocyte responses – that also require CD14 for efficient signalling – are not affected, as indicated by the absence of a negative effect of sTLR2 on LPS or poly I:C stimulated Mono Mac-6 cells (see section 2.2.3, Fig. 2.2.4, page 85). It is possible that, when the effect of sTLR2 depends solely on its capacity to interact with CD14 (no decoy activity, i.e. TLR3 and TLR4 signalling), the extent of sTLR2 inhibition critically depends not only on the local concentration of sTLR2, but also on the expression levels of CD14 (mCD14 or sCD14) and the mTLR involved (i.e. TLR3 or TLR4/MD2) as well as on the affinity and stoichiometry of the interactions of mTLR, CD14 and sTLR2 and those of the ligand with mTLR and CD14. In support of this possibility, results of very recent experiments in our laboratory show that overexpressing sTLR2 in human astrocytoma (U373) cell transfectants – which express very low levels of TLR4, do not express mCD14 and require sCD14 for sensitive signalling – significantly reduces CD14-dependent LPS-induced (TLR4-mediated) NF-κB activation (Fig. 2.4.6), suggesting that sTLR2 may be
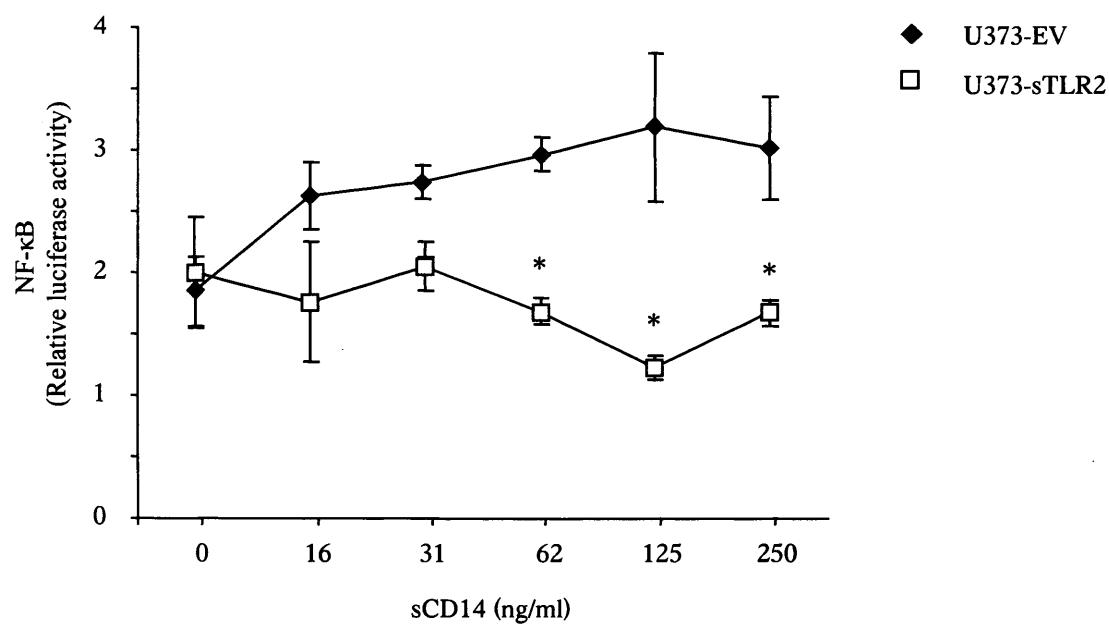
Figure 2.4.6. Overexpression of sTLR2 renders U373 cells hyposensitive to CD14-dependent TLR4-mediated stimulation.

Triplicate cultures (3 x 10^5 cells/well) of U373 cells stably expressing sTLR2 (U373 + sTLR2) or the empty vector (U373-EV), were transiently transfected with firefly and *Renilla* luciferase reporter plasmids. Forty-eight hours post-transfection, the cells were stimulated (8 h) with LPS (60 ng/ml) in the presence of the indicated concentrations of sCD14. Subsequently, luciferase activity was measured as indicated under *Materials and Methods* (section 4.16, page 141). Results shown are of one experiment (±SD) representative of three. Differences in NF-κB activity between U373-EV and U373-sTLR2 were significant: *, p < 0.05.

targeting sCD14. Clearly, a better knowledge of the parameters governing the interactions beween TLRs, CD14, the ligands, and sTLR2, will improve our understanding of the sTLR2's activity. With regard to TLR3, its mostly intracellular location and function (Lee et al., 2006) may limit the activity of sTLR2.

The demonstrated capacity of sTLR2 to interact with CD14 and disrupt its co-receptor activity left open the question of which region(s) of the TLR2 ECD are involved in such interaction. It was anticipated that the identification of such region(s) could inform the design of novel tools to reduce pro-inflammatory responses, which might work by exclusively blocking CD14's co-receptor function. The next phase of this study addressed that possibility.

2.5 Identification and functional characterisation of the TLR2 ECD regions involved in the TLR2-CD14 interaction

It was considered important to identify the region(s) of the TLR2 molecule involved in its interaction with the co-receptor, CD14, not only because it would improve our understanding of the mechanisms underlying the modulatory activity of sTLR2, but also because it would have therapeutic potential. Indeed, it was reasoned that such identification would enable the design of soluble peptides, incorporating the critical amino acid sequences, that could be tested for their ability to disrupt the CD14's co-receptor activity by binding to CD14. In this way, TLR2-mediated pro-inflammatory responses would be reduced – thus avoiding the deleterious effects associated with excessive inflammation – while TLR2's capacity to recognise and respond to microbial ligands would be preserved.

2.5.1 Mutational analysis of the TLR2 ECD

2.5.1.A   Generation and expression of TLR2 LRR mutants

The human TLR2 ECD contains a total of twenty leucine-rich repeats (LRR; Fig. 1.3 and Fig. 2.5.3), which are known to be critically involved in protein-protein interaction and ligand recognition. Thus, in order to identify the TLR2 LRRs involved in the interaction with CD14, the systematic point mutation of each of the 20 LRRs of the TLR2 ectodomain was conducted, and then the capacity of the mutants to mediate ligand-induced cell activation in the absence and presence of CD14 was evaluated.
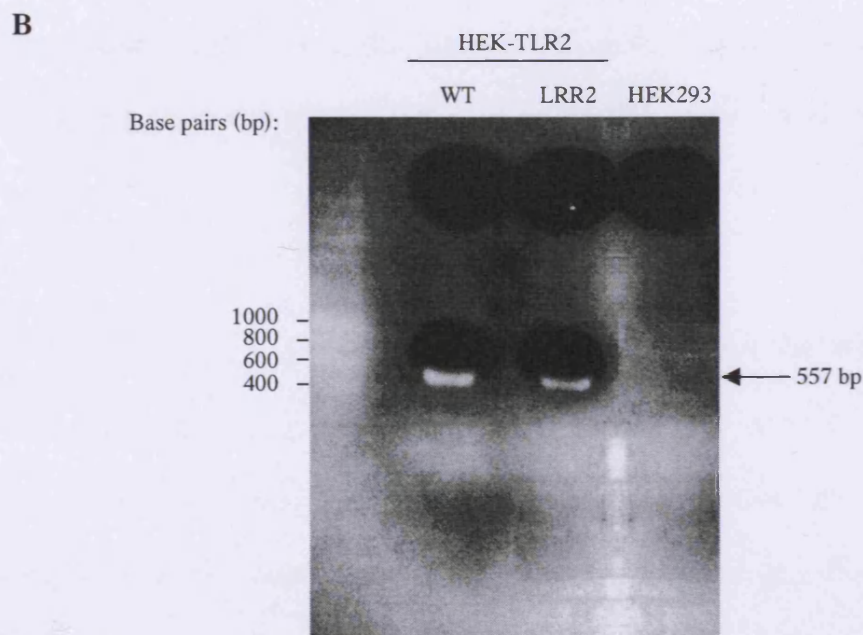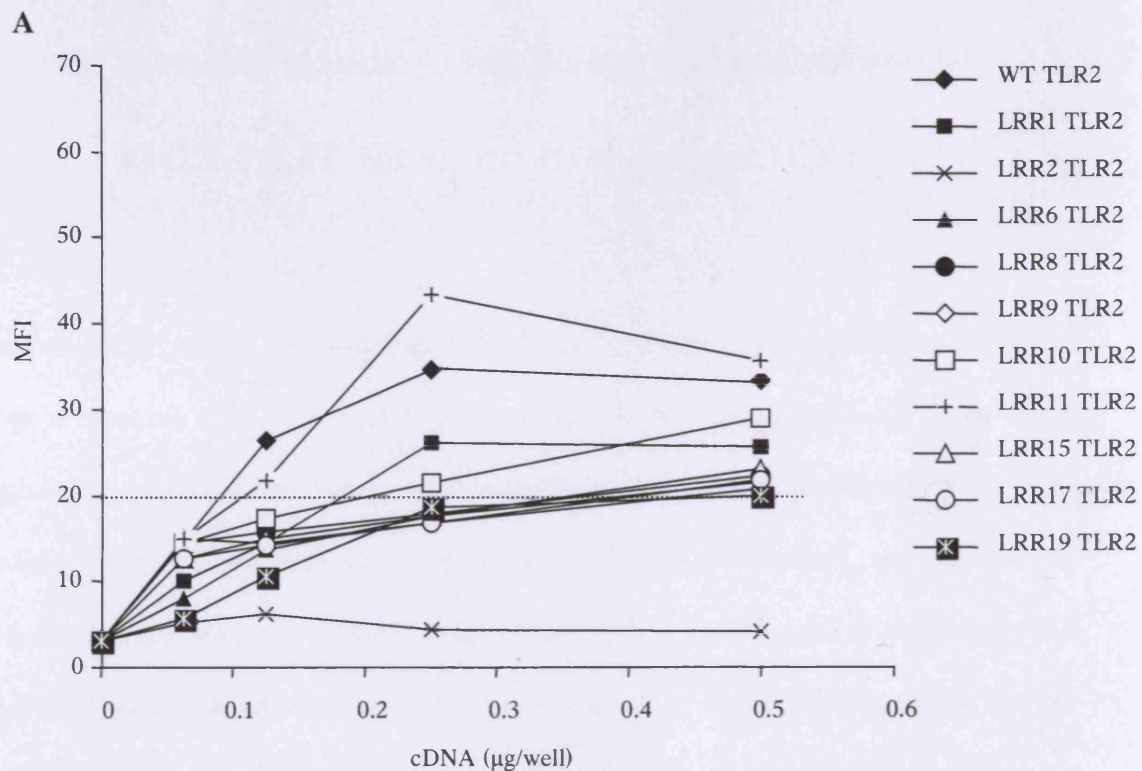
Figure 2.5.1. Expression of LRR TLR2 mutants.
A, Cell-surface expression of LRR TLR2 mutants and WT TLR2 in HEK293 cells (3 x 10^5 cells/well) transiently transfected with the indicated amounts of the WT and mutant cDNAs. Surface expression of the different TLR2 proteins was assessed by flow cytometry using the anti-TLR2 mAbs T2.5 (this figure) or TL2.1 (not shown) A488-conjugated. Results are representative of 3 independent experiments. The dotted line indicates the minimum level of expression selected to carry out subsequent functional experiments. **B,** Detection of LRR2 TLR2 and WT TLR2 mRNAs following extraction of total RNA from the corresponding HEK293 cell transfectants (3 x 10^6 cells), reverse transcription, and cDNA amplification using human TLR2 specific primers, as described under *Materials and Methods* (section 4.20, page 146). The expected size of the corresponding PCR product is 557 pb.

The strategy for the mutation of the LRRs consisted in substituing valine for 2 or 3 leucine residues of a particular LRR. Valine was chosen for substitutions as it is a non-polar, neutral amino acid, structurally close to leucine. This strategy thus minimised possible changes in the tertiary structure of the protein, which could affect the mutants functional activity. The 20 LRR mutated TLR2 cDNAs were obtained by PCR using an appropriate pair of primers and the pCRII®-TOPO® plasmid containing the full-length TLR2 cDNA as a template (*Materials and Methods*, section 4.20, page 145).

The expression in HEK293 cells (which do not express native TLR2, TLR4 or CD14) of the TLR2 cDNAs coding for the TLR2 mutant and wild-type (WT) proteins was tested next. A comparable level of expression between the mutants and WT TLR2 was required to make possible the comparison between the functional activity of the TLR2 mutants and that of WT TLR2. To this aim, cells were transiently transfected with increasing concentrations of each TLR2 cDNA (mutants and WT, 0.062-0.5 μg), and the amount of cDNA required to reach a comparable and significant level of expression (MFI = 20-30) of the different TLR2 proteins was determined by flow cytometry. Figure 2.5.1, A shows a representative TLR2 cDNA titration experiment. Based on this experiment, 0.5 μg cDNA/well (3 x 10^5 cells) was used in subsequent studies. Notably, the result of the TLR2 cDNA titration also indicated that the LRR2 TLR2 mutant (Fig. 2.5.1, A) as well as LRR3, 4, 5, 7, 12, 13, 14, 15, 16, 18 and 20 TLR2 mutants (not shown) were not expressed at the cell surface at levels detectable by flow cytometry. This was confirmed by using two different human TLR2-specific mAbs (TL2.1 and T2.5), indicating that the lack of detection of these mutants was most likely not due to the loss of the epitope recognised by the mAb in the mutants. However, RT-
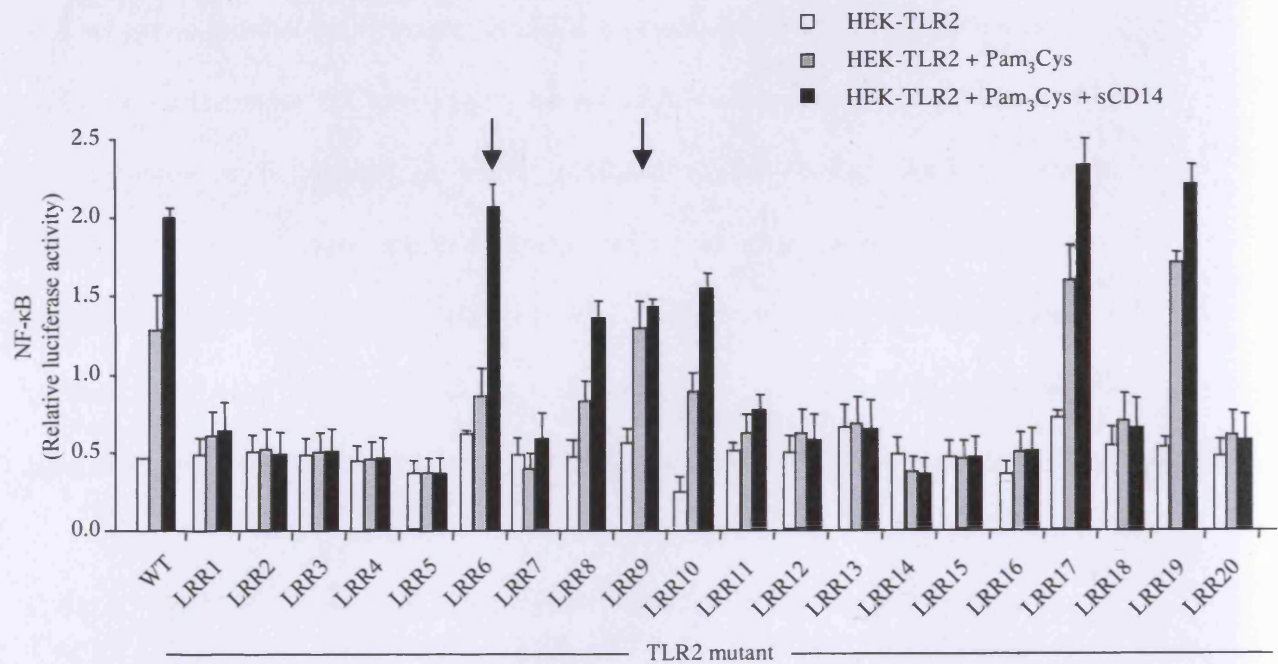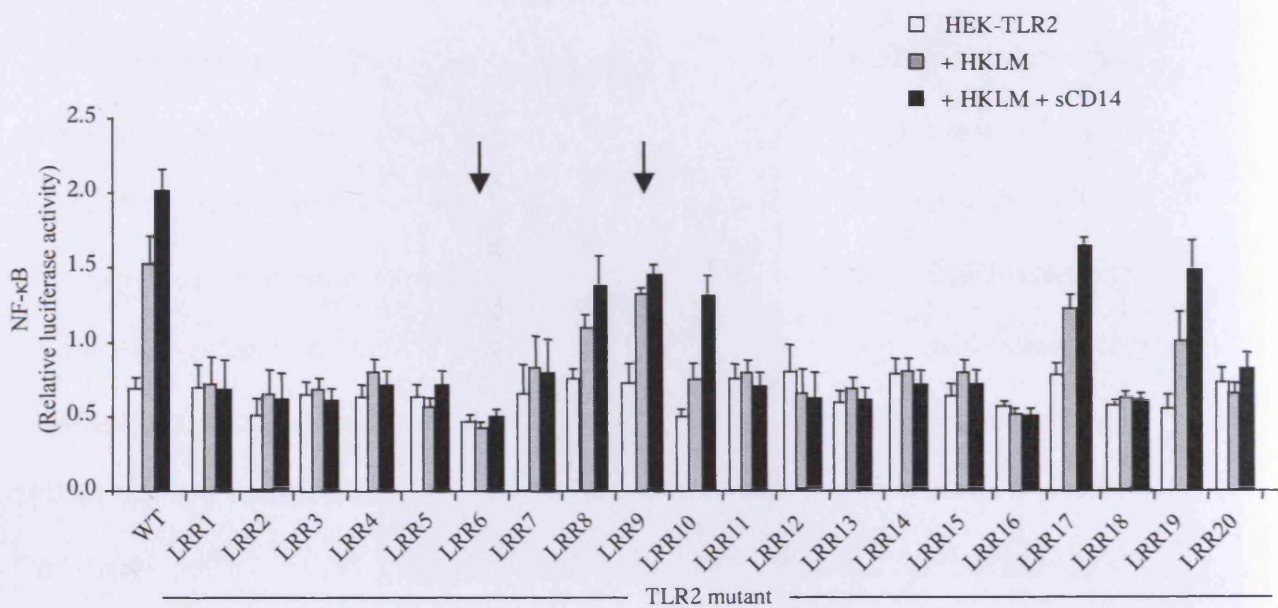
Figure 2.5.2. Effect of TLR2 LRR mutations on ligand recognition and CD14 co-receptor activity.
HEK293 cells transiently expressing the wild-type (WT) TLR2 protein or a TLR2 mutated at the indicated leucine-rich repeat (LRR) were stimulated (16 h) with Pam$_3$Cys lipopeptide (50 ng/ml) or whole heat-killed *Listeria monocytogenes* (HKLM; 10:1, bacteria:cell ratio) in the absence or presence of 500 ng/ml sCD14. Cell activation was measured by using an NF-κB reporter assay. Results are from one experiment (±SD) representative of three. Arrows point at LRR TLR2 mutants showing a marked effect of the mutation on co-receptor activity.

PCR analysis of RNA extracted from each TLR2 mutant and WT cell transfectants showed readily detectable levels of the corresponding TLR2 mRNA. Figure 2.5.1, B, shows a representative example of the detection of the mRNA of one of the TLR2 mutants whose cell-surface expression was undetectable (LRR2 TLR2 shown) and that of WT TLR2. This finding indicated that the lack of cell-surface expression of a number of mutants was not due to a deficient transfection or transcription of the corresponding cDNAs. The study, therefore, focused on the functional activity of the cell-surface expressed mutants LRR1, 6, 8, 9, 10, 11, 17 and 19 of TLR2.

2.5.1.B   Selection of candidate regions of the TLR2 ECD to be involved in the CD14-TLR2 interaction

In order to identify the human TLR2 LRR(s) involved in the interaction with the co-receptor CD14, a comparative study of the capacity of WT TLR2 and each mutant to mediate ligand-induced NF-κB activation in the absence and presence of sCD14 was performed next. The TLR2-mutants whose cell-surface expression was undetectable were included in the study as controls.

Figure 2.5.2, A and B, shows that HEK293 cells transiently transfected with WT TLR2 were sensitive to stimulation with either the TLR2 agonist synthetic bacterial lipopeptide Pam_3Cys (Fig. 2.5.2, A) or the whole Gram-positive bacterium heat-killed *Listeria monocytogenes* (HKLM; Fig. 2.5.2, B), as judged by the activation of the transcription factor NF-κB. Sensitivity to stimulation was enhanced in the presence of the co-receptor, sCD14. Most cell-surface expressed TLR2 mutants were sensitive to stimulation by Pam_3Cys and HKLM. However, the enhancing effect of the co-receptor was found affected in the LRR6 TLR2 and LRR9 TLR2 mutants (Fig. 2.5.2). The

mutation of the LRR6 made the HEK-LRR6 TLR2 transfectants less responsive to Pam$_3$Cys stimulation, but more dependent on sCD14's co-receptor activity, as compared to the wild-type TLR2 cell transfectants (Fig. 2.5.2, A). The responsiveness of this mutant to HKLM, either in the absence or presence of sCD14, was completely lost (Fig. 2.5.2, B). The mutation of the LRR9 affected only the co-receptor-dependent cell sensitivity of LRR9 TLR2, since the response of the HEK-LRR9 TLR2 transfectants to Pam$_3$Cys or HKLM in the absence of sCD14 was comparable to that of WT TLR2 transfectants, while their response to either of the two ligands was not enhanced by sCD14 (Fig. 2.5.2). LRR1 and LRR11 TLR2 mutants did not mediate cell activation in response to either Pam$_3$Cys or HKLM (Fig. 2.5.2), suggesting that these LRRs may be involved in ligand recognition by TLR2. Indeed, previously published work showed the involvement of LRR1 in PGN recognition (Mitsuzawa et al., 2001). Pam$_3$Cys or HKLM did not induce activation of the LRR2, 3, 4, 5, 7, 12, 13, 14, 15, 16, 18 and 20 TLR2 cell transfectants. This finding was consistent with the observation that cell surface expression of these mutants was not detected (see previous section).

Given the effect of the mutations in LRR6 and LRR9 on co-receptor activity, the LRR6 TLR2 and LRR9 TLR2 mutants were selected for further studies.

2.5.2 Functional activity of TLR2-derived peptides

2.5.2.A Effect of peptides representing TLR2 LRRs on TLR2-mediated cell activation

It was reasoned that the mutations in LRR6 and 9 may have affected the activity of the co-receptor directly, by modifying the TLR2 ECD regions primarily involved in the TLR2-CD14 interaction, or indirectly, by inducing a modification in the
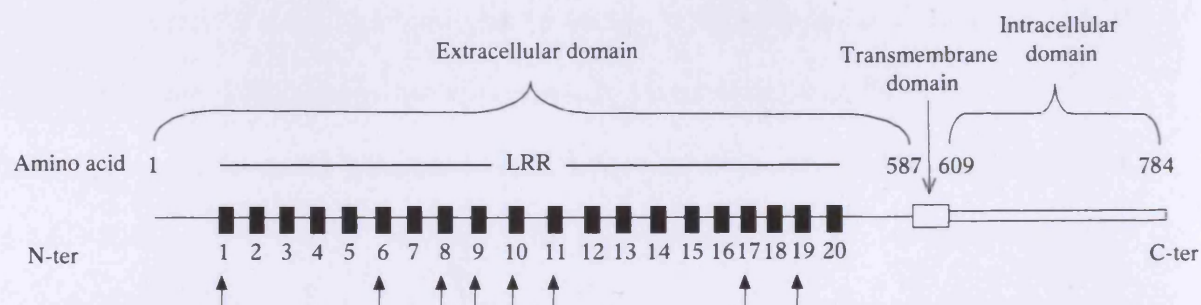
Figure 2.5.3. The human TLR2 molecule.
Schematic representation of the human TLR2 protein showing the 20 leucine-rich repeats (LRR) present in the extracellular domain (the separation shown between LRRs is representative of the distance between domains). Arrows point at the LRR tested for their involvement in the TLR2-CD14 interaction (Figs. 2.5.2, 2.5.4, and 2.5.5.). N-ter and C-ter, N-terminus and C-terminus of the TLR2 protein.

Table 2.5.1. Human TLR2 ECD-derived peptide sequences.

TLR2 LRR (amino acid position*)	Corresponding peptide sequence	Peptide molecular mass (Da)
LRR6 (173-196)	LTFLEELEIDASDLQSYEPKSLKS	2756.08
LRR9 (247-275)	TNSLIKKFTFRNVKITDESLFQVMKLLNQ	3457.13
LRR10 (276-305)	ISGLLELEFDDCTLNGVGNFRASDNDRVID	3298.62
LRR15 (412-434)	LKNLTNIDISKNSFHSMPETCQW	2707.1
LRR17 (456-476)	PKTLEILDVSNNNLNLFSLNL	2371.74
LRR20 (522-545)	FHTLKTLEAGGNNFICSCEFLSFT	2680.08

* Position of the amino acids on the human TLR2 precusor sequence – i.e. including the signal peptide (Met[1] to Glu[20]).

A

Calu-3 cells

B
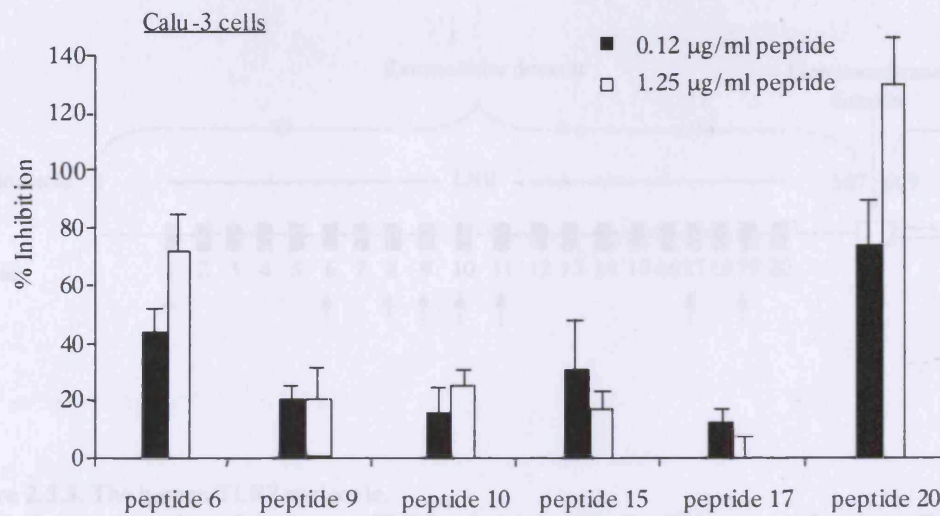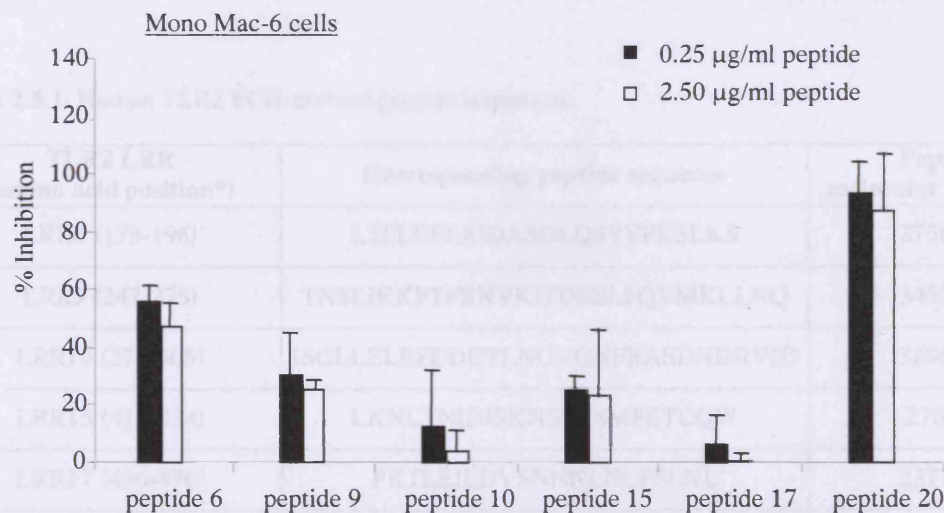
Mono Mac-6 cells

Figure 2.5.4. Effect of TLR2 ECD-derived peptides on TLR2-mediated cell activation.
Triplicate cultures (1.5×10^5 cells/well) of Calu-3 **(A)** or Mono Mac-6 **(B)** cells were stimulated (16h) with a suboptimal concentration of 250 ng/ml (Calu-3) or 400 ng/ml (Mono Mac-6) of Pam$_3$Cys in the presence or absence of the indicated concentrations of TLR2 ECD-derived peptides representing the indicated LRR of human TLR2. Following incubation, the cell culture supernatants were tested for IL-8 levels by ELISA. Results are expressed as % inhibition of IL-8 release induced by each peptide compared to that exerted by the anti-CD14 mAb MY4 (10 µg/ml) – which blocks CD14 co-receptor activity completely – used as 100% inhibition control. Results are from one experiment (±SD) representative of four.

tertiary structure of TLR2 that results in increased (LRR6 mutation) or decreased (LRR9 mutation) capacity (avidity) to interact with the co-receptor. Therefore, to test whether the LRR6 and LRR9 were directly involved in the interaction with CD14, the peptides corresponding to LRR6 and LRR9 were generated (Table 2.5.1) and first tested for their capacity to inhibit the ligand-induced TLR2-mediated activation of cells that naturally express both CD14 and TLR2: 1) the human respiratory epithelial cell line Calu-3, which expresses very low levels of CD14 and TLR2, and 2) the human monocyte-like cell line, Mono Mac-6, which expresses high and moderate levels of CD14 and TLR2, respectively. Although LRR15 TLR2 and LRR20 TLR2 mutants could not be expressed and tested (see section 2.5.1.A), the peptides corresponding to LRR15 and LRR20 were also generated and tested, as the LRR15 of human TLR2 shows considerable sequence similarity to that of mouse TLR2 and human and mouse TLR4 – suggesting that LRR15 is involved in a critical function – , and LRR20 is closest to the immediate juxtamembrane region of the ectodomain, which appears to be involved in receptor interactions and signalling (Gay et al., 2006; Fig. 2.5.3 and Table 2.5.1). In addition, the peptides corresponding to LRR10 and LRR17 were generated and used as negative controls (Fig. 2.5.3; Table 2.5.1), as mutations in these LRR did not affect the sensitivity of the corresponding cell transfectants (Fig. 2.5.2).

Figure 2.5.4, A and B, shows that only the peptides corresponding to LRR6 (peptide 6) and LRR20 (peptide 20) exerted a marked inhibition on the Pam_3Cys-mediated release of the typical pro-inflammatory chemokine, IL-8, by Calu-3 and Mono Mac-6 cells, as compared to that exerted by the anti-CD14 mAb MY4 – which blocks CD14 co-receptor activity completely – used as 100% inhibition control. Peptide 9 (LRR9) did not affect cell activation at the two concentrations tested, indicating that LRR9 is not
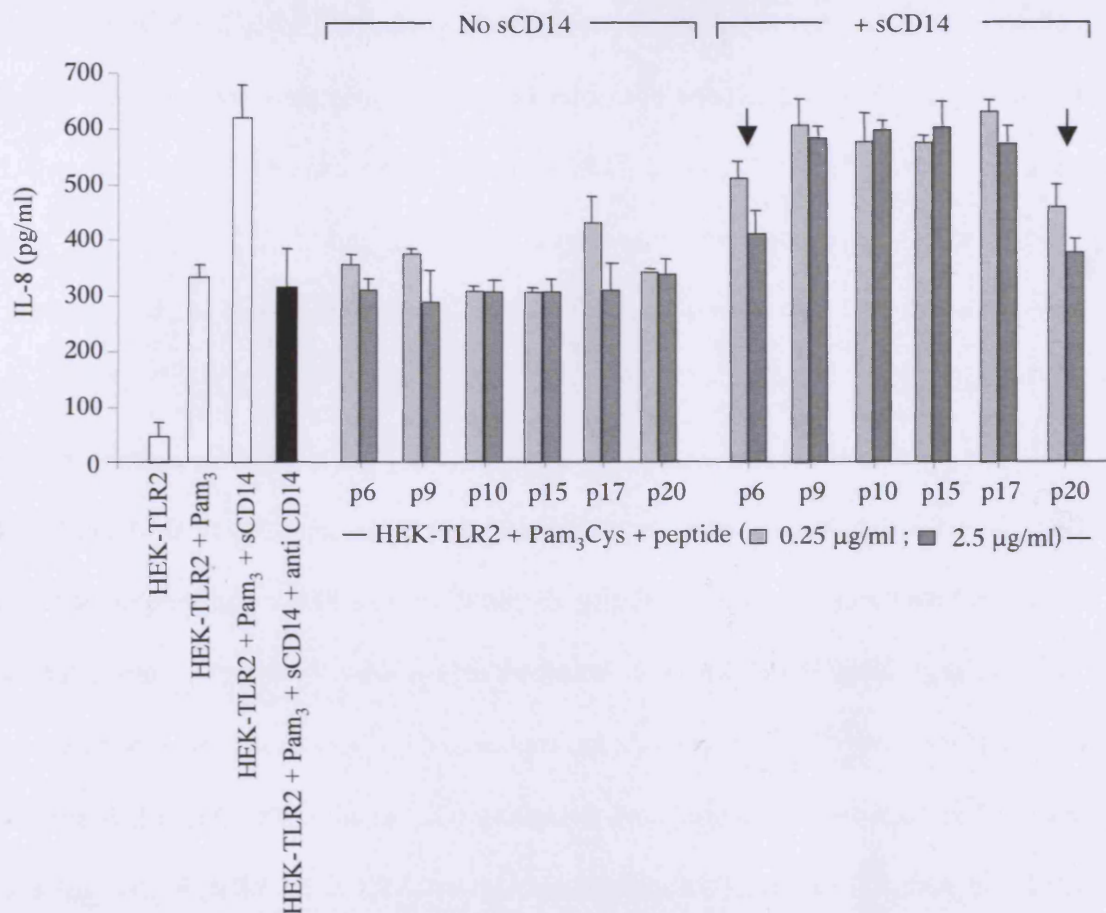
Figure 2.5.5. Peptides 6 and 20 specifically inhibit CD14 co-receptor activity.
Triplicate cultures (1.5 x 10^5 cells/well) of HEK-WT TLR2 cell transfectants were stimulated (16 h) with Pam$_3$Cys (50 ng/ml) in the absence or presence of sCD14 (500 ng/ml), the anti CD14 mAb MY4 (10 µg/ml) and the indicated concentrations of TLR2 ECD-derived peptides. Following incubation, the cell culture supernatants were tested for IL8 by ELISA. Results are from one experiment (±SD) representative of three. Arrows point at peptides 6 and 20, which inhibited the CD14-dependent, but not the CD14-independent cell activation.

directly involved in the TLR2-CD14 interaction, and that the loss of the enhancing effect of CD14 in the ligand-induced activation of the HEK-LRR9 TLR2 transfectant (Fig. 2.5.2) most probably resulted from an indirect effect of the mutation on the structure of the TLR2 molecule.

2.5.2.B   Peptides 6 and 20 specifically inhibit CD14 co-receptor activity

In order to confirm that the inhibitory effect of peptides 6 and 20 on the lipopeptide-induced cell stimulation described previously (Fig. 2.5.4) resulted from the specific disruption of CD14 co-receptor activity, the effect of peptides 6 and 20 on the Pam_3Cys-induced stimulation of HEK-WT TLR2 cell transfectants in the absence and presence of sCD14 was tested (Fig. 2.5.5). The Pam_3Cys-induced release of IL-8 by HEK-TLR2 cells was substantially enhanced by sCD14. This enhancing effect was completely abrogated by the anti-CD14 mAb MY4, as the levels of IL-8 released were reduced to those in the absence of the co-receptor (Fig. 2.5.5, left). Peptides 6 and 20 – at two concentrations – did not affect Pam_3Cys-induced IL-8 release in the absence of sCD14 (Fig. 2.5.5, centre), since the levels of IL-8 were similar to those released by the cells in the presence of the control peptides, and comparable to those released by cells stimulated in the presence of Pam_3Cys only. However, in the presence of sCD14, peptides 6 and 20, but not the control peptides, inhibited cell stimulation in a dose-dependent manner (Fig. 2.5.5, right). These results confirmed that LRR6 and LRR20 are directly involved in the TLR2-CD14 interaction, and that peptides 6 and 20 are capable of modulating TLR2-mediated cell activation and pro-inflammatory responses by specifically targeting the TLR co-receptor, CD14.

2.5.3 Discussion

In the last phase of this project, the systematic mutation of the twenty LRR domains of the human TLR2 molecule was conducted to define the regions involved in the interaction of TLR2 with the co-receptor, CD14. The results presented in this section demonstrated that LRR6, 9 and 20 are involved in such interaction. LRR9, however, seems to be indirectly involved, since a peptide representing LRR9 did not reproduce the effect of the mutation.

The mechanism underlying the inhibitory effect of peptide 6 and peptide 20 remains to be determined and a number of experiments are being conducted to address this issue (see *Concluding Remarks and Future Work*, page 119, (8)). It is possible that peptides 6 and 20 inhibit the enhancing effect of CD14 on cell activation by binding to the co-receptor, like sTLR2, and thus affect CD14's capacity to interact with mTLR2. It is also possible that one or both peptides interfere with the binding of the microbial ligand to the co-receptor. In this regard, it is interesting to note that the region of CD14 involved in its interaction with TLR2, i.e. amino acids 57-64 (Iwaki et al., 2005), is within the N-terminal side of the molecule, which is involved in ligand binding.

Nevertheless, peptide 6 and peptide 20 proved to be capable of reducing the TLR2-mediated activation of cells that naturally express both CD14 and TLR2, i.e. Calu-3 and Mono Mac-6 cells, as well as that of HEK-TLR2 cell transfectants. Significant levels of inhibition in Mono Mac-6 and HEK-TLR2 cells were obtained by using higher doses of peptide (0.25 and 2.5 µg/ml) than those used for Calu-3 cells (0.12 and 1.25 µg/ml). The higher level of mTLR2 expression in Mono Mac-6 and HEK-

TLR2 cells compared with that in Calu-3 cells most likely requires a higher amount of peptide to compete favourably with mTLR2 for interaction with CD14.

The inhibitory capacity of peptides 6 and 20, as well as that of peptide 9, remains to be tested *in vivo*. The findings described in this section, however, pave the way for future studies to evaluate the strategy of using peptides, or small molecules mimicking the peptides' activity, to target and neutralise the TLR co-receptor CD14, thus reducing pro-inflammatory responses.

Most of the therapeutic strategies to control TLR responses tested so far are based on the use of TLR antagonists to inhibit TLR ligand recognition. The antagonists can be structural analogs of agonists that bind to the TLR but fail to signal, TLR blocking antibodies, or small molecule antagonists selected from compound libraries (Kanzler et al., 2007). Alternative strategies use small molecules to target intracellular TLR signal intermediates (O'Neill, 2003). Many of these approaches have failed due to factors such as cost, toxicity, high dose requirements, the half-life of the antagonist or negative effects on bacterial clearance. Current trials still using such approaches are at the pre-clinical or phase I to III stages. A phase I trial of an anti-human CD14 mAb has been conducted although a very high mAb dose is required for effect (Verbon et al., 2001). Current therapeutic approaches increasingly aim to reduce the side effects resulting from potentially excessive TLR-mediated inflammatory responses without abrogating TLR's capacity to recognise and respond to the invading microorganism. In this context, the results presented in this section define a potential strategy to reduce inflammation without abrogating TLR recognition capacity that is novel in that it targets the co-receptor molecule by using small peptides. By modulating TLR triggering, this strategy is expected to preserve TLR ligand recognition, and by extension, microbial

clearance capacity, while reducing the level of the inflammatory response. By targeting the co-receptor and not the microbial ligand, this strategy offers the possibility of modulating responses to three different TLRs, TLR2, 3 and 4, by targeting their common co-receptor. Notably, this strategy has the advantage of using small peptides, thus avoiding the high cost of Ab production and the problems associated with the use of high doses of relatively large proteins.

3. CONCLUDING REMARKS

and

FUTURE WORK

Following the initial description of the crucial involvement of TLRs in acute inflammation and septic shock (Poltorak et al., 1998; Hoshino et al., 1999) and the more recent well-documented observations implicating TLRs in a number of autoimmune and chronic inflammatory diseases – such as lupus, arthritis, inflammatory bowel disease (IBD) and atherosclerosis (Zuany-Amorim et al., 2002; Barrat et al., 2005; Tobias and Curtiss, 2007) – it has become clear that TLR overactivation plays a prominent role in the pathogenesis of a variety of acute and chronic inflammatory conditions (Kanzler et al., 2007). The different levels at which TLR activity can be regulated highlight the importance of such regulation to the maintenance of immune homeostasis. sTLR2 is the only soluble form of a mammalian TLR so far identified that occurs naturally. It has been hypothesised that sTLR2 may protect the host from deleterious TLR2-mediated innate immune responses by preventing the excessive initial triggering of TLR2 (Zuany-Amorim et al., 2002; LeBouder et al., 2003; Heggelund et al., 2004; Liew et al., 2005). This study was therefore conducted to test this hypothesis by: 1) evaluating the full extent of sTLR2's regulatory capacity and its biological relevance *in vivo*, 2) studying the mechanism underlying such regulatory capacity, and 3) evaluating the therapeutic potential of sTLR2's activity.

The results of this study demonstrated that sTLR2 regulates TLR2-mediated pro-inflammatory responses induced by microbial components and whole Gram-positive bacteria *in vitro* and *in vivo*, and that sTLR2 also has the potential to modulate critical effector functions, namely phagocytosis and superoxide production. Notably, the modulatory capacity of sTLR2 does not seem to affect bacterial clearance. Two mechanisms contributing to such regulatory activity were identified; firstly, the capacity of sTLR2 to act as a decoy microbial receptor, and secondly its capacity to disrupt the

interaction of TLR2 with its co-receptor, CD14, by binding to CD14. The therapeutic

potential of these findings was highlighted by the identification of three LRRs of TLR2

which appear to be involved in the interaction with CD14 – namely LRR6, 9 and 20 –

and the demonstration that peptides representing LRR6 and LRR20 are capable of

reducing CD14-dependent TLR2-mediated pro-inflammatory responses *in vitro*, most

likely by targeting CD14 and disrupting its co-receptor activity. These findings thus

establish sTLR2 as a regulator of TLR2-mediated inflammatory responses and pave the

way for future studies evaluating novel therapeutic strategies to control inflammation.

Future work will address a number of issues and questions raised by the present

study:

1. The improvement of the yield of rhsTLR2 will be the subject of future

work. Recently, the crystallographic structure of the TLR2-TLR1 heterodimer was

achieved using a novel strategy for the production of soluble TLRs that is based on the

generation of hybrid molecules consisting of fragments of TLRs and hagfish variable

lymphocyte receptors fused at conserved sites (Jin et al., 2007). The use of this strategy

will be evaluated in our laboratory.

2. The elution profile of sTLR2 was monitored using anti-His and anti-TLR2

Abs and showed a major ~72-kDa polypeptide band, and additional minor ~83-kDa and

~90-kDa bands only detectable by the anti-TLR2 (polyclonal) Ab. By contrast, the

Coomassie blue staining of the eluted fractions and final preparations showed the ~90-

kDa sTLR2 isoform as the main sTLR2 polypeptide. Based on previous findings (Iwaki

et al., 2002; LeBouder et al., 2003; Weber et al., 2004), it is speculated that the three

sTLR2 isoforms may be glycoforms, sharing the same backbone polypeptide – the TLR2 ectodomain. To test this possibility, deglycosylation experiments of purified sTLR2 followed by immunoblotting with anti-His and anti-TLR2 Abs, as well as Coomassie blue staining, may be conducted.

3.   The results of the *in vitro* studies show that sTLR2's modulatory effect is not limited to the release of IL-8, since NF-κB activation – which results in the induction of a number of immune mediators – was markedly affected by sTLR2. Notably, the modulatory effect on NF-κB appears to be stronger than that on IL-8 release. Differences in sensitivity between the ELISA (used for IL-8 determinations) and the NF-κB reporter assay may contribute to the observed difference in modulatory effect. It is also possible, however, that this difference reflects a differential effect of sTLR2 on the signalling pathways leading to IL-8 release. It would therefore be of interest to test and compare the effect that sTLR2 exerts on NF-κB and AP-1 – transcription factor activated through the MAP kinases pathway, whose activation may also lead to IL-8 production (Hipp et al., 2002; Kang et al., 2007).

4.   For the *in vivo* studies, two mouse models of acute peritoneal inflammation were used to prove the concept that sTLR2 can have a significant modulatory effect on bacterial-induced pro-inflammatory responses. The use of these models also showed that sTLR2 can perform this activity without compromising bacterial clearance. The mouse models were based on the peritoneal injection of a *S. epidermidis*-derived cell-free supernatant or live *S. epidermidis* (5×10^7 CFU). Current work in our laboratory is

addressing the issue of whether sTLR2 can show a similar modulatory effect when a higher bacterial innoculum is used or when other bacterial strains are tested.

5.   The demonstrated capacity of sTLR2 to regulate pro-inflammatory responses poses the question of whether sTLR2 confers protection during bacterial-induced sepsis. Comparative survival rate studies in mouse models of Gram-positive sepsis will help to address this question. Home Office authorisation is being sought to perform these studies.

6.   sTLR2 may not only modulate an acute inflammation, but also influence the course of chronic inflammatory conditions. It would therefore be of interest to test the effect of sTLR2 in mouse models of chronic inflammatory processes such as atherosclerosis, arthritis, and IBD.

7.   The study of the mechanism responsible for sTLR2's modulatory effect demonstrated the capacity of sTLR2 to act as a decoy microbial receptor. TLR2 is known to heterodimerise with TLR1 or TLR6 for lipopeptide recognition (Takeuchi et al., 2001; Takeuchi et al., 2002), and crystallographic studies showed that Pam_3Cys serves as a link or bridge between TLR2 and TLR1 (Jin et al., 2007). It is therefore possible that sTLR2 performs its decoy activity, in our experimental systems, by binding to TLR1 in the presence of the tri-acyl lipopeptide and consequently preventing TLR1 from associating with mTLR2 – which is crucial to signalling. This possibility may be tested by evaluating the effect of sTLR2 on the lipopeptide-induced stimulation of cells from TLR1-deficient mice.

8.   The description of peptides capable of reducing CD14-dependent TLR2-mediated responses poses the question of the underlying mechanism. At present, experiments are being conducted in our laboratory to test: 1) the binding of peptide 6 and peptide 20 to CD14 (Biacore and plate assays), 2) the possible disruption of the interaction between mCD14 and mTLR2 by peptide 6 and/or 20 (FRET and co-immunoprecipitation experiments), and 3) the effect of peptides 6 and 20 on the binding of Pam_3Cys to CD14 (flow cytometry analysis of the binding of biotinylated Pam_3Cys to CHO-CD14 transfectants in the presence and absence of the peptides).

9.   The therapeutic potential of using peptides to target the co-receptor, CD14, deserves further investigation: 1) It will be important to test the modulatory capacity of peptides 6, 9 and 20 in the *in vivo* models of acute inflammation used here to study sTLR2, as well as in models of chronic inflammation (e.g. arthritis, IBD, atheroschlerosis). 2) The use of small molecules, selected from compound libraries, with the capacity to mimic the effect of peptides 6 and 20 is being considered. This would avoid the problems associated with the use of peptides, i.e. high dose, solubility and short life span.

10.   The demonstrated capacity of sTLR2 and peptides 6 and 20 to modulate inflammatory responses by targeting CD14 and disrupting its co-receptor activity suggested that sTLR2 and/or the peptides may be used to modulate responses mediated not only by TLR2, but also by TLR3 and TLR4, by targeting their common co-receptor. The results of recent preliminary experiments showing the hyporesponsiveness of LPS-stimulated cells overexpressing sTLR2 (section 2.4.4, Fig. 2.4.6) supports this

possibility. Thus, following confirmation of these preliminary findings, the effect of sTLR2 and peptides 6, 9 and 20 on mouse models of LPS – or live Gram-negative bacteria – induced acute peritoneal inflammation will be tested.

In conclusion, this study defines sTLR2 as an efficient regulator of TLR2-mediated inflammatory responses, as it is capable of reducing inflammation by controlling PMN influx while preserving monocyte/macrophage recruitment and without compromising bacterial clearance. The capacity of sTLR2 and peptides derived from its ectodomain to exert regulatory effects by targeting the TLR co-receptor, CD14, may inform the design of novel therapeutics against acute and chronic inflammatory conditions that will aim at disrupting the co-receptor's activity, thus blunting, but not abrogating, microbial recognition and host immune responses.

4. MATERIALS and METHODS

Table 4.1. List of antibodies and immunoreagents

Antibodies to	Clone/code	Isotype	Source
Human CD14	MY4 (and MY4-FITC)	Mouse IgG2b	Coulter, Luton, U.K.
Human CD14	69.4	Rabbit Igs	Sanofi-Synthelabo, France (Labéta et al., 2000)
Human TLR2 peptide (N-terminus 20-mer peptide [18]SKEESSNGASLSGDRNGIGK[37])	TLR2p	Rabbit Igs	Provided by Prof. B.P. Morgan, Dept. of Medical Biochemistry and Immunology, Cardiff University,U.K. (Le Bouder et al., 2003)
Human TLR2 peptide (N-terminus 17-mer peptide)	sc8689	Goat IgG	Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.
Human TLR2	IMG 319	Mouse IgG1	Imgenex, San Diego, CA, U.S.A.
Human TLR2	TL2.1 (and T2.1-PE)	Mouse IgG2a	eBioscience, San Diego, CA, U.S.A.
Human/mouse TLR2	TL2.5 (and T2.5-A488)	Mouse IgG1	eBioscience
cMyc epitope	9E10	Mouse IgG1	Sigma-Aldrich, Dorset, U.K.
His_5 tag	anti-His_5 mAb	Mouse IgG1	Qiagen Valencia, CA, U.S.A.
Mouse F4/80	CI:A3-1-FITC	Rat IgG2b	Serotec, Oxford, U.K.
Mouse CD11b	M1/70-APC	Rat IgG2b	BD Bioscience, Oxford, U.K.
Mouse IgGs	HRP-conjugated anti mouse IgG Ab	Rabbit Igs	Amersham/GE Healthcare, Little Chafont, U.K.
	Biotin-conjugated anti mouse IgG Ab		Dako Ltd, High Wycombe, U.K.
Rabbits IgGs	HRP-conjugated anti rabbit IgG Ab	Mouse Igs	Amersham/GE Healthcare,
Human IgGs	Biotin-conjugated anti human IgG Ab	Mouse Igs	Southern Biothec. Birmingham, AL, U.S.A.
Immunoreagents	**Clone/code**	**Isotype**	**Source**
Mouse IgG (purified)	MOPC-21	IgG1	Sigma-Aldrich
Mouse IgG (purified)	UPC-10	IgG2a	Sigma-Aldrich
Mouse IgG (purified)	MOPC-141	IgG2b	Sigma-Aldrich
Mouse IgG-A488	-	IgG1	eBioscience
Mouse IgG-PE	UPC-10	IgG2a	Sigma-Aldrich
Rat IgG-FITC	MCA1125F	IgG2b	Serotec
Rat IgG-APC	A95-1	IgG2b	BD Bioscience
HRP-conjugated cholera toxin B	-	-	List Biologicals, Campbell, CA, U.S.A.
HRP-conjugated streptavidin	-	-	Jackson Immunoresearch Lab., PA, U.S.A.
APC- conjugated streptavidin	-	-	Southern Biotech

4.1 Antibodies and reagents

The antibodies and immunoreagents used and their sources are listed in Table 4.1. RPMI-1640, DMEM and Express Five SFM media, L-glutamine, sodium pyruvate, non-essential amino acids (NEAA), penicillin, streptomycin and insulin were from Invitrogen Ltd (Paisley, U.K.). Bac Vector Insect Cell Medium and X-gal were from Novagen (San Diego, CA, U.S.A.). Low-endotoxin foetal calf serum (FCS) was from Hyclone (Logan, UT, USA; < 0.06 U/ml endotoxin). Ficoll-Histopaque-1077, bovine serum albumin (BSA), protein G-Sepharose, Nonidet P-40 (NP-40), Triton X-100, PMA, ionomycin and ampicillin were from Sigma-Aldrich (Dorset, U.K.). Ni-nitrilotriacetic acid (Ni-NTA) Superflow resin was from Qiagen (Valencia, CA, U.S.A). Hygromycin B was from Calbiochem (San Diego, CA, U.S.A.). Ultra-pure LPS (*Escherichia coli* O111:B4 strain), heat-killed *Listeria monocytogenes* (HKLM), peptidoglycan (PGN), polyinosinic-polycytidylic acid (poly I:C) and flagellin were purchased from Invivogen (San Diego, CA, U.S.A.). The synthetic bacterial lipopeptide Pam_3-Cys-Ser-$(Lys)_4$ HCl (Pam_3Cys) was from EMC microcollections GmbH (Tübingen, Germany). The human cytokines IL-1β and TNF-α were from R&D Systems (Minneapolis, MN, U.S.A.). All chemicals were reagent grade.

4.2 Cells and cell cultures

Sf9 and High Five ovarian insect cells (Invitrogen Ltd) − derived from *Spodoptera frugiperda* and *Trichoplusia ni,* respectively − were cultured at 27°C in Bac Vector Insect Cell Medium (Sf9) and Express Five SFM (High Five) medium as adherent (Sf9) or suspension (High Five) cultures.

Chinese hamster ovary (CHO), mouse RAW264 (American Type Culture Collection-ATCC, Rockville, MD, U.S.A), and CHO-CD14 (previously generated in our laboratory, Labéta et al., 2000) cells were cultured in RPMI-1640 medium supplemented with 10 % FCS (Hyclone), 2 mM glutamine, and specific additives for the CHO-CD14 cells: 1 mM pyruvate, 0.5% (v/v) NaHCO₃, and 50 µg/ml L-proline. Human embryonic kidney (HEK) 293 cells, the astrocytoma cell line U373 (ATCC), and the human airway epithelial cell line Calu-3 (kindly provided by Prof K.P. Jones, School of Health Sciences, University of Wales Institute, Cardiff, U.K.) were cultured in DMEM medium supplemented with 10% FCS. The medium was further supplemented with 200 µg/ml hygromycin B for culturing HEK-TLR2, U373-TLR2 and U373-TLR2/sTLR2 cell transfectants. The human monocytic cell line, Mono Mac-6 (kindly provided by Prof. H.W.L. Ziegler-Heitbrock, Dept. of Immunology, Leicester University, U.K.), was cultured in RPMI-1640 medium supplemented with 10 % FCS, 2 mM glutamine, 1 mM pyruvate, 1 % non-essential amino acids and 10 µg/ml insulin. Human peritoneal mesothelial cells were isolated by serial tryptic digestion of omental tissue from consenting patients undergoing elective abdominal surgery, and cultured as previously described (Topley et al., 1996).

Peripheral blood mononuclear cells (PBMC) were obtained following Ficoll density-gradient centrifugation of buffy coats from heparinised blood of healthy donors (Welsh Blood Bank, Cardiff, U.K.). The blood was diluted 1:1 with room temperature RPMI-1640 medium, overlaid (12 ml) onto 8 ml of room temperature Ficoll-Histopaque to a blood:Ficoll final ratio of 3:2, and centrifuged at 750 x g for 20 min at room temperature. The upper (plasma) and lower (red cells and PMN) phases were discarded, and the interphase containing the PBMC was recovered and washed three times at room

temperature with RPMI-1640 medium (first wash, 370 x g, 12 min; second, 190 x g, 10 min; third, 120 x g, 8 min). Monocytes were obtained by adherence (2 h, 37°C) of PBMC resuspended in RPMI-1640 medium supplemented with 1% FCS, penicillin (50 U/ml) and streptomycin (50 µg/ml). The purity of the monocyte preparations was always > 95%, as evaluated by flow cytometry with anti-CD14, -CD19 and -CD3 mAbs.

Human neutrophils (PMN) were prepared by dextran sedimentation and Ficoll density-gradient centrifugation of citrated blood from healthy donors (60 ml). Blood (12 x 5 ml) was incubated (90 min, 37°C) with 12 x 350 µl of citrate buffer (76 mM citric acid, 169 mM sodium citrate, pH 5.6) and 12 x 1 ml 70% dextran (Baxter Healthcare, Newbury, U.K.), and the lower erythrocyte plasma layer was discarded. The upper, platelet and leukocyte-rich, layers were pooled and washed with PBS (2 x, 300 x g, 8 min, room temperature). Cells were then resuspended in 5 ml of PBS and overlaid onto 5 ml of Ficoll Hypaque (Amersham/GE Healthcare, Little Chafont, U.K.). After centrifugation (400 x g, 35 min, room temperature), the interface (MNC) was discarded and the pellet (PMN) was washed with PBS (2 x, 300 x g, 8 min, room temperature). Erythrocytes contaminating the PMN preparations were removed by hypotonic lysis (3 ml of ice cold 0.2% NaCl, 1 min). To restore iso-osmolarity, 1.6% NaCl (3 ml) was added prior to washing with PBS (2 x, 300 x g, 8 min, room temperature). PMN were then resuspended in PBS (4 x 10^6 cells/ml) in preparation for superoxide production assays (section 4.18). The purity of the PMN preparation was always > 95%, as evaluated by differential cell count on cytospin preparations.

4.3 Cell transfections

Cells were transfected using the Lipofectamine™ Transfection system (Invitrogen), according to the manufacturer's instructions. For all transient transfection experiments, cells (HEK293 or HEK-TLR2) were seeded in a 24-well plate (1.5×10^5 cells/well) and transfected 24 h later (approximately 3×10^5 cells/well) using 2 µl of Lipofectamine™ per well and 2 µl of Plus Reagent™ per µg of construct.

For NF-kB reporter assays, HEK293, HEK-TLR2, U373-EV or U373-sTLR2 cells were transiently transfected with 0.25 µg of a construct directing the expression of the firefly luciferase reporter gene under the control of the NF-κB promoter (pNF-κB-Luc; Stratagene, La Jolla, CA, U.S.A.) and 0.05 µg of a construct directing the expression of the *Renilla* luciferase independently of NF-κB (pRL-SV40; Promega, Southampton, U.K.). Twenty four hours post-transfection, cell activations and NF-κB measurements were conducted as described below (section 4.16).

4.4 SDS-PAGE, Western blotting and Coomassie blue staining

SDS-PAGE was carried out using the Bio-Rad Mini Protean II gel apparatus (Bio-Rad, Hercules, CA, U.S.A). All samples were diluted with Laemmi reducing sample buffer (2% SDS, 100 mM DTT, 50 mM Tris-HCl, pH 6.8, 10 % glycerol and 0.1% bromophenol blue, final concentration) and boiled for 4 min prior to 10% SDS-PAGE. Pre-stained molecular weight markers (Bio-Rad) were run in parallel. Electrophoresis was carried out using SDS running buffer (25mM Tris base, 192 mM

Table 4.2. List of antibodies used for Western blotting and corresponding experimental condition

Specificity of primary Ab	Blocking * conditions	Primary Ab dilution */ BSA concentration	Secondary Ab * (1:2000 in 2% milk)
His$_5$ (mAb)	3%BSA	1/1000 in 3%BSA	Anti-mouse-HRP
Human TLR2 (TLR2p, polyc. Ab)	2% milk	1/3000 in 2% milk	Anti-mouse-HRP
Human TLR2 (IMG319, mAb)	5% milk	1/500 in 2% milk	Anti-mouse-HRP
Human TLR2 (sc8689, polyc. Ab)	5% milk	1/2000 in 2% milk	Anti-goat-HRP
Human CD14 (MY4, mAb)	5% BSA	1μg/ml in 2% BSA	Anti-mouse-HRP
Human CD14 (69.4, polyc. Ab)	5% BSA	1/2000 in 2% BSA	Anti-rabbit-HRP
cMyc (mAb)	5% milk	1/500 in 2% milk	Anti-mouse-HRP
HRP-conjugated cholera toxin B	2% milk	1/1000 in 2% milk	-

*Reagents were diluted in PBS-T

glycine, 0.1% SDS). Gels were briefly washed twice with PBS, before processing to Western blotting or Coomassie blue staining.

In preparation for Western blots, following electrophoresis, the gels were incubated with transfer buffer (48 mM Tris base, 39mM glycine, 20% (v/v) methanol) at room temperature for a total of 20 min with 3 changes of buffer. Extra thick filter paper (Bio-Rad) and the nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Uk Ltd., Little Chafont, U.K.) used for transfer were kept in transfer buffer for 20 min before transfers. Electrotransfer was conducted in a semi-dry transfer cell apparatus (Transblot SD, Bio-Rad) for 30 min at 13 volts. Following transfer, the membranes were blocked for 1 h at room temperature with blocking buffer consisting of PBS/0.1% Tween-20 (PBS-T) supplemented with BSA as indicated in Table 4.2. Membranes were then washed (1 x 15 min and 2 x 5 min) and incubated with the appropriate first Ab (Table 4.2) overnight at 4°C with gentle rocking, and following washing (see above), the membranes were incubated (1 h, room temperature) with the second Ab-horseradish peroxidase (HRP) conjugated (ECL system, Amersham). Subsequently, the membranes were washed (PBS-T, 1 x 15 min and 4 x 5 min at room temperature) before detection by enhanced chemiluminescence (ECL system, Amersham).

To test the specificity of the detection of sTLR2 in the mice peritoneal lavages, peptide competition experiments were performed (Fig. 2.3.3). Before immunoblotting, TLR2p was incubated (2 h, room temperature) with a 10 x mass excess of the peptide used for immunisation or BSA as a control.

Table 4.3. List of primers

Name	Position (5' → 3')	Sequence (5' → 3')	Use
TLR2 -77 (fwd.)	- 77 → - 58	AGGTACCTGTGGGGCTCATT	Sequencing
TLR2 158 (fwd.)	TLR2: 158 → 177	CTGTAAAAAGCCTTGACCTG	Sequencing, screening
TLR2 527 (rev.)	TLR2: 548 → 527	GCATCAATCTCAAGTTCCTCAA	Sequencing, screening
TLR2 638 (fwd.)	TLR2: 638 → 658	TGCTGGAGATTTTTGTAGATG	Sequencing
TLR2 1048 (fwd.)	TLR2: 1048 → 1068	CTGGTTCCTTGTTTACTTTCA	Sequencing
TLR2 1195 (rev.)	TLR2: 1215 → 1195	GGTTTTTTCCAATGATGCCAA	Sequencing
TLR2 1598 (fwd.)	TLR2: 1598 → 1618	ATAACTTCATTTGCTCCTGTG	Sequencing
TLR2 1737 (rev.)	TLR2: 1755 → 1737	ACATTCCGACACCGAGAGG	Sequencing
TLR2 2094 (rev.)	TLR2: 2114 → 2094	TCAGAAAGCACAAAGACAGTT	Sequencing
M 13 5' (fwd.)	pCRII-TOPO™ (5')	CATTTTGCTGCCGGTC	Sequencing
M 13 3' (rev.)	pCRII-TOPO™ (3')	TAATACGACTCACTATAGGG	Sequencing
Q_T	-	CCAGTGAGCAGAGTGACGA GGACTCGAGCTCAAGC(T)$_{17}$	RT
pdN6	-	Random hexamer	RT
pDR2 5' (fwd.)	pDR2ΔEF1α™ (5')	CAAGCCTCAGACAGTGGTTC	Sequencing, screening
pDR2 3' (rev.)	pDR2ΔEF1α™ (3')	ATGTCTGGATCGGTGCGGGC	Sequencing

Table 4.4. Thermal cycling settings for PCR

Step	T°C	Time	Nr of cycles
Initial denaturation	95°C	5 min	1
Denaturation	94°C	40 sec	
Annealing	[a]55°C-68°C	40 sec	30
Extension	72°C	[b]1-2$^{1/2}$ min	
Final extension	72°C	10 min	1

[a] The annealing temperature depended on the melting T°C of primers.
[b] The extension time depended on the size of the PCR product (minimum 1 min/1000 bases)

For protein stainings, following electrophoresis, the gels were Coomassie blue stained (Coomassie Brilliant Blue R-250 Staining Solutions Kit, Bio-Rad) according to the manufacturer's instructions. The sensitivity of the Coomassie blue staining was between 8 and 28 ng of protein.

4.5 Polymerase Chain Reaction (PCR)

To screen bacterial colonies for the presence of the pMel Bac B-His rhsTLR2 or pDR2ΔEF1α-TLR2 mutant constructs (sections 4.8 and 4.20, respectively), individual colonies were picked from the LB/Agar plates and resuspended into 20 μl of water. 10 μl of this solution was then used as a template, and amplified using the TLR2-specific primer TLR2 158 (forward; pMel Bac B-His rhsTLR2) or the pDR2ΔEF1α 5' primer (forward; pDR2ΔEF1α-TLR2 mutant) and the TLR2 527 (reverse). The sequences and positions of the primers are given in Table 4.3.

To amplify the TLR2 mutant cDNA from total RNA by PCR, a mixture of 2 μl of pdN6 and 2 μl of Q_T reverse-transcribed RNA was used as template, and amplified using the TLR2-specific primers 638 (forward) and 1195 (reverse; Table 4.3).

For PCR, mixtures (50 μl) containing 0.3 mM dNTPs (Bioline), 1 mM $MgCl_2$ (Bioline), 25 pmol of forward and reverse primers (Table 4.3), and 2.5 U of Taq DNA polymerase (Bioline) in PCR buffer (Bioline, 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 67 mM Tris-HCl pH 8.8), were subjected to the cycling conditions shown in Table 4.4. All PCRs were performed using a Hybaid Omnigene thermocycler (Hybaid Ltd., Teddington, U.K.). PCR products were analysed by DNA agarose gel electrophoresis.

4.6 DNA agarose gel electrophoresis

DNA products were resolved on a 1% (w/v) agarose (Invitrogen) gel in TAE buffer (40 mM Tris Acetate pH 7.2, 1 mM EDTA). Agarose was dissolved in TAE buffer by boiling. The solution was allowed to cool to approximately 55°C before ethidium bromide (Sigma-Aldrich) was added at a final concentration of 50 ng/ml. The solution was poured into the gel casting tray of an Electro-4 gel tank (Hybaid Ltd.), and left for at least 30 min to set. The gel was transferred to the gel tank and TAE buffer was added. Before loading, DNA products were mixed with DNA loading buffer (0.25% (w/v) bromophenol blue, 30% glycerol in dH_2O) at a final DNA product: loading buffer ratio of 10:1. Electrophoresis was carried out at constant voltage (100 V) until separation of the DNA fragments. The SmartLadder (Eurogentec, Southampton, U.K.) was used as a DNA size marker. PCR products were visualised, and cut out from the gel using an UV transilluminator lamp. DNA was purified using a DNA-binding column (QIAquick® gel extraction kit, Qiagen) following the manufacturer's instructions. The extent of the purification was evaluated by performing 1% gel agarose electrophoresis.

4.7 DNA sequencing

DNA sequences were verified using the ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq® Polymerase FS (PerkinElmer, Waltham, MA, U.S.A.). The PCR mixture (8 µl final volume) contained 700 ng of plasmid, 5 pmol of primer (Table 4.3), and 3 µl of Terminator mixture containing the enzyme, the buffer, dNTPs and the Big Dye Terminators. Thermal cycling (25 cycles)

conditions were: denaturation, 96°C for 30 secs; annealing, 50°C for 15 secs; extension, 60°C for 4 min. Following thermal cycling, 40 µl of sterile water was added, and the PCR product was precipitated by adding 5 µl 3 M Na acetate buffer pH 5.4, 140 µl of ethanol, and incubating 20 min at −70°C. The sample was then centrifuged at 16,000 x g for 20 min at room temperature, and the precipitated DNA was washed with 75% (v/v) ethanol. DNA sequencing was carried out in our DNA sequencing facility (CBS, Cardiff University, School of Medicine) by using an automated ABI 377 DNA sequencer (Applied Biosystems, Warrington, U.K.). TLR2 mutants sequences were then compared to the reported "Homo sapiens toll-like receptor 2 sequence" (NCBI: NM 003264) using the sequence comparison software: Megalign™ DNA.

4.8 Production of recombinant human sTLR2 (His-rhsTLR2)

4.8.1 Generation of the His-rhsTLR2 cDNA

To generate His-rhsTLR2, the baculovirus expression system was used. A TLR2 construct consisting of the putative human TLR2 extracellular domain (Glu^{21}-Arg^{587}) with a C-terminal 6x histidine (His) tag tail was generated. The TLR2 cDNA was obtained by RT-PCR using RNA isolated from Mono Mac-6 monocytes, and cloned into the pCR®II-TOPO® cloning vector, as previously described (LeBouder et al., 2003). The plasmid pCR®II-TOPO® containing the full-length TLR2 cDNA was used as a template to generate a PCR fragment corresponding to amino acids 21-587 of TLR2 with a C-terminal His tag. The sense and antisense primers used were:

5'-CGC*GGATCC*GGAATCCTCCAATCAGGCTTCT-3' and

5'-CGC*AAGCTT*CTA<u>GTGATGGTGATGGTGATGG</u>AGGGGGCCTTGAAAC

AGAACTTCTAACCTGTGACATTCCGACACCGAGAGGCG-3', underlined and

bold type are the His tag and PreScission-Amersham-protease cleavage site,

respectively. In italics, are the BamHI (*GGATCC*) and HindIII (*AAGCTT*) restriction

sites. PCR products were analysed by DNA gel electrophoresis (1768 base pairs)

followed by DNA extraction and purification.

The purified PCR products were then digested in a 30 μl final reaction mixture

containing 3 μl of NEB2 buffer (10 mM Tris-HCl, 10 mM MgCl$_2$, 50 mM NaCl, 1 mM

dithiothreitol, pH 7.9, New England Biolabs (NEB) Ltd., Hitchin, U.K.), 3 μl of 10x

BSA (10 mg/ml, NEB), 1.5 μl of BamHI (15 U/μl, Amersham Pharmacia) and 1.5 μl of

HindIII (12 U/μl, Bioline, London, U.K.). The mixture was incubated 2 h at 37°C, and

the digestion was stopped by incubating the samples for 15 min at 65°C. The digested

PCR products were purified using the QIAquick® Nucleotide removal kit (Qiagen)

following the manufacturer's instructions. In preparation for cloning, 1 μg of pMelBac

B expression vector was digested in a 20 μl mixture containing 2 μl of NEB2 buffer, 2

μl of 10x BSA, 1 μl of BamHI and 1 μl of HindIII. The mixture was incubated 2 h at

37°C, and the reaction was stopped by incubating 15 min at 65°C. One microlitre of

Calf Intestinal Phosphatase (CIP) (10 U/μl, NEB) was added, and the vector was

dephosphorylated for 90 min at 37°C. The digested and dephosphorylated plasmid was

then purified using the Geneclean® III DNA purification kit (Anachem UK Ltd., Luton,

U.K.) according to the manufacturer's instructions.

The ligation was carried out by mixing 55 ng of the digested purified pMelBac B

plamid with 50 ng of the purified His-rhsTLR2 PCR products (1:3, plasmid: insert

molar ratio, approximately), 1.5 μl of ligase buffer (30 mM Tris-HCl pH 7.8, 10 mM

CGC**GGATCC**GGAATCCTCCAATCAGGCTTCTCTGTCTTGTGACCGCAATGGTATCTGCAAGGGCAGCTCAGGATCTTTAAACTCC
ATTCCCTCAGGGCTCACAGAAGCTGTAAAAAGCCTTGACCTGTCCAACAACAGGATCACCTACATTAGCAACAGTGACCTACAGA
GGTGTGTGAACCTCCAGGCTCTGGTGCTGACATCCAATGGAATTAACACAATAGAGGAAGATTCTTTTTCTTCCCTGGGCAGTCT
TGAACATTTAGACTTATCCTATAATTACTTATCTAATTTATCGTCTTCCTGGTTCAAGCCCCTTTCTTCTTTAACATTCTTAAAC
TTACTGGGAAATCCTTACAAAACCCTAGGGGAAACATCTCTTTTTTCTCATCTCACAAAATTGCAAATCCTGAGAGTGGGAAATA
TGGACACCTTCACTAAGATTCAAAGAAAAGATTTTGCTGGACTTACCTTCCTTGAGGAACTTGAGATTGATGCTTCAGATCTACA
GAGCTATGAGCCAAAAAGTTTGAAGTCAATTCAGAATGTAAGTCATCTGATCCTTCATATGAAGCAGCATATTTTACTGCTGGAG
ATTTTTGTAGATGTTACAAGTTCCGTGGAATGTTTGGAACTGCGAGATACTGATTTGGACACTTTCCATTTTTCAGAACTATCCA
CTGGTGAAACAAATTCATTGATTAAAAAGTTTACATTTAGAAATGTGAAAATCACCGATGAAAGTTTGTTTCAGGTTATGAAACT
TTTGAATCAGATTTCTGGATTGTTAGAATTAGAGTTTGATGACTGTACCCTTAATGGAGTTGGTAATTTTAGAGCATCTGATAAT
GACAGAGTTATAGATCCAGGTAAAGTGGAAACGTTAACAATCCGGAGGCTGCATATTCCAAGGTTTTACTTATTTTATGATCTGA
GCACTTTATATTCACTTACAGAAAGAGTTAAAAGAATCACAGTAGAAAACAGTAAAGTTTTTCTGGTTCCTTGTTTACTTTCACA
ACATTTAAAATCATTAGAATACTTGGATCTCAGTGAAAATTTGATGGTTGAAGAATACTTGAAAAATTCAGCCTGTGAGGATGCC
TGGCCCTCTCTACAAACTTTAATTTTAAGGCAAATCATTTGGCATCATTGGAAAAAACCGGAGAGACTTTGCTCACTCTGAAAA
ACTTGACTAACATTGATATCAGTAAGAATAGTTTTCATTCTATGCCTGAAACTTGTCAGTGGCCAGAAAAGATGAAATATTTGAA
CTTATCCAGCACACGAATACACAGTGTAACAGGCTGCATTCCCAAGCACACTGGAAATTTAGATGTTAGCAACAACAATCTCAAT
TTATTTTCTTTGAATTTGCCGCAACTCAAAGAACTTTATATTTCCAGAAATAAGTTGATGACTCTACCGATGCCTCCCTCTTAC
CCATGTTACTAGTATTGAAAATCAGTAGGAATGCAATAACTACGTTTTCTAAGGAGCAACTTGACTCATTTCACACACTCGAAGAC
TTTGGAAGCTGGTGGCAATAACTTCATTTGCTCCTGTGAATTCCTCTCCTTCACTCAGGAGCAGCAAGCACTGGCCAAAGTCTTG
ATTGATTGGCCAGCAAATTACCTGTGTGACTCTCCATCCCATGTGCGTGGCCAGCAGGTTCAGGATGTCCGCCTCTCGGTGTCGG
AATGTCACAGG**TTAGAAGTTCTGTTTCAAGGCCCCCTC**<u>CATCACCATCACCATCAC</u>**TAG*****AAGCTT***GCG
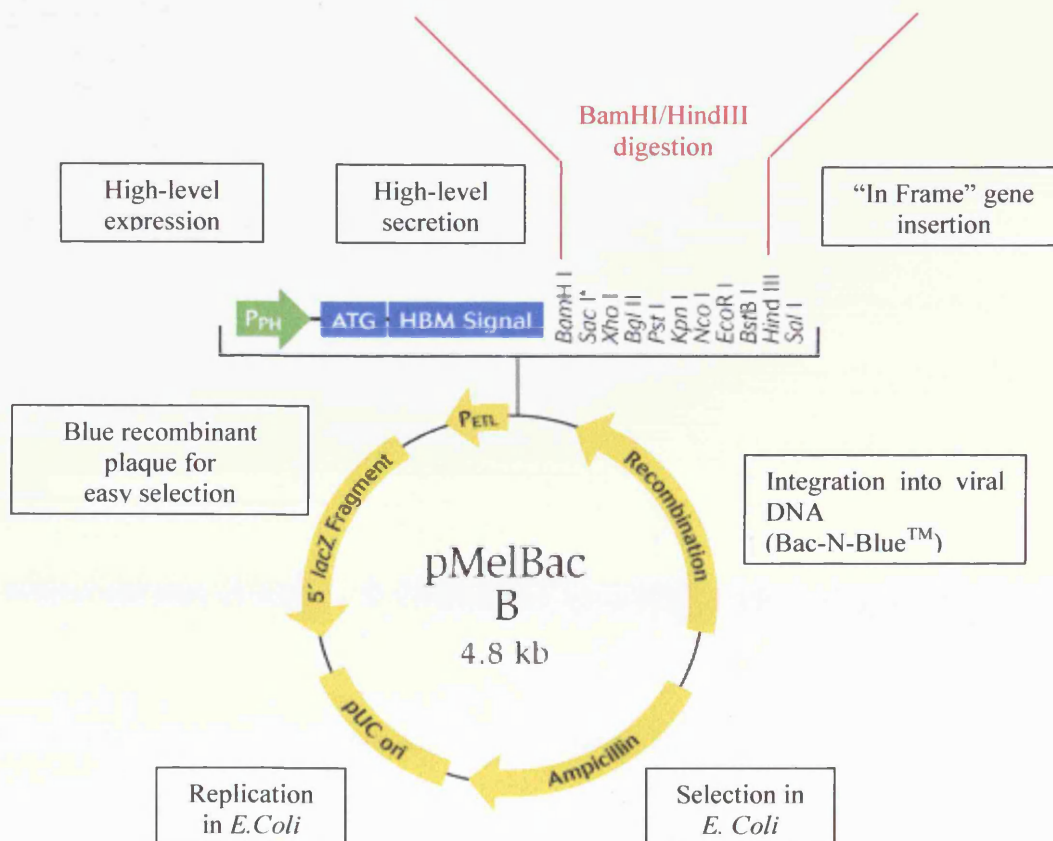
Figure 4.1. Cloning of the His-rhsTLR2 cDNA into the pMelBac B vector.
The cDNA coding for the His-rhsTLR2 was cloned into the pMelBa B vector using the BamHI and HindIII restriction enzymes (restriction sites in red and italics). An extra nucleotide (G, green and bold) was inserted after the BamHI restriction site for the His₆-rhsTLR2 to be in frame with the honeybee-mellitin (HMB) signal. The stop codon (blue and bold), the cleavage sequence (black and bold) and the 6x histidine tag (underlined) are indicated. HBM signal, Honeybee melitin signal; P_{PH}, Polyhedrin promoter.

MgCl$_2$, 10 mM DTT, 1 mM ATP; Promega), and 1.5 µl of ligase (1 U/µl; Invitrogen).

The reaction mixture (15 µl final volume) was incubated overnight at 16°C. An aliquot

(2 µl) was electroporated into 100 µl of electrocompetent DH5α *Escherichia coli* at 2.5

kV, 25 µF and 200 Ω using a Bio-Rad Gene pulser™ II. After electroporation, 200 µl of

SOC medium (2% (w/v) Bacto-Tryptone, 0.5% (w/v) Yeast extract, 10 mM NaCl, 2.5

mM KCl, 10 mM MgCl$_2$, 20 mM Glucose, pH 7) were immediately added, and bacteria

were incubated 30 min at 37°C. Bacteria were then spread on Luria Bertani (LB)/Agar

plates (1% (w/v) Bacto-Tryptone, 0.5% (w/v) Yeast extract, 172 mM NaCl, pH 7.7 /

1.5% (w/v) Agar) containing 100 µg/ml ampicillin and 50 µg/ml X-gal, and incubated

overnight at 37°C. The following day, five clones were screened by PCR using TLR2-

specific primers (Table 4.3) to test for the presence of the His-rhsTLR2 construct. A

positive clone was selected, cultured, and the pMelBac B-His-rhsTLR2 construct (Fig.

4.1) was extracted and purified using the QIAprep® spin miniprep kit (Qiagen)

following the manufacturer's instructions. The full sequence of the His-rhsTLR2 cDNA

was then verified by sequencing.

4.8.2   Production of His-rhsTLR2

Sf9 cells were co-transfected with 3 µg of the recombinant pMelBac B-His-

rhsTLR2 cDNA construct and 0.5 µg of Bac-N-Blue™ DNA by using the Bac-N-

Blue™ transfection and expression system (Invitrogen), according to the manufacturer's

instruction. After 72 h, supernatants from the infected Sf9 were collected and tested

(plaque assays) to determine the titre of, and select the recombinant virus from the

remaining wild-type virus, as indicated by the manufacturer. Once the presence of

recombinant virus in the initial supernatant was confirmed, high-titre viral stocks were

prepared. This was carried out by series of low-multiplicity infections of Sf9 cells, following the Bac-N-Blue^{TM} transfection and expression protocol. Briefly, Sf9 cells were infected with the initial viral stock to increase the viral titre, and after 72 h the cell supernatant was collected, and the viral titre determined by a plaque assay. This step was repeated until having a supernatant with an optimal viral titre, i.e. 5 x 10^7 to 2 x 10^8 PFU/ml.

The cells were infected with viral stock of a known titre, and the volume of viral stock to be used was determined by the following formula:

$$V= (n \times MOI)/ t$$

Where V is the volume (ml) of viral stock to be used for the infection, n the number of cells to be infected, MOI the multiplicity of infection (for a low multiplicity of infection, MOI=0.3) and t, the titre of the viral stock (PFU/ml).

High Five cells were used to produce the recombinant sTLR2 protein, and the volume of high-titre viral stock to be used for the infection was determined according to the formula described previously. Unlike the generation of the high-titre viral stock, for protein production a higher MOI was used (MOI=5). This ensured a synchronous infection of all cells in the culture, thus allowing maximum amounts of recombinant protein to be harvested at a given time point. Exponentially growing High Five cell cultures (2.5 x 10^6 cells/ml) were infected with the appropriate volume of viral stock, cultured for 3 days (27°C, 120 rpm), and the supernatants were then collected and processed in preparation for His-rhsTLR2 purification.

4.9 Purification of His-rhsTLR2

After a 72-hour infection, the High Five culture supernatants (typically, 200 ml) were collected, filtered (22 μm filters) and concentrated 25 times using Centricon Plus 70 (Millipore, Billerica, MA, U.S.A.; 2000 x g, 4°C). The concentrated supernatant was then dialysed (Seamless cellulose tubing, high retention, 23 mm diameter, Sigma-Aldrich) in the binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole pH 8.0). Buffer exchange was achieved by 2 x 90 min and 1 x 16 h dialysis at 4°C (1:250, supernatant: binding buffer ratio).

His-rhsTLR2 was batch-purified by metal-affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA)-coated beads. Routinely, 200 μl of beads (bead volume) were used to purify His-rhsTLR2 contained in the concentrated supernatant corresponding to 200 ml of High Five cell supernatant. Prior to binding, the beads were washed 5 times with 400 μl of binding buffer (1000 x g, 30 sec), and then added to the dialysed and concentrated supernatant. After a 2 h incubation at 4°C with gentle rotation, the beads were collected by centrifugation (1000 x g, 2 min), and washed 16 times with 250 μl of binding buffer (1000 x g, 30 sec).

Preliminary experiments were carried out to determine the optimal concentration of imidazole in the binding and washing buffers. While a low concentration of imidazole in the washing buffer ensures efficient elimination of contaminants non-specifically bound to the Ni-NTA resin, a relatively high concentration may lead to a loss of His-tagged protein in the washes. Thus, preliminary washing experiments with buffer supplemented with 10, 20 or 40 mM imidazole were conducted following binding. The presence of His-rhsTLR2 and proteins in the washes – a total of 16 – was monitored by Western blotting with an anti-TLR2 specific polyclonal Ab (TLR2p) and

A

W15   W16   W15   W16   W15   W16

← ~ 90-kDa rhsTLR2

← ~ 72-kDa rhsTLR2

Imidazole (mM) :—— 10 ——— 20 ——— 40 ——

B

W15   W16   W15   W16   W15   W16

← ~ 90 kDa
← ~ 83 kDa
← ~ 72 kDa

Imidazole (mM) :— 10 —— 20 —— 40 —–—
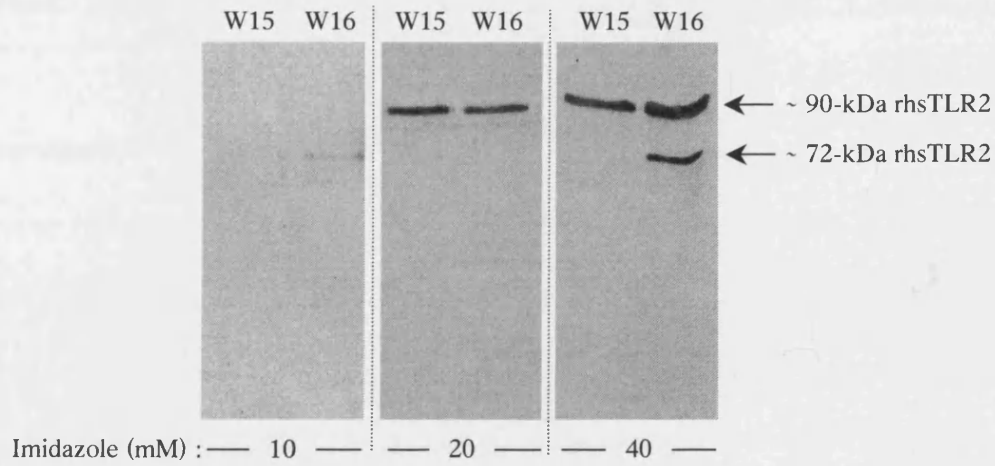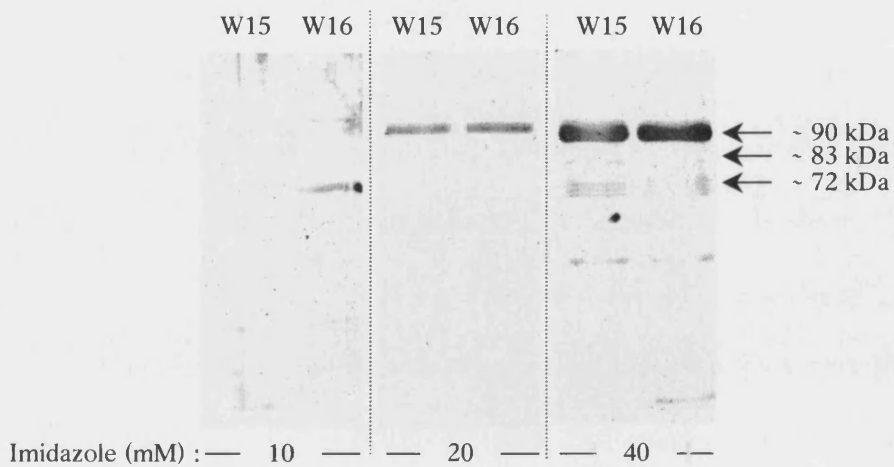
Figure 4.2. The effect of different concentrations of imidazole in the washing buffer.
A, Western blot analysis following 10% SDS-PAGE of the last two washes (W15 and W16) of the Ni-NTA-coated beads carried out – following binding – with washing buffer supplemented with 10, 20 or 40 mM imidazole. Immunoblottings were performed with the anti-TLR2 polyclonal Ab, TLR2p. The approximate molecular mass of the sTLR2 polypeptides is indicated. A duplicate 10% SDS-PAGE gel run in parallel was Coomassie blue stained and is shown in **B**.

Coomassie blue G250 staining (Fig. 4.2). As shown in Figure 4.2, A, the Western blot analysis revealed the presence of significant amounts of His-rhsTLR2 polypeptides in the last two washes carried out with buffer supplemented with either 20 or 40 mM of imidazole. By contrast, a negligible amount of His-rhsTLR2 was detected in the washes when 10 mM imidazole was used. These observations were confirmed by Coomassie blue staining of the washes (Fig. 4.2, B). Therefore, a 10 mM imidazole concentration in the binding and washing buffer was used.

Following washing, the His-rhsTLR2 was eluted from the Ni-NTA beads by increasing the concentration of imidazole in the buffer to 250 mM. The beads were washed 4 times (1000 x g, 30 sec) with 200 µl of elution buffer (binding buffer supplemented with 250 mM imidazole), and the 4 fractions collected were pooled (800 µl total) and dialysed twice (90 min, 4°C) in 500ml of PBS using a dialysis chamber (D-tube™ Dialyser Midi, MWCO: 6-8 kDa, Novagen).

The protein content of the preparation was determined (Dc Protein Assay, Bio-Rad) before aliquoting and storage at −70°C until use. Typically, 200 ml of High Five cell culture supernatant yielded 200-240 µg of purified His-rhsTLR2. The purity of the preparation was analysed as described in *Results*, section 2.1.2 page 79.

4.10 Co-immunoprecipitation experiments

For mCD14-mTLR2 co-immunoprecipitation experiments, 5 x 10^6 freshly-isolated human monocytes were washed and resuspended in phenol red-free RPMI-1640 medium, and incubated for 30 min at 37°C in the presence of 5 µg/ml His-rhsTLR2 or 10 µg/ml BSA. After washing, the cells were lysed for 30 min at 4°C with lysis buffer (1% (v/v) Nonidet P-40, 50 mM Tris-HCl, 150 mM NaCl, 1 µg/ml

leupeptin and pepstatin, 1 mM PMSF, pH 7.4). The cell lysate was precleared by incubation (overnight, 4°C, orbital rotation) with 80 μl of a 50% suspension of protein G-Sepharose, followed by a further 1 h, 4°C incubation with 4 μg of the isotype-matched control, mouse IgG2b (MOPC-141), and finally with protein G-Sepharose (50 μl, 1 h, 4°C, orbital rotation). The precleared samples were incubated (1 h, 4°C) with 5 μg of the anti-CD14 mAb, MY4, and the immunocomplexes precipitated with 50 μl of the protein G-Sepharose bead suspension. Following washing, samples were analysed by Western blotting with the anti-TLR2 mAb, IMG 319.

4.11 Crosslinking experiments

For chemical crosslinking experiments, 5×10^6 cells were resuspended in 500 μl of cold phenol red-free RPMI-1640 medium and incubated with 5 μg of His-rhsTLR2 or the irrelevant His-tagged soluble protein sCD55 (kindly donated by Dr. C. Harris, Dept. of Medical Biochemistry and Immunology, Cardiff University, U.K.) for 30 min at room temperature. Immediately after washing (cold RPMI-1640), 3 mg/ml (final concentration) of the membrane impermeable and non-cleavable crosslinker, bis(sulfosuccinimidyl)suberate (BS^3, Pierce, Perbio Science, Northumberland, U.K.) were added to the samples, and the mixture was incubated for an additional 30 min at room temperature. Crosslinking was stopped by the addition of 10 mM Tris-HCl pH 7.4 buffer and incubation on ice for 15 min. The cells were then lysed as described in section 4.10, and the cell lysates incubated with 10 μl Ni-NTA beads per 100 μl lysate (2 h, 4°C, orbital rotation). The beads were then washed, and the protein eluted with Laemmli reducing sample buffer containing 250 mM imidazole. The eluate was

analysed by 7.5 % SDS-PAGE and Western blotting using an anti-CD14 (69.4) or anti-TLR2 (sc8689) polyclonal Ab.

4.12 Preparation of lipid rafts

Freshly-isolated human monocytes (1×10^8 cells) were resuspended at 1×10^7 cells/ml in warm phenol red-free RPMI-1640 medium and stimulated (1h, 37°C) with 5 μg/ml Pam$_3$Cys in the absence or presence of 5 μg/ml sTLR2. Subsequently, protein solubilisation was carried out with lysis buffer (1% (v/v) Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, 1 mM PMSF, 1 μg/ml leupeptin and pepstatin pH 7.4) for 1 h at 0°C. Cell lysates (1.5 ml) were then mixed with an equal volume of cold 90% sucrose solution (45% final concentration) prepared in sucrose gradient buffer (50 mM Tris-HCl, 150 mM NaCl pH 7.4) and placed at the bottom of a centrifuge tube. Samples were overlaid with 7 ml of 30% followed by 2 ml of 5% cold sucrose solutions (both in sucrose gradient buffer) and centrifuged at 200,000 x g for 16 h at 4°C. One millilitre fractions were gently removed from the top of the gradient, and the non-ionic detergent, n-octylglucoside (60 μM final concentration), was added to each fraction. Equal aliquots of each fraction were run in 10% SDS-PAGE gels and analysed by Western blotting using the anti-CD14 mAb, MY4, or the anti-TLR2 mAb, IMG 319.

To define the lipid raft-containing fractions in the sucrose gradient, aliquots of each fraction were analysed by dot blotting to determine the presence of the raft-associated ganglioside, GM1. Presence of GM1 in the blots was tested with HRP-conjugated cholera toxin B (List Biologicals, Campbell, CA), and the detection visualised by enhanced chemiluminescence (ECL system, Amersham)

4.13 Fluorescence resonance energy transfer (FRET) experiments

4.13.1 Sample preparation for FRET

For FRET experiments, human monocytes (3×10^6 cells/spot) were allowed to adhere (1 h, 37°C) to multi-spot slides (Shandon Multi-spot, Thermo Electron Corp. Basingstoke, U.K.) in the presence of 5 μg/ml sTLR2 or BSA in phenol red-free RPMI-1640 medium. Subsequently, the cells were incubated with 20 % normal rabbit serum for 15 min at room temperature before washing (2 x brief washes with PBS-0.01% NaN₃). Labelling (1 h, 4°C) with the anti-CD14 mAb My4-Cy3 or its isotype-matched IgG2b-Cy3 control (acceptor fluorophore, 0.25 μg/spot) was performed in the absence or presence of 5 μg/ml His-rhsTLR2 or BSA. Both antibodies were Cy3-conjugated using the FluoroLink mAb Cy3 labeling kit (GE healthcare U.K. Ltd, Bucks, U.K.) following the manufacturer's instructions. The slides were then washed as described previously, fixed (2% paraformaldehyde, 15 min, room temperature) and washed again before staining with the anti-TLR2 mAb, T2.5-Alexa 488 (donor fluorophore), as described for MY4-Cy3. After washing and fixing, the slides were mounted (Vectashield mounting fluid, Vector, laboratories, Peterborough, U.K.) in preparation for FRET analysis.

4.13.2 FRET data acquisition and analysis

FRET was measured by the release of quenched donor fluorescence after acceptor photobleaching using a previously described technique (Kenworthy, 2001). In

A
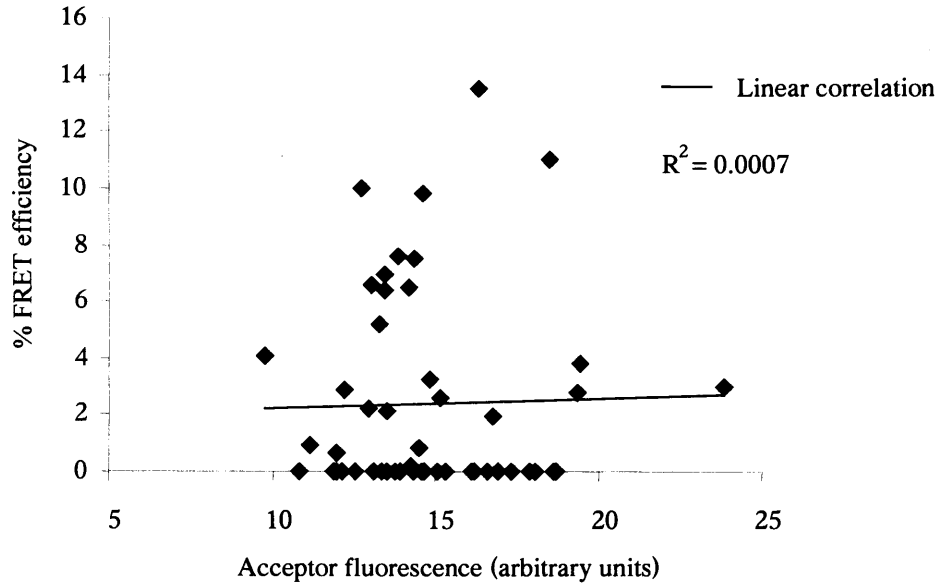
B
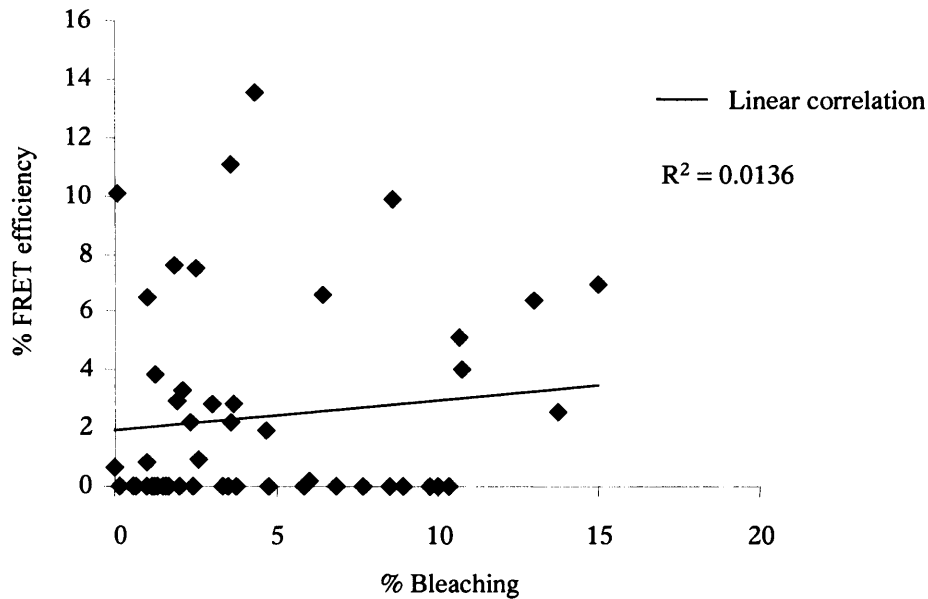
Figure 4.3. Absence of correlation between FRET efficiency and acceptor fluorescence or FRET efficiency and bleaching efficiency.

Percentage of FRET efficiency between TLR2 (T2.5-A488) and CD14 (MY4-Cy3) on the surface of human monocytes, in resting conditions and in the absence of sTLR2. Each dot represents a cell event. Results are of one experiment representative five. Calculation of the linear correlation coefficient (R^2) between FRET efficiency and acceptor fluorescence (before bleaching) **(A)**, or FRET efficiency and bleaching efficiency **(B)**, shows no correlation between these parameters (-0.1< R^2 <0.1). This indicates that the energy transfer observed between TLR2 and CD14 results from a genuine interaction, and not from random proximity.

this technique, the donor fluorescence intensity before and after acceptor photobleaching – in the same cell – is compared. FRET efficiency was quantified by:

E (%) = [(I_{DA}-I_{DB})/I_{DA}] x 100, where E represents % FRET efficiency; I_{DA} and I_{DB}, the donor's intensity after and before photobleaching of the acceptor, respectively. As bleaching of the acceptor fluorophore constitutes the basis of this FRET technique, the events for which the bleaching of the acceptor was unsuccessful, i.e. the intensity of the acceptor after bleaching was superior or equal to the intensity before bleaching, were not considered in the data analysis of the present study. The cells (100-150 cells/condition) were imaged using a Leica TCS SP2 spectral confocal and multiphoton system (Leica Microsystem Ltd., Bucks, U.K.).

Random proximity effects that arise from confining donors and acceptors to a surface can lead to substantial non-specific energy transfer. A hallmark of FRET arising from such random proximity is a characteristic increase in E (% FRET efficiency) as a function of acceptor surface density. In contrast, E for specifically interacting proteins should not depend on acceptor surface density (Kenworthy, 2001) or bleaching efficiency. To confirm that the transfer of energy observed in the experiments described in this study was due to a genuine interaction between CD14 and TLR2, and not to a random proximity effect, the correlation between E and acceptor intensity (Fig. 4.3, A), and between E and bleaching efficiency (Fig. 4.3, B) was determined. Figure 4.3 shows that there was no correlation between E and acceptor intensity nor between E and bleaching efficiency, indicating that the transfer of energy observed was due to a genuine interaction between CD14 and TLR2.

4.14 Binding of sTLR2 to Pam₃Cys, LPS and bacteria

4.14.1 Binding to Pam₃Cys and LPS

Triplicate wells of microtiter well plates (high binding, Costar, Corning, NY, U.S.A.) were coated (20 µl) with the indicated amounts (Fig. 2.3.2) of Pam₃Cys or LPS dissolved in ethanol. Following solvent evaporation at room temperature, non-specific binding in the wells was blocked by incubation (2 h, room temperature) with a PBS solution containing 1% BSA, 5% sucrose and 0.05% sodium azide. The plates were then washed three times with washing buffer (PBS/0.05% Tween-20) and incubated (4 h, 37°C) with 5 µg/ml of His-rhsTLR2 or sCD46-Fc (50 µl/well; kindly provided by Dr. C. Harris) diluted in 0.05% Tween-20, 0.1% BSA, 20 mM Trizma base, 150 mM NaCl pH 7.3. Subsequently, the wells were washed and incubated with an anti-His₅ mAb (0.2 µg/ml) or biotin-conjugated anti-human Igs Ab (0.5 µg/ml) for 2 h at room temperature. Following washing, the wells incubated with sTLR2 and the anti-His mAb were incubated further (2 h, room temperature) with a biotin-conjugated anti mouse IgG Ab, before washing and incubation (20 min, room temperature) with streptavidin-HRP (2 µg/ml). The wells were then washed before adding 100 µl/well of tetramethylbenzidine substrate (TMB, SureBlue™, KPL, MD, U.S.A.). The colour reaction was stopped (1 M HCl; 50µl/well), and colour developed measured at 450 nm.

Binding of LPS to the wells (used in control experiments) was confirmed by the binding of purified sCD14 to the wells (250 ng/well), which was detected using the biotin-cojugated anti-CD14 mAb, MY4, followed by incubation with streptavidin-HRP and TMB substrate, as described previously.

4.14.2   Binding to bacteria

In order to test the binding of sTLR2 to bacteria, 5×10^4 heat-killed *Staphyloccocus epidermidis* PCI 1200 strain were resuspended in 100 μl of PBS/0.5% BSA and preincubated (30 min, room temperature) with 500 ng of sCD14. Subsequently, 1 μg of recombinant mouse sTLR2-human Fc (R&D Systems) or control CD46-Fc fusion protein was added, and the samples were incubated for a further 30 min at room temperature. Following washings (2 x PBS/ 0.5% BSA), samples were incubated (0°C, 30 min) with 0.5 μg of a biotin-conjugated anti-human IgG Ab. Following washing and fixing (2 % paraformaldehyde, 10 min, room temperature), APC-conjugated streptavidin (0.1 μg) was added, and the samples were incubated for 20 min on ice before washing and analysis by flow cytometry.

4.15 Immunofluorescence and flow cytometry

In preparation for flow cytometric analyses, cells were washed twice and resuspended in binding buffer (PBS, 0.5% BSA, 0.05% NaN_3), at a density of 5×10^6 cells/ml, and 100 μl aliquots were distributed in wells of round bottom 96-well plates. Fc receptors on monocytes and neutrophils were blocked by incubating the samples with a 20% normal rabbit serum solution (in binding buffer; 100 μl/ 5×10^5 cells) for 15 min at room temperature. Subsequently, cells were incubated with the specific mAbs (0.5 μg mAb per 5×10^5 cells in 100 μl binding buffer) for 30 min at 4°C. Following incubation and washes (2 x 200 μl binding buffer), samples were fixed in 2% p-formaldehyde (Sigma-Aldrich). The stained cells were analysed by flow cytometry, 10 000 events were acquired for each sample using a FACSCalibur cytometer (Becton Dickinson, San jose, CA), equiped with the CellQuest™ Pro software.

4.16 Cell activation

For all activation experiments, cells were resuspended in phenol red-free RPMI-1640 medium, and cultured in round bottom 96-well plates, unless stated otherwise.

For the cell activation experiments shown in Figure 2.2.2 (*Results*, effect of overexpressing sTLR2) and Figures 2.5.4 and 2.5.5 (*Results*, effect of the TLR2-derived peptides), 1.5×10^5 cells/well were cultured in serum-free medium supplemented or not with 500 ng/ml sCD14 (purified from human milk, as previously described, Labéta et al., 2000) and stimulated with the indicated amounts of the different TLR2 ligands (Pam$_3$Cys, PGN, HKLM). For the experiments testing the effect of the TLR2-derived peptides (Figs. 2.5.4 and 2.5.5), the peptides were solubilised in an acetonitrile/water solution according to their solubility characteristics, before aliquoting and storage at – 70°C (10 mg/ml stock solution) until further use. After a 16 h incubation (37°C), cell culture supernatants were collected, and the IL-8 concentration was determined by ELISA (Human CXCL8/IL-8 DuoSet, R&D Systems) according to the manufacturer's instructions.

For the cell activation experiments described in Figure 2.2.3 (*Results*, effect of purified rhsTLR2), cell aliquots (1.5×10^5 cells/well) were cultured in serum-free medium supplemented or not (Mono Mac-6) with 500 ng/ml sCD14 and stimulated with the indicated concentrations of TLR ligands, non-TLR ligands or PMA + ionomycin. Cells were stimulated in the absence or presence of 5 µg/ml purified rhsTLR2. Following overnight (16 h) incubation, the cell culture supernatants were tested for IL-8 by ELISA.

For NF-κB reporter assays, HEK293, HEK-TLR2, U373-EV and U373-sTLR2 cells were seeded at 1.5×10^5 cells/well in 24-well plates the day before transfection and

then transiently transfected with reporter genes (as well as TLR2 constructs, HEK293 and HEK-TLR2) as indicated previously (section 4.3). Twenty-four hours post-transfection, HEK293 and HEK-TLR2 cell transfectants were stimulated (16 h, 37°C) with the indicated amounts of Pam$_3$Cys or HKLM, in the absence or presence of 500ng/ml of sCD14 and 5 µg/ml of purified rhsTLR2, as indicated in the corresponding *Results* section. U373-EV and U373-sTLR2 cell transfectants were stimulated (8 h, 37°C) with 60 ng/ml of LPS and the indicated concentrations of sCD14 (Fig. 2.4.6) Luciferase activity was measured using the Dual-Luciferase® reporter assay system (Promega), following the manufacturer's instructions.

4.17 Phagocytosis experiments

To evaluate the effect of sTLR2 on phagocytosis, RAW264 murine macrophages (4 x 10^5) were resuspended in 400 µl of binding buffer (phenol red-free RPMI-1640, 1% sodium azide, 2.5% HEPES) and incubated with FITC-labelled *S. epidermidis* (Molecular Probes, Leiden, The Netherlands) at a bacteria:cell ratio of 10:1 for 30 min at either 0°C or 37°C, to test for bacteria binding or phagocytosis, respectively. To test the effect of sTLR2, the bacterial suspension was preincubated with 5 µg/ml sTLR2 or BSA (2x sTLR2 molarity) for 30 min at 37°C. Following binding, the samples were washed (4x) with cold washing buffer (PBS, 1% sodium azide), the cells were resuspended in 100 µl of washing buffer, fixed (2% paraformaldehyde, 15 min, room temperature), and analysed by flow cytometry. To distinguish between cell-surface bound and phagocytosed bacteria, the cell-surface fluorescence was quenched by adding 125 µg/ml trypan blue to the samples before analysis.

4.18 Superoxide production experiments

For superoxide production assays, 150 μl of assay buffer (13 mM Na_2HPO4, 3 mM NaH_2PO4, 120 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO4$, 11 mM dextrose, 0.71 mM $CaCl_2$ pH 7.4) containing 5 μg/ml Pam_3Cys or heat-killed *S. epidermidis* strain PCI 1200 (ATCC; 5 x 10^6/well) , with or without 5 μg/ml sTLR2, were added in triplicate to microtiter well plates (microlite 2, Thermo LabSystems, Franklyn, MA) and kept at 37°C. To this mixture, 2 μM Luminol (from 10 μM stock solution) and freshly-isolated human PMN (2 x 10^5) resuspended in 50 μl PBS were added, and the chemiluminescence generated was immediately measured, and then every 2 min for 1 h using a fluorometer (Fluostar Optima plate reader, BMG Labtech, Aylesbury, U. K.).

4.19 *In vivo* experiments

Inbred 8 to 12-week-old C57/BL6 mice (Harlan U.K. Ltd, Oxon, U.K.) were maintained under barrier conditions and pathogen free, as assessed by regular microbiological screenings. All procedures were carried out following Home Office approval, under project license number PPL-40/2131.

4.19.1 Preparation of *S. epidermidis* cell-free supernatant (SES)

Cell-free supernatant from *S. epidermidis*, termed SES, was prepared as previously described (Hurst et al., 2001). Briefly, suspension cultures of *S. epidermidis* – isolated from an end-stage renal failure patient under continuous ambulatory peritoneal dialysis – were centrifuged, filtered and dialysed (H_2O). Fractions were freeze-dried and aliquots stored at -70°C until use. SES preparations were free from live

bacteria, as assessed by the lack of bacterial growth on DST agar (Oxoid Ltd, Basingstoke, U.K.). SES preparations were reconstituted in PBS prior to *in vivo* use, and their potency standardized by quantifying the release of IL-6 by RAW264 cells exposed to defined doses of SES.

4.19.2 SES-induced peritoneal inflammation model

SES-induced mouse peritoneal inflammation was induced by i.p. injection of a defined 500 µl dose of SES with or without 100 ng of sTLR2. Control animals were administered sterile PBS, with or without 100 ng of sTLR2. At the indicated time points, mice were sacrificed, and their peritoneal cavities lavaged with 2 ml of ice-cold PBS. Neutrophil and monocyte numbers in the lavages were assessed by differential cell count on cytospin preparations and Coulter counting (Coulter Z2, Beckman-Coulter, High Wycombe, U.K.), or by double staining of leukocytes with a FITC-conjugated anti-mouse F4/80 mAb (macrophages) and an APC-conjugated anti-mouse CD11b mAb (macrophages/monocytes) followed by flow cytometry analysis. To evaluate PMN survival in the peritoneal cavity, leukocytes were double stained with Annexin V-FITC (1/40) and propidium iodide (1/20; BD Bioscience), and analysed by flow cytometry. Levels of KC, MIP-2, and MCP-1 in the cell-free peritoneal lavage samples were quantified by ELISA (R&D Systems). To test for mouse sTLR2 in the peritoneal lavages, equal aliquots were diluted with 3x concentrated Laemmli reducing sample buffer and analysed by Western blotting using the TLR2-specific polyclonal Ab, TLR2p.

Table 4.5. Pairs of primers used to generate TLR2 LRRs valine for leucine point substitutions

LRR		Sequence (5′–3′)
LRR1	Forward	GCTGTAAAAAGCGTTGACGTGTCCAACAACAGG
	Reverse	CCTGTTGTTGGACACGTCAACGCTTTTTACAGC
LRR2	Forward	AGGTGTGTGAACGTCCAGGCTGTGGTGGTGACATCCAATGGA
	Reverse	TCCATTGGATGTCACCACCACAGCCTGGACGTTCACACACCT
LRR3	Forward	TCTTTTTCTTCCGTGGGCAGTGTTGAACATGTAGACTTATCCTAT
	Reverse	ATAGGATAAGTCTACATGTTCAACACTGCCCACGGAAGAAAAAGA
LRR4	Forward	TCCCAGTAAGTTTACGAATGTTACAGAAGAAACGGGCTTGAACCA
	Reverse	TGGTTCAAGCCCGTTTCTTCTGTAACATTAGTAAACTTACTGGGA
LRR5	Forward	CTTTTTTCTCATGCTCACAAAAGTGCAAATCGTGAGAGTGGGAAT
	Reverse	ATTCCCACTCTCACGATTTGCACTTTTGTGAGCATGAGAAAAAAG
LRR6	Forward	GATTTTGCTGGAGTTACCTTCGTTGAGGAAGTTGAGATTGATGCT
	Reverse	AGCATCAATCTCAACTTCCTCAACGAAGGTAACTCCAGCAAAATC
LRR7	Forward	AACGTAAGTCATGTGATCGTTCATATGAAGCAG
	Reverse	CTGCTTCATATGAACGATCACATGACTTACGTT
LRR8	Forward	TCCGTGGAATGTGTGGAAGTGCGAGATACTGAT
	Reverse	ATCAGTATCTCGCACTTCCACACATTCCACGGA
LRR9	Forward	GAAACAAATTCAGTGATTAAAGTGTTTACATTTAGA
	Reverse	TCTAAATGTAAACACTTTAATCACTGAATTTGTTTC
LRR10	Forward	CAGATTTCTGGAGTGGTAGAAGTAGAGTTTGATGAC
	Reverse	GTCATCAAACTCTACTTCTACCACTCCAGAAATCTG
LRR11	Forward	AAAGTGGAAACGGTAACAATCCGGAGGGTGCATATTCAAAGG
	Reverse	CCTTTGAATATGCACCCTCCGGATTGTTACCGTTTCCACTTT
LRR12	Forward	TTATTTTATGATCTGAGCACTTTATATTCACTTACAGAAAGA
	Reverse	TCTTTCTGTAAGTGAATATAAAGTGCTCAGATCATAAAATAA
LRR13	Forward	CAAATTTTCACTGACATCCACGTATTCTACTGATTTTAAATG
	Reverse	CATTTAAAATCAGTAGAATACGTGGATGTCAGTGAAAATTTG
LRR14	Forward	GCCTGGCCCTCTGTACAAACTGTAATTGTAAGGCAAAATCAT
	Reverse	ATGATTTTGCCTTACAATAACAGTTTGTACAGAGGGCCAGGC
LRR15	Forward	GGAGAGACTTTGGTCACTGTGAAAAACGTGACTAACATTGAT
	Reverse	ATCAATGTTAGTCACGTTTTTCACAGTGACCAAAGTCTCTCC
LRR16	Forward	AAGATGAAATATGTGAACGTATCCAGCACACGA
	Reverse	TCGTGTGCTGGATACGTTCACAATTTTCATCTT
LRR17	Forward	ATTCCCAAGACAGTGGAAATTGTAGATGTTAGCAAC
	Reverse	GTTGCTAACATCTACAATTTCCACTGTCTTGGGAAT
LRR18	Forward	AATTTATTTTCTGTGAATGTGCCGCAAGTGAAAGAACTTTAT
	Reverse	ATAAAGTTCTTTGACTTGCGGCACATTCACAGAAAATAAATT
LRR19	Forward	GATGCCTCCCTCGTACCCATGGTACTAGTAGTGAAAATCAGTAGG
	Reverse	CCTACTGATTTTCACTACTAGTACCATGGGTACGAGGGAGGCATC
LRR20	Forward	TCATTTCACACAGTGAAGACTGTGGAAGCTGGT
	Reverse	ACCAGCTTCCACAGTCTTCACTGTGTGAAATGA

4.19.3   Live bacteria-induced peritoneal inflammation model

Peritoneal inflammation was also induced in mice by i.p. injection (500 µl) of 5 x 10^7 CFU *S. epidermidis* PCI 1200 strain (ATCC) in the absence or presence of sTLR2 (100 ng). At the indicated time points, the mice were sacrificed and their peritoneal cavity lavaged, as described above. Blood was obtained by cardiac puncture. Bacterial CFU were determined by culturing blood and peritoneal lavage samples on Mueller-Hinton agar plates (Oxoid) overnight at 37°C. PMN numbers in the peritoneal lavages were determined as described for the SES model.

4.20 Generation of TLR2 mutants

The 20 leucine-rich repeats (LRR) TLR2 mutants were generated by point mutation, the strategy consisted in substituting a valine for 2 or 3 of the leucines of a particular LRR to create a TLR2 mutant cDNA. Twenty pairs of mutated complementary primers were designed, so that the 2 or 3 point mutations were centered, with 12 non-mutated bases at each end of the primer (MWG Biotech, London, U.K; Table 4.5). Point mutations were obtained using the Quick Change II™ kit (Stratagene), following the manufacturer's instructions. Briefly, the pCRII®-TOPO® vector containing the TLR2 WT cDNA (TOPO-TLR2 WT), previously generated in our laboratory (LeBouder et al., 2003), was used as a template and the desired point substitutions were introduced by PCR using the appropriate pair of mutated primers. PCR conditions were: initial denaturation: 95°C, 1 min followed by 18 cycles of : denaturation (95°C, 50 sec), annealing (50°C, 50 sec), extension (68°C, 7 min 30 sec), and a final extension step (68°C, 7 min). The PCR products were then digested (37°C, 90 min) with 10 U of DpnI (Promega) to cut the non-methylated TOPO-TLR2 WT used
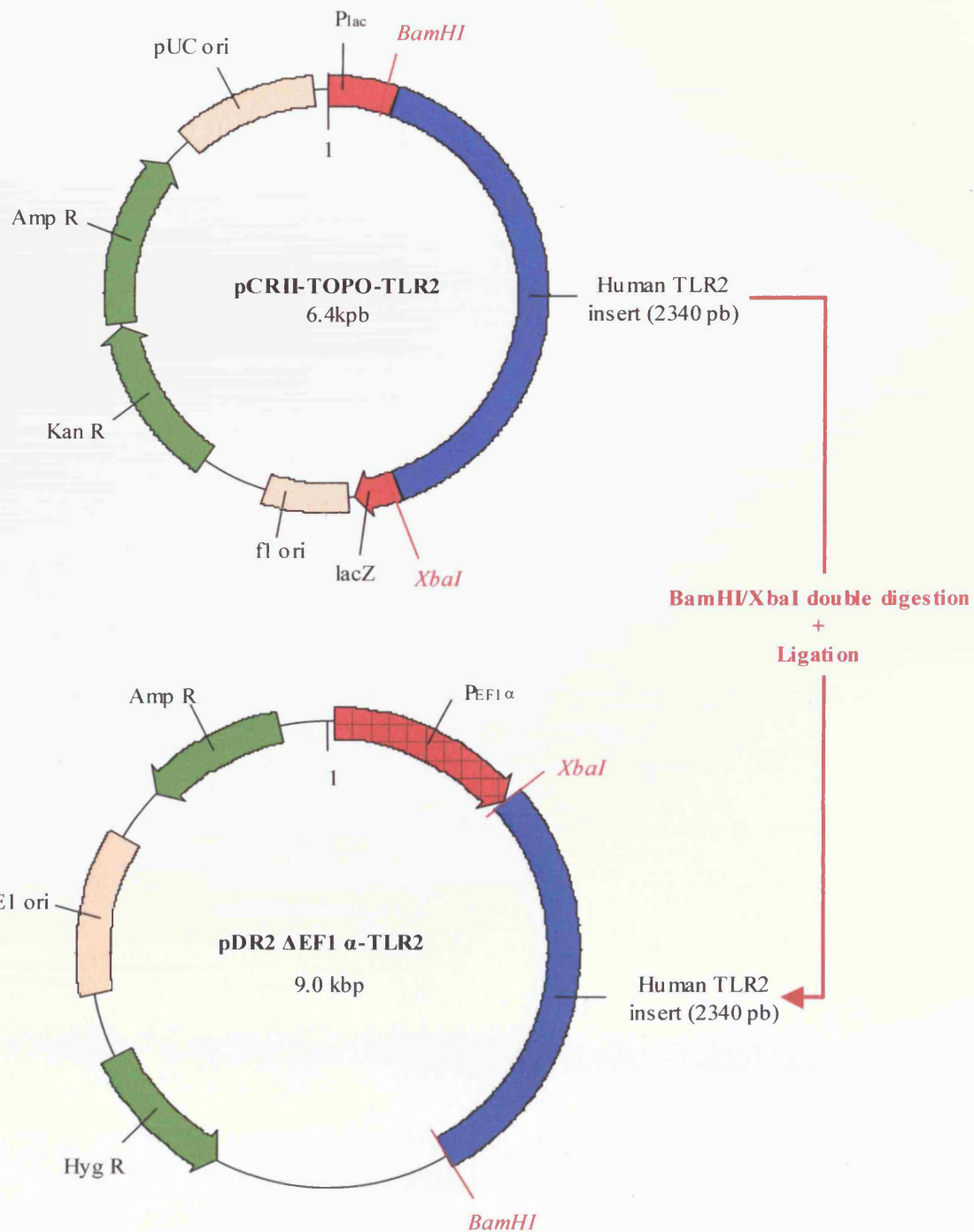
Figure 4.4. Subcloning of the human TLR2 cDNA from the pCR®II-TOPO® transfer vector into the pDR2ΔEF1α mammalian expression vector.
The cDNAs encoding WT TLR2 or LRR TLR2 mutants were subcloned from the pC®RII-TOPO® plasmid - used as a template to carry out the point mutation - to the pDR2ΔEF1α plasmid - used to transfect HEK293 cells – using the *XbaI* and *BamHI* restriction enzymes.

as a template. Chemically competent bacteria were transfected with the DnpI-digested

PCR product and screening of individual colonies was carried out by DNA sequencing

to check that the point mutation was introduced. The full TLR2 mutant sequence was

then similarly verified.

The TLR2 mutants cDNAs were transferred from the pCR®II-TOPO® cloning

vector to the pDR2ΔEF1α mammalian expression vector using the BamHI and XbaI

restriction enzymes (Fig. 4.4), as previously described for the subcloning of His-

rhsTLR2 cDNA into the pMelBac™ B vector (section 4.8.1). The pDR2ΔEF1α plasmid

contains the ampicillin and hygromycin resistance genes, allowing the selection of

transformed bacteria and transformed eukaryotic cells, respectively. The expression of

the gene inserted in the vector is under control of the elongation factor-1α (EF1α)

promoter, which allows gene transcritpion in mammalian cells (Fig. 4.4).

To test for the presence of WT TLR2 or LRR mutant TLR2 mRNAs in transfected

cells, total RNA was extracted from the HEK293 cells (used as a control), HEK-WT

TLR2 or HEK-TLR2 mutant transfectants (3 x 10^6 cells) using the RNA extraction kit:

RNeasy Mini® from Qiagen, according to the manufacturer's instructions.

The TLR2 mutants cDNA was obtained by reverse transcription (RT) of the extracted

mRNAs, using the Omniscript™ Reverse Transcriptase kit (Qiagen). To eliminate any

secondary structures, total RNA was incubated for 5 min at 70°C and then ice-cooled

for 5 min. RT was carried out on 3 μg of denaturated total RNA in the presence of 30 U

RNasin (Promega), 0.5 mM dNTPs (Qiagen kit), 150 pmol of random hexamer primers

(pdN6) or 40 pmol of oligo-dT primers (Q$_T$) (Table 4.3), and 6 U Omniscript™ Reverse

Transcriptase (Qiagen kit) in RT buffer (Qiagen kit). RT was performed at 42°C for 90

146

min in a 30 µl final reaction volume. The RT-PCR products were then analysed by PCR

for the presence of TLR2 mutants or TLR2 WT cDNA (section 4.5).

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