



Michael B. Drennan

Mycobacterium tuberculosis and *Trypanosoma brucei* as
models for the TLR-dependent activation of the innate
immune system

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Mycobacterium tuberculosis and *Trypanosoma brucei* as
models for the TLR-dependent activation of the innate
immune system

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The most exciting phrase to hear in science, the one that heralds new discoveries, is not, “Eureka!” (“I found it”) but rather, “Hmm.....that’s funny....”

-*Isaac Asimov*

Declaration

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I have been in a green field, with my arms outstretched, and haven't touched sides. I would like to thank Bernhard Ryffel and Stefan Magez for this freedom.

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And finally, to my family, for the art.

University of Cape Town

Publications

The following are publications directly associated with the thesis, as well as studies performed outside the context of its philosophy:

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Am J Pathol. 2004 Jan;164(1):49-57.

The induction of a Type 1 immune response following a *Trypanosoma brucei* infection is MyD88-dependent
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Valerie J.F. Quesniaux, C. Fremond, M. Jacobs, S. Parida, D. Nicolle, V. Yeremeev, F. Bihl, F. Erard, T. Botha, M. Drennan, M.N. Soler, M. Le Bert, B. Schnyder, and B. Ryffel
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Stefan Magez, M. Radwanska, M. Drennan, L. Fick, T.N. Baral, M. Merimi, X. Van Hoang, P. Brouckaert, F. Brombacher, and P. De Baetselier
J Immunol *submitted*

Abbreviations

<i>A. fumigatus</i>	<i>Aspergillus fumigatus</i>
Ag	antigen
APC	antigen presenting cell
<i>B. burgdorfei</i>	<i>Borrelia burgdorfei</i>
BSA	bovine serum albumin
<i>C. albicans</i>	<i>Candida albicans</i>
<i>C. neoformans</i>	<i>Cryptococcus neoformans</i>
<i>C. parvum</i>	<i>Cryptosporidium parvum</i>
CCR5	C-C chemokine receptor 5
CD	cluster of differentiation
CFU	colony forming unit
DAB	3,3 diaminobenzidine tetrahydrochloride
DC	dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule-grabbing nonintergrin
DE	diethylaminoethyl
DMEM	Dulbecco's modified Eagle's medium
DMG	dimyristoylglycerol
dsRNA	double-stranded RNA
<i>E. coli</i>	<i>Escherichia coli</i>
<i>E. tenella</i>	<i>Eimeria tenella</i>
ELISA	enzyme linked immunosorbent assay
FACS	fluorescent activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GIP	glycosylinositolphosphate
GM-CSF	granulocyte/macrophage colony-stimulating factor
GPI	glycosylphosphatidylinositol
<i>H. influenzae</i>	<i>Haemophilus influenzae</i>
HRP	horseradish peroxidase
h-Toll	human Toll
IFN	interferon
Ig	immunoglobulin
IKK	inhibitor of nuclear factor- κ B-kinase complex
IL	interleukin
iNOS	inducible nitric oxide synthase
IRAK	IL-1R-associated kinase
IRF3	interferon regulatory factor 3
JNK	JUN N-terminal kinase
<i>L. major</i>	<i>Leishmania major</i>
<i>L. monocytogenes</i>	<i>Listeria monocytogenes</i>

<i>L. pneumophila</i>	<i>Legionella pneumophila</i>
LAM	lipoarabinomannan
LPS	lipopolysaccharide
RRR	leucine-rich repeats
<i>M. avium</i>	<i>Mycobacterium avium</i>
<i>M. bovis</i> BCG	<i>Mycobacterium bovis</i> bacillus Calmette-Guérin
<i>M. fermentans</i>	<i>Mycoplasma fermentans</i>
<i>M. tuberculosis</i>	<i>Mycobacterium tuberculosis</i>
Mal	MyD88-adaptor-like
MD-2	myeloid differentiation protein 2
ME	mercaptoethanol
mfVSG	membrane fraction variant surface glycoprotein
MHC	major histocompatibility complex
MyD88	myeloid differentiation factor 88
n.d.	not detected
N.D.	not determined
NF	nuclear factor
NK	natural killer
NO	nitric oxide
OADC	oleic acid-albumin-dextrose-catalase
OspA	outer-surface lipoprotein
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
<i>P. berghei</i>	<i>Plasmodium berghei</i>
<i>P. falciparum</i>	<i>Plasmodium falciparum</i>
PAMP	pattern associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phyco-erythrin
PGN	peptidoglycan
PIM	mannosylated phosphatidylinositol
PMSF	phenylmethyl Sulfonyl Fluoride
poly(I:C)	polyinosinic-polycytidyllic acid
PRR	pattern recognition receptor
R-848	imidazoquinoline resiquimod
RF	rheumatoid factor
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>S. pneumonia</i>	<i>Streptococcus pneumonia</i>
SLE	systemic lupus erythematosus
sVSG	soluble variant surface glycoprotein
<i>T. brevifolia</i>	<i>Taxus brevifolia</i>
<i>T. brucei</i>	<i>Trypanosoma brucei</i>
<i>T. cruzi</i>	<i>Trypanosoma cruzi</i>
<i>T. gondii</i>	<i>Toxoplasma gondii</i>
<i>T. palladium</i>	<i>Treponema palladium</i>
T _H	T-helper
TIR	Toll/IL-1R-domain-containing transmembrane receptor

TIRAP	TIR domain-containing adaptor molecule
TLCK	N α -p-tosyl-L-lysine chloromethyl ketone
TLR	toll like receptor
TMB	trimethylbenzene
TNF	tumour necrosis factor
T _R	regulatory T cell
TRAF6	TNF-receptor-associated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	TIF-domain-containing adaptor protein inducing IFN- β
VSG	variant surface glycoprotein

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Abstract

The aims of this study were to address the importance of TLR signaling in the development of immunity following infection with either *Mycobacterium tuberculosis* or *Trypanosoma brucei*.

Results presented here discuss the following:

- a. Evaluate the contribution of CD14, TLR2, TLR4 and MyD88 to the generation of a protective immune response following either low- or high-dose mycobacterial aerosol challenge. Collectively, a requirement for Toll signaling during a mycobacterial infection is shown using animals deficient in the adaptor protein MyD88. Consequently, screening of the individual PRR-deficient animals revealed a more pronounced role for TLR2 during a mycobacterial infection as opposed to CD14 or TLR4. Here, TLR2-deficient animals were unable to clear systemic bacteria as well as wild-type controls, a result attributed to defective granulomatous responses in the lungs of TLR2-deficient animals. Dissection of the TLR2 phenotype revealed that animals lacking this receptor developed pulmonary immunopathology during an *M. tuberculosis* infection, characterized by elevated levels of TNF, IFN- γ and IL-12p40, as well as increased numbers of CD4 $^{+}$ and CD8 $^{+}$ T cells. Data presented here indicates that TLR2 is required by macrophages to control a pulmonary *M. tuberculosis* infection, and in its absence the host attempts to compensate for this deficiency by upregulating a Type 1 inflammatory response, ultimately resulting in immunopathology and chronic pneumonia.

- b. Evaluate the contribution of CD14, TLR1, TLR2, TLR2 & 4, TLR9 and MyD88 to the generation of a protective immune response following a standard *T. brucei* intraperitoneal challenge. Here, MyD88-deficient macrophages are non-responsive towards both soluble variant-specific surface glycoprotein (sVSG) as well as membrane bound VSG (mfVSG) purified from *T. brucei*. Infection of MyD88-deficient mice with either clonal or non-clonal stocks of *T. brucei* resulted in elevated levels of parasitemia. This was accompanied by reduced plasma IFN-gamma and TNF levels during the initial stage

of infection, followed by moderately lower VSG-specific IgG2a antibody titers during the chronic stages of infection. Analysis of several Toll-like receptor (TLR)-deficient mice revealed a partial requirement for TLR9 in the production of IFN-gamma and VSG-specific IgG2a antibody levels during *T. brucei* infections. The MyD88-dependent induction of an inflammatory response was also associated with infection-induced pathology in that animals lacking MyD88 were less anemic than wild-type controls. These results implicate the mammalian TLR family and MyD88 signaling in the innate immune recognition of *T. brucei* but also in infection-associated pathology.

Chapter 1

General introduction

Chapter 1

1. General introduction

1.1 Prologue

The immune system protects the host from a range of microorganisms and toxins, but must overcome several complicated problems in order to achieve this. Firstly, the host should be able to distinguish molecular structures present on a diversity of pathogens, a task which should encompass a large variety of environmental microorganisms. Secondly, the ensuing response should be mediated within a relatively short period of time, particularly because of the rate of proliferation of organisms such as viruses and bacteria. However, although this response should be dynamic enough to eliminate the pathogen, it should not be so vigorous so as to damage host tissue. And finally, a system should be in place such that a mechanism of tolerance exists to prevent healthy tissues of the host from coming under attack.

The course of evolution has moulded the host's response to invading organisms such that very many of the host's earliest solutions remain in place along with the most recent. As a result, the host's immune response can be said to consist of an innate immune system forming the core of host defenses, and an acquired or adaptive immune system. In this regard, the innate immune system has been evolving for a far longer period of time than the acquired immune system, and is therefore in certain respects, more complicated and refined.

The innate immune system comprises several independent lines of defense which function collectively to safeguard the integrity of the host. Quintessentially, the innate immune system is responsible for creating an environment in which host cells' survive and invading exogenous life forms do not. Such an inhospitable environment is aided by the secretion of various antimicrobial peptides by host cells, the function of which remains an integral part retained by the innate immune system to this day. In general, these peptides are encoded within the host's genome and with time, have become inducible rather than constitutively expressed.

In vertebrates, the development of a system used to eradicate toxins produced by microbes or other organisms involved the secretion of pathogen-specific soluble receptors, or immunoglobulins. The importance for the generation of an adaptive immune response therefore plays an important role in the battle against organisms such as viruses and bacteria, pathogens once thought to fall within the domain of protective innate immunity. Nevertheless, the development of a protective adaptive immune response is critically dependent on the innate immune system. Here, the production of cytokines such as tumor necrosis factor (TNF), IL-1, IL-6, or IL-12 by cells such as macrophages and dendritic cells are essential for the generation of adaptive immunity, without which the adaptive immune system is severely weakened. Thus, the initiation of an innate immune response is essential to multicellular life on this planet, and effectively tailors the developing adaptive immune response. Indeed, several primitive organisms such as subvertebrates are able to survive without an adaptive immune response. Furthermore, circumstances that generate innate immunodeficiency disorders are more life-threatening than those that produce adaptive immune impairment.

1.2 A brief history of Toll

Studies performed on embryonic patterning in *Drosophila* initially identified Toll as a component of the Dorsal group, the Dorsal group being a set of genes responsible for encoding mutually complementing participants in a signaling cassette required for the optimal dorsal-ventral polarization of fly embryos (Anderson *et al.*, 1985^a; Anderson *et al.*, 1985^b). The fact that Toll was a central component in this signaling cassette led Nusslein-Vollhardt and colleagues to the realization that Toll in fact encoded a plasma membrane receptor bearing specificity for a protein ligand called Spätzle (Morisato & Anderson, 1994; Schneider *et al.*, 1994). Indeed, it was found that Spätzle encoded a novel protein, which upon proteolytic processing became active, thereby determining where Toll was active (Morisato & Anderson, 1994). Thus, the identification of Toll was discovered in an area initially unrelated to the realm of immunity.

Despite the establishment of Toll as a regulator of early embryogenesis, it was not until the early 1990s that a link between the Toll pathway in *Drosophila* and the

interleukin-1 receptor (IL-1R)-NF- κ B-signaling cascade in mammals was made (Gay & Keith, 1991; Heguy *et al.*, 1992). In *Drosophila*, activation of the Toll pathway following contact with Spätzle propagated nuclear translocation of an NF- κ B-like transcription factor via an intracellular signaling cascade (Letsou *et al.*, 1991; Shelton *et al.*, 1993; Grosshans *et al.*, 1994). Here, the contact between the adaptor Tube and a serine kinase called Pelle resulted in the phosphorylation and degradation of the I κ B orthologue Cactus (Geisler *et al.*, 1992), an inhibitor of a transcription factor called Dorsal (Ip *et al.*, 1991). Dorsal was then able to regulate maternal-effect genes involved in dorsoventral patterning (Thisse *et al.*, 1991; Pan *et al.*, 1991). In mammals, signaling via the IL-1R-NF- κ B-cascade proceeds via a Toll/IL-1R (TIR)-domain-containing transmembrane receptor, and it was this cytoplasmic domain that was found to have homology with Toll (Gay & Keith, 1991).

At the time, it was known that in mammalian systems, IL-1 could induce NF- κ B translocation to the nucleus (Nonaka *et al.*, 1990; Anisowicz *et al.*, 1991; Bomsztyk *et al.*, 1991). This fact spurred two groups of *Drosophila* geneticists to independently identify κ B motifs in the promoter sequences of insect genes known to encode antimicrobial peptides (Reichhart *et al.*, 1992; Rosetto *et al.*, 1995). Thus, apart from its role in embryonic development, Toll was suspected to control the expression of antimicrobial peptides in the insect host. In 1996, the requirement for Toll to protect an adult *Drosophila* from an *Aspergillus fumigatus* infection was the first report to link the Toll pathway with the regulation of an antifungal response (Lemaitre *et al.*, 1996). Here, in response to *A. fumigatus*, the production of an antifungal peptide called Drosomycin is dependent on the translocation of a rel family member called Dif, a homolog of Dorsal (Rutschmann *et al.*, 2000). As was observed for the developmental cascade (Geisler *et al.*, 1992), Spätzle was also required for the activation of Toll in response to *A. fumigatus* (Rutschmann *et al.*, 2000). Therefore, a model was established in which Toll functioned to control the expression of genes encoding antimicrobial peptides which in turn promoted studies aimed at linking activation of the Toll pathway and the cytokine-induced activation of NF- κ B.

In 1997, a human homologue of the *Drosophila* Toll termed human Toll (h-Toll) was cloned and characterized (Medzhitov *et al.*, 1997), in so doing becoming the first

member of what was to be called the mammalian Toll-like receptor family of proteins. h-Toll (subsequently known as Toll-like Receptor 4) was shown to consist of an extracellular domain laden with leucine-rich repeats (LRR), and a cytoplasmic domain which shared homology with the cytoplasmic domain of the human interleukin (IL)-1 receptor. As was seen for its *Drosophila* counterpart, h-Toll could stimulate the translocation of NF- κ B to the nucleus and induce the expression of genes coding for the inflammatory cytokines IL-1, IL-6 and IL-8. The subsequent molecular cloning of a class of human receptors in 1998 showed these proteins to have intra- and extracellular domains homologous to that of *Drosophila* Toll (Rock *et al.*, 1998), indicating that these proteins could constitute an important component of immunity in humans.

It was at this point in time that a series of purely genetic approaches identified Toll-like Receptor 4 (TLR4) as the mammalian protein required for recognition of LPS and transduction of the LPS signal across the plasma membrane (Poltorak *et al.*, 1998^{a,b}). Here, Poltorak and colleagues were able to show that mutations in the *Tlr4* gene present in two strains of mice, namely C3H/HeJ and C57BL/10ScCr, were responsible for their LPS hyporesponsiveness. Through the application of positional cloning methods, the mutation in C3H/HeJ mice was attributed to the replacement of a conserved proline residue in the TLR4 cytoplasmic domain with that of a histidine (Poltorak *et al.*, 1998^b), while the *Tlr4* locus in C57BL/10ScCr mice was entirely deleted (Poltorak *et al.*, 1998^b; Poltorak *et al.*, 2000). By 1999, studies using gene-targeted mice in which the transmembrane and cytoplasmic regions of TLR4 were deleted, confirmed the results of Poltorak and colleagues showing that the induced *Tlr4* mutation abolished responses to LPS (Hoshino *et al.*, 1999).

Subsequently, thirteen mammalian TLR paraloques have been indentified (Beutler *et al.*, 2004). These constitute 10 TLR proteins in humans and 12 in mice, all of which have been shown to function as recognition receptors for various protozoal (Campos *et al.*, 2004; Scanga *et al.*, 2002), bacterial (Poltorak *et al.*, 1998^a; Takeuchi *et al.*, 1999; Hayashi *et al.*, 2001; Zhang *et al.*, 2004), fungal (Meier *et al.*, 2003) and viral (Tabeta *et al.*, 2004; Heil *et al.*, 2004; Diebold *et al.*, 2004) ligands, thereby controlling various aspects of both innate and adaptive immune responses.

1.3 TLR ligand specificity

The genetic approaches used by Poltorak and colleagues to identify the TLR involved in LPS hyporesponsiveness in mice spurred other labs to search for possible polymorphisms within the human *Tlr4* gene. Indeed, TLR4 polymorphisms have been associated with endotoxin hyporesponsiveness in humans (Arbour *et al.*, 2000). Here, the TLR4 polymorphism was identified as an amino acid substitution, from aspartic acid to glycine at position 299 (D299G). Similar polymorphisms have subsequently been associated with predisposition towards septic shock resulting from gram-negative bacterial infections (Lorenz *et al.*, 2002^a), as well as an increased risk of premature birth (Lorenz *et al.*, 2002^b). Nevertheless, human TLR4 has been shown to be critical in the innate immune response to LPS from Gram-negative bacteria and the receptor has subsequently been shown to be involved in signaling in response to a broad range of exogenous and endogenous molecules (Table 1). For example, several exogenous molecules that use TLR4 for signal transduction include the F protein of respiratory syncytial virus (Kurt-Jones *et al.*, 2000), the anti-tumour agent Taxol found in the Western yew *Taxus brevifolia* (Kawasaki *et al.*, 2000), and an envelope protein found in a murine retrovirus (Rassa *et al.*, 2002). A few endogenous ligands have been shown to signal via TLR4 (Table 1), although it is suspected that contaminating LPS was present in several of the protein preparations (Vabulas *et al.*, 2002; Okamura *et al.*, 2001; Smiley *et al.*, 2001). This being said, although the principal TLR4 activator is LPS, some controversy exists as to whether LPS actually binds to TLR4.

Preliminary experiments which suggested that an additional molecule was required for TLR4-mediated LPS signaling were proposed by Shimazu *et al.* (1999) in which overexpression of TLR4 did not confer LPS responsiveness on human embryonic kidney 293 cells. This additional molecule was subsequently identified as MD-2 (Shimazu *et al.*, 1999), and conferred LPS responsiveness by physically associating with the extracellular domain of TLR4. It is now known that not only TLR4 and MD-2 are involved in LPS responsiveness, but it has been shown that CD14 and MD-2 function collectively to present LPS to TLR4 (da Silva Correira *et al.*, 2001). This receptor complex not only functions for LPS signaling but is involved in the LPS-mimetic action

of Taxol (Kawasaki *et al.*, 2000). Although Taxol bears no structural similarity to LPS, it possesses several LPS-like activities including signal induction mediated by the TLR4-MD2 complex (Kawasaki *et al.*, 2000).

Table 1.1 | Toll-like receptors and their ligands

Receptor	Ligand	Origin of ligand	Reference
Exogenous ligands			
TLR1	Triacyl lipopeptides Soluble factors	Bacteria and mycobacteria <i>Neisseria meningitidis</i>	Takeuchi <i>et al.</i> , 2002 Wyllie <i>et al.</i> , 2000
TLR2	Lipoprotein/lipopeptides Peptidoglycan Lipoteichoic acid Lipoarabinomannan Phenol-soluble modulin Glycoinositolphospholipids Glycolipids Porins Atypical lipopolysaccharide Atypical lipopolysaccharide Zymosan	Various pathogens Gram-positive bacteria Gram-positive bacteria Mycobacteria <i>Staphylococcus epidermidis</i> <i>Trypanosoma cruzi</i> <i>Treponema maltophilum</i> <i>Neisseria</i> <i>Leptospira interrogans</i> <i>Porphyromonas gingivalis</i> Fungi	Aliprantis <i>et al.</i> , 1999 Takeuchi <i>et al.</i> , 1999 Schwandner <i>et al.</i> , 1999 Means <i>et al.</i> , 1999 Hajjar <i>et al.</i> , 2001 Campos <i>et al.</i> , 2001 Opitz <i>et al.</i> , 2001 Massari <i>et al.</i> , 2002 Werts <i>et al.</i> , 2001 Hirschfeld <i>et al.</i> , 2001 Underhill <i>et al.</i> , 1999a
TLR3	Double-stranded RNA	Viruses	Alexopoulou <i>et al.</i> , 2001
TLR4	Lipopolysaccharide Taxol Fusion protein Envelope protein Heat-shock protein 60*	Gram-negative bacteria Plants Respiratory syncytial virus Mouse mammary-tumour virus <i>Chlamydia pneumoniae</i>	Poltorak <i>et al.</i> , 1998a Kawasaki <i>et al.</i> , 2000 Kurt-Jones <i>et al.</i> , 2000 Rassa <i>et al.</i> , 2002 Bulut <i>et al.</i> , 2002
TLR5	Flagellin	Bacteria	Hayashi <i>et al.</i> , 2001
TLR6	Diacyl lipopeptides Lipoteichoic acid Zymosan	Mycoplasma Gram-positive bacteria Fungi	Takeuchi <i>et al.</i> , 2001 Takeuchi <i>et al.</i> , 1999 Ozinsky <i>et al.</i> , 2000
TLR7	Imidazoquinoline Loxoribine Bropirimine Single-stranded RNA	Synthetic compounds Synthetic compounds Synthetic compounds Viruses	Hemmi <i>et al.</i> , 2002 Heil <i>et al.</i> , 2003 Heil <i>et al.</i> , 2003 Diebold <i>et al.</i> , 2004
TLR8	Imidazoquinoline Single-stranded RNA	Synthetic compounds Viruses	Jurk <i>et al.</i> , 2002 Heil <i>et al.</i> , 2004
TLR9	CpG-containing DNA	Bacteria and viruses	Hemmi <i>et al.</i> , 2000
TLR10	N.D.	N.D.	-
TLR11	N.D. Profilin-like protein	Uropathogenic bacteria <i>Toxoplasma gondii</i>	Zhang <i>et al.</i> , 2004 Yarovinsky <i>et al.</i> , 2005
Endogenous ligands			
TLR2	Heat-shock protein 70*	Host	Asea <i>et al.</i> , 2002
TLR4	Heat-shock protein 70* Domain A of fibronectin* Oligos. of Hyaluronic acid* Fibrinogen*	Host Host Host Host	Vabulas <i>et al.</i> , 2002 Okamura <i>et al.</i> , 2001 Termeer <i>et al.</i> , 2002 Smiley <i>et al.</i> , 2001

* It is possible that these ligand preparations, particularly those of endogenous origin, were contaminated with lipopolysaccharide and/or other potent microbial components, and as a result careful analysis is required to conclude that TLRs recognize the indicated endogenous ligands. N.D., not determined; TLR, Toll-like receptor. Table adapted from the following article: Akira & Takeda, 2004.

Several reports have proposed that TLR2 is also involved in LPS signaling (Kirschning *et al.*, 1998; Yang *et al.*, 1998), although it has been suggested that overexpression of TLR2 promotes certain cell lines to become sensitized to minor non-LPS contaminants in LPS preparations (Heine *et al.*, 1999; Takeuchi *et al.*, 1999; Underhill *et al.*, 1999^a). Indeed, it was later shown that highly purified LPS does not activate cells in a TLR2-dependent manner (Hirschfeld *et al.*, 2000), although the receptor is involved in responses towards a structural variant of LPS from *Leptospira* and *Prophyromonas* (Werts *et al.*, 2001; Hirschfeld *et al.*, 2001).

TLR2 is however the primary receptor involved in responses towards lipoproteins. The immunostimulating activities of lipoproteins can be attributed to the presence of lipoylated NH₂ termini present in a variety of gram-negative and gram-positive bacteria, as well as mycoplasmas. TLR2 mediates cellular responses to lipoproteins derived from *Borrelia burgdorferi*, *Treponema pallidum*, *M. tuberculosis* and *Mycoplasma fermentans* (Hirschfeld *et al.*, 1999; Aliprantis *et al.*, 1999; Brightbill *et al.*, 1999; Takeuchi *et al.*, 2000^a), but is also involved in mediating responses to several infectious pathogens and their products (Table 1). As can be seen in the table, these include whole mycobacteria, mycobacterial lipoarabinomannan, whole Gram-positive bacteria, *Treponema* glycolipid, yeast cell walls, peptidoglycan (PGN) and the *Trypanosoma cruzi* glycophosphatidylinositol anchor (Lien *et al.*, 1999; Yoshimura *et al.*, 1999; Schwandner *et al.*, 1999; Means *et al.*, 1999; Flo *et al.*, 2000; Opitz *et al.*, 2001; Campos *et al.*, 2001).

It was later discovered that TLR2 did not function independently in response to several ligands. Here, TLR2 ligand specificity and signal transducing ability was determined by heterodimeric interactions with TLRs such as TLR1 and TLR6 (Ozinsky *et al.*, 2000). In this case, cytokine production by a macrophage cell line was dependent on the cytoplasmic portion of TLR2 functionally pairing with either TLR1 or TLR6, while dimerization of the TLR2 cytoplasmic domain did not induce cytokine production. This effect was however dependent on the stimulus used. The detection of Gram-positive bacteria, PGN and zymosan is mediated by cooperative signaling between TLR2 and TLR6, while TLR2 functioned independently or with a TLR other than TLR6 to detect bacterial lipopeptides (Ozinsky *et al.*, 2000). More recently, heterodimeric complexes

between TLR1 and TLR2 were shown to be required for ara-lipoarabinomannan- and tripalmitoyl cysteinyl lipopeptide-stimulated cytokine secretion from mononuclear cells (Sandor *et al.*, 2003), as well as responses towards the outer-surface lipoprotein (OspA) of *Borrelia burgdorferi* (Alexopoulou *et al.*, 2002). Thus, although specific ligands activate cells in a TLR2-dependent manner, the formation of complexes consisting of additional TLRs or helper proteins such as CD14 enhance the signaling capacity of the cell.

The capacity of a few TLRs such as TLR2 and 4 to recognize a variety of ligands is not shared by all of the members of the mammalian TLR family. In the case of TLR5, a 55-kD protein monomer obtained from both Gram-positive and Gram-negative bacterial flagella was identified as the only TLR5 ligand (Hayashi *et al.*, 2001). However, the uptake and translocation of flagellin by cells requires that the flagellin filaments undergo depolymerization before affording recognition by TLR5 (Smith *et al.*, 2003). Specific ligand affinities can also be afforded to TLRs involved in the recognition of nucleic-acid-like structures (Table 1). The first of the TLRs found to recognize a nucleic acid structure was TLR9 (Hemmi *et al.*, 2000). In these studies, TLR9-deficient mice were found to be non-responsive towards bacterial DNA. Here, TLR9 was found to specifically recognize unmethylated CpG motifs present in bacterial but not vertebrate DNA. As a result, the subsequent administration of CpG DNA is sufficient to protect against infections by intracellular pathogens such as *Leishmania major* and *Listeria monocytogenes* in mice (Elkins *et al.*, 1999; Krieg *et al.*, 1998; Zimmermann *et al.*, 1998). In terms of recognition of double-stranded (ds) RNA produced by several viruses during their replicative cycle, induction of type 1 interferons (IFN- α and - β) is believed to be mediated in part by TLR3 (Alexopoulou *et al.*, 2001). Studies showed that expression of human TLR3 in a dsRNA-nonresponsive cell line induced NF- κ B activation following stimulation with either dsRNA, or the synthetic dsRNA analogue polyinosinic-polycytidylic acid [poly(I:C)]. Furthermore, animals deficient in TLR3 showed reduced responses towards both dsRNA and poly(I:C) (Alexopoulou *et al.*, 2001), although some doubt exists as to whether TLR3 is actually involved in the direct recognition of dsRNA. This stems from the fact that plasmacytoid dendritic cells are the principal cells in both human and mouse blood that produce type 1 interferon in response to viral challenges (Siegal *et al.*, 1999; Asselin-

Paturel *et al.*, 2001), the problem being that TLR3 is not expressed in this cell type (Kadowaki *et al.*, 2001). This would indicate that TLR3 is not the only receptor involved in the recognition of viral nuclear material. Indeed, it was later shown that the physiological ligand for TLR7 and TLR8 is in fact single-stranded RNA (Heil *et al.*, 2004; Diebold *et al.*, 2004), thereby linking virus recognition by plasmacytoid dendritic cells to IFN production.

Although TLR7 and TLR8 participate in the discrimination of nucleic-acid structures in viruses, there is evidence that they are also able to confer responsiveness towards several synthetic compounds (Hemmi *et al.*, 2002; Jurk *et al.*, 2002; Heil *et al.*, 2003). The first synthetic agonist shown to activate a TLR was the antiviral agonist imidazoquinoline resiquimod (R-848; Hemmi *et al.*, 2002). Here, TLR7 was required for responsiveness towards imidazoquinoline R-848 in mice. At the same time however, another group showed that both TLR7 and TLR8 could independently mediate recognition of imidazoquinoline R-848 (Jurk *et al.*, 2002), thereby suggesting a certain amount of redundancy among these receptors. Since then, the immunomodulators loxoribine and bropirimine have been shown to activate cells via TLR7 (Heil *et al.*, 2003), thereby introducing the concept of TLR-activating agents as tools that can be used in clinical applications.

TLRs 7, 8 and 9 not only have homologous structures and nucleic-acid-like ligand specificities, but all are localized intracellularly during cellular activation (Ahmad-Nejad *et al.*, 2002; Heil *et al.*, 2003). Here, the development of a monoclonal antibody raised against TLR9 enabled the intracellular localization of endogenous TLR9 in a mouse macrophage cell line (Ahmad-Nejad *et al.*, 2002). Subsequently, TLR7 and 8 localized in the endosomal and/or lysosomal compartments (Heil *et al.*, 2003), indicating that TLRs 7, 8 and 9 formed a functional subgroup within the TLR family. In contrast to these findings, TLRs 1, 2 and 4 are located on the cell surface (Shimazu *et al.*, 1999; Hajjar *et al.*, 2002; Yang *et al.*, 1999; Visintin *et al.*, 2001), and are recruited to phagosomes only after activation by their respective ligands (Underhill *et al.*, 1999^a; Ozinsky *et al.*, 2000).

Therefore, the subcellular localization of different TLRs can be correlated to some extent with the molecular patterns of their ligands, which would indicate that in terms of specific TLRs, internalization of TLR ligands may be required for full activation of

immune cells by TLRs. This imparts an additional layer of complexity with respect to TLR-dependent activation of cells in that signaling pathways used by individual TLR proteins may very well be tailored not only to the stimulus at hand, but also the cellular localization of the stimulus.

1.4 TLR-signaling pathways

Upon ligand binding, initiation of TLR signaling in mammals proceeds via a common cytosolic TIR domain (Dunne & O'Neill, 2003). This domain not only has sequence homology with the TIR domain of the IL-1R family, but when activated, enables the recruitment of cytosolic TIR domain-containing adaptor proteins. The first of these proteins to be described was myeloid differentiation primary-response protein 88 (MyD88; Lord *et al.*, 1990), initially identified as a gene induced during the differentiation of M1 myeloleukaemic cells into macrophages. The activation of MyD88 ultimately results in the induction of an intracellular signaling cascade consisting of two separate signaling pathways, namely JUN N-terminal kinase (JNK) and NF- κ B (Fig. 1.1; Akira & Takeda, 2004).

1.4.1 The MyD88-dependent signaling pathway

Initial studies using macrophages deficient in MyD88 showed that this adaptor molecule had a central role in the production of inflammatory cytokines induced by lipoproteins, peptidoglycan, CpG DNA, dsRNA, flagellin and imidazoquinolines (Takeuchi *et al.*, 2000^{a,b}; Alexopoulou *et al.*, 2001; Hemmi *et al.*, 2002; Hacker *et al.*, 2000; Schnare *et al.*, 2000; Hayashi *et al.*, 2001). These results indicated that microbial components recognized by the majority of TLRs relay that activation signal via MyD88. The adaptor protein MyD88 was found to consist of two separate domains, namely a TIR domain in the C-terminal portion of the protein that interacted with the TLR and IL-1R TIR domains, as well as an N-terminal death domain. In its activated state, the N-terminal domain of MyD88 interacts with a death domain-containing IL-1R-associated kinase (IRAK), which consists of four family members, namely IRAK-1, IRAK-2, IRAK-M,

and IRAK-4 (Li *et al.*, 2002). Individually, IRAK-1 and IRAK-4 were shown to be important components in the cellular response towards IL-1 and LPS (Kanakaraj *et al.*, 1998; Thomas *et al.*, 1999; Swantek *et al.*, 2000; Suzuki *et al.*, 2002), although IRAK-4 proved to be the more essential component in the IL-1- and TLR4-dependent signaling pathways.

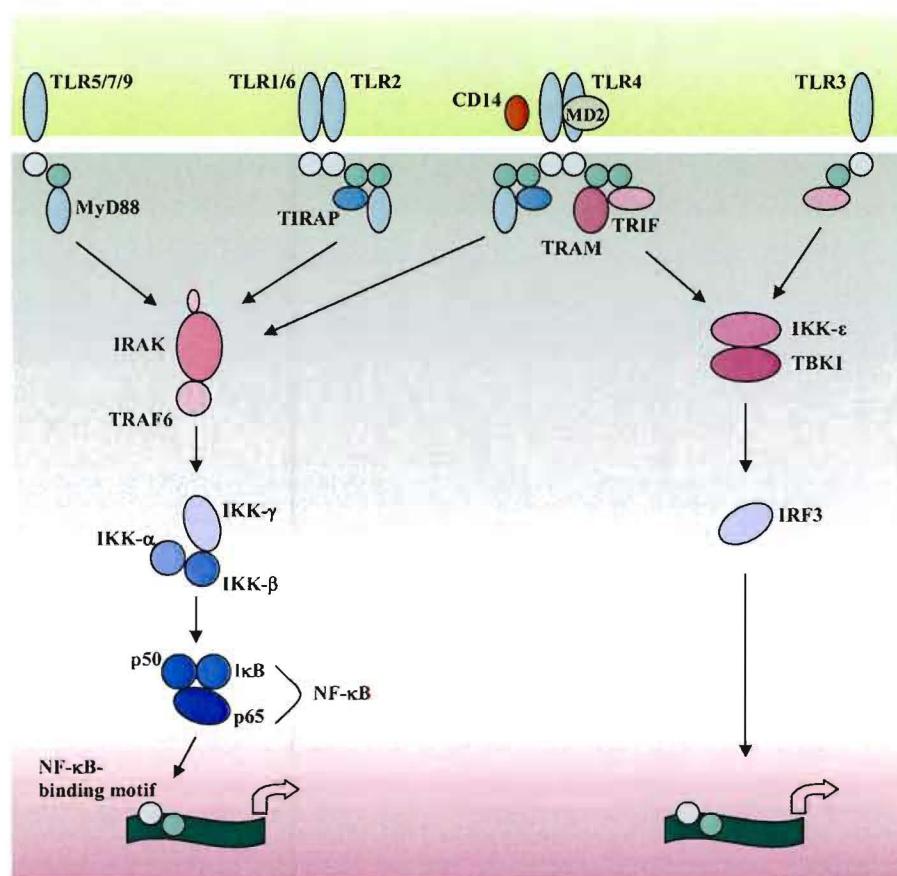


Figure 1.1 TLR-signaling: MyD88-dependent and –independent pathways

Stimulation of different TLRs facilitates the activation of two pathways: the MyD88-dependent and-independent pathways. The MyD88-dependent pathway involves the early phase of nuclear factor- κ B (NF- κ B) activation, which leads to the production of inflammatory cytokines. The MyD88-independent pathway activates interferon-regulatory factor (IRF3) and involves the late phase of NF- κ B activation, both of which lead to the production of IFN- β and the expression of IFN-inducible genes. (Figure adapted from Akira & Takeda, 2004).

Ultimately, the IRAK homologs promoted association with tumour-necrosis factor (TNF)-receptor-associated factor 6 (TRAF6), which then lead to the activation of the IKK complex (inhibitor of nuclear factor- κ B ($I\kappa B$)-kinase complex). The IKK complex in turn sequestered NF- κ B in the cytoplasm resulting in dissociation and nuclear translocation of NF- κ B (Akira & Takeda, 2004). As a result, cells deficient in MyD88 are non-responsive towards ligands for TLR1, 2, 3, 4, 5, 7 or 9 in terms of TNF and IL-12 induction.

1.4.2 The MyD88-independent signaling pathway

There were however some inconsistencies in the notion that NF- κ B activation was solely a result of MyD88-dependent signaling. This stemmed from experiments in which LPS was still capable of inducing NF- κ B activation, albeit with delayed kinetics (Kawai *et al.*, 1999). This led to the conclusion that an additional signaling pathway must exist, even though MyD88 was shown to be important for LPS-induced production of inflammatory cytokines. Analysis of the MyD88-independent activation of LPS signaling revealed a second adaptor protein named TIR domain-containing adaptor molecule (TIRAP) or MyD88-adaptor-like (Mal; Fitzgerald *et al.*, 2001; Horng *et al.*, 2001). It was found that Mal participated in TLR4, but not TLR9 signaling, which suggested that it played a role in the LPS-induced NF- κ B activation in MyD88-deficient cells. In these experiments, the addition of a cell-permeable TIRAP/Mal peptide abolished LPS-induced maturation in both wild-type and MyD88-deficient dendritic cells, indicating that TIRAP/Mal was involved in LPS-induced MyD88-independent signaling. Indeed, the generation of Mal/TIRAP deficient mice revealed a role for Mal/TIRAP in TLR4 signaling (Yamamoto *et al.*, 2002^a), however, it was also evident that neither Mal nor MyD88 were required for the TLR3-dependent activation of interferon-inducible genes.

The MyD88-independent activation of interferon-inducible genes can therefore be attributed to the TLR3- and TLR4-dependent activation of transcription factor IFN regulatory factor-3 (IRF-3). IRF-3 regulates IFN- β production, which then regulates a range of other genes (Sato *et al.*, 2000; Iwamura *et al.*, 2001; Taniguchi *et al.*, 2002). In this case, two additional TIR domain-containing adaptors called TRIF and TRAM, are

believed to regulate the distinct TLR-dependent pathways leading to IRF-3 and NF- κ B activation (Yamamoto *et al.*, 2002^b; Fitzgerald *et al.*, 2003).

Thus, depending on the stimulus at hand, activation of individual TLRs not only drive distinct signaling pathways, but this combined action ultimately affects the maturation of the cell in question.

1.5 TLR activation of cells

Most tissues of the body, in particular those present at mucosal surfaces, are interlaced with resident innate leukocytes macrophages, dendritic cells (DCs), and mast cells. In the event of a pathogen entering the body, pathogen recognition mediated by TLRs orchestrates the recruitment of leukocytes to the site of infection, in so doing activating tissue stromal cells, tissue-resident innate cells, and circulating leukocytes.

TLRs expressed on epithelial cells of the respiratory, intestinal and urogenital tracts (Ashkar *et al.*, 2003; Zhang *et al.*, 2004), represent the first line of defense against invading pathogens. Activation of these TLRs by an invading pathogen results in the production of cytokines, antimicrobial peptides and chemokines (Kagnoff & Eckmann, 1997; Hertz *et al.*, 2003). Cellular recruitment is regulated by a combination of direct recognition of pathogen-associated molecular patterns (PAMPs) by the vascular epithelium, as well as epithelial chemokines produced by the luminal surfaces of both the local tissue as well as the draining lymph nodes. One such example is a mouse model of septic shock induced by systemic administration of LPS (Andonegui *et al.*, 2003). In this model, the accumulation of neutrophils into the lungs is associated with pulmonary failure that results in sepsis-related death. Andonegui and colleagues showed that TLR4 responsiveness by the vascular epithelial cells, but not the neutrophils, was required for the neutrophil sequestration into the lungs. In another study, the presence of TLR4 on both the epithelial and hematopoietic cellular compartments was required for a protective inflammatory response following infection with uropathogenic *Escherichia coli* (Schilling *et al.*, 2003). Therefore, TLRs present on epithelial, endothelial and hematopoietic cells recognize PAMPs present in invading pathogens and play an important role in inflammation-induced cellular recruitment.

Cells which comprise this initial inflammation-induced cellular infiltrate include neutrophils, NK cells, monocytes, basophils and eosinophils. Attempts to determine the extent of TLR expression on cells either recruited due to an inflammatory response or cells stimulated *in vitro* has been met with some difficulty. Discrepancies in TLR expression profiles have been attributed to species-specific differences in TLR expression, mRNA expression and responsiveness towards various TLR agonists, as well as the lack of reliable TLR antibodies. Nevertheless, analysis of TLR expression at the mRNA level has revealed that some TLRs are either positively or negatively regulated, depending on the cell type. In human neutrophils, mRNAs for TLRs 1 through 10 are expressed, with the exception of TLR3 (Hayashi *et al.*, 2003). Neutrophils were responsive towards LPS, zymosan and R-848, but could not be stimulated by CpG (Neufert *et al.*, 2001). Similarly, NK cells have been shown to express TLR9 mRNA, but are non-responsive towards CpG stimulation (Hornung *et al.*, 2002). Both eosinophils and mast cells express mRNAs for several TLRs, but are only responsive towards a few agonists. Here, eosinophils only respond to R-848 via TLR7, despite constitutive expression of TLRs 1, 4, 7, 9 and 10 mRNA (Nagase *et al.*, 2003), while mast cells express TLRs 1, 2, 4 and 6 but only respond to TLR2 and TLR4 agonists (McCurdy *et al.*, 2003; Supajatura *et al.*, 2001 & 2002). Monocytes on the other hand express a variety of TLRs that ultimately drive their differentiation into either tissue macrophages or DCs, thereby contributing to the generation of adaptive immunity.

The majority of papers investigating TLR expression profiles on DC subsets have used RT-PCR as a tool to detect individual TLRs. In addition to this, some studies have used inhibitors of endosomal pathways such as baflomycin and chloroquine to determine the functional location of individual TLRs. Collectively, these reports have shown that TLRs 3, 7, 8 and 9 require an acidic environment for endosome maturation, while TLRs 1, 2, 4, 5 and 6 are solely expressed on the cell surface (Heil *et al.*, 2003; Lee *et al.*, 2003; Matsumoto *et al.*, 2003; Ahmed-Nejad *et al.*, 2002). Furthermore, murine and human DCs exhibit differential TLR expression patterns (Kadowaki *et al.*, 2001; Edwards *et al.*, 2003). In humans, CD11c⁺ myeloid DCs (mDCs) express TLRs 1, 2, 3, 5, 6 and 8, while freshly isolated plasmacytoid DCs (pDCs) express TLR7 and 9 (Hornung *et al.*, 2002; Jarrossay *et al.*, 2001; Kadowaki *et al.*, 2001). The stage of cellular maturation also

influences TLR expression profiles. During the differentiation of human blood monocytes into immature DCs, expression of TLRs 1, 2, 4 and 5 are progressively lost when cultured in the presence of IL-4 and granulocyte-macrophage colony-stimulating factor (Visintin *et al.*, 2001). Here, the differentiation into immature DCs is also associated with the acquired expression of TLR3. In mice, TLR expression not only varies between DC subsets, but TLR expression and responsiveness also depends on whether the DCs are freshly isolated or derived *in vitro* (Table 1.2).

Table 1.2 | Toll-like receptor expression by mouse and human dendritic-cell subsets*

DC subset	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8	TLR9	TLR10
<i>Mouse</i>										
CD11c ⁺	+	+	+	+	+	+	+	+	+	ND
CD11c ⁺ CD3 ⁺	+	+	+	+	+	+	+	+	+	ND
Plasmacytoid DCs	+	+/-	+/-	-‡	+	+	++*	+	++‡	ND
<i>Human</i>										
Blood DCs	++	+	++‡	+†	+	+	++‡	+	-‡	+
Monocyte-derived DCs (GM-CSF)	ND	ND	++‡	++‡	ND	ND	++‡	ND	ND	ND
Monocyte-derived DCs (GM-CSF + IL-4)	+	++‡	+	+/-‡	+	+	+/-	+	-‡	+
Blood plasmacytoid DCs						+/-	+/-	+/-	+/-	+/-
Plasmacytoid DCs (IL-3)	+	-‡	++‡	-‡	-	+	++	-	++‡	+/-

* Data compiled from Degli-Esposti & Smyth (2005) and is representative of numerous studies investigating TLR expression and function in DC subsets in mice and humans. Cells were either directly isolated or generated *in vitro*. The cytokines used to generate the latter populations are indicated in parentheses.

† Values indicate that the expression of the TLR was confirmed by a functional response to the relevant ligands.

‡ The splenocyte population did not respond to TLR4 ligands, but bone-marrow-derived DCs cultured with granulocyte/macrophage colony-stimulating factor (GM-CSF) did (Boonstra *et al.*, 2003).

|| This DC subset was non-responsive towards R-848 (Edwards *et al.*, 2003), but did produce CC-chemokine ligand 5 (CCL5/RANTES) in response to R-848 (Ruggeri *et al.*, 2002).

¶ Blood DCs do not express TLR4, but LPS responsiveness in this case might be mediated by TLR2 (Hertz *et al.*, 2001).

This is observed for murine splenic DCs which express low amounts of TLR4, whereas freshly isolated murine DC subsets do not respond when stimulated with LPS (Boonstra *et al.*, 2003). In contrast, myeloid DCs generated with granulocyte-macrophage colony-stimulating factor respond robustly when stimulated with LPS as a result of their high level of TLR4 expression (Boonstra *et al.*, 2003). Nevertheless, the differential functions of DC subsets imparts a certain amount of elasticity in the generation of multiple effector

cell types such as T_H1, CTL or B cell responses and depends on the cellular environment as well as the stimulus received (Kalinski *et al.*, 1999; Vieira *et al.*, 2000; Tanaka *et al.*, 2000; Nagai *et al.*, 2003).

The generation of an adaptive immune response coincides with the maturation of DCs and the presentation of processed peptides to naïve T lymphocytes in the context of MHC molecules. Stimulation of naïve T lymphocytes initially occurs in the draining lymph nodes, and is associated with the TLR-induced downregulation of inflammatory chemokine receptors and an upregulation of the receptors for lymphoid chemokines (Sallusto *et al.*, 1998; Dieu *et al.*, 1998; Forster *et al.*, 1999; Gunn *et al.*, 1999). Once inside the lymphoid tissue, DCs induce the activation and differentiation of T cells by presenting the peptide to the T cell receptor in the context of an MHC molecule. CD28 expression on naïve T cells is triggered by upregulating the co-stimulatory molecules CD40, CD80 and CD86 (Hoebe *et al.*, 2004). The differentiation of CD4⁺ T cells into either T_H1 or T_H2 cells depends on the density of peptides presented by DCs, the cytokines secreted, and the types of costimulatory molecules expressed (Constant & Bottomly, 1997). A similar process occurs for the activation of CD8⁺ T cells, although memory CD8⁺ T cell responses require CD4⁺ T cell help (Janssen *et al.*, 2003; Shedlock & Shen, 2003; Sun *et al.*, 2003; Bourgeois *et al.*, 2002). Thus, TLR recognition of PAMPs promotes the activation and maturation of DCs which in turn induce T cell activation and differentiation.

Although the role of DCs in instigating an immune response is well established, recent reports suggest that natural killer (NK) cells may have a role to play as initiators of immunity (Carayannopoulos & Yokoyama, 2004). While the initial signals seem to originate from DCs, NK cells are thought to coordinate immunity through the activation and elimination DCs (Degli-Esposti & Smyth, 2005). This DC-NK-cell crosstalk occurs with DCs that have already been activated through TLR-mediated pathogen recognition, although some reports suggest that NK cells have a role to play as initiators of immunity in that they express some members of the TLR family (Hornung *et al.*, 2002; Muzio *et al.*, 2000; Chalifour *et al.*, 2004). Direct activation of NK cells through TLRs can induce the production of IFN- γ and TNF thereby acquiring cytolytic activity against immature DCs (Sivori *et al.*, 2004), further highlighting the complexity of DC-NK-cell interactions.

Finally, B-cell activation and antibody production can be linked to DC-B-cell interactions (MacPherson *et al.*, 1999), but also to TLR-mediated activation of B cells (Martin & Kearney, 2002). Here common bacterial components such as phosphatidylcholine can interact with TLRs present on B cells and promote T cell-independent antibody responses (Martin & Kearney, 2002). Antigens derived from microbial organisms such as LPS and CpG DNA can also trigger polyclonal B cell activation (Poltorak *et al.*, 1998^b; Krieg *et al.*, 2002), which may potentially result in the production of autoreactive antibodies. Thus, in the case of B cells that express autoreactive membrane immunoglobulin, mechanisms have developed that control against autoantibody production elicited by TLR agonists (Rui *et al.*, 2003).

Collectively, TLRs not only alert the immune system to the presence of microbial infection, but tailor the appropriate immune response towards a specific pathogen. Although TLRs play an important role in the induction of an adaptive immune response against infectious diseases, the activation of TLRs can also result in the development of autoimmune disorders. The balance between a protective or a harmful immune response is addressed in the following sections.

1.6 Bacterial infection and septic shock

Although TLR-mediated pathogen recognition is required for the initiation of an effective immune response, excessive TLR signaling during an acute bacterial infection can lead to sepsis, one of the primary causes of death in intensive care units in the United States (Bochud & Calandra, 2003). Sepsis and its most severe form, septic shock, are characterized as an uncontrolled systemic inflammatory response which can ultimately result in tissue injury, vascular instability and multiple organ failure.

The development of septic shock can be directly linked to a hyperinflammatory immune response mediated by the activation of TLR4 and CD14 (Miller *et al.*, 2005). Here, endotoxin released in large quantities from Gram-negative bacteria results in excessive production of TNF and IFN- γ , the major cytokines contributing to polymicrobial sepsis. Therefore, strategies aimed at blocking the toxic effects of LPS have in part focused on a temporary blockade of TLR4 signaling. The validity of such an

approach is substantiated by the fact that mice having either a natural or induced *Tlr4* gene mutation are resistant to LPS-induced endotoxic shock (Poltorak *et al.*, 1998^{a,b}; Hoshino *et al.*, 1999). Furthermore, animals lacking CD14 are resistant to LPS toxicity as well as Gram-negative-induced sepsis (Haziot *et al.*, 1996). The feasibility of inducing protection against sepsis in animals by administration of anti-CD14 monoclonal antibodies has been evaluated in rabbit models (Schimke *et al.*, 1998), although treatment with monoclonal antibodies alone is not sufficient for full protection (Opal *et al.*, 2003). Similar approaches aimed at blocking TLR4 signaling during sepsis have focused on the use of specific analogues of LPS, which function as LPS antagonists, in so doing interfering with the recognition of LPS by TLR4. One such example is the LPS lipid A analogue E5564 which has been shown not only to pharmacologically interfere with TLR4-induced LPS signaling *in vitro* (Mullarkey *et al.*, 2002), but appears to be a potential therapeutic agent for Gram-negative sepsis (Lynn *et al.*, 2003).

The use of TLR4 antagonists as a tool to curb septic shock should however be approached with caution. In humans, an amino acid substitution in TLR4 (D299G), has been associated with an increased risk of Gram-negative infections (Agnese *et al.*, 2002; Lorenz *et al.*, 2002^a), as well as an increased incidence of systemic inflammatory syndrome (Child *et al.*, 2003). This being said, the effect of the human D299G polymorphism on sepsis can only be linked to Gram-negative infections as the same polymorphism does not affect polymicrobial sepsis (Feterowski *et al.*, 2003).

1.7 Allergic disease

Over the past three decades there has been an increase in the prevalence of allergic diseases such as asthma. Presently, the design of treatments aimed at controlling the transition from allergic disease to asthma has been limited by a lack of understanding of the essential mechanisms involved in triggering chronic inflammatory disease.

In asthmatics, chronic inflammatory disease has been associated with a prevailing T_H2 response, although no mechanistic explanation is available for the selective activation of T_H2 cells (El Biaze *et al.*, 2003; Hussain & Kline, 2003; Kuchroo *et al.*, 2003). Mouse models of allergic airway disease have shown that stimulation of lung DCs

with low doses of inhaled LPS have induced a T_{H2} response and allergic inflammation, whereas increased doses of LPS have shifted the T_{H2} -type inflammation to a T_{H1} response (Eisenbarth *et al.*, 2002). Physiologically, these experimental setups have correlated the low LPS doses to the amount of LPS present on inhaled environmental antigens, while higher LPS doses are associated with amounts present during Gram-negative bacterial infections. In terms of activation of the immune response, it is thought that the induction of a T_{H2} response following inhalation of environmental LPS is TLR4-dependent, while the induction of a T_{H1} response after Gram-negative infection is also TLR4-dependent. Nevertheless, one of the aims of treating asthma is believed to involve changing the T_H -cell balance from a dominant T_{H2} -type inflammation to a T_{H1} -type response.

LPS has however been shown to have opposing effects on allergic asthma. Studies on people with allergic asthma have not only shown that the severity of asthma induced by house mite allergen can be more closely correlated to LPS levels as opposed to the allergen itself (Michel *et al.*, 1996), but are also more sensitive to the bronchoconstrictive effects of inhaled endotoxin (Michel *et al.*, 1989). In contrast, exposure of LPS in early childhood may decrease the incidence of asthma later in life (Braun-Fahrlander *et al.*, 2002; Gehring *et al.*, 2002). This being said, the ability of LPS to exacerbate asthma and decrease atopy is not observed for individuals with the D299G polymorphism in that these individuals have a decreased risk of bronchoreactivity (Werner *et al.*, 2003), and increased atopic severity (Yang *et al.*, 2004).

Mechanistic roles proposed for TLR4 in the development of asthma and atopy include the production of IL-10 by DCs which limit T_{H1} inflammatory pathology (Higgins *et al.*, 2003), and the LPS-induced activation of regulatory T cells which serve to curb T_{H1} inflammatory responses (Caramalho *et al.*, 2003). This being said, a search for compounds that can be used for the treatment of allergy has identified R-848, the TLR7 and TLR8 agonist, as a potential candidate (Brugnolo *et al.*, 2003). Here, the characteristic T_{H2} response associated with allergen-specific CD4⁺ lymphocytes was shifted to a protective T_{H1} response, indicating that the use of imidazoquinolines could have beneficial effects in the treatment of allergic disorders.

1.8 Autoimmune disease

The TLR-dependent activation of a pathogen-specific immune response has also been linked to the development of autoimmunity. In this setting, potentially self-reactive T cells in the peripheral circulation are kept in check by regulatory T cells (T_R cells). T_R cells therefore have a dual function in that they suppress the inflammatory responses of activated T cells, but also maintain tolerance to self antigens. Here, IL-6 produced by DCs in response to TLR activation makes antigen-specific T cells refractory to suppression by T_R cells, allowing a pathogen-specific immune response to be mounted (Pasare *et al.*, 2003), but also relieves suppression of auto-reactive T cells.

The MRL-lpr murine model of systemic lupus erythematosus (SLE) and rheumatoid arthritis has supported a link between TLRs and autoimmune disease. Here, MRL-lpr mice produce exceedingly high titres of IgG anti-IgG rheumatoid factor (RF), a B-cell receptor with specificity toward self-IgGs (Theofilopoulos *et al.*, 1985; Wolfowicz *et al.*, 1988). Complexes of IgG bound to nucleosomes activate B cells from mice that express RF, a phenomenon also seen in SLE patients who have auto-antibodies that recognize nucleosomes and DNA (Tan *et al.*, 1989). The link to TLR signaling comes from the fact that B cells from mice expressing RF but deficient in MyD88 do not respond to anti-nucleosome IgGs (Leadbetter *et al.*, 2002). More specifically, TLR9 has been implicated in this as the TLR9 antagonist S-ODN 2088 (Leadbetter *et al.*, 2002; Lenert *et al.*, 2001) inhibits B cell proliferation. Furthermore, stimulation of TLR9 with CpG oligonucleotides is shown to break tolerance in an experimental autoimmune encephalomyelitis model of multiple sclerosis (Ichikawa *et al.*, 2002), substantiating the role of TLRs in breaking peripheral tolerance towards self-antigens.

Apart from potential TLR antagonists that can be used to ameliorate autoimmune responses, a more dangerous approach would be to use high doses of LPS as a tool to induce suppression of auto-reactive T cells by T_R cells (Caramalho *et al.*, 2003). In this model, excessive doses of LPS increase the immunosuppressive effects of T_R cells, in so doing describing a potential mechanism used by the immune system to minimize potential systemic damage resulting from massive inflammation.

1.9 Cancer

The stimulation of an immune response such that it resembles an infection, termed immunotherapy, is a strategy that has been used in several studies as a means to eliminate tumours (Heckelsmiller *et al.*, 2002; Carpentier *et al.*, 1999; Egeter *et al.*, 2000). The general concept behind this type of approach is to prime an antigen-presenting cell such as a DC with a tumour-specific antigen, and then administer these cells together with microbial molecules such as CpG oligodeoxyribonucleotides. Indeed, such approaches have proved effective in reversing immune tolerance towards tumours (Heckelsmiller *et al.*, 2002), ultimately curing animals of large solid tumours that are resistant towards chemotherapy. The TLR-dependent development of an immune response towards adjuvants such as CpG oligodeoxyribonucleotides has also been effective against neuroblastoma, lymphoma, melanoma, glioma, fibrosarcoma as well as lung carcinoma (Carpentier *et al.*, 1999; Carpentier *et al.*, 2000; Egeter *et al.*, 2000; Ballas *et al.*, 2001; Kawarada *et al.*, 2001).

1.10 TLR signaling and infection

Although several studies have shown that TLRs expressed on various cell types are involved in the recognition of a large variety of PAMPs (Table 1.1), it has been difficult to identify roles for individual TLRs during *in vivo* infectious models. The majority of work done implicating TLR-signaling and its importance in an innate immune response have done so using animals deficient in MyD88.

In terms of bacterial infections, MyD88 was shown to be essential for clearance of *Staphylococcus aureus* (Takeuchi *et al.*, 2000), *Pseudomonas aeruginosa* (Skerret *et al.*, 2004), group B streptococci (Mancuso *et al.*, 2004), and *Mycobacterium avium* (Feng *et al.*, 2003), susceptibility being associated with impaired inflammatory cytokine production and elevated bacterial loads. The requirement for MyD88 during infection is not only restricted to bacterial models, but can be extended to protozoal infections as well. Here MyD88-deficient animals have reduced resistance towards *Toxoplasma gondii* (Scanga *et al.*, 2002), *Plasmodium berghei* (Adachi *et al.*, 2001), *L. major* (Muraille *et al.*,

al., 2003), as well as the spirochete *B. burgdorferi* (Liu *et al.*, 2004). In terms of the *Leishmania* model, susceptibility was associated with a polarized T_H2 response that developed in the absence of the MyD88-dependent IL-12-mediated T_H1 response (Muraille *et al.*, 2003; Debus *et al.*, 2003).

Although attempts to dissect the MyD88-dependent activation of the innate immune response in terms of the contributions made by individual TLRs have proved less successful, immune defects have been observed in some TLR models. TLR2-deficient animals infected with *Streptococcus pneumonia* (Echchannaoui *et al.*, 2002; Koedel *et al.*, 2003; Mancuso *et al.*, 2004), *S. aureus* (Takeuchi *et al.*, 2000^c) and *L. monocytogenes* (Torres *et al.*, 2004), have shown that TLR2 contributes to host resistance against these infections. In a model investigating immune responses towards *M. avium* (Feng *et al.*, 2003), TLR2-deficient animals showed impaired host resistance and neutrophil responses to the bacterium, although such defects were not as severe as those observed for MyD88-deficient animals. Although TLR4 appeared to have a negligible role in resistance towards an *M. avium* infection (Feng *et al.*, 2003), studies have shown that TLR4 plays a role in host defence against *Haemophilus influenzae* (Wang *et al.*, 2002), *Salmonella* infections (Bernheiden *et al.*, 2001), as well as fungal infections caused by *Candida albicans* (Netea *et al.*, 2002). Individually, functional roles for TLRs 3, 5 and 11 have also been identified *in vivo* (Wang *et al.*, 2004; Hawn *et al.*, 2003; Zhang *et al.*, 2004). Here, encephalitis caused by West Nile virus was attributed to viral entry into the brain mediated by TLR3 (Wang *et al.*, 2004), a stop codon polymorphism in the ligand-binding domain of TLR5 was associated with susceptibility to *Legionella pneumophila* (Hawn *et al.*, 2003), and preventing infection of internal organs of the urogenital system by uropathogenic bacteria has been linked to TLR11 (Zhang *et al.*, 2004).

It should be noted however that the *in vivo* models in which individual TLRs contributed to overall host resistance were models where the pathogen of interest caused infection within a relatively short period of time. The more chronic the infection, the more difficult it has been to relate overall host resistance to an individual TLR (Feng *et al.*, 2003; Scanga *et al.*, 2002; Adachi *et al.*, 2001; Reiling *et al.*, 2002). Here, pathogens such as *Mycobacterium* spp. and protozoan parasites have developed sophisticated

strategies for evading host immune responses. Indeed, these organisms produce long-lasting chronic infections in order to maximize their opportunities for successful transmission. Results presented here discuss the bacterium *Mycobacterium tuberculosis* and the protozoan parasite *Trypanosoma brucei*, and the requirement for the TLR-dependent activation of the host's immune system following infection with these pathogens.

Chapter 2

Materials and methods

2. Materials and methods

2.1 Mice

C3H/HeJ (containing *Tlr4* point mutation) and C3H/HeN (C3H/HeJ wild-type controls) were obtained from Charles River Breeding Laboratories, Kisslegg, Germany. Animals deficient in TLR1 (Takeuchi *et al.*, 2002), TLR2 (Michelsen *et al.*, 2001), TLR9 (Hemmi *et al.*, 2000), MyD88 (Kawai *et al.*, 1999), TNF (Taniguchi *et al.*, 1997), IFN- γ R1 (Huang *et al.*, 1993), IL-1R1 (Labow *et al.*, 1997), and Caspase-1 (Kuida *et al.*, 1995) were backcrossed onto a C57Bl/6 background 5 times or more. Mice deficient in TLR2/4 were on a 129/SvJxC57Bl/6 background (Quesniaux *et al.*, 2004^b). CBA/J mice were used as the corresponding wild-type controls for animals deficient in CD14 (Haziot *et al.*, 1996). Age matched control littermate mice of the corresponding genetic background were used in all experiments. Mice were obtained from either the University of Cape Town (Cape Town, South Africa) or the Transgenose Institute animal breeding facility (Orléans, France). All experiments performed were in accordance with the guidelines of the Animal Research Ethics Board of the University of Cape Town, South Africa, and the Regional Ethics Committee for Animal Experiments of Toulouse, France.

2.2 Bacteria and infections

M. tuberculosis H37Rv (Abel *et al.*, 2002) was grown in Middlebrook 7H9 broth (Difco, Detroit, MI) supplemented with Middlebrook OADC enrichment medium (Life Technologies, Gaithersburg, MD), 0.002% glycerol and 0.05% Tween 80. Pulmonary infection with *M. tuberculosis* H37Rv of either 100 or 500 live bacteria was performed using a Glas-Col Inhalation Exposure System, Model A4224. Inoculum size was checked 24 h after infection by determining the bacterial load in the lung of infected mice.

2.3 Colony enumeration assay

Bacterial loads in the lung, liver and spleen of infected mice were evaluated at different time points after infection with *M. tuberculosis* H37Rv. Organs were weighed and defined aliquots were homogenized in 0.04% Tween 80 saline. Tenfold serial dilution of organ homogenates were plated in duplicates onto Middlebrook 7H10 agar plates

containing 10% OADC and incubated at 37°C for 19-21 days. Colonies on plates were enumerated and results are expressed as log₁₀ CFU per organ.

2.4 Microscopic investigation of the lungs

For analysis mice were sacrificed by carbon dioxide inhalation. Organs were weighed and fixed in 4% phosphate buffered formalin and paraffin-embedded. Two to 3-μm sections were stained with haematoxylin and eosin and a modified Ziehl-Nielsen method as described (Jacobs *et al.*, 2000). For the immunostaining formalin-fixed paraffin-embedded sections were deparaffinized and rehydrated and stained with rabbit anti-mouse antibody specific for iNOS as described (Garcia *et al.*, 2000). The sections were then washed in PBS and incubated for 30 min at room temperature with the biotinylated secondary antibody. The sections were incubated with avidin-biotin complexes (ABC vector kit) for 30 min, washed and incubated with DAB substrate (Dako).

2.5 Lung homogenate preparations and FACS analysis of cell surface markers

Whole lungs were removed from infected mice at different time points and were homogenized in 1 ml 0.04% Tween 80 saline and supernatants were collected after low-speed centrifugation, aliquoted and frozen at -80°C. Isolated lung cells were obtained by collagenase and DNase treatment as described previously (Sallusto *et al.*, 1999). The cells were counted and incubated with antibodies against CD3 (anti-CD3 PE, clone 145.2C11), CD4 (anti-CD4 FITC, clone H129.19), CD8 (anti-CD8 FITC, clone 53-6.7), CD11a (anti-CD11a PE, clone M17/4), CD44 (anti-CD44 PE, clone IM7), CD11c (anti-CD11c FITC, clone HL3), Ly-6G (anti-Ly6G FITC, clone RB6-8C5), I-A/I-E (anti-I-A/I-E PE, clone M5/114.15.2), CD16/32 (clone 2.4G2). All staining procedures were performed in PBS containing 0.1% BSA, and 0.1% sodium azide (FACS buffer) for 20 min at 4°C. All Abs were used at 0.2 μg/10⁶ cells and obtained from BD PharMingen (San Diego, CA). Cells were fixed with 4% paraformaldehyde for at least 1 h and analysed by flow cytometry. Cells were gated on the lymphocyte or monocyte population by forward and side scatter, and the data analysed using CellQuest software (BD Systems, San Jose, CA).

2.6 Preparation of elicited peritoneal macrophages

Mice were injected with 1 ml of 4% thioglycolate (Difco). Five days later, peritoneal exudate cells were isolated from the peritoneal cavity by washing with ice-cold DMEM supplemented with 10% FCS (Life Technologies). Cells were cultured overnight and washed with DMEM/10% FCS to remove non-adherent cells. Adherent monolayer cells were used as peritoneal macrophages. Peritoneal macrophages (5×10^5 cells/ml) were cultured in DMEM/10% FCS and stimulated with either LPS (*E. coli*, serotype O111:B4, Sigma; 100ng/ml) or *M. tuberculosis* H37Rv (multiplicity of infection 2:1). Supernatants were harvested after 4 h.

2.7 Antigen-specific IFN- γ production

For measuring antigen-specific production of IFN- γ , single cell suspension of splenocytes were prepared from wild-type and TLR2-deficient mice 4 weeks after infection with *M. tuberculosis* H37Rv. Cells were resuspended in DMEM supplemented with 10% FCS, and penicillin and streptomycin (100U/ml and 100 μ g/ml; Life Technologies). Splenocytes were cultured at 5×10^5 cells/well in 96-well round-bottom microplates (Nunc, Naperville, IL), and stimulated with either 5 μ g/ml Con A (Sigma) or live *M. tuberculosis* H37Rv (multiplicity of infection 2:1) at 37°C and 5% CO₂. Supernatants were harvested after 3 days and stored at -80°C.

2.8 CD4 $^+$ and CD8 $^+$ T cell enrichment

Peripheral lymph nodes were obtained 4 wk after infection with *M. tuberculosis* H37Rv and single cell suspensions were prepared in DMEM supplemented with 10% FCS, and penicillin and streptomycin (100U/ml and 100 μ g/ml; Life Technologies). Enrichment of CD4 $^+$ and CD8 $^+$ T cells was performed by negative selection with magnetic mouse anti-B220-specific Dynabeads (Dynal, Robbins-Scientific, Mountain View, CA). Negatively enriched CD3 $^+$ T cell suspensions contained > 90% CD3 cells, as determined by flow cytometry analysis (Holscher *et al.*, 1998).

2.9 Functional CD4⁺ and CD8⁺ T cell assay

In vitro responses to mycobacterial Ag were measured as described previously (Kirman *et al.*, 1999), with slight modifications: 4×10^5 CD3⁺ enriched lymph node cells obtained from C57BL/6 infected mice were cultured with 2×10^4 peritoneal macrophages pulsed with 2×10^5 *M. tuberculosis* H37Rv or 1µg of PPD for 4 h in antibiotic-free DMEM. Resident peritoneal macrophages were obtained 1 day before the experiment from peritoneal lavages of uninfected C57BL/6 and TLR2-deficient mice and incubated in 96-well round-bottom microplates (Nunc) in complete DMEM. CD3⁺ T cells and pulsed macrophages were incubated for 96 h at 37°C and 5% CO₂. To determine mycobacterial Ag-specific IFN-γ production, 100 µl of supernatants from cultures were taken and kept frozen at -80°C.

2.10 Parasites and experimental infection

For clonal infections, the Anttat1.1E clone of the EATRO 1125 stock of the pleiomorphic blood-stream form was originally provided by Dr. N. Van Meirvenne (Institute of Tropical Medicine, Antwerp, Belgium). Frozen stabilate stocks of AnTat1.1 *T. brucei* used for infections were stored at -80°C at the Free University of Brussels. Mice were infected intra-peritoneally with 5×10^3 parasites diluted in PBS (pH 8.0) supplemented with 1.6% glucose. Animals were bled from the tail at intervals of either 1 or 2 days for the duration of the infection, and parasites were counted using a light microscope.

2.11 Parasites and natural infection

For infections of tsetse flies, male flies from the *Glossina morsitans morsitans* colony maintained at ITMA (Institute of Tropical Medicine, Antwerp) were used throughout the study. This colony was maintained on rabbits at 25°C and 65% relative humidity and is characterized by a high intrinsic vectorial capacity. Teneral flies (8-32 hrs after emergence) were fed their first bloodmeal on immune-suppressed NMRI mice (cyclophosphamide (Endoxan ®), 80mg/kg), showing a parasitaemia of 10^8 parasites/ml of a pleiomorphic *T. brucei brucei* AnTAR1 population, containing at least 70% short stumpy forms. After the infective meal, flies were maintained on uninfected rabbits for 28 days, with 3 days/week feeding-regime. 28 days after the infective bloodmeal, flies

were starved for 72 hours and were forced to salivate on a pre-warmed (37°C) glass slide. This drop of saliva was examined for the presence of metacyclic trypanosomes. Metacyclic-infected tsetse flies were retained for the non-clonal infection experiments.

2.12 sVSG, mfVSG and genomic DNA preparation

Trypanosomes were harvested from infected blood by DE52 chromatography (Lanham, 1968), using sterile PBS (pH 8.0) supplemented with 1.6% glucose for equilibration and elution. After separation, parasites were washed and resuspended in RPMI 1640 medium at a concentration of 10⁹ parasites/ml. sVSG was prepared from DE52-purified parasites by osmotic lysis for 5 min at 37°C in 10 mM sodium phosphate (pH 8.0) containing 0.1 mM TLCK and 0.1 mM PMSF (Boehringer Mannheim). The supernatant was passed through a column of DE52-equilibrated in 10 mM sodium phosphate (pH 8.0). sVSG was further purified on a column of Sephadryl-S200 (Pharmacia Biotech), dialyzed against water overnight at 4°C, and freeze-dried. mfVSG was prepared according to the method previously described (Jackson *et al.*, 1985). VSG samples were incubated under gentle shaking for 2 h at room temperature with Prosep-Remtox (Bioprocessing, Princeton, NJ.) glass beads to remove possible LPS contamination. Following this, beads were separated by sample filtration over a 22-μm sterile Spin-X centrifuge tube filter (Costar). Protein concentration of VSG was estimated by a detergent-compatible protein assay kit (Bio-Rad Laboratories, Hercules, CA) using BSA as a standard. sVSG was resuspended in PBS (pH 8.0) and mfVSG was resuspended in PBS containing 0.02% N-octylglucoside (Sigma). For isolation of *T. brucei* DNA, DE52-purified parasites were lysed using TRIZOL® (Invitrogen), and DNA was purified according to manufacturer's recommendations. DNA was resuspended in water and concentrations were determined by spectrophotometric analysis. Endotoxin units less than 0.5pg/mg VSG or DNA was determined using the LAL test (Bio Whittaker, Walkersville, MD). VSG preparations contained no *T. brucei* DNA.

2.13 Primary macrophage cultures

MyD88-deficient (Kawai *et al.*, 1999), TLR9-deficient (Hemmi *et al.*, 2000) and control murine bone marrow cells were isolated from femurs and cultivated (10⁶/ml) for 7 days in

DMEM supplemented with 2 mM L-glutamine, 0.2 µM 2-ME, 20% horse serum and 30% L929 cell-conditioned medium. Following this, cells were resuspended in cold PBS and recultured for 3 days in fresh medium at 37°C and 5% CO₂. MyD88-deficient bone marrow-derived macrophages were plated in 96-well micro-culture plates at a density of 10⁵ cells/well. Culture medium used was DMEM containing 100U/ml penicillin, 100 µg/ml streptomycin, 0.2 µM 2-ME and 2 mM L-glutamine. Cells were stimulated with 1 µg/ml LPS (*Escherichia coli*, serotype O111:B4, Sigma-Aldrich, St. Louis, MO), 5 or 10 µg/ml sVSG and 5 or 10 µg/ml mfVSG both in the presence or absence of 30 U/ml IFN-γ. Similarly, TLR9-deficient bone marrow-derived macrophages were cultured with 1 µg/ml LPS or 1.0 mg/ml, 2.5 µg/ml or 12.5 mg/ml *T. brucei* DNA in the presence of 30 U/ml IFN-γ. All cells were stimulated for 24 in the presence of 50 U/ml polymyxin B sulphate following which supernatants were harvested and frozen at -80°C.

2.14 Quantification of anti-VSG serum titers

Infection-induced anti-VSG serum titers were determined in a VSG solid phase ELISA. ELISA plates (Nunc) were coated in PBS (pH 8.0) with purified sVSG (10 µg/ml – 100 µL/well) by overnight incubation at 4°C. Free binding sites were blocked by an additional overcoat of BSA (1 mg/ml – 300 µL/well, 1 hour incubation at 37° C). Serial serum dilutions were added to the plates and incubated overnight, following which plates were extensively washed with PBS. Detection of bound serum antibodies was done using 1/1000 dilutions of IgM, and IgG isotype-specific HRP coupled antibodies (SouthernBiotech), followed by TMB substrate addition. After 20 minutes, the substrate conversion reaction was stopped by addition of 50µl 1N H₂SO₄ and ODs were measured at 450 nm.

2.15 Cytokine ELISA

Supernatants were harvested and assayed for cytokine content using commercially available ELISA reagents for TNF-α, IFN-γ and IL-12p40 (R&D Systems, Abingdon, UK and BD PharMingen, San Diego, CA).

2.16 Nitrite Measurements

Nitrite concentrations in freshly obtained lung homogenate supernatants were determined using the Griess reagent (3% phosphoric acid, 1% p-aminobenzene-sulphonamide, 1% n-1-naphthylethylenediamide) as described (Reis e Sousa *et al.*, 1997).

2.17 Statistical Analysis

Data were analysed by comparison of wild-type and gene-deficient mice at each time point, with 4-6 mice per time point. All graphic-result presentations were prepared using the GraphPad Prism software. The same software was used for statistical analysis of data (student *t* test) and comparative analysis of survival data using a designated GraphPad Prism statistical module, here values of $p \leq 0.05$ were considered significant. Each experiment was repeated at least once to ensure reproducibility.

Chapter 3
Mycobacterium tuberculosis as a model

Chapter 3

***Mycobacterium tuberculosis* as a model for innate immunity**

Summary

Recognition of *Mycobacterium tuberculosis* by the innate immune system is essential in the development of an adaptive immune response. Innate pattern recognition receptors (PRRs) implicated in the recognition of *M. tuberculosis* include CD14, TLR2 and TLR4. However, a large variety of mycobacterial cell wall components activate macrophages through TLR2, suggesting that this innate immune receptor might have a predominant role in the host response to *M. tuberculosis* infection. Results presented here show that MyD88, and by inference TLRs, are required by the host to control an aerogenic *M. tuberculosis* infection. Individually, animals lacking TLR2 were more susceptible to an aerogenic mycobacterial infection than animals lacking either TLR4 or CD14. Here, TLR2-deficient animals were unable to clear systemic bacteria as well as wild-type controls, a result attributed to defective granulomatous responses in the lungs of TLR2-deficient animals. Dissection of the TLR2 phenotype revealed that animals lacking this receptor developed pulmonary immunopathology during an *M. tuberculosis* infection, characterized by elevated levels of TNF, IFN- γ and IL-12p40, as well as increased numbers of CD4 $^{+}$ and CD8 $^{+}$ T cells. Data presented here indicates that TLR2 is required by macrophages to control a pulmonary *M. tuberculosis* infection, and in its absence the host attempts to compensate for this deficiency by upregulating a Type 1 inflammatory response, ultimately resulting in immunopathology and chronic pneumonia.

3.1 Introduction

The global resurgence of tuberculosis has intensified research efforts directed at identifying the role of immune cell activation by *M. tuberculosis*. Protective cell-mediated type 1 immune responses play a critical role in host defense against intracellular *M. tuberculosis* infection. The secretion of type 1 cytokines by Ag-specific T cells play an important role in protective granuloma formation and stimulate the antimicrobial activity of infected macrophages, allowing intracellular microbial killing (Flynn *et al.*, 2001).

The discovery of the TLR protein family and its importance in mediating immune responses has provided novel insights into mechanisms linking innate and adaptive immunity. The number of members of human and mouse TLRs thought to participate in innate immunity has grown, and evidence suggests a role for TLRs in the activation of immune cells by *M. tuberculosis* (Takeda *et al.*, 2003; Underhill *et al.*, 1999^b). In this context it has been proposed that initial recognition of mycobacterial components may involve other PRRs such as CD14, scavenger and complement receptor and DC-SIGN in addition to members of the TLR family (Takeda *et al.*, 2003; Quesniaux *et al.*, 2004^a). In terms of the TLR protein family, TLRs 2 and 4 have been implicated in the recognition of mycobacterial ligands (Brightbill *et al.*, 1999; Jones *et al.*, 2001; Means *et al.*, 1999), although these results were largely based on *in vitro* studies. More specifically, it was shown that *M. tuberculosis*-induced TNF production was largely TLR2-dependent (Underhill *et al.*, 1999^b; Takeuchi *et al.*, 1999), and that immunostimulatory responses to *M. tuberculosis* 19-kDa lipoprotein, lipoarabinomannan (LAM) and mannosylated phosphatidylinositol (PIM) could be attributed to TLR2 signaling (Brightbill *et al.*, 1999; Jones *et al.*, 2001; Means *et al.*, 1999). In addition to the aforementioned TLR2 agonists, heat-sensitive membrane-associated factors associated with *M. tuberculosis* have been shown to activate cells via TLRs 2 and 4 (Lien *et al.*, 1999; Means *et al.*, 1999). This being said, the accumulation of *in vitro* data points towards TLR2 as the more dominant TLR involved in recognition of *M. tuberculosis* ligands. This was further corroborated by direct killing of *M. tuberculosis* in both mouse and human macrophages as a direct result of 19-kDa lipoprotein-induced cellular activation mediated by TLR2 signaling (Thoma-

Uszynski *et al.*, 2001). Here, the TLR2-dependent activation of murine macrophages led to NO-dependent growth inhibition of intracellular *M. tuberculosis*, whereas human monocyte cultures displayed antimicrobial activity dependent on TLR2 but independent of both NO and TNF. It should be noted however that in addition to recognizing various mycobacterial ligands, several reports suggest that TLR2 may have a role to play in immune evasion by *M. tuberculosis* (Noss *et al.*, 2000 & 2001; Ramachandra *et al.*, 2001; Pai *et al.*, 2003; Tobian *et al.*, 2003).

One of the mechanisms used by *M. tuberculosis* that allow the bacillus to survive for prolonged periods of time in the presence of vigorous CD4⁺ T cell responses is to prevent Ag processing or MHC-II expression by infected macrophages, thereby preventing recognition of infected macrophages by effector CD4⁺ T cells (Gercken *et al.*, 1994; Hmama *et al.*, 1998; Noss *et al.*, 2000). TLR2 has been implicated in this process of immune evasion in that *M. tuberculosis* 19-kDa lipoprotein was identified as a major inhibitor of macrophage MHC-II expression and Ag processing (Noss *et al.*, 2001). Furthermore, inhibition of IFN- γ -induced class II transactivator expression and alternate class I MHC antigen processing by *M. tuberculosis* 19-kDa lipoprotein (Pai *et al.*, 2003; Tobian *et al.*, 2003), demonstrate that PAMPs can also induce counter-regulatory or inhibitory effects via PRRs such as TLR2.

Results presented in this chapter argue that animals require MyD88 to control an *M. tuberculosis* infection. At the level of individual PRRs, *in vitro* studies implicated CD14, TLR2 and TLR4 in innate recognition of mycobacterial bacilli. Upon mycobacterial infection, TLR2 is shown to have a more pronounced role during infection than either CD14 or TLR4, although both CD14 and TLR4 are likely to contribute to overall host resistance. Dissection of the TLR2 phenotype revealed that animals lacking this receptor developed pulmonary immunopathology during an *M. tuberculosis* infection, characterized by inflamed lungs and an increased T-cell infiltrate. The lack of functional granulomas during the chronic stages of infection accompanied by chronic pneumonia resulted in TLR2-deficient animals succumbing to the infection.

3.2 Results

3.2.1 *MyD88*-deficient mice are unable to control an aerosol *M. tuberculosis* infection

Infection of *MyD88*-deficient mice with 100 CFU live mycobacteria resulted in a bacterial burden in the lungs consistently higher than that observed for wild-type controls at each time point examined (Fig. 3.1a), reaching approximately 3 \log_{10} difference at the time of death. The elevated pulmonary infection in *MyD88*-deficient animals was accompanied by bacterial dissemination into the liver and spleen with the resulting bacterial load approximating 1.5 \log_{10} higher than that observed for wild-type controls (Fig. 3.1b-c). All *MyD88*-deficient mice died by 12 weeks post-infection (Fig. 3.1d; $p < 0.0001$), but survived longer than animals deficient in TNF (Fig. 3.1d; $p < 0.0001$). Collectively, the enhanced susceptibility of *MyD88*-deficient mice towards an *M. tuberculosis* infection suggests a role for PRRs/TLRs in the initial activation of an innate immune response.

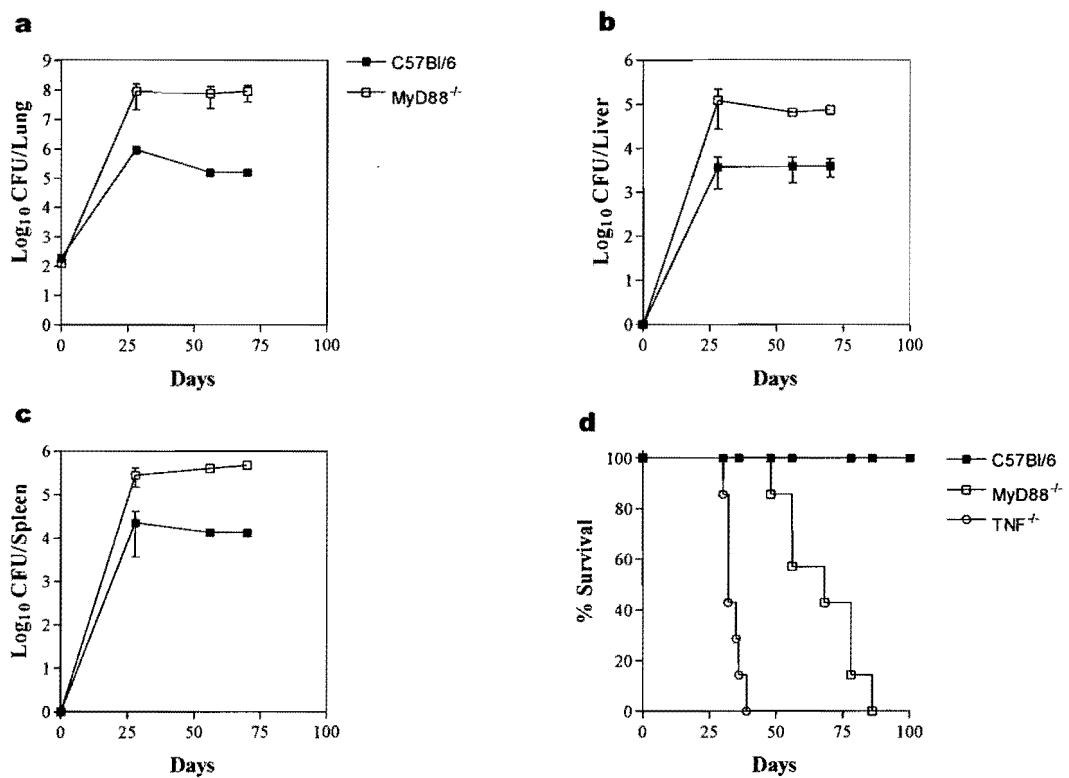


Figure 3.1 *MyD88*-deficient mice are susceptible to an aerogenic *M. tuberculosis* infection. Control (■), *MyD88*- (□) and TNF-deficient mice (○) were aerogenically infected with 100 CFU *M. tuberculosis*. CFU counts were determined at the indicated time points in the lungs (a), liver (b) and spleen (c). Survival of wild-type, *MyD88*- and TNF-deficient mice (d).

3.2.2 Progression of an aerosol M. tuberculosis infection in animals deficient in either CD14 or TLR2

Gene-deficient animals and their corresponding wild-type controls were infected aerogenically with 100 live mycobacteria per animal. Animals deficient in CD14 controlled the infection as well as wild-type controls and exhibited no differences in bacterial burden in the lungs, liver or spleen for the duration of the experiment (Fig 3.2a-c).

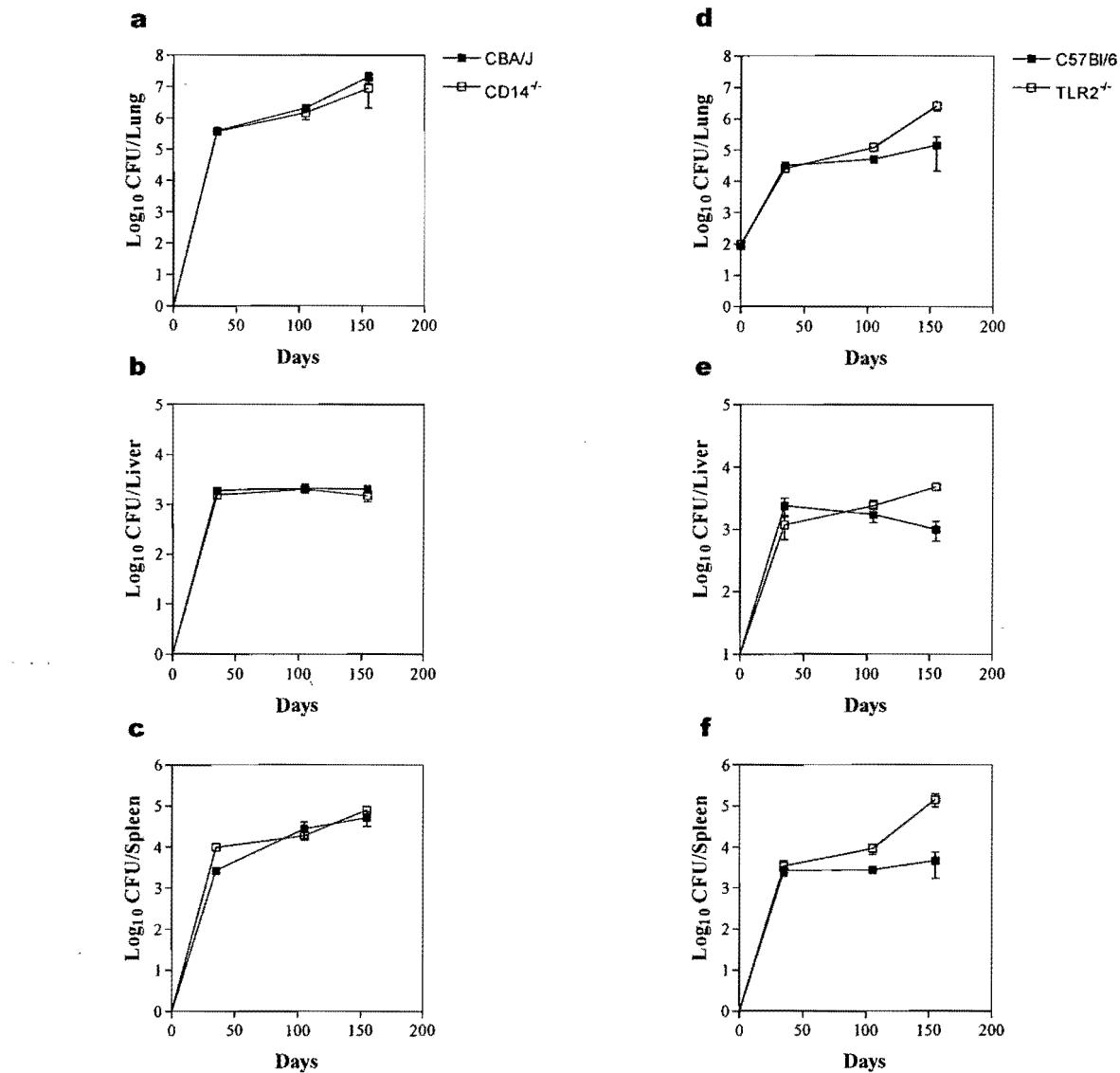


Figure 3.2 M. tuberculosis CFU counts in the lungs, livers and spleens of aerosol-infected animals deficient in CD14 and TLR2. Control (■) and deficient mice (□) mice were aerogenically infected with 100 CFU M. tuberculosis. CFU counts were determined at the indicated time points in the lungs (a & d), liver (b & e) and spleen (c & f).

Animals deficient in TLR2 infected with the same dose of *M. tuberculosis* experienced no change in clinical status but exhibited 1 log₁₀ higher bacterial loads in the lungs compare to wild-type controls at 5 months post-infection (Fig 3.2d). The heightened pulmonary infection in TLR2-deficient animals was accompanied by bacterial dissemination into the liver and spleen with the resulting bacterial load exceeding that found in wild-type controls (Fig. 3.2e-f). A partial requirement for TLR4 in controlling local growth and dissemination of an aerosol *M. tuberculosis* infection was previously shown by Abel *et al.* (2002). Here, infection of C3H/HeJ and C3H/HeN mice with 100 CFU mycobacteria resulted in approximately 1 log₁₀ higher bacterial loads in C3H/HeJ mice in the lung, liver and spleen during the chronic stages of infection. Thus, a similar disease phenotype is observed for animals deficient in either TLR2 or TLR4 during the chronic stages of infection when infected with 100 CFU mycobacteria.

3.2.3 High dose aerosol *M. tuberculosis* infection of CD14-, TLR2- and TLR4-deficient mice

Aerosol *M. tuberculosis* infections delivering 100 CFU per animal are used as a standard/low dose in the majority of models investigating progression of the disease (Turner *et al.*, 2003; Saunders *et al.*, 2002; Cowley *et al.*, 2003; Feng *et al.*, 1999). In these systems, distinct phenotypes only become apparent should the missing molecule have a nonredundant role. In terms of infections investigating molecules involved in the innate immune recognition of *M. tuberculosis*, experimental setups using higher doses of mycobacteria would allow compensatory processes to be minimized. This type of approach has been supported by infectious models investigating roles of individual TLRs involved in host response towards bacilli such as *S. aureus* (Takeuchi *et al.*, 2000) and group B streptococci (Mancuso *et al.*, 2004).

In this regard, we increased the infectious dose of aerogenically delivered mycobacteria from 100 CFU to 500 CFU per mouse. Animals deficient in CD14, TLR4, TLR2 and the corresponding wild-type controls were infected with 500 CFU mycobacteria in an attempt to determine whether an individual PRR had a more prominent role during the infection (Fig. 3.3).

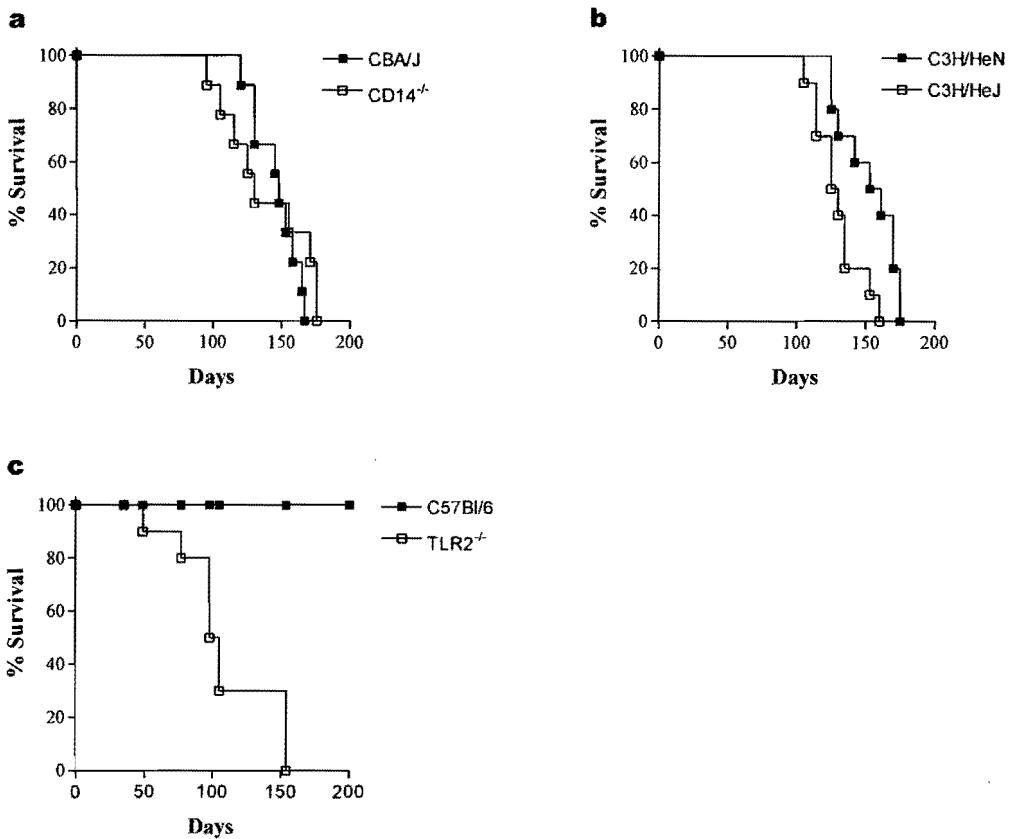


Figure 3.3 Survival of CD14-, TLR2- and TLR4-deficient mice following aerosol infection with *M. tuberculosis*. Wild-type (■) and deficient mice (□) were aerogenically infected with 500 CFU *M. tuberculosis*. CBA/J and CD14^{-/-} mice (a; 9 mice per group), C3H/HeN and C3H/HeJ (b; 10 mice per group) or C57Bl/6 and TLR2^{-/-} mice (c; 10 mice per group) were monitored for survival. Moribund mice were sacrificed. Survival rates of C57Bl/6 and TLR2^{-/-} mice were significantly different ($p < 0.0005$).

No difference in survival was observed for either CD14- or TLR4-deficient animals when compared to wild-type controls ($p > 0.05$; Fig. 3.3a & b). However, all TLR2-deficient mice were unable to control the infection (Fig. 3.3c), with all animals succumbing to infection by 22 weeks (Fig. 3.3c; $p < 0.0005$). Wild-type mice controlled the same infectious dose and appeared healthy at 7 months post-infection at which point the experiment was terminated. This would indicate that TLR2 contributes to host resistance against *M. tuberculosis* more so than either CD14 or TLR4, although genetic backgrounds do play a role in increased susceptibility towards high dose aerosol infections (Pan *et al.*, 2005; Fig. 3.3). Nevertheless, due to the more susceptible phenotype observed for the deficiency in TLR2 during 500 CFU challenges, experiments were performed in an attempt to characterize the immune response towards an *M. tuberculosis* infection in the absence of TLR2. In terms of bacterial loads, the increased

susceptibility observed for TLR2-deficient mice was accompanied by 1 \log_{10} higher mycobacterial burdens in the lungs at weeks 8 and 14 post-infection when compared to wild-type controls (Fig. 3.4a). The augmented pulmonary infection present in TLR2-deficient animals resulted in dissemination of bacilli into the liver at week 8 (Fig. 3.4b) as well as the spleen at week 14 (Fig. 3.4c).

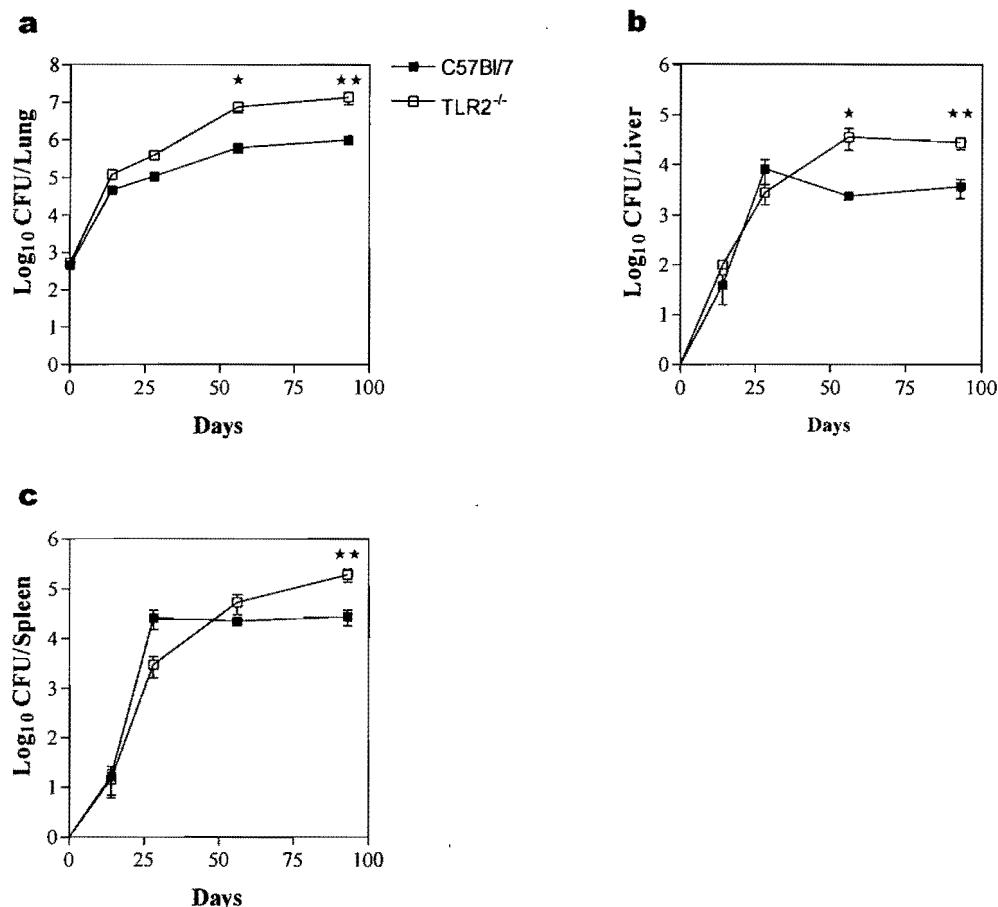


Figure 3.4 TLR2-deficient mice are unable to control an aerosol *M. tuberculosis* infection effectively. Wild-type (■) and TLR2-deficient mice (□) were aerogenically infected with 500 CFU *M. tuberculosis*. CFU counts were determined at the indicated time points in the lungs (a), liver (b) and spleen (c). *, p < 0.05; **, p < 0.01.

Therefore, a deficiency in TLR2 resulted in elevated bacterial burdens in the lungs, liver and spleen following either 100 or 500 CFU mycobacterial infections. To determine whether this could be partly attributed to a defective granulomatous response in the lungs, lungs sections were examined histologically to assess the progression of disease.

3.2.4 TLR2-deficient mice develop a defective granulomatous response

Following a standard/low dose 100 CFU mycobacterial challenge, animals deficient in TLR2 showed an increased number of bacilli in the lungs at 5 months post-infection (Fig. 3.2d). The lungs of wild-type and TLR2-deficient mice were examined histologically at this time point to determine whether the elevated bacterial numbers could be correlated with a defective granulomatous response (Fig. 3.5). Lung micrographs of wild-type animals showed robust mononuclear cell infiltration with abundant focal lymphocytes, suggesting that the infection was controlled (Fig. 3.5a). However, microscopic investigation of the lungs of TLR2-deficient mice revealed coalescent inflammatory lesions, reduced airspace and an absence of proper granuloma formation (Fig. 3.5b).

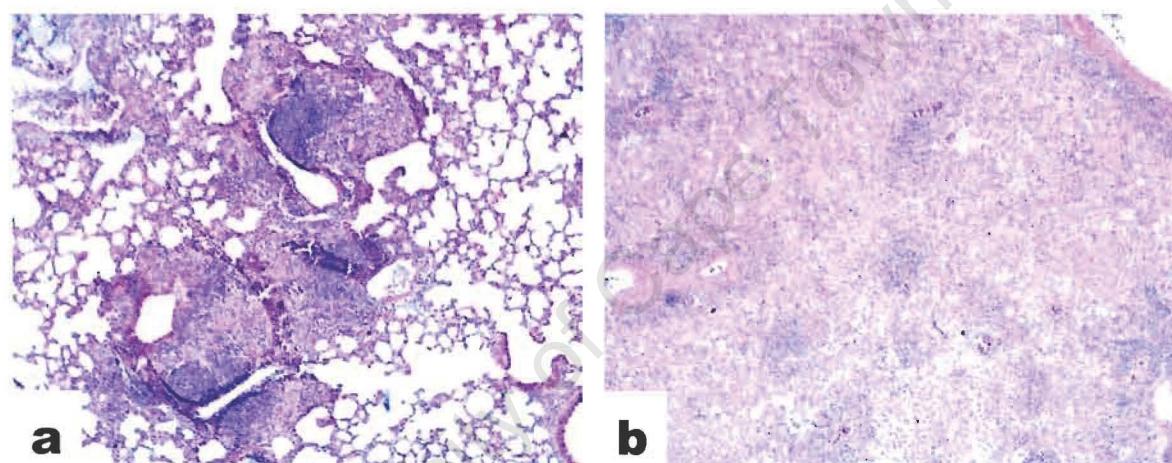


Figure 3.5 TLR2-deficient mice exhibit defective granuloma formation in response to *M. tuberculosis* infection. Lung tissue taken from wild-type (a) and TLR2^{-/-} mice (b) 5 months after aerosol infection with 100 CFU *M. tuberculosis*. Low-power micrographs of representative lung sections are shown (magnification, x40).

The presence of inflammatory pathology in TLR2-deficient animals at 5 months post-infection warranted examination of the granulomatous response in these animals following 500 CFU *M. tuberculosis* infection (Fig. 3.6). Here, microscopic analysis of the lungs of wild-type and TLR2-deficient mice at 2 weeks post-infection showed comparable thickening of alveolar septae, with no discernable granulomatous structures (Fig. 3.6a & b). By 4 weeks post-infection, wild-type mice had developed typical granulomatous lesions, displayed robust mononuclear cell infiltration characterized by

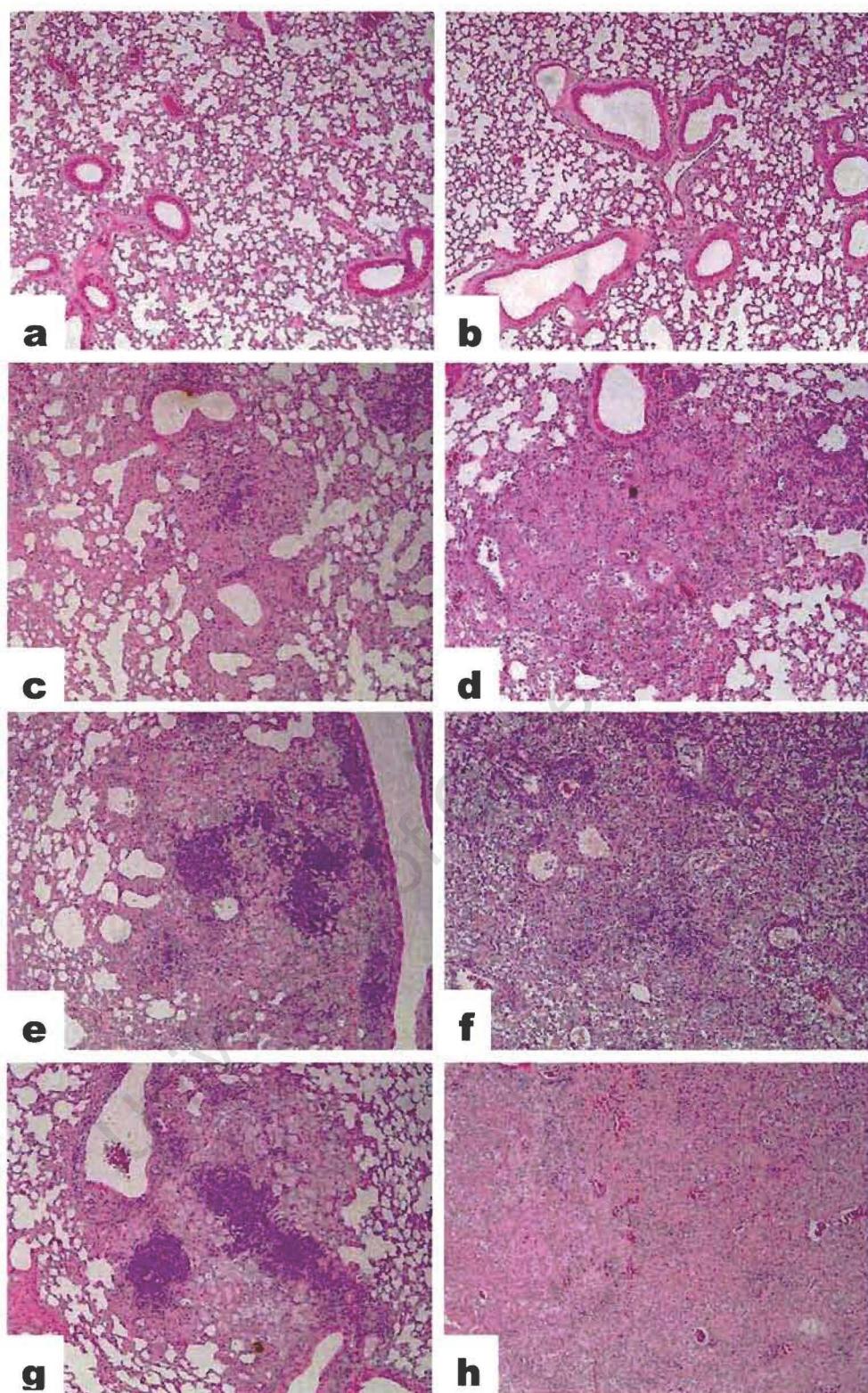


Figure 3.6 Defective granuloma formation in TLR2-deficient mice is associated with an exaggerated inflammatory response. Lung tissue from wild-type (left column) and TLR2-deficient mice (right column) was analysed following 500 CFU *M. tuberculosis* infection. Lungs removed at days 14 (a & b), 28 (c & d), 56 (e & f) and 98 (g & h) post-infection. Low-power micrographs of representative lung sections are shown (magnification, x40).

epithelioid macrophages, as well as lymphocytic perivascular and peribronchiolar cuffing (Fig. 3.6c). TLR2-deficient mice exhibited a diffuse recruitment of inflammatory cells, thickening of the alveolar septae and interstitial pneumonia (Fig. 3.6d). A significant difference in lung architecture was however seen at 8 weeks post-infection (Fig. 3.6e & f). While wild-type mice lung sections displayed compact epithelioid granulomas containing dense infiltrations of lymphocytes (Fig. 3.6e), TLR2-deficient lungs showed massive coalescent inflammatory lesions, focal necrosis, diffuse foamy macrophages, substantial perivascular cuffing and very few granulomatous structures (Fig. 3.6f). Macroscopically, TLR2-deficient lungs were swollen, displayed pleural adhesions, and were significantly larger than the lungs of wild-type controls (Fig. 3.9a). This phenotype was also seen in TLR2-deficient animals at 5 months post-infection (Fig. 3.5b). During the late stages of infection in the 500 CFU model, moribund TLR2-deficient animals developed chronic pneumonia and extensive pleuritis (Fig. 3.6h). The elevated numbers of bacilli in the lungs of TLR2-deficient mice at this stage of infection was attributed to decreased pulmonary macrophage activation. To show that pulmonary macrophages were indeed less activated in TLR2-deficient animals, iNOS immunostaining was performed on lungs sections of chronically infected animals (Fig. 3.7).

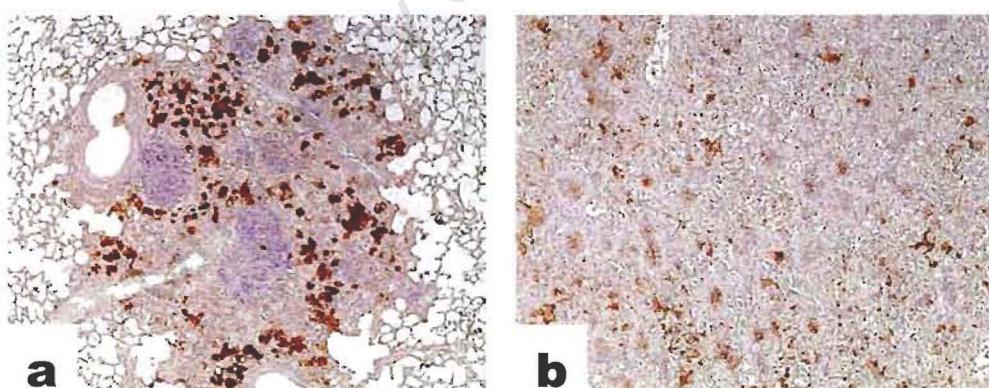


Figure 3.7 Diffused iNOS expression in the lungs of *M. tuberculosis* infected TLR2-deficient mice. Immunohistochemical staining was performed on lung sections of wild-type and TLR2-deficient mice infected with 500 CFU *M. tuberculosis*. Lungs were removed at 98 days post-infection and stained using a polyclonal rabbit anti-mouse antibody (see Materials and methods). Brown staining shows iNOS-positive epithelioid macrophages. Low-power micrographs of representative lung sections are shown (magnification, x40).

Pulmonary macrophages present in wild-type lungs expressed iNOS within the granuloma structure (Fig. 3.7a), and although TLR2-deficient macrophages also expressed iNOS, these macrophages were dispersed throughout the tissue (Fig. 3.7b). In addition to iNOS staining, levels of nitric oxide (NO) in lung tissue homogenates were lower in TLR2-deficient mice during the chronic stages of infection (Table 3.1), indicating that the capacity to kill mycobacteria was reduced in the absence of TLR2.

TABLE 3.1.

Production of NO in wild-type and TLR2^{-/-} mice following aerosol M. tuberculosis infection

Time post-infection (days)	C57BL/6 ($\mu\text{M/g}$) ^a	TLR2 ^{-/-} ($\mu\text{M/g}$) ^a
56	433.3 \pm 44.6	303.1 \pm 96.7
98	491.1 \pm 43.1	273.8 \pm 14.8 ^b

^a Total pulmonary nitrites in the lungs of wild-type and TLR2-deficient mice infected with 500 CFU *M. tuberculosis*. Total pulmonary nitrate was reduced by nitrite reductase to nitrite and measured by the Greiss reagent.

^b $p < 0.001$

Therefore, the decreased levels of NO present in lung homogenates of TLR2-deficient mice indicated that activation of macrophages in response to tubercle bacilli was dependent on TLR2.

3.2.5 Macrophages require TLR2 to respond optimally to *M. tuberculosis* bacilli

Stimulation of macrophages with mycobacterial bacilli induces the production of TNF and IL-12p40, both of which have important roles in the control of local immune responses against *M. tuberculosis* (Flynn *et al.*, 1995; Holscher *et al.*, 2001). In this regard, we sought to determine whether TLR2 was required by macrophages to respond optimally to live mycobacteria. Here, peritoneal macrophages from wild-type and TLR2-deficient mice were stimulated with either LPS or live *M. tuberculosis* bacilli (Fig. 3.8). Indeed, macrophages deficient in TLR2 produced significantly less TNF (Fig. 3.8a) and IL-12p40 (Fig. 3.8b) than wild-type controls, while no significant difference was observed between the two groups when stimulated with LPS. The results therefore imply that the decreased NO levels in the lungs of TLR2-deficient mice could be due to defective macrophage activation. Nevertheless, the absence of TLR2 resulted in a

defective macrophage response *in vitro* in terms of TNF and IL-12p40 production, a result which would indicate that the pulmonary inflammatory response in TLR2-deficient mice would also be reduced.

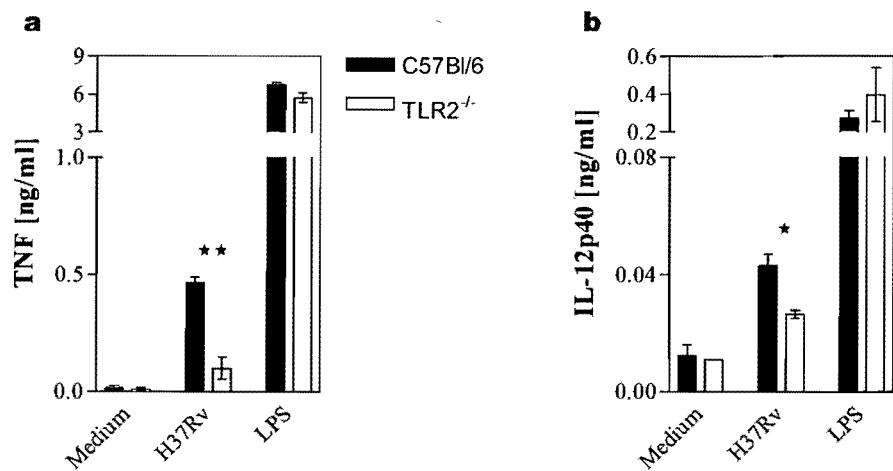


Figure 3.8 Reduced cytokine production on TLR2-deficient macrophages with an in vivo phenotype. Elicited peritoneal macrophages from wild-type (■) and TLR2-deficient mice (□) were incubated with LPS (100 ng/ml) or live *M. tuberculosis* (MOI = 2:1). Supernatants were harvested 4 hours after stimulation and assayed for TNF (a) and IL-12p40 (b) by ELISA. *, p < 0.01; **, p < 0.001.

3.2.6 Analysis of pulmonary immune responses in TLR2-deficient mice

Macroscopically, the lungs of TLR2-deficient mice became swollen and larger than wild-type controls at days 56 and 98 post-infection (Fig. 3.9a), weighing significantly more than control mice at these time points. The increase in lung mass at day 56 post-infection corresponded with the development of lung pathology in TLR2-deficient animals at the same time point (Fig. 3.6f). The inflammatory lung pathology was not a result of lower proinflammatory cytokines but was associated with increased amounts of both TNF and IL-12p40 in lung homogenates of TLR2-deficient animals. Here, the levels of TNF in lung homogenates of TLR2-deficient animals were initially higher at 4 weeks post-infection (Fig. 3.9b), and remained so for the duration of infection. Levels of IL-12p40 were also elevated in TLR2-deficient animals, however significant differences between the two groups were only seen at day 28 (Fig. 3.9c).

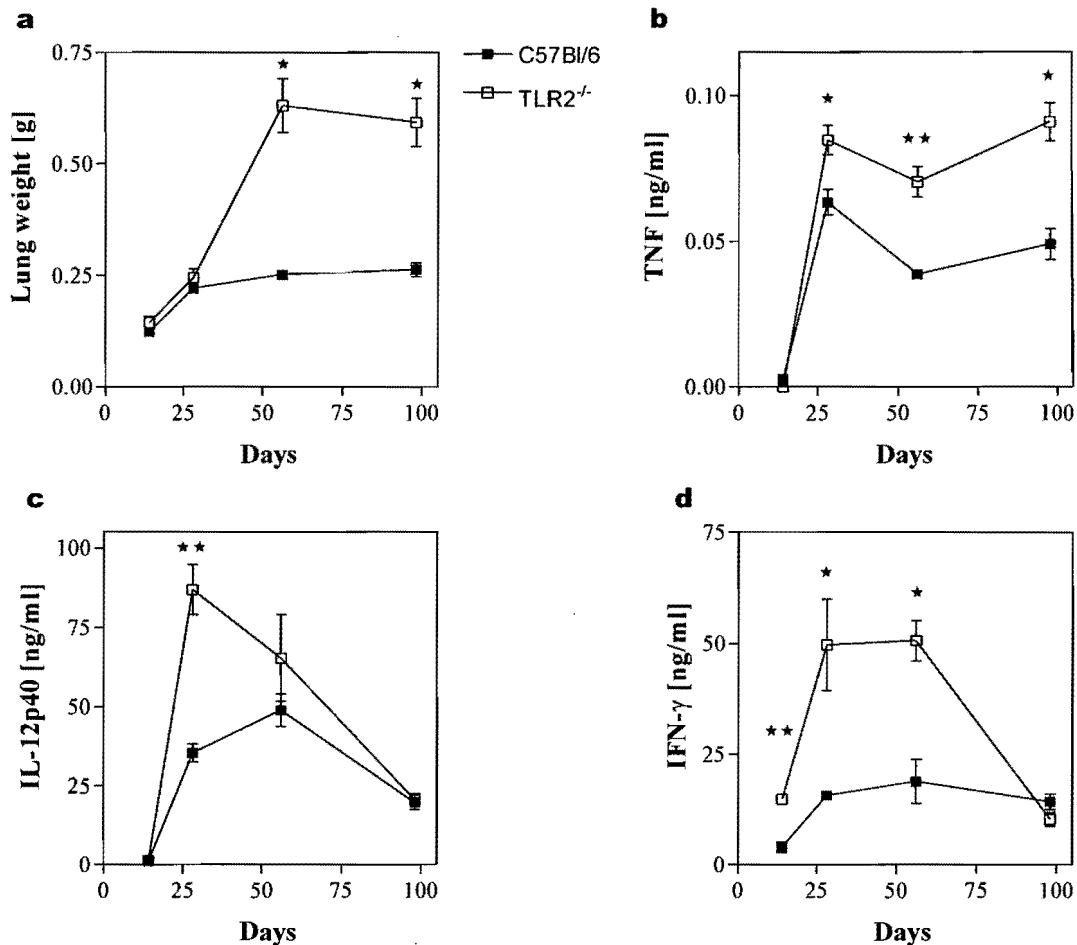


Figure 3.9 Increased lung weight and inflammatory cytokine production in TLR2-deficient mice infected with *M. tuberculosis*. Wild-type (■) and TLR2-deficient mice (□) were infected aerogenically with 500 CFU *M. tuberculosis*. Animals were sacrificed at days 14, 28, 56 and 98 post-infection, following which lung weights (a), TNF (b), IL-12p40 (c), and IFN- γ (d) in lung homogenates were recorded. *, $p < 0.01$; **, $p < 0.001$.

In addition to elevated levels of TNF and IL-12p40 in lung homogenates of TLR2-deficient mice, levels of IFN- γ present in the lungs of these animals remained higher than those observed in wild-type controls at days 14, 28 and 56 post-infection (Fig. 3.9d). Production of IFN- γ is a key effector mechanism against *M. tuberculosis* and is mediated by T cells at the site of infection. To determine whether the elevated levels of IFN- γ present in the lungs of TLR2-deficient mice could be correlated with an increase in the amount of infiltrating T cells, flow-cytometric analysis of the recruitment of lymphocytes into the lungs was performed (Fig. 3.10).

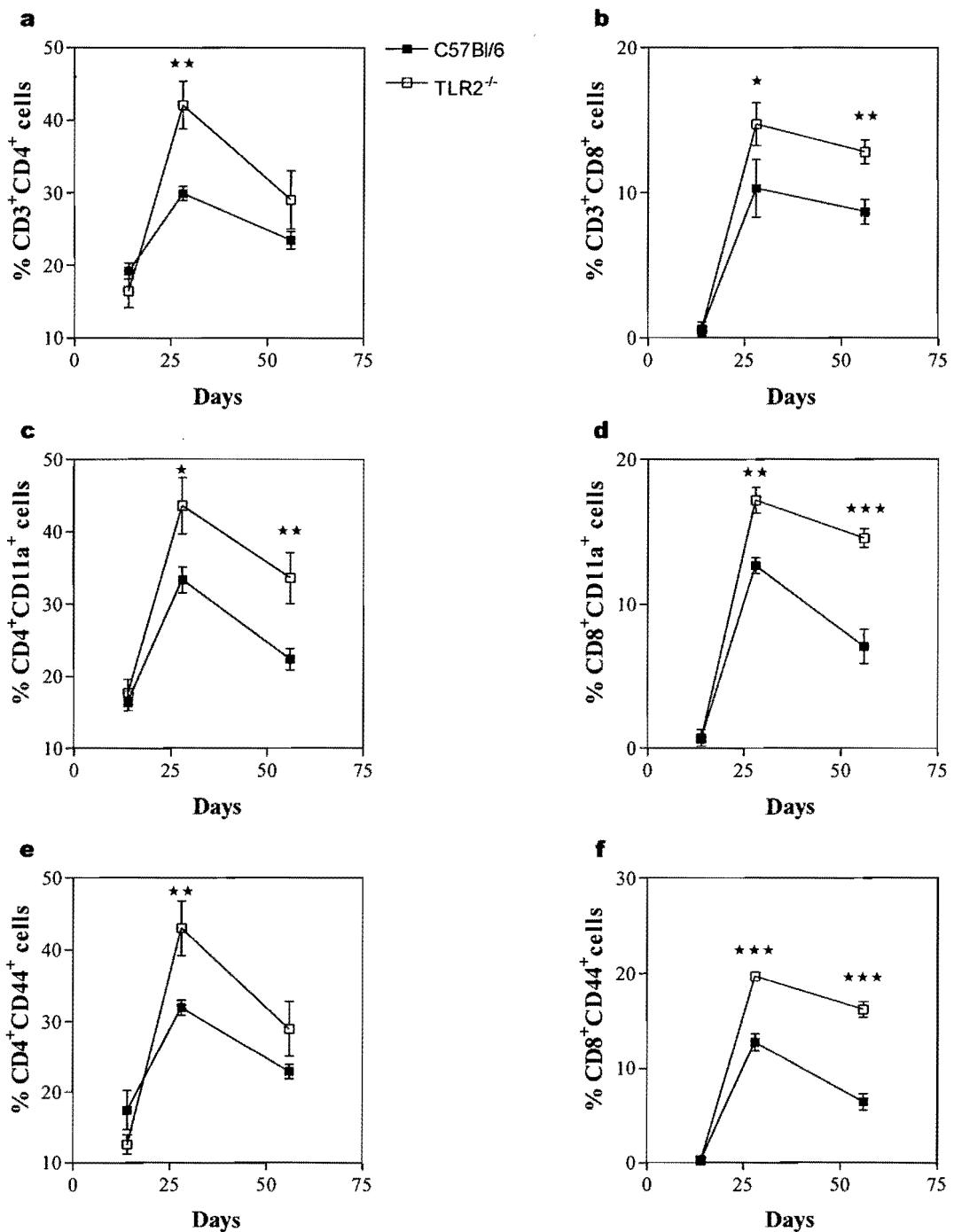


Figure 3.10 Increased recruitment and activation of T cells in TLR2-deficient mice infected with *M. tuberculosis*. Wild-type (■) and TLR2-deficient mice (□) were infected aerogenically with 500 CFU *M. tuberculosis*. Lung cells were harvested 2, 4 and 8 weeks post-infection and stained for CD3, CD4, CD8, CD11a and CD44. (a-f) Percentages of activated CD4⁺ and CD8⁺ cells in the lymphocyte gate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Higher numbers of CD4⁺ and CD8⁺ T cells were present in the lungs of TLR2-deficient animals after 3 weeks of infection (Fig. 3.10a & b). These cells also showed increased expression of the activation markers CD11a and CD44 at weeks 4 and 8 post-infection (Fig. 3.10c-f), indicating that the elevated levels of IFN- γ found in the lungs of TLR2-deficient mice was associated with increased recruitment and activation of both CD4⁺ and CD8⁺ T cell populations. To determine whether the elevated levels of IFN- γ present in the lungs of TLR2-deficient were due to the increased activation of the lymphocyte compartment, *in vitro* Ag-specific restimulation assays were performed.

3.2.7 Antigen-specific T cell responses in TLR2-deficient mice infected with *M. tuberculosis*.

Recall responses of Ag-specific T cells are optimal during the third and fourth week of infection, after which these responses decline. Animals aerogenically infected with 500 CFU *M. tuberculosis* were therefore sacrificed at 4 weeks post-infection, following which spleens were removed. Restimulation of wild-type and TLR2-deficient splenocytes with either ConA or mycobacterial bacilli (Fig. 3.11a), showed higher levels of IFN- γ produced by TLR2-deficient cell cultures. This was observed for the Ag-specific bacillus stimulus as well as the non-specific mitogen ConA. To determine whether the increased IFN- γ levels observed in TLR2-deficient splenocyte cultures was a result of increased T cell numbers, enriched T cell suspensions were prepared from infected mice and restimulated *in vitro* (Fig. 3.11b). Here, enriched CD3⁺ T cell suspensions were prepared from wild-type mediastinal lymph nodes on day 28 after infection with *M. tuberculosis* by magnetic cell sorting. Enriched CD3⁺ T cells with a purity of >95% were restimulated *in vitro* with Ag-pulsed wild-type or TLR2-deficient peritoneal macrophages, and IFN- γ production was measured (Fig. 3.11b). In the case of TLR2-deficient peritoneal macrophages pulsed with either PPD or viable *M. tuberculosis*, the resulting restimulation of wild-type enriched CD3⁺ T cells produced more IFN- γ than T cells restimulated by wild-type macrophages. This would indicate that TLR2 signaling might influence the outcome of either CD4⁺ or CD8⁺ T cell differentiation. Indeed, down-regulation of cell surface molecules such as MHC-II by *M. tuberculosis* might be a mechanism used by the bacillus to evade the host immune response. Several reports have

implicated TLR2 in the inhibition of macrophage MHC-II expression by 19-kDa lipoprotein from *M. tuberculosis* (Noss *et al.*, 2001; Tobian *et al.*, 2003; Pai *et al.*, 2003; Ramachandra *et al.*, 2001). Thus, increased levels of IFN- γ present in TLR2-deficient restimulation assays may be partly due to skewed antigen presentation. In the case of TLR2-deficient animals infected with 500 CFU *M. tuberculosis*, elevated levels of IFN- γ present in lung homogenates at 2 weeks post-infection (Fig. 3.9d), could not be correlated with increased numbers of CD4 $^+$ T cells (Fig. 3.10a). Analysis of pulmonary CD11c $^+$ and Ly-6G $^+$ cells present in the lungs of TLR2-deficient animals at 2 weeks post-infection showed that although there were no differences in the numbers of cells present (Fig. 3.11a), cell surface expression of MHC-II was higher in animals deficient in TLR2 when compared to wild-type controls (Fig. 3.11b).

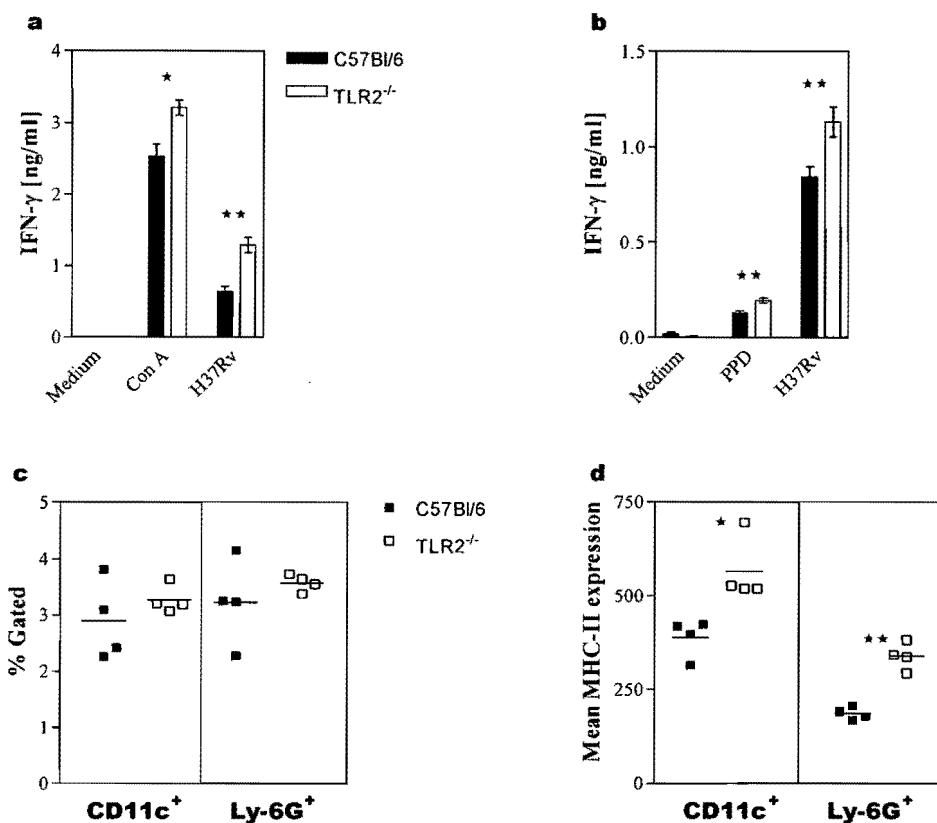


Figure 3.11 Ag-specific IFN- γ production and MHC-II cell-surface expression is elevated in TLR2-deficient animals. (a) Splenocyte restimulation assay from 4 week-infected wild-type (■) and TLR2-deficient mice (□). (b) Ag-specific stimulation of CD3 $^+$ T cells from pooled mediastinal lymph nodes of 4 mice 28 days after infection. (c-d) Cell-surface analysis of MHC-II expression on pulmonary CD11c $^+$ and Ly-6G $^+$ cells in wild-type and TLR2-deficient mice at 2 weeks post-infection. One representative of two experiments performed is shown.*, $p < 0.05$; **, $p < 0.001$.

3.3 Discussion

Microbial ligands have been shown to activate various mammalian TLRs, facilitating the transcription of genes that activate the adaptive immune response, including cytokines and various co-stimulatory molecules (Takeda *et al.*, 2003; Underhill *et al.*, 1999^b; Takeuchi *et al.*, 1999; Brightbill *et al.*, 1999; Stenger *et al.*, 2002). Globally, animals deficient in the adaptor protein MyD88 have been used as a tool to evaluate the importance of TLR-signaling in the initiation of an innate immune response. In the context of a virulent *M. tuberculosis* infection, the absence MyD88 results in an increased systemic mycobacterial infection which ultimately results in the host succumbing to the disease before congenic wild-type controls (Fig. 3.1). Collectively, this result has been confirmed by other groups investigating the *in vivo* contribution of MyD88 towards an *M. tuberculosis* infection (Scanga *et al.*, 2004; Fremond *et al.*, 2004; Sugawara *et al.*, 2003), although the models used do differ individually. Results presented by Scanga *et al.* (2004) and Sugawara *et al.* (2003) reveal that the infectious dose, strain of *M. tuberculosis* used, and genetic background of gene-deficient animals largely affect the experimental outcome. However, the results presented by Sugawara *et al.* (2003) suggested that the development of an adaptive immune response during an *M. tuberculosis* infection was not entirely dependent on the presence of MyD88. This concept was proven by Fremond *et al.* (2004) in which vaccination with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) conferred substantial protection to a virulent *M. tuberculosis* infection despite the absence of MyD88. The authors concluded that MyD88 is an essential component of an innate immune response against *M. tuberculosis*, but that the development of adaptive immunity can proceed in its absence.

Although the role of MyD88 in innate immunity following an *M. tuberculosis* infection is largely associated with TLR-signaling, a role for IL-1 and IL-18 signaling during infection should not be ignored. In this regard, both IL-1 and IL-18 signal via MyD88 (Adachi *et al.*, 1998) and in their absence, could partially account for the susceptibility of MyD88-deficient mice towards mycobacterial infections (Juffermans *et al.*, 2000; Yamada *et al.*, 2000). Nevertheless, within the repertoire of ligands recognized by the TLR family, TLRs 1, 2, 4 and 6 have been associated with recognition of

mycobacterial antigens (Thoma-Uszynski *et al.*, 2001; Bulut *et al.*, 2001; Takeuchi *et al.*, 2002). Within this group of TLRs, TLR2 appears to have a more dominant role in that agonists of this receptor have been identified to be whole mycobacteria, mycobacterial cell wall glycolipid lipoarabinomannan (LAM), mannosylated phosphatidylinositol, and a 19-kDa Mtb lipoprotein (Thoma-Uszynski *et al.*, 2001; Aliprantis *et al.*, 1999; Underhill *et al.*, 1999^b; Gilleron *et al.*, 2003; Quesniaux *et al.*, 2004^b). More specifically, *in vitro* experiments have confirmed that TLR2 activation is directly linked to killing of mycobacterial bacilli (Thoma-Uszynski *et al.*, 2001), an event that corresponds with increased NO production by murine macrophages, and that live mycobacterial bacilli induce TNF and IL-12p40 production in a TLR2-dependent manner (Fig. 3.8). Extrapolation of these findings into an *in vivo* model would suggest that animals lacking TLR2 would be more susceptible to a mycobacterial infection. Results presented here would argue that TLR2 is indeed required for optimal control of a virulent aerogenic *M. tuberculosis* infection. In an animal lacking TLR2, disease progression is associated with elevated mycobacterial numbers in the lungs as well as increased dissemination of bacilli into both the spleen and liver (Fig. 3.2 & 3.4). Furthermore, these animals are unable to form mycobactericidal granulomas (Fig. 3.5 & 3.6), and succumb to the infection before wild-type controls (Fig. 3.4).

Comparison of these results with other models investigating the *in vivo* role of TLR2 during mycobacterial infections indicates that TLR2-signaling is not redundant during disease progression. In an acute model of *M. bovis* BCG infection, intraperitoneal challenge with BCG resulted in a tenfold higher bacterial load in the lungs of TLR2-deficient mice (Heldwein *et al.*, 2003). In models using an aerogenic route of infection, TLR2-deficient mice infected with the Kurono strain of *M. tuberculosis* displayed a reduced capacity to control the pulmonary infection (Sugawara *et al.*, 2003), while in a high-dose infection model using *M. tuberculosis* H37Rv, TLR2-deficient mice were more susceptible than wild-type controls (Reiling *et al.*, 2002). Differences in these models can be ascribed to the initial route of infection, the strain of *M. tuberculosis* used (Dormans *et al.*, 2004; Lopez *et al.*, 2003; Manabe *et al.*, 2003), and the genetic background of animals used in experiments (Pan *et al.*, 2005; Mitsos *et al.*, 2000; Kamath *et al.*, 2003)

however, the general conclusion is that TLR2 has a more prominent role during an *M. tuberculosis* infection than TLRs 4 or 6.

The results presented by Reiling *et al.* (2002) more closely reflect the results presented in this chapter. Animals deficient in CD14, TLR2 or TLR4 control a low-dose aerogenic *M. tuberculosis* infection at 15 weeks post-infection, although a TLR2-deficiency results in an increased systemic infection and poor granuloma formation by 22 weeks post-infection (Fig. 3.2 & 3.5). In a high dose *M. tuberculosis* infection model, animals deficient in TLR2 succumb to the disease before wild-type controls (Reiling *et al.*, 2002; Fig. 3.4), mortality being associated with an increased systemic infection (Fig. 2.4) and chronic pneumonia (Fig. 3.6). Histologically, the lungs of TLR2-deficient animals showed accelerated tissue pathology associated with elevated levels of proinflammatory cytokines (Fig. 3.9), causing extensive pulmonary inflammation. The elevated levels of proinflammatory cytokines present in the lungs of TLR2-deficient mice may however be the consequence of an inability of the host to control replication of the bacillus at the level of the macrophage. Here, production of TNF, IL-12p40 and NO by macrophages is dependent on TLR2 signaling (Fig. 3.8; Reiling *et al.*, 2002; Thoma-Uszynski *et al.*, 2001). The resulting NO produced by the macrophage is then required by the cell to kill intracellular bacilli, a process which is mediated by TLR2 (Thoma-Uszynski *et al.*, 2001). The reduced expression of iNOS in the lungs of TLR2-deficient animals (Fig. 3.7) along with lower levels of pulmonary NO (Table 3.1) suggest that pulmonary macrophages produce less related nitrogen intermediates than wild-type controls and would therefore contain more mycobacterial bacilli (Fig. 3.4a). The increased recruitment of activated CD4⁺ and CD8⁺ T cells to the lungs of TLR2-deficient animals could be a result of the host attempting to compensate for the decreased effector functions of TLR2-deficient macrophages. Indeed, it has been shown that the addition of TNF and IFN- γ to macrophage cell cultures effects killing of intracellular mycobacteria to the same extent as 19-kDa-induced TLR2-mediated activation (Thoma-Uszynski *et al.*, 2001), indicating that TNF, IFN- γ and at least TLR2 work in concert to kill intracellular mycobacterial bacilli. However, in the absence of TLR2, increased amounts of TNF and IFN- γ are unable to restore full macrophage effector functions as evident by the reduced

levels of pulmonary NO and increased bacillary burden present in TLR2-deficient mice (Table 3.1; Fig. 3.4).

An increased proinflammatory response has however been shown to be detrimental for the host. Here, high levels of TNF have been associated with cachexia and septic shock (Beutler *et al.*, 1988) and high-dose therapy using IL-12 has been shown to have toxic inflammatory side effects (Ryffel *et al.*, 1997). In the *M. tuberculosis* model, TNF has been associated with host-mediated destruction of lung tissue (Rook *et al.*, 1989; Moreira *et al.*, 2002), the increased lung pathology correlating with elevated levels of pulmonary TNF. In a recent report a role for IL-27 was described in limiting the pathological sequelae of chronic inflammation induced by an aerogenic *M. tuberculosis* infection (Holscher *et al.*, 2005). In this study, the absence of IL-27/WSX-1 resulted in increased production of the proinflammatory cytokines TNF and IL-12p40 as well as an increased pulmonary recruitment of activated CD4⁺ T cells. The higher levels of protective immunity were however associated with a chronic inflammatory response which subsequently resulted in the accelerated death of WSX-1-deficient mice. Histologically, the lungs of these animals showed advanced inflammatory cell infiltration and interstitial fibrosis, an accelerated lung pathology that was associated with increased levels of TNF and IL-12p40. These animals also exhibited increased Ag-specific CD4⁺ T-cell responses towards *M. tuberculosis* which accompanied increased macrophage effector functions such as NOS2-dependent reactive nitrogen intermediates. In TLR2-deficient animals an increased Ag-specific T-cell response was also mounted (Fig. 3.11), which was unable to compensate for the defective macrophage effector functions due to the absence of TLR2. It remains to be determined whether the increased expression of MHC-II on pulmonary CD11c⁺ and Ly-6G⁺ could account for the elevated Ag-specific T-cell responses (Fig. 3.11), particularly due to the fact that mycobacterial bacilli are able to regulate MHC-II Ag-processing via TLR2 (Noss *et al.*, 2001 & 2002). The regulation of MHC-II expression is not only restricted to the receptor itself, but extends to the induction of mRNA for class II transactivator (CIITA), IFN regulatory factor-1 (IRF-1), and MHC-II by IFN- γ (Pai *et al.*, 2003). Here, *M. tuberculosis* 19-kDa lipoprotein directly inhibits IFN- γ -induced MHC-II expression, thereby linking TLR2 activation to IFN- γ -inducible genes. It is therefore clear that TLR2 not only induces proinflammatory

responses towards *M. tuberculosis* antigens, but is also involved in counterregulatory or inhibitory processes such as inhibition of MHC-II expression.

Apart from TLRs 1, 2, 4 and 6, several other receptors have been shown to mediate interactions of *M. tuberculosis* with macrophages. Receptors found to participate in phagocytosis of *M. tuberculosis* by macrophages include CD14, complement receptors, scavenger receptors, mannose receptor, dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN), CD44, and Fc γ receptors (Ernst *et al.*, 1998; Leemans *et al.*, 2003). *M. tuberculosis* induces the production of reactive nitrogen intermediates and matrix metalloproteinase-9 upon engagement of mannose receptor (Rivera-Marrero *et al.*, 2002; Ezekowitz *et al.*, 1990). CD14 has been implicated in the uptake of nonopsonized tubercle bacilli as well as the LAM-stimulated release of TNF, IL-1, and IL-8 (Peterson *et al.*, 1995; Zhang *et al.*, 1993; Pugin *et al.*, 1994). DC-SIGN has been shown to mediate the uptake of tubercle bacilli by human monocyte-derived dendritic cells (Tailleux *et al.*, 2003; Geijtenbeek *et al.*, 2003), and has been implicated in transmitting *M. tuberculosis*-induced signals in dendritic cells and macrophages (Geijtenbeek *et al.*, 2003; van Lent *et al.*, 2003). There are therefore several cell-surface receptors other than those belonging to the TLR protein family that are involved in mediating macrophage responses towards *M. tuberculosis*. The balance between the use of these cell-surface receptors as a means to activate a proinflammatory innate immune response and potential mechanisms used by the bacillus to evade that response ultimately results in either protective immunity or chronic disease. Studies using individual PRR-deficient animals should therefore take into account the fact that much redundancy exists within recognition of mycobacterial components, and pronounced phenotypes in such models can depend on several factors. Although genetic background and strain of *M. tuberculosis* used can influence the experimental outcome, results presented here indicate that TLR2 plays a more prominent role during an *M. tuberculosis* infection than either CD14 or TLR4.

Chapter 4

***Trypanosoma brucei* as a model**

Chapter 4

***Trypanosoma brucei* as a model for innate immunity**

Summary

The initial host response towards the extracellular parasite *Trypanosoma brucei* is characterized by the early release of inflammatory mediators associated with a Type 1 immune response. In this chapter, results argue that this inflammatory response is dependent on activation of the innate immune system mediated by the adaptor molecule MyD88. Here, MyD88-deficient macrophages are non-responsive towards both soluble variant-specific surface glycoprotein (sVSG) as well as membrane bound VSG (mfVSG) purified from *T. brucei*. Infection of MyD88-deficient mice with either clonal or non-clonal stocks of *T. brucei* resulted in elevated levels of parasitemia. This was accompanied by reduced plasma IFN-gamma and TNF levels during the initial stage of infection, followed by moderately lower VSG-specific IgG2a antibody titers during the chronic stages of infection. Analysis of several TLR-deficient mice revealed a partial requirement for TLR9 in the production of IFN- γ and VSG-specific IgG2a antibody levels during *T. brucei* infections. The MyD88-dependent induction of an inflammatory response was also associated with infection-induced pathology in that animals lacking MyD88 were less anemic than wild-type controls. These results implicate the mammalian TLR family and MyD88 signaling in the innate immune recognition of *T. brucei* as well as infection-associated pathology.

4.1 Introduction

To date, several reports have investigated the role of TLRs in initiating an innate immune response against experimental parasitic infections including *T. cruzi*, *L. major*, *P. berghei* and *T. gondii* (Campos *et al.*, 2004; Debus *et al.*, 2003; Adachi *et al.*, 2001; Scanga *et al.*, 2002). Collectively, the MyD88-dependent production of inflammatory cytokines was shown *in vitro* as well as *in vivo*, thereby implicating the TLR protein family in the initial recognition event. More specifically, *in vitro* experiments using APC deficient in MyD88 showed that the adaptor molecule was required for the initial induction of IL-12 following stimulation with parasite antigen (Scanga *et al.*, 2002; Campos *et al.*, 2004), while animals deficient in MyD88 were more susceptible to live infections when compared to wild-type controls (Muraille *et al.*, 2003; Adachi *et al.*, 2001). The majority of these models therefore indicated that the induction of inflammatory cytokines required the MyD88-dependent activation of an innate immune response, the absence of which resulted in susceptibility towards parasitic infection.

The induction of an early polarized variant surface glycoprotein (VSG)-specific Type 1 immune response following an African trypanosome infection is generally associated with overall host resistance (Schleifer *et al.*, 1993; Mansfield, 1994; Schopf *et al.*, 1998; Magez *et al.*, 2002). More specifically, control of a *T. brucei* infection at the level of parasitemia has been associated with the early induction of Type I cytokines such as TNF and IFN- γ , as well as a VSG-specific B cell response (Cross *et al.*, 1990; Borst *et al.*, 1994; Hertz *et al.*, 1998; Magez *et al.*, 1999). At the level of the macrophage, the production of TNF is the result of the combined activation by circulating sVSG released by the parasite due to stress, as well as membrane-bound VSG (mfVSG) that consists of the dimyristoylglycerol (DMG) lipid moiety of the glycosylphosphatidylinositol (GPI) anchor (Ferguson *et al.*, 1988; Magez *et al.*, 1998). Individually, while sVSG induces macrophages to secrete TNF in an IFN- γ -dependent manner, mfVSG can overactivate macrophages in an IFN- γ -independent manner (Magez *et al.*, 1998). The induction of both TNF and IFN- γ are therefore critical cytokines required for the initial control of the disease, their absence being associated with a severely diminished capacity to control proliferation of the parasite (Hertz *et al.*, 1998; Magez *et al.*, 1999). The production of

TNF due to an infection is however also believed to be related to the severity of *T. brucei*-induced pathology (Magez *et al.*, 2002). Here, TNF-induced pathology has been associated with anemia and inflammation of the brain in both the human and animal host (Okomo-Assoumou *et al.*, 1995; Lucas *et al.*, 1993; Magez *et al.*, 2002).

The requirement for MyD88 in mediating cellular inflammatory responses towards various parasitic antigens indicated that macrophage activation and TNF production by *T. brucei* sVSG and mfVSG was dependent on MyD88-signaling. Results presented here show that *T. brucei*-derived sVSG and mfVSG do indeed activate macrophages in a MyD88-dependent manner, and mice lacking the adaptor are unable to control either clonal or non-clonal *T. brucei* infections. Dissection of this response indicated that macrophages required TLR9 to respond to *T. brucei*-derived genomic DNA, while TLR9 was found to partially mediate immune inflammatory responses during the later stages of infection. Although MyD88 is required by the host to effectively control a *T. brucei* infection, activation of an inflammatory response via this adaptor protein was also associated with infection-induced anemia. Thus, while the host requires MyD88 to control the development of parasitemia, this event is also responsible for infection-associated immunopathology.

4.2 Results

4.2.1 Macrophages require MyD88 to respond optimally to sVSG and mfVSG

It has previously been shown that the glycosylinositolphosphate (GIP) moiety of sVSG is the main trypanosome-derived TNF-inducing component (Magez *et al.*, 1998). However, the TNF-inducing capacity of sVSG required IFN- γ priming, suggesting that in an *in vivo* setting, macrophage responsiveness towards sVSG occurs after an IFN- γ prestimulation event. Here, all sVSG macrophage stimulation experiments were performed using a concentration of protein no higher than 10 μ g/ml, a dose corresponding to a trypanosome load of approximately 2×10^7 parasites during infection. To determine whether macrophage TNF responses towards sVSG might be mediated by TLRs, bone marrow-derived macrophages deficient in the adaptor molecule MyD88 were stimulated with sVSG either in the presence or absence of IFN- γ (Fig. 4.1a). Neither wild-type nor MyD88-deficient macrophages responded to sVSG in the absence of IFN- γ . Upon prestimulation with IFN- γ , wild-type macrophages produced TNF in a dose-dependent manner while macrophages deficient in MyD88 produced none. TNF production in response to LPS was reduced in MyD88-deficient macrophages (Fig. 4.1a), but not absent. sVSG fractions were free of endotoxin, indicating that TNF production was solely due to sVSG stimulation. The production of nitrite by wild-type macrophages in response to sVSG was dependent on IFN- γ priming, but was reduced in MyD88-deficient macrophages (Fig. 4.1b). In contrast to TNF and nitrite, no IL-6 or IL-12p40 were detected in culture supernatants (Fig. 4.1c & d), and prestimulation with IFN- γ did not induce macrophages to secrete IL-6 or IL-12p40 in response to sVSG. In general, macrophages were far less responsive towards sVSG than they were to LPS (Fig. 4.1a-d). Here, cytokine production induced by 1 μ g/ml LPS far exceeded that produced by 10 μ g/ml sVSG.

In response to environmental stress, trypanosomes release their VSG via a VSG lipase (Rolin *et al.*, 1996). Once this occurs, the dimyristoylglycerol (DMG) VSG anchor remains in the trypanosomal membrane while the GIP-VSG is released (Fox *et al.*, 1986). However, during a trypanosomal infection, it is also likely that macrophages are exposed to the VSG-GPI anchor due to lyses of trypanosomes.

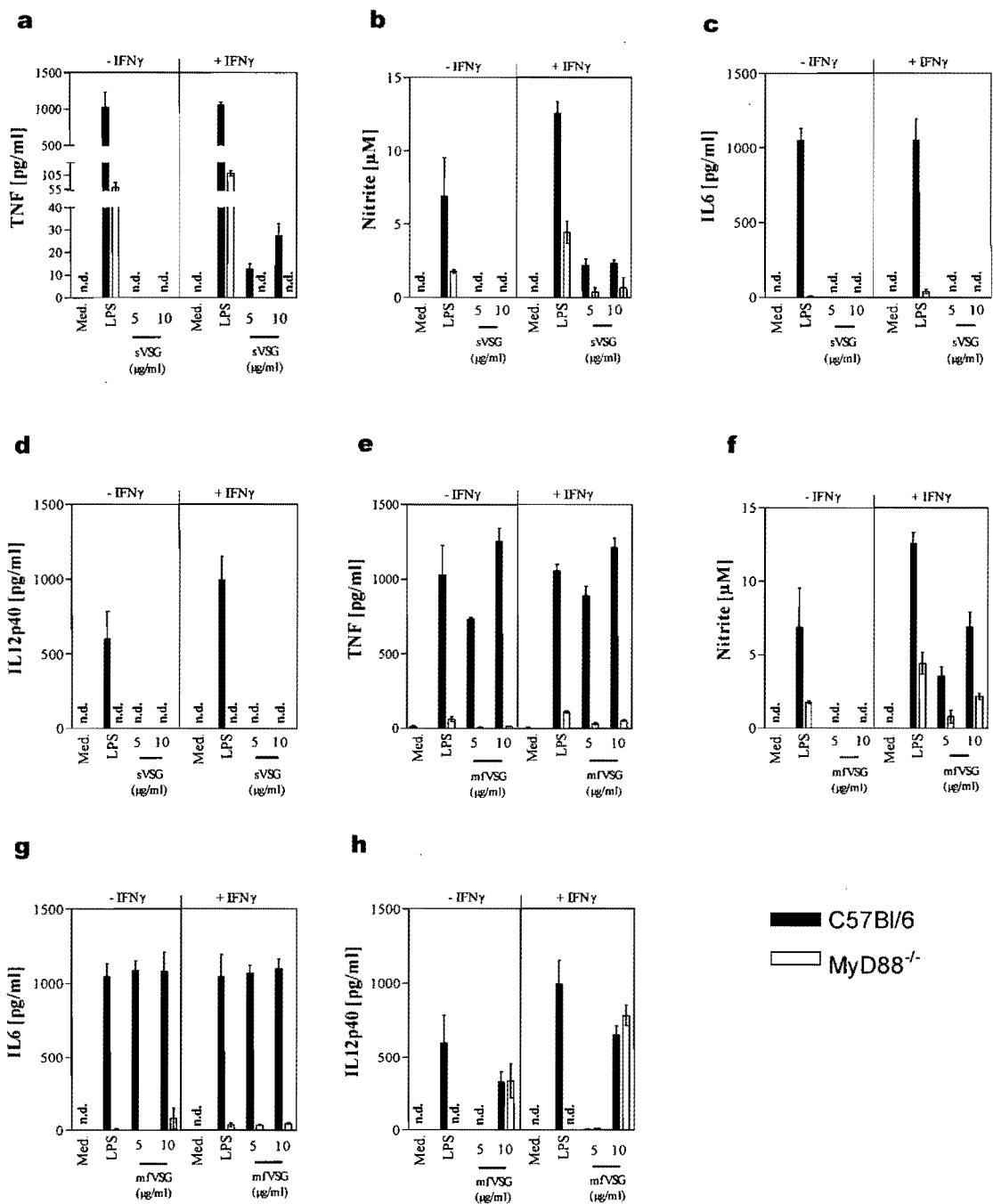


Figure 4.1 Impaired responses to *T. brucei* antigen in *MyD88 $^{-/-}$* macrophages. (a-d) Bone marrow derived macrophages from wild-type (filled bars) or *MyD88 $^{-/-}$* (open bars) mice were stimulated with LPS (1 μ g/ml) or AnTat1.1 *T. brucei* soluble VSG (5 or 10 μ g/ml) in the presence or absence of 30 U/ml IFN- γ for 24h. TNF, Nitrite, IL-6 and IL-12p40 in culture supernatants were determined by ELISA. (e-h) Bone marrow derived macrophages from wild-type and *MyD88 $^{-/-}$* mice were stimulated with LPS (1 μ g/ml) or AnTat1.1 *T. brucei* membrane-fraction VSG (5 or 10 μ g/ml) in the presence or absence of 30 U/ml IFN- γ for 24h. Concentrations of TNF, Nitrite, IL-6 and IL-12p40 in culture supernatants were determined by ELISA. n.d., not detected.

Within this context, it has already been shown that mfVSG can elicit a macrophage response and that this response is independent of IFN- γ prestimulation (Magez *et al.*, 1998). To determine whether mfVSG-induced macrophage responsiveness was dependent on MyD88 signaling, macrophages deficient in MyD88 were stimulated with mfVSG either in the absence or presence of IFN- γ (Fig. 4.1e-h). Firstly, macrophages required MyD88 to produce TNF in response to mfVSG stimulation (Fig. 4.1e), but in contrast to sVSG, this response was not dependent on prestimulation with IFN- γ . Secondly, macrophages did require MyD88 and IFN- γ prestimulation to produce nitrite upon mfVSG stimulation (Fig. 4.1f). Thirdly, unlike macrophages stimulated with sVSG (Fig. 4.1c & d), macrophages stimulated with mfVSG secreted both IL-6 and IL-12p40 (Fig. 4.1g & h), the production of which was not dependent on IFN- γ prestimulation. However, while MyD88 was required by macrophages to produce IL-6 in response to mfVSG stimulation (Fig. 4.1g), the production of IL-12p40 was MyD88-independent (Fig. 4.1h). Therefore, macrophages in general were far more responsive towards mfVSG than sVSG, the response required MyD88 for the production of TNF, IL-6 and nitrite but not IL-12p40.

4.2.2 Mice require MyD88 signaling to control a *T. brucei* infection

To evaluate the role of MyD88 in host resistance to *T. brucei*, mice deficient in MyD88 were infected with clonal pleiomorphic AnTat1.1 *T. brucei*. The development of parasitemia and survival of these animals were compared to wild-type controls, as well as mice deficient in either IFN- γ R1 or TNF (Fig. 4.2). In terms of development of parasitemia, mice deficient in MyD88 could not efficiently control the height of first peak parasitemia, a phenotype similar to mice deficient in either IFN- γ R1 or TNF (Fig. 4.2a & c). Following clearance of the first peak of parasitemia, wild-type animals developed a reduced second peak of parasitemia at days 13-15 post-infection, and succumbed to the final lethal peak of parasitemia at approximately 40-50 days post-infection (Fig. 4.2a). Animals deficient in either MyD88 or IFN- γ R1 had significantly elevated parasitemia levels post first-peak (Fig. 4.2a), which remained elevated for the duration of the infection with both gene-deficient animals succumbing to infection before wild-type controls (Fig. 4.2b; $p < 0.01$ & $p < 0.001$, respectively). Mice deficient in TNF controlled

parasitemia development post first-peak more effectively than either MyD88- or IFN- γ R1-deficient animals (Fig. 4.2c), and displayed similar survival kinetics when compared to wild-type controls (Fig. 4.2d; $p > 0.10$). Thus, MyD88-deficient animals succumbed to the infection before TNF-deficient animals (Fig. 4.2d; $p < 0.01$).

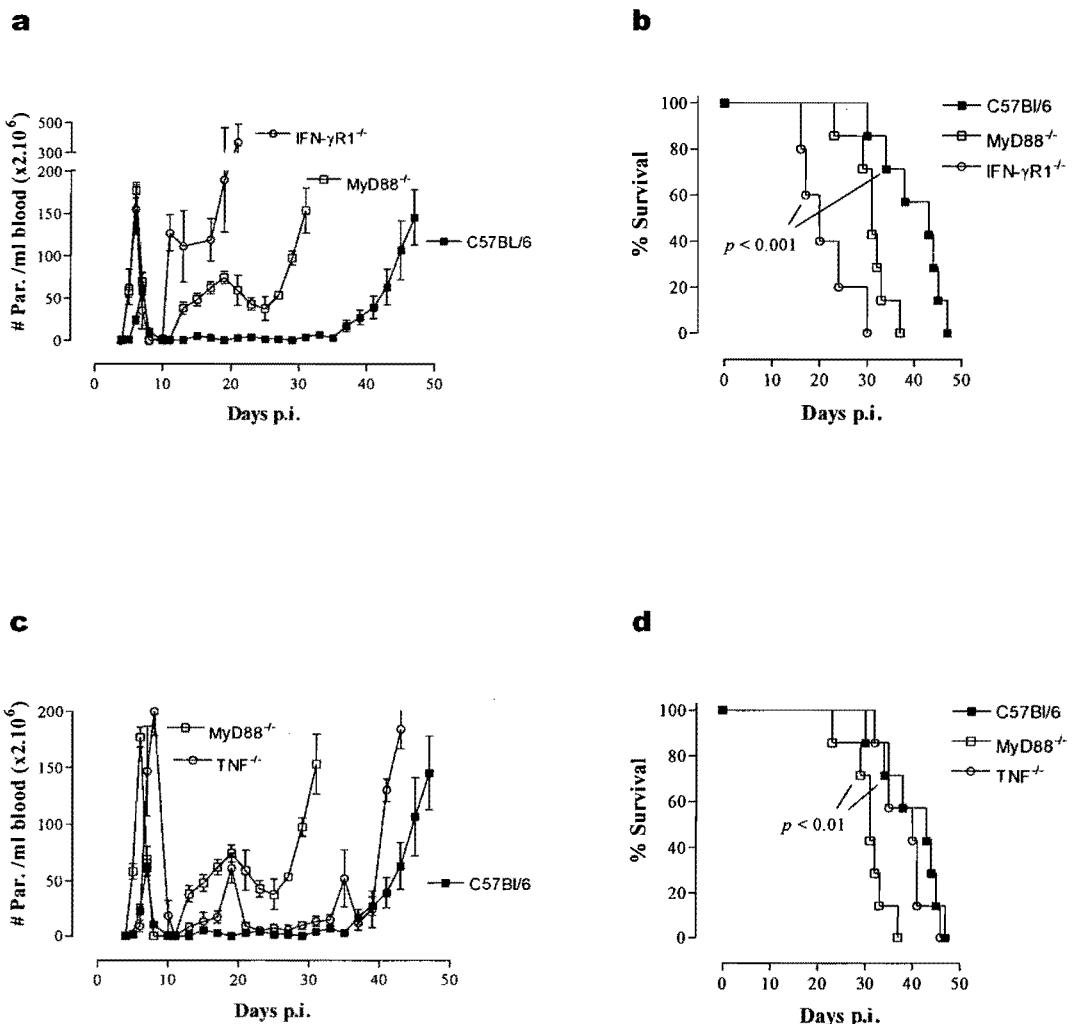


Figure 4.2 *MyD88* is required to control a pleomorphic *T. brucei* *AnTat1.1* infection. Parasitemia development and survival in (a-b) wild-type (■), MyD88^{-/-} (□), IFN- γ R1^{-/-} (○) and (c-d) wild-type (■), MyD88^{-/-} (□), and TNF^{-/-} (○) mice. Five mice per group were infected at day 0 intraperitoneally with 5×10^3 *AnTat1.1* *T. brucei* parasites. Parasitemia data indicate mean \pm of 5 mice per group, while survival data is representative of two to three pooled experiments.

In addition, IL-12p40 did not contribute significantly to first-peak parasitemia control as animals deficient in this cytokine controlled the first peak of parasitemia as well as wild-type controls (Table 4.1). To determine whether the impaired resistance of MyD88-deficient mice towards a *T. brucei* infection could be attributed to a reduced Type 1 immune response, IFN- γ and TNF were measured in the plasma at various time-points post-infection (Fig. 4.3a & b). At 4 days post-infection, MyD88-deficient mice had reduced plasma IFN- γ levels when compared to wild-type controls (Fig. 4.3a), which then increased marginally at day 6 post-infection. A similar trend was observed for plasma TNF levels in MyD88-deficient mice (Fig. 4.3b).

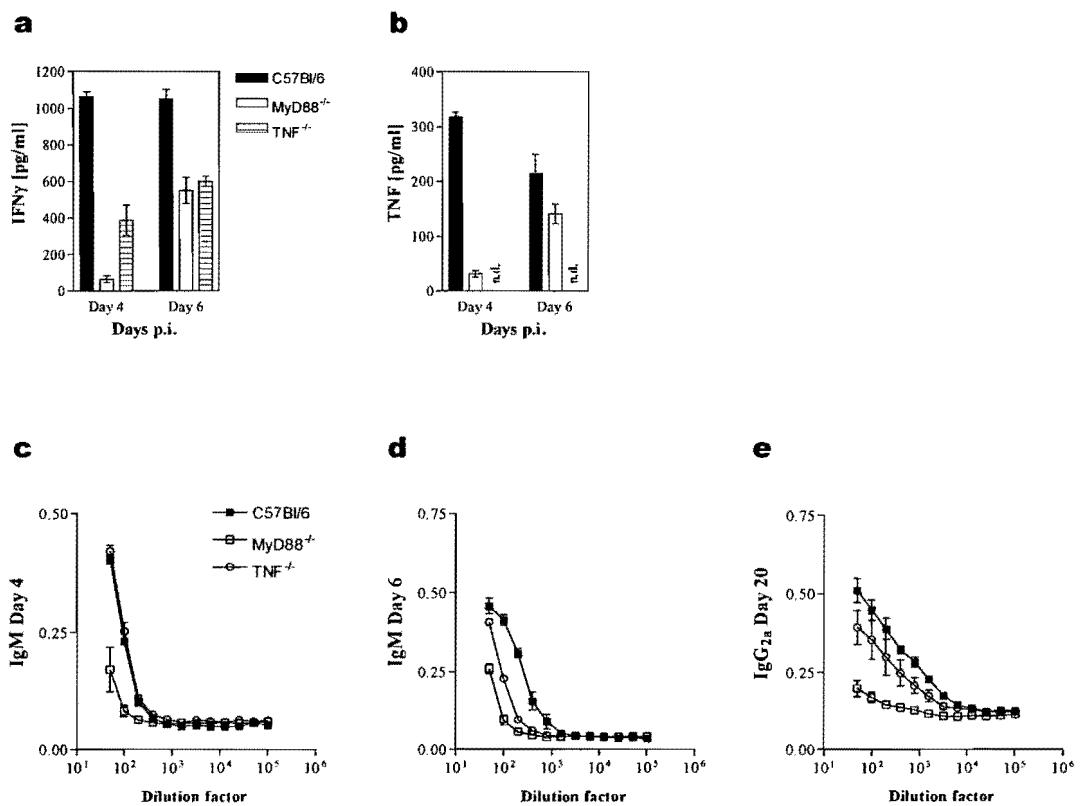


Figure 4.3 *MyD88* is required to mount an early inflammatory response following a *T. brucei* infection. (a-b) wild-type (filled bars), MyD88^{-/-} (open bars), and TNF^{-/-} (striped bars) were infected intraperitoneally with 5×10^3 AnTat1.1 *T. brucei* parasites. Sera was collected at days 4 and 6 post-infection and analysed for IFN- γ and TNF by ELISA. n.d., not detected. (c-e) Sera was collected from wild-type (■), MyD88^{-/-} (□) and TNF^{-/-} (○) mice at days 4, 6 and 20 post-infection and analysed for VSG-specific IgM and IgG2a antibodies.

For comparative purposes, plasma IFN- γ levels were analyzed in TNF-deficient animals. Here, plasma IFN- γ levels were also reduced in mice lacking TNF at days 4 and 6 post-

infection (Fig. 4.3a). Furthermore, parasite clearance is also mediated by a VSG-specific Ab response. Here, VSG-specific IgM and IgG2a titers at days 4, 6 and 20 post-infection were moderately reduced in MyD88-deficient animals (Fig. 4.3d, e & f). Antibody titers for IgG1, IgG2b and IgG3 in MyD88-and TNF-deficient mice were comparable to wild-type controls for the duration of infection (data not shown). Thus, the development of a Type 1 cytokine and VSG-specific IgG2a response during a clonal *T. brucei* infection is mediated in part by the MyD88-dependent activation of the innate immune system.

4.2.3 *T. brucei* induces immunopathology by MyD88-dependent mechanisms

The induction of TNF by *T. brucei*-derived sVSG and mfVSG is an important component of parasitemia control and host survival (Magez *et al.*, 1998; Magez *et al.*, 1999), but is also largely responsible for infection-associated immunopathology such as anemia (Magez *et al.*, 2002; Magez *et al.*, 2004). The development of anemia in laboratory mice following an experimental *T. brucei* infection has been shown to be largely mediated by TNF (Magez *et al.*, 2004), the production of this cytokine being a consequence of macrophage activation by parasite-derived glycoproteins and GPI moieties. Results presented here show that the induction of TNF following stimulation of macrophages with sVSG or mfVSG is MyD88-dependent (Fig. 4.1), and that animals deficient in MyD88 have lower systemic TNF levels during the first peak of infection than those observed for wild-type controls (Fig. 4.3b). The development of anemia was therefore assessed in animals lacking MyD88 to determine whether the adaptor molecule had a role to play in infection-associated pathology. Wild-type animals infected with pleiomorphic AnTat1.1 *T. brucei* become progressively more anemic during the chronic stages of infection (Fig. 4.4), with animals losing on average 47% of red blood cells compared to the first day of infection. Infection of animals deficient in TNF were considerably more resistant to the development of anemia (Fig. 4.4a), and only lost 13% of red blood cells following clearance of the first peak of parasitemia. Similarly, animals lacking MyD88 (Fig. 4.4b) or IFN- γ R1 (Fig. 4.4c) are also more resistant to infection-associated anemia, losing 17% and 15% of red blood cells during infection respectively. Therefore, the MyD88-dependent activation of an innate immune response following infection with *T. brucei* results in the production of TNF and IFN- γ (Fig. 4.3a & b), both of which

contribute to the control of parasitemia and development of anemia in wild-type C57Bl/6 animals. Collectively, the MyD88-dependent induction of a Type 1 inflammatory response following a *T. brucei* infection suggests a role for TLRs in the initial activation of this innate immune response.

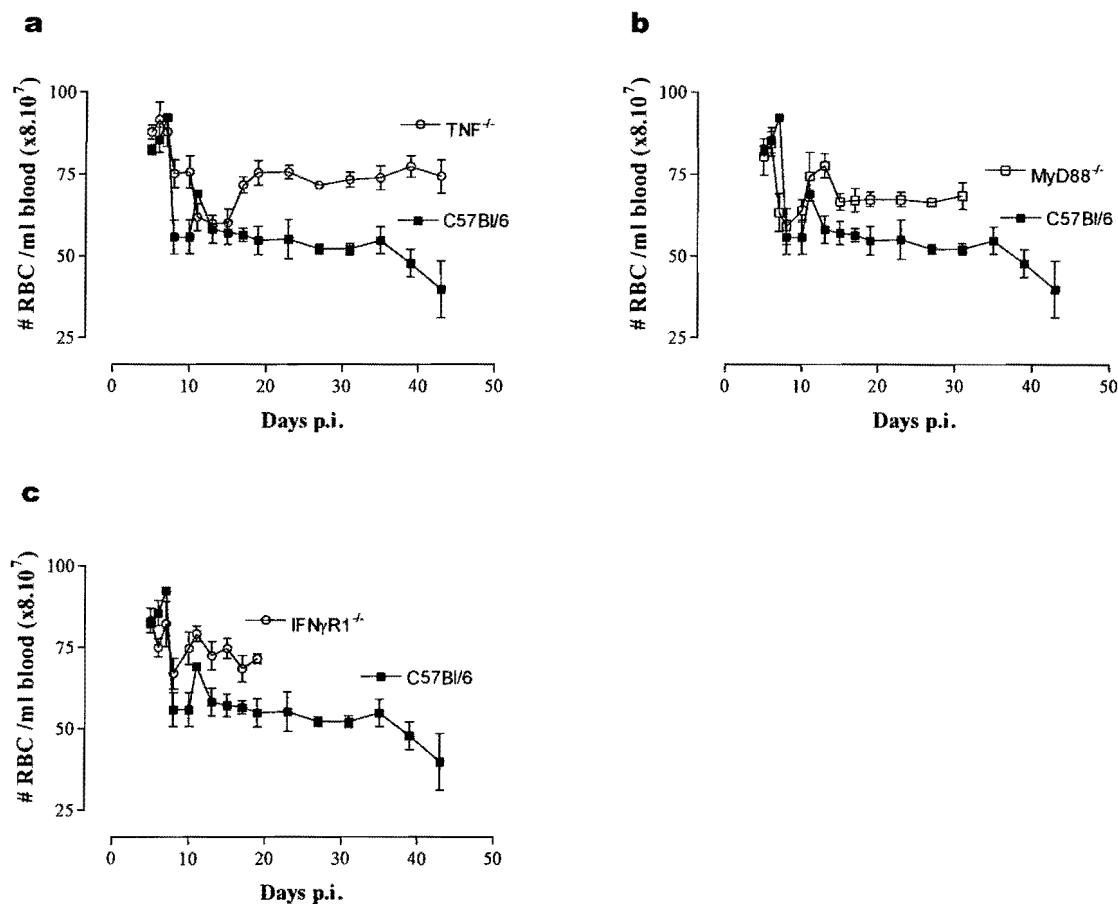


Figure 4.4 Development of anemia following a pleomorphic *T. brucei* AnTat1.1 infection is MyD88-dependent. Anemia expressed as numbers of red blood cells/ml blood in (a) wild-type (■), and TNF^{-/-} (○) mice; (b) wild-type (■), and MyD88^{-/-} (□) mice; and (c) wild-type (■), and IFN- γ R1^{-/-} (○) mice. Five mice per group were infected at day 0 intraperitoneally with 5×10^3 AnTat1.1 *T. brucei* parasites.

4.2.4 Analyses of several TLR-deficient mice reveal a role for TLR9 in *T. brucei* parasitemia control

Although MyD88 is an integral component of the TLR-signaling pathway (Kaisho *et al.*, 2001), the role this adaptor molecule has in conferring responsiveness towards IL-1 and IL-18 should be addressed in infectious models (Medzhitov *et al.*, 1998; Wesche *et al.*, 1997; Adachi *et al.*, 1998). To determine whether the increased susceptibility of MyD88-

deficient animals during a clonal *T. brucei* infection was associated with defective IL-1 and/or IL-18 signaling, mice deficient in IL-1R1 and caspase-1 were infected with clonal *T. brucei*. In contrast to MyD88-deficient mice, these mice controlled the first and second peaks of parasitemia as well as wild-type controls (Table 4.1), indicating that the increased susceptibility towards *T. brucei* observed for MyD88-deficient animals was due to defective TLR-signaling. Here, screening of several PRR/TLR-deficient animals revealed that neither CD14, TLR1, TLR2, the combination of both TLR2 and 4, nor TLR9 were required by the host to control the first peak of parasitemia following a clonal *T. brucei* infection (Table 4.1). However, animals deficient in TLR9 developed elevated levels of parasitemia following clearance of the first peak of parasites (Table 4.1), indicating that TLR9 could be involved in parasitemia control post first-peak.

TABLE 4.1 Systemic responses of PRR- and cytokine-deficient mice following a clonal *T. brucei* infection¹

Mouse strain	1 st Peak parasitemia (x2.10 ⁶ Par./ml)	2 nd Peak parasitemia (x2.10 ⁶ Par./ml)	% RBC loss
² C57Bl/6			
TLR1 ^{-/-}	56 ± 7	9 ± 5	47
TLR2 ^{-/-}	58 ± 6	10 ± 7	43
TLR9 ^{-/-}	37 ± 4	12 ± 9	44
MyD88 ^{-/-}	51 ± 14	25 ± 4	46
TNF ^{-/-}	167 ± 47	38 ± 14	17
IFN-γR1 ^{-/-}	197 ± 41	15 ± 13	13
IL-1R1 ^{-/-}	141 ± 38	111 ± 83	15
Caspase-1 ^{-/-}	43 ± 10	14 ± 8	48
IL-12p40 ^{-/-}	55 ± 20	7 ± 2	47
IL-12p40 ^{-/-}	53 ± 9	16 ± 4	unknown
³ TLR2/4 ^{+/+}	135 ± 48	10 ± 5	47
TLR2/4 ^{-/-}	121 ± 23	5 ± 4	36
⁴ CBA/J	66 ± 31	15 ± 10	12
CD14 ^{-/-}	58 ± 18	8 ± 5	13

¹ Five wild-type and PRR/cytokine-deficient mice were infected intraperitoneally with 5x10³ AnTat1.1 *T. brucei* parasites. Results are representative of 2-3 similar experiments.

² Gene deficient mice backcrossed onto a C57Bl/6 background 5 times or more.

³ Gene deficient mice on a 129/SvJxC57Bl/6 background.

⁴ Gene deficient mice on a CBA/J background

Bold = wild-type parasitemia counts

Underline = gene deficient mice with elevated parasitemia and reduced anemia

In terms of the progression of anemia, the aforementioned gene-deficient animals developed severe anemia during the chronic stages of infection (Table 4.1) indicating that individual contributions made by these TLRs and cytokines during anemia are negligible compared to that seen for TNF, IFN- γ and MyD88.

4.2.5 *T. brucei* DNA stimulates macrophages in a TLR9-dependent manner

Following the first wave of parasitemia, lysis of the parasite results in the release of several parasite components into the blood stream. Apart from sVSG and mfVSG that promote a macrophage response, it has been reported that DNA from *T. brucei* is not only mitogenic for B lymphocytes (Shoda *et al.*, 2001), but induces macrophages to produce TNF, nitric oxide and IL-12p40 in the presence of IFN- γ . Here, the extent of B cell proliferation and macrophage activation was associated with CG dinucleotide content, a feature associated with bacterial DNA and TLR9 responsiveness (Hemmi *et al.*, 2000). It was therefore postulated that induction of an inflammatory response following a *T. brucei* infection might be mediated, in part, by TLR9-dependent macrophage activation. Indeed, when stimulated with *T. brucei* DNA, macrophages deficient in TLR9 produced less TNF (Fig. 4.5a), IL-6 (Fig. 4.5b), IL-12p40 (Fig. 4.5c) and nitrite (Fig. 4.5d) than their wild-type counterparts. Macrophages deficient in TLR9 were unresponsive towards CpG DNA (Fig. 4.5a-d), but responded normally to stimulation with LPS. In general, macrophages were far less responsive towards *T. brucei* genomic DNA than they were to CpG DNA (Fig. 4.5a-d), possibly due to the fact that the frequency of CG dinucleotides in *T. brucei* genomic DNA is only 3.9% (Shoda *et al.*, 2001). These results suggest that macrophage responsiveness towards *T. brucei* DNA is mediated in part by TLR9.

4.2.6 Mice require TLR9 to control parasitemia development following first peak clearance

Not only did macrophages deficient in TLR9 respond poorly to *T. brucei* genomic DNA, but animals deficient in TLR9 infected with clonal *T. brucei* had increased numbers of parasites following clearance of the first peak of parasitemia (Fig. 4.6a), although no significant difference was observed in the mean survival time of both wild-type and TLR9-deficient animals (Fig. 4.6b; $p > 0.10$).

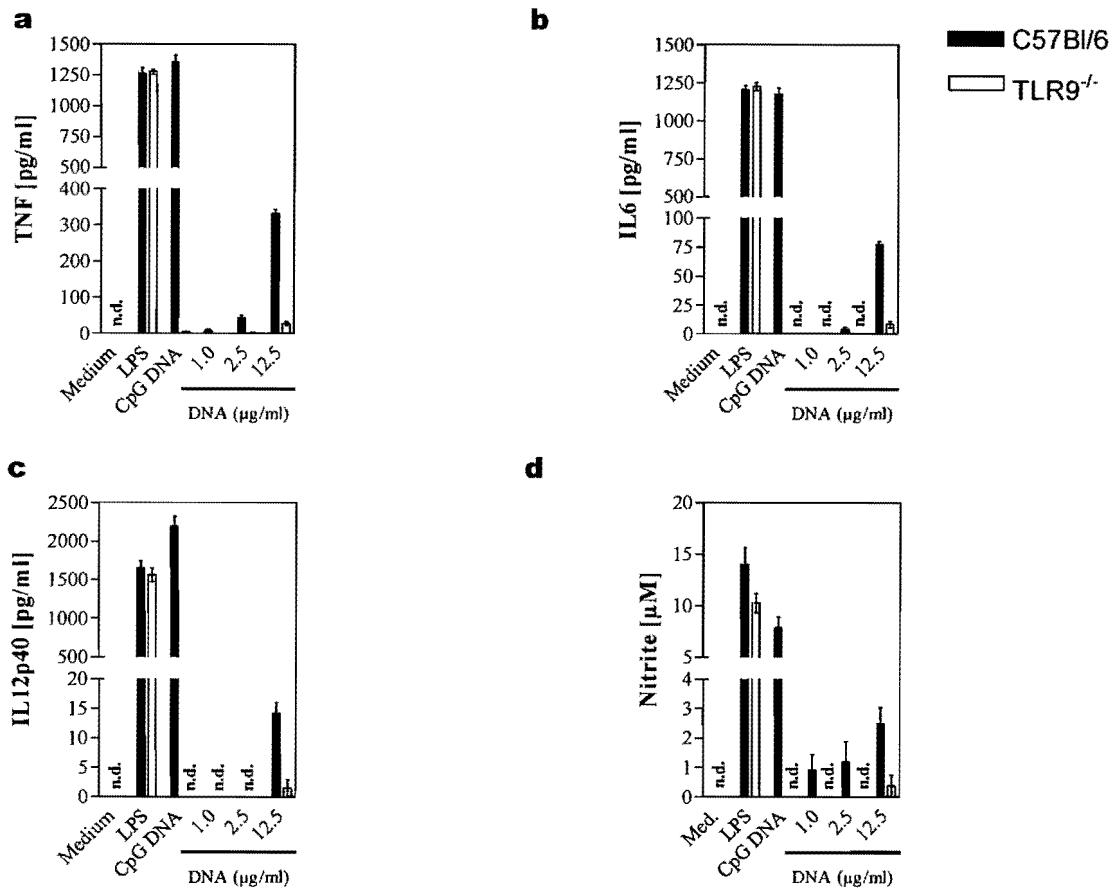


Figure 4.5 Impaired responses to *AnTat1.1* *T. brucei* genomic DNA in TLR9^{-/-} macrophages. (a-d) Bone marrow derived macrophages from wild-type or TLR9^{-/-} mice were stimulated with CpG ODN (1.0 µM), LPS (1.0 µg/ml) or the indicated concentrations of *T. brucei* genomic DNA in the presence of 30 U/ml IFN-γ for 24h. Concentrations TNF, IL-6, IL-12p40 and Nitrite in culture supernatants were determined by ELISA. n.d., not detected.

IFN-γ is a critical factor in contributing to parasitemia control following first peak clearance (Fig. 4.2a; Table 4.1). Analysis of plasma IFN-γ levels in TLR9-deficient mice showed reduced IFN-γ levels during the second parasitemia peak (Fig. 4.6c). Reduced plasma IFN-γ levels coincided with clearance of the first peak of parasitemia at days 10-13 at a point where 10⁸ parasites would release genomic DNA into circulation. This was accompanied by lower VSG-specific IgG2a antibody titers at day 13 post-infection (Fig. 4.6d), which were restored to wild-type levels by day 16 post-infection (Fig. 4.6e). The reduction in plasma IFN-γ levels in TLR9-deficient mice following clearance of the first

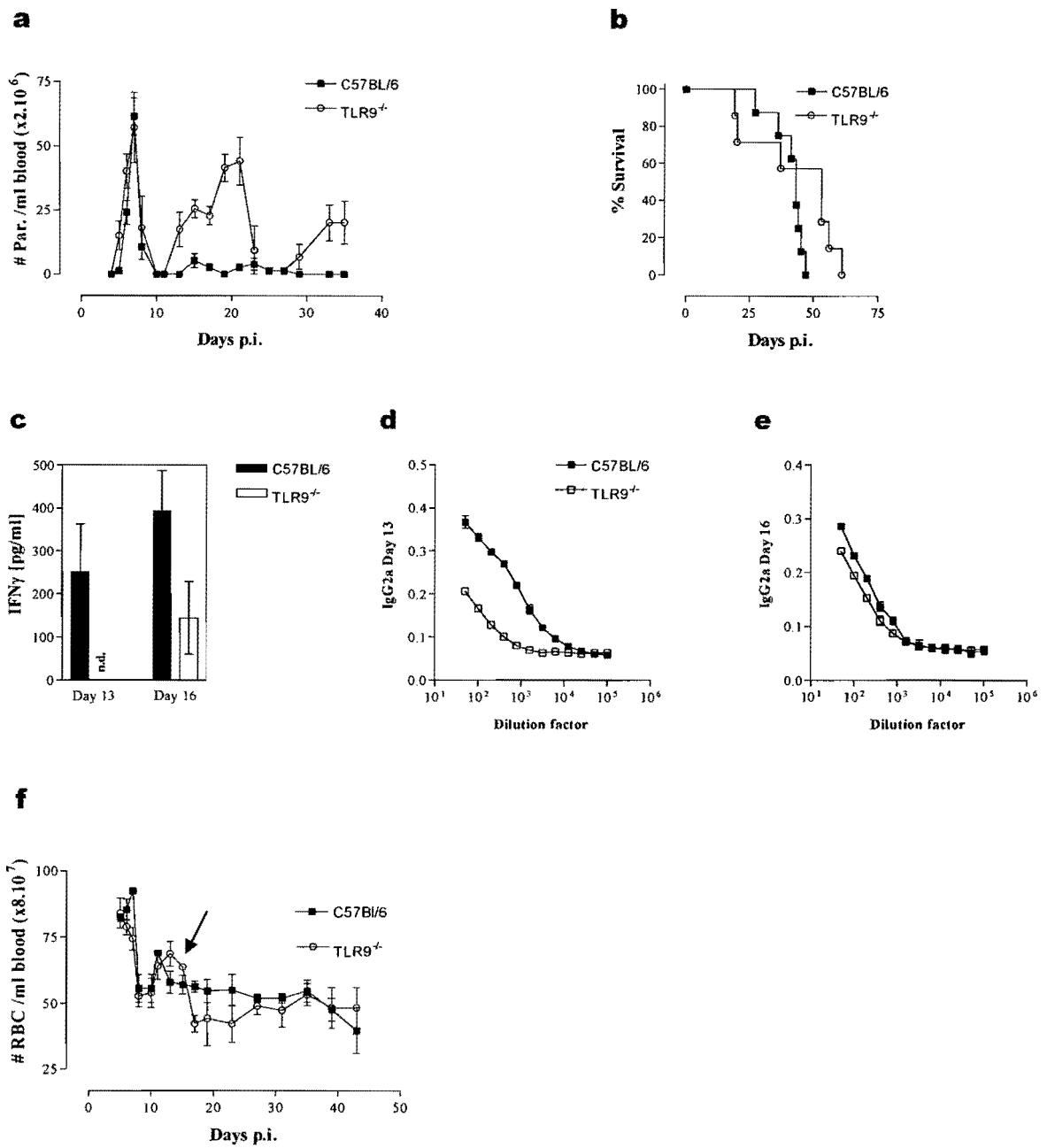


Figure 4.6. Reduced resistance of TLR9^{-/-} mice following a *T. brucei* infection. (a-b) Parasitemia development and survival in wild type (■) and TLR9^{-/-} (○) mice infected intraperitoneally with 5×10^3 AnTat1.1 *T. brucei* parasites. (c-e) Sera was collected at days 13 and 16 post-infection and analysed for IFN- γ , VSG-specific IgG2a antibodies by ELISA. n.d., not detected. (f) Anemia development in wild-type (■) and TLR9^{-/-} (○) mice and expressed as numbers of red blood cells/ml blood.

peak of parasitemia coincided with a moderate reduction in anemia at days 10-13 post-infection (Fig. 4.6f), although this was not significant and TLR9-deficient animals became as anemic as wild-type controls during the later stages of infection.

4.2.7 Mice require MyD88 and TLR9 to control a non-clonal *T. brucei* infection

In the field, *T. brucei* parasites that infect livestock initially express metacyclic VSG and not the clonal form of VSG first seen by the immune response as present on AnTat1.1 *T. brucei* parasites (Magez *et al.*, 2002; Barry *et al.*, 1984; Graham *et al.*, 1990). Seeing that the murine host required both TLR9 and MyD88 to control a clonal *T. brucei* infection, we sought to determine whether this was also true for a non-clonal metacyclic *T. brucei* infection. Using a tsetse fly as a vector, mice deficient in either TLR9 or MyD88 were infected with metacyclic AnTat1.1 *T. brucei* parasites (Fig. 4.7a). When compared to the clonal AnTat1.1 *T. brucei* infection (Fig. 4.2a & 4.6a), the non-clonal AnTat1.1 *T. brucei* infection resulted in similar parasitemia profiles (Fig. 4.7a). Mice deficient in MyD88 were unable to control both first and second parasitemia peaks. Here, non-clonal parasitemia levels in MyD88-deficient animals reached 4×10^8 parasites/ml blood at day 20 post-infection (Fig. 4.7a), as opposed to 2×10^8 parasites/ml blood for the clonal infection (Fig. 4.2a). Additionally, all MyD88-deficient animals succumbed to non-clonal infection by day 20 post-infection (Fig. 4.7b), while these animals survived for 40 days following a clonal infection (Fig. 4.2b). Mice deficient in TLR9 controlled the first peak of parasitemia as well as wild-type controls, but were unable to effectively clear parasites following first peak parasitemia (Fig. 4.6a). Both MyD88- and TLR9-deficient mice succumbed to the infection before their wild-type controls (Fig. 4.7b; $p < 0.002$ & $p < 0.005$, respectively). In a similar set of experiments, animals lacking TNF displayed a profound loss in control of parasitemia (Fig. 4.7c), and succumbed to infection before wild-type controls (Fig. 4.7d; $p < 0.005$). The progression of parasitemia was also associated with the development of anemia (Fig. 4.7e-g), and this at a rate similar to that observed for non-clonal *T. brucei* infections (Fig. 4.4). Here, animals lacking MyD88 showed a reduction in anemia during the first peak of infection (Fig. 4.7e), a result not observed in animals lacking TNF (Fig. 4.7g). The role of TNF in anemia could not be determined following clearance of the first peak of parasitemia as all TNF-deficient mice succumbed to the infection at this time-point (Fig. 4.7d & g). Clearance of the first peak of parasitemia coincided with moderately reduced anemia in TLR9-deficient animals (Fig. 4.7e & f). The results therefore demonstrate that MyD88, TNF and TLR9 contribute to the control of parasitemia following a non-clonal *T. brucei* infection, and that MyD88

and TLR9 are partly involved in the induction of anemia both during and after the first wave of parasitemia.

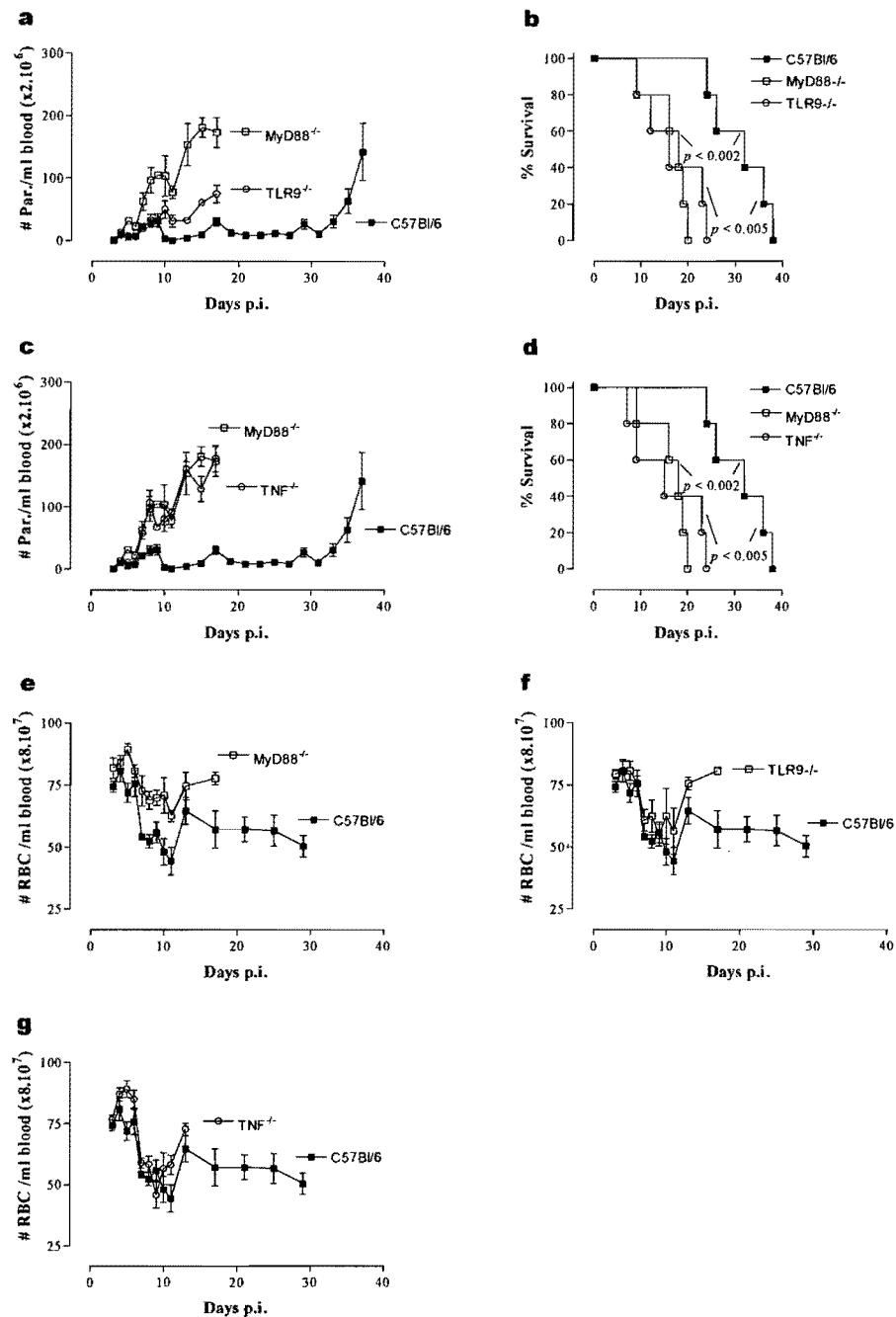


Figure 4.7. Differential requirement for MyD88, TLR9 and TNF during a non-clonal *T. brucei* infection. (a-d) Parasitemia development and survival of wild-type (■), MyD88^{-/-} (□), TLR9^{-/-} (□) and TNF^{-/-} (○) mice. (e-g) Anemia development in wild-type (■) and TLR9^{-/-} (○) mice and expressed as numbers of red blood cells/ml blood. Five mice per group were infected with metacyclic AnTar1.1 *T. brucei* parasites using *Glossina spp.* as vector. Results are expressed as means \pm standard deviations.

4.3 Discussion

There is a growing body of evidence which indicates that recognition of several parasitic infections leads to the MyD88-dependent activation of an innate immune response (Campos *et al.*, 2004; Debus *et al.*, 2003; Muraille *et al.*, 2003; Adachi *et al.*, 2001; Del Rio *et al.*, 2004; Scanga *et al.*, 2002). In models analyzing experimental *T. gondii*, *T. cruzi*, *P. berghei*, *L. major* or *B. burgdorferi* infections, a deficiency in MyD88 was correlated with reduced plasma IL-12p40 and/or IFN- γ levels during the infection. This was accompanied by decreased production of IL-12p40 and IFN- γ following splenocyte restimulations with parasitic antigen (Campos *et al.*, 2004; Muraille *et al.*, 2003; Adachi *et al.*, 2001; Scanga *et al.*, 2002). Thus, in these models, the activation of the MyD88 signaling pathway results in the early production of IFN- γ and IL-12, both of which are essential components in host resistance against parasitic infections.

The cytokines TNF and IFN- γ have been shown to be critical factors in determining the relative level of host resistance against the extracellular parasite *T. brucei* (Hertz *et al.*, 1998; Magez *et al.*, 1999; Namangala *et al.*, 2001). Deficiencies in either of these cytokines have been associated with elevated levels of parasitemia and, in terms of IFN- γ , shorter survival times. Results presented here link the initial production of TNF and IFN- γ following a *T. brucei* infection to the MyD88-dependent activation of an innate immune response. The production of TNF by macrophages in response to either sVSG or mfVSG requires signaling via MyD88. Thus, the release of parasite antigen in the time period preceding the first peak of parasitemia stimulates macrophages to produce TNF, which in turn contributes to controlling the height of first-peak parasitemia. Lower systemic TNF levels present in mice lacking MyD88 is indicative of a failure by the host to respond to parasite antigens. However, the activation state of macrophages during infection may influence the ability of the host to respond to a Trypanosome infection (Coller *et al.*, 2003). Indeed, macrophage responsiveness towards *T. brucei* sVSG required IFN- γ prestimulation, which suggests that direct sensitization of host T cells by *T. brucei* to produce IFN- γ occurs before macrophages are fully responsive to sVSG (Olsson *et al.*, 1993). However, IL-12p40-mediated activation of the host T cell compartment does not appear to play a crucial role during a *T. brucei* infection as mice

deficient in this cytokine control first-peak parasitemia as well as wild-type controls (Table 4.1). In addition, stimulation of macrophages with mfVSG did induce the production of IL-12p40, but this response was independent of MyD88-signaling. These results suggest that the induction of an IFN- γ environment following a *T. brucei* infection is independent of the action of IL-12, as previously shown in experimental infection models using *T. cruzi* (Muller *et al.*, 2001), *L. monocytogenes* (Brombacher *et al.*, 1999) and *M. tuberculosis* (Holscher *et al.*, 2001), but that the induction of IFN- γ requires signaling via MyD88. This initial IFN- γ environment not only controls first peak parasitemia, but also modulates VSG-specific IgM to IgG2a switching (Schopf *et al.*, 1998), thereby contributing to parasitemia control post first-peak.

Within this context, MyD88 is required for responsiveness towards IL-1 α , IL-1 β as well as IL-18 (Medzhitov *et al.*, 1998; Wesche *et al.*, 1997; Muzio *et al.*, 1997; Adachi *et al.*, 1998). Deficiencies in these cytokines have been associated with a diminished capacity to clear intracellular pathogens and fungi (Tsuji *et al.*, 2004; Juffermans *et al.*, 2000; Mencacci *et al.*, 2000), the reports attributing a reduced early protective response to decreased levels of IFN- γ . Mice deficient in IL-1R1 and caspase-1 were used to control for possible decreased responsiveness towards IL-1 α , IL-1 β and IL-18, respectively. The protease caspase-1 is required for processing of IL-1 β and IL-18 into their biologically active forms (Fantuzzi *et al.*, 1999). Infection of mice deficient in either IL-1R1 or caspase-1 showed little or no differences in terms of parasitemia control when compared to wild-type animals (Table 4.1), and although a role for MyD88 in IL-1 and IL-18 signaling during infection cannot be excluded, the necessity for these cytokines appear to be minimal when compared to TNF and IFN- γ . Therefore, we attribute the reduced early protective response observed in MyD88-deficient animals to defective Toll signaling. However, deficiencies in several individual TLRs failed to reproduce the elevated parasitemia levels observed during first-peak parasitemia in MyD88-deficient animals (Table 4.1). This would indicate that the control of parasitemia is not dependent on activation of an innate immune response via TLRs 1, 2 or 4. Although it is possible that these receptors function cooperatively with one another in response towards parasite components such as sVSG and mfVSG, this study shows no individual role *in vivo* for these receptors either before or after the first peak of parasitemia. However, a partial role

for TLR9 was found to contribute to parasitemia control following first peak clearance. During this stage of infection, lyses of 10^8 parasites would release more nuclear material into circulation when compared to lyses of parasites before the first peak of infection. Here, IL-12p40 produced by macrophages in response to *T. brucei* DNA could in turn induce IFN- γ production by PBMC (Shoda *et al.*, 2001), and could account for the reduced systemic IFN- γ levels found in TLR9-deficient mice following clearance of the first peak of parasitemia. In addition to activating macrophages via TLR9, parasite DNA may also be involved in triggering B cells to proliferate and differentiate (Shoda *et al.*, 2001; Shirota *et al.*, 2002), thereby contributing to the differentiation of Th1 cells and isotype switching. Therefore, both sVSG and parasitic DNA released systemically during infection contribute to macrophage activation and the resulting inflammatory response. The extent to which they contribute to macrophage activation and the inflammatory response could be more substantially addressed once a receptor for sVSG has been identified. This being said, attempts to identify individual TLRs involved in the activation of a protective innate immune response against other parasitic infections have been inconclusive. In some cases, purified parasitic components such as *T. cruzi*-derived GPI anchor were shown to activate TLR2 from both mouse and human origin *in vitro* (Campos *et al.*, 2001), although within an *in vivo* setting, mice deficient in this TLR do not display a severely reduced capacity to control a *T. cruzi* infection compared to mice deficient in MyD88 (Campos *et al.*, 2004). Furthermore, in murine infection models using *P. berghei*, *T. gondii* and *L. major*, investigators have been unable to formally demonstrate that individual, or combinations of TLRs, are involved in innate recognition of the aforementioned parasites.

Although activation of an innate immune response is required to control a *T. brucei* infection, uncontrolled growth of the parasite in the absence of MyD88 is not necessarily the causative agent for infection-associated pathology. In this regard, data indicates that the general inflammatory state induced by the infection results in pathologies such as meningoencephalitis (Okomo-Assoumou *et al.*, 1995) and anemia (Magez *et al.*, 1999). Meningoencephalitis is however poorly characterized in mice (Sileghem *et al.*, 1995), while the development of anemia in the mouse model has received more attention. Here, studies describing a mechanism for red blood cell lysis

have focused on the release of lytic factors by the parasites themselves (Tizard *et al.*, 1978), antibody-mediated lysis of RBCs loaded with VSG molecules (Rifkin *et al.*, 1990; Rickman & Cox, 1979 & 1980) as well as erythrophagocytosis (Witola *et al.*, 2001). However, seeing that the development of anemia corresponds with the general inflammatory state induced by the infection, it is not surprising that a role for TNF has been found in this process (Sileghem *et al.*, 1994; Magez *et al.*, 1999). The exact mechanism linking TNF to the induction of anemia remains to be shown, but it has been postulated that the capacity of TNF to modulate the activation, growth, and phagocytic potential of macrophages links this cytokine to RBC loss through erythrophagocytosis (Branch *et al.*, 1996; Collins *et al.*, 1992). This finding is however complicated by the fact that the level of anemia is influenced by the background of the host (Magez *et al.*, 2004; Taiwo *et al.*, 2000), a result attributed to p75 TNF receptor shedding in a murine model as a means to prevent inflammatory pathology (Magez *et al.*, 2004). Results presented here expand on the finding that the murine host is not only less anemic in the absence of TNF (Fig. 4.4a), but that the development of anemia can be linked to the MyD88-dependent activation of macrophages by parasite components such as sVSG and mfVSG (Fig. 4.1 & 4.4b). This not only holds true for clonal experimental *T. brucei* infections (Fig. 4.4b), but for non-clonal metacyclic *T. brucei* infections as well (Fig. 4.7e). A lesser role in anemia can be attributed to the TLR9-dependent activation of macrophages by parasitic DNA (Fig. 4.5), which upon comparison to clonal and non-clonal infections in MyD88-deficient mice, effects an intermediary phenotype (Fig. 4.6f & 4.7f). Therefore, the resulting MyD88-dependent activation of the innate immune system by parasite components serves to control parasite numbers, but in doing so contributes to the infection-associated pathology as well.

Collectively, it is likely that the induction of an innate immune response following a *T. brucei* infection is mediated by interaction between several TLRs that signal via MyD88. Indeed, the requirement for the MyD88-dependent activation of an innate immune response is far more pronounced in non-clonal *T. brucei* infections. In the field, this would indicate that the VSG repertoire presented by the parasite is far greater, emphasizing the requirement for the TLR protein family in initiating an immune response. This being the case, a recent report has described a ligand for TLR11, a TLR

initially found to confer protection against uropathogenic bacteria (Zhang *et al.*, 2004). Although in this paper the phenotype was restricted to the urogenital system, Yarovinsky *et al.* (2005) show that TLR11 is also involved in the recognition of a protozoan profilin-like protein. Profilin homologs can be found in several apicomplexan parasites including *T. gondii*, *P. falciparum*, *Cryptosporidium parvum*, and *Eimeria tenella* (Yarovinsky *et al.*, 2005; Rosenberg *et al.*, 2005), and is also present in *T. brucei* (Wilson *et al.*, 1997). However, it is feasible that recognition of *T. brucei* is not solely mediated by the TLR family, but could include pattern recognition receptors involved in recognizing sugar moieties within the variant surface glycoprotein. An example of this type of complex formation between the TLR protein family and proteins unrelated to the TLR family exists for recognition of endotoxin by the CD14-TLR4-MD2 complex (Miyake *et al.*, 2004; Akashi *et al.*, 2003). In the case of *T. gondii*, parasite-induced IL-12 production by DCs was found to be mediated by the dual interaction between MyD88 and the C-C chemokine receptor 5 (CCR5) (Scanga *et al.*, 2002; Aliberti *et al.*, 2000), indicating that TLRs function in concert with additional cell-surface receptors to effect the appropriate immune response. Analysis of the VSG coat covering *T. brucei* revealed that the galactose side chain composition appears to be important in terms of the TNF-inducing capacity of sVSG (Magez *et al.*, 1998). Thus, while the generation of a protective Type 1 immune response following a *T. brucei* infection is MyD88-dependent, it is likely that the initial recognition event is mediated by member/s of the TLR family functioning cooperatively with a surface protein/s involved in galactose recognition.

Chapter 5

General discussion & outlook

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The requirement for TLRs in conferring protective immunity in insects and mice suggested a central role for TLRs in the primary recognition of infectious pathogens LeMaitre *et al.*, 1996; Poltorak *et al.*, 1998^a). An early hypothesis held that individual TLR recognized or sensed specific microbial molecules, such that collective TLR recognition would enable the host to detect most microbes. Initially, TLR ligand specificities were determined using overexpression studies, and although such studies revealed useful information about the functioning of signal transduction molecules (Takeda *et al.*, 2003), it was found that the results of these studies might in fact be misleading. For example, overexpression studies suggested that TLR2 played a role in LPS signaling (Yang *et al.*, 1998; Kirschning *et al.*, 1998), a result that was later attributed to cell lines becoming extremely sensitive to minor non-LPS contaminants in LPS preparations due to the overexpression of TLR2. The more recent advent of TLR knockout mice enabled a more thorough characterization of individual TLR ligands (Akira *et al.*, 2001). The results obtained from these studies suggested that the individual TLR-ligand specificities determined *in vitro* could be extrapolated to *in vivo* infectious models where the host would require TLRs to initiate an immune response. Collectively, the majority of infectious disease models investigating the contribution made by TLR-signaling in the initiation of immunity have done so using animals deficient in MyD88. In mammals, ligand binding by TLRs induces a signaling event that proceeds via MyD88, an intracellular adaptor protein. Although the majority of TLRs signal via MyD88, some TLRs have also been shown to signal via MyD88-independent pathways (see introduction), both of which ultimately induce NF-κB activation. In the work presented here, the contribution of TLR signaling towards the control of infectious diseases is assessed in the context of *M. tuberculosis* and *T. brucei*, addressing the following questions: i) during an infection, is the requirement for individual TLRs influenced by the duration of the infection?, ii) do different routes of infection influence the TLR-

dependent activation of an immune response?, and iii) is there a correlation between the TLR-dependent control of an infection and immunopathology?

The requirement for individual TLRs in activating an innate immune response does appear to be proportional to the rate of proliferation of the pathogen. This statement is corroborated when comparing acute infections caused by bacteria and viruses to more chronic infections such as those caused by *M. tuberculosis* and *T. brucei*. For example, in acute bacterial and viral infections, individual TLR-deficiencies have been shown to severely compromise an innate immune response (Wang *et al.*, 2004; Takeuchi *et al.*, 2000^c; Echchannaoui *et al.*, 2002). In these experimental systems, the rapid induction of inflammatory cytokines such as TNF are essential in controlling these infections which indicates that the induction of an inflammatory response by individual TLRs is sufficient to control shorter more acute infections. However, when comparing these results to those obtained for more chronic infections such as those induced by *M. tuberculosis* and *T. brucei*, it appears that collective TLR-signaling is required to elicit a controlled inflammatory response. This was corroborated by the fact that animals lacking MyD88 were unable to control either *M. tuberculosis* or *T. brucei* infections. This being said, although collective TLR-signaling is required to control more chronic infections, work presented here also shows that specific TLR-signaling is not redundant in such infections. It was shown that TLR2 contributed significantly to controlling mycobacterial growth *in vivo*, proving that this TLR is an important member of the TLR family in the context of mycobacterial infections. The same was seen for TLR9 and the importance of this TLR in controlling growth of the parasite during the more chronic stages of *T. brucei* infections. Thus, there are members of the TLR protein family that have more prominent roles in the development of immunity during chronic infections, but it is their collective signaling that prevents progression to disease.

Activation of the immune response by various TLRs is however not solely dependent on the pathogen at hand, but appears to be modulated by the initial route of infection as well. In the case of *M. tuberculosis* infections, several reports indicate that intraperitoneal as opposed to intravenous or aerogenic infection can severely compromise the resulting phenotype (Heldwein *et al.*, 2003; Feng *et al.*, 2003; Reiling *et al.*, 2002), a result also observed for intraperitoneal and subcutaneous experimental parasitic

infections (Kropf *et al.*, 2004; Adachi *et al.*, 2001; Scanga *et al.*, 2002; Alexopoulou *et al.*, 2002). Delineating the role of individual TLRs in activating an innate immune response is therefore made more complicated due to the use of different routes of infection, a fact which is more evident in shorter acute infections. For example, animals deficient in MyD88 were highly susceptible to an intravenous *S. aureus* infection (Takeuchi *et al.*, 2000^c), but resistant to an aerogenic *S. aureus* challenge (Skerret *et al.*, 2004). The authors ascribe this discrepancy to the MyD88-independent activation of alveolar macrophages and neutrophils which imparts a certain amount of tissue specificity towards staphylococcal infections. Secondly, models investigating the role of TLRs in the innate recognition of *S. pneumonia* show that the control of an intranasal infection proceeds in a TLR2-independent manner (Knapp *et al.*, 2004), with more protection conferred by TLR4 during both nasopharyngeal and intranasal routes of infection (Malley *et al.*, 2003; Branger *et al.*, 2004). In contrast, pneumococcal meningitis induced by intracerebral injection of *S. pneumonia* was shown to be TLR2-dependent (Echchannaoui *et al.*, 2002; Koedel *et al.*, 2003), which suggests that the role of TLR2 in the host response towards *S. pneumonia* is dependent on the route of infection. Thirdly, in infectious studies using the encapsulated yeast *Cryptococcus neoformans*, activation of the immune response was shown to be independent of TLR4 (Yauch *et al.*, 2004; Biondo *et al.*, 2005), although significant differences were observed in the survival times of wild-type and TLR4-deficient animals infected intraperitoneally as opposed to intranasally (Yauch *et al.*, 2004). These results therefore show that the initiation of an immune response is also dependent upon the site of infection, and that the requirement for individual TLRs in this response varies accordingly. Although this has been observed for the TLR-dependent activation of the immune system during acute infections, this system of immune activation should be taken into account for pathogens that cause more chronic infections as well.

In terms of the *M. tuberculosis* model presented here, all infections were initiated by delivering the bacillus directly into the lungs, and all conclusions pertaining to the TLR-dependent activation of the immune response were based on an experimental set-up that closely reflects the natural route of infection. All experiments were performed using one strain of *M. tuberculosis*, and although various mycobacterial strains have been

shown to influence pathogenesis of the disease (Dormans *et al.*, 2004; Lopez *et al.*, 2003; Manabe *et al.*, 2003), differences in immune responses in the *M. tuberculosis* model were not influenced by the use of different strains of *M. tuberculosis*. Such uniformity could not be employed in the *T. brucei* model as experiments used to investigate the TLR-dependent activation of an immune response varied in terms of the parasite strain used, as well as the initial route of infection. The differences in progression of infection were apparent when comparing the clonal and non-clonal infections. Gene-deficient animals infected with non-clonal parasites had higher levels of parasitemia and succumbed to the infection before animals infected with clonal stabilates of the parasite. This would indicate that the non-clonal form transmitted by the tsetse fly is more virulent than the clonal form. However, mice infected with non-clonal parasites had the parasites delivered sub-cutaneously or intra-dermally by the fly, while the clonal infections were initiated intra-peritoneally. Therefore, in addition to the use of two strains of parasite, the initial route of infection with these strains does differ and may contribute to the TLR-dependent activation of an immune response. It remains to be determined whether sub-cutaneous as opposed to intra-peritoneal *T. brucei* infections elicit a differential TLR-response in terms of the route of infection. However, no differences were observed in terms of the role for TLR9 in either clonal or non-clonal infections in that the requirement for this TLR was restricted to the second peak of parasitemia, and argues that differing routes of infection have little or no effect on the requirement for TLR9 in this model.

The fact that activation of innate immunity is dependent on both the nature of the microbe and the site(s) of infection suggests the preferential activation of different cell types in a pathogen- and tissue-specific manner. A recent report describing the *in vivo* production of TNF by specific leukocyte subsets showed that local as opposed to systemic TNF production was not only dependent on different cellular subsets, but on the physiological location as well as the cellular stimulus used (Grivennikov *et al.*, 2005). Here, in an intracellular bacterial model using *L. monocytogenes*, TNF derived from either macrophages or neutrophils was sufficient for protection against a low dose infection, with no discernable role for either B- or T-cell-derived TNF. However, a role for T-cell-derived TNF became apparent once higher doses of *L. monocytogenes* were used, indicating that T-cell-derived TNF is required for protection against high

intracellular bacterial loads that cannot be solely provided by innate mechanisms. In contrast to this, the deleterious role of TNF in LPS/D-Gal-induced toxic shock was solely due to its production by macrophages and neutrophils, with no role for either B- or T-cell-derived TNF in this process. The corollary with the TLR-dependent activation of innate mechanisms becomes more apparent here. In the intracellular bacterial model using *M. tuberculosis*, a deficiency in TLR2 impairs the capacity of the macrophage not only to produce TNF, but also to kill intracellular bacteria. Once the dose of bacteria is increased or uncontrolled due to the defective macrophage compartment, T-cell-derived TNF is a function used by the host to control the infection. This was evident in the *M. tuberculosis* model where increased levels of pulmonary TNF were associated with an increased recruitment of activated T-cells. It remains to be formally demonstrated that macrophage-derived TNF plays a more prominent role during pulmonary *M. tuberculosis* infection as opposed to T-cell-derived TNF, although a pronounced role for T-cell-derived TNF was recently shown in long-term mycobacterial infections (Saunders *et al.*, 2004). These results therefore suggest that T-cells are the major producers of TNF during mycobacterial infections and that a defect at the level of the macrophage results in an upregulation of the host T-cell response during an *M. tuberculosis* infection. In the model investigating innate mechanisms required to control the extracellular parasite *T. brucei*, data presented here shows that macrophages require TLRs to produce TNF in response to parasite antigens. This inflammatory response is however restricted to the macrophage as it is macrophage-derived TNF and not T-cell-derived TNF that is responsible for control of parasitemia (unpublished observations). Therefore, control of parasitemia appears to be dependent on the production of TNF by macrophages and not T-cells, thereby more closely resembling the model of LPS/D-Gal-induced toxic shock (Grivennikov *et al.*, 2005). Collectively, these results indicate that recognition of both *M. tuberculosis* and *T. brucei* is initially orchestrated by the macrophage, but that the major cellular producers of TNF are T-cells during mycobacterial infections and macrophages during *T. brucei* infections.

Although the induction of TNF is associated with a protective immune response in an infectious setting, the cytokine has been implicated in pathology associated with infectious and sterile inflammation, as well as several autoimmune diseases, in so doing

contributing to swelling, tissue injury and influx of immunologically active cells. Although treatment of inflammatory diseases caused by sterile inflammation initially focused on the blockade of cytokines such as TNF, new approaches are leaning towards comprehensive ‘upstream’ blockade of signaling molecules such as TLRs (Ulevitch, 2004). These strategies not only apply to the downregulation of TLR function in the context of pathologies such as sepsis and autoimmune disease, but also extend to the upregulation of TLR function in diseases such as cancer, allergy and disease epidemics. However, the specific targeting of innate immune pathways such as TLRs as a means to control pathologies induced by diseases such as autoimmunity and sterile inflammation should be approached with caution. Work presented here shows that manipulation of host TLR-signaling pathways can result in both uncontrolled disease progression as well as infection-induced immunopathology. In the model of *M. tuberculosis*, deletion of TLR2 impaired the immune response such that the host attempted to compensate for this deficiency by upregulating an inflammatory response. The resulting pulmonary phenotype was one similar to that observed for sterile inflammation, namely tissue injury and an influx of immunologically active cells. In terms of the *T. brucei* model, blocking TLR signaling certainly reduced the infection-associated anemia, but also resulted in the inability of the host to control growth of the parasite. Collectively, these results indicate that the blockade of TLR signaling as a means to quell pathologies such as those caused by sterile inflammation would ultimately endanger the host’s ability to control infectious disease. This would not only apply to pathogens that cause acute infections, but should be taken into account for pathogens that cause more chronic infections as well.

The work presented in this thesis therefore shows that the mammalian host requires TLRs to recognize molecular structures present on *M. tuberculosis* and *T. brucei*. Infection of the host with either pathogen showed that the innate immunological response initiated by TLR signaling regulated the adaptive immune response during the chronic stages of infection. Furthermore, results presented here show that although the TLR-dependent activation of an immune response was dynamic enough to eliminate the pathogen, this response can be responsible for the development of immunopathology as well.

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