UNIVERSITY OF LIVERPOOL

The role of TLR2 in cutaneous leishmaniasis and as a target for vaccine adjuvants

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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Abstract

After the introduction of clean water, vaccination is thought to be the most effective public health tool ever introduced, responsible for preventing millions of cases of disease, disability and death each year. Unfortunately there remain a number of important human diseases for which we have no vaccine, particularly parasitic diseases, such as leishmaniasis, which primarily affect poor communities in tropical regions. There are many complex reasons why we have failed to develop effective vaccines for parasitic diseases, but there is hope that with our improved understanding of the immune system alongside the development of a new generation of vaccines, we will soon develop new vaccines which are effective enough to prevent such diseases. Toll-like receptors (TLRs) are major targets for adjuvants and have been shown to be crucial for defence against a number of infections. TLR2 recognises bacterial lipopeptides in a heterodimer with either TLR1 or TLR6, and its function has been linked to protection against various bacterial infections and to the efficacy of the BCG vaccine. TLR2 has been shown to recognise surface glycoconjugates of *Leishmania* parasites *in vitro*, particularly lipophosphoglycan (LPG). In this study, *in vivo* experimental infections show that TLR2 has a protective role in controlling cutaneous leishmaniasis (CL), as shown by increased lesion sizes and parasite burdens in $TLR2^{-/-}$ mice infected with *L. major* and *L. mexicana*. Furthermore, it appears that LPG is not the major mediator of TLR2 activation during infection with *L. mexicana*, as parasites lacking LPG also resulted in exacerbated disease in $TLR2^{-/-}$ mice. Mice lacking TLR2 co-receptors TLR1 and TLR6 did not show increased susceptibility to infection, suggesting either mono-TLR2 function or alternative co-receptor involvement. Infected $TLR2^{-/-}$ mice show a skewed Th2 immune response to *Leishmania*, as demonstrated by elevated IL-4, IL-13 and IL-10 production by draining lymph node (DLN) cells in response to antigen. These results suggest that TLR2 is involved in promoting protective immune responses to *Leishmania* parasites during primary infection *in vivo*, and is a potential target for protective and therapeutic vaccine adjuvants. Paradoxically, however, TLR2-targeting lipopeptides Pam2 and Pam3 were ineffective adjuvants for use in a whole-cell vaccine to protect against CL, as whole-cell autoclaved *L. major* (ALM) vaccines containing lipopeptides resulted in exacerbated disease upon challenge when compared to unvaccinated controls and in contrast to effective vaccination when CpG adjuvants were used. The ratio of antigen specific IgG1:IgG2c antibody isotypes, which is a marker of the type of adaptive immune response (Th1 or Th2), was elevated in mice that received vaccines containing lipopeptide adjuvants, suggesting that these adjuvants drive non-protective Th2 responses to *Leishmania*. In a Th2-dependent vaccine model using *Brugia malayi*, the use of Pam2 as an adjuvant resulted in an enhanced protective phenotype with similar efficacy to the Th2-driving adjuvant Alum. Thus, in the context of CL infection TLR2 has a protective role in late-stage primary infections with *L. major* and *L. mexicana*, yet when targeted with lipopeptide adjuvants in whole-cell vaccines promotes exacerbated disease in challenge infections, through driving Th2 immune responses. Lipopeptides that target TLR2, such as Pam2, are therefore more appropriate for use as adjuvants in vaccines where Th2 protective immunity is required.

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Contributors Statements

In accordance with the University of Liverpool's rules, I hereby present the contributions to all chapters:

Chapter 1. Alice Halliday wrote this and Professor Mark Taylor supervised the writing.

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Chapter 3. Alice Halliday performed the scientific experiments, with assistance from Dr Michael Chance with the first infection experiment with *L. major* FV1, and Miss Pamela Pask and Miss Joanna Herriot with the animal work. Professor Mark Taylor supervised the experimental design, work and the writing.

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Publications, Presentations and Conferences

Publications

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Oral Presentations

Halliday A & Taylor MJ., *Leishmania vaccine adjuvants and Toll-like receptors* (May 2011), LSTM Postgraduate Research Day, Marriot Hotel, Liverpool, UK.

Halliday A (one of 5 speakers)., *'Bon Voyage: Travel, Health and Disease': an evening of talks, discussion and demonstrations by scientists* (December 2011), British Society of Immunology Public Event, World Museum, Liverpool, UK.

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Halliday A, Bates PA & Taylor MJ., *Vaccines for Leishmania: The use of lipopeptides as vaccine adjuvants* (March 2011), University of Liverpool Postgraduate Poster Day, University of Liverpool.

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List of Abbreviations and Acronyms

aaMΦ	- Alternatively Activated Macrophage
ALM	- Autoclaved *Leishmania major*
AUC	- Area Under the Curve
BmFE	- *Brugia malayi* female extract
BmL3E	- *B. malayi* L3 extract
BmMfE	- *B. malayi* Mf extract
BSA	- Bovine Serum Albumin
BSU	- Biomedical Services Unit
$CaCl_2.2H_20$	- Calcium chloride dehydrate
CCL3	- Chemokine (C-C motif) ligand 3
CD	- Cluster of differentiation
CL	- Cutaneous leishmaniasis
CLR	-C-type Lectin Receptor
ConA	- Conconavalin A
cMΦ	- Classically Activated Macrophage
CTL	- Cytotoxic T Lymphocyte
CXCL1	- Chemokine (C-X-C motif) ligand 1
CXCL2	- Chemokine (C-X-C motif) ligand 2
DAMP	- Damage associated molecular pattern
DC	- Dendritic Cell
DCL	- Diffuse cutaneous leishmaniasis
DC-SIGN	- Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DLN	- Draining Lymph Node
DMEM	- Dulbecco's Modified Essential Medium
DNA	- Deoxyribose nucleic acid
DPBS	- Dulbecco's Phosphate Buffered Saline
DMF	- Dimethylformamide
DMSO	- Dimethyl sulfoxide
ELISA	- Enzyme-linked immuno-sorbent assay
EN	- Endemic normals
EU	- Endotoxin Units
FBS	- Foetal Bovine Serum
Fc	- Fragment, crystallisable
FcR	- Fc receptor
FTAg	- Freeze-Thaw Antigen
GIPL	- Glycoinositol Phospholipid
GP	- Glycoprotein
GPI	- Glycosylphosphatidylinositol
HBSS	- Hank's Balanced Salt Solution
HEPA	- High-efficiency particulate air
HEPES	- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	- High performance liquid chromatography
H_2SO_4	- Sulphuric acid
HCl	- Hydrochloric acid
HI	- Heat-inactivated
IFN	- Interferon
Ig	- Immunoglobulin
IL	- Interleukin
i.p.	- Intraperitoneal
IPTG	- Isopropylthio-β-galactoside
i.v.	- Intravenous
IVC	- Individually ventilated cages
KCl	- Potassium chloride
KH_2PO_4	- Potassium phosphate monobasic
KMP-11	- Kinetoplastid Membrane Protein – 11
LACK	- *Leishmania* homolog of receptors for activated C-kinase
LBP	- LPS Binding Protein
LF	- Lymphatic filariasis
LHF	- Left Hind Foot
lmr	- *L. major* response gene
LN	- Lymph Node
LRR	- Leucine rich repeat
LRV	- *Leishmania* RNA virus
LSTM	- Liverpool School of Tropical Medicine
MΦ	- Macrophage
MAC	- Membrane Attack Complex
Mal	- MyD88 adaptor-like protein
mDC	- Monocyte-derived Dendritic Cell
2-ME	- 2-Mercaptoethanol
Mf	- Microfilariae
MFI	- Median Fluorescent Intensity
MOPS	- 3(-N-morpholino)propanesulfonic acid
MPL	- 3-*O*-desacyl-4'-monophosphoryl lipid A
MyD88	- Myeloid differentiation primary response 88
NaCl	- Sodium chloride
Na_2CO_3	- Sodium carbonate
$NaHCO_3$	- Sodium bicarbonate
Na_2HPO_4	- Sodium phosphate dibasic
NaH_2PO_4	- Sodium phosphate monobasic
NEAA	- Non-essential amino acids
NLR	- NOD-like receptor
NLRP3	- NOD-like receptor family, pyrin-domain-containing 3
NOD	- Nucleotide-binding oligomerization domain-containing protein
NTC	- No Template Control

NTD	- Neglected Tropical Disease
nTreg	- Naturally-occurring Treg
MCL	- Mucocutaneous leishmaniasis
$MgSO_4$	- Magnesium sulphate
$MnCl_2$	- Manganese (II) chloride
MOPS	- 3-(N-Morpholino) propanesulfonic acid, 4-Morpholinepropanesulfonic acid
MR	- Mannose Receptor
NLRP3	- NOD-like receptor family, pyrin-domain-containing 3
ODN	- Oligodeoxynucleotide
Pam	- Palmitoyl
Pam2	- S-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteine
Pam3	- N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteine
PAMP	- Pathogen-Associated Molecular Pattern
PBS	- Phosphate Buffered Saline
PCR	- Polymerase Chain Reaction
pDC	- Plasmacytoid DC
PG	- Phosphoglycan
PKDL	- Post Kala-azar dermal leishmaniasis
PRR	- Pathogen Recognition Receptor
PSG	- Promastigote Secretory Gel
qPCR	- Quantitative PCR
RbCl	- Rubidium Chloride
RHF	- Right Hind Foot
RNA	- Ribonucleic acid
RPMI	- Roswell Park Memorial Institute
RT	- Room Temperature
s.c.	- Subcutaneous
SDS	- Sodium dodecyl sulphate
SDS-PAGE	- SDS Polyacrylamide Gel Electrophoresis
TBE	- Tris-Borate EDTA
TBST	- Tris-Buffered Saline-Tween 20
TIR	- Toll/interleukin-1 receptor
TLCK	- N-tosyl-L-lysinechloromethyl ketone
TNFα	-Tumour necrosis factor alpha
TLR	- Toll-like Receptor
TMB	- 3,3′,5,5′-Tetramethylbenzidine]
TRAM	- TRIF-related adaptor molecule
Treg	- T regulatory cell
TRIF	- TIR domain-containing adaptor inducing IFNβ
Tris	- Tris base / Trizma
VL	- Visceral leishmaniasis
WMAg	- Washed Membrane Antigen
WT	- Wildtype

Chapter 1. Introduction

Leishmaniasis

Leishmaniasis is a neglected tropical disease (NTD), currently affecting at least 12 million people, with 350 million at risk in 98 countries across the globe (1, 2). Protozoan parasites from the genus *Leishmania* are the causative agents for leishmaniasis, which encompasses a spectrum of disease types which can affect humans and other animal species. Species of the *Leishmania (Sauroleishmania)* subgenus infect lizards, whilst the *Leishmania (Leishmania)* and *Leishmania (Viannia)* parasites infect mammals (3). *Leishmania* parasites are transmitted to mammal hosts via the bite of an infected female sandfly. Manifestations of the disease range from minor self-healing lesions to the fatal visceral disease. The main disease types are: cutaneous leishmaniasis (CL) where lesions form on the skin tissue and are usually self-contained and heal; diffuse cutaneous leishmaniasis (DCL) where cutaneous lesions spread across the cutaneous skin tissue; mucocutaneous leishmaniasis (MCL) where parasites preferentially replicate in the mucosal tissue causing severe tissue destruction; and finally visceral leishmaniasis (VL, also known as Kala-azar) where parasites migrate to the liver and spleen and replicate in macrophages (MΦs) within these organs, and which can be fatal if untreated (4, 5). A rare form of leishmaniasis that can manifest several years after successful treatment of VL is post Kala-azar dermal leishmaniasis (PKDL), which occurs in a varying proportion of VL patients. The outcome upon infection is largely dependent upon the species of *Leishmania*, although other factors also have important roles in disease outcome such as immunity and nutritional status (4). There are 20 identified species which can infect humans, and they can be classified according to their geographical location, their vector species, the sub genus of *Leishmania*, the clade and the disease they are associated with (1, 5). The characteristics of the 12 most important human *Leishmania* pathogens are given in Table 1; note that *L. infantum* and *L. chagasi* are the same species, but they are named differently depending on the geographical location.

Epidemiology and global burden of leishmaniasis

Leishmaniasis is primarily a disease which affects poor communities in developing countries, and as there are very limited resources available for the diagnosis and treatment of the disease, it is considered by some to be one of the most neglected of NTDs (6). Although VL accounts for almost 100% of all deaths attributed to leishmaniasis, CL accounts for the majority of cases. Whilst 98 countries have recently been identified as having endemic transmission of human leishmaniasis, the burden of disease is largely confined to major foci in a smaller number of countries (2). For VL, 90% of cases are found in six countries: India, Bangladesh, Sudan, South Sudan, Brazil and Ethiopia; CL is more widely distributed, with up to 75% cases occurring in Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica and Peru (2). The species responsible for causing the majority of cases of MCL are confined to South American countries, particularly Bolivia, Peru and Brazil (1). It is likely that the real

global burden of leishmaniasis is far greater than the estimated 12 million infected worldwide, as underreporting and a lack of surveillance are major problems in many endemic areas (6). Furthermore, there is diversity in the disease manifestations, transmission sites, vector hosts, animal reservoirs and levels of asymptomatic infection within the field of leishmaniasis, making predicting the burden extremely difficult (6).

Sub-genus	Clade	Species	Associated disease/s	Geographical location
Leishmania (Leishmania)	*L. major*	*L. major*	CL	Old world
		L. tropica	CL	
		L aethiopica	CL	
	L. donovani	*L. donovani*	VL, PKDL	
		L. infantum *	VL	
		L. chagasi *	VL	New world
	L. mexicana	*L. mexicana*	CL, DCL	
		L. amazonensis	CL, DCL	
		L. venezuelensis	CL	
Leishmania (Viannia)		*L. braziliensis*	CL, MCL	
		L. peruviana	CL	
		L. panamensis	CL, MCL	
		L guyanensis	CL	

Table 1. The 12 major *Leishmania spp* to infect humans, classified according to subgenus, clade, associated disease manifestations and geographical location. * N.B. *L. infantum* and *L. chagasi* are the same species, but are named differently depending on the geographical location.

Life cycle

The life cycle of *Leishmania* parasites is shown in Figure 1, and consists of two developmentally distinct stages: the promastigote stage which exists within the sandfly and is extracellular, and the amastigote stage which exists intracellularly within phagocytic cells in the mammalian host. *Leishmania* parasites are transmitted to mammalian hosts by an infected female sandfly when it takes a blood meal. During the feeding process infective metacyclic promastigotes are transmitted to the site of the sandfly bite and are readily engulfed by phagocytic cells such as neutrophils and MΦs. Once inside host MΦs, *Leishmania* parasites transform into the small, non-flagellated amastigote stage and are able to survive and replicate asexually within the phagolysosome compartment. After several rounds of replication, infected cells rupture and release amastigotes, which then infect other surrounding MΦs and cause tissue pathology at the site of parasite replication. Infected MΦs can be taken up into an uninfected female sandfly during feeding on mammalian blood. Within the midgut of the sandfly, amastigotes are released from MΦs and will transform to the extracellular procyclic promastigote stage (7).

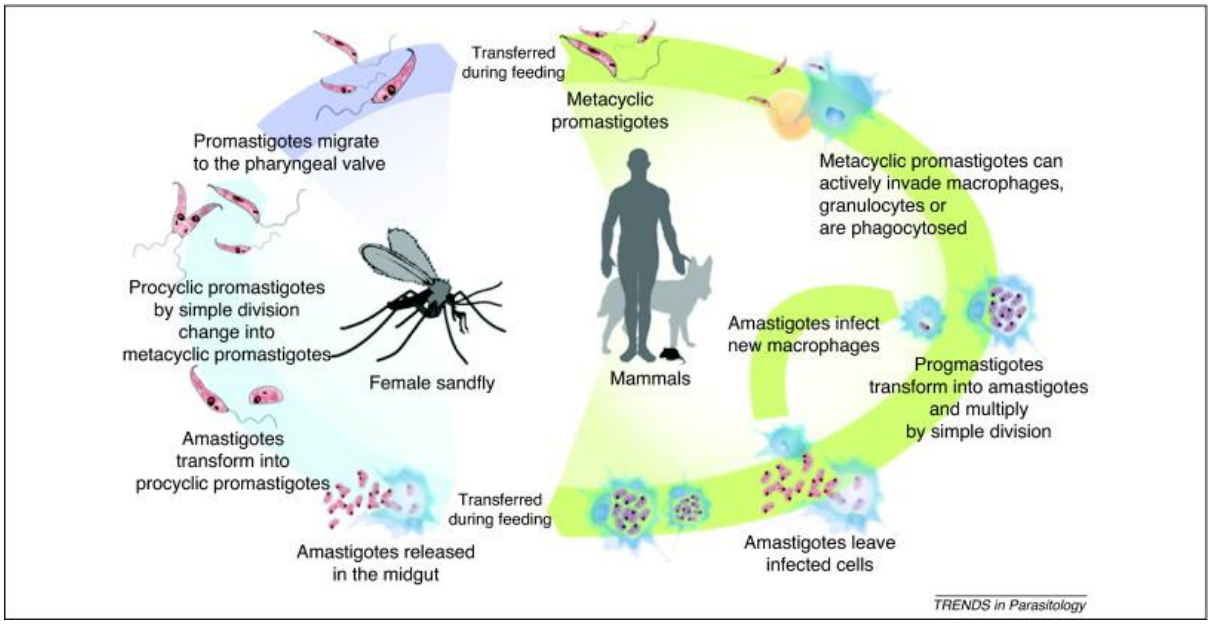

Figure 1. Life cycle of *Leishmania* parasites. Figure taken from (8) with permission.

The first promastigote development stage is a procyclic promastigote, with short flagella, which are able to attach to the midgut wall and divide asexually. As the parasites migrate towards the mouthparts, they divide and mature through distinct morphological forms, until finally transforming into the infective metacyclic promastigote form, which are very motile with long flagella and do not divide (3, 7). These metacyclics are able to infect a new mammal host and continue the life cycle when the sandfly takes the next blood meal.

Overview of *Leishmania* cell biology with a focus on the biochemistry of the parasite surface

The surface of the *Leishmania* parasite plays an important role in the interaction with host cells in both the sandfly and the mammalian host (9, 10). The promastigote and the amastigote have varying quantities and modifications of surface glycoconjugate molecules (see Figure 2), and these differences influence the events after exposure to specific cell types and other immune components. Promastigotes possess a thick outer surface called the glycocalyx, which is almost entirely absent on the amastigote parasite. During metacyclogenesis, modifications are made to the composition of molecules that comprise the glycocalyx, which relate to increased infectivity of the metacyclics to the mammalian host (11, 12). The major surface components are anchored to the parasite membrane by a glycosylinositolphospholipid (GPI) anchor.

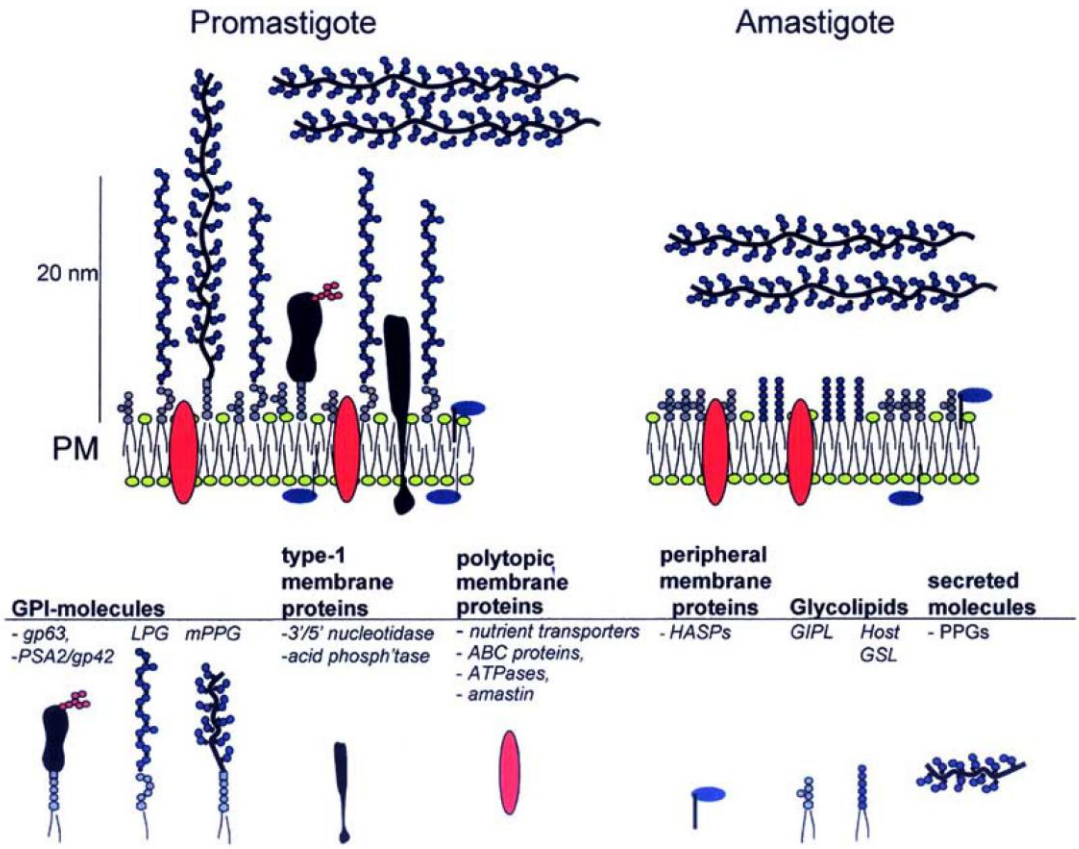

Figure 2. The major surface molecules of *Leishmania* promastigotes and amastigote parasites. This figure is a schematic representation, taken from (9) with permission.

Lipophosphoglycan

Lipophosphoglycan (LPG) is the major macromolecular component of the glycocalyx of the flagellated promastigote form of *Leishmania,* but it is not detectable on the glycocalyx of amastigotes (13). Analysis of purified LPG from *L. major* has shown that the molecule has an unusual *lyso*-1-0-alkylphosphatidylinositol lipid anchor (14), which unlike other GPI-anchored molecules, contains only one alkyl chain. The alkyl chain is an unsaturated hydrocarbon of either C_{24} or C_{26} in length (12). The GPI anchor is linked via the inositol to heptasaccharide glycan core, which is attached to the large phosphoglycan (PG) domain containing 15-30 Gal-Man-P repeating units. The Gal-Man-P repeats can have additional substitutions, which differ between strains and species of *Leishmania* (12). At the end of the PG backbone is a mannose-containing cap. The *lpg1* gene encodes a putative galactofuranosyltransferase (Gal_f transferase), which is involved in the biosynthesis of LPG. Studies with *Leishmania* parasites that specifically lack *lpg1* have shed light on the roles of LPG in different *Leishmania* species. An *L. major* *lpg1*$^{-/-}$ mutant was generated by Späth *et al* which was shown to lack LPG expression, but retain GPI anchored proteins, secreted phosphoglycans (PGs) and GIPLs (15). The *lpg1*$^{-/-}$ parasites had lost the Gal_f –mannose (Gal_f-Man) linkage in the LPG molecule, whereas the same linkage was present in the GIPLs. Indeed, biochemical characterisation of the *lpg1*$^{-/-}$ parasites showed that they had normal levels of secreted phosphoglycans

and GIPLs, suggesting only LPG is affected. Thus, the Gal_f transferase encoded by the *lpg1* is important for biosynthesis of LPG, but not the other major groups of glycoconjugates (15).

It is established that LPG plays an important role in parasite viability within the sandfly vector. *L. major* and *L. donovani* parasites lacking LPG show reduced survival in the sandfly midgut. Whilst the LPG deficient (*lpg1*$^{-/-}$) parasites were able to survive and replicate initially (albeit at reduced levels), they were unable to persist past midgut meal excretion (16). This effect is linked to the ability of LPG to mediate binding to midgut epithelial cells (16).

Several roles have been attributed to LPG within the mammal host, including protection against complement mediated lysis, prevention of phagolysosomal fusion and attachment and uptake to MΦs (12, 15, 17-19). Many species of *Leishmania* drastically modify their LPG structure during metacyclogenesis, and the increased resistance of metacyclic (and log-phase) *L. major* was linked to increased branching of LPG on the surface, and the release of the C5b-9 complex (membrane attack complex, MAC) into culture by the resistant parasites was speculated to be associated with LPG shedding (20). Furthermore, *L. major lpg1*$^{-/-}$ parasites were found to be more susceptible to human serum (i.e. complement mediated lysis) than wildtype (WT) parasites (19). The *L. major lpg1*$^{-/-}$ promastigotes were found to enter mammalian MΦs normally, but unlike the wild type parasites they were destroyed within 2 days (21). Furthermore, although *L. major lpg1*$^{-/-}$ parasites were found to produce lesions in susceptible BALB/c mice, formation of these lesions was delayed (15). However, when the size of inoculum was increased to 5 x 10^7 parasites, there was little difference between *L. major* WT and *lpg1*$^{-/-}$ mutants on onset of lesion formation in susceptible BALB/c mice. Despite the reduction in virulence displayed in the experiments, the mutants were still able to establish lesions, which grew, and mutant amastigotes that were isolated from such lesions are as infectious as the WT, which is perhaps to be expected as LPG is not expressed in this life stage. *L. major lpg1*$^{-/-}$ parasites are more susceptible to complement mediated lysis than WT parasites (19), and were able to infect phox-deficient MΦs as well as WT, suggesting LPG protects *L. major* promastigotes against oxidative burst during phagocytosis by MΦs (19). However, it appears that unlike previous suggestions (11), *L. major* LPG plays no role in complement mediated uptake to macrophages (19).

In contrast to *L. major*, *L. mexicana lpg1*$^{-/-}$ promastigotes are as able as WT parasites to infect and replicate in macrophages *in vitro*, and to infect and replicate in mice as effectively as the WT (22). Consistent with the finding that LPG is not crucial for *L. mexicana* virulence, is the fact that LPG is not altered during metacyclogenesis of *L. mexicana*, whereas it is substantially modified in *L. major* and *L. donovani* (23). In fact, expression of LPG is down-regulated on the surface of infective *L. mexicana* promastigotes (24).

It is unclear why LPG is down-regulated in the mammalian amastigote stage, but as LPG displays stimulatory activities (25-27), it is hypothesised that this is a mechanism of avoiding immune activation in the mammalian host.

GIPLs

GPI anchored glycolipids (GIPLs), are smaller than LPG and are expressed by both promastigote and amastigote forms of *Leishmania*, and are the predominant glycolipid on both the promastigote and amastigote surface. GIPLs are also variable in both their glycan and lipid structures, as with LPG, and differences in abundance and modification exist between the different *Leishmania* species (28). However, a conserved core structure is present, which is a Manα1-4GlcN core linked to an alkyl-acylglycerol or a *lyso*-alkylglycerol through a phosphatidylinositol (28). GIPLs are usually subdivided into three groups (Type I, II and hybrid) based on the location of the R-Manα1-substitution on the proximal mannose. There has been no precise function assigned to GIPLs, although they are likely to be important for the parasite as GIPLs of some form are found on all trypanosomatids (29). It has not been possible to generate mutants that lack all GIPLs specifically, which has hampered our understanding of their functional significance, so the conclusions that can be made about LPG functions are not as easily made for GIPLs.

Gp63

Gp63 is a GPI anchored surface metalloproteinase, which is highly conserved amongst all *Leishmania* species and plays an important role in resistance against the mammalian immune response, particularly in the initial stages after infection. Gp63 binds serum complement components, and is able to convert complement protein C3b to the inactive iC3b form, which acts to both opsonise the parasite for phagocytosis as well as avoid the mechanisms of complement mediated lysis (discussed further below) (30). This is demonstrated partly by the observation that gp63 is down-regulated in the intracellular amastigote stage (9). However, isoforms of gp63 are expressed at low levels in the amastigote stage (31). In addition to its roles in interacting with complement, gp63 has also been reported to cleave host cell surface proteins, such as MHC class I and cluster of differentiation (CD)4 (which suggests a mechanism of host immune response modulation), as well as components of the extracellular matrix (9, 32). However, as the parasite does not exist as a promastigote in the host for long, it is unlikely that gp63 plays a major role in immune modulation during chronic stages of infection. In support of this speculation, *L. major* parasites with the gp63 gene cluster deleted showed more susceptibility to complement mediated lysis in the promastigotes form, but amastigotes were as virulent as WT (33).

Multiple mechanisms of immune subversion

Leishmania parasites possess many other 'virulence factors' which promote their survival in the host, many of which have properties relating to immune subversion, as reviewed in (9, 32, 34). Examples include the cysteine peptidase enzymes, which modulate MΦ activation by interfering with cell signalling pathways (35-37). Importantly, different species rely on individual components to differing extents, and some possess unique virulence factors which relate to the pathology of the disease they cause (21, 32, 38).

Immunology of leishmaniasis

Mouse models of *Leishmania spp.* infection

As *Leishmania* parasites are often able to infect several mammal hosts, it is possible to carry out experimental infections of mice with *Leishmania* species that cause disease in humans, and many of these models closely resemble human clinical disease. In particular, models of CL using *L. major* have been widely exploited as tools for immunological research and have greatly increased out understanding of the immunopathology of leishmaniasis, as well as the adaptive immune responses to intracellular pathogens in general (39). The finding that non-healing BALB/c mice develop a CD4+ T helper 2 (Th2) cell type of immune response to infection, whilst C57BL/6 mice, which heal their lesions, develop a Th1-type response, was instrumental in our understanding of how the adaptive immune response can be tailored towards different types of pathogen. It is now a fundamental understanding in immunology that a Th1 cell-mediated type of response is appropriate for combating infection by intercellular pathogens (e.g. *Leishmania* parasites), whilst Th2 responses are required for protection against extracellular pathogens (39). It is interesting that the understanding of the adaptive immune response involved in *Leishmania* infection preceded our understanding of the innate immune responses in different cell types, which has been a major focus of *Leishmania* immunological research in recent years (40, 41). It is now clear that the mechanisms involved in the immunopathological processes of *Leishmania* infections are extremely complex, involving a number of different host cell types as well as parasite and sandfly factors, and can vary dramatically depending on the parasite species and host genetic background. In this review of *Leishmania* immunology, I will focus on the studies exploring immune responses in CL infection. While some mouse strains (such as the C57BL/6 or CBA mice) develop lesions upon infection with the old-world species *L. major,* which heal within a period of a few weeks (so are called 'healing' mice), they develop non-healing but contained lesions upon infection with new-world species which cause CL, such as *L. mexicana* and *L. amazonensis* (42-44). Contrastingly, BALB/c mice are more susceptible to most species which cause CL, and develop uncontrolled lesion development which leads eventually to the death of the mouse (i.e they are 'non-healing' (42, 44).

The protective Th1 response

As introduced above, the adaptive immune response and CD4 T cells in particular, play a major role in the outcome to infection with *Leishmania* parasites. The early events after infection with *L. major* and how this can lead to a protective Th1 response are summarised in Figure 3 below. At the time of infection, *Leishmania* parasites are first exposed to cells of the innate immune system, such as phagocytic cells and antigen presenting cells (APCs) which play an important role in shaping the adaptive immune response. The roles of different innate immune components are discussed in more detail later, but their role in terms of promoting a protective Th1 response is to drive the expansion of naïve T cells to a Th1

phenotype. CD4 Th1 cells are characterised by their production of the cytokines interleukin 12 (IL-12), interferon-γ (IFNγ) and tumour necrosis factor alpha (TNFα).

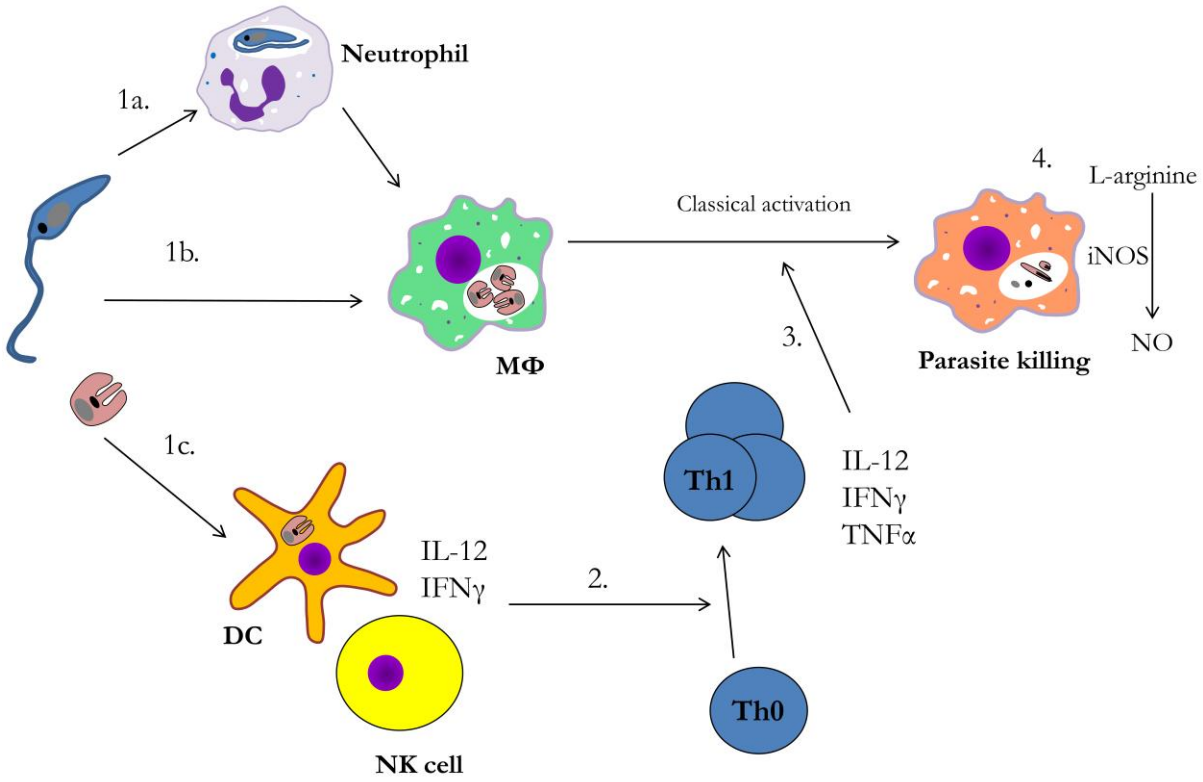

Figure 3. The protective immune response in *Leishmania* infection. Upon infection with *Leishmania*, parasites are taken up by phagocytic cells such as neutrophils, MΦs or monocyte-derived dendritic cells (DCs) (1a, b, c. respectively). Within the phagolysosome of infected MΦs, amastigote parasites can replicate. mDCs and NK cells are activated by *Leishmania* parasites to produce IL-12 and IFNγ (respectively), which promotes the expansion naïve T cells (Th0) to antigen specific Th1 cells (2). Th1 cells produce cytokines such as IFNγ, TNFα which promote the classical activation of infected MΦs (3). Classically activated MΦs produce inducible nitric oxide synthase (iNOS), which breaks down L-arginine to form nitric oxide (NO), which is required for parasite killing (4).

IL-12 is particularly important for development of cellular immunity and a robust Th1 response to *L. major* infection, and indeed to infection with other intracellular pathogens (45). Production of IL-12 by immune cells drives the development of an adaptive Th1 response, and is also important for the maintenance of an effective Th1 response during the course of *L.major* infection (46). IL-12 receptor depleted mice on a resistant/healing background are unable to control *L. major* infection (47), and administration of IL-12 to susceptible BALB/c mice confers resistance (48, 49). In more natural low-dose infections, it is likely that IL-12 acts to redirect early Th2 responses to *L. major*, as IL-12 production does not occur at high levels in the initial stages of *L. major* infection even in healing mice, and growth of *L. major* is the same for the first 4-5 weeks of infection, in the presence or absence of IL-12 (50). The role of IL-12 in protection against new world CL is not as clear as for *L. major*. Administration of IL-12 cannot cure infection of C57BL/6 mice with *L. amazonensis* as it can with BALB/c mice infected with *L. major*

(51). Furthermore, mice lacking IL-12p40 showed progression of lesions to the same extent as WT mice when infected with *L. mexicana*. The lack of IL-12 did not affect the production of IFNγ by draining lymph node (DLN) cells collected 7 weeks after infection, whereas production of IFNγ is substantially diminished in IL12p40$^{-/-}$ mice infected with *L. major* when compared to WT mice at the same time point (52). The production of IL-4, a non-protective Th2 cytokine, was slightly increased in response to parasite antigen in *L. mexicana* infected mice which lacked IL-12p40 compared to WT mice (52). A vaccination study in a mouse model of *L. amazonensis* infection showed that mice lacking IL-12 were just as protected as WT mice when vaccinated with the killed *Leishmania* vaccine 'Leishvacin ®' in association with *Corynebacterium parvum* as an adjuvant (53). Therefore, IL-12 does not appear to be as crucial for the control of *L. mexicana* complex parasites as it is for *L. major* (54).

Key effector cytokines in the Th1 response that leads to parasite clearance are IFNγ and TNFα. Both of these cytokines are important in activating infected MΦs for intracellular killing (classical activation), by causing an upregulation of inducible nitric oxide synthase (iNOS) production, which breaks down L-arginine to producing nitric oxide (NO) (55) (Figure 3). Healing mice with the TNFα gene deleted are very susceptible to even low numbers of *L. major*. Furthermore, these mice develop a disease in which the parasite disseminates as in VL, and the infection is eventually fatal (56). The importance of NO in controlling *Leishmania* has long been known, as mice lacking iNOS are extremely susceptible to infection with *L. major* (55). In models of leishmaniasis where lesions heal, a robust Th1 response is found, characterised by increased production of IFNγ, TNFα and iNOS (57). Early IFNγ production is also important for the development of a robust Th1 response. Natural killer (NK) cells are known to be an important source of IFNγ during infection with *L. major*, and of particular importance in the early innate immune response to infection (58, 59). In addition to its role in enabling parasite killing by NO, production of IFNγ plays an important role in down regulation of Th2 and regulatory immune responses, which are detrimental to controlling infection. This is demonstrated in mouse models where anti-IFNγ antibodies administered to genetically susceptible mice prior to infection with *L. major* resulted in a Th2 response to infection and more severe disease (44). Similarly, a protective role for TNFα was demonstrated when administration of anti-TNFα antibodies caused exacerbated infection in C3H and BALB/c mice infected with *L. major*, whilst recombinant TNFα reduced disease severity in both strains (60).

In general, levels of IFNγ are much lower in resistant/healing mice infected with *L. mexicana* parasites compared to those found in *L. major* infection of resistant/healing mice (52). As with *L. major* infection, iNOS is an important factor for control of *L. mexicana*, as iNOS$^{-/-}$ mice develop lesions which grow uncontrollably and are progressive, and parasite numbers continue to increase throughout the infection, as opposed to being contained at a steady state (52). STAT4 is a transcription factor, which is important for development of Th1 responses. As with iNOS and IFNγ, a lack of STAT4 leads to uncontrolled

parasite growth and lesion progression in *L. mexicana* infected mice on a C57BL/6 background (52). Thus, a Th1 response, which occurs independently of IL-12, appears to be responsible for control of *L. mexicana* and other new world species. It therefore appears that whilst effector cytokine responses are relatively low during *L. mexicana* and *L. amazonensis* infection compared to *L. major*, these low levels are required to control the slow growing *L. mexicana* parasites, and limit lesion progression to controllable levels.

Immune responses that promote parasite survival

The production of IL-4 early on in infection is key to the development of an inappropriate Th2 response to *L. major* infection in BALB/c mice, as IL-4$^{-/-}$ BALB/c mice heal infection, as do BALB/c mice treated with anti-IL-4 monoclonal antibody at the time of infection (61, 62). The source of this early IL-4 in this model has been identified as an oligoclonal population of T cells possessing a Vβ4Vα8 T cell receptor, which are responsive to the *Leishmania* homolog of receptors for activated kinase (LACK) antigen (63). IL-13 is also important for maintenance of a Th2 response to *L. major* infection, and acts in much the same way as IL-4 to potentiate Th2 development (64), and can compensate for IL-4 in experiments where IL-4 is depleted. Resistant/healing mouse strains, however, also show production of IL-4 in the initial phase of *L. major* infection from the same T cell population, but are able to redirect an early Th2 response by production of IL-12. Thus, it is the inability of BALB/c mice to redirect an initial Th2 response to a Th1 response, which is key to their susceptibility to *L. major*. A number of factors have been implicated in the sustained Th2 responses in BALB/c mice, including a down-regulation of IL-12 receptor (IL-12R), an increased inflammatory cell infiltrate, intrinsic defects in Th1 differentiation by CD4 T cells and innate immune cells, and an inability to prevent parasite dissemination (reviewed in (39, 65)).

The regulatory cytokine IL-10 is now known to play as important a role in susceptibility to *L. major* as IL-4. While for some strains of *L. major*, removal of both IL-4 and IL-13 responses from BALB/c mice is not enough to confer resistance, removal of IL-10 in addition to these two Th2 cytokines does render mice resistant to *L. major*, and IL-10$^{-/-}$ mice on a BALB/c background show reduced lesions and parasite burdens (66). Naturally occurring CD4+ CD25+ T regulatory cells (nTregs) which produce IL-10 are able to confer susceptibility to *L. major* infection in otherwise healing mice, suggesting that nTregs are a major source of suppressive IL-10 (65). However, other studies indicate that IL-10 derives from other cell types also, and that nTregs may suppress resistant mechanisms in other ways, such as contact dependent mechanisms (67). Interestingly, in addition to their roles in suppression of protective responses, it appears that nTregs are important in maintaining concomitant immunity to *L. major*, by suppressing responses that clear parasites, but allowing development of immunological memory and protection against re-infection with *L. major* (68). Whilst C57BL/6 mice are considered resistant to *L. major* (lesions show an acute phenotype and heal), *L. major* parasites persist in the skin of these mice after healing. This persistence is achieved due to the induction of nTregs, which suppress effector Th1 CD4 T cells from

completely clearing the infection (68). However, when mice lack suppressive mechanisms in the form of IL-10, the immunity to re-infection is lost, compared to WT mice which show a significantly reduced parasite load upon a second infection (68). Thus, regulatory activity appears to have a role for both parasite persistence, and long term immunity for the host (Figure 4). In addition, Th1 cells can produce high levels of regulatory IL-10 when they are over-activated in a highly inflammatory setting, when infected with a virulent strain of *L. major* (69).

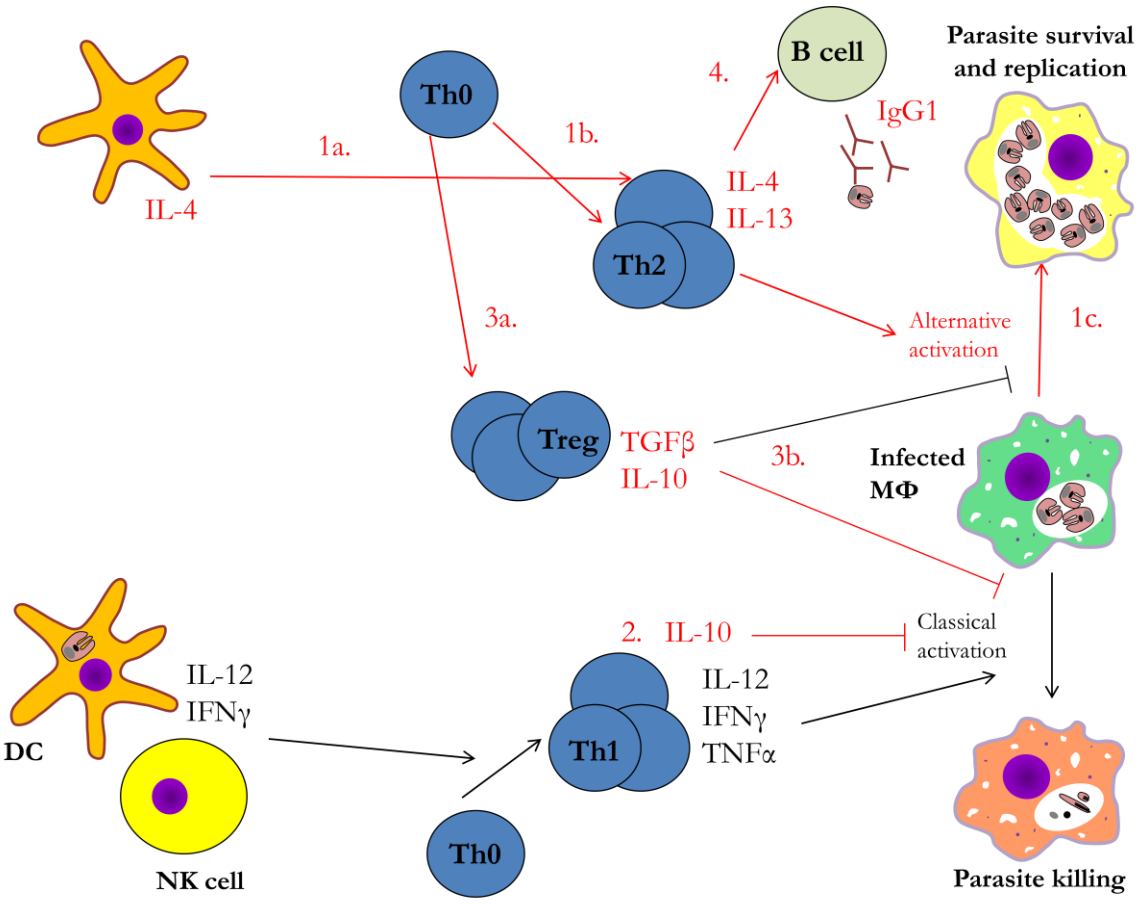

Figure 4. The adaptive immune responses associated with susceptibility and non-healing in CL. The adaptive immune responses that leads to parasite killing is indicated below (labelled in black), with the production of IL-12 and IFNγ promoting the expansion of Th1 cells, which produce cytokines (IFNγ, TNFα) that drive the classical activation of MΦs leading to parasite killing. Alternatively (labelled in red), DCs and other APCs exposed to *Leishmania* parasites may drive the expansion of specific Th2 cells by production of IL-4 (1a.); Th2 cells producing IL-4 and IL-13 (1b.) drive the alternative activation of infected MΦs, which by producing arginase, breaks down L-arginine to polyamines, which allows for parasite survival and growth (1c.). Parasite killing is also suppressed by the production of IL-10 by highly activated Th1 cells (2). In addition, the expansion of T regulatory cells (Treg) (3a.) leads to the production of regulatory cytokines TGFβ and IL-10 which act to suppress either or both Th1 and Th2 cells (3b), which can lead to either increased parasite survival or reduced disease exacerbation depending on the model. B cells producing antigen specific IgG1 antibody (4) facilitates parasite survival in a mechanism whereby ligation of IgG1 bound parasites to the FcγRIII receptor promotes IL-10 production.

In the C57BL/6 and *L. mexicana* infection model, IL-10 plays an important role in the chronicity of the infection (70). A healing response was observed in mice lacking IL-10 that was mediated in part by IL-

12p40, which has previously been shown to play no role in disease progression. This suggests that IL-10 acts to suppress the Th1 response mediated by IL-12p40 in WT C57BL/6 mice (70). IL-10 production during infection with *L. mexicana* is thought to be due to ligation of antibody specific to amastigotes binding to receptors which bind the crystallisable fraction of antibody (Fc receptors, FcRs) of MΦs. Primed MΦs produce greater levels of IL-10 when exposed to antibody opsonised *L. mexicana* amastigotes, when compared to un-opsonised amastigotes (70). Furthermore, mice deficient in the antibody receptor FcRγ showed the same phenotype as IL-10$^{-/-}$ mice when infected with *L. mexicana* (70). Thus, this specific immune response mechanism, whereby antibody binding to amastigotes promotes dissemination of parasites into uninfected MΦs and promotion of IL-10, greatly contributes to the chronicity of *L. mexicana* complex infections (52, 70, 71).

Recent studies have added further complexity to the adaptive immune factors that are involved in the immunopathogenesis of CL infections. The Th17 pro-inflammatory CD4$^+$ subset has been associated with susceptibility to *L. major*; the IL-17$^+$ CD4$^+$ T cells present in high numbers in *L. major*-infected susceptible BALB/c mice are thought not to influence the Th1/Th2 development, but instead to increase susceptibility in these mice due to increasing neutrophil recruitment (72, 73). Th9 cells, which are considered by some as a subset of Th2 cells, have been linked to increased susceptibility in *L. major* infection, as have follicular helper T cells (Tfh) which act to facilitate B cell activation which has been previously linked with increased susceptibility to infection (70, 71, 73-75).

Early events after infection, and interaction of *Leishmania* parasites with different cell types

Once within the mammalian host, *Leishmania* promastigotes immediately encounter the complement system, and are quickly taken up by phagocytic cells, particularly neutrophils, MΦs and dendritic cells (DCs). The mechanism of uptake has been attributed to a number of different receptor mediated interactions, which differ between parasite species and morphological forms, and between different host cell types in different environments, as reviewed in (10).

MΦs are the important location of parasite replication in leishmaniasis

Leishmania parasites are phagocytosed by three main cell types: MΦs, neutrophils and some DCs. But it is within MΦs that *Leishmania* parasites are able to replicate, and they are able to do this within the harsh environment of the phagosome, which has adapted to destroy ingested pathogens. MΦs actively phagocytose pathogens, apoptotic cells and other debris and they also play an important role in priming adaptive immune response by presenting antigen to T cells. *Leishmania* parasites can be taken up by MΦs by a number of different mechanisms and they then become the final definitive site for parasite replication (76). Promastigotes are less able to withstand the harsh conditions of the MΦ compared to amastigotes, and so instead they delay the maturation of the phagosome to allow them to transform to

the amastigote, which is better adapted for survival in the MΦ. The characteristics of the promastigote-containing phagosome therefore differ to those which harbour amastigotes, as reviewed in detail by Moradin and Descoteaux (34). LPG is the major promastigote molecule that has been linked with the ability to delay phagosomal maturation (77).

In their steady state, MΦs exist in tissues and are very efficient at phagocytosis. They can be activated by internal and external signals and depending on the signals they will assume a particular activated phenotype (Figure 4). The two major types of activated MΦ are: 1) classically activated MΦs (cMΦ), which develop in response to TLR ligands and Th1 cytokines (particularly IFNγ) and are designed to kill intracellular pathogens via production of NO and 2) alternatively activated MΦs (aaMΦ), which are associated with Th2 responses and are important in wound healing and immune responses to helminth infections (78). Whereas cMΦs produce the enzyme iNOS, which breaks down L-arginine to form NO, aaMΦs use the enzyme arginase, which breaks down L-arginine to form polyamines. In *Leishmania* infections, the phenotype of the infected MΦ has a crucial impact on infection, with cMΦs allowing for sufficient parasite killing (55) and aaMΦs being associated with increased parasite growth (76, 79) (Figure 4). Indeed, in addition to the multiple mechanisms of immune subversion employed by *Leishmania* to prevent or shift the immune response away from the classical activation of MΦs, it has recently been shown that *Leishmania* possess their own arginase enzyme which augments aaMΦ activation and promotes parasite survival (79, 80). Infected MΦs can promote a protective Th1 response by acting as APCs and producing IL-12, but the ability of MΦs to produce IL-12 is impaired upon infection with *Leishmania* parasites (17, 81). The balance of the activation status of the infected MΦs in *Leishmania* infection, which is influenced by many different host and parasite derived factors, is therefore what determines the eventual outcome of infection with *Leishmania* parasites (76).

The role of neutrophils

Neutrophils are innate immune cells that are particularly important for early defence against pathogens and are rapidly recruited to inflammatory sites. In leishmaniasis, a massive recruitment of neutrophils to the site of infection occurs following inoculation of parasites whether infection is mediated via needle or sandfly (and in either case in the absence of parasites) (82, 83). Recruitment of neutrophils to the infection site is achieved by the local production of IL-8 in humans, and chemokine (C-X-C motif) ligand 1 (CXCL1)) and CXCL2 in mice (84). Interestingly, even at this early stage the phenotypes of neutrophils exposed to *L. major* infection appear to be different between healing and non-healing mouse strains. For example, the expression levels of the Toll-like receptors (TLRs) TLR2, TLR7 and TLR9 are greater in *L. major*-exposed neutrophils from mice which heal leasions, when compared to those from a non-healing strain (85). Furthermore, the ability of the exposed neutrophils to attract DCs to the site of infection is greater in healing C57BL/6 mice, compared to non-healing BALB/c mice (86). The mechanism of DC recruitment by neutrophils is believed to be via the production of the chemokine (C-C motif) ligand 3

(CCL3), as CCL3 deficient mice on a resistant background failed to recruit DC to the infection site at the level of WT mice, an effect which was reversed upon transfer of WT neutrophils (86). Lack of neutrophil-derived CCL3 during this initial phase of infection hindered the development of an effective Th1 response mediated by $CD4^+$ T cells in mice resistant to *L. major* (86). A study using a model of *L. braziliensis* infection found that neutrophils were important mediators for control of parasite growth *in vivo* and *in vitro* via their ability to induce MΦs to kill intracellular amastigotes via cell-contact as well as production of TNFα (87). Furthermore, neutrophils can account for the earliest source of IL-12 in response to infection (85).

Whilst the presence of neutrophils at the site of infection has been known for a while, the importance of phagocytosis of promastigotes by neutrophils occurring prior to ingestion by MΦs in the initial phase of infection has only recently been realised. A number of studies have shown that *L. major* and other species are able to survive inside neutrophils and remain infective to other cells (82, 88, 89). *In vivo* imaging of *L. major* injection to the mouse ear dermis has revealed that injected promastigotes are relatively immobile after they have been injected into the mammalian host, and they are rapidly ingested by the neutrophils which have migrated to the site of infection. Mice depleted of neutrophils before infection with *L. major* showed a reduction of viable parasites in the infected tissue, both immediately after infection, but also weeks after, suggesting that the early presence of neutrophils is important for disease progression in this model (82). Specific markers can be used to identify which of the distinct neutrophil granules are employed in the attack against *Leishmania* within the infected neutrophil. It appears that azurophilic (primary) granules are selectively fused with the phagosome containing the *Leishmania* parasite, but that fusion with specific and tertiary granules does not occur (89). Thus, *L. major* is able to avoid parasite killing via respiratory burst and acidification in the neutrophil, as in the MΦ. *L. major* is also known to delay the apoptosis of neutrophils, perhaps by interfering with the caspase-3 pathway (90). Nevertheless, neutrophils infected or exposed to *L. major* do still undergo apoptosis, which allows a mechanism of 'silent' uptake into MΦs, which rapidly engulf apoptotic cells without activating antimicrobial effector mechanisms (91). Indeed, it has been shown that human MΦs are able to ingest *L. major* infected apoptotic neutrophils, and that this resulted in the release of TGF-β, an anti-inflammatory cytokine (88). Thus, in addition to their role as an innate effector cells in fighting the infection, it is believed that neutrophils can also exacerbate infection by acting as 'trojan horses' for *Leishmania* parasites, by providing them with a temporary home as well as a safe entry into the MΦs, in the initial stages of infection (91). However, *in vivo* studies have yet to provide evidence for MΦ ingestion of infected neutrophils (82). Whether neutrophils play a protective or exacerbating role in leishmaniasis therefore appears to depend on the model used, and may be species dependent (41).

DC subsets play distinct roles in *Leishmania* infection

DCs are professional APCs which play an important role in antigen uptake and presentation to T cells, and thereby act as the sentinels of the immune system. It is therefore unsurprisingly that in CL, DCs have been shown to be crucial for the priming of a protective Th1 response. There are many different subsets of DCs which are present in different tissue sites and can play differential roles in initiating adaptive immune responses. Different subsets of DCs have been shown to have differential ability to produce IL-12 upon infection with *Leishmania* parasites *in vitro* (92), and the interactions between parasites and DCs vary greatly between species of *Leishmania*, and also depend the host genetic background (reviewed in (93)). Langerhans cells reside in the epidermis and were initially thought to play an important role in *Leishmania* infection *in vivo* (94), but this was disputed by another study which found that antigen presentation by Langerhans cells, MΦs or B cells was not required for resistance to *L. major* and instead dermal DCs were the crucial DC subset for priming Th1 cells (95). Plasmacytoid DCs (pDCs) are recruited to the infection site, but they do not phagocytose *Leishmania* parasites. Despite this, pDCs are likely involved in controlling infection to *L. major*, as adoptively transferred pDCs were able to provide protection to recipient mice (96). pDCs are activated by *Leishmania* antigens to produce IL-12 and IFNαβ, and this is the mechanism by which pDCs are believed to contribute to the control of infection (97). Leon *et al* reported that monocytes recruited to the site of *L. major* infection in C57BL/6 mice differentiate into monocyte-derived DCs (mDCs) and it was found to be these mDCs that were crucial for priming Th1 cell development, via the production of IL-12, after they had migrated to draining lymph nodes from the site of infection (98). Differences between DC functions in healing and non-healing mouse strains has been explored in mouse models of CL, but there is yet to be conclusive evidence that such differences account for the differential phenotypes that occur upon infection (76).

Natural killer cells are important for the protective response to *Leishmania*

Natural killer (NK) cells are innate immune cells which play an important role in clearance of tumour cells, rejection of tissue transplants and pathogens that infect host cells. The effector functions of NK cells are the destruction of target cells and release of inflammatory cytokines such as IFNγ, TNFα and GM-CSF (99). NK cells are recruited to the infection site within 24 hours of infection with *L. major*, and their recruitment is mediated in part by CXC10 (84). IL-12 production is crucial for activating this early NK cell response (97). Activated NK cells act to control *Leishmania* infection by providing an early source of IFNγ, and by associating directly with myeloid DCs (mDCs) to activate them and help promote a Th1 response (100, 101). In turn, mDCs are crucial for mediating the NK cell response, as mice depleted of mDCs, but not pDCs, depleted *L. infantum* infected mice of an NK cell response (97).

Wound repair as an alternative mechanism involved in parasite clearance

The polarization of host immunity has become a dogma for explaining the patterns of disease progression in models of leishmaniasis. This has recently been questioned by an analysis of the genes from 3 distinct

loci involved in infection with *L. major,* which differ between healing C57BL/6 and non-healing BALB/c mice. The loci were termed *L. major* response - (*lmr-*) *1, lmr-2* and *lmr-3*. Studies on the effects of these distinct loci on the course of *L. major* infection have shown that their influence on disease outcome are independent of adaptive immune responses (102). Mice congenic for *lmr* genes from donor strains have been used to explore the functions of these loci. Resistant C57BL/6 mice with the *lmr* genes from BALB/c mice are less able to control disease with *L. major*, whilst BALB/c mice with *lmr* genes from C57BL/6 mice are more resistant (103, 104). Interestingly, healing of non-infected wound tissue is also affected by the *lmr* loci genes, attributing the role of these loci to a healing response in general (104). Resistant mice are able to deposit collagen at a faster rate than non-healing mice, and this activity is linked to the *lmr1* and *lmr2* loci (103). When a natural model of infection was used to explore the role of the *lmr* genes, where small numbers of *L. major* were injected intradermally into the ear, the *lmr* loci were shown to exert their effects on wound healing locally at the site of infection, and independently of the lymph node and systemic responses (103). Thus, it appears that a wound healing responses to *Leishmania* infection can influence disease progression and outcome, and that this occurs independently of the immune response.

The role of the sandfly bite and promastigote secretory gel (PSG)

An important role for the sandfly bite in the outcome of *Leishmania* infection was first demonstrated in 1988 by Titus *et al*, when it was found that the co-injection of lysates of the salivary glands of the sandfly vector *Lutzomyia longipalpis* resulted in the exacerbation of infection with *L. major* (105). This effect was later attributed in part to a peptide from sandfly saliva called maxadilan, which is a vasodilator and promotes Th2 cytokine production over Th1 cytokines by exposed human PBMCs (106). It has also been shown that maxadilan can alter the phenotype of MΦs towards an alternatively activated state (107), as well as influence DC function and T cell activation (108), which may help explain its ability to exacerbate infection. Other components of saliva have also been linked to its immunmodulatory properties, such as AMP and adenosine (108). It is important to note that sandfly saliva is immunogenic, and individuals living in areas where sandflies are prevalent will likely have been exposed and so have secondary immune responses to salivary proteins. These immune responses will also play an important role in the immune responses to an infection. In some cases, levels of antibodies to salivary components have been linked to enhanced protection (such as for protective responses to VL infection in infants in Brazil (109), whilst several studies have linked increased anti-saliva antibodies to increased susceptibility to CL in different settings(108).

In addition to the effects of the sandfly saliva, an important feature of *Leishmania* infected sand flies is the presence of the promastigote secretory gel (PSG), which forms a 'plug' in the anterior regions of the midgut around which the infective metacyclic promastigotes accumulate in the latter stages of infection (110). Importantly, when a comparison was made between the injection of *L. mexicana* parasites by needle

or sandfly to murine hosts (using a comparable dose), the sandfly-transmitted infections showed more profound disease progression in two strains of mouse. Furthermore, sandfly transmission resulted in the formation of chronic non-healing lesions in CBA/Ca mice, whereas an acute disease phenotype was observed upon needle challenge (111). The main factor involved in this sandfly bite-mediated disease exacerbation is the parasite-derived filamentous PPG (fPPG), which comprises most of the PSG plug. More specifically, the glycan moieties of fPPG are responsible for exacerbating disease (111). The mechanism of disease exacerbation has been attributed to the ability of PSG to recruit MΦs to the infection site in the skin, and further to facilitate survival within MΦs by increasing alternative activation and arginase production, which enhance parasite growth (112). The sandfly derived infection was also found to increase the recruitment of neutrophils to the site of infection, which acted to favour parasite survival in a model of *L. major* infection (82).

These findings demonstrate a clear role for the transmission of *Leishmania* parasites by sandfly vectors in the outcome of infection. The infectious dose administered by sandfly can range from a few hundred to around 10,000 parasites per bite, and averages at approximately 1000 per bite (111, 113). This is lower than the doses administered in most experimental models where a needle is used, where doses can be as high as 10^7 per injection. Furthermore, sandfly-transmitted promastigotes were found to be highly enriched in metacyclic forms (111). The mode of transmission should therefore be taken into account when considering the outcomes of infection in an experimental model setting.

Current methods of treatment and control of leishmaniasis

Leishmaniasis in humans is primarily treated in a clinical setting by using one of a handful of chemotherapy options available. Pentavalent antimonials (e.g. sodium stibogluconate and meglumine antimoniate) are the most widely used treatments, as they are the WHO recommended first-line of treatment (4), and they have been used to treat the disease for over 70 years. Miltefosine is also a common treatment choice, particularly as resistance to antimony is increasing in major endemic areas such as the Indian subcontinent. A newer chemotherapy option is Amphotericin B, which was initially inappropriate for widespread use due to its high cost and toxic effects. Fortunately, these issues have in recent years been alleviated by the development of the less toxic liposomal form of delivery (AmBisome ®), and the reduction in price of the drug to 10% of the original cost in developing countries (114). Despite these developments, treatments are costly and difficult to administer, and are still not accessible for many affected individuals living in remote areas and/or in poverty.

The most widely used methods of prevention focus on interruption of transmission by eliminating/treating animal reservoirs, and/or by targeting the sandfly vectors. There is currently no vaccine for human leishmaniasis which is widely available. Our in-depth understanding of the immune responses involved in *Leishmania* infection, and those which are required for control of infection, together

with evidence of long-term protective immunity following natural exposure, should facilitate and promote the development of an effective vaccine, or a variety of vaccines, for the diseases associated with leishmaniasis.

Vaccine development for leishmaniasis

Leishmanisation

Leishmanisation is the practice of purposefully inoculating uninfected individuals with live *Leishmania* parasites, and it has been practiced for centuries in many endemic areas, in order to prevent natural infection in visible sites on the body (115-117). In most cases, this practice was highly successful in causing very mild disease which healed and provided long-term immunity to subsequent infection. In fact, this approach is still the most effective method of prevention ever tried in human populations, as up to 100% protection was reported in one small clinical trial using challenge with live *L. major* as a vaccine in Iran (116). However, due to issues over quality control, safety and the increasing prevalence of immune disorders such as HIV, the use of Leishmanisation has been discontinued in many endemic countries (118). Nevertheless, it provides important proof-of-concept that vaccination is a viable intervention for leishmaniasis.

Killed *Leishmania* vaccines

First generation vaccines containing killed parasites have been explored extensively over decades in an attempt to develop a safe and effective vaccine for leishmaniasis. Whilst this approach may be feasible and scalable, issues over standardisation exist with the use of cultured parasites, which would be a major barrier for the registration of a vaccine (118). Studies in mice and humans have found killed parasites to be safe and immunogenic vaccines, but with poor efficacy in protecting against leishmaniasis (41, 118).

New strategies for leishmaniasis vaccine design

Developments in vaccine design in recent years have led to the use of many different types of experimental vaccine in models for leishmaniasis, some of which are outlined below.

Subunit vaccines

Subunit vaccines involve the use of defined immunogenic molecules, singularly or in combination, which have advantages over the use of killed parasites in terms of standardisation, scalability and cost. Several candidate antigens have been identified by different research groups. Gp63 was one of the first candidate antigens, which when delivered with various adjuvant formations demonstrated efficacy in some, but not all, mouse models and human immunogenicity studies (119-122). Other antigens that have been used singularly in experimental subunit vaccines include LACK, gp46, cysteine proteases and kinetoplastid membrane protein 11 (KMP-11), which have also demonstrated varying levels of efficacy in models (41,

120, 121, 123, 124). In recent years the field has moved towards using more than one antigen in subunit vaccines. A recombinant polyprotein containing several immunogenic and conserved proteins of *Leishmania*, Leish-111f, has been used in many experimental models and in Phase I and II human clinical trials, and has been found to be safe and immunogenic (125-128), and improve treatment outcome when used in combination with treatment with antimonials (126). Other vaccines containing a combination of protein antigens, such as the Leish-KSAC vaccine, are being evaluated in pre-clinical studies (129).

DNA vaccines

A relatively new approach to vaccine design has been the use of genetically modified DNA containing the candidate antigen(s) of interest. Many of the single or polymeric antigens that have been explored for use in subunit vaccine preparations have been tested as DNA vaccines. This method has several attributes which make it desirable for a *Leishmania* vaccine, such as being able to activate cytotoxic T lymphocyte (CTL) responses (130, 131). Whilst this approach seems favourable given the promising findings in terms of efficacy and long term immunity elicited from DNA vaccines in rodent experimental models (130-136), DNA vaccination has not yet been approved for use in humans, and it is not clear if similar levels of efficacy are achievable in a DNA vaccine in humans (114, 123).

Sandfly saliva as a vaccine target

Given that the immune response to saliva has been shown to influence outcome upon infection in various endemic areas, as well as in rodent models of infection (108), components of saliva have become an exciting target for *Leishmania* vaccine development. Several groups have explored different approaches to anti-saliva vaccines, and as a result some key target antigens have been identified. Interestingly, a study by Oliveira *et al* found that DNA immunisation with two distinct saliva antigens conferred different immune responses in mice, which lead to either increased resistance or disease exacerbation upon challenge (137). Antigens which have shown promising results in vaccine studies are LJM11 and LJM19 of *Lutzomyia longipalpis*, and PpSP15 of *Phlebotomus papatasi* (108). However, there are issues that need to be overcome in terms of the influence of long term exposure to sandfly bites, genetic and antigenic variation in salivary proteins amongst sandfly populations and as with other Leishmania vaccines, defining reliable immune correlates of protection.

Other approaches

Other approaches that have been adopted in vaccine studies for *Leishmania* include DC vaccines (138, 139), vaccines against salivary components, the use of attenuated/genetically modified *Leishmania* parasites, and incorporation of *Leishmania* antigens into a viral vector, all of which have resulted in good efficacy in pre-clinical vaccine studies (123, 129, 140).

Immune correlates of protection

An important aspect of vaccine discovery and design is identification of measureable immune responses which relate/correlate to the level of protection against the disease in question. Identifying so-called immune correlates of protection is important in vaccine development as it is not always desirable or possible to test all vaccines in terms of efficacy to protect against disease (such as for HIV infection), but it may be possible to test for the immune correlates of protection. It also greatly informs the design of new vaccines by allowing researchers to choose vaccine approaches and components which promote the responses that are associated with protection.

In recent years, multiparameter flow cytometry has been used to analyse the type of immune response which confers protection post vaccination to infectious agents (141). This technique allows for analysis of qualitative cytokine production at a single cell level and has revealed that multifunctional $CD4^+$ T cells which simultaneously produce IL-2, TNFα and IFNγ show the highest correlation with protection against infection with *L. major* post vaccination (142). Darrah *et al* used a live *L. major* inoculation as a positive control (as this remains the best known method of protection), as well as other Leish-111f vaccine formulations. They found that the number of triple cytokine producing (multifunctional) $CD4^+$ T cells generated in response to vaccination correlated with the level of protection (142). Raman *et al* developed a mouse model for immunotherapeutic vaccines, in which Leish-111f formulations were tested on *L. major* infected mice. In contrast, this study found that multifunctional $CD4^+$ T cells were not produced in the highest levels in the group that showed the best responses to the therapeutic vaccine (143). Instead, the mice that received the most effective therapeutic vaccine showed the lowest number of multifunctional $IL2^+$ $TNF\alpha^+$ $IFN\gamma^+$ $CD4^+$ T cells, but the greatest number of terminal effector, IFNγ-producing $CD4^+$ cells (144). Furthermore, contrary to the view that a strong Th1 response will be required for protective immunity with *Leishmania* vaccines, the immune response generated by vaccination with the attenuated *L. major lpg2*$^{-/-}$ vaccine strain, was not associated with a strong Th1 response (145). In a vaccine model exploring the use of a live vaccine (comparable to that used in humans for leishmaniasation) in mice, which was shown to be more effective than a killed vaccine at protecting against sandfly-mediated challenge with *L. major*, the enhanced protection was linked to an absence of neutrophils at the site of infection upon challenge (146). A study using a DNA vaccine encoding the LACK antigen observed that protection and long term immunity was dependent on CD8+ T cells (131). Therefore, there is currently little or no consensus on the optimal immune correlates that are associated with protective immunity to *Leishmania* vaccines and our current knowledge demonstrates a complex array of diverse immune responses associated with different vaccination approaches and models. Further studies to define the characteristics of protective immune responses against *Leishmania* vaccination that are predictive of protective immunity in humans is therefore warranted.

Adjuvant discovery and design

A key development which is required for all new vaccines is adjuvant discovery and design. It is hoped that our increasing understanding of innate immune responses to *Leishmania* and their role in disease outcome will inform vaccine adjuvant design and selection for new *Leishmania* vaccines. Toll-like receptors (TLRs) are important components of the innate immune system and targets for adjuvant action and development. Investigating the role of TLRs in *Leishmania* should inform vaccine adjuvant design for *Leishmania* vaccines.

Toll-like receptors

In 1991 Charles Janeway speculated that the immune system must use a mechanism to discriminate between 'infectious non-self and non-infectious self', and proposed that this was achieved by receptors that were able to recognise microbial patterns (147). Since then, there have been major developments in immunology research to corroborate this, including the discovery of many types of pattern recognition receptors (PRRs) which are responsible for sensing molecular patterns from external sources, particularly microbial organisms. TLRs were the first group of PRRs to be discovered, and they include 13 different receptors (10 found in humans, 12 in mice) responsible for recognising a wide range of pathogen associated molecular patterns (PAMPs), from nucleic acids to large surface glycoprotein structures, from all types of microbe (148, 149). TLRs can be subdivided into groups according to their cellular location. TLR3, TLR7, TLR8 and TLR9 are intracellular TLRs, whilst TLR1, TLR2, TLR4, TLR5, TLR6, TLR10 and TLR11 are present on the cell membrane. In this review of TLRs, their ligands, activation and function, special attention will be given to TLR2 and its co-receptors.

Pathogen-derived TLR ligands

Many different microbial PAMPs have been identified as ligands for TLRs, and they are summarised in Figure 5 below. Intracellular TLRs recognise the DNA and RNA of viruses, as well as the DNA of bacterial and protozoan microbes. Surface glycogen and protein containing PAMPs of all classes of microbe are recognised by extracellular TLRs. TLR2 has been implicated in the recognition of a wide range of PAMPs from all four major classes of microbe, including virus glycoproteins, bacterial lipoproteins and lipopeptides, zymosan from fungi and GPI anchored molecules from protozoan parasites (148).

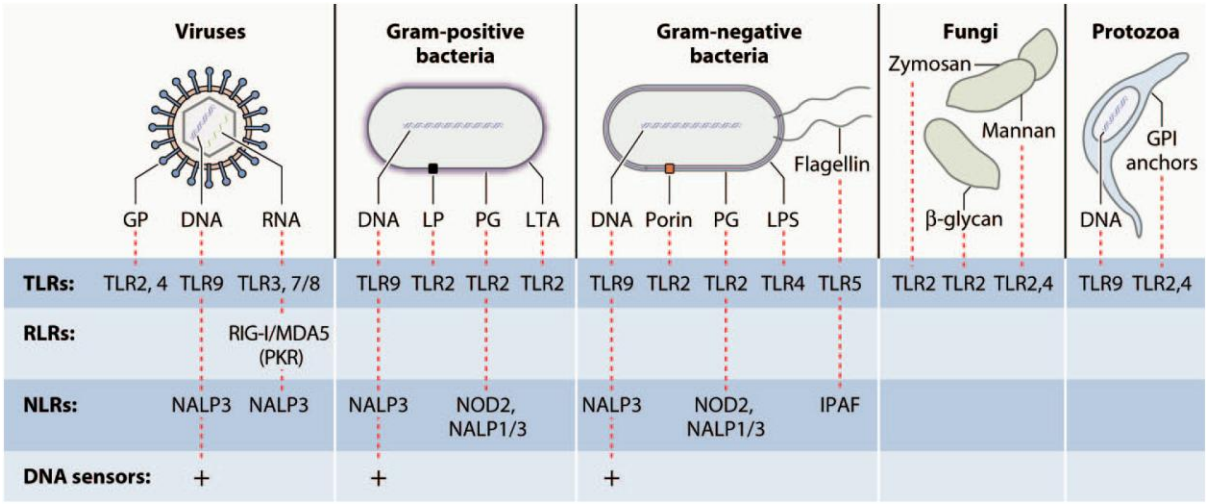

Figure 5. TLRs and other PRRs recognise a diverse range of PAMPs from different classes of microbes. Figure taken from (150) with permission.

Endogenous TLR ligands

The term 'endogenous ligand' was coined when heat shock proteins (HSP) were found to cause the production of proinflammatory cytokines by host cells, and this activity was linked to TLR4 complex activation (151). Since then, many other non-microbial molecules which are capable of activating TLRs and other PRRs, termed damage-associated molecular patterns (DAMPs), have been identified (152). However, the ability of HSP preparations to activate TLR4 was later attributed to LPS contamination (153). Indeed, a number of proposed endogenous TLR ligands may also have shown TLR activity as a result of contaminating bacterial ligands (154).

The majority of the DAMPs that have been linked to TLR activation are either extracellular matrix (ECM) components, released intracellular components, or modified lipids. In most cases the reported TLR activity is via TLR2 or TLR4 (152), which are the receptors for the common contaminants of biological preparations, lipopeptides and endotoxin (LPS). Whilst many studies have used routine tests to exclude the activity of such contaminants, many of these tests have been found to lack the specificity and robustness to definitively exclude contamination with some of the most potently active molecules known. Indeed, there are reports that when using only low endotoxin reagents, no TLR activity was found when using endogenous oxidised phospholipids or low density lipoproteins (LDL) in their assays (155). Erridge proposes that many endogenous ligands may act to increase TLR activity to exogenous ligands such as LPS, either by directly binding ligands and enhancing their binding to TLRs, or by increasing cellular sensitivity to the ligands (155). Nevertheless, there remains a growing body of evidence from more recent studies that some host-derived molecules can directly stimulate immune responses in a TLR dependent fashion. Such endogenous ligands are often produced upon cell death or injury (hence the term DAMPs), although ligands from tumour cells have also been reported (152).

TLR structure, activation and signalling

The structure of TLRs can be broken down into 3 main components: the extracellular domain, which contains leucine-rich repeats (LRRs), the transmembrane domain, and the intracellular Toll/interleukin-1 receptor (TIR) domain. In response to recognition of a ligand, the extracellular domains of TLRs form dimers, an event that is required for activation and subsequent downstream signalling. TLRs can either form homodimers or heterodimers, depending on the specific TLR. Note that intracellular TLRs do not strictly speaking have an 'extracellular' domain, as this domain exists within the endosome inside the cell.

TLR2 forms heterodimers with either TLR1 or TLR6 in response to presence of a ligand. The current paradigm in relation to the specificity of TLR2 to lipopeptides, is that recognition is dependent on the heterodimer formed, with TLR2/1 responding to triacylated lipopeptides and TLR2/6 to diacylated lipopeptides (156). Until recently, these two complexes were believed to be the only functional TLR heterodimers, but in 2009 Stewart *et al* reported the formation of the TLR4/6 heterodimer in response to CD36 binding of altered endogenous components (157), and TLR11/12 heterodimers have recently been purified from mouse cells (158). Binding of ligands to TLRs often requires cooperation from soluble components and/or co-receptors. For example, for LPS activation of TLR4, a series of components are needed for binding and activation (159). LPS binding protein (LBP) is a serum glycoprotein which can bind LPS and cause its removal from the bacterial membrane, LBP subsequently presents LPS to the cell surface receptor CD14. CD14-bound LPS can be presented to the LPS receptor complex, which consists of a homodimer of TLR4 and an MD-2 bound to each TLR4 molecule. Both CD36 and CD14 are also co-receptors for TLR2 heterodimers. Soluble CD36, binds negatively charged diacylglycerol ligands extracellularly, and can deliver these ligands to TLR2/6 via membrane bound CD14 (160), but is not thought to be involved in the binding of triacylglycerol ligands of TLR2 (161). The discrimination of different lipopeptide structures has been explored by Omueti *et al* with the aid of synthetic structures with varying numbers, and positioning, of acyl groups. Surprisingly, it was not the number of acyl groups that defined TLR1 or TLR6 co-receptor activity, but the chirality of the group(s) in relation to the central carbon atom (162). A monoacylated synthetic lipopeptide $PamCSK_4$ was found to weakly activate TLR2/1, and R isomers were found to be more potent ligands than S isomers (162). Naturally occurring lipopeptides of bacteria possess specific chiralities and acylation patterns, and thus the dogma that diacyl lipopeptides are recognised by TLR2/1 and triacyl lipopeptides by TLR2/6 remains true for these ligands. Whether TLR2 can be activated without either TLR1 or TLR6, and if this occurs *in vivo*, remains unclear. Whilst diacylated lipopeptides have been shown to activate cells deficient of TLR6 or TLR1 (163), this does not rule out any compensatory co-receptor activity from the TLR which is not knocked out. The crystal structure of the TLR2/1 complex, bound to the synthetic triacylated lipopeptide ligand, Pam_3CSK_4 (Pam3) was determined by Jin *et al* in 2007 (164). Structural studies of ligand-TLR complexes have shed light on the specific components involved, and interactions between the various components in the

binding complex. The mininal TLR2 stimulatory component of lipopeptides has been identified as the N-terminal acyl glyceryl cysteine (165).

There is a need for structural studies of more TLR-ligand complexes, such as for parasite GPI anchors and TLR2, in order to determine exactly which patterns are recognised by each complex, and how additional factors such as co-receptors are involved in complex formation with different ligands. Thus, there remain gaps in our knowledge about the structural determinants involved in TLR2 activation by different PAMPs. It is thought that when TLRs dimerise upon activation, a subsequent dimerisation of the intracellular TIR domains occurs, and this initiates recruitment of the adaptor proteins (166) (Figure 6).

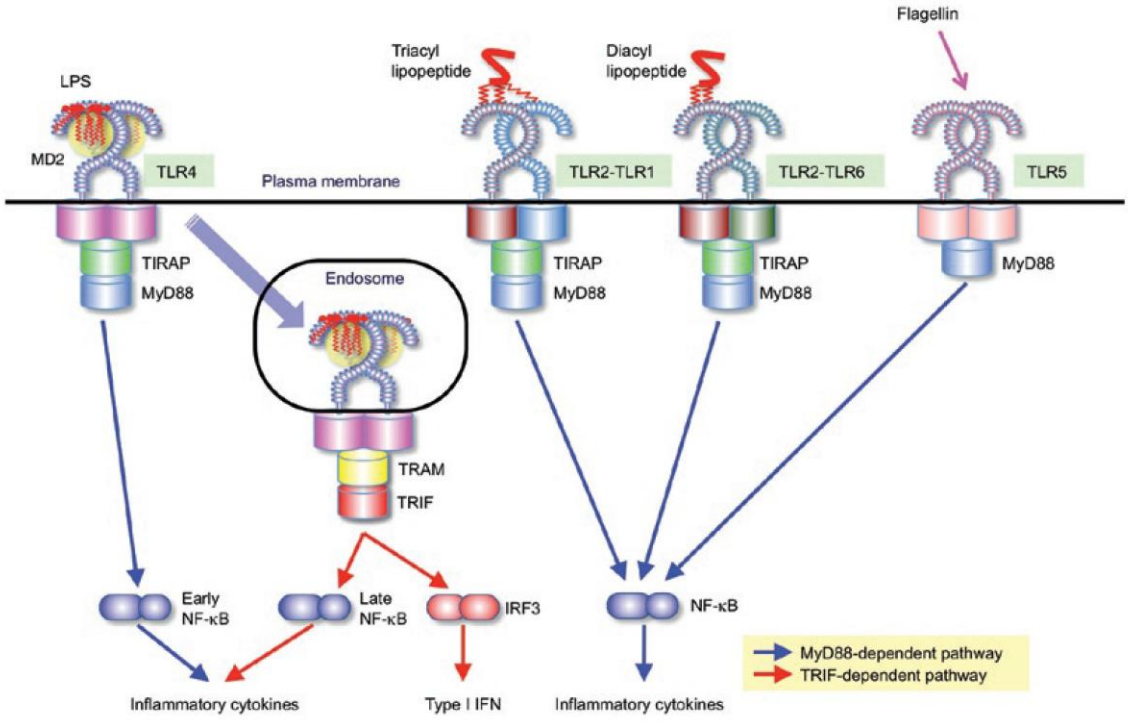

Figure 6. Recognition of bacterial PAMPs by surface TLR complexes. LPS is recognised by a homodimer of TLR4 in combination with MD-2, diacylated and triacylated lipopeptides are recognised by TLR2/1 and TLR2/6 heterodimers respectively, and flagellin is recognised by a homodimer of TLR5. Upon dimerization of the TLR receptors and formation of these complexes, activation of adapter molecules such as TIRAP and MyD88 (all these TLR complexes), or TRAM and TRIF (TLR4 only) results in the activation of NF-κB, leading to production of inflammatory cytokines. Figure taken from (148) with permission.

Adaptor proteins are recruited to the TIR domains leading to induction of a signalling cascade; these include Myeloid differentiation primary response 88 (MyD88), MyD88 adaptor-like protein (Mal), TIR domain-containing adaptor inducing IFNβ (TRIF), and TRIF-related adaptor molecule (TRAM). These adaptor molecules contain a TIR domain, which allows for their binding to the TIR domain of activated TLRs. MyD88 is involved in the signalling events associated with all TLRs with the exception of TLR3,

where a TRIF-dependent pathway is utilised. TLR4 mediates signalling via either MyD88 or TRIF, whilst all remaining TLRs (TLR2/1, TLR2/6, TLR5, TLR7, TLR8, TLR9) signal via a MyD88-dependent pathway (148). After recruitment of the various adaptor molecules, a number of kinases are activated, and eventually transcription factors (such as NF-κβ) are activated to enter the nucleus and upregulate expression of genes encoding pro-inflammatory mediators.

TLRs play a role in adaptive immune responses

Although speculated by Janeway in 1989 (167), it wasn't until 1997 that Medzhitov and Janeway found that adaptive immune responses could be triggered by the recognition of PAMPs via a 'human homologue of Drosophila Toll', now known as TLR4, by triggering the expression of co-stimulatory molecules and proinflammatory cytokines (168). This was later confirmed *in vivo* by the characterisation of the TLR4 gene locus in mice, and the finding that defective TLR4 genes in two strains of mice made them susceptible to *E. coli* infection due to their inability to respond to LPS and mount an appropriate immune response to infection (169). Since the discovery of multiple other TLRs, their ligands, and the exploration of their role *in vivo*, it is now clear that TLRs play crucial roles in the priming of adaptive immune responses in many different settings (170). Given their crucial role in innate and adaptive immune responses to pathogens, it is unsurprising that TLRs have been found to play roles in resistance and/or susceptibility to infection with a wide range of microorganisms (discussed further below). However, despite great progress in terms of the characterisation of TLR ligands, binding complexes and signalling pathways, the precise factors involved in the initiation of different types of adaptive immune response *in vivo* still require further elucidation (170, 171).

TLR crosstalk and interaction with other PRR signalling

It is now becoming clear that the scale and type of resulting immune response to a pathogen or other stimulus is determined by a combination of different signals recognised by a wide variety of PRRs (172). C-type lectins (CLRs) are a sub-family of carbohydrate-binding proteins, which contain a conserved calcium-dependent carbohydrate recognition domain (173). Important CLRs that are involved in the innate immune recognition of microbes are Dectin-1, DC-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN; present in humans, not mice), SIGN-R1 and Langerin. Both DC-SIGN and SIGN-R1 are CLRs, which have been linked with promoting Th2 responses (173). NOD-Like Receptors (NLRs) are intracellular proteins, expressed mainly by APCs and epithelial cells, which can bind to microbial proteins, usually bacterial cell-wall components, within the host cell cytosol. Upon binding of PAMPs to NLRs, signalling cascades are activated which result in changes of gene transcription, and results in inflammation. Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is an NLR, which recognises muramyl dipeptide of bacterial peptidoglycan, and upon activation up-regulates the transcription factor NF-κβ and promotes inflammatory responses (174).

Zymosan and β-1,3-glucan are fungal PAMPs, which activate innate immune cells by interacting with both TLR2 and Dectin-1 (175). Initially reported to result in inflammatory responses, these TLR2/Dectin-1 activating fungal ligands have recently been shown to activate regulatory responses in APCs, which promote antigen specific tolerance (176). However, β-glucan ligands from fungal microbes have also been shown to activate Th1 and Th17 responses through Dectin-1, in the absence of TLR/MyD88 signalling (177). In M*ycobacterium tuberculosis* infection, NOD2 and TLR2 act synergistically to induce inflammatory cytokine production. But as NOD2 and TLR2 double deficient mice are still able to control *M. tuberculosis* infection, it is believed other PRRs are also important in this infection (172). Thus, multiple PRRs from different families can be activated at the same time within one cell, and the interactions between the signalling events that occur can result in various different responses to the stimulus/stimuli. This allows the innate immune system to act as a comprehensive and selective surveillance network that can sense microbial invasion and potential danger signals in a number of different ways.

The role of TLR2 in bacterial, viral and fungal infections

The major TLR2 ligands characterised so far are lipoproteins of bacteria. Whilst all bacteria express lipoproteins, they are found abundantly on the surface membrane of Gram-positive bacteria in particular (178). TLR2 activation has been linked to the outcome of infection with different pathogenic bacteria. TLR2$^{-/-}$ mice infected with *M. tuberculosis* presented with increased bacterial loads and succumbed more readily to infection (179). In *Staphylococcus aureus* infection, TLR2 activation during infection was linked with susceptibility to disease, as LTA binding to TLR2 increased IL-10 production, which resulted in decreased MHC II presentation (180). There is also increasing evidence that TLR2 plays an important role in the protective immune response to certain viral infections. For example, TLR2$^{-/-}$ mice are more susceptible to infection with Cytomegalovirus (CMV), and this increased susceptibility was attributed to an impaired NK cell response (181).

In fungal infections, TLR2 is involved in the innate recognition of fungal cell walls in addition to TLR4 and Dectin-1 (178). The combined activation of TLR2, Dectin-1 and the NOD-like receptor family, pyrin-domain-containing 3 (NLRP3) inflammasome by fungal pathogens is important for an adequate induction of IL-1β response, which helps leads to clearance (182), indicating an importance for PRR synergy in immune responses to fungal pathogens. Studies using TLR2 deficient mice have yielded opposing results in terms of the importance of TLR2 in clearance of infection with fungal pathogens, but they consistently report a role for TLR2 in a pro Th1 inflammatory response to infection (178).

TLRs in parasitic disease

Parasite derived TLR ligands

Many *in vitro* studies have attributed TLR activation activity to parasite PAMPs. One of the more studied ligand groups are GIPLs and GPI anchors from Trypanosomes, which have been known to stimulate inflammatory immune responses for some time. In 1997 Camargo *et al* found that *T. cruzi* derived GPI mucin preparations were found to stimulate NO and IFNγ production by exposed MΦs and could also facilitate intracellular killing of *T. cruzi* or *Leishmana spp* (in conjunction with IFNγ), but that *Leishmania* derived LPG or GIPLs were unable to potentiate NO responses (183). Almeida *et al* demonstrated that highly purified GPI mucins from *T. cruzi* trypomastiogotes, but not epimastigotes, were potent activators of inflammatory responses, and are active at nanomolar concentrations (184). Structural comparisons between trypomastigote and epimastigote GPIs suggest that the activity of the bioactive GPIs can be attributed to the unsaturated fatty acid in the alkyacylglycerolipid component (184). Subsequent studies have attributed the pro-inflammatory activity of GPI anchors and GIPLs from *T. cruzi* to activation of TLR2 in combination with CD14 (185), and as a heterodimer with TLR6 (186). Although earlier studies suggest that TLR4 is not involved in GIPL recognition (185), TLR4 in combination with MD-2 has since been reported to respond to ceramide-containing GIPLs from *T. cruzi* (187).

A number of *in vitro* studies have identified TLR2-dependent activation of inflammatory responses by *Leishmania* LPG (26, 27). Similarly to *T. cruzi* GIPLs, *L. major* LPG was unable to elicit a TLR2 mediated response from MΦs when the lipid portion was removed (26), indicating that the lipid of the unique GPI anchor of LPG is responsible for TLR2 signalling. However, de Veer *et al* reported that TLR2 activation by other *Leishmania* glycolipids, which also contain GPI anchors, did not occur. Another interesting finding from this study was the lack of TLR2 activation from *L. mexicana* LPG, in comparison to *L. major* LPG, which contradicts the idea that the lipid moiety is responsible for TLR2 signalling, as this is identical in both species (29). Becker *et al* report TLR2-dependent activation of NK cells by *L. major* LPG (27). This study demonstrates that LPG from infective metacyclic *L. major* was a more potent activator of TLR2 than LPG from procyclic *L. major*. As the modifications of LPG that occur during metacyclogenesis involve addition of glycan chains to the phosphosaccharide repeats, this finding suggests that this region is somehow involved in TLR2 recognition of LPG (27). There is a need to elucidate further whether LPG is an authentic ligand of TLR2, and what specific components of LPG are required for this activity.

GPI anchors from *Plasmodium falciparum* (causative agent of malaria) have been shown to activate TLR2/1 heterodimers, and both TLR2 and TLR4 have been linked to inflammatory responses mediated by *Toxoplasma gondii* GPI anchor structures (188). There is increasing evidence therefore, that protozoan GPI-anchored molecules can activate cells in a TLR-dependent fashion. As with all studies on TLR ligand

characterisation, the risk of contaminating structures or the use of supraphysiological concentrations may lead to erroneous designations of authentic TLR-ligand binding.

TLR9 is an intracellular TLR, which is activated by unmethylated CpG DNA, and was first identified as a receptor for bacterial DNA. However, recent evidence indicates that TLR9 is also stimulated by protozoan DNA, as *L. infantum, T. cruzi* and *T. brucei* DNA activated DCs and MΦs in a TLR9 dependent fashion (97, 189, 190). A non-DNA ligand for TLR9 has been proposed in *Plasmodium spp* infections, in the form a haemozoin, a product of digestion of haemoglobin by the malaria parasite (191). However, it has since been shown that haemozoin does not activate TLR9 directly, but can enhance TLR9 activation by delivering malaria DNA to the receptor (188, 192). As discussed above, there is a need for the structural components involved in TLR-parasite ligand interaction to be determined, as they have been for some bacterial ligands.

In vivo evidence for a role of TLRs in parasitic disease

There is increasing evidence therefore, that protozoan ligands can activate TLRs using *in vitro* studies. Infection experiments employing knockout mice lacking TLRs or signalling pathway components have explored the roles of TLRs in parasite infection and disease dynamics.

Trypanosoma cruzi

TLR4 has been implicated in the control of *T. cruzi in vivo*. C3H/HeJ mice, which do not express functional TLR4, were found to be more susceptible to *T. cruzi* infection than C3H/HeN mice, which have functional TLR4 molecules (193). A role for GIPLs in TLR4 responsiveness was determined *in vivo*, as injection of GIPLs into C3H/HeJ mice resulted in the absence of an inflammatory response (193). Despite *in vitro* evidence that GPI mucins from *T. cruzi* trypomastigotes stimulate TLR2, infected TLR2$^{-/-}$ mice developed *T. cruzi* infection in a similar way to WT mice, and were able to generate robust immune responses (194). MyD88$^{-/-}$ mice however, show reduced immune responses during *T. cruzi* infection, and were more susceptible to infection than WT and TLR2$^{-/-}$ mice (194). TLR9$^{-/-}$ mice are more susceptible to *T. cruzi* infection than WT mice, showing an increased parasitaemia and mortality levels, although not to the same extent as MyD88$^{-/-}$ mice. When both TLR2 and TLR9 are absent from the murine host (TLR2$^{-/-}$/TLR9$^{-/-}$ mice) however, the parasitaemia increases to levels equivalent to MyD88$^{-/-}$ mice (189). These results are important for indicating a cumulative effect of TLR signalling during parasitic infection; whilst TLR2 signalling alone appears to have no effect on disease outcome in *T. cruzi* infection, TLR2 and TLR9 signalling combined is linked to an increased level of resistance (189).

Toxoplasma gondii

T. gondii is an apicomplexan protozoan parasite which resides intracellularly in infected mammalian hosts. It can infect many types of mammal and is known to be present in over 60% of the human population, causing asymptomatic infection in most cases. Disease can occur after *T. gondii* infection in certain

settings, such as when the host is immunosuppressed or when primary infection occurs during pregnancy when infection of the unborn child can result in preterm abortion (195). TLRs were implicated in resistance to *T. gondii* in mouse models by the finding that MyD88$^{-/-}$ mice are susceptible to *T. gondii* infection and rapidly succumb to the disease, and this is linked with their inability to produce IL-12, when compared to resistant WT mice, which have a strong IL-12 response to the parasite (196). TLR2, TLR4 and TLR11 were implicated as they have been shown to recognise the *T. gondii* parasite, or parasite-derived PAMPs *in vitro* (197, 198). TLR11 was found to recognise a profilin-like protein from *T. gondii* and is required for IL-12 production in response to *T. gondii* by DCs (198). However, a recent study found that the previously uncharacterised TLR12 was also involved in the recognition of *T. gondii* profilin-like protein and could function as either a homodimer or heterodimer with TLR11 to respond to the parasite (158). Its function as a homodimer in pDCs in upregulating IL-12 production was found to be crucial for resistance in mice, and was more important than the TLR11 function (158).

Leishmaniasis

The TLR signalling components that have been linked to *Leishmania* infections are summarised in Figure 7. The first studies to indicate a role for TLRs during *Leishmania* infection involved the use of mice deficient in MyD88. Mice lacking MyD88 (on a C57BL/6 background) were found to have increased susceptibility to *L. major* infection (26, 199). Interestingly, the disease progression in these MyD88$^{-/-}$ mice was similar to that of the susceptible BALB/c strain, where progressive lesion development is coupled with an inappropriate Th2 immune response (199). Thus MyD88 signalling is an important part of developing a Th1 response in mice which are able to heal lesions. MyD88$^{-/-}$ mice also had exacerbated disease when infected with *L. braziliensis* in a separate study (200), and MyD88 signalling was found to be necessary for maturation of DCs during *L. donovani* infection (201), indicating that MyD88 is important for protection in different species of *Leishmania*. These results indicate that TLRs may play an important role in resistance to *Leishmania* infection, but as MyD88 is also involved IL-1 signalling, a TLR-independent role for MyD88 in these models cannot be ruled out.

Two studies by Kropf *et al* explored a possible role for TLR4 in *L. major* infection and found that TLR4$^{-/-}$ mice on a C57BL/6 background had larger lesions and greater parasite burdens during the early stages of infection (202, 203). However, these mice did resolve the lesions, indicating that TLR4 alone does not contribute to the level of susceptibility seen in MyD88$^{-/-}$ mice. Infection with *L. pifanoi* resulted in an increased parasite burden in mice lacking TLR4 compared to WT mice, at one week post infection (204). The disease progression was not monitored for longer than 1 week in this study, so the full infection and disease dynamics of the TLR4$^{-/-}$ mice in this model remains unclear. Interestingly, a novel glycoprotein complex of *L. pifanoi*, proteoglycolipid complex P8 (P8 PGLC), was found to be a ligand of TLR4, indicating a direct role for TLR4 in parasite recognition during infection (204). These results should be

taken with caution however, as it is well-known that proposed TLR ligands are often mistaken findings due to the contamination of trace endotoxin or other PAMPs (154).

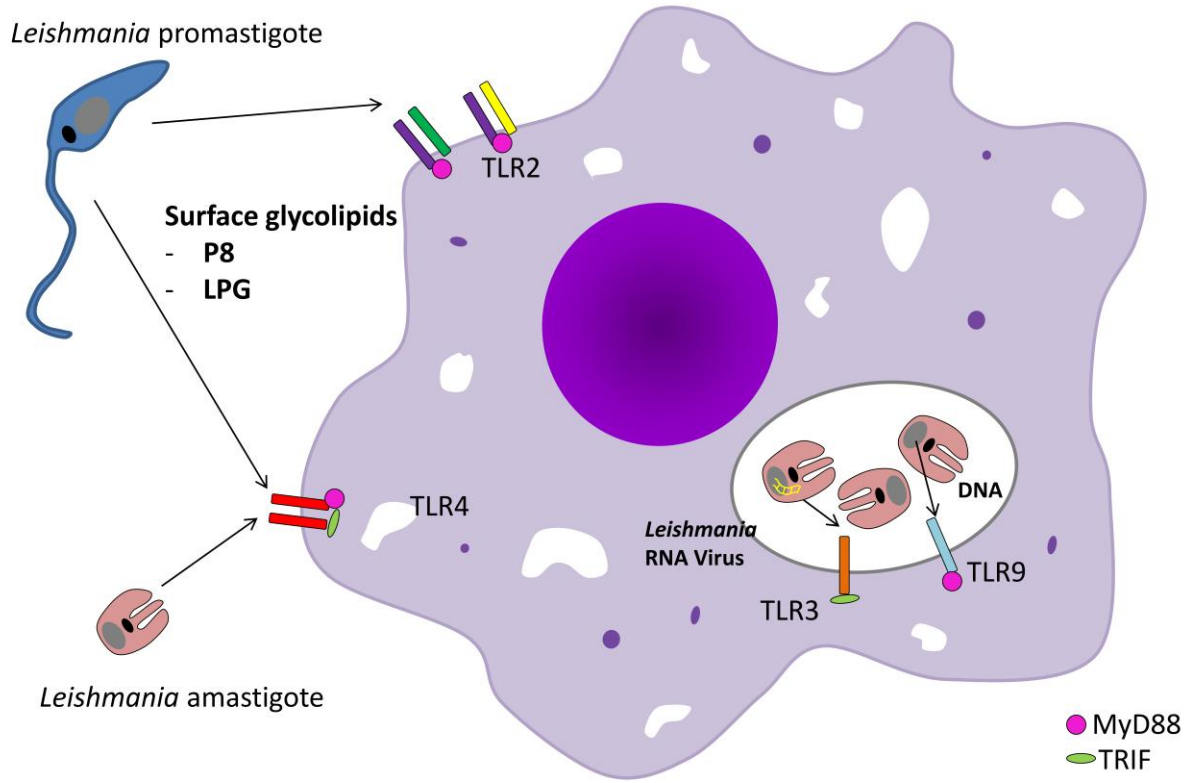

Figure 7. The role of TLRs in *Leishmania* infection. Various studies have identified a role for TLRs in the recognition of *Leishmania* parasites *in vitro* and *in vivo*. The surface TLRs TLR2 and TLR4 can be activated by surface glycolipid and glycolipid complexes, such as LPG and P8 (26, 204, 205) and TLR4 plays a role in disease outcome *in vivo* (202, 203). Intracellular TLRs TLR9 and TLR3 sense DNA from the *Leishmania* parasite: TLR9 recognises unmethylated DNA from the parasite and this plays an important role in priming a protective immune response to infection *in vivo* (97, 206, 207), whilst TLR3 is stimulated by the *Leishmania* RNA virus and this has been linked to the increased immunopathology (metastases) in infections by parasites with higher titres of the virus (208, 209).

It has been widely hypothesised that TLR2 may be responsible for the role of MyD88 during infections with *Leishmania spp*, as LPG has been found to act as a ligand of TLR2 *in vitro* (see above). However, contrary to expectations, the lack of TLR2 increased resistance to infection with *L. braziliensis*, as TLR2$^{-/-}$ mice developed smaller lesions and TLR2$^{-/-}$ dendritic cells induced stronger immune responses to parasites *in vitro* (200). These results suggest that TLR2 somehow exacerbates disease during *L. braziliensis* infection, and perhaps that the parasite exploits TLR2 signalling to promote its growth. However, as TLR2$^{-/-}$ mice showed only increased lesion size, but no increase in parasite burden or duration of lesion formation (200), the role of TLR2 in this model appears to be one of reducing tissue damage in the lesion, as opposed to direct control of parasite growth and dissemination.

Abou Fakher *et al* explored the role of TLR9 during *L. major* infection of C57BL/6 mice by using knockout strains and found that TLR9$^{-/-}$ mice had higher levels of parasitaemia and larger lesions than

WT mice (207). Furthermore, DCs lacking TLR9 were not activated to prime a protective Th1 immune response when exposed to *L. major* parasites or DNA, further indicating an important role for TLR9 in this model, and that TLR9 activation may be the mechanism for driving protective immune responses (207). In a model of *L. infantum* infection, TLR9 activation of mDCs was found to be crucial for the activation of NK cells, and their production of IFNγ (97).

Recently, a role for an RNA virus, which infects some species and strains of *Leishmania* (the *Leishmania* RNA virus, LRV) in the immunopathology of new world MCL has been identified (208). Although LRV was detected in *Leishmania* parasites several decades ago, it was not thought to play any role in the outcome of *Leishmania* infection until recently (209). Ives *et al* identified that *L. guyanensis*, a new world species of *Leishmania* that causes CL and MCL, results in differing pathologies depending on the strain of parasite. A strain that was associated with increased metastatic disease (MCL) was found to have higher titres of LRV, and this resulted in hyper-activation of immune response via activation of TLR3 by the LRV, leading to exacerbated disease (208).

TLR ligands as vaccine adjuvants

Advances in our understanding of innate immune responses and the crucial role they play in shaping adaptive immunity has led to increased interest in trying to understand how existing effective vaccines, and in particular adjuvant components, work. Whilst adjuvants used to be considered 'the immunologist's dirty little secret' (167) as it was not understood how they were able to boost immune responses to antigens, we are now uncovering the mechanisms of adjuvants which have been previously used, or are currently licensed for use in vaccines for humans (such as Alum and MF59) (210, 211). It appears that all adjuvants function in some way to activate the innate immune response at the site of vaccination, and that this is likely to be the most important mechanism of action, as opposed to allowing for sustained release of antigen (the depot effect) which had previously been thought the important mechanism of action of many adjuvants (210). For example, Hutchinson *et al* showed that the ability for Alum to boost antigen presentation by various cell types, T cell expansion, antibody production and memory responses, was unaffected by the removal of the injection site at as early as 2 hours after injection (212), strongly disputing any need for a depot effect at the injection site. Indeed, other studies have disputed whether Alum remains adsorbed to antigen for a sustained period of time, and whether this is linked to adjuvanticity (213, 214). However, licensed and experimental adjuvants have been consistently shown to be strong inducers of local chemokine and cytokine production, to increase recruitment of cells to the injection site and to increase uptake and presentation and antigen by APCs, as well as increase APC maturation (particularly DCs) (210, 212, 215-217).

As a major development in innate immune research in recent years has been on TLR activation and signalling, TLR ligands have become an area of intense interest in vaccine and adjuvant design (218). They

are the most well-defined activators of PRRs known, and offer a great deal of potential for adjuvant development (173). Many existing vaccines employ the use of killed or attenuated microbes, which containing many different PAMPs intrinsically, and thereby activate TLRs and other PRRs (i.e. they contain natural adjuvants). For example, the yellow fever vaccine acts to stimulate DCs by the activation of TLR2, 7, 8 and 9 (219), and the BCG vaccine activates immune response by interaction with many PRRs including TLR2, 4 and 9 (220, 221). It is hoped that our increased understanding of the mechanism of adjuvants, combined with the function of PRRs and other innate immune processes, we can design and develop adjuvants and delivery systems, consisting of basic PRR agonists and particulates, which are capable of promoting a strong antigen specific immune response, with the desired effector and memory responses (210). One TLR ligand, the TLR4 ligand 3-*O*-desacyl-4'-monophosphoryl lipid A (MPL), a non-toxic derivative of LPS from *Salmonella minnesota* is incorporated in the Adjuvant System 04 (ASO4; GlaxoSmithKline) oil-in water adjuvant, which has recently been licensed for use in two human vaccines (217).

Project aims

This thesis aims to investigate the role for TLR2 and its co-receptors in the control of cutaneous leishmaniasis *in vivo*, in order to inform rational vaccine and adjuvant design for future leishmaniasis vaccine candidates.

The aims of this project can therefore be summarised as:

1. To determine the role of TLR2 and co-receptors TLR1 and TLR6 in CL infection
2. To design a vaccine for CL containing TLR2 ligand adjuvants
3. To explore the efficacy of the vaccines + TLR2 ligand adjuvants in a vaccine models for CL (Th1 immunity) and lymphatic filariasis (Th2 immunity) in comparison to standard adjuvants, and determine immune correlates of protection in these models.

Chapter 2. Methods

This chapter provides detailed methodology for all of the experiments performed and presented in this thesis. In individual results chapters (Chapters 3-7), brief descriptions of the methods used are given, which refer back to the methodology given in this chapter, as well as providing specific details that apply only to those chapters.

Reagents and equipment

Details of powder and liquid reagents that were used in this study are given in full in Table 2, along with their sources. All were kept in the conditions required as stated in the manufacturer's storage instructions.

Category	Name	Company
Powders	Agarose	Sigma
	Bovine Serum Albumin (BSA)	Sigma
	Calcium chloride dehydrate ($CaCl_2.2H_20$)	Sigma
	Concanavalin A (ConA)	Sigma
	Glycine	Sigma
	Heparin sulphate	Sigma
	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma
	Imidazole	Qiagen
	Isopropylthio-β-galactoside (IPTG)	Qiagen
	Kanamycin sulphate	Invitrogen
	LB Broth	Miller
	Leupeptin hydrochloride	Sigma
	Lysozyme	Sigma
	Magnesium sulphate ($MgSO_4$)	Sigma
	Manganese (II) chloride ($MnCl_2$)	Sigma
	3-(N-Morpholino)propanesulfonic acid, 4-Morpholinepropanesulfonic acid (MOPS)	Sigma
	N-tosyl-L-lysinechloromethyl ketone (TLCK)	Sigma
	Potassium acetate	Sigma
	Potassium Chloride (KCl)	BDH
	Potassium phosphate monobasic (KH_2PO_4)	VWR
	Rubidium Chloride (RbCl)	Sigma
	Sodium dodecyl sulphate (SDS)	Fisher
	Sodium bicarbonate ($NaHCO_3$)	Sigma
	Sodium carbonate (Na_2CO_3)	Sigma
	Sodium chloride (NaCl)	Sigma
	Sodium phosphate dibasic (Na_2HPO_4)	BDH
	Sodium phosphate monobasic (NaH_2PO_4)	BDH
	Tris Base (Tris, or Trizma)	Sigma
	Tris.Borate EDTA Buffer 5 x sachets	Sigma
	Tris.HCl	Sigma
	Urea	Qiagen
	X-gal (Ultra-pure)	Invitrogen
Liquids	100 x BME vitamins	Sigma
	2-Mercapthoethanol (2-ME) 50 mM	Invitrogen
	2-Propanol (isopropanol)	Sigma
	3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA	Sigma
Liquids (continued)	Ampicillin solution	Invitrogen
	Dimethyl sulfoxide (DMSO)	Sigma

Category	Name	Company
	Dimethylformamide (DMF)	Sigma
	Dulbecco's Modified Essential Medium (DMEM)	Invitrogen
	Dulbecco's Phosphate Buffered Saline (DPBS)	Sigma
	Ethidium Bromide solution	Sigma
	Foetal bovine serum "gold", heat-inactivated (HI-FBS gold)	PAA
	Formaldehyde	Sigma
	Gentamicin sulphate	Sigma
	Giemsa staining solution	BDH
	Glycerol	Sigma
	Grace's Insect Medium	Invitrogen
	Hank's Balanced Salt Solution (HBSS)	Sigma
	Hydrochloric acid (HCl)	AnalaR
	L-glutamine solution 200 mM	Sigma
	Medium 199 1x	Invitrogen
	Methanol (HPLC grade)	Fisher
	Non-essential amino acids (NEAA), 100 x	Sigma
	Nuclease-free water	Ambion / Sigma
	Penicillin-Streptomycin	Invitrogen
	RBC lysis buffer, 1 x	eBioscience
	RNA later	Ambion
	Roswell Park Memorial Institute (RPMI) 1640 medium	Invitrogen
	SOC medium	Invitrogen
	Sodium hydroxide (NaOH) solution, 5 M	Prepared in-house
	Sulphuric acid (H_2SO_4)	AnalaR
	TE Buffer	Ambion
	Trypan blue solution, 4 %	Invitrogen
	Tween-20	Sigma

Table 2. List of powder and liquid reagent names and their sources.

The details of the solutions, buffers and media reagents made freshly in the laboratory are given in Table 3. Double distilled water (dH_20) was prepared in-house and sourced from specialised taps in the laboratories. To measure and alter pH, an UltraBasic UB-10 benchtop pH meter was used (Denver Instruments) and pH was adjusted using HCl and/or NaOH unless stated otherwise.

Solution name	Components	Concentration	Diluent	Adjustments
10 x PBS	NaCl KCl Na_2HPO_4 KH_2PO_4	80 g/l - 1.37 M 2 g/l - 27 mM 14.4g/l – 81 mM 2.4 g/l – 15 mM	dH_20	
PBS	10 x PBS	1 x	dH_20	pH 7.2 Sterile filtered
70 % Ethanol	Ethanol	70 % v/v	dH_20	
Complete M199	HI-FBS gold gentamicin sulphate BME vitamins	20 % 25 μg/ml 1 x	Medium 199	Sterile filtered, kept at 4°C
Complete Grace's medium	HI-FBS gold gentamicin sulphate BME vitamins	20 % v/v 25 μg/ml 1 x	Grace's Insect Medium	pH 5.5 Sterile filtered, kept at 4°C

Solution name	Components	Concentration	Diluent	Adjustments
Complete DMEM extra	HI-FBS gold Penicillin-Streptomycin L-glutamine 2-ME NEAA	10 % v/v 50 U/ml, 50 µg/ml 2 mM 0.1 mM 1 x	DMEM	Sterile filtered, kept at 4°C
Complete DMEM	HI-FBS gold Penicillin-Streptomycin	10 % v/v 50 U/ml, 50 µg/ml	DMEM	Sterile filtered, kept at 4°C
Complete RPMI extra	HI-FBS gold Penicillin-Streptomycin L-glutamine 2-ME NEAA	10 % v/v 50 U/ml, 50 µg/ml 2 mM 0.1 mM 1 x	RPMI	Sterile filtered, kept at 4°C
Complete RPMI	HI-FBS gold Penicillin-Streptomycin	10 % v/v 50 U/ml, 50 µg/ml	RPMI	Sterile filtered, kept at 4°C
Basic RPMI	Penicillin-Streptomycin	50 U/ml, 50 µg/ml	RPMI	Sterile filtered, kept at 4°C
ELISA coating buffer	Na_2CO_3 $NaHCO_3$	3.03 g/l 6 g/l	dH_20	pH 9.6
ELISA blocking buffer	BSA	1 % w/v	PBS	Made fresh each use
Cytokine ELISA reagent diluent	BSA	1 % w/v	PBS	pH 7.2 – 7.4 Sterile filtered
IFNγ reagent diluent	Tris NaCl BSA	20 mM 150 mM 0.1 %	dH_20	pH 7.2 -7.4 Sterile filtered
Ab ELISA reagent diluent	Tris NaCl BSA Tween 20	50 mM 0.14 M 1% 0.05%	dH_20	Kept at 4°C, made fresh each use
Stop solution	H_2SO_4	0.9 M / 1.8 N	dH_20	
LB medium	LB Broth Powder (Tryptone Yeast extract NaCl)	2.5 % (w/v) 1 % (w/v) 0.5 % (w/v) 1 % (w/v)	dH_20	Autoclaved
LB Agar	LB Broth Powder Agar	2.5 % (w/v) 1.5 % Agar	dH_20	Autoclaved
5 X TBE Buffer	5 x TBE Buffer blend (Tris-borate EDTA)	5 x / 1 sachet/litre (0.445 M 10 mM)	dH_20	
TBE Buffer	5 x TBE buffer	1 x / 20% v/v	dH_20	
10 x Running Buffer	Tris HEPES SDS	1 M 1 M 1 % w/v	dH_20	Dissolve Tris base first and adjust to pH 8.3
Running Buffer	10 x Running buffer	1 x / 10% v/v	dH_20	
10 x Transfer Buffer	Tris Glycine	250 mM 1.92 M	dH_20	Dissolve Tris base first and adjust to pH 8.3
Transfer Buffer	10 x Transfer Buffer Methanol	1 x / 10% v/v 20% v/v	dH_20	Keep at 4°C or below
Tris-Buffered Saline-Tween 20 (TBST)	Tris NaCl Tween 20	50 mM 150 mM 0.05% v/v	dH_20	
TFB1 Buffer	RbCl $MnCl_2$ Potassium acetate	100 mM 50 mM 30 mM	dH_20	Adjust to pH 5.8, sterile filter

Solution name	Components	Concentration	Diluent	Adjustments
	$CaCl_2.2H_20$ Glycerol	10 mM 15 % v/v		
TFB2 Buffer	MOPS RbCl $CaCl_2.2H_20$ Glycerol	10 mM 10 mM 75 mM 15% v/v	dH_20	Adjust to pH 6.8 using KOH, sterile filter
Psi Broth	$MgSO_4$ KCl	4 mM 10 mM	LB medium	
Native Lysis Buffer	NaH_2PO_4 Tris.Cl Imidazole Lysozyme	100 mM 10 mM 10 mM 1 mg/ml	dH_20	Adjust to pH 8.0, lysozyme is added to buffer just before use.
Denaturing Lysis Buffer	NaH_2PO_4 Tris.Cl Urea	100 mM 10 mM 8 M	dH_20	Adjust to pH 8.0
Native Protein Wash Buffer	NaH_2PO_4 NaCl Imidizole	50 mM 300 mM 20 mM	dH_20	Adjust to pH 8.0
Native Protein Elution Buffer	NaH_2PO_4 NaCl Imidazole	50 mM 300 mM 250 mM	dH_20	Adjust to pH 8.0
Denaturing Wash Buffer	-	-	Denaturing Lysis Buffer	Adjust to pH 6.3
Elution Buffer D	-	-	Denaturing Lysis Buffer	Adjust to pH 5.9
Elution Buffer E	-	-	Denaturing Lysis Buffer	Adjust to pH 4.5
FACS Buffer	BSA EDTA	0.5 % w/v 2 mM	PBS	Sterile filtered, kept at 4°C

Table 3. Buffers, solutions and cell culture media

The details of plastic consumables used are given in Table 4 below.

Name	Company
0.5, 1.5 and 2 ml microcentrifuge tubes (sterile)	Starlab
1, 1.2 and 2 ml cryopreservation tubes	NUNC, Starlab
1, 2, 5, 10 and 25 ml serological pipettes (sterile)	Starlab / VWR
1, 2.5, 5, 10, 20 and 50 ml luer-lock syringes	BD Plastipak
10, 100, 200 and 1000 µl graduated pipette tips (sterile, with or without filters)	Starlab
100 x 15 mm^2 sterile petri dishes	NUNC/ VWR
15 ml centrifuge tubes (sterile)	Starlab
20 ml conical falcon tubes (sterile)	Sterilin
25, 75 and 175 cm2 culture flasks	Fischer Scientific / NUNC
2HB High-binding plates for ELISA	Immulon
48-well and 96-well flat bottom plates (sterile)	Thermo Scientific / NUNC
50 ml centrifuge tubes (sterile)	Starlab
70 µm cell strainer (sterile)	BD
8-well PCR tube strips (clear)	Starlab
96-well high-profile white PCR plates	Starlab
96-well U bottom plate (sterile)	Thermo Scientific / NUNC
Glass microscope slides	VWR
Poly-L-lyseine frosted microscope slides	
Syringe needles (various sizes) (sterile)	Terumo Neolus

Table 4. Details of plastic consumables

Details of the origin and other descriptives related to the type and preparation of animals, parasites, antibodies, machines, software, kits, and other such "specific-use" reagents, are given in the appropriate methods sections below.

Animals, cell culture, and parasite life-cycle maintenance

Mice and Gerbils

TLR1$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$ and TLR6$^{-/-}$ mice were originally obtained from Professor Akira's laboratory (Laboratory of Host Defense, Osaka University, Japan) and have since been maintained in the Biomedical Services Unit, Duncan Building, University of Liverpool. C57BL/6 mice were purchased from Charles River and BALB/c mice were purchased from Harlan. Naive Mongolian Jirds were purchased from Charles River, whilst Jirds already infected with *Brugia malayi* were purchased from TRS Laboratories, USA. All procedures involving animals were approved by the Home Office and the LSTM and University of Liverpool Ethics Committees. Procedures took place either in the Biomedical Services Unit (BSU), Duncan Building, University of Liverpool, or the BSU animal facility in the Ronald Ross Building, University of Liverpool where animals are housed in individually ventilated cages (IVCs) with air filtered with High-efficiency particulate air (HEPA) filters. The location of animals used in individual experiments is detailed in the methods sections for each result chapter.

Cell culture

Cells and parasites were cultured *in vitro* in the conditions indicated in Table 5 below:

Cell type	Culture media	Incubator conditions	Volumes and containers
Leishmania promastigote parasites	Complete M199	26°C	1-7.5 ml (25 cm^2 flask) 5-20 ml (75 cm^2 flask) 20 – 75 ml (175 cm^2 flask)
Leishmania amastigote parasites	Complete Grace's	32°C	1-7.5 ml (25 cm^2 flask) 5-20 ml (75 cm^2 flask) 20 – 55 ml (175 cm^2 flask)
Mouse splenocytes or DLN cells	Complete DMEM extra or complete RPMI extra	37°C, 5 % CO_2	100 – 200 µl (96 well flat-bottom plates)
Mouse peritoneal exudate cells (PECs), MΦs, HEK cells, *B. malayi* parasites	Complete RPMI	37°C, 5 % CO_2	100 – 200 µl (96 well flat-bottom plates) 1-7.5 ml (25 cm^2 flask) 5- 20 ml (75 cm^2 flask or petri dish)
E. coli	SOC medium or Complete LB with supplements	37°C with continual shaking	5-6 ml (20 ml conical flask)

Table 5. Culture conditions for different cell types and parasites.

Counting of cells

Mammalian and parasite cells were counted using either a haemocytometer or an automated cell counter (the method used in each experiment is indicated in corresponding methods sections). The cell suspension to be counted was gently disrupted to allow for equal distribution of the cells before dilution, if necessary, in DPBS. Then 10 µl was removed and placed in either a well of a 96-well flat-bottom plate or a microcentrifuge tube and mixed with an equal volume of trypan blue solution by gentle pipetting. For motile cells, such as cultured *Leishmania* promastigotes, cells were fixed prior to staining in 4 % formalin in PBS solution (at a ratio of 1:1). Cells which form clumps in culture, such as *Leishmania* amastigotes, were disrupted using a 1 or 2.5 ml syringe and a blunt-end dosing needle prior to counting.

Haemocytometer

For counting using a haemocytometer, the counting chambers were prepared by placing thick coverglass over the two grids of the haemocytometer (Assistent) and ensuring a tight grip. Then 10 µl of the cell/trypan blue mix was placed next to the counting chamber of a haemocytometer to allow for it to fill by capillary action. Cells were allowed to settle for 1 minute prior to counting using the x20 objective of a light microscope. The number of cells per 1 mm^2 was counted by recording the number of cells in the central grid; this was repeated and the average of two readings was multiplied by the conversion factor (x 10^4) for the number of cells per ml.

Automatic cell counter

In some cases, mammalian cells were counted using a TC-10 ™ Automated Cell Counter (BioRad). In such instances, cells were prepared as described above, and 10 µl cell/trypan blue mix was placed in the well of a TC-10 cell counting slide (BioRad) and allowed to settle for 1 minute. The slide was then placed in the Automatic Cell Counter and the number of live cells per ml was calculated and displayed on the screen.

Cryopreservation of cells

Cultured cells were frozen in media solutions (as detailed in Table 5) containing either glycerol (10 %) or DMSO (10 %) as a cryoprotectant, depending on the cell type. *Leishmania* promastigotes were cryopreserved by placing log-phase parasites into complete M199 containing 7.5 – 10% glycerol, in cryopreservation tubes, and freezing slowly to -80°C using a Mr Frosty container (Nalgene) containing isopropanol. *Leishmania* amastigotes were cryopreserved as above but instead using complete Grace's containing 10% DMSO as the freezing medium; mammalian cells were also cryopreserved using culture media containing DMSO. After freezing, the vials were kept in long-term storage in either liquid nitrogen or at -80°C. Cryopreserved promastigotes were recovered from storage by defrosting and slowly diluting (drop-by-drop) into fresh complete M199. Frozen amastigotes or mammalian cells were recovered from

storage by defrosting the vials quickly, centrifuging and removing freezing medium quickly, and placing cells into fresh culture medium.

Mycoplasma detection

Cell cultures (kept for longer than 7 days in culture) were regularly checked for mycoplasma contamination using the MycoAlet Mycoplasma™ Detection Kit (Lonza) according to manufacturer's instructions. Briefly, 100 µl cleared cell supernatant from each cell culture sample was added to a well of a 96-well flat-bottom plate (Wallac – for use with 1450 MicroBeta™) along with 100 µl reconstituted MycoAlert Reagent and allowed to incubate for 5 minutes. Using a 1450 MicroBeta Trilux liquid scintillation and luminescence counter (Wallac) a 1 second reading was taken for each well before adding 100 µl MycoAlert substrate to each sample and waiting 10 minutes at RT before taking another reading. The ratio of the second reading to the first is used to determine presence of mycoplasma contamination, with values over 1.2 considered positive, those under 0.9 considered negative and those in between considered borderline and therefore needing to be retested. A positive control (MycoAlert Assay control Set, Lonza) and negative control (culture medium alone) was included for each assay.

Leishmania life cycle maintenance

Leishmania cell culture

Leishmania major FV1 (MHOM/IL/80/Friedlin; clone V1), *L. major* LV39 (MRHO/SU/59/P), *L. mexicana* (MNYC/BZ/62/M379) and the genetically modified *L. mexicana lpg1*$^{-/-}$ (also M379) were provided by Professor Paul Bates and Dr Rod Dillon. Promastigote parasites were cultured in complete M199 medium at 26ºC. Axenic amastigotes (of *L. mexicana* parasites only) were cultured in complete Grace's medium at 32ºC. In the case of both promastigotes and amastigotes, parasites were kept in volumes of 5 – 55 ml volumes and were sub-passaged at a ratio of 1:2 – 1:20 in fresh medium every 5 – 10 days according to growth rate (typically 1:10 every 7 days). Growth was monitored by counting the concentration of parasites in culture using a haemocytometer and/or by visual inspection of cultured cells using an inverted microscope.

Passage of parasites through animals

Infectivity of parasites was maintained by regular passage of parasites through a susceptible animal. For this purpose, stationary phase promastigotes (day 6+ of culture) or axenic amastigotes were washed in HBSS twice, and resuspended in sterile HBSS. Between 10^5 and 10^6 parasites in a 100 µl volume of HBSS were injected into the shaven rump of female BALB/c mice and lesions were allowed to develop for 6 weeks or more. Mice were then sacrificed to obtain parasite infected tissues. Lesion-derived amastigotes were obtained by mechanically disrupting cutaneous lesion tissue or infected draining lymph nodes (DLNs) using sterile scissors, forceps, a metal gauze (for cutaneous lesion tissue) or a 70 µm cell strainer

(for DLN) and cells were collected into either complete M199 or complete Grace's medium for the generation of promatigotes or amastigotes respectively.

B. malayi life cycle maintenance

The life cycle of *B. malayi* was maintained by the Filariasis laboratory staff (Andrew Steven and Dr Darren Cook) at LSTM and the BSU staff, University of Liverpool. For *B. malayi* life cycle maintenance, all procedures on live animals were performed in the BSU in the Duncan Building, University of Liverpool. Jirds infected in the peritoneum with adult *B. malayi* parasites were originally purchased from TRS laboratories, USA. Microfilariae (Mf) produced by *B. malayi* adults in these animals were obtained every 2 weeks (maximum) by a peritoneal tapping method, as described by Griffiths *et al*, 2010, (222) and performed by Dr Darren Cook and/or Mrs Pamela Pask. After counting, 15,000 mf/ml were mixed with human blood for feeding to adult female mosquitoes. L3 infective larval stages were collected after 14 days by crushing the mosquitoes into basic RPMI medium and allowing the L3 larvae to pass through a Baermann's apparatus. L3 were counted and collected by manually picking individual L3 into 50 – 100 µl volumes of fresh basic RPMI.

Infections

Experimental infection with *Leishmania spp*

Age-matched female mice (8-12 weeks at beginning of experiment) from each group of mice (5-8 per group) were infected on the same day for each infection experiment. Stationary-phase promastigotes (day 7-10 of culture) of *L. major* (FV1 or LV39) or *L. mexicana* (WT or *lpg1*$^{-/-}$ M379), or axenic amastigotes of *L. mexicana* (WT) were used for infections. Parasites used for infections were passaged only once or twice after recovery from lesion amastigotes to reduce the loss of infectivity in culture. Furthermore, the proportion of metacyclics was increased in some promastigote cultures by transferring promastigotes grown in complete M199 medium for 7 days to complete Grace's medium and cultured at 26ºC for a further 3 days as described elsewhere (223); these are referred to as metacyclic-enriched promastigotes (the use of this method for individual infection experiments is indicated in the methods sections of each chapter). For infections of the rump, the area was shaved and wiped with 70% Ethanol prior to injection, then 100 µl HBSS containing the parasite inoculum (10^5 parasites) was injected subcutaneously approximately 1 cm above the base of the tail using a 1 ml syringe and a 27 gauge needle. For infections of the foot, parasites were resuspended in a volume of 20 µl HBSS per mouse and were injected subcutaneously to the central part of the upper hind right foot using an insulin needle and syringe.

Slides were made of samples used for infections by placing 10 – 20 µl parasites on a poly-lysine slide and allowing it to air dry. Parasites were fixed on to the slide by incubation in methanol for 1 minute, before staining with 10% Giemsa solution for 10 minutes. After slides had dried, they were preserved by

mounting a coverslip using Low Viscosity DPX mounting solution (BIOS Europe). The proportion of metacyclic parasites in promastigote samples was determined by inspecting Giemsa stained slides using the x100 objective of a light microscope, and recording the number of cells of different forms, as described by Rogers *et al* (110).

Measurement of cutaneous lesions

Lesion sizes were monitored by measuring the diameter of lesions in the infected area using a metric dial calliper every 7 days. Diameter measurements were recorded for lesions on the rump; multiple measurements of diameter were recorded for those lesions with a non-circular circumference to gain and average diameter value. Mice often had more than one lesion present, and in this case diameter measurements of each lesion were taken. Lesion size was defined as the total area of lesion present (mm^3, calculated using diameter measurements), in order to account for variation in lesion shape and numbers (see Figure 8). Lesion area was found to correlate more closely with parasite burdens than the diameter measurement (data not shown), which is more frequently used in other published studies.

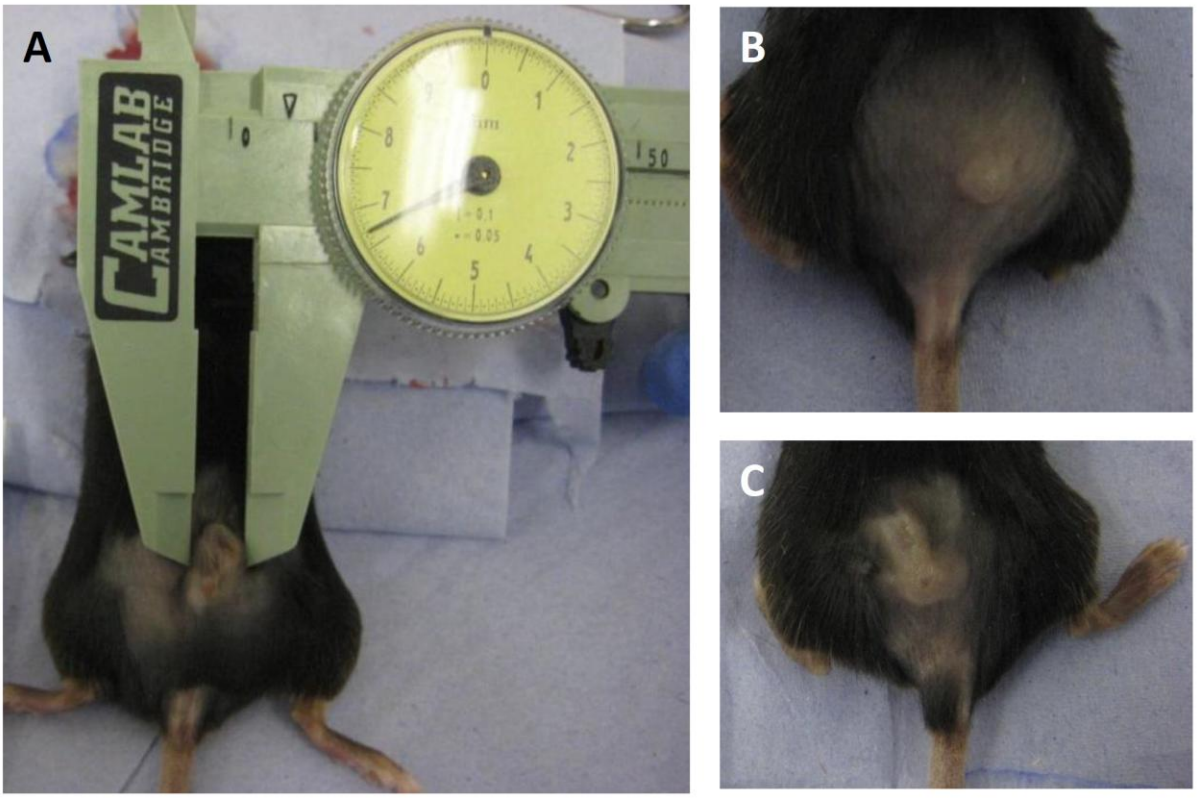

Figure 8. Examples of the appearance of lesions on mice infected with *L. major* or *L. mexicana*. Diameter measurements of lesion were taken using a dial calliper (A), and where the lesion was non-circular, several diameter measurments were taken. Some lesions were a regular, circular shape (B), whilst other were irregular (A), or there were multiple lesions (C). These mice were infected with *L. mexicana* promastigotes for 14 weeks.

For lesions of the foot, the thickness of the infected foot (from central part of footpad to central part of upper foot) was recorded as well as the thickness of the contralateral uninfected foot. The difference

between these two measurements was taken as the lesion size. The presence of ulcerated lesions was also recorded each week.

Removal of cutaneous lesions and limiting dilution

The *Leishmania*-infected lesion tissue was removed from the mouse immediately after schedule 1 killing. For lesions of the rump, the area was wiped with 70% ethanol solution before incisions were made to the cutaneous tissue surrounding the lesion with sterile stainless steel dissection scissors. The area of skin (including the lesion) was then removed from the subcutaneous layer using sterile forceps and scissors. For foot lesions, the foot was removed by excision at the ankle to ensure collection of all lesion tissue. Lesion tissues were then processed in one of three ways according to their intended use. The lesion tissue was then used either for establishment of parasite culture and/or limiting dilution analysis by mechanical homogenisation, or was placed in RNA later solution for subsequent DNA extraction and parasite burden estimation by qPCR, as detailed later.

Parasite burden of infected tissues was measured in some instances by limiting dilution analysis as described elsewhere (224). Briefly, the infected tissue was cut into small sections into complete M199 medium using sterile scissors and forceps, a sterile syringe plunger and placed over a sterile metal gauze. The cell suspension was collected, whilst removing large sections of remaining tissue or debris by allowing these to settle, and serial 1 in 2 dilutions were made of each cell suspension across a 96 well flat-bottom plate in complete M199 medium, in duplicate. After 7 days the wells of the plates were checked for the presence of motile parasites. The highest dilution showing the presence of parasites for each series was used to calculate the original parasite concentration and tissue parasite load.

Experimental vaccine model for *B. malayi*

The vaccination model of *B. malayi* in BALB/c mice was developed with Dr Joseph Turner and Miss Ana Guimaraes in LSTM and in based on modifications to previously described models (225). Fifty infective *B. malayi* L3 were collected into 50 – 100 µl warm basic RPMI and were injected into the peritoneal cavity of male BALB/c mice using a sterile 1 ml syringe and a 25 gauge needle.

Measurement of *B. malayi* parasite burden and collection of PECs

The collection of parasite and PECs was carried out with assistance from Dr Joseph Turner and Miss Ana Guimaraes. Infected BALB/c mice were killed 6 days after infection with *B. malayi* L3 parasites and the peritoneal cavity was washed thoroughly 3 times with 5 ml sterile basic RPMI medium and collected into a 15 ml centrifuge tube. Parasites were allowed to settle at the bottom of the tube and were then transferred to a 48-well culture plate for counting using a dissection microscope. Motile parasites were counted whilst immotile worms and those encased in granulomas were not included for parasite burden data. The remaining media sample contained PECs, which were washed in basic RPMI, enumerated using the automated cell counter, and kept on ice for further use as described later.

Parasite antigens and vaccine components

Leishmanial antigens

Leishmania freeze-thaw antigen

Freeze-thaw antigen (FTAg) was made from cultured promastigotes as described and developed elsewhere (51, 71). Stationary-phase promastigotes were washed three times in DPBS and resuspended at a concentration of 10^9/ml, and were then subjected to 5 rapid freezing and thawing cycles at -80°C and 37°C respectively. Protein concentration was measured using the BCA assay (described later) and aliquots of FTAg were kept at -80°C until use.

Amastigote washed membranes

For *L. mexicana* parasites only, washed membrane antigen (WMAg) was generated from cultured axenic amastigotes using hypotonic lysis as described by Thomas *et al*, 2008 (71). Axenic amastigotes were washed three times in PBS and counted using a haemocytometer before lysing in nuclease-free water containing 0.1 mM TLCK and 1 μg/ml leupeptin at 10^9 parasites/ ml for 5 minutes on ice. The lysed parasites were then frozen at -80°C after addition of an equal volume of 0.1 mM TLCK, 1 μg/ml leupeptin, 20 % glycerol. After freezing, the lysed parasites were thawed and centrifuged at 6,100 **g** for 10 minutes (4°C) to remove PBS containing soluble protein and protease inhibitors before resuspending membranes at 10^9/ml in PBS. The WMAg solution was assayed for protein concentration using the BCA assay and aliquots were kept at -80°C until use.

Autoclaved *Leishmania major*

Autoclaved *L. major* (ALM) antigen was made using a method described first by Bahar *et al* (226). Briefly, *L. major* promastigote cultures were grown to log-phase (day 5) in complete M199 and a 50 ml volume was transferred into larger cultures by addition of 200 ml complete Grace's medium and allowed to grow for a further 4 days. The promastigote parasites (approximately 10^9) were then washed three times in sterile DPBS and resuspended in 2ml DPBS. This highly concentrated volume of parasites was placed in a glass container and autoclaved at 151°C for 15 minutes. Protein concentration was measured using the BCA assay and aliquots were stored at -80°C until further use.

B. malayi antigens

The methodology for preparation of *B. malayi* parasite extracts was based on that used for obtaining *B. malayi* adult female extract (BmFE) as described in Turner *et al*, 2006 (227) with some adjustments. Mf obtained from infected Jirds (described above) were separated from cells and other debris using a PD-10 desalting column (GE Life Sciences). Briefly, a peritoneal exudate sample in a volume of 2.5 ml basic RPMI medium, containing 0.5 – 5 x 10^6 Mf, was loaded on to a prepared column and allowed to pass through the column by gravity. Fractions were collected by gradual addition of warm basic RPMI to the

column, and were regularly checked for presence of Mf and cells by examination of drops using a glass slide and an inverted microscope. Those fractions containing only Mf were pooled and washed twice in sterile DPBS by centrifugation at 300 – 400 **g** for 5 minutes. Mf were enumerated and resuspended in sterile DPBS at a concentration of approximately 2 x 10^6 /ml prior to protein extraction. *B. malayi* L3 parasites were collected from an infected mosquito crush, as described above, and were washed by centrifugation at 300 – 400 **g** (5 minutes) and transfer into fresh microcentrifuge tubes, washed 4 times in, and resuspended in, sterile DPBS a concentration of 2000 Mf /ml.

For generation of soluble protein extracts of *B. malayi* Mf (BmMfE) and L3 (BmL3E), parasites collected as indicated in DPBS were sonicated five times for periods of 15 seconds on ice, interrupted by periods of resting on ice for 20 seconds, using a Vibracell sonicator and a sterile endotoxin-free sonicator probe. The soluble proteins were then extracted from the sonicated parasites into the DPBS by incubating at 4ºC overnight with gentle agitation using a rotary shaker. Insoluble material was removed by centrifugation at 13,000 rpm (microcentrifuge) for 30 minutes at 4ºC. Dead L3 were obtained by heat-killing the L3 larvae (in aliquots of 50 L3 in 100 µl basic RPMI) at 65°C for 10 minutes as used in vaccine studies as previously reported (228).

Vaccine formulations

All individual doses of vaccines were made to a volume of 100 µl or 20 µl in DPBS, with the exception of the Live and Dead L3 vaccines where parasites were kept in basic RPMI (also 100 µl). The antigen and adjuvant components present in each vaccine are given below (Table 6). Sham inoculated or challenge control mice were given 100 µl DPBS. The schedules for inoculations used in the vaccine experiments are detailed in the methods section for the corresponding results chapters. The unmethylated CpG Oligodeoxynucleotide (ODN) 1826 adjuvant of the sequence 5'- TCCATGACGTTCCTGACGTT -3' (referred to as CpG from hereon) was a kind gift from Lyn Jones and Matthew Selby at Coley (Pfizer). Lipopeptide adjuvants S-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteine (Pam_2CSK_4, or Pam2 from hereon) and N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteine (Pam_3CSK_4 or Pam3) were purchased from EMC Microcollections. CpG, Pam2 and Pam3 were dissolved in nuclease-free water and kept in 1-10 mg/ml stocks at -80°C before use. Imject® Alum Adjuvant (Alum) was purchased from Thermo Scientific and was mixed with antigen according to manufacturer's instructions. Briefly, Alum was added dropwise to protein antigen mixtures until a 1:1 ratio was reached and was continually mixed for a further 30 minutes at 4°C, using a rotary shaker.

Vaccine name	Volume /dose	Antigen	Quantity /dose	Adjuvant	Quantity / dose
Adjuvants only	20 µl	-	-	CpG Pam2 Pam3	50 µg 10 µg 10 µg
CpG	100 µl	-	-	CpG	50 µg
rKMP-11 + CpG	100 µl	rKMP-11	10 µg	CpG	50 µg
ALM	20 µl	ALM	50 µg	-	-
ALM + CpG	20 µl	ALM	50 µg	CpG	50 µg
ALM + Pam2	20 µl	ALM	50 µg	Pam2	10 µg
ALM + Pam3	20 µl	ALM	50 µg	Pam3	10 µg
ALM + Adjuvants	20 µl	ALM	50 µg	CpG Pam2 Pam3	50 µg 10 µg 10 µg
Dead L3	100 µl	Heat killed L3	50	-	-
BmMfE	100 µl	BmMfE	50 µg	-	-
BmMfE + Alum	100 µl	BmMfE	50 µg	Alum	50 µl
BmMfE + Pam2	100 µl	BmMfE	50 µg	Pam2	10 µg

Table 6. Vaccine formulations used in vaccination experiments. The volume and the quantities of antigen and adjuvant/s for each dose are given.

Tissue/cell collection and immunological techniques

Collection of blood plasma from mice

For the collection of blood plasma, mice were killed by a schedule 1 method which was confirmed by performing a cardiac puncture and blood was collected into a 1.5 ml microcentrifuge containing 10 µl heparin sulphate in PBS (10,000 U/ml, sterile filtered). Samples were kept on ice and then spun down at 13,000 rpm for 30 minutes at 4°C. Plasma was removed from the blood cell pellet into aliquots and at -20°C until further use.

Obtaining splenocytes and draining lymph node cells from mice

The spleen and/or DLNs were removed from mice after collection of blood and parasite tissues, using sterile scissors and forceps, and were placed in complete medium (either DMEM or RPMI depending on the experiment). The tissue was then disrupted using a sterile syringe plunger and a cell strainer (70 µM) and a single cell suspension was collected into a petri dish before transferring to a centrifuge tube and placing on ice. Cells were then washed in cold medium twice before being counted.

Culture and stimulation of immune cells

The splenocytes and DLN cells were resuspended into either complete RPMI extra or complete DMEM extra at a concentration of 4 or 8 x 10^6 cells/ml and were plated in duplicate or triplicate on 96 well plates in 100 µl volumes (i.e. 4 or 8 x 10^5 cells/well). Antigens and control stimulations were diluted in the same medium to double the final concentration in 100 µl and added to the cells. The controls, antigens and the final concentrations used were as follows: negative control (media alone); 10 µg/ml *Leishmania* FTAg; 10 µg/ml *Leishmania* WMAg; 20 µg/ml BmL3E, 20 µg/ml BmMfE, 2.5 µg/ml Con A; 10 µg/ml anti-mouse

CD3 antibody (eBioscience). For the anti-CD3 stimulations, wells were first coated with the antibody, by diluting in DPBS and incubating on the plates for 30 minutes at 37°C to allow for coating of the wells, before removal and gentle washing with DPBS, and addition of cells and media. After addition of all antigens, plate containing cells were incubated at 37°C and 5 % CO_2 for 72 hours before removal of the supernatants for storage at -20°C.

Cytokine Enzyme-Linked Immune-sorbent Assay (ELISA)

Cytokine levels in supernatants from cell cultures were measured for the following cytokines by ELISA DuoSet kits (all R&D) according to manufacturer's instructions: mouse IFNγ, IL-10, IL-13, IL-4 and IL-5. Briefly, wells of 96-well 2HB high binding plates were coated with 50 µl/well capture antibody diluted in PBS overnight at room temperature (RT). Plates were washed three times in ELISA wash buffer. Plates were blocked for 1 hour at RT with 300 µl reagent diluent then washed three times before addition of samples and standards, diluted in medium (complete RPMI or DMEM depending on which was used in the original cell culture) and plated in duplicate, and incubated for 2 hours at RT. Plates were washed three times before addition of 50 µl/well detection antibody and incubated at RT for 2 hours before washing a further three times and incubating with the working concentration of streptavidin-HRP in reagent diluent and incubation at RT for 20 minutes. Plates were washed thoroughly a further 3 times before addition of 50 µl/well TMB substrate and incubation in the dark for up to 20 minutes. The reaction was stopped by addition of 25 µl/well stop solution to the wells.

Antibody isotype ELISA

Mouse plasma samples were tested for levels of antigen specific antibody isotypes IgG1, IgG2a (for BALB/c mice) and IgG2c (for C57BL/6 mice) using Immunoglobulin Quantitation kits from Bethyl Labs according to manufacturer's instructions with minor modifications. Wells of 96-well 2HB high binding ELISA plates were coated with 100 µl/well of either coating antibody in the appropriate concentration (for the standard wells only) or parasite antigens FTAg, WMAg or BmMfE at a concentration of 10µg /ml (for the sample wells), all diluted in ELISA coating buffer, and incubated overnight at 4°C. Plates were washed 3 times in ELISA wash buffer between each step as described above. Wells were blocked with 200 µl ELISA blocking buffer for 1 hour at RT, then washed before addition of plasma samples or standards diluted in Ab ELISA reagent buffer. For some experiments, plasma samples were initially pooled for each experimental group and a dilution series of between 1/10 and 1/1280 (8 x 2-fold serial dilutions) of sample were used, to identify the concentration of plasma sample appropriate for use in an ELISA including all individual samples. All samples were plated in duplicate, as well as standard plasma samples if concentrations of antibody were being measured. Plates were then washed before addition of detection antibody linked to HRP, diluted in Ab ELISA reagent buffer and incubated for 2 hours at RT. Plates were washed for the final time before addition of TMB

substrate solution, and the reaction was allowed to develop for approximately 15-20 minutes before addition of stop solution.

ELISA measurements and working

After addition of stop solution to plates, the absorbance of light at 570 and 450 nm was measured in each well by reading the plate in a Varioskan plate reader (Thermo Electron Corporation). The absorbance values of the 570 reading were subtracted from the 450 reading to give an overall absorbance value. For determination of the absorbance values and concentration of cytokine/antibody in each sample, the average absorbance values of the wells which had only diluent in for the sample step (blanks) were first subtracted from all other wells. The average values for each sample were then calculated, before the standard curve (absorbance vs. concentration) was generated. A line of best fit (2 parameter curve in most cases) was fitted on a plot of absorbance against concentration to generate an equation for calculating concentration in the sample-containing wells for that plate. Concentrations recorded for each samples then multiplied by the dilution factor to give the final concentration of cytokine/antibody per sample.

Tissue fixation and staining

Tissues from adjuvant swelling reactions were removed using sterile dissection scissors and embedded in 4% formalin. Sections of 4 μm fixed tissue were cut by microtome and mounted by electrothermal bath at 45°C on Poly-L-lysine slides by the Department of Veterinary Pathology, Animal and Population Health, University of Liverpool. Haematoxylin-Eosin (H&E) staining was performed by serial passages in Harris Haematoxylin (2 minutes), 1% Acid Alcohol (5 seconds), Scott's Tap Water (30 seconds) and Eosin (2 minutes). Each step was followed by a rapid wash in running tap water. Slides were then dehydrated by rapid passages in 70%, 90%, 100% ethanol and xylene (all by staff at the Department of Veterinary Pathology, Animal and Population Health, University of Liverpool.

Flow cytometry

Mouse PECs were characterised using flow cytometry. After washing and counting of cells using the automated cell counter, PECs were washed three times in, and resuspended into, FACS buffer, and kept on ice for the rest of the staining process. Fc receptors were blocked on cells by incubating cells in FACS buffer containing 1% v/v normal rat serum (Invitrogen) and 0.5% v/v anti-CD16/CD32 (eBioscience) for 30 minutes (at a concentration of 1 x 10^6 cells/ 100 μl). Cells were then stained with conjugated antibodies specific for surface markers of interest, or isotype controls, in FACS buffer at a concentration of 1 x 10^6 cells/ 100 μl, for 30 minutes in the dark. The antibodies used were the following (company, clone, final concentration used): Rat anti-mouse F4/80 Antigen eFluor® 450 (eBioscience, BM8, 1 μg/ml); Rat IgG2a K Isotype Control eFluor® 450 (eBioscience, eBR2a, 1 μg/ml); Rat anti-mouse Siglec-F PE (BD Pharmingen, E50-2440, 1 μg/ml); Rat IgG2a K Isotype Control PE (BD Pharmingen, R35-95, 1 μg/ml); Rat anti-mouse CD206 (MΦ Mannose Receptor) Alexa Fluor® 647 (AbD Serotec, MR5D3, 2.5

μg/ml); Rat IgG2a Isotype Control Alexa Fluor® 647 (AbD Serotec, IgG2a, 2.5 μg/ml). Each sample was stained with marker-specific antibodies, or isotype controls, or a combination of the two, to assess for non-specific staining. Samples were either analysed straight away or fixed in FACS buffer containing 2% formaldehyde and kept at 4°C before analysis within 3 days. Stained samples of cells were run through a BD™ LSRII (BD Biosciences) flow cytometer using FACSDiva™ (BD Biosciences) software. At least 10,000 cells were collected for each sample. Unstained and single stained cell samples were used for compensation for each experiment, and isotype control stained cells were used to determine levels non-specific staining. FCS data files were analysed using FlowJo (Tree Star) software.

Wound healing

To assess wound healing in mice, a sterile 4 mm punch biopsy tool was used to make a clean circular wound to the shaven rump (sterilised with 70 % ethanol). Healing was then measured by taking regular measurements of the diameter of the lesion using a dial caliper.

Molecular techniques

DNA Extraction

DNA was extracted from mouse tissue (including leishmanial lesions) and cell pellets using the DNA Blood and Tissue Kit (Qiagen) according to manufacturer's instructions, with some modifications. Mouse tissue was weighed and 25 mg of tissue per MiniSpin column was used as instructed. The tissue lysis step was performed for tissue samples only, with incubation of tissue in ATL buffer and proteinase K at 56ºC overnight for mouse tissues. After addition of AL buffer a 10 minute incubation was performed at 72ºC for 10 minutes for tissue samples, and at 56°C for cell pellet samples. In an attempt to increase throughput, the use of 96 well DNA column plates was compared to that of the Qiagen MiniSpin columns in terms of quality and quantity of DNA extracted. It was found that the MiniSpin columns gave higher yields and greater purity of DNA, thus this method was used for the extraction of DNA from all tissue and cell preparations.

Checking DNA concentration

The concentration of DNA in samples was determined using a NanoDrop machine (ThermoScientific) and supporting ND-1000 v.7 software.

Primers, PCR equipment and general PCR procedure

A list of the primers used and their sequences is given in Table 7 below. All primers were produced by Integrated DNA Technologies (IDT) using desalting conditions. Upon receipt, primers were resuspended in TE Buffer at a stock concentration of 100 μM, before further dilution in nuclease-free water and storage in aliquots at -20ºC.

Primer name	Sequence	Origin
TLR1 a	5'-GAT GGT GAC AGT CAG CAG AAC AGT ATC-3'	(229)
TLR1 b	5'-AAG GTG ATC TTG TGC CAC CCA ACA GTC-3'	
TLR1 c	5'-ATC GCC TTC TAT CGC CTT CTT GAC GAG-3'	
TLR2 a	5'-GTT TAG TGC CTG TAT CCA GTC AGT GCG-3'	
TLR2 b	5'-TTG GAT AAG TCT GAT AGC CTT GCC TCC-3'	
TLR2 c	5'-ATC GCC TTC TAT CGC CTT CTT GAC GAG-3'	
TLR4 a	5'-CGT GTA AAC CAG CCA GGT TTT GAA GGC-3'	
TLR4 b	5'-TGT TGC CCT TCA GTC ACA GAG ACT CTG-3'	
TLR4 c	5'-TGT TGG GTC GTT TGT TCG GAT CCG TCG-3'	
TLR6 a	5'-GAA ATG TAA ATG AGC TTG GGG ATG GCG-3'	
TLR6 b	5'-TTA TCA GAA CTC ACC AGA GGT CCA ACC-3'	
TLR6 c	5'-ATC GCC TTC TAT CGC CTT CTT GAC GAG-3'	
JW11	5'-CCT ATT TTA CAC CAA CCC CCA GT-3'	(230)
JW12	5'-GGG TAG GGG CGT TCT GCG AAA-3'	
KMP-11 FWD	5'-CGG GAT TCA TGG CCA CCA CGT ACG-3'	Designed in-house
KMP-11 REV	5'-CGC AAC CTT TTA CTT GGA CGG GTA C-3'	
AM1	5'CGC GTG TCG TTC GGC TTT ATG TG 3'	Sequences provided by Paul Bates (unpublished)
AM2	5'CTT ACG GAG CTT GCT GAG GTG AGG 3'	

Table 7. List of primers. The names and sequences of primers are given, along with a reference from the publication they were taken from, if applicable.

PCR procedures were optimised by varying the following (in this order): primer concentration, amount of DNA template per reaction, the annealing temperature, followed by $MgCl_2$ concentration (only if deemed necessary) to find the best conditions for optimal product amplification and reduced primer-dimer formation. PCR reactions contained the following components: target DNA (usually 5-10% final volume), forward and reverse primers (0.05 -1 μM), DNA polymerase (Taq, Phusion (both NEB) or SybrGreen (Qiagen)), dNTPs (if no pre-made master mix is used), $MgCl_2$ (NEB, if needed in addition to any contained within mastermix), and nuclease-free water. The components for each PCR reaction are detailed in later methods sections. In all cases, the master mix for each PCR was made in a designated DNA-free room, by combining all of the products needed in each reaction, but without the template DNA, before aliquoting the desired quantity into wells/tubes. DNA was then added to appropriate wells in a separate room, within a safety cabinet, before the plates/wells were sealed. For normal PCR reactions, a BioRad iCycler machine was used whilst for quantitative PCR (qPCR), a Chromo 4™ System for real-time PCR detection (BioRad) was used and data was collected using MJ Opticon Monitor Analysis Software Version 3.1 (BioRad).

Gel electrophoresis for DNA

Agarose gels were prepared with 100 – 150 ml of 0.5 – 1 % TBE Buffer from the 5 x TBE buffer stock in a glass container of at least 2 x the volume of liquid. Between 1-2 % w/v agarose powder was added before the solution was heated by microwaving on full power for 2 minutes using a 800 W (E) Proline Powerwave machine, until the solution was clear. After the solution had cooled sufficiently, 4 μl/ 100 ml ethidium bromide was added and the solution was mixed before pouring into the gel mould. PCR/DNA samples and 100bp and/or 1kb ladder markers were mixed with loading dye (both NEB) before dispensing into wells of the gel, which was submerged in 0.5 – 1 % TBE buffer within a gel tank. DNA fragments within each sample were then separated (100 V, 150 mA) for approximately 40 minutes. DNA fragments were then visualised under UV light and image capture using an Ingenius UV Illuminator and Genesnap software (both Syngene).

Genotyping of TLR$^{-/-}$ mice

To routinely check the genotype of TLR$^{-/-}$ mice kept in out colonies, and to confirm that the mice used in experiments were of the expected genotype, mouse lesion or tail snip DNA was checked for presence of either the WT gene or the KO construct, using primer pairs that were designed by the Akira lab where the TLR KO mice were originally generated (229), see Table 7. Primers a and b were used to amplify the WT TLR gene, whilst primers b and c were used to amplify the construct used to generate the knock-out. Reactions were carried out in a total volume of 25 μl containing 1 x Taq Mastermix (NEB) 200 μM each primer, 1 μl DNA and nuclease-free water. The reaction conditions for all reactions were as follows: 4 minutes at 95ºC followed by 35 cycles of 94ºC for 30 seconds, 55ºC for 1 minute and 74ºC for 1 minute. A final incubation at 74ºC for 10 minutes was included to ensure full extension of the PCR products. The presence or absence of each product was determined by visualising on agarose gels under UV light.

Genotyping of parasite DNA

To confirm that parasites used in experiments were of the correct species, DNA preparations were used to amplify an intergenic region between the RPS7 genes on chromosome 1 of the *Leishmania* parasite genome. Sequences for the AM1 (forward) and AM2 (reverse) primers were provided by Professor Paul Bates, and are given in Table 7. Reactions were carried out in a total volume of 25 μl containing 1 x Taq Mastermix (NEB) 0.5 μM each primer, 2 μl DNA and nuclease-free water. The reaction conditions for all reactions were as follows: 30 seconds at 95ºC followed by 30 cycles of 95ºC for 30 seconds, 57ºC for 30 seconds and 68ºC for 1 minute. A final incubation at 68ºC for 5 minutes was included to ensure full extension of the PCR products. The 1100- 1400 bp product was then digested with MspI enzyme (in the presence of Buffer 4, both NEB) at 37°C for 2 hours. The presence or absence of each product was determined by visualising the products on agarose gels under UV light. The pattern of bands formed

differs between species: 2 bands of different sizes between the range of 300-350 bp indicates *L. mexicana*, whilst 1 band of approximately 500bp and another of approximately 300bp indicates *L. major*.

Cloning and Expression

PCR for cloning

For cloning purposes PCR products were amplified from target DNA using a high fidelity DNA polymerase (Phusion, NEB) to ensure errors in amplification were kept to a minimum. Primers for amplifying the *L. mexicana* KMP-11 gene were designed to incorporate restriction digest sites (BamHI for the forward primer; HindIII for reverse), a clamp region (C or Gs) at the 5' end, and correspond to the opposing ends of the KMP-11 gene. Thus the primers KMP-11 forward (5'-CGG GAT TCA TGG CCA CCA CGT ACG-3'), and KMP-11 reverse (5'-CGC AAC CTT TTA CTT GGA CGG GTA C-3') (Table 7) were designed, and were purchased from Integrated DNA Technologies. The PCR reaction components in either a 20 or 50 µl volume were as follows: 0.02 U/ml Phusion DNA Polymerase (NEB), 1 x Phusion HF Buffer (NEB), 200 µM dNTPs, 500nM forward and reverse KMP-11 primer, 1-2.5 µl *L. mexicana* DNA, and nuclease-free water. The PCR conditions were as follows: denaturation for 30 seconds at 98˚C, followed by 35 cycles of denaturation for 10 seconds, annealing for 20 seconds and extension for 30 seconds at 98˚C, 67˚C and 72˚C respectively. An additional extension step at 72˚C for 10 minutes was included to ensure proper elongation of the amplicon.To allow for incorporation into the TOPO cloning vector, A-overhangs were added by incubating the PCR product with 0.05 U/ml Taq DNA polymerase (Qiagen) for 9 minutes at 72˚C. The amplified PCR product with A overhangs was purified using a QIAquick column (Qiagen) according to manufacturer's instructions.

Cloning into TOPO2.1

For the first cloning attempt, amplicons were incorporated into the TOPO2.1 vector (Invitrogen) by mixing DNA product (4 µl) with 1 µl TOPO2.1 and 1 µl salt solution gently at room temperature for 5 minutes, before transformation into 10-beta *E. coli* chemically competent cells (NEB) according to manufacturer's protocol. Briefly, 4 µl of the TOPO reaction was added to *E. coli* 10-beta cells (NEB) that had been defrosted on ice, the tube was flicked 4-5 times then incubated on ice for 30 minutes prior to heat-shock at 42ºC for 30 seconds and a further 5 minute incubation on ice. SOC medium (950 µl) was added to cells and incubated at 37ºC for 1 hour with continual shaking. Before use, 40 µl of 40 mg/ml X-gal in DMF was prepared and added to LB Agar plates, which were pre-warmed to 37°C. Cells were diluted into SOC medium at different concentrations and were incubated overnight on LB Agar plates. Positive clones were selected first by blue-white screening and were then selected for further tests after subsequent growth of individual colonies in 5-10 ml LB medium supplemented with 100 µg/ml ampicillin. Pelleted cells from *E. coli* [TOPO] colonies grown overnight could then be used for extraction of plasmid and further analysis. For this, TOPO2.1+construct vectors were eluted from pellet cultured cells into EB Buffer using a QIAprep mini prep kit (Qiagen) according to manufacturer's instructions.

Sub cloning, and expression of KMP-11 in pQE-30 plasmid

To prepare competent cells, *E. coli* M15 [pREP4] cells (supplied in QIAexpress Type IV kit) were streaked onto LB agar plates containing 25 μg/ml and cultured overnight at 37°C. One colony was selected for growing in liquid LB broth containing 25 μg/ml kanamycin up to a total volume of 100 ml at 37°C with continual agitation, until an OD_{600} of 0.5 was reached. The OD_{600} of the bacterial culture was determined by placing 1 ml of culture in a cuvette and measuring using a UV Visible Spectrophotometer (CARY /Varian) The culture was then cooled on ice and centrifuged at 400 **g**, at 4°C for 5 minutes before resuspending cells in 30 ml ice-cold TFB1 Buffer (see Table 3) and kept on ice for 90 minutes. Cells were then centrifuged as before and resuspended in 4 ml ice-cold TFB2 buffer and aliquotted into 100 -200 μl volumes in 1.5 ml tubes and frozen on dry ice before storing at -80°C.

Stock samples of pQE-30 were linearised by digesting with BamHI and HindIII enzymes at 37°C for 2 hours, ran on an agarose gel before being removed and cleaned using the QIAquick gel extraction kit. The target gene KMP-11 for subcloning was removed from selected TOPO vector by digesting TOPO.KMP-11 constructs with BamHI and HindIII enzymes for 2 hours at 37°C. The digested DNA was ran on an agarose gel to visualise the separated BamHI-KMP-11-HindIII construct, which was excised from the gel and purified using a QIAquick column (Qiagen) according to manufacturer's instructions. The construct could then be incorporated into the expression vector pQE-30 by mixing insert and vector DNA at 1 7:1 molar ratio, in the presence of 1.5 U T4 DNA ligase (NEB) and DNA Ligase Buffer at 16°C overnight. Competent *E. coli* M15 [pREP4] cells were transformed to harbour either the expression vector pQE-30, the ligated pQE-30.KMP-11 reaction, or the control pQE-40 plasmid, by mixing 100 μl cells with the approximately 1 μl vector DNA and leaving on ice for 20 minutes before incubating at 42°C for 90 seconds. Psi broth (500 μl) was then adding to transformed cells and they were grown at 37°C for 60-90 minutes with continual shaking, before being spread on LB agar plates containing 25 μg/ml kanamycin and 100 μg/ml ampicillin. Selected colonies were grown up in 10 ml LB medium with 25 μg/ml kanamycin and 100 μg/ml ampicillin and checked for presence of the KMP-11 gene as above.

Selected positive *E. coli* [pQE-30-KMP-11] colonies were grown in the presence of IPTG to induce expression of rKMP-11 and 2 ml of each culture was taken for screening for rKMP-11 expression. Cells were pelleted and resuspended in Native Lysis Buffer at 2ml /g wet weight and left on ice for 30 minutes before sonicating on ice for 6 x 10 seconds at 200-300 W with a 10 second rest between each burst. Lysates were centrifuged at 10,000 **g** for 25 minutes at 4°C to remove insoluble material. For a positive control *E. coli* [pQE40] cells were grown and collected as above, but were lysed using Denaturing Lysis Buffer at 5 ml/g wet weight with gentle vortexing at RT; lysates were collected by centrifugation as above. All lysates were ran on SDS PAGE gels and stained with GelCode blue for visualisation of proteins. Uninduced cultures (i.e where no IPTG was added) were prepared as above as controls. Colonies were

screened for production of recombinant proteins (rKMP-11 at 11 kDa and rDHFR at 26 kDa). Once a positive colony was selected, a 100 ml culture of *E. coli* [pQE-30-KMP-11, pREP4] (or control E. coli [pQE40, pREP4]) was grown in LB medium containing 25 μg/ml kanamycin and 100 μg/ml ampicillin was prepared at 37°C, before IPTG was added at a final concentration of 1M and cultures were grown at 30°C overnight with continual shaking throughout. These cultured cells were centrifuged at 4,000 **g** at 4°C for 20 minutes. Pellets were lysed in the appropriate Lysis Buffer as described previously.

Checking for transformation by PCR and restriction enzyme analysis

In some cases, small samples of individual *E. coli* colonies were added directly to PCR tubes containing the PCR mastermix using a 10 μl pipette tip, prior to the PCR cycles being performed in the PCR machine (i.e. without extraction of DNA from bacterial cells). Alternatively, plasmid DNA extracted from selected colonies was used in PCR reactions and resulting products were checked for product amplification by running on an agarose gel.

For restriction digest analysis, plasmid DNA samples to be tested were incubated with one of the following restriction enzymes along with the appropriate buffer according to manufacturer's instructions: *EcoRI*, *HindIII, SalI* and *BamHI* (all NEB). Typically, samples were incubated at 37°C for at least 40 minutes. The DNA products from the digest were visualised on an agarose gel under UV light.

Sequencing

For sequencing analysis, plasmids containing inserts were eluted using a mini prep kit as described above, but into nuclease-free water and according to the guidelines of the DNA Sequencing Core, Cardiff University, along with appropriate primer preparations. Results were returned in the form of ab1 files, which could then be assessed for sequence and quality using ChromoLite software. Satisfactory sequences could be checked for alignment against a target sequence using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

qPCR for preparations containing *Leishmania* DNA

For quantification of parasites in lesion tissue, a qPCR method was developed based on that described by Nicolas *et al* (230) with modifications. The protocol was adapted by optimising of the following: primer concentration, reaction volume, annealing temperature, $MgCl_2$ concentration, DMSO concentration. The following components were used in each 20 μl reaction: 1 x SybrGreen Mastermix (Qiagen), 500 nM JW11 and JW12 primers, nuclease-free water and 2 μl DNA (samples had concentration between 35 and 150 ng/μl), to amplify a 120 bp region of kinetoplastid DNA. Reactions were performed in duplicate for each sample, in wells of a 96-well high profile white PCR plate (Starlab). The reaction conditions were as follows: 95ºC for 15 minutes, followed by 40 amplification cycles of 95ºC for 15 seconds, 60ºC for 15 seconds and 72ºC for 15 seconds. A melting curve was then generated by increasing the temperature from 50 – 95 °C and reading the plate at each 1 degree increment. A standard curve was included on each

plate, where 8 x 10-fold serial dilutions of DNA from cultured *L. major* or *L. mexicana* parasites were diluted in nuclease-free water and spiked with DNA from naive mouse tissue. The number of parasites per 2 μl sample of standard parasite DNA was plotted against CT value and a standard curve was generated which represented 0.01 – 1 x 10^5 parasites, with a typical CT range of 16 – 36 and a cut off Ct of 35 (< 0.1 parasites). The following controls were included on each plate in duplicate: no template control (NTC), nuclease-free water, DNA from *Leishmania*-positive lesion, and DNA from naive mouse tissue. Average parasite numbers for reactions were used to estimate total parasite burdens per lesion, by adjusting for total DNA volume from the initial DNA extraction. The results of the melting curve were used to determine the presence of product and/or any primer dimer formation.

Protein purification and analysis

Purification of His-tagged recombinant proteins rKMP-11 and rDHFR

Recombinant proteins were generated using the QIAexpressionist system (Qiagen), using the expression vector pQE-30 and *E. coli* M15 cells, producing His-tagged recombinant proteins which were purified from lysates using Nickel column chromatography, according to manufacturer's instructions, with minor modifications. To purify rKMP-11 a 50% Ni-NTA slurry was added to lysate at a ratio of 1:4 and mixed gently at 4°C for 1 hour. The mixture was first purified by loading onto a polypropylene column and allowed to flow through. The column was washed twice in Native Protein Wash Buffer, before eluting in Native Protein Elution Buffer containing increasing concentrations of imidazole. The control protein rDHFR was purified as above but under denaturing conditions by washing with Denaturing Purification Buffer, then Eluting first in Protein Buffer D and then Protein Buffer E. To increase the purity of the rKMP-11, a large batch of rKMP-11 purified using the column method was further purified using the ÄKTA prime plus system (GE Healthcare), according to manufacturer's instructions, to allow for gradual increase in imidazole concentration. The eluents were collected and stored after each step (or every 0.5 ml for AktaPrime) and checked for protein content and purity by SDS PAGE. Eluted fragments containing sufficient purified protein were collected and dialysed to remove imidazole or urea using a Slyde-A-Lyzer Casette (ThermoScientific) by first washing in the corresponding Lysis Buffer without imidazole or urea for 2 hours at 4°C with continual stirring, and then placing in DPBS overnight at 4°C with continual stirring. Endotoxin was removed and samples were checked for endotoxin levels, and yield was determined using the BCA Assay, as described.

Protein concentration

Concentration of protein-containing solutions was determined using the BCA Assay (Pierce), according to manufacturer's instructions. Briefly, the sample of interest was placed, either neat or diluted further in diluent, in duplicate wells of a 96 well plate and in a volume of 10 or 25 μl, before addition of 200 μl of BCA working reagent (WR) to give a ratio of 1:20 or 1:8 sample:WR respectfully. A standard curve of

protein (albumin) diluted in the diluent as the sample (usually DPBS) was used on each plate. The sample/WR mix was left to incubate at 37°C for 30 minutes before the absorbance at 562 nm was measured using a plate-reader. The absorbance values from the wells containing diluted standards were used to generate a standard curve by fitting a line of best fit; this was used to calculate the concentration of protein in sample wells.

SDS PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was performed by mixing protein-containing samples were mixed with NuPAGE sample loading buffer (Invitrogen) and NuPAGE reducing buffer (Invitrogen), and heated at 96°C for 10 minutes before transferring to ice for immediate loading on to the SDS PAGE apparatus. The SDS PAGE mini cell apparatus (BioRad) was assembled to include pre-made Precise Protein Tris-HEPES-SDS Gels (Pierce; with either 10, 12 or 15 lanes and either a 12 or 4-20% gradient of SDS gels) submerged in Running buffer (see Table 3). Protein solutions were then loaded into individual lanes on the pre-cast gels. For estimation of protein size, a pre-stained Kaleidescope Precision Plus Protein Standard (BioRad) was also loaded. The gel ran at 150 V for 30 – 40 minutes.

Gels were washed 3 times in dH_20 with continual agitation for 15 minutes, before submerging in GelCode Blue (ThermoScientific) and agitating at RT for 1 hour. Gels were de-stained by rinsing several times in dH_20 for approximately 1-2 hours.

Western Blot

For immunoblotting /western blot, SDS PAGE gels were generate containing proteins of interest, as described above. These were immediately submerged in ice-cold Transfer Buffer, along two pieces each of sponge and filter paper, for 15 minutes. The PVDF membrane was activated by submerging in methanol for 15 seconds, in dH_20 for 2 minutes, then in Transfer Buffer for 15 minutes. A blot module was assembled in a cassette, in which the gel and PVDF membrane were placed next to each other, surrounded by filter paper and sponges on each side. Cassettes were placed in a mini cell submerged in ice-cold Transfer Buffer. To keep the reagents cool, an ice pack was also placed in the cell. The tank ran at 350 mA for 50 minutes to transfer proteins from the gel from the PVDF membrane.

PVDF membranes could then be stained using immunoblotting. The general procedure for this was as follows: firstly, membranes were blocked in 4 % milk in PBST for 2 hours with continual agitation. The membranes were then stained with primary antibody, diluted in 4% milk in TBST, and incubated at 4°C overnight with continual agitation. The membrane was then rinsed in TBST at RT for 15 minutes once, then for 5 minutes three times, before incubation with secondary antibody diluted in 4% milk for 1 hour, before a period of washing in PBST as before. Details and concentrations of antibodies used are indicated in individual methods and results sections.

Removal of endotoxin

Endotoxin was removed from rKMP-11 preparations using Detox-Gel and Columns (ThermoScientific) according to manufacturer's instructions. Samples were tested before and after endotoxin removal for levels of endotoxin by using a pre-paid endotoxin testing service (Lonza).

Statistical Analysis

Data was collected into excel spreadsheets and explored and analysed using both SPSS 20 © and R (version 2.15.2) © software packages. Datasets were tested for normality and other typical distributions, before deciding on the appropriate statistical methods. Parametric tests were used when data was found to be normal, to fit another typical distribution (e.g. Poisson or negative binomial), or could be normalised by transformation. However parametric tests were only used if all comparable datasets could also be analysed in the same way. As indicated in the results chapters, in many cases both approaches were taken and the findings were compared. To explore differences in variation in outcomes between groups, ANOVA or Kruskall-Wallis tests were used, for parametric or non-parametric analysis respectively. However, to compare averages between two groups, the student's t test or Mann-Whitney U test was used, for parametric or non-parametric analysis respectively, instead of using post-hoc tests using the multivariate techniques. This is because, after considerable exploration of the datasets and discussion with a statistician, it was decided that multivariate analyses to compare groups were not appropriate for this type if study where small sample sizes are generally used. Although this approach risks increasing the number of type I errors recorded, this is only an issue in those cases where the significance is low ($p = 0.1 – 0.5$). Indeed, bivariate analysis is also an approach widely used by many others exploring the outcomes of parasitic infection in mouse models (43, 70, 86, 203, 231, 232) . Correlation of non-parametric variables was achieved using Spearman's rank test. Graphs were generated using GraphPad Prism 5 © software.

Chapter 3. The role of TLRs in *Leishmania major* infection

Abstract

The role of TLR2 and its co-receptors during infection with *L. major* is not currently known, despite several studies reporting a role for TLR2 in the recognition of surface glycolipids from the parasite. In this study, a role for TLR2 in control of *L. major in vivo* was confirmed, as mice deficient in TLR2 presented with larger lesions and higher parasite burdens than their WT counterparts at the height of infection. A role for TLR4 was also found in reducing disease after *L. major* infection, as has been reported in other studies. Recall immune responses by DLN cells *in vitro*, and heightened levels of antigen specific IgG1 in the plasma of infected TLR2$^{-/-}$ mice suggests that TLR2 acts to reduce development of regulatory and Th2 responses to *L. major*, which act to exacerbate infection. Surprisingly, neither of the known co-receptors for TLR2 (TLR1 and TLR6), was found to have a role in controlling *L. major* in combination with TLR2, as mice lacking these co-receptors did not develop exacerbated disease. Interestingly, TLR6$^{-/-}$ mice appear to have increased resistance to *L. major* and are able to heal lesions faster than WT mice and other groups, suggesting TLR6 exacerbates infection in WT mice. Together these results demonstrate an important role for TLR2 in the control of *Leishmania in vivo* and regulation of protective Th1 immunity and show TLR2 recognition occurs independently of the canonical TLR2 co-receptors, TLR1 and TLR6, and that paradoxically TLR6 deficiency promotes a more rapid healing phenotype.

Introduction

Background

The mouse model of *L. major* infection in mice (particularly BALB/c and C57BL/6 strains) has been extensively studied for markers of resistance and susceptibility and has given insight into the nature of immune responses, which are required for control of intracellular pathogens such as *Leishmania* spp. In particular the adaptive immune response has been comprehensively examined in C57BL/6 and BALB/c mice infected with *L. major*, and from this we now understand the key elements that are required for control of infection (39). The most important protective response is a robust Th1 response characterised by production of IFNγ, leading to classical activation of MΦs, production of TNFα and NO, and finally to intracellular killing of parasites. Whilst many studies have explored the innate immune response to *L. major* parasites by distinct cell subsets *in vitro*, a limited number of *in vivo* studies exploring the role of the innate immune response during chronic infection have been reported. Such *in vivo* studies would help us to understand a role for innate immune responses to parasites in controlling disease during infection and how these influence adaptive immune responses. Studies using knockout mouse strains have identified a role for TLR pathways as mice lacking the adaptor molecule MyD88 were highly susceptible to *L. major* and mounted an inappropriate Th2 response (26, 199, 233). This suggested that TLR activation during infection may have a role in priming the production of IL-12 and a switching to a protective Th1 response. A role for TLR4 in controlling *L. major* infection has been suggested by *in vivo* studies by one group (202, 203), although disputed by another (207), and TLR9 has been shown to play a role in controlling *L. major* infection *in vivo* (206). TLR2 has been implicated in the recognition of *Leishmania* parasites *in vitro*, and in particular the recognition of LPG, the major surface glycolipid present on the infective promastigote stage (26). It has been reported that activation of TLR2 by LPG results in both a pro-inflammatory phenotype as shown by increased Th1 cytokine production by NK cells (27) and NO production in MΦs (205), but also a regulatory phenotype as shown by increased expression of suppressors of cytokine signalling (SOCS) molecules SOCS-1 and SOCS-3 in murine MΦs (26). Furthermore, different forms of LPG (i.e. soluble or membrane bound) have been shown to result in differently activated phenotypes of PBMCs after TLR2 engagement (234).

Aim of the study

In this study, mice lacking TLR2, TLR1, TLR6 and TLR4, were infected with *L. major* to determine the role of TLR2 and its known co-receptors *in vivo*, and to compare this to TLR4, which has previously been reported to control *L. major*.

Methods

Mice, parasites and infections

All procedures involving live animals were performed at the BSU in the Duncan Building, University of Liverpool. Female age matched WT (C57BL/6), TLR2$^{-/-}$, TLR1$^{-/-}$, TLR6$^{-/-}$ and TLR4$^{-/-}$ mice were infected with either 10^5 *L. major* FV1 stationary-phase promastigotes by s.c. injection to the shaven rump in a 100 μl volume of HBSS, or with 10^5 *L. major* LV39 to the upper side of the LHF. These parasites cultures were confirmed to be negative for mycoplasma contamination. Lesion progression was monitored by taking weekly measurements of lesion size using a metric dial calliper, and these measurements were used to generate area under the curve (AUC) values. At the end of infection experiments, mice were culled via cardiac puncture to allow for the collection of blood for plasma samples (with the exception of the first experiment). The lesion was removed and either processed for limiting dilution or placed in RNA later, and the spleens and DLNs were removed under sterile conditions and processed for cell stimulation experiments or parasite burden analysis (DLN only, experiment 1).

Parasite burden

The parasite burden of tissues was estimated by qPCR using JW11 and JW12 primers as described. This method was first validated against the more widely used method of limiting dilution. For this validation, lesion (n = 4) or DLN tissues (n = 29) were homogenised and passed through a cell strainer to obtain a single cell suspension in a fixed volume of complete M199. Half of this was used for limiting dilution, whilst the other half was used for qPCR by centrifugation of cells and extraction of DNA using a Qiagen Blood and Tissue Kit.

Cell stimulations and immunological techniques

For these experiments, DLN cells and splenocytes were used a concentration of 4 x 10^5 cells/ well. Cells were cultured for 72 hours in the presence of either 20 μg/ml *L. major* FV1 FTAg, 2.5 μg /ml ConA or media alone in a total volume of 200 μl/well. Culture supernatants were then removed and stored at -20°C until analysis for IFNγ and IL-10 levels using cytokine ELISA. The levels of antigen specific IgG1 and IgG2c in plasma samples from mice were measured using antibody ELISA with *L. major* FV1 FTAg as the capture antigen.

Wound healing

To assess wound healing in WT and TLR6$^{-/-}$ mice, 6 mice of each genotype were given a 4 mm circular wound using a punch biopsy tool, and healing was measured by recording the diameter of the wound at regular timepoints as indicated.

Results

Lesion development

To measure development of *L. major* infection, the lesions of mice were measured every week until the end of the infection in two experiments; in the first experiment mice were all culled at week 12 and in the second experiment some mice were culled at either week 10 or 18; results are displayed in Figure 9 and Figure 10. All groups showed similar patterns of lesion development, with appearance of lesions between 4-6 weeks p.i. and a peak in lesion size at 10 – 12 weeks, before reduction in lesion size and healing at approximately 18 weeks. Mice lacking TLR2 showed larger lesions than WT mice at week 10 in experiment 2 and at week 12 in experiment 1 but were able to control the infection eventually as shown in Figure 9A and Figure 10A, suggesting that these mice are less able to control lesion development but that TLR2 is not crucial for the eventual healing of *L. major* lesions in C57BL/6 mice. Similarly, $TLR4^{-/-}$ mice had larger lesions at time points between week 9 and week 12 post infection in both experiments, but were also able to eventually control the growth of lesions after infection with *L. major* (Figure 9D and Figure 10C).

Mice which lacked TLR1, a known co-receptor of TLR2, did not show any difference in development of lesions compared to WT mice in the first experiment. The average lesion size was smaller than that of $TLR2^{-/-}$ mice in the later stages of infection, but this difference did not reach significance ($p = 0.064$ for lesion area at 12 weeks p.i.). As there was no difference to control mice in experiment 1, a group of $TLR1^{-/-}$ mice was not included in experiment 2. $TLR6^{-/-}$ mice showed a different phenotype of lesion development to the other groups, with lesions being larger than in WT mice soon after their initial appearance (weeks 4-7), but healing earlier so having smaller lesions in the later stages of infection (week 11-12 in experiment 1 and week 13 in experiment 2), as shown in Figure 9C and Figure 10B.

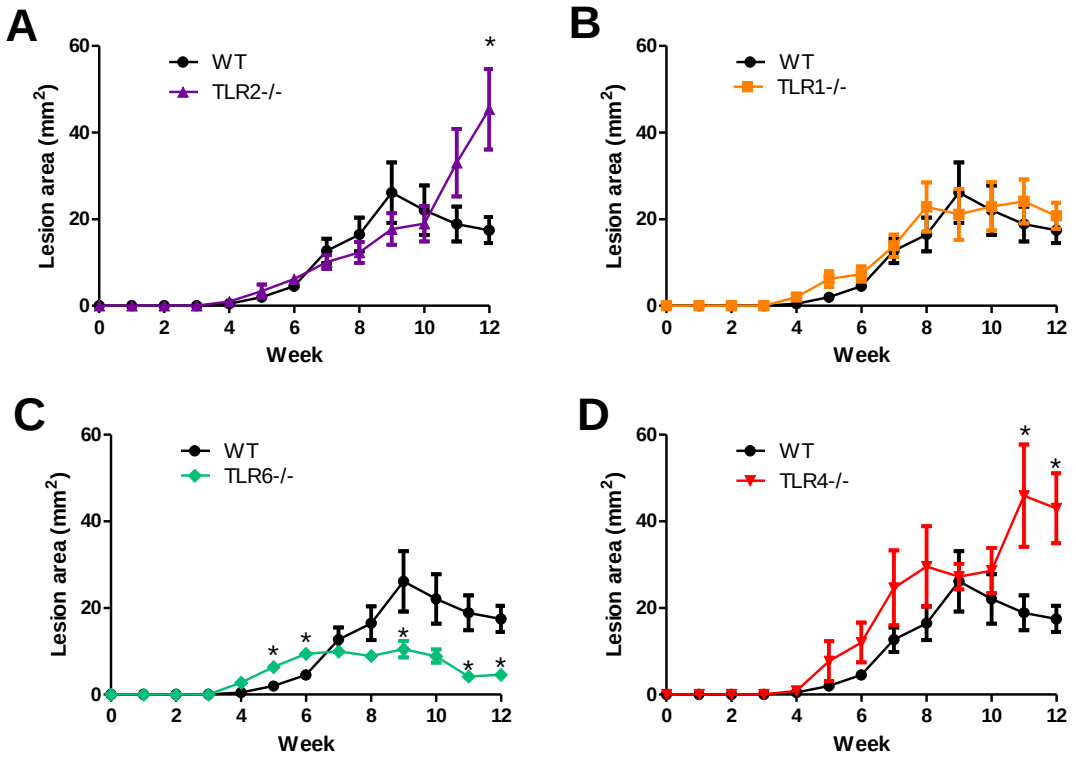

Figure 9. Lesion development in WT and TLR$^{-/-}$ mice infected with *L. major* (experiment 1). Lesion development in WT mice is presented in all charts, and was compared to that of TLR2$^{-/-}$ (A), TLR1$^{-/-}$ (B), TLR6$^{-/-}$ (C) and TLR4$^{-/-}$ (D) mice infected with *L. major*, experiment 1. Data points indicated the mean values for each group +/- SEM (n = 5-7). Groups were compared using the Mann-Whitney U test (* p<0.05).

The overall lesion development seen in such infection experiments can be represented by calculating the AUC for the lesion sizes over time for each individual. The average AUC values are displayed in Figure 11. The datasets were found to be log-normally distributed so parametric tests were possible for the logged values, and groups were compared using the independent samples t test. These data indicate more severe disease overall in TLR4$^{-/-}$ mice in particular, with AUC values being significantly greater than WT mice at week 10, 12 and 18 p.i. in this group. Whilst no other significant differences in AUC values were seen, the general pattern was found to be the same as indicated by the lesion sizes over time – with TLR2$^{-/-}$ mice having exacerbated disease compared to WT, and TLR6$^{-/-}$ mice having reduced disease in the later timepoints, especially compared to TLR2$^{-/-}$ and TLR1$^{-/-}$ mice ($p=0.011$ at week 12).

The AUC values for the first experiment (week 12) show a decoupling of TLR6$^{-/-}$ mice from either TLR2$^{-/-}$ or TLR1$^{-/-}$ mice – with significantly lower AUC values in this group (Figure 11B). The reduction in lesion size and development was not as evident for TLR6$^{-/-}$ mice in the second experiment, although the TLR6$^{-/-}$ mice did heal lesions earlier than the other groups (Figure 10B), which is reflected in the AUC values.

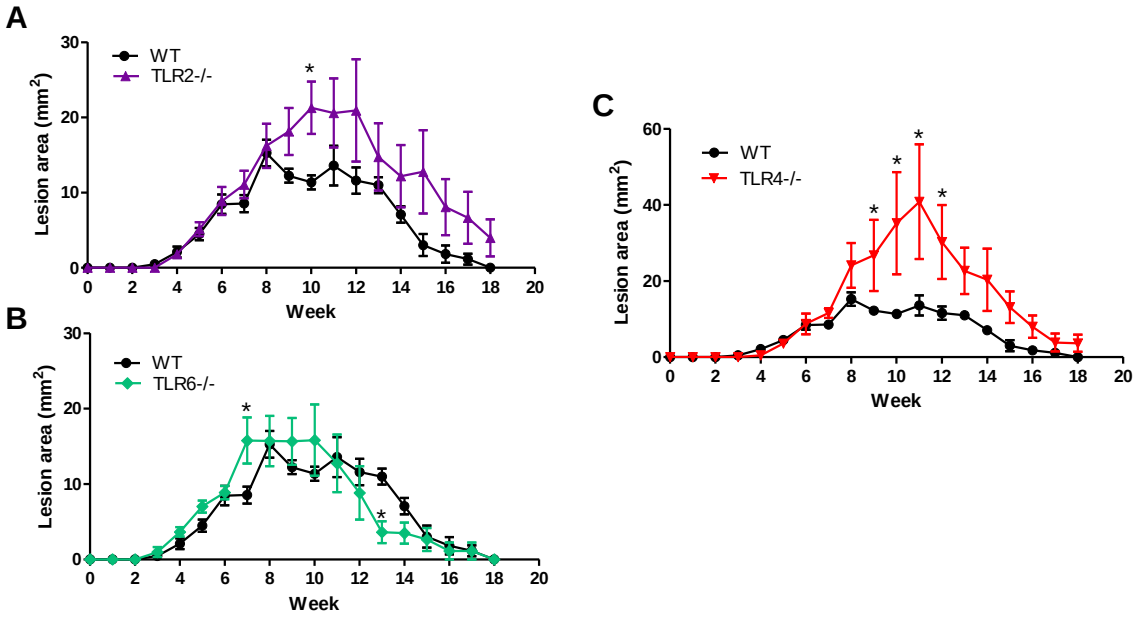

Figure 10. Lesion development in WT and TLR$^{-/-}$ mice infected with *L. major* (experiment 2). The lesion development of WT mice is presented in all charts, and was compared to that of TLR2$^{-/-}$ (A), TLR6$^{-/-}$ (B), and TLR4$^{-/-}$ (C) mice, infected with *L. major* for 18 weeks. Data points indicated the mean values for each group +/- SEM (n = 4-9); note that some mice were sacrificed at week 10. Groups were compared using the Mann-Whitney U test (* p<0.05).

In the first experiment, the number of lesions that ulcerated was recorded at week 8 and week 9 when the ulcerations were first noticed. At week 10 in the first experiment no lesions remained ulcerated. In the second experiment the presence of ulceration was recorded at every time point, so that a % of lesions that ulcerated could be calculated (Figure 11 E) for each group. The level of ulceration recorded was generally greater in the second experiment, and TLR2$^{-/-}$ mice showed the greatest tendency towards ulcerations, with 100% have a lesion which was ulcerated at some point during infection with *L. major* FV1 in the second experiment.

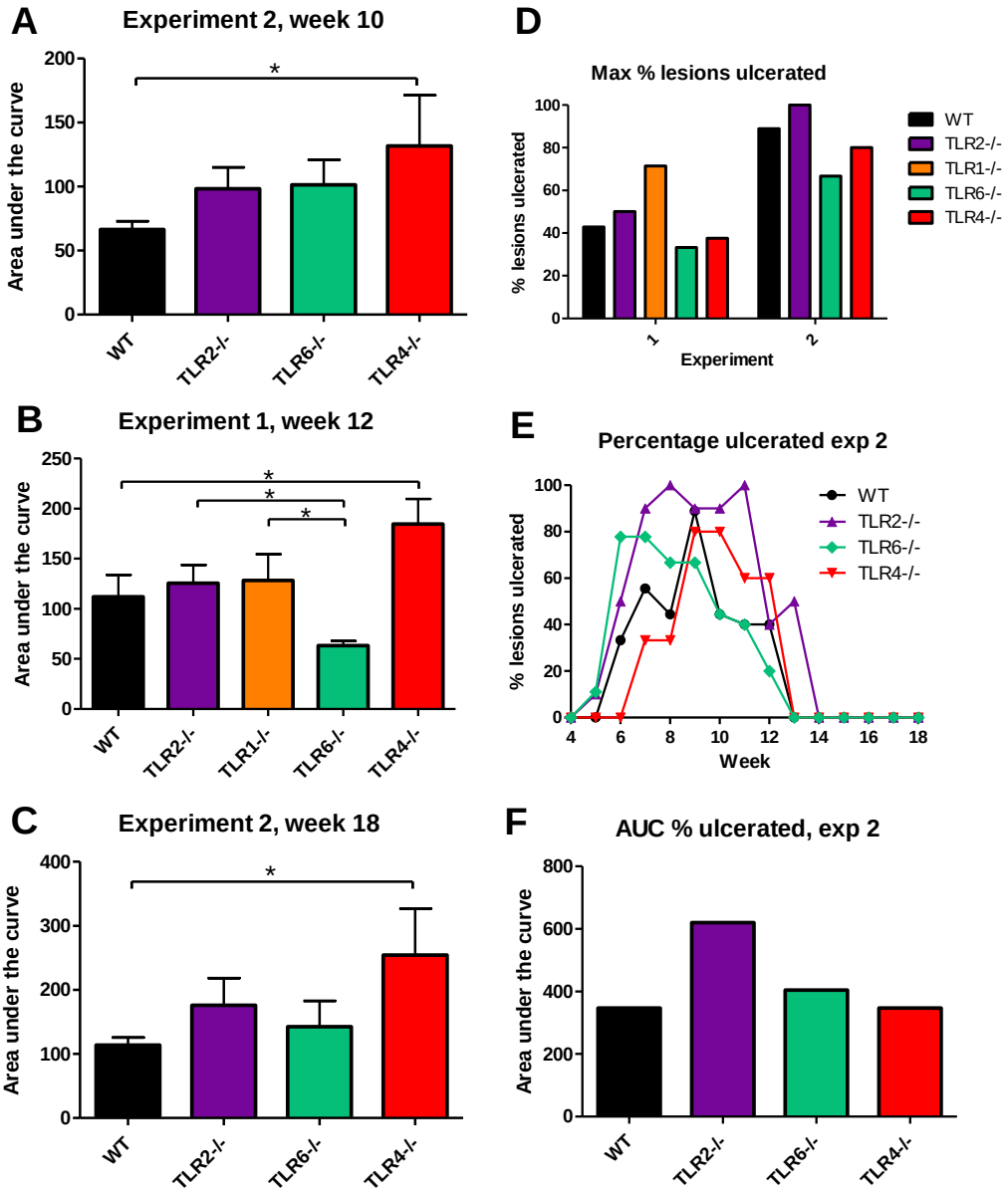

Figure 11. **AUC analysis and levels of ulceration for *L. major* infection experiments 1 and 2. Average AUC values are shown (mean + SEM) for each group in the different experiments and at different timepoints (A-C) Bars represent mean values + SEM. Groups were compared using the Mann-Whitney U test (* $p<0.05$). The maximum percentage of lesions that ulcerated was recorded in each group of mice, at any one time during the infection experiments, was calculated and is presented in (D). The percentage of lesions that ulcerated was recorded for each group at each time point, and the percentages of the groups used in experiment 2 are given in (E), along with the area under the curve values for the ulcerated lesion percentage data, over the entire of experiment 2 (F).**

Development of qPCR method for quantifying parasites in infected tissue samples

In order to accurately quantify parasite burden in lesion tissues, a qPCR method was developed based on that published by Nicolas *et al* (230) but with modifications for use with a real-time thermocycler machine

and SybrGreen. To compare this method with a more widely used method of limiting dilution, two types of infected tissue (DLN and rump lesion) were first homogenised and processed for limiting dilution, and a proportion of each sample was also taken for parasite quantification by qPCR. In both cases the average test result was used to calculate the overall parasite burden in the starting sample. The results of the qPCR quantification for each tissue were much greater (roughly 100 x greater) than that given by limiting dilution, suggesting the method is more sensitive at detecting individual parasites compared to limiting dilution However, whereas limiting dilution is a measure of the number of viable parasites within the lesion tissue, the qPCR method will also potentially measure the DNA of dead parasites (prior to sufficient DNA degeneration) as well as living viable parasites in the lesion.

A plot of limiting dilution against qPCR parasite burden values is given in Figure 12. Neither variable follows a normal distribution so the relationship between the two measurement methods was measured using the Spearman rank-order correlation method, which found there was a significant monotonic relationship between the two measurements (Correlation coefficient = 0.633; $p<0.001$). Thus it was felt the qPCR method was both more sensitive, and validated against, the standard method of limiting dilution.

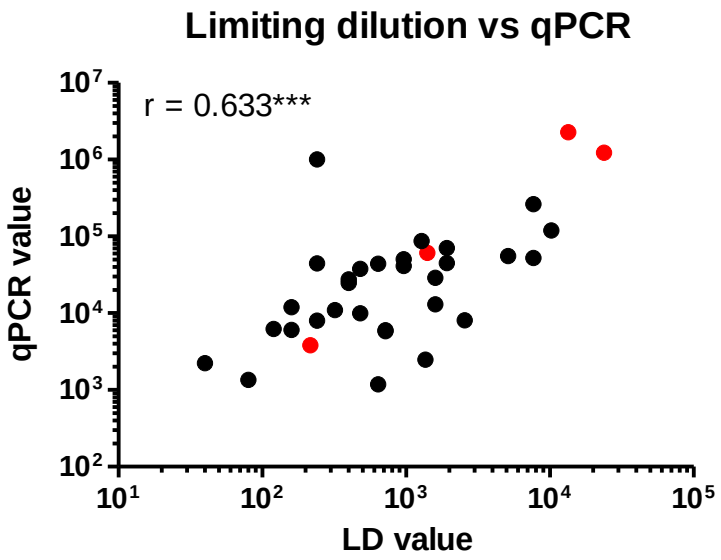

Figure 12. Limiting dilution vs qPCR. *L. major* infected tissues (DLN = black; lesion = red) were disrupted and analysed for parasite burden using both limiting dilution and qPCR methods. The values obtained using each method were plotted against each other and correlated using the spearman's rank-order correlations as neither population was normally distributed. This gave an r value of 0.633, $p<0.001$.

Parasite burden data

The parasite burdens in collected lesion tissue samples were quantified by using the validated qPCR method. CT values obtained from duplicate reactions for each lesion DNA sample were used to quantify parasites per µl DNA using the standard curve containing DNA extracted from quantified parasites

spiked with naïve mouse DNA. The results were then adjusted for total DNA volume to give an overall value for parasites per lesion.

Analysis using parametric methods

The mean and standard deviations of the parasite burden data per genotype is given in Table 8.

Group	Week 10 (experiment 2)			Week 12 (experiment 1)			Week 18 (experiment 2)		
	n	Mean	SD	n	Mean	SD	n	Mean	SD
C57BL/6	4	1003933.25	1966764.268	6	235495.50	468475.433	5	395.49	547.499
TLR2$^{-/-}$	5	3588890.00	134342.421	7	6557664.29	9403765.772	4	7827.67	12838.64
TLR1$^{-/-}$	-	-	-	6	64100.17	81510.392	-	-	-
TLR6$^{-/-}$	5	319478.14	427381.360	5	5784.00	8051.109	4	10.65	9.727
TLR4$^{-/-}$	-	-	-	7	1011047.57	1742798.361	5	47595.09	67541.265

Table 8. Average parasite burdens in lesions of WT and TLR$^{-/-}$ mice at different time points post infection with *L. major*. Means and standard deviations are given for each timepoint.

The parasite burden data show extra-Poisson dispersal (i.e. the variation is greater than the mean), as is typical for count data, so groups were compared by fitting parasite count data to a negative binomial model using a generalised linear model function, which seemed to fit well. Comparisons between groups are made by generating incidence rate ratios and identifying the confidence intervals, as displayed in Table 9.

Comparison	Week 10 (experiment 2)			Week 12 (experiment 1)			Week 18 (experiment 2)		
	IRR	95% CI	P value	IRR	95 % CI	P value	IRR	95 % CI	P value
TLR2$^{-/-}$ / WT	3.575	0.66 – 19.37	0.140	27.85	4.82 – 160.99	***<0.001***	19.80	3.39 – 115.68	***0.001***
WT / TLR1$^{-/-}$	-	-	-	3.67	0.66 – 20.61	0.139	-	-	-
WT / TLR6$^{-/-}$	3.142	0.44 – 22.44	0.254	40.72	6.62 – 250.59	***<0.001***	37.66	9.88 – 143.55	***<0.001***
TLR4$^{-/-}$ / WT	-	-	-	4.24	0.66 – 27.95	0.127	120.37	25.43 – 569.67	***<0.001***
TLR2$^{-/-}$ / TLR1$^{-/-}$	-	-	-	102.30	26.45 – 395.73	***<0.001***	-	-	-
TLR2$^{-/-}$ / TLR6$^{-/-}$	11.23	3.77 – 33.46	***<0.001***	1133.76	260.92 – 4926.42	***<0.001***	745.50	151.01 – 3680.38	***<0.001***
TLR2$^{-/-}$ / TLR4$^{-/-}$	-	-	-	6.49	1.39 – 30.19	***0.017***	-	-	-
TLR4$^{-/-}$ / TLR2$^{-/-}$	-	-	-	-	-	-	6.08	1.02 – 36.13	***0.047***
TLR1$^{-/-}$ / TLR6$^{-/-}$	-	-	-	11.08	2.64 – 46.45	***0.001***	-	-	-
TLR4$^{-/-}$ / TLR1$^{-/-}$	-	-	-	15.77	3.51 – 70.93	***<0.001***	-	-	-
TLR4$^{-/-}$ / TLR6$^{-/-}$	-	-	-	174.80	34.98 – 873.53	***<0.001***	4532.86	1163.37 – 17661.43	***<0.001***

Table 9. Incidence Rate Ratios (IRRs) comparing average lesion parasite burden between genotypes. Only the IRRs where values are greater than 1 were displayed to avoid duplication.

The parasite burden data, graphically displayed in Figure 13, indicate that parasite burdens in the infected mice generally follow the same trend as seen with lesion sizes – i.e. TLR2$^{-/-}$ and TLR4$^{-/-}$ mice display larger parasite burdens than WT mice, and this is more evident in the later timepoints post infection,

whereas TLR6$^{-/-}$ mice show smaller parasite burdens compared to WT and other groups, and again this is more prominent at the later time points. As with the lesion and AUC results, TLR1$^{-/-}$ mice showed no difference in parasite load compared to WT mice at week 12 (experiment 1), but had larger parasite burdens compared to TLR6$^{-/-}$ mice and smaller parasite burdens compared to TLR2$^{-/-}$ mice.

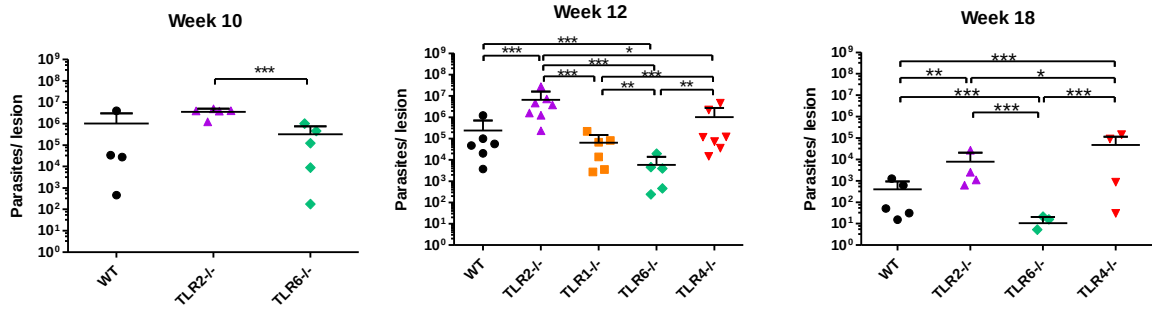

Figure 13. Parasite burden of lesions of WT and TLR$^{-/-}$ mice at 10, 12 and 18 weeks post infection with *L. major,* compared using parametric methods (* p<0.05; **p<0.005; ***p<0.001). Individual parasite burdens are displayed, as well as the mean + SD.

Analysis using non-parametric methods

As the datasets in this study are quite small, it is not entirely clear whether the negative binomial model is the best method for analysing the data, and the convention elsewhere in similar studies is to use non-parametric tests. Thus, groups were also compared to each other using the Mann-Whitney U test, and the results are given in Table 10 below.

	Week 10			Week 12			Week 18		
Comparison	**U/W**	**z**	**p**	**U /W**	**z**	**p**	**U /W**	**z**	**p**
WT vs TLR2$^{-/-}$	3 / 13	-1.715	0.111	1 / 22	-2.857	***0.002***	2 /17	-1.96	0.063
WT vs TLR1$^{-/-}$	-	-	-	14 /35	-0.641	0.589	-	-	-
WT vs TLR6$^{-/-}$	10 /25	0	1.00	3 /18	-2.191	***0.030***	2 /12	-0.196	0.063
WT vs TLR4$^{-/-}$	-	-	-	13 /34	-1.143	0.295	10 /25	-0.522	0.690
TLR2$^{-/-}$ vs TLR1$^{-/-}$	-	-	-	0 /21	-3.00	***0.001***	-	-	-
TLR2$^{-/-}$ vs TLR6$^{-/-}$	0 /15	-2.611	***0.008***	0 /15	-2.84	***0.003***	0 /10	-2.309	***0.029***
TLR2$^{-/-}$ vs TLR4$^{-/-}$	-	-	-	7 /35	-2.236	***0.026***	9 /24	-0.245	0.905
TLR1$^{-/-}$ vs TLR6$^{-/-}$	-	-	-	7 /22	-1.461	0.177	-	-	-
TLR1$^{-/-}$ vs TLR4$^{-/-}$	-	-	-	10 /31	-1.571	0.138	-	-	-
TLR6$^{-/-}$ vs TLR6$^{-/-}$	-	-	-	1 /16	-2.68	***0.005***	3.5/13.5	-1.599	0.111

Table 10. Comparison of parasite burdens in WT and TLR$^{-/-}$ mice using non-parametric tests.

The results from these tests give similar findings to when fitting the negative binomial model, i.e. TLR2$^{-/-}$ mice had significantly greater parasite burdens compared to WT (week 12), TLR1$^{-/-}$ (week 12), TLR6$^{-/-}$

(week 10, 12 and 18) and TLR4$^{-/-}$ (week 12) mice. TLR6$^{-/-}$ mice have reduced parasite burdens, with a significantly lower burden compared to WT at week 12 and at all time points when compared to TLR2$^{-/-}$ mice.

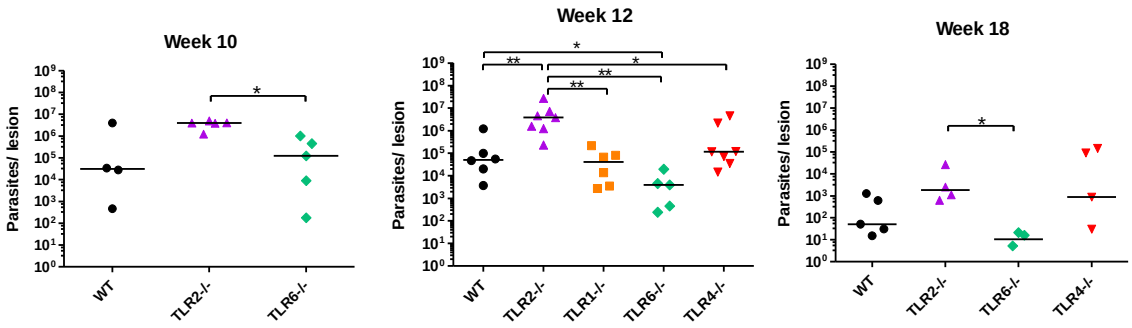

Figure 14. Parasite burdens in WT and TLR$^{-/-}$ mice at 10, 12 and 18 weeks post infection with *L. major*, compared using non-parametric methods (* p<0.05; **p<0.005; ***p<0.001). Individual parasite burdens are displayed, along with the median value for each group. Zero values were reported for the week 18 time point (for one TLR6$^{-/-}$ and one TLR4$^{-/-}$ mouse) but do not appear on the charts due to the use of logarithmic scale on the y axis.

One major exception in the findings using non-parametric comparison tests (compared to parametric) is that no differences in parasite burden are found between WT and TLR4$^{-/-}$ mice at the time points measured, despite the elevated lesion sizes and AUC values found in this group. Also, TLR2$^{-/-}$ mice did not show significantly greater parasite burdens when compared to WT mice at week 18 when compared using the Mann-Whitney U test, although the p value indicates this difference is almost significant (p=0.063).

Parasite burden in DLNs of mice, week 12

As the DLN tissue was taken for validation of the qPCR method of quantifying parasite burden, the values could be used to quantify the parasite burdens in the DLN of mice from the different groups. The results indicated extra-Poisson dispersal as with the lesion parasite data, thus the data was fitted to a generalised linear model using a negative binomial model to compare parasite burdens by group of mice using parametric methods, as described above. As shown in Figure 15, TLR2$^{-/-}$ mice had the highest parasite burdens in the DLN tissue compared to other groups, and the burden was significantly greater than in WT mice when compared using both parametric and non-parametric tests. In addition, TLR2$^{-/-}$ displayed higher DLN parasite burdens compared to both TLR1$^{-/-}$ and TLR6$^{-/-}$ mice according to the parametric tests.

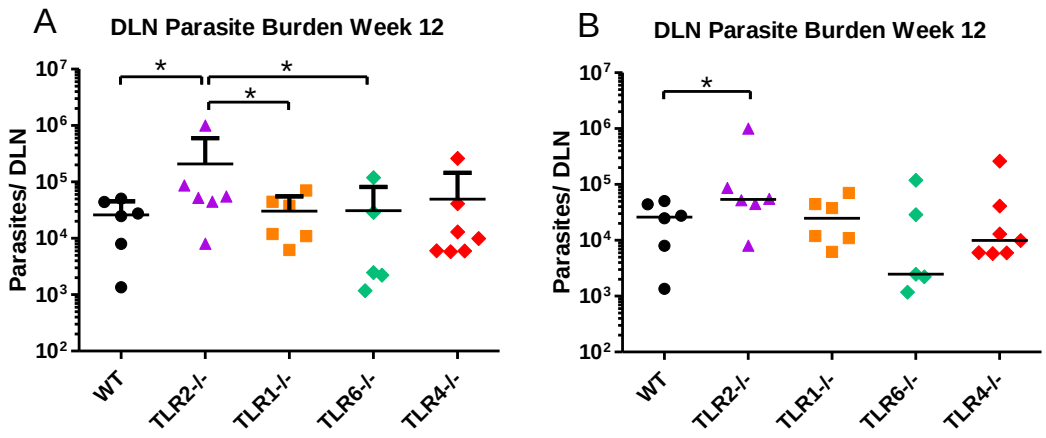

Figure 15. Parasite burden in DLN tissue in WT and TLR$^{-/-}$ mice at week 12 p.i. with *L. major* FV1, analysed using parametric and non-parametric tests (* p<0.05; **p<0.005; ***p<0.001). A shows the individual parasite burden data plotted, with mean + SD and significant differences as identified by parametric methods (using generalised linear model and negative binomial distribution), whilst B shows data with median values and significant difference as identified by non-parametric methods (Mann-Whitney U test).

Cytokine responses in WT, TLR2$^{-/-}$ and TLR6$^{-/-}$ mice at week 10 post infection

To explore the immune responses in the different mice during infection with *L. major*, immune cells of mice were taken for stimulation assays at the 2 time points when mice were culled in experiment 2: week 10 and week 18. DLN cells and splenocytes were processed and stimulated with *L. major* FTAg or controls for 72 hours. The supernatants from these stimulations were then used to measure cytokine levels using ELISA. The two cytokines chosen for analysis were IFNγ and IL-10, given their known roles in resistance and susceptibility, respectively, in this model of infection with *L. major*. Splenocytes from naïve mice of each genotype were used as controls (n=6 total) and there was no detectable cytokine response to FTAg in these stimulations, whereas ConA gave increased cytokine production in all cases (data not shown). The results for the cytokine responses in stimulations of cells from infected mice at week 10 are shown in Figure 16. The results of the cytokine levels found in all experimental groups is given in Appendix 2.

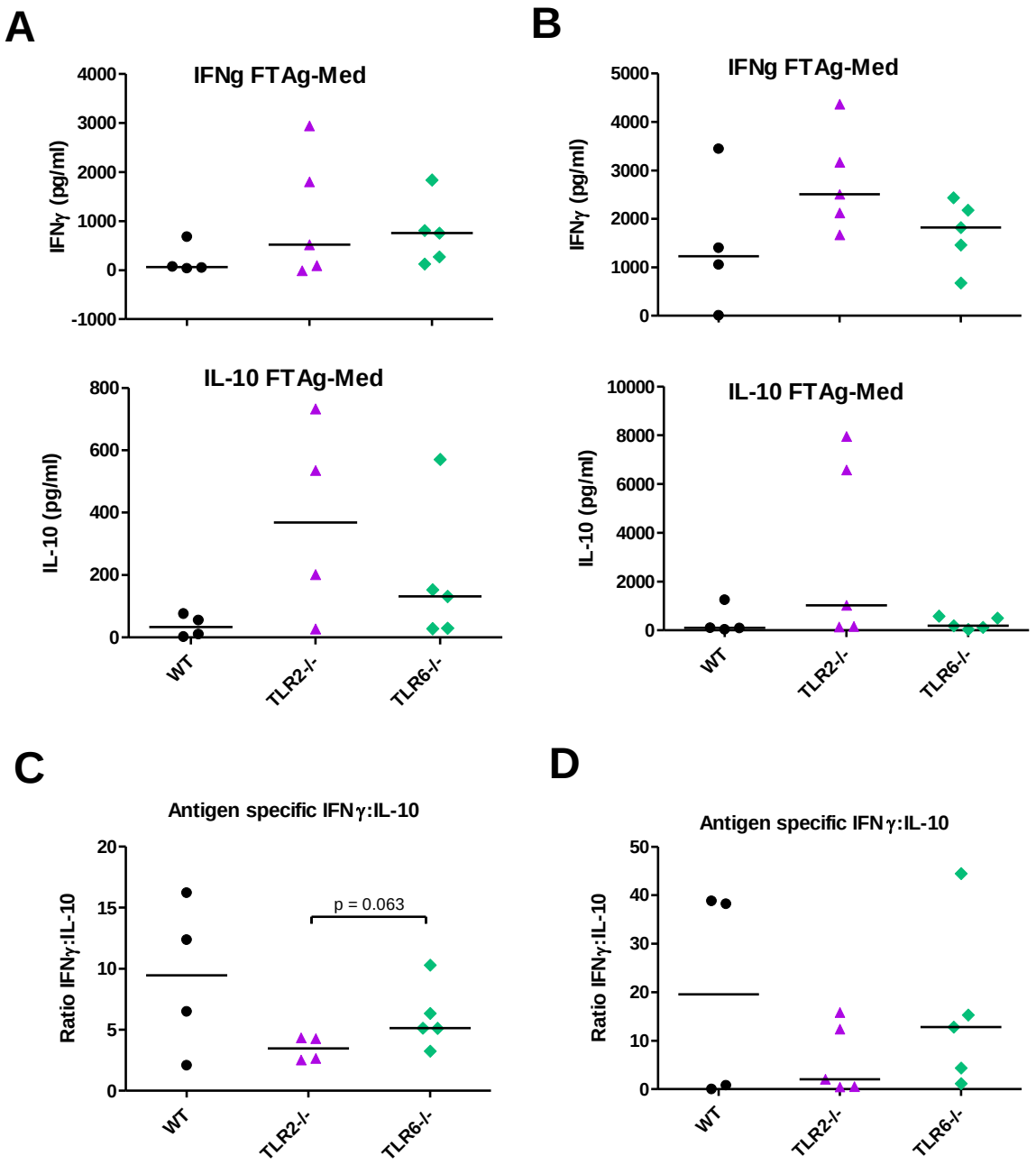

Figure 16. Cytokine responses at week 10 post infection. WT, TLR2$^{-/-}$ and TLR6$^{-/-}$ mice were infected with *L. major* and infection was allowed to develop for 10 weeks. At the end of the experiment, DLN cells and splenocytes were recovered and stimulated *in vitro* with *L. major* FTAg antigen and controls. After 72 hours the supernatants were removed and were analysed by ELISA for the presence of IFNγ and IL-10. Individual points represent the mean average levels of cytokine calculated from duplicate cultures per mouse, and horizontal bars represent median averages for each group. The results of IFNγ and IL-10 production in FTAg stimulated cells (with media control values subtracted) are shown for DLN (A) and spleen (B) cells. The ratio of antigen specific IFNγ:IL-10 produced by DLN cell (C) and splenocytes (D) is also shown.

Although not significant, the TLR2$^{-/-}$ mice showed a trend for elevated antigen specific IFNγ and IL-10 responses compared to WT and TLR6$^{-/-}$ mice in DLN cells and splenocyte stimulations (Figure 16 A, B),

perhaps indicating a greater number of T cells specific for *L. major* in these mice, although further information is required to concluded this (e.g. by performing ELISPOTs). When these values were used to measure the ratio of these cytokines produced in response to antigen, the TLR2$^{-/-}$ mice had a reduced amount of IFNγ to IL-10 produced in response to the parasite antigen when compared to that seen for WT and TLR6$^{-/-}$ mice (Figure 16 C, D), and this was almost significant when compared to TLR6$^{-/-}$ in the DLN cell stimulations ($p=0.063$). This suggests a more regulated immune responses in the TLR2$^{-/-}$ mice at week 10 post infection. Indeed, two individuals in the TLR2$^{-/-}$ group produced more IL-10 in response to FTAg than in response to the ConA positive control (Appendix 2), suggesting a high level of regulatory immune responses to *L. major* occurring in these mice.

Cytokine responses in WT, TLR2$^{-/-}$, TLR6$^{-/-}$ and TLR4$^{-/-}$ mice at week 18 post infection

Immune responses were also recorded for mice at week 18 p.i. with *L. major*, and the results from the cell stimulation cytokine ELISAs are given in Figure 17. As with the week 10 cytokine response, a high level of variation was seen in the cytokine responses. Some spontaneous cytokine production (i.e. media alone wells) occurred in the stimulations for some DLN and splenocyte cultures. In particular, spontaneous IFNγ was produced by DLN cells from all the TLR4$^{-/-}$ mice but none was detected in WT mice, and elevated spontaneous IL-10 production was seen in TLR2$^{-/-}$ mice with both DLN and spleen cultures, with the latter being significantly greater than that seen for WT mice (Figure 17 A, C). TLR2$^{-/-}$ mice showed elevated antigen specific IFNγ and IL-10 responses in the splenocyte stimulations, when compared to WT mice (Figure 17D). The IL-10 response was also elevated in the positive control ConA-stimulated splenocyte cultures from TLR2$^{-/-}$ mice, indicating a universal regulatory response compared to the other groups. TLR6$^{-/-}$ mice, which had the lowest parasite burdens at week 18, showed the highest ratio of antigen specific IFNγ:IL-10 in the splenocyte cultures, which was significantly greater than WT and TLR2$^{-/-}$ mice. However, these mice also surprisingly had relatively low IFNγ:IL-10 responses to antigen in the DLN cultures.

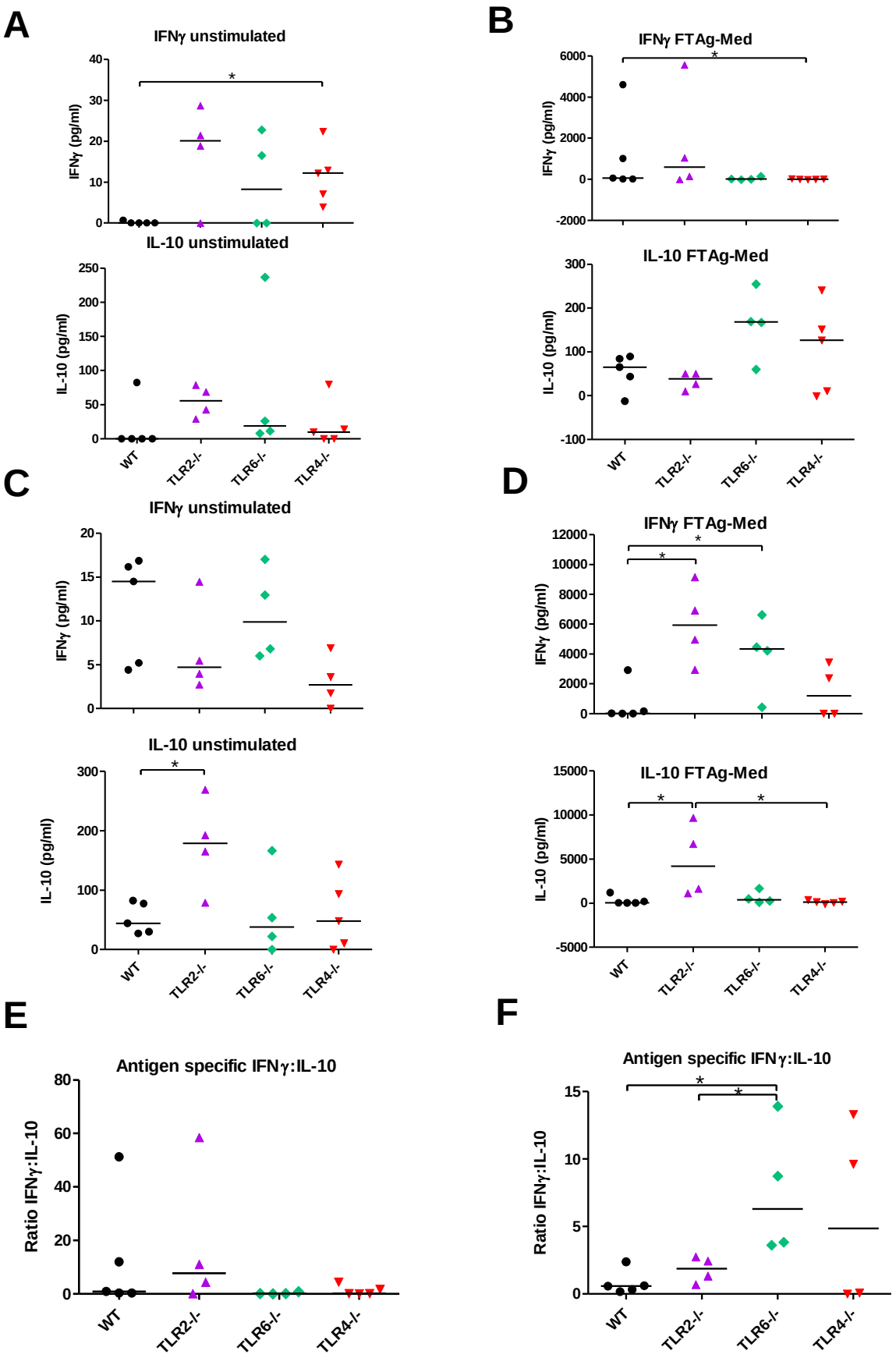
A
IFNγ unstimulated
IFNγ (pg/ml)
IL-10 unstimulated
IL-10 (pg/ml)
B
IFNγ FTAg-Med
IFNγ (pg/ml)
IL-10 FTAg-Med
IL-10 (pg/ml)
C
IFNγ unstimulated
IFNγ (pg/ml)
IL-10 unstimulated
IL-10 (pg/ml)
D
IFNγ FTAg-Med
IFNγ (pg/ml)
IL-10 FTAg-Med
IL-10 (pg/ml)
E
Antigen specific IFNγ:IL-10
Ratio IFNγ:IL-10
F
Antigen specific IFNγ:IL-10
Ratio IFNγ:IL-10
WT
TLR2-/-
TLR6-/-
TLR4-/-

Figure 17. Cytokine responses in WT, TLR2$^{-/-}$, TLR6$^{-/-}$ and TLR4$^{-/-}$ mice at week 18 post infection with *L. major*. WT, TLR2$^{-/-}$, TLR6$^{-/-}$ and TLR4$^{-/-}$ mice were infected with *L. major* and infection was allowed to develop for 18 weeks. At the end of the experiment, DLN cells and splenocytes were recovered and stimulated *in vitro* with *L. major* FTAg antigen and controls. After 72 hours the supernatants were removed and were analysed by ELISA for the presence of IFNγ and IL-10. Individual points represent the mean average levels of cytokine calculated from duplicate cultures per mouse, and horizontal bars represent median averages for each group. Unstimulated production (A, C), and antigen specific (B, D) IFNγ and IL-10 production (calculated by subtracting the cytokine produced in media-control wells from those in stimulated well) was measured (A, B – DLN cells, C, D – splenocytes). The ratio of antigen specific IFNγ:IL-10 produced by DLN cell (G) and splenocytes (H) is also shown. Groups were compared using the Mann-Whitney U test (*p<0.05).

Antigen specific antibody isotype levels

Blood plasma samples were taken at the two time points to measure levels of antigen specific IgG antibody isotypes using *L. major* promastigotes antigen (FTAg) for capture in ELISAs. Isotype switching of antigen specific antibodies by B cells is influenced by the production of different cytokines. The two major isotypes of circulating IgG are therefore biomarkers of the type of immune response, with IgG1 isotype indicating a Th2–biased response and IgG2a/c indicating a Th1 response in mice (due to a requirement of IL-4/IFNγ in IgG1/IgG2a-c isotype switching (235)). The results are displayed in Figure 18 and indicate that for all groups, the level of antigen specific IgG1 antibody did not change from week 10 to week 18 (Figure 18 A), whereas the concentration of antigen specific IgG2c increased in all groups from week 10 to week 18. Thus the ratio of IgG1:IgG2c decreased in all groups from week 10 to week 18 as shown in Figure 18 C indicating a shift towards a dominant Th1 type of immune response. This corresponds with a reduction in lesion size and a controlling of the infection in all groups. The most evident shift between these two time points is in TLR6$^{-/-}$ mice where a significant increase in the concentration of antigen specific IgG2c is seen, and a significant reduction in the ratio of IgG1:IgG2c specific for *L. major* antigen was measured. The overall levels of antigen specific IgG1 collected at both time points was significantly higher in the TLR2$^{-/-}$ group compared to WT mice ($p = 0.040$), indicating an overall elevated Th2 response in these mice.

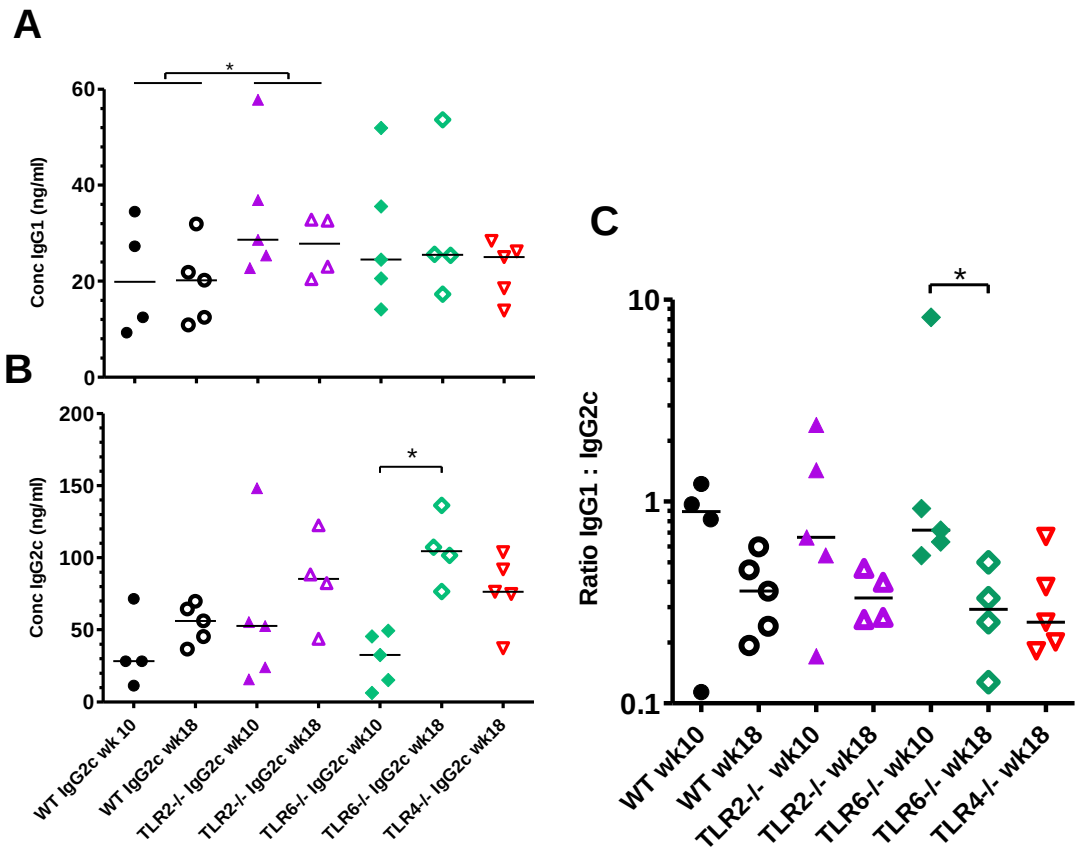

Figure 18. IgG antibody isotypes in mice infected with *L. major*. WT (black circles), TLR2$^{-/-}$ (purple triangles), TLR6$^{-/-}$ (green diamonds) and TLR4$^{-/-}$ (red triangles) mice were infected with *L. major* and blood plasma samples were collected at either 10 or 18 weeks after infection (when experiments were ended). The levels of IgG1 (A) and IgG2c (B) antibodies specific to the *L. major* FTAg were determined by ELISA and the concentrations were determined. Individual points represent the mean average levels of antibody from duplicate wells per mouse, and horizontal bars represent median averages for each group. The ratio of antigen specific IgG1:IgG2c for each individual was then calculated (C). Groups were compared using the Mann-Whitney U test (*p<0.05).

TLR6$^{-/-}$ mice do not heal cutaneous wounds faster than WT mice

Models of cutaneous leishmaniasis in mice have implicated various immune factors in skewing towards either resistance or susceptibility to infection. Other factors however, such as differences in wound healing processes, can also have a role in leading to differential ability to cure *Leishmania* infection in different strains of laboratory mice. It was observed that a proportion (approximately 25-35%) of TLR6$^{-/-}$ mice in the colony developed blindness by a process that appeared to be one of healing in the tissue surrounding the eye. Thus, it was hypothesised that TLR6$^{-/-}$ mice may have an increased ability to heal, and that this may explain the increased rate of cure in these mice in *L. major* infection. Indeed, TLRs have been implicated in the process of wound healing in mouse knockout studies (236). To explore whether the increased resistance to *L. major* observed in TLR6$^{-/-}$ mice is related to an enhanced ability to heal wounds, WT and TLR6$^{-/-}$ were given cutaneous wounds at the same site used for the *L. major* infection

(shaven rump) using a punch biopsy tool, and healing was monitored by daily measurements of the wound diameter. Results are presented in Figure 19 below.

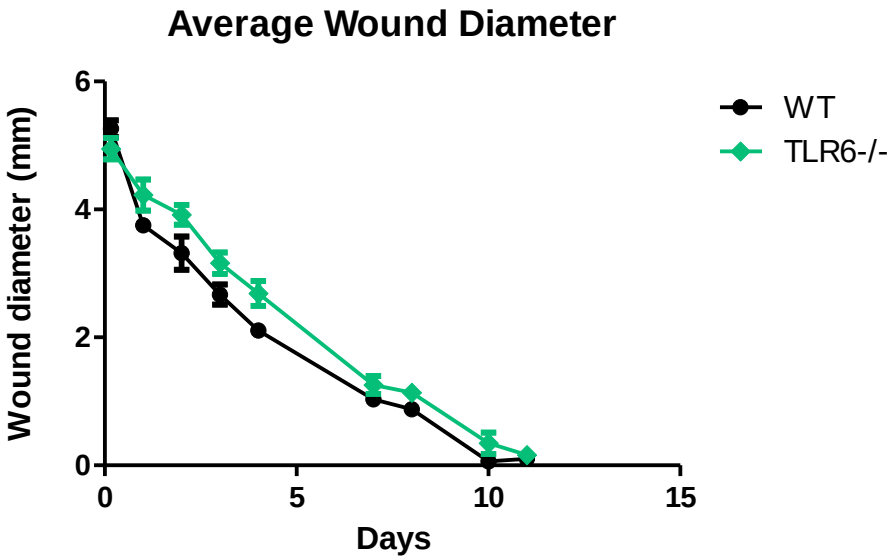

Figure 19. Rate of healing of punch biopsy wounds in WT and TLR6$^{-/-}$ mice. WT or TLR6$^{-/-}$ (n=6) mice were given a circular wound on the shaven rump using a punch biopsy tool. The healing was measured by taking regular measurements of the lesion diameter using a dial calliper. The average lesion diameter +/- SEM is displayed.

In all mice, the wounds reduced in size within the first 24 hours and healed completely within 11 days. There was no difference in the ability of TLR6$^{-/-}$ and WT mice to heal the wound created by the punch biopsy, and if anything the rate of healing was slightly faster in the WT mice. Thus, it appears that TLR6$^{-/-}$ mice do not have an increased ability to heal wounds when compared to WT mice, using this method.

Discussion

The data presented indicate a role for TLR2 during infection with *L. major* FV1 in helping to control infection, as mice lacking this receptor develop more severe disease. In addition the results confirm a role for TLR4 as previously reported (202, 203). However, neither TLR2 nor TLR4 is crucial for eventual control over *L. major* infection as mice lacking either receptor eventually healed their lesions. What is clear from the results presented here is that TLR4$^{-/-}$ mice present with exacerbated disease in the form of larger lesions and elevated AUCs over the course of infection. Kropf *et al* found similar results in terms of kinetics of infection when using *L. major* LV39 in a similar infection model, where lesions in C57BL/10ScN mice which lack a functional TLR4 gene had larger lesions just after the acute phase of infection (day 53), and higher parasite burdens at several timepoints (early and late stages of infection), when compared to their WT counterparts (C57BL/10ScSn) (202, 203). The 10ScN mice were found to produce elevated Th1 and Th2 cytokine responses to *L. major* in DLN restimulations (compared to ScSn or WT) including both IFNγ and IL-10, which was not repeated in this study as only elevated IFNγ was

observed, although the timepoints post infection at which DLN were taken was very different: week 4 p.i. in (203) and week 18 p.i in this study. An additional finding was that MΦs from mice lacking TLR4 were found to produce more arginase in response to *L. major* infection when compared to TLR4-competent MΦs, suggesting that TLR4 plays a role in preventing alternative activation of MΦs during infection independently of the adaptive immune response (203). A role for neutrophil elastase (NE) in the activation of *L. major* infected MΦs to kill via TLR4 was provided in a study by Ribeiro-Gomes *et al*, where it was demonstrated that neutrophils were able to induce intracellular killing in a TNFα and TLR4 dependent manner, and NE was responsible for this effect (237). Thus a host derived TLR4 ligand, or DAMP, is potentially linked with the role of TLR4 in *L. major* control.

In the two *L. major* infection experiments presented TLR2 was found to have a role in controlling development of lesions caused by *L. major*, and importantly in controlling parasite replication, as mice lacking this receptor showed larger lesions and elevated parasite burdens compared to WT mice. Another study reported that unpublished experiments showed TLR2-deficient mice were as resistant as WT C57BL/6 mice to *L. major* LV39 strain (207), however as the details of these experiments have not been fully reported it is not possible to compare the findings with those reported in this study. These unpublished studies may not have been conducted over a long enough time period to detect the point at which disease is exacerbated in TLR2$^{-/-}$ mice. It may also be possible that differences in TLR function occur between infections with different strains of *L. major*. Indeed, Revaz-Breton *et al* reported that the immune responses in MyD88$^{-/-}$ mice were markedly different during infection with either *L. major* LV39 or *L. major* IR75, indicating that pathways involving this adaptor molecule (i.e. TLR and/or IL-1) have distinct roles even between very closely related *L. major* strains (238). Thus, it is possible that TLR2 could play a more significant role in infection with *L. major* FV1 when compared with *L. major* LV39.

Certain immune responses were also elevated in TLR2$^{-/-}$ mice, such as antigen-specific IgG1 in the plasma and antigen specific cytokine responses, both protective and regulatory (IFNγ and IL-10) from splenocytes. Several studies have linked *Leishmania*-specific IgG (74, 75), and specifically IgG1 antibody isotypes (71) to susceptibility to infection with *L. major* or other *Leishmania spp.* It is believed that during infection, amastigotes are able to infect new MΦs via IgG antibody receptors (FcγRs), which results in production of IL-10, thereby regulating protective responses at the site of infection (e.g. cMΦ activation) and allowing further parasite replication (70). At the earlier time point of week 10, the ratio of antigen specific cytokine production by DLN cells and splenocytes suggested a skew towards a regulatory phenotype (lower IFNγ:IL-10 production), although further evidence is needed to confirm this as significance was not reached. The overall elevation of antigen specific responses suggests expansion of antigen specific T cells was elevated in TLR2$^{-/-}$ mice, which may be secondary to the increased antigen exposure due to higher parasite load, or related more directly to the function of TLR2 during infection. Interestingly, a study using mice infected with *L. braziliensis*, a parasite from the *Leishmania (Viannia)*

subgenus causing CL, found that while MyD88$^{-/-}$ mice were more susceptible to infection, TLR2$^{-/-}$ mice were more resistant than WT C57BL/6J mice during the acute phase of infection (as measured by lesion size, but not parasite burden where no differences were seen) (200). Whilst this finding differs markedly from the increased lesion sizes and parasite burdens found in *L. major* infected TLR2$^{-/-}$ mice in this study, Vargas-Inchaustegui *et al* also reported that TLR2$^{-/-}$ infected mice had elevated numbers of IFNγ producing CD4 T cells at week 8, and a sustained production of IFNγ by DLN cells in response to parasite antigen, whereas WT mice showed a reduction in IFNγ between week 4 and week 8. These results are similar to those reported here, where TLR2$^{-/-}$ mice showed enhanced IFNγ and IL-10 responses to FTAg in splenocyte restimulations at week 18 p.i. (Figure 17) (200). The same study also showed that DCs from TLR2$^{-/-}$ mice were more able to activate CD4 T cells to proliferate and produce IFNγ than WT DCs, after infection with *L. amazonensis* and *L braziliensis* parasites *in vitro* (200). Why a lack of TLR2 should increase the ability of DCs to activate T cells is unclear, but may be an example of a parasite-derived TLR2 ligand which acts to suppress activation of DCs. Whilst we also found evidence for an increased number of antigen specific T cells in TLR2$^{-/-}$ mice during *L. major* infection, this was associated with larger lesions and increased parasite burdens. This may suggest that a TLR2-mediated downregulation of immune activation during *Leishmania* infection is able to assist control of *L. major*, but is detrimental during infection with *L. braziliensis*.

Studies utilising TLR9$^{-/-}$ mice infected with *L. major* showed similar disease kinetics to those reported here with TLR2$^{-/-}$ and TLR4$^{-/-}$ mice, with increased lesion sizes and parasite burdens during the acute phase of infection, but eventual control of the disease (206, 207). In these models, it was found that TLR9 in DCs is activated by *L. major* DNA and this activation promotes priming of a protective Th1 response via production of IL-12, activation of NK cells and of IFNγ production, which all act to promote parasite killing by iNOS production by MΦs and to suppress non-protective Th2 responses (206, 207). Nevertheless, infected TLR9$^{-/-}$ mice were able to mount an appropriate Th1 response and heal their lesions, and the deficiency appeared to be a delayed ability to control non-protective Th2 responses. Thus, neither TLR2 nor TLR4 nor TLR9 is solely responsible for the important role of MyD88 in mounting a protective response to *L. major*, where mice deficient in MyD88 develop uncontrollable disease and insufficient Th1 or entirely inappropriate responses (199, 238). It is known that activation of more than one TLR can have either a complimentary, synergistic or antagonistic effect on innate immune responses (and subsequent adaptive immune responses) (239), and it may well be that it is a combination of TLRs that cooperate synergistically, all via MyD88 signalling, to allow for protective responses. Such a phenomenon appears to be the case in infection with a related intracellular protozoan parasite, *T. cruzi,* where mice deficient in both TLR2 and TLR9 were found to be more susceptible than mice deficient in either one receptor, and the TLR2$^{-/-}$TLR9$^{-/-}$ dual deficient mice had levels of susceptibility comparable to that of mice deficient in MyD88 (189). It would be interesting to use such double knockout mice, or even triple knockout mice (e.g. TLR2$^{-/-}$TLR4$^{-/-}$TLR9$^{-/-}$) in *L. major* infection experiments to explore whether

the combined effect of the different TLRs is able to account for the requirement of MyD88 in protective responses and eventual control of infection. As MyD88 is an adaptor molecule for the IL-1R pathway, it may also be that there is an important role for this receptor in protection to *L. major*, in addition to a role for TLR activation.

Given that TLR2 is known to function as a heterodimer, with either TLR1 or TLR6, it was hypothesised that one or other of the mice lacking these co-receptors would display the same disease phenotype as that of the TLR2$^{-/-}$ mice, thus demonstrating a role for either co-receptor. However, it was found that the kinetics and parasite burdens of infected mice lacking either co-receptor differed both from each other and from TLR2, suggesting a decoupling of the roles of these three receptor molecules in *L. major* infection. This finding is surprising given our current understanding of TLR2 function and may suggest an as-yet unidentified mechanism of TLR2 function, such monomeric TLR2 ligand recognition. We attempted to produce mice lacking both TLR1 and TLR6 by cross breeding these two transgenic strains to address this question, but no double knockout TLR1$^{-/-}$TLR6$^{-/-}$ progeny were produced in several progenies (data not shown), suggesting that this genotype is not viable.

Another unanticipated finding was the unique phenotype of infected TLR6$^{-/-}$ mice, which presented with lower parasite burdens in the latter stages of infection, and healed lesions faster than WT and other groups, suggesting TLR6 has a role in exacerbating infection with *L. major* and delaying healing. It is unclear in what capacity TLR6 exerts this effect, as it would appear to be independent of its known function as a co-receptor for TLR2. Furthermore, it does not appear that this increased resistance in TLR6$^{-/-}$ is associated with any difference in ability to heal wounds in the absence of *L. major* infection. It would be interesting to determine whether any differences in innate immune signalling occur in WT, TLR2$^{-/-}$, TLR1$^{-/-}$ and TLR6$^{-/-}$ MΦs and/or DCs after infection with *L. major*, as this may help to explain the differences in parasite infection/disease profiles observed.

In the C57BL/6 model of infection with *L. major*, nTregs have been shown to play an important role in allowing for the persistence of a small number of parasites at the site of infection after healing, which allows for continued immune activation and enhanced immune memory and greater protective immunity (68). It could therefore be possible that the TLR6$^{-/-}$ mice used in this study had reduced numbers of *L. major* specific nTregs, thus allowing for faster reduction in parasite numbers and lesion sizes, and would explain why the highest number of parasites reported in TLR6$^{-/-}$ mice at week 18 was 21 (Figure 14). Furthermore, the TLR6$^{-/-}$ mice presented with a more dramatic shift towards a protective Th1 phenotype, suggesting that the mice possessing a functional TLR6 (i.e. all other groups) were less able to switch readily to a Th1 response, perhaps due to inhibition by nTregs. It would therefore be interesting to phenotype the T cell populations in the infected mice using flow cytometry and determine if there are any difference in quantities of Th1, Th2, Th17 and nTregs. In addition, it would be interesting to explore if

there are any differences in the phenotype of MΦs at the infection site, as the individual TLRs have an influence on the activation of MΦs towards a cMΦ or aaMΦ phenotype (78, 240).

Chapter 4. The role of TLRs in *Leishmania mexicana* infection

Abstract

TLR2 has been shown to play a role in the recognition of *Leishmania* parasites *in vitro* and this activation has been linked to the activation of both protective and suppressive immune responses. We have demonstrated in *L. major* infection experiments, that TLR2 plays a role in controlling disease cause by *L. major in vivo*, as mice lacking TLR2 develop more severe disease. It appears that TLR2 is playing a role in *L. major* infection without a need for either TLR1 or TLR6. It is unclear which ligand/s of TLR2, whether parasite-derived or otherwise, is responsible for this observed susceptibility in TLR2$^{-/-}$ mice. To explore whether TLR2 plays a similar role in infection with *L. mexicana*, we infected WT, TLR2$^{-/-}$, TLR1$^{-/-}$, TLR6$^{-/-}$ and TLR4$^{-/-}$ mice with *L. mexicana*. As with *L. major*, TLR2 and not TLR1 or TLR6 appears to play a protective role in controlling *L. mexicana* infection as TLR2$^{-/-}$ mice develop more severe disease. We also show that TLR2$^{-/-}$ mice show a skewed Th2 response to *L. mexicana* during chronic infection suggesting that TLR2 activation during infection promotes protective responses. Given that infection of TLR2$^{-/-}$ mice with *L. mexicana* parasites lacking the TLR2 ligand LPG also resulted in exacerbated disease, we show that this is not a major or exclusive ligand involved in the TLR2 mediated control during chronic infection.

Introduction

Background

In Chapter 3, data was presented that implicated both TLR4 and TLR2 as having a role in controlling *L. major* infection in C57BL/6 mice. Surprisingly, neither of the known TLR2 co-receptors (TLR1 and TLR6) appeared to act as co-receptors for TLR2 in *L. major* infection, indicating a novel mechanism of TLR2 function in *Leishmania* infection. Furthermore, TLR6$^{-/-}$ mice were found to heal *L. major* infection faster than the other groups, indicating that these mice have increased resistance to infection.

Aim of the study

In order to test whether these findings could be replicated in another species that causes CL, we carried out similar experiments in the TLR$^{-/-}$ mice using *L. mexicana*, a causative agent of new-world CL. Furthermore, it is unclear whether the role for TLR2 during infection is linked to the recognition of LPG, which is the most widely reported ligand of TLR2 from *Leishmania* parasites (26, 27). Therefore we tested whether *L. mexicana* parasites, which have been genetically modified to lack the surface glycoprotein LPG (*L. mexicana lpg1*$^{-/-}$), reverted to a WT phenotype in TLR2$^{-/-}$ mice. It is also possible to culture axenic amastigotes of *L. mexicana* (in contrast to *L. major*), which express almost no LPG on their surface (13) and use these for infections to compare with LPG-rich promastigotes. These two approaches allowed us to explore whether the TLR2 dependent role in parasite control is restricted to either the promastigote stage (during the initial stage of infection before transformation into amastigotes) or the amastigotes stage, and more specifically whether LPG is the ligand for TLR2 *in vivo*.

Methods

Mice, parasites and infections

All procedures involving live animals were performed at the BSU in the Duncan Building, University of Liverpool. Female age matched (8-12 weeks old) WT (C57BL/6), TLR2$^{-/-}$, TLR1$^{-/-}$, TLR6$^{-/-}$ and TLR4$^{-/-}$ mice were infected with 10^5 *L. mexicana* WT or *lpg1*$^{-/-}$ metacyclic-enriched promastigotes, or *L. mexicana* WT amastigotes, by s.c. injection to the shaven rump, in a 100 µl volume of HBSS. These cultures were confirmed to be negative for mycoplasma contamination. The percentage of metacyclics in parasites used for infection was found to be 43% in the WT promastigote experiment and for *lpg1*$^{-/-}$ infection it was 38%. Lesion progression was monitored by taking weekly measurements of lesion size (mm^2) using a metric dial calliper and the measurements were used to generate AUC values. At the end of infection experiments, mice were culled via cardiac puncture to allow for the collection of blood for plasma samples (experiment 2 only). The lesion was removed and placed in RNA later, and the spleens and DLNs were removed under sterile conditions and processed for cell stimulation experiments.

Parasite burden

The parasite burden of tissues was estimated by qPCR alone, using JW11 and JW12 primers as described.

Cell stimulations and immunological techniques

For these experiments, DLN cells and splenocytes were used a concentration of 8 x 10^5 cells/well. Cells were cultured for 72 hours in the presence of either 20 μg/ml *L. mexicana* FTAg, 20 μg/ml WMAg, 2.5 μg/ml ConA or media alone in a total volume of 200 μl/well. Culture supernatants were then removed and stored at -20°C until analysis for IFNγ, IL-10, IL-4 and IL-13 levels using cytokine ELISA. The levels of antigen specific IgG1 and IgG2c in plasma samples from mice were measured using antibody ELISA with either *L. mexicana* FTAg or WMAg as the capture antigen.

Results

Parasite burden analysis

As with the *L. major* experiments presented in Chapter 3, the data from the *L. mexicana* infection was explored and groups were compared using both parametric and non-parametric tests. The mean and standard deviations of the parasite burden results are given in Table 11 below.

Genotype	Promastigote WT			Amastigote WT			Promastigote *lpg1*$^{-/-}$		
	n	Mean	SD	n	Mean	SD	n	Mean	SD
WT	8	20848487.5	18913227.1	7	1638655.4	4293812.2	5	755780000.0	845855774.9
TLR2$^{-/-}$	5	51862000.0	27219992.0	8	2016332.5	2330005.0	5	1321980000.0	988824125.9
TLR1$^{-/-}$	8	24613910.0	22056274.4	6	262703.3	251328.7	-	-	-
TLR6$^{-/-}$	8	16429200.0	9808354.5	7	536218.6	716523.9	-	-	-
TLR4$^{-/-}$	5	13626686.0	6578240.0	8	284747.5	782377.1	-	-	-

Table 11. Mean and standard deviations of parasite burdens in WT and TLR$^{-/-}$ mice infected with *L. mexicana* promastigotes, amastigotes or *lpg1*$^{-/-}$ promastigotes.

Interestingly, the variance in the lesion parasite burdens from the promastigote infection were smaller than their mean averages, whilst they were larger for lesions from the amastigote infection and comparable in the *lpg1*$^{-/-}$ infection experiment, indicating a different distribution of data in these datasets. Nevertheless, it was possible to fit the data from all experiments to a negative binomial model using R, and in all cases this was found to fit better than an intercept only model or using a Poisson distribution. The results from the comparisons of IRR values obtained by fitting a negative binomial distribution using a generalised linear model function are displayed in Table 12.

Comparison	Promastigote WT			Amastigote WT			Promastigote *lpg1*$^{-/-}$		
	IRR	**95 % CI**	**P**	**IRR**	**95 % CI**	**P**	**IRR**	**95 % CI**	**P**
TLR2$^{-/-}$ / WT	2.488	1.21-5.10	***0.013***	1.482	0.22- 10.08	0.681	1.749	0.61–5.03	0.299
TLR1$^{-/-}$ / WT	1.18	0.52-2.70	0.694	-	-	-	-	-	-
WT / TLR1$^{-/-}$	-	-	-	5.407	0.69 -42.46	0.102	-	-	-
WT / TLR6$^{-/-}$	1.269	0.63-2.57	0.507	2.648	0.37 -19.18	0.355	-	-	-
WT / TLR4$^{-/-}$	1.530	0.76-3.08	0.233	4.988	0.73 -33.93	0.094	-	-	-
TLR2$^{-/-}$ / TLR1$^{-/-}$	2.107	1.03-4.29	***0.040***	8.015	1.08 -59.25	***0.038***	**-**	**-**	**-**
TLR2-$^{-/-}$ / TLR6$^{-/-}$	3.157	1.79-5.55	***<0.001***	3.927	0.58 -26.71	0.154	-	-	-
TLR2$^{-/-}$ / TLR4$^{-/-}$	3.806	2.18-6.66	***<0.001***	7.395	1.16 -12.81	***0.031***	**-**	**-**	**-**
TLR1$^{-/-}$ / TLR6$^{-/-}$	1.498	0.75-3.01	0.256	-	-	-	-	-	-
TLR6$^{-/-}$ / TLR1$^{-/-}$	-	-	-	2.041	0.26 -16.03	0.489	-	-	-
TLR1$^{-/-}$ / TLR4$^{-/-}$	1.806	0.90-3.61	0.095	-	-	-	-	-	-
TLR4$^{-/-}$ / TLR1$^{-/-}$	-	-	-	1.084	0.15 -8.01	0.936	-	-	-
TLR6$^{-/-}$ / TLR4$^{-/-}$	1.206	0.70-2.07	0.498	1.883	0.28 -12.81	0.509	-	-	-

Table 12. Comparisons of parasite burdens in WT and TLR$^{-/-}$ mice infected with *L. mexicana* promastigote (WT or *lpg1*$^{-/-}$) or amastigote parasites using parametric methods. Parasite burdens for mice of different genotypes (n=5-8) infected with *L. mexicana* parasites were determined for each experiment. These values were fitted to a generalised linear model using a negative binomial function, to allow for comparison of means between groups. Incidence rate ratios (IRRs) are presented (IRR = mean1/mean2) with their confidence intervals. The average values of groups are considered significantly different when the CI values of IRRs do not encompass 1; instances where this is the case are highlighted in bold and italics in the P column.

When non-parametric tests were used to compare parasite burdens from different groups (see Table 13) those that were found to be different differed greatly from those found using the parametric tests.

Comparison	*L. mexicana* promastigote			*L. mexicana* amastigote			*L. mexicana lpg1-/-*		
	U/W	**z**	**p**	**U /W**	**z**	**p**	**U /W**	**z**	**p**
WT vs TLR2$^{-/-}$	4 /40	-2.342	***0.019***	8 /36	-2.316	***0.021***	9 /24	-0.731	0.548
WT vs TLR1$^{-/-}$	27/63	-0.525	0.645	6 /34	-2.143	***0.035***	-	-	-
WT vs TLR6$^{-/-}$	31/67	-0.105	0.956	13 /41	-1.469	0.165	-	-	-
WT vs TLR4$^{-/-}$	17/32	-0.439	0.724	26 /62	-0.231	0.867	-	-	-
TLR2$^{-/-}$ vs TLR1$^{-/-}$	7/43	-1.903	0.065	5 /26	-2.453	0.013	-	-	-
TLR2$^{-/-}$ vs TLR6$^{-/-}$	0/36	-2.928	***0.002***	14 /42	-1.62	0.121	-	-	-
TLR2$^{-/-}$ vs TLR4$^{-/-}$	0/15	-2.611	***0.008***	6 /42	-2.731	***0.005***	-	-	-
TLR1$^{-/-}$ vs TLR6$^{-/-}$	27/63	-0.525	0.645	20 /48	-0.143	0.945	-	-	-
TLR1$^{-/-}$ vs TLR4$^{-/-}$	17/32	-0.439	0.724	6 /42	-2.324	***0.020***	-	-	-
TLR6$^{-/-}$ vs TLR6$^{-/-}$	15/30	-0.732	0.524	12 /48	-1.852	0.072	-	-	-

Table 13. Comparisons of parasite burdens in WT and TLR$^{-/-}$ mice infected with *L. mexicana* promastigote (WT or *lpg1*$^{-/-}$) or amastigote parasites using non-parametric methods. Median values of 2 groups (n=5-8) were compared to each other using the Mann-Whitney U test. Instances where medians were found to be significantly different to each other are highlighted in bold and italics in the P column.

The parasite burden results from all groups of mice infected with WT *L. mexicana* promastigotes or amastigotes are presented in Figure 20, and the significant different between groups are displayed according to both parametric and non-parametric methods of analysis. The parasite burdens of WT and TLR2$^{-/-}$ mice infected with *L. mexicana lpg1*$^{-/-}$ promastigotes are not displayed here as neither method of comparing groups used detected a significant difference.

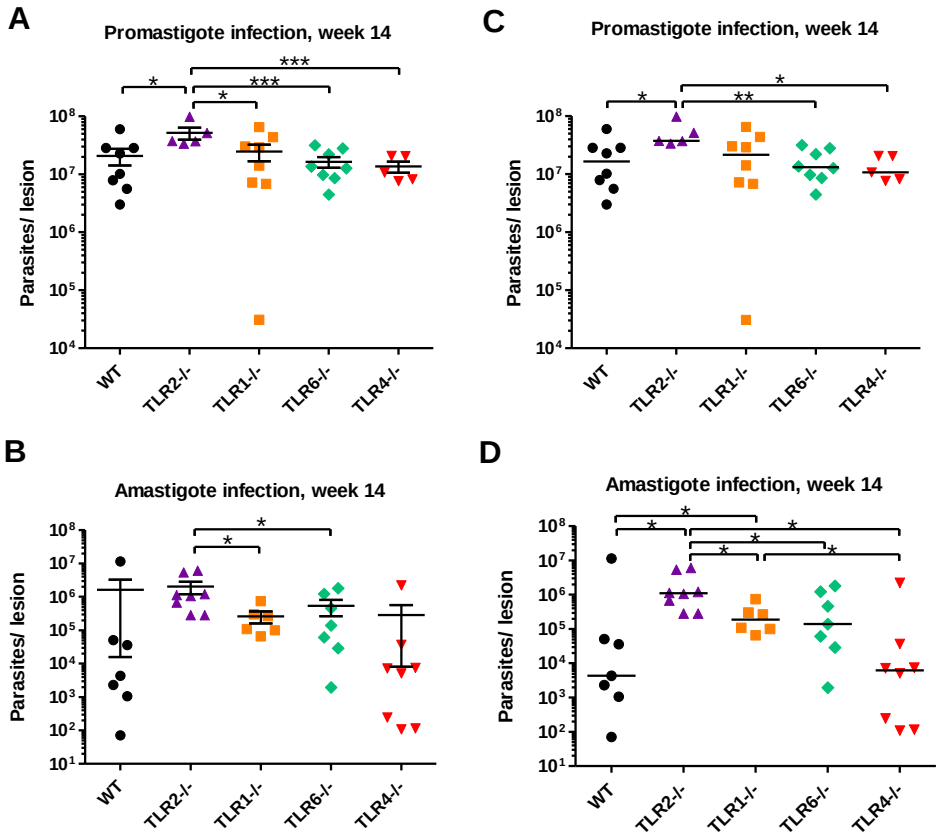

Figure 20. Parasite burdens in promastigote (A, C) and amastigote (B, D) *L. mexicana* infected lesions of WT and TLR$^{-/-}$ mice, with comparisons between groups displayed. For parametric analysis, groups were compared by fitting a generalised linear model (A, B), mean values +/-SEM are shown; using non-parametric methods groups were compared using the Mann-Whitney U test (C, D) horizontal bars represent medians (* p <0.05; ** p<0.005; *** p<0.001).

From exploring these data, it was evident that the group size and high variance in the parasite burden datasets in these experiments influenced the different outcomes between parametric and non-parametric statistical analysis. A good example of this is in the amastigote experiment, where despite 2-log-fold greater parasite burdens of TLR2$^{-/-}$ infected mice compared to the majority of WT mice, no difference between the two groups was found when a parametric test was used, but the Mann-Whitney U test found the two groups to be significantly different (Figure 20). Given that it is still not standard practice to use parametric methods to compare these kind of results in *Leishmania* infection experiments, especially using

a generalised linear model approach, it was decided after consultation with a statistician to continue analysis on results from these experiments using a non-parametric approach, by comparing medians between two groups using the Mann-Whitney U test.

The roles of TLR2, 1 and 6 in controlling *L. mexicana* infection

In this study, infection experiments with *L. mexicana* parasites showed that mice that lack TLR2 developed larger lesions than WT mice upon infection (Figure 21 A, B), and have larger numbers of parasites in lesion tissue at 14 weeks p.i. (Figure 20 C, D and Figure 22). This indicates that TLR2 plays a role in controlling parasite replication in the lesion during chronic infection, in a similar way to that found for *L. major*. Furthermore, no clear role was indicated for either TLR1 or TLR6 in controlling infection with *L. mexicana* as co-receptors for TLR2, as mice lacking either co-receptor developed similar disease kinetics and parasite burdens to that of WT mice, rather than $TLR2^{-/-}$ mice, with the exception of slightly larger lesions in $TLR1^{-/-}$ at some early time points, and larger parasite burdens in these mice after infection with amastigotes (Figure 21 & Figure 22).

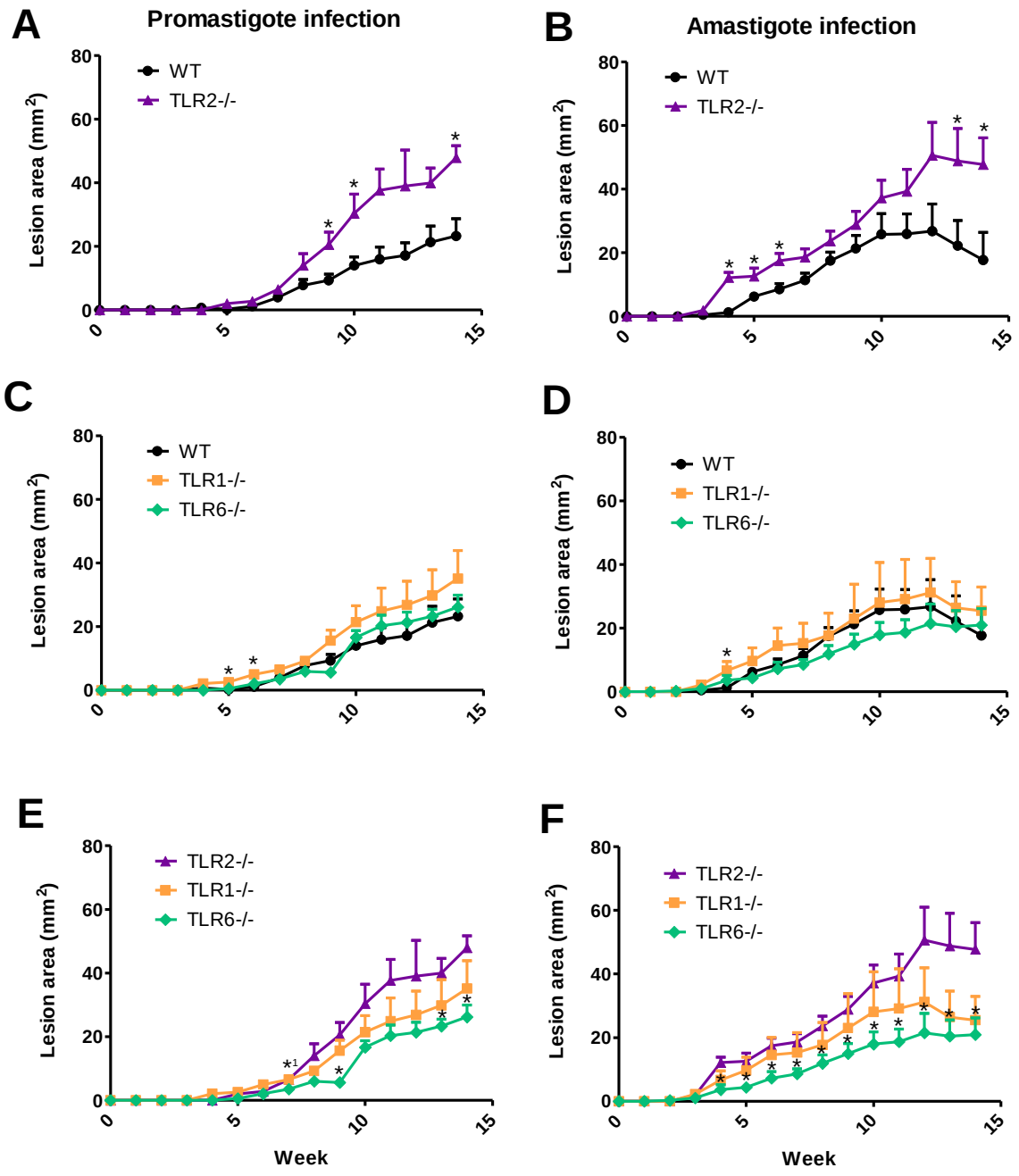

Figure 21. Lesion progression in WT, TLR2$^{-/-}$, TLR1$^{-/-}$ and TLR6$^{-/-}$ infected with *L. mexicana* parasites. Mice (n= 6-8 /group) were infected with 10^5 promastigotes (A, C, E) or amastigotes (B, D, F) to the shaven rump and lesion development was monitored by taking weekly measurements of lesion size using a dial caliper. Graphs show average lesion area + SEM, groups were compared using the Mann-Whitney U test at each time point (* p <0.05). In A, B, C, D groups were compared to WT whereas in E and F groups were compared to TLR2$^{-/-}$. *[1] indicates TLR6$^{-/-}$ vs TLR2$^{-/-}$.

When compared to TLR2$^{-/-}$ mice, lesion size was smaller in TLR6$^{-/-}$ mice at several timepoints during infection with both promastigote and amastigote *L. mexicana* parasites. Whilst no significant difference in lesion size was observed between TLR2$^{-/-}$ and TLR1$^{-/-}$ mice in infection experiments, the average lesion

size in TLR1$^{-/-}$ mice was consistently smaller than in TLR2$^{-/-}$ mice, and followed the same lesion development pattern as the WT mice. Furthermore, infected TLR1$^{-/-}$ mice had significantly lower parasite burdens than TLR2$^{-/-}$ mice at 14 weeks post infection with *L. mexicana* amastigotes (Figure 21 & Figure 22).

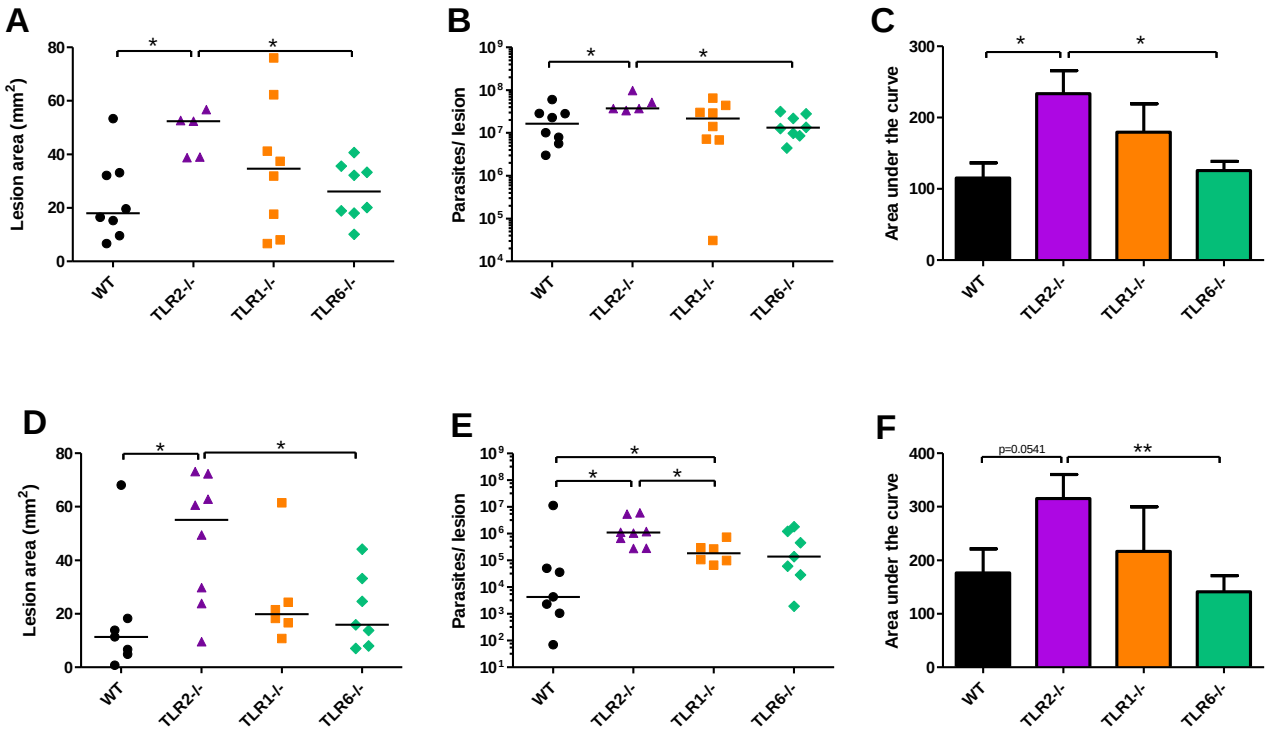

Figure 22. Final measurements at 14 weeks post infection in WT TLR2$^{-/-}$, TLR1$^{-/-}$ and TLR6$^{-/-}$ mice infected with *L. mexicana* promastigotes (A, B, C) and amastigotes (D, E, F). The lesion areas at the end of the experiment (A, D), the parasite burden of lesions (B, E) and the AUC values (C, F). In A, B, D, E, individual measurements for each mouse are shown, and horizontal bars represent median values. In C, F, bars represent means + SEM. Groups were compared using the Mann-Whitney U test (* p <0.05).

Infection of TLR2$^{-/-}$ mice with *L. mexicana lpg1*$^{-/-}$

As several studies have shown that *Leishmania* LPG is a ligand for TLR2, we tested whether amastigote stages, which lack expression of LPG, would give a different phenotype in TLR2$^{-/-}$ infected mice (i.e. revert to the WT phenotype). However, the phenotypes of both amastigote and promastigote infections were strikingly similar between the two experiments, with TLR2$^{-/-}$ mice developing larger lesions in the later stages of infection and presenting with higher parasite burdens than WT mice and other groups (Figure 22). To explore this finding further, we carried out an infection experiment with *L. mexicana lpg1*$^{-/-}$ parasites, which specifically lack the LPG molecule on their surface. TLR2$^{-/-}$ mice also developed larger lesions than WT mice when infected *L. mexicana lpg1*$^{-/-}$ parasites, suggesting that activation of TLR2 by LPG is not the mechanism of TLR2 mediated control of parasite replication *in vivo* (see Figure 23). The average parasite burden in TLR2$^{-/-}$ mice was also greater in TLR2$^{-/-}$ mice infected with *L. mexicana lpg1*$^{-/-}$

parasites, but this was not found to be significant, perhaps due to the low group size in this experiment (n=5).

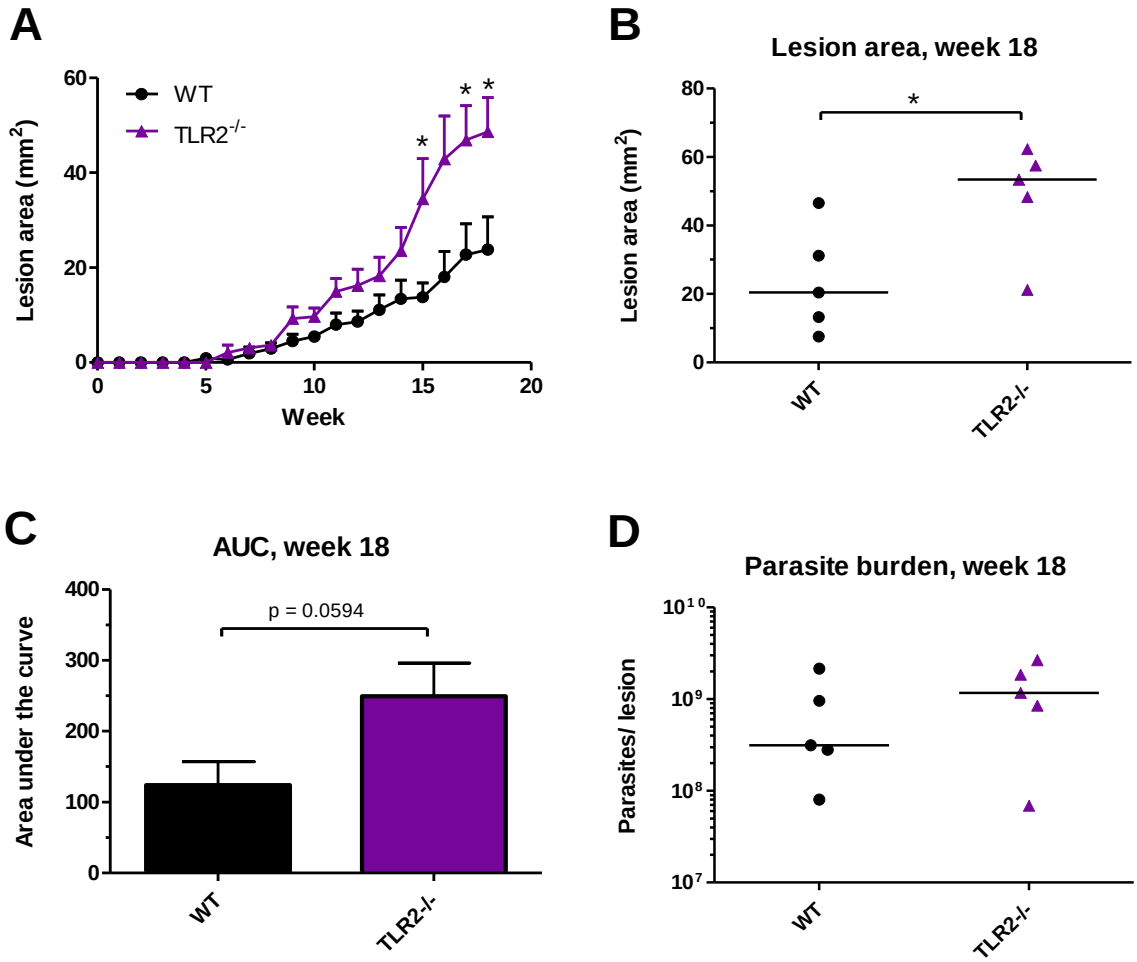

Figure 23. Infection of WT and TLR2$^{-/-}$ mice with *L. mexicana lpg1*$^{-/-}$ promastigote parasites. C57BL/6 (WT) and TLR2$^{-/-}$ mice (n=5) were infected with *L. mexicana lpg1-/-* promastigotes and lesions were allowed to develop for 18 weeks. The lesion size at weekly time points (A), at the end of the experiment (B), AUC values (C) and parasite burden of lesions at week 18 (D). In A, C, bars represent means + SEM. In B, D individual measurements for each mouse are shown, and horizontal bars represent median values. Groups were compared using the Mann-Whitney U test (* p <0.05).

The role of TLR4 in *L. mexicana* infection

TLR4 has been implicated in the recognition and control of *Leishmania* parasites in several studies (202-204, 237), but its role in *L. mexicana* infection *in vivo* has not yet been explored fully in a chronic setting or using model that closely mimics immune responses found in human disease (such as in C57BL/6 mice). In this study, mice which lacked the TLR4 receptor (TLR4$^{-/-}$) developed lesions that did not differ significantly from WT mice when infected with either promastigotes or amastigotes of *L. mexicana* at any time point over a 14 week infection (Figure 24 A, D). In fact, the trend tended towards smaller lesions in TLR4$^{-/-}$ infected mice, although no significant differences were seen. Furthermore, there were no

differences in either parasite burden or AUC values at 14 weeks post infection (Figure 24 B, C, E, F). These results suggest that TLR4 plays no role in controlling *L. mexicana* infection in this model, in contrast to that seen for *L. major* in the previous chapter.

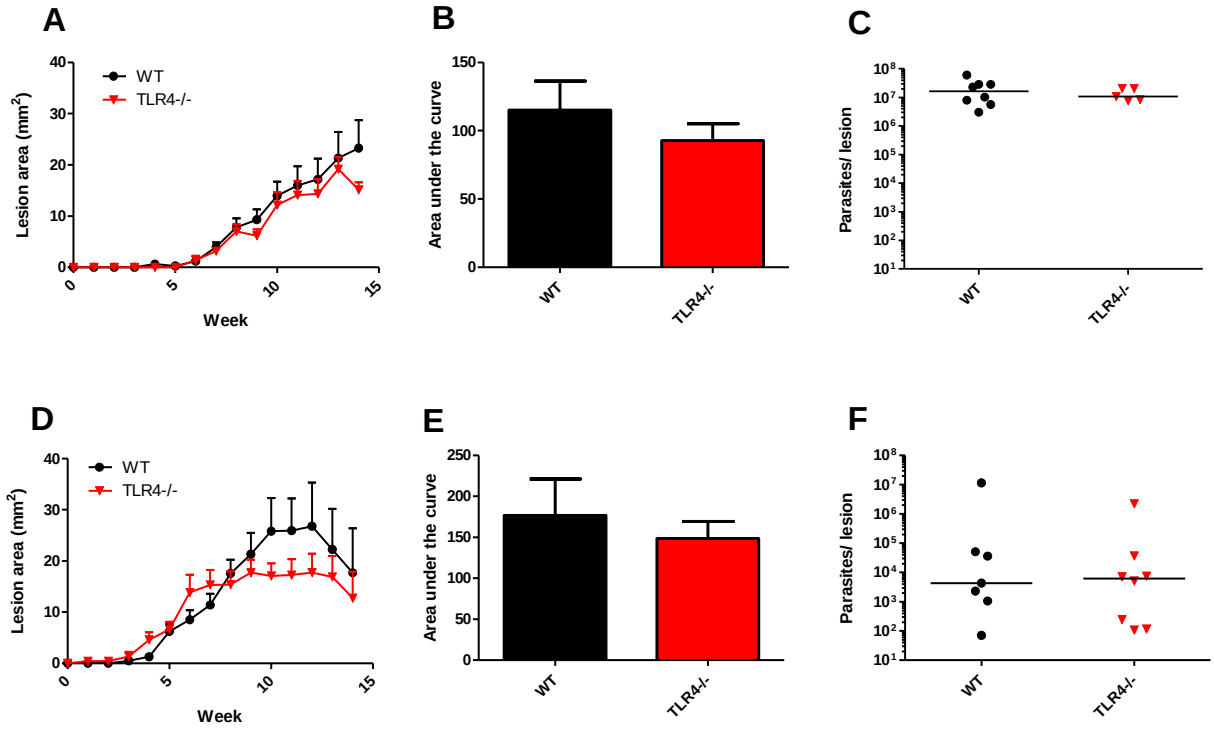

Figure 24. Infection of TLR4$^{-/-}$ mice with *L. mexicana* promastigotes (A, B, C) or amastigotes (D, E, F). C57BL/6 (WT) and TLR4$^{-/-}$ mice (n=7-8) were infected with *L. mexicana* promastigotes or amastigotes and lesions were allowed to develop for 14 weeks. The lesion size at weekly time points (A, D), AUC values (B, E) and parasite burden of lesions at week 14 (C, F) are displayed for promastigote infection and amastigote infection experiments, respectively. In A, B, D, E bars represent means + SEM are shown. In C, F individual measurements for each mouse are shown, and horizontal bars represent median values. Groups were compared using the Mann-Whitney U test.

Spontaneous cytokine production

To explore any differences in adaptive immune responses in the infected TLR$^{-/-}$ and WT mice, DLN cells were recovered and cultured for 72 hours in the presence of *L. mexicana* promastigote (FTAg) and amastigote (WMAg) antigens and the supernatants were analysed for cytokine levels using ELISA. LN cells from naïve controls were included in experiments, and these were not found to produce detectable levels of cytokine in media controls or in response to either *Leishmania* antigen, but did produce cytokines in response to ConA as expected (data not shown). Interestingly, many unstimulated DLN cell cultures from infected mice produced cytokine responses; this was particularly noticeable for DLN cells from the *L. mexicana* promastigote infection experiment (Figure 25).

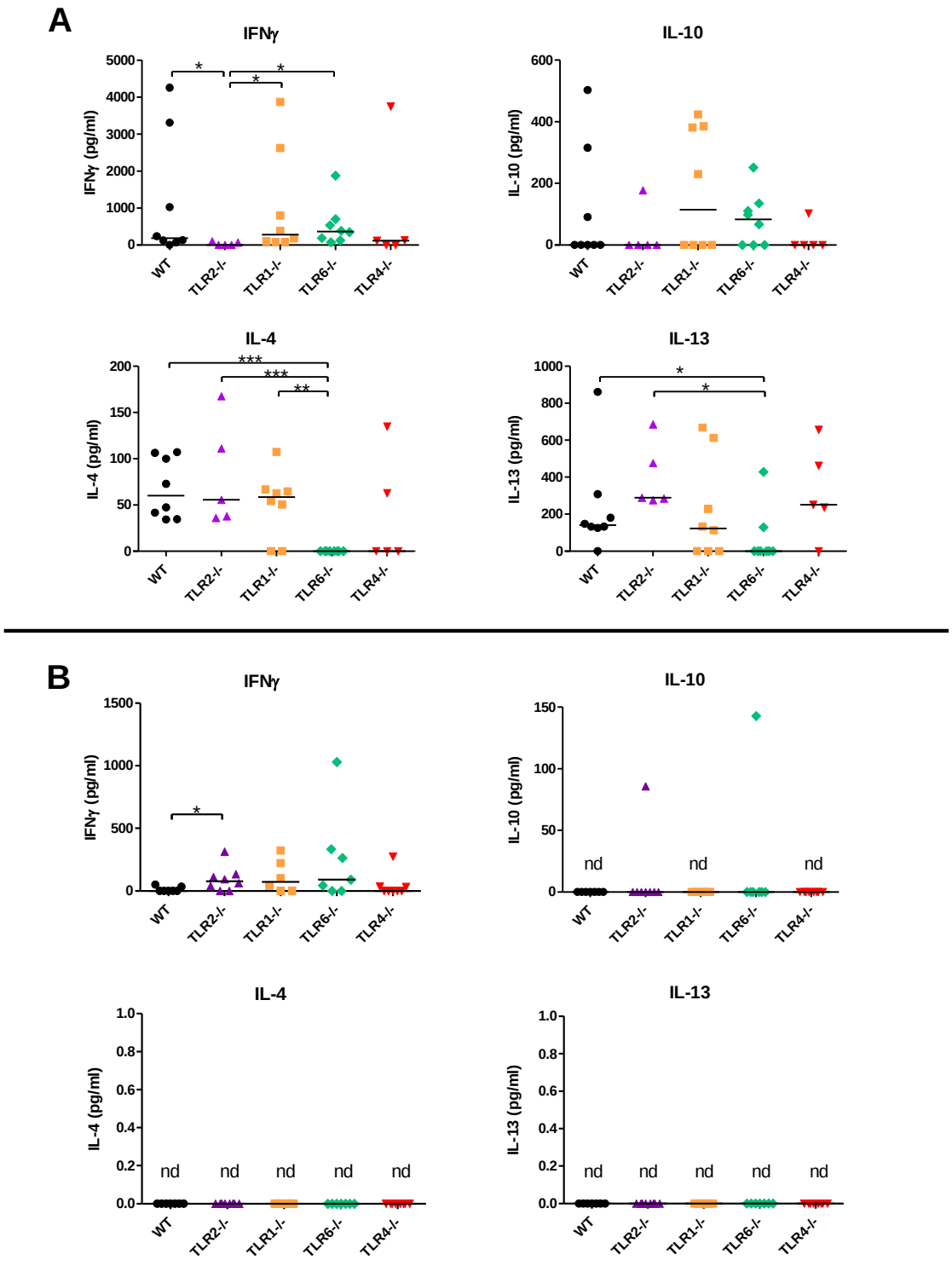

Figure 25. Cytokine production by unstimulated DLN cells in culture, from mice infected with *L. mexicana* promastigotes (A) or amastigotes (B), 14 weeks after infection. At the end of the infection experiments, DLN cells were collected and cultured *in vitro* for 72 hours and cytokine levels in the supernatants were measured for levels of IFNγ, IL-10, IL-4 and IL-13 by ELISA. Individual points represent the mean average levels of cytokine from triplicate wells per mouse, and horizontal bars represent median averages for each group. Groups were compared using the Mann-Whitney U test (* $p < 0.05$; nd = none detected).

As the DLN in infected mice are likely to contain high numbers of live parasites at the time of recovery, the cytokine production in these cultures may reflect the stimulation of the DLN cells by native parasites present in the DLN, and may therefore reflect the parasite burden in the DLN. The reduced lesion sizes and parasite burdens in the amastigote infection experiment at week 14 is likely linked to reduced parasite burdens in the DLN also, which may explain the lack of spontaneous cytokine production in the DLN cultures when compared to those from promastigote infected mice.

These results may therefore reflect the profile of cytokine responses in DLN of infected mice at this time point *ex vivo*. Promastigote infected TLR2$^{-/-}$ mice showed reduced levels of IFNγ produced spontaneously in culture compared to WT, TLR1$^{-/-}$ and TLR6$^{-/-}$ mice, whereas TLR6$^{-/-}$ mice had comparatively lower spontaneous production of the Th2 cytokines IL-4 and IL-13 when compared to WT, TLR2$^{-/-}$ and TLR1$^{-/-}$ mice. This suggests that the immune response in the DLN of TLR2$^{-/-}$ mice is skewed towards a Th2 response, whilst comparatively the DLN of infected TLR6$^{-/-}$ mice is skewed towards a Th1 response (N. B. ratios were not possible to calculate due to the high number of individuals with no detectable cytokine). In the amastigote infection experiment, in most cases the only detectable cytokine produced in the media control cultures was IFNγ, suggesting that DLN is more skewed towards a Th1 response than in the promastigote infected mice. No noticeable differences were observed between spontaneous cytokine production by DLN cells from TLR4$^{-/-}$ compared to WT mice.

FTAg and WMAg specific cytokine production

The antigen specific cytokine responses by DLN cells were calculated by subtracting the levels produced spontaneously from those produced in response to *in vitro* stimulation with either a promastigote (FTAg) or amastigote (WMAg) *L. mexicana* antigen; the results are displayed in Figure 26 and Figure 27 respectively. These results again show that TLR2$^{-/-}$ mice have a skewed immune response towards a Th2 and regulatory type when compared to WT mice, presenting with comparable levels of IFNγ, yet higher levels of IL-10, IL-4 and IL-13 in response to either antigen after DLN stimulations from both promastigote and amastigote infections. This is further supported when the results are displayed in terms of ratios of Th1:regulatory or Th1:Th2 cytokines, where the ratio of IFNγ to either IL-10, IL-4 or IL-3 was significantly reduced in TLR2$^{-/-}$ mice compared to WT mice, as well as comparing to other TLR$^{-/-}$ groups in many cases (see Figure 28). These results suggest that a presence of TLR2 acts to reduce the Th2 adaptive immune response, as well as regulatory IL-10, during *L. mexicana* infection.

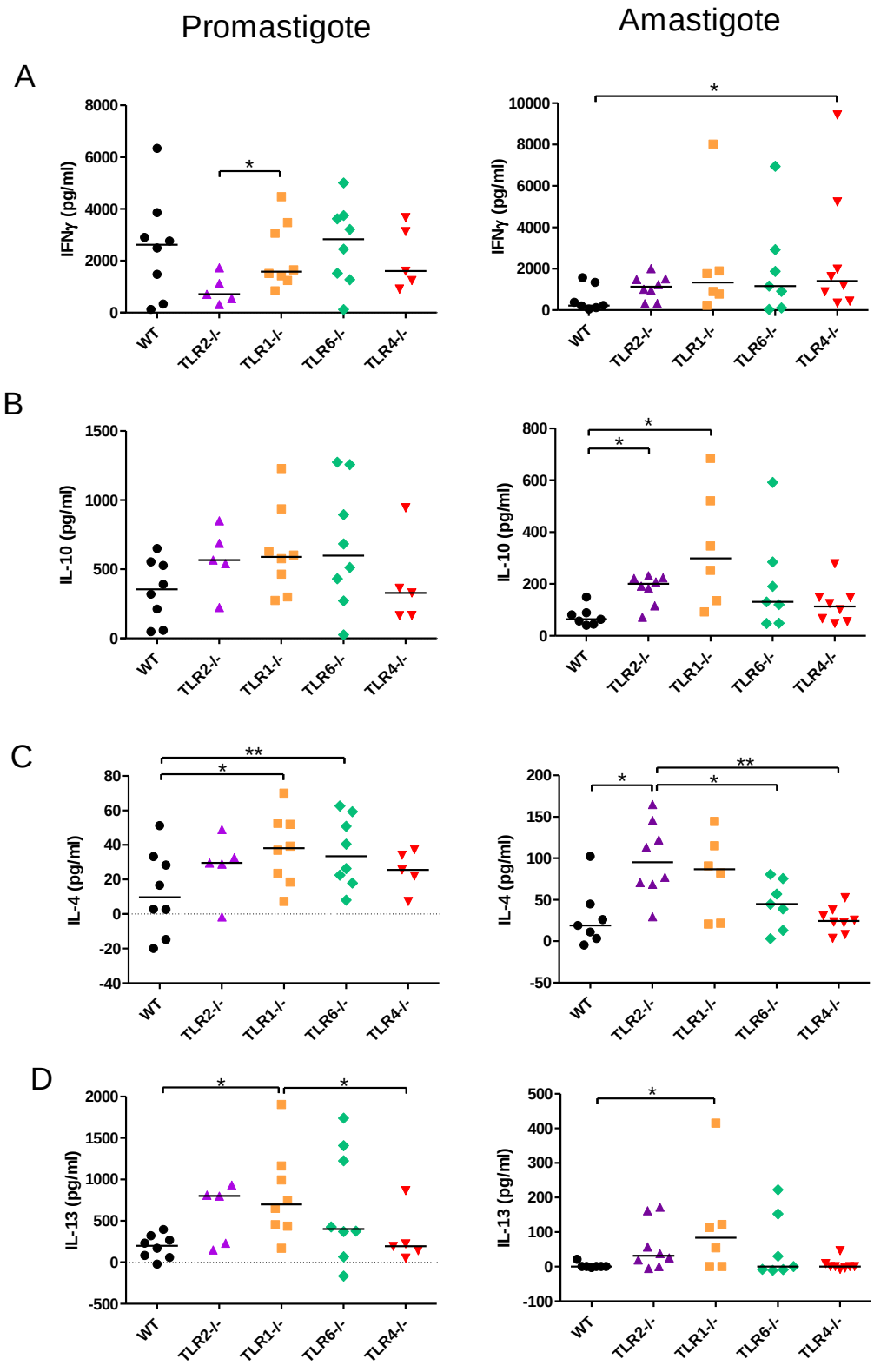

Figure 26. **FTAg specific production of cytokines by DLN cells from WT and TLR$^{-/-}$ mice infected with *L. mexicana* promastigotes (left) or amastigotes (right). At the end of the infection experiments, DLN cells were collected and cultured *in vitro* for 72 hours and cytokine levels in the supernatants were measured for levels of IFNγ (A), IL-10 (B), IL-4 (C) and IL-13 (D) by ELISA. Individual points represent**

the mean average levels of cytokine from triplicate wells per mouse, and horizontal bars represent median averages for each group. Groups were compared using the Mann-Whitney U test (* p <0.05; nd = none detected).

In some cases an elevated level of Th2 cytokines was produced by mice lacking TLR2 co-receptors TLR1 and TLR6 as well as TLR2 in response to *L. mexicana* antigens (when compared to WT mice, Figure 26), even though TLR1$^{-/-}$ and TLR6$^{-/-}$ mice did not develop the same increased susceptibility phenotype as seen in TLR2$^{-/-}$ mice. However, when comparing ratios of protective IFNγ to the regulatory and Th2 cytokines IL-10, IL-4 and IL-13, DLN from TLR1$^{-/-}$ and TLR6$^{-/-}$ mice produced comparable ratios of IFNγ to IL-10, IL-4 or IL-13 as WT mice, with the exception of an elevated IFNγ:IL-4 ratio (i.e. Th1 skewed) in response to WMAg in the case of TLR1$^{-/-}$ mice infected with *L. mexicana* amastigotes (Figure 28). Thus, despite elevated immune responses as detected by ELISA, TLR1$^{-/-}$ and TLR6$^{-/-}$ mice do not appear to have a consistently altered type of adaptive immune response to *L. mexicana* during the chronic stage of infection, unlike TLR2$^{-/-}$ mice which are more skewed towards responses which exacerbate infection. The adaptive immune responses in TLR4$^{-/-}$ mice infected with *L. mexicana* did not differ from WT in most cases, with the exception of *L. mexicana* amastigote infected mice where TLR4$^{-/-}$ mice showed highly elevated levels of IFNγ produced in response to *L. mexicana* WMAg (Figure 27), and the levels of IFNγ produced by these mice were also greater in relation to IL-10, IL-4 and IL-13 production (Figure 28), suggesting stronger protective Th1 responses in these mice. Interestingly, the immune response in TLR4$^{-/-}$ mice was skewed greatly towards a protective Th1 response when compared to TLR2$^{-/-}$ infected mice, as demonstrated by lower production of Th2 cytokines and higher ratio of IFNγ to IL-10, IL-4 and IL-13 in many of the restimulation experiments.

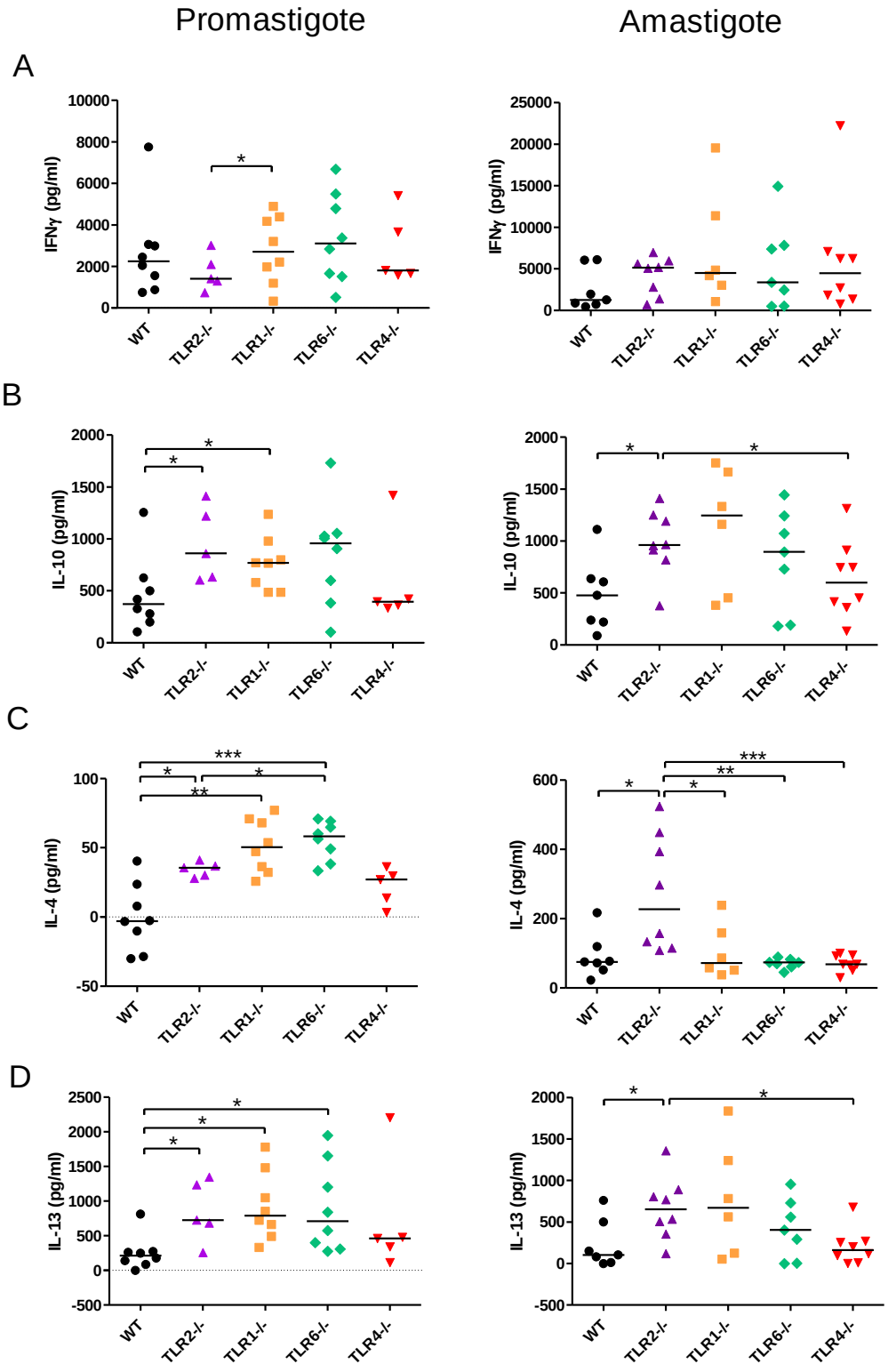

Figure 27. **WMAg specific production of cytokines by DLN cells from WT and TLR$^{-/-}$ mice infected with *L. mexicana* promastigotes (left) or amastigotes (right). At the end of the infection experiments, DLN cells were collected and cultured *in vitro* for 72 hours and cytokine levels in the supernatants were measured for levels of IFNγ (A), IL-10 (B), IL-4 (C) and IL-13 (D) by ELISA. Individual points represent the mean average levels of cytokine from triplicate wells per mouse, and horizontal bars represent median**

averages for each group. Groups were compared using the Mann-Whitney U test (* p <0.05, **p<0.005; ***p<0.001).

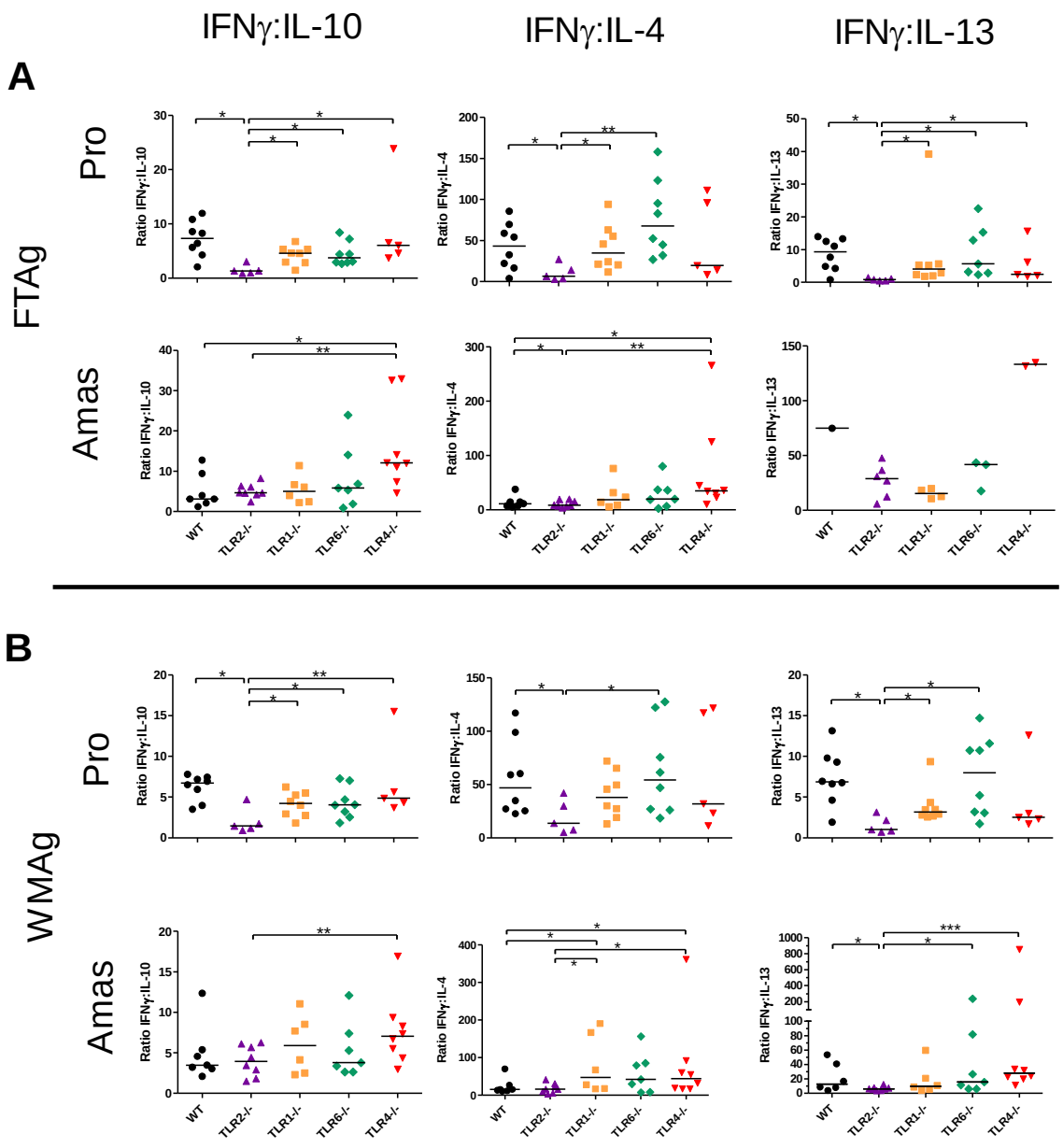

Figure 28. Ratio of antigen specific Th1 to regulatory and Th2 responses in DLN stimulations from WT and TLR$^{-/-}$ mice infected with *L. mexicana* parasites. The ratio of production of IFNγ to IL-10, IL-4 and IL-13 was calculated using cytokine levels calculated by ELISA from DLN cells stimulated with *L. mexicana* FTAg (A) and WMAg (B) antigen. Individual points represent the ratio of the mean average levels of cytokine from triplicate cultures per mouse, and horizontal bars represent median averages for each group. Groups were compared using the Mann-Whitney U test (* p <0.05, **p<0.005; ***p<0.001).

TLR2$^{-/-}$ mice showed increased susceptibility to infection with *L. mexicana lpg1*$^{-/-}$ parasites as well as WT promastigotes and amastigotes, as described earlier. The adaptive immune response in these mice was measured by stimulating DLN cells from mice infected for 18 weeks, using only FTAg as the antigen. The overall trend was that all cytokines were elevated in the cultures with stimulated TLR2$^{-/-}$ DLN cells,

perhaps reflecting the higher parasite burden in these mice. The only cytokine with significantly increased levels compared to WT was IL-4. No significant differences in the ratios of IFNγ to other cytokines produced was found (data not shown).

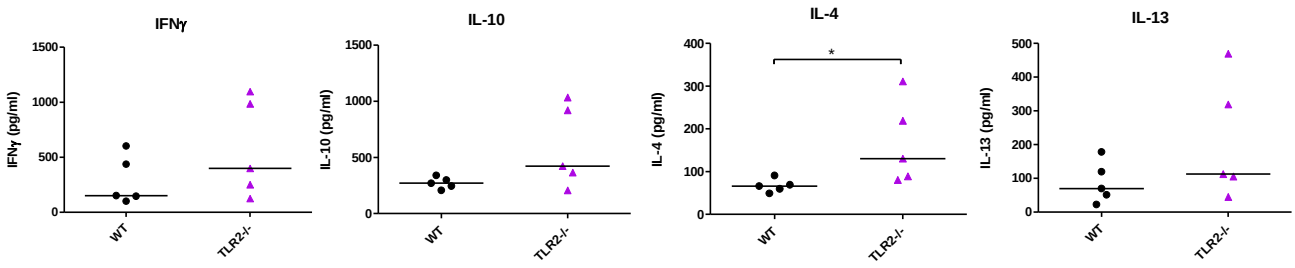

Figure 29. Immune responses in DLN cells from WT and TLR2$^{-/-}$ mice infected with *L. mexicana lpg1*$^{-/-}$ promastigotes, week 18. DLN cells were isolated from infected mice at 18 weeks p.i. and were restimulated *in vitro* with parasite antigen and controls for 72 hours. Supernatants were analysed for the presence of cytokine using ELISA: IFNγ, IL-10, IL-4, IL-13. Individual points represent the mean average levels of cytokine from triplicate wells per mouse, and horizontal bars represent median averages for each group. Groups were compared using the Mann-Whitney U test (* p <0.05).

Levels of antigen specific antibody

Antibody responses have been closely linked with the chronicity of *L. mexicana* infection, in particular antigen specific IgG1 has been linked with IL-10 production and the chronicity of infection in the C57BL/6 model. To explore whether levels of IgG1 and IgG2c are influenced by the absence of any of the surface TLRs explored in this study, levels of antigen specific IgG1 and IgG2c in plasma samples taken from week 14 infected mice were measured by ELISA. Surprisingly, despite elevated IL-10 and Th2 responses, infected TLR2$^{-/-}$ mice did not show increased levels of antigen specific IgG1, or ratio of IgG1:IgG2c in their plasma compared to WT, TLR1$^{-/-}$ or TLR6$^{-/-}$ mice. The only difference of note was the increased levels of both IgG isotypes in TLR2$^{-/-}$ mice compared to TLR6$^{-/-}$ mice (data not shown). No differences in levels of IgG1 or IgG2c specific to *lpg1*$^{-/-}$ FTAg or WT WMAg antigen specific levels were detected in the WT and TLR2$^{-/-}$ mice infected with *L. mexicana lpg1*$^{-/-}$ promastigotes, or in the ratio of these two isotypes (data not shown). Plasma from naïve mice was not found to have any antigen specific IgG antibody by ELISA.

Discussion

This study explored the role of TLR2, TLR1, TLR6 and TLR4 in infection with *L. mexicana*. The findings reproduce many of those found for *L. major* infection (presented in Chapter 3), particularly in relation to TLR2 where mice lacking this receptor developed more severe disease after infection with both species of *Leishmania*. We were therefore able to explore whether this mechanism of TLR2 mediated control was due to activation by LPG by using parasites lacking LPG for infection in the form of both the naturally occurring lpg-lacking form (amastigotes) and genetically modified parasites which lack the expression of a

full LPG molecule. The initial hypothesis was that activation of TLR2 on APCs during the early stages of infection is able to boost the activation of immune responses which help to control parasite replication during the acute phase of infection (i.e. Th1 type responses), as proposed by Kavoosi *et al* (241). As TLR2$^{-/-}$ mice developed more severe disease (as measured by increased lesion size) when infected with these LPG-lacking parasites as well as WT promastigotes, it appears that the activation of TLR2 by LPG is not the sole mechanism of TLR2 mediated control in this model. However, as the increase in parasite burden in the *lpg1*$^{-/-}$ infected TLR2$^{-/-}$ mice was not found to be significant, and the increased disease severity in TLR2$^{-/-}$ mice (as measured by parasite burden, lesion size and AUC) was in general more apparent in the promastigote infection experiment, this may point to a partial role for LPG-TLR2 interaction (i.e. in addition to non-LPG activation of TLR2). However, when looking at the lesion progression curves from two *L. mexicana* WT experiments (Figure 21) it appears that the lesions started to heal more in the amastigote infection than in the promastigote infection at week 18, which may explain the greater variance seen in the parasite burden data from this experiment. For the *L. mexicana lpg1*$^{-/-}$ infection experiment, it is likely that the smaller group size (n=5 compared to n=6-8 in the WT experiments) affected the lack of statistical significance in parasite burden being observed in the lesions from TLR2$^{-/-}$ mice.

Although the *lpg1*$^{-/-}$ parasites used in this experiment lack a full LPG molecule, they retain the ability to synthesize the membrane anchor of LPG, which includes the acyl group that was found to be crucial for TLR2 activation (26). It is not known whether the anchor of LPG is still expressed in high levels in the promastigotes of *lpg1*$^{-/-}$ parasites. Furthermore, it has been suggested that the phosphoglycan chain, which is absent in the *lpg1*$^{-/-}$ parasites, has an important role in the ability to activate TLR2, as shown by other studies comparing LPG isolated from different *Leishmania* species (241). Osanya *et al* showed that synthetically produced tri-mannose molecules based on the cap of LPG (and ManLAM of *M. tuberculosis*), when coated onto the surface of synthetic beads, were able to signal through TLR2 and MR and enhance protective Th1 responses when administered with *L. major* parasites *in vivo* (242). However, the aforementioned study is the first to attribute the TLR2 activating ability of LPG to the mannose cap, and is in contrast to most studies using purified LPG which attribute the ability to activate TLR2 to the lipid moiety of the GPI anchor (26, 27), and indeed to other studies of TLR2 ligands which have determined the crucial acyl group required for efficient TLR2 activation (156, 162, 163, 243). To determine the precise mechanism of TLR2 activation by LPG and/or other parasite derived glycosylated molecules, it is important to determine the crystal structure of the ligand-receptor complex, as has been achieved for LPS-TLR4-MD2, Pam2-TLR2/6, Pam3-TLR2/1 and dsRNA-TLR3 (164, 166).

It would be interesting to explore infection of WT and TLR2$^{-/-}$ mice with LPG-deficient parasites of other *Leishmania* species (e.g. *L. major*) to determine if differences found in the ability to activate Th1 response via TLR2 influences disease severity, particularly as it has been shown that TLR ligands from

related but different species of microbe can induce contrasting adaptive immune responses upon TLR activation (244). Nevertheless, these data do suggest at least that there is activation of TLR2 during infection with *L. mexicana* and this activation occurs when LPG is not present in its typical abundance and form. Whether the responsible TLR2 ligand/s involved is/are of parasite origin (e.g GIPLs), or a host derived DAMP released in the lesion, is unclear. In addition, whether LPG of other species of *Leishmania* play a role in TLR2 activation also remains unknown, as differences in the host and immune factor components involved infections with *Leishmania* species are numerous and complex, such that generalisation across all species cannot be made using the findings from one model (245). For example, the role for LPG in virulence differs between species, and appears to be far less important as a virulence factor for *L. mexicana* than it is for *L. major* and *L. donovani* (21). Furthermore, a study exploring the activation of immune responses to the TLR4 bacterial ligand LPS *in vivo*, found contrasting responses induced by LPS from two species of bacteria even though both were potent activators of DCs: *E. coli* LPS promoted a Th1 type response while *Porphyromonas gingivalis* LPS promoted a Th2 type response (244). The innate immune response therefore possesses an additional level of discrimination between microbes: i.e. not only can innate immune receptors act to promote different responses when different combinations of PAMP/s and DAMP/s are present, but differences in species-specific structures of PAMPs can influence these responses.

The absence of an apparent role for TLR1 or TLR6 in the TLR2-mediated control of *L. mexicana* and *L. major* points towards a ligand for TLR2 which has an alternative interaction with the receptor to that known for bacterial acylated TLR2 ligands, where the ligand-receptor complex has been elucidated in great detail. In these cases, the heterodimerisation of TLR2 with either co-receptor determines the specificity of the receptor for its ligand, with TLR2/6 recognising triacylated lipoproteins/lipopeptides (156) and TLR2/1 recognising diacylated lipoproteins/lipopeptides (243, 246). Whilst the increased resistance to *L. major* by mice lacking TLR6 was observed in Chapter 3, TLR6$^{-/-}$ mice did not have any reduced disease severity or parasite burdens upon infection with *L. mexicana* in this study. This may suggest that TLR6 acts to exacerbate infection with *L. major*, but not *L. mexicana,* or may perhaps be a reflection of the more chronic nature of *L. mexicana* infection, and in the reduced Th1 response involvement when compared to *L. major*.

This study was able to explore more comprehensively the adaptive immune responses in mice infected with *L. mexicana* compared to the experiments presented in Chapter 3 for *L. major* infection. These recall-response results show that mice lacking TLR2 showed increased production of IL-10, IL-4 and IL-13 in response to *L. mexicana* antigen, and display a shift towards a regulatory/Th2 phenotype in terms of cytokines produced. All of these cytokines have been linked to exacerbating infection in models of *L. mexicana* (37, 247, 248). Interestingly, whilst the ratio of immune responses to antigen clearly demonstrate a diminished IFNγ in comparison to IL-10, IL-4 and IL-13 when compared to other groups, the IFNγ

responses to antigen by DLN did not differ from other groups. This suggests that TLR2 activation during infection acts to reduce expansion of Th2 cells (and perhaps other subsets producing IL-10), rather than driving the protective IFNγ response during infection. As IL-10 can be produced by many different T cell subsets, as well as APCs (67), it is not possible to determine which cells are responsible for the elevated IL-10 levels reported here. Given this skewing towards a Th2 response, it is surprising that infected TLR2$^{-/-}$ mice did not also present with heightened *L. mexicana* specific IgG1 levels in plasma, as was found in the *L. major* infections and which has been linked to elevated IL-10 and non-healing in *L. mexicana* infection (70, 71). Further studies exploring the expansion of adaptive immune cell lineages are needed to investigate the differences in adaptive immune response in TLR2$^{-/-}$ mice. Furthermore, the cell type responsible for the TLR2 mediated control of infection is unknown, and could be determined using conditional knockout mice. Whilst studies have reported differences in activation by DCs of WT and TLR2$^{-/-}$ DCs by *Leishmania* parasites (200), TLR2 is also expressed on monocytes, neutrophils, T cells and B cells as well as on keratinocytes in the skin. Therefore several of these cell types may be important for TLR2 function during infection with *L. major* and *L. mexicana*.

This study did not identify any role for TLR4 in *L. mexicana* infection, as mice lacking TLR4 did not present with any difference in lesion sizes or parasite burden compared to WT mice upon infection with either promastigote or amastigotes of *L. mexicana*. This is in contrast to the findings with *L. major* in the previous chapter, as well as with other studies using *L. major* (202, 203) and *L. pifanoi* (204) (the latter of which is closely related to *L. mexicana*). The study by Whitaker *et al* where TLR4 deficient mice were found to be more susceptible to infection with *L. pifanoi* amastigotes only measured the parasite burden at 1 week post infection in BALB/c mice as the disease outcome (204), whereas in the experiments presented here the lesion size and parasite burdens during the chronic stages of infection with *L. mexicana* (week 14) in C57BL/6 mice were comparable to WT (Figure 24). The difference in the disease phenotypes between *L. major* and *L. mexicana* we have observed may indicate a differential role for TLR4 in acute and chronic infection or relate to differences in the expression or presentation of putative TLR4 ligands between species.

A recent study by Naik *et al* demonstrated a crucial role for cutaneous tissue commensal bacteria in shaping the immune responses to *L. major* infection (249). By using either specific pathogen free or germ free mice, it was demonstrated that cutaneous tissue commensal bacteria are required for lesion development upon infection with *L. major*, but that commensal bacteria are also required for the development of an appropriate adaptive immune responses to *L. major* infection leading to control of parasite numbers (249). Thus, commensal bacteria present on the cutaneous tissue play an important role in the immune response in CL infection. Furthermore, this role for the skin bacteria in the immune response development was dependent on MyD88 and IL-1R signalling (249). It is therefore possible that exposure of host cells to bacteria at the site of the lesion may be responsible for activating TLR2 (via

ligation of bacterial TLR2 PAMPs such as lipopeptides) at the site of the *Leishmania* lesions, and this activation has a positive effect on the control of the infection, although the lack of dependency for TLR1 or TLR6 would rule out commensal bacteria being the sole cause of the TLR2-dependent disease exacerbation. Determining the relative roles of exogenous (commensal bacteria) and endogenous PAMPs (*Leishmania* TLR2 ligands) and DAMPs (host TLR2 ligands) will require further experimental approaches, and suggest a complex interplay of one or all of these potential sources of TLR2 ligands on *Leishmania* infection and immune response dynamics. The observation that the difference between WT and TLR2$^{-/-}$ mice occurs late on in the infection with *Leishmania* parasites may suggest a role for a commensal bacterial or host derived PAMP/s, to which the host is exposed to more in the later stages of infection, and which acts to promote a healing response via activation of TLR2, rather than a role for recognition of parasite derived ligands during the initial establishment of infection, although further experimental approaches are required to test this hypothesis.

Whilst many questions are raised by our experiments comparing infections of *L. major* and *L. mexicana* in mice lacking TLR2, 1, 6 and 4 presented in this Chapter and in Chapter 3, a consistent finding is that there is a clear role for TLR2 in controlling disease during infection with these two parasite species, and that TLR2 activation during infection promotes a protective immune response through the regulation or modulation of Th2 immunity. These findings encouraged us to investigate the use of TLR2 ligands as potential vaccine adjuvants for use in vaccines for CL.

Chapter 5. Development of a vaccine model using recombinant kinetoplastid membrane protein 11 (rKMP-11)

Abstract

KMP-11 is a small 11 kDa protein that is conserved amongst kinetoplastid parasites and is expressed on the surface of amastigotes and promastigotes of *Leishmania*, making it an attractive vaccine antigen candidate and one that has induced protection in several vaccination models. Furthermore, KMP-11 has been shown to be associated with the putative TLR2 ligand present on the *Leishmania* surface, LPG. For these reasons, KMP-11 was chosen as the antigen for use in the development of lipopeptide vaccines for protection against *L. mexicana* infection in mice. Recombinant KMP-11 of *L. mexicana* was successfully cloned and expressed in *E. coli,* and was purified using nickel affinity chromatography. The rKMP-11 preparation generated was found to react to the sera from C57BL/6 mice infected with *L. mexicana*, indicating that KMP-11 is immunogenic in this infection model. However, when mice were vaccinated with rKMP-11 together with CpG as an adjuvant, they developed similar lesion sizes of a challenge infection with *L. mexicana* to those seen in the control group of mice treated with only CpG adjuvant. These results suggest that this experimental vaccine model requires further development for the assessment of vaccines comprising of KMP-11 and lipopeptides, as no protective immunity was generated using this vaccination regimen with gold standard CpG adjuvants.

Introduction

Background

As demonstrated in Chapters 3 & 4, TLR2 plays a role in chronic infection with *L. major* and *L. mexicana* suggesting that TLR2 signalling during infection leads to an immune response, which is favourable for control of CL. Thus, it is a rational approach to target TLR2 in a vaccine for CL by using TLR2 ligands as adjuvants.

Rationale for the development of a lipopeptide vaccine comprising KMP-11 epitopes

Lipopeptides are the archetypal ligands for TLR2 and their ability to stimulate immune responses has been widely reported (250, 251). They are produced naturally, particularly by bacteria, but can also be synthetically made to incorporate known epitopes into the peptide moiety, which are linked to the acyl moiety responsible for activation of TLR2 heterodimers (see Figure 30). Thus, it is possible to create self-adjuvanting lipopeptide vaccines which are small, cheap to produce and do not require administration with other adjuvants (252).

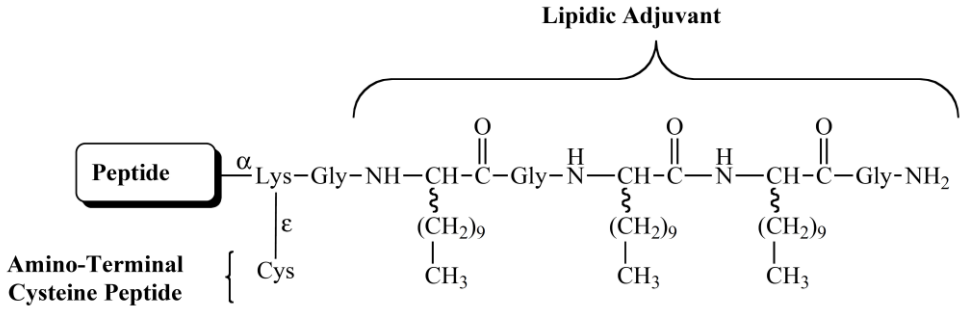

Figure 30. The basic structure of a lipopeptide vaccine. Figure taken from (253), with permission.

Lipopeptides are capable of inducing enhanced immune responses to associated antigens, due to the lipid moiety activating and recruiting DCs and other APCs, and increasing the uptake of the vaccine into these sentinel cells (251, 252). Importantly, lipopeptide vaccines have been shown to be strong inducers of cellular mediated immunity, as well as humoral responses (250-252, 254). Whilst TLR ligands have been incorporated as adjuvants in a number of new experimental vaccines for CL, few have explored the use of lipopeptides. In one report, Cote-Seirra *et al* successfully linked part of the *Leishmania* antigen gp63 to a lipoprotein from *Pseudomonas aeringinosa* and showed it could elicit good levels of protection to disease caused by *L. major* (255), suggesting that such an approach can be successful in a model of CL. This study also demonstrated that the lipid moiety is crucial for adjuvancy, as vaccine formulations which lacked this portion were unable to elicit strong Th1 responses or confer the same level of protection against infectious challenge (255). Thus, lipopeptides are a group of new vaccines, which have several advantages

over other vaccine approaches in terms of cost, safety and diverse immunogenic properties, which should be evaluated in experimental *Leishmania* vaccines. The ability of lipopeptides to activate TLR2 mimics the stimulatory activities reported for *Leishmania* LPG, which can activate TLR2 to produce inflammatory responses *in vitro* (26, 27), although our previous results suggest this does not play a major role *in vivo*. The finding that TLR2 adjuvants have been shown to promote greater immunological memory than TLR4 adjuvants (256), which have been used to some success in *Leishmania* vaccine studies, further suggests that lipopeptide vaccines are worth evaluating.

The chosen vaccine antigen to adopt for this approach was kinetoplastid membrane protein 11 (KMP-11). KMP-11 is a small 11 kDa membrane associated protein which is abundantly expressed on the surface of kinetoplastid parasites (257, 258). *Leishmania* KMP-11 was originally discovered due to its tendency to co-purify with LPG, and was first termed 'LPG-associated protein' (258-260). LPG was found to be highly immunogenic in early studies and was considered a promising vaccine candidate, as mice primed with *L. major* LPG showed elevated numbers of T cells responsive to *L. major* (261). However, the immunogenic activity of LPG was later attributed to the KMP-11 molecules present in the LPG preparations (260), and KMP-11 was subsequently shown to be a potent immunogen and stimulator of T cell proliferation in infection models of *Trypanosoma* and *Leishmania* parasites (258). Thus, KMP-11 is a conserved and important membrane molecule of *Leishmania* parasites, which is antigenic during infection, making it a potential vaccine candidate. Epitope mapping of KMP-11 has shown there are 30 MHC class-I restricted epitopes within the protein that can induce production of IFNγ by human CD8+ T cells (262). KMP-11 is expressed in large quantities on the parasite cell surface (259) of both promastigotes and amastigotes (263). A DNA vaccine containing the gene for KMP-11 showed strong levels of protection against infection of highly susceptible golden hamsters by two strains of *L. donovani* (134). Another KMP-11 vaccine which was generated by making hybrid dendritic cells gave protection in a mouse model of *L. donovani* and induced a strong CTL response (139). KMP-11 has shown strong promise as a vaccine antigen in *L. donovani* models of infection, some promise for use in a vaccine for *L. major* although rIL-12 is required (135), but has yet to be tested in models of new-world CL.

Aim of the study

To overall aim of this study was to develop self-adjuvanting lipopeptide vaccines, comprising of an antigenic epitope of KMP-11 and acyl moiety capable of activating the immune system via TLR2 signalling, thus mimicking the natural activation of the immune system by LPG and KMP-11 in *Leishmania* infection. However, as KMP-11 has not been tested before as a vaccine for a *L. mexicana* model of CL, we first sought to clone and purify recombinant *L. mexicana* KMP-11 and explore its use as a vaccine antigen in this model in recombinant protein form, in combination with TLR ligand adjuvants.

Methods

Cloning, expression and purification of recombinant KMP-11

The KMP-11 gene was cloned from *L. mexicana* DNA using a high fidelity enzyme and was then incorporated into the TOPO2.1 vector (Invitrogen), as described. After confirmation of correct KMP-11 gene incorporation using PCR, restriction digest and sequencing analysis, the gene was cleaved from TOPO2.1 and sub-cloned into the expression vector pQE-30 (Qiagen). The expression and purification of rKMP-11 was then carried out, as described in detail in Chapter 2.

Immunoreactivity of antibodies to recombinant proteins

To explore the immunoreactivity of rKMP-11, rDHFR, and *L. mexicana* lysates to antibodies, western blots were performed after protein separation by SDS PAGE (see Chapter 2). The following antibodies were used at the indicated concentrations: anti-His antibody (Sigma, 1:3,000 dilution), and pooled plasma samples from *L. mexicana* infected mice (collected as indicated in Chapter 2; 1:150).

Mice, vaccinations, parasites and infection

All procedures involving live animals were performed at the BSU in the Duncan Building, University of Liverpool. Female C57BL/6 mice (8-10 weeks) were used for the vaccine experiment. For the pilot study, 3 mice per group were vaccinated subcutaneously with either 10 μg rKMP-11 and 50 μg CpG adjuvant, or 50 μg CpG adjuvant alone, in a total volume of 20 μl DPBS. Each mouse was given two doses of the same vaccine; the first dose was administered s.c. to the upper side of the LHF, and the second dose was administered s.c. to the shaven rump two weeks later. *L. mexicana* M379 promastigotes were grown from lesion amastigotes in complete M199 as described, and were sub-passaged twice before use as an infectious challenge. Four weeks after the second dose, mice were challenged with 10^5 *L. mexicana* promastigotes in 20 μl HBSS s.c. to the upper side of the RHF. Lesion development was monitored by taking weekly measurements of the thickness of the infected RHF and uninfected contralateral foot (LHF) using a dial caliper.

Results

Cloning of rKMP-11

To explore the use of KMP-11 as a vaccine antigen for *L. mexicana* infection of C57BL/6 mice, we generated a recombinant *L. mexicana* KMP-11 protein. To make recombinant KMP-11, the KMP-11 gene was first amplified from *L. mexicana* DNA by PCR using a high fidelity polymerase and the presence of a product of the correct size (c. 300 bp) was determined by visualisation on an agarose gel (Figure 31 A). After the addition of poly-A tails and purification of the product, the KMP-11 gene was inserted into the TOPO 2.1 vector and the ligated vector was used to transform competent *E. coli* cells. To screen for

uptake of TOPO-KMP-11, colonies were screened by blue-white screening (not shown), PCR for the KMP-11 gene, and restriction enzyme analysis (Figure 31 B and C respectively).

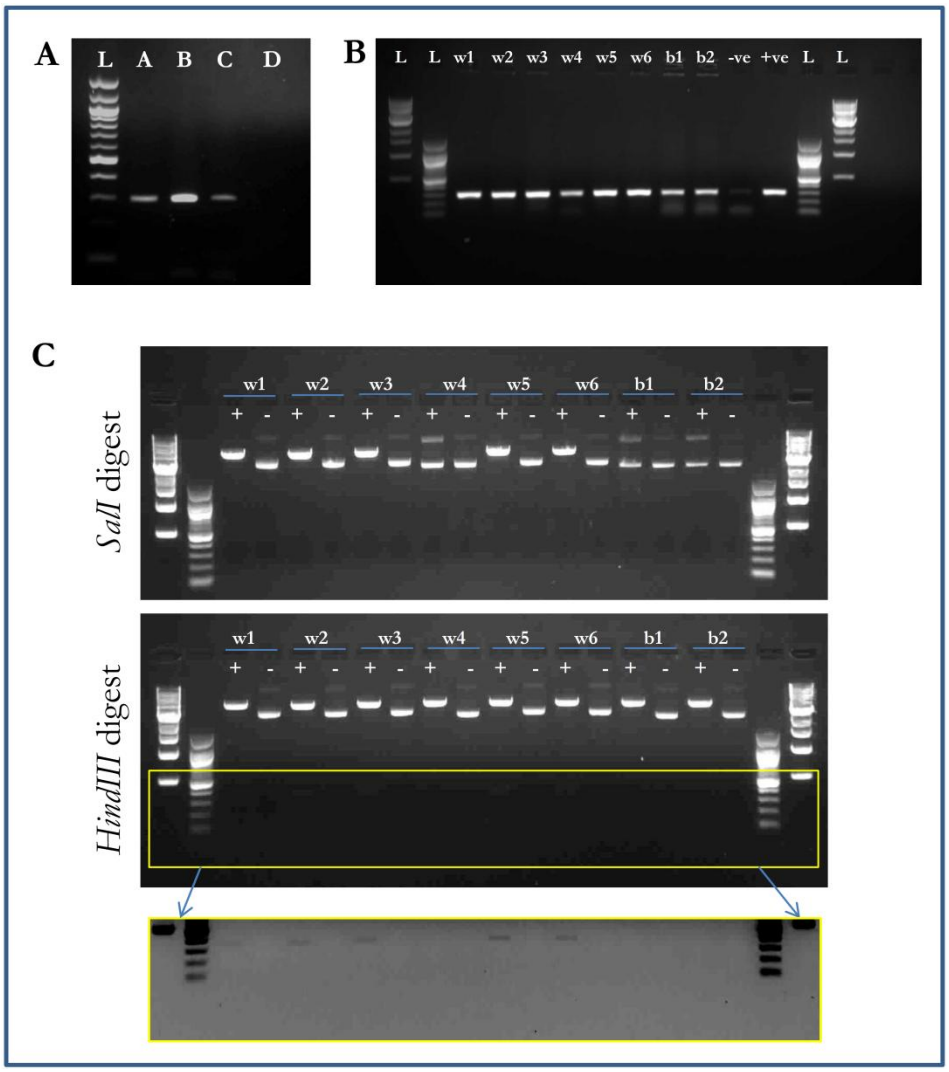

Figure 31. Cloning of *Leishmania* KMP-11 into TOPO 2.1 vector. The 273 bp KMP-11 gene was amplified from genomic DNA of *L. mexicana* (A) before ligation into the TOPO vector. White and blue colonies were selected for further analysis after transformation of *E. coli* cells with ligated TOPO-KMP-11 vector. Each was tested for presence of the KMP-11 gene by PCR (B) and for the presence of TOPO-KMP-11 by restriction digest analysis using the *SalI* and *HindII* enzymes (C).

In screening by PCR, all of the white colonies as well as two blue colonies (expected to be negative for KMP-11 gene but which have taken up TOPO vector) showed a positive result for the presence of the KMP-11 gene. In the restriction digest analysis, two enzymes were used: *SalI* was chosen as the only target site for this enzyme is within the KMP-11 gene, and *HindIII* as it had two target sites in TOPO-KMP-11 and only one in TOPO alone. The restriction digest maps for the two plasmids are shown in Appendix 3. Despite testing positive for the KMP-11 gene, colonies w4, b1 and b2 did not harbour TOPO-KMP-11 as they are uncut by the *SalI* enzyme and did not give 2 distinct bands when cut with

HindIII (Figure 31C). This suggests that the KMP-11 gene was taken up by these colonies but not in the complete TOPO-KMP-11 form desired. Therefore, the colonies w1, w2, w3, w5 and w6 were selected for sequencing analysis. All of the sequenced TOPO-KMP-11 plasmids showed the presence of a KMP-11 gene from *L. mexicana* with 100% homology to one of the published genes of *L. mexicana* (LmxM.34.2221 GeneDB) and 98 or 99% homology with the other two *L. mexicana* genes and 97% homology to *L. major* KMP-11 (LmjF.35.2210, GeneDB) at the nucleotide sequence level.

Expression and purification of rKMP-11

For expression of the *L. mexicana* KMP-11 gene, the BamHI-KMP-11-HindIII construct was cleaved from TOPO-KMP-11 by restriction digest and ligated into the pQE30 expression vector. The ligated pQE30-KMP-11 vector was used to transform competent *E. coli* M15 [pREP4] cells. As with TOPO-KMP-11, colonies which grew on the antibiotic containing plates were selected for further growth and analysis to check for transformation with pQE30-KMP-11 by PCR, restriction digest analysis and sequencing. The sequencing alignment results for one of the selected plasmids are shown below in Figure 32. All of the purified pQE-30-KMP-11 plasmids from screened colonies were found to align with 100% homology with the KMP-11-3 gene of *L. mexicana* (Accession number LmxM.34.2221). Furthermore, transformed colonies were found to express a protein of approximately 11kDa after induction of rKMP-11 expression by IPTG, as assessed by SDS PAGE (not shown).

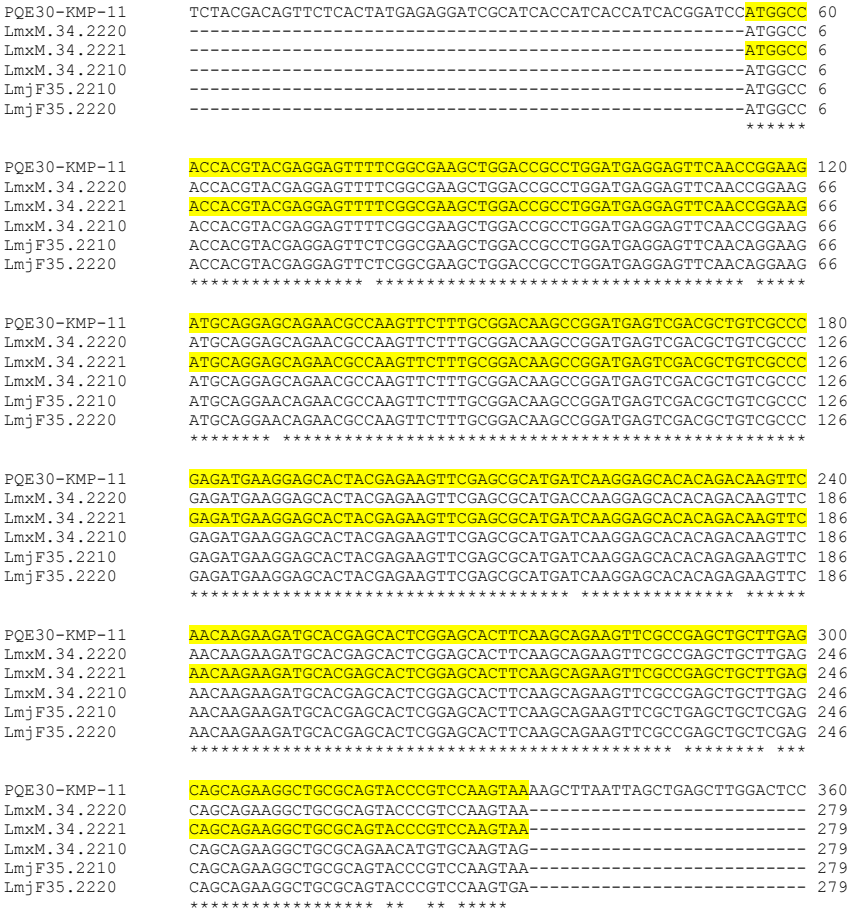

Figure 32. Alignment of pQE30-KMP-11 sequence with *Leishmania* KMP-11 genes. The plasmids from three pQE30-KMP-11 positively selected colonies were sequenced by Cardiff DNA Sequencing Core. The sequences were aligned against *L. mexicana* and *L. major* KMP-11 genes as published on GeneDB. All of the pQE30-KMP-11 plasmids shown 100% homology with LmxM.34.2221 (KMP-11 3 gene of L. mexicana) – this image shows the alignment of one pQE-30-KMP-11 sample with the KMP-11 genes; the matching sequences are highlighted in yellow.

Large volumes of *E. coli* [pQE30-KMP-11, pREP4] cells were therefore grown in the presence of IPTG to induce production of rKMP-11. The cell lysates of these colonies were passed through Ni-NTA columns and purified as indicated, and the purity was assessed by SDS PAGE. Figure 33 shows an SDS PAGE of rKMP-11 collected from a Ni-NTA column by eluting with increasing concentrations of imidazole. A strong band of rKMP-11 protein is visible at the appropriate size in many of the fractions collected after eluting with 100 mM imidazole and above (c 11 kDa), and a faint band at approximately 25 kDa in size is also visible in the fractions, which is likely to indicate dimers of rKMP-11.

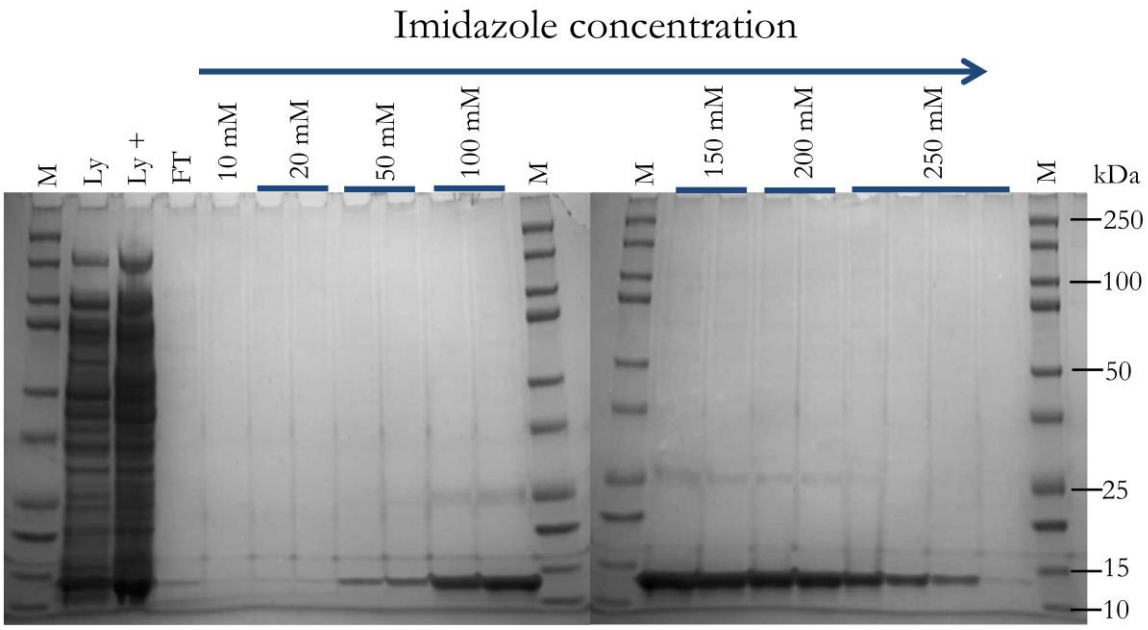

Figure 33. Purification of rKMP-11. Fractions were collected after addition of buffer containing increasing concentrations of imidazole. Codes: M = Marker; Ly = Lysate of culture; Ly+ = Lysate of induced culture; FT = flow-through.

After first purifying rKMP-11 using a gravity column, and then with the AktaPrime system, the purity was found to be satisfactory by SDS PAGE analysis. Selected fractions were pooled, dialysed to exchange into a DPBS buffer, depleted of endotoxin and filter sterilised. The endotoxin levels of rKMP-11 was found to be between 380 -810 EU/mg protein before, and 14.4 EU/mg after removal of endotoxin. According to Brito and Singh of Novartis Vaccines, levels of up to 20 EU/mg are acceptable for recombinant protein preparations for use in pre-clinical vaccines (264).

Immunogenicity of rKMP-11 in *L. mexicana* infection

To test the immunogenicity of KMP-11 in the *L. mexicana* model of infection of C57BL/6 mice, the reactivity of sera obtained from *L. mexicana* infected C57BL/6 mice to rKMP-11 was analysed by western blot. It was found that the sera from infected C57BL/6 mice reacted to rKMP-11, as shown in Figure 34. As controls, samples were also checked for reactivity to the anti-His tag and anti-KMP-11 antibodies. The anti-His antibody showed reactivity to rKMP-11 and control rDHFR, but not to the parasite lysates, as expected (data not shown).

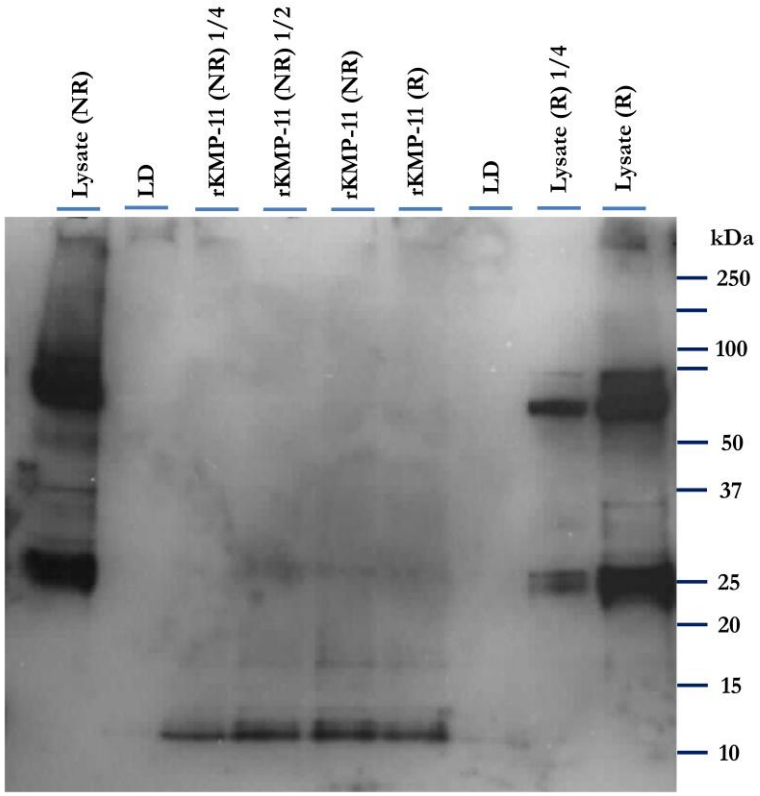

Figure 34. Plasma from *L. mexicana* infected mice recognises rKMP-11 in reduced and non-reduced forms. Lysates from *L. mexicana* and rKMP-11 samples in different dilutions were either reduced (R) or not (NR) before separating by SDS-PAGE and then transferring onto a PVDF membrane. The membrane was incubated with pooled plasma (1:150) collected from *L. mexicana* infected C57BL/6 mice, prior to incubation with secondary anti-mouse–HRP conjugated antibody and developed for 1 minute. Representative of two experiments.

Vaccine efficacy – pilot study

As KMP-11 has not previously been tested as the vaccine antigen in a model of *L. mexicana* infection, we carried out a small pilot experiment using two groups of 3 mice. The test group was vaccinated with rKMP-11 plus the gold standard adjuvant for *Leishmania* vaccines, TLR9 ligand CpG, whilst the control group received CpG adjuvant alone. CpG was chosen as it is a TLR ligand which drives strong Th1 responses and has been shown to improve the efficacy of *Leishmania* protein and whole cell vaccines (265, 266). Mice were vaccinated twice with the same vaccine preparation and then challenged 4 weeks after the final dose by s.c. injection of 10^5 *L. mexicana* promastigotes to the top of the RHF. As shown in Figure 35, lesions developed in both groups of mice beginning at approximately 11 weeks post infection. Both groups then showed a progressive increase in lesion size until the end of the experiment at 19 weeks p.i.

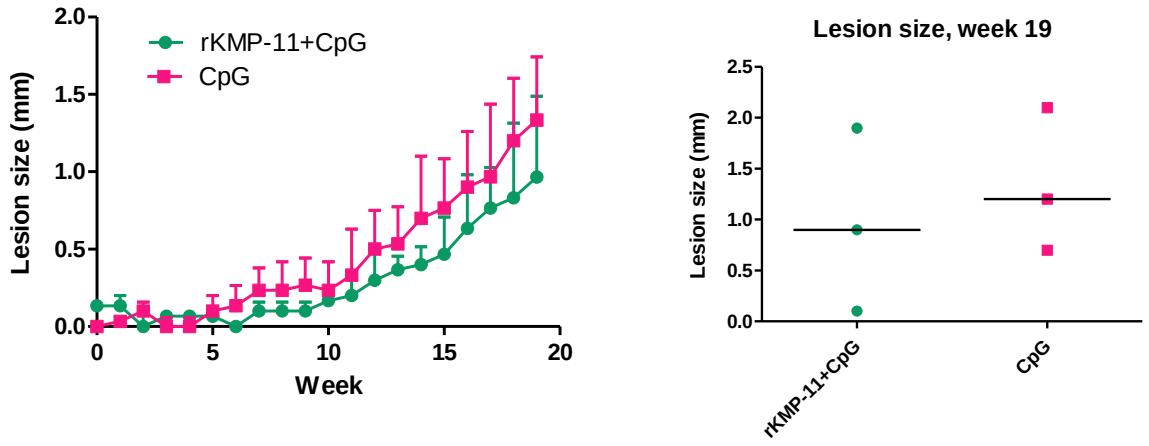

Figure 35. Lesion development in mice vaccinated with rKMP-11 + CpG adjuvant (green) or CpG adjuvant alone (pink) after needles challenge with 10^5 *L. mexicana* promastigotes. Groups were compared using the Mann-Whitney U test.

Despite the average lesion size of the rKMP-11 + CpG vaccinated mice being consistently smaller than those that were vaccinated with CpG alone, the difference was not significant. Whilst this may be due to a the small group size (n=3) used in this trial experiment, the lack of a significant level of protection suggests that vaccination with rKMP-11 did not replicate the efficacy reported in other vaccination models.

Given the poor efficacy of rKMP-11 vaccination in this pilot experiment, further experimentation was suspended due to time constraints.

Discussion

KMP-11 has been tested as a vaccine candidate in several different types of experimental vaccines, mostly using *L. donovani* or *L. chagasi* models of infection in mice. Whilst KMP-11 of *L. mexicana* has not been explored as a vaccine candidate, it has more than 95% homology to other *Leishmania spp.* KMP-11, and studies using the closely related *L. amazonensis* have shown that KMP-11 is abundantly expressed in the amastigote stage as well as the promastigote stage, and is an important membrane component (263), suggesting that it is also a potential vaccine candidate for new-world CL. Here, we have shown that *L. mexicana* rKMP-11 can be successfully produced in an *E. coli* expression system, as has been achieved with KMP-11 from other *Leishmania* species elsewhere (267). Furthermore, KMP-11 appears to be immunogenic in *L. mexicana* infection, as indicated by the binding of antibodies from infected C57BL/6 mice to the rKMP-11 protein in western blots. However, our pilot study suggested that rKMP-11 did not provide significant protection against disease caused by *L. mexicana* when administered with the gold-standard CpG adjuvant, although a small reduction in lesion size was observed compared to the control vaccinated mice.

The CpG adjuvant has proven successful at improving efficacy in protein vaccines for *Leishmania* infection in mice by others (266, 268). However, these studies used either *L. donovani* or *L. major* for the challenge infection, and therefore the efficacy of CpG as an adjuvant has not been tested in vaccines against *L. mexicana*. In addition to this, KMP-11-containing vaccines, which have had success in inducing protection by other groups have typically used DNA vaccination, rather than in a recombinant protein form (134), which may be a more robust vaccination strategy.

It is possible that despite lesion development in all mice in this experiment, the control group used was protected to some extent by the CpG adjuvant alone. Others have reported a protective effect of the CpG adjuvant when used alone as a vaccine in mice (265, 269). Furthermore, Verthelyi *et al* found that in Rhesus macaques, CpG ODNs reduced parasite burden and lesion development when used as a prophylactic vaccine for protection against *L. amazonesis* (closely related to *L. mexicana*) in the absence of antigen (270). A study also using *L. amazonensis* infection (but in BALB/c mice) identified a similar protective effect of the TLR4 ligand ONO-4007 when used alone in a vaccine, compared to either a PBS or antigen alone vaccination (271). Thus, we may have observed no difference between the rKMP-11 + CpG and CpG groups in this study due to both benefitting from a protective prophylactic effect of the CpG adjuvant alone. It would be interesting to repeat the experiment using a PBS or antigen alone vaccine group, to test this hypothesis.

The aim of the pilot study was to determine whether a vaccine consisting of rKMP-11 and CpG adjuvant could provide sufficient protection in our model to act as a positive control, against which to compare other vaccine formulations consisting of lipopeptide adjuvants and rKMP-11. As we did not demonstrate efficacy with our rKMP-11 vaccine, we decided to investigate the use of lipopeptide vaccines with whole cell antigen vaccines (ALM) that have consistently demonstrated efficacy in several *Leishmania* vaccine models.

Chapter 6. Lipopeptides as adjuvants for a *Leishmania* vaccine

Abstract

TLR2 plays a role in the control of CL as demonstrated by exacerbated disease in TLR2$^{-/-}$ mice upon infection with either *L. major* or *L. mexicana* parasites. Studies exploring the immune response in infected TLR2$^{-/-}$ mice suggest that TLR2 activation plays a role in promoting a protective immune response to *Leishmania*, which helps clear the infection faster and reduce disease severity. We therefore proposed that TLR2 ligands could be beneficial as adjuvants for use in *Leishmania* vaccines. To test this hypothesis, we explored the use of Pam2 and Pam3 lipopeptide adjuvants, which activate TLR2/6 and TLR2/1 heterodimers respectively, in a vaccine model for CL using the whole cell antigen ALM. The lipopeptides were compared to the gold-standard Th1-inducing TLR ligand adjuvant CpG in their ability to provide protection against challenge with *L. major* in C57BL/6 mice. Surprisingly, we found that the use of lipopeptides in ALM containing vaccines did not provide any protection for mice upon infection with *L. major*, and in the case of Pam2, the adjuvant even exacerbated the disease severity in vaccinated mice, in contrast to protective immunity induce by vaccination with ALM + CpG. Assessment of the immune response in these mice indicated that Pam2, and to a lesser extent Pam3, were able to elevate antigen specific immune responses to *L. major*, but the immune response displayed a skewed Th2 phenotype, particularly characterised by elevated IgG1 levels. We observed that vaccines containing CpG and Pam2 adjuvants resulted in local reactions at the site of vaccination, but that this did not relate to vaccine efficacy. When the local response was further explored in mice exposed to lipopeptides alone, we noticed a difference in the time course of localised reactions between Pam2 and Pam3. In conclusion, Pam2 and Pam3 were not suitable adjuvants in an ALM containing vaccine for protection against *L. major*, as they both elicited a Th2 immune response, and Pam2 resulted in exacerbated disease.

Introduction

Background

In the previous chapter we attempted to develop a vaccine using rKMP-11 in a model using *L. mexicana*, but we were unable to show significant protection when the gold-standard Th1 adjuvant, CpG was included in a vaccine with rKMP-11. We therefore decided to test the use of lipopeptides as vaccine adjuvants in the well characterised whole cell antigen vaccine (ALM) model against *L. major*.

The use of ALM vaccines in mouse vaccine models for *L. major* infection

A widely used vaccine model for CL is where heat-killed autoclaved *L. major* (ALM) parasites, are given in two doses (prime and boost) prior to challenge with *L. major* promastigotes (53, 265, 268, 272). Killed, whole-cell *Leishmania* preparations have been widely used for several decades in human and mouse vaccine studies for protection against infection with *Leishmania* parasites (226, 268, 273-275). ALM is obtained by heat-killing large numbers of cultured parasites, and this is the most widely used approach for the preparation of whole cell vaccine antigens (others include freeze-thawing or formalin fixing parasites (265, 275)). Whilst the efficacy of ALM vaccines in humans studies was found to be very poor overall, the success was greater in trials conducted in South American countries when compared to the middle east or Africa (118, 123, 273, 276).

The BCG vaccine has been explored as an adjuvant for the ALM vaccine in human trials, but ALM + BCG was not found to significantly improve upon the efficacy of protection to VL compared to BCG alone (118, 273). However, this may be related to a prophylactic effect of BCG alone, due to either cross-reactivity of antigens in BCG and *Leishmania* parasite, or to immune-stimulatory (adjuvant) effects of BCG on those who were naturally exposed to infection during or around the time of BCG exposure (273).

In mice, the ALM vaccine has shown to provide good efficacy to needle challenge of infection with *Leishmania* parasites, in a number of different studies (268, 277). Whilst the ability of ALM vaccines to provide long term protective immunity has been disputed, Okwor *et al* recently demonstrated that repeated inoculation with ALM could result in the expansion of sufficient Th1 memory T cells specific for *L. major* and this strategy was as effective as live parasites at providing protection to challenge up to 13 weeks after the final dose was given (277). This demonstrates that the use of first generation killed parasite vaccines can provide protection to *L. major* when delivered in the appropriate manner. The use of immune responses boosting adjuvants may be an alternative strategy to multiple doses in increasing the efficacy of ALM vaccines. Furthermore, this model of vaccination provides a good foundation on which to compare the use of TLR2-stimulating lipopeptide adjuvants to the TLR9-stimulating gold-standard CpG adjuvant. Walker *et al* explored the use of CpG as an adjuvant in a vaccine containing killed *L. major*

parasites (in the form of freeze-thawed parasites), which elicited a strong Th1 response to *L. major* in susceptible BALB/c mice, and provided protection in 40% of individuals receiving the vaccine (265).

Aim of the study

In this study, we aimed to explore the efficacy of Pam2 and Pam3 as adjuvants in a vaccine against *L. major*. Their efficacy was compared to the gold standard Th1-driving TLR9 ligand CpG. We also explored the use of TLR2 ligands Pam2 and Pam3 in combination with CpG, as synergy of TLR responses has proved effective in boosting Th1 responses in other studies (278). The C57BL/6 model of infection was chosen, as this strain of mouse mounts immune responses which are more comparable to those seen in human infections, as opposed to the Th2 responses which occur in the BALB/c model (73).

Methods

Mice and parasites

All procedures involving live animals were performed at the BSU in the Duncan Building, University of Liverpool. Female C57BL/6 mice were purchased from Charles River, and were 8-10 weeks at the start of each experiment. *L. major* FV1 promastigote parasites were cultured in complete M199 medium, and were sub-passaged no more than two times after culture was established from lesion-derived amastigotes. For the infectious challenge, parasites were enriched for metacyclics as described in Chapter 2.

Vaccinations and challenge infection

C57BL/6 mice (7-8 per group) were vaccinated twice with the same vaccine dose in a 20 μl volume; the first dose was given s.c to the upper side of the LHF, and the second dose was given s.c to the shaven rump two weeks later. There were 7 different groups each receiving one of the following vaccines (the components of which are given in Table 6): PBS; Pam2 + Pam3 + CpG (Adjuvants only); ALM; ALM + CpG; ALM + Pam2; ALM + Pam3; ALM + Adjuvants. Four weeks after the second vaccine dose, mice were challenged by s.c. injection of 10^5 metacyclic-enriched *L. major* FV1 parasites, in 20 μl HBSS, to the upper side of the RHF. The vaccination schedule and groups are indicated in Figure 36.

To explore the local reactions to lipopeptides adjuvants, C57BL/6 mice (3/group) were given s.c. injections of the following in 20 μl HBSS: 50 μg ALM + 16.14 μg Pam2; 16.14 μg Pam2; 16.19 μg Pam3; 8.07 μg Pam2; 8.10 μg Pam3; 1.61 μg Pam2; 1.62 μg Pam3.

Measurement of local reactions at the site of immunisation on the foot, and lesion size on the infected foot, were measured using the same method, i.e. by measuring the thickness of the two hind feet using a dial calliper and subtracting the thickness of the unexposed/uninfected foot from that of the exposed/infected foot (mm).

Immune responses

To assess recall responses in the infected vaccinated mice, DLN cells and splenocytes were collected, processed and used at a concentration of 8 x 10^5 cells/ well. Cells were cultured for 72 hours in the presence of either 20 μg/ml *L. major* FTAg, 2.5 μg/ml ConA or media alone in a total volume of 200 μl/well. Culture supernatants were then removed and stored at -20°C until analysis for IFNγ, IL-10, IL-4 and IL-13 levels using cytokine ELISA. The levels of antigen specific IgG1 and IgG2c in plasma samples from mice were measured using antibody ELISA with *L. major* FTAg as the capture antigen.

Results

Vaccine efficacy – disease severity

Mice were divided into seven groups and were vaccinated with two doses of the same vaccine, as indicated by the vaccine schedule outlined in Figure 36. At four weeks after the second dose of vaccine was given, mice were challenged in the RHF with 10^5 *L. major* promastigotes. Lesion development was monitored by taking weekly measurements of the infected and uninfected hind feet and recording the difference. These results are displayed in Figure 37A. "Sham vaccinated" mice received two doses of PBS in their vaccination regime, so are effectively naïve controls. In these mice, lesions were evident at 2 weeks p.i. and peaked at week 7, before beginning to reduce in size. Mice inoculated with the adjuvants only were included as a control group to assess for any non-specific protective effect of the adjuvant components in absence of antigen. These mice showed lesions that were not significantly different in size from naïve controls, suggesting that the combined adjuvants alone did not have a protective effect on lesion development when administered prior to infection in the absence of antigen.

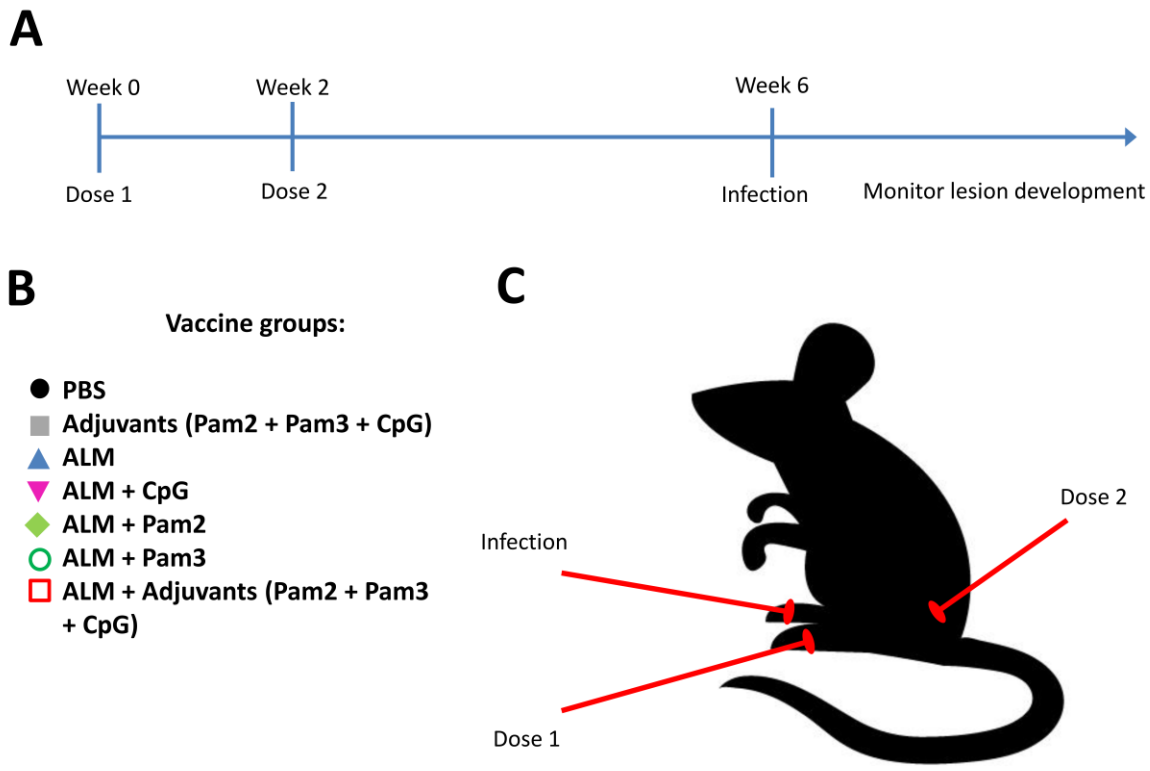

Figure 36. The vaccination and challenge schedule. All mice received two doses of the same vaccine, with a two week interval, and were then challenged with *L. major* 4 weeks after the last dose, as displayed on the schedule (A). There were 7 groups of mice (n = 7-8) in the experiment, and the components in the vaccines are indicated in (B) along with the symbol used for each group (as used in Figures in the Chapter from hereon). Vaccine doses and infectious challenge were administered s.c. to distinct tissue sites, as indicated in (C).

Those vaccinated with the killed cell vaccine ALM alone, showed a reduced lesion size in the later stages of infection, but this was not found to be significant at any time point. Consistent with these results, the AUC values for the Adjuvants only and ALM vaccinated groups did not differ from the PBS group (Figure 37B). When the gold standard CpG adjuvant was included in the ALM vaccine, however, mice developed smaller lesions compared to either PBS or ALM vaccinated mice, and a lower average AUC, indicating that this adjuvant acts to increase protection and reduce lesion development. There was no difference in lesion development when ALM + Pam3 was used as the vaccine, compared to either the naïve control group or the ALM vaccinated group, indicating that this adjuvant had no effect on the efficacy of the ALM vaccine. Notably, when Pam2 was used as an adjuvant with ALM, the lesion sizes were increased when compared to PBS and ALM vaccinated groups at several time points post 4 weeks p.i., and the AUC values were also increased in this group over the course of the experiment. However, when both lipopeptide adjuvants were included in the ALM vaccine in addition to the protective CpG adjuvant, lesions sizes and AUC values were reduced when compared to the PBS group, but not the ALM vaccinated group, demonstrating little or no impact on the addition of lipopeptides to protective CpG efficacy.

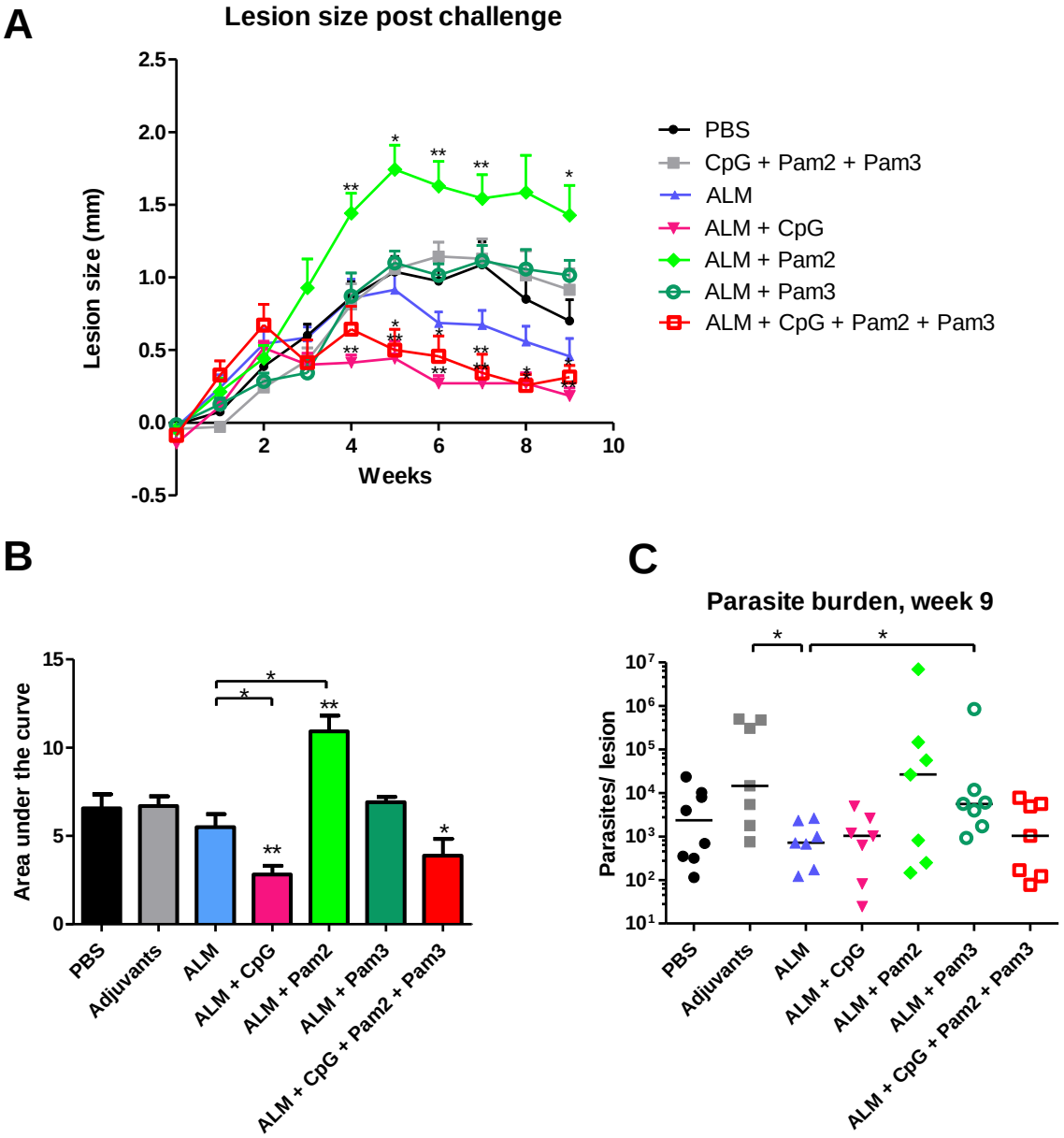

Figure 37. Development of disease in mice vaccinated with ALM and adjuvants. Lesion development was monitored by measuring the difference in thickness of the infected and uninfected feet (RHF-LHF) in mm (A); mean values + SEM are shown. The overall disease severity is summarised by calculating the area under the curve (AUC) from weekly lesion size data sets (B) (mean + SEM is shown), and by calculating the parasite burden in infected tissue after 10 weeks, using qPCR (C) (points represent individual parasite burdens and horizontal bars represent median values). Groups were compared using the Mann-Whitney U test (* p <0.05).

Vaccine efficacy – parasite burden

The parasite burdens of infected feet were determined by qPCR at the end of the experiment, after 9 weeks of infection with *L. major*, as shown in Figure 37C. The results show a high degree of variation in parasite burdens, between and within groups. As a result, few statistically significant differences were

found between groups when they were compared using the non-parametric bivariate analysis (Mann-Whitney U test). Those vaccines which displayed protective effect as determined by a reduction in lesion size and AUC (i.e. ALM + CpG and ALM + Adjuvants) did not show a reduction in parasite burden compared to either PBS or ALM vaccinated controls. In fact the lowest parasite burdens were seen in the ALM vaccinated group. The groups which received vaccines containing lipopeptides displayed the highest parasite burdens, with the mice vaccinated with just adjuvants (Adjuvants only) or ALM + Pam3 having significantly greater parasite burdens than the ALM vaccinated controls. Surprisingly, the ALM + Pam2 vaccinated mice did not have significantly greater parasite burdens compared to either control group, despite the greatest average parasite burden being in this group as well as the individual with the highest burden. The variation in parasite burden within the ALM + Pam2 vaccinated group of mice was notable, with almost a 5-log difference between those presenting with the highest and lowest parasite loads.

As with the parasite burden data from the *L. major* infection experiments in Chapter 3, the variation in the dataset from the vaccinated mice was found to be greater than the mean values for each group, suggesting extra-Poisson dispersal. The differences between groups were then also estimated using parametric methods, by fitting the data to a negative binomial model using a generalised linear function. The results are outlined in Appendix 4. This approach to statistical analysis found significantly elevated parasite burdens in the ALM + Pam2, ALM + Pam3 and Adjuvants only vaccinated groups (*** $p<0.001$ in all cases) when compared to ALM and PBS vaccinated controls. Furthermore, the ALM vaccinated mice had significantly reduced parasite burdens when compared to the PBS vaccinated group (* $p<0.05$).

To summarise, the vaccines which showed efficacy in reducing lesion size and AUC (i.e. those containing both ALM and CpG) did not give significant reduction in parasite burden at the lesion site at 9 weeks p.i., whilst those vaccines containing lipopeptides (except where ALM and CpG were both present also), resulted in elevated average parasite burdens at 9 weeks p.i.

Spontaneous production of cytokines by splenocytes from vaccinated mice infected for 9 weeks with *L. major*

After 9 weeks of infection, the splenocytes of infected vaccinated mice were recovered and processed for *in vitro* stimulation with antigen. Spontaneous production of Th1, regulatory and Th2 cytokines in culture was detected for individuals across all vaccination groups, as shown in Figure 38A. Surprisingly, the ALM vaccinated group, which had the lowest parasite burdens at week 9 p.i., presented with spontaneous production of the regulatory cytokine IL-10 which was significantly greater than the PBS control group, and those vaccinated with ALM + Pam3 or ALM + Adjuvants. Low levels of IL-4 were spontaneously produced by splenocytes of mice from all groups, although these were significantly lower in the ALM +Pam3 vaccinated group, compared to others.

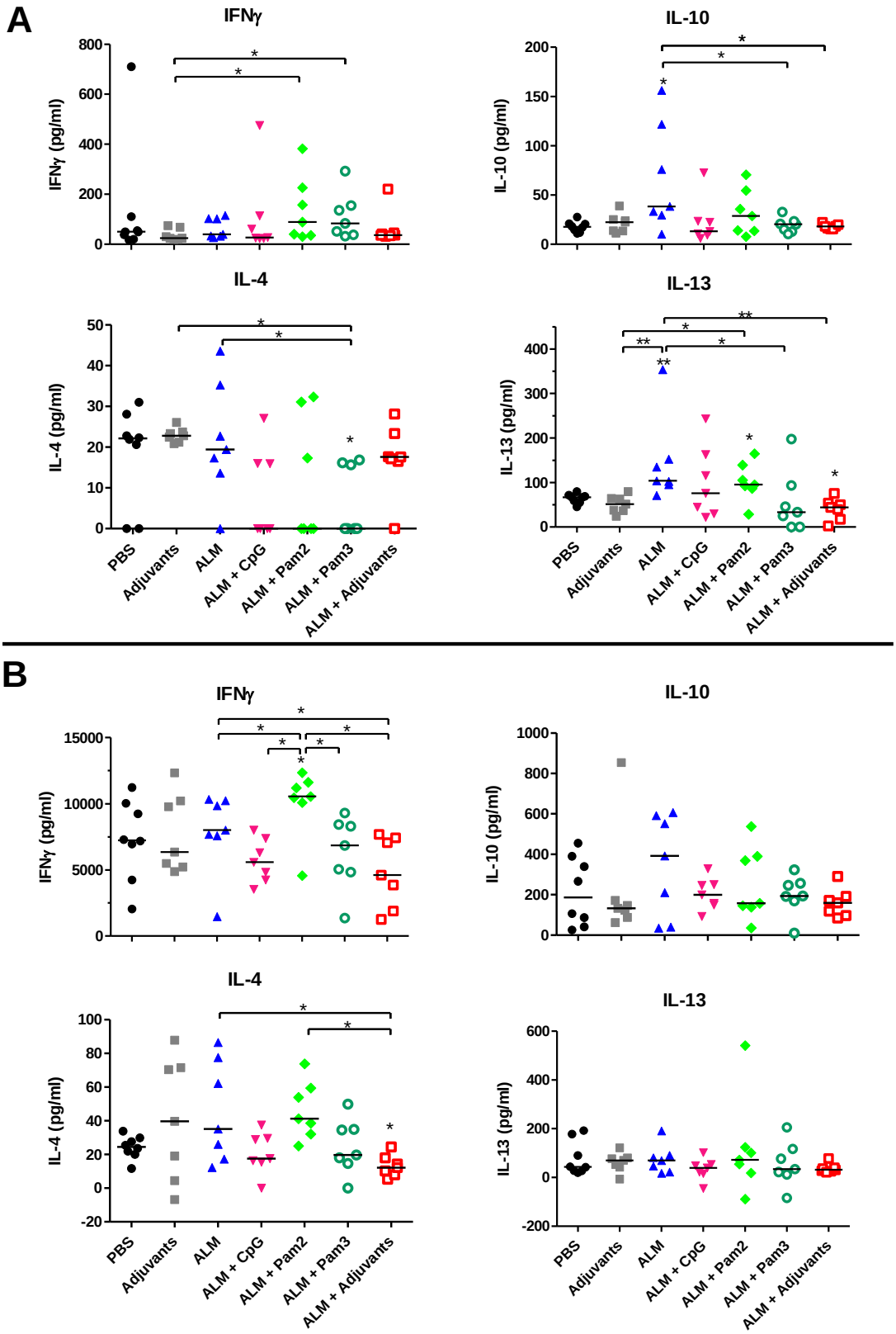

Figure 38. Cytokine responses after 9 weeks of infection with *L. major* in mice vaccinated with ALM vaccines +/- adjuvants. Mice were vaccinated twice with the indicated vaccines, and were challenged 4 weeks after the final dose with *L. major*. After 9 weeks of infection, mice were culled and their splenocytes

were processed and cultured in the presence of FTAg and controls. The levels of IFNγ, IL-10, IL-4 and IL-13 were measured by ELISA; (A) shows the spontaneous production of these cytokines in cultures, (B) represents the FTAg specific responses (adjusting for spontaneous production). Individual points represent the mean average levels of cytokine from triplicate wells per mouse, and horizontal bars represent median averages for each group. Groups were compared using the Mann-Whitney U test: differences to the control PBS group are indicated above the group data points, whilst differences between vaccinated groups are indicated by connecting bars (p<0.05 *; p<0.005 **; p<0.001*).**

Some mice produced high levels of IL-13 spontaneously in culture, and mice vaccinated with ALM or ALM + Pam2 produced significantly more IL-13 compared to the sham vaccinated (PBS) mice. Interestingly, the pattern of spontaneous production of IL-4 and IL-13 was not comparable, despite these both being Th2 cytokines.

In vitro recall responses from splenocytes, at 9 weeks post infection

The antigen specific responses by splenocytes were determined by subtracting the spontaneous production of cytokines from the levels of cytokine produced in the presence of *L. major* FTAg (Figure 38B). Mice vaccinated with ALM + Pam2 showed elevated production of the protective Th1 cytokine IFNγ compared to 5 of the 6 other groups of mice. No differences in antigen specific IL-10 or IL-13 were detected between groups. However, notably a number of individuals produced lower levels of IL-13 in response to FTAg than was produced spontaneously in culture, which suggests an antigen-specific downregulation of production of IL-13 in response to *L. major* in these mice. Those mice vaccinated with ALM + Adjuvants produced significantly lower levels of antigen specific IL-4 when compared to other groups. ALM + CpG vaccinated mice also produced lower levels of IL-4 in response to antigen, although this was not found to be significantly lower than other groups. These findings suggest that vaccines containing CpG and ALM act to reduce IL-4 responses to antigen after exposure to the infectious *L. major* parasites.

Given that the overall lesion development in the ALM + CpG and ALM + Adjuvants was lower than other groups from 4 weeks post infection with *L. major*, it is likely that the level of exposure of immune cells to parasite antigen was lower compared to other groups where lesions developed to a greater extent (and therefore are likely to have had higher parasite burdens), which may help to explain the lower antigen specific responses we detected in individuals from these vaccine groups. The ratio of IFNγ to IL-10, IL-14 and IL-13 produced in response to antigen was also determined for each individual. There were no detectable differences in the ratio of protective (i.e. IFNγ) to non-protective (IL-4, IL-13, IL-10) cytokines produced by mice from each group, indicating that the overall type of cytokine response to the *L. major* antigen was similar across groups, despite elevated cytokine responses being evident in some cases (data not shown). This likely reflects that fact that at this stage of the infection the mice have all begun to control the infection and reduce the parasite burden, and have broadly comparable immune responses to the parasite.

Levels of antigen specific IgG antibody isotypes in the plasma of vaccinated mice infected for 9 weeks with *L. major*

As the cytokine responses at week 9 p.i. were perhaps sub-optimal (due to timing in relation to the infection) for exploring differences between groups in terms of the immune responses, we used antibody isotypes (IgG1/IgG2c) from infected mice as markers of the type of Th1 and Th2 immune responses. After 9 weeks of infection with *L. major*, plasma was collected from the blood of vaccinated mice and the levels of antigen specific IgG isotypes were determined by ELISA (Figure 39). Whilst concentration levels were calculated using standard curves on ELISA plates, not all samples fitted within this range, and therefore absorbance values were used for displaying and final group comparison analysis even though the patterns observed were found to be the same. When the Kruskall-Wallis test was used to assess for differences in populations across the entire datasets for each of the antibody isotypes, the levels of IgG1 were found to significantly differ across all groups (** $p<0.0016$) whereas levels of IgG2c were found to be the same ($p = 0.1484$). Therefore, results indicate that the different vaccines differ markedly in their ability to drive an antigen specific IgG1 response, compared to the IgG2c response where differences between vaccinated groups were not detected (Figure 39A). The IgG1 antibody subclass was significantly elevated in the ALM + Pam2 and ALM + Pam3 vaccinated mice, when compared to sham vaccinated controls, suggesting that both lipopeptide adjuvants result in elevated Th2 antibody responses. The highest IgG1 levels were recorded in the group vaccinated with ALM + Pam2; the levels of IgG1 in this group were significantly greater than all other groups, except for those which received ALM alone or ALM + Adjuvants, suggesting that the presence of Pam2 in an antigen containing vaccine drives an elevated antigen specific IgG1 response compared to CpG and Pam3 adjuvants, but this is reduced to some extent by the addition of the other two adjuvants. Given that the lowest levels of IgG1 were recorded in the group vaccinated with ALM + CpG, it is likely that this reduction of IgG1 levels in the ALM + Adjuvants group compared to the ALM + Pam2 group is a result of the inclusion of CpG. The presence of CpG adjuvant alone in a vaccine containing ALM acted to reduce the concentration of antigen specific IgG1 levels compared to the ALM vaccine alone, suggesting this adjuvant restricts the class switching of antigen specific IgG to an IgG1 isotype, which is consistent with its known Th1 promoting activity.

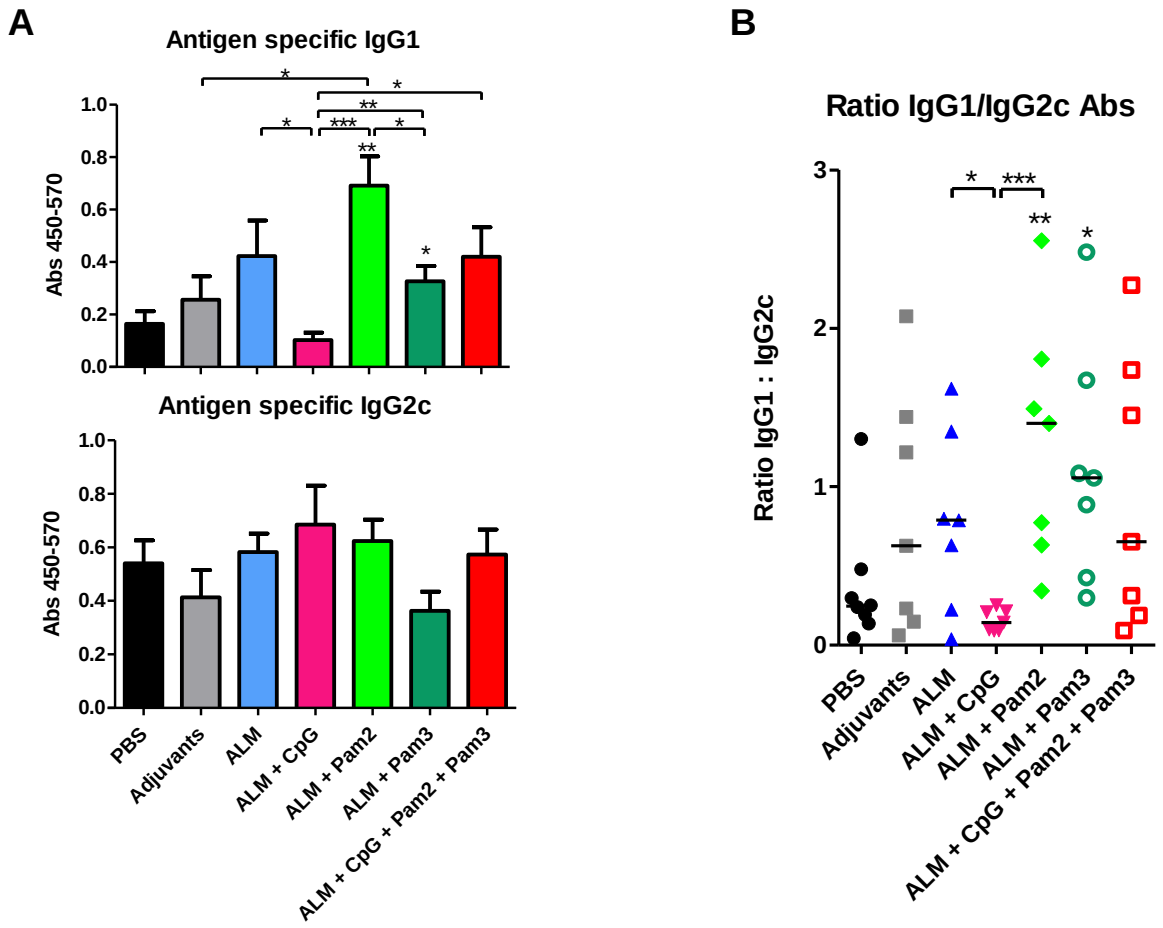

Figure 39. Antigen specific IgG antibody responses to FTAg in mice vaccinated mice infected with *L. major* for 9 weeks. Plasma samples were collected after 9 weeks of infection with *L. major* in mice that were vaccinated with ALM containing vaccines, with or without adjuvants, or controls. Levels of IgG1 and IgG2c antibodies specific for *L. major* FTAg were determined by ELISA, results are displayed as Absorbance (Abs) at 450-570 nm (A); bars represent the mean + SEM values for the average levels of antibody (from duplicate samples for each individual) in each group. The ratio of IgG1:IgG2a was estimated using the mean absorbance values for each individual (from supplicate values) and is displayed in (B); points represent the ratio of mean antibody levels (IgG1:IgG2c) for each individual. Groups were compared using the Mann-Whitney U test: differences to the control PBS group are indicated above the group data points, whilst differences between vaccinated groups are indicated by connecting bars ($p<0.05$ *; $p<0.005$ **; $p<0.001$*).**

The ratio of antigen specific IgG1:IgG2c antibody levels in plasma or serum is often used as a marker of the type of antigen specific adaptive immune response, with higher values indicating a Th2-skewed response, and lower values indicating a Th1-skewed response (235). The ratios of IgG1:IgG2c antibody levels were all below 1 when concentration values were used (data not shown), indicating that in general, all mice were producing more of a Th1 than Th2 type of adaptive immune response to *L. major* antigens. This fits with the observation that at this point in the infection (week 9 p.i.), all the mice had begun to control the infection and reduce lesion sizes. When either Pam2 or Pam3 was included in an ALM vaccine on their own, there was a significant shift towards a Th2 response to *L. major* antigen when compared to sham vaccinated (PBS) mice, or mice vaccinated with ALM + CpG (Figure 39B). Thus,

despite the finding that both antibody isotypes were significantly elevated by ALM + Pam2 adjuvant when compared to ALM + Pam3, both the lipopeptide adjuvants shifted the immune response towards a Th2 type when compared to CpG adjuvant or when mice were unexposed to antigen prior to infection. The CpG adjuvant was included in the experiment as a gold standard Th1-driving adjuvant, and in this experiment we found that it was able to shift the adaptive immune responses significantly towards a Th1 response, as indicated by a significant reduction in IgG1:IgG2c values when compared to those found in ALM vaccinated mice.

Local reactions to lipopeptide containing vaccines

After mice were vaccinated with the first dose, local reactions were observed with some groups developing a significant swelling at the site of the first injection (the LHF). In order to monitor this reaction, the thickness of both hind feet was measured, as is done for the measurement of *Leishmania* foot lesions. Figure 40 shows the swelling reactions at the injection site of mice from each vaccine group at one and three weeks after the first dose was given. No reaction was seen in the PBS vaccinated or ALM vaccinated mice. Those mice that received the ALM + Pam3 vaccine showed a marginal increase in the average level of swelling after 1 week, compared to PBS or ALM vaccinated mice, but this was not significant. Mice which received either the ALM + CpG or ALM + Pam2 vaccine, however, showed significantly elevated swelling at 1 and 3 weeks post exposure, when compared to either PBS or ALM vaccinated animals. This indicated that addition of either of these adjuvants to ALM was sufficient to induce a long-lasting local swelling at the injection site. Furthermore, ALM + CpG resulted in an increased swelling when compared to ALM + Pam3. However, the adjuvants alone (i.e. Pam2 + Pam3 + CpG) were also sufficient to induce a local reaction, as mice vaccinated with Adjuvants only also showed significantly increased swelling at these two time points. The most severe swelling was recorded in mice vaccinated with ALM + Adjuvants (i.e. when all vaccine components were present) where an average increase of over 1.5 mm in thickness was seen at the vaccinated foot site after one week; the swelling in this group of mice was significantly greater than every other group. These findings indicate that the addition of either 10 μg Pam2 or 50 μg CpG to the ALM antigen is sufficient to induce an localised swelling response, which is present for up to 3 weeks post vaccination. The presence of antigen does not appear to be a requirement for induction of the reaction, but addition of antigen to the three adjuvants resulted in a further increase in swelling, indicating that there is an additive response after the addition of all whole cell antigen vaccine and adjuvant components.

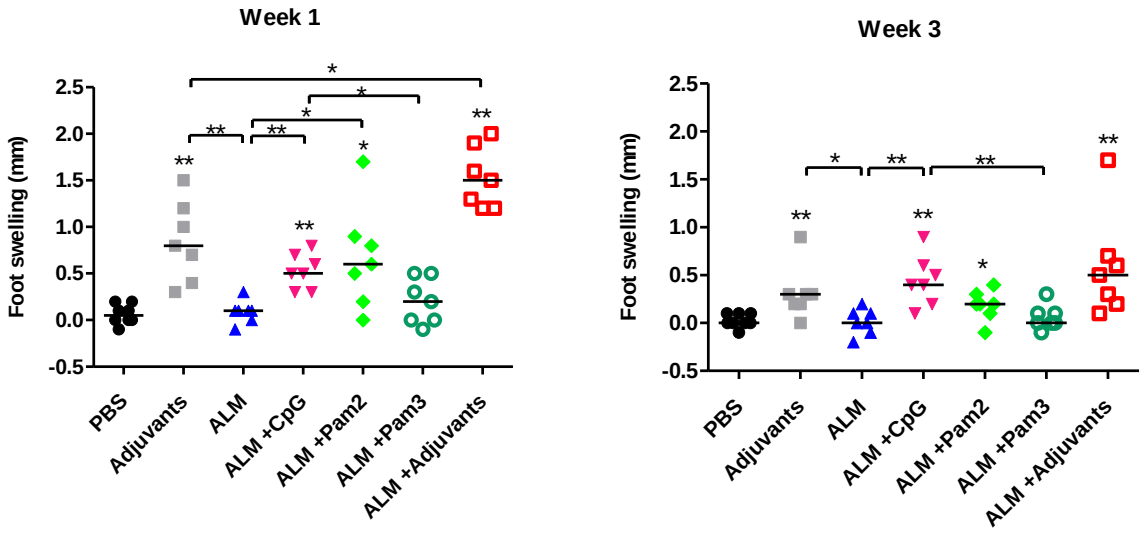

Figure 40. Local reactions at the injection site after s.c. immunisation with experimental vaccines including ALM and/or TLR ligand adjuvants, at 1 or 3 weeks post exposure. Data points represent swelling on the exposed foot of individuals, as determined by subtracting the size of the contralateral foot (RHF-LHF, mm), with median values shown as horizontal bars. Groups of vaccinated mice were compared against sham inoculated (PBS) controls using the Mann-Whitney U test (*p<0.05; **p<0.005).

Two weeks after the first vaccine dose, mice received the second dose s.c. to the shaven rump. Some local responses were also recorded at this site, in the form of redness but not swelling. As the area of tissue exposed at this site is less confined than that of the upper side of the foot, it is possible that a local swelling response could dissipate more readily compared to the confined space of the foot. The swelling responses in the feet had subsided in all mice by 4 weeks post first vaccination, allowing for the infectious challenge to be administered to the contralateral foot and for the lesions on the infected feet to be measured by comparing to the contralateral foot (which received the first vaccine dose).

It is intriguing that the presence of the Pam2 adjuvant induced a significant local reaction at the vaccination site, yet the presence of Pam3 did not. To further explore the swelling response to lipopeptide adjuvants, a small study was carried out in naive mice where groups of mice (n=3) received equimolar (10, 5 or 1 mmole) doses of either Pam2 or Pam3 subcutaneously to the upper side of the LHF. The sizes of the exposed and unexposed feet were measured a regular intervals after injection and the difference between the two gave a measurement of foot swelling (mm). The results are displayed in Figure 41 and they demonstrate that the dynamics of local reactions are different between the two lipopeptides.

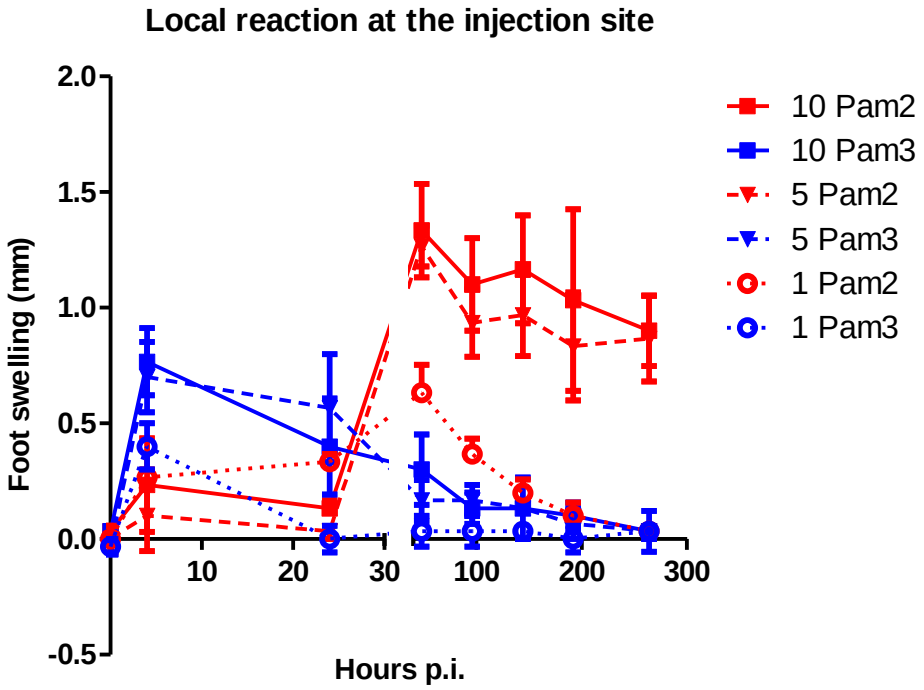

Figure 41. Local reaction to lipopeptide adjuvants. The swelling response to the s.c administration of Pam2 (red) and Pam3 (blue) lipopeptides at differing concentrations was measured by comparing the thickness of the exposed foot to the contralateral unexposed foot. Data points represent mean values at each time point post injection (p.i.) +/- SEM (squares with solid lines = 10 nmole; triangles with dashed lines = 5 nmole; open circles with dotted lines = 1 nmole).

No significant differences were found between groups receiving equimolar doses of Pam2 and Pam3. However, clear patterns in the local response to these lipopeptides were observed. Pam3 induced a rapid swelling of the exposed foot resulting in a peak increase in foot size after 4 hours which exceeded that caused by Pam2 at the corresponding dose. After 24 hours, the levels of swelling caused by Pam3 had already reduced, whereas those mice that received Pam2 showed the greatest swelling after 48 hours. The swelling caused by 5 or 10 nmole of Pam2 was sustained above 0.5 mm for up to 11 days after the initial injection, whereas the swelling caused by the other treatments had subsided by 4-5 days after injection. Thus, the local reactions caused by lipopeptides are dose dependent, but the dynamics of the swelling responses induced by the triacylated and diacylated lipopeptide molecules differ considerably: Pam3 induces an immediate swelling which subsides after 4 hours, whilst Pam2 induces a more prolonged localised swelling response which peaks after 2 days and is sustained for over a week at higher doses. A control group was included which receive the same vaccine as was used in the initial experiment, i.e. ALM + Pam2 (50 and 20 μg respectively), to control for the effect of ALM. This presented with the same reaction pattern as the 10 and 5 μg doses of Pam2 alone, but was not shown in Figure 41 in order to increase clarity.

The lipopeptide exposed animals were sacrificed after 11 days (264 hours) post injection, and the exposed foot tissue was preserved in formalin and processed for histology by staining slide sections with H&E. However, no noticeable differences in cell composition or tissue structure were detected.

Discussion

As in other studies using similar vaccine models for CL, we found that CpG was effective at providing reduction in disease severity (in terms of lesion size and AUC) when included in a leishmanial whole cell vaccine (266, 268, 279). However, parasites were still present at week 9 p.i., and were not significantly reduced when compared to other vaccine groups, indicating this vaccine was not sufficient to provide complete protection and did not reduce parasite burden as measured by qPCR at this timepoint. The use of Pam2 in the ALM vaccine resulted in exacerbated infection in the form of larger lesions and a higher average AUC value. The average parasite burden in the ALM + Pam2 vaccinated group was also elevated compared to the other groups, although this was only significant using the parametric statistical approach (see Appendix 4). Therefore it cannot be ruled out that the increased lesion sizes observed are a result of increased swelling, instead of an indication of higher parasite loads during infection. As the mice in all groups had reduced their lesions sizes from the peak point by the time at which parasite burden was measured, a lack of significant differences between parasite burden at this stage may not reflect the situation at the time of peak parasite burden (likely to be at weeks 3 -5 based on other studies exploring parasite burden and lesion development in *L. major* infection (50)), when differences between the groups are likely to have been greatest.

Raman *et al* have recently shown that the use of both a TLR4 and TLR9 ligand in a polyprotein vaccine for CL can enhance efficacy compared to the use of either ligand adjuvant alone (144), suggesting that synergy of TLR activation by vaccine adjuvants can promote enhance protection. When Pam2 and Pam3 were used in combination with CpG in an ALM vaccine, they did not act to bolster or reverse the vaccine induced protection provided by the CpG adjuvant in the vaccine, although the lowest disease severity was observed in those mice which were exposed only to antigen and CpG. Nevertheless, this suggests that the addition of TLR2 ligands to the Th1 promoting TLR9 ligand CpG does not reduce its ability to promote vaccine induced protection to a disease which requires a cell-mediated Th1 immune response. The only vaccine available in humans which provides cell-mediated immunity to an infection is BCG, which has been shown to act in part by activation of both TLR2 and TLR9 (220, 221). Combined, this suggests that the activation of both TLR9 and TLR2 by adjuvants in a vaccine favours an overall Th1 type of immune response.

Surprisingly, those mice which showed the lowest parasite burdens and disease severity did not have the greatest IFNγ responses, and instead presented with increased IL-10 and Th2 responses, especially in the

case of the ALM vaccinated mice. This may reflect the late stage of the infection, i.e. all of these mice have begun to control parasite replication and reduce lesion sizes from their maximum. Whilst Th1 responses are required for controlling parasite replication, they are also inflammatory and delay wound healing, whereas Th2/IL-10 responses can assist wound healing and reduce inflammation/pathology at the wound site (68). It would be useful to explore antigen specific responses at the time of challenge, instead of several weeks after exposure. The immune responses at the time of challenge may be a better measure to explore immune correlates of protection, and may correspond better with studies which have explored cytokine responses to Pam2 and Pam3, or related lipopeptides, in other models (such as (254, 280)). In addition, other studies exploring immune correlates of protection in *Leishmania* vaccines have used the time of challenge at which to explore the immune responses in mice given different vaccines combinations (141-143).

The immune response elicited by Pam2 and Pam3 in this study appears to be one where the Th2 immune response was favoured over the Th1 response. This is best demonstrated by the ratio of IgG1:IgG2c levels in the vaccinated mice, which are skewed in favour of IgG1 in Pam2 and Pam3 vaccinated mice. Pam3 has been shown to drive Th2 responses to antigen in C57BL/6 in a study where the OVA peptide was used as the antigen. This Th2 response to Pam3 was found to be mediated by increased phosphorylation of ERK1/2 and expression of c-Fos in DCs, acting to promote IL-10 production and suppress IL-12 (281). However, we didn't observe an elevated IL-10 response in the recall responses by splenocytes in mice that received the Pam3 containing vaccine.

Studies of lipopeptide-containing vaccines in mice have yielded conflicting results regarding the adaptive immune response elicited. When an antigenic peptide epitope of *M. tuberculosis* was attached to a Pam2 diacyl moiety and used as a vaccine in mice, DC activation was enhanced compared to administration of antigen alone, and both a protective Th1 response and a long-lasting memory response to *M. tuberculosis*, was elicited *in vivo*. Thus, the lipopeptide vaccine was able to provide efficacy and this was found to be better than the existing vaccine, BCG (254). This study suggests that lipopeptides can be developed to provide cell-mediated immunity to an intracellular pathogen, which requires a similar immune response for protection than *Leishmania*. In fitting with we these findings, Pam3 was found to enhance protection in a mouse study using a DNA/MVA prime-boost vaccine approach for protection to *L. braziliensis* (282). This enhanced protection was linked with increased protective Th1 (IFNγ and Granzyme B production) and memory T cell responses, as well as reduced IL-10 and IL-13 responses (282). BCG acts in part by activation of TLR2, and has been used as an adjuvant in immunotherapy for the treatment of cancer for years. Yamazaki *et al* therefore wished to explore the use of Pam2 and related diacyl lipopeptides for their ability to promote anti-tumour responses and reduce tumour size in a mouse model of melanoma. As with *M. tuberculosis* and *L. major* infections, effective immune destruction of melanoma tumour cells requires NK cell activation and a Th1 response (especially IFNγ). Upon s.c. administration of Pam2

lipopeptides to mice with melanomas on their back, tumour growth was not reduced, but was slightly increased compared to PBS injected controls (280). This response was found to be due to an elevated production of IL-10 which led to the expansion of tumour specific Tregs, which act to suppress protective tumour specific immune responses (280). Despite the similarity between the activity of Pam2 in driving exacerbated disease in both the model presented here and by Yamazaki *et al*, we did not observe an elevated IL-10 response (or decreased IFNγ) in the Pam2 vaccinated mice and the immunological readouts point instead towards a skewed Th2 response to infection. Nevertheless, this disparity in the adaptive immune responses may be in part due to the timing at which the immunological parameters were assessed, as Yamazakai *et al* explored the immune response elicited by Pam2 in a period of hours to 3 days, whilst here we are looking at the responses after 9 weeks of infection after challenge. Thus, an elevated IL-10 production may have occurred at the time of vaccination with Pam2 in our study, and the reduced expansion of specific phenotypes of T cells may well have followed this and impacted on the lack of resistance to *L. major* upon challenge.

The differences observed therefore may be related to the attachment of the TLR2 ligand to the vaccine antigen of interest. Studies have shown that linking peptides to acyl group moieties can enhance delivery of the antigen to immune cells, such as T cells, by providing a mechanism of endocytosis via TLR2 (251, 283). In our lipopeptide-containing vaccines, the complex antigen mixture was co-administered with the lipopeptides but not attached, and therefore the delivery of antigen to immune cells may not result in adequate innate immune stimulation, antigen processing and presentation and co-stimulatory molecule expression when compared to where the antigen/s and lipopeptide are physically attached. However, Kamath *et al* recently explored the influence of co-administration of antigen and adjuvant (CFA in this case) components in the shaping of Th1 and Th17 immune responses to vaccines, and determined that it is the timing of DC exposure to antigen and adjuvants which is crucial, as opposed to there being a physical interaction between the two components. Exposure of DCs to free antigen prior to their exposure to the adjuvant lead to suppression of Th1 T cell expansion in mice, whereas increased numbers of DCs that had been activated by adjuvant at the same time as exposure to the antigen led to Th1/Th17 expansion (284).

The antibody responses elicited in our experiment by CpG are similar to studies exploring the type of immune response elicited by CpG, which found a strong Th1 bias in immune responses to co-injected antigen and an absence/suppression of Th2 responses (285). However, whilst others have reported significant increases in antigen specific IgG2 antibodies to vaccine antigen when CpG was used as an adjuvant (212, 266), we saw no difference to those vaccinated with ALM antigen alone. This may be explained by the use of different strains of mice (BALB/c mice were used in (212, 266)). The effect of including Pam2 and Pam3 when used in combination with CpG was to increase the levels of antigen specific IgG1 in vaccinated mice, when compared to when CpG was used alone. This suggests that

lipopeptide adjuvants are able to shift some of the adaptive immune responses to vaccines elicited by the strong Th1-promoting CpG adjuvant, and the response that was most significantly altered in this study was the level of IgG1. Hutchison *et al* have also reported elevated IgG1 levels when CpG adjuvant was used in combination with the Th2-driving adjuvant Alum (212). A combination of CpG and Pam2/3 adjuvants may therefore be useful in a vaccine where a Th1 biased responses is required but elevated antibody responses are also favourable.

The ability of TLR ligands to enhance antibody responses has been linked to the activation of both DCs and B cells (173, 286). Other studies exploring the use of TLR ligand adjuvants in promoting antibody responses have reported an increase in antigen specific antibody titres when two TLR ligand adjuvants are used, in comparison to one. Kasturi *et al* reported that IgG1 and IgG2 were both significantly enhanced by the use of TLR4 and TLR7 ligand adjuvants combined in a vaccine for use against H5N1 influenza in mice, when compared to the use of single TLR ligands (286). These findings differ from our observations, where the highest levels of IgG1 were elicited when the TLR2/6 ligand Pam2 was used alone, and these levels were not altered by the addition of Pam3 and CpG (TLR2/1 and TLR9 ligands). This suggests that effect of activation of multiple TLRs does not have a simple synergistic effect in relation to antibody production (i.e. that a combination of endocytic and extracellular TLRs being activated leads to enhance antibody production).

Differences between the activation of antigen specific responses by the different TLR ligand adjuvants used in this study could be explained in part by the distribution of the TLRs on different subsets of cells. TLR9 which recognises the CpG adjuvant is expressed by pDCs and B cells specifically in humans, but is also expressed by myeloid lineage cells (including MΦs) in mice (287). TLR2/1 and TLR2/6 heterodimers are expressed by many more cell types including neutrophils, T and B cells, NK cells and immature DCs (218). The activation of innate immune responses in the different vaccines was therefore likely to target different subsets of cells, which may influence the type and magnitude of an immune response to the vaccine. By directly activating pDCs and B cells, K-class CpG adjuvants (such as the CpG ODN 1826 used in this experiment) results in an immune response which is characterised by the production of proinflammatory and Th1 cytokines (TNFα and IL-6) as well as the production of polyreactive antibodies (287). A study exploring the use of CpG in a vaccine containing the LACK antigen in protection against *L. major*, found that CpG enhanced efficacy and that the protective response elicited by the CpG containing vaccine could be transferred to naive recipient mice by CD11c+ DC cells from vaccinated mice (288), indicating that activated DCs in CpG vaccinated mice are sufficient to develop a protective response. The specific immune responses elicited *in vivo* by Pam2 and Pam3 have not been as comprehensively characterised as they have for CpG ODNs. However, it is known that lipopeptide adjuvants are also capable of activating DC populations *in vivo*, leading to enhanced adaptive immune responses to antigen (280, 289). Our experiments add to the published results so far by describing an

elevation in antigen specific antibody by lipopeptide adjuvants; specifically, the IgG1 isotype was elevated by Pam2, and an overall skewed isotype response towards increased IgG1 over IgG2a by both Pam2 and Pam3 was observed, when used in a whole cell vaccine model.

It is interesting that Alum, an adjuvant which promotes Th2 and antibody responses to vaccines (particularly in mice), has shown improved efficacy in *Leishmania* vaccine studies, including with ALM and other whole-cell preparations in BALB/c mice (275). Alum was even able to reduce lesion development greater than the use of BCG (275), which is the only licensed vaccine known to stimulate cell mediated immunity. This suggests that an archetypal Th2 driving adjuvant can promote protective responses to *L. major* in BALB/c mice, but in C57BL/6 mice, we observed that Pam2 resulted in an elevated Th2 response, which exacerbated disease. In conclusion, we found lipopeptides to be ineffective adjuvants for use in an ALM vaccine to protect against *L. major*, and the inefficacy was linked to an enhanced Th2 adaptive immune response during infection, particularly with respect to elevated IgG1.

Marked and persistent local swelling reactions to the adjuvants Pam2 and CpG were observed with the primary inoculum. Furthermore, we identified a differential swelling reaction to the lipopeptides adjuvants Pam2 and Pam3 in the absence of antigen, with the former inducing a more delayed onset and more sustained swelling reaction than the latter. The doses used here were comparable to other studies (254, 280). Wiedemann *et al* compared the inflammatory infiltrates after s.c. injection of different adjuvants, complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), LPS and Pam3 (290). After a dose of 50 μg Pam3 the inflammatory response in the tissue site of exposed mice was characterised by the accumulation of inflammatory cells including MΦs, neutrophils and epithelial cells, which differed to that induced by CFA and IFA where oil accumulation was associated collagen formation and necrosis. LPS also induced a local inflammatory response over a sustained period. Despite the obvious induction of an inflammatory response by the TLR ligands used in this study, the authors describe that in comparison to the potent CFA and IFA adjuvants, which are associated with tissue damage and side effects, Pam3 and LPS induced responses which healed over time and were associated with improved efficacy in terms of adaptive immune response priming (290).

Here we identified a different pattern of swelling reaction in exposed mouse tissues after s.c. injection with Pam2 and Pam3. Interestingly, a recent phase 2 clinical trial exploring the immunogencitity and safety of a (diacylated) lipopeptide vaccine developed for HIV, reported dose dependent local reaction in the human participants, following i.m. injection (291), which is fitting with our observations. Our comparison of the reactions two Pam2 and Pam3 suggests that the local reaction to the lipopeptide differs depending of the acylation pattern. A comprehensive study by Farhat *et al*, showed no differences in signalling events between activation of TLR2 in combination with either TLR1 or TLR6 (246), and others have reported no distinct immune responses to Pam2 and Pam3 (292). Turner *et al* reported that diacylated lipopeptides were more potent stimulators of TNFα production by peritoneal MΦs of

C57BL/6 mice, and that the dynamics of TNFα production different between the two types of lipopeptide: with diacylated lipopeptides demonstrating a complete dependency on TLR6, whereas triacylated lipopeptides were only partially dependent on TLR1 (293). Therefore, differences in the binding kinetics and a complete or partial dependency of the ligand-TLR complexes, may account for some of our findings. DePaolo *et al* observed a regulatory immune response after stimulation of the TLR2/6 complex, whilst an inflammatory immune responses after stimulation of the TLR2/1 complex in bacterial infection, and they speculate that pathogens such as the bacterium *Yersinia pestis* selectively activate the regulatory TLR2/6 complex (294). The crystal structures of The TLR2/6-Pam2 and TLR2/1-Pam3 complexes have been determined and these findings indicate that the TLR2/6 structure may have a stronger interaction than the TLR2/1 structure upon ligand binding (295), which may account in part for the prolonged local reaction observed in the mice treated with Pam2 compared to Pam3. It is also possible that the observed differences in local reactions is due to a difference in hydrophobicity between the two lipopeptides.

Given that the ability of adjuvants to drive enhanced protective responses has been consistently linked with an ability to drive innate immune responses (296), it was initially hypothesised that the localised swelling response may correlate with efficacy of the vaccines and could act as an early marker or correlate of vaccine efficacy. If this was the case, the most effective antigen-containing vaccine would have been ALM + Adjuvants, followed by ALM + CpG and ALM + Pam2, then ALM + Pam3 and ALM. However, we did not find this to be the so, showing that the ability to drive a localised response in the form of swelling is not a useful indicator of the ability of an adjuvant to improve efficacy. This is not surprising as swelling responses can manifest in many different forms (i.e. inflammation, innate immune cell recruitment or oedema for example), and distinct innate inflammatory responses promote different adaptive responses (210, 296, 297).

In summary, this study identified that lipopeptides are ineffective adjuvants for use in a whole-cell containing vaccine for protection against *L. major*, and can even exacerbate disease in challenge infections through promoting Th2 or regulatory responses that can compromise Th1 dependent protective immunity (280). However, the ability of Pam2 in particular to drive elevated antigen specific immune responses (especially IgG1 levels), indicates that this adjuvant may be useful in other vaccines where a Th2 and antibody response is desired.

Chapter 7. Lipopeptides as adjuvants in a Th2 disease model

Abstract

A protective immune response to helminth infections comprises mainly of a robust Th2 response. As we previously found that the Pam2 adjuvant promoted a strong Th2-type response, which was found to be inappropriate for protection against the intracellular protozoa *L. major*, we tested the use of Pam2 as an adjuvant for a helminth vaccine where Th2 responses would be favourable. Thus, a murine *Brugia malayi* infection model was utilized to test the relative efficacy of vaccination with soluble *B. malayi* extracts in the presence or absence of Pam2. The gold standard Th2-driving adjuvant Alum was able to improve the efficacy of a vaccine containing Mf antigens (BmMfE), so that it provided enhanced protection against *B. malayi* challenge infection, when compared to sham vaccinated mice or mice vaccinated with BmMfE alone. The use of Pam2 as an adjuvant also reduced parasite burdens, achieving similar levels of efficacy to vaccination with the Alum adjuvant. Pam2 and Alum were both found to enhance antigen specific IgG1 antibody production in vaccinated mice, although unlike Alum, Pam2 also induced IgG2a responses and did not alter the ratio of IgG1:IgG2a produced in response to parasite antigens. This suggests that Pam2 drives different adaptive immune responses to Alum with a broader range of immuno-potentiating activity. In conclusion, this data indicates that adjuvants targeting TLR2 pathways could be considered as novel therapeutic options for use in vaccines that require Th2 and enhanced antibody-dependent immunity.

Introduction

The previous chapter presented data which identified lipopeptides, and in particular the diacylated lipopeptide Pam2, as adjuvants that are able to induce significant antigen specific immune responses to parasite antigens when used in vaccines. However, the use of Pam2 resulted in exacerbated disease upon challenge with *L. major*. The use of Pam2 as an adjuvant in a whole-cell vaccine, skewed the immune response towards a Th2-type, characterised by high IgG1:IgG2 antibody isotype ratio and enhanced IL-4 and IL-13 production in response to antigen. Such an immune response to an intracellular parasite such as *L. major* is detrimental to control of infection, and resulted in exacerbated disease in this model. However, a strong Th2 response is required for the control of other pathogens, including parasitic nematodes. We therefore tested the use of Pam2 as an adjuvant for vaccines that require a strong Th2 response, by utilising a mouse vaccination model of *Brugia malayi*, which causes lymphatic filariasis (LF) in humans.

Lymphatic filariasis

LF is an NTD caused by three species of filarial nematode: *Wuchereria bancrofti*, *B. malayi*, and *B. timori*. It affects an estimated 120 million people worldwide (298), and results in severe disability in many of those afflicted.

The parasites which cause LF are transmitted to human hosts by infected female mosquitoes when they take a blood-meal, during which time infective L3 larvae exit the mosquito and enter the dermis of the host at the wound site. The life cycle of *B. malayi* is given in Figure 42, which is similar for *W. bancrofti* and *B. malayi* with the exception that the species of mosquito vectors differs between parasites (299). Within the human host, adult parasites reside within the lymphatics and are the life stage responsible for causing pathology. However, not all infected individuals present with disease. Pathology ranges from mild lymphoedema in affected tissues (usually the leg or scrotum), to severe and prolonged lymphoedema associated with skin hardening and infections of the skin and lymph tissues (elephantiasis).

Immunity to LF and the use of mouse models

In humans, a range of disease manifestations are observed, and as with leishmaniasis, the host immune response to infection has a major impact on disease presentation and progression. Generally, individuals living in an endemic area will fall in to one of three main categories: (1) those with clinical pathology (with or without signs of active infection in the form of circulating filarial antigen and/or Mf); (2) those with infection but no pathology (i.e. asymptomatic infection); and (3) those with no signs of infection and no pathology (known as endemic normals, ENs) (298, 300). ENs are often studied to gain information on the protective immune responses that provide resistance to infection. Such studies have highlighted that a robust mixed Th1/Th2 response in humans, as well as low IgG4/IgE ratios, are associated with an apparent resistance to infection and the associated pathology (300).

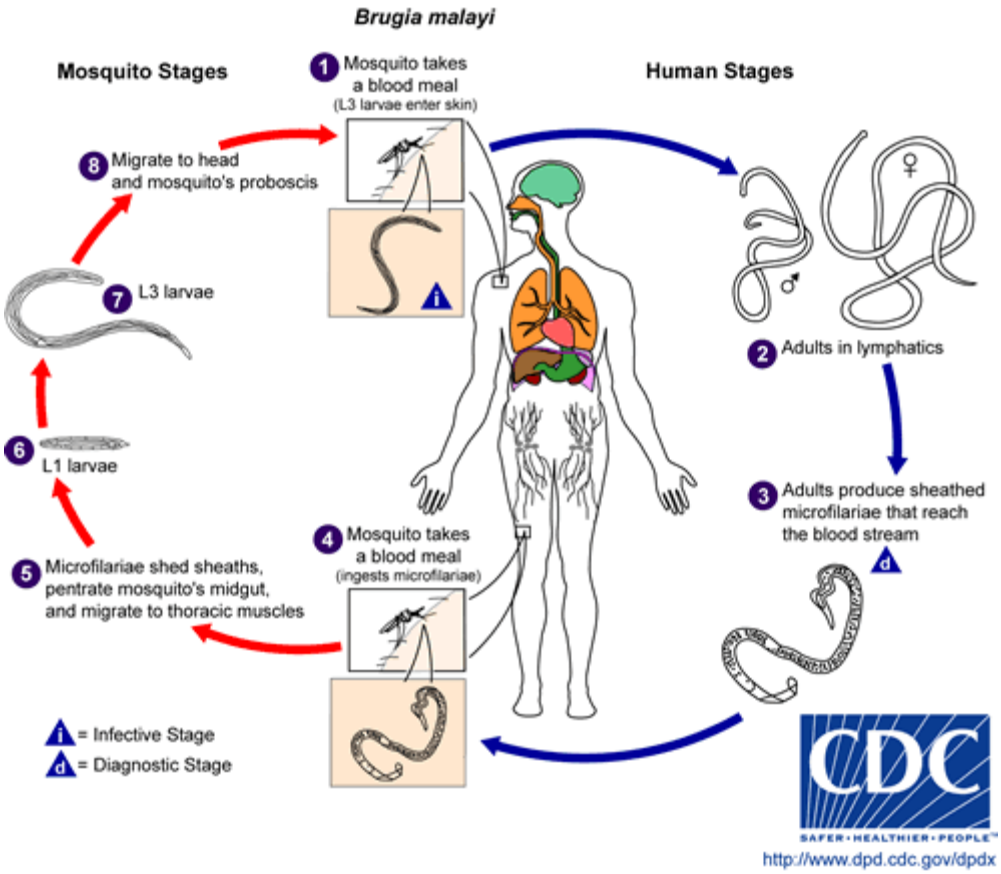

Figure 42. The life cycle of *Brugia malayi*. An infected mosquito transmits infective L3 larvae to the skin of a human and parasites enter at the wound site (1). Once within the human host, the larvae moult twice (i.e. from L3 to L4 and finally to the adult stage), over a period of approximately 1 year (2). Adult male and females live within the lymphatics and reproduce, producing millions of microfilariae (Mf) which then circulate in the bloodstream (3). Mf can be taken up by uninfected mosquitoes when they take a blood meal (4). Within the mosquito, Mf migrate through various tissues whilst moulting twice, and eventually become infective L3 after approximately 14 days (5-8). Figure taken from (299).

The cytokines IL-5 and IFNγ appear to be important for resistance in ENs, and are reduced in PBMCs responses from other groups (301). However, those with clinical pathology are also capable of mounting strong Th1 and Th2 responses to filarial antigens and TLR agonists (302).

Rodent models of filarial infections have provided further insight into the type of immune responses required for resistance to infection. Models have demonstrated the importance of an adaptive immune response in the clearance of parasites (303) and both B and T cells have been shown to play crucial roles (304). As in many other helminth infections, an important role for Th2 responses in resistance to filarial infection has been identified. IL-4 and IL-13 appear to have a role in clearance of larval stages in either *Litomosoides sigmondontis* or *Brugia spp.* infection models in some cases (300, 305), but these cytokines are not always crucial for protection. For instance, IL-4$^{-/-}$ mice did not differ in their ability to clear developing *B. malayi* worms in the peritoneum when compared to WT mice, but Mf production by adults was increased suggesting that IL-4 acts to reduce fecundity during infection (306). The cytokine IL-5 is also important to resistance, and its function is to allow for recruitment of eosinophils to the infection

site (307). However, a role for Th1 responses is also indicated, as is observed in human infections, as C57BL/6 mice which are able to clear parasites quicker than other inbred strains, mount a stronger Th1 response to infection than BALB/c mice, and IFNγ$^{-/-}$ mice are more permissive to infection compared to WT on a C57Bl/6 background (305).

The effector cells important for parasite killing in LF include eosinophils (308-311). Mice which are deficient in eosinophil peroxidase (EPO) or the major basic protein 1 developed significantly greater parasite burdens upon infection (310). Whilst eosinophils have been consistently linked with an immune response associated with clearance in most studies, a model of adult *B. malayi* infection in mice has also disputed this. Mice receiving implanted adults have been reported to survive in the peritoneal cavities of mice despite a prolonged eosinophilia at the infection site, and an absence of IL-5 (although greatly reducing eosinophilia) in C57BL/6 mice did not impact on parasite survival (312). It therefore appears that the mechanisms involved in clearance are more complex, and can vary between different parasite life-cycle stages. The role of MΦs in parasite clearance is unclear. Induction of MΦs with an alternatively activated phenotype (aaMΦs) are coincidently elicited as part of the predominant Th2 response to filarial experimental infections (313, 314), and monocytes with aaMΦ-like characteristics are observable in naturally infected human populations (315). These nematode-elicited aaMΦs have been studied extensively for their ability to suppress proliferation of lymphocytes *in vitro* and *in vivo*, which is presumed to promote parasite survival (313, 314). Recent evidence suggests, however, that in contrast to their role in promoting adult worm survival through driving immune tolerance, aaMΦs are essential for coordinating eosinophil recruitment and mediating protection against *B. malayi* infection (Turner *et al.* pers. com.).

Vaccine studies in mouse models of LF

A number of different rodent models have been used to explore the use of vaccination to protect against filarial infections. Radiation-attenuated larval vaccines have consistently provided the most potent protection in these models (300, 316). As with resistance to primary infections, both T and B cell responses appear to be crucial for enhanced clearance in vaccinated mice (225, 317). Importantly, recent studies have revealed that the key role for B cells in providing vaccine induced immunity is not linked to antibody production, but instead to production of cytokines which promote a robust Th2 response, and recruitment of cells to the infection site (317). In the *L. sigmodontis* model, vaccine induced reduction of parasite burden has been linked to IL-5 and eosinophilia, as neutralisation of IL-5 in vaccinated mice reduced eosinophilia to the infection site and prevented vaccine mediated reduction in parasite burden (308).

Aim of the study

As a Th2 response is required for vaccine efficacy for filarial parasites and as our previous work identified Pam2 as a potent driver of Th2 immune responses, we tested the use of Pam2 as an adjuvant for vaccination against *B. malayi*. We compared the use of Pam2 with Alum, which is the archetypal adjuvant for use in promoting Th2 responses to vaccine antigens (296).

Methods

Mice, vaccinations and challenge infection

Methods used in this study were developed with advice and assistance from Dr Joseph Turner and Miss Ana Guimaraes (Filariasis Laboratory, LSTM). All procedures involving live animals were performed at the BSU in the Ronald Ross Building, University of Liverpool. Male BALB/c mice were purchased from Harlan, and were aged 8-10 weeks at the start of each experiment. Mice were vaccinated with one of the following vaccines, in a 100 μl volume, via s.c. route to the nape of the neck: PBS; Dead L3 (gold-standard, two doses 2 weeks apart) or BmMfE; BmMfE + Alum; BmMfE + Pam2 as a single dose. Mice (4-5/group) were challenged 2 weeks after the last vaccine dose with 50 live *B. malayi* L3 (Chapter 2). After 6 days p.i., mice were humanely culled, and the following were collected and processed accordingly as described in Chapter 2: blood via cardiac puncture (for plasma); parasites from the peritoneal cavity, peritoneal exudate cells (PEC) cells and splenocytes. The parasite burden was determined by counting the number of free, motile parasites collected from the peritoneum of each mouse using a dissecting microscope.

Immune responses in *B. malayi* infected mice

To phenotype the inflammatory cells recruited to the site of infection, PEC cells were collected from infected mice at necropsy 6 days post challenge infection. After washing, cells were stained for the following surface markers as described in Chapter 2: F4/80, Siglec-F and Mannose Receptor. Fluorescently labelled PECS were analysed by flow cytometry as described in Chapter 2.

The splenocytes were washed and resuspended into either complete RPMI extra at a concentration of 8 x 10^6 cells/ml and were plated in a total volume of 200 μl in the presence of BmL3E (20 μg/ml), BmMfE (20 μg/ml), anti-mouse CD3 antibody (10 μg/ml), or media alone for 72 hours. Culture supernatants were then removed and stored at -20°C until analysis for IFNγ, IL-10, IL-4 and IL-13 levels using cytokine ELISA.

The levels of antigen specific IgG1 and IgG2c in plasma samples from mice were measured using antibody ELISA with BmMfE used as the capture antigen.

Results

Vaccine efficacy

The percentage of live parasites recovered was recorded for each individual and the average (median) percentage recoveries for each vaccine group are displayed in Figure 42B. The group vaccinated with two doses of Dead L3 displayed a 47% reduction in median parasite recovery when compared to challenge control mice, but this was not found to be significant, perhaps due to the wide variation in parasite recoveries from vaccinated animals. Mice vaccinated with the filarial extract BmMfE alone showed no significant reduction in the average parasite recovery. When mice were vaccinated with BmMfE in the presence of Alum, a 35% reduction in median parasite recovery compared to challenge controls was observed and this was found to be significant, when compared to BmMfE alone ($p = 0.018$). When the Pam2 adjuvant was used, a 47% reduction in parasite recovery was observed compared to challenge controls, but this was not significantly different, again perhaps due to a wide variation in parasite recoveries. The average parasite recovery in mice vaccinated with BmMfE + Pam2 was reduced to similar levels as the gold standard Dead L3 vaccine (20% of challenge dose) and better than mice vaccinated with BmMfE + Alum (16%), but this reduction was not significant compared to any other group. Additional experiments with larger group sizes to account for the high variability in parasite recoveries observed in vaccinated mice and with evaluation of challenge infections at a later time point are warranted to confirm these observations.

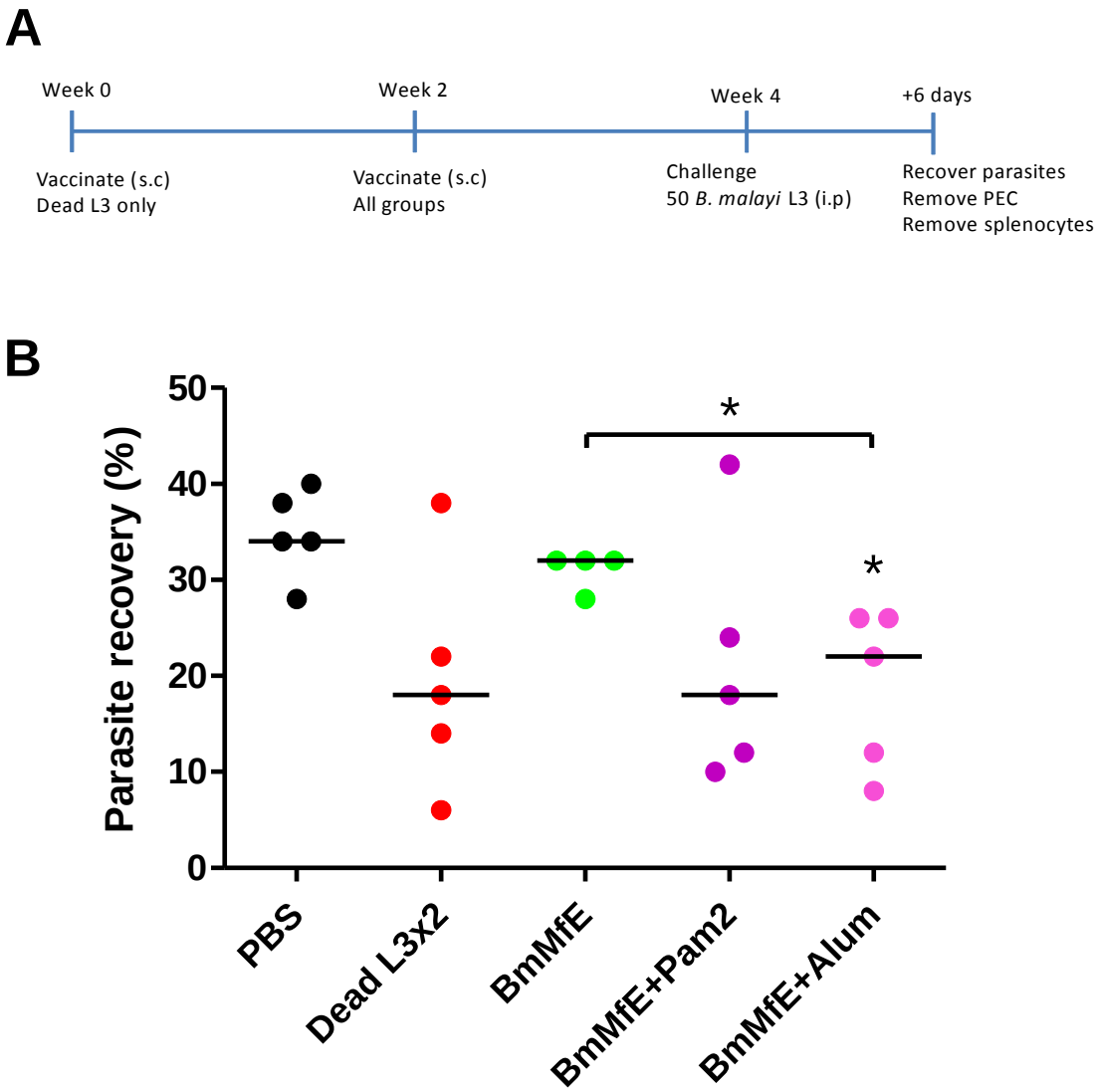

Figure 43. Efficacy of vaccines containing filarial extracts +/- adjuvants. Groups of mice (5 groups, n = 5) were given s.c. vaccinations with the indicated vaccines 2 weeks before infection with 50 *B. malayi* L3 i.p. (with the exception of the group vaccinated with Dead L3 which received a prior vaccination 2 weeks before the final dose), parasites were recovered along with other cells at 6 days p.i. (A). Live parasites were recovered and the individual recoveries from mice in each group are given in (B) as a percentage recovery of the initial parasite inoculum. Horizontal bars represent the median values. Significant differences between the average recovery from groups of vaccinated mice and the control PBS group are indicated above the data points for each group, significant differences between recoveries in vaccinated groups are indicated by connector bars (Mann Whitney U test, * $p<0.05$).

Immune responses

To assess the immune responses in the different vaccinated groups of mice, the PEC infiltrate was characterised in the site of infection by determining total cell counts and phenotyping the cells using flow

cytometry. Splenocytes and blood were also collected to analyse the cytokine profile and antibody responses to parasite antigen respectively.

Peritoneal cell recruitment

On average 2.68 x 10^6 (+/- 1.72 x 10^5) PECs were recovered from naïve mice, which increased to a mean average of 8.62 x 10^6 (+/- 6.03 x 10^5) upon infection with *B. malayi* for 6 days (sham vaccinated mice). When comparing vaccinated groups of mice to sham vaccinated (PBS) mice, an elevated number of PECs were recovered from vaccinated mice, which was significant for the Dead L3, BmMfE and BmMfE + Alum vaccinated mice (Figure 44A). The BmMfE + Pam2 vaccinated group presented with fewer PECs than the other vaccinated mice, which was significantly less than the Dead L3 vaccinated group. Thus, Pam2 did not act to increase the cellular infiltrate at the site of infection when used in combination with a BmMFE antigen vaccine.

The PECs were stained with fluorescently conjugated antibodies and analysed by flow cytometry to determine the proportion of cell types recruited to the infection site (peritoneal cavity) in the different groups of mice. As two major effector cells associated with *B. malayi* infection in mouse models are eosinophils and MΦs (225), we focused on these populations of cells. The marker used for eosinophils was Siglec F and the marker for MΦs was F4/80, and cells were gated using these markers (Figure 44B). Gated MΦs were further assessed for expression of Mannose Receptor (MR, CD206), which is a marker of alternative activation (78). The percentages of eosinophils and MΦs in individual PEC samples, and the proportion of MR positive (MR+) and highly MR expressing (MR^{high}) MΦs within each sample, were used to determine the average percentage of each cell type in the cellular infiltrates for each group (Figure 44C). The cells termed "other" within PEC populations are $F4/80^-$ and $Siglec\text{-}F^-$ and consist mainly of small and large lymphocytes as judged by forward and side scatter characteristics (225).

The proportion of eosinophils in the PEC samples increased in vaccinated mice, particularly in those mice vaccinated twice with Dead L3 (Figure 44C). The proportions of MR+ and MR^{high} MΦs were also elevated in vaccinated groups of mice, and seemed to be related to increased eosinophil numbers/proportion of PECs, and were again elevated in particular in mice vaccinated with the Dead L3 vaccine. These findings suggest that all vaccines, especially the Dead L3 vaccine, were able to result in a selected elevated recruitment of effector cells to the site of infection, which are known facets of a protective anti-*B. malayi* response, but did not relate directly with the level of efficacy observed. These findings indicate enhanced Th2-associated effector cell responses in all vaccinated mice.

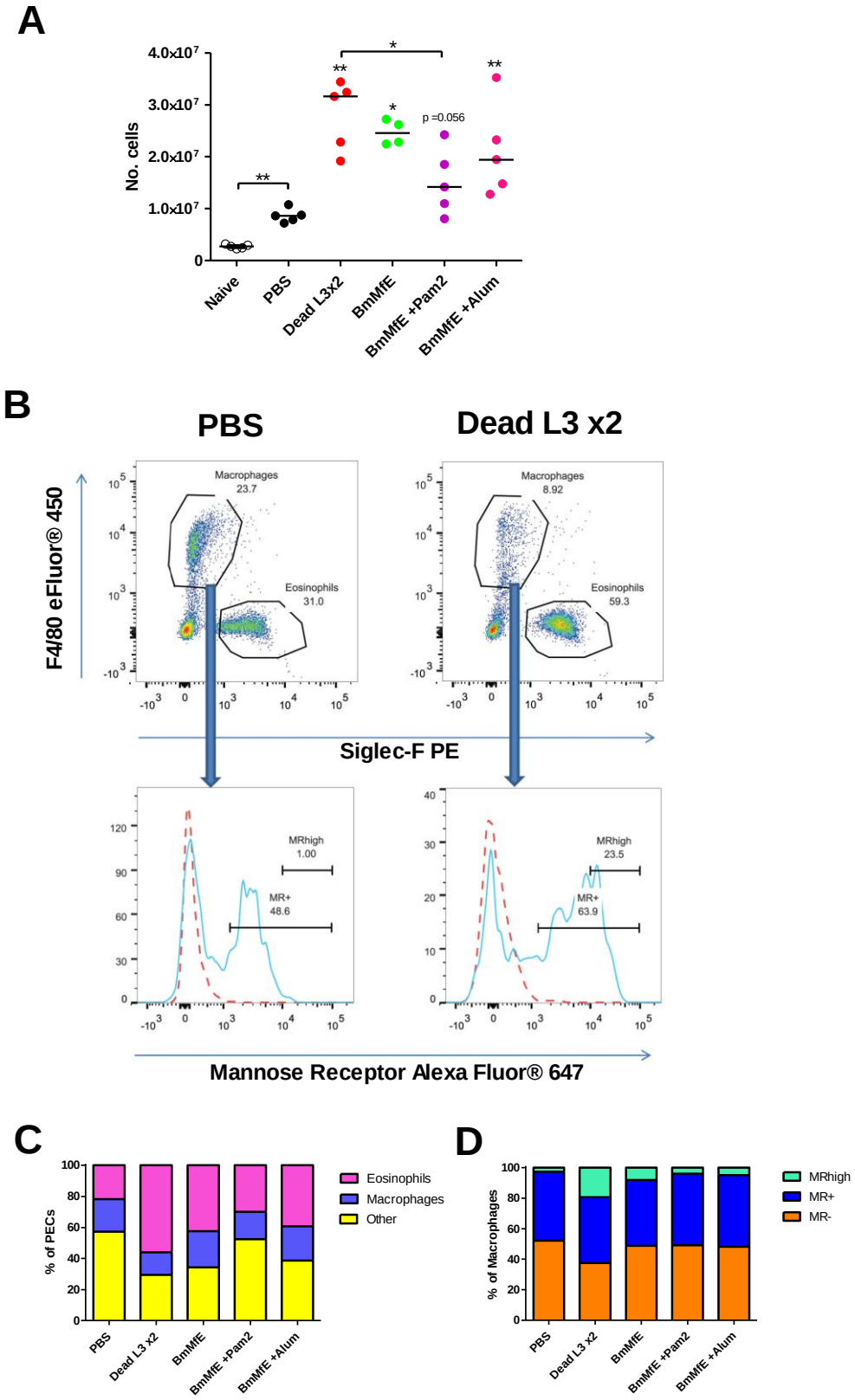

Figure 44. **Characterisation of the cellular infiltrate in the peritoneal cavity of vaccinated mice infected with *B. malayi* for 6 days. The total number of PECs was counted (A), results show cell counts for individual mice in each group, and horizontal bars represent median values. Significant differences**

between naïve controls and PBS vaccinated mice are indicated by arrow bars; where levels of cytokines produced by mice vaccinated with antigen-containing vaccines differed to PBS vaccinated mice, this is indicated above the data points for that group, whilst other difference between vaccine groups are indicated by connector bars (Mann-Whitney U test, P<0.05 *; p<0.005 **). The proportion of cellular phenotypes analysed by flow cytometry. The gating strategy of MΦs (F4/80 positive) and Eosinophils (Siglec-F positive) is shown, as well as the gating for Mannose Receptor positive and high populations of MΦs (red dashed lines indicate sample incubated with isotype control) (B). The resulting percentages were used to determine the mean average percentages of each cell type within the PEC and MΦ populations for each group of (n=5) mice (D).

The percentages of gated cells was used to determine the total numbers of each cell type in the PEC samples recovered, thereby allowing for a quantitative measure of recruitment of these cells in each individual (Figure 45). The greatest numbers of eosinophils were recruited to the peritoneal cavity of Dead L3 vaccinated mice, although BmMfE and BmMfE + Alum vaccinated mice also recruited significantly greater numbers of eosinophils compared to sham vaccinated mice (Figure 45A). The BmMfE + Pam2 vaccinated mice did not show elevated numbers of eosinophils, indicating that this vaccine was not effective at bolstering recruitment of eosinophils to the infection site. Overall, numbers of eosinophils recruited to the site of challenge infections did not relate with levels of efficacy. The BmMfE and BmMfE + Alum vaccinated mice recruited elevated numbers of MΦs, but this was reduced significantly by the addition of the Pam2 adjuvant (Figure 45B). The expression levels of MR, a marker of alternative activation, were measured on MΦ populations. MR positive and MRhigh MΦs were generally elevated in vaccinated mice, with the exception of mice vaccinated with BmMfE + Pam2. Overall numbers of MΦs or MR+ or MRhigh phenotypes did not correlate precisely with vaccine efficacy.

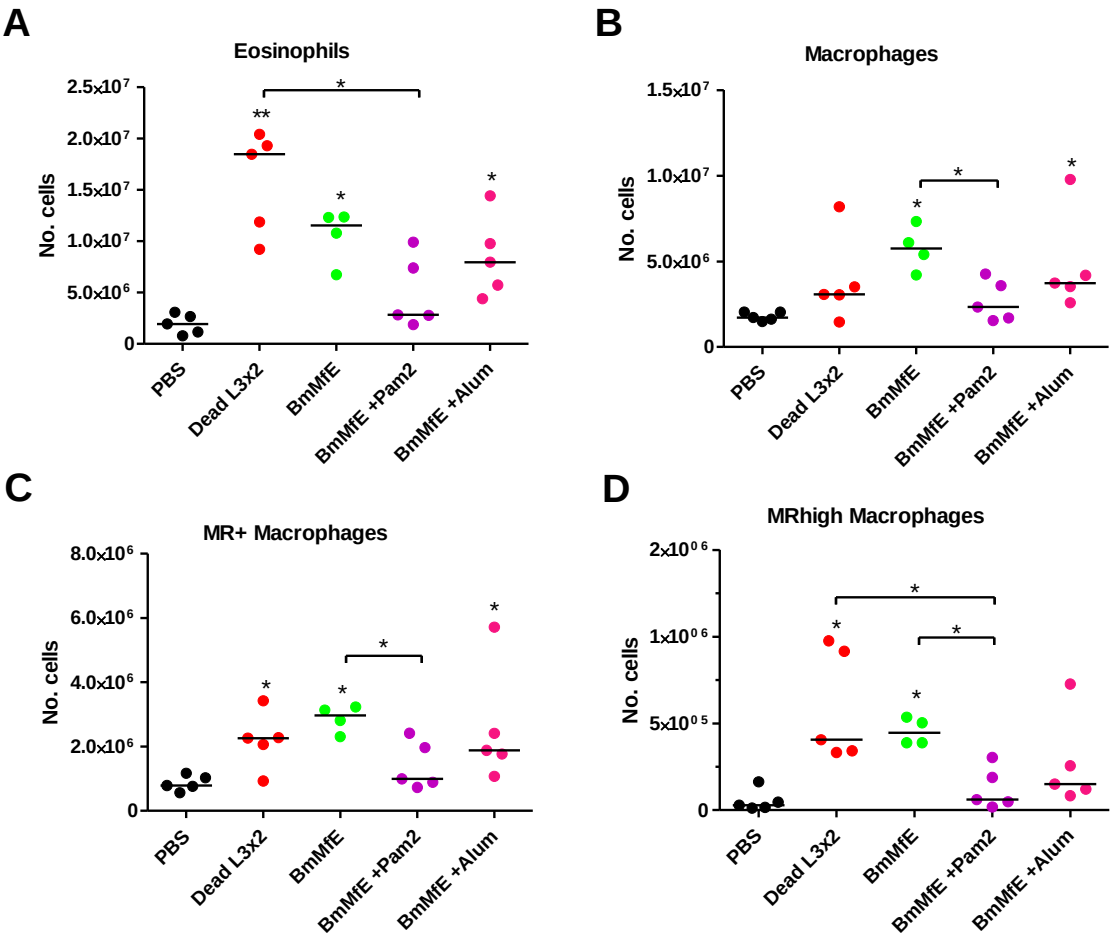

Figure 45. Recruitment of effector cells to the infection site. The total number of eosinophils (A) macrophages (B), MR+ macrophages (C), and MRhigh macrophages (D) recruited to the peritoneal cavity of vaccinated mice 6 days after challenge infections with *B. malayi* L3. Individual counts are shown for mice in each group, horizontal bars represent the median values; significant increases in mean number of cells produced by vaccinated mice compared to PBS sham-vaccinated mice is indicated above the data points for that group, whilst other differences between vaccine groups are indicated by connector bars (Mann-Whitney U test, P<0.05 *; p<0.005 **).

Splenocyte cytokine production

In order to assess the adaptive immune responses to the infective *B. malayi* L3 parasite, splenocytes were cultured *in vitro* for 72 hours in the presence of soluble antigen from *B. malayi* L3, BmL3E. The levels of cytokines produced by unstimulated cells were subtracted from the levels produced when cells were stimulated with BmL3E, in order to quantify the levels of antigen specific cytokine production to L3 antigens (Figure 46). The results indicate a general elevation of Th2 response to L3 antigen upon infection with *B. malayi* in sham vaccinated mice, and this is boosted by vaccination with *B. malayi* antigen-containing vaccines. In the case of IL-4, IL-13 and IL-5, all groups challenged with L3 infection showed elevated responses to BmL3E when compared to naïve mice (N.B. these comparisons are not indicated on the figures). In the case of IFNγ, however, none of the groups produced IFNγ in response to BmL3E compared to naïve mice. Interestingly, in some cases the levels of IFNγ produced to BmL3E was lower

than that produced by unstimulated splenocytes, thereby resulting in negative values, and indicating the background levels of IFNγ produced by splenocytes from these mice were reduced upon exposure to BmL3E, i.e. the Th1 response was down-regulated in these individuals. This could indicate the presence of a regulatory population of cells, but may also be a result of regulation of Th1 response by the dominant Th2 response.

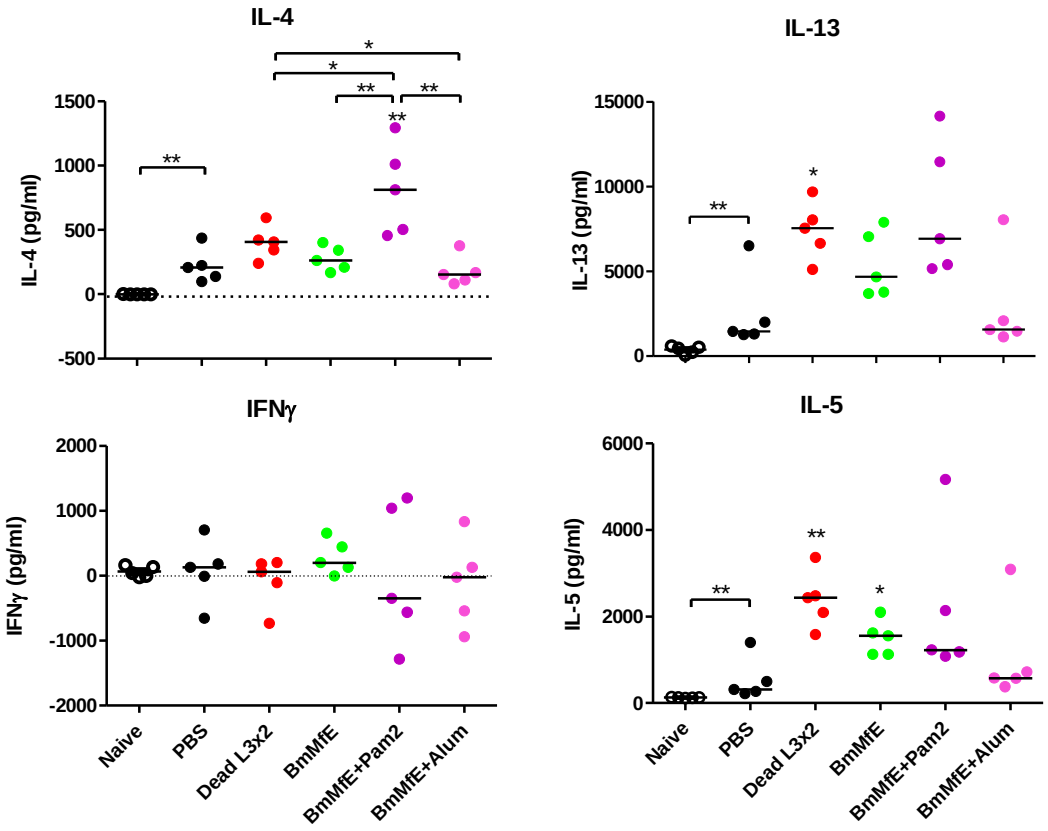

Figure 46. L3 specific immune responses from splenocytes of vaccinated mice and controls. Levels of cytokine (IL-4, IL-13, IFNγ and IL-5) produced by unstimulated cells were subtracted from those produced in the presence of BmL3E. Control groups of naïve mice and those vaccinated with PBS but challenged with L3 are included (white and black points respectively). Individual points represent the mean average levels of cytokine calculated from triplicate splenocyte cultures per mouse, and horizontal bars represent median averages for each group. Where levels of cytokines produced by mice vaccinated with antigen-containing vaccines differed to PBS vaccinated mice, this is indicated above the data points for that group whilst other differences between vaccinated groups, or between naïve mice and PBS vaccinated mice are indicated by connector bars (Mann-Whitney U test, P<0.05 *; p<0.005 **). Note that differences between vaccinated groups and naïve controls are not indicated, to allow for greater clarity in the figure.

The Dead L3 vaccine significantly increased the IL-13 and IL-5 responses to L3 antigen when compared to sham vaccinated controls, and the IL-4 response was also elevated. The BmMfE vaccines did not perform as well overall as the Dead L3 vaccine in terms of boosting Th2 cytokine responses to L3

antigen, with the notable exception of the BmMfE + Pam2 vaccine where a significantly greater production of IL-4 was observed in this group compared to all other vaccinated groups (Figure 46).

Antigen specific IgG isotype responses

There were no detectable levels of antigen specific IgG antibody isotypes specific for BmMfE from naïve or sham vaccinated mice, whilst those vaccinated with the Dead L3 vaccine did have delectable levels of both isotypes specific for BmMfE (data not shown). The highest levels of BmMfE specific antibodies recorded were from mice vaccinated with BmMfE, and in the case of both isotype subclasses, the greatest responses were recorded in individuals vaccinated with BmMfE + Pam2 (mean absorbance of 0.83 +/- 0.26, compared to a mean of 0.071 +/- 0.019 in the BmMfE vaccinated group and 0.413 +/- 0.129 for the group vaccinated with BmMfE + Alum), indicating that Pam2 is a strong driver of IgG responses generally.

Both Pam2 and Alum adjuvants significantly elevated the levels of IgG1 antibody specific for the antigen compared to the groups vaccinated with BmMfE antigen alone ($p=0.008$ in both cases). Thus Pam2 is able to induce a comparable antigen specific IgG1 response when compared to Alum. The levels of antigen specific IgG2a antibody were elevated in the BmMfE + Pam2 group, but not the BmMfE + Alum group, indicating that Pam2 is able to drive an elevated IgG2a response to antigen, whereas Alum does not. When the ratio of IgG1:IgG2a levels were determined for each group, it appears that Alum drives a specific elevated IgG1 (i.e. Th2) responses as displayed by an elevated IgG1:IgG2a ratio of antigen specific isotypes (although not significant), whereas the average IgG1:IgG2a levels induced by Pam2 were no different to the mice vaccinated with antigen alone. Thus, Pam2 is a strong driver of both IgG1 and IgG2a responses, and does not skew the antigen specific humoral response towards a Th2 subtype when compared to the Alum antigen, where only IgG1 levels are elevated.

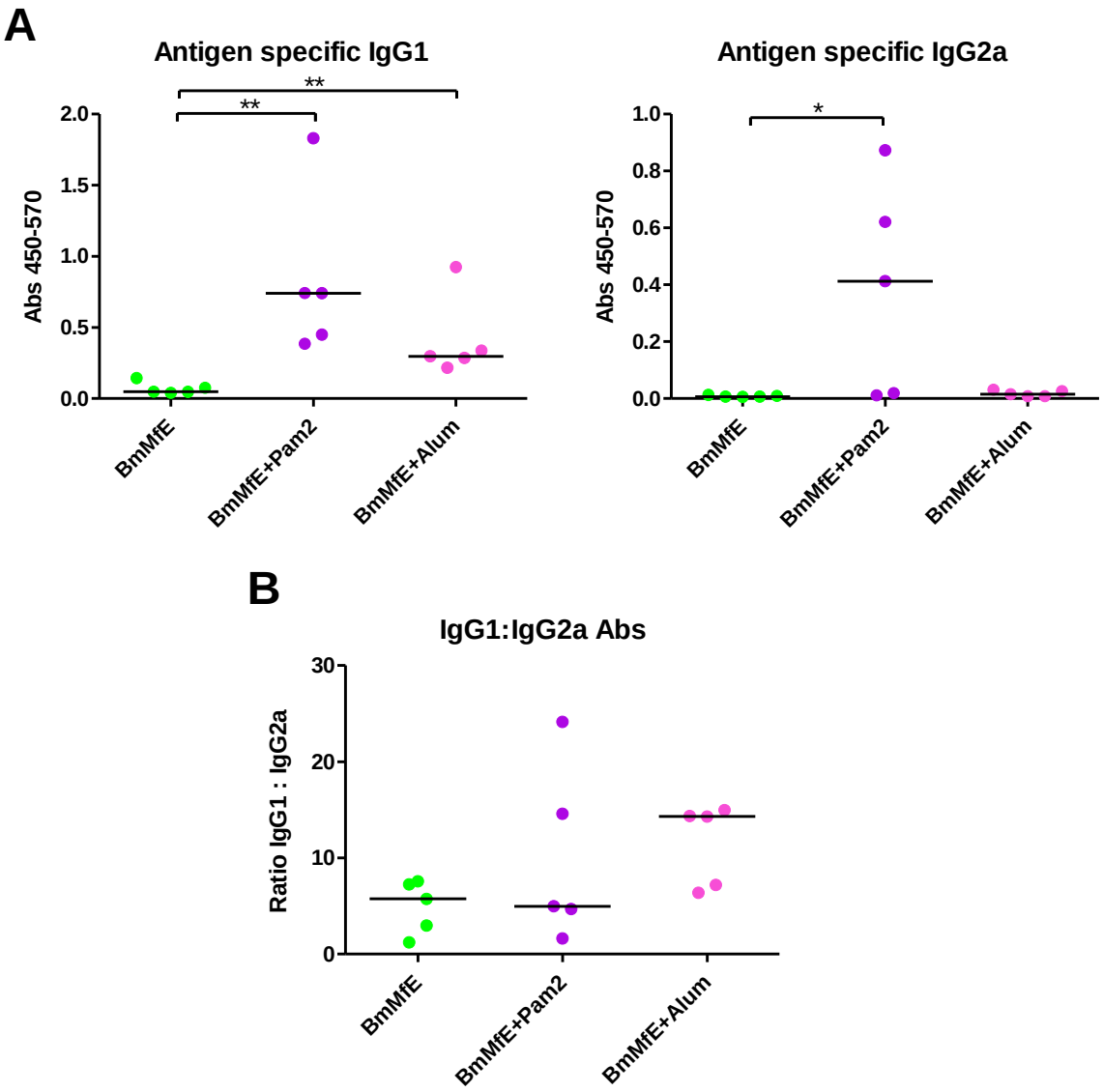

Figure 47. Levels of Mf antigen specific IgG antibody isotypes in mice vaccinated with BmMfE in the presence or absence of adjuvants. The levels of IgG1 and IgG2a isotypes specific to BmMfE were determined for individuals in each group and from naïve mice by ELISA: Absorbance values (Abs) at 450-570 nm are shown. Just those mice vaccinated with BmMfE vaccines (i.e. with no adjuvant, with Pam or with Alum) were compared for the levels of antigen specific IgG1 and IgG2a in their plasma (A). Individual points represent the mean average levels of antibody in duplicate wells per mouse, and horizontal bars represent median averages for each group. In addition, the ratio of IgG1:IgG2a was calculated using the Abs values (B). Groups were compared using the Mann-Whitney U test (*p<0.05; **p<0.005).

Discussion

This study aimed to test the use of Pam2 as an adjuvant in a vaccine where Th2 responses are required for vaccine efficacy. We observed a significant reduction in parasite burden when BmMfE + Alum was

used as a vaccine. However, even though vaccines using Pam2 as an adjuvant showed greater a reduction in parasite recoveries compared to the Alum adjuvant, and to the same level as the Dead L3 'positive control' vaccine, a high variability in parasite recoveries from these vaccination groups failed to produce statistically significant differences. High variability in parasite recovery has been reported from *B. malayi* models in mouse peritoneal cavities, and the variability reported by others was often greater than what we have found (e.g. from 0 to 60% recovery within the same group of mice (225)), suggesting that it was not a variability unique to our experimental set up. Nevertheless, the levels of vaccine efficacy were encouraging as they indicated that the use of Pam2 as an adjuvant in this model was able to improve upon an antigen only vaccination (BmMfE) and produce comparable reductions in parasite burdens to Alum, which should be confirmed with further experiments including larger group sizes.

Although correlates of protection in vaccine models for LF have yet to be comprehensively defined, the role of effectors which are important for clearance of primary infection appear to differ to some extent compared to those for clearance in vaccinated mice or upon secondary infection (307, 318). Nevertheless, recruitment of eosinophils and other effector cells (e.g macrophages) appears to be a common factor linked to resistance (225, 308, 316). By counting and phenotyping the cellular infiltrate at the site of the challenge infection we were able to assess the effect of vaccination on the local immune response to *B. malayi* challenge infection. Challenge infection resulted in a recruitment of large numbers of cells to the peritoneal cavity as previously reported (225). Vaccination acted to increase the recruitment of cells to the peritoneal cavity in all cases, with the notable exception of mice vaccinated with BmMfE + Pam2, which showed reduced cellular recruitment compared to all other vaccine groups. The finding that the Dead L3 vaccine consistently recruited the greatest numbers of Th2-associated effector cells (i.e. eosinophils and aaMΦs) upon challenge indicates that intact parasites drive local Th2/eosinophilic responses to a greater extent than soluble protein preparations when used in vaccines.

The role of aaMΦs in LF infection remains unclear. Loke *et al* identified a population of adherent PEC cells recruited to the peritoneal cavity by adult *B. malayi* which were able to suppress proliferation of antigen specific T cells and reduce antigen specific IFNγ, via an IL-4 independent and TGFβ dependent mechanism; and these cells were identified as aaMΦs (314). Therefore recruited aaMΦs play an important role in the suppression of an early Th1 response and promotion of a dominant Th2 response to *B. malayi* adult parasites. However, in our laboratory, aaMΦs in the PEC population has been linked to increased resistance to *B. malayi* (Turner *et al*, unpublished), and they appear to have an important and essential role in the recruitment of eosinophils (319, 320) (Turner *et al*, unpublished).

No apparent effect on the magnitude or composition of the inflammatory infiltrate was observed by using Alum adjuvants with a BmMfE vaccine, in contrast to other vaccine models, where Alum is associated with increasing the recruitment of eosinophils, monocytes, neutrophils and other cells (297, 321, 322). When Pam2 was used as an adjuvant, a reduction in most cell numbers recruited was observed

in comparison to the use of BmMfE antigen alone. In general, neither the total amount, or relative proportions, of different cellular types or activation phenotypes recruited to the site of challenge infection were consistently related with the level of efficacy and suggest that under the conditions used in this vaccination model, they do not serve as useful correlates of vaccine efficacy. Further studies determining the dynamics of effector cell recruitment during challenge infection over longer time frames may help to more precisely define correlates of immune protection.

Our results indicate a polarised Th2 response to L3 is present in the spleen cells of mice after a six day exposure to *B. malayi* challenge infections, as indicated by the elevated production of IL-4, IL-13 and IL-5 cytokines by sham vaccinated mice in response to *B. malayi* antigens, when compared to naïve controls. It is known that *B. malayi* L3 are strong drivers of systemic Th2 responses, and this is a characteristic feature of helminth infections in general (301). The splenocyte immune responses to filarial antigens indicate boosted cytokine responses in all vaccinated mice, and a generally skewed Th2 response to L3 antigen in all exposed mice. The observation that restimulation of splenocytes with parasite antigen was unable to drive the production of an IFNγ response from vaccinated mice confirmed the restriction of *B. malayi* vaccines to driving Th2 immunity. When comparing vaccinated groups of mice, the IL-4 response to BmL3E was boosted by the use of Pam2 compared to all other vaccine groups, providing further evidence of the potent Th2-driving capacity of this lipopeptide as an adjuvant.

Alum adjuvants are clinically approved adjuvants which have been used for in vaccines for decades to boost immune responses, and typically drive strong antibody and Th2 responses (296). They consist of aluminium salts (aluminium phosphate and aluminium hydroxide), which adsorb to the antigen components of the vaccine. Despite being used in many different human and animal vaccines, the cellular and molecular mechanisms by which Alum is able to boost immune responses has only recently been studied in detail (210, 296). Alum adjuvants are able to stimulate enhanced innate immune responses at the site of exposure, in a mechanism independent of TLR and signalling (via MyD88 and/or Trif) (323). Thus, while Alum and Pam2 both act to enhance innate and adaptive immune responses, the mechanism of action by which they do this is strikingly different, as the effects of Pam2 on immune responses are elicited via TLR2/6 and MyD88 signalling (163). Mechanisms which have been attributed to the activity of Alum to activate immune responses include activation of the NOD-like receptor family, pyrin-domain-containing 3 (NLRP3) inflammasome (322), and induction of cell death followed by subsequent release of endogenous danger signals (296). However, the importance of each of these in the ability of Alum to drive adaptive immune responses is still a subject of debate, as some groups have yet to find evidence of the involvement of the NLRP3 inflammasome upon exposure to Alum (296, 297, 324). Flach *et al* propose that by binding to plasma membrane lipids, Alum results in alterations in lipid structures on DCs, which leads to increased antigen uptake and presentation (324). Thus, the initial mechanism/s of action of the most widely used adjuvant in human vaccines is still unclear. What is clear, however, is that

Alum results in elevated production of cytokines and chemokines at the injection site, which leads to the recruitment of various different cell subsets, including APCs, and an increased presentation of antigen (210, 215, 216, 324, 325).

We found the Alum adjuvant was able to drive an elevated IgG1 response to the vaccine antigen, as was expected given its use in vaccines to enhance antigen specific antibody levels (296), and a reported ability to promote IgG1 responses in mice (212). We also showed that Pam2 was found to elevate antigen specific IgG1 in vaccinated mice, which is consistent with the results of the previous chapter where Pam2 elevated antigen specific IgG1 to *Leishmania* antigens when used as an adjuvant in an ALM vaccine. In this study, however, an elevated level of antigen specific IgG2a was also detected in mice vaccinated with a BmMfE + Pam2 vaccine, which was not observed in the previous study (for IgG2c). Importantly, the ratio of IgG1:IgG2a was reduced compared to Alum, indicating that Alum is a more selective driver of Th2 rather than Th1 responses (reported before in mice (212)), whilst Pam2 appears to drive both antibody isotypes. Humoral responses have been reported to have important roles in parasite clearance in LF. Antibodies are required for adequate clearance of Mf, as mice lacking B cells (μMT) or the FcγR ($FcγR^{-/-}$) showed a dramatically reduced ability to clear *B. malayi* Mf from blood (326), and B cell deficient mice were unable to reduce *L. sigmodontis* parasite burden after pre exposure to irradiate L3 vaccine (327). However, it has also been reported that antibodies have no effect on parasite clearance, as in a C57BL/6 model of *B. pahangi* infection mice deficient in FcγRs were not impaired in their ability to clear parasites (317), perhaps suggesting different requirements for antibodies in different models. It would be interesting to determine the levels of antigen specific IgE in these mice, as this has been linked with vaccine induced clearance of filarial parasites in mouse models (300, 327). $IgE^{-/-}$ mice were more permissive to primary infection with *B. malayi* L3 than BALB/c mice, and parasites were able to develop to adulthood in the absence of IgE, although they could not produce Mf (318). Unlike in humans, where eosinophil degranulation can be mediated by antibody-dependent cell-mediated cytotoxicity (ADCC) via the Fc receptors for IgE (FcεR), mice do no express FcεRs on eosinophils, and so their function in response to filarial infection is not directly linked to antigen specific IgE production (328). Interestingly, $IgE^{-/-}$ mice were deficient in IgG1 and IgG2 levels in serum upon primary infection, whilst the PEC infiltrate was unaltered compared to WT. Upon secondary infection with *B. malayi* L3 however, $IgE^{-/-}$ mice were as resistant as WT and the serum levels of IgG1 were also comparable (318). This perhaps suggests a role for IgG1 in clearance of parasites in secondary infection (or prior to previous exposure). IgG is recognised by mouse eosinophils and has been shown an ADCC upon binding to FcγRs (328), but this mechanism has not been linked with the effector function of eosinophils in filarial infection. The roles of complement, ADCC and degranulation in eosinophil-mediated killing of *B. malayi* L3 is currently being researched in our laboratory.

In summary, this study strongly indicates that Pam2 is able to drive improved efficacy in a vaccine model where a Th2 response is favourable for enhanced immunity to the pathogen of interest, and that levels of efficacy were comparable to that of the archetypal Th2-driving adjuvant Alum. However, the immune response readouts suggest that the two adjuvants differ in the type of immune responses they elicit; whilst both are capable of driving elevated antigen specific IgG1 responses, Pam2 also resulted in increased antigen specific IgG2a levels and an elevated systemic IL-4 antigen recall response by splenocytes.

Chapter 8. Discussion

The role of TLR2 in CL

This study has identified a role for the receptor TLR2 in controlling disease severity in CL infection in mice, and this role was linked to driving a favourable protective Th1 response to infection (Chapters 3&4). The dynamics of the role of TLR2 on *Leishmania* infection are such that the effect of TLR2 activation on disease severity manifests several weeks after initial infection, and appears to function to promote an effective healing response. However, a lack of TLR2 does not prevent the eventual resolution of the infection. This finding improves our understanding of how *Leishmania* parasites interact with TLRs during infection *in vivo*, and how this interaction impacts on immune responses and disease outcome. A picture emerges whereby TLR2, TLR4 and TLR9 are all involved in the recognition of *Leishmania* parasites *in vitro* and *in vivo*, in addition to TLR3 in recognition of the dsRNA of the LRV. Whilst the studies by others using TLR deficient mice indicated TLR9 as having the more dominant role in driving protective immune responses to *Leishmania* infections (207), our findings suggest that TLR2 activation may act to bolster this protective immune response during infection. Although the protective influence of TLR2 was consistently observed in our experiments for both *L. major* and *L. mexicana,* other studies using the mucocutaneous species *L. braziliensis* and *L. amazonenesis*, have demonstrated an exacerbatory role for TLR2 during infection (40, 200), illustrating the influence of TLR2 is complex and can exert profoundly different species-dependent outcomes. Our findings further suggest that the ligand for the TLR2-mediated effects *in vivo* is not, at least exclusively, LPG, and that if the ligand in question is parasite derived, it is expressed by amastigotes in *L. mexicana* infection. Whilst others have shown activation of TLR2 by LPG preparations *in vitro* resulting in inflammatory responses (26, 27, 234), it is important to note that this is in contrast that many of the known functions of LPG *in vivo,* which are related to the down regulation of inflammatory responses (15, 19, 32, 329), so it would be paradoxical for LPG to also promote protective immune responses in the context of an *in vivo* infection. Further research is needed to understand which host cells are involved in the TLR2 interaction with *Leishmania*, and to determine whether that activator of TLR2 is in fact derived from the parasite, or an alternative source, such as other microbes present at the infection site, or host DAMPs. An interesting area of research is the impact of resident skin microflora at the lesion site (249), which play an important role in lesion development and immunity to *L. major*. It would be interesting to further explore the role of the skin microbiota in relation to TLR2 activation during CL infection in mice.

In addition, whilst the role of the sandfly bite was not explored in this thesis, it is an extremely important aspect of *Leishmania* transmission, and many aspects of this natural mode of transmission have implications on disease outcome. As it is likely that salivary components, including sandfly gut microbiota and their products, also possess multiple PAMPs and thus potentially ligands for TLR2 and its co-receptors, it would be very interesting to explore the outcome of infection in TLR2$^{-/-}$ mice (and indeed

those lacking other TLRs) infected by the *Leishmania*-infected sandfly bites. In addition, immunomodulatory components of the saliva may impact on and/or modify any parasite-derived activation of TLR2 on host cells at the infection site.

This study also suggests that TLR2 may have a function independent of its known requirement for a co-receptor (TLR1 or TLR6), as neither of these were found to have a protective role in *Leishmania* infection, which matched that observed for TLR2. In addition, TLR6$^{-/-}$ mice were found to be more resistant to *L. major*, but not *L. mexicana*, infection, suggesting an opposing role to TLR2 during infection. These findings are intriguing, and may have implications for the current dogma accepted for TLR2 activation by bacterial PAMPs for TLR2. Although TLR10 has been shown to act as an additional co-receptor for TLR2 in humans, it is not present in mice. However, the presence of an unknown additional co-receptor is possible, and the ability of TLR2 to act as a homodimer in some settings cannot be ruled out. TLR12, which is part of the same family of surface TLRs as TLR2, has recently been shown to function as a homodimer in some settings, or as a heterodimer with TLR11 in others, and this differential receptor formation has implications for its role in *T. gondii* infection *in vivo* (158).

When comparing the findings from the first two results Chapters (3&4), which focused on the roles of TLR2 and co-receptors during *Leishmania* infection, to Chapter 6, where TLR2 driving adjuvants were explored for use in vaccines for parasitic infection, two contrasting roles for TLR2 in terms *Leishmania* specific responses emerge. During chronic infection, TLR2 appears to have a role in driving protective Th1 responses, which act to control parasite replication and reduce lesion size. When activated in a prophylactic vaccine setting however, TLR2 activation resulted in a predominantly Th2 response to *L. major* infection upon challenge, which acted to increase disease severity. Thus, stimulation of TLR2 prior to infection appears to drive detrimental immune responses whereas TLR2 activation during chronic infection acts to promote protective immune responses and assist in controlling infection. This thesis therefore demonstrates the divergent complexity of TLR2 functions, and is an example of how TLR2 activation can have differing consequences in relation to infection, even with the same pathogen. The ability for TLR2 to form heterodimers with TLR1, TLR6 and TLR10, as well as interact with other TLRs, may help to account for this complexity to some extent (178). It is also likely that different cell types, concurrent activation of other TLRs and PRRs, and cytokine/chemokine environment in the tissues, all have roles to play in influencing the outcome of TLR2 activation in different settings. For example, fungal β-1,3-glucan is known to signal via interacting with both TLR2 and the CLR Dectin-1 (175), and it could be that *Leishmania* PAMP/s can signal via TLR2 in combination with other PRRs (with CLRs being a likely candidate group of PRRs, given the abundance of glycoproteins on the surface of *Leishmania*).

Development of a vaccine for CL containing lipopeptides

The second aim of this project was to develop a vaccine for CL, which included the use of TLR2 ligands as adjuvants. Given that both TLR4 and TLR9 have also been shown to play a role in disease outcome *in vivo* (97, 202, 203, 206, 207), and have proven effective adjuvant targets in experimental and clinical studies for leishmaniasis vaccines (122, 125, 127, 143, 146, 265, 268, 270, 279, 288, 330), our findings for a protective role for TLR2 during infection suggested TLR2 could be a valid target for vaccine adjuvants. Our results, however, suggest that the use of TLR2 ligand lipopeptide adjuvants in *Leishmania* vaccines are ineffective (Chapter 6), and in the case of Pam2, acted to exacerbate disease upon challenge infection with *L. major*. However, whether a self-adjuvanting lipopeptide vaccine would give similar vaccine induced responses to that observed in our study using ALM and lipopeptide adjuvants cannot be predicted from these findings. The formulation, composition and delivery site of the vaccine, which contains a TLR2 ligand appears to have an impact on TLR2 activation and the extent of the immune response elicited. In our hands and in others, the TLR9 ligand CpG promotes enhanced vaccine efficacy in vaccine models of leishmaniasis, and has been shown to promote strong Th1 and memory responses. CpG is likely to be a highly favourable adjuvant in future vaccine development studies for leishmaniasis, and may also be useful in a therapeutic vaccine setting. The topical adjuvant, imiquimod, which activates TLR7 and has been approved for use as topical treatment of cervical warts, for example, is also a potent stimulator of Th1 responses and has shown promising results when used for prophylactic vaccines and as a therapeutic treatment for leishmaniasis (218, 279). The TLR4 ligand MPL, and other LPS/Lipid A derivatives, have also shown promising results in other studies on leishmaniasis vaccines, and MPL has the advantage in that it has already been already approved for use in humans vaccines (in the AS04 adjuvant) (125, 127, 271, 279, 330). Indeed, Reed *et al* are currently exploring using an alternative TLR4 ligand structure, GLA-SE, with the hope that this will be likely to also be approved for human use given its similarity to MPL (218, 279). With the hope that increasing numbers of novel adjuvants which stimulate TLRs are approved for use in humans, activation of multiple TLRs (TLR synergy) may also be a favourable approach to take in future studies (144).

A potential caveat of the *Leishmania* studies conducted for this thesis is the lack of use of the sandfly bite in the infection studies, and in particular for the challenge infections in the vaccine studies. Rogers *et al* demonstrated the importance of using a more natural sandfly bite model for challenge in mice vaccine studies, by showing that a vaccine which is protective against a needle challenge was effective at reducing disease upon sandfly challenge, and vice, versa (331). Indeed, the vaccine used in this study which provided the best protection against needle challenge, ALM + CpG, was found to provide no protection against sandfly bite challenge in another study (146).

There are a number of groups exploring different types of vaccine design in the development of a protective vaccine for *Leishmania*. Many consider that an effective vaccine requires continual stimulation

of the immune system, which is most likely to be achieved by using attenuated parasites (123), but the finding that even a vaccine using LPG2$^{-/-}$ parasites was only able to provide protection when a CpG adjuvant was co-administered (140), highlights the important role in adjuvants in a future *Leishmania* vaccine development. Indeed, some have taken the approach of using adjuvants such a CpG to improve on the safety of live parasites (Leishmanisation) as a vaccine approach, as this still remains by far the most effective method of vaccination to date (41, 269). Blander and Sander recently speculated that the immune responses to PAMPs and other innate signals may differ in terms of magnitude and quality, due to a system whereby the 'microbial' threat is measured according to whether certain criteria have been met. They propose that immune responses to pattern recognition are scaled according to the following five checkpoints: 1) whether PAMPs are soluble or particulate; 2) whether PAMPs are associated with a dead or alive microbe; 3) whether the microbe is a pathogen or non-pathogen (i.e. does it possess active virulence factors?); 4) whether the microbe has invaded or colonised the tissue site; and 5) whether to have regulation of inflammatory responses (this decision is dependent on tissue location) (171). Thus, recognition of soluble immune PAMPs by cells at the epithelial surface results in relatively weak innate immune response, compared to the recognition of multiple PAMPs in a tissue site which is normally sterile, and by cells which also detect viability of a living microbe (via recognition of 'vita-PAMPs' such as microbial mRNA) (171). If this hierachical approach to innate immune activation leads to differing responses, it will have major implications for the design of vaccines, which seek to balance these outcomes in favour of the host. It suggests that the use of living and/or attenuated microbes, containing multiple intact PAMPs, which are targeted to the appropriate cell type and tissue, will be the best strategy for ensuring the highest levels of immune activation, if inflammatory responses are required. This would support the suggestion by Okwor *et al* that the development of attenuated parasites is a worthwhile approach for the development of a *Leishmania* vaccine (123).

The lack of a consensus about the appropriate immune correlates of protection to monitor the efficacy of vaccines in CL models is another are of research that requires further study. Although *Leishmania* immunology is among the most intensively studied areas of research and a paradigm system for our fundamental understanding of host-parasite immunity, even well-established mouse models have identified a range of different factors that are associated with enhanced protection to CL after vaccination, and these vary considerably between studies (131, 142, 143, 145, 146) . This suggests that factors depending on the nature of the vaccine, as well as the model system, can impact on immune correlates, which maybe complex, dynamic, diverse and multifactorial. A priority should therefore be to define which of these immune correlates best predicts protective immunity in humans, and to identify consistent markers of protective immunity in the most suitable vaccine models.

TLR ligands and adjuvant development

Adjuvants act in various different mechanisms to directly or indirectly activate DCs and promote enhanced immune responses to antigens (210). Compared to many of the TLR ligand adjuvants, such as CpG, which is known to drive strong Th1 responses, there is no consensus in the literature on the type of immune responses driven by lipopeptides adjuvants, or TLR2 ligands in general. From the findings of this study and others, it appears that the immune response elicited by lipopeptides depends largely on the vaccine approach that is adopted. For Th1 responses, peptide/protein epitopes which are physically attached to the TLR ligand (the acyl moiety) appear to be favourable (251, 254, 332), as is the addition of basic lipopeptides to DNA vaccine formulations (282, 333). In contrast, the addition of basic lipopeptide structures to protein or whole cell antigen preparations, or the use of lipopeptides structures alone (i.e. without antigen) appears to drive a Th2 and/or regulatory response (280, 334). Furthermore, the route of administration is likely to have an effect on the type of immune response elicited. The above observations were all made in models where s.c. injection with a needle was used, but TLR2 ligands administered by an i.p or i.v route have also been shown to induce Th2 and regulatory responses (176, 281). However, when administered via i.d. route with a DNA vaccine in a prime boost DNA/MVA approach, Pam3 enhanced Th1 and memory responses and improved efficacy of a vaccine for *L. braziliensis* (282). These combined observations suggest that the activation of TLR2 in a vaccination setting is more complex than activation of other TLRs, such as TLR9, and that the use of TLR2 ligands as adjuvants may need to be understood in the context of the formulation and site of exposure.

A trend that exists between the two main sections of this study (i.e. the infection experiments in TLR$^{-/-}$ mice and the lipopeptide adjuvant studies) is that the immune responses elicited by TLR2, TLR1 and TLR6 appear to be different. This conclusion is based on three major observations: Firstly, TLR2 appears to have a role in controlling parasite replication and pathogenesis during CL infection, whereas neither TLR1 or TLR6 played a similar role; Secondly, a lack of TLR6$^{-/-}$ during *L. major* infection led to increased healing and a stronger protective immune response, whilst a lack of TLR1 had no effect; Finally, we observed differences in magnitude of swelling reactions to the TLR2/6 and TLR2/1 ligands, Pam2 and Pam3 (respectively) upon exposure to mice, and enhanced antigen specific Th1/Th2 immune responses elicited by Pam2. The field would benefit from more in depth analysis of the immune responses that are elicited by activation of TLR2/1 and TLR2/6. For example, it would be interesting to compare the phenotypes of cells recruited in response to exposure to Pam2 and Pam3 upon s.c. injection, important cell subsets to look at would be neutrophils, MΦs, DCs and eosinophils. In addition, whether there are qualitative differences in the type of innate immune responses elicited by Pam2 and Pam3 remains to be determined *in vivo*. Whilst they have both been shown to result in the release of inflammatory cytokines by APCs (246, 253, 293), whether there are any differences in the upregulation of costimulatory molecules, ability to present antigen or promote memory responses remains to be elucidated *in vivo*. Studies comparing the Th1-driving TLR9 ligand CpG to the Th2-driving TLR-independent Alum adjuvant *in vivo*,

showed no differences in the magnitude and kinetics of the T cell responses induced by these two adjuvants, and there were no differences in the uptake and presentation of antigen to CD4 T cells by various different APC subsets (212). Instead, differences in the initial mechanism of innate immune triggering, the pathways exploited, and resulting genes modulated (e.g. "adjuvant core response genes") are likely to be important to influencing the quality of the immune response (210). A study comparing various TLR ligand adjuvants (MPL, R-848 and CpG, which activate TLR4, TLR7/8 and TLR9 respectively) in primates identified differences in the transcription of genes involved in various innate immune pathways in PBMCs recovered from exposed individuals. In addition, whilst rapid expansion of DCs and other APCs by ligands of TLR when injected *in vivo*, differences in the dynamics of activation and phenotypes of these APCs were observed (335). It would be useful to have studies comparing lipopeptide adjuvants to other TLR ligand adjuvants, in a similar style of study.

It is now widely accepted that multiple TLRs ligands/innate stimuli are preferable to single components when used as vaccine adjuvants (296). Understanding adjuvant specific responses, when used singularly or in combination, is important in choosing the appropriate adjuvants, and already we are seeing vaccines licensed which use more than one adjuvant in order to enhance and shape the immune response (e.g. using alum and MPL) (218). Our findings with Pam2 and Pam3 adjuvants, has improved our knowledge in relation to the use of these adjuvants in vaccines, as more suitable for vaccines requiring Th1/Th2 and antibody responses.

Conclusion

Vaccines remain the most effective, economical and efficient tools for combatting infectious diseases, and the effort to develop vaccines for important human parasitic diseases should remain a research priority. We are moving towards an era of rational vaccine design, whereby immune correlates of protection will be identified for the disease of interest, which can inform vaccine design and adjuvant choice (211, 336, 337). Current gaps in our understanding of the biology of parasitic infections hamper our efforts in taking the right approach to vaccine development. The challenge for vaccine development for CL and other parasitic infections is to better understand how to promote the immune responses that provides long lasting and if possible, sterile protection, outcomes which challenge even the best experimental vaccines to date. The complexities of the role of TLR2 and its co-receptors that have been uncovered in this thesis, in CL infection and in a vaccination setting, provide further insight into the prospect of TLR2 ligands as a target for vaccine adjuvants. The findings also uncovered many new questions, and indicate that there remain gaps in our understanding of the role of distinct PRRs in infectious disease immunology, and in vaccinology. A greater understanding of how innate immunity drives protective adaptive immunity and how this can be exploited in adjuvant and vaccine design is required to achieve the ambition of rational vaccine design leading to effective vaccines for future generations.

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Appendix 1 – Genotyping

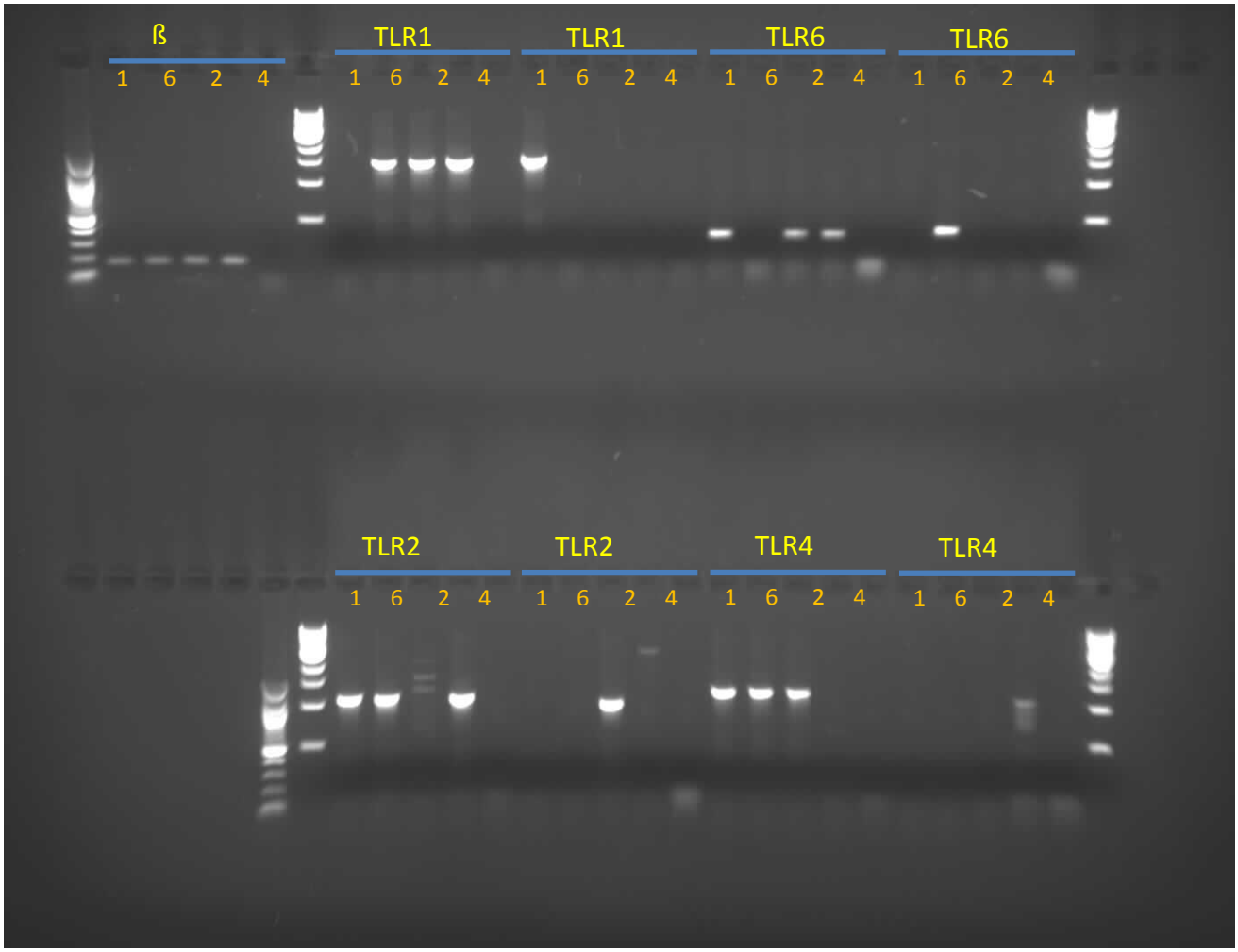

Figure 48. Genotyping of WT and TLR$^{-/-}$ mice using PCR.

Example of results of genotyping of DNA from tissue of naive mice from TLR$^{-/-}$ colonies to ensure for correct genotype. DNA from mice were used in PCR reactions for the amplification of the control target (ß actin) and WT and KO targets of TLR1, TLR2, TLR4 and TLR6 genes. The different targets are highlighted in yellow, and the expected genotype of each sample is indicated above each lane in orange (i.e. TLR1$^{-/-}$ samples are identified by '1'). A no template control ('B') was included for each reaction. Products from PCR reactions were ran on an agarose gel and results are displayed above. TLR1$^{-/-}$, TLR6$^{-/-}$, TLR2$^{-/-}$ and TLR4$^{-/-}$ samples all gave positive results for the ß-actin PCR and all gave the expected presence/absence of WT and KO products (i.e. TLR1$^{-/-}$ sample was negative for TLR1 WT product, positive for TLR1 KO product, positive for all other TLR WT products and negative for all other KO products).

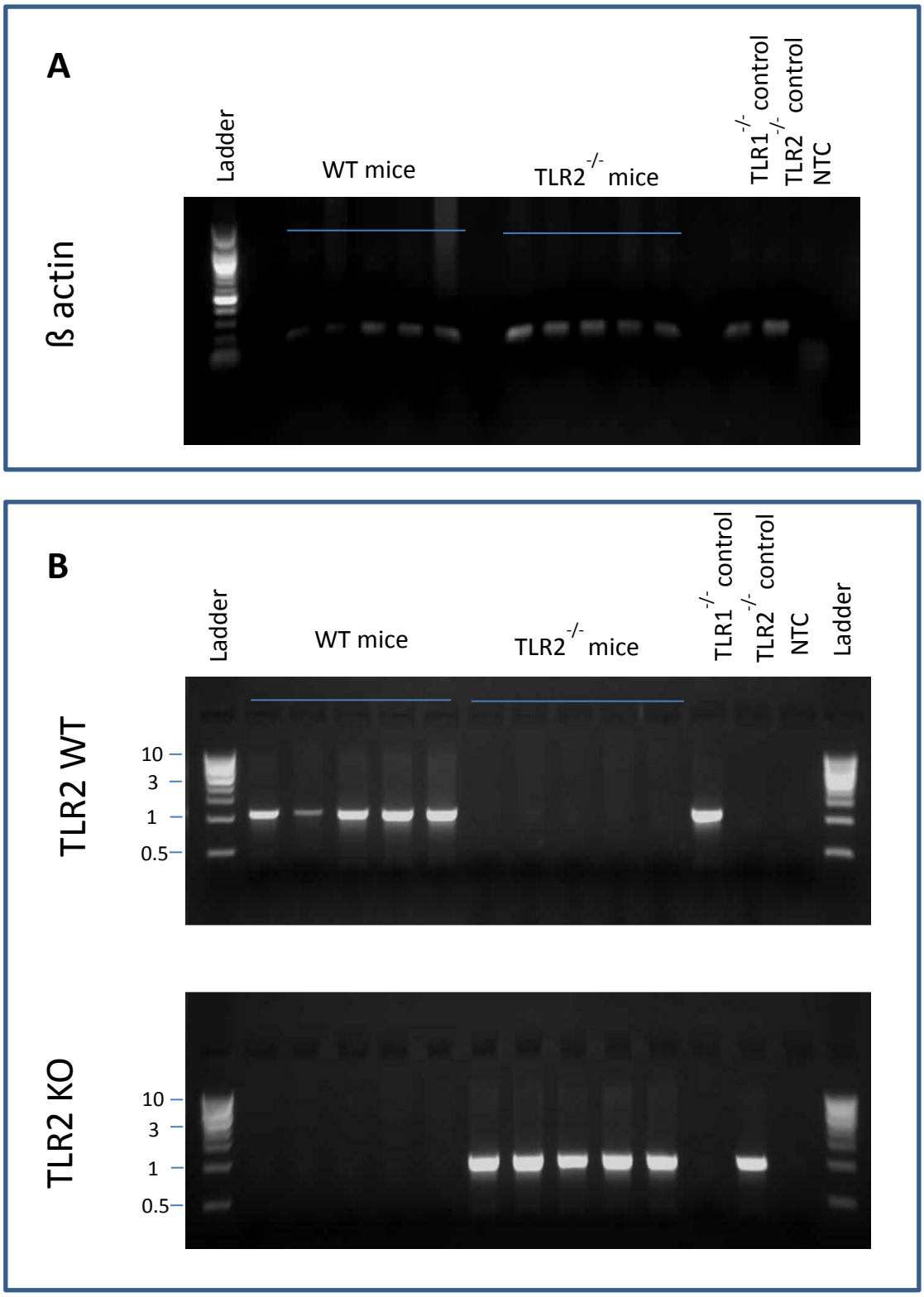

Figure 49. Genotyping of WT and TLR2-/- mice by PCR.

Example of results from genotyping of DNA from lesion tissue of mice used in an infection experiment. Lesion DNA from mice infected with *L. mexicana lpg1*-/- promastigotes was used to check for the genotype of the mice used. Lesion DNA was used to check for presence of ß-actin gene (control gene,

shown in A), and WT and KO targets of the TLR2 gene (shown in B). It was expected that 5 WT (C57BL/6) mice and 5 TLR2$^{-/-}$ mice were used in the experiment. The results indicated that the expected products were found in each sample. Thus the mice used were the correct genotype.

Appendix 2 – Cytokine response, *L. major* infected mice

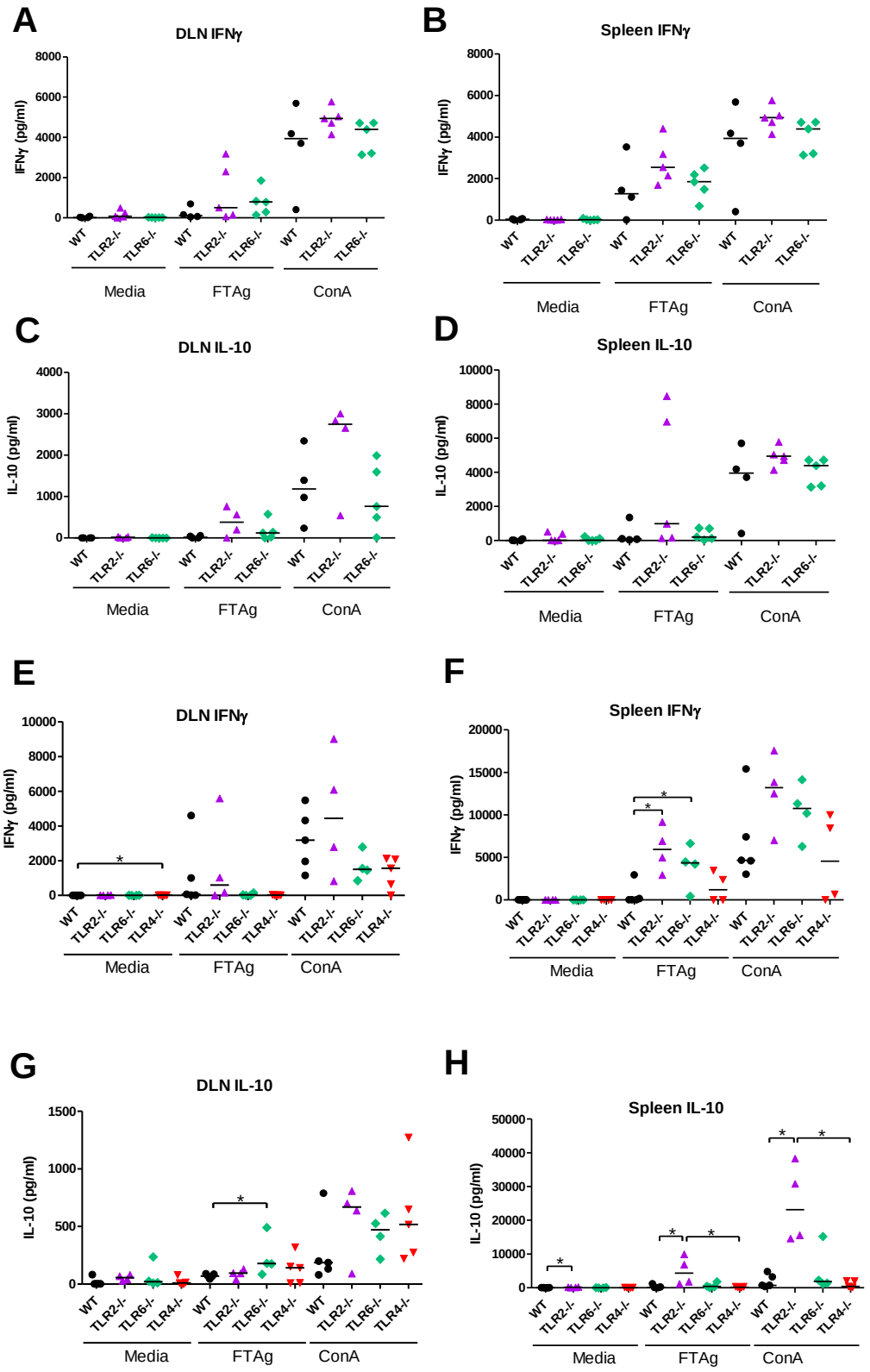

Figure 50. Cytokine responses in DLN and splenocyte restimulation experiments from *L. major* infected WT and TLR$^{-/-}$ mice, including FTAg stimulated cultures and negative (Media) and positive (ConA) controls. Results are given for immune cells harvested at week 10 (A-D) and week 18 (E-H) from DLN (A, C, E, G) and splenocyte (B, D, F, H) cell cultures. Groups were compared using the Mann-Whitney U test (*$p<0.05$).

Appendix 3 – Restriction Digest Maps

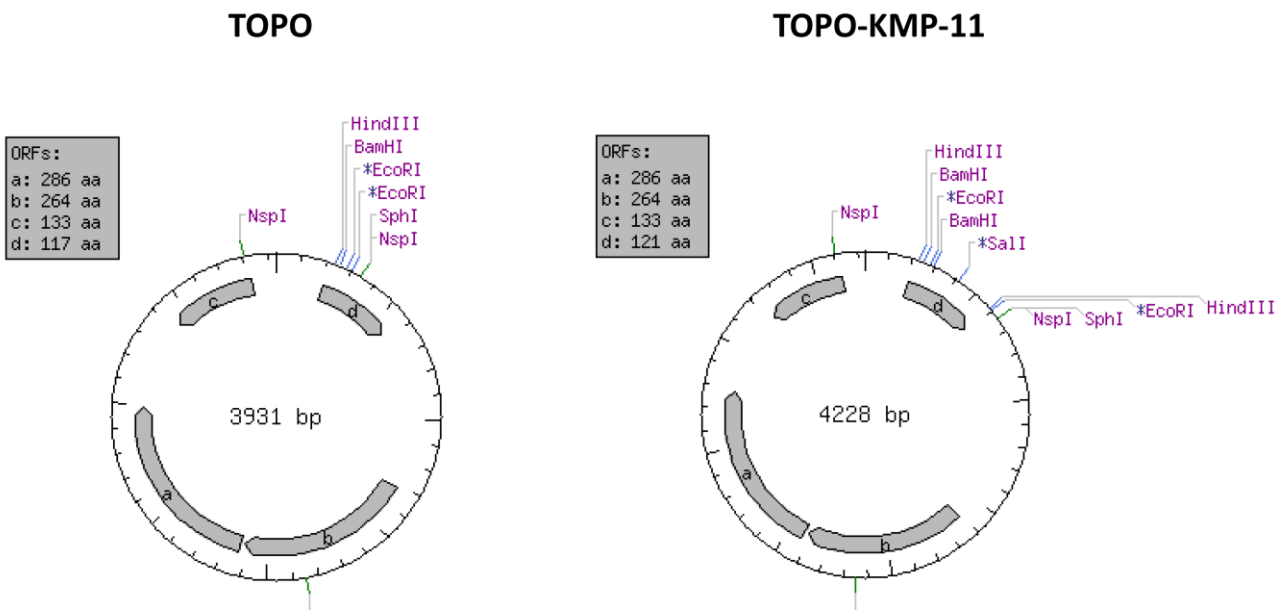

Figure 51. Restriction digest maps of TOPO and TOPO-KMP-11 showing the digest sites of different restriction enzymes.

Appendix 4 – Parametric analysis of parasite burden in ALM vaccinated mice infected with *L. major*

The mean, median and standard deviations of the parasite burdens from the different vaccinated groups are given in Table 14 below. The distributions are not all Gaussian, as there are greater than 10% differences between the mean and median averages in the majority of cases. The overall dataset population, and most of the individual populations (the ALM group being the exception), follow a negative binomial distribution, as the SD values exceed the mean averages.

Vaccine group	Parasite number			
	n	Mean	Median	SD
PBS	8	6160.38	3189.00	7929.102
Adjuvants	7	187316.43	14730.00	234687.270
ALM	7	1099.71	718.00	1007.230
ALM +CpG	7	1530.29	1039.00	1788.918
ALM +Pam2	7	1044241.14	26880.524	2660286.524
ALM +Pam3	7	125337.29	5666.00	318337.450
ALM +Adjuvants	7	2820.14	1041.00	3212.658
Total	*50*	*191713.96*	*2357.00*	*1005628.059*

Table 14. Average parasite burdens at 9 weeks p.i. in groups groups of vaccinated mice infected with *L. major*.

Parasite burdens of different groups could be compared by fitting a negative binomial distribution and using a generalised linear model approach, and by calculated the IRRs and CIs for each. A p value of < 0.05 was considered significant. Results are presented in Table 15 and Figure 52 below.

Comparison	IRR	95% CI	P value
Adjuvants / PBS	30.40782571	5.703 - 162.121	<0.001 ***
PBS / ALM	5.601867364	1.050 - 29.873	0.0436 *
PBS / ALM +CpG	4.025704795	0.755 - 21.468	0.103
ALM +Pam2 / PBS	169.5079791	31.793 - 903.742	<0.001 ***
ALM +Pam3 / PBS	20.34631878	3.816 - 108.478	<0.001 ***
PBS / ALM +Adjuvants	2.184528466	0.410 - 11.647	0.36
Adjuvants / ALM	170.3235733	30.234 - 959.507	<0.001 ***
Adjuvants / ALM +CpG	122.4006891	21.732 - 689.402	<0.001 ***
ALM +Pam2 /Adjuvants	5.574485355	0.990 - 31.397	0.0514
Adjuvants / ALM +Pam3	1.4945124	0.265 - 8.418	0.649
Adjuvants / ALM +Adjuvants	66.4201185	11.793 - 374.100	<0.001 ***
ALM +CpG / ALM	1.391524627	0.247 - 7.839	0.708
ALM +Pam2 /ALM	949.561216	168.558 - 5349.29	<0.001 ***
ALM +Pam3 /ALM	113.9773791	20.232 - 642.085	<0.001 ***
ALM +Adjuvants /ALM	2.564337088	0.455 - 14.446	0.2856
ALM +Pam2 / ALM +CpG	682.3890843	121.156 - 3843.44	<0.001 ***
ALM +Pam3 / ALM +CpG	81.90827307	14.543 - 461.335	<0.001 ***
ALM +Adjuvants / ALM +CpG	1.842825515	0.327 - 10.381	0.488
ALM +Pam2 / ALM +Pam3	8.331137488	1.479 - 46.924	0.0162 *
ALM +Pam2 / ALM +Adjuvants	370.2950055	65.745 - 2085.630	<0.001 ***
ALM +Pam3 / ALM +Adjuvants	44.44266805	7.891 - 250.316	<0.001 ***

Table 15. **Comparisons of parasite burdens in groups of vaccinated mice infected with *L. major* promastigotes using parametric methods. These values were fitted to a generalised linear model using a negative binomial function, to allow for comparison of means between groups. Incidence rate ratios (IRRs) are presented (IRR = mean1/mean2) with their confidence intervals. The average values of groups are considered significantly different when the CI values of IRRs do not encompass 1; instances where this is the case are highlighted in bold and italics in the P column.**

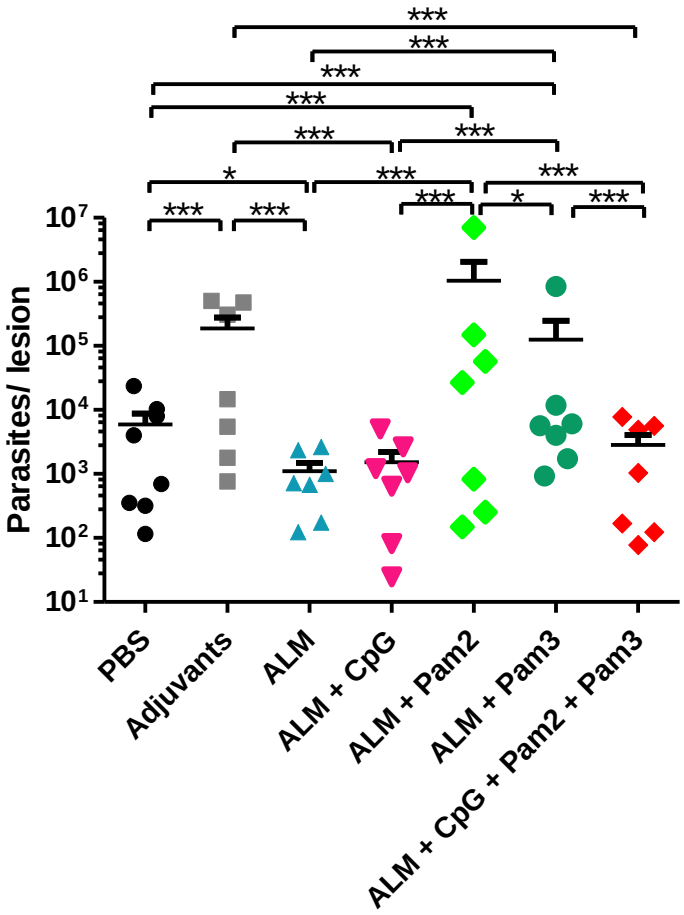

Figure 52. **Parasite burdens in mice vaccinated with ALM and adjuvants, compared using parametric methods. Individual parasite burdens for mice in each are shown, as well as mean values +SEM. Where means were found to be significantly different between groups, this is indicated by connector bars and asterisks (* p<0.05; **p<0.005; p<0.001).**

Using parametric analysis on this dataset, significant differences between most of the vaccinated groups were found. ALM vaccinated mice had the lowest burden, and this was significantly lower than the PBS, Adjuvants alone, ALM + Pam2 and ALM + Pam3 groups. The group with the next lowest burden was the ALM + CpG vaccinated group, which was not significantly reduced compared to the PBS group, but was compared to the Adjuvants alone, ALM + Pam2 and ALM + Pam3 groups. The group which receive only Adjuvants had significantly greater parasite burdens than the naïve mice, and higher than when ALM was also included in the vaccine. This was surprisingly and suggests this adjuvant cocktails acts to

exacerbate infection, but only when used in the absence of antigen. The parasite burdens in the group vaccinated with ALM + Pam2 were highest, and were greater than all other groups, except for those which received just the adjuvants. The parasite burdens in the group that receive the ALM + Pam3 vaccine were significantly greater that received the ALM, ALM + CpG, PBS and ALM + Adjuvants vaccines. It therefore appears that the Adjuavnts only vaccine, as well as the ALM + Pam2 and ALM + Pam3 vaccines, all exacerbate parasite burdens compared to the control and other groups. The ALM is efficacious at significantly reducing burden, and ALM + CpG and ALM + Adjuvants reduced burdens to comparable levels to this vaccine.