Plasticity of Macrophages From Helminth Infection

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A thesis submitted for the degree of Doctor of Philosophy of the University of Edinburgh

by

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Declaration

I declare that the contents of this thesis are entirely my own work, unless otherwise stated.

Katharine (Katie) Jude Louise Mylonas 23rd January 2008

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Abstract

The prime function of classically activated macrophages (CAM ϕ ; activated by Th1-type (or 'type 1') signals such as IFN- γ) is microbial destruction. Alternatively activated macrophages (AAM ϕ ; activated by Th2 (or 'type 2') cytokines, such as IL-4) play important roles in allergy and responses to parasite infection. A murine model for filarial infection has been used as an *in vivo* source of AAM ϕ , which we have termed nematode-elicited Macrophages (NeM ϕ). Mice are surgically implanted into the peritoneal cavity with adult *Brugia malayi*. By one week post-infection, the PEC population is dominated by macrophages that display IL-4 dependent features such as the expression of Arginase1, RELM- α and Ym1.

In light of the increasing evidence that macrophages show functional adaptivity, it was decided to study the NeM ϕ response to pro-inflammatory Th1 activating signals as a model to investigate whether the switch between alternative and classical activation can occur in macrophages differentiated in an *in vivo* infection setting. Despite the long-term exposure to Th2 cytokines and anti-inflammatory signals *in vivo*, we found that NeM ϕ were not terminally differentiated but could switch from alternative activation to a more classically activated phenotype in response to LPS/IFN- γ . This was reflected by a switch in the enzymatic pathway for arginine metabolism from arginase to iNOS and the reduced expression of *RELM-\alpha* and *Ym1*.

To ask whether these AAM ϕ could be induced to become antimicrobial, we also carried out infections with "type 1"-inducing pathogens, It was found that LPS/IFN- γ , treated NeM ϕ were able to control infection with *Leishmania mexicana* as effectively as LPS/IFN- γ activated thioglycollate-elicited macrophages (ThioM ϕ) and parasite killing was mediated by nitric oxide production. NeM ϕ were also infected with the mycobacterium *Mycobacterium bovis* BCG. It was found that NeM ϕ responded to low doses of BCG by controlling it for the entire timeframe of the study, i.e. 6 days. NeM ϕ responded to high doses of BCG infection with early control of infection and high levels of apoptosis, and that this phenotype is independent of IL-4.

Next we asked whether the macrophage phenotype would alter during co-infection with both a 'type 1' and 'type 2'- inducing pathogen. Ym-1 and RELM- α were looked at in the lungs of mice co-infected with malaria (*Plasmodium chabaudi*; 'type 1' pathogen) and the helminth worm *Nippostrongylus brasilisensis* ('type 2' pathogen). After 7 days of infection it was found that there was less Ym-1 and RELM- α present in co-infected, as compared with *Nippostrongylus*-only infected mice, which suggested that the immune system could switch from an alternative to a more classically activated state in order to deal with a type-1 infection or that the malarial infection was causing a delay, or decrease in the Th2 response.

Lastly, we wanted to look at what innate signals determine the AAM ϕ phenotype and its ability to switch towards classical activation. It is currently unknown whether signaling through toll-like receptors (TLRs) plays any role in the alternative activation of macrophages. In light of this, it was decided to investigate, using MyD88-/- animals, whether alternative activated macrophages could be recruited in the absence of the important adaptor molecule, MyD88, and thus determine whether TLR signals impact negatively or positively on the AAM ϕ . Of the NeM ϕ present in the peritoneal cell populations (PEC) of both wild type (WT) and MyD88-deficient mice we found no difference in terms of arginase production or the expression of RELM- α or YM1. Therefore, lack of MyD88 had no apparent effect on the NeM ϕ phenotype.

Abbreviations

ΑΑΜφ	Alternatively activated macrophage
AMCase	Acidic mammalian chitinase
APC	Antigen presenting cell
Argl	Arginase 1
a.u.	Arbitrary units
BCG	Bacille Calmette-Guerin
BM	Bone marrow
BM-TGH	Brugia malayi TGF-β homologue
b.p.	Base Pair
BSA	Bovine serum albumin
САМф	Classically activated macrophage
CFSE	Carboxyfluorescin diacetate succeinimidyl ester
ConA	Concanavalin A
cpm	Counts per minute
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbant assay
ES	Excretory/secretory
FCS	Foetal calf serum
FITC	Fluorescein isothiocyantate
FIZZ	Found in inflammatory zone
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
GM-CSF	Granulocyte –macrophage colony-stimulating factor
ICOS	Inducible co-stimulatory molecule
IFN	Interferon
IL	Interleukin
IL-4R	IL-4 receptor
iNOS	Inducible nitric oxide synthase

	i.p.	Intraperitoneal
	ISPF	Isonitroso-propiophenone
	LN	Lymph node
	L-NMMA	NG-monomethyl-L-arginine
	LPS	Lipopolysaccaride
	Mf	Microfilaria
	MHC	Major histocombatibility complex
	MIF	Macrophage migration inhibitory factor
	MMR	Murine Mannose Receptor
	Μφ	Macrophage
	NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
	NeMø	Nematode-elicited macrophage
	NES	Nippostrongylus brasiliensis excretory/secretory product
	NGF	Nerve growth factor
	NO	Nitric oxide
	NOHA	N ^w -hydroxyl-L-arginine
	OAT	Ornithine amino transferase
	ODC	Ornithine decarboxylase
	PAMP	Pathogen-associated molecular pattern
	PD-L	Programmed death ligand
	PEC	Peritoneal exudates cell
	PRR	Pattern recognition receptor
	RAG	Recombination activating gene
	RELM	Resistin-like molecule
	STAT	Signal transduction and activators of transcription
·	TCR	T Cell receptor
	TGF	Transforming growth factor
	Th	T helper cell
	TLR	Toll-like receptor
	WT	Wild type

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Table of Contents

.

Chap	ter 1	1
Intro	duction	1
1.	Innate Vs Adaptive Immunity	
2.	Diversity of CD4+ T cell Subsets	2
	2.1 Th1/Th2 Paradigm	2
	2.2 Regulatory T cells	5
	2.3 Th17 Cells	6
3.	The Th2 Cytokines IL-4 and IL-13	8
4.	Parasitic Helminths and the Th2 Response	9
5.	Lymphatic Filariasis	12
6.	Macrophages	
7.	The Th2 Response and Macrophage Activation: Alternatively A	Activated
	Macrophages (AAM ϕ)	21
8.	The Functions of AAM ϕ	
	8.1 Wound Healing	23
	8.2 Regulation	
	8.3 Effector Function	25
9.	The Th1 Response and Macrophage Activation: Classical Activ	ation of
	Macrophages (AAM)	
10	. iNOS and Nitric Oxide (NO)	
11.	iNOS/Arginase	
12.	Innate Recognition of Microbial Products	
13.	Co-infection	
14.	Aims of PhD	

Chap	oter 2	. 38
Plast	icity of Macrophage Function	. 38
1.	Introduction	. 38
2.	Materials and Methods	. 40
	2.1 Mice	. 40
	2.2 Brugia malayi infection	. 40
	2.3 Macrophage Activation	. 41
	2.4 FACs staining	. 42
	2.5 Proliferation Assay	. 43
	2.6 Quantification of NO and arginase activity	. 43
	2.7 RNA extraction and real-time RT PCR	. 44
	2.8 Immunofluorescence Assay	. 45
	2.9 Cytokine quantification	. 45
	2.10 MFB-F11 TGF-beta Bioassay	46
3.	Results	47
	3.1 LPS/IFN-γ causes a switch in L-Arginine metabolism from Arginase	;
	to iNOS and downregulates the expression of alternative activation	
	markers	47
	3.2 F4/80 expression on NeM ϕ in response to LPS/IFN- γ	. 53
	3.3 Suppressive phenotype of NeM ϕ is not reversed by LPS/IFN- γ	. 53
	3.4 NeMø express cell surface activation markers that can be further	
	upregulated in response to LPS/IFN-γ	. 57
	3.5 TNF- α , IL-6, IL-10 and IL-27, but not IL-12p40 or TGF- β , producti	on
	are enhanced in response to LPS/IFN-γ	59
4.	Discussion	61
	·	

.

LPS/IFN-y treatment confers resistance of NeMø to infection with Leishmania mexicana promastigotes	67
Leishmania mexicana promastigotes	67
1. Introduction	67
2. Materials and methods	69
2.1 Mice	69
2.2 Brugia malayi infection	70
2.3 Macrophage activation	70
2.4 Quantification of NO	70
2.5 RNA extraction and real-time RT-PCR	70
2.6 Immunofluorescent Assay (IFA)	70
2.7 Cytokine quantification	70
2.8 Macrophage infection with L. mexicana	71
2.9 Data analysis	72
3. Results	72
3.1 LPS/IFN-γ enables NeMφ to control <i>Leishmania</i> infection	72
3.2 TNF- α , IL-6 but not IL-12p70 production are enhanced in response	se to
infection in NeMø	77
3.3 The pattern of C3 expression by cultured ThioMo and NeMo corre	elates
with "uptake" of <i>L. mexicana</i> parasites	79
4. Discussion	81

Chapter 4 85		
lycobacterial infection: NeMø exhibit control of infection at low dose early		
nd control of infection and apoptosis at high doses of Mycobacterium		
ovis BCG		
1. Introduction		
2. Materials and methods		
2.1 Mice		
2.2 Brugia malayi infection		
2.3 Macrophage activation		
2.4 Quantification of NO 88		
2.5 Cytokine quantification		
3. Results		
3.1 NeMø control a low dose of BCG infection		
3.2 At a high infection dose NeM control mycobacterial growth in the		
early stages if infection and are highly susceptible to apoptosis 89		
3.3 NeM¢ from low dose BCG infection produce Nitric Oxide in		
response to IFN- γ but NeM ϕ from the high dose infection do not		
(Day 6)		
3.4 NeMø demonstrate a distinct cytokine profile following		
mycobacterial infection		
4. Discussion		

•

Chapter 5 102	
AAM¢ markers in the lung from an <i>in vivo</i> co-infection setting: Co	-infection
with the helminth Nippostrongylus brasiliensis and rodent malaria	
Plasmodium chabaudi	102
1 Takan darakina	100
2. Materials and methods	105
2.1 Mice and infections	105
2.2 Immunohistochemistry (IHC)	106
2.3 RNA extraction and real-time RT PCR	107
2.4 Western blotting	107
3. Results	108
3.1 A timecourse of Ym-1 and RELM- α expression in lungs of	f mice
infected with N. brasiliensis	108
3.2 Gross pathology of lungs in co-infection	110
3.3 At day 3 post-infection, co-infection is not affecting Ym-1	and
RELM-α expression	112
3.4 At day 5 post-infection singly Nippostrongylus-infected m	ice have
begum to express higher levels of Ym-1 and RELM- $lpha$ than	those co-
infected with malaria	114
3.5 At day 7 post-infection singly Nippostrongylus-infected m	ice express
higher levels of Ym-1 and RELM- α than co-infected animal	als.
reflected by the Th2-type, cytokine production in the draining	ing lymnh
node	116
2.6 Dy 20 days post infaction symposium of Vm 1 and DELM	
burge of an infected with 1	
lungs of co-infected mice has now overtaken that in singly	
Nippostrongylus-infected mice	119
5. Discussion	121

Chapter 6 127
MyD88 and the Alternative Activation of Macrophages 127
1. Introduction 127
2. Materials and methods 129
2.1 Mice 129
2.2 Brugia malayi infection 129
2.3 Macrophage Activation 130
2.4 Quantification of NO and arginase activity
2.5 RNA extraction and real-time RT-PCR
2.6 Proliferation Assay 130
2.7 Western Blotting 130
2.8 Flow Cytometry
2.9 Cytocentrifuge preparations 132
2.10 Counting of Microfilaria
2.11 Parasite Extract
2.12 <i>in vitro</i> splenocyte cultures
2.13 Cytokine Assay
2.14 Data Analysis
3. Results
3.1 MyD88-/- bone marrow-derived macrophages display deficiencies in
classical activation but can become alternatively activated when
treated with IL-4
3.2 The Th2 bias is not significantly altered in <i>B. malayi</i> -implanted mice
in the absence of MyD88 136
3.3 Absence of MyD88 does not affect the cell recruitment after B.
malayi implant but does result in reduced thioglycollate-elicited
recruitment 138

	3.4 The absence of MyD88 does not affect recruitment of different cell
	types to peritoneal cavity140
	3.5 Absence of MyD88 does not affect arginase production or suppressive
	ability of the NeM resulting from <i>B. malayi</i> implant
	3.6 Absence of MyD88 does not affect numbers of microfilaria, or
	expression of the alternative activation markers $Ym1$ or RELM- α , in
	the peritoneal fluid of implanted mice
4.	Discussion
Chapt	er 7 154
Genera	al Discussion
1.	NeMø are not terminally differentiated but can respond to Th-1 type
	stimuli 155
2.	Infection of NeM ø with intracellular pathogens 157
3.	AAM
4.	NeM do not require signalling through toll-like receptors to achieve an
	alternatively activated phenotype 162
5.	Conclusions 163
Appen	dices
Append	lix 1- Various Real-time RT-PCR results for $Ym-1$ and RELM- α
	expression
Append	lix 2. Effect of LPS/IFN- γ on the cell surface activation markers in naïve
	macrophages and NeMø166
Append	lix 3. Chapter 4 – work of Dr. Ian Faribairn 167
Append	lix 4 Chapter 6 -infection of mice 169
Append	lix 5. Anaemia, body weight and malaria parasitemia day 0-20 171
Append	lix 6. Day 7 cytokine production 172

.

.

17	13
1	17

Index of Figures and Tables

Chapter 1

Figure 1. Key transcription factors and cytokines involved in	
Th1/Th2 polarisation	4
Figure 2. Diversity of CD4+ T cell subsets	7
Figure 3. Receptors for IL-4/IL-13	9
Figure 4. Life cycle of Brugia malayi	14
Figure 5. The spectrum of filarial disease in endemis areas	15
Figure 6. Brugia malayi implant model for the generation of NeM	17
Figure 7. Pathways of macrophage activation	20
Figure 8. Multifactorial mechanism of suppression by macrophages from	
helmnith infection	26
Figure 9. Difference in L-arginine metabolic pathways used by iNOS	
and Arginase and negative feedback of opposing pathways	30

Chapter 2

Figure 1. Reversal of the NeMophenotype from alternative to classical
activation
Figure 2. Reversal of the NeMø phenotype from alternative to classical activation
continued 50-52
Figure 3. F4/80 expression on NeMφ in response to LPS/IFN-γ
Figure 4. NeMø can produce nitric oxide in response to LPS/IFN-y but will still
retain the ability to suppress cell proliferation in a nitric oxide
independent manner 55
Figure 5. Effect of LPS/IFN- γ on the cell surface activation markers in control

	macrophages and NeMo	58
Figure 6.	. TNF- α , IL-6, IL-27 and IL-10, but not IL-12p40 or TGF- β , productior	1
	are enhanced in response to LPS/IFN-γ	50

Chapter 3

Figure 1. NeMø contain the largest number of viable Leishmania mexicana
parasites after 3 hours73
Figure 2. LPS/IFN-y or IFN-y alone confer resistance of NeMo to L. mexicana
Infection75
Figure 3. LPS/IFN-y confer resistance of NeMo to L. mexicana infection 76
Figure 4. Expression of cyto-and chemokines during <i>L. mexicana</i> infection 78
Figure 5. Similar pattern of infection with L. mexicana promastigotes after 3
hours and expression of complement 3

Chapter 4

Figure 1. Infection with Mycobacterium bovis BCG (low dose)	90
Figure 2. Infection with <i>M. bovis</i> BCG (high dose)	91
Figure 3. NeMø undergo apoptosis when infected with high doses of BCG	93
Figure 4. Nitric Oxide assays from supernatants of BCG infected macrophages	95
Table 1. Cytokine expression of BCG infected Macrophages	96

Chapter 5

•

Figure 1. Ym1 and RELM- α expression in the lung in response to	
Nippostrongylus brasiliensis infection	. 109
Figure 2. Pathology of lungs	. 111
Figure 3. Day 3 Ym-1 and RELM- α mRNA and protein expression	113
Figure 4. Day 5 Ym-1 and RELM- α mRNA and protein expression	. 115
Figure 5. Day 7 Ym-1 and RELM- α mRNA and protein expression 117	-118
Figure 6. Day 20 Ym-1 and RELM- α mRNA and protein expression	. 120

Figure 7. Coinfection with malaria and helminth causes an impairment of	
expression of the alternative activation markers at day 7, but this	
reverses by day 20	123

Chapter 6

Figure1.	Upon stimulation with IL-4 BMM from MyD88-/- mice produce
	arginase but only display iNOS activity when stimulated with LPS and
	IFN-γ together
Figure 2.	The Th1 response is impaired in MyD88-/- mice but the Th2 bias is not
	significantly altered in <i>B. malayi</i> -implanted animals
Figure 3.	No impairment in cell recruitment in MyD88-/- mice after implantation
,	with Brugia malayi worms139
Figure 4.	Similar numbers of macrophages and eosinophils are recruited in wild
	type (WT) and MyD88-/- mice (KO) 141
Figure 5.	Similar numbers of T and B cells are recruited in Wild Type (WT) and
	MyD88-/- (KO) mice
Figure 6.	There was no difference in the arginase activity or suppressive ability of
	recruited NeM
	malayi worms 144
Figure 7.	The absence of MyD88 had no effect on numbers of microfilaria, or the
	production of RELM- α or Ym1 proteins by cells in the peritoneal cavity
	after B. malayi implant 146
Figure 8.	The absence of MyD88 had no effect on Ym1 or Arginase1 mRNA
	expression by purified macrophages from the peritoneal cavity after B.
	malayi implant. There was an increase in RELM- α mRNA expression in
	NeMφ in that absence of MyD88 147
Figure 9.	Possible scenario to explain need for LPS/IFN-y together for the
	induction of NO in the absence of MyD88 152

CHAPTER 1

INTRODUCTION

1

1. Innate Vs Adaptive Immunity

The immune system has evolved to protect against an array of pathogens that range from the microscopic that live within host cells, such as mycobacteria, to large parasites such as helminth worms that can be several metres long living in the gut of its host. Different parasites/pathogens use distinct methods for growth and survival, and so the host must also employ and activate diverse effector mechanisms in order to deal with them. For example, to respond to pathogens that live extracellularly, such as helminth worms, an immune response must be mounted that contains the parasite through encapsulation in the tissues or expulsion from the gut (Diaz & Allen, 2007; Nawa *et al*, 1994). This same response would not be effective against bacteria or protozoa that have carved out a niche within the cells of the host. In addition to pathogen control, collateral damage to the host is always a possible side effect of an aggressive immune response to a given pathogen, so the optimal response must also be one that minimises this damage, while still being the correct effective response against the invading organism (Graham *et al*, 2005a; O'Garra *et al*, 2004).

There are two arms to the vertebrate immune system. The innate immune system is the first arm and initially detects any foreign invasion through the recognition of common components of microbial pathogens in a relatively non-specific manner. Recognition of foreign particles leads to the development of inflammation through the release of cytokines and chemokines, and this innate response is quite often sufficient in clearing the infection (Janeway & Medzhitov, 2002). However, when and if an infecting organism survives this initial response, the second arm of the immune response comes

into play. This adaptive immune response involves B and T lymphocytes recognising foreign molecules in a precise way through antigen-specific extracellular receptors. T-helper (Th) cells, specific for the cell surface marker CD4, orchestrate the employment of the suitable immune response, whether this is one particular type of response for the clearance of intracellular pathogens or another type for the expulsion of extracellular parasites such as helminths (Janeway, 2001).

2. Diversity of CD4+ T Cell Subsets

2.1 Th1/Th2 Paradigm

CD4+ T cells coordinate the immune response by differentiating into discrete subsets and secreting distinct cytokines. This differentiation is thought to determine the success of the immune response against any particular pathogen, while limiting damage to the host. In the 1970-80s, many groups were working with the hypothesis that more than one subset of helper CD4+ T cell existed with differing cytokine profiles induced after stimulation with activating agents. It was thought that different T helper cell (Th) subsets had divergent and contrasting roles in the immune response with regards to regulation and effector function. By the mid-1980s, CD4+ T cells had been divided into the Th1 and Th2 subtypes based on cytokine profiles. Researchers, particularly Mossman, Coffman and colleagues had produced stable antigen-specific mouse T-cell clones and found certain patterns of cytokine production (Mosmann *et al*, 1986). The Th1/Th2 paradigm had been born. The type 1 T cell clones produced interleukin (IL)-2, interferon (IFN)- γ and granulocyte-macrophage colony-stimulating factor (GM-CSF) in response to antigen presenting cells with antigen or ConA. The type 2 subtype was found to produce IL-4 and IL-5 (Mosmann *et al*, 1986). It has been found that Th1 development is regulated by transcription factors, such as signal transducer and activator of transcription-4 (STAT-4) and T-bet. These are different to the transcription factors controlling Th2 development, STAT-6, GATA-3 and c-maf, and the two sets are mutually antagonistic (Figure 1) (Romagnani, 2006) (Szabo *et al*, 2003). STAT-4 and T-bet can be activated when IL-12 is produced by antigen presenting cells (more about that later). Cytokines produced by Th1 cells, in particularly IFN- γ , generally favour the production of IgG2a and IgG3 opsonising and complement fixing antibodies, and mediate protection against intracellular pathogens. On the other hand, Th2 cytokines induce IgE production by B cells and eosinophil differentiation and activation (Abbas *et al*, 1996).

It is now known that after a T-cell receptor (TCR) encounters the appropriate peptide-MHC complex, clonal expansion is triggered and the helper T cells undergo rapid differentiation into one of at least 2 functional phenotypes (Th1 or Th2). Th1 cells are responsible for cell-mediated immunity, and in terms of control of infection, this is thought to provide protection against intracellular pathogens, such as mycobacteria and *Leishmania spp*, as previously mentioned. Broadly speaking, Th2 cells are considered to be responsible for extracellular immunity, often against helminth infections (Janeway, 2001). The effects of Th1 and Th2 responses on macrophage phenotype will be discussed below. As well as roles in host defence against pathogens, both types of T helper cell have been implicated in pathological immune responses. Th1 cells have been found to be involved in organ-specific autoimmunity and Th2 cells have been implicated in the pathogenesis of asthma, allergy and fibrosis (Murphy & Reiner, 2002; Wynn, 2004). This immune dichotomy is not so clear-cut, however, and further CD4+ T cell populations exist.



Figure 1. Key transcription factors and cytokines involved in Th1/Th2 polarisation

Modfied from: Neurath, M.F., Finotto, S. and Glimcher, L.H. (2002) The role of Th1/Th2 polarization in mucosal immunity. *Nature Medicine*. 8 567-573

2.2 Regulatory T cells

Regulatory T cell (Tregs) subsets have been described that have roles in immunomodulation and protection against self-antigens (Figure 2) (O'Garra et al. 2004). Tregs are a heterogeneous family of CD4+ T cells that are involved in regulating both Th1 and Th2 responses. Naturally occurring CD4+ Tregs arise during T cell development in the thymus and constitute approximately 10% of peripheral CD4+ T cells (Jordan et al, 2001; Walsh et al, 2004). This population can be defined by constitutive expression of the α chain of the IL-2 receptor, CD25 (Sakaguchi et al, 1995). Cell surface markers such as CTLA-4, glucocorticoid-induced TNF receptor family-related receptor (GITR), OX40 and CD62L have been identified and relative expression levels used to define and isolate CD4+CD25+ Tregs. However, as with CD25, none of these markers alone represent a definite marker for naturally occurring Tregs. More recently, a molecule known as Foxp3 has been shown to be uniquely expressed by these Tregs and is thought to be an important transcription factor in controlling Treg differentiation (Fontenot et al, 2003; Hori et al, 2003; Walsh et al, 2004). These Tregs are thought to exert their immunosuppressive effect on T cell proliferation in vitro through a contact-dependent mechanism that is also largely cytokine dependent. CTLA-4 and GITR expressed on the surface of these Tregs have been implicated to play a role in this suppression (Shimizu et al, 2002; Takahashi et al, 2000). In in vivo models, blockade of both IL-10 and TGF-B have been reported to reverse the suppressive phenotype of these Tregs (Walsh et al, 2004).

Type 1 regulatory (Tr1) cells are an inducible population of T cells that specifically produce IL-10 and, to a lesser extent, TGF- β (Battaglia *et al*, 2004; Groux *et al*, 1997; van Roon *et al*, 2006). They are induced *in vitro* by stimulation with IL-10 and mediate their suppression primarily by an IL-10-dependent pathway. Tr1 cells have been shown to be involved in preventing the development of both Th1-mediated experimental

autoimmune diseases (Groux *et al*, 1997), as well as Th2-mediated allergy (Cottrez *et al*, 2000; van Roon *et al*, 2006). Th3 cells are another subset of regulatory T cells that produce large amounts of TGF- β and have been shown to suppress experimental autoimmune encephalomyelitis (EAE; mouse model of multiple sclerosis) induction. Th3 cells were originally generated and identified in mice orally tolerised to myelin basic protein (MBP) (Chen *et al*, 1994). They are induced *in vitro* upon mitogen stimulation in combination with IL-2 and TGF- β .

2.3 Th17 Cells

Most recently, another subset of effector CD4+ T cell has been described distinct from Th1 or Th2 cells. They produce IL-17 and are known as Th17 cells. These cells have a suggested role in certain inflammatory and autoimmune diseases, such as systemic lupus erythematosus and EAE (Romagnani, 2006). It is also thought that IL-17 may play an important role in the protection against extracellular bacteria, since this cytokine induces the recruitment of neutrophils (Mangan et al, 2006; Romagnani, 2006; Steinman, 2007). Th17 cells are activated by the combined activity of IL-6 and TGF-β production by DCs and maintained by IL-23 (Bettelli et al, 2006). Th17 cells do not express either T-bet nor GATA-3 transcription factors but upon stimulation with IL-6 and TGF- β the orphan nuclear receptor RORyt is expressed. (Ivanov et al, 2006). TGF-ß is also involved in the generation of Tregs, but IL-6 has been shown to inhibit their development. This suggests that the presence/absence of IL-6 will dictate whether a pathogenic Th17 response will be generated inducing autoimmunity, or Treg cells initiated inhibiting autoimmune disease (Romagnani, 2006). IL-4 and IFN-y also inhibit the development of Th17 cells (Iwakura & Ishigame, 2006) but it is uncertain what the effects of Tregs are on this subset (Figure 2).

6





Modfied from Romagnani, S. (2006). Regulation of the T cell response. Clin. and Exp. Allergy, 36, 1357-1366

3. The Th2 Cytokines IL-4 and IL-13

The Th2 cytokines IL-4 and IL-13 have many roles in immunity including mediating resistance to many gastrointestinal parasites, promoting allergic inflammation, fibrosis and asthma (Chiaramonte *et al*, 1999; Grunig *et al*, 2002; Padilla *et al*, 2005; Wills-Karp *et al*, 1998). These cytokines have overlapping and also distinct properties. Their unique properties can be shown in asthma and parasite models where IL-13 plays a more important role than IL-4 in the development of airway hyperresponsiveness (AHR) (Wills-Karp, 1999), pulmonary fibrosis (Zhu *et al*, 1999) and the expulsion of *Nippostrongylus brasiliensis* (Urban *et al*, 1998). IL-4 plays a central role in Th2 cell development. IL-4 and IL-13 exert their effects on many different cell types, including macrophages (see below Alternatively Activated Macrophages), fibroblasts, eosinophils, mast cells, B and T cells (Ramalingam *et al*, 2008; Wynn, 2004).

IL-4 and IL-13 bind to, and send signals through, receptors composed to various combinations of four receptor subunits: IL-4R α , IL-13R α 1, IL-13R α 2 and the common γ -chain (γ c) (Ramalingam *et al*, 2008) (Figure 3). IL-4R α can pair with the γ c to form the type 1 IL-4 receptor (IL-4R) or with IL-13R α 1 to form the type 2 IL-4R. IL-4 can signal through both the type 1 and type 2 receptors, whereas IL-13 can only signal through the type 2 receptor (Murata *et al*, 1999). It is thought that the type 1 IL-4R mediates STAT6 signalling in haematopoietic cells and is responsible for the expansion of CD4+ Th2 cells. The type 2 IL-4R is thought to be the main route of STAT6 signalling in nonhaematopoietic cells (Murata *et al*, 1999; Ramalingam *et al*, 2008). IL-13R α 2 binds IL-13 with high affinity, lacks a signaling motif and exists in membranebound and soluble forms. These findings have led to the belief that IL-13R α 2 is a decoy receptor for IL-13 (Chiaramonte *et al*, 2003). Indeed, it has been found to be an inhibitor of IL-13-induced inflammatory and remodeling responses in a murine ova-inflamed lung (Zheng *et al*, 2008).



Figure 3. Receptors for IL-4/IL-13

4. Parasitic Helminths and the Th2 Response

Even though individual species of helminth worms may be very distantly related to each other, inhabit distinct habitats within their hosts and have a range of different strategies of infection/evasion (Knoll & Carroll, 1999; Maizels *et al*, 2004) they consistently induce Th2 immune responses in their hosts (Maizels *et al*, 2004; Maizels *et al*, 1993;

Sher & Coffman, 1992). The mechanisms whereby these multicellular parasites drive a Th2 bias have not been clearly defined and a combination of factors are likely involved. These factors may include parasite antigens present on the surface, or excreted/secreted from the worm, that may cause signalling through pattern recognition receptors (PRR)s (discussed in more detail below) on cells of the innate immune response and mediate the early skewing towards the type-2 response (Tawill et al, 2004). For example, it has been shown that the excretory-secretory products from Nippostrongylus brasiliensis (NES), which are made up of glycoproteins collected from adult worms cultured in vitro, can drive Th2 responses in mice without the requirement of infection with the helminth (Holland et al, 2000). Also, soluble extracts of the filarial nematode Brugia malayi induce Th2 responses and this has been shown to depend on the presence of intact glycans (Tawill et al, 2004). Schistosome soluble egg antigens (SEA) are strong activators of Th-2 responses. Carbohydrates present on these antigens have been found to be important for this process (Pearce & MacDonald, 2002). Lacto-N-fucopentaose III in particular acts as a Th2 adjuvant (Okano et al, 2001). Proteins, such as protease enzymes (potentially produced by helminth parasites) have also been implicated in driving Th2 responses (Sokol et al, 2007).

Signalling through PRRs could be responsible for the early and abundant production of IL-4, possibly from NK cells, in the response to helminths, or their extracts (Balmer & Devaney, 2002; Holland *et al*, 2000; Medzhitov & Janeway, 2000; Osborne & Devaney, 1998; Sabin *et al*, 1996). More recently eosinophils, basophils and mast cells have been implicated as early sources of IL-4 (Loke *et al*, 2007; Sokol *et al*, 2007; Voehringer *et al*, 2006; Voehringer *et al*, 2004). CD4+ T cells activated in the presence of this early IL-4 tend to differentiate into Th2 cells. A Th2 immune response is then mounted which typically involves the production of more IL-4, and also IL-5, IL-9 and IL-13, among others. IL-10 is also secreted by this subset of T helper cells (also secreted by Th1 cells, T regulatory cells, B cells and macrophages; (O'Garra & Vieira, 2007). Th2 cells promote the production of immunoglobulin (Ig) G1 and IgE from B cells and, also the

10

mobilisation of specific effector cells, e.g. macrophages, eosinophils, mast cells and basophils (Maizels *et al*, 2004).

Although Th2 responses are protective for most infections with gastrointestinal nematodes in mice, such as with N. brasiliensis, their role in tissue infection is less clear-cut. Th2 response are protective in the gut since type 2 cytokines, such as IL-4 and 13, act on mast cells and goblet cells to induce expulsion of the parasite through, for example, mucus production and muscle contraction (Maizels & Yazdanbakhsh, 2003; Nawa et al, 1994; Urban et al, 1998). It has been suggested that helminths have evolved mechanisms to induce Th2 immunity in order to direct a response that benefits both parasite and host. The benefit to host may be protection against immune pathology due to Th1 cell mediated inflammation (MacDonald et al, 2002). Another idea proposed is that Th2 responses have evolved in mammals to be mounted upon innate recognition of worm molecules (shared among many taxa) and the parasites have evolved to downregulate the host immunity in order to evade this response (discussed in the following paragraph). The ideal Th2 response for the host is one that contains the parasite, while at the same time, healing tissue damaged by these tissue migratory mutlicellular animals (Diaz & Allen, 2007) (Maizels & Yazdanbakhsh, 2003). Indeed, the Th2 response induces wound-healing functions in certain cell types, for example macrophages (Goerdt et al, 1999; Wynn, 2004).

Parasitic helminths, as opposed to microbial intracellular pathogens, are large multicellular animals and are able to live for decades within a host. More than a third of the world's population are infected with parasitic helminths and this can be the cause of severe morbidity and disability. Although severe pathology, such as granulomas and organ failure can occur, it is more common for infections not to be overtly symptomatic (Maizels & Yazdanbakhsh, 2003). This is because many helminth infections are associated with a suppression of the host immune response and the long lifespan of these infections may be due to their ability to modulate or turn off their host's protective immunity. Also, although Th1 and Th2 responses are required for resolution of various

infections, an exaggeration of either one can be damaging to the host (Specht et al, 2004). Regulatory T cells produced by the host have been shown to be involved in downmodulating inflammatory and protective immune responses through production of IL-10 and TGF- β (Maizels *et al*, 2004). Suppressive macrophages have also been shown to be induced by nematode infection, as will be discussed in more detail below (Loke et al, 2000b). Helminths can also modulate the immune response in many other ways. They may be able to exploit TGF- β mediated immune downregulation by secreting homologues to this cytokine, such at the B. malayi TGF-B-like protein BM-TGH2 (Gomez-Escobar et al, 1998). They may also inhibit the recruitment of immune cells from the blood. For examples, some hookworms produce proteases that degrade eotaxin (Culley et al, 2000; Maizels & Yazdanbakhsh, 2003). The most well characterised example of nematode mediated suppressive agent is probably ES-62, a glycoprotein produced by filarial parasites that has recently been demonstrated to have therapeutic potential in allergic diseases, such as asthma. ES-62 has been found to suppress mast cell function by blocking important signal transduction events through the Fce receptor and this action is protective against mast cell-dependent hypersensitivity (Melendez et al, 2007).

5. Lymphatic Filariasis

The work of the Allen parasite immunology lab involves the study of the interactions between nematode parasites and their hosts. The group is particularly interested in filarial nematodes that live in or migrate through the tissues, such as those that cause lymphatic filariasis (Elephantiasis). Filariasis is a mosquito-borne disease that currently affects approximately 120 million people in tropical regions. This thesis has mainly been concerned with *Brugia malayi* infection settings.

Lymphatic filariasis has a large economic impact. As well as the direct expense due to medical treatment, communities are indirectly affected by the cost associated with reduced work capacity and labour loss. In fact, this disease has been identified by the World Health Organisation (WHO) as the second leading cause of long-term and permanent disability in the world (WHO, 1997). The nematode *Brugia malayi* is found in Southeast and Eastern Asia and *Brugia* spp. are thought to account for about 10% of cases of lymphatic filariasis, with *Wucheria bancrofti* responsible for the remaining 90% (Melrose, 2002). It has been estimated that 44 million people have overt clinical disease due to filarial infection including lymphoedema and elephantiasis (WHO, 1997).

Infection of human hosts occurs when an infected mosquito pierces the skin. The thirdstage larvae (L3) of the parasite enter the body and migrate through the tissue subcutaneously to the nearest lymphatic vessel. They mature by moulting to become fourth stage larvae at around 7 days post-infection (p.i.) and then moult to adults by around 30 days p.i. The male and female adults mate within the lymphatics and begin to produce millions of microfilariae (Mf) by 60 days p.i. These are released out of the lymphatic system and pass into the bloodstream of the host (Freedman, 1998), from which some are taken up by feeding mosquitoes. The Mf mature in the mosquito when they leave the mid-gut and move to the flight muscles. Here they become L3s and move to the mosquito's proboscis where they are passed to the next host during a blood meal (outlined in Figure 4) (Lawrence, 2001).

The average life span of filarial worms in infected humans is thought to be 8-16 years. The established view is that three groups of people are to be found in regions endemic for filarial nematodes (Figure 5). Firstly, 'endemic normals' are those who have been exposed to these helminths but have no evidence of disease, including no microfilariae in the bloodstream and no clinical evidence of infection. Secondly, there are those individuals with circulating microfilariae (Mf+) but no disease symptoms. This 'asymptomatic microfilaraemia' is often the most common manifestation of filariasis. This form of filarial infection may not be quite so 'asymptomatic', however, as recent



Figure 4. Life cycle of Brugia malayi

Thanks to Henry McSorley for this figure

studies have shown that large-scale cryptic lymphatic and tissue damage may be occurring in the absence of obvious disease symptoms (Freedman *et al*, 1994; Melrose, 2002). Lastly, there are those with chronic disease such as lymphoedema and elephantiasis. These people are generally without circulating microfilariae and are thought to be resistant to L3s and some may even be able to kill adult nematodes. However, this immunity appears to come with immunopathological consequences. Individuals in all three groups may also suffer from episodic acute filarial disease, which includes debilitating fevers and lymphadenitis (Lawrence, 2001; Melrose, 2002; WHO, 1997).





Modified from: Lawrence, R.A. (2001) Immunity to filarial nematodes. Vet. Parasit. 100,33-44

It has been found that Mf+ patients have extremely raised levels of IL-4 driven filiarialspecific IgG4, which can be as high as 95% of serum antibody (as opposed to 5% in uninfected individuals). IgE levels, also upregulated by IL-4, are raised in those with chronic pathology. Mf+ people also have raised IgE levels but have higher IgG4: IgE ratios than those with chronic pathology, suggesting that relative IgE levels may have a role in this filarial-induced pathology. It may be that Mf- individuals with chronic filarial pathology and lots of IgE may have killed their adult parasites. The antibody isotypes IgG2 and IgG3 have also been associated with the chronic pathology of elephantiasis (Lawrence, 2001).

When peripheral blood mononuclear cells (PBMC) are taken from actively infected individuals (Mf+), an immunosuppressive response is seen. Their PBMC fail to proliferate *in vitro* in response to filarial antigen. PBMC taken from endemic normals and those with chronic pathology do usually proliferate. Although PBMC from Mf+ individuals do not proliferate, they do produce cytokine and IL-4 responses tend to predominate. There is little IFN- γ produced. IFN- γ : IL-4 ratios are high in patients with chronic pathology but low in Mf+ individuals. Thus, downregulation of IFN- γ seems to be a critical factor in the maintenance of filarial infection. There is also evidence that IL-10 plays a key role in immunosuppression by filarial nematodes, which may or may not come from regulatory T cells (Lawrence, 2001).

B. malayi harbour the endosymbiont *Wolbachia* that is an obligate intracellular bacterium most closely related to *W. pipientis* of arthropods (Bandi *et al*, 2001). They play an important role in the biology of filarial nematodes, with roles in sex determination and speciation. Targeting this bacterium with antibiotics also results in decreased fecundity, impaired molting and even worm death both *in vitro* and *in vivo* (Hise *et al*, 2007). *Wolbachia* bacteria are present in large numbers throughout all life stages of filiarial parasites of humans. This suggests that the host will be exposed to this endosymbiont following the death of the helminth or release of bacterial products (Taylor *et al*, 2001). Therefore, it is thought that individuals infected with *B. malayi* may respond to these bacteria, as well as to nematode products, and that *Wolbachia* can initiate or promote inflammation (Hise *et al*, 2007). Inflammatory responses can also be caused by filarial chemotherapy, which causes large quantities of parasite material to be released. The key pro-inflammatory cytokines IL-1β and TNF- α are produced mainly by macrophages and these potentiate further inflammation caused by *B. malayi* is mediated by

LPS-like activity from *Wolbachia* (Daehnel *et al*, 2007; Hise *et al*, 2007; Taylor *et al*, 2000). This idea will be discussed further in chapter 6 of this thesis.

Although mice cannot support the full developmental cycle of *Brugia malayi*, single stage, short-term infections of mice have proven to be useful in dissecting immune responses to these filarial parasites. Indeed, these types of experiments have shown that L3 and adult stages of the parasite induce a Th2 response, whereas Mf injections primarily induce a Th1 response and only after prolonged infection induce Th2 cytokines, such as IL-4, as well as IFN- γ (Lawrence *et al*, 1994). We have been using a mouse model of infection with *B. malayi* to understand host-parasite interactions and aspects of immunomodulation during filariasis. In order to do this, mice are surgically implanted with adult *B. malayi* filarial worms into the peritoneal cavity (Figure 6). These parasites survive for several weeks and result in recruitment of immune cells to the peritoneal cavity, of which approximately 70% are macrophages (MacDonald *et al*, 1998). My work has involved the investigation of macrophages activated in the type-2 environment brought on by this helminth, which have been termed nematode-elicited macrophages (NeM ϕ ; Loke *et al*, 2000b).

Figure 6. *Brugia malayi* implant model for the generation of NeMo



5 adult *B. malayi* worms

6. Macrophages

Macrophages are present in every tissue in the body. For example, they are found in the liver as Kupffer's cells and in the lung as alveolar macrophages, and indeed as the most abundant mononuclear cell in the intestines. Macrophages are mononuclear phagocytes with various roles in homeostasis, immunological processes, as well as in inflammation (Sasmono & Hume). Since they are so widely distributed in the body, they provide a first line of defence against invading organisms. As part of innate (non-specific) immunity they have the ability to recognise, engulf and kill potential pathogens such as mycobacteria and intracellular protozoan parasites such as Leishmania (Chan et al, 1992; Reiner & Locksley, 1995). They may also be involved in protection against larger invading organisms such as helminths, as has been found in vitro (Taylor et al, 1996; Thomas et al, 1997). Macrophages can also cause the destruction of tumour cells through the production of tumour necrosis factor (TNF)- α and nitric oxide (NO) (Duerksen-Hughes et al, 1992). They can also recognise some virus-infected cells as well as cells undergoing apoptosis (Henson et al, 2001; Sasmono & Hume). Macrophages also act as professional scavengers, phagocytosing microbes, apoptotic and necrotic cells, modified lipoprotein particles, and thus play important roles in homeostasis, as well as immunity (Ricote et al, 2004).

Through their function as antigen presenting cells (APC), macrophages bridge the gap between the innate and adaptive (specific) immune responses (Unanue, 1984). Upon phagocytosis, macrophages break down proteins and process the antigens for presentation on MHC molecules, where T-helper cells can recognise the substances as "foreign" and mount an adaptive immune response (Brodsky & Guagliardi, 1991). T cells require this antigen-specific signal through their T cell receptor (TCR) but also a second co-stimulatory signal for optimal T-cell activation. Co-stimulatory signal pathways promote the proliferation, effector function and cytokine production of T cells. One example is the B7-CD28 pathway, which provides essential signals for T cell activation. Inducible co-stimulatory molecule (ICOS) has also been found to be essential
for T cell activation and function (Dong *et al*, 2001) and it is thought to promote T cell/B cell collaboration through the CD40/CD40L pathway (McAdam *et al*, 2001). Macrophages also provide additional signals for the process of T cell activation. For example, if antigen presentation on the surface of infected macrophages (and other APCs, in particular dendritic cells; DCs), is in the context of MHC class II, along with Interleukin (IL)-12 from these APCs, this stimulates Th1 (type 1 helper t-cells) to proliferate (Trinchieri, 1994). The link between innate and adaptive immunity comes full circle when signals, such as Interferon (IFN)- γ , that macrophages receive from activated T cells allow them to become effector cells (see below "classical activation of macrophages") (Dalton et al, 1993).

Since macrophages participate in both specific immunity via antigen presentation and nonspecific immunity against bacterial, viral, fungal, and protozoan pathogens, it is not surprising that macrophages display a range of functional and morphological phenotypes. Macrophages develop from blood monocytes, which also give rise to dendritic cells and osteoclasts. Although monocytes are often considered immature macrophages, they already possess migratory (Imhof & Aurrand-Lions, 2004), chemotactic (Marra *et al*, 1999), pinocytic and phagocytic activities, as well as receptors for IgG Fc-domains (Fc γ R) (Wyss *et al*, 1990) and iC3b complement (Payne & Horwitz, 1987). Monocytes originate in the bone marrow from a common myeloid progenitor shared with neutrophils. Monocytes undergo further differentiation (at least one day) to become multifunctional tissue macrophages and it is signals that they encounter during migration to inflamed/infected sites in tissues of the body that induce highly distinct phenotypes and functions (Gordon, 2003; Gordon & Taylor, 2005).

Figure 7. Pathways of macrophage activation



Modified from: Gordon, S. (1999) Chap. 15. Macrophages and the immune response. In Fundamental Immunology. W.E. Paul, ed. (Lippincott-Raven, Philadelphia) pp. 533-545.

AND Gordon, S and Taylor, P. R. (2005). Monocyte and macrophage heterogeneity. *Nat. Rev.* 5, 953-964. AND Martinez, F. O., Sica, A., Mantovani, A. and Locati, M. (2008). Macrophage activation and polarization. *Front. Biosci.*, 13, 453-461.

7. The Th2 Response and Macrophage Activation: Alternatively Activated Macrophages (AAMφ)

The function of macrophages once they reach the tissues is determined by signals they receive from the local environment. Possibly the most important of these are cytokine signals from T helper cells also recruited to the site of infection or injury. Macrophages activated by the Th2 cytokines IL-4 and IL13 have been termed alternatively activated macrophages (AAM or, Figure 7) (Gordon, 2003). More recently, the IL-21 receptor has also been identified as an important amplifier of the AAM phenotype, mainly by increasing IL-4Ra and IL-13Ra expression (Pesce et al, 2006). The importance of AAM of in type 2 conditions is strongly suggested by their prevalence in chronic Th2type inflammatory conditions such as helminth infection (Loke et al, 2000b; Nair et al, 2005; Rodriguez-Sosa et al, 2002) and allergy (Holcomb et al, 2000; Lee et al, 1999; Zimmermann et al, 2003). The first report that suggested the use of the term "alternative activation" came from Siamon Gordon's lab to describe cellular changes to macrophages induced by IL-4 that were distinct from those induced by IFN-7 (Stein et al, 1992). Both of these cytokines had been previously shown to upregulate MHC class II molecules on macrophages, but whereas IFN-y had been shown to downregulate murine mannose receptor (MMR) expression (Mokoena & Gordon, 1985), this in vitro study described IL-4-mediated upregulation of the MMR on elicited peritoneal macrophages (Stein et al, 1992). Prior to these studies, the effects of IL-4 and IL-13 were grouped with IL-10, as "deactivating" macrophages. Gordon and colleagues demonstrated that this is not the case. They showed that IL-4 and IL-13, in contrast to IL-10, were "activating" and stimulated antigen presentation through upregulation of MHC class II, co-stimulatory molecules and mannose receptor expression (Gordon, 2003).

A few years later, Modolell and colleagues showed that the enzyme arginase (later found to be the isoform arginase 1 (Munder *et al*, 1999)) can be another marker of alternatively activated macrophages as it is potently induced by IL-4 in bone marrow-derived macrophages (BMM ϕ) (Modolell *et al*, 1995). There are two isoforms of arginase in vertebrates with distinct expression profiles. Arginase 1 is found in the cytosol of cells and is predominantly expressed in the liver of naïve mice and it has an important role in the urea cycle. Arginase 2 is a mitochondrial enzyme and is expressed in tissues such as the brain and kidneys at low levels, but its function is not well characterised (Mori & Gotoh, 2000). Macrophages can express both arginase 1 and 2, and expression of both isoforms may be driven by Lipopolysachharide (LPS). However, only arginase 1 has been shown *in vitro* to be inducible by IL-4 and IL-13 in a Stat6 dependent manner (Louis *et al*, 1999; Nair *et al*, 2006). Work from the Allen lab using a murine model for filarial infection with *B. malayi* implantation as a source of macrophages, has shown that *Arginase 1* is an IL-4 dependent gene *in vivo* as NeM ϕ from IL-4-/- mice are impaired in their ability to produce *Arginase 1* mRNA (Loke *et al*, 2002).

As well as Arginase 1, two other abundantly expressed genes are found expressed by NeM ϕ from *B. malayi* infection, which are also highly dependent on IL-4 *in vivo*, *Ym-1* and *RELM-* α (Loke *et al*, 2002). Ym-1 and RELM- α can also be upregulated in macrophages *in vitro* by IL-4 and IL-13 (Edwards *et al*, 2006; Nair *et al*, 2003; Raes *et al*, 2002). Since then the expression of Arginase 1, YM-1 and RELM- α have been found to be a generalised feature of helminth infection. These proteins are induced at the sites of infection with the nematodes *Litomosoides sigmodontis* and *N. brasiliensis* (Nair *et al*, 2005; Pesce *et al*, 2006; Reece *et al*, 2006; Taylor *et al*, 2006) and *Heligmosomoides polygyrus* (Anthony *et al*, 2006). Expression of these AAM ϕ markers has also been associated with the platyhelminthic infections of *Schistosoma mansoni* (Pesce *et al*, 2006) and *Taenia crassiceps* (Terrazas *et al*, 2005).

Ym-1 is a secreted 45-kDa chitinase-like lectin found in mice (Chang *et al*, 2001; Jin *et al*, 1998). Chitinase enzymes, with known protective function against chitin containing pathogens, have been well studied in plants, fish and microbes. Chitinase genes have been identified in mammals and are together known as the chitinase-like mammalian protein family (Boot *et al*, 2001). Of these, only two functional chitinases have been

uncovered; chitotriosidase (Renkema *et al*, 1995) and acidic mammalian chitinase (Boot *et al*, 2001). Ym-1 is member of this family but lacks chitinase function (Jin *et al*, 1998). RELM- α (Resistin-like molecule; also known as Fizz1 (found in inflammatory zone), is a member of the RELM family of proteins, which share sequence homology to resistin, a protein secreted by adipocytes that can regulate responsiveness to insulin (Steppan *et al*, 2001).

8. The Functions of AAMφ8.1 Wound Healing

Increasing evidence suggests involvement of AAM oin wound healing and tissue remodelling (Loke et al, 2007; Sandler et al, 2003). They may also play a role in tumorigenesis (Liu et al, 2003; Sinha et al, 2005) and fibrotic scarring (Hesse et al, 2001) both of which have been described as wound-healing gone awry (Wynn, 2004). Larginine metabolism through arginase enzyme activity can eventually lead to the generation of polyamines and prolines (see iNOS/Arginase below and Figure 3). Proline has a role in collagen production and polyamines in cell proliferation (Albina et al, 1993; Bronte & Zanovello, 2005; Igarashi & Kashiwagi, 2000; Jackson et al, 1986). These properties originally lead to the hypothesis that AAM are involved in wound healing (Goerdt et al, 1999; Hesse et al, 2001; Wynn, 2004). Helminths are multicellular organisms and there is the potential for damage to tissue of infected hosts. Therefore, it is perhaps not surprising that an immune response would be induced with tissue repair and remodelling as an important function. For example, N. brasiliensis has a migratory route through the lung and this causes substantial damage in the form of pulmonary haemorrhaging, which is quickly repaired (McNeil et al, 2002). Indeed, Ym-1, RELM- α and Arginase 1 gene expression, as well as known markers of lung remodelling such as Elastin and Firbronectin 1, are rapidly upregulated in the lung 2-4 days after infection

with *N. brasiliensis* indicating a role for AAM ϕ in innate immunity and repair (Reece *et al*, 2006).

The induction of AAM ϕ also appears to be an innate response to injury in the *B. malayi* implant model (Loke *et al*, 2007). Early IL-4 or IL-13 production has been found to be essential for AAM ϕ induction, but use of RAG-deficient animals has shown that CD4+ Th2 cells are not required for this early response to tissue damage. Mast cells were proposed as a possible source of this early IL-4/13 (Loke *et al*, 2007). Other evidence that AAM ϕ are involved in the response to tissue injury comes from the possible functions of highly expressed proteins Ym-1 and RELM- α . Ym-1 has been shown to bind to the glycan heparin, which is abundant on cell surfaces and extra-cellular matrix. Through this binding it is possible that Ym-1 may have a role in remodelling of tissue by coordinating cell-to-cell and cell to matrix contacts (Chang *et al*, 2001; Nair *et al*, 2006). RELM- α has been found associated with fibrosis in the lung (Liu *et al*, 2004). This protein has mitogenic properties and so may be involved in causing this fibrosis through inducing the proliferation of smooth muscle cells and actin production (Teng *et al*, 2003). RELM- α also has angiogenic properties (Teng *et al*, 2003), an essential component of wound healing.

8.2 Regulation

AAM ϕ have long been considered "anti-inflammatory" or downregulatory but direct evidence for this was scarce. A role for AAM ϕ in immune regulation has been demonstrated by an experimental model using conditional gene knockout mice in which IL-4R α chain is absent in macrophages and neutrophils. AAM ϕ were shown to be essential for protection against organ injury during schistosomiasis. They may help to protect against hepatocellular damage during schistosomiasis by regulating nitric oxide production (see section 9. iNOS/Arginase). Therefore, data from this paper suggest that, in contrast to CAM ϕ , one function of AAM ϕ is the regulation or dampening down of the immune response (Herbert *et al*, 2004). In relation to this, work from our lab has shown that NeM ϕ from *B. malayi*-implanted mice are potent suppressors of proliferation of not just immune cells (Allen *et al*, 1996) but a range of murine and human tumour cell lines. This suppression is dependent on IL-4 and is mediated by cell-to-cell contact (Loke *et al*, 2000b; MacDonald *et al*, 1998). NeM ϕ from a *L. sigmodontis* infection model have also been shown to have suppressive properties, which is at least partially dependent on TGF- β expression (Taylor *et al*, 2006). The suppression associated with AAM ϕ from the platyhelminthic infections with *T. crassiceps* (Terrazas *et al*, 2005) and *S. mansoni* (Smith *et al*, 2004) is associated with the upregulation of programmed death ligand 1 (PD-L1) on the macrophages. In both cases, blockage of PD-L1 blocked the ability of these AAM ϕ to dampen down T cell responses (Smith *et al*, 2004; Terrazas *et al*, 2005). However, other mechanisms have also been proposed, including the production of 12/15-lipoxygenase, reactive oxygen species (Brys *et al*, 2005) and arginase 1 (chapter 2 of this thesis). These suppressive mechanisms are outlined in Figure 8.

8.3 Effector Function

Anthony *et al.* have shown AAM ϕ to be effector cells in the protective memory response to the helminth parasite *H. polygyrus*. During this infection, AAM ϕ have a role in parasite expulsion, by impairing larval health and mobility in an arginase-dependent manner. Animals, in which the AAM ϕ population have been depleted with clodronate liposomes, cannot expel the parasite compared to PBS liposome treated controls (Anthony *et al*, 2006). Effector function is also suggested by the finding that in *S. mansoni*-infected mice AAM ϕ are associated with the egg-induced liver granulomas. However, this same paper showed that AAM ϕ were not required to the expulsion of *Nippostongylus brasiliensis* (Herbert *et al*, 2004). They may also indirectly cause killing of helminth parasites through the recruitment of eosinophils, as mice deficient for



AAM ϕ had reduced recruitment of these effector cells when implanted with *B. malayi* (Loke *et al*, 2007). Related to this, Ym-1 may have eosinophil chemotactic properties (Owhashi *et al*, 2000). It has also been suggested that RELM- α , due to its fibrotic properties, may play a role in the killing of helminth parasites by sequestering them in tissues, thus reducing their motility and facilitating killing by effector eosinophils or neutrophils at the site of infection (Nair *et al*, 2006). RELM- α could also contribute to expulsion of worms from the gastro-intestinal tract through its ability to regulate nerve

growth factor (NGF) and possibly influencing enteric nerve cell function (Zhao *et al*, 2003). With the exception of the Anthony study, all this data is circumstantial and definitive evidence for AAM ϕ involvement in killing of helminth parasites is still needed.

9. The Th1 Response and Macrophage Activation: Classical Activation of Macrophages (CAMφ)

Classical activation is the archetypal mode of activation of macrophages (Figure 2). It is well defined and is dependent on the products of activated Th1 lymphocytes and natural killer cells, in particular IFN- γ (Dalton *et al*, 1993; Wherry *et a*l, 1991). Other factors involved in this activation include danger signals, such as heat shock proteins (Byrd *et al*, 1999; Gordon, 2003) and innate recognition of microbial pathogen-associated molecular patterns (PAMPS; e.g. LPS) (Aderem & Ulevitch, 2000). Toll-like receptors (TLRs) that recognise PAMPs such as LPS as part of the innate immune system (Rock *et al*, 1998) will be discussed in more detail in chapter 6.

Classically activated macrophages (CAM ϕ) are the major effector cells of the Th1 arm of the immune response and their prime function is microbial destruction. Activated murine macrophages generate a variety of oxygen- and nitrogen-derived radicals with anti-microbial properties (Chanock *et al*, 1994; Nathan, 1983). The enzyme complex of NADPH oxidase produces reactive oxygen intermediates via reduction of molecular oxygen, such as hydrogen peroxide (H₂0₂), hydroxyl radicals (OH·) and superoxide anion (O₂⁻) (Wientjes & Segal, 1995). Inducible nitric oxide synthase (iNOS/NOSII) produces the primary reactive nitrogen intermediate, NO (Mori & Gotoh, 2000). These oxygen and nitrogen radical-based cytotoxic pathways can be triggered by proinflammatory cytokines, such as IFN- γ and TNF- α (Ding *et al*, 1988). Destruction of intracellular microbial parasites such as *Leishmania* spp. and *Mycobacterum bovis* (BCG) are know to require the classical activation of macrophages by a Th1 response. Leishmanicidal activity is known to require NO generation by CAM ϕ (Evans *et al*, 1993; Lemesre *et al*, 1997; Liew *et al*, 1990). Mycobacteria that go on to survive and replicate within macrophages are thought to do so by a variety of strategies, including the avoidance of killing by reactive oxygen (Manca *et al*, 1999) and nitrogen intermediates (Yu *et al*, 1999). A strong early Th1 response is also mounted towards the protozoan parasites of the *Plasmodium* genus. IFN- γ , and CAM ϕ induction by this cytokine seems to be important in the initial phase of infection with malaria parasites (Li *et al*, 2001). Infections with these microbial pathogens will be discussed further in the subsequent chapters.

10. iNOS and Nitric Oxide (NO)

iNOS (or NOS2) has been found in macrophages from human, cow, sheep, mouse and chicken (MacMicking *et al*, 1997). NO is produced by this enzyme from the substrate L-arginine and is a lipid and water-soluble radical gas. iNOS oxidises L-arginine in two steps; L-arginine is hydroxylated firstly to N^{∞}-hydroxyl-L-arginine (NOHA), which is then oxidised to L-citrulline and NO (Figure 9). NO reacts with oxygen in water and its reactive intermediates to produce other radicals such as NO₂, and other conversion products such as NO₂⁻ (nitrogen dioxide), NO₃⁻ (nitrate) and ONOO⁻⁻ (peroxynitrite anion) (MacMicking *et al*, 1997; Stamler, 1995). In infected individuals, NO has be shown to have antiviral, antimicrobial, pro- and anti-inflammatory, cytotoxic and cytoprotective effects and these has been shown to be due to iNOS-derived NO through the use of iNOS-/- mice (Bogdan *et al*, 2000a; Bogdan *et al*, 2000b; Hesse *et al*, 2000; Nathan & Shiloh, 2000).

The cytotoxic effects of NO are mediated by the inhibition of iron-containing enzymes such as mitochondrial electron transfer proteins (Nathan, 1992). These effects lead to the

mutation of DNA, inhibition of DNA repair and synthesis, inhibition of protein synthesis and inactivation of enzymes amongst other things (Bogdan *et al*, 2000a; DeGroote, 1999). In the case of *Leishmania* parasites, several parasite targets may be affected by NO toxicity including metabolic enzymes, such as GAPDH (glyceraldehydes-3-phosphate dehydrogenase) (Mauel & Ransijn, 1997) and aconitase (Lemesre *et al*, 1997) or cysteine proteinase (Salvati *et al*, 2001).

11. iNOS/Arginase

The iNOS/Arginase balance in activated macrophages remains the best way to differentiate between AAM and CAM Arginase 1, as well as iNOS, metabolises Larginine and the distinction between AAM and CAM can be best understood in terms of L-arginine metabolism (Figure 9). Under type 1 conditions, macrophages produce NO as a result of the up-regulation of iNOS, which is a catalyst of the L-arginine substrate. In AAMo, IL-4 or Il-13 upregulate the enzyme Arginase 1, which converts L-arginine to L-ornithine and urea (Munder et al, 1998). While the NO produced by CAM has antimicrobial and cytotoxic properties, L-ornithine produced by AAMo is the substrate for two additional enzymes, ornithine decarboxylase (ODC) and ornithine amino transferase (OAT) which generate polyamines and prolines respectively. As mentioned above, proline has a role in collagen production and polyamines in cell proliferation (Albina et al, 1993; Bronte & Zanovello, 2005; Igarashi & Kashiwagi, 2000; Jackson et al, 1986), making it likely that AAMo are involved in wound healing (Goerdt et al, 1999: Hesse et al, 2001; Wynn, 2004). The induction of either arginase or iNOS is usually coupled with suppression of the opposing enzyme, indicating a competitive nature in these alternative states of macrophage metabolism (Modolell et al. 1995). Indeed, the intermediate iNOS pathway product, NOHA, is a competitive inhibitor of arginase (Hecker et al, 1995; Wu & Morris, 1998). Conversely, polyamines produced by arginase metabolism of L-arginine are known iNOS inhibitors (Blachier et al, 1997).

Figure 9. Difference in L-arginine metabolic pathways used by iNOS and Arginase and negative feedback of opposing pathways



Competition for the L-arginine substrate and other negative feedback loops also contribute to this suppression of opposing enzymes (Figure 9). Thus, the counter regulation of M ϕ phenotype occurs not only at the level of opposing T cell phenotypes (i.e. Th1 vs. Th2) but also at the level of the enzymatic pathway itself.

This separation of classical versus alternative activation of macrophages by their iNOS-Arginase balance may be an oversimplification. For example, LPS, often referred to as an inducer of Th1 cytokines, can activate both iNOS and arginase expression in macrophages (Bronte *et al*, 2003). Indeed, if both enzymes were to be co-expressed, kinetic considerations indicate that there would not be great affect on NO production. Even though arginase has a lower affinity for the substrate (K_m about 3000 fold that of iNOS; lower K_m = higher affinity) it catalyses the reaction (V_{max}) 1000-fold faster than iNOS, and this means that L-arginine can be metabolised by these two enzymes at approximately the same rate (Bronte & Zanovello, 2005; Fligger *et al*, 1999). Therefore, production of either or both of these enzymes would depend on levels of external signals the macrophages were receiving and availability of L-arginine.

12. Innate Recognition of Microbial Products

Whereas adaptive immunity is mediated by B and T cells, and is characterised by specificity and memory, the innate immune system is mediated by phagocytes, including macrophages, neutrophils and DCs, and has until relatively recently been considered non-specific. However, it has been shown that the innate immune system must be sufficiently specific in order to discriminate between self and pathogenic organisms (Akira & Hoshino, 2003; Medzhitov, 2001). The innate immune system detects the presence of pathogens following infection by recognising conserved motifs in the microorganism. PRRs of innate immunity recognise these molecules that are broadly shared amongst pathogens, and are distinguishable from host molecules, called pathogen-associated molecular patterns (PAMPs). PAMPs have common features that make them targets for the innate immune system. They must be essential for microbial survival, incapable of developing mutations and conserved between a given class of microorganism (Medzhitov, 2001). PAMPs are usually produced only by invading

microbes, although some PRRS may recognise unique molecules displayed on stressed or injured mammalian cells, such as heat shock proteins (Vabulas *et al*, 2001). Examples of PAMPS include LPS from the outer membrane of gram-negative bacteria (Ulevitch & Tobias, 1999), flagellin found in the flagella of certain bacterial species. (Hayashi et al, 2001) and double-stranded RNA, which is unique to many viruses at certain stages of their replication (Alexopoulou *et al*, 2001).

There are many kinds of PPR that are expressed in different locations. These locations include the cell surface, intracellular compartments and in blood and tissue fluids. PRRs are involved in opsonisation, phagocytosis, activation of complement cascades and proinflammatory signalling pathways, among other actions of innate immunity (Medzhitov, 2001). Secreted PPRs include mannan binding lectin (MBL), which recognises terminal mannose residues and is involved in the activation of lectin complement pathway (Holmskov, 2000), and LPS-binding protein (LBP), which, as the name suggests, recognises LPS (Wright *et al*, 1989). Among PRRs present on the cell surface are CD14, which is a cell surface receptor that also recognises LPS, and also peptidoglycan (Wright *et al*, 1990), and the macrophage mannose receptor (MMR), which recognises terminal mannose residues (Fraser *et al*, 1998). Intracellularly, nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRS) are found in the cytoplasm. NLRS include NOD-1 and NOD-2 that allow recognition of peptidoglycan components of bacteria and leads to the induction of proinflammatory cytokines such as IL-1, TNF- α and IL-12 (Inohara *et al*, 1999; Medzhitov, 2001).

Toll-like receptors (TLRs) are a family of PRRs that are present both intracellularly and on the cell surface of mammalian cells and recognise bacteria, viruses, fungi and protozoa (Creagh & O'Neill, 2006). Toll was originally identified in *Drosophila* as a gene involved in early development and later was found to be essential for anti-fungal immunity (Lemaitre *et al*, 1996). This discovery led to the search for Toll-related molecules in mammalian immune responses. At least 13 TLRs have been identified in humans and mice together, and the ligands for some have not been elucidated as yet (Bowie & Haga, 2005). TLRs are part of a superfamily along with IL-1 receptor (IL-1R) members (Rock *et al*, 1998) and are believed to function as dimers (mostly homodimers) (Xu *et al*, 2000). TLR4 was the first characterised mammalian Toll and it has an essential role in LPS-dependent responses (Hoshino *et al*, 1999). Other TLRs include TLR5 that recognises flagellin (Hayashi *et al*, 2001) and TLR9, which identifies bacterial DNA (unmethylated CpG DNA) (Hemmi *et al*, 2000). TLR2 is involved in the recognition of a wide range of microbial products, including peptidoglycan from Grampositive bacteria, yeast cell walls and atypical LPS, which is structurally different from Gram-negative LPS. TLR2 forms heterodimers with at least two other TLRs, TLR1 and TLR6, and this dictates the specificity of the ligand recognition (Medzhitov, 2001).

Signalling via the TLRs leads to the activation of various genes with functions in host defence, including proinflammatory cytokines, co-stimulatory molecules and effector molecules, such as iNOS and anti-microbial peptides (Medzhitov, 2001). The signalling cascades brought about via TLRs originate from the intracellular region known as the Toll/IL-1 receptor (TIR) domain (Xu *et al*, 2000). This leads to the recruitment of the myeloid differentiation factor 88 (MyD88), and downstream signalling to the nucleus and inflammatory cytokine induction (Figure 10; Adachi *et al*, 1998). MyD88 is a critical adaptor molecule shared by many TLRs and signalling through many of these receptors is completely dependent on it (Akira *et al*, 2001). It is also an important component in the signalling cascades mediated by IL-1R and IL-18R (Adachi *et al*, 1998).

MyD88-independent pathways also exist for some TLRs, i.e. TLR3 and TLR4 (Akira & Hoshino, 2003). The TRIF cascade is the MyD88-independent signalling pathway associated with TLR3 and TLR4 (Figure 10). TRIF-related adaptor molecule (TRAM) is an essential link between TRIF and TLR4. This MyD88-independent pathway leads to the activation of IRF-3 and the induction of IFN- α and β , which lead to an inflammatory response (Kawai *et al*, 2001; Figure 10).



Figure 10. TLR signalling

Modified from: Robert, C., Franklin, B. S., Gazzinelli, R. T. (2008). Role of TLRs/MyD88 in host resistance and pathogenesis during protozoan infection: lessons from malaria. *Semin Immunopathol* 30:41-51

Classical activation of macrophages is not only dependent on the products of activated T helper 1 (Th1) cells, in particular IFN- γ (Dalton *et al*, 1993), but also the recognition of microbial PAMPS through TLRs (e.g. LPS and TLR4) (Aderem & Ulevitch, 2000). In

the absence of MyD88, Th1 responses are greatly diminished and this indicates a role for TLRs and MyD88-dependent signalling in the control of adaptive Th1 immunity (Adachi *et al*, 1998). Th2 responses, on the other hand, have been shown in some studies to be intact or even augmented in the absence of MyD88 (Kaisho *et al*, 2002; Muraille *et al*, 2003; Schnare *et al*, 2001) and this suggests that Th2 responses are elicited in a MyD88-independent manner. However, other work suggests that TLR signaling does play a role in Th2 responses (Eisenbarth *et al*, 2002). The effect of MyD88 signalling on the alterntative activation of macrophages will be discussed in chapter 6 of this thesis.

13. Co-infection

Ultimately, the dichotomy between AAM ϕ vs. CAM ϕ is important because it will determine the outcome of infection with metazoan vs. microscopic pathogens respectively. However, this "decision" of which pathway to select becomes more complicated when one considers co-infection with both type-1 and type-2 pathogens simultaneously. Estimates put those infected with parasitic helminths at over 2 billion people. Helminth infections in humans, such as gastrointestinal nematodes e.g. Ascaris *lumbricoides*, are highly prevalent in sub-Saharan Africa, South America and Southeast Asia, where the protozoan parasites, such as malaria are also endemic (Su *et al*, 2005). These are also the regions associated with high morbidity and mortality from infection with other microbial pathogens, such as *Mycobacterium tuberculosis* (Elias *et al*, 2001). Therefore, common locations result in high rates of co-infection between helminths and other pathogens. Different organisms occurring in the same host usually influence one another directly or indirectly (Cox, 2001). This may be especially apparent with helminths and microbial parasites since Th1 responses, that clear intracellular pathogens, and Th2 induced by helminths, inhibit one another.

With relevance to this thesis, malaria-helminth co-infection has been found to either intensify (Graham *et al*, 2005b; Helmby *et al*, 1998; Su *et al*, 2005) or improve (Briand *et al*, 2005; Nacher *et al*, 2000) disease severity. Concurrent infection with the nematode *H. polygyrus* renders otherwise resistant mouse strains susceptible to *Plasmodium chabaudi* parasites, by impairing the development of protective immunity against malaria (Su *et al*, 2005). Infection with *Ascaris lumbricoides*, however, seems to confer some protection against cerebral malaria in humans (Nacher *et al*, 2000). Other factors, such as the presence or absence of a particular parasite stage, may also have an effect on the severity of the disease outcome with malaria. Graham *et al* found that mice co-infected with *L. sigmodontis* and *P. chabaudi* have more severe malarial disease when they do not have blood-circulating microfilaraemia, compared to when they do (Graham *et al*, 2005b).

With other protozoan parasites of the *Leishmania* genus, it has been found that prior infection with the cestode *T. crassiceps* favours infection with *Leishmania major* and *L. mexicana*. This is thought to be through the induction of AAM ϕ (Rodriguez-Sosa *et al*, 2006) as arginase 1 induced in these macrophages supports the growth of intracellular *Leishmania* parasites (Iniesta *et al*, 2002; Kropf *et al*, 2005). In the case of mycobacteria, *S. mansoni* infection makes mice more susceptible to *M. bovis* BCG by impairing antigen specific Th1 responses (Elias *et al*, 2005b). However, when mice are co-infected with BCG and *N. brasiliensis* the helminth infection does not affect elimination of the mycobacteria from the lung, although the Th1 response is impaired. This shows that an ongoing Th2 response in the lung does not necessarily lead to increased susceptibility to BCG (Erb *et al*, 2002).

As well as the implant model with *B. malayi*, infection with *N. brasiliensis* a rodent model of hookworm, has also been used during this PhD. *N. brasiliensis* has a lung migration phase of its lifecycle and has been used to look at the effects of nematode infection on the phenotype of macrophages in the lungs of mice co-infected with *Plasmodium chabaudi* rodent malaria. This work will be discussed in chapter 5.

14. Aims of PhD

Although our knowledge of macrophage function in the Th2 setting is rapidly increasing a number of questions remain to be addressed about their function, their development and their level of phenotypic plasticity. The main focus of this PhD was to consider Th2activated AAM ϕ in the context of Th1 settings, both *in vitro* and *in vivo*. The aim was thus to address the level of M ϕ plasticity with relevance to "real" infection. For these reasons the following questions were asked:

- How does treatment of AAM with type1 signals alter their phenotype? Chapter 2.
- How do AAMφ deal with intracellular pathogens where a Th1 response is required in order to clear infection- *Leishmania mexicana* and Bacille Calmette-Guerin (BCG)? – Chapters 3 and 4.
- How does treatment of AAMφ with type 1 signals alter their susceptibility to infection? Chapters 3 and 4.
- What happens to the AAMφ phenotype during an *in vivo* co-infection between a Th1-inducing intracellular pathogen, *Plasmodium chabaudi*, and a Th2-inducing helminth, *Nippostrongylus brasiliensis*? Chapter 5
- What role, if any, do Toll-like Receptors (TLRs), and more specifically the adaptor protein MyD88, have in the development of the alternatively activated phenotype in macrophages both *in vitro* and *in vivo*? Chapter 6.

CHAPTER 2

Plasticity of Macrophage Function

1. Introduction

Signals encountered by developing macrophages during migration determine their functional properties at sites of inflammation or infection. Among these signals, cytokines, which can act synergistically or have opposing effects, are responsible for the development of highly divergent macrophage phenotypes. As previously mentioned, classical activation of macrophages is dependent on the products of activated T helper 1 (Th1) cells, in particular interferon-y (IFN-y) (Gordon, 2003), while alternatively activated macrophages (AAM) are activated by the Th2 cytokines interleukin (IL)-4 and IL-13. Although macrophages have been usefully classified in this way, the range of actual phenotypes is likely to be much broader with AAM and CAM representing two points in a wide spectrum. Unlike T helper cells where activation leads to terminal differentiation into Th1 and Th2 cells, macrophages appear to display a high degree of flexibility (Edwards et al, 2006; Mantovani et al, 2004; Porcheray et al, 2005; Stout & Suttles, 2004). Plasticity of function may be an economical strategy for the immune system since, in contrast to the high turnover of T cells, macrophages are longer-lived and may need to adapt their function to different pathogens or environments they will face during their lifespan.

Many studies have shown that macrophage responsiveness to a given cytokine can be altered and/or suppressed by the cytokines to which it was previously exposed (Joyce & Steer, 1996; Lang *et al*, 2002). However, the flexibility of the AAM ϕ phenotype is still a subject of controversy. Whilst some reports show that IL-4 pre-treatment of macrophages renders them unresponsive to Th1 activation (Erwig *et al*, 1998; Herbert *et*

al, 2004; Joyce & Steer, 1996; Lang et al, 2002; Modolell et al, 1995), others have found that the previous IL-4 exposure enhanced the responsiveness to Th1 activating signals (Major et al, 2002). As these results were obtained by *in vitro* activation with IL-4, the differences may be due to variation in the length and intensity of the activation stimuli. Indeed, Stout et al. recently demonstrated that two cytokines could have either antagonistic or synergistic effects on murine macrophage function dependent on the order and length of each cytokine treatment (Stout et al, 2005). In another study, human macrophages were stimulated with either pro- (e.g. TNF- α) or anti-inflammatory (e.g. IL-10) cytokines and then cultured with a counterstimulatory cytokine or medium alone. They found that macrophages stimulated towards a specific activation state could switch their phenotype rapidly when given counterstimulatory signals or return to a quiescent state after signal arrest (Porcheray et al, 2005). Due to conflicting *in vitro* data, it is difficult to determine whether macrophages demonstrate functional adaptivity *in vivo*, and the physiological relevance of this phenomenon.

Investigating macrophage plasticity in vivo could have important implications for therapeutic targeting of macrophages in chronic diseases but also for our general understanding of how the immune system copes with multiple infections that may require differing immune responses. In support of the hypothesis that macrophage plasticity may confer increased efficiency and flexibility for the immune response, Gratchev et al. recently demonstrated that in response to a second stimulation with IFN- γ , in vitro derived human AAM ϕ displayed significantly higher bactericidal activity (Gratchev *et al*, 2006). Also, in a murine model, pre-treatment with the Th2 cytokine IL-13 was found to prime macrophages in their LPS-induced anti-*Toxoplasma gondii* ability (Authier *et al*, 2007). However, the degree of flexibility of macrophages recruited to sites of infection is still unknown.

In light of the increasing evidence that macrophages show functional adaptivity, we decided to study the NeM ϕ (from the *B. malayi* implant model) response to Th1 activating signals. In this chapter, we have used this to investigate whether the switch

between alternative and classical activation can occur in macrophages differentiated in an *in vivo* infection setting and, in subsequent chapters 3 and 4, whether this would translate into an ability to control an intracellular microbial infection. Despite the longterm exposure to Th2 cytokines and anti-inflammatory signals *in vivo*, we found here that NeM ϕ were not terminally differentiated but could switch from alternative activation to a more classically activated phenotype in response to LPS/IFN- γ .

2. Materials and Methods

2.1 Mice

All experiments used WT or IL-4-/- mice on the C57BL/6 background, and these were bred in house or purchased from Harlan, UK. Mice were 6-8 weeks old at the start of the experiment.

2.2 Brugia malayi infection

Adult parasites were removed from the peritoneal cavity of infected gerbils purchased from TRS Laboratories (Athens, GA) or maintained in house. C57BL/6 males were surgically implanted intra-peritoneally (i.p.) with 5-6 live adult female *B. malayi*. Three weeks later, the mice were euthanized. The peritoneal exudate cells (PEC) were harvested by thorough washing of the peritoneal cavity with 15 ml of ice-cold Dulbecco's Modified Media (DMEM) (Gibco). As a control for non-Th2 polarised inflammation, mice were injected i.p. with 0.8 ml of 4% thioglycollate medium brewer modified (Becton Dickinson). Three days later, the PECs were harvested, as above.

2.3 Macrophage Activation

The recovered PECs were cultured in DMEM, supplemented with 10% Foetal Calf Serum (FCS), 2mM L-glutamine, 0.25U/ml penicillin and 100mg/ml streptomycin (Gibco). Thioglycollate-elicited PECs were plated in 9 cm petri dishes and left untreated or treated for 18-24 hours with IL-4 (20ng/ml; BD Pharmingen). The non-adherent cells were subsequently washed off and the remaining adherent macrophages were left untreated or treated with LPS (100ng/ml; *Escherichia coli* 0111:B4 Sigma-Aldrich) and IFN-γ (10U/ml; BD Pharmingen) together or separately for 18-24 hours. NeMφ were similarly recovered and plated in medium alone or with LPS and/or IFN-γ. Following treatment, the non-adherent cells were washed off and the adherent macrophages were recovered by a 15 minute incubation at 37 °C in warm 10mM glucose and 3mM EDTA in PBS.

In order to obtain 'control' (but not necessarily completely naïve) macrophages from the same site as the *Brugia* implant i.e. the peritoneal cavity, thioglycollate-elicted macrophages (ThioM ϕ) were prepared (see materials and methods). Thioglycollate medium is widely uses as a stimulatory agent to induce non-infectious peritoneal inflammation and elicit macrophages in mice. It is a rich nutrient medium containing proteins and carbohydrates (Li *et al*, 1997). Thioglycollate stimulus causes the recruitment of large numbers of cells to the site of inflammation but does not increase the microbicidal activity of macrophages and, therefore by this measure, does not activate them (Leijh *et al*, 1984). Injection with this inflammatory agent is also a very quick and efficient method for eliciting macrophages and helps to reduce the number of animals used for research in this field (Li *et al*, 1997). This is unlike the use of resident peritoneal macrophages as controls whereby large numbers of animals must be sacrificed in order to obtain similar cell numbers.

Bone marrow derived macrophages (BMM ϕ) were prepared by harvesting the bone marrow from the femur and tibia of C57BL/6 mice. Erythrocytes were lysed using 3 ml red blood cell lysis buffer (Sigma-Aldrich) for 5 minutes. Differentiation into macrophages was preformed according to published protocols (Dransfield, 1996). Cells were plated onto petri-dishes at 7.5 X 10⁶ cells/plate and cultured in DMEM, supplemented with 25% Foetal Calf Serum (FCS) (GIBCO), 25% L929 supernatant (as a source of M-CSF), 2mM L-glutamine, 0.25U/ml penicillin and 100µg/ml streptomycin. The medium was replaced after four and six days to allow a pure population of macrophages to be present at day seven. They were then plated in medium alone or with LPS and/or IFN- γ (as above).

2.4 FACS staining

For intracellular staining, cells were treated with brefeldin A (10 μ g/ml) for 4-6 hours at 37°C before incubation at 4°C for 15 minutes in blocking buffer (1:20 mouse serum, 0.5 mg/ml rat IgG in FACS buffer; PBS supplemented with 2mM EDTA and 0.5% BSA). This was followed by staining for 20 minutes on ice with the antibodies (Ab) of interest at the appropriate dilution as determined by titration. The antibodies were generally directly fluorochrome conjugated or biotinylated. When using biotinylated antibodies, an additional step involving incubation of the cells with fluochrome-conjugated streptavidin beads (in this case APC; Pharmingen) was performed. The Abs included anti-F4/80-biotinylated (1:100); anti-MHC II-FITC (1:200) and PE-conjugated anti-CD86 (1:100) (BD), as well as the appropriate isotype control Abs (anti-IgG2a-FITC, anti-IgG2a-PE, anti-IgG2a-biotinylated). The cells were then fixed in 2% paraformaldehyde before resuspension in cytofix/cytoperm (BD) and washing in perm wash. The saponin in the perm wash permeabilises the cells. Perm wash also serves as antibody diluent and wash buffer during the rest of the intracellular cytokine staining process, as permeabilisation with saponin is reversible. Anti-Ym1 (1:25; Stem Cell technologies) and anti-RELM- α

(1:100; Peprotech) antibodies (or isotype control- IgG from rabbit serum; Sigma) were then added (in perm wash) for one hour, before addition of Alexaflour488 (1:300 also in perm wash; Molecular Probes). The cells were then washed 2x in perm wash and once in FACS buffer before acquisition and analysis (BD FACStation and FlowJo software).

2.5 Proliferation Assay

Macrophages purified by adherence were co-cultured $(1 \times 10^5 \text{ cells/well})$ in 96-well flatbottomed plates with the EL-4 cells $(1 \times 10^4 \text{ cells/well})$ in the presence or absence of the inhibitor NG-monomethyl-L-arginine (L-NMMA; 100 μ M; Sigma-Aldrich) or Nhydroxy-nor-l-arginine (nor-NOHA; 250 μ M; Sigma-Aldrich). Following a 48-hour incubation, 100 μ l supernatant was removed to measure nitric oxide (NO) production. 1μ Ci of [³H]TdR in 10 μ l complete medium was then added to each well, and plates were incubated overnight before harvesting and counting using a liquid scintillation counter (Microbeta 1450, Trilux). Quadruplicate measurements per sample were performed. Results were plotted in counts per minute (cpm).

2.6 Quantification of NO and arginase activity

NO production was assessed by nitrite accumulation in the culture media using the Greiss Reagent. In brief, 100μ l culture supernatant was mixed with 100μ l of 5.8% phosphoric acid, 1% sulphanilamide, 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride. Absorbance was measured at 540 nm or 490nm (with background correction at 650nm) using a microplate reader. Concentration was determined according to a standard curve of sodium nitrite solution.

Arginase activity was measured according to previously published protocols (Munder JI 1998). Briefly, $1-2x10^5$ cells were lysed with 100 µl 0.1% Triton X-100. Following a 30 minute incubation with shaking, 100 µl of 25 mM TrisHCL and 20 µl of 10 mM MnCl₂ were added and the enzyme activated by heating to 56°C for 10 minutes. L-Arginine hydrolysis was carried out by incubating 100 µl of this lysate with 100 µl of 0.5M L-Arginine (pH 9.7) at 37°C for various time-points between 15 and 60 minutes. The reaction was then stopped with 800 µl H₂SO₄ (96%)/H₃PO₄ (85%)/H₂O (1/3/7, v/v/v), and 40 µl of 9% isonitroso-propiophenone (ISPF) added, followed by heating to 99°C for 30 minutes before reading on the microplate reader at 540 nm. A standard curve of urea solution was used to determine concentrations. One unit of arginase enzyme activity is defined as the amount of enzyme that catalysed the formation of 1µmol of urea per minute at 37°C. Unless otherwise stated, all reagents were obtained from Sigma-Aldrich.

2.7 RNA extraction and real-time RT PCR

RNA was recovered from cells by re-suspension in TRizol (Invitrogen). Total RNA was extracted according to the manufacturer's instructions. Following DNAse1 treatment (Ambion) to remove contaminating genomic DNA, 1 µg of RNA was used for the synthesis of cDNA using MMLV reverse transcriptase (Stratagene). Relative quantification of the genes of interest was measured by real-time PCR, using the LightCycler (Roche Molecular Biochemicals). Five serial 1:4 dilutions of a positive control sample of cDNA were used as a standard curve in each reaction and the expression levels were estimated from the curve. Real-time PCR of the housekeeping gene β -actin allowed normalisation of the expression of the genes of interest. PCR amplifications were performed in 10 µl, containing 1 µl cDNA, 4mM MgCl₂, 0.3mM primers and the LightCycler-DNA SYBR Green I mix. The amplification of β -Actin, Fizz1, Arginase1 and iNOS was performed in the following conditions: 30s denaturation at 95°C, 5s annealing of primers at 55°C and 12s elongation at 72°C, for 40-50 cycles.

For *Ym1* amplification, the annealing temperature was increased to 63°C. Primers for lightcycler PCR analysis were:

 β -Actin: TGGAATCCTGTGGCATCCATGAAAC and TAAAACGCAGCTCAGTAACAGTCCG. Arginase I: CAGAAGAATGGAAGAGTCAG and CAGATATGCAGGGAGTCACC. RELM- α : GGTCCCAGTGCATATGGATGAGACCATAGA, CACCTCTTCACTCGAGGGACAGTTGGCAGC. Ym1: TCACAGGTCTGGCAATTCTTCTG and TTTGTCCTTAGGAGGGGCTTCCTCG.

iNOS: GCATTTGGGAATGTAGACTG and GTTGCATTGGAAGTGAAGCGTTTC.

2.8 Immunofluorescence Assay

For immunofluorescence assay (IFA), macrophages (on coverslips) were fixed in 1-2ml 3% paraformaldehyde (in PBS) for 20 minutes and then washed in PBS. They were permeabilized in 1-2ml 0.25% Triton X100 (in PBS) for 10 mins and washed in PBS again. Blocking for 30 minutes was carried out with 1-2ml 1-3% w/v BSA in PBS. Binding of the primary antibody (α -YM1: Stemcell Technologies; 1:25 dilution in BSA/PBS or RELM- α ; Peprotech 1:50 dilution BSA/PBS) was performed for 1 hour. Coverslips were washed three times in PBS for 5 min with shaking. Binding of secondary antibody (anti-rabbit Alexafluor488 (Molecular Probes); 1:200 in BSA/PBS) was carried out for 1 hour and then cells washed in PBS. For nuclear staining 1µl 10ml/ml DAPI during first wash and then cells washed three more times for 5 min with shaking. Coverslips were washed in dH₂O and placed on top of gel/mount on slide and allow to dry in the dark. Cells visualised using an Olympus BX50 microscope and photographs taken with a Microcolor model RGB-MS-C camera.

2.9 Cytokine quantification

For measurement of cytokine production, $5x10^5$ PECs were plated in 24-well plates for 2-4 hours followed by removal of non-adherent cells. The remaining adherent

macrophages were left untreated or treated for 24 hours with LPS/IFN- γ as described above, followed by recovery of the supernatants for cytokine quantification. TNF- α was measured using the DuoSet TNF-a ELISA kit (R&D Systems) according to manufacturer's instructions. IL-6 and IL-12p40 were measured according to standard sandwich ELISA protocols, using Ab pairs (unconjugated and biotinylated) from BD Pharmingen, and ExtrAvidin-alkaline phosphatase conjugate in conjunction with Sigma FastTM p-nitrophenyl phosphate tablet substrate (both from Sigma-Aldrich). IL-10 and MCP-1 were quantified using the cytokine bead array (CBA) kit (BD Pharmingen) according to manufacturer's instructions. IL-27 was measured using the Quantikine ELISA kit (R&D Systems) according to manufacturer's instructions.

2.10 MFB-F11 TGF-beta Bioassay

This assay was carried out by Henry McSorley. Levels of TGF- β in the macrophage supernatants were measured using the MFB-F11 luciferase reporter bioassay, where levels of TGF- β signaling are detected by *Tg/b1-/-* mouse fibroblasts which have been stable transfected with a plasmid containing a secreted alkaline phosphatase reporter gene coupled to a Smad-binding element promoter (SBE-SEAP) (courtesy of Tony Wyss-Coray, Stanford University School of Medicine) (Tesseur et al, 2006). Cells were allowed to adhere to 96-well tissue culture plates at 4 x 10⁴ cells/well in 50 µl DMEM with 10 % FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2mM L-glutamine and 15 µg/ml hygromycin B (Invitrogen), at 37 °C for 4 h. 50 µl samples were then added, and incubated at 37 °C overnight. 10 µl samples of culture supernatant were then collected into white 96-well luminometer plates (BMG Biotechnologies), and were assayed using the SEAP detection kit (Clontech) and read on a LUMIstar luminometer (BMG Biotechnologies).

3. Results

3.1 LPS/IFN- γ causes a switch in L-Arginine metabolism from Arginase to iNOS and downregulates the expression of alternative activation markers

Preferential expression of arginase over iNOS is a consistent feature of NeM ϕ (Loke et al, 2002). We decided to investigate whether the Th1 activating signals (LPS and/or IFN- γ) could alter the NeM ϕ iNOS/arginase balance. ThioM ϕ controls and NeM ϕ were left untreated or treated overnight with LPS and IFN- γ , together or separately, and recovered for gene expression analysis by real-time RT-PCR (Figure 1 A, B). As expected, untreated NeM ϕ showed high ArginaseI expression but low iNOS RNA levels, while untreated ThioM ϕ showed none or low expression of both genes. Both IFN- γ and LPS could act alone or in concert to reduce the Arginase 1 expression in NeM ϕ . This decrease was paralleled by an increase in iNOS expression. In contrast, ThioM ϕ required both signals for the induction of iNOS. This could reflect the less mature activation state of this macrophage type (Leijh *et al*, 1984), which may require signalling through a pathogen recognition receptor such as TLR-4 before becoming responsive to IFN- γ . For subsequent experiments we decided to use LPS and IFN- γ together to ensure classical activation of the ThioM ϕ group.

Although ThioM ϕ exhibited increases in Arginase 1 mRNA in response to LPS/IFN- γ , consistent with previous studies (Munder *et al*, 1998), this induction in gene expression did not result in increased arginase enzyme activity (Figure 1 C). However, arginase and iNOS enzyme activities of NeM ϕ did reflect the gene expression data (Figure 1 C, D). This included a downregulation in arginase activity in LPS/IFN- γ treated NeM ϕ and a significant increase in NO production. In contrast to the mRNA levels, the NO produced by LPS/IFN- γ treated NeM ϕ was lower than by LPS/IFN- γ -treated ThioM ϕ . These differences may reflect timing, as mRNA levels precede protein production, or other



Figure 1. Reversal of the NeMø phenotype from alternative to classical activation.

Thioglycollate-elicited M ϕ or NeM ϕ from C57BL/6 mice were left untreated (clear) or treated overnight with LPS and IFN- γ together or separately as indicated. The cells were recovered for RNA expression analysis of Arginase1 (A), iNOS (B), by realtime RT-PCR, and assessed for arginase activity (C). The supernatants were also recovered to assess the iNOS activity by measurement of nitrite production by the cells (D). Results are shown as the mean of replicate samples (+/- S.E.M) and are representative of three experiments. post-translational controls. For example, the higher level of arginase in LPS/IFN- γ treated NeM ϕ may compete with iNOS for L-Arginine. Nevertheless, the significant increase in nitric oxide production coupled by a decrease in arginase activity demonstrates that NeM ϕ can switch to a more classical activation phenotype.

Having identified *RELM-\alpha* and *Ym1* as the main IL-4 dependent genes expressed in NeM ϕ (Loke et al, 2002), we investigated whether *RELM-\alpha/Ym1* expression was also altered by LPS/IFN- γ treatment. As a control for non-differentiated macrophages, we measured *RELM-\alpha* and *Ym1* expression in ThioM ϕ (Figure 2 A). Intriguingly, we observed induction of both genes by LPS/IFN- γ in a similar pattern to Arginase 1 expression, although the expression levels were lower than in untreated NeM ϕ . Upon overnight treatment with LPS/IFN- γ , *RELM-\alpha* and *Ym1* expression in NeM ϕ was reduced by up to 90% (Figure 2 A). However, the extent of this reduction was variable in subsequent experiments usually ranging from 25% to 90%. These are shown in Appendix 1.

We next decided to determine whether these decreases in *Ym-1* and *RELM-α* mRNA were reflected in decreased protein expression after treatment with LPS/IFN-γ. In a separate experiment, levels of YM-1 and RELM-α protein expression were investigated by IFA (Figure 2 B & C) and flow cytometry (Figure 2 D; another separate experiment) to detect intracellular levels. No reduction in Ym1 was seen in NeM ϕ treated with LPS/IFN-γ (Figure 2 B (by eye) and D; 66.8% +ve cells in untreated compared to 69.3% in LPS/IFN-γ treated). Indeed, expression of Ym1 was still observed in NeM ϕ (untreated or LPS/IFN-γ treated) after 3 days in culture (data not shown). RELM- α was also detected by IFA and as shown in figure 2 C, there was less RELM- α protein observed in NeM ϕ after overnight treatment with LPS/IFN-γ than in NeM ϕ that were left untreated (Figure 2 C- by eye), consistent with the RNA data. However, we did not see this reduction by flow cytometry (Figure 2 D- 6.75% +ve cells in untreated NeM ϕ compared to 7.01% in LPS/IFN-γ treated), although this was a separate experiment. Perhaps the

reduction of mRNA of these alternative activation markers in NeM ϕ fails to decrease at a protein level within cells in the time frame of these experiments.



A. RELM- α and Ym1 expression

Figure 2. Reversal of the NeMø phenotype from alternative to classical activation continued. Thioglycollate-elicited Mo or NeMo from C57BL/6 mice were left untreated (clear) or treated overnight with IL-4 or LPS/IFN- γ as indicated. The cells were recovered for RNA expression analysis of RELM-a and Ym1 (A) by real-time RT-PCR. Results are shown as the mean of replicate samples (+/- S.E.M) and are representative of three experiments. Shown over page: In a separate experiment, immunofluorescent assays (IFAs) were carried out to visualise Ym1 (B) and RELM-a protein expression (C) (both green). Nuclei were stained with DAPI (blue). In another experiment, flow cytometry analysis for Ym1 and RELM-a expression was carried out and is also shown (D).

(B) Ym1 IFA





(C) RELM-α IFA



Fig 2. Continued



Figure 2 D. Flow cytometry. Number in pink gate represents % +ve cells.

3.2 F4/80 expression on NeMo in response to LPS/IFN-y

The median fluorescence intensity (MFI) for F4/80 was higher in untreated NeM ϕ than ThioM ϕ (Figure 3). Since F4/80 has been implicated in immunological tolerance through the generation of regulatory T cells (Lin *et al*, 2005), the higher surface expression on NeM ϕ points to a potential regulatory function. Macrophages are a prominent cell type in other filarial nematode infection models such as *Litomosoides sigmodontis*, where regulatory T cells play an important role in immunoregulation (Taylor *et al*, 2006; Taylor *et al*, 2005). LPS/IFN- γ treatment of NeM ϕ did not affect F4/80 staining in this experiment, but it did show a reduction in LPS/IFN- γ treated ThioM ϕ (Figure 3). The significance of this result will not be clear until the function of F4/80 is known. However, it suggests that not all aspects of the NeM ϕ phenotype can be altered by LPS/IFN- γ treatment.

3.3 Suppressive phenotype of NeMφ is not reversed by LPS/IFN-γ

Since proliferative suppression is one of the well-defined regulatory properties of NeM ϕ (Loke *et al*, 2000b; MacDonald *et al*, 1998), we decided to investigate whether LPS/IFN- γ affected the ability of NeM ϕ to suppress the proliferation of co-cultured EL-4 thymoma cells (Figure 4). Although we have previously shown that nitric oxide is not involved in the NeM ϕ suppressive phenotype (MacDonald *et al*, 1998) we included the nitric oxide inhibitor L-NMMA as a control since LPS/IFN- γ treatment resulted in nitric oxide production from all macrophage groups (see Fig 1), and we wanted to distinguish the known suppressive effects of nitric oxide (Albina et al, 1991; Albina & Henry, 1991; Mills, 1991) from the suppressive mechanism mediated by NeM ϕ . Having previously shown that the NeM ϕ suppressive phenotype is dependent on IL-4 (MacDonald *et al*, 1998), we also included NeM ϕ generated in IL-4-/- mice as a negative control for



Figure 3. F4/80 expression on NeMø in response to LPS/IFN-y

C57BL/6 mice were implanted intraperitoneally (i.p.) with B. malayi adult worms and the peritoneal exudate cells recovered three weeks later. As a control, C57BL/6 mice were injected i.p. with thioglycollate (thio) and the cells recovered at day 3. The cells were left untreated or treated with LPS/IFN- γ overnight followed by flow cytometry analysis to identify the M ϕ population by F4/80. These results are representative of three experiments.


Figure 4. NeM ϕ can produce nitric oxide in response to LPS/IFN- γ but will still retain the ability to suppress cell proliferation in a nitric oxide independent manner. ThioM ϕ , ThioM ϕ +IL-4 and NeM ϕ from C57BL/6 mice were left untreated or treated with LPS/IFN- γ overnight followed by replacement of the medium and co-culture with EL-4 thymoma cells with or without the nitric oxide inhibitor L-NMMA. After 48 hours, the EL-4 cell proliferation was assessed by [3H] thymidine incorporation (A) and supernatants were recovered for measurement of the nitric oxide levels (B). Data is representative of three separate experiments and is plotted as the mean of triplicate wells (+ S.E.M.). In a separate experiment, carried out only once, the arginase inhibitor nor-NOHA was used after LPS/IFN- γ treatment of ThioM ϕ and NeM ϕ . They were co-cultured with EL-4 thymoma cells and again after 48 hours, the EL-4 cell proliferation was assessed by [3H] thymidine incorporation (C).

proliferative suppression. Finally, we included ThioMø pretreated with IL-4, allowing a comparison between *in vitro*-derived AAMø and *in vivo*-derived NeMø.

As expected, the EL-4 cell proliferation was suppressed when co-cultured with NeM ϕ in comparison to control ThioM ϕ (Figure 4 A, clear bars). ThioM ϕ pre-treated with IL-4 (*in vitro* AAM ϕ) were equally suppressive while IL-4-/- NeM ϕ were unable to suppress EL-4 proliferation, confirming that IL-4 was essential for the development of this suppressive phenotype (Loke *et al*, 2000b). When treated with LPS/IFN- γ (black bars), all macrophage cell types could significantly suppress proliferation. However, L-NMMA treatment (grey bars) reversed the LPS/IFN- γ induced suppressive phenotype in the ThioM ϕ and the IL-4-/- NeM ϕ , demonstrating that this suppression was nitric oxide mediated. In response to LPS/IFN- γ treatment NeM ϕ and *in vitro* AAM ϕ still suppressed EL-4 thymoma proliferation. This was not mediated by nitric oxide since it was not altered by L-NMMA. LPS/IFN- γ is thus not sufficient to reverse the suppressive function of either *in vitro* or *in vivo* derived AAM ϕ .

While all macrophage groups produced NO in response to LPS/IFN- γ , NeM ϕ produced significantly less NO than ThioM ϕ (Figure 4 B), consistent with our previous data (Figure 1 D). Of note, WT NeM ϕ showed increased responsiveness to LPS/IFN- γ in comparison to IL-4-/- NeM ϕ and produced higher levels of NO. This implies that activation by IL-4 may in fact enhance responsiveness to classical activation stimuli as previously reported (Major *et al*, 2002; Stout *et al*, 2005).

The IL-4 dependent mechanism of suppression in still unknown but one possibility is | that arginase acts by depleting arginine needed for cell growth (Munder *et al*, 2006). To test this WT NeM ϕ were treated with nor-NOHA in order to inhibit arginase activity. This led to partial reversal of the suppressive phenotype, with nor-NOHA treatment enhancing proliferation of the EL-4 cells co-cultured with NeM ϕ (Figure 4 C). However, EL-4 cells co-cultured with nor-NOHA treated ThioM ϕ , also exhibited

dramatically enhanced proliferation. This suggests that inhibition of even constitutive levels of arginase has significant effects on cellular proliferation. In the presence of nor-NOHA, NeM ϕ permitted only one fifth the level of EL-4 proliferation as similarly treated ThioM ϕ suggesting arginine consumption is not the dominant mechanism of suppression.

3.4 NeM ϕ express cell surface activation markers that can be further upregulated in response to LPS/IFN- γ .

We studied the surface expression of MHC class II (MHCII; Figure 5 A), and the costimulatory molecule CD86 (Figure 5 B) to determine the activation status of NeM ϕ before and after LPS/IFN- γ treatment. Untreated NeM ϕ expressed higher levels of MHCII and CD86 than ThioM ϕ , as shown by mean fluorescence intensity (MFI). However, the isotype controls were also higher in NeM ϕ (Figure 5 A, B) and thus comparing MFI directly may overestimate the difference. The expression of these markers is consistent with the original classification of AAM ϕ as "activated" (Stein *et al*, 1992). It is also consistent with previous studies showing that NeM ϕ are efficient antigen presenting cells (Loke *et al*, 2000a). Upon LPS/IFN- γ stimulation, the MFI of both markers was increased significantly in NeM ϕ but not in ThioM ϕ . Indeed, MHC II and CD86 on ThioM ϕ do not appear to be upregulated by LPS/IFN- γ but have been in a previous experiment (shown in Appendix 2 A). However, in both these experiments (Figure 5 and Appendix 2 A) the upregulation of MHCII and CD86 was highest in LPS/IFN- γ treated NeM ϕ .

PD-L1 and PD-L2 are the most recently identified members of the B7 family of costimulatory molecules and have been reported as useful markers to distinguish between classical and alternative activation of macrophages in vitro. Additionally, they have been implicated in the proliferative suppression observed by macrophages during infection



Figure 5. Effect of LPS/IFN- γ on the cell surface activation markers in control macrophages and NeM ϕ . Untreated ThioM ϕ or NeM ϕ from C57BL/6 mice were left untreated or treated overnight with LPS/IFN- γ . The cells were recovered and double-stained for F4/80 and MHC Class II (A) and CD86 (B). Flow cytometry graphs show histograms of F4/80-gated macrophages. Results are representative of three experiments.

with the platyhelminths, Taenia crassiceps and Schistosoma mansoni (Smith et al, 2004; Terrazas et al, 2005). We have previously measured surface expression of both PD-L1 and PD-L2 on NeMo. It has been found that PD-L1 surface expression was similar in both ThioM\u00f6 and NeM\u00f6 and was upregulated in both macrophage groups in response to LPS/IFN-y (Appendix 2 B), consistent with reports that classically activated macrophages show increased PD-L1 expression (Loke & Allison, 2003). Surprisingly, despite previous studies reporting PD-L2 as a marker for in vitro AAM\$\$\$\$ (Loke & Allison, 2003), both untreated and LPS/IFN-y treated NeMo did not express PD-L2 (Appendix 2 B). To confirm that the PD-L2 staining was optimal, it was found that in shown (Loke & Allison, 2003). Our finding that in vivo AAMo display different surface expression profiles to *in vitro* derived AAM is consistent with previous studies that reported differences in gene expression profiles and cell morphology (Nair et al, 2003), and reiterates the importance of in vivo models for the study of these activated macrophage subsets. Further, in contrast to suppressive macrophages found in platyhelminth infection (Smith et al, 2004; Terrazas et al, 2005), this data suggests that the PD-L1/PD-L2 costimulatory molecules may not be responsible for the suppression we observe in nematode infection.

3.5 TNF- α , IL-6, IL-10 and IL-27, but not IL-12p40 or TGF- β , production are enhanced in response to LPS/IFN- γ .

ThioM ϕ , *in vitro* AAM ϕ and NeM ϕ were again left untreated or treated overnight with LPS/IFN- γ and then supernatants taken for cytokine measurement by sandwich ELISA, cytometric bead array (CBA) or MFB-F11 TGF- β Bioassay. Figure 6 shows that TNF- α was absent in all groups but was induced in response to LPS/IFN- γ in both ThioM ϕ and ThioM ϕ + IL-4 and to a much lesser extent in NeM ϕ . Therefore, pre-treatment with IL-4, or induction in an IL-4 environment, did not prevent TNF- α being induced by type-1



Figure 6 TNF- α , IL-6, IL-27 and IL-10, but not IL-12p40 or TGF- β , production are enhanced in response to LPS/IFN- γ . Thioglycollate elicited macrophages (ThioM ϕ), ThioM ϕ + IL-4 and NeM ϕ From C57BL/6 mice were untreated or treated overnight with LPS/IFN- γ . Supernatants were recovered and the levels of various cytokines were measured by sandwich ELISA (TNF- α , IL-6, IL-27p28 and IL-12p40) or cytometric bead array (CBA; IL-10) or TGF- β assay carried out- see materials and methods. Results are shown as the mean of replicate samples (+/- S.E.M) and are representative of three experiments.

stimuli, consistent with previous reports from Stout *et al* (Stout *et al*, 2005). This was also true for IL-27 production, which followed a similar pattern to TNF- α . Untreated NeM ϕ produced relatively high levels of IL-6 relative to ThioM ϕ and all groups displayed an increase in IL-6 expression with LPS/IFN- γ treatment (Figure 6). Prior to LPS/IFN- γ treatment, both NeM ϕ and IL-4 exposed ThioM ϕ produced TGF- β but this was reduced following treatment (Figure 6).

Enchancement of TNF- α and IL-6 together with reduction in TGF- β levels provided more evidence that NeM ϕ can be reprogrammed to increase their pro-inflammatory capacity. However, the switch to a more classical activation state was not complete. Whereas ThioM ϕ and *in vitro* derived AAM ϕ produced IL-12p40 in response to LPS/IFN- γ , NeM ϕ completely failed to do so (Figure 6). Additionally the downregulatory cytokine IL-10 followed a pattern more similar to IL-6 than TGF- β . Consistent with previous reports that IL-10 is produced by AAM ϕ (Katakura *et al*, 2004) IL-10 was produced to the greatest extent by NeM ϕ and increased following LPS/IFN- γ treatment. Enhancement may not be surprising as LPS/IFN- γ are known to induce IL-10 production by macrophages (Correa *et al*, 2005).

4. Discussion

Macrophages can be involved in both pro- and anti-inflammatory responses, in tissue destructive as well as restorative activities (Gordon, 2003; Stout & Suttles, 1997). Increasing evidence suggests that macrophages activated in a Th2 setting have tissue repair as a primary function. This hypothesis has been driven by the knowledge that proline, which is an important precursor of collagen and polyamines, is produced by AAM ϕ under the control of arginase (Hesse *et al*, 2001) and supported by subsequent data that AAM ϕ are involved in fibrosis (Wynn, 2004). Recent data from our lab strongly supports this hypothesis as the key features of alternative activation are induced

solely in response to tissue injury (Loke et al. 2007; Nair et al. 2005). It is understandable that a strong wound healing response would occur in the context of helminth infection as tissue migratory or tissue invasive parasite often lead to physical trauma to host tissue. Additionally, helminth infections are generally not life threatening and can live for decades within a human host by downregulating the host immune response and protecting themselves from elimination while minimizing severe damage to the host (King et al, 1993; Maizels & Yazdanbakhsh, 2003; Muller, 2002). Importantly, the entire process of tissue repair requires the production of antiinflammatory factors (Eming et al, 2007). Consistent with this, NeMo produce many factors associated with tissue repair and reduced inflammation (e.g. arginase1, IL-10 and TGF-β; Figure 1 A and 6 respectively) (Albina et al, 1990; Pierce et al, 1989). We wanted to ask if the immune system, faced with a potentially more life-threatening situation, had the capacity to rapidly switch from a wound healing to a pro-inflammatory response (e.g. against a bacterial infection). Such functional plasticity would allow AAM¢ to take on a more classically-activated phenotype that might be beneficial prior to the recruitment of new cells to the site of infection.

We show here that a M ϕ population generated in the Th2 environment of helminth infection *in vivo* can respond to pro-inflammatory stimuli *ex vivo*. This was reflected by a switch in the enzymatic arginine metabolism pathway of NeM ϕ for from arginase to iNOS (Figure 1), as well as reduced expression of *RELM-\alpha* and *Ym1* mRNA transcripts in response to the Th1 activating signals LPS/IFN- γ (Figure 2 A). However, the reduction of mRNA did not translate into a reduction of protein (Figure 2 B-D) within the timeframe studied *in vitro*. Even so, this switch allowed resources to be devoted to the expression of antimicrobial factors, such as iNOS (observed in Figure 1 C). We also observed an increase in surface expression of class II and the co-stimulatory molecule CD86 (Figure 5 and Appendix 2 A) under the influence of LPS/IFN- γ suggesting that these AAM ϕ have enhanced APC function.

Analysis of the NeMø cytokine profile (Figure 6) presented an intriguing picture with implications for APC function. Although LPS/IFN-y treatment appeared to promote a more classically activated phenotype in the AAM ϕ through elevated TNF- α , IL-6 and iNOS, this switch was not complete, and they failed to produce any detectable IL-12p40 (Figure 6). Thus, although these cells had increased markers of classical activation, they could not promote Th1 cell development (Hsieh et al, 1993). LPS/IFN-y treatment also caused the upregulation of IL-10 in NeM
(Figure 6), which inhibits Th1 responses (Hsieh et al, 1992). This is consistent with a recent study of lamina propria macrophages from the intestine of mice, which like NeM ϕ express relatively high IL-10 and TGF- β . These Mø also produce little or no IL-12 after stimulation with Toll-like receptor ligands. IL-10 produced by these lamina propria macrophages may act in an autocrine manner to mediate this lack of IL-12 since lamina propria Mo from IL-10-deficient mice produce more IL-12p40 and p70 after stimulation with TLR ligands then wild type animals (Denning et al, 2007). This study concludes that IL-10 producing Mo cannot respond to TLR signals. However, no other pro-inflammatory mediators apart from IL-12 were studied. Therefore, it is not possible to say whether these lamina propria macrophages would have been unable to produce other pro-inflammatory cytokines (apart from Il-12) upon stimulation with TLR ligands or other type-1 cytokines (Denning et al, 2007).

Also in relation to the finding that NeM ϕ cannot be induced to produce IL-12, recent work from Foster *et al* has shown that TLR-induced genes with different functions may have diverse regulatory requirements. On an epigenetic level this means that, with a continuing exposure to a toll-like stimulus (i.e. LPS), gene-specific modifications of chromatin lead to the priming of antimicrobial effectors (e.g. *Cnlp*) while proinflammatory mediators, such as IL-6, are transiently silenced. This would potentially allow the immune system to effectively deal with microbial pathogens, while controlling the pathology associated with inflammation (Foster *et al*, 2007). The fact that we have seen the products of some genes associated with an anti-microbial response upregulated in response to LPS/IFN- γ in NeM ϕ (i.e. iNOS) but not IL-12, suggest that these AAM ϕ could have undergone similar epigenetic modifications *in vivo*. Therefore, IL-12 may not be the definitive pro-inflammatory marker to measure when assessing a switch of macrophage phenotype from alternative activation.

Untreated NeM ϕ produced abundant quantities of both TGF- β and IL-6 (Figure 6), which are known to be involved in tissue repair (Pierce et al, 1989) and chronic helminth infection (Rodriguez-Sosa et al, 2002) respectively. It has also been recently recognized that these two cytokines act cooperatively and non-redundantly to achieve Th17 commitment (Bettelli et al, 2006; Mangan et al, 2006; Veldhoen & Stockinger, 2006). Recently, it has also be found that although they drive Th-17 lineage commitment, they also induce the upregulation of IL-10 which is probably important in regulating this potentially pathogenic Th-17 mediated response (McGeachy et al, 2007). However, as they were unable to produce IL-12p40, they would not have the ability to make IL-23 (Oppmann et al, 2000) and therefore could not sustain the Th17 induction process (Aggarwal et al, 2003). The reduction in TGF- β and increase in TNF- α following IFN-y and LPS are more modest than the changes in the arginase/iNOS balance or other markers associated with healing such as Ym1 and RELM-a. Thus, although the macrophages appear to change functional phenotype, the complete absence of IL-12p40 suggests that are not contributing to the further development of either Th1 or Th17 responses.

One question to ask next would be whether NeM ϕ could actively inhibit the Th17 response as part of their anti-inflammatory wound healing function. In such circumstances, induction of a proinflammatory Th17 response would be undesirable (Kolls & Linden, 2004). IL-27 acts directly upon naïve T cells to suppress the development of Th17 effectors (Batten *et al*, 2006; Stumhofer *et al*, 2006). It may carry this out in part through competition with IL-6 for binding of the receptor component gp130, which is shared by both IL-6 and IL-27 (Heinrich *et al*, 1998; Pflanz *et al*, 2004).

64

If NeM ϕ did produce IL-27 they could inhibit the development of an unwelcome proinflammatory Th17 response. We thus measured IL-27 but did not detect any from unstimulated NeM ϕ (Figure 6), although they did produce some IL-27 after LPS/IFN- γ treatment (although only around 11% of that produced by ThioM ϕ treated with LPS/IFN- γ), which correlated with an increase in IL-6 (Figure 6). However, IL-27 could possibly act to inhibit Th17 development under these circumstances.

A consistent feature of NeM and other helminth induced macrophages is that they act as potent suppressors of cellular proliferation (Loke et al, 2000b; MacDonald et al, 1998), and several different mechanisms for the suppression have been proposed. We recently provided evidence that TGF- β may be involved to some degree (Taylor *et al*, 2006) and our ability to detect production of this cytokine by NeMø is consistent with this (Figure 6). We have now seen that arginase contributes to this suppression since inhibition with nor-NOHA caused a partial reversal of this phenotype. However, both arginase (Figure 1 A+C) and TGF- β (Figure 6) production were partially reversed upon treatment with LPS/IFN-y and yet these cells retained full suppressive abilities (Figure 4 A), so additional factors must be involved. PD-L1 has been implicated in the proliferative suppression observed by macrophages during infection with the platyhelminths, T. crassiceps and S mansoni (Smith et al, 2004; Terrazas et al, 2005) and would be consistent with a contact dependent mechanism. However, evidence for an upregulation of this ligand on NeMo compared with the ThioMo controls has not been found in this lab (Appendix 2 B). Also, NeMo did not upregulate another marker of AAM¢ generated in *in vitro*, PD-L2 (Appendix 2 B). Data now emerging from several labs would suggest that the profound suppression observed may be due to multiple antiproliferative mechanisms at play (Matlack et al, 2006; Bronte et al, 2003; O'Connor et al, 2000; Smith et al, 2004; Taylor et al, 2006).

Here we showed that macrophages in the context of chronic nematode infection were not terminally differentiated but could switch from alternative activation to a more classically activated phenotype in response to the Th1 activating signals LPS/IFN- γ . This was despite the long-term exposure to Th2 cytokines and anti-inflammatory signals *in vivo*.

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CHAPTER 3

LPS/IFN- γ treatment confers resistance of NeM ϕ to infection with *Leishmania mexicana* promastigotes.

1. Introduction

All parasites of the genus *Leishmania* are obligate, intracellular parasites that infect cells of the mononuclear phagocyte lineage of their vertebrate hosts (Alexander *et al*, 1999). Leishmanicidal activity is known to require NO generation by (classically) activated M¢ (Evans *et al*, 1993; Lemesre *et al*, 1997; Liew *et al*, 1990). *Leishmania* parasites are responsible for the disease leishmaniasis, which emcompasses a wide spectrum of disease from the self-healing cutaneous lesions to fatal visceral infections. Their primary hosts are vertebrates, including dogs, rodents, and humans. *Leishmania* currently affects 12 million people in 88 countries (WHO). Since these parasites live within macrophages they require the ability to withstand or prevent their killing functions. To sustain a chronic infection, these parasites must also subvert degradation, and the antigen presenting and accessory cell functions of macrophages, to prevent the development of adaptive immunity.

The sandfly vector of the *leishmania* parasite is the blood-sucking female of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World. These insects are found throughout the tropical and temperate regions of the world. The sandfly ingest macrophages containing the round, non-motile form of the parasite while feeding on blood from an infected animal. These amastigotes are released into the stomach of the insect, where they develop quickly into the flagellated, elongated and motile promastigotes. The promastigotes then migrate to the alimentary tract, where they live extracellularly and multiply by binary fission. After some days (4-5), the promastigotes

move to the oesophagus and salivary glands and then are injected into a new host along with the saliva when the insect's proboscis pierces the skin during a blood meal. The promastigotes are taken up by macrophages and rapidly convert to amastigotes (Alexander *et al*, 1999; Farrell, 2002).

In the murine models of Leishmania infection, it has been observed that there is often dichotomy in resistance/susceptibility to infection depending on the strain of mouse and species of parasite. An oversimplification of this would be to state that Th1 responses are induced by IL-12 and lead to the production of IFN-y and this confers protection in resistant strains (e.g. C57BL/6) to *L. major*. Susceptible mouse strains (e.g. BALB/c) produce a skewed Th2-type response, producing IL-4 (Reiner & Locksley, 1995). This paradigm of Th1/Th2 induced resistance/susceptibility to intracellular infection, although useful, is predominately based on infection with L. major. However, different Leishmania species display different virulence factors and, therefore, there are profound differences in the immune mechanisms that mediate resistance/susceptibility to infection and pathology in response to different Leishmania species. For example, most mouse strains are resistant to L. major but not to L. mexicana (Alexander & Bryson, 2005; McMahon-Pratt & Alexander, 2004). Also, although IL-4 has been shown to have a predominant role in susceptibility to L. major in BALB/c mice, this cytokine does not necessarily indicate non-healing infection, as resistant C57BL/6 mice produce IL-4 early in infection, but can still develop a protective Th1 response (Alexander & Bryson, 2005; Scott et al, 1996). Linked to this, C57BL/6 mice can control L. mexicana independently of IL-12 (Buxbaum et al, 2002), further illustrating how much of an oversimplification the Th1/Th2 paradigm of resistance/susceptibility is. Human visceral leishmaniasis is associated with a mixed Th1-Th2 response whereby both IFN-y and IL-10 are coexpressed. Therefore, the outcome of infection is probably determined by the balance between the effects of protective (IFN- γ , IL-12) and nonprotective (IL-10, TGF- β) cytokines during the early phases of infection (Holaday et al, 1993; Karp et al, 1993; Saha et al, 2007).

Predominantly, the promastigote form of *Leishmania* gains entry to host M ϕ after opsonisation with the complement component C3b and, more particularly a breakdown product of C3b, C3bi. These bind to the macrophage receptors CR1 and CR3 respectively. gp63, a metalloprotease upregulated in metacyclic promastigotes, promotes this uptake by cleaving C3 to C3bi, and also inhibiting complement-mediated lysis (Alexander *et al*, 1999; Brittingham *et al*, 1995; Brittingham & Mosser, 1996). By preferentially accessing macrophages via these receptors, the promastigotes also fail to trigger the respiratory burst (Brittingham & Mosser, 1996). Other receptors involved in the macrophage uptake of promastigotes include the mannose receptor (Wilson & Pearson, 1986), CR4 (Alexander *et al*, 1999), and the Fc receptor (Chang, 1981).

Leishmania have been used widely in recent years as model organisms for the study of intracellular infection. Since they produce a wide spectrum of disease in mice, they have provided good models of for the study of the mechanisms by which successful intracellular parasitism occurs and how the mammalian immune system deals with such infections. In Chapter 2 it was shown that the macrophages were not terminally differentiated but could switch from alternative activation to a more classically activated phenotype in response to the Th1 activating signals LPS/IFN- γ , despite the long-term exposure to Th2 cytokines and anti-inflammatory signals *in vivo*. So we next wanted to elucidate whether this would translate into an ability to control an intracellular microbial infection. NeM ϕ from our *Brugia malayi* infection setting were therefore infected with *L. mexicana* parasites and it was found that they could switch to an NO-producing classically activated macrophage phenotype that effectively dealt with an intracellular *Leishmania* infection.

2. Materials and Methods

2.1 Mice

As in Chapter 2

2.2 Brugia malayi infection

As in Chapter 2

2.3 Macrophage Activation

As in Chapter 2

2.4 Quantification of NO

As in Chapter 2

2.5 RNA extraction and real-time RT PCR

As in Chapter 2. Real-time PCR of the housekeeping gene β -actin allowed normalisation of the expression of the gene of interest i.e. C3. *C3*: CACCGCCAAGAATCGCTAC and GATCAGGTGTTTCAGCCGC β -Actin: TGGAATCCTGTGGCATCCATGAAAC and TAAAACGCAGCTCAGTAACAGTCCG.

2.6 Immunofluorescent Assays (IFA)

As in Chapter 2-Ym-1

2.7 Cytokine quantification

IL-12p70, IL-6, TNF- α , IL-10 and MCP-1 were quantified using the cytokine bead array (CBA) kit (BD Pharmingen) according to manufacturer's instructions. 50 µl of antibodybead reagent and 50 µl of antibody phycoerythrin reagent were added to 25 µl sample. The mixture was incubated for 2 hours at room temperature and washed to remove the unbound phycoerythrin before further analysis. Two-color flow cytometric analysis was performed using a FACSCalibur flow cytometer (BD Biosciences). Data were acquired and analyzed using BD cytometric bead array software.

2.8 Macrophage infection with L. mexicana

L. mexicana (strain MNYC/BZ/62/M379) or MNYC/BZ/62/M379 (DsRed integrated into sRNA locus) (Sorensen et al, 2003) promastigotes were cultured in vitro in Semi-Defined Medium (SDM; (Misslitz et al, 2000) with 10% FCS and 1% penicillinstreptomycin (complete SDM) at 26°C. Promastigotes were added to macrophages. purified by adherence and plated on coverslips (BDH) in 24-well plates, at a ratio of 10:1 for 3 hours or 30:1 for 3 hours, in the case of the red strain. The macrophages were then washed (2x) with complete DMEM to remove non-phagocytosed parasites and wells were replenished with complete medium. Where indicated, the nitric oxide inhibitor L-NMMA was added to the final concentration of 400µM. At 3 hours or day 3 post infection, cells were fixed with 2% formaldehyde for 20 min at room temperature, washed (2x) with PBS, then stained with 0.5-1 ml Giemsa for 5-10 minutes, followed by washing (2x) with distilled H₂O. Coverslips were removed from wells, allowed to dry and mounted onto slides with DPX mount (BDH), before microscopic examination. Approximately 200 cells per group were counted and percentage infection recorded. Alternatively, a leishmanicidal assay was carried out at 3 hours after infection as previously described (Proudfoot et al, 1995). The cells were lysed using 0.01% SDS in 100ul DMEM (FCS free) for 30 min. To assist this lysis, pipetting up and down 5-10 times was carried out. Released amastigotes were resuspended in SDM (10% FCS, 1% penicillin-streptomycin) in a total of 600 µl per well and cultured for 72 hours at 26°C. Four aliquots of 150µl for each sample was then transferred to quadruplicate wells of a 96-well plate and pulsed with methyl-[³H]thymidine (1µCi/well) for a further 18 hours at 26°C. Leishmanicidal activity was measured as reduction in the incorporation of this ³H] thymidine by surviving parasites as described. Counts were conducted on a liquid scintillation counter (Mictobeta 1450, Trilux). Unless otherwise stated, all reagents were obtained from Sigma-Aldrich. For immunofluorescence assay (IFA), macrophages infected with on coverslips were fixed in 1-2ml 3% paraformaldehyde (in PBS) for 20

minutes and then washed in PBS. They were permeabilized in 1-2ml 0.25% Triton X100 (in PBS) for 10 mins and washed in PBS again. Blocking for 30 minutes was carried out with 1-2ml 1-3% w/v BSA in PBS. Binding of the primary antibody, α -YM1 (Stemcell Technologies; 1:25 dilution in BSA/PBS) was performed for 1 hour. Coverslips were washed three times in PBS for 5 min with shaking. Binding of secondary antibody (anti-rabbit Alexafluor488 (Molecular Probes); 1:200 in BSA/PBS) was carried out for 1 hour and then cells washed in PBS. For nuclear staining 1µl 10ml/ml DAPI during first wash and then cells washed three more times for 5 min with shaking. Coverslips were washed in dH₂O and placed on top of gel/mount on slide and allow to dry in the dark. Cells were visualised using an Olympus BX50 microscope and photographs taken with a Microcolor model RGB-MS-C camera.

2.9 Data analysis

Graphs were prepared using PRISM (GraphPad software, Berkeley, CA).

3. Results

3.1 LPS/IFN-γ enables NeMφ to control Leishmania infection.

As LPS/IFN- γ treatment could switch alternatively activated NeM ϕ to a NO-producing classically activated macrophage phenotype (Chapter 2), we asked whether this flexibility of function is reflected in the ability to control infection by an intracellular pathogen. To address this question, infections were carried out with *L. mexicana* promastigotes, and parasite survival after three hours was determined in order to give an

estimate of parasite uptake. This was done by lysis of the M ϕ and measurement of parasite proliferation by [³H] thymidine incorporation (Figure 1).



Leishmania viability after 3 hours

Figure 1. NeM ϕ contain the largest number of viable *Leishmania mexicana* parasites after 3 hours. Bone marrow derived macrophages (BMM ϕ) and NeM ϕ from C57BL/6 mice were untreated (clear) treated overnight with LPS/IFN- γ (black) followed by infection with *L. mexicana* at 10:1 parasite to macrophage ratio. Parasites that were not taken up were washed off 3 hours post infection, and parasite viability determined by lysis of the M ϕ and measurement of the parasite proliferation by [³H] thymidine incorporation. Results are shown as the mean of replicate samples (+/- S.E.M) and are representative of three experiments.

Untreated NeM ϕ had the most surviving promastigotes after three hours since this group recorded more than 10,000 counts per minute (CPM) reflecting parasite number, compared to bone marrow-derived M ϕ (BMM ϕ ; +/- LPS/IFN- γ), which had less than a 10th of this (Figure 1; 1,000 CPM). NeM ϕ may have encountered signals *in vivo* that increase expression of the receptors involved in the phagocytosis of *Leishmania* (e.g. complement receptors) (Mosser & Edelson, 1985). The thymidine count for NeM ϕ treated with LPS/IFN- γ was 2500 CPM. There are two possibilities for this difference with untreated NeM ϕ . Firstly, LPS/IFN- γ may downregulate receptors for *Leishmania* uptake or the NO produced in the first three hours is rendering the parasites unviable, so that it appears that the uptake for this groups is lower (Figure 1). However, the latter is likely to be the case, as will be discussed below (Figure 5 A).

To assess the capacity of different M ϕ populations to control *L. mexicana* growth we determined the percent of M ϕ infected after three days by microscopy. Figure 2 A shows that LPS/IFN- γ treatment, and to a lesser extent with IFN- γ alone, conferred resistance to *L. mexicana* in both BMM ϕ and NeM ϕ . Indeed NeM ϕ seemed better able to clear infection when treated with IFN- γ alone than BMM ϕ .

In a separate experiment we chose to examine the role of NO in the leishmanicidal activity observed by NeM ϕ treated with LPS/IFN- γ . L-NMMA was used to inhibit iNOS activity as verified by nitrate levels of the day3 supernatant (Figure 2 B) and the macrophages were again examined after three days of infection (Figure 2 C). In agreement with the previous experiment, LPS/IFN- γ treatment of NeM ϕ conferred an increased ability to kill *Leishmania* but in the presence of iNOS inhibitors, NeM ϕ treated with LPS/IFN- γ were rendered as susceptible to infection as those not treated (Figure 2 C). These data demonstrate that NO is playing an important role in this microbicidal activity of LPS/IFN- γ activated NeM ϕ .

A. Microscopic examination (day 3)



Figure 2. LPS/IFN- γ or IFN- γ alone confer resistance of NeM ϕ to *L. mexicana* infection. BMM ϕ and NeM ϕ from C57BL/6 mice were untreated (clear) or treated overnight with IFN- γ (grey) or LPS/IFN- γ (black). The nitric oxide inhibitor L-NMMA was also added (B+C). Infection with *L. mexicana* was at a 10:1 parasite to macrophage ratio. Parasite levels after 3 days measured by microscopic examination of the number of infected macrophages (approx. 200 macrophages counted per group) (A+C) The supernatants were recovered for measurement of the nitric oxide levels (B). Results are representative of one experiment.



A. Immunofluorescence images- Day3

Figure 3. LPS/IFN- γ confer resistance of NeM ϕ to *L. mexicana* infection. In another experiment, ThioM ϕ , ThioM ϕ +IL-4 and NeM ϕ from C57BL/6 mice were untreated (clear) or treated overnight with LPS/IFN- γ (black) followed by a 30:1 infection ratio. Parasite levels after 3 days again measured by giemsa examination (B). Immunofluoresence Assays (IFAs) were carried out using a *L. mexicana* strain that displayed red fluoresence, along with Ym-1 staining (green) (A). Results are representative of three experiments.

Experiments were also carried out with promastigotes of a *L. mexicana* strain that displays red fluorescence (Sorensen *et al*, 2003) at a 30:1 ratio, this time using ThioM ϕ as controls. Figure 3 A shows that in the untreated groups there is an abundance of red intracellular parasites at day three but in all LPS/IFN- γ treated groups we see an almost complete absence of *L. mexicana* parasites. Thus, these type-1 stimuli were again conferring full resistance to this intracellular parasite, regardless of macrophage history. These results were confirmed by microscopic examination (Figure 3 B). M ϕ were also stained with α -Ym1 antibody to confirm their AAM ϕ status prior to infection. Ym1 staining was still detectable following LPS/IFN- γ treatment (Figure 3 A-green). We have already shown in other experiments that while *Ym1* mRNA decreases upon LPS/IFN- γ treatment (Chapter 2, Figure 2 A), protein can still be detected by IFA (Chapter 2-Figure 3 B&C).

3.2 TNF- α , IL-6 but not IL-12p70 production are enhanced in response to infection in NeM ϕ .

We next decided to assess the cytokine profiles of our *Leishmania* infected macrophages by measuring levels of cytokines in the supernatants after 24 hours of infection from the same experiment as is shown in Figure 3 i.e. red promastigotes, 30:1 ratio. Figure 4 shows that NeM ϕ were unable to produce IL-12, even after infection with this parasite (Figure 4). This is in agreement with the lack of IL-12p40 produced by NeM ϕ , after treatment with LPS/IFN- γ (Chapter 2, Figure 7). ThioM ϕ and *in vitro* derived AAM ϕ produced IL-12 in response to infection alone (Figure 4 A). The fact that NeM ϕ failed to do so shows that the switch to a more classical activation state was not complete.

IL-6 was produced in high quantities by LPS/IFN- γ -treated NeM ϕ , which increased further with *Leishmania* infection (Figure 4 B). Untreated/uninfected NeM ϕ did not produce IL-6 (Figure 4 B), which is contrast to our previous data. In chapter 2 figure 4,



Figure 4. Expression of cyto- and chemokines during *L. mexicana* infection. ThioM ϕ , ThioM ϕ +IL-4 and NeM ϕ from C57BL/6 mice were untreated (clear) or treated overnight with LPS/IFN- γ (black) followed by a 30:1 infection ratio. IL-12p70 (a), IL-6 (b), TNF-a (c) and MCP-1 (d) levels after 24 hours measured by cytometric bead arrayTM (BD). Inf:: infected with *L. mexicana*, uninf:uninfected. Results are shown as the mean of replicate samples (+/- S.E.M) and are representative of three experiments.

it is clear that NeM ϕ produce a lot of this cytokine initially (~ 400ng/ml). Thus, it seems that prolonged culture (48 hours, with washing of cells during that time) causes a decrease in the production of IL-6.

TNF- α was increased with LPS/IFN- γ treatment in all groups regardless of infection status. Only in untreated NeM ϕ was infection alone sufficient to induce production of this proinflammatory cytokine. This, along with the fact that MCP-1 chemokine levels are elevated in LPS/IFN- γ /infected cells (Figure 4), indicates that NeM ϕ had the potential to induce a pro-inflammatory response after infection with *L. mexicana*, albeit a limited one.

3.3 The pattern of *C3* expression by cultured ThioM ϕ and NeM ϕ correlates with "uptake" of *L. mexicana* parasites

Infections were once again carried out with *L. mexicana* promastigotes at a ratio of 10 parasites per macrophage after treatment with LPS/IFN- γ , and pre-treatment with IL-4 in some cases. After three hours, cells were washed, fixed and Giemsa stained (see materials and methods). In this case the proportion of parasites taken up by the macrophages was quantified by microscopic examination rather than determined by lysis of the M ϕ and measurement of the parasite proliferation by [³H] thymidine incorporation. This may give a more accurate measurement of the actual "uptake" of *Leishmania* by infected macrophages after three hours, since it also takes into account parasites that have gained access to the M ϕ but may have been rendered unviable subsequently by antimicrobial mediators.

The results of this microscopic examination are shown in Figure 5 A. Untreated ThioM ϕ contained relatively few parasites (~25%) compared with those pretreated with IL-4 and

Figure 5. Similar pattern of infection with *L. mexicana* promastigotes after 3 hours and expression of complement 3. ThioM ϕ (pretreated or not with IL-4) and NeM ϕ were left untreated or treated overnight with IFN- γ and LPS. They were then infected with *Leishmania* promastigotes at a 10:1 ratio. Microscopic examination was carried out after an infection time of 3 hours (a). Cells not infected with *Leishmania* were recovered for RNA expression analysis of *C3* (b). Results of B are shown as the mean of replicate samples (+/- S.E.M) and are representative of one experiment.





80

then LPS/IFN- γ . NeM ϕ had the highest proportion of parasite-infected cells (~70%) and this was increased with LPS/IFN- γ treatment to about 80% of macrophages infected. NeM ϕ may have encountered signals *in vivo* that increased expression of the receptors involved in the phagocytosis of *Leishmania* e.g. CR3 (Mosser & Edelson, 1985). This result appears to contradict the result in Figure 1 whereby LPS/IFN- γ treated NeM ϕ contain fewer viable *L. mexicana* parasites after three hours than untreated NeM ϕ . However, microscopic examination (as in Figure 5 A) takes into account all parasites taken up by the macrophages, including those rendered unviable after uptake. Therefore, Figures 1 and 5 may both be true indicators of what is occurring. Whereas Figure 5 indicates total uptake, Figure 1 shows how many up-taken parasites remain alive and able to replicate after three hours of exposure to different M ϕ groups.

As mentioned before, the promastigote form of *Leishmania* gains entry to host M ϕ after opsonisation with the complement component C3b (Brittingham & Mosser, 1996; Mosser & Edelson, 1985). Heat-treated foetal calf serum used in the cell culture medium should not contain any complement proteins. Therefore, we decided to investigate whether the macrophages themselves could be producing the complement proteins that were enabling the parasites to gain access to their intracellular environments. Indeed, real time RT-PCR showed that *C3* expression of ThioM ϕ , *in vitro*-derived AAM ϕ and NeM ϕ was upregulated in response to LPS/IFN- γ (Figure 5 B). The pattern of *C3* RNA expression (Figure 5 B) did correlate with the percentage of infected M ϕ (Figure 5 A,B), implying that complement produced by M ϕ may indeed be playing some role in allowing *Leishmania* to enter the cells.

4. Discussion

We have shown that LPS/IFN- γ treatment could confer resistance of NeM ϕ to infection with *L. mexicana* promastigotes. The prototypical model of murine *Leishmania spp.*

clearance involves a dominant Th1 response leading to the classical activation of macrophages and elimination of the parasites through the production of NO (Alexander *et al*, 1999). NeM ϕ could clear the parasites almost as well as naïve M ϕ given the same treatment of LPS/IFN- γ (Figure 2 A). In fact, when given IFN- γ alone, they were possibly less susceptible to infection with this parasite than the control M ϕ under the same conditions (Figure 2 A). Upregulation of IFN- γ R on NeM ϕ may have already occurred *in vivo* and we have previously seen nitric oxide produced from NeM ϕ treated with IFN- γ , but not ThioM ϕ (data not shown), which supports this notion.

Previous work in the field has shown that helminth infection may favour Leishmania establishment by inducing alternative activated macrophages (Kropf et al, 2005; Rodriguez-Sosa et al, 2006). This is because L-ornithine synthesised from arginase1 activity, which is largely controlled by IL-4, can be used by the parasites to generate polyamines and proliferate (Iniesta et al, 2002; Kropf et al, 2005). The role of IL-4 in promoting susceptibility in non-healing murine L. mexicana infections has been demonstrated, since normally susceptible mice lacking IL-4 or STAT6 acquire a Th1 response and fail to develop lesions (Noben-Trauth et al. 1996; Satoskar et al. 1995; Satoskar et al, 1997). Although these findings may seem at odds with the results presented here, IL-4 does not always promote susceptibility. IL-4 has been shown to provide a strong stimulus for the killing of intracellular amastigotes of L. major, as long as low concentrations of IFN-y are present (Bogdan et al, 1991). Indeed, we found that susceptibility of NeMo to L. mexicana decreased dramatically when treated with even IFN- γ alone, as well as LPS/IFN- γ (Figure 2 A), as already discussed (above). Although NeMø from our B. malayi infection model produced lower levels of nitric oxide after LPS/IFN-y treatment than ThioM\u00f6 (Chapter 2, Figure 1 D) or IL-4 pretreated ThioM\u00f6 (data not shown), it was still enough to confer resistance. This is illustrated in Figure 2 C whereby LPS/IFN- γ treated NeM ϕ became as susceptible to infection as untreated when the metabolic inhibitor of NO (L-NMMA) was added, if not more so. Even when NeMø were infected at a 30:1 ratio of parasite: $M\phi$, they still managed to clear the parasite upon

LPS/IFN- γ treatment (Figure 3 A&B). The presence of Ym1 protein in the macrophages controlling infection demonstrates that it was the NeM ϕ that were indeed dealing with the parasites (Figure 3 A).

Although IL-12 is an important contributor for the induction of protective immunity against Leishmania (Li et al, 1996), Figure 4 A demonstrates that NeMo were still unable to produce this cytokine, even after infection with this parasite (Figure 4), and thus would be unable to promote Th1 development. Leishmania promasitoges are known to be potent inhibitors of macrophage IL-12 production, both in vitro (in bone marrow derived M_{\$\phi\$}; Carrera *et al*, 1996) and *in vivo* (Belkaid *et al*, 1998), although this may depend on species of Leishmania (McMahon-Pratt & Alexander, 2004). Lipophosphoglycan (LPG), present on the surface of the parasite is thought to be involved in the regulation of IL-12 production in macrophages by promastigotes of Leishmania parasites (Alexander et al, 1999). Given all the information thus far gleaned from this experimental study, it appears that LPS/IFN-y exposure confers the ability to NeM to kill the parasites, but not the ability to promote a protective adaptive response. Instead one could postulate that NeMo might function to control infection with an intracellular pathogen until such time that a more suitable phenotype of Mo, and other leukocytes, could be recruited to the site of infection.

NeM ϕ did have some pro-inflammatory ability following LPS/IFN- γ stimulation, however, since they could produce TNF- α and MCP-1 (Figure 4). MCP-1 is a chemoattractant with an essential role in the recruitment of monocytes to sites of inflammation by the production of a chemotactic gradient. LPG from *Leishmania* parasites is also thought to have a role in suppressing this chemokine, (Lo *et al*, 1998) but this does not appear to be the case here. NeM ϕ were just as able as ThioM ϕ and ThioM ϕ +IL-4, to produce this protein (Figure 4 D) and would thus be involved in recruiting more cells to the site of infection. IL-6 was produced to the highest degree in NeM ϕ treated with LPS/IFN- γ and infected with *L. mexicana*, implying that infection was causing this increase (Figure 4 B). Although IL-6 is often thought of as a pro-inflammatory cytokine (Hirano et al, 1990), in the context of *Leishmania* infection, it is known to downregulate IFN- γ and therefore downmodulate leishmanicidal activity by in macrophages, and may play a role in the pathogenesis of leishmaniasis (Alexander *et al*, 1999; Hatzigeorgiou *et al*, 1993). It may therefore be the case that the parasite is in some circumstances promoting the production of IL-6 in order to escape killing.

Finally, we showed in Figure 5 that the NeM ϕ are producing complement products (complement 3), which may have aided the entry of the *L. mexicana* promastigotes. Indeed it has been previously suggested that when *Leishmania* parasites successfully bind to intact macrophages without exogenous complement present, they may fix the small amount of C3 that has been generated endogenously by macrophages (Mosser *et al*, 1992). The pattern of *C3* RNA expression (Figure 5 B) correlated with the percentage of infected M ϕ (Figure 5 A,B), which supports this hypothesis. Given more time it would also be informative to measure levels of the relevant complement receptors (i.e. CR1 and CR3), and perhaps block the actions of these receptors with antibody antagonists to investigate whether this would lower the uptake of parasites into the macrophages.

The work from this and chapter 2 has shown that macrophages were not terminally differentiated but could switch from alternative activation to a more classically activated phenotype in response to the Th1 activating signals LPS/IFN- γ , despite the long-term exposure to Th2 cytokines and anti-inflammatory signals *in vivo*. NeM ϕ could switch to a NO-producing classically activated macrophage phenotype that effectively dealt with an intracellular *Leishmania* infection.

CHAPTER 4

Mycobacterial infection:

NeMφ exhibit control of infection at low dose, and early control of infection and apoptosis at high doses of *Mycobacterium bovis* BCG

This study was carried out in collaboration with Dr. Ian Fairbairn of the University of Edinburgh Medical School. Dr. Fairbairn carried out all the work involving infection with *Mycobacterium bovis* Bacille Calmette Guerin (BCG) due to access to the appropriate facilities.

1. Introduction

M. tuberculosis and *M. bovis* are facultative intracellular parasites, which inhabit macrophages and can cause tuberculosis in humans and livestock (Erb *et al*, 2002). *M. tuberculosis* infects approximately one-third of the world's population and TB kills more than 2 million people a year, therefore it is a major health issue. These facts emphasise the need to understand the interaction of mycobacteria with host phagocytic cells i.e. macrophages.

Macrophages infected with these pathogens interact with both CD4+ and CD8+ T cells by presenting antigen on class II and class I MHC molecules respectively. The T cells in turn activate macrophages to become antimicrobial. Macrophages promote Th1 activity by producing IL-12 (Altare *et al*, 1998), IL-18 (Sugawara *et al*, 1999) and IL-23 (Fairbairn *et al*, 2001) and the T cells activate macrophages though the release of IFN- γ (Jouanguy *et al*, 1996) and TNF- α (Fairbairn *et al*, 2001; Flynn *et al*, 1995). These effector macrophages and T cells are thought to interact by positive feedback and this usually leads to the control of the mycobacterial infection (Erb *et al*, 2002; Fairbairn *et al*, 2001). This immune response to mycobacterial infection can more often than not contain, rather than completely eliminate, the infection (Flynn & Chan, 2001). During tuberculosis infection of the lung, granulomas are formed at sites of mycobacterial infections and are an essential part of this control by host immunity. They consist of macrophages, T cells, B cells and fibroblasts. Cells are positioned in granulomas in such a way that it allows infected macrophages to become surrounded by effector cells and allows for the activation of the antimicrobial mechanisms in these macrophages by the cytokines derived from T cells (see above). Granulomas contain mycobacterial infection and prevent spread to other tissue, but also cause immunopathology in the lung if persistent infection leads to chronic granuloma infection (Roach *et al*, 2002).

But how do mycobacteria enter and survive in the macrophages in the first place? Several receptors involved in the internalisation of mycobacteria by macrophages have been characterised. Binding of mycobacteria to host cells is thought to involve the complement receptors CR3 and CR4, as well as other molecules including the mannose receptor (MR), CD14 and TLR2 (Sendide *et al*, 2005). The mycobacterial cell surface glycolipid lipoarabinomannan (LAM) is known to enhance the phagocytosis of these pathogens (Strohmeier & Fenton, 1999). LAM is a complex polysaccharide composed of arabinan and mannan linked to the cell membrane (Hunter *et al*, 1986) which also has roles in inhibiting IFN- γ -induced functions including macrophage microbicidal activity (Sibley *et al*, 1988), in diminishing T cell activation (Kaplan *et al*, 1987), and scavenging of potential cytotoxic oxygen free radicals (Chan *et al*, 1991).

Mycobacteria that go on to survive and replicate within macrophages are thought to do so by a variety of strategies, including the avoidance of killing by reactive oxygen (Manca *et al*, 1999) and nitrogen intermediates (Yu *et al*, 1999). For example, *M. tuberculosis* has been shown to be highly resistant to killing by up to mmol of H_2O_2 and this ability is thought to be mediated by the mycobacterial catalase-peroxidase protein (KatG) and the akyl hydroperoxide reductase protein (AhpC), which are both enzymes with H_2O_2 as a substrate that neutralise its killing capacity (Manca *et al*, 1999). Although NO and NO₂ exhibit strong antimycobacterial activity, more virulent strains of the mycobacteria *M. tuberculosis* and *M. bovis* are resistant to the reactive nitrogen intermediate ONOO⁻ which may also enhance intracellular survival of these pathogens (Yu *et al*, 1999).

Mycobacterial species are also thought to survive intracellularly by actively inhibiting phagolysosome fusion (Clemens & Horwitz, 1995; Hart *et al*, 1987). Phagocytosed microorganisms can be degraded by the acidic hydrolases present in lysosomes when fusion with phagosomes takes place. The antimicrobial effect of fusion may also be in part due to the direct or indirect effects of acidification (Flynn & Chan, 2001). It has been reported that *M. tuberculosis* generate large amounts of ammonia which helps prevent phagolysosome fusion through undefined mechanisms (Gordon *et al*, 1980). This ammonia production also leads to alkalinisation of the acidic environment, which decreases the potency of the degradative enzymes which function best at an acidic pH (Flynn & Chan, 2001). If these pathogens survive and undergo intracellular replication, infected macrophages are thought to become progressively unresponsive to further activation by cytokines released by Th1 cells (Fairbairn *et al*, 2001; Reiner, 1994).

In Chapter 2 it was shown that NeM ϕ were not terminally differentiated but could switch from alternative activation to a more classically activated phenotype and in chapter 3 this was found to translate into an ability to control an intracellular microbial infection with *Leishmania mexicana* parasites in response to LPS/IFN- γ . As with *Leishmania* infection, it is thought that Th2 immune responses, with high IL-4, IL-5 and IL-10 levels, may promote disease during infections caused by mycobacteria (Cooper & Flynn, 1995; Erb *et al*, 2002). Therefore, to assess how *in vivo*-derived AAM ϕ cope with another "type 1" pathogen, NeM ϕ were infected with the mycobacterium *M. bovis* BCG. *M. bovis* BCG is an attenuated vaccine strain of the mycobacterium produced by sequential passage of a virulent *M. bovis* strain by Calmette and Guerin in the 1920s (Smith, 2003)

It was found that NeM ϕ responded to low doses of BCG by controlling it for the entire timeframe of the study, i.e. 6 days. NeM ϕ responded to high doses of BCG infection with early control of infection and high levels of apoptosis, and that this phenotype is independent of IL-4.

2. Materials and Methods

2.1 Mice

As in Chapter 2

2.2 Brugia malayi infection

As in Chapter 2

2.3 Macrophage Activation

As in Chapter 2, except that cell culture was carried out in the absence of any antibiotic.

2.4 Quantification of NO

As in Chapter 2

2.5 Cytokine quantification

IL-6, TNF- α , IL-10, IL-12p70 and MCP-1 were quantified using the cytokine bead array (CBA) kit (BD Pharmingen) according to manufacturer's instructions. As in Chapter 3.

Cells and bacteria, BCG viability assay, TUNEL staining –all work of Ian Fairbairn.-Appendix 3 material and methods

3. Results

3.1 NeM control a low dose of BCG infection

ThioMo, WT and IL-4-/- NeMo were exposed to a set number of bacteria per macrophage (1, 2 or 10 bacteria). It was found that phagocytosis was approximately 10%, and was similar between both ThioMo and NeMo. To infect the macrophages at a "low dose", they were exposed to 2 bacteria for every cell, which resulted in a final infection rate of 1 BCG per 5 macrophages. Mø were lysed on days 1, 3 and 6 and BCG survival was assessed by the number of colony forming units (CFU) in the lysate. Results are expressed as growth indices. An index of above 1 indicated the mycobacteria were surviving and their numbers are increasing, and values below one indicated that they are being killed. At a low dose of infection, WT and IL-4-/- NeMø could control the BCG infection up until day 6. The growth indices were below 1 up until day 3 and it rose only slightly above 1 thereafter (Figure 1). Control of the BCG was more efficient infections was lower that than of the ThioMo up to day 6. Indeed, until day 3 NeMo were killing the BCG since the growth index was below 1 (Figure 1). This shows that this type 1 pathogen, at least at this low dose of infection with BCG.

3.2 At a high infection dose NeM ϕ control mycobacterial growth in the early stages of infection and are highly susceptible to apoptosis.

For the "high dose" experiments, macrophages were exposed to 10 mycobacteria per cell. At the 10% phagocytosis rate, the final infection was 1 bacterium per M ϕ . NeM ϕ had the ability to control and even kill the BCG for up to three days (Figure 2 A and B) since the growth indices of the BCG at this time point was below 1 in both the WT and

Figure 1. Infection with Mycobacterium bovis BCG (low dose).

ThioM ϕ , WT and IL-4-/- NeM ϕ from C57BL/6 mice were infected with BCG to give a final infection ratio of approximately 1 BCG per 5 macrophages. Cells were incubated for the indicated times and the cell lysate then evaluated for BCG colony forming units. Results are expressed as the growth index so that values above 1 indicate the BCG is growing and values below one indicate it is being killed. Infection of M ϕ was performed by Ian Fairbairn who produced this figure. Results are representative of three experiments.


Figure 2. Infection with *M. bovis* BCG (high dose). ThioM ϕ , WT and IL-4-/-NeM ϕ from C57BL/6 mice were infected with BCG to give a final infection ratio of approximately 1 BCG per macrophage. Cells were incubated for the indicated times and the cell lysate then evaluated for BCG colony forming units. Where IFN- γ was present, it was given immediately after infection. Figure 2A shows the course of infection over 6 days. B and C show the extent of infection with or without IFN- γ treatment at 3 and 6 days respectively. Again the results are expressed as the growth index as in Figure 1. Results are representative of three experiments. Infection of M ϕ was performed by Ian Fairbairn who produced this figure.



0.1

ThioMø

NeM ϕ

A. Infection timecourse

0.1

ThioMø

91

IL-4-/- NeMø

IL-4-/- NeM groups. When the high dose-infected M were treated with IFN-y at the time of infection, this appeared to benefit the ThioMo in terms of killing BCG at both days 3 and 6, as indicated by the growth indices of about 1 and below 1 respectively (Figure 2 B and C). This means that treatment with IFN-y was conferring the ability to kill these intracellular pathogens in ThioMo. This was at least in part due to NO since L-NMMA, an inhibitor of nitric oxide production, limited the ability of IFNy stimulated ThioM\u00f6 to control mycobacterial infection (data not shown). However, IFN-\u00c7 treatment did not lead to enhanced killing capacity in the NeMø at this infection dose (Figure 2 B and C) and killing ability was unaffected by L-NMMA treatment (data not shown), so NO must not have been involved here. We saw little difference in the growth indices of the mycobacteria between the untreated and IFN-y treated NeMo from either WT or IL-4-/- animals (Figure 2 B and C). From day 3 to day 6 there is large growth in BCG for both WT and IL-4-/- NeMo (growth indices increased exponentially; Figure 2 A and C), but microscopic examination showed that the NeMo were dead and dying, and this was the result of uncontrolled growth of the bacteria in the media (Ian Fairbairn personal communication).

Cell death was very pronounced, and unique to the NeM ϕ , so it was decided to determine the nature of this cell death. While death by necrosis is accidental and caused by physical damage to the cell, apoptosis is a strictly regulated programme of suicide with biochemical and morphological features very distinct from those seen in necrosis. When cells are undergoing apoptosis their DNA becomes fragmented. Terminal transferase-mediated dUTP-biotin nick end labelling (TUNEL) detects DNA fragmentation and therefore stains cells undergoing apoptosis (Janeway, 2001). TUNEL staining was carried out on M ϕ with a high dose infection after 16 hours. When TUNEL is carried out, apoptotic cells appear green when observed under a fluorescent microscope. Both WT and IL-4-/- NeM ϕ appeared green (Figure 3 C and E), but not ThioM ϕ , even after IL-4 pre-treatment (Figure 3 A and G). This apoptosis occurred in IL-4-/- NeM ϕ phenotype is IL-4 independent.



Figure 3. NeMø undergo apoptosis when infected with high doses of BCG. ThioMø, WT and IL-4-/- NeMø from C57BL/6 mice were infected with BCG to give a final ratio of approximately 1 BCG per macrophage. 16 hours later they were fixed, permeabilized and DNA fragmentation detected with the TUNEL assay. Apoptotic cells stain green using florescent microscopy (a, c, e, g). Phase contrast microscopy of the relevant fields is shown (b, d, e, h). Infection of Mø and microscopy was performed by Ian Fairbairn. Results are representative of one experiment.

3.3 NeM ϕ from low dose BCG infection produce Nitric Oxide in response to IFN- γ but NeM ϕ from the high does infection do not (Day 6).

Figure 4 shows the results of NO assays carried out from the supernatants of M ϕ infected for 6 days at either a high (a) or low (b) dose of infection with BCG. The results in figure 4 B are expressed as optical density units (O.D) since there was a failure in the standard curve and nitrite concentrations could not be assessed. There was no opportunity to repeat this particular assay, but the O.D. value gave an indication of the relative NO production between the M ϕ groups. ThioM ϕ from both high and low infections produced the most NO when treated with IFN- γ (Figure 4 A and B). Infected WT and IL-4-/- NeM ϕ upregulated NO production upon IFN- γ stimulation at low dose infection, although this is quite a lot less than ThioM ϕ (approximately 3.5-fold less; Figure 4 B). At a high dose of infection, WT NeM ϕ did not upregulate the production of NO upon infection and IFN- γ treatment (Figure 4 A). The induction of apoptosis by high dose infection maybe have been so rapid in NeM ϕ (Figure 3) that they did not have time to produce NO.

3.4 NeMφ demonstrate a distinct cytokine profile following mycobacterial infection

A cytometric bead array was carried out to assess the cytokine profile in the supernatants of BCG infected NeM ϕ and this was compared to ThioM ϕ at a high infection dose after 48 hours (Table 1). Both ThioM ϕ and NeM ϕ produced TNF- α upon infection, although this was less in NeM ϕ . This indicates that NeM ϕ have the ability to induce a proinflammatory response when infected with BCG. NeM ϕ produced more than three times as much IL-6 as ThioM ϕ during BCG infection, and we have already shown that NeM ϕ already produce large amounts of IL-6 before infection (Chapter 2 Figure 6). Figure 4. Nitric Oxide assays from supernatants of BCG infected macrophages. ThioM ϕ , WT and IL-4-/- NeM ϕ from C57BL/6 mice were infected with BCG at a high dose (a) or low dose (b). These were incubated with or without IFN- γ for 6 days and the supernatants taken for measurement of nitrite production. Results are shown as the mean of replicate samples (+/- S.E.M) and are representative of three experiments.



A. NO assay from high dose infection

B. NO assay from low dose infection



95

IL-10 levels were also elevated in NeM ϕ , as compared to ThioM ϕ , although these levels of cytokine (3.3pg/ml in ThioM ϕ and 50.85 pg/ml in NeM ϕ) are very low considering the fact that we have seen NeM ϕ producing up to 15 ng/ml of IL-10 in Chapter 2 (Figure 6; although this was only after overnight culture compared to 2 days here). This may indicate that NeM ϕ are in fact switching from an anti- to a more pro-inflammatory phenotype upon infection with mycobacteria. NeM ϕ were also producing the chemokine MCP-1 (Table 1), albeit nearly four times less than ThioM ϕ . Expression of IL-12p70 could not be detected in this array to any extent in either ThioM ϕ or NeM ϕ .

Table 1. Cytokine expression of BCG infected Macrophages. ThioM ϕ and NeM ϕ from C57BL/6 mice were infected with BCG to give an approximate final ratio of 1 BCG per macrophage. They were incubated for 48 hours and than the supernatants were removed and the indicated cytokines assessed by cytometric bead assay. This experiment was carried out once.

		Mean (pg/ml)	SEM
TNF-α	ThioMø	1454.	473.9
	NeMø	1184	253.3
MCP-1	ThioMø	1306.4	1077.2
	NeMø	343.5	51.3
IL-10	ThioMø	3.3	
	NeMø	50.85	15.75
IL-6	ThioMø	173.3	83.8
	NeMø	636.75	352.10

4. Discussion

Despite the fact that TB affects so many people, the vast majority of people exposed can effectively control infection with *M. tuberculosis*. The mechanisms responsible for effective host immunity to, as opposed to immune escape by mycobacteria is only partially understood (Verreck *et al*, 2004). As previously mentioned, it is supposed that Th2 immune responses with high IL-4, IL-5 and IL-10 levels promote disease during infections caused by mycobacteria (Cooper & Flynn, 1995; Erb *et al*, 2002). However, the impact of a Th2 response on the course of mycobacterial disease has been insufficiently characterised and is the subject of some controversy. Using gene-deficient mice, IL-10 has been found to cause a delay in mycobacterial clearance, probably through its role in downmodulating macrophage function (Bogdan & Nathan, 1993; Jacobs *et al*, 2000), although other studies have failed to repeat this finding (Erb *et al*, 1998; Erb *et al*, 2002). It has also been proposed that the alternative activation of macrophages leaves them without a coordinated defence programme to *M. tuberculosis* (Kahnert *et al*, 2006).

In general, however, it is actually unclear what the impact of Th2 responses are on the efficient elimination of mycobacteria *in vivo*. Indeed, it was found that previous infection with a Th2-inducing helminth *Nippostrongylus brasiliensis* did not interfere with the elimination of *M. bovis* BCG from the lungs of mice (Erb *et al*, 2002). Perhaps in support of this, we found that AAM ϕ from an *in vivo* type 2 setting (NeM ϕ) could effectively control infection with *M. bovis* BCG up until day 6 when they were infected at a low dose (Figure 1). We have shown in previous chapters that NeM ϕ can produce antimicrobial mediators such as NO when given appropriate stimulus (Chapter 2, Figure 1 B and D). Indeed, Figure 4 shows that infected NeM ϕ could produce NO upon IFN- γ stimulation at low dose infection (Figure 4 B). However, NeM ϕ were also controlling BCG infection both without IFN- γ (Figure 1) and NO production (Figure 4 B). Contrary to this, ThioM ϕ at high dose of infection did control BCG to a greater degree after IFN- γ

treatment (Figure 2 B), and this was shown to be through the generation of NO, since treatment with L-NMMA impaired killing (data not shown). Although the role of iNOS in host defence against *M. tuberculosis* is well established, it is not essential for early control of infection (Cooper *et al*, 2000a) and a role for reactive oxygen intermediates (ROIs) in mycobacterial killing cannot be excluded (Flynn & Chan, 2001). Indeed, mice deficient in NADPH oxidase exhibit enhanced susceptibility to *M. tuberculosis* infection (Cooper *et al*, 2000b) so perhaps in this case reactive oxygen intermediates are playing a role in killing the mycobacteria. To test this theory it would be necessary to prevent production of these with an inhibitor such as superoxide dismutase (SOD) and see if NeM¢ could no longer control infection.

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As previously mentioned, an effective Th1 response clears mycobacterial infections, and this has been attributed to the ability of the mediators IFN- γ and TNF- α to induce NO generation in macrophages in mice (Chan et al, 1992). However, with human macrophages these mediators may be unable to induce NO (although this inability of human macrophages to make NO is controversial (Fang & Nathan, 2007; Schneemann & Schoeden, 2007)) but mycobacterial infection is nonetheless controlled for the majority of individuals (Fairbairn et al, 2001). Other mechanisms may also exist in order to clear infection. One such antimycobacterial mechanism may be apoptosis-mediated killing (Fairbairn et al, 2001). It has been reported that macrophages undergoing apoptosis can kill intracellular mycobacteria whereas those dying by necrosis do not. Indeed during tuberculosis infection macrophage apoptosis within the granuloma is essential for eradication of the pathogen (Fairbairn, 2004). What is more, the avoidance of macrophage apoptosis induction has been found to be a virulence factor in mycobacteria (Keane et al, 2000). Here it was found that at earlier timepoints during the high dose infection rates NeMo could control BCG infection (Figure 2 A and B) while at the same time dying by apoptosis (Figure 3 C). Perhaps this means that an alternatively activated Mo population generated in vivo due to a nematode infection, could control an intracellular pathogen for a number of days by apoptosing, after which time a more specialised Mø type would have to be recruited to the site. Infected apoptotic macrophages are also known to release apoptotic blebs with mycobacterial antigens, which are taken up by uninfected APCs. These are processed and presented to T-cells and thus apoptosis is also essential for stimulation of the adaptive immune response (Fairbairn, 2004; Schaible *et al*, 2003).

It has been found that IL-12 is an important cytokine for controlling infection with M. tuberculosis (Cooper et al, 1995) and, therefore, it was surprising not to find any in infected ThioM ϕ (data not shown). In previous chapters it has been shown that uninfected ThioM ϕ do not tend to produce any IL-12p70 (Chapter 3, Figure 4) or IL-12p40 (Chapter 2, Figure 6), except after LPS/IFN- γ treatment. However, we did find some IL-12p70 induced in *L. mexicana* infected ThioM ϕ (Chapter 3, Figure 4). It seems that BCG infection did not cause an upregulation of this cytokine in ThioM ϕ . Less surprising was the lack of IL-12 being produced by NeM ϕ since we were unable to detect any IL-12p40 in response to treatment with LPS/IFN- γ (Chapter 2, Figure 6) or IL-12p70 in response to *L. mexicana* infection (Chapter 3 figure 4). These infected M ϕ would therefore presumably be unable to drive the development of a Th1 response.

Another cytokine required for the control of mycobacterial infections is TNF- α , as mice deficient in TNF- α , or its receptor, die rapidly and with higher bacterial burdens than control mice when infected with *M*, tuberculosis (Bean *et al*, 1999; Flynn *et al*, 1995). Both ThioM ϕ and NeM ϕ produced this cytokine (Table 1). TNF- α 's importance in the control of mycobacterial infection is probably due in part to its role in macrophage activation, but it is also involved in the important process of granuloma formation. Evidence for this has come from studies using TNF-/- animals, which have shown that the granulomatous response is deficient following acute *M. tuberculosis* and the granulomas that do form are disorganised with fewer activated macrophages. Although our experiments were carried out *in vitro* they show that NeM ϕ could potentially aid granuloma formation in an *in vivo* setting through the production of this important cytokine. Cell recruitment and lymphocyte co-localisation with the macrophages is also impaired in TNF- α deficient animals (Flynn & Chan, 2001; Roach *et al*, 2002). Related to this TNF- α is thought to upregulate chemokine expression, including MCP-1 which effects the recruitment of macrophages (Czermak *et al*, 1999). NeM ϕ produced less TNF- α than ThioM ϕ , which correlates with less MCP-1 production, so perhaps these facts are related (Table 1) i.e. the TNF- α maybe be having an autocrine effect on the macrophages, inducing MCP-1 production.

IL-6 has also been implicated in the response to mycobacteria, with a potential role in suppression of T cell responses (VanHeyningen *et al*, 1997). NeM ϕ produced much higher levels of IL-6 than ThioM ϕ during BCG infection (Table 1). IL-6 has been shown to downregulate RNA expression of the anti-apoptotic signal bcl-2, thus leading to apopotosis (Tanaka et al, 2000). Therefore, these elevated levels of IL-6 observed in NeM ϕ (Table 1) may have contributed to the programmed death of these cells upon infection with high doses of BCG. This may also be why ThioM ϕ were not dying by apoptosis, since they produced 3.6X less IL-6 (Table 1).

IL-10 is anti-inflammatory and it has been suggested that IL-10 may prevent IFN- γ activation of M ϕ . However, as mentioned above IL-10 gene-deficient mice are not more resistant to *M. tuberculosis* (Erb *et al*, 1998; North, 1998). Indeed, NeM ϕ produce more IL-10 than ThioM ϕ at 48 hours (16X more; Table 1) but actually control infection at high dose better at this time point (Figure 2 A). However, levels of IL-10 cytokine are relatively low for both ThioM ϕ and NeM ϕ , so IL-10 and its anti-inflammatory affect may actually be downregulated in response to mycobacterial infection.

From this work we have shown that AAM ϕ generated in a Th2 environment *in vivo* can control infection with a second intracellular microorganism, *M. bovis* BCG. In this case, type-1 stimuli such as IFN- γ are not required for control of the potential pathogens. NeM ϕ , generated *in vivo*, have an ability to die by apoptosis for successful control of

mycobacterial infection, that ThioM ϕ and *in vitro*-derived AAM ϕ do not. It is hoped that this study may contribute to the fundamental understanding of the important process of mycobacterial clearance, which could lead to more effective treatments for TB.

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CHAPTER 5

AAMφ markers in the lungs from an in vivo co-infection setting: Co-infection with the helminth *Nippostrongylus brasiliensis* and rodent malaria *Plasmodium chabaudi*

This work was carried out in collaboration with, and data shown in appendices performed by, Dr. Andrea Graham, Dr. Simmi Majahan and Karen Grocock.

1. Introduction

The parasitic nematode Nippostrongylus brasiliensis is a potent activator of Th2 responses. In murine models of infection, mice are infected with third stage larvae through the skin. These larvae then migrate to the lungs, move up the trachea and down the oesophagus to the intestinal tract where they develop into mature adults in the small intestine. The adult worms live within the gut lumen and produce eggs, which are excreted in the faeces. A Th2 response is required to clear this parasite, which is characterised by the production of IL-4, IL-5, and, perhaps most importantly, IL-13 (Urban et al, 1998). These lead to increased IgE and eosinophilia in the blood (Liesenfeld et al, 2004). Within the intestine, mast cell hyperplasia and goblet cell proliferation take place, along with expulsion of the parasite. Goblet cells, and the mucins they discharge into the lumen of the intestine, have been shown to be involved in this expulsion of the worms (Nawa et al, 1994). It is less clear whether mucosal mast cell are required for this, although these cells are thought to mediate immunity to other intestinal nematodes, such as Heligmosomoides polygyrus and Trichuris spiralis (Lawrence et al, 1996). High levels of alternative activation markers, Ym-1 and RELM- α , have been found in the lungs of *N. brasiliensis*-infected mice (Nair *et al*, 2005; Reece et al, 2006), although it has been shown, through the use of macrophage/neutrophil IL-4 receptor α -deficient mice, that AAM ϕ are not required for expulsion of this worm (Herbert *et al*, 2004).

Helminth parasite infections in humans, such as gastrointestinal nematodes e.g. Ascaris lumbricoides and hookworm species e.g. Neactor americanus (of which N. brasilensis serves as a model) are highly prevalent in sub-Saharan Africa, South America and Southeast Asia, where the protozoan parasite malaria is also endemic (Su et al, 2005). Immune responses to protozoan parasites can be modulated by a concurrent helminth infection, as observed in both epidemiology studies in humans and animal models (Cox, 2001). With relevance to this work and as mentioned in the introductory chapter, malaria-helminth co-infection has been found to either intensify (Graham et al, 2005b; Helmby et al, 1998; Su et al, 2005) or improve (Briand et al, 2005; Nacher et al, 2000) disease severity. Infection with, and control of, malaria is associated with an early Th1 response, which is maintained for the first 14 days of infection (Li et al, 2001). An overly vigorous response can result in immunopathology, so the response must also be controlled by immunomodulatory cytokines such as TGF-B (Omer & Riley, 1998). In this way the parasite is killed but hyperinflammation avoided. This suggests that resolving malaria infection requires a fine balance within the immune response, which may be disrupted by concurrent infection with a helminth parasite (Graham et al, 2005b).

In previous chapters we have focussed on the ability of *in vivo*-derived NeM ϕ to switch from an alternatively activated to a more classically activated phenotype upon *in vitro* treatment with type-1 signals LPS/IFN- γ , to better cope with intracellular infection by *Leishmania mexicana* (Chapter 3) or *Mycobacterium bovis* (BCG; Chapter 4). While these *ex vivo* experimental settings have been very useful in deciphering the functional plasticity of macrophages, it is still important to determine the relevance of this *in vivo*. In order to investigate macrophage activation during co-infection *in vivo* we collaborated with the Graham lab in experiments using the Th1-inducing blood-stage malaria parasite *Plasmodium chabaudi* and the Th2-inducing helminth *N. brasiliensis*. As mentioned above, many studies have focussed on the effect of concurrent helminth infection on malarial disease severity. Less emphasis has been placed on the effects of infection with a protozoan parasite on a developing Th2 response, although some studies have looked at Th2 responses in co-infection settings (Helmby et al, 1998; Liesenfeld et al, 2004). We investigated the effect of co-infection on the expression of alternative activation markers Ym-1 and RELM- α in the lung over time. As these markers are highly dependent on Th2 cytokines, we took this as an indication of the effect of a concurrent malaria infection on a developing Th2 response, as well as assessing the capacity of animals to cope with both infections. We decided to focus on the lung since both parasites are known to be present in this organ for at least a portion of their lifespan. As previously mentioned N. brasiliensis worms migrate through the lung in the first 1-3 days, inducing Ym-1 and RELM-a expression (Nair et al, 2005). Infected red blood cells (RBC) of many species of malaria parasites adhere to the endothelial cells of the microvasculature of various organs, including the lung, and the rodent malaria P. chabaudi has been found to sequester there (Coquelin et al, 1999). Indeed, the lung may be a preferred site for the invasion of erythrocytes by the malaria parasite, since blood circulation in the alveoli has been shown to be slow compared to other sites and merozoites (the RBC infective stage) are often found to be free and dispersed within the lung (Coquelin et al, 1999).

Mice were infected simultaneously with *P. chabaudi* parasites and *N. brasiliensis* L3 larvae, either together or separately. It was found that by day 7 post infection, there was a reduction in expression of Ym-1 and RELM- α in co-infected compared to animals infected with *N. brasiliensis* alone. This correlated with Th2-cytokine levels in the draining lymph nodes, with co-infected animals releasing less IL-4, IL-13 and IL-5 than those singly infected with the helminth. 20 days after infection, this pattern had reversed, with co-infected animals expression higher levels of Ym-1 and RELM- α than singly-helminth infected. This work indicates that animals co-infected with parasites that elicit opposite responses may deal with the malaria infection by down-modulating the Th2

response to the helminth at earlier timepoints, when perhaps the malarial infection is most critical (e.g. in terms of anaemia) but may be able to compensate for this later on, when the malaria parasite has been successfully cleared.

2. Materials and Methods

2.1 Mice and infections

8-10 week old female BALB/c mice were purchased from Harlan UK. For the timecourse experiment, mice were injected with 200 (in 50 μ l in PBS) *N. brasiliensis* L3 larvae only s.c on Day 0 and sacrificed on days 3, 5, 7, 15, 20 and 26. Controls were injected with 50 μ l PBS. For co-infection experiments mice were divided into 4 treatment groups: Uninfected, infected with malaria only (i.p injection of 1x 10⁵ *P.c.chabaudi* parasitised red blood cells; pRBC), infected with *N. brasiliensis* only (200 *N. brasiliensis* L3 larvae were injected s.c) and co-infected (Appendix 4). The animal infections were carried out by Karen Gilmour, Simmi Mahajan and Andrea Graham. As a control for the *Nippostrongylus* infection, mice were injected with PBS only (50 μ l) and for malaria infection, they were injected with naïve blood cells (100 μ l). At days 3, 5, 7 and 20 mice were sacrificed as usual.

To obtain broncho-alveolar lavage (BAL), the trachea was cannulated and lungs lavaged with 1ml PBS. Cannulae were prepared from fine bore polythene tubing (Portex) cut into the appropriate length (approximately 4 cm) and a 23 G needle (0.6mm X 25mm; BD) inserted into one end. The small incision was made in the trachea at an appropriate point and the cannula inserted and tied in position with Mersilk black braided silk suture thread (Ethicon) before perfusion to remove RBCs and lavage. Occasionally, one lobe was cut off with suture thread before lavage and placed in RNAlater (Ambion) before homogenisation and TRIZOL (Invitrogen) RNA extraction. Otherwise, whole lungs

were treated in this way for RNA extraction. Recovered BAL cells were counted, RNA extracted and realtime PCR analysis carried out (see below). BAL fluid was retained for western blotting analysis of Ym-1 and RELM- α protein content (see below). Other lungs were placed in 4% formalin and processed (placed in paraffin blocks and sections made by in-house facility at the QMRI, Little France) and sections prepared for immunohistochemistry (see below). Photographs were taken of whole lungs using a Nikon Coolpix 4500 digital camera. The gross pathology of day 3 and day 7 lungs from one experiment was scored blindly. Score values of 1 to 4 were given as follows- 1-completely intact, pink and smooth, 2- slightly lumpy, some blood spots, 3- very bumpy, lots of blood spots, 4- shrunken and very bumpy, almost black in appearance.

2.2 Immunohistochemistry (IHC)

Sections were placed in 300ml Xylene for 10 min and then rehydrated from absolute alcohol to 64 OP for 1 min each. The sections were rinsed in deionised water for 1 min. They were then placed (in a plastic rack) into 1% antigen retrieval solution (Vector) and microwaved for a total of 15min at 1000W. The sections were transferred into running tap water to cool for 20min and then placed in 2% H₂0₂ block (Sigma) for 15min with rocking. The sections were then transferred into PBS and loaded into Sequenza racks and washed 2x with PBS. 3 drops of protein block (Dako) were added to each slide for 10min. After this time 125µl of the primary antibody (diluted in Dako diluent) was added to each slide for 60 min (Ym-1 used 1:100; Stemcell technologies; RELM- α was produced in house and used at 1:400 dilution). As a negative control, Dako diluent only was added to slides. Slides were washed twice in PBS and then $125\mu l$ of the secondary antibody (Goat arabbit IgG*^{biotin}; Dako) diluted in Dako diluent added to each slide for 30min. Slides were again washed twice in PBS. 3 drops of Vector ABC elite reagent was added to each slide for 30min. They were washed three times in PBS, before 125µl of DAB (Dako) was added to each slide for 5min. The slides were washed once in PBS and then removed from sequenza racks and transfered into H₂0. The sections were counterstained for 30 seconds in haematoxylin, rinsed in tap water, then in Scott's water

and then transfered back into tap water, before dehydration back through the alcohols into xylene for 10 secs each. Slides were finally mounted in Pertex and allowed to dry overnight. Slides were examined microscopically and given "blind" scores based on "protein level" (darkness of brown stain) and "area" of staining. A protein level score of 1 =no staining, 2 = weakly stained, 3 = moderate staining and 4 = strong staining. For the area of staining, 1 = 0, 2 = < 30% of the area had stained, 3 = 30-60% and 4 = > 60%of the area stained. These values were averaged over 5 fields and multiplied together to give a final score in arbitrary units (i.e. if a slide stained for Ym-1 had an average 'protein level' score of 3 (moderate) and this was over 50% of the area (i.e. area score of 3) over 5 fields the combined score would be 3 x 3 = 9. The highest score possible is therefore 4 x 4 (darkest staining over >60% area) = 16. This gave an indication of the relative protein levels of Ym-1 and Fizz1 from the lungs of the various groups. Figure 5 C shows representative slides from IHC using the α -Ym-1 antibody at 7 days post infection. Generally speaking most alveolar macrophages in the lung were positive for Ym-1 and, as most of the visible differences in staining intensity occurred in the bronchial epithelia, so we decided to focus on this area for future work.

2.3 RNA extraction and real-time RT PCR

Whole lung tissue was firstly placed in RNAlater (Ambion) and homogenised, before being placed in TRIZOL for RNA extraction and real-time RT PCR, as in Chapter 2. Control cDNA was taken from NeM¢ from the *Brugia malayi* implant model.

2.4 Western Blotting

The BAL fluid was analysed for Ym-1 and RELM- α protein expression. This procedure was undertaken by Marieke Hoeve in this instance but the method is outlined in Chapter 6.

3. Results

3.1 A timecourse of Ym-1 and RELM- α expression in lungs of mice infected with *N. brasiliensis*

To provide baseline information prior to the main co-infection experiments the patterns of Ym-1 and RELM- α protein expression in *N. brasiliensis* infected mice (also referred to as nippo-infected or helminth-infected) were ascertained over the timecourse planned for the main experiment i.e. beyond 20 days. Female BALB/c mice were infected with 200 L3s or injected with 50 μ l PBS as a control. At days 3, 5, 7, 15, 20 and 26 mice were sacrificed and BAL fluid recovered for Western blot analysis of the proteins in question.

Ym-1 protein started to appear in the BAL fluid (BALF) at day 3, but it increased significantly by day 5 and was at its peak by day 7 (Figure 1 A). By day 15, Ym-1 levels had decreased and continued to do so until day 20 (Figure 1 A). RELM- α expression followed a similar expression pattern to Ym-1 (Figure 1 B). RELM- α protein levels had also started to rise by day 3 and the peak of expression was at days 5-7 levels. RELM- α levels were almost absent by day 15 (Figure 1 B). These peaks of expression for both Ym-1 and RELM- α (around days 5-7) probably correlate with the adaptive immune response and arrival of Th2 cells in the lung (Voehringer *et al*, 2004). This peak of expression at days 5-7, and the inflammation of the lung tissue due to helminth infection, are illustrated in the pictures of anti-Ym1 IHC (Figure 1 C). There was little to no protein detected by days 20 and 26. This illustrated the kinetics of expression in the lungs of Ym-1 and RELM- α in mice infected with *N. brasiliensis*.



Figure 1. Ym1 and RELM- α expression in the lung in response to *N.* brasiliensis infection. BAL fluid was recovered from the lungs of BALB/c mice after various days of infection and western blot analysis of Ym1 (a) and RELM- α (b) protein expression carried out. Significant differences were determined by the Mann-Whitney test *p<0.05. BALF: bronchoalveolar lavage fluid. This experiment was carried out once. Sections of lungs were prepared and IHC carried out. Section photographs of IHC for Ym-1 are shown (c)

3.2 Gross pathology of lungs from co-infections

In the next experiment, mice were infected with malaria only, *N. brasiliensis* or both parasites. Control animals were uninfected but injected with PBS alone and naïve red blood cells (materials and methods). At days 3 and 7, whole lungs were removed and after lavage was carried out, photographs of the lungs taken. In this way we could observe the gross damage caused to the lung by infection. Damage to the *Nippostrongylus*-infected lungs was expected but we wished to see whether co-infection with malaria had any effect on this damage. These photographs were blind-scored and the results shown in Figure 2. Representative photographs are also presented.

Infection with this helminth was detrimental to the overall health of the mice, causing transient anaemia in singly and doubly infected animals between days 4 and 6 post infection (Appendix 5 A). Helminth-infected animals also had lower minimum body mass than uninfected and malaria only mice (Appendix 5 B). *N. brasiliensis* migration caused considerable damage to the lungs, with the worst affected becoming shrunken and bloody in appearance (Figure 2 A; bottom left co-infected). There was no significant difference found between the lung damage of those singly infected with the helminth and the co-infected ones at day 3, although more co-infected lungs were given values at the higher end of the pathology scores (four of the co-infected lungs had scores above 3 AUs, as opposed to only one from the singly-infected group; Figure 2 A). By day 7, no difference was seen (Figure 2 B).

(A) Day 3 lungs





(B) Day 7 lungs



Figure 2. Pathology of lungs. Whole lungs were removed from BALB/c mice after 3 (a) and 7 (b), photographs taken (right) and gross pathologies scored (left). Nippo = N. *brasiliensis*-infected.

3.3 At day 3 post-infection, co-infection is not affecting Ym-1 and RELM- α expression

Three days after infection, whole lung tissue was taken for RNA extraction. Figure 3 A shows the Realtime RT-PCR results of Ym-1 and $RELM-\alpha$ expression from the various experimental groups. Ym-1 RNA was found in whole lung tissue from Nippostrongylusand co-infected animals, but only at low levels (up to approx. 0.5% control cDNA; Figure 3 Ai). There were higher levels of $RELM-\alpha$ present (up to approx. 200% control cDNA) than YM-1, but again only in Nippostrongylus- and co-infected groups (Figure 3 A ii). There was a slight trend towards higher levels of both Ym-1 and $RELM-\alpha$ in co-infected than Nippostrongylus-infected lungs.

Ym-1 protein levels in the lung were measured at day 3 in this particular experiment (Figure 3 B), as measured by immunohistochemistry (IHC; Figure 3 B i) and western blots of BAL fluid (Figure 3 B ii) and did not go above background levels of Ym-1. There is always detectable Ym-1 protein in alveolar M ϕ of naïve mice (personal observation and Nio 2004 (Nio *et al*, 2004)). RELM- α protein expression from western blots of BALF (Figure 3 B iv) correlated with the expression of RNA from whole lung tissue, with some expression in *Nippostrongylus*-only infected mice, which was lower than that in co-infection.

We presume that Ym-1 and RELM- α expression at day 3 are mainly a reflection of the innate response to *N. brasiliensis* migration as seen previously (Reece *et al*, 2006). These early increases in YM-1 and RELM- α RNA or protein show an insignificant trend towards higher levels in the lungs of mice co-infected (with malaria and *N. brasiliensis*) than those infected with the helminth alone.





Figure 3. Day 3 Ym-1 and RELM- α mRNA and protein expression. RNA was recovered from whole lung tissue of BALB/c mice and real time RT-PCR carried out for *Ym1* and *RELM-\alpha* RNA expression (a). Sections of lungs were prepared and IHC carried out (b; left), along with western blots of BALF for Ym1 and RELM- α protein expression (b; right). Nippo: *N. brasiliensis*-infected. BALF: broncho-alveolar lavage fluid. ep.: epithelium. -----: level of background stain. Results are representative of three experiments.

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3.4 At day 5 post-infection singly *Nippostrongylus*-infected mice have begun to express higher levels Ym-1 and RELM- α than those co-infected with malaria

By 5 days post infection, Ym-1 mRNA was present in *Nippostrongylus*- and co-infected lungs, with a trend towards a higher level in *Nippostrongylus*-only (Figure 4 A i). This trend was reflected in protein expression where *Nippostrongylus*-only infected lung sections expressed Ym-1 above background and this was significantly higher than in coinfected lung sections (Figure 4 B i). This pattern was also seen from protein in the BALF as measured by western blot of Ym-1 (Figure 4 B ii), although in this instance the difference between *Nippostrongylus*-only and co-infected lungs was not significant. *RELM-α* mRNA expression (Figure 4 A ii) and protein expression (Figure 4 B iii and iv) also followed this trend, whereby expression tended to be higher in *Nippostrongylus*only than co-infected mice, although again this was also only significant when measured by IHC (Figure 4 B iii).

These data demonstrate that Ym-1 and RELM- α expression began to increase in the lungs of mice after 5 days of infection with *N. brasiliensis* (either singly or co-infected with malaria), as indicated by an increase in both mRNA (Figure 4 A) and protein levels (Figure 4 B). This was in agreement with the initial timecourse (Figure 1) and perhaps reflects an influx of helminth-specific Th2 cells in the lung at this timepoint. There was also a definite trend towards higher expression of these proteins in *Nippostrongylus*-only than in co-infected animal, indicating that the Th2 response may be downregulated in the co-infected mice.



Whole lung tissue

Figure 4. Day 5 Ym-1 and RELM- α mRNA and protein expression. RNA was recovered from whole lung tissue of BALB/c mice and real time RT-PCR carried out for Ym1 and RELM- α RNA expression (a). Sections of lungs were prepared and IHC carried out (b; left), along with western blots of BALF for Ym1 and RELM- α protein expression (b; right). Nippo: N. brasiliensis-infected. BALF: broncho-alveolar lavage fluid. ep.: epithelium. Significant differences were determined by the Mann-Whitney test. *p<0.05. ________: level of background stain. Results are representative of three experiments.

3.5 At day 7 post-infection singly *Nippostrongylus*-infected mice express higher levels Ym-1 and RELM- α than co-infected animals, reflected by the Th2-type cytokine production in the draining lymph node

Similarly to day 5, there was a trend for higher Ym-1 expression in Nippostrongylusonly infected than in co-infected lungs at day 7. In the whole lung, Ym-1 expression was higher in Nippostrongylus-only than in co-infected animals, although this did not quite reach significance (p=0.059; Figure 5 B ii). Ym-1 protein expression was significantly higher in animals singly infected with *N. brasiliensis* than those co-infected with malaria, shown by both IHC (Figure 5 B i and 5 C) and western blot of the BALF (Figure 5 B ii). With regards to *RELM-* α mRNA expression, Nippostrongylus-only infected lungs again displayed significantly higher levels of *RELM-* α RNA expression than co-infected (Figure 5 A ii). This was also reflected in protein expression, but did not reach significance (Figure 5 B iii and iv).

Of note, IHC showed that alveolar macrophages were positive for Ym-1 in animals from all treatment groups on all days (including naives; data not shown). In general, alveolar macrophages were not positive for RELM- α in any treatment group. Any differences in expression levels of Ym1 and RELM- α were only clearly seen in the bronchial epithelial cells (see Figure 5 C for Ym1 IHC example). Therefore, it was these cells that were used to intensity of staining scores for both Ym1 and RELM- α , as previously mentioned (materials and methods).

The trend from day 5 post infection, with higher expression of both Ym-1 and RELM- α in the lungs of mice singly infected with *N. brasiliensis* than in mice co-infected along with the rodent malaria *P. chabaudi*, continued and became significant at day 7, adding further evidence that the Th2 response is downregulated in the co-infected mice. Since this pattern was so pronounced it was decided to measure the cytokine production of the thoracic lymph nodes. Cultured lymph node cells from co-infected animals produced

Whole lung tissue



Figure 5. Day 7 Ym-1 and RELM- α mRNA and protein expression. RNA was recovered from whole lung tissue of BALB/c mice and real time RT-PCR carried out for Ym1 and RELM- α RNA expression (a). Sections of lungs were prepared and IHC carried out (b; left), along with western blots of BALF for Ym1 and RELM- α protein expression (b; right). Section photographs of IHC for Ym-1 are shown (c). Nippo: *N. brasiliensis*-infected. BALF: bronchoalveolar lavage fluid. ep.: epithelium. Significant differences were determined by the Mann-Whitney test. *p<0.05 -----: level of background stain. Results are representative of three experiments.

(C) α -Ym-1 immunohistochemistry



Figure 5 continued. At day 7 post-infection mice infected with *Nippostrongylus brasiliensis* only display greater expression of Ym-1 in lung sections than those coinfected with the helminth and *Plasmodium chabaudi* rodent malaria. X 200 magnification

less IL-4, 13, 5, and 10 both spontaneously (Appendix 6 A-D) and in response to ConA (Appendix 6 F-I) than *Nippostrongylus*-only mice, reflecting the pattern seen with AAM ϕ markers, Ym-1 and RELM- α . Conversely, IFN- γ was produced by co-infected animals as well as the malaria-only infected mice (Appendix 6 E and J). This is reflected in the fact that malaria parasitemia is starting to rise at this time in malaria infected animals, as seen by % parasitized red blood cells (Appendix 5 C).

3.6 By 20 days post-infection, expression of Ym-1 and RELM- α in the lungs of co-infected mice has now overtaken that in singly *Nippostrongylus*-infected mice.

As reflected in the original timecourse experiment Ym-1 and RELM- α mRNA and protein levels had diminished substantially by day 20. However there was a shift in the pattern of expression. *Ym-1* mRNA expression in whole lung now showed a trend towards higher expression in co-infected than in *Nippostrongylus*-only (Figure 6 A i). Ym-1 protein was now also higher in the BAL fluid of co-infected mice (Figure 6 B ii), but not as measured by IHC (Figure 6 B i). RELM- α expression also followed this pattern. Realtime RT-PCR of the whole lung both showed higher levels of *RELM-\alpha* in co-infected animals compared to *Nippostrongylus*-only (Figure 6 A ii). There was a trend towards this in terms of protein expression of RELM- α in the lungs, but this did not reach significance (Figure 6 B iii and iv).







Figure 6. Day 20 Ym-1 and RELM- α mRNA and protein expression. RNA was recovered from whole lung tissue of BALB/c mice and real time RT-PCR carried out for Ym1 and RELM- α RNA expression (a). Sections of lungs were prepared and IHC carried out (b; left), along with western blots of BALF for Ym1 and RELM- α protein expression (b; right). Nippo: N. brasiliensis-infected. BALF: broncho-alveolar lavage fluid. ep.: epithelium. Significant differences were determined by the Mann-Whitney test *p<0.05. ----- : level of background stain. Results are representative of three experiments.

4. Discussion

Most work involving helminth-malaria co-infection situation has seen malaria parasites introduced to a setting where the helminth is already established (Graham et al, 2005b; Helmby et al, 1998; Su et al, 2005). Our work differs in that respect since both parasites are introduced simultaneously, and also in the fact that we have used alternative activation markers of macrophages, Ym-1 and RELM- α , as a read-out of the Th2 effector response, as well as cytokine measurements, in the tissue in which both parasites are present. Direct evidence for the presence of malaria parasites in the lung came from the fact that high numbers were detected in the lung tissues of infected animals by quantitative PCR of malaria DNA (Marieke Hoeve -personal communication). Before we looked at how malaria was affecting N. brasiliensis-induced Ym-1 and RELM- α in the lung, a timecourse experiment was carried out to investigate the patterns of expression of these proteins in singly infected mice (Figure 1). Peak expression of both occurred at a time when an adaptive Th2 immune response would be coming into play around day 5-7. This is in agreement with work by Voehringer et al. Using IL-4 reporter mice, they have found that Th2 cells have begun entering the lung by day 5 post-infection in N. brasiliensis-infected animals and this reaches a peak at day 9 (a timepoint we did not look at). Th2 cell numbers in the lung then begin to decline by day 13 (Voehringer et al, 2004). We also showed that Ym-1 upregulation lasted longer in the BALF of the lung than RELM- α in that particular experiment. Co-infection with malaria prolonged the expression of both of these proteins in the lung (Figure 6 B ii and iv), perhaps due to a delayed or prolonged Th2 response.

The markers of alternatively activated M ϕ , Ym-1 and RELM- α , were used here to demonstrate that malarial parasites are able to modulate responses to helminth coinfections. Seven days after infection with blood stage malaria parasites and L3 of *N*. *brasiliensis*, co-infection with malaria was having the effect of down-regulating the Th2 immune response (and the resultant alternative activation markers) elicited against the helminth (Figure 5 and Appendix 6), when compared to animals singly infected. Downregulated Th2 responses to a helminth infection due to malaria infection have previously been observed with *Schistosoma mansoni* and *P. chabaudi*. In that study Th2 responses to the schistosome antigens were suppressed for up to 1 month after malaria infection (Helmby *et al*, 1998). In our experiments, RELM- α and Ym-1 expression was higher in co-infected individuals at day 20 post-infection (Figure 6) than helminth only infected mice (although these levels of expression are lower overall than day 7; Figure 5 e.g. *RELM-\alpha* mRNA levels peak at 1000% control cDNA on day 7, and only approx. 50% control cDNA on day 20). This suggests that the Th2 response may have recovered after clearance of the Th1-inducing malaria by Day 15 (Appendix 5 C). Another possibility is that the type 2 response began in the co-infected animals at a similar time to the singly *N. brasiliensis* infected mice but rose more slowly, peaked at a lower level but lasted a little longer. These two possible scenarios are outlined in Figure 7.

On the other hand, the co-infected individuals in this study were still able to elicit a sufficient Th1 response against the potentially life threatening malaria, as measured by IFN-y production (Appendix 6 E & J) and the ability to clear the malaria within 15 days (the same amount of time as malaria-only mice; Appendix 5 B). The malarial disease severity was not detrimentally affected by concurrent helminth infection, and may even have been improved, as measured by slightly higher red blood cell density (less anaemia), and lower parasitemia, in co-infected compared with malaria-only mice (Appendix 5 A & B). It has been suggested that the transient anaemia seen early on in the Nippostrongylus-infected mice around day 5 post-infection (Appendix 5 A) may have resulted in the lower peak parasitemia seen in co-infected mice (Appendix 5 C). This is because a lower availability of RBCs early on would result in fewer malaria parasites gaining access to a host cell and thus, peak parasitemia would indeed be lower in the co-infected individuals (Andrea Graham, in press). Indeed, previous co-infection studies involving other helminths and malaria have shown that these worms can limit RBC availability to malarial parasites (Fagbemi et al, 1985; Lwin et al, 1982). Therefore, the early N. brasiliensis-induced transient anaemia (Appendix 5 A), coupled



Figure 7. Coinfection with malaria and helminth causes an impairment of expression of the alternative activation markers at day 7, but this reverses by day 20. Comparison of the levels of Ym-1 protein in the lungs at various timepoints after infection with N. brasiliensis either alone (nippo) or at the same time as P. chabaudi (coinfected), as a representation of what is happen to AAM ϕ markers in the lungs.

with a sufficient Th1 response (as measured by day 7 IFN- γ production; Appendix 6 E & J), may allow co-infected individuals to clear the malaria parasites as well as, if not better, than singly malaria-infected mice.

In the early stages of malaria infection in resistant mouse strains there is a rapid production of IFN- γ , produced by CD4+ T cells and this is maintained for the first 14 days of infection (Meding *et al*, 1990). One of the major ways in which IFN- γ is thought to be beneficial against malaria parasites is through the classical activation of macrophages and the consequent production of TNF- α , IL-1, IL-6 and soluble mediators such as nitric oxide (NO) and reactive oxygen species (ROS) (Li *et al*, 2001). Despite these facts, we did not detect any *iNOS* or *TNF*- α mRNA in the lung tissue of malariaonly or co-infected mice at any timepoint (data not shown), so these do not seem to be involved in malaria clearance, at least from within the lung. This may be consistent with data that NO is not important for control of parasitemia in blood-stage malaria, although it is important against liver-stage parasites (Favre *et al*, 1999; Nussler *et al*, 1993; Saeftel *et al*, 2004).

It may be considered surprising that we did not detect the pro-inflammatory mediators TNF- α or iNOS in the lung, despite the presence of malaria parasites. However, there is little inflammation in the lungs even under normal conditions, which is surprising considering the potential of inhaled microbes to gain access to the interior of the lung during breathing. Indeed, a pool of alveolar macrophages can handle up to one billion bacteria injected intratracheally before adaptive immunity is induced (Lambrecht, 2006). Alveolar macrophages are important in the defence of the lung but may be kept in a quiescent state, by consistent exposure to TGF- β in the alveolar spaces induced by the integrin $\alpha V\beta \beta$, in order to prevent collateral damage to the alveolar epithelial cells involved in gaseous exchange. Perhaps the numbers of malaria parasites that made their way to the lung were not enough to cause the upregulation of TNF- α or iNOS. Just as likely is that they were induced but upregulation was transient, and turned off

sufficiently quickly, that we did not detect these pro-inflammatory mediators at the timepoints investigated. Indeed, it is known that the pro-inflammatory activation of alveolar macrophages caused by signalling through TLRs is quickly turned off by IFN- γ production (Takabayshi *et al*, 2006). Malaria parasites are thought to cause signalling through TLRs, such TLR9 (Coban *et al*, 2005) and, as we saw in Appendix 6 E, IFN- γ was produced in the draining lymph nodes at day 7 post-infection. IFN- γ stimulates production of matrix metalloproteinase (MMP)-9 by alveolar macrophages. This may activate latent TGF- β , which again inhibits macrophage activation (Takabayshi *et al*, 2006). Another reason why we may have not seen iNOS or TNF- α in the lung is the possibility that activation of alveolar macrophages does not occur if the malaria parasites stay in the microvasculature of the lung and do not cross into the tissue. This is unlikely to be the case, however, given the extensive damage to the lung due to *Nippostrongylus*, at least in the co-infected mice, and the evidence from other labs that merozoites are often found to be free and dispersed within the lung (Coquelin *et al*, 1999).

TNF- α has been shown to be important against malaria, despite the fact that we did not detect any in the lungs of infected mice. Treatment with exogenous TNF- α protects susceptible strains against otherwise lethal infection with *P. chabaudi* (Stevenson & Ghadirian, 1989). This suggests that TNF- α was probably present in malaria-infected mice but did not exert its affects in the lung (or at least was undetectable here by our measures). Increases in levels of TNF- α could be an alternative explanation (other than RBC availability) for why there was reduced parasitemia in co-infected animals relative to those infected with malaria alone (Appendix 5 C). Production of TNF- α has been reported to be increased shortly after *N. brasiliensis* infection (Benbernou *et al*, 1992). This may mean that animals co-infected with *N. brasiliensis* and malaria parasites (at the same time) have a higher level of TNF- α initially than mice singly infected with malaria, and this may be having the protective effect in the co-infected animals.

TNF- α cytokine has been shown to play a major role in pathogenesis of malarial disease and levels in the serum of individuals infected with the human malaria *P. falciparum* correlates with disease severity (Mordmuller *et al*, 1997). For example, TNF- α may play a role in the anaemia associated with disease through suppression of erythropoiesis (Li *et al*, 2001). TNF- α may also be a cause of cerebral malarial pathology, since TNF- α production causes the upregulation of adhesion molecules, such as ICAM-1 and CD36, on endothelial cells and this is thought to be involved in the sequestration of monocytes and parasites in the brain, amongst other organs (Lucas *et al*, 1997). Regulating the production of this cytokine during co-infection with a helminth parasite may contribute to the protection against cerebral malaria in individuals with concurrent infections.

The importance of studying co-infection between malaria and helminths is clear when thinking about the design of possible vaccine strategies against these often deadly protozoan parasites. Vaccines are supposed to ideally evoke an efficient Th1 response and induce specific antibodies against the pathogens. It is possible that concurrent helminth infection would interfere with the induction, or modulate, a Th1 response to a potential vaccine candidate, as has been shown to be the case with BCG (Elias *et al*, 2005a). These considerations should be taken into account before vaccination plans are implemented.

In this chapter, it has been shown that infection with *P. chabaudi* malaria parasites could modulate the Th2 response against a concurrent helminth infection with *N. brasiliensis*, as measured by Ym-1 and RELM- α expression, as well as cytokine measurements. Co-infected individuals could mount a Th1 response sufficient to clear the protozoan parasite as well as singly infected animals. This shows that the immune system can respond to both types of infection simultaneously and deal adequately with a potentially life threatening Th1 inducing pathogen, malaria, by perhaps delaying or reducing the peak Th2 response against the concurrent helminth infection.
CHAPTER 6

MyD88 and the Alternative Activation of Macrophages

1. Introduction

The immune system must detect and destroy invading pathogenic microorganisms by discriminating between self and non-self. Adaptive immunity is carried out through the detection of non-self through the recognition of peptide antigens using receptors expressed on the surface of B and T cells. This adaptive system is only observed in vertebrates, whereas the innate immune system is phylogenetically conserved and is present in even primitive multicellular organisms (Hoffmann et al, 1999; Takeda et al, 2003). The mechanisms by which the innate immune system recognises non-self have been the subject of intense research for the past ten years (Medzhitov et al, 1997). Pattern recognition receptors (PRRs) of innate immunity recognise molecules that are broadly shared amongst pathogens, and are distinguishable from host molecules, called pathogen-associated molecular patterns (PAMPs). These PRRs include the mannose receptor, NOD-like receptors (NLRs) and Toll-like Receptors (TLRs), among others. Antigen presenting cells, such as macrophages, express a range of TLRs and thus help regulate the activation of the innate and, further down the line, adaptive immune responses (Janeway & Medzhitov, 2002; Medzhitov, 2001). Myeloid Differentiation Factor 88 (MyD88) is a critical adaptor molecule shared by many TLRs and signalling through many of these receptors is completely dependent on MyD88. However, MyD88independent pathways also exist for some TLRs, e.g. TLR4 (Akira & Hoshino, 2003).

Classical activation of macrophages is dependent on the products of activated T helper 1 (Th1) cells, in particular interferon- γ (IFN- γ) (Dalton *et al*, 1993) and, importantly, the

recognition of microbial PAMPS through TLRs (e.g. LPS and TLR4) (Aderem & Ulevitch, 2000). In the absence of MyD88, Th1 responses are greatly diminished and this indicates a role for TLRs and MyD88-dependent signalling in the control of adaptive Th1 immunity (Adachi et al, 1998). Th2 responses, on the other hand, have been shown in some studies to be intact or even augmented in the absence of MyD88 (Kaisho et al, 2002; Muraille et al, 2003; Schnare et al, 2001) and this suggests that Th2 responses are elicited in a MyD88-independent manner. However, other work suggests that TLR signaling does play a role in Th2 responses. For example, Eisenbarth et al, found that low levels of LPS-induced signaling through TLR4 is necessary to induce Th2 responses to inhaled antigens (OVA) in a mouse model of allergic sensitization (Eisenbarth et al, 2002). This was later found to be MyD88-dependent in a site-specific manner (i.e. governed by initial route of antigen exposure) (Piggott et al, 2005). Therefore, whether signaling through MyD88 is a requirement of a Th2 response may depend on the particular model under investigation. However, it is still not known whether signalling through TLRs is required for the induction of the alternative macrophage activation pathway.

Another reason to investigate MyD88 in the context of filarial infection is the extensive literature implicating TLRs in macrophage activation by the bacterial endosymbiont *Wolbachia*. During lymphatic filiariasis caused by the nematode *Brugia malayi*, inflammatory pathology can lead to lymphoedema and elephantiasis. Inflammatory responses can also be caused by filarial chemotherapy, which causes large quantities of parasite material to be released. The key pro-inflammatory cytokines IL-1 β and TNF- α are produced mainly by macrophages and these potentiate further inflammatory mediator expression (Taylor *et al*, 2000). It has been proposed that the inflammation caused by *B. malayi* is mediated by LPS-like activity from *Wolbachia* signalling through TLRs in a MyD88-dependent manner (Daehnel *et al*, 2007; Hise *et al*, 2007; Taylor *et al*, 2000). It was proposed originally that that *Wolbachia* contained LPS activity acting through TLR-4 (Taylor *et al*, 2000). However, it was subsequently found that it contains no LPS and fails to signal through TLR-4. The pro-inflammatory activity of *Wolbachia*

(at least *in vitro*) has more recently be found to occur through TLR-2 in a MyD88dependent manner (Hise *et al*, 2007), although the ligand is not known.

Other than the requirement for IL-4/IL-13 little is actually known about what determines the alternative activation phenotype in macrophages. In light of these studies above, we decided to investigate whether alternatively activated macrophages could be recruited in the absence of the important adaptor molecule, MyD88, and thus determine whether TLR signals impact negatively or positively on the AAM . We first investigated AAM o generated in vitro by using bone marrow-derived Mo (BMMo) treated with IL-4. We found that there was some reduction of arginase activity in the absence MyD88, but intact levels of Arginase1, RELM- α and YM-1 mRNA. Interestingly, we also found that MyD88-/- BMMø, unlike WT, only produced NO when treated with LPS and IFN-γ together, but not separately. For the in vivo studies, B. malayi worms were implanted into both WT and MyD88-/- mice and the peritoneal cell populations investigated after 19 days. The results were somewhat surprising, considering previous published reports showing evidence of significantly enhanced Th2 responses during nematode infection (Helmby & Grencis, 2003). No differences in cell numbers and type (with regards to proportions of Mo, eosinophils and lymphocytes) were found. We also found no difference in terms of arginase activity, suppressive ability or the expression of RELM- α or YM1 in purified NeMo. In agreement with this, the Th2 bias was not significantly affected in the MyD88-/- implanted mice when compared to the wild type.

2. Materials and Methods

2.1 Mice

All experiments used were C57BL/6 or MyD88-/- male mice, bred in house or purchased from Harlan, UK. Mice were 6-10 weeks old at the start of the experiment.

2.2 Brugia malayi infection

As in Chapter 2

2.3 Macrophage Activation

As in Chapter 2

2.4 Quantification of NO and arginase activity

As in Chapter 2

2.5 RNA extraction and real-time RT PCR

As in Chapter 2

2.6 Proliferation Assay

As in Chapter 2

2.7 Western Blotting

17 μ l samples were mixed with 9.5 μ l of loading mix (6.8 μ l NuPAGE LDS sample loading buffer + 2.7 μ l Nu PAGE Sample Reducing Agent) in eppendorf tubes. The lids were pierced and vials heated to 99°C on heat block for at least 10 minutes. Each sample was run on SDS-PAGE gel, with NuPAGE MES running buffer (20mls x20 NuPAGE MES buffer plus 380 mls dH2O) at 150 volts (V) for 55 min. The blotting apparatus was assembled and filter paper pre-soaked in NuPAGE transfer buffer (12.5ml x20 Transfer buffer, 25ml Methanol, 212.5 ml dH2O). NuPage reagents were from Invitrogen. The gel and nitrocellulose membrane (kept moist with transfer buffer) were placed in the blotting device and transfer carried out at 30V for 1hour. The transfer filter (BioRad Trans Blot) was then stained with Ponceau Red (Sigma) to check that the transfer was successful. Washing with TBS-Tween (100mls x10 TBS, 2ml Triton-X100 (Sigma), 500 μ l Tween 20 (Sigma), 897.5mls dH2O for 1 litre) was then carried out twice for 10 minutes. Another wash with x1TBS was carried out for 10 minutes. The blot was then blocked for 30 minutes in Pierce StartingBlock at room temperature with rocking. This was carried out in plastic bags (4 mls per bag). Primary Abs were then made to required strength in Pierce StartingBlock + 0.05% Tween-20: Anti-Ym1 (Allen lab (Nair et al, 2005)): 1/2500 (0.3 μ g/ml, 0.12 ng/ml)) and Anti-RELM α Peprotech 0.2 μ g/ml (=1/500) (made up again in 4 mls Ab-solution per filter/per bag). These were then incubated overnight at 4°C with rocking. They were then washed 3 times in TBS-Tween for 10 minutes and the secondary Ab made up to the required strength in Pierce StartingBlock + 0.05% Tween-20; Goat-anti-Rabbit HRP: 1/2000 (4 mls Ab-solution per filter/per bag). These were then incubated at room temperature for 1 hour with rocking and washed twice for 5 minutes in TBS-Tween and then 3x5 minutes in TBS. For detection with ECL kit (Amersham) the solution was prepared using 4 ml reagent A and 100 μ l Reagent B. The blots were incubated with this for 5 minutes. Any signal produced was detected using film (*Hyperfilm*: Amersham ECL Hyperfilm) (generally exposure of 10 - 180 seconds sufficient). A MultiImage light cabinet along with the Fluorchem programme (Alphainotech) were used to measure the relative concentrations of proteins on the blots.

2.8 Flow Cytometry

1 X 10⁶ cells per group were incubated at 4°C for 15 minutes in blocking buffer; 2% mouse serum, in FACS buffer (PBS supplemented with 2mM EDTA and 0.5% BSA), followed by staining for 20 minutes on ice with the antibodies (Ab) of interest at the appropriate dilution as determined by titration. The antibodies were generally directly fluorochrome conjugated or biotinylated. When using biotinylated antibodies, an additional step involving incubation of the cells with fluorochrome-conjugated streptavidin beads (Pharmingen) was performed. The Abs included anti-F4/80-biotinylated (1:100); anti-MHC II-FITC (1:200); PE-conjugated anti-CD86 (1:100); anti-CD4-APC (1:100); anti-CD8-PE (1:100); anti-B220-PCP (1:100) and anti-SiglecF-PE (1:100), as well as the appropriate isotype control Abs (anti-IgG2a-FITC, anti-IgG2a-biotinylated). The cells were then washed 3x in FACS buffer before acquisition and analysis (BD FACStation and FlowJo software).

2.9 Cytocentrifuge preparations

Cytocentrifuge preparations from 400μ l cells ($2x10^6$ cells/ml) in complete medium were made using a Shandon Cytospin. The slides were air-dried overnight and fixed for 10 minutes in cold methanol, followed by staining with Diff-Quik (Dade) according to the manufacturer's instructions. The cell populations were determined by microscopic examination (x40 objective) of at least 100 cells per slide.

2.10 Counting of Microfilaria

Peritoneal lavages were spun for 10 mins at 1250rpm and then cells resuspended in 2 ml DMEM. 10 μ l of the cell suspensions was added to 190 μ l FACS lysing solution (1X BD-Biosciences) to fix mfs. This was spun for 5 mins at 4000 rpm. 100 μ l was removed and the cells/mfs resuspended in the remaining 100 μ l of supernatant, which is spread on a slide. Counts are carried out by microscopic examination.

2.11 Parasite Extract

B. malayi adult male and female worms were used to make parasite extracts. Briefly, the worms in PBS were homogenised in a glass homogeniser on ice and the soluble fraction was collected after centrifugation twice at 13000 rpm for 15 minutes. The protein concentration was determined using the Coomassie plus protein assay (Pierce). Extracts were stored at -20° C until use for immunisations and in vitro cultures.

2.12 in vitro splenocyte cultures

The spleens were removed and single cell suspensions prepared. These were cultured in 96-well round bottom plates at 10^6 cells per well containing either 10μ g/ml parasite extract (BMA) or 1μ g/ml Concanavalin A (ConA) or medium alone (complete RPMI) at 37° C. After 72h of culture, supernatants were removed for cytokine assay.

2.13 Cytokine Assay

The amount of cytokines (IL-5, IL-4, IL-13, IFN γ and IL-10) in the culture supernatants was measured using BD Cytometric Bead Array Flex sets, with slight modifications from the manufacturer's instructions (BD Biosciences). Briefly, 50µl of sample or standard were incubated in round-bottom 96-well plates (Costar) with 25µl of cytokine capture bead mixture (anti-cytokine-coated microspheres, 0.5µl/sample/cytokine) with protection from the light and gentle shaking for 10 minutes. They were then incubated on bench top for 50 mins (1h total incubation) at room temperature. Plates were washed with 200µl of wash buffer and spun at 200g for 5min. Samples and standards were then incubated with 25µl of PE detection reagent (phycoerythrin-conjugated anti-mouse cytokine antibodies) in darkness for 1h (again gently rocking for 10 mins and then 50 minutes on bench). After another wash, beads were resuspended in 150µl of wash buffer. Samples were acquired on FACSArray analyser (BD Biosciences) and amount of cytokines obtained using FCAP analysis software (BD Biosciences).

2.14 Data analysis

Graphs were prepared using PRISM (GraphPad software, Berkeley, CA). The Mann-Whitney test was carried out to test for significance.

3. Results

3.1 MyD88-/- bone marrow-derived macrophages display deficiencies in classical activation but can become alternatively activated when treated with IL-4.

Arginase 1 expression is a consistent feature of alternatively activated macrophages and its expression is generally dependent on Th2-type signals, such as IL-4 (Louis et al, 1999). To determine whether signalling through TLR, or more specifically MyD88, was necessary for the alternative activation of macrophages, we decided to investigate whether bone marrow-derived macrophages (BMM ϕ), cultured from both WT and MyD88 deficient mice, could be activated in this way. The activity of the opposing enzyme, iNOS, which is a signature enzyme associated with the classical activation of macrophages was also measured (Mori & Gotoh, 2000). To further characterise the AAM ϕ phenotype, we measured the expression of other markers of alternative activation, *RELM-α* and *YM-1*.

Macrophages were treated +/- IL-4 overnight before treatment with LPS and IFN- γ , either together or separately, or media alone, as indicated for 16-20 hours. After this time, arginase and iNOS enzyme activities were monitored (Figure 1 A and B), as well as mRNA expression of *Arginase 1*, RELM- α and *YM-1* (Figure 1 C). Both WT and MyD88-/- cells exhibited arginase activity in response to IL-4 and the MyD88-/- mice appeared to produce less than the WT mice in response to IL-4 (Figure 1 A). However, untreated WT BMM φ also produced arginase and this response was abolished in MyD88-/- mice. This suggests that some MyD88-dependent stimulus in the culture medium may have been contributing to arginase production by these macrophages (see discussion). Indeed, LPS is shown to induce arginase activity over the media background but only in WT animals.



Figure 1. Upon stimulation with IL-4 BMM ϕ from MyD88-/- mice produce arginase but only display iNOS activity when stimulated with LPS and IFN- γ together. BMM ϕ from WT and MyD88-/- mice on the C57BL/6 background were grown *in vitro* as described in the materials and methods. M ϕ were preteated o/n with IL-4 and then given LPS and IFN- γ together or separately for 16-20 hours. Urea concentration is shown as a measure of arginase activity (a) and nitrite as a measure of iNOS activity (b). mRNA was extracted and realtime RT-PCR for *Arginase 1*, *RELM-\alpha* and *YM-I* expression carried out (c). mRNA expression is shown as a % of a positive control sample and was normalised to β -actin. Results are shown as the mean of replicate samples (+/- S.E.M) and are representative of three experiments.

135

Nitrite in the supernatants of the cultured macrophages was measured using the Greiss reagent and was used as a measure of iNOS activity. As expected, WT BMM ϕ produced nitrite when treated with LPS and/or IFN- γ . The two stimuli together had an additive effect on iNOS activity. WT BMM ϕ , pretreated with IL-4, did not produce NO when stimulated with LPS alone (Figure 1 B). This may be due to the contribution of LPS to the activity of the opposing enzyme arginase, which would have a negative effect on NO production. Intriguingly, MyD88-/- BMM ϕ only produced NO when treated with LPS and IFN- γ together but not either stimulus alone.

There was no impairment in the ability of MyD88-/- BMM ϕ treated with IL-4 to produce other markers of alternative activation, including *RELM-\alpha* and *Ym-1* (Figure 1 C). Notably, there was only a slight trend towards decreased *Arginase 1* expression in MyD88-/- BMM ϕ , which was at odds with the arginase activity data (Figure 1). This RNA data implies that there is no deficiency in the ability to generate AAM ϕ in MyD88-/- animals and that enhanced arginase enzyme activity seen *in vitro* by untreated WT cells depends on a MyD88-dependent mechanism.

3.2 The Th2 bias is not significantly altered in *B. malayi*-implanted mice in the absence of MyD88.

Before determining the impact of MyD88 deficiency on macrophage activation status *in vivo*, it was important to first ascertain if there would be any impairment or enhancement in the overall Th2 response in *Brugia* implanted mice. For this, the Th2 cytokines IL-4, IL-5, IL-10 and IL-13, as well as IFN- γ as a marker of Th1 activation, were measured from the supernatants of cultured splenocytes treated with media alone, ConA or BMA (Figure 2 B-E). As expected, all Th2 cytokines were increased in an antigen-specific manner to BMA in WT implanted mice. The antigen-specific Th2 cytokines, IL-4, 5, 10 and 13 were further elevated in the MyD88-/- implanted mice, but this did not reach



Figure 2. The Th1 response is impaired in MyD88-/- mice but the Th2 bias is not significantly altered in *B. malayi*-implanted animals. Splenocytes from mice on the C57BL/6 background were treated with media alone, Concanavalin A (ConA) or *Brugia malayi* antigen (BMA) for 72 hours before the supernatants were removed and levels of IFN- γ (a), IL-4 (b), IL-5 (c), IL-10 (d) and IL-13 (d) measured by cytometric bead array. Significant differences were determined by the Mann-Whitney test * p<0.05, **p<.01. These results are representative of three experiments.

statistical significance as measured by a Mann-Whitney test (Figure 2 B-E). In agreement with previous reports, the Th1 response was impaired in MyD88-/- animals, as measured by IFN- γ production by cultured splenocytes (Figure 2 A). This was true for both MyD88-/- implanted mice and thioglycollate injected mice compared to their WT counterparts. The difference in IFN- γ production between WT and MyD88-/- implanted mice was found to be statistically significant in response to both ConA and BMA. This trend was also seen between the WT and MyD88-/- thioglycollate-treated mice. However, there were too few mice in these control groups (n=3) in this particular experiment to carry out the Mann-Whitney test with accuracy. Overall, these results showed that the Th2 response was not impaired in MyD88-/- mice that had been implanted with *B. malayi* and that there may have been some enhancement.

3.3 Absence of MyD88 does not affect the cell recruitment after *B. malayi* implant but does result in reduced thioglycollate-elicited recruitment.

Peritoneal exudates cells (PEC) were recovered and counted from WT and MyD88-/mice surgically implanted with *B. malayi* adult worms (d19) and some injected i.p. with thioglycollate for three days. There was no difference in cell numbers between WT and MyD88-/- mice implanted with *B. malayi* (Figure 3 A). However, there were significantly fewer cells recruited into the gene-deficient animals than WT after thioglycollate injection (Figure 3). In two further repeats of this experiment this difference in thioglycollate recruitment was not significant, but the trend continued. This indicates that a TLR stimulus may be at least partially required for cell recruitment by thioglycollate and is not surprising considering that it is likely to contain TLR ligands (see discussion).



Figure 3. No impairment in cell recruitment in MyD88-/mice after implantation with *Brugia malayi* worms. 19 days after implant (imp), and 3 days after thioglycolate i.p. injection (thio) PECs (mice on C57BL/6 background) were recovered and counted. Significant differences were determined by the Mann-Whitney test * p<0.05. These results did not repeat in subsequent experiments.

3.4 The absence of MyD88 does not affect recruitment of different cells types to peritoneal cavity.

Although there were no overall differences in cell numbers recruited to the peritoneal cavity in implanted WT vs. MyD88-/- mice, it was possible that a lack of MyD88 affected the profile or quality of cells recruited. Therefore, we examined the proportion of F4/80 (Figure 4 A) and SiglecF positive cells (Figure 4 B) in the PEC by flow cytometry to determine the % macrophages and eosinophils present respectively. The cell populations were also determined by microscopic examination of cytocentrifuge preparations (cytospins; Figure 4 D & E). There was a discrepancy between the proportion of F4/80 +ve cells found by FACs staining (Figure. 4a) and macrophages enumerated under the microscope (Figure 4 D). Nonetheless, there was no difference in the proportion of macrophages recruited i.p. between WT and KO animals. The relative percentages of macrophages seen by cytospin (Figure 4 D) are more in agreement with previous work in the lab than the measure of F4/80 +ve cells by FACs (Figure 4a). We do not know the reasons for the differences between the FACs and cytospin data but they could reflect cell death during the FACs preparation or suboptimal staining of particular populations. There was also no difference seen between the WT and MyD88-/- in terms of eosinophilia, although again there was a slight disparity between the FACS staining and cytospins (Figures 4 B & E).

Figure 5 also shows there was no difference between WT and KO mice in terms of lymphocytes. Similar proportions of CD8 and CD4 T cells were found in the PECs of both WT and KO implanted mice (Figure 5 A & B). B220 was used as a marker of B cells and there may have been a slight decrease in these cells in the implanted animals that lacked MyD88 (Figure 5 C). Total lymphocyte populations were determined by microscopic examination of cytocentrifuge preparations and there was no difference between the WT and KO mice (Figure 5 D).

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Figure 4. Similar numbers of macrophages and eosinophils are recruited in wild type (WT) and MyD88-/- mice (KO). 19 days after implant (3 days after thioglycollate injection; Thio) PEC from mice on the C57BL/6 background were recovered and double-stained for F4/80 (A) and SiglecF given in С. **(B)**. Sample plots are Cytocentrifuge preparations were also made and macrophages (D) and eosinophils (E) enumerated by microscopic examination. imp; from implanted mice. These results are representative of three experiments.



Figure 5. Similar numbers of T and B cells are recruited in Wild Type (WT) and MyD88-/- (KO) mice. 19 days after implant (3 days after thioglycollate injection; Thio) PEC were recovered from mice on the C57BL/6 background and stained for CD8 (A), CD4 (B) and B220 (C). Cytocentrifuge preparations were also made and total lymphocytes (D) enumerated by microscopic examination. These results are representative of three experiments.

142

3.5 Absence of MyD88 does not affect arginase production or suppressive ability of the NeM₀ resulting from *B. malayi* implant.

To determine the phenotype of the recruited cells, macrophages were purified from the PEC by adherence and the levels of arginase activity measured (Figure 6 A). As predicted, NeM ϕ produced more arginase than ThioM ϕ but there was no difference in the levels of arginase between WT and MyD88-/- mice NeM ϕ , but there was a significant difference between the WT and MyD88-/- ThioM ϕ populations (Figure 2 B). Although not as apparent as the *in vitro* result, thioglycollate induces a low level of arginase production in the absence of MyD88 after IL-4 treatment in BMM ϕ (Figure 1 A) and in response to thioglycollate (Figure 6 A) was not evident in macrophages from a chronic *in vivo* Th2-type setting.

Since proliferative suppression is a well-defined characteristic of NeM ϕ (Loke *et al*, 2000b) we decided to investigate whether the absence of MyD88 would affect this feature. As expected, EL-4 cell proliferation was suppressed when co-cultured with NeM ϕ in comparison to control ThioM ϕ (Figure 6 B). This was still true for NeM ϕ generated in MyD88-deficient animals. Therefore, deficiency of signalling through TLRs did not have an effect on this NeM ϕ trait either. WT ThioM ϕ can also exhibit proliferative suppression, although this is not IL-4 dependent (unpublished data not shown). This suppressive activity was seen here (Figure 6 B) and, in contrast to NeM ϕ mediated suppression, was MyD88-dependent.



Figure 6. There was no difference in the arginase activity or suppressive ability of recruited NeM ϕ (WT or MyD88-/- mice) after implantation with *B. malayi* worms. 19 days after implanting, PEC were recovered from mice on the C57BL/6 background and macrophages were purified by adherence and arginase enzyme activity calculated (A). Suppressive ability was measured by replacement of the medium and co-culture with EL-4 thymoma cells. After 48 hours, the EL-4 cell proliferation was assessed by [³H] thymidine incorporation (B) In all cases thioglycollate (thio) was also injected i.p. for 3 days as a control. Significant differences were determined by the Mann-Whitney test * p<0.05. These results are representative of three experiments.

3.6 Absence of MyD88 does not affect numbers of microfilaria, or expression of the alternative activation markers Ym1 or RELM- α , in the peritoneal fluid of implanted mice.

We next wanted to address whether absence of MyD88 affected worm viability. A count of the first larval stage of a filarial worm, the microfilariae produced by the implanted female worms, can be an indication of worm viability (Rao & Well, 2002). After 19 days of *B. malayi* infection, the peritoneal lavage fluid was extracted and the numbers of microfilaria were counted from both WT and MyD88-/- animals (Figure 7 A). No difference was detected between the two, suggesting that the absence of signalling through TLRs had neither a positive nor negative effect on worm survival and fecundity in this model of filarial nematode infection.

Since the AAM ϕ markers Ym1 and RELM- α are secreted proteins, western blots were carried out in order to detect these proteins in the peritoneal lavage fluid of implanted mice (Figure 7 B-C). Once again no detectable significant difference was found between the WT and MyD88-/- animals. If MyD88 was required for the alternative activation of macrophages we might expect less protein expression in the MyD88-/- mice, but on the contrary, there was a slight trend towards higher RELM- α expressed by the MyD88-deficient mice (Figure 7 B), consistent with the trend towards higher Th2 induction.

RNA was also extracted from purified macrophages and Realtime RT-PCR carried out for Ym-1, RELM- α and Arginase1 mRNA expression (Figure 8 A-C) to see whether these levels would correlate with YM-1 and RELM- α protein production (Figure 7) and arginase activity (Figure 6). Ym-1, RELM- α and Arginase1 (Figure 8 A-C) mRNA levels correlated with what was measured for protein expression. In one experiment a large significant increase in RELM- α mRNA expression was measured in NeM ϕ in the absence of MyD88 (data not shown). However, this result did not repeat in subsequent experiments.

145



Figure 7. The absence of MyD88 had no effect on numbers of microfilaria, or the production of RELM- α or Ym1 proteins by cells in the peritoneal cavity after *B. malayi* implant. At day 19 after *B. malayi* implant of mice on the C57BL/6 background, i.p. lavages were carried out and numbers of microfilaria present enumerated (A). Western blots were carried out using the lavage fluid for RELM- α (B) and YM-1 (C). These results are representative of three experiments.



Figure 8. The absence of MyD88 had no effect on Ym1, RELM- α or Arginase1 mRNA expression by purified macrophages from the peritoneal cavity after *B. malayi* implant. At day 19 after *B. malayi* implant of mice on the C57BL/6 background, i.p. lavages were carried out and macrophages purified by adherence. ~ RNA extraction and realtime RT-PCR was carried out for Ym1 (A), RELM- α (B) and Arginase1 (C). mRNA expression is shown as a % of a positive control sample and was normalised to β -actin. These results are representative of three experiments.

147

Despite a subtle enhancement in some aspects of the AAM\$\$\$\$\$ phenotype, taken together these results indicate that an absence of MyD88 does not have a marked effect on the numbers or types of cells recruited to the peritoneal cavity due to the presence of a helminth infection.

4. Discussion

These results indicate that macrophages from a chronic in vivo Th2-type setting do not require signaling through TLRs for the induction of the alternative activation of macrophages. We found no impairment in the Th2 response in the absence of MyD88 in B. malayi implanted mice compared to WT when spleen cells were treated with antigen from this nematode (BMA; Figure 2), and even found a trend towards augmentation of the Th2 response, although this was not statistically significant. Previous studies using MyD88-deficient mice showed evidence of significantly enhanced Th2 responses (Eisenbarth et al, 2002; Muraille et al, 2003). This is in agreement with work carried out with the gastrointestinal nematode Trichuris muris (Helmby & Grencis, 2003). Resistance to this parasite requires a Th2 response, which is impaired in susceptible strains. MyD88-/- animals are highly resistant to chronic infection with this parasite and display enhanced Th2 responses relative to wild type counterparts, as measured by IL-4 and 13 production from mesenteric lymph node cells stimulated in vitro with T. muris antigen (Helmby & Grencis, 2003). This augmentation of type 2 cytokines is far greater than the trend we observed in this study. T. muris worms burrow within cecal epithelial cells, exposing these cells to commensal bacteria. It is likely that this acts as a powerful stimulus of the Th1 response, through MyD88-dependent pathways. Thus the Th2 response to the nematode may normally be impaired due to an elevated Th1 response (deSchoolmeester et al, 2006). In the absence of MyD88, the Th2 response would be unleashed and these animals able to expel the parasite. Therefore, the increase in Th2 response in the MyD88-/- animals infected by T. muris over the WT is likely due to the fact that they cannot mount an effective Th1 response against the bacteria to which they are exposed. This is supported by reports that in the absence of MyD88, signalling through TLR4 (by the MyD88-independent pathway) can confer the ability to support Th2 responses (Kaisho *et al*, 2002).

Relative to *T. muris*, the *B. malayi* implant model is essentially sterile, with no expectation of a type 1 response due to commensal bacteria. Therefore, it follows logically that we did not see a significant increase in the Th2 response in the absence of MyD88. However, as previously mentioned, *B. malayi* contains endosymbiotic bacteria, which may be expected to influence the immune response. Even so, despite the strong *in vitro* evidence that *Wolbachia* ligands can signal through TLRs in a MyD88-dependent fashion (Hise *et al*, 2007), we saw little effect of MyD88 deficiency. Thus, in the context of live infection, the role of *Wolbachia* may not be as great as previously presumed.

Although we found no significant differences in the magnitude of the immune response elicited between WT and MyD88-/- implanted mice, MyD88 deficiency may still have influenced the ability of macrophages to respond appropriately to signals in vivo. However, this did not seem to be the case as similar numbers (Figure 3) and types of cells (Figure 4 and 5) were recruited to the peritoneal cavity in both stains. In terms of functionality of AAM\$, MyD88 deficiency had no effect on the ability of NeM\$ to suppress the proliferation of co-cultured EL-4 cells (Figure 6 B). Also, levels of arginase activity were the same in WT and MyD88-/- NeM
(Figure 6 A). Protein expression of YM-1 and RELM- α in the peritoneal cavity were also unaffected by MyD88-deficency (Figures 7 B & C). mRNA expression of YM1 (Figure 8 A) and Arginase 1 (Figure 8 C) correlated exactly with protein expression (Figure 7 C, YM-1 and Figure 6 A; arginase respectively). Not surprisingly, the absence of any change in effector cell function or numbers translated into no effect on Brugia worm viability, as we found similar numbers of microfilariae in the peritoneal cavity of both WT and MyD88-/- animals (Figure 7 A). One significant difference was identified in that we found that RELM- α mRNA expression was greatly increased in NeMø in the absence of MyD88 (Figure 8

B). However, this did not translate into changes in protein levels, as we have already seen.

Although, for the most part, MyD88 deficiency had a limited impact on parasiteimplanted mice, we did see some effects on the response to thioglycollate treatment. There was a trend towards an impairment in cell recruitment to the peritoneal cavity in thioglycollate treated MyD88-/- mice (Figure 2). There was also a decrease in arginase activity in MyD88-deficient ThioM ϕ (Figure 6 A) and also a decrease in the suppressive ability of these M ϕ compared to WT (Figure 6 B), as measured by an increase in proliferation of co-cultured EL-4 cells. These facts may be linked, since in chapter 2 we observed that arginase could contribute to the suppressive ability of NeM ϕ (Chapter 2, Figure 4 C). However, this effect of arginase is likely to reflect only a small part of the story, given the relatively large difference in suppressive ability of WT ThioM ϕ , in relation to MyD88-/- ThioM ϕ (WT approx. 10 X more suppressive; Figure 6 B), compared to the differences in arginase enzyme activity (WT display approx. 2X more arginase activity than MyD88-/-; Figure 6 A).

Although the *in vivo* work from this study implied that MyD88 is not required for an optimal Th2 response, and resulting AAM ϕ , the *in vitro* work using BMM ϕ treated with IL-4 tells a somewhat different (but not contradictory) story about the importance of MyD88. In figure 1 A we saw an impairment in the arginase activity in MyD88-/-BMM ϕ compared to that of WT animals, even in the absence of IL-4. WT BMM ϕ also produced arginase suggesting that some stimulus in the culture medium was contributing to arginase production by these macrophages. The complete absence of arginase activity in MyD88-/- untreated cells demonstrates that this media-induced activity is MyD88 dependent. Consistent with this, treatment with LPS, which signals through TLR4, also increased arginase production (Figure 1a: left), which is in agreement with published reports that LPS can induce production of arginase, and indeed, both isoforms of this enzyme (arginase 1 and 2) can be induced by LPS (Louis *et al*, 1999). There was only a slight trend towards a decrease in *Arginase 1* mRNA expression in MyD88-/- BMM ϕ and thus the increased MyD88-dependent arginase activity seen in WT animals (Figure 1a) may have been due to arginase 2, as the enzyme assay does not distinguish between the two. In both WT and MyD88-/- macrophages IL-4 was able to increase the level of arginase. Similar levels of the other markers of alternative activation, *RELM-α* and *Ym-1*, were produced in WT and MyD88-/- BMM ϕ in response to IL-4 providing further evidence that there was no deficiency in the ability to produce AAM ϕ in MyD88-/animals. This also illustrates the importance of looking at several M ϕ readings, as measuring arginase activity alone could give a skewed impression of what was actually happening.

Another intriguing aspect of the *in vitro* studies using BMM ϕ was the fact that nitric oxide was only produced by MyD88-/- M ϕ when LPS and IFN- γ were given together but not separately (Figure 1 B), whereas WT BMM ϕ produced nitric oxide with LPS and IFN- γ independently. One interpretation of why this might be is as follows: In the absence of MyD88 (Figure 9 right) LPS signals through TLR4 via the MyD88-independent pathway (through interferon regulatory factor (IRF)-3), which causes the upregulation of IFN- β , but not iNOS. IFN- β in turn causes the upregulation of the transcription factor IRF-1 (Fujita et al, 1989). IRF-1 expression coupled with the IFN- γ in the media then leads to the production of NO. This is supported by previous reports showing that LPS-dependent augmentation of *iNOS* mRNA expression by IFN- γ is due to IRF-1 upregulation by LPS (Koide *et al*, 2007). IFN- γ cannot induce iNOS alone because there is a lack of IRF-1 (or some other molecule involved in IFN- γ signalling) in the absence of MyD88.

This theory relies on the presupposition that there is, for example, no IRF-1 present in the absence of MyD88, and that it is upregulated by LPS through the MyD88-independent pathway. When MyD88 is intact (Figure 9 left), LPS induces iNOS in a MyD88-dependent manner. IFN- γ causes the upregulation of iNOS through IRF-1,



Figure 9. Possible scenario to explain need for LPS/IFN- γ together for the induction of NO in the absence of MyD88. When MyD88 is intact, LPS signaling through TLR4 causes the upregulation of iNOS and also IRF-1. IFN- γ signaling through it's receptor produces NO if IRF-1 is already present. In the absence of MyD88, LPS cannot produce NO but can produce IRF-1 through the MyD88-independent pathway. IFN- γ alone cannot produce NO due to a lack of IRF-1. However, when LPS and IFN- γ are together IRF-1 is present and therefore IFN- γ treatment can now lead to the upregulation of iNOS.

which is present since MyD88 is intact. Treating the macrophages with both LPS/IFN- γ together causes a synergistic increase in iNOS, due to increased IRF-1 expression by LPS (IRF-1 must be produced by LPS whether MyD88 is there or not).

In summary, these results in this chapter have shown that MyD88 was not required for the alternative activation of macrophages either *in vitro* or *in vivo*. Further, the Th2 response was fully intact in MyD88-/- mice implanted with the filarial nematode *B*. *malayi*.

CHAPTER 7

General Discussion

Macrophages display a wide range of phenotypes, which are dependent on the signals they have encountered during migration to sites of infection. Macrophages have been usefully classified as classically or alternatively activated depending on their exposure to Th1- or Th2-type stimuli respectively (Gordon, 2003). However, the range of macrophage phenotypes is likely to be much broader. It may be an economical strategy for macrophages to display plasticity of function, since they are long-lived and may need to adapt their function to different pathogens or environments faced during their lifespan. Investigating macrophage plasticity in vivo could have important implications for therapeutic targeting of macrophages in chronic diseases but also for our general understanding of how the immune system copes with multiple infections that may require differing immune responses. The main aim of this PhD was firstly to establish the level of functional plasticity in NeMo when treated with Th-1 type stimuli, such as LPS/IFN-y, and then to elucidate how these AAM behaved when infected with Th1inducing intracellular parasites. We then wished to investigate the possible in vivo relevance of this through a co-infection experiment involving a Th2-inducing helminth infection (N. brasiliensis) coupled with the Th-1 inducing protozoan malaria parasite P. chabaudi. Finally, it was decided to use animals deficient in the ability to respond to type-1 signals. This work, using MyD88-deficicent mice, would address whether there was a requirement for this adapter molecule, important in TLR signalling, in the alternative activation of macrophages.

1. NeMø are not terminally differentiated but can respond to Th-1 type stimuli

In chapter 2 of this thesis it was found that, despite the long-term exposure to Th2 cytokines and anti-inflammatory signals in vivo, NeM ϕ were not terminally differentiated but could switch from alternative activation to a more classically activated phenotype in response to LPS/IFN- γ . This was reflected in the ability of NeM ϕ , from the *B. malayi* implant model, to rapidly swap L-arginine metabolism from the arginase enzymatic pathway to iNOS activity. This phenotype change was further demonstrated by the downregulation of messenger RNA for two AAM ϕ markers, *Ym1* and *RELM-\alpha*, in NeM ϕ treated with LPS/IFN- γ . However, protein expression remained elevated, suggesting that regulation occurs more rapidly at a transcriptional level. This could also mean that NeM ϕ can retain aspects of their AAM ϕ phenotype, while directing their transcriptional machinery to other priorities. These data imply that NeM ϕ can redirect their phenotype to allow resources to be devoted to antimicrobial factors, such as NO, and also pro-inflammatory cytokines, such as TNF- α and IL-6, which also increased upon treatment with the type-1 stimuli LPS/IFN- γ (chapter 2).

LPS/IFN- γ treatment also led to an increase in MHC II and the co-stimulatory molecule CD86 in NeM ϕ , suggesting that they would have enhanced APC function. However, the phenotypic switch from AAM ϕ to CAM ϕ was not complete. LPS/IFN- γ treated NeM ϕ retained their anti-proliferative capacity and failed to upregulate IL-12p40 and so would be unable to promote Th1 or sustain Th17 cell development. This implies that NeM ϕ may have undergone epigenetic changes *in vivo*, which prevent them from producing IL-12. This possibly reflects work from Foster and colleagues who have shown that continuing exposure to toll-like stimulus (such as LPS) can lead to gene-specific modifications of chromatin, causing the priming of antimicrobial effectors while silencing pro-inflammatory mediators (Foster *et al*, 2007). It would be of interest to investigate whether NeM ϕ have indeed undergone specific epigenetic modification at the promoters for the IL-12 genes, by using chromatin immunoprecipitation (ChIP) assays, as Foster *et al.* have done for other pro-inflammatory cytokines, such as IL-6. A lot of progress has been made in recent years in understanding the epigenetic control of gene expression of helper T cell differentiation (Reiner, 2001), as opposed to macrophages, where relatively little is known apart from the work mentioned above (Foster *et al*, 2007). Further investigation of factors affecting chromatin remodelling, or other epigenetic changes, in macrophages will be essential to fully understand macrophages development and plasticity.

This thesis has also added to our understanding of proliferative suppression by macrophages. We recently provided evidence that TGF-B may be involved to some degree (Taylor et al, 2006) and our ability to detect production of this cytokine by NeMø is consistent with this (Chapter 2, Figure 6). We have now seen that arginase contributes to this suppression since inhibition with nor-NOHA caused a partial reversal of this phenotype (Chapter 2, Figure 4 C). However, we also found that both arginase (Chapter 2, Figure 1 A+C) and TGF- β (Chapter 2, Figure 6) production were partially reversed upon treatment with LPS/IFN-γ and yet NeMφ retained full suppressive abilities (Figure 4 A), suggesting that these are not the main players in NeMo-mediated suppression. In addition to this, we found that PD-L1 and 2, members of the B7 family implicated in the proliferative suppression observed by macrophages during infection with the platyhelminths, T. crassiceps and S. mansoni (Smith et al, 2004; Terrazas et al, 2005) are probably not responsible for the suppression we observe during infection with B. malayi (Chapter 2 and Appendix 2). We also found that, despite previous studies reporting PD-L2 as a marker for in vitro AAM\$ (Loke & Allison, 2003), NeM\$ did not express PD-L2 (Appendix 2 B).

2. Infection of NeMo with intracellular pathogens

Given that AAM ϕ generated *in vivo* could switch to a more CAM ϕ phenotype in response to Th1 activating signals LPS/IFN- γ , we decided to investigate whether this would translate into an ability to control the intracellular parasites *L. mexicana* and *M. bovis* BCG. We found that treated NeM ϕ could effectively control *L. mexicana* promastigotes, and that they did so through LPS/IFN- γ -induced NO production, as shown through use of the NO inhibitor L-NMMA (chapter 3). We were able to visualise parasite infection using a strain of *L. mexicana* promastigotes that display red fluorescence. Simultaneous expression of Ym-1 fluorescent staining with red parasites demonstrated that AAM ϕ do not display a full switch in phenotype, despite gaining the ability to control *Leishmania* parasites. NeM ϕ were also shown to produce the complement product *C3*, expression of which increased upon LPS/IFN- γ treatment. The pattern of *C3* expression correlated with uptake of these parasites and this adds evidence that complement produced may be contributing to *Leishmania* entrance.

As with *Leishmania* infection, Th2 responses are thought to promote disease during mycobacterial infection (Cooper & Flynn, 1995; Erb *et al*, 2002). In order to investigate how NeM ϕ cope with another type-1 pathogen, we infected them with *M. bovis* BCG (chapter 4). NeM ϕ were able to control infection with BCG at a low dose with or without IFN- γ treatment (and subsequent NO production). It would be possible to test whether reactive oxygen intermediates (ROIs) were involved in the NeM ϕ control of BCG by treating them with superoxide dismutase, which disrupts ROI production. IFN- γ treated ThioM ϕ were more effective at controlling BCG than untreated cells. Unlike NeM ϕ , this killing was dependent on NO, as L-NMMA treated IFN- γ -treated ThioM ϕ could no longer to control BCG.

Macrophages undergoing apoptosis can kill intracellular mycobacteria, while those dying by necrosis do not and it has been found that macrophage apoptosis is an

important mechanism to clear the parasite during tuberculosis infection (Fairbairn, 2004). Indeed, at high doses of BCG infection, NeM ϕ were dying by apoptosis, whereas ThioM ϕ were not. This was independent of IL-4, as IL-4-/- NeM ϕ still underwent apoptosis at high infection dose.

High IL-6 production may be involved in favouring apoptosis in NeM ϕ , since they were producing much higher levels of this cytokine than ThioM ϕ , but this is unlikely to be the whole story. It would be interesting to investigate why the combination of NeM ϕ with high dose of infection with BCG uniquely led to apoptosis. M ϕ do not readily undergo apoptosis and thus determining these factors would be of interest. This might be tested by treating ThioM ϕ with various cytokines (with or without BCG infection) and then carrying out TUNEL staining to see if any cytokines induced apoptosis along with infection.

Chapters 3 and 4, taken together, indicate that NeM ϕ retain the ability to switch to an anti-microbial phenotype, either by LPS/IFN- γ induction of NO against *Leishmania* parasites, or other mechanisms against mycobcateria including apoptosis-mediated killing. These abilities could confer the ability in AAM ϕ to effectively deal with a potentially life-threatening type-1 infection until a more specialised cell could be recruited. To take these types of studies forward, it would be necessary to investigate NeM ϕ response to type-1 pathogens in an *in vivo* setting. To carry this out, we could expose mice, already implanted with *B. malayi*, to type-1 pathogens that can establish i.p. infections, such as *Samonellae* spp. We could establish whether AAM ϕ in a real type-2 setting could control a type-1 pathogen, and perhaps measure the different levels of exposure to these differing infections the NeM ϕ could tolerate. This type of study would also finally allow us to test the hypothesis that NeM ϕ could potentially control infection with an intracellular pathogen until such time that a more suitable phenotype of M ϕ , and other leukocytes, could be recruited to the site of infection. To investigate this,

the influx of macrophages and other cells types, plus their activation status, could be measured over time of co-infection.

3. AAM₀ markers from an *in vivo* co-infection setting

There are definite limitations to the conclusions one can make from these *in vitro* infections of macrophages with *L. mexicana* and BCG. As mentioned above, *in vivo* studies would have to be carried out to question the validity of our finding that NeM ϕ can control these pathogens. An ecological/evolutionary analysis of co-infection with type-1 versus type-2 parasites had been planned (by Andrea Graham's group, in collaboration with our lab) and we took this opportunity to study *in vivo* interactions using two well-established laboratory models. This involved the co-infection with the Th1-inducing malaria parasite *P. chabaudi* and the Th2-inducing helminth *N. brasiliensis*. A drawback for this PhD project was that this study could not address the question of how NeM ϕ handle intracellular pathogens directly. Nonetheless, malaria does require the classical activation of macrophage activation status in a situation where type 1 and type-2 immunity co-exist could be asked.

The effect of co-infection on markers of alternative activation in the lungs of mice was principally investigated in chapter 5. It was found that concurrent infection with malaria parasites could modulate the Th2 response to the helminth. By day 7 post-infection (a timepoint when the Th2 lymphocyte response against the helminth would be coming into play) singly infected *N. brasiliensis*-infected mice expressed higher levels of Ym1 and RELM- α than co-infected animals. This was reflected by the cytokine production in the draining lymph nodes, in which singly-*N. brasiliensis* infected mice produced more IL-4. IL-5. IL-13 and IL-10 than those co-infected with malaria. This trend was reversed by 20 days post infection, where there was higher expression of Ym1 and RELM- α in

co-infected than in *Nippostrongylus*-only lungs, although mRNA and protein levels had diminished substantially. The reason for these patterns could be that malaria infection caused the Th2 response to the helminth to be delayed. Another possibility could be that the Th2 response is induced at the same time in the co-infected animals, but to a lesser extent, and persists longer in the co-infected animals. These two hypotheses could be tested by carrying out a timecourse experiment of Ym1 and RELM- α expression in singly- and co-infected lungs and measuring the Th2 cytokines more frequently (e.g. every day for the first 7 days and then every second day thereafter) for the length of the experiment, i.e. 20 days.

The importance of co-infection studies is evident when one considers that 740 million people are thought to be affected by hookworm infections, such as Neactor americanus, in the tropics where malaria is also endemic (WHO). It is now known that damage to the lung of mice previously infected with N. brasiliensis is evident even at very late time points after infection (>300 days) and this ongoing tissue damage is associated with the presence of AAMo. Marsland et al. postulate that these macrophages are either part of the host tissue response or may be perpetuating continuing disease (Marsland et al, 2008). We do not yet know how the Th2 response or AAM¢ contribute to healing of lung tissue, or indeed on-going lung disease in the case of Nippostrongylus infection. Nonetheless, our data (Chapter 5) suggest that co-infection with malaria, with a lowered Th2 response, could affect the repair process in the lung and, depending on the role of the AAM of in this setting, concurrent malaria infection along with this helminth could have detrimental or advantageous consequences. This knowledge could have important implications when considering treatment of people with these types of concurrent infections. When the role of AAM ϕ in lung damage/repair is discovered, they could be used as potential therapeutic targets (for induction or destruction).

Although the focus of this thesis has been on macrophages, it is important to consider that many of the findings may apply to other cells that respond to the Th2 cytokines IL-4 or IL-13. This is emphasised in chapter 5 as, in the malaria-*Nippostronglyus* co-

infection, we found that the markers associated with the alternative activation of macrophages Ym-1 and RELM- α were found by another cell type, namely the bronchial epithelial cells of the lungs (chapter 5, materials and methods, Figure 5 C). Studies from this, and other labs, have also suggested that Ym-1 may also be expressed by neutrophils and eosinophils, and RELM- α also by eosinophils (Harbord *et al*, 2002; Loke *et al*, 2007; Voehringer *et al*, 2004). This suggests that other cell types can become "alternatively activated" perhaps as part of an innate response to injury (Loke *et al*, 2007), and so may have importance during wound healing.

Another interesting finding from this experiment was that the malaria parasitemia was lower in the animals co-infected with the helminth and malaria, than those infected with malaria alone. One reason proposed for this is that the transient anaemia early on caused by *N. brasiliensis* migration through the lungs is leaving fewer red blood cells (RBC) to be inhabited by malaria merozoites. To test this, co-infected animals could be treated with erythropoiten, which stimulates RBC formation in the bone marrow, at the time when this transient anaemia would usually be occurring. If a lack of RBC were causing this lowered parasitemia in co-infected, then erythropoietin treatment would cause co-infected mice to have parasitemia levels as high as singly malaria-infected. Another reason for this lower parasitemia could be enhanced TNF- α in co-infected animals resulting in enhanced malaria parasite killing (discussed in chapter 5).

Different organisms occurring in the same host usually influence one another directly or indirectly (Cox, 2001). This may be especially apparent with helminths and microbial parasites since Th1 responses, that clear intracellular pathogens, and Th2 induced by helminths, inhibit one another. Anti-malaria vaccines are supposed to ideally evoke an efficient Th1 response and induce specific antibodies against malaria pathogens. It is possible that concurrent helminth infection would interfere with the induction, or modulate, a Th1 response to a potential vaccine candidate. Therefore, studying co-infection between malaria and helminths is important when thinking about the design of possible vaccine strategies. Here we discovered that concurrent infection with malaria

and *N. brasiliensis* did not have a negative effect on the Th1 response necessary to clear the merozoites and actually reduced the parasitemia in co-infected animals when compared to animals infected with malaria alone. Factors like this could have implications when considering deworming strategies in malaria-endemic regions.

4. NeM ϕ do not require signalling through toll-like receptors to achieve an alternatively activated phenotype

One of the aims of this thesis was to understand the development of AAM ϕ in the context of type-1 and Type-2 immunity. As part of this, we investigated the role of MyD88, the important adaptor protein in TLR signalling, in the *in vivo* induction of alternatively activated macrophages from the *B. malayi* implant model. This was undertaken to ask whether certain type 1 signals mediated via MyD88 contribute normally to AAM ϕ development. We found no impairment of the Th2 response or the NeM ϕ phenotype in MyD88-/- relative to WT animals. Both groups produced similar levels of arginase, Ym1, RELM- α and also had an equally suppressive phenotype, when co-cultured with EL-4 cells. This lack of any difference was perhaps surprising considering published data indicating that a lack of MyD88 should enhance Th2 response to helminth parasites (Helmby & Grencis, 2003). This difference likely lies with the different habitats of these parasites. Whereas the *B. malayi* implant model is essentially sterile, with no expectation of a type 1 response, infection with *T. muris* in the gut is associated usith burrowing into cecal epithelial cells and exposure to commensal bacteria and associated LPS (Helmby & Grencis, 2003).

For future experiments it would be interesting to investigate how NeM ϕ lacking MyD88 would respond to type-1 stimuli, such as LPS. If MyD88-/- NeM ϕ were found to respond to LPS (e.g. by upregulating *iNOS* production) antagonistic antibodies for TLR4, through which our *E. coli*- derived LPS signals, could be used to test whether this
was signalling was mediated through the MyD88-independent pathway. As we saw in chapter 6, naïve BMM ϕ only produced nitric oxide when treated with LPS/IFN- γ together, but not separately. It would be interesting to investigate whether NeM ϕ , unlike BMM ϕ , could respond to IFN- γ in the absence of MyD88 (through potential signals encountered *in vivo*). Most importantly, we could ask whether the ability of these *in vivo*-derived AAM ϕ to switch to a more CAM ϕ phenotype, in response to LPS and IFN- γ , is MyD88 dependent and how a deficiency in TLR signalling would translate into the ability of NeM ϕ to control intracellular pathogens, such as *L. mexicana*.

5. Conclusions

This PhD has contributed to the knowledge of macrophage biology, through the illustration that the macrophage phenotype may be more flexible than previously realised. More specifically it has added to the understanding of macrophages from a Th2 environment associated with helminth infection, and has shown that AAM ϕ can be induced to become anti-microbial, which may turn out to have importance when targeting macrophages in disease therapeutics. The work also contributed to the understanding of macrophage-mediated suppression and has shown that TLR-signalling does not contribute to the AAM ϕ phenotype.

APPENDICES



Appendix 1. Various realtime RT-PCR results of Ym-1 and RELM- α RNA expression on macrophages treated with LPS and IFN- γ together or separately. Thioglycollate-elicited M ϕ , bone marrow-derived macrophages (BMM ϕ) or NeM ϕ from C57BL/6 mice were left untreated or treated overnight with IL-4 or LPS together or separately with IFN- γ as indicated. The cells were recovered for RNA expression analysis of Ym1 and RELM- α .



Appendix 2. Effect of LPS/IFN- γ on the cell surface activation markers in naïve macrophages and NeM ϕ . Untreated ThioM ϕ , IL-4 treated ThioM ϕ (tinted) or NeM ϕ were left untreated (bold line) or treated overnight with LPS/IFN- γ (dotted line). The cells were recovered and double-stained for F4/80 and MHC Class II, B7.1, B7.2 (A) or PD-L1 and PD-L2 (B) Flow cytometry graphs show histograms of F4/80-gated macrophages and dashed lines show the isotype control. Mean fluorescent intensity of histograms for untreated or treated (bold) are displayed. Experiment by Meera Nair.

APPENDIX 3

Chapter 4 - work of Dr. Ian Faribairn

Materials and Methods

Cells and bacteria

M. bovis BCG Danish Strain 1331 was obtained from Statens Serum Institut and maintained in log phase growth in 7H9 broth supplemented with 10% albumin dextrose catalase (ADC) enrichment medium and 0.2% Tween 80. Prior to infection BCG were passed through a 26 gauge needle and underwent water bath sonication for 60 seconds to reduce clumping.

BCG viability assay

Macrophages were plated in 96-well flat-bottom microtitre plates at a density of 5×10^4 cells per well. Either 5×10^4 (MOI 1:1) or 5×10^5 (MOI 10:1) per well were added. To aid infection plates were centrifuged at 700g for 5 minutes and incubated at 37° C for 2 hours. Excess BCG were removed by washing. As assessed by CFU, ~10% of BCG were taken up, giving ratios of 0.1 and 1 viable BCG per macrophage. Where indicated IFN γ (Sigma) (100u/ml) and N^G-Monomethyl-L-arginine monoacetate (L-NMMA) (Sigma) (400 μ M) were added immediately following infection. Following incubation at 37° C with 5%CO₂ for the indicated number of days, 150 μ l supernatant was removed from each well and replaced with ice cold 0.1% Triton-100. Cell lysis was promoted 10 minute incubation on ice followed by vigorous pipetting. 50 μ l aliquots from each well

were serially diluted in 7H9 supplemented with 10%ADC enrichment and 0.2% glycerol, and plated in 96-well flat-bottom microtitre plates. The plates were then incubated at 37°C for 12 days. Microcolonies were counted at 100x magnification. All experiments were performed in quadruplets.

Tunel staining (Boehringer Mannheim)

 $5x10^5$ macrophages were plated on 13mm glass coverslips in 24-well plates, infected with BCG as described above and incubated for 16 hours. Macrophages were then fixed, permeabilised and stained as per manufacturer's instructions.

Appendix 4

Chapter 6 -infection of mice

Materials and Methods

Hosts, parasites and experimental infection

Age- and sex-matched female BALB/c mice were used in all experiments. All mice were purchased from Harlan UK and housed in filter top or individually ventilated cages (IVC), maintained in a 12h: 12h light- dark cycle. The mice were fed 41B maintenance diet and water *ad libitum* (Harlan UK). *Plasmodium chabaudi chabaudi* clone AS was originally isolated from thicket rats (*Thamnomys rutilans*) and was cloned by serial dilution and passage. Parasites, from frozen blood stabilates, were passaged twice through donor mice prior to experimental infection. *Nippostrongylus brasiliensis* worms were maintained by Yvonne Harcus by serial passage through Sprague-Dawley rats. L3 larvae were obtained by culturing the faeces of infected rats at 26°C for a minimum of 5 days. Infection was initiated by subcutaneous (s.c) injection of 200 infective (L3) larvae.

Co-infection with N. brasiliensis and P.c.chabaudi:

8-10 week old BALB/c mice were divided into 4 treatment groups: Uninfected, infected with malaria only, infected with *N. brasiliensis* only and co-infected. For the helminth infected groups 200 *N. brasiliensis* L3 larvae were injected s.c on Day 0. An i.p injection of 1×10^5 P.c.chabaudi parasitised red blood cells (pRBC) was also given on Day 0 to

mice scheduled for malaria infection. Mice were sacrificed on Day 20 post-infection under terminal anaesthesia. Whole blood was collected from the brachial artery and was separated using Sera Sieve (Hughes & Hughes Ltd). Mice were monitored daily for body weight and red blood cell density was measured on a Beckman Coulter Counter by diluting 2µl tail blood into 80ml Isoton solution (Beckman Coulter). Asexual malaria parasites were monitored daily throughout the infection, from day 2 to day 20 post infection (p.i) inclusive. Thin blood smears were taken with tail blood fixed in methanol and stained with 20% Giemsa and parasites were counted using x1000 microscopy. The number of parasites in 500 red blood cells (RBC) was counted per smear and the percentage calculated to give a measure of parasitemia.

In vitro lymph node cultures

The thoracic lymph nodes were removed and single cell suspensions prepared. These were cultured in 96-well round bottom plates at 10^6 cells per well containing either 1µg/ml Concanavalin A (ConA) or medium alone (complete RPMI) at 37°C. After 72h of culture, supernatants were removed for cytokine assay.

Cytokine Assay

The amount of cytokines (IL-5, IL-4, IL-13, IFNy and IL-10) in the culture supernatants was measured using BD Cytometric Bead Array Flex sets by Simmi Mahajan (as in Chapter 6 material and methods).







(C) Malaria parasitemia time course



Appendix 5 Anaemia, body weight and malaria parasitemia day 0-20. Mice were monitored daily for red blood cell density (a), body weight (b) and asexual malaria parasites (c) by Karen Grocock and Andrea Graham. Results are representative of three experiments.



Appendix 6. Day 7 cytokine production. Thoracic lymph nodes (TLN) were removed and single cell preparations cultured for three days +/- ConA ($1\mu g/ml$) Cytokine measurements were taken using the BD Cytometric Bead Array Flex sets by Simmi Mahajan. Results are representative of three experiments.

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