Mechanisms involved in type II macrophage activation and effector functions

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Abstract

Autoimmunities are extremely difficult to treat and involved in their pathogenesis are pro-inflammatory immune responses redirected against one's own tissues. Studies in our lab have shown macrophages that are induced to become type II macrophages protect against an animal model of MS, experimental autoimmune encephalomyelitis (EAE), with protection due to immune deviation. Another way to deviate immune responses away from inflammation is by infection with the parasitic helminth *Schistosoma mansoni*, which also protects against EAE. The contribution of type II macrophages in this protection is unknown, as are the mechanisms involved in promoting the phenotype induced by type II activation. This project investigates key mechanisms involved in type II activation, while also elucidating the possible effect of schistosome exposure on the induction of this activation state.

Using a validated model of type II activation *in vitro*, we compared the effects of schistosome immune complexes on various macrophage properties such as cytokine, surface marker and enzymatic profiles. This thesis identified that exposure to schistosome complexes induces a macrophage state with characteristics of two distinct activation states (type II and alternative activation), as well as completely novel characteristics. This activation state shows many phenotypic properties associated with immune regulation, and may have important consequences for understanding mechanisms involved in protection against inflammatory illnesses.

We also investigated key mechanisms involved in the anti-inflammatory responses induced by type II activation. Cytokine, chemokine and surface marker profiles of macrophages were assessed in response to type II activation *in vitro,* with the main emphasis on determining the effects of IL-10 and CD40 on the type II activation phenotype and function. This investigation found that type II activated macrophages depend on low levels of CD40/CD40L signalling to polarise Th2 development, as the expression of receptors for Th2-inducing cytokines are significantly impaired in the absence of this interaction. This suggests an important role for the low but maintained levels of CD40 on type II activated macrophages, in aiding the deviation of immune responses, while maintaining Th2 polarization. We also suggest a suppressive role of CD40/CD40L in IL-10 production, which is a novel find.

The requirement of new treatments for MS is escalating as more people are affected each year. The impact of MS on the quality of life is severe and long lasting. Having a greater understanding of the mechanisms involved in deviating pro-inflammatory or anti-inflammatory responses will enable the development of much more effective treatments and therapies in the future.

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List of abbreviations:

ab –Antibody

Ag- Antigen

ANOVA- Analysis Of Variance

APC- Antigen Presenting cell(s)

BMMØ- Bone marrow derived macrophage

CFSE-5,6-carboxyfluorescein succinimidyl ester

c-Maf- Leucine zipper transcription factor

CNS- Central Nervous System

CO_2 – Carbon dioxide

CTCM- Complete T cell medium

CTLA-4- Cytotoxic T Lymphocyte Antigen 4

Cyc- Cychrome

DC – Dendritic Cell(s)

Derp-1- Dermatophagoides Pteronyssinus group1

dPBS- Dulbeccos Phosphate Buffered Saline

EAE- Experimental Autoimmune Encephalomyelits

Egg:Ser- Schistosome egg opsonised with Serum from schistosome infected mice

ELISA- Enzyme-linked immunosorbant assay

eNOS- Endothelial Nitric Oxide Synthase

FACs- Flourescence-activated cell sorting

FcγR- Immunoglobulin G Receptor (Fc gamma receptor)

FCS- Foetal Calf Serum

Fitc- Fluorescein Isothiocyanate

Foxp3- Forkhead Box P3

GA- Glatiramer Acetate

GATA-3- Trans-acting T cell specific transcription factor

GITR-Glucocorticoid induced tumour necrosis factor receptor family related

gene

GM-CSF- Granulocyte/Macrophage-Colony Stimulating Factor

IC-Immune complex

IDO- Indoleamine 2,3-dioxygenase

IFNγ- Interferon gamma

IgG- Immunoglobulin G

Ig-Immunoglobulin

IL- Interleukin

IL-2Rα- Interleukin 2 receptor alpha

IL-4Rα- Interleukin 4 Receptor alpha

iNOS- Inducible Nitric Oxide Synthase

iTregs- Inducible Regulatory T cell(s)

LPS- Lipopolysaccharide

MCP-1- Monocyte chemotactic protein-1

MFI-Mean Flourescene Intensity

MHC - Major Histocompatibility Complex

MMP- Matrix metalloprotease

MØ- Macrophage

mRNA- messenger RNA

ms- mouse

MS- Multiple Sclerosis

NFκB- Nuclear Factor-Kappa B

NK cells- Natural Killer

nNOS- Neuronal Nitric Oxide Synthase

NO- Nitric Oxide

nTreg- Natural Regulatory T cell(s)

OVA- Ovalbumin Protein

PBS- Phosphate Buffered Saline

PD-1- Programmed Death-1

PDL-1- Programmed Death Ligand-1

PDL-2- Programmed Death Ligand-2

PE- Phycoerythrin

PRRs- Pathogen Recognition Receptors

rb- rabbit

RBC- Red blood cell

RELMα -Resistin-like molecule-alpha

rIL-10- recombinant interleukin 10

rIL-4- recombinant Interleukin 4

RT- Room temperature

SDS PAGE- Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEA- Schistosome Soluble Egg Antigen

SEA-IgG- Soluble egg antigen opsonised with purified immunoglobulin G

SPHK1- Sphingosine Kinase-1

SRBC- Sheep Red Blood Cell

SRBC:IgG- SRBC opsonised with immunoglobulin G

STAT-1- Signal Transducer and Activator of Transcription protein 1

STAT-5-Signal Transducer and Activator of Transcription protein 5

STAT-6- Signal transducer and activator of transcription protein 6

TAM- Tumour Associated Macrophage

T-bet- Member of T-box family of transcription factor expressed in T cells

TCR- T Cell Receptor

TGFβ- Transforming Growth Factor beta

Th- T helper cell

Th17- T helper 17 cell

Th1-T helper 1 cell

Th2- T helper cell 2

TLR- Toll-like receptor

TNFα- Tumour Necrosis Factor-alpha

TNFRI- Tumour Necrosis Factor Receptor type 1

TNFRII- Tumour Necrosis Factor Receptor type 2

Tregs- Regulatory T Cell(s)

Ym-1- Chitinase-like secetory lectin

Units and measurements:

µg/ml- micrograms per millilitre

h- hour

mM- micro moles

U/ml- units per millilitre

ng/ml- nonograms per millilitre

Chapter 1: Introduction

1.1 Innate and adaptive immune responses

Adequate protection against pathogens requires an efficient innate and adaptive immune response (Abbas *et al.*, 1996; Bowdish *et al.*, 2007; Janeway *et al.*, 2002). The innate arm of the immune response is the first to recognise pathogens, create an initial defense, and alert the adaptive arm of the immune response when extra help is required. Cells involved in innate immune responses include macrophages (MØ), dendritic cells (DC), mast cells, basophils, neutrophils and natural killer (NK) cells (Janeway *et al.*, 2002). Innate immune cells express a repertoire of germ-line encoded receptors. Upon recognition of general pathogen associated molecular patterns (PAMPs), innate cells elicit rapid responses such as the phagocytosis of bacteria, uptake of cellular debris and secretion of reactive oxygen species. Innate responses also interact with cells of the adaptive immune system through the presentation of antigen and production of cytokines and mediators which influence the adaptive responses (Vyas *et al.*, 2008). Unlike the innate system, cells of the adaptive immune response elicit more targeted responses against a single antigen to which their assembled receptors are specific (Janeway *et al.*, 2002). Cells of the adaptive immune system include CD4 T cells, NK.T cells, Regulatory T cells, and B cells. Although the responses of the adaptive immune system is slower than that of the innate arm, the cells of the adaptive system can elicit memory responses such that upon re-exposure to the same antigen, a much faster, more effective and stronger response can occur. The innate and adaptive immune systems work together to provide host protection, with each system aiding the other for optimal anti-pathogenic responses (Campbell *et al.*, 1996; Stenger *et al.*, 1996).

1.2 Macrophages

1.2.1 Derivation

Macrophages are innate immune cells, and are the first line of defense against invading pathogens (Dunn *et al.*, 1985). They are derived from haemopoietic cells that develop into monocytes, which upon entering tissues from the blood, differentiate into macrophages (Brem-Exner *et al.*, 2008; Gordon *et al.*, 2005; Mosser *et al.*, 2008). Macrophages are distributed in essentially every tissue such as the lungs, liver, bone, brain, connective tissue, intestines and skin. Depending on the tissue environment, they are capable of eliciting different physiological functions (Stout *et al.*, 2004) . These functions can include host defence, wound repair, or immune regulation (Albina *et al.*, 1990; Anderson *et al.*, 2002b; MacMicking *et al.*, 1997). Macrophages are extremely heterogeneous and exhibit a variety of functions including homeostatic mechanisms such as the recycling of red

blood cells (RBCs) and removal of debris, as well as functions involving the activation of immune responses against pathogens (Mosser *et al.*, 2008). Much of their immune responses are derived by the recognition of conserved molecular patterns on pathogens, which are detected by pathogen recognition receptors (PRRs) such as scavenger receptors and toll-like receptors (TLR) (Janssens *et al.*, 2003; Pluddemann *et al.*, 2007). There are also receptors such as FcγRs and complement receptors which are involved in recognising opsonised particles (Gordon, 2007). Upon recognition of pathogens, macrophages can elicit a variety of functions depending on the receptors involved in the particular response. Receptors such as FcγRs, scavenger receptors and complement receptors can elicit phagocytosis (Aderem *et al.*, 1999). In contrast, TLR signalling alters the production of inflammatory mediators involved in immune activation, which in the context of antigen presentation, can initiate appropriate T cell responses. These only represent some functions of macrophages, and each function often crosses over into the other, as seen by the ability of phagocytosis to sometimes induce the production of inflammatory mediators or enhance T cell proliferation (Barker *et al.*, 2002; Janeway *et al.*, 2002).

1.2.2 Function

1.2.2.1 Phagocytosis

Phagocytosis is an ancient function of macrophages (Sanjuan *et al.*, 2007), and involves the recognition of pathogen associated molecular patterns (PAMPs) through the expression of PRRs (Akira *et al.*, 2004). These PRRs enable macrophages to recognize and phagocytose particles or pathogens, and include a wide array of different receptors such as scavenger receptors, mannose receptors, Fcγ receptors and complement receptors (Aderem *et al.*, 1999; Janeway *et al.*, 2002). In addition to taking up and killing pathogens, macrophages also use phagocytosis for homeostatic mechanisms like the clearance of erythrocytes and apoptotic or necrotic debris (Barker *et al.*, 2002; Mosser *et al.*, 2008). Involved in the degradation of pathogens upon cellular uptake into the phagosome, are processes involved in phagosome maturation, phagosome-lysosome fusion and acidification. Although TLRs are non-phagocytic receptors, they contribute to the phagocytic process through alterations of macrophage responses, and are important in the maturation of phagosomes (Sanjuan *et al.*, 2007). As well as clearing pathogens by uptake and degradation, the phagocytic process can often result in the production of inflammatory mediators, and enhanced expression of co-stimulatory molecules associated with T cell activation (Barker *et al.*, 2002). This is seen by the ligation of mannose receptors and Fcγ receptors (Aderem *et al.*, 1999), which either alone or in conjunction

with TLR signalling can activate the transcription factor NFκB and result in the production of inflammatory mediators (Gordon, 2007).

1.2.2.2 Pathogen/danger recognition

The ability of macrophages to respond to microbial infection lies in the wide array of PRRs capable of recognising different PAMPs, thus enabling responses to numerous pathogens such as bacteria, fungi and viruses (Creagh *et al.*, 2006). PAMPs are conserved molecular motifs that are present on bacteria and helminths, as well as host cells, and enable immune cells to distinguish non-self (pathogen) from self (host cells) (Janssens *et al.*, 2003). These PRRs include TLRs, lectin receptors, scavenger receptors and complement receptors (Pluddemann *et al.*, 2007). Some of these PRRs are involved in the homeostatic mechanisms (e.g scavenger receptors), while others are involved in the activation of macrophages (e.g. TLR) through the production of inflammatory mediators (Janeway *et al.*, 2002; Mukherjee *et al.*, 2009). To date, 13 TLRs have been identified in mice and 11 in humans (Zhang *et al.*, 2008). Different responses can be elicited by the downstream signalling of various TLRs, and some TLRs can co-operate with each other. An example of this is TLR2 co-operating with either TLR1 or 6 to significantly enhance inflammatory cytokine production (Ozinsky *et al.*, 2000). In response to TLR signalling, macrophages produce a vast array of inflammatory mediators (Akira *et al.*, 2004; Janeway *et al.*, 2002).

The most well characterised TLR is TLR4, which is stimulated by bacterial components such as lipopolysaccharide (LPS) from the outer membrane of gram negative bacteria (Akira *et al.*, 2004). TLR4 signalling leads to NFκB activation and results in increased expression of MHC II, the co-stimulatory molecule CD40, as well as the production of pro-inflammatory cytokines such as IL-6, TNFα and IL-12 (Chow *et al.*, 1999; Gordon, 2007). The production of TNFα in concert with IFNγ increases the anti-microbial functions of macrophages by enhancing the production of superoxide anions and nitric oxide (Chow *et al.*, 1999; Hoshino *et al.*, 1999; Modolell *et al.*, 1995). The multitude of cytokines produced can elicit a variety of responses ranging from activation to inhibition of immune responses, depending on the cytokine repertoire induced (Gerber *et al.*, 2001; Janeway *et al.*, 2002; O'Shea *et al.*, 2008; Rahim *et al.*, 2005).

1.2.2.3 Other functions

Macrophages are extremely plastic and multi-functional (Mosser, 2003; Stout *et al.*, 2004). Macrophages are capable of a wide variety of different functions, some of which include tissue remodelling and antigen presentation. In the presence of helminths for example, macrophages alter their functions to produce cytokines and mediators involved in tissue

remodelling and repair, as opposed to exhibiting anti-microbial functions (Corraliza *et al.*, 1995; Kane *et al.*, 2008; Pesce *et al.*, 2009b). Macrophages involved in wound repair and tissue remodelling express enzymes diametrically opposed to those produced by macrophages involved in responses to danger signals (Modolell *et al.*, 1995). One such enzyme is arginase1, which converts the substrate for nitric oxide production, into precursors for collagen and polyamines that are involved in the deposition of the extracellular matrix (Corraliza *et al.*, 1995; Modolell *et al.*, 1995).

Another important function of macrophages is the presentation of antigen to T cells in the context of major histocompatability complexes (MHC), to evoke responses of T cells carrying T cell receptors (TCR) specific for recognising the particular antigen (Russell *et al.*, 2009). Dendritic cells are highly specialised at antigen presentation, however macrophages also contribute to this process. Pathogens are taken up, processed, and presented on the cell surface in the context of MHC I (for intracellular-derived pathogens) , or MHC II (for extracellular constituents) (Smith-Garvin *et al.*, 2009). TLR signalling plays an important role in the activation of APCs, resulting in the high levels of expression of MHC molecules, as well as co-stimulatory molecules on APCs, required for the full activation of T cells (Banchereau *et al.*, 1994; Vyas *et al.*, 2008).

Macrophages are highly plastic, and are able to significantly deviate T cell responses depending on the environmental signals (e.g cytokines and chemokines), which are predominantly produced by macrophages (Attwood *et al.*, 1999; Barker *et al.*, 2002; Curtsinger *et al.*, 1999). Macrophages in an inflammatory state are able to present antigen and induce the development of CD4 T cell subsets of an inflammatory phenotype (Th1,Th17) (Schulz *et al.*, 2009; Seder *et al.*, 1996). Macrophages involved in immune regulation elicit an environment dominated by anti-inflammatory cytokines/mediators which results in more polarized Th2 responses (Anderson *et al.*, 2002a; Gerber *et al.*, 2001). Therefore, macrophages have important roles in directing not only innate responses, but also adaptive immune responses, through the different immune environments elicited by the various macrophage phenotypes.

1.2.3 Production of immune mediators

1.2.3.1 IL-12

IL-12 is a pro-inflammatory cytokine most prominently produced by APCs. IL-12 consists of a heterodimer of p40 and p35, which together form IL-12p70 (Oppmann *et al.*, 2000), and is produced by multiple cell types although the major source appears to be from dendritic cells and macrophages (DeKruyff *et al.*, 1997; Kelsall *et al.*, 1996). IL-12 is required for the ability of macrophages to protect the host from intracellular pathogens

such as *Leishmania major*, and *Mycobacterium avium* (Campbell *et al.*, 1996; Florido *et al.*, 2004; Li *et al.*, 1997), with the addition of recombinant murine IL-12 shown to cure such infections (Heinzel *et al.*, 1993). This protection occurs primarily through the induction of Th1 cells producing IFNγ (Seder *et al.*, 1993). In concert with CD40 ligation, this induces the activation of macrophages resulting in the production of nitro oxide (NO) and superoxide anions required for parasite killing (Campbell *et al.*, 1996).

1.2.3.2 IL-10

IL-10 is secreted by many different cell types including Th1 cells (Abbas *et al.*, 1996), Th2 cells (Sornasse *et al.*, 1996), Tregs (Groux *et al.*, 1997), and innate cells such as macrophages (Anderson *et al.*, 2002a). Although IL-10 was first thought to suppress immune responses as seen in its suppressive effect on macrophages (Bogdan *et al.*, 1991), it soon became evident that its functions extended beyond simple inhibition. IL-10 has been shown to suppress Th1-associated responses by inhibiting pro-inflammatory cytokines such as IL-6, TNFα, IL-1β and IL-12p40 (Anderson *et al.*, 2004; de Waal Malefyt *et al.*, 1991; Howard *et al.*, 1993). Additionally it is also able to promote the development of regulatory T cell subsets (Tregs). Furthermore, an essential role for IL-10 in Th2 induction was demonstrated by Groux *et al* 1997, where IL-10 deficient mice suffered from severe morbidity during helminth infection due to the absence of a protective Th2 response. IL-10 induces the expression of IL-4Rα on macrophages (Lang *et al.*, 2002), which are essential in mediating the protective effects of Th2-derived IL-4 during schistosomiasis. In the context of pro-inflammatory diseases such as endotoxemia and EAE, macrophage-produced IL-10 has been shown to be essential in conferring protection or increasing recovery (Gerber *et al.*, 2001; Howard *et al.*, 1993; Kennedy *et al.*, 1992).

1.2.3.3 TNFα

Tumor necrosis factor alpha (TNFα) is a pro-inflammatory cytokine produced most predominantly by macrophages (Ma, 2001). TNFα production is produced early in response to inflammatory stimuli such as IFNγ or LPS (Crume *et al.*, 2007; Vila-del Sol *et al.*, 2007). The autocrine production of TNFα is essential for the activation of macrophages, as seen by the inability of macrophages to elicit anti-microbial functions in the absence of TNFα (Vila-del Sol *et al.*, 2007). This is due, in part, to the activation of transcription factors such as NFκB, which are required for the production of inflammatory mediators (e.g iNOS), cytokines, and chemokines involved in the recruitment of leukocytes (Ciesielski *et al.*, 2002; Vila-del Sol *et al.*, 2007). Some of the inflammatory consequences of TNFα production include its involvement in septic shock, tumoricidal functions, as well as the pathogenesis of autoimmunities including MS, where high levels

of TNFα production are seen at the peak of disease (Abu-Amer *et al.*, 1994; Begolka *et al.*, 1998).

In addition to initiating inflammatory responses, TNFα also exhibits regulatory functions. This is seen by the suppressive effects on inflammatory mediators such as IL-12 (Ma, 2001), which are significantly enhanced in the absence of TNFα. The functions of TNFα vary, dependant on the environmental setting, the location, as well as the differential signalling pathways elicited through the receptors TNFRI or TNFRII.

1.2.3.4 IL-6

IL-6 is a pleiotropic cytokine that is produced by a variety of cells including T cells, dendritic cells, macrophages, and astrocytes (Akira *et al.*, 1990; Jang *et al.*, 2004; Linkhart *et al.*, 1991; Yasukawa *et al.*, 2003). The functions of IL-6 range from bone resorption to the proliferation of lymphocytes, differentiation of B cells, and the induction of both pro-inflammatory (Veldhoen *et al.*, 2006) and anti-inflammatory responses (Rincon *et al.*, 1997). IL-6 has been shown to inhibit the production of other pro-inflammatory cytokines such as TNFα and IL-1β by monocytes (Maimone *et al.*, 1997; Tilg *et al.*, 1994). Overall, IL-6 seems to have a role in both the initiation and down-regulation of inflammatory responses. This idea is supported by the induction of IL-6 by IL-12 early on, but the inhibition of IL-12 and Th1 responses by IL-6 during later stages of schistosomiasis (La Flamme *et al.*, 2000).

1.2.3.5 Nitric Oxide

The production of nitric oxide (NO) occurs through the enzyme nitric oxide sythase (NOS), to which three isoforms exhist; neuronal NOS(nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Alderton *et al.*, 2001). nNOS and eNOS are primarily produced in neuronal or endothelic tissue (Alderton *et al.*, 2001), however most NO occurs primarily through inducible nitric oxide sythase (iNOS). This can be induced in many different cell types although macrophages are believed to be a major producer of NO during immune responses (MacMicking *et al.*, 1997). All three NOS enzymes exhibit different catalytic functions and can lead to various consequences, and this can also be demonstrated *in vivo*, where iNOS but not eNOS or nNOS is essential for maintaining the production of NO in order to control infection with the intracellular parasite *Leishmania spp* (Stenger *et al.*, 1996).

1.2.4 Macrophage activation states

A recent system of classification was proposed by Mosser *et al* in 2008 to separate activated macrophages based on their functional phenotypes: host defence, wound

healing, and immune regulation. Mosser *et al* (2008) and Stout *et al* (2004), in a similar review, also emphasize the importance of understanding that macrophages exhibit not only the three currently recognized states of activation (i.e. classical, alternative and type II), but a broad spectrum of activation states. Because macrophages are highly plastic (Stout *et al.*, 2004), and are not simply restricted to a single activation state, there may be distinct macrophage states which exhibit characteristics of two or more activation states, termed 'hybrid' macrophages (Mosser *et al.*, 2008). However, for the purpose of this thesis only the three well-characterized types of macrophage activation states, namely classical, type II (regulatory), and alternative, will be covered.

1.2.4.1 Classical

In response to microbial products, macrophages are activated via TLR signalling pathways, into a state that exhibits anti-microbial actions (Campbell *et al.*, 1996; Janeway *et al.*, 2002). Macrophages activated in this manner are termed classically activated. Classically activated macrophages are efficient at presenting antigen to T cells, and once activated, can induce both the proliferation of T cells, and Th1 polarisation (Campbell *et al.*, 1996; Mosser, 2003; Seder *et al.*, 1996). *In vitro* studies inducing classically activated macrophages, often stimulate them into such a state with interferon gamma (IFNγ), followed by LPS (Held *et al.*, 1999; Sutterwala *et al.*, 1997). *In vivo*, IFNγ can be derived from cells of both the innate immune system (e.g. NK T cells), and adaptive immune system (e.g. Th1 cells) (Germann *et al.*, 1993). TNFα is one of the first cytokines produced by classically activated macrophages, with secretion occurring as early as two hours after stimulation (Crume *et al.*, 2007). IFNγ can stimulate the production of TNFα by macrophages, which further enhances the anti-microbial functions of classically activated macrophages through increased production of NO (MacMicking *et al.*, 1997; Vila-del Sol *et al.*, 2007). Classical activation requires these two signals, as IFNγ primes the cell to enable a full response to LPS (Held *et al.*, 1999). Such macrophages then produce high amounts of pro-inflammatory cytokines (e.g IL-12, TNFα, and IL-6), and chemokines (e.g MCP-9, MCP-1) (Anderson *et al.*, 2002b; Grazia Cappiello *et al.*, 2001; Steube *et al.*, 1999). Classically activated macrophages up-regulate the expression of surface molecules that are involved in antigen presentation, such as MHC II (Edwards *et al.*, 2006), as well as co-stimulatory molecules like CD40 and CD80 which are involved in regulating and maintaining the interaction between macrophages and T cells (Nolan *et al.*, 2008; Qin *et al.*, 2005; Tierney *et al.*, 2009).

During infections with intracellular pathogens, the classical activation of macrophages is essential. For example, infection of macrophages with the intracellular parasite *Leishmania major*, renders them incapable of producing NO and killing the parasite. This

inhibition results in exacerbation of disease (Liew *et al.*, 1990; Stenger *et al.*, 1996). However, although the pro-inflammatory responses induced by classical macrophages are required for protection from invading pathogens, the mediators can be detrimental when the production is uncontrolled. Such an uncontrolled pro-inflammatory response occurs in septic shock, which is associated with the overproduction of TNFα and reactive oxygen intermediates (Victor *et al.*, 1998), or in autoimmunity when the response is inappropriately directed at self antigens. In particular, during the autoimmune disease multiple sclerosis (MS), classically activated macrophages cause damage to the central nervous system (CNS) as a result of IL-23, IL-12p40 and IL-6 production, as well as the production of proteases such as matrix metalloproteases (MMPs) (Becher *et al.*, 2002; Langrish *et al.*, 2005; Tran *et al.*, 1998; Veldhoen *et al.*, 2006).

1.2.4.2 Alternative

In the presence of IL-4, IL-13 or glucocorticoids, macrophages are activated into a state distinct from those which exhibit the classical anti-microbial functions described above (Modolell *et al.*, 1995; Stein *et al.*, 1992). These macrophages are believed to be primarily involved in wound healing rather than microbial killing (Pesce *et al.*, 2009a). Central to this difference in function is the altered metabolism of L-arginine to precursors for collagen synthesis as opposed to NO for microbial killing (Albina *et al.*, 1990; Modolell *et al.*, 1995). Alternatively activated macrophages dominate during infection with *Schistosoma spp*, and are involved in the generation of granulomas that surround the parasite eggs which become trapped in tissues (Hesse *et al.*, 2001). Alternatively activated macrophages are essential to host survival during schistosomiasis as in their absence, schistosome-infected mice succumb to severe mobidity and death (Herbert *et al.*, 2004).

In addition to their contribution to tissue remodelling, alternatively activated macrophages also differ from classically activated macrophages in several other regards. For instance, these macrophages are not efficient at presenting antigen to T cells, do not produce significant amounts of NO, and do not produce pro-inflammatory cytokines like IL-12 (Corraliza *et al.*, 1995; Edwards *et al.*, 2006; Schebesch *et al.*, 1997). In contrast, they produce anti-inflammatory cytokines such as IL-10. *In vitro* and *in vivo*, alternatively activated macrophages can be identified by their high levels of arginase1 (Hesse *et al.*, 2001), RELMα (FIZZ1) (Raes *et al.*, 2002), and chitinase (YM-1) (Edwards *et al.*, 2006). Furthermore, alternatively activated macrophages do not up-regulate the activation markers CD40, CD80 or MHC II, but can be identified through their high expression of programmed death ligand-2 (PDL-2) (Loke *et al.*, 2003; Tierney *et al.*, 2009).

1.2.4.3 Type II

A third distinct state of macrophage activation is type II or regulatory activation. In contrast to classical activation where macrophages are stimulated with TLR agonists such as LPS, type II activation occurs when TLR stimulation is combined with ligation of FcγRs by immune complexes (Anderson *et al.*, 2002b; Gerber *et al.*, 2001). These macrophages exhibit anti-inflammatory effects, with increased production of IL-10 and reduced production of IL-12. Unlike alternatively activated macrophages, type II macrophages elicit the activation of T cells, as demonstrated by increased CD25 expression, and the biasing of T cell responses towards a Th2 dominated subset (Anderson *et al.*, 2002b; Edwards *et al.*, 2006).

This pathway of activation by FcγR ligation was the first identified; however, it is important to note that since this first discovery, other means of type II or regulatory macrophage activation have been found. For example, glucocorticoids or IL-10, inhibit the production of pro-inflammatory cytokines by macrophages and enhance anti-inflammatory responses (Elenkov, 2004; Martinez *et al.*, 2008). Also macrophages in the tumor microenvironment termed tumor associated macrophages (TAM) elicit characteristics similar to regulatory macrophages. They produce high levels of IL-10 and low levels of pro-inflammatory cytokines, but do not express iNOS or successfully present antigen to T cells (Dinapoli *et al.*, 1996; Mantovani *et al.*, 2002). Although these conditions can elicit regulatory macrophages, some characteristics differ between regulatory macrophages induced by these different pathways, such as iNOS expression (Martinez *et al.*, 2008). Despite these differences, the main functional characteristic which appears to define regulatory macrophages is the preferential production of IL-10 over IL-12.

The protective, anti-inflammatory effects of type II activated macrophages have been demonstrated in several animal models of pro-inflammatory disease such as septic shock (i.e. lethal endotoxemia) and multiple sclerosis (Gerber *et al.*, 2001; Tierney *et al.*, 2009). In these studies, transfer of type II activated macrophages protected mice from lethal doses of LPS (Gerber *et al.*, 2001; Sutterwala *et al.*, 1998), or CNS inflammation and disease (Tierney *et al.*, 2009).

Although beneficial against pro-inflammatory diseases (Gerber *et al.*, 2001; Tierney *et al.*, 2009), as with classically activated macrophages, excessive regulatory macrophage responses can be detrimental to the host. For example, tumor associated macrophages, through their immunosuppressive effects, promote tumor growth (Lin *et al.*, 2006). Furthermore, during *Leishmania* infection, high levels of circulating antibodies form

complexes with *Leishmania*, therefore eliciting FcγR ligation and inducing type II macrophage activation (Miles *et al.*, 2005). This activation state is not beneficial to the host as it impairs the elimination of the parasite.

1.3 CD4 T cells

In the thymus, T cells undergo developmental changes involving the recombination of their T cell receptor (TCR) chains to generate unique TCRs (Swain, 1995). After TCR recombination takes place, T cells undergo processes which result in distinct populations of either CD4 or CD8 T cells (Li *et al.*, 2007). Each population is involved in different immunological processes. CD8 T cells (Cytotoxic T cells) are involved in the killing of virally-infected or cancerous cells (Barry *et al.*, 2002), whereas CD4 helper T cells play an important role in providing support to other immune cells such as B cells, macrophages and CD8 T cells, as well as directing and regulating immune responses (Wan *et al.*, 2009).

T cells leave the thymus as naïve cells that have never encountered their TCR-specific antigen. To activate a naive CD4 T cell successfully, at least two signals are required. First, the TCR must recognize a peptide antigen in the context of an MHC II molecule expressed on the APC, and secondly, this interaction must be accompanied by a co-stimulatory signal (discussed in more detail later) (Lechler *et al.*, 2001). Upon activation, CD4 T cells proliferate, aided by the cytokine IL-2 (Jelley-Gibbs *et al.*, 2000; Swain, 1995). After proliferation, T cells differentiate into effector T cells, capable of producing cytokines and mediators that activate or regulate other immune cells, or become memory T cells. The effector T cell subset into which naive CD4 T cells differentiate, largely depend on the cytokine environment at the time of activation, as can be determined by the activation state of the antigen presenting cell (Le Gros *et al.*, 1990; Manetti *et al.*, 1993; Mosser *et al.*, 2008; Wan *et al.*, 2009).

CD4 T helper cells can be divided into several subsets based on their different effector functions. Mossman *et al* (1986) demonstrated the ability of two helper T cell populations (Th1 and Th2) to be identified based on the production of the cytokines IFNγ or IL-4, respectively. Furthermore, not only could the T cell subsets be defined by the cytokines they produced, but the cytokines also exhibited counter-regulatory activity on the differentiation of other T cell subsets (Maggi *et al.*, 1992; Parronchi *et al.*, 1992; Seder *et al.*, 1992). Since then, more CD4 T cell subsets have been identified, and the major and most well-defined of which include Th1, Th2, Th17, and regulatory T cells (Mills, 2008; Zhu *et al.*, 2008). As this thesis will focus mainly on T cell differentiation into Th1 and Th2 cells, only these subsets will be discussed in depth.

1.3.1 Th1 cells

Th1 cells function in cell mediated immunity and are essential for protection from intracellular pathogens such as *Mycobacterium avium* and *Leishmania major* (Campbell *et al.*, 1996; Florido *et al.*, 2004), as well as eliciting anti-tumour activity (Micallef *et al.*, 1997). However, the same responses which are beneficial during bacterial infections are detrimental when the responses are directed at targeting 'self antigen'. This is seen in the dominant role Th1 cells have in eliciting pro-inflammatory responses during an animal model for multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), and rheumatoid arthritis (Becher *et al.*, 2002; Leung *et al.*, 2000).

IL-12 is an essential cytokine in the development, proliferation and survival of Th1 cells (Manetti *et al.*, 1993; Seder *et al.*, 1993). In the absence of IL-12, such as that seen in mice lacking the IL-12 gene (Magram *et al.*, 1996), or from macrophages defective in IL-12 production (Anderson *et al.*, 2002b), there is a severe impairment in type1 responses and the development of Th1 cells. The strength of the ability of IL-12 to induce the development of Th1 cells was demonstrated by Manetti *et al* (1993), where in the presence of a Th2-promoting antigen-Dermatophagoides pteronyssinus group1 (Derp1)-T cells elicited a Th1 rather than Th2 response after prior exposure to IL-12. At the level of transcription, the commitment to Th1 differentiation occurs via inhibition of GATA-3, a transcription factor associated with Th2 commitment (Nurieva *et al.*, 2003), and the induction of STAT-1/T-bet (Szabo *et al.*, 2000), involved in suppressing Th2 development.

IFNγ is the most prominent cytokine derived from Th1 cells and mediates a range of inflammatory responses (Abbas *et al.*, 1996). IFNγ activates macrophages into a state which produces higher amounts of toxic free radicals as well as pro-inflammatory cytokines essential to host protection from pathogens (Seder *et al.*, 1993). The interaction of Th1 cells with APCs in the presence of IFNγ induces the activation and increased anti-microbial activity of APCs (MacMicking *et al.*, 1997). This interaction further enhances the production of IL-12 and other pro-inflammatory cytokines by the APCs. The production of IFNγ not only has important roles in activating macrophages and other APCs, but can also support cytotoxic T cell and B cell responses. Specifically, after exposure to Th1 subsets, the primary antibody isotypes produced by B cells are IgG2a and IgG3, which are isotypes involved in the opsonisation and phagocytosis by activated APCs (Coffman *et al.*, 1993; Manetti *et al.*, 1993).

1.3.2 Th2 cells

In an environment dominated by IL-4, CD4 T helper cells develop into the Th2 subset (Le Gros *et al.*, 1990; Seder *et al.*, 1992). There is no single cell type responsible for the

production of this cytokine, and the main source still remains a mystery. Cells hypothesized to contribute to the production of IL-4 include NKT cells, basophils, eosinophils and Th2 cells (Bjerke *et al.*, 1996; Le Gros *et al.*, 1990; Macaulay *et al.*, 1997). Even in the context of an infection with a Th1 inducing pathogen, *L. major*, IL-4 administration induces a potent Th2 response (Chatelain *et al.*, 1992). The production of IL-4 by T cells works in an autocrine and paracrine manner, signalling through the IL-4 receptor, a heterodimer of IL-4Rα and the common gamma chain (i.e. IL-2Rγ). This signal initiates a cascade of signalling events involving STAT-6, which results in the activation of transcription factors driving Th2 commitment (Kaplan *et al.*, 1996). These transcription factors include GATA-3 and c-maf (Kurata *et al.*, 1999), and just as T-bet inhibits IL-4 production, GATA-3 inhibits IFNγ production (Ouyang *et al.*, 1998; Szabo *et al.*, 2000).

Other cytokines are also thought to play a role in Th2 differentiation, including IL-2 and IL-10. IL-2 was first identified for its effects on promoting T cell proliferation and differentiation (Smith, 1988), and is produced by a variety of cells including Th1 cells. Recently, IL-2 has been identified as a major cytokine determinant of early differentiation into the Th2 subset. Signalling via IL-2Rα induces STAT-5 dependant increased accessibility of the IL-4 locus, required for IL-4 production (Cote-Sierra *et al.*, 2004), and enhances the expression of IL-4Rα (Liao *et al.*, 2008), without which, no Th2 development would occur. In addition to the deviation towards a Th2 subset, IL-2 is important in the survival and function of regulatory T cells (Sakaguchi, 2004; Setoguchi *et al.*, 2005). IL-2 was also found important in preventing overzealous inflammatory responses, and was found capable of limiting T cell responses directly through various mechanisms such as the induction of apoptosis, termed activation induced cell death (Jelley-Gibbs *et al.*, 2005). IL-10 is also thought to play a role in Th2 cell biasing, as IL-10 knockout mice are unable to elicit Th2 responses to the Th2-inducing parasitic worm, *Schistosoma mansoni* (Wynn *et al.*, 1998). Furthermore, macrophages defective in IL-10 production are likewise unable to drive Th2 differentiation *in vitro* (Anderson *et al.*, 2002b).

The primary cytokines produced by fully differentiated Th2 cells include IL-4, IL-5 and IL-13 (Wan *et al.*, 2009). IL-4 is produced by basophils, NK cells, mast cells, eosinophils, T cells, and macrophages under specific conditions (Mukherjee *et al.*, 2009; Pouliot *et al.*, 2009). Not only does IL-4 induce the development of Th2 cells (Le Gros *et al.*, 1990; Seder *et al.*, 1992), but also activates macrophages into an altered state such that they no longer produce pro-inflammatory cytokines, but instead elicit anti-inflammatory responses involved in wound healing and repair (Albina *et al.*, 1990; Raes *et al.*, 2002). In the setting of pro-inflammatory diseases such as EAE, IL-4 is associated with protection rather than pathogenesis (Begolka *et al.*, 1998; Shaw *et al.*, 1997).

In contrast to Th1 cells, Th2 cells takes longer to differentiate as demonstrated by the dependency of IL-4 production on cell cycle progression (Bird *et al.*, 1998). Effector Th2 cells have major roles in humoral immunity, elimination of extracellular pathogens (e.g. parasitic helminths) (Sher *et al.*, 1992), and through cytokine production, counter-regulate or "suppress" the pro-inflammatory responses elicited by Th1 or Th17 cells (Zhu *et al.*, 2008). IL-12 and IFNγ aid Th1 development and inhibit Th2 responses, whereas IL-10 and IL-4 are potent antagonists to Th1 responses. Furthermore, IL-4 and IL-10 are capable of inhibiting the development and proliferation as well as the effector functions of Th1 cells through the inhibition of a range of pro-inflammatory cytokines involved in driving Th1 responses (D'Andrea *et al.*, 1993; Fiorentino *et al.*, 1991; Murray, 2006). As with Th1 cells, CD4 T cells of the Th2 subset can induce antibody production by B cells, however the predominant antibody isotypes produced are IgG1 and IgE (Manetti *et al.*, 1993).

Although Th2 responses have been associated with recovery from pro-inflammatory diseases (Gerber *et al.*, 2001; Shaw *et al.*, 1997), as with Th1 responses, the same mechanism which elicits protection can lead to pathology under different environmental settings. For example, excessive Th2 responses induces atopic asthma and dermatitis (Wierenga *et al.*, 1990), and during schistosomiasis, can lead to excessive fibrosis through the production of IL-13 (Herbert *et al.*, 2008; Ramalingam *et al.*, 2009). Thus it is the balance between these diverse immune responses that is critical to maintain.

1.3.3 Th17 cells

The discovery that another subset of CD4 T cells was involved in the pathogenesis of the pro-inflammatory disease, EAE, led to the identification of Th17 cells. IL-6 and TGFβ enable de novo development of Th17 cells (Veldhoen *et al.*, 2006), and IL-23 plays an important role in the stabilisation of this T cell subset (Langrish *et al.*, 2005). After activation, Th17 cells produce high amounts of IL-6, TNFα, IL-21, IL-22 and IL-17. Unlike Th1 cells, they produce very little IFNγ (Jager *et al.*, 2009; Langrish *et al.*, 2005; Stockinger *et al.*, 2007). Effector Th17 cells elicit anti-pathogenic roles, eliminating various infections with which Th1 cells require help, such as *Leishmania*, *Salmonella* and *Mycobacterium tuberculosis* (Wan *et al.*, 2009) . Th17 cells also promote pro-inflammatory autoimmune responses and have been implicated in the pathogenesis of EAE, psoriasis and T cell mediated Colitis (Langrish *et al.*, 2005; Yen *et al.*, 2006).

1.3.4 Regulatory T cells

Another distinct subset of CD4 T cells are regulatory T cells (Tregs). Naturally occurring regulatory T cells (nTregs) develop in the thymus (Sakaguchi, 2004; Wan *et al.*, 2009). However naive CD4 T cells can also be induced to develop into a regulatory state in the periphery, termed inducible Tregs (iTreg) (Zhu *et al.*, 2008). Naturally occurring Tregs

express high levels of CD25 (IL-2Rα), glucocorticoid induced tumour necrosis factor receptor family related gene (GITR), and the transcription factor FOXp3 (Bettelli *et al.*, 2006; Nakamura *et al.*, 2001), with FOXp3 being a widely used marker to identify nTregs (Fontenot *et al.*, 2005). FOXp3 was found to not only act as a marker, but also play a role in the development of nTregs in the thymus (Fontenot *et al.*, 2003). Although the discovery of FOXp3 in nTregs has been extremely beneficial in their identification, it has also become clear than FOXp3 can also be induced in non-nTreg effector T cells, through STAT-5 signalling (Roncarolo *et al.*, 2008). Inducible Tregs can be induced by IL-10 and TGFβ (Zhang *et al.*, 2010). Unlike nTregs, iTregs do not constitutively express FOXp3, but can do so after induction, and only express it transiently.

Upon activation, Tregs produce cytokines such as IL-10 and TGFβ, and suppress T cell responses (Sakaguchi, 2004). The suppression of T cell responses is believed to occur through a range of mechanisms. For example, high levels of CD25 (IL-2Rα) on Tregs can induce T cell death, due to deprivation of IL-2 in the environment which is required for CD4 T cell survival (Pandiyan *et al.*, 2007). Also, apoptosis can be induced by the production of granzymes (Vignali *et al.*, 2008). Immune responses can also be suppressed by the inhibitory cytokines IL-10 and TGFβ, and through the ability of Tregs to alter the function of APCs to produce immunosuppressive molecules such as indoleamine 2,3-dioxygenase (IDO), which directly inhibits T cell proliferation (Munn *et al.*, 1999). Overall, Tregs function primarily to promote self tolerance and prevent exacerbated inflammatory responses (Vignali *et al.*, 2008). The role of Tregs in limiting self reactive immune responses has been seen in mice and humans, where a lack of Tregs results in severe autoimmune lymphoproliferative diseases. Patients with mutations with FOXp3, suffer from x-linked diabetes melitis, and enteropathy (Wildin *et al.*, 2001).

1.4 Macrophage-T cell Interactions

Macrophages are one of the main professional APCs in addition to DCs and B cells. This process results in the direct interaction between the macrophage and T cell in an antigen-specific manner as described in Section 1.2.2.3. Although the recognition of peptide in the context of a self-MHC molecule by the T cell's TCR forms the foundation of this interaction, other molecules are important in determining the outcome of this exchange in terms of the level of T cell activation, the type of subset differentiation, or type of macrophage effector functions. These molecular interactions include but are not limited to CD28:CD80/86, CD40L:CD40, and PD-1:PDL-1/PDL-2.

1.4.1 CD28/CD80 co-stimulation

Naïve T cells cannot be activated by TCR recognition of peptide bound to MHC alone, but instead require a second signal – co-stimulation (Lamb *et al.*, 1983; Lechler *et al.*, 2001).

The first co-stimulatory molecules to be identified were CD80 and CD86, which are expressed on APCs, and their T cell-expressed ligands, CD28 and CTLA-4 (Linsley *et al.*, 1991). The interaction between CD28 and CD80 or CD86 sends a positive signal and leads to T cell activation (Alegre *et al.*, 2001), while CTLA-4 binding to CD80 or CD86 sends an inhibitory signal and limits T cell activation (Waterhouse *et al.*, 1995). CD28 is constitutively expressed on T cells, however co-signalling molecules alter their levels of expression depending on the environment. Therefore, APCs presenting self antigen to self-reactive T cells in the absence of activation, should not be expressing high levels co-stimulatory molecules. In this circumstance, T cells are not receiving the required activation signals and instead become un-responsive (Lechler *et al.*, 2001). This is beneficial in the prevention of autoimmunities. Conversely, TLR (or another PRR) stimulation, which occurs during an infection, results in the up-regulation of CD80 or CD86 on APCs and thus promotes the activation of antigen-specific naïve T cells to fight the invading organism (Janeway *et al.*, 2002).

1.4.2 The dual role of CD40/CD40L co-stimulation

Another co-signalling molecule is CD40 present on B cells, macrophages, monocytes, platelets, astrocytes and endothelial cells. This molecule was identified in 1986 as a co-stimulatory molecule involved in the induction of B cell growth and isotype switching (Armitage *et al.*, 1993; Banchereau *et al.*, 1994; Clarke, 2000). Its' ligand, CD40L (CD154), is present on activated T cells, and was identified in 1992 by Armitage *et al*. The interaction between CD40L on T cells and CD40 on B cells is essential for isotype switching by B cells. This was demonstrated in patients with a genetic mutation of CD40L termed X-linked hyper IgM syndrome, whereby B cells were unable to elicit isotype switching. They only produced IgM antibodies, which resulted in recurrent infections (Hill *et al.*, 1993). However the role of the CD40/CD40L interaction goes beyond B cells, and has also shown to have important roles in the activation of many cell types including T cells, monocytes/macrophages and epithelial cells (Grewal *et al.*, 1998; Grewal *et al.*, 1996).

CD40/CD40L interaction is important in both T cell and APC activation (Stout *et al.*, 1996b). In the absence of CD40L, antigen-specific T cells show reduced capacity to produce cytokines in response to antigen *in vitro* (Grewal *et al.*, 1998), implicating this interaction as playing a role in the induction of T cell unresponsiveness in the periphery. Ligation of CD40 on macrophages and monocytes via CD40L, induces the up-regulation of cytokines/chemokines and surface markers associated with the induction of T cell responses, and cell-mediated immunity (Stout *et al.*, 1996a; Toes *et al.*, 1998). For example, CD40 ligation by CD40L up-regulates CD80, MHC II and cytokines such as TNFα, IL-6, and IL-12 (Alderson *et al.*, 1993; Campbell *et al.*, 1996; Caux *et al.*, 1994),

involved in promoting inflammatory Th1 responses. The CD40/CD40L interaction not only enhances the ability of APCs to activate T cells through the production of IL-12 and expression of MHC II, but is also essential for the induction of anti-microbial activity and activation of the APC itself (Campbell *et al.*, 1996; Stout *et al.*, 1996b). Th1-derived IFNγ along with CD40/CD40L signalling increases the production of nitric oxide (NO) (Tian *et al.*, 1995), essential for the killing of intracellular pathogens. CD40/CD40L interactions can also induce the up-regulation of other co-stimulatory molecules such as CD80/86 on B cells, making them more efficient at presenting antigen and stimulating T cells (Wu *et al.*, 1995).

The role of CD40/CD40L in directing T cell subset differentiation has been under intense investigation, as some studies have demonstrated the role is not strictly limited to the induction of Th1 responses. The production of prototypic Th1 and Th2 cytokines (IFNγ and IL-4 respectively) are significantly abrogated in the absence of CD40L in mice; implying CD40 plays a role in both Th1 and Th2 immune responses (Poudrier *et al.*, 1998). This has been further supported by MacDonald *et al* (2002), who demonstrated *in vitro* and *in vivo* that the CD40/CD40L interaction between the T cell and DC was essential to induce an immune response by Th2- but not Th1-inducing stimuli (MacDonald *et al.*, 2002). In addition, Hancock *et al* (1996) found that administration of anti-CD40L antibody resulted in a reduction in IFNγ and IL-12, and an increase in Th2 associated cytokines IL-4 and IL-10, suggesting that CD40/CD40L alterations can promote Th2 induction. Therefore, while it seems CD40 is involved in both Th1 and Th2 responses, many studies also support a preferential role for CD40 in Th2 biasing.

This reciprocal role of CD40 in regulating both Th1 and Th2 responses may be down to the level of signalling received. Indeed, corroborating with this is *in vitro* data by Mathur *et al* (2004), performed on primary microglia and macrophage cell lines. Mathur *et al* demonstrate high levels of CD40 signalling induces the production of the pro-inflammatory cytokine IL-12, while low level signalling elicits a reduction in IL-12 and increase in the anti-inflammatory cytokine IL-10. Thus CD40/CD40L signalling represents a single interaction which elicits dual counter-regulatory roles. The exact mechanisms involved in inducing these reciprocal responses are unknown, and studies are currently underway. In this thesis, the mechanisms involved in altering the CD40/CD40L interaction and the effects of this altered interaction on macrophage as well as CD4 T cell phenotypes are investigated.

1.4.3 PD-1/PDL-1 co-signalling

Co-inhibitors as well as co-stimulators exist to limit the extent of immune activation. Like CTLA-4, Programmed Death 1 (PD-1) is an inhibitor of T cell responses. PD-1 was first

identified in 1992 by Ishida *et al*, in studies on the programmed death of cells. PD-1 is not constitutively expressed, but as with CTLA-4, is induced by T cell activation. Its' role in peripheral tolerance and prevention of autoimmunites has become apparent, with PD-1 deficient mice suffering from spontaneous autoimmunities such as lupus-like arthritis (Nishimura *et al.*, 1999), and autoimmune mediated cardiomyopathy (Nishimura *et al.*, 2001b).

There are two ligands for PD-1; Programmed Death ligand 1 and 2 (PDL-1 and PDL-2) (Freeman *et al.*, 2000; Latchman *et al.*, 2001), and the functions of these ligands remain controversial. Some studies indicate the PD-1 ligands inhibit T cell activation and proliferation (Freeman *et al.*, 2000), however there have also been conflicting evidence that they are involved in T cell stimulation (Tseng *et al.*, 2001). Other studies have shown that the PD-1 ligands may inhibit the response of APCs through reverse signalling as seen in DCs (Kuipers *et al.*, 2006). PDL-1 is constitutively expressed on a range of cells including T cells, DCs, B cells and macrophages as well as non-haemopoietic tissue, with its up-regulation occurring by exposure to inflammatory stimuli such as LPS (Yamazaki *et al.*, 2002). PDL-2, a second ligand for PD-1 (Latchman *et al.*, 2001) is less widely expressed, and its expression on DCs and macrophages only occurs after stimulation with cytokines such as IFNγ, GMCSF, or IL-4 (Loke *et al.*, 2003; Yamazaki *et al.*, 2002). PDL-1 and PDL-2 exhibit some overlapping functions, where both induce inhibition of T cell responses as exhibited by reduced IFNγ and IL-2 by T cells (Keir *et al.*, 2006). The ligands for PD-1 seem to exhibit functions both similar and distinct from each other, having effects not only on the inhibition/stimulation of T cell responses, but also having diametrically opposed roles in Th1/Th2 responses (Loke *et al.*, 2003), implying they may elicit differential roles in deviating immune responses.

1.5 Schistosomiasis

1.5.1 Lifecycle

More than 250 million people are affected by schistosomiasis world wide (Chitsulo *et al.*, 2000). Schistosomiasis is infection with the trematode worm of the *schistosoma* genera (Butterworth *et al.*, 1994). The species which cause the majority of infection in humans are *S.mansoni*, *S.japonicum*, and *S. Haematobium*. All are endemic in tropic and subtropical equatorial areas (Kurtzke, 2000), and although the risk of mortality is less than that of other infectious diseases such as *M.tuberculosis*, schistosomiasis as with other helminth diseases, results in severe long lasting morbidity (Ouma *et al.*, 2001).

The infective cercariae derived from fresh water snails residing in contaminated water, penetrate the skin of humans while gathering water, catching fish, or washing. After

travelling through the blood stream, matured worms reside in the hepatic portal vasculature where they mate and produce their eggs about 5-6 weeks post infection (Dunne *et al.*, 2005). Eggs must then traverse the intestine to exit out through the feces (*S.mansoni , S.Japonicum*) or pass through the urine in the case of urinary schistosomiasis caused by *S. Haematobium* (Hu *et al.*, 2004). After deposition of the egg-containing feces or urine into the waterways, miracidia hatch from the eggs and infect the snail host continuing the infectious cycle.

1.5.2 Schistosomiasis immunology

Although the schistosome worm itself can live for up to 40 years undetected by the immune system (Chabasse *et al* 1985 as cited in Dunn *et al*., 2005 and Hu *et al.,* 2004), the eggs they produce induce a strong inflammatory response which is required for the eggs to exit out through the intestine/bladder to continue their life cycle. However, many eggs get lodged in organs such as the liver (*S.mansoni*) where they reach a dead end in their life cycle progression. The inflammatory response which ensues is largely driven by polarised Th2 responses (Grzych *et al.*, 1991; Pearce *et al.*, 2004), which orchestrates a granulomatous/fibrotic response at these foci of eggs (Pearce *et al.*, 2004). This Th2 driven response is both essential for host protection, and at the same time is the cause of host pathology. In *S.mansoni* infection, IL-4 prevents excessive damage to the liver induced by reactive oxygen radicals produced in response to bacterial components which seep out of the intestine, as shown in IL-4 deficient mice which suffer severe liver damage associated with significant increases in NO (La Flamme *et al.*, 2001), as well as in mice deficient in the IL-4 receptor (Herbert *et al.*, 2004). However, in excess, the same response can be detrimental to the host. At the site of granuloma formation, mediators and cytokines in the local environment, derived predominantly by macrophages, induce Th2 cells to secrete IL-4 and IL-13, which are pro-fibrotic cytokines (Wynn *et al.*, 2004). As well as having important roles in altering macrophage and T cell responses into an anti-inflammatory state and perpetuating responses involved in tissue remodelling, IL-4 and IL-13 also directly induce collagen synthesis from fibroblasts (Jakubzick *et al.*, 2003; Sempowski *et al.*, 1994) which when in excess, results in severe pathologies as seen during the chronic stages of schistosomiasis. Such pathologies include liver fibrosis, hepatosplenomegaly, the development of portal ascites, as well as portal hypertension, all of which may lead to organ failure (Ouma *et al.*, 2001; Pearce *et al.*, 2004). However, the immune responses to eggs aid in the movement though the gut/bladder wall for those destined to those sites. It also prevents systemic deposition of trapped eggs within the liver/intestine and through its counter regulation of Th1 responses, prevents exacerbated Th1-driven inflammation (Herbert *et al.*, 2008; Pearce *et al.*, 1991). Therefore, Th2

dominant immune responses, although pathology-inducing, are essential for host protection from severe schistosomiasis.

1.5.3 Macrophage activation during Schistosomiasis

Macrophages and DCs are altered in the presence of schistosome egg or soluble egg antigen (SEA). Both DCs and macrophages show a significant reduction in the ability to promote Th1 responses and produce pro-inflammatory cytokines such as IL-12 (La Flamme *et al.*, 2004; Zaccone *et al.*, 2003). This effect of reducing the activation in response to pro-inflammatory conditions occurs independently of MyD88 in DCs, a characteristic signalling molecule involved in the production of pro-inflammatory cytokines (Kane *et al.*, 2008). In the presence of LPS *in vitro*, which is a likely contaminant during infection with schistosomiasis, macrophages exposed to SEA produce lower levels of the pro-inflammatory cytokine IL-12 and increased production of IL-10 (La Flamme *et al.*, 2004). Therefore, SEA can induce cytokine deviation at the level of the macrophage, such that Th1 inducing cytokines are reduced while Th2 or Treg-inducing cytokines are increased. This ability to counter-regulate the response to LPS is likely a mechanism which occurs *in vivo,* in the setting of contaminating bacterial components which seep out of the intestine from pores created by the traversing of eggs through the gut wall (Herbert *et al.*, 2008).

Alternatively activated macrophages are essential for host survival during schistosomiasis (Herbert *et al.*, 2004), and IL-4 in the absence of alternatively activated macrophages does not elicit protection, indicating macrophages as the major elicitors of IL-4 induced protection. Further studies identified that in the absence of IL-4Rα on bone marrow derived cells such as macrophages, severe mortality ensues (Herbert *et al.*, 2008). This was associated with severe inflammation in the liver, which may have been caused by the lack of anti-inflammatory mediators and cytokines, and the removal of the suppressive effect of alternatively activated macrophages on T cell responses (Herbert *et al.*, 2008). As well as alternatively activated macrophages inducing Th2 responses, they are also capable of suppressing these same Th2 responses, and this dual effect is essential in maintaining a balance of Th2 induced protection and Th2 induced pathology (Nair *et al.*, 2009; Pesce *et al.*, 2009a).

The effect of schistosome infection on type II activation is currently unknown. As with type II macrophage activation (Tierney *et al.*, 2009), schistosome infection either with worm, egg, or soluble egg antigen (SEA), protects mice against EAE (La Flamme *et al.*, 2003; Sewell *et al.*, 2003; Zheng *et al.*, 2008). Furthermore, some of the responses associated with protection are similar. As with type II activation, protection by schistosome infection is dependent on immune deviation resulting in polarised Th2 responses, and reductions in

Th1 responses (La Flamme *et al.*, 2006; La Flamme *et al.*, 2003; Sewell *et al.*, 2003; Tierney *et al.*, 2009; Zheng *et al.*, 2008). Schistosome egg antigen complexes elicit greater anti-inflammatory responses than schistosome egg antigen alone, with high levels of IL-10 and reduced production of IL-12 (La Flamme *et al.*, 2004), which is a characteristic shared by type II macrophage activation (Anderson *et al.*, 2002b). During infection, schistosome eggs are potent inducers of inflammation, aiding in the exit of eggs into the intestine across the gut wall (Pearce *et al.*, 2004). This results in increased intestinal permeability, and as the intestine is a reservoir of bacteria, leakage of bacterial components such as LPS occurs. Furthermore, this is accompanied by high levels of inflammatory mediators such as IFNγ and IL-12 (Herbert *et al.*, 2008). In addition to the presence of IFNγ and LPS, schistosomiasis is also accompanied by circulating immune complexes (de Jesus *et al.*, 2002; Santoro *et al.*, 1979). Type II activation is induced by exposure to immune complexes in the presence of inflammatory mediators such as LPS and IFNγ (Gerber *et al.*, 2001). Therefore schistosome infection elicits an environment in support of type II macrophage activation, and it is therefore possible such an activation state occurs *in vivo*.

1.6 Objectives of this investigation

It is essential to identify different macrophage phenotypes as particular phenotypes are associated with disease pathogenesis, or more importantly, with disease prevention/recovery. To date, the number of distinct markers for differentiating type II activation from other regulatory states is quite limited. Furthermore, very little is understood in terms of the mechanisms involved during type II activation, and whether this activation state contributes to the protection schistosome infection provides against EAE.

This thesis investigates the effects of schistosome immune complexes on macrophage activation with the hypothesis that schistosome immune complexes are able to induce type II macrophage activation, similarly to opsonised SRBCs. Understanding the effects of schistosome products on macrophages, and whether the induction of type II activation is involved, will provide further insight into the possible mechanisms involved in protection from inflammatory diseases.

This thesis also investigates mechanisms involved in promoting the type II macrophage phenotype. In particular, the role CD40 alterations have in the effector functions elicited by type II activation is investigated. The hypothesis is that low levels of CD40 present on type II macrophages promote the anti-inflammatory profile elicited by this activation state and are involved in T cell biasing in favour of the Th2 subset.

The goal of this investigation is to provide a greater understanding of mechanisms involved in anti-inflammatory responses elicited by macrophage activation, with the notion that a greater understanding of mechanisms involved in immune deviation will aid the development of more effective treatments for autoimmunities.

Chapter 2: General Methods

2.1 Animals

All experiments involving animals were approved by the Victoria University Animal Ethics Comittee (AEC). C57BL/6 mice were obtained from and bred at eitherthe Biomedical Research Unit of the Malaghan Institute of Medical Research (MIMR), or the Otago School of Medicine Biomedical Research Unit, Wellington, New Zealand.

Transgenic OTII mice with a transgenic TCR for OVA $_{323-339}$ on a C57BL/6 background (Robertson *et al.*, 2000) were generously donated by Ian Hermans and Franca Ronchese, and were bred at the Biomedical Research Unit of the Malaghan Institute of Medicine, Wellington, New Zealand.

Following guidelines specified by the ministry of agriculture and forestry (MAF), mice were housed at Victoria University of Wellington in the animal facility. Mice aged 6-12 weeks were used for experiments following AEC guidelines.

2.2 Primary Cell culture

2.2.1 General Macrophage culture

Bone marrow derived macrophages (BMMØ) were cultured from C57BL/6 mice. After euthanasia, mice were soaked with 70% ethanol as were the instruments used for dissection. With the use of a 23 g needle and syringe, bone marrow was flushed from the femurs and tibias with divalent cation free Dulbecco's PBS (dPBS-Invitrogen, Auckland, NZ) with collections made into a 50 ml falcon tube (BD Bioscience, Franklin Lakes, NJ, USA). Cells were broken apart by homogenisation, washed by centrifugation at 760xg in wash buffer (Appendix B), followed by lysis of red blood cells with 2 ml of RBC lysing solution (Sigma, St Louis, MO, USA) for 2 minutes. After further washing cells in wash buffer, cells were then seeded at 10^6cells /ml in T75 tissue culture flasks (BD Bioscience, Franklin Lakes, NJ, USA) in complete T cell media (CTCM-In appendix B). After overnight culture at 37^{o}C in 5% CO_2, adherent cells were removed and the remaining progenitor cells were cultured on 90 mm non-tissue culture-treated, gamma-sterilized petri dishes (Raylab, NZ) in CTCM with 5 ng/ml rGMCSF and rIL-3 (Peprotech, N.J) at 37^{o}C in 5% CO_2 for 8-10 days until fully differentiated into macrophages. Cells were then removed by carrying out several rounds of incubation in ice-cold dPBS at 4^{o}C followed by vigorous pipetting.

For stimulation assays, macrophages were cultured in 96 well plates (BD Bioscience) at 10^5 cells/well in CTCM, and were primed overnight in 20 U/ml IFNγ (BD Biosciences)

followed by exposure to the various culture conditions (as specified in figures). Macrophages were stimulated with 200 ng/ml lipopolysaccharide (LPS- Sigma) in the presence or absence of opsonised sheep red blood cells (SRBC-IgG), un-opsonised SRBC (Taylor Preston Limited), opsonised schistosome whole egg, un-opsonised schistosome egg (Dalton *et al* 1997), opsonised soluble egg antigen (SEA), or un-opsonised SEA (Boros *et al* 1970). Cells were incubated at 37^{o}C in 5%$CO_{2.}$

2.2.2 Generation of immune complexes

2.2.2.1 SRBC immune complexes

For each batch of new sheep red blood cells (SRBC), an agglutination assay was carried out in order to determine the optimal concentration of rabbit anti-SRBC IgG (Sigma, St Louis, MO, USA) for opsonisation. Two fold serial dilutions of rabbit anti-SRBC antibody were made in round bottom 96 well plates (BD Bioscience) starting with a 1:50 dilution in 1xPBS (Appendix B). Fresh SRBCs were added to all wells at a final concentration of 10^8 cells/ml, and incubated at room temperature for 1-2 hours. The highest non-agglutinating concentration was determined by visualising the plate under a light source. Optimal concentrations ranged between 1:200 to 1:400 of rabbit anti-SRBC IgG.

Opsonised SRBCs were generated by incubating SRBCs (10^8 cells/ml) with rabbit anti-SRBC antibody at predetermined non-agglutinating titres. Incubation of the SRBCs and antibody was carried out at room temperature with rotation of the mixture for 30-40 minutes. Cells were then washed and the remaining SRBC-IgG complex was added to the macrophage culture at a ratio of 10 SRBC: 1 macrophage.

2.2.2.2 Schistosome whole egg immune complexes

Schistosome whole egg complex was generated by incubation of 1000 *Schistosoma mansoni* eggs with serum from schistosome infected C57BL/6 mice at a 1:100 dilution of serum. The solution was incubated at room temperature while rotating, for 30 minutes. Following incubation, non-bound serum was removed by washing the solution in PBS, and resuspending the remaining schistosome egg-complex at the desired concentration such that there were 1000 eggs per well.

2.2.2.3 Schistosome soluble egg antigen (SEA) immune complexes

Antibody (IgG) from schistosome infected mice was purified on a protein G sepharose column (Sigma), by Dr J. Tierney. To obtain the SEA-immune complex, SEA (either 5 or 10 µg/ml) was incubated with anti-SEA IgG (either 10 or 20 µg/ml) in 1xdPBS. The solution was incubated for 1 hour while rotating at room temperature.

2.2.3 Obtaining single cell splenocyte suspensions

After removal from OTII C57BL/6 mice, spleens were mechanically mashed using the back of a 1 ml plunger belonging to a 1ml syringe (BD Bioscience), through 70 µm cell strainers (BD Bioscience) into 50 ml falcon tubes to create single cell suspensions. After washing in wash buffer followed by CTCM (Appendix B), splenocytes were incubated for 2 minutes in red blood cell lysing solution. Cells were washed at 760xg centrifugation and resuspended in CTCM at a desired concentration for CD4 T cell isolation.

2.2.4 Isolation of CD4 T cells from OTII positive mice using Dynabeads and DETACHaBEAD (Brief outline of manufacturer's instructions)

Splenocytes from OTII positive mice were isolated (described above) and resuspended at $1x10^7$ cells/ml in isolation buffer (Appendix B). Mouse CD4 (L3T4) Dynabeads (Invitrogen Dynal, Norway) were washed and added to the resuspended cells at 25 µl per $1x10^7$ splenocytes, and incubated for 20 minutes while rotating at 4^oC. CD4 negative T cells were then removed by carrying out several rounds of placing the tube on a DynaMag magnet (Invitrogen) for 2 minutes before discarding the supernatant and resuspending the cells in 100 µl CTCM per $1x10^7$ cells. The remaining bead-bound CD4 T cells were isolated by adding 10 µl of mouse CD4 DETACHaBEAD (Invitrogen) per $1x10^7$ cells, and incubated while rotating at room temperature for 45 minutes. The CD4 T cells were then isolated by placing the tube on the magnet and collecting the supernatant. This was carried out for several rounds to obtain a maximum amount of CD4 T cells. These were then resuspended at the desired concentration for later use in functional assays or flow cytometry (specified in figures). For stimulation assays with macrophages, the CD4 T cells were plated at approximately $1x10^6$ cells/ml ($2.5x10^5$cells/well) in 96 well plates containing the macrophage cultures, and incubated at 37^oC in 5% CO_2 for 72 hours.

2.3 CFSE staining protocol of CD4 T cells.

CD4 T cells, purified as described above (2.2.4), were resuspended in sterile 1xdPBS at a concentration of $2x10^7$ cells/ml before incubating with Carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) used at 625 nM. After incubation for 8 minutes at room temperature while wrapped in foil, an equal volume of 100% foetal calf serum (FCS- ICP biologicals, Auckland) was added to quench the CFSE and terminate the reaction. Cells were thoroughly washed to remove extracellular CFSE by several rounds of centrifugation in CTCM, after which cells were resuspended at the desired concentration of 2.5×10^6 cells/ml prior to culture in 96 well plates containing macrophage cultures.

2.4 Cytokine detection

2.4.1 Enzyme linked immunosorbant assay (ELISA)

To determine levels of cytokines in the supernatants collected from cell cultures, a sandwich enzyme-linked immunosorbent assay was carried out following manufacturer's recommendations (BD Pharmingen, Franklin Lakes, NJ). All ELISA antibodies were purchased from BD Bioscience unless otherwise stated. In brief:

The cell culture supernatant was removed and stored at -20 ^{o}C until used. 96 well ELISA plates were coated with 50 µL of capture antibody specific to the cytokine of interest (specified in figures). Dilutions of capture antibody were made in capture buffer before transfer to the ELISA plate according to the manufacturer's guidelines for that antibody (See Appendix A table1). ELISA plates were incubated at 4 ^{o}C overnight.

Plates were then flicked to remove any unbound antibody in solution, and incubated in blocking solution (1 x dPBS containing 5% FCS) at room temperature for approximately 2 hours to block non-specific binding. Plates were washed 2-3 times in a washing solution of 1xPBS (Appendix B) containing 0.05% Tween-20 (Sigma) (TPBS), after which samples were added to the wells either as neat or diluted in blocking solution. Cytokine standards were added to the top two rows in 2 fold serial dilutions with the concentration of the first well specified in appendix A. Plates were incubated for either 2 hours at room temperature, or overnight at 4^{o}C.

After washing plates in TPBS approximately 4 times, biotinylated detection antibody (BD Bioscience) was diluted in blocking solution at the recommended concentration, and 50 µl was added to the wells. Plates were incubated for 1 hour at room temperature. Plates were then washed in TPBS, and incubated in Streptavidin-HRP (SA-HRP-BD Bioscience) for 30min-1 hour depending on the cytokine of interest.

Directly after washing plates in TPBS, equal volumes of substrate reagent A and B (BD Bioscience) were added to each well and allowed to develop at room temperature under visual supervision. The substrate induced a colour change which allowed one to be able to decide the appropriate time to stop the reaction, at which time 0.18 M sulphuric acid (STOP solution) was added to each well.

Absorbance of each well was then read at 450 nm using a VersaMax plate reader.

2.4.2 Cytometric Bead array (CBA)

For the detection of MCP-1, IL-6, and TNFα in addition to IL-10 and IL-12, a CBA mouse inflammation kit (BD Bioscience) was performed as per manufacturers instructions.

In brief:

BD Standards were prepared in BD assay diluent, with a range of 0-1667 pg/ml. The 'mixed capture beads' were prepared by mixing together 2.5 µl aliquots of each of the 6 capture beads per well to be analysed, in a 1:1 dilution with assay buffer. BD PE detection was diluted in assay buffer in a 1:1 dilution. Following this, 25 µl of the 'Capture bead mix' was added to all wells in a 96-well round bottom plate. This was followed by the addition of 25 µl of samples and 25 µl of standards to the appropriate wells. Additionally, 25 µL of the diluted PE detection was added to all wells, followed by a 2 hour incubation at room temperature in the dark. The plate was then washed in BD wash buffer (BD Bioscience) two times, prior to resuspending the bead-sample pellet in 300 µl of wash buffer for analysis on the flow cytometer as reported in 2.5.

2.5 Flow Cytometry

Cells were harvested from 96 well plates at the end of the culture period. For macrophage cultures, cells from duplicate cells were combined in order to acquire enough cells for flow cytometry. Prior to staining, cells were washed to remove excess CTCM. The Fc receptors of cells were blocked by incubation with rat anti-CD16/32 Fc block (1µg/ml) for 10 minutes. Cells were stained with fluorescently labelled antibodies (BD, Serotec, eBioscience-Appendix B) at dilutions determined by either optimisation within the lab, or recommended by the manufacturers guidelines. Cells were stained and incubated with the fluorescently labelled antibodies for the markers of interest as well isotype controls following the manufacturers recommendations (BD Pharmingen). After staining for markers of interest, cells were analysed using the FACScan flow cytometer (BD Bioscience). For analysis of primary macrophages, 900-1500 live events were collected per assay. For examination of splenocytes or CD4 T cells, 5000- 10,000 live events were collected.

The live cell population was determined using the forward scatter (FSC) and side scatter (SSC) data of the non-stained population, which was gated around. Isotype controls and single stains were used to determine background fluorescence and compensation respectively.

Analysis of results was carried out using the CELLQuest pro software (BD, Franklin Lakes, NJ, USA).

2.6 Western Blot

2.6.1 Obtaining lysate

Macrophages were removed 9 hours following primary stimulation under the different culture conditions (in figures). Supernatant was removed and macrophages were incubated for 5 minutes in trypsin (TrypLE express, Invitrogen) at 37^{o}C in 5% CO_2. Fetal calf serum (FCS-ICP biologicals, Auckland) was added to quench the reaction. Cells were washed two times to remove excess trypsin, with the final wash in 1xdPBS. Cells were then incubated with 100 µl of lysing buffer (10 µl of protease inhibitor cocktail (Sigma) per 1 ml of RIPA buffer (Appendix B)) for 1 hour at 4^{o}C while shaking. The solution was then centrifuged at 2000xg for 25 minutes to remove debris. Protein concentrations were assessed using a BCA kit (Pierce). The supernatant containing proteins of interest was removed and stored at -20 or -80^{o}C for future assessment.

2.6.2 Running western blot

Preparation of lysates for running in gels was carried out as follows; 80 µg of each lysate (or 22 µl max) from 2.6.1 were transferred into fresh tubes, and incubated with 8 µl of the reducing buffer mix (1:10 dilution of 2-mercaptoethanol (Sigma) in 5 x reducing buffer-containing bromophenol blue in running buffer), at 90^{o}C for 10 minutes.

SDS PAGE (Sodium dodecyl sulfacte polyacrylamide gel electrophoresis) was performed with 30 µl of lysates containing reducing buffer along with 3 µl of dual colour ladder (Biorad) .This was run at 200V for 45 minutes (Appendix B for gel recipe). Proteins were transferred onto a PVDF membrane (GE Healthcare, Amersham) by running the transfer for 4 hours at 50 V in a 4^{o}C cold room. Immunoblots were removed and blocked using 5% non-fat skim milk in TTBS (Appendix B) for 1 hour at room temperature. Membranes were then probed with the primary antibody of interest (Dilutions in Appendix A table2). Membranes were probed with the primary antibody for 2 hours at room temperature, or overnight at 4^{o}C. This was followed by 3 x 5 minute washes in TTBS. Membranes were probed with a secondary fluorescently labelled antibody against the species for which the primary antibody was derived from, for 2 hours at room temperature. Depending on the primary antibody, this was anti-rabbit cy3, or anti-mouse cy5 (GE Healthcare). Immunoblots were washed with 3 x 5 minute washes in TTBS. Images of the probed membranes were then obtained on a Fuji Film FLA-5100 scanner. The same membranes were then washed with 3 x 5 minute washes in TTBS and the same process was repeated for each primary antibody used. Dependant on the experiment, the primary antibodies included rabbit anti-SPHK1 (Abcam), rabbit anti-RELMα (Abcam) or mouse anti-iNOS

(Transduction laboratories). Scanned images were analysed using Image Quant. Western blots were normalised to α tubulin.

2.7 Graphing and statistical analysis

Results were analysed and graphed in GraphPad, Prism v. 4.0 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Statistical analysis was carried out using one way ANOVA or two-way ANOVA with Bonferroni correction.

Chapter 3: Defining key mechanisms of type II macrophage activation

3.1 Introduction

Macrophages are highly plastic innate cells capable of altering their physiology and therefore their functions depending on changes in the micro-environment (Stout *et al* 2004). As such, they are able to elicit a broad spectrum of activation states (Mosser *et al.*, 2008) of which the major states include classical, alternative, and type II. Macrophages activated in response to pro-inflammatory stimuli are termed classically activated macrophages. These macrophages elicit cell mediated, Th1-promoting pro-inflammatory responses (Liew *et al.*, 1990; Stenger *et al.*, 1996), and can be characterised by high levels of NO, essential in the killing of intracellular pathogens, as well as the production of inflammatory cytokines and chemokines such as IL-12, TNFα, IL-6 and MCP-1 (Steube *et al.*, 1999). Also associated with the activation of classically activated macrophages is the expression of co-signalling molecules like CD80 and CD40, which are up-regulated during inflammation (Alderson *et al.*, 1993; Caux *et al.*, 1994b; Tierney *et al.*, 2009). In the absence of co-stimulatory markers, cell-mediated responses are significantly impaired (Campbell *et al.*, 1996; Florido *et al.*, 2004). This phenotype has been shown to be detrimental in autoimmunities such as MS, and reductions in the level of macrophages exhibiting classical activation, or inhibition of the inflammatory mediators produced by these macrophages, are associated with protection against CNS inflammation (Cua *et al.*, 2003; Huang *et al.*, 2001b; Sewell *et al.*, 2003).

In 1992 Stein *et al* identified a novel macrophage state induced by IL-4, which elicited distinct functions from that of classically activated macrophages (Stein *et al.*, 1992). Unlike classically activated macrophages, alternatively activated macrophages convert L-arginine into precursors for collagen synthesis and are involved in tissue remodelling and wound repair as opposed to pathogen killing (Raes *et al.*, 2002). This pathway is in contrast to the conversion of L-arginine to NO by iNOS in classically activation macrophages (Modolell *et al.*, 1995). Also expressed in alternatively activated macrophage is RELMα, which along with arginase 1, exhibits roles in wound repair as well as the suppression of overzealous inflammatory responses (Nair *et al.*, 2009; Pesce *et al.*, 2009a; Pesce *et al.*, 2009b). Alternatively activated macrophages are poor at presenting antigen to T cells, unlike classically activated macrophages (Edwards *et al.*, 2006), and are more inhibitory than stimulatory of T cell responses (Schebesch *et al.*, 1997).

Adding to the repertoire of macrophage subsets was the discovery of type II activated macrophages (Anderson *et al.*, 2002; Gerber *et al.*, 2001; Sutterwala *et al.*, 1997). These macrophages were classed under the same category as alternatively activated macrophages, due to their differences from classically activated macrophages; however the pathway to activation is completely different. Type II activation occurs via the ligation of Fcγ receptors in the presence of LPS and significantly alters the responses to inflammatory stimuli by reducing the expression of co-stimulatory markers (Tierney *et al.*, 2009) and deviating the cytokine profile to promote anti-inflammatory cytokines such as IL-10, over pro-inflammatory cytokines such as IL-12 (Gerber *et al.*, 2001). Unlike alternatively activated macrophages, type II macrophages express iNOS and can be distinguished from classically and alternatively activated macrophages by high expression of the enzyme sphingosine kinase 1 (SPHK1) (Edwards *et al.*, 2006). Furthermore, type II macrophages have the ability to present antigen to T cells and induce Th2 cell biasing (Anderson *et al.*, 2002; Lucas *et al.*, 2005). It is this ability to elicit a Th2 polarized immune response that is believed to counter-regulate Th1-mediated inflammatory responses and provide protection against inflammatory diseases such as endotoxemia and EAE, an animal model of MS (Gerber *et al.*, 2001; Tierney *et al.*, 2009). However, the exact mechanisms involved in type II activation and how these effects provide protection against EAE are currently unknown. Because type II activation is a strong polarizer of anti-inflammatory Th2 responses, understanding the mechanisms involved in the ability of type II activation to deviate immune responses would provide greater understanding of how to increase or prevent beneficial or pathological processes respectively.

Aims:

To better understand the mechanisms involved in type II activation, we examined several aspects of type II macrophage activation. These aspects included assessing changes in cytokines and surface markers over a period of time following stimulation, and the involvement of IL-10 and CD40 in regulating the phenotypic alterations in type II macrophages. Overall, there were three specific aims to be addressed:

1. To assess the kinetic changes in the expression of co-signalling molecules and cytokine profiles, in order gain an insight into the kinetics involved in type II activation.

2. Investigate the contribution of IL-10 in the ability of type II macrophages to reduce inflammatory cytokines and surface markers, to determine whether the increase in IL-10 is essential for this macrophage phenotype.

3. Study the role of CD40 signalling, with regards to the phenotypic changes elicited by type II activation, and investigate whether increased signalling impairs the anti-inflammatory phenotype.

3.2 Results

3.2.1 Ability of opsonised SRBCs to induce type II macrophage activation

In order to investigate the mechanisms involved in type II macrophage activation, we first characterized the phenotype of the type II activated macrophages in our experimental system. Additionally, we wished to identify broader changes occurring in response to type II activation to aid in a greater understanding of how type II activation protects against EAE as well as other inflammatory disorders. For all experiments, we used opsonised sheep red blood cells (SRBCs) in the presence of LPS to induce type II macrophage activation, while LPS alone or in the presence of un-opsonised SRBC was used to induce classical activation. The production of various cytokines was assessed 8 h following stimulation.

SRBC or SRBC:IgG (opsonised SRBC) in the absence of LPS elicited similar effects to medium alone with respect to cytokine production (IL-10, IL-12, MCP-1, IL-6, TNFα; Figure 3.1). Moreover, stimulation with SRBC in the presence of LPS elicited a similar level of cytokine production to that seen by stimulation with LPS alone, indicating SRBC alone has no effect on macrophage activation (Figure 3.1). In contrast to the effect of un-opsonised SRBC, the effects seen by opsonised SRBC in the presence of LPS differed significantly from LPS stimulation alone. For example, IL-10 production in response to SRBC:IgG + LPS is significantly higher than that induced by LPS or SRBC + LPS (un-opsonised). Furthermore, macrophages stimulated with LPS produced high levels of IL-12p40 while the addition of SRBC:IgG to cultures with LPS resulted in a significant reduction in IL-12. These results confirm that the addition of SRBC:IgG has induced type II as opposed to classical macrophage activation in our system.

Exploring the effect of type II activation on the production of other cytokines, we assessed MCP-1, IL-6, IL-4, and TNFα. MCP-1, also known as CCL2, was up-regulated by macrophages stimulated with LPS, and this level of production remains largely unaffected by the addition of SRBC, with only a slight reduction observed. However, MCP-1 levels are reduced by the addition of SRBC:IgG to LPS-stimulated cultures. Although statistically non-significant, this is similar to the effect seen on IL-12 production. LPS stimulated macrophages produce very high levels of IL-6, and in contrast to the effects seen on IL-10, MCP-1 and IL-12, SRBC in the presence of LPS appear to reduce the production of IL-6, to a similar degree to that observed by SRBC:IgG + LPS (Figure 3.1). This finding

indicates the reduction in LPS-induced IL-6 is not due specifically to the ligation of Fcγ receptors, but rather due to an unknown mechanism involving the presence of SRBC. Similarly, high levels of TNFα are also produced by LPS stimulated macrophages, but appear to be reduced by the presence of un-opsonised SRBC in LPS cultures. Interestingly, no reduction in TNFα is observed by stimulation of macrophages with LPS in the presence of SRBC:IgG. However as the data are from only 1 experiment, further investigation is required to confirm these results since this effect has not been reported previously. In response to LPS, macrophages produced nearly undetectable levels of IL-4 similar to non-activated macrophages (Figure 3.1). Macrophages stimulated with LPS in the presence of SRBC:IgG showed a higher production compared to non-activated and LPS-activated macrophages, however the difference was not statistically significant. These cytokine profiles indicate stimulation with LPS in the presence of SRBC:IgG increases the production of IL-10, while reducing pro-inflammatory cytokines IL-12 and MCP-1, as well as having effects on IL-6 and possibly TNFα. MCP-1 reductions by type II activation have not been previously reported, nor have reductions in any other chemokines. As chemokines such as MCP-1 contribute to the pathogenesis of inflammatory illnesses, further investigation into the effects of type II activation on chemokines is warranted.

In order to further validate the activation state induced upon stimulation with LPS in the presence of SRBC:IgG, the enzymatic profile of macrophages activated either with LPS or LPS + SRBC:IgG was assessed via western blotting. The levels of iNOS and sphingosine kinase 1 (SPHK1) enzymes were normalised to the expression of α tubulin, one of an array of house-keeping proteins. Figure 3.2 shows the normalised % of control for the expression of iNOS and SPHK1 in comparison to the levels seen in macrophages stimulated with LPS alone. Macrophages stimulated with LPS have high levels of iNOS indicating successful induction of classically activated macrophages (Figure 3.2), and as expected, macrophages stimulated with LPS + SRBC:IgG express similar iNOS levels as LPS alone (figure 3.2). Sphingosine kinase 1 (SPHK1), a marker for type II macrophage activation, is significantly up-regulated in macrophages stimulated by LPS in the presence of SRBC:IgG compared to stimulation with LPS alone, indicating the induction of type II macrophage activation. As with the cytokine profile, results from the western blot further support the notion that our system of activating macrophages with opsonised SRBC plus LPS induces type II macrophage activation. The ability of these type II activated macrophages to enhance anti-inflammatory cytokines (e.g. IL-10) while reducing its inflammatory counterparts (e.g. IL-12p40 and MCP-1), along with the enhanced expression of SPHK1 generates a viable means to assess type II macrophage activation for further studies within this project. Finally, this work is the first report of type II

macrophages reducing the expression of MCP-1, or any chemokine, therefore illustrating type II macrophages reduce a wider but selective range of inflammatory mediators than IL-12.

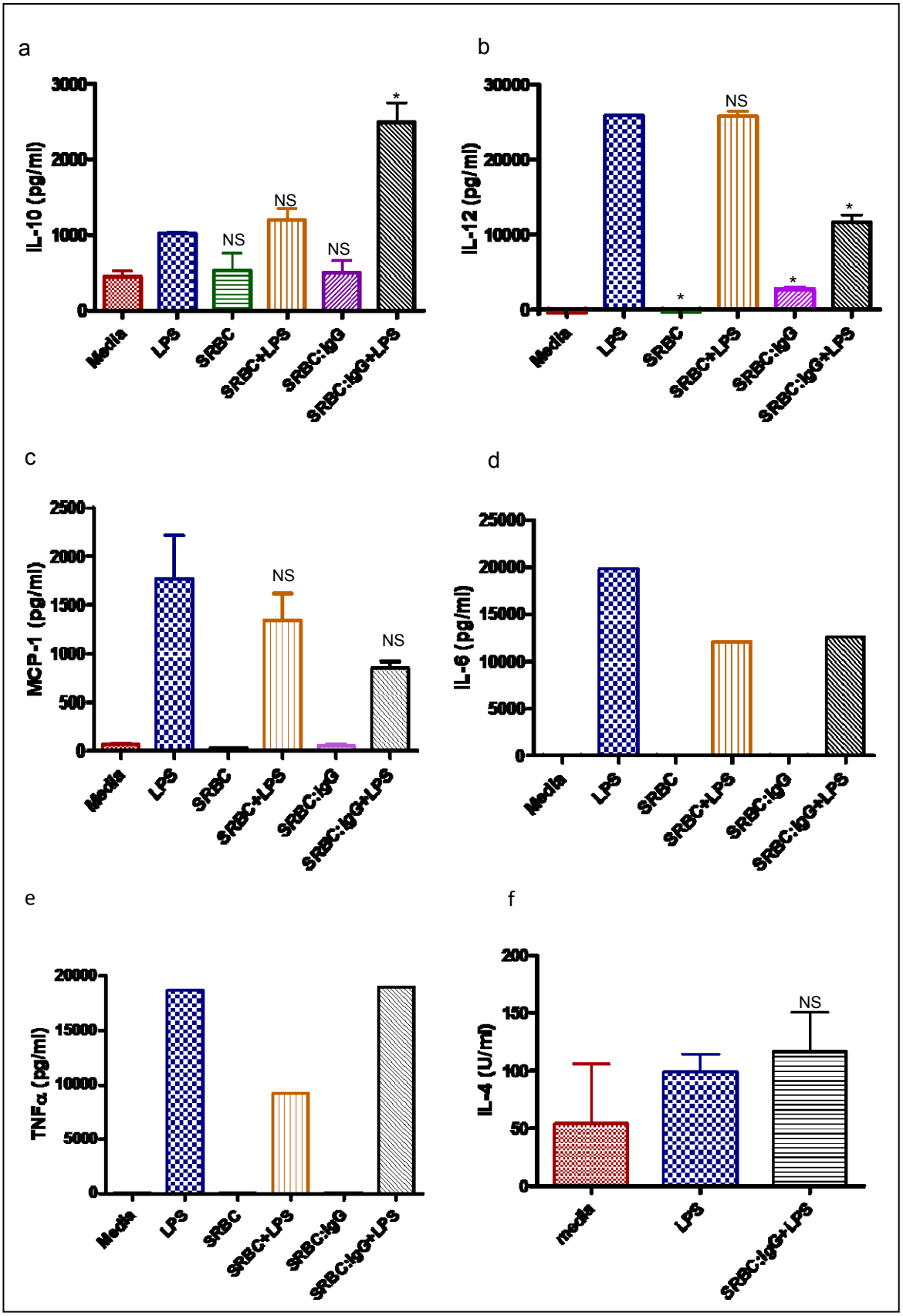


Figure 3.1 SRBC:IgG + LPS elicit different cytokine profiles by macrophages than LPS alone.

Primary BMMØ (10^6 cells/ml) derived from C57BL/6 mice were primed with 20 U/ml IFNγ overnight followed by stimulation with un-opsonised SRBC (ratio of 10 SRBC: 1 MØ), opsonised SRBC

(SRBC:IgG), 200 ng/ml LPS alone, or LPS plus un-opsonised or opsonised SRBC. After 8 h culture, IL-10 (a), IL-12 (b), MCP-1 (c), IL-6 (d), TNFα (e) and IL-4 (f) production were measured via ELISA or a CBA assay (sec 2.4). *P<0.05: One way ANOVA followed with Bonferroni post test; Conditions versus LPS. Data points (a-c) represent mean +SEM of combined duplicate wells of two experiments. (D-F) represent data points of single wells, resulting in no error bars. (F) represents a single ELISA containing samples for the three conditions of Medium, LPS and SRBC:IgG+LPS.

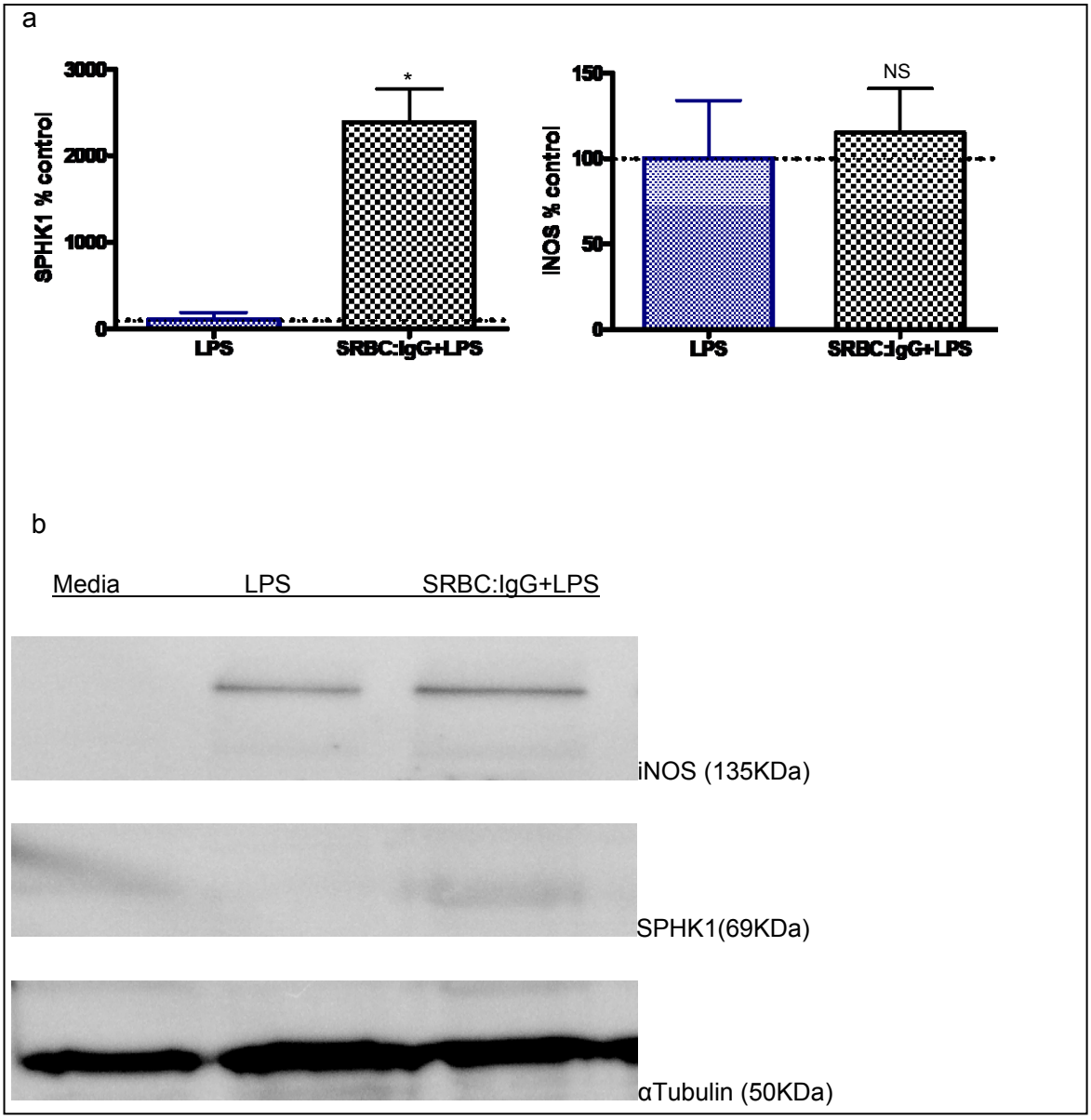


Figure 3.2 Macrophages exposed to SRBC:IgG up-regulate SPHK1 and iNOS expression. Primary BMMØ (10^6 cells/ml) derived from C57BL/6 mice were primed with 20 U/ml IFNγ overnight followed by stimulation with 200 ng/ml LPS (classical-inducing) or opsonised SRBC plus LPS (10 per MØ) (type II-inducing). After 9 h culture, expression levels of SPHK1, iNOS and αTubulin were assessed via western blotting (sec 2.6) with anti-SPHK1 pAb (1:700), anti-α Tubulin pAb (1:1000), and anti-iNOS pAb (1:700). Band densities were normalised to the expression of α tubulin, and presented as a % control compared to LPS. * P<0.05: One way ANOVA followed with Bonferroni post test;

SRBC:IgG+LPS versus LPS. Data represents the mean+SEM of 2-3 experiments. Diagram (b) is a single membrane representative of the membranes assessed to derive graph (a).

3.2.2 Kinetics of cytokines and co-stimulatory markers altered by type II macrophage activation

Previous studies identified that type II activated macrophages not only alter their cytokine profile, but also significantly change the expression of surface markers in response to inflammatory stimuli (Tierney *et al.*, 2009). Other than the study by Tierney *et al*, there has been only one other report of alterations in surface markers (Edwards *et al.*, 2006). In order to gain an understanding of possible mechanisms involved during type II macrophage activation, this thesis investigated the kinetics of cytokine and surface marker alterations by assessing changes over a period of time after classical of type II macrophage stimulation. By understanding which alterations occur at which time, the aim was to identify mechanisms involved in determining the type II activated macrophage phenotype.

Represented in this section (Figure 3.3-3.4) are the effects seen by the addition of various stimulants in the presence of LPS. Unless otherwise stated, the effects of SRBC or SRBC:IgG in the absence of LPS mimic that seen by the media controls. Figure 3.3 a-b show the changes in IL-10 and IL-12 concentration over time, while Figure 3.3 c-d show the same conditions represented as a % of control (LPS alone). Unless otherwise stated, there was very little difference in the response of macrophages to LPS stimulation in the presence of SRBC and stimulation with LPS alone. From 2-8 h, the production of IL-12 by classically activated macrophages steadily increased with high levels maintained 24 h post-stimulation. Production of this pro-inflammatory cytokine was significantly reduced by type II activation (SRBC:IgG+LPS) at 24 h post stimulation. Additionally, at 8 -24 h post stimulation, type II activated macrophages produced higher levels of IL-10 than LPS stimulation alone, and these levels peaked at 8 h post stimulation. Together these results indicate that the changes in cytokine production are apparent at all time points post stimulation and are not a result of an accelerated time course in the type II activated cultures.

In addition to cytokine assessments, the expression of CD40, CD80, MHC II, and PDL-1 was assessed at the different time points via flow cytometry (Figure 3.4). Data is presented as a % control (a-d) in comparison to classical activation (LPS), with the raw data in a2-c2 representing changes over time for all conditions. Exposure of macrophages to SRBC in the presence of LPS induced the same levels of expression of surface markers to that of LPS stimulation alone. Classically activated macrophages (LPS alone) had high levels of CD40 present on their surface, and as with IL-12, levels of CD40 increased over time with the highest levels measured 30 h post-stimulation (Figure 3.4). By 8 h post stimulation, differences in CD40 expression were observed. Type II

macrophages (SRBC:IgG+LPS) had higher levels of CD40 than non-activated macrophages, which barely expressed CD40, but had significantly lower expression of CD40 compared to LPS stimulation. Moreover, the levels of CD40 on type II activated macrophages remained constant 8-24 h post stimulation, whereas by 30 h post stimulation, the levels of CD40 on type II macrophages began to increase towards the levels expressed on classical macrophages. In terms of CD80 expression, macrophages exposed to LPS or medium alone exhibited the same levels of expression. Type II macrophages (SRBC:IgG+LPS) had levels of CD80 significantly lower than levels seen on classically activated macrophages, and the levels on these macrophages remained nearly constant over time from 8-30 h with the greatest % reduction in comparison to LPS-stimulation occurring at 24 h post-stimulation. PDL-1 expression occurred much later than the expression of CD40 and CD80 on activated macrophages. By 24 h of culture, significant changes are observed with LPS-treated macrophages exhibiting increased expression of this surface marker. Type II activated macrophages showed reduced expression of PDL-1 at 24 and 30 h post stimulation with the most notable reduction in PDL-1 compared to LPS-stimulation occurring 24 h after stimulation.

By 8 h after stimulation, up-regulation of MHC II (IA^b) expression is observed on all macrophages treated with LPS. No real difference in the levels of MHC II on macrophages from the three different treatment groups were seen until 24 and 30 h post stimulation. Exposure to SRBC:IgG significantly reduced the expression of MHC II in response to LPS, most significantly at 24-30 h of culture. This data is representative of only one experiment for the 8-30 h post stimulation time points. Therefore, caution must be used in drawing conclusions.

Observations from these kinetic analyses indicate alterations in cytokine production occur at an earlier time point than the alterations in expression of co-signalling markers. This finding may suggest a causal relationship whereby the increased IL-10 or reduced IL-12 plays an important role in the alterations of CD80, CD40, and PDL-1 induced by type II macrophage activation. Understanding mechanisms involved in perpetuating the phenotype elicited by type II macrophage activation will expand our knowledge on mechanisms involved in the induction of anti-inflammatory responses.

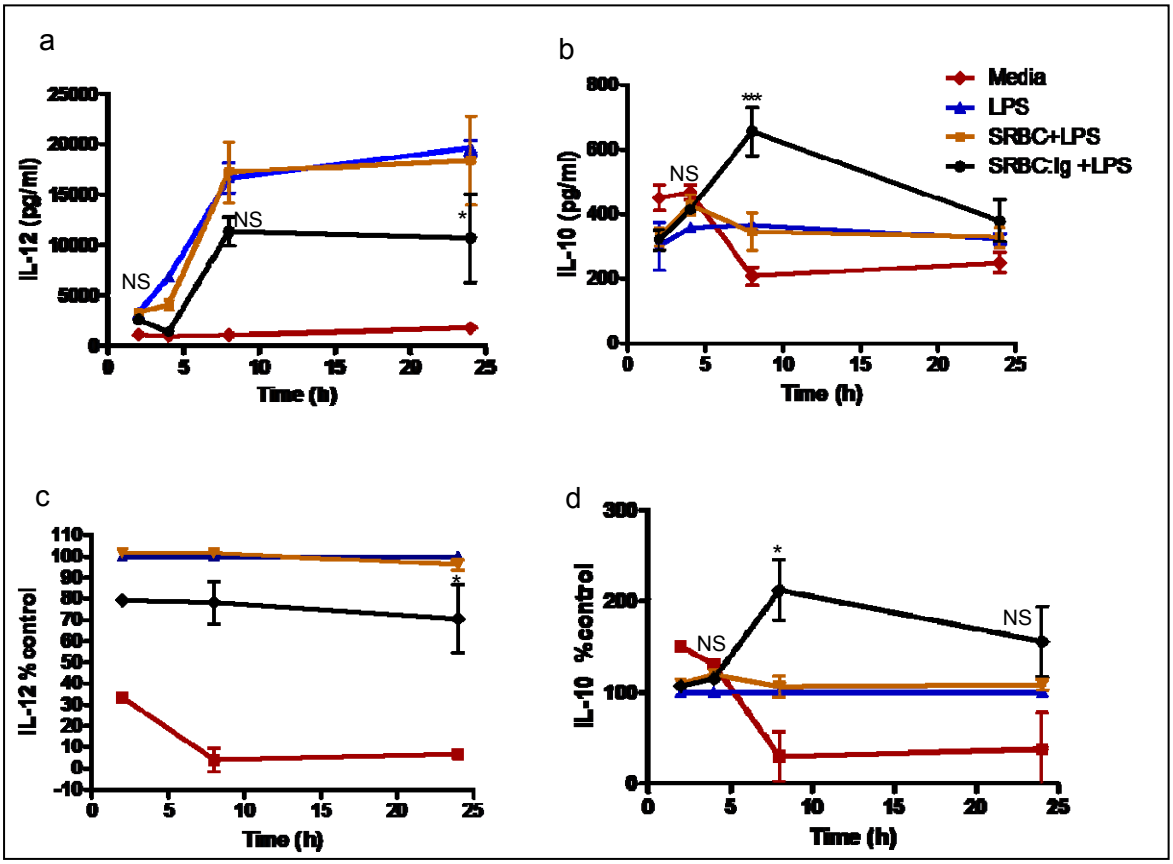


Figure 3.3 Type II activation induces the most significant alteration in the IL-10/IL-12 ratio at 8 h post stimulation. Macrophages derived from C57BL/6 mice were primed overnight with 20 U/ml IFNγ and stimulated with 200 ng/ml LPS alone (classical-inducing stimuli) or LPS in the presence of SRBC or opsonised SRBC (type II-inducing stimuli). Cells were also left non-stimulated in media. After 2, 4, 8, and 24 h culture, IL-12 (a,c) and IL-10 (b,d) were assessed via ELISA. (c-d) represent the compilation of data, presented as % compared to LPS (classical activation). *P<0.05 **P<0.01 ***P<0.001: Two way ANOVA with Bonferoni post test; SRBC:IgG+LPS versus LPS. Data points represent the mean+SEM of two experiments (c-d), or duplicate wells from one experiment (a-b).

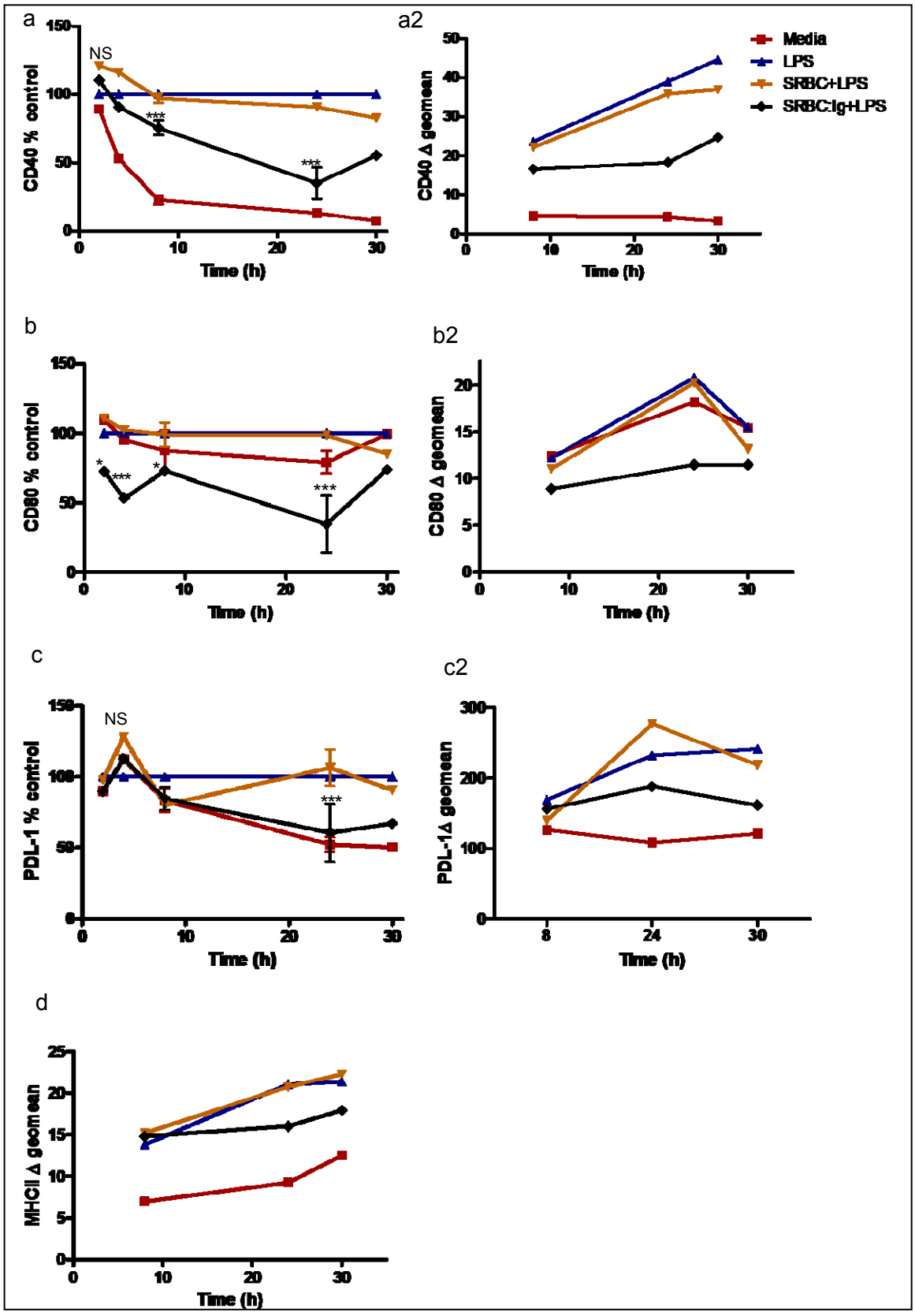


Figure 3.4 Type II activation induces the most significant alterations in surface marker expression at 24 h post stimulation. Macrophages derived from C57BL/6 mice were treated as described in

Figure 3.3. After 2, 4, 8, 24, and 30 h culture, CD40 (a), CD80 (b), PDL-1 (c) and MHC II (d) were assessed via flow cytometry. * P<0.05, **P<0.01, ***P<0.001: Two way ANOVA with Bonferroni post test; SRBC:IgG+LPS versus LPS. Data points represent the mean +SEM of two experiments, but in some instances (d) data points represent a single experiment.

3.2.3 Involvement of IL-10 in the type II macrophage profile

The kinetic analysis (sec 3.2.2) revealed that type II activation results in significant increases in IL-10 and reductions in IL-12 prior to reductions seen in the expression of co-signalling molecules. Previous literature emphasizes the suppressive effects of IL-10 on inflammatory cytokines/chemokines such as TNFα, IL-12 and MCP-1 (de Waal Malefyt *et al.*, 1991), with reports of IL-10 also reducing the expression of CD40 in response to LPS (Qin *et al.*, 2006). To investigate possible mechanisms involved in determining phenotypic characteristics of type II activated macrophages, the role of the increase in IL-10 elicited by type II activation was investigated in order to elucidate whether it is responsible for some of the observed characteristics.

Classical and type II activated macrophages were cultured in the presence of either 5 ng/ml recombinant IL-10 to enhance IL-10 levels, or 2 µg/ml anti-IL-10 antibody (ab) to block IL-10. Cytokine and cell surface markers were assessed 8 and 24 h post stimulation. Upon the addition of anti-IL-10 ab (Figure 3.5), no IL-10 was detected in any of the cultures indicating IL-10 has been successfully inhibited. Looking at IL-12 production, it can be seen that the inhibition of IL-10 led to an increase in IL-12 production by both classically and type II activated macrophages. In contrast, the addition of rIL-10 to cultures led to an almost complete inhibition of IL-12 production with only very low levels of IL-12 detected in cultures with classical and type II macrophages. Importantly, the absence of IL-10 did not impair the ability of type II activated macrophages to reduce IL-12 in comparison to classically activated macrophages.

MCP-1 and IL-6 production were also assessed in response to IL-10 inhibition with anti-IL-10 ab, although not in response to the addition of IL-10. In the absence of IL-10, type II activation maintained reduced MCP-1. However the % reduction was not as significant as seen under normal conditions. Both classically and type II activated macrophages increased MCP-1 production in the absence of IL-10, indicating IL-10 may cause some suppression of MCP-1 either directly or through the alterations of other important cytokines. While IL-6 was only assessed for one experiment, it appeared that IL-6 production was unaffected by the blockage of IL-10.

We wished to assess whether the increased IL-10 by type II activation was essential for the observed reductions in CD40 and CD80 expression. Contrary to expectations, the blockage of IL-10 had very little effect on CD40 or CD80 expression by both classically and type II activated macrophages. Moreover, the addition of rIL-10 to cultures also had no effect on the expression of these co-stimulatory markers with classical and type II macrophages exhibiting the same response seen under normal circumstances. As a

whole, these data indicate that IL-10 alone does not play an essential role in regulating the cytokine and co-stimulatory marker profile elicited by type II activation.

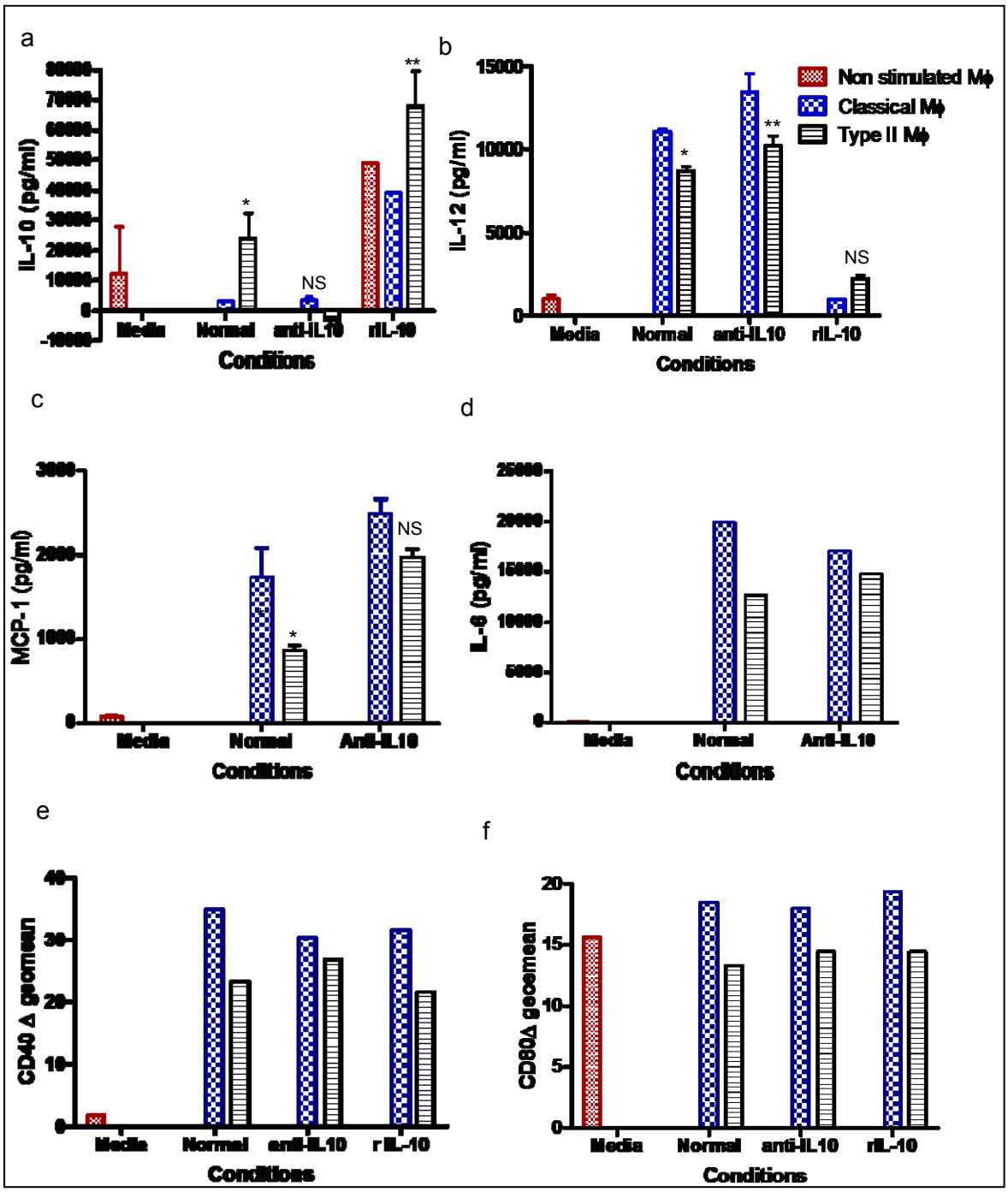


Figure 3.5 IL-10 inhibition does not alter the phenotype of type II activated macrophages. Bone marrow derived macrophages derived from C57BL/6 mice were exposed to classical and type II-inducing stimuli (Figure 3.3), with the addition of anti-IL-10 antibody (2 µg/ml) or recombinant IL-10 (5 ng/ml), or rat IgG as an isotype control (5 ng/ml). After 8 h culture IL-10 (a), IL-12 (b), MCP-1 (c) and IL-6 (d) were assessed via ELISA. *P<0.05 **P<0.01: Two way ANOVA with Bonferroni post test; Effects of classical versus type II activation under Normal conditions or IL-10 blockage. Data points (a-d) represent mean +SEM from duplicate wells. Data points in (d) represent single wells. After 24 h culture CD40 (e) and CD80 (f) were assessed via flow cytometry. Data points represent a single experiment.

3.2.4 Involvement of CD40 signalling in changes to the type II macrophage profile

CD40 ligation induces the up-regulation of inflammatory cytokines such as IL-6 and IL-12, as well as co-stimulatory markers including CD80 (Alderson *et al.*, 1993; Caux *et al.*, 1994a). Studies have shown low levels of CD40 signaling induce IL-10 and inhibit IL-12 production, while strong signaling does the opposite in altering the cytokine environment to drive pro-inflammatory responses (Mathur *et al.*, 2004). Our studies have shown that type II activated macrophages elicit an anti-inflammatory profile with significantly lower expression of CD40 compared to classically activated macrophages. Thus, we wished to determine the effect of the reduced CD40 expression on the anti-inflammatory profile by looking particularly at whether low levels of CD40 signalling were involved in perpetuating the anti-inflammatory phenotype of type II macrophages.

An agonistic anti-CD40 antibody (8 μg/ml) was added to cultures of classical and type II activated macrophages, and IL-12 and IL-10 levels were assessed 8 and 24 h following primary stimulation. Figure 3.6 illustrates the effects of additional CD40 stimulation on the expression of IL-12 and IL-10. IL-12 production by both classical and type II activated macrophages increased slightly upon additional CD40 stimulation. Addition of the isotype control had no effect on IL-12 or IL-10 production, indicating the effects were not due to non-specific binding. Although CD40 stimulation enhanced IL-12 production, the levels produced by type II activated macrophages in comparison to classical macrophages was still significantly reduced. Furthermore, IL-10 production by type II activation remained higher than levels produced by classical activation, both under normal circumstances and with the addition of the CD40 stimulatory antibody suggesting that the stimulation through CD40 did not shift the activation state of the macrophage from type II to classical or vice versa.

To identify the effects of altered CD40 signalling on the expression of co-signalling markers, CD80, MHC II and CD40 levels were assessed via flow cytometry. The expression of CD80 on type II activated macrophages remained unaltered by the addition of the stimulatory CD40 antibody, as did MHC II expression (Figure 3.6). CD40 expression on classical and type II activated macrophages was significantly reduced after the addition of stimulatory CD40 antibody. However, this reduction was most likely due to the agonistic stimulatory antibody preventing binding of the fluorescently labelled CD40 antibody used for flow cytometry. Irrespective of this, it can be seen that the levels of CD40 on type II activated macrophages remain lower than levels on classical macrophages, indicating enhanced CD40 signalling did not ablate the ability of type II activation to reduce this

expression. However, because the data presented in Figure 3.6 is from one experiment, careful considerations must be made in drawing strong conclusions before confirmation of the findings. Together, these experiments indicate that CD40 signalling in type II activated macrophages has very little effect on the anti-inflammatory profile, despite the lower levels of CD40 expression. This suggests the macrophage phenotype occurs via a different pathway other than through altered CD40 signalling in the macrophage. This conclusion does not however exclude the possible role of reduced CD40 expression and thus signalling, through CD40L on T cells in response to type II activated macrophages, and requires further investigation.

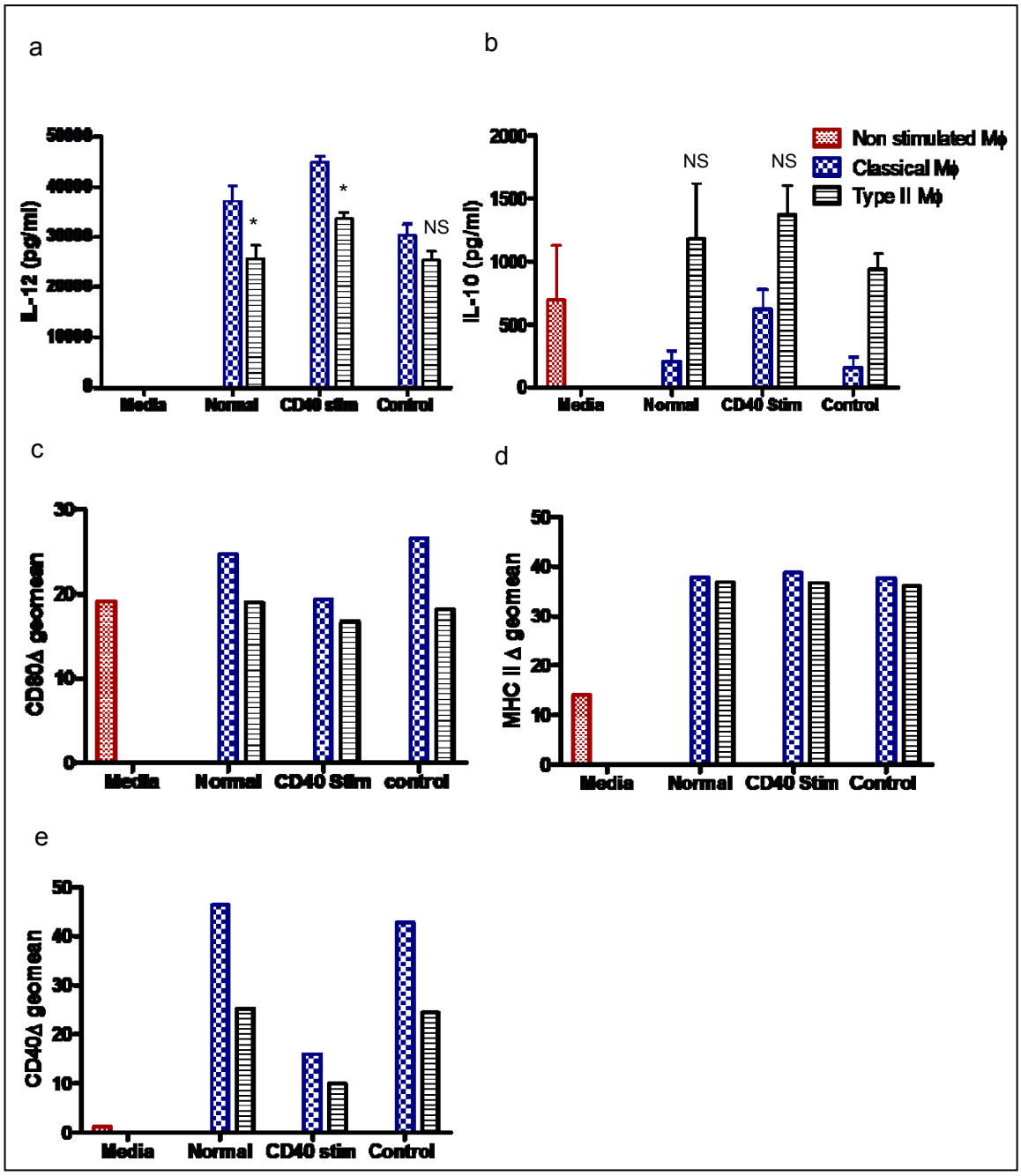


Figure 3.6 CD40 stimulation does not alter the phenotype of type II activated macrophages. Macrophages derived from C57BL/6 mice were exposed to classical and type II-inducing stimuli (Figure 3.3), with the addition of 8 µg/ml stimulatory rat-anti-mouse CD40 antibody (3/23) and 8 µg/ml IgG isotype control. After 8 h culture, IL-12 (a) and IL-10 (b) levels were accessed via ELISA. *P<0.001: Two way ANOVA with Bonferroni post test; Type II versus classical before and after CD40 stimulation. Bars represent duplicates from one experiment. After 24 h culture, CD80 (c), MHC II (d) and CD40 (e) were assessed via flow cytometry. Bars represent one experiment.

3.3 Discussion

Type II activation deviates immune responses in a manner which significantly prevents the onset of inflammatory diseases (Gerber *et al.*, 2001; Tierney *et al.*, 2009). The mechanisms involved in type II activation are currently unknown, and full characterisation of the type II macrophage phenotype has yet to be elucidated. Therefore, this thesis investigated the effects of type II activation on a broader range of characteristics including cytokines, chemokines and surface markers. Until 2006, many researchers relied heavily on the IL-10/IL-12 ratio, and the Th2 biasing effect to identify type II activation (Anderson *et al.*, 2002; Lucas *et al.*, 2005; Sutterwala *et al.*, 1997). Although there has been the identification of additional biochemical markers including enzymes and surface markers, the characterisation of this activation state is still limited. Therefore this thesis investigates the effects of type II activation on an array of markers to add to our existing repertoire of cytokines and surface markers altered by type II activation, while utilizing enzymatic profiling for further validation of this activation state.

In order to verify that our system induced type II macrophage activation, both the cytokine and enzymatic profile of primary macrophages stimulated classically with LPS was compared to those stimulated with opsonised SRBC in the presence of LPS. Classically activated macrophages stimulated with LPS, induced high production of IL-12, IL-6 and MCP-1 with little IL-10, in accordance with previous studies (Mantovani *et al.*, 2004; Mosser *et al.*, 2008). A more anti-inflammatory cytokine profile was elicited by exposure to LPS in the presence of opsonised SRBC. As seen previously seen by Gerber *et al* and Lucas *et al*, FcγR ligation via immune complexes in the presence of LPS suppressed the production of the pro-inflammatory cytokine IL-12, in parallel to increased production of the anti-inflammatory cytokine IL-10. The increase in IL-10 was specific to the ligation of FcγRs in the presence of TLR signalling via LPS, as no significant IL-10 production is seen in the presence of FcγR ligation alone (opsonised SRBC without LPS). These results support previous observations (Lucas *et al.*, 2005; Zhang *et al.*, 2006), which demonstrated the requirement for the two signals for IL-10 up-regulation.

We also investigated the effect of FcγR ligation on the response of macrophages to the production of other cytokines/mediators. We found that as with IL-12p40, FcγR ligation reduced the production of MCP-1 and IL-6 in response to LPS. The effect of type II activation on chemokine production has not been previously reported, however the effects seen on IL-6 somewhat contradict previous literature (Gerber *et al.*, 2001; Grazia Cappiello *et al.*, 2001). Capiello *et al* reported the reduction in pro-inflammatory cytokines was specific to IL-12, and not IL-6, TNFα, or IL-1β. However this conclusion came from the expression of IL-6 mRNA, not IL-6 protein secretion which we show here. It is possible

that although IL-6 mRNA is unaffected (Grazia Cappiello *et al.*, 2001), repressive effects may be occurring at the post transcriptional stages leading to reduced IL-6 secretion in response to type II activation. Additional work is required to verify whether this hypothesis is correct.

Th2 responses are associated with protection against EAE, through the counter-regulation of inflammatory immune responses (Cua *et al.*, 1995; Kennedy *et al.*, 1992; Shaw *et al.*, 1997). Although type II activation increases the production of the prototypic Th2 cytokine IL-4 by CD4 T cells (Anderson *et al.*, 2002; Tierney *et al.*, 2009), it was unknown whether macrophages themselves were a contributor to Th2 development through macrophage-derived IL-4 production. IL-4 has often been regarded as a T cell-derived cytokine with important roles in B cell proliferation/antibody production, as well as the commitment of T cells towards a Th2 subset via STAT-6 signalling (Kaplan *et al.*, 1996; Le Gros *et al.*, 1990). Until recently, it was not believed that macrophages could be a major source of IL-4. However, IL-4 production in alveolar macrophages and macrophage cell lines have been reported (Buttner *et al.*, 1997; Mukherjee *et al.*, 2009; Pouliot *et al.*, 2009), leaving open the question of whether type II macrophages can produce IL-4. Macrophages treated with LPS plus opsonised SRBC produced levels of IL-4 slightly greater than that of LPS treated macrophages; however, this increase did not reach significance suggesting that in this system, IL-4 is not produced to any great extent. Previous literature has reported the production of IL-4 by lung resident macrophages (Buttner *et al.*, 1997), in the course of fibrosis induced by irradiation. However it was not fully understood how this occurred and what the implications were. Other than IL-4 production by alveolar macrophages and the RAW264.7 cell line, no other literature to date has fully examined the ability of macrophages to produce IL-4. Therefore, the appearance of IL-4 production, albeit at a low level, by the macrophages stimulated with LPS plus opsonised SRBC, indicates the possibility of type II macrophages producing IL-4. As IL-4 is largely involved in Th2 responses and protection against EAE (Le Gros *et al.*, 1990; Shaw *et al.*, 1997), further investigations into the effects of type II macrophages in IL-4 production and IL-4 responsiveness is warranted, especially with regards to IL-10 production which has been shown to up-regulate IL-4 receptor expression (Lang *et al.*, 2002; Rückerl *et al.*, 2006), as type II macrophages are very strong inducers of IL-10.

MCP-1 (monocyte chemotactic protein-1) is a chemokine involved in the chemotaxis of inflammatory cells such as monocytes, macrophages, and lymphocytes, and exacerbates inflammatory responses which are detrimental in the context of allograft rejection or EAE (Huang *et al.*, 2001a; Lee *et al.*, 2003; O'Shea *et al.*, 2008). IL-6 is a pro-inflammatory cytokine which, when in the presence of TGFβ, promotes the development of Th17 cells which are highly pathogenic during EAE (Langrish *et al.*, 2005; Perona-Wright *et al.*, 2009;

Serada *et al.*, 2008). High levels of IL-6, IL-12, and MCP-1 are associated with the peak of disease during EAE or MS (Maimone *et al.*, 1997; McManus *et al.*, 1998). Importantly, mice lacking MCP-1 are protected against EAE, associated with a lack of macrophage recruitment to the CNS, required for the induction of inflammatory Th1 responses (Huang *et al.*, 2001a). Furthermore, inhibition of IL-6 protected mice against EAE through inhibition of Th17 development (Okuda *et al.*, 1998; Serada *et al.*, 2008). IL-12p40, through either IL-12p70 (IL-12p40/p35) or IL-23 (IL-12p40/p19) (Oppmann *et al.*, 2000), also contributes to disease, through its induction of pathogenic Th1 or Th17 cells respectively (Becher *et al.*, 2002; Cua *et al.*, 2003). Reductions in IL-12-producing macrophages in the CNS and inhibition of IL-23 protect mice from developing EAE (Cua *et al.*, 2003; Sewell *et al.*, 2003). Therefore, the reduction in these pro-inflammatory mediators (IL-12p40, IL-6 and MCP-1), may be a mechanism which contributes to the protection type II activated macrophages elicit against inflammatory illnesses (Gerber *et al.*, 2001; Tierney *et al.*, 2009). Understanding mechanisms involved in perpetuating anti-inflammatory responses by type II activated macrophages is therefore key to better understanding how to treat MS.

Edwards *et al* (2006) identified the differential expression of the enzyme sphingosine kinase 1 (SPHK1), specifically up-regulated in type II activated macrophages. We therefore underwent enzymatic profiling, comparing the effects of LPS to that of opsonised SRBC plus LPS on the production of iNOS and SPHK1. Figure 3.2 demonstrated the high expression of SPHK1 in cells stimulated with opsonised SRBC in the presence of LPS in comparison to LPS alone. Furthermore, iNOS was produced by macrophages in response to LPS, as well as LPS + opsonised SRBC, with similar levels of expression. These enzymatic profiles correlate to the enzymatic profile of type II activation, previously reported (Edwards *et al.*, 2006). Therefore both the cytokine profile and enzymatic profile from our experiments correlate with previous studies, validating the successful induction of type II macrophage activation. How these enzymes contribute to the effector functions of type II macrophages is not yet known, but given the unique profile, further work is merited.

Although it was observed that type II activation significantly alters the cytokine profile away from inflammatory responses, it was unknown which processes contributed to the anti-inflammatory phenotype. To address this, changes in cytokine and surface marker expression on type II activated macrophages were assessed over a period of time after stimulation. As IL-10 and IL-12 production show the most distinctive alterations in response to type II activation, kinetic analyses were carried out on these two cytokines in order to identify which time point the most notable changes occurred. Over a period of time from 2-24 h, type II activation maintains reduced levels of IL-12 compared to classical

macrophages, with no significant changes in the % difference over time. However, there is a significant difference in the % increase in IL-10 over time. Type II activated macrophages exhibit the most significant increase in IL-10 at 8 h post-stimulation, illustrating the optimal time point of 8 h for assessing the immune-modulating effects of type II activation on cytokine production.

In addition to cytokines, co-signalling molecules are also important for regulating the type of immune response which follows (Lenschow *et al.*, 1996). The effects of type II macrophage activation on the expression of co-signalling markers CD40, CD80, PDL-1 and MHC II was assessed via flow cytometry. As with IL-12, expression of these surface markers are up-regulated on macrophages stimulated classically with LPS and IFNγ (Edwards *et al.*, 2006; Qin *et al.*, 2006; Tierney *et al.*, 2009). In correlation with these observations, data presented in this thesis also show classically activated macrophages up-regulate these surface markers. Compared to the media controls, the increased expression of MHC II on LPS-stimulated macrophages correlates with observations by Edwards *et al* (2006). Levels of MHC II are up-regulated on antigen presenting cells such as macrophages in response to the prototypic cytokine IFNγ, and are an indicator of the ability of macrophages to induce CD4 T cell responses (Edwards *et al.*, 2006; O'Keefe *et al.*, 2001). At the 8 h time point, there is very little difference seen in the levels on type II activated macrophages compared to classical macrophages, as also demonstrated by Tierney *et al* (2009). However, this effect changes with time, as type II activation elicits reductions in MHC II in response to LPS at 24-30 h. These results contrast previous studies, which indicated MHC II levels for type II activated macrophages increased 24 h post stimulation (Edwards *et al.*, 2006). This difference may be due to slight differences in the experimental setting under which type II activation was induced, leading to differences in the timing of certain responses. Also, as this data on MHC II is from a single experiment, repeats are required to confirm this observation. Aberrant MHC II expression is associated with the induction of autoimmunities such as MS, where certain MHC II haplotypes are associated with increased risk of developing multiple sclerosis (MS) (Dyment *et al.*, 2005). High levels of MHC II are expressed in the CNS during inflammatory disorders such as EAE, and Alzheimer's, implicating their role in inflammatory immune responses (Baranzini *et al.*, 2000; Perlmutter *et al.*, 1992). In mice immunized to exhibit EAE, a deficiency in the transactivator of MHC II expression, which is involved in regulating the expression of inducible MHC II genes, resulted in protection from disease (Stuve *et al.*, 2002). This demonstrates the possible benefits of type II macrophages reducing their expression of MHC II in the context of preventing inflammation.

The expression of a co-stimulatory molecule CD80 was also assessed. CD80 is an important co-stimulatory molecule involved in T cell responses. Through the binding of CD80 to CD28 or CTLA-4 on T cells, CD80 is able to promote or inhibit the activation of CD4 T cells in collaboration with MHC II (Alegre *et al.*, 2001). Corresponding with previous observations by Tierney *et al* (2009), no significant difference in the expression of CD80 in media-treated or LPS-treated macrophages was seen 2, 4, 8, 24 and 30 h after stimulation. Again correlating with Tierney *et al*, type II activated macrophages exhibited a significant reduction in the expression of CD80. This reduction in CD80 expression could be observed not only at 8 h post stimulation (Tierney *et al.*, 2009), but also at 2, 4, 24 and 30 h post stimulation. The most significant reduction in CD80 expression was observed 24 h after stimulation. CD80, as with CD40, is an important co-stimulatory molecule which binds to CD28 on T cells, and aids in determining the outcome of the macrophage-T cell interaction. CD80 and CD40 both play synergistic roles in the induction of pro-inflammatory responses, as demonstrated in a cecal ligation by puncture (CLP) model of polymicrobial sepsis where mice deficient in CD80 exhibited a reduced rate of mortality, accompanied by reduced pro-inflammatory cytokines (Nolan *et al.*, 2009). On the other hand, wild type mice with CLP had high levels of CD80 accompanied by increased mortality. In patients with MS, increased levels of CD80 are seen at lesion sites (Windhagen *et al.*, 1995), and in EAE, high levels of CD80 have been correlated with disease progression (Karandikar *et al.*, 1998). The fact that the levels of CD80 and MHC II expression on type II activated macrophages are below that seen on classical macrophages indicates type II activation may be impairing the ability to drive T cell responses, and present antigen to T cells. This could be beneficial in the context of autoimmunities whereby a reduction in the activation of autoreactive T cells can prevent progression of disease.

PD-1 is a molecule present on T cells, which has a role in limiting T cell responses and maintaining peripheral tolerance (Nishimura *et al.*, 1999). PDL-1 is a ligand for PD-1, which is constitutively expressed on a variety of cells including macrophages, and is up-regulated in response to inflammatory stimuli (Freeman *et al.*, 2000; Yamazaki *et al.*, 2002). We investigated PDL-1 expression on type II and classically activated macrophages. Previous experiments (Tierney *et al.*, 2009) looking at an 8 h time point have demonstrated a significant reduction in PDL-1 by type II activation in comparison to classically activated macrophages. Correlating with Tierney *et al*, at 8 h post stimulation a reduction in PDL-1 on type II activated macrophages can be seen in comparison to classical activation. However, this reduction is minimal in comparison to the significant decrease exhibited 24 or 30 h post stimulation. This emphasizes the importance of looking at multiple time points when assessing the effects of type II activation on surface marker

expression. PD-1, to which PDL-1 binds to, is associated with inhibition of T cell responses through the inhibition of proliferation and cytokine production (Freeman *et al.*, 2000; Latchman *et al.*, 2001), and has been associated with the induction of peripheral tolerance where its absence has lead to the development of autoimmunites such as arthritis, lupus and cardiomyopathy (Nishimura *et al.*, 1999; Nishimura *et al.*, 2001). Due to the role of PD-1 ligation in inhibiting T cell proliferation, it was previously presumed type II activated macrophages which have reduced levels of the ligand for PD-1, would support T cell proliferation. However, in addition to inhibition of T cell proliferation, other effects of the PD-1 ligands have been noted, where their involvement in enhancing, rather than inhibiting T cell responses has been observed (Tseng *et al.*, 2001; Wang *et al.*, 2003). Furthermore PDL-1 and PDL-2 have shown differential responses to Th1/Th2 cytokines (Loke *et al.*, 2003). Therefore it is possible that the reduction in PDL-1 plays a role in inhibiting or promoting Th1/Th2 biasing. If PDL-1 was associated with Th1 biasing, as has been previously suggested as a possibility (Loke *et al.*, 2003), a reduction in PDL-1 by type II activation may be beneficial during inflammatory disease. Studies are currently underway to try and further understand the complexity of the PDL-1/PD-1 interaction and their different functions.

CD40 is an important co-signalling molecule involved in the activation of Th1 cells (Campbell *et al.*, 1996; Kelsall *et al.*, 1996). At 24-30 h where CD40 is most highly expressed in response to LPS (Figure 3.4), type II activated macrophages exhibited the most significant reduction in the expression of this co-stimulatory molecule. The observation corresponds to that previously reported at an 8 h time point (Tierney *et al.*, 2009). The level of CD40 on type II activated macrophages is above that seen on non-activated macrophages, but significantly lower than levels on classically activated macrophages. CD40 is an essential co-stimulatory molecule for the induction of Ag-specific T cell responses (Grewal *et al.*, 1998). Although CD40 is associated with cell-mediated inflammatory responses (Stout *et al.*, 1996), studies have also shown in the absence of this co-stimulatory molecule, Th2 development is impaired (MacDonald *et al.*, 2002b), illustrating the dual role of CD40 in promoting both Th1 and Th2 counter regulatory responses. CD40 ligation has also been attributed to increased production of inflammatory mediators and surface molecules such as CD80, which aids Th1 responses (Alderson *et al.*, 1993; Caux *et al.*, 1994b). Furthermore, CD40 promotes the development of pathogenic Th17 cells through the production of IL-6 (Perona-Wright *et al.*, 2009). A reduction in CD40 on type II activated macrophages may therefore promote the suppression of inflammatory mediators, thus reducing inflammatory disease. Some studies have also shown the ability of CD40 to promote anti-inflammatory or pro-inflammatory responses depending on their level of signalling (Mathur *et al.*, 2004;

Murugaiyan *et al.*, 2006). Therefore the particular low level of CD40 expressed on type II activated macrophages may be instrumental in preventing the development of inflammatory responses by Th1 and Th17 cells, while promoting anti-inflammatory Th2 responses, to provide protection against EAE. The role of CD40 requires further investigation, as does the contribution of the increased IL-10 by type II activation.

We investigated whether the increase in IL-10 is essential for the type II activation phenotype, which consists of a reduction in CD40 ,CD80, PDL-1 as well as decreased IL-12 and MCP-1. Our finding that rIL-10 addition significantly decreases IL-12 production correlates with previous studies demonstrating a direct suppressive effect of IL-10 on IL-12 (Schottelius *et al.*, 1999). IL-12 production from cells including monocytes (de Waal Malefyt *et al.*, 1991), the RAW264.7 cell line (Rahim *et al.*, 2005), as well as primary macrophages (Anderson *et al.*, 2002), are inhibited by IL-10. More importantly, in agreement with previous studies (Anderson *et al.*, 2002), the reduction in IL-12 production observed with type II activation was in no way affected by the blockage of IL-10. The ability of type II activated macrophages to reduce the production of MCP-1 and IL-6 was also largely unaffected by the absence of IL-10, again implicating an independent mechanism involved in the reduction of inflammatory cytokines by type II activation. The mechanism whereby additional IL-10 is able to inhibit IL-12, yet IL-10 is not required for the inhibition of IL-12, IL-6 or MCP-1 by type II activation, is yet to be understood.

The role of IL-10 on alterations in surface markers by type II activation was also assessed. Previous literature reported a reduction in CD40 on macrophage cell lines in response to LPS plus IL-10 (Qin *et al.*, 2006). However, this investigation found, as with the cytokine profile, reductions in the expression of co-stimulatory markers CD40 and CD80 was not dependant on IL-10, although the data on the expression of these surface markers is only from one experiment and must be interpreted with caution. However, these preliminary results are a good indicator that even though IL-10 and IL-12 alterations occur prior to changes in surface marker expression, these alterations do not solely direct reductions in the expression of surface markers and other pro-inflammatory cytokines. This indicates type II activation reduces inflammatory cytokines/surface markers through an alternative mechanism other than IL-10. It is necessary to identify these mechanisms, which may in fact be occurring in synergy with IL-10. Once these essential mechanisms are identified, it may be possible to enhance the ability of macrophages to promote anti-inflammatory responses.

Due to the dual role of CD40 previously mentioned (Mathur *et al.*, 2004), we wished to investigate if CD40 stimulation was involved in the other phenotypic properties of type II activated macrophages. We hypothesized that this reduction in CD40 by type II activation

causes a reduced level of signaling such that low levels induce the production of anti-inflammatory cytokines and suppress pro-inflammatory responses. Previous studies have demonstrated low level signaling up-regulates IL-10 in comparison to high level signaling which promotes IL-12 production by DCs (Murugaiyan *et al.*, 2006). To my knowledge, the dual role of CD40 signaling in primary macrophages from C57BL/6 mice has not been investigated. This investigation utilized an agonistic stimulatory antibody for CD40, in order to enhance signaling on type II activated macrophages. The theory being, that if the reduction in CD40 occurred with the goal to reduce signaling and elicit anti-inflammatory characteristics, by increasing CD40 signaling, the anti-inflammatory effect of type II activation will be impaired.

High levels of CD40 stimulation had no effect on the ability of type II activated macrophages to reduce the production of IL-12 and increase IL-10 in comparison to classically activated macrophages. These observations conflict those seen by Mathur *et al* (2004). Mathur *et al* demonstrated low levels of CD40 signalling with 2-4 μg/ml of anti-CD40 ab increased IL-10 in BALB/c derived peritoneal macrophages. We observed that at high levels of CD40 signalling, type II activated macrophages maintained higher levels of IL-10 and reduced IL-12 in comparison to classical macrophages. This indicates the increased production of IL-10 and reduced IL-12 occurred independently of any reductions in CD40 signalling by type II activation.

Previous studies have demonstrated enhanced expression of surface markers in response to CD40 signalling (Alderson *et al.*, 1993). Data presented in this thesis demonstrates enhanced CD40 stimulation has no effect on the expression of CD80 or MHC II by both classically activated and type II activated macrophages. Furthermore, type II activated macrophages maintained reduced CD40 levels compared to classical macrophages, as seen under normal stimulation conditions. The significant reduction in CD40 detected by both macrophage subsets is most likely due to the CD40 stimulatory antibody remaining bound to CD40 present on macrophages. This preliminary data indicates low levels of CD40 signalling is not essential for the type II macrophage phenotype, as indicated by the effects of enhanced CD40 signalling. These observations correlate to the previously described lack of effect of IL-10 alterations on the phenotype induced by type II activation. However, one explanation for differences between Mathur *et al* and this data may be that the responses by bone marrow-derived C57BL/6 macrophages and peritoneal BALB/c macrophages differ in the specific levels of CD40 which define low level and high level signalling. This possibility requires further investigation.

Although this thesis identified increased CD40 signalling did not impair the ability of type II activation to elicit an anti-inflammatory phenotype, identifying the effect of reducing CD40 expression or signalling on classical macrophages would provide more information on the importance of CD40 expression levels. This investigation identified additional effects of type II activation on cytokine/chemokine and surface marker expression in addition to illustrating that the anti-inflammatory profile occurs independently of IL-10. These findings expand our current knowledge of the type II activation state, and further emphasise the importance of understanding the mechanisms involved in promoting the phenotypic properties elicited by type II activation, as many of the characteristics are indicative of eliciting beneficial effects against inflammatory disease.

Chapter 4: Elucidating the effects of type II macrophages on T cell responses

4.1 Introduction

T cell differentiation into Th1, Th2, or other subsets is largely determined by the cytokine environment under which the T cells are stimulated. Antigen presenting cells such as macrophages and dendritic cells which produce a variety of cytokines in response to different micro-environments play a major role in determining the outcome of T cell differentiation (Stein *et al.*, 1992; Stout *et al.*, 2004). Once differentiated, the subset functions are characterised by the cytokines the T cells subsequently produce (Abbas *et al.*, 1996; Wan *et al.*, 2009). Th1 cells produce IFNγ and support cell-mediated responses whereas Th2 cells can be characterised by their production of regulatory cytokines such as IL-13, IL-5, and IL-4 (Abbas *et al.*, 1996; Wan *et al.*, 2009). Strong promoters of Th1 development are IL-12, and Th2 development IL-4 and IL-2 (Cote-Sierra *et al.*, 2004; Le Gros *et al.*, 1990; Liao *et al.*, 2008). In addition to cytokines in the environment, direct interaction between T cells and APCs through MHC:TCR and co-stimulatory molecules is essential in the development of different T cell subsets. Of particular interest is the co-stimulatory molecule CD40 ligand (CD40L) present on T cells and its' receptor CD40 on APCs (Grewal *et al.*, 1998).

CD40 signalling exhibits dual roles. It has a well documented role in promoting cell mediated immunity and driving pro-inflammatory responses which are essential for the clearance of intracellular pathogens and anti-tumour responses (Campbell *et al.*, 1996; DeKruyff *et al.*, 1997; Florido *et al.*, 2004; Stout *et al.*, 1996). It has also been reported as an essential mechanism of Th2 development (MacDonald *et al.*, 2002b). Benefits of blocking the Th1-promoting effects of CD40L signalling have been demonstrated in animal models of EAE (Girvin *et al.*, 2000; Howard *et al.*, 1999), yet in terms of Th2 development, the exact effects of CD40 signalling between T cells and macrophages is less well understood. In the absence of CD40 or CD40L as seen in knockout mice, Th2 responses are inhibited (MacDonald *et al.*, 2002a). Furthermore, studies have found that CD40 inhibition *in vivo* by administration of anti-CD40L antibodies, polarises immune responses from a Th1 dominant one into a Th2 biased response (Hancock *et al.*, 1996). This dual role of CD40 may lie in the length (Lee *et al.*, 2002), or strength of signal, where strong CD40 signalling induces Th1 dominant responses which are useful for inducing anti-tumour responses (Schoenberger *et al.*, 1998), whereas low level signalling increases the production of regulatory cytokines such as IL-10 by APCs, which promote Th2 and anti-inflammatory responses (Mathur *et al.*, 2004; Murugaiyan *et al.*, 2006). Much still remains

to be investigated in order to understand the exact mechanisms which determine the ability of CD40/CD40L interactions to drive counter-regulating Th1/Th2 immune responses.

Type II activated macrophages are capable of biasing T cell responses towards the development of Th2 cells (Anderson *et al.*, 2002; Gerber *et al.*, 2001), and mechanisms involved in this biasing need elucidation. The role of CD40 in the context of type II activation is unknown; however, recent studies have shown that type II activation reduces CD40 as well as PDL-1 expression on macrophages (Tierney *et al.*, 2009). The ability of type II activated macrophages to alter adaptive immune responses has beneficial consequences in the protection from inflammatory illnesses such as endotoxemia (Gerber *et al.*, 2001) and EAE (Tierney *et al.*, 2009), and the type of interaction which occurs between T cells and macrophages can determine whether a protective Th2 response occurs (Cua *et al.*, 1995), or pathology-inducing Th1/Th17 response ensues (Becher *et al.*, 2002; Langrish *et al.*, 2005).Therefore, it is important to understand the different mechanisms involved in regulating the outcome of T cell subset differentiation.

This thesis investigates the mechanisms involved in Th2 biasing by type II activated macrophages with the long term goal of understanding mechanisms that may lead to protection against pro-inflammatory diseases.

Aims:

The ability of type II activated macrophages to induce Th2 cell biasing has previously been reported (Anderson *et al.*, 2002; Gerber *et al.*, 2001), and has been shown to promote protection from EAE (Tierney *et al.*, 2009). However, the mechanisms by which type II activated macrophages drive such responses are still unclear. Both Th1 and Th2 responses have demonstrated a dependency of CD40/CD40L signalling (Campbell *et al.*, 1996; MacDonald *et al.*, 2002a). Other studies demonstrate enhanced Th2 development by CD40L inhibition (Hancock *et al.*, 1996). These contradictory observations may be due to differences in the level of CD40 inhibition, whereby a particular low level of inhibition elicits Th2 polarisation, which is otherwise inhibited in the complete absence of CD40L. This idea is supported by studies that show different levels of CD40 signalling on dendritic cells and macrophage cell lines, promote counter-regulatory Th1/Th2 responses both *in vitro* and *in vivo* (Mathur *et al.*, 2004; Murugaiyan *et al.*, 2006).

As discussed in chapter 3, type II activated macrophages exhibit significant reductions in the expression of CD40 and PDL-1, while still being a potent inducer of Th2 responses (Anderson *et al.*, 2002). The goal of this thesis was to investigate mechanisms involved in T cell biasing by type II activated macrophages. To further understand mechanisms

involved in promoting Th2 development, we examined how altering the level of signalling between CD40 and CD40L influences Th (T helper) cell subset differentiation. In particular, we addressed three specific aims:

1. To compare the effects of type II activation and classical activation on T cell proliferation and activation, given the reduced expression of PDL-1 on type II macrophages.
2. To determine if low levels of CD40 signalling promote the generation of Th2 cells.
3. To investigate whether Th2 cell biasing can be enhanced by reducing the CD40/CD40L interaction.

4.2 Results

4.2.1 Effects type II activation has on CD4 T cell proliferation

In Chapter 3, the difference in the phenotype of type II and classically activated macrophages was investigated. As with previous studies (Tierney *et al.*, 2009), a significant reduction in the expression of several co-stimulatory molecules including CD40 and PDL-1 expression was observed on type II activated macrophages. PDL-1 has been associated with the induction of T cell anergy, as well as T cell stimulation (Keir *et al.*, 2006; Latchman *et al.*, 2001; Tseng *et al.*, 2001). Given that a reduction in PDL-1 would be expected to enhance T cell proliferation, investigations were carried out to assess if T cell proliferation was altered in our experimental system, given the observed reduction in PDL-1 on type II activated macrophages.

To investigate T cell subset differentiation, we used naïve T cells isolated from OTII mice whose T cells express a transgenic TCR specific for $OVA_{323-339}$ peptide in the context of MHC II. Macrophages were non-activated or were classically or type II activated as previously described (sec 2.2). Purified CD4 OTII cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) and added to the macrophage cultures 2-3 h post-stimulation, either in the absence or presence of OVA protein or $OVA_{323-339}$ peptide. 72 h following co-culture, T cells were removed and assessed by flow cytometry for their degree of proliferation as determined by CFSE dilution. Because CFSE is a cytoplasmic dye which is split evenly between daughter cells upon mitosis, the reduction in the fluorescence of cells correlates to the level of proliferation that has occurred (Lyons *et al.*, 1994).

All macrophages were able to present antigen and activate T cell proliferation (Figure 4.1). T cells exposed to either classical or type II activated macrophages presenting OVA protein or $OVA_{323-339}$ peptide exhibited similar levels of antigen-specific proliferation (Figure 4.1). The response to the different antigen forms were similar, except that $OVA_{323-339}$ peptide was more antigenic and induced greater T cell proliferation. T cells exposed to non-stimulated macrophages presenting $OVA_{323-339}$ peptide had similar levels of antigen-specific proliferation as those induced by classical and type II activation; however, no proliferation was exhibited in response to non-stimulated macrophages that presented OVA protein. Only background proliferation was detected in co-cultures of T cells and macrophages containing only medium. Therefore, T cell proliferation in response to antigen presented by macrophages was similar irrespective of whether they were classical or type II activated.

IL-2 cytokine production was also assessed (Figure 4.2a), as another indicator of a T cell activation. T cells cultured alone did not produce significant amounts of IL-2, nor did T cells cultured with macrophages in the absence of antigen. In contrast, T cells exposed to macrophages in the presence of antigen, showed significant production of IL-2, and T cells cultured in the presence of non-stimulated macrophages had the highest amounts of IL-2 detected in their supernatant. In comparison, T cells exposed to classical macrophages showed lower levels of IL-2, and T cells cultured with type II activated macrophages had the lowest levels of antigen-specific IL-2 detected in the supernatant. These results may suggest that less IL-2 is produced by T cells stimulated by type II activated macrophages; however, increased reuptake of IL-2 may be another explanation. This possibility was further explored by assessing the expression of IL-2Rα on T cells in these cultures. Interestingly the expression of this high affinity IL-2 receptor is increased and may account for increased reuptake of IL-2 (Figure 4.2b).

Overall, this data suggests type II activated macrophages elicit similar proliferative responses by CD4 T cells to that induced by classically activated macrophages.

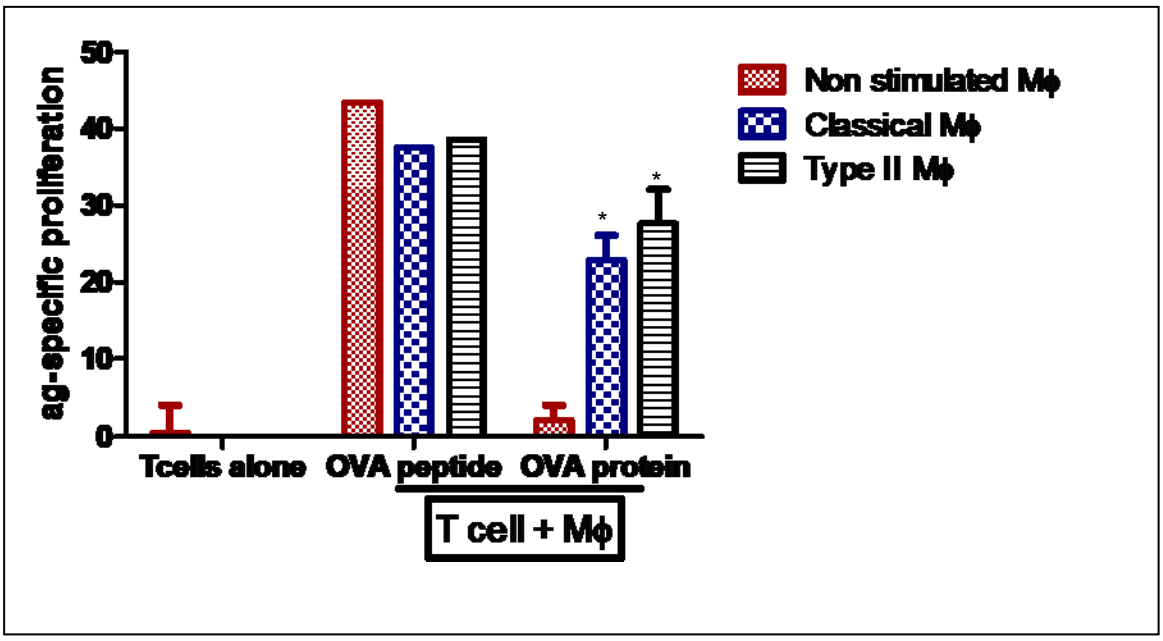


Figure 4.1 Type II and classical activation support similar levels of antigen-specific T cell proliferation. Macrophages (10^6 cells/ml) were exposed to classical or type II activating stimuli. CD4 T cells were isolated from OTII mice (sec 2.2) and stained with CFSE. CD4 T cells ($2.5x10^6$ cells/ml) plus 1 μM $OVA_{323-339}$ peptide or 150 μg/ml OVA protein were added to macrophage cultures 3 h after classical or type II activation. After 72 h culture, CFSE was assessed by flow cytometry and presented as relative proliferation compared to cultures without antigen. The data from two experiments is shown. Note: Bars for 'OVA peptide' represent one experiment. *P<0.05: Two way ANOVA with Bonferroni post-test; Responses to macrophage states with antigen present versus antigen absent. Bars represent data points (mean+SEM) of two experiments. Relative proliferation = % decrease in CFSE of co-culture conditions in the presence of antigen relative to conditions in the absence of antigen.

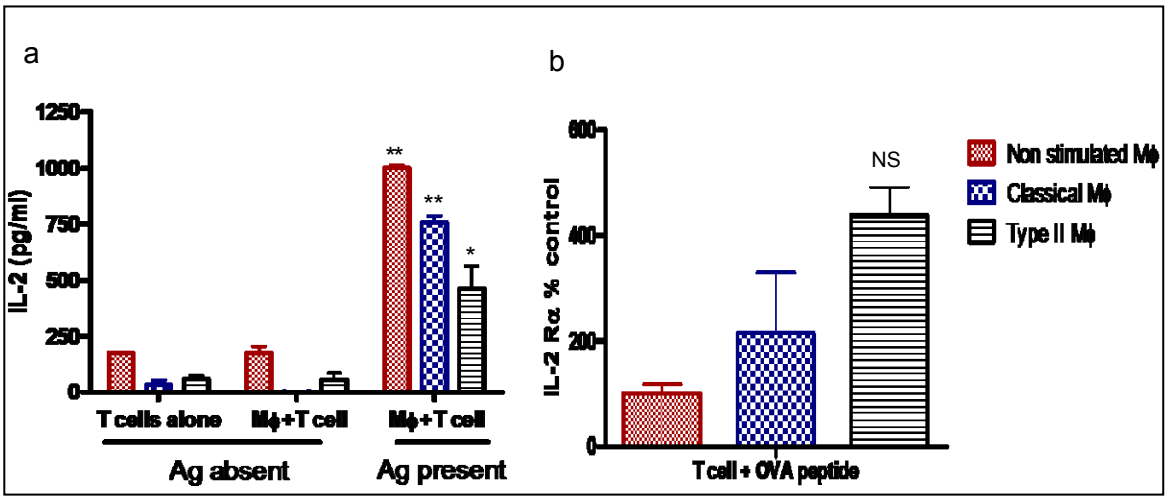


Figure 4.2 Type II and classical activation induce different levels of antigen-specific IL-2 production. Macrophages (10^6 cells/ml) were co-cultured with CD4 T cells ($2.5x10^6$ cells/ml) as described in Figure 4.1. After 72 h culture, IL-2 was assessed via ELISA (a), and IL-2Rα was assessed via flow cytometry (b), and presented as % of control compared to cultures with non-activated macrophages. The data from two experiments is shown. *P<0.01, **P<0.001: Two way ANOVA with Bonferroni post-test; (a) Responses to macrophage states with antigen present vs antigen absent. (b) P> 0.05: One way ANOVA; responses to type II versus classical MØ. Bars represent data points (mean+SEM) from duplicate wells of two experiments.

4.2.2 Effect of type II activation on CD4 T cell biasing

Previous studies have identified the ability of type II activated macrophages to alter adaptive immune responses through the polarisation of a pro-inflammatory Th1 response into a Th2 dominant response (Anderson *et al.*, 2002; Gerber *et al.*, 2001; La Flamme *et al.*, 2006; Tierney *et al.*, 2009). In order to identify ways of enhancing Th2 biasing, we first needed to understand the effects of type II activated macrophages on naive CD4 T cells in the context of our experimental system. To this end, naïve CD4 T cells from OTII mice were cultured with either non-stimulated, classically activated or type II activated macrophages in the presence of $OVA_{323-339}$ peptide. Cultures were maintained for 72 h, after which IFNγ and IL-4 were analysed via ELISA and IL-4Rα and IL-2Rα (CD25) expression were assessed via flow cytometry.

Figure 4.3 demonstrates that similar levels of IFNγ were detected by T cells cultured with non-stimulated or classically activated macrophages (a). In comparison, IFNγ production by CD4 T cells exposed to type II activated macrophages was significantly reduced, indicating a biasing against differentiation into IFNγ-producing Th1 cells. Although IL-4 production is the best marker of Th2 cells, only very low levels of IL-4 were detected in the cultures and no difference between the presence of the different macrophage activation states was found. These results confirm that classically activated macrophages and not type II activated macrophages promote Th1 differentiation.

As another measure of Th2 biasing, we investigated the responsiveness of T cells to IL-4 by measuring the expression level of its receptor IL-4Rα (b). T cells exposed to non-stimulated and classically activated macrophages had much lower levels of IL-4Rα expression in comparison to those exposed to type II activated macrophages, suggesting that type II macrophages induce T cells to become more responsive to this prototypic Th2 cytokine. Additionally, IL-2Rα was highly expressed on T cells exposed to type II activated macrophages (Figure 4.2). While IL-2Rα expression on T cells was also up-regulated in response to classically activated macrophages, the levels were below those of T cells exposed to type II activated macrophages. These results indicate that type II activation increases CD4 T cell responsiveness to IL-4 and IL-2 and thus may promote the differentiation of these cells to the Th2 phenotype.

As previously seen in other systems (Anderson *et al.*, 2002; Edwards *et al.*, 2006; Gerber *et al.*, 2001), the findings presented here indicate type II activated macrophages are capable of inducing Th2 cell biasing as identified by increased responsiveness to IL-4Rα and IL-2Rα as well as reduced production of IFNγ. Investigations are still underway to identify the exact role of IL-2 in Th1/Th2 biasing, but our results further support the notion that IL-2Rα expression in addition to other factors such as IL-4Rα, have an active role in

inducing Th2 biasing (Cote-Sierra *et al.*, 2004; Liao *et al.*, 2008). As IL-4 cytokine production cannot be reliably detected due to the low levels at the time points tested, the use of IL-2Rα expression in conjunction with other markers makes it possible to carry out further experiments elucidating mechanisms involved in T cell biasing by type II activated macrophages.

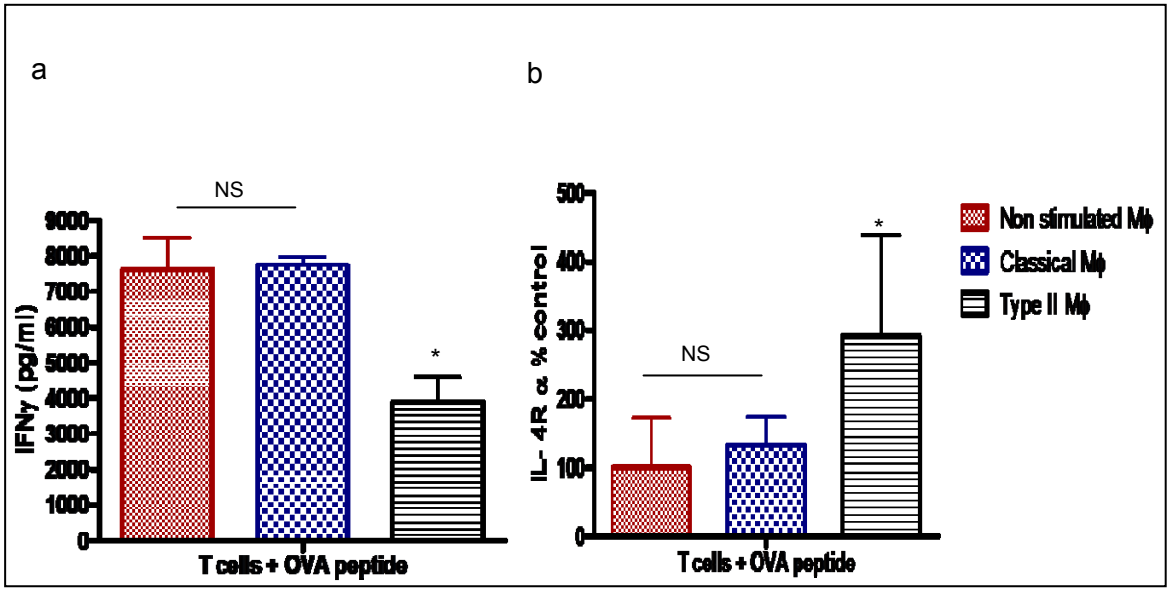


Figure 4.3 Type II activation reduces Th1 while increasing Th2 responsiveness. Macrophages (10^6 cells/ml) were co-cultured with CD4 T cells ($2.5x10^6$ cells/ml) as mentioned in Figure 4.1. After 72 h culture, IFNγ was assessed via ELISA (a), and IL-4Rα was assessed via flow cytometry (b) and presented as % of control. * $P<0.05$: One way ANOVA with Bonferroni post test; Antigen specific responses to type II versus classical MØ. Bars represent data points (mean+SEM) from two experiments.

4.2.3 Role of CD40/CD40L interactions during CD4 T cell biasing

Studies have demonstrated the dual effects of CD40 on dendritic cells and macrophage cell lines, in perpetuating both pro-inflammatory and anti-inflammatory responses depending on the level of CD40 signaling (Murugaiyan *et al.*, 2006). We previously demonstrated type II activated macrophages have reduced levels of CD40, suggesting that the reduced interaction between CD40/CD40L may have a possible role in Th2 cell biasing. In order to identify mechanisms of enhancing Th2 biasing in response to classical and type II activated macrophages, we investigated the effect of reducing the CD40/CD40L interaction between CD4 T cells and macrophages.

IFNγ production by T cells exposed to classical and non-activated macrophages is equal, whereas T cells exposed to type II activated macrophages have a significant reduction in IFNγ (Figure 4.4). This finding indicates there are less developing Th1 cells in cultures with type II activated macrophages. Upon addition of 0.1-2 μg/ml inhibitory CD40L antibody (MR1), IFNγ production is increased under all conditions of macrophage activation (Figure 4.4). At higher levels of the inhibitory antibody (4-8 μg/ml), IFNγ production is decreased under all macrophage activation conditions (a), such that levels are lower than those seen in the absence of CD40/CD40L inhibition. Comparing the effect of CD40/CD40L inhibition on the relative reduction in IFNγ induced by type II macrophages in comparison to classical macrophages, no significant effect can be seen (b). However, with increasing concentrations of inhibitory antibody, T cells exposed to type II activated macrophages maintain reduced IFNγ production compared to exposure to classically activated macrophages. This indicates the ability of type II activated macrophages to reduce the development of Th1 cells independently of interactions between CD40 and CD40L.

The effects of CD40/CD40L inhibition on the expression of Th2-associated receptors were also assessed. As shown previously (Figure 4.3), IL-4Rα expression on CD4 T cells in response to classical macrophages is similar to levels in response to non-stimulated macrophages. Furthermore, the level of IL-4Rα expression in response to classically activated macrophages remains unchanged irrespective of CD40/CD40L inhibition (Figure 4.5). IL-4Rα expression in response to type II macrophages is much higher than other culture conditions, but while 2 μg/ml of inhibitory antibody had no real effect, 4-8 μg/ml of inhibitory antibody reduced the expression of IL-4Rα on T cells. IL-2Rα expression, which is normally high in response to type II activation, was similarly reduced in the presence of 0.5-8 μg/ml of inhibitory CD40L antibody (Figure 4.5). At 8 μg/ml of antibody, IL-2Rα expression on T cells exposed to type II activated macrophages decreased to levels expressed in response to classical and non-stimulated macrophages.

IL-10 production was assessed in response to CD40/CD40L inhibition. Anti-CD40L antibody at 0.1 μg/ml had no effect on IL-10 in response to type II or classically activated macrophages (Figure 4.6), although IL-10 production in co-cultures with type II activated macrophages was much higher than co-cultures with classically activated macrophages. At 0.5 μg/ml a slight decrease in IL-10 was seen; however at 2-8 μg/ml, the levels of IL-10 in response to type II activated macrophages were similar to the levels seen prior to inhibition. At 2-8 μg/ml, in an almost dose dependant fashion, IL-10 production in response to classically activated macrophages significantly increased compared to IL-10 produced in the absence of CD40/CD40L inhibition. This illustrates a possible suppressive effect of CD40/CD40L signaling on IL-10 production. In contrast, IL-12 production remained unchanged by CD40/CD40L inhibition (Figure 4.6), and co-cultures with type II activated macrophages had far lower levels of IL-12 compared to co-cultures with classically activated macrophages. Taken together, these studies demonstrate a necessary role for CD40/CD40L signaling in the induction of Th2 responses by type II activated macrophages, as previously described in dendritic cells (MacDonald *et al.*, 2002a) and found that type II activated macrophages are able to depress Th1 responses irrespective of the level of CD40/CD40L signaling. Also illustrated in this investigation is the suppressive effect of CD40/CD40L signaling on IL-10 production in response to inflammatory stimuli, presenting a new avenue of investigation.

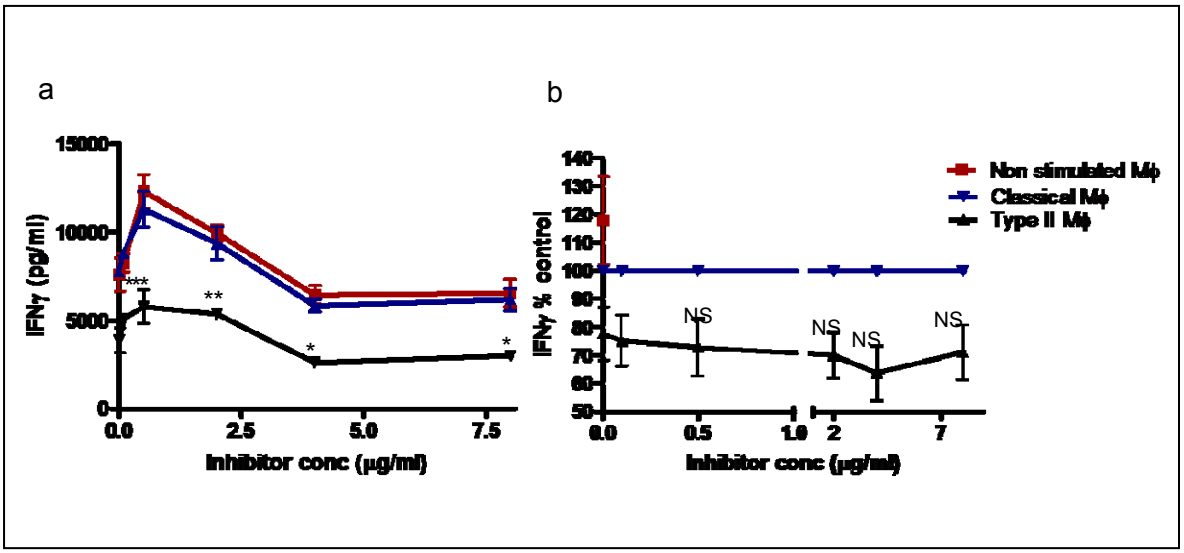


Figure 4.4 Type II macrophages reduce Th1 development independant of CD40/CD40L interactions. Macrophages (10^6 cells/ml) were exposed to classical or type II inducing stimuli. CD4 T cells were isolated from OTII mice (sec 2.2.4) and stained with CFSE. CD4 T cells ($2.5x10^6$ cells/ml) plus 1 µM $OVA_{323-339}$ peptide was added to macrophage cultures, along with varying concentrations (0.1 - 8 µg/ml) of anti-mouse CD40 ligand antibody (MR1) 3 h after classical or type II stimulation. After 72 h culture, IFNγ was assessed via ELISA. *P<0.05 **P<0.01 ***P<0.001: Two way ANOVA with Bonferroni post test ; Responses to Type II versus Classical MØ. (a) Represents data points (mean + SEM) from duplicate wells of one experiment. (b) Represents combined datapoints (mean + SEM) from two experiments presented as % of control compared to cultures with classical macrophages.

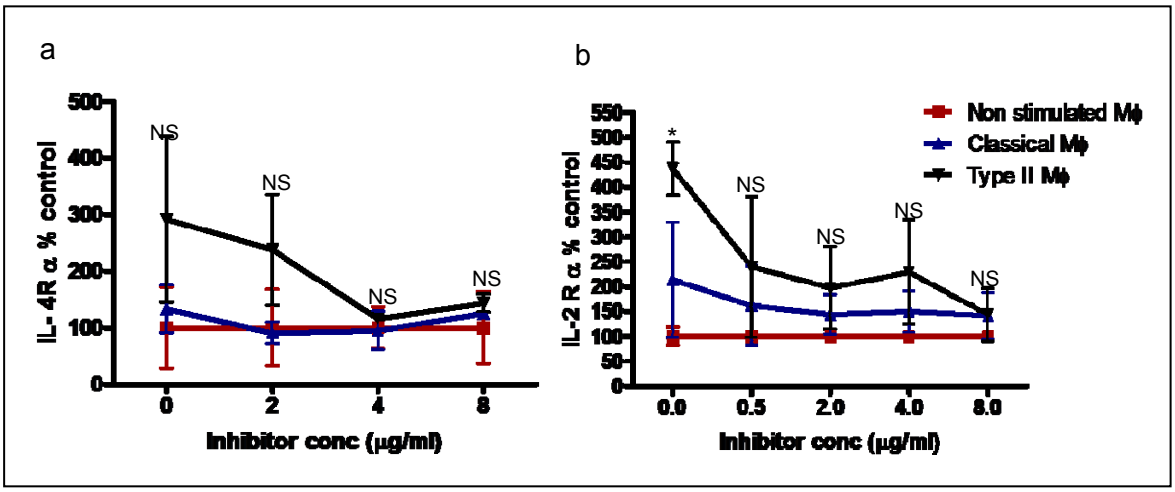


Figure 4.5 CD40/CD40L inhibition reduces Th2 responsiveness by type II macrophages. Macrophages were co-cultured with CD4 T cells as mentioned in Figure 4.4. After 72 h culture IL-4Rα (a) and IL-2Rα (b) were assessed via flow cytometry. *P<0.05: Two way ANOVA with Bonferroni post test; Responses to Type II versus Classical MØ. Graphs represent data points (mean + SEM) from two experiments presented as % of control compared to cultures with classical macrophages.

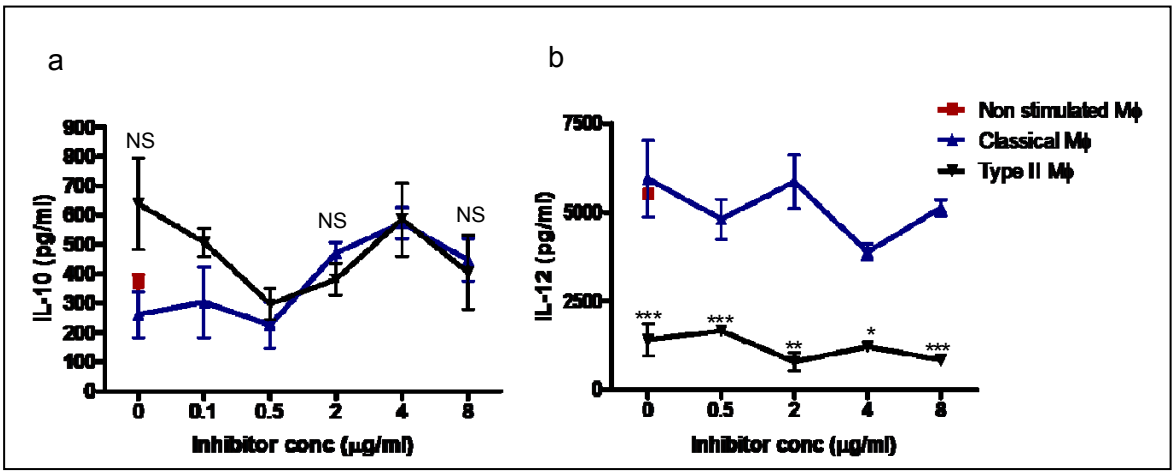


Figure 4.6 CD40/CD40L inhibition increases IL-10 in response to classical macrophages. Macrophages were co-cultured with CD4 T cells as mentioned in Figure 4.4. After 72 h culture, IL-10 (a) and IL-12 (b) were assessed via ELISA. *P<0.05 **P<0.01 ***P<0.001: Two way ANOVA with Bonferroni post test: Responses to Type II versus Classical MØ. Graphs represent data points (mean + SEM) from two experiments, presented as % of control compared to cultures with classical macrophages.

The red dot in (4.4b) and (4.6a-b) represent the average level of cytokines in co-cultures with non-stimulated macrophages. [see appendix A for complete graphs]

4.3 Discussion

Earlier investigations found that signalling through CD40 and IL-10 did not play an important role in defining the phenotypic characteristics of type II macrophages (Chapter 3). However, these experiments did not assess the effects of the CD40/CD40L interaction between macrophages and CD4 T cells in co-cultures. CD40L signalling on CD4 T cells is essential for promoting an appropriate response to antigen (Grewal *et al.*, 1998). In recent years, it has come to light that CD40L is not only essential for the induction of inflammatory Th1 responses (Alderson *et al.*, 1993; Florido *et al.*, 2004), but also in the development of Th2 responses (MacDonald *et al.*, 2002a; Poudrier *et al.*, 1998), whereby a deficiency in CD40L results in an inability to elicit protective Th2 responses during infection with a parasitic helminth. These different observations on the effect of CD40L inhibition on T cell responses may correspond to the dual ability of macrophages to perpetuate pro-inflammatory and anti-inflammatory responses (Mathur *et al.*, 2004). Type II activated macrophages protect against EAE with Th2 biasing implicated in the protective effects (Tierney *et al.*, 2009). Because CD40 levels are significantly reduced on type II activated macrophages, it is possible that type II activated macrophages have low level CD40/CD40L interactions with T cells, which influences T cell biasing (La Flamme *et al.*, 2006; Tierney *et al.*, 2009). Therefore, identifying the role of the reductions in CD40 on T cell biasing may enhance our understanding of how type II activated macrophages protect against pro-inflammatory diseases.

In order to further investigate mechanisms involved in T cell biasing by type II activation, studies were carried out to elucidate the effects of type II activated macrophages on naive T cell function (e.g. proliferation, cytokine production) upon first antigenic exposure. A previous study demonstrated that type II activated macrophages had a significantly reduced expression of PDL-1 (Tierney *et al.*, 2009). This co-signalling molecule has been associated with T cell suppression through its interaction with PD-1 (Keir *et al.*, 2006), but has also been implicated in T cell stimulation under certain conditions (Tseng *et al.*, 2001), thus making it difficult to predict the role of PDL-1 reductions by type II activation. As type II activated macrophages exhibit a significant reduction in this molecule in comparison to classical activation, it was suggested by Edwards *et al* (2006) and Tierney *et al* (2009) that type II activation may promote greater T cell proliferation than classical activation. However, we found that CD4 T cells exposed to classical and type II activated macrophages proliferated similarly in response to OVA peptide or protein (antigens). This finding conflicts with those of Edwards *et al* where a slight increase in proliferation in response to the type II activated macrophages was reported (Edwards *et al.*, 2006).

One possible explanation for the difference may be in the experimental systems used. Edwards *et al* used IgG-coated OVA as the immune complex and used T cells from the

DO11.10 TCR transgenic mouse line while our system utilized IgG-coated SRBC as the immune complex and T cells from OTII mice. Although both recognise the same core amino acid within OVA protein, it is possible that differences in other TCR contact residues between the two cells resulted in different responses (Robertson *et al.*, 2000). Additionally, Edwards *et al* reported enhanced T cell proliferation to type II activation, however as PDL-1 was not the focus of their investigation, levels were not assessed. Thus, no direct correlation between PDL-1 and proliferation was discussed.

This investigation indicates that the significant reduction observed in PDL-1 expression by type II activation does not lead to enhanced CD4 T cell proliferation over that observed with classically activated macrophages. This finding could suggest a role of PDL-1 other than its inhibitory functions on T cells. Such a role may include T cell activation and/or T cell biasing. In support of this idea, previous literature demonstrate effects of PDL-1 that are unrelated in their anti-proliferative role (Loke *et al.*, 2003; Tseng *et al.*, 2001; Zhu *et al.*, 2006). Using different murine models of EAE, Zhu *et al* (2006) demonstrated instances in which PDL-1 blockage had no effect on disease progression, irrespective of the reported role of PDL-1 in limiting auto-reactive responses (Keir *et al.*, 2006). Furthermore, Loke *et al* (2003) illustrated the differential regulation of PDL-1 and PDL-2 by counter-regulatory Th1/Th2 responses respectively, implicating a possible role for the different ligands in the counter-regulatory T cell responses. Therefore, the observed reductions in PDL-1 on type II activated macrophages may serve a role in Th1/Th2 cell biasing as opposed to T cell proliferation.

Prior to carrying out further investigations into the mechanisms involved in T cell biasing, another goal was to validate the induction of T cell biasing by type II activation using our system. This investigation also set out to identify ways of assessing T cell biasing for future investigations using the same system.

IFNγ is a prototypic cytokine produced by Th1 cells (Abbas *et al.*, 1996; Szabo *et al.*, 2000), involved in the induction of Th1 responses which aid the activation of anti-microbial, pro-inflammatory responses by macrophages and surrounding T cells (Alderton *et al.*, 2001; Campbell *et al.*, 1996; Li *et al.*, 1997; MacMicking *et al.*, 1997). The prototypic cytokine for developing Th2 cells is IL-4 (Abbas *et al.*, 1996; Kaplan *et al.*, 1996; Le Gros *et al.*, 1990; Seder *et al.*, 1992). Our studies showed that T cells exposed to either non activated or classically activated macrophages produce high amounts of IFNγ, indicative of Th1 cell biasing. In contrast, T cells exposed to type II activated macrophages produced significantly less IFNγ compared T cells cultured with classically activated macrophages. This reduction in IFNγ-producing cells is indicative of a reduction in Th1

development in response to type II macrophage activation, corroborating with previous literature (Gerber *et al.*, 2001; La Flamme *et al.*, 2006).

Although IL-4 is the best marker for Th2 polarization, only very low levels of IL-4 were detected in the T cell cultures. These low levels of IL-4 may be due to the long time IL-4 takes to be produced by T cells (Bird *et al.*, 1998), along with re-uptake of IL-4 through the IL-4 receptor on T cells. As we were unable to assess Th2 development by IL-4 detection, the effects of type II activated macrophages on the expression of surface markers associated with developing Th2 cells was assessed. IL-4Rα as well as IL-2Rα are implicated in increasing the responsiveness of CD4 T cells to Th2-inducing cytokines. Signalling through IL-2Rα via the STAT-5 signalling molecule is essential in the expression of IL-4Rα (Liao *et al.*, 2008), as well as the production of IL-4 in CD4 T cells (Cote-Sierra *et al.*, 2004). In this investigation, very little IL-4Rα was expressed on cells exposed to non-activated or classically activated macrophages. In contrast, IL-4Rα expression was up-regulated on T cells exposed to type II activated macrophages, and this increase in IL-4Rα corresponded to an increase in IL-2Rα expression, further supporting the relationship between IL-4Rα and IL-2Rα expression. Although type II activation has previously demonstrated the increase in IL-2Rα (Edwards *et al.*, 2006), this is the first demonstration of such a large increase in IL-4Rα by type II activation. Furthermore, previous studies have not assessed the involvement of IL-2Rα expression in T cell biasing as opposed to being used as an indicator of general activation by type II activated macrophages. Although much remains to be investigated to fully elucidate a direct correlation in the setting of type II activation, previous reports have demonstrated a direct link between IL-2Rα, IL-4Rα and increased IL-4 responsiveness (Cote-Sierra *et al.*, 2004; Liao *et al.*, 2008). This suggests type II activation increases the responsiveness of CD4 T cells to Th2-inducing cytokines, while reducing the development of IFNγ-producing Th1 cells. This Th2 cell biasing corresponds with previous observations (Anderson *et al.*, 2002; Tierney *et al.*, 2009), and validates the use of IL-4Rα as well as IL-2Rα in conjunction with cytokine profiles, to identify Th2 cells.

Although data presented on IL-2Rα expression is preliminary, it warrants further investigation in light of recent literature supporting a role of IL-2 in many regulatory responses not associated with proliferation. This project has not yet investigated the effects of type II activated macrophages on regulatory T cell induction which is also associated with increased IL-2 responsiveness (Pandiyan *et al.*, 2007); however, the lack of inhibition on T cell proliferation supports the conclusion that Tregs are not the dominant subset induced. Further studies looking at FoxP3, a widely used marker of Tregs, is required to ascertain the exact involvement of inducible Tregs (iTregs) in protection mediated by Type II activated macrophages (Roncarolo *et al.*, 2008), as it is possible that

the cytokine environment and phenotype induced by type II activation supports the development of Th2 as well as iTregs, both of which are associated with protection against EAE.

Th1 cells are involved in promoting pathological processes during EAE (Becher *et al.*, 2002), with administration of the Th1 cytokine IFNγ resulting in exacerbations of MS , and levels of IFNγ detected at the peak of disease (Begolka *et al.*, 1998). Therefore the reduction in IFNγ demonstrated by type II activated macrophages is a contributing factor to the prevention of Th-1 mediated disease. Th2 responses are associated with protection from inflammatory diseases such as EAE (Cua *et al.*, 1995; Shaw *et al.*, 1997; Weber *et al.*, 2007), and the protection type II activation provides against EAE is associated with Th2 polarisation (Tierney *et al.*, 2009). Treatments which enhance Th2 development, such as glatiramer acetate (GA), are also beneficial in the treatment of MS (Weber *et al.*, 2007). Therefore, as well as the ability of type II activated macrophages to reduce inflammatory responses by macrophages, the reduction in Th1 T cell responses and increase in Th2 T cell responses elicited by type II activation presents beneficial effects against inflammation. Given that these findings demonstrate the ability of type II activated macrophages to alter T cell biasing, the next step is to identify mechanisms involved in promoting this effect.

This thesis focused on the role of CD40, as a role for CD40 in promoting both Th1 and Th2 responses has been reported (Becher *et al.*, 2000; MacDonald *et al.*, 2002a; MacDonald *et al.*, 2002c). Because CD40 expression is significantly reduced on type II activated macrophages, we set forth to investigate the effect of altered levels of CD40/CD40L signalling on T cell biasing. The different levels of CD40/CD40L signalling were regulated by titrating the concentration of anti-CD40L blocking antibody. Blockage between CD40 and CD40L resulted in reduced general responses of T cells as exhibited by the reduced levels of IFNγ detected in all co-cultures. This reduction agrees with previous literature emphasizing the requirement for CD40 signaling to elicit IFNγ production by Th1 cells (Alderson *et al.*, 1993; Campbell *et al.*, 1996). However, contradictory to this is the lack of effect of full CD40/CD40L inhibition on the production of IL-12. Additionally, co-cultures with type II activated macrophages maintained significantly lower levels of IL-12 compared to classically activated macrophages, and their general production of IL-12 in response to either type II or classical activation remained unaltered irrespective of the level of CD40 inhibition. This finding corresponds to the previous observation where alterations in CD40 signaling did not affect the cytokine profiles of type II activated macrophages, but contradicts studies which found that CD40/CD40L signaling was essential for antigen-specific IL-12 production derived from activated macrophages, dendritic cells and microglia (Becher *et al.*, 2000; DeKruyff *et al.*, 1997; Kelsall *et al.*,

1996). One possible reason for the lack of alteration in IL-12 production by CD40/CD40L inhibition, is that IL-12 production in the presence of a strong Th1-inducing stimulus such as LPS, occurs independent of CD40 ligation (Reis e Sousa *et al.*, 1997). As LPS is involved in the induction of both type II and classical activation, this explanation has merit.

The ability of type II activated macrophages to reduce IFNγ-producing Th1 cells is maintained irrespective of full inhibition of CD40/CD40L interactions, indicating this reduction occurs independently of the CD40/CD40L interaction, as does the reduction in IL-12 production. This result illustrates a possible relationship whereby the ability of type II activated macrophages to maintain reduced IL-12 production is associated with the maintained reduction of Th1 development. In addition to Th17 cells, Th1 cells constitute an important cell type involved in the pathogenesis of EAE (Becher *et al.*, 2002; Langrish *et al.*, 2005). Since IL-12 is important in the development of IFNγ-producing Th1 cells (Howard *et al.*, 1999; Kelsall *et al.*, 1996; Seder *et al.*, 1996), the maintained reduction of this cytokine by type II activation may be key to inhibiting Th1 development and promoting protective effects against EAE.

Even though the reduction in Th1 development mediated by type II activation occurs independently of CD40/CD40L, we wished to identify whether this interaction was essential for Th2 biasing by type II activated macrophages, as seen previously by dendritic cells (MacDonald *et al.*, 2002c). With 2 μg/ml of inhibitory CD40L antibody, there is a slight but non-significant reduction in the expression of IL-4Rα on T cells exposed to type II activated macrophages. Under these conditions, these cells still exhibit much higher levels of expression compared to T cells exposed to non-activated or classically activated macrophages indicating low level inhibition of CD40/CD40L still enables Th2 biasing by type II activation while maintaining the inhibitory effect on Th1 development. However 4-8 μg/ml of inhibitory antibody reduces the expression of IL-4Rα expression in response to type II activation, to levels detected on T cells exposed to non-activated macrophages. A similar effect can be seen with the expression of IL-2Rα, which dramatically decreases upon CD40/CD40L inhibition. At full inhibition of the CD40/CD40L interaction (8 μg/ml), a complete reduction in the expression of IL-2Rα can be seen, illustrating the dependency of Th2 responsiveness on CD40/CD40L signaling. The lack of effect low levels of CD40/CD40L inhibition have on IL-4 responsiveness suggests a possible explanation for the induction of Th2 polarization upon administration of anti-CD40L antibody reported by Hancock *et al* (2006). Although this is not the first report of a requirement for CD40L signaling in Th2 development (MacDonald *et al.*, 2002a), it is the first study to demonstrate the requirement for this interaction in Th2 biasing by type II activation.

Our previous studies indicated IL-10 derived from macrophages did not have a significant effect on the macrophage phenotype elicited by type II activation. However, IL-10 derived from macrophages and T cells can be different both in their regulation (Shoemaker *et al.*, 2006), and their function (Roers *et al.*, 2004). Therefore, we assessed the effect of CD40/CD40L alterations on IL-10 production by T cells exposed to type II activated macrophages. IL-10 production in co-cultures with type II activated macrophages remained higher than levels detected in co-cultures with classically activated macrophages and were not further enhanced by CD40/CD40L inhibition. However, co-cultures with classically activated macrophages exhibited increased production of IL-10, in a dose dependant manner with increasing inhibition of CD40/CD40L. At full CD40/CD40L inhibition, the level of IL-10 in co-cultures with classically activated macrophages equaled the levels of IL-10 in co-cultures with type II activated macrophages. This indicates CD40/CD40L interactions may be having a suppressive effect on IL-10 production, which has not been previously reported.

In summary, these studies uncovered a suppressive effect of CD40/CD40L on IL-10 production, which could be overcome by inhibiting the CD40/CD40L interaction. The resulting alterations in IL-10 may be a contributing factor to the regulation of Th1 and Th2 responses seen at high and moderate levels of CD40/CD40L signaling respectively. These findings also provide a greater understanding into how CD40 inhibition prevents Th2 development, as previous studies have not investigated the effects of CD40 inhibition on the receptors for IL-4 and IL-2, or on the production of the suppressive cytokine IL-10. Although we were unable to enhance Th2 biasing by type II activated macrophages, we now have a greater understanding of some of the critical mechanisms involved in Th2 and Th1 biasing.

Chapter 5: Effects of schistosome complexes on macrophage activation

5.1 Introduction

In recent times, a strong negative correlation between the exposure to infectious diseases and the development of autoimmunity has been reported (Cook, 1996; Fleming *et al.*, 2007), suggesting that chronic infection with some parasites provides a level of protection against autoimmune diseases. For example, schistosomiasis has been shown to provide protection from autoimmune diseases such as type1 diabetes, graves thyroiditis and multiple sclerosis, in mouse models of these diseases. Interestingly, exposure to the schistosome egg (Cooke *et al.*, 1999; Sewell *et al.*, 2003) or soluble egg extract (SEA) (Zaccone *et al.*, 2003; Zheng *et al.*, 2008), and not just live infection (La Flamme *et al.*, 2003), provides similar protection. This suggests that it is the immune response to the eggs which regulate autoimmune responses. While it has been shown that this protection is dependent on a polarised Th2 response (Sewell *et al.*, 2003), the mechanism by which schistosome infection provides protection is not known.

Previous research (Herbert *et al.*, 2004) found that alternatively-activated macrophages were important during schistosomiasis as their absence resulted in severe morbidity despite the presence of a strong Th2 response. This study indicates that during schistosomiasis, macrophages regulate immune responses and promote survival. To date, the main role for alternatively activated macrophages has been associated with their role in wound repair/fibrogenesis (Albina *et al.*, 1990) and the down-regulation of inflammatory responses (Herbert *et al.*, 2008; Pesce *et al.*, 2009a). These macrophages can be identified by various markers, some of which include up-regulation of arginase1 and RELMα (Loke *et al.*, 2003; Modolell *et al.*, 1995; Raes *et al.*, 2002). Thus, one possible mechanism by which schistosome infection may protect from autoimmunity is by altering macrophage activation.

Macrophages exhibit extreme plasticity (Mosser *et al.*, 2008; Stout *et al.*, 2004), and can alter their phenotypic properties accordingly with the situation at hand. While it has been shown that schistosome infection leads to alternative activation of macrophages, it is also possible that other states of activation occur as well, such as type II activation. Furthermore, if these different activation states do occur during schistosomiasis, it is reasonable to presume that these states may also be contributing to protection from pro-inflammatory diseases such as EAE. In support of this idea, recent studies have shown that altering macrophage activation to support type II macrophages protects from EAE and the protection is Th2-dependant (La Flamme *et al.*, 2006; Tierney *et al.*, 2009). Thus,

we wished to examine whether schistosome immune complexes can induce type II macrophage activation *in vitro*.

Aims:

The mechanism by which schistosome infection protects from EAE and other pro-inflammatory diseases is still under study, although it is clear that it is Th2-dependant (La Flamme *et al.*, 2003; Sewell *et al.*, 2003; Zheng *et al.*, 2008). Type II macrophages induce Th2-biased responses (Anderson *et al.*, 2002), but no study to date has identified whether macrophages are activated into a type II state during schistosome infection. Type II activation is typically induced via Fcγ receptor ligation by exposure to immune complexes in the presence of inflammatory stimuli. During schistosome infection, high levels of schistosome-specific antibodies and immune complexes are in the blood and tissue (Dunne *et al.*, 2005; Santoro *et al.*, 1979), as well as inflammatory stimuli caused by leakage of bacterial components due to egg passage through the intestine (Herbert *et al.*, 2008). Therefore, the environment generated during schistosome infection is capable of inducing type II macrophage activation. Indeed, it has been demonstrated that macrophages exposed to LPS in the presence of SEA or SEA complexed with serum from an infected mouse, produce far less IL-12 and increased IL-10 (La Flamme *et al.*, 2004), suggesting that type II activation does occur. We aim to investigate fully the effects of schistosome immune complexes on macrophages *in vitro* to determine whether type II activation occurs. To this end, we addressed 2 specific aims:

1. To identify the cytokine and surface marker profiles on murine bone barrow derived macrophages induced by schistosome whole egg complexes (Egg:Serum), and soluble egg antigen immune complexes (SEA :IgG), in order to elucidate responses indicative of type II activation.
2. To determine if schistosome egg antigen complexes (SEA:IgG) induce a similar enzymatic profile to that of type II macrophages or to that of other known activation states (e.g. alternative or classical).

5.2 Results

5.2.1 Anti-inflammatory effect of schistosome egg

To investigate the effects of schistosome eggs on macrophage activation, primary bone marrow-derived macrophages were stimulated with LPS (i.e. classically activated) in the presence of either schistosome egg, or egg opsonised with serum from a schistosome infected mouse (i.e. immune complex). Because schistosome eggs are very large and polyvalent, it was expected that they would be good at ligating Fcγ receptors on macrophages and inducing type II activation. Cytokine analysis of culture supernatants showed that while LPS-stimulated macrophages produced high levels of IL-12 and low levels of IL-10, stimulation of macrophages with LPS in the presence of either egg or egg complex resulted in reduced levels of IL-12 and increased IL-10 (Figure 5.1), similar to the effect seen with type II activation by opsonised SRBC (Chapter3). In the absence of LPS, stimulation with either egg or egg complexed with infected serum (Egg:Ser) elicited the same response as non-stimulated macrophages. Kinetic studies indicate the most significant reduction in IL-12 and increase in IL-10 occur 8 h post stimulation as was noticed previously with type II activated macrophages. Interestingly, the modulating effects of schistosome eggs or eggs complexed with infected serum on the responses to LPS were very similar (Figure 5.1), suggesting that the alterations in macrophage responses may be occurring via a non-FcγR pathway.

Unlike stimulation with opsonised SRBC, stimulation with LPS plus schistosome eggs or eggs complexed with infected serum only moderately alters the expression of co-stimulatory molecules (Figure 5.2). While LPS-induced CD80 expression was slightly reduced by egg or egg complex 24 h post-stimulation, CD40 expression was unaffected except at 24 h where a reduction can be seen by both egg and egg complex in the presence of LPS. PDL-1, like CD40, remained unaffected 2-8 h post stimulation but reduced slightly 24 h after stimulation with schistosome egg plus LPS. This effect was not seen with schistosome egg complex. Overall, there is a small reduction in the expression of activation/co-stimulatory markers 24 h after treatment with schistosome egg or egg complex compared to classical activation. However, these reductions in expression are significantly less than those seen by type II activated macrophages. Moreover, because the 24 h time point was only examined once, the consistency of this reduction needs verification.

Taken together, these data indicate that schistosome whole eggs either alone or as an immune complex, are capable of activating macrophages into a state which exhibits an anti-inflammatory cytokine profile. Although the kinetics of the cytokine alterations are similar to type II activation, the expression profile of co-stimulatory markers differs to that

of type II activated macrophages. These findings, in addition to the similarity in responses to the egg immune complex and eggs alone in the presence of LPS, suggest that type II macrophage activation may not be induced by schistosome egg complexes. Rather, they indicate that the anti-inflammatory profile is generated through another unknown mechanism that warrants further investigation.

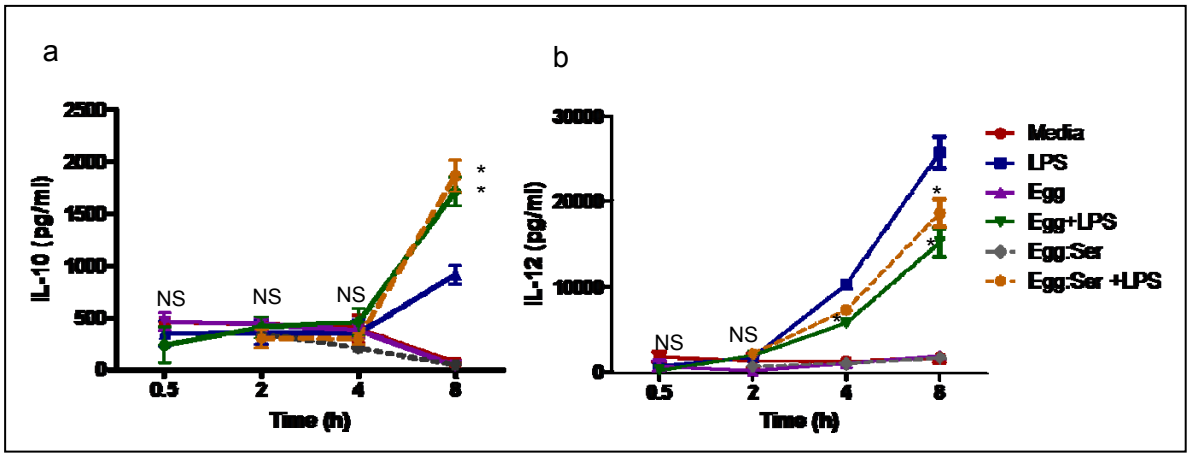


Figure 5.1 Schistosome egg and egg complex reduce LPS-induced IL-12 and increase IL-10. Primary BMMØ (10^6 cells/ml) derived from C57BL/6 mice were primed with 20 U/ml IFNγ overnight followed by stimulation with Media, LPS (200 ng/ml), Schistosome egg (1000/well), Egg + LPS, opsonised Egg (Egg:Serum from schistosome infected mouse), or opsonised Egg + LPS. After 0.5, 2, 4 and 8 h culture IL-10 (a) and IL-12 (b) were assessed via ELISA. *P<0.001: Two way ANOVA followed by Bonferroni post test; Egg + LPS or Egg:Serum + LPS versus LPS. Graphs represent the mean + SEM from combined duplicate wells of two experiments.

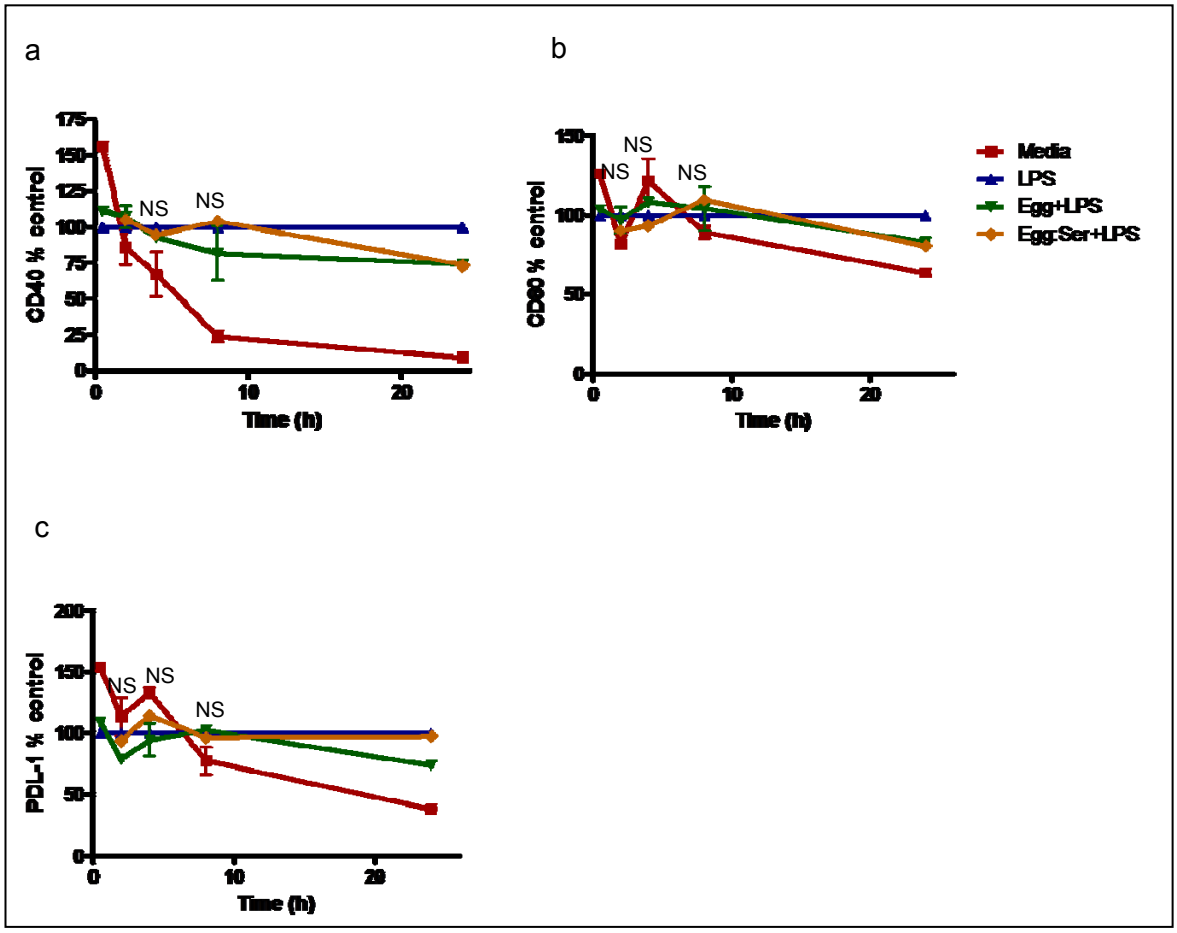


Figure 5.2 Schistosome egg and egg complex induce no significant alterations in macrophage surface markers. Primary BMMØ (10^6 cells/ml) derived from C57BL/6 mice were treated as described in Figure 5.1. After 0.5, 2, 4, 8 and 24 h culture CD40 (a), CD80 (b) and PDL-1 (c) were assessed via flow cytometry. Difference between Egg + LPS or Egg:Ser + LPS versus LPS is NS P>0.05: Two way ANOVA with Bonferroni post test. Data represents the mean + SEM of two experiments. The 24 h time point represents one experiment. Data is presented as % of control compared to cultures with LPS. See Appendix A for full graphs with stimulants in the absence of LPS.

5.2.2 Ability of SEA/SEA complexes to activate macrophages of an anti-inflammatory nature

Because immune complexes consisting of soluble schistosome antigen (SEA) and not necessarily whole eggs circulate in the blood of infected subjects, the effect of schistosome soluble egg antigen immune complexes on macrophage activation was assessed. Primary murine bone marrow-derived macrophages were stimulated for 8 h and 24 h with LPS in the presence or absence of SEA or SEA complexed with purified IgG (SEA-IgG) from schistosome-infected mice, and compared to type II activation induced by opsonised SRBC. To determine the optimum ratio of SEA to purified IgG, various ratios of SEA and IgG were used and the reduction in LPS-stimulated IL-12 production was assessed. Although SEA alone induced a reduction in IL-12, the addition of purified IgG enhanced this reduction. Figure 5.3 indicates the optimal ratio of SEA to IgG is 5 µg/ml SEA to 20 µg/ml of purified IgG, as SEA-IgG ratios of 5-10 µg ,10-10 µg/ml or 10-20 µg/ml induce a less significant reduction in IL-12 compared to the 5-20 µg/ml SEA-IgG ratio.

Using this optimal ratio of SEA-IgG, IL-10 production was assessed by macrophages treated with SEA complexes. Figure 5.4 indicates at the optimised ratio of SEA complex, the reduction in IL-12 was greater than that seen with type II activation and the increase in IL-10 was equal to the increase by type II activation. As type II activated macrophages are known to induce Th2 biasing, IL-4 expression was also assessed under the same conditions. As expected, classically activated macrophages (LPS) produced nearly undetectable levels of IL-4. However, while type II activated macrophages (SRBC complex plus LPS) produced low but detectable levels of IL-4, stimulation of macrophages with SEA complexes in the presence of LPS induced a significant increase in IL-4 production. This is a novel finding, and the potential role of macrophage-derived IL-4 during schistosome infection warrants further investigation.

In order to investigate the effects of SEA complexes on co-stimulatory marker expression, expression levels were compared to that seen with type II activated macrophages and alternatively activated (i.e. IL-4-stimulated) macrophages. Figure 5.5 shows LPS stimulation induces a strong up-regulation in CD40 and a modest increase in CD80 and PDL-2 expression compared to non-stimulated macrophages. In contrast, macrophages exposed to LPS in the presence of SEA-complexes showed reduced levels of CD40 and CD80 expression compared to classical activation, and reduced the expression of PDL-2, which was up-regulated upon exposure to rIL-4. Based in this data, SEA-complexes induce a co-stimulatory marker and cytokine profile similar to that of type II activated macrophages, suggesting that SEA complexes may be inducing type II macrophage activation. Furthermore, there was a significant increase in IL-4 in response to SEA-

complexes, which was not found in the other activation conditions (classical, type II or alternative). Therefore further investigations are merited to investigate whether type II activation occurs or whether a novel activation state has been induced.

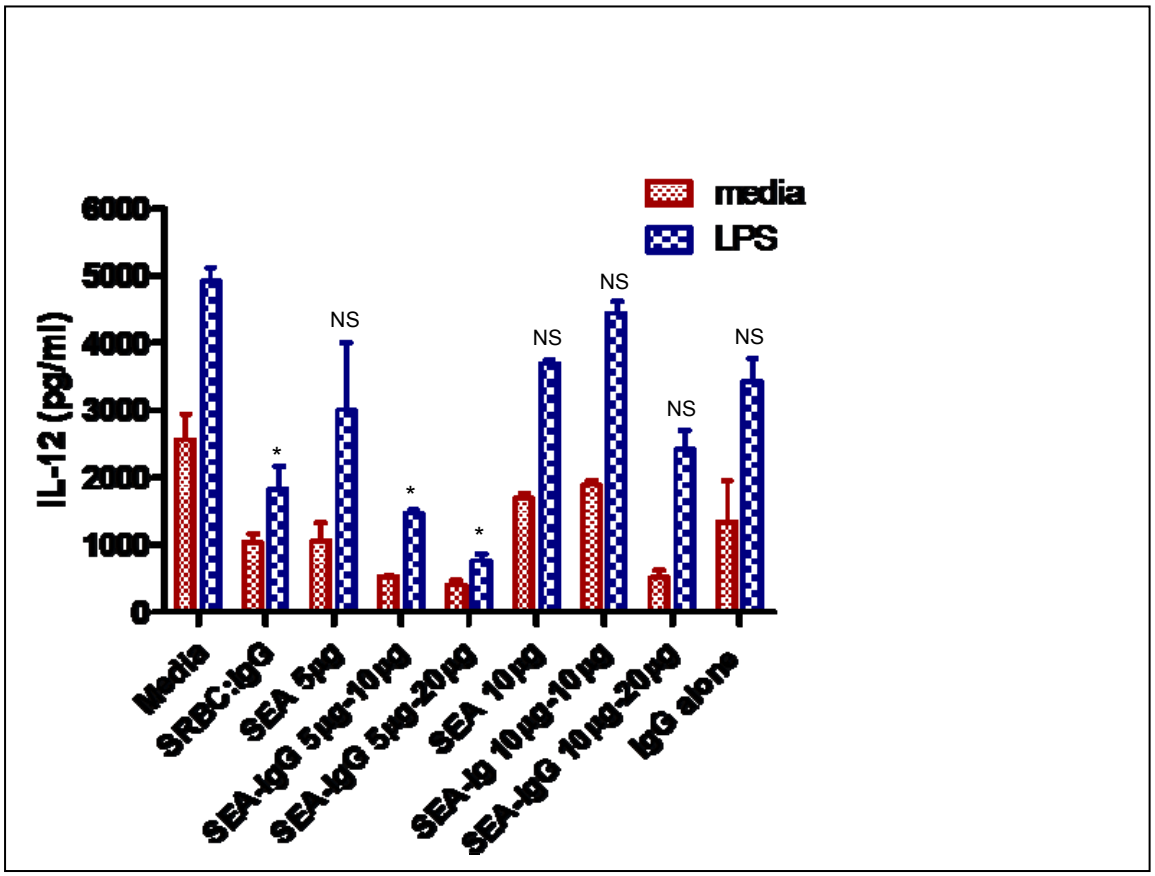


Figure 5.3 Schistosome complex of 5-20 µg/ml elicits the greatest reduction in LPS-induced IL-12. As mentioned in Figure 5.2, primary BMMØ were primed overnight. Macrophages were then stimulated both in the presence and absence of LPS (200 ng/ml) , with 5 µg/ml or 10 µg/ml SEA, or SEA-IgG complex at various ratios of SEA bound to purified IgG (5 µg/ml or 10 µg/ml SEA, with 10 µg/ml or 20 µg/ml IgG). Cells were also classically (LPS) or type II activated (SRBC:IgG+LPS). After 8 h culture, IL-12 was assessed via ELISA. *P<0.05: One way ANOVA; Conditions versus LPS. Data represents the mean+SEM from duplicate wells of one experiment.

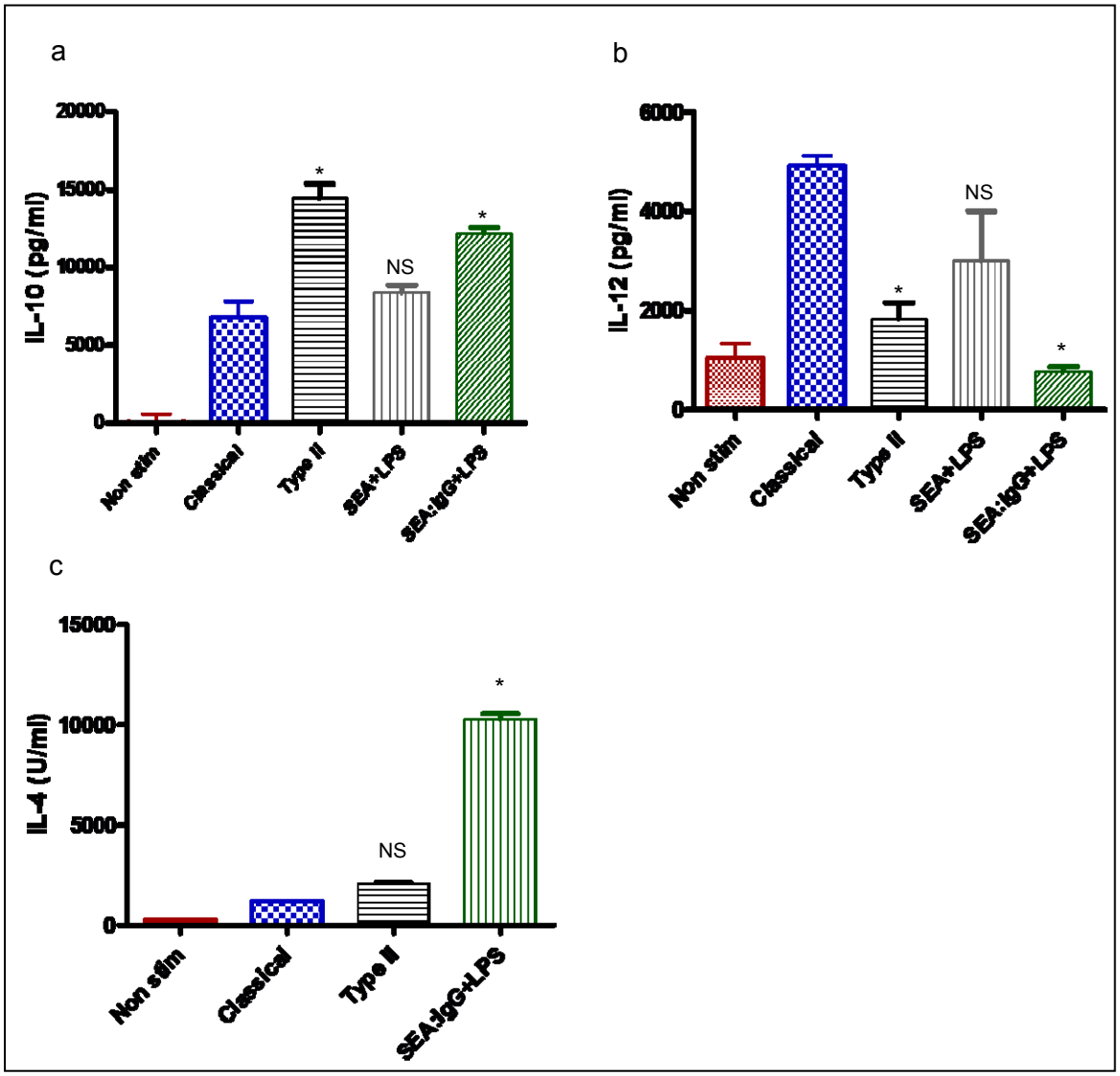


Figure 5.4 SEA complexes elicit a similar IL-10/IL-12 ratio to type II macrophages but differ significantly in IL-4 induction. Primary BMMØ (10^6 cells/ml) derived from C57BL/6 mice were primed with 20 U/ml IFNγ overnight followed by exposure to 5 µg/ml SEA or SEA-IgG (5 µg/ml -20 µg/ml) in the presence and absence of LPS (200 ng/ml). Macrophages were also exposed classical and type II–inducing stimuli (Fig 5.3). After 8 h culture IL-10 (a), IL-12 (b), and IL-4 (c) were assessed via ELISA. IL-4 was measured in the absence of the treatments with un-opsonised SRBC and un-opsonised SEA. *P<0.05: One way ANOVA with Bonferroni post test; Conditions versus Classical. Data represents the mean+ SEM from duplicate wells of one experiment. NS represents P>0.05.

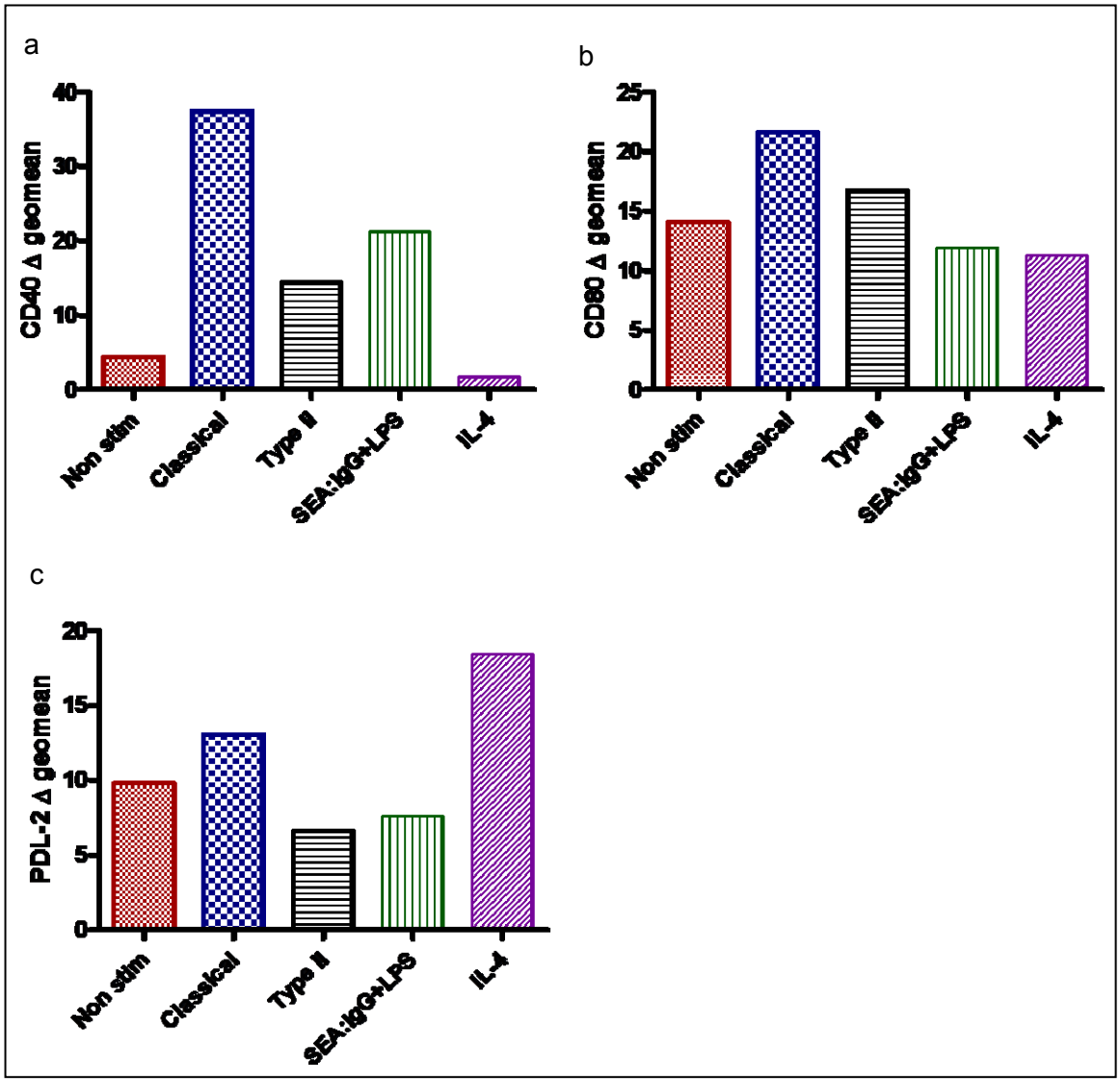


Figure 5.5 SEA complexes elicit similar alterations in surface markers to type II macrophages. Primary BMMØ (10^6 cells/ml) derived from C57BL/6 mice were treated as mentioned in Figure 5.4. Additionally, macrophages were stimulated with 20 U/ml rIL-4. After 24 h culture CD40 (a), CD80 (b), and PDL-2 (c) were assessed via flow cytometry. Bars represent one experiment.

5.2.3 Enzymatic profile of macrophages exposed to SEA complexes

In addition to cytokine production and surface marker expression, enzyme markers for classical, type II and alternative macrophage activation were investigated using western blotting to assess the activation state of macrophages induced by schistosome immune complexes plus LPS. Macrophages were stimulated for 9 h and 24 h, and lysates were probed by western blotting for the expression of SPHK1 as a marker for type II activation, RELMα (FIZZ1) for alternative activation, and iNOS for classical activation. Alpha tubulin expression was used as a loading control and all samples were normalized to this protein. Similar to cytokine production, 9 h was found to be the optimal time to assess intracellular proteins of interest.

Representative gels and calculated band densities are shown in Figures 5.6 and 5.7 for iNOS, SPHK1, and RELMα. Band densities are presented as a percentage of control compared to LPS, in order to compare the expression of the various markers to classical stimulation. Graphs were generated from 2-4 western blots for most samples, although SEA complex plus LPS-stimulated macrophages (SEA:IgG+LPS) were only run once. Figure 5.6 shows a similar expression of iNOS in type II activated macrophages to that of classical macrophages. iNOS was not detected in samples stimulated with SEA alone, but was expressed in macrophages exposed to SEA or SEA complexes in the presence of LPS. Unfortunately the samples including the lysate from macrophages stimulated with SEA complex alone were unsuccessful, so the direct effect of this immune complex on macrophages cannot be assessed at this time. However, this condition will be an essential control to include in all future work based on this study. Taken together, these results indicate that SEA or SEA complex-activated macrophages in the presence of LPS, produce iNOS similarly to classical and type II activated macrophages.

To evaluate a type II-specific marker, the production of sphingosine-kinase 1 (SPHK1) was assessed in macrophages exposed to SEA complexes in the presence or absence of LPS and in type II activated macrophages. As expected, there was a significant increase in SPHK1 expression by type II activation in comparison to classical macrophages, further supporting its role as a marker for type II activation (Figure 5.6). Macrophages exposed to SEA alone or SEA in the presence of LPS exhibit higher SPHK1 expression than classical macrophages, but lower than that expressed in type II activated macrophages (Figure 5.6). The production of SPHK1 in response to SEA alone, indicated the SPHK1 expressed in response to SEA plus LPS, is primarily driven by the effect of SEA, and not necessarily by the addition of LPS, unlike iNOS expression. In contrast, there is no detectable SPHK1 expression in macrophages stimulated with SEA complexes plus LPS. However, as this condition was done only once, further work needs to be done to confirm this result.

RELMα expression was also assessed as a marker for alternative activation which is known to occur during schistosome infection, and is induced by IL-4. As shown previously, RELMα expression in classical and type II activated macrophages was very low (Figure 5.7). In contrast, rIL-4-induced alternatively activated macrophages produced significant amounts of RELMα, validating its high expression as a marker for alternatively activated macrophages. Additionally, SEA complexes in the presence of LPS induced much higher levels of RELMα compared to alternatively activated macrophages, suggesting that this condition may share some of the features of alternatively activated macrophages. Furthermore, given that IL-4 is produced by macrophages stimulated with SEA complexes and LPS, this elevation in RELMα is likely to be induced by this cytokine and not directly induced by the SEA complexes and LPS. Overall, the analysis of the enzymatic profile of macrophages indicates SEA either alone or in a complex is capable of inducing a distinct activation state from classical macrophages. The expression of markers for type II activation as well as alternative activation indicates SEA complexes are inducing the activation of macrophages into a 'hybrid' state, which exhibits characteristics from more than one known activation state.

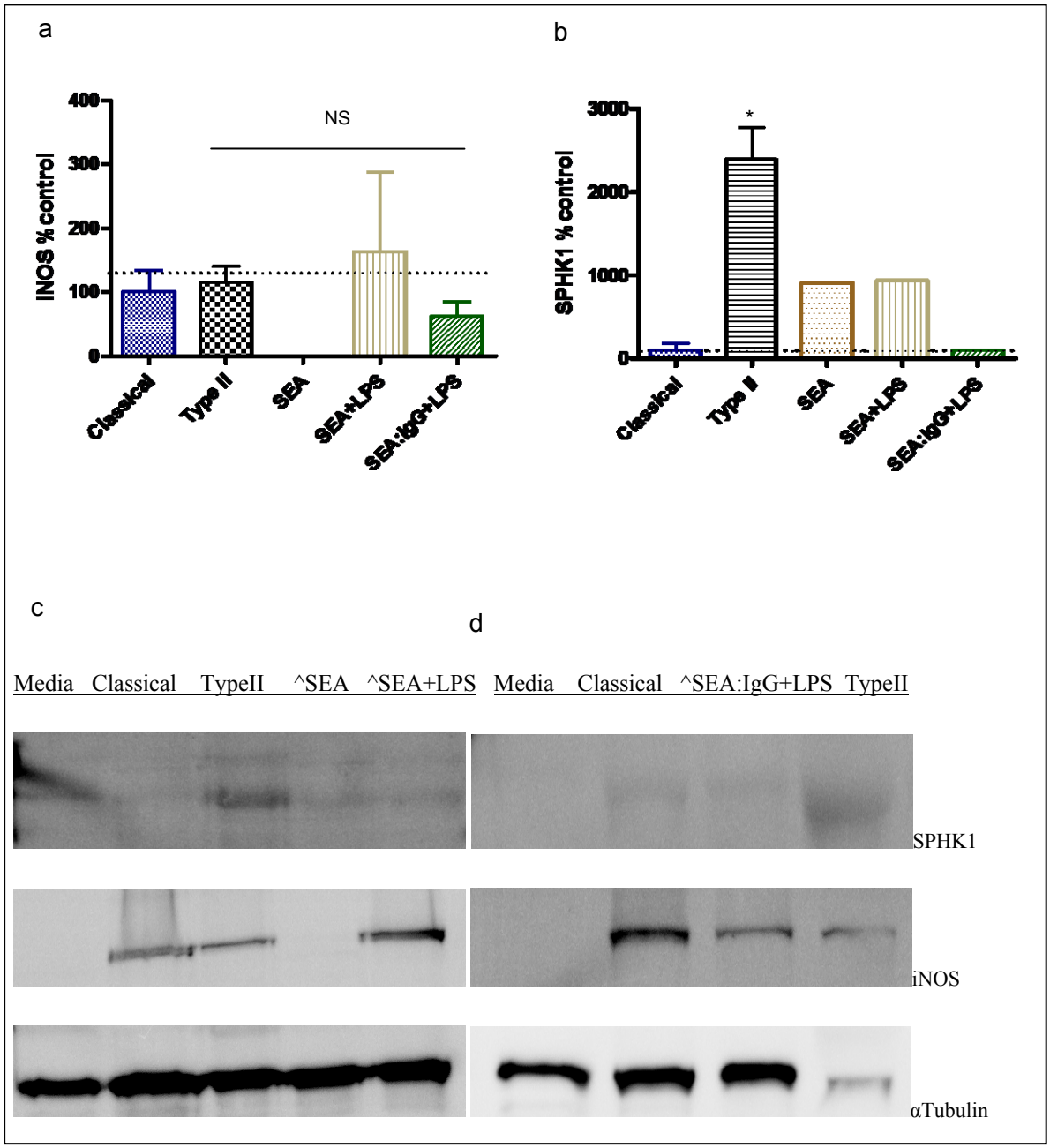


Figure 5.6 SEA induces SPHK1 expression and iNOS in macrophages. Primary BMMØ (10^6 cells/ml) derived from C57BL/6 mice were primed with 20 U/ml IFNγ followed exposure to 5 μg/ml SEA, or SEA-IgG in the presence or absence of 200 ng/ml LPS (SEA-IgG: 5 μg/ml-20 μg/ml), or classical or type II-inducing stimuli. After 9 h culture, iNOS (a) and SPHK1 (b) were assessed via western blotting as mentioned in section 2.6, with anti-SPHK1 pAb (1:700), anti-iNOS pAb (1:700) and anti-αtubulin (1:1000). Bars represent the band densities of 1-3 experiments normalised to αtubulin. *P<0.05: One way ANOVA with Bonferroni post test; Conditions versus Classical activation (LPS). Bars for SEA:IgG + LPS represent a single experiment. Data is presented as % of control compared to cultures with LPS. (c-d) are representative of gels used to elucidate (a-b). Differences between the gels are represented by (^). Molecular weights: SPHK1 (69KDa), iNOS (135KDa), αTubulin (50KDa).

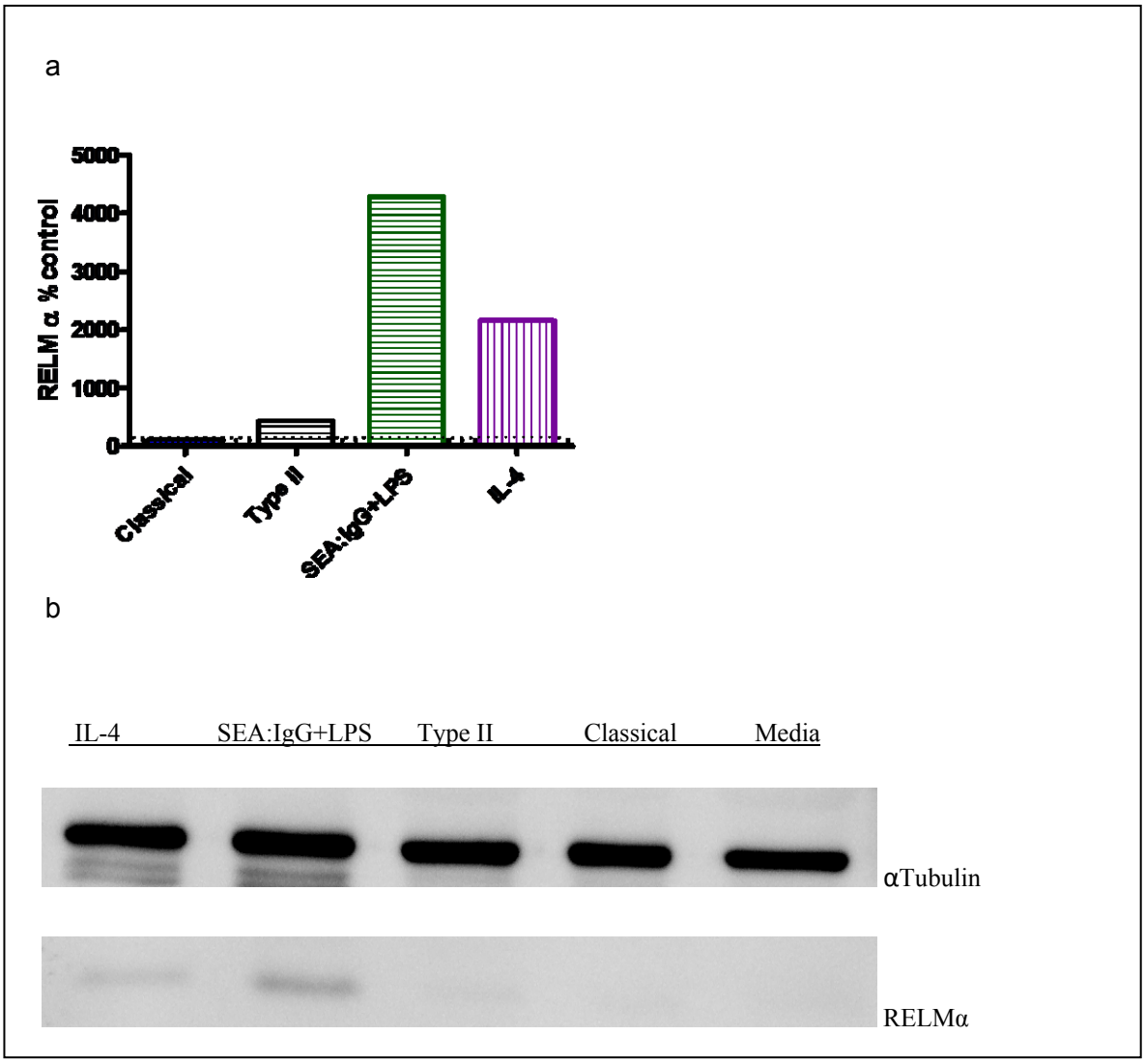


Figure 5.7 SEA complexes induce the expression of RELMα in macrophages. Primary BMMØ (10^6 cells/ml) derived from C57BL/6 mice were primed with 20 U/ml IFNγ followed by exposure to SEA-IgG + LPS (5 μg/ml-20 μg/ml), 20 U/ml rIL-4, or classical or type II-inducing stimuli. After 9 h culture, RELMα was assessed via western blotting (section 2.6), with anti-RELMα (1:700) followed by anti-αtubulin (1:1000). Bars represent the band densities of one experiment normalised to α tubulin. Data is presented as % of control compared to cultures with LPS. Diagram (b) is the gel used to elucidate the graph in (a). Molecular weights: αTubulin (50KDa), RELMα (12-15KDa).

5.3 Discussion

During infection with the parasitic helminth of the *Schistosoma* genera, a polarized Th2 response dominates in the host (Grzych *et al.*, 1991; Pearce *et al.*, 2004). Studies demonstrating an inverse correlation between areas prevalent in schistosome infection, and those prevalent in autoimmunities such as MS, has prompted researchers to hypothesise that schistosome infection may provide protection against MS (Fleming *et al.*, 2007). Indeed, schistosome infection or exposure to isolated schistosome eggs or soluble egg antigen elicits protection against animal models of MS and type 1 diabetes (Cooke *et al.*, 1999; La Flamme *et al.*, 2003; Sewell *et al.*, 2003; Zaccone *et al.*, 2003; Zheng *et al.*, 2008). The protection was accompanied by a reduction in Th1 inflammatory responses and an increase in Th2-type responses, and was abolished in the absence of this Th2 response (La Flamme *et al.*, 2003; Sewell *et al.*, 2003). In a similar manner, inducing type II macrophage activation *in vivo* protects from the animal model of MS in a Th2 dependent manner (Tierney *et al.*, 2009). Therefore, this thesis investigated the macrophage response to schistosome egg or egg antigen immune complexes, in order to determine whether type II macrophage activation may be induced during schistosome infection and may thus contribute to the reported protective effect against autoimmune diseases.

The involvement of macrophages in driving protective Th2 responses to whole schistosome eggs is unknown; hence, we wished to identify the anti-inflammatory effects of exposure to schistosome eggs on macrophages. Furthermore, we assessed whether these egg immune complexes induced type II activated macrophages similar to those induced by SRBC complexes. We found that similar to opsonised SRBCs, schistosome egg and egg immune complexes, in the presence of LPS, significantly reduced IL-12 and increased IL-10 production in comparison to classically activated macrophages. However, unlike type II activation induced by opsonised SRBC, the effects seen with schistosome egg complexes were mirrored by the effects of schistosome egg in a non-complexed (i.e. non-opsonised) form. This result indicates the response is not specific to FcγR ligation as type II activation is (Lucas *et al.*, 2005; Zhang *et al.*, 2006a). However, macrophages activated in this manner by schistosome eggs may still be an important contributor to host protection due to their production of IL-10, given that IL-10 is essential for protection against severe disease caused by schistosomiasis (Hoffmann *et al.*, 2000). Finally, previous work has clearly shown that the increased IL-10 in conjunction with reduced IL-12 plays an important role in immune deviation and host protection during schistosomiasis (Hesse *et al.*, 2004) and EAE (Sewell *et al.*, 2003). Our findings indicate that schistosome egg either alone or in a complex with serum from a schistosome infected mouse has the ability to induce anti-inflammatory cytokine profiles similar to that observed with type II

activation, however the mechanisms involved differ from that of type II macrophage activation.

Type II activation by opsonised SRBC induces a significant reduction in the expression of CD80, CD40 and PDL-1 in response to LPS (Tierney *et al.*, 2009) (Chapter 3), but the effect of schistosome egg on the expression of these surface markers is currently unknown. Assessing the changes in CD80, PDL-1 and CD40 by schistosome eggs and egg complexes in the presence of LPS, we found the expression profile more similar to classical macrophages than that of type II activated macrophages. While there were slight reductions in the expression of CD40 and CD80 at the 24 h time-point, these reductions occurred independent of whether or not schistosome egg was in a complex form. Furthermore, the expression of PDL-1 in comparison to classical macrophages remained largely unchanged by stimulation with schistosome egg or egg complexes in the presence of LPS.

Thus, macrophages exposed to schistosome egg and egg complexes do not exhibit the same characteristics of type II activation in terms of the changes seen in activation marker expression. In addition to this, the similarity in response to the egg or egg complex suggests that the response induced by these products in the presence of LPS may be occurring through a different mechanism than that of Fcγ receptor ligation which evokes type II activation. It is more likely that components of the schistosome egg itself were eliciting the observed anti-inflammatory effects. These components may include glycosylated products (e.g. proteins, lipids) on the schistosome egg, which promote Th2 cell biasing (Okano *et al.*, 1999). Schistosome eggs have been demonstrated to promote highly polarised Th2 immune responses, capable of protecting against EAE (Sewell *et al.*, 2003), and this investigation further supports this finding by demonstrating that immune deviation can occur by direct interaction of the schistosome egg with macrophages. However, we have not shown that this effect on macrophages elicits Th2 cell biasing, but based on the cytokine profile, and previous *in vivo* observations (Sewell *et al.*, 2003), it is highly possible. The ability of schistosome egg antigens, but not whole schistosome eggs, to induce Th2 biasing through the interaction with APCs has been previously reported (MacDonald *et al.*, 2002c; Steinfelder *et al.*, 2009). Dendritic cells exposed to a schistosome egg component, omega-1, altered their phenotype to elicit Th2 cell biasing upon contact with CD4 T cells (Steinfelder *et al.*, 2009). It was concluded that the immune deviation was elicited by egg-induced changes in the level of signalling by DCs. Furthermore, in addition to the effects on DCs, the interaction of schistosome egg components with basophils has also been shown to induce immune deviation (Schramm *et al.*, 2003) by up regulating IL-4, a strong inducer of Th2 development (Le Gros *et al.*, 1990). The exact mechanism involved in deviating the macrophage response presented in

this thesis is unknown; however further investigations using specific egg components like omega-1 should be carried out.

In contrast to whole schistosome eggs, the majority of published work focuses on schistosome soluble egg antigen (SEA), which is extremely antigenic and induces strong Th2 responses (Pearce *et al.*, 2004; Zheng *et al.*, 2008). Investigations have been carried out to elucidate the components of SEA which provide them with Th2-inducing properties, and some of the suggested components include IL-4 inducing principle of *Schistosoma mansoni* (IPSE) and N-fucopentaose III (Okano *et al.*, 1999; Schramm *et al.*, 2003), both of which induce highly polarised Th2 responses. Studies using SEA derived from *Schistosoma japonicum* have also shown that the SEA-specific Th2 responses are protective against EAE (Zheng *et al.*, 2008). Because SEA immune complexes are generated during schistosomiasis (Rezende *et al.*, 1997), it is possible that these complexes can promote type II activation as well as Th2 responses. Therefore, this investigation examined the effects of SEA complexes on macrophages in order to understand possible effects associated with prevention of inflammatory diseases like EAE.

Using the SEA immune complex, we found a significant increase in IL-10 and reduction in IL-12 in the presence of LPS. This finding complements *in vivo* data, in which protection against EAE by schistosome infection is associated with reductions in IL-12 and increases in IL-10 (La Flamme *et al.*, 2003). It also correlates with the IL-10/IL-12 profile elicited by exposure to SEA complexed with serum (La Flamme *et al.*, 2004), and the cytokine profile induced by type II activation as demonstrated in Chapter 3. In contrast to SEA immune complexes, SEA and LPS had only a minor effect on cytokine production suggesting that, as with type II activation, the alternation in IL-10 and IL-12 occurred through ligation of FcγRs.

SEA has been demonstrated to contain components which strongly induce the production of IL-4 by immune cells such as basophils (Schramm *et al.*, 2003). IL-4 production was assessed in the cultures of classically activated and type II activated macrophages, with very little IL-4 detected. In contrast to the nearly undetectable levels noted by classical and type II activated macrophages, macrophages stimulated with SEA complexes in the presence of LPS produced very high levels of IL-4. Although IL-4 cytokines are increased during schistosomiasis (Grzych *et al.*, 1991), to date there have been no reports of macrophages producing IL-4 in response to schistosome infection or exposure to schistosome products. In fact, the only studies reporting the production of IL-4 by macrophages used the RAW 264.7 cell line derived from a BALB/c mice and alveolar macrophages during pulmonary fibrosis (Buttner *et al.*, 1997; Mukherjee *et al.*, 2009; Sempowski *et al.*, 1994). Due to the important role of IL-4 in T cell biasing as well as in

differentially activating alternatively activated macrophages and perpetuating anti-inflammatory responses, the ability of SEA complexes to initiate IL-4 production merits further investigation as this pathway may be an important mechanism involved in driving Th2 responses.

Also investigated were the effects of SEA complexes on the expression of CD40, CD80, and PDL-2. In accordance with type II activation, SEA complexes in the presence of LPS induced a reduction in CD80 and CD40. Studies have previously demonstrated the requirement for CD40/CD40L signalling in the induction of Th2 responses (MacDonald *et al.*, 2002c). As with type II activation, the reduction in CD40 but absence of complete inhibition in response to SEA complexes emphasizes the possible role of this particular level of CD40 signalling in promoting protective responses. As with type II activation, the reduction in the expression of these co-stimulatory markers was most significant in response to the complex form of SEA.

As IL-4 promotes alternative activation of macrophages (Raes *et al.*, 2002; Stein *et al.*, 1992), we assessed the expression of PDL-2, a ligand for PD-1 differentially up-regulated on alternatively activated macrophages (Loke *et al.*, 2003; Tierney *et al.*, 2009). Although rIL-4-induced alternatively activated macrophages induced PDL-2, neither type II activated nor SEA complex plus LPS-activated macrophages up-regulated PDL-2 expression. Given the significant increase in IL-4 by exposure to SEA immune complexes and LPS, this finding was contrary to expectation. It is unclear as to why there is such a difference between the effects of IL-4 derived from SEA-complex treated macrophages and rIL-4, which induced alternatively activated macrophages with respect to PDL-2 expression. This difference may be because the concentration of IL-4 produced by SEA complex-activated macrophages was below the threshold needed to drive all aspects of alternative macrophage activation or due to a difference in the mechanisms involved. The differences in the expression of PDL-2, CD80, CD40 and IL-12 by SEA complexes and LPS compared to rIL-4 indicate that these stimuli induce activation states with distinct profiles, whereas the SEA complex plus LPS condition follows a similar pattern of expression as type II activated macrophages.

In order to aid our understanding of the activation state induced by SEA complexes, the expression levels of enzyme markers for classical, type II and alternative macrophage activation were assessed. SPHK1 has been identified as an indicator of type II activation (Edwards *et al.*, 2006), RELMα (FIZZ1) of alternative activation (Edwards *et al.*, 2006; Raes *et al.*, 2002) and iNOS production of classically and type II activated macrophages (Edwards *et al.*, 2006).

The similar production of iNOS by type II and classically activated macrophages corresponds to previous reports (Edwards *et al.*, 2006), indicating both cell types are capable of eliciting immune responses against intracellular pathogens. This high expression of iNOS is in contrast to alternatively activated macrophages which do not express detectable levels of iNOS or NO but instead arginase 1 or RELMα (Modolell *et al.*, 1995; Raes *et al.*, 2002). If SEA complexes were inducing alternative activation, it would be expected that iNOS expression would be reduced or absent. However, iNOS was produced by macrophages exposed to both SEA and SEA complexes in the presence of LPS, but was not expressed under the same conditions in the absence of LPS. This indicates SEA or SEA complex-activated macrophages in the presence of LPS are likely capable of carrying out processes involved in intracellular killing, a mechanism associated with classical and type II activated macrophages (Campbell *et al.*, 1996; Edwards *et al.*, 2006), whereas in the absence of LPS, they are not.

In agreement with previous work (Edwards *et al.*, 2006), SPHK1 expression was significantly higher in type II activated macrophages in comparison to classically activated macrophages. To date, there is no published data on the expression of SPHK1 by SEA or SEA complex treated macrophages; thus, the finding that there is an increase in SPHK1 expression in response to SEA compared to classically activated macrophages is a novel find. This SPHK1 expression was seen upon exposure to not only SEA alone, but also SEA in the presence of LPS, indicating SEA alone was driving this response rather than the presence of LPS. This is in contrast to the expression of iNOS which does depend on LPS exposure. It is important to note that the level of SPHK1 expression is less than that produced by type II activated macrophages, but much greater than any levels produced by classical macrophages. As such, its presence should not be used as a sole determinant of type II activation. It suggests the macrophages may be able to carry out similar functions to type II activated macrophages even if type II activation is not actually induced.

The role of SPHK1 in type II activation is currently unclear. Sphingosine-1-phosphate, produced via SPHK1, has been implicated to have an inhibitory role on Th1 cytokine production (Yang *et al.*, 2005), but also in aiding survival of macrophages during pro-inflammatory stress (Wu *et al.*, 2004). Recent studies have also indicated a role for sphingosine-1-phosphate in lymphocyte egress (Melendez, 2008), which may seem somewhat contradictory, but overall indicates a possible role in maintaining immune balance. The lack of expression of SPHK1 by SEA-complex and LPS-activated macrophages is an interesting finding as it does not follow the trend, in which SEA alone was seen to drive its production. This may indicate that when the SEA is in a complex form, the mechanism allowing SEA to induce SPHK1 expression is neutralised in some

manner by the functional characteristic induced by SEA complexes. However, this action does not correlate with the other phenotypic characteristics induced by SEA complexes such as CD80/CD40/PDL-2 reductions and iNOS expression, which resemble those of type II activated macrophages. Therefore, further investigation in the effect of SEA complexes and LPS on SPHK1 production by macrophages is merited.

RELMα expression was assessed in order to elucidate whether alternative activation was occurring given the high production of IL-4 by SEA complex plus LPS-stimulated macrophages and the role of IL-4 in driving alternative activation (Stein *et al.*, 1992). Type II activated macrophages produced nearly undetectable levels of RELMα, as did classically activated macrophages and this finding agrees with previous reports (Edwards *et al.*, 2006). In contrast to classical and type II activation, macrophages exposed to recombinant IL-4 produced significant amounts of RELMα, corresponding with the induction of alternative activation (Raes *et al.*, 2002). Thus, the appearance of high production of RELMα in response to SEA complexes in the presence of LPS, suggests the induction of alternative activation, with RELMα levels much higher than that expressed by macrophages exposed to even rIL-4.

Based solely on this western blot analysis, one would conclude that SEA complexes induced alternative activation of macrophages. However, this conclusion is not justified given the expression of SPHK1 and the cytokine profile, which suggest that neither type II nor alternative activation is induced. Altogether our findings indicate that SEA complexes induce a novel activation state exhibiting a hybrid of characteristics, blended from type II activation and alternative activation as well as some characteristics from neither state. Although ‘hybrid’ activation states have been previously suggested (Mosser *et al.*, 2008), no induction of such a state as this has been previously reported.

The induction of this unique ‘hybrid’ activation state may have important beneficial implications to the regulation of autoimmunity as well as schistosomiasis. IL-4 is able to provide protection against EAE (Shaw *et al.*, 1997) and schistosomiasis (Brunet *et al.*, 1999), through the induction of Th2 responses. Understanding mechanisms involved in increasing IL-4 production may have extremely beneficial consequences in the treatment of inflammatory diseases. For example, RELMα aids wound repair (Raes *et al.*, 2002) as well as suppresses excessive Th2 responses (Nair *et al.*, 2009; Pesce *et al.*, 2009b), and during schistosomiasis, RELMα is essential for the prevention of lethal levels of hepatic fibrosis induced by Th2 responses (Nair *et al.*, 2009). Similarly, RELMα expression may also benefit EAE by aiding in repair of the CNS and preventing excessive T cell responses. This thesis presents a novel activation state induced by schistosome egg antigen complexes and LPS, which exhibits a range of characteristics involved in

maintaining a balance between the Th2 polarised fibrogenic responses required for host survival during schistosomiasis (Herbert *et al.*, 2004), and reducing overzealous inflammatory responses which would otherwise induce pathology (Pesce *et al.*, 2009b). Therefore this state of activation may be beneficial in EAE as well, where the balance of immune responses is essential.

Chapter 6: General discussion

6.1 Overall Summary of thesis

Various studies have demonstrated the potent protective effects of immune deviation on inflammatory disorders (Tierney *et al.*, 2009; Weber *et al.*, 2007; Zheng *et al.*, 2008). Macrophages are capable of deviating immune responses into pro-inflammatory or anti-inflammatory responses dependant on their activation status (Edwards *et al.*, 2006; Mosser *et al.*, 2008a). In autoimmunites, studies have shown that protective immune deviation can be induced by pre-exposure to a Th2–inducing parasite *Schistosoma mansoni*, as well as pre-treatment with type II activated macrophages which elicit Th2 biasing (La Flamme *et al.*, 2003; Sewell *et al.*, 2003; Tierney *et al.*, 2009). The exact mechanisms involved in type II activation and the induction of protective anti-inflammatory responses during schistosomiasis are unknown and currently under investigation. Similarities exist between the effects of type II activated macrophages and schistosome infection in protection against an animal model of MS, EAE. It is unknown what effect schistosome infection has on macrophage activation, however high levels of circulating immune complexes as well as inflammatory mediators occur during infection (Rezende *et al.*, 1997; Santoro *et al.*, 1979), making it likely that type II activation is a contributing factor in the protection schistosomiasis elicits. Understanding the immunological events involved in protection by either type II activation or other macrophage phenotypes which may be induced during schistosomiasis, may benefit the development of drugs which mimic or enhance the protective effects against autoimmune diseases.

This thesis investigates mechanisms involved in perpetuating the phenotypic profile of type II activated macrophages, and promoting the deviation of T cell responses into polarised Th2 responses, with particular emphasis on IL-10 and CD40. This investigation also encompasses the effects of schistosome immune complexes on macrophage activation, in order to elucidate whether type II activation may be induced in the course of schistosome infection.

A ‘hybrid’ activation state

Although results from the schistosome investigations are preliminary and require validation, they provide new insight into possible effects of schistosome infection on macrophage activation. Mosser *et al* reported the notion that some macrophages elicit traits of more than one activation state, termed ‘hybrid macrophages’ (Mosser *et al.*, 2008b). To my knowledge, there are been no reports to date which have induced such a state. However this investigation may have identified such a ‘hybrid’ state, induced by exposure to schistosome egg antigen complexes. By only looking at surface markers, the

SPHK1 enzyme, and cytokines IL-10 and IL-12, it would suggest schistosome complexes induce type II macrophage activation. However, the high levels of RELMα (a marker of alternative activation (Raes *et al.*, 2002)) and high IL-4 production (a characteristic unknown of any activation states) make this macrophage phenotype distinct from both type II activation and alternative activation. The high production of IL-4 was a novel find, as to date the only reports of IL-4 production by macrophages include alveolar macrophages (Buttner *et al.*, 1997; Sempowski *et al.*, 1994) and macrophage cell lines (Mukherjee *et al.*, 2009).

This activation state may elicit more beneficial effects compared to either type II or alternative activation states alone. The high levels of IL-4 and IL-10 and reduced IL-12 may induce anti-inflammatory immune responses, promoting the development of Th2 and Treg cells as has been previously reported by this cytokine environment (Le Gros *et al.*, 1990; Roncarolo *et al.*, 2008; Skapenko *et al.*, 2005), while inducing the activation of other innate cells into regulatory states (Mantovani *et al.*, 2007). Furthermore, this macrophage phenotype may pose as one of the main initiators of Th2 responses, as the initial source of IL-4 during schistosome infection has previously been questioned. Understanding mechanisms involved in IL-4 production will be extremely beneficial in the context of inflammatory illnesses. IL-4, as opposed to IL-12, is associated with protection from EAE (Shaw *et al* 1997). Through the expression of RELMα and SPHK1, this activation state may be beneficial in both wound healing (Raes *et al.*, 2002) as well as suppression of overzealous T cell responses (Nair *et al.*, 2009; Pesce *et al.*, 2009). These characteristics may therefore be beneficial in protection against severe schistosomiasis as well as EAE and other pro-inflammatory diseases which require immunoregulation.

Features of anti-inflammatory macrophages

Classically activated macrophages, IFNγ producing Th1 cells and IL-23-induced Th17 cells are implicated as major cell types associated with the pathogenesis of EAE (Cua *et al.*, 2003; Langrish *et al.*, 2005; O'Connor *et al.*, 2008; Tran *et al.*, 1998). Conditions which alter the immune response and establish an anti-inflammatory environment to prevent the accumulation of inflammatory cells, protect against EAE. This has been demonstrated in mice, by the administration of type II activated macrophages or pre-infection with parasites of the *Schistosoma spp.*, which deviate the immune response into a polarised Th2/Treg/anti-inflammatory status and prevent the initiation of inflammatory disease (Sewell *et al.*, 2003; Tierney *et al.*, 2009). Only minimal information on the mechanisms of protection provided by schistosome infection or type II macrophages is known, therefore we investigated a broader range of effects of type II activation in order to expand our current knowledge, and to gain a greater understanding of mechanisms of action.

Type II activation reduced the production of the chemokine MCP-1 in an inflammatory environment, as well as IL-6. High levels of IL-6 and MCP-1 are attributed to disease progression (Maimone *et al.*, 1997; McManus *et al.*, 1998), and inhibition of MCP-1 as well as IL-6 have proven successful in preventing the infiltration of inflammatory cells into the CNS (Huang *et al.*, 2001; Serada *et al.*, 2008). Although this investigation, in accordance with previous literature, demonstrated the suppressive effects of IL-10 on the expression of pro-inflammatory cytokines and chemokines (Anderson *et al.*, 2002; Bogdan *et al.*, 1991), we found IL-10 was not responsible for the anti-inflammatory profile elicited by type II activation. This has been previously demonstrated by Anderson *et al*, but only with regards to IL-12 reductions. Our investigations support that of Anderson *et al*, while further illustrating that reductions in the expression of co-stimulatory markers, and inflammatory mediators MCP-1 and IL-6 by type II activation, occur independently of IL-10. This implies the existence of a novel mechanism whereby selective pro-inflammatory responses are reduced by Fcγ receptor ligation. Also demonstrated here is the broader effect of type II activation on chemokine and cytokine production. Although previous studies (Anderson *et al.*, 2002; Gerber *et al.*, 2001; Grazia Cappiello *et al.*, 2001) have concluded type II activation has no effect on other pro-inflammatory cytokines such as IL-6 and TGFβ, the observations made were at the mRNA level, and not the secreted protein. This is the first study to my knowledge where a reduction in MCP-1 and IL-6 protein by type II activation is demonstrated, illustrating additional mechanisms other than through reduced IL-12, which may aid in protection against EAE.

As with CD40 reductions, another shared characteristic of anti-inflammatory phenotypes induced by type II activation or exposure to schistosome complexes, was the expression of spingosine kinase 1 (SPHK1), an enzyme whose activity *in vivo* is still controversial. SPHK1 is expressed in a variety of cells including T cells, mast cells, and basophils (Kihara *et al.*, 2006; Yang *et al.*, 2005). However, its significant up-regulation in macrophages has so far only been attributed to type II activation (Edwards *et al.*, 2006). In this investigation, we reported a significant increase in this enzyme in response to schistosome immune complexes. Our investigations, as well as previous literature, illustrate the anti-inflammatory profile of macrophages elicited by exposure to schistosome immune complexes as well as type II-inducing stimuli, with both implicated in the biasing of T cell responses into the Th2 subset (MacDonald *et al.*, 2002b; Okano *et al.*, 1999; Zheng *et al.*, 2008). Therefore, it is possible that in the context of the macrophages, SPHK1 supports functions involved in promoting Th2 development. SPHK1 activation in Th1 cells has demonstrated a role in reducing Th1 associated cytokines IFNγ and IL-2 (Yang *et al.*, 2005), therefore it is possible that similar effects occur, caused by macrophage-derived SPHK1. Humans and mice express three isoforms of SPHK1, all of

which exhibit slight variations in their catalytic activities (Billich *et al.*, 2003; Kihara *et al.*, 2006). Therefore, it is possible that some of the conflicting results observed *in vivo*, such as its' role in promoting inflammatory responses and cell migration (Melendez, 2008), were due to the activity of the different isoforms under different environmental conditions, as well as the level, location and source of SPHK1. To date, the exact role of SPHK1 in macrophages remains elusive, especially with regards to type II activated macrophages. As SPHK1 is expressed in type II activated macrophages as well as macrophages activated into the 'novel' state by exposure to schistosome complexes, it seems likely that SPHK1 contributes in some way to the anti-inflammatory outcome, and therefore warrants further investigation.

These investigations also illustrated a reduction in CD40 associated with increased anti-inflammatory functions. Supporting this notion is the reduction in LPS-induced CD40 expression which occurred in response to both schistosome immune complexes and SRBC immune complexes. Studies have shown that an active component of schistosome egg antigen (SEA), Omega-1, significantly increases Th2 responses and reduces Th1 development, with a reduction in CD40 expression on dendritic cells observed (Steinfelder *et al.*, 2009). However, the investigators did not assess the significance of this reduction at the time. As with Steinfelder *et al*, we saw a reduction in CD40 in response to schistosome immune complexes as well as type II-inducing stimuli on macrophages, as opposed to dendritic cells. CD40 promotes the production of inflammatory cytokines such as IL-6, IL-12 and IL-23, all of which are associated with the induction of pathology-inducing Th1 and Th17 subset development (Langrish *et al.*, 2005; Perona-Wright *et al.*, 2009). In mice deficient in CD40, a significant inhibition of Th17 and Th1 development is noted, through the reduction of IL-6, IL-23 and IL-12 production, which therefore protects against EAE (Perona-Wright *et al.*, 2009). The reduction in CD40 on macrophages exposed to type II-inducing stimuli or schistosome complexes, may therefore significantly impair the development of Th17 cells through the reductions in inflammatory cytokines, as well as reducing the infiltration of inflammatory cells through reduced chemokine production. Therefore, this investigation puts into focus another possible mechanism contributing to the protection type II activation and schistosome infection provide against EAE.

Involvement of CD40 signalling in T cell biasing

Although CD40 expression has long been associated with Th1-driven cell mediated responses (Alderson *et al.*, 1993; Campbell *et al.*, 1996), studies in 2002 by MacDonald *et al* illustrated the essential requirement of CD40 signalling for the induction of Th2 responses by dendritic cells. Type II activation or schistosome infection protect against EAE (La Flamme *et al.*, 2003; Sewell *et al.*, 2003; Tierney *et al.*, 2009), and as

demonstrated in this thesis, macrophages express reduced CD40 in the absence of complete inhibition upon exposure to either stimuli. Furthermore, both are capable of polarising Th2 responses (Gerber *et al.*, 2001; La Flamme *et al.*, 2006; Zheng *et al.*, 2008). This suggests to us that low but maintained levels of CD40 on macrophages (i.e. not completely abolished) have a role in deviating T cell responses. This thesis therefore assessed the effect of altered levels of CD40/CD40L signalling between T cells and macrophages, with the aim of identifying if there was a particular low level which enhances Th2 cell biasing.

Although we did not enhance type II activation through the inhibition of CD40/CD40L signalling, we did see significant differences in T cell responses. These differences depended on the level of CD40/CD40L signalling. An example was the loss of IL-10 suppression with T cells that were exposed to classical macrophages after the signalling was reduced. Furthermore, low levels of CD40/CD40L inhibition had no significant effect on Th2 polarisation by type II activation, while Th1 development was still significantly impaired. An inhibitory effect on both Th2 and Th1 responses was seen in the complete absence of CD40/CD40L signalling however. Further studies may be required; however this investigation suggests that at low levels of CD40L signalling, Th1 development is reduced, whereas there is little effect on the responsiveness of T cells to Th2-inducing stimuli by type II activation. In the setting of inflammatory illnesses, macrophages which elicit this low level of CD40/CD40L interaction seen by type II macrophages may be beneficial in depressing detrimental Th1 responses while maintaining protective Th2 development. This would somewhat explain why in some circumstances the absence of CD40 results in the ablation of Th2 development (MacDonald *et al.*, 2002b), whereas in other situations, CD40L inhibition protects against disease by increasing Th2 development, as seen in the prevention of graft rejection (Hancock *et al.*, 1996). This may be a result of the preferential inhibition of Th1 and not Th2 responses, when there is incomplete blockage of CD40L.

Major producers of IL-10 include Th2 cells (Sornasse *et al.*, 1996) as well as Tregs (Groux *et al.*, 1997). Although IL-2Rα and IL-4Rα expression are commonly associated with the induction of Th2 responses (Cote-Sierra *et al.*, 2004), it is also possible that type II activated macrophages induce Tregs. Studies have shown IL-4 to induce Tregs from naive CD4 T cells, resulting in high expression of IL-2Rα (CD25) and increased IL-10 (Skapenko *et al.*, 2005), which are characteristics seen in our studies. Furthermore, McKee *et al* demonstrated the role of both Tregs and Th2 cells in the suppression of Th1 development during schistosomiasis (McKee *et al.*, 2004), illustrating the possible effect of schistosome complexes or type II activation on Treg development. Further investigations would be required to assess this induction, however as proliferation was not inhibited by T

cells exposed to type II macrophages, it may be fair to assume the responses were that of a Th2 polarized subset.

Our results support previous reports (MacDonald *et al.*, 2002b), whereby the complete absence of CD40 results in a reduction in the development of Th2 cells, as well as supporting the notion that differential levels of CD40 signalling alter counter-regulatory responses (Mathur *et al.*, 2004). Also provided in this thesis are some novel insights into the suppressive effects of CD40/CD40L signalling on IL-10 production, a mechanism which may be a contributing factor to the suppression of both Th1 and Th2 responses seen at high levels of CD40 inhibition, and may possibly be involved in the induction of suppressive regulatory T cells (Wan *et al.*, 2009).

6.2 Summary

Further supporting previous literature (Mathur *et al.*, 2004; Murugaiyan *et al.*, 2006), this thesis illustrates the intricate balance a single co-signalling molecule can elicit. As with MacDonald *et al*, CD40 was found essential for the induction of Th2 responses, which in our setting was elicited via type II activation. IL-10, although suppressive of IL-12 as also previously reported (D'Andrea *et al.*, 1993), was not required for the cytokine and surface marker profile elicited by type II activation. This supports studies by Grazia Cappiello *et al*, who saw no effect in the ability of type II macrophages to reduce IL-12, while extending the knowledge to the effects on other cytokines/chemokines and surface markers which were not previously investigated. As well as providing further insight into alterations type II activation induces at both the level of macrophages and T cells, we present previously unreported effects of schistosome complexes on macrophage activation, illustrating the induction of what seems to be a novel activation state exhibiting a hybrid of characteristics of type II and alternative activation. The findings from this thesis bring us a step closer to understanding mechanisms involved in promoting anti-inflammatory immune responses, which is essential for identifying more effective treatments for autoimmunities. Furthermore, although much remains to be validated, provided here are some new avenues of research for future investigations into understanding essential components of immune deviation.

6.3 Future

Future studies may involve investigating the roles of SPHK1, a shared marker of both type II activation and the ‘hybrid’ state induced by schistosome immune complexes. As both type II activation and schistosome infection protect against EAE, SPHK1 and RELMα may be important contributors to this effect (La Flamme *et al.*, 2003; Sewell *et al.*, 2003; Tierney *et al.*, 2009; Zheng *et al.*, 2008). Investigations may also be carried out to elucidate whether the activation state induced by schistosome complexes *in vitro*, occurs

in vivo, and whether it contributes to protection against EAE. As T cell proliferation in response to type II activation and classical activation were similar despite the reduction in PDL-1 on type II macrophages, further investigations are required to elucidate the role of PDL-1/PD-1 signalling during type II activation. This co-signalling interaction, as with CD40L signalling, may be required for Th2 cell biasing. Further understanding the effect of co-signalling molecules on T cell biasing may have important implications in the treatments of not only pro-inflammatory associated diseases, but also those diseases such as leishmaniasis which require efficient Th1 responses (Stenger *et al.*, 1996). The suppressive effect of CD40/CD40L signalling on anti-inflammatory cytokines such as IL-10 should also be further investigated, as should the effects of increasing CD40 signalling on T cell responses in contrast to CD40 inhibition. Although CD40 inhibition was unable to increase the anti-inflammatory Th2-biasing capabilities, it would still be interesting to investigate the outcomes of different levels of increased CD40/CD40L signalling.

Identifying ways to enhance anti-inflammatory responses against EAE will not only benefit the development of treatments for MS, but will also have positive implications in the treatment of other inflammatory illnesses. Furthermore, the increase in knowledge about mechanisms involved in different macrophage phenotypes will enable us to better identify mechanisms involved in promoting or preventing diseases.

Appendix A-Supplementary Tables and figures

TableA1 Final concentrations/dilutions of ELISA reagents

IL-12p40 (BD Biosciences)

Reagent	Dilution/concentration	Diluent
Capture	1:1000	0.1 M Na_2HPO_4, pH=9.0
Standards	4 ng/ml	5% FCS in PBS pH=7.4
Detection	1:1000	5% FCS in PBS pH=7.4
SA-HRP	1:2000	5% FCS in PBS pH=7.4

IL-10 (BD Biosciences)

Reagent	Dilution/concentration	Diluent
Capture	1:500	0.1 M Na_2HPO_4, pH=9.0
Standards	25 ng/ml	10% FCS in PBS pH=7.4
Detection	1:2500	10% FCS in PBS pH=7.4
SA-HRP	1:1000	10% FCS in PBS pH=7.4

IFNγ (BD Biosciences)

Reagent	Dilution/concentration	Diluent
Capture	1:1000	0.1 M Na_2HPO_4, pH=9.0
Standards	4 ng/ml	5% FCS in PBS pH=7.4
Detection	1:4000	5% FCS in PBS pH=7.4
SA-HRP	1:2000	5% FCS in PBS pH=7.4

IL-4 (BD Biosciences)

Reagent	Dilution/concentration	Diluent
Capture	1:2000	PBS, pH=7.4
Standards	50 U/ml	5% FCS in PBS pH=7.4
Detection	1:4000	5% FCS in PBS pH=7.4
SA-HRP	1:2000	5% FCS in PBS pH=7.4

Table A2 Western blot antibody dilutions

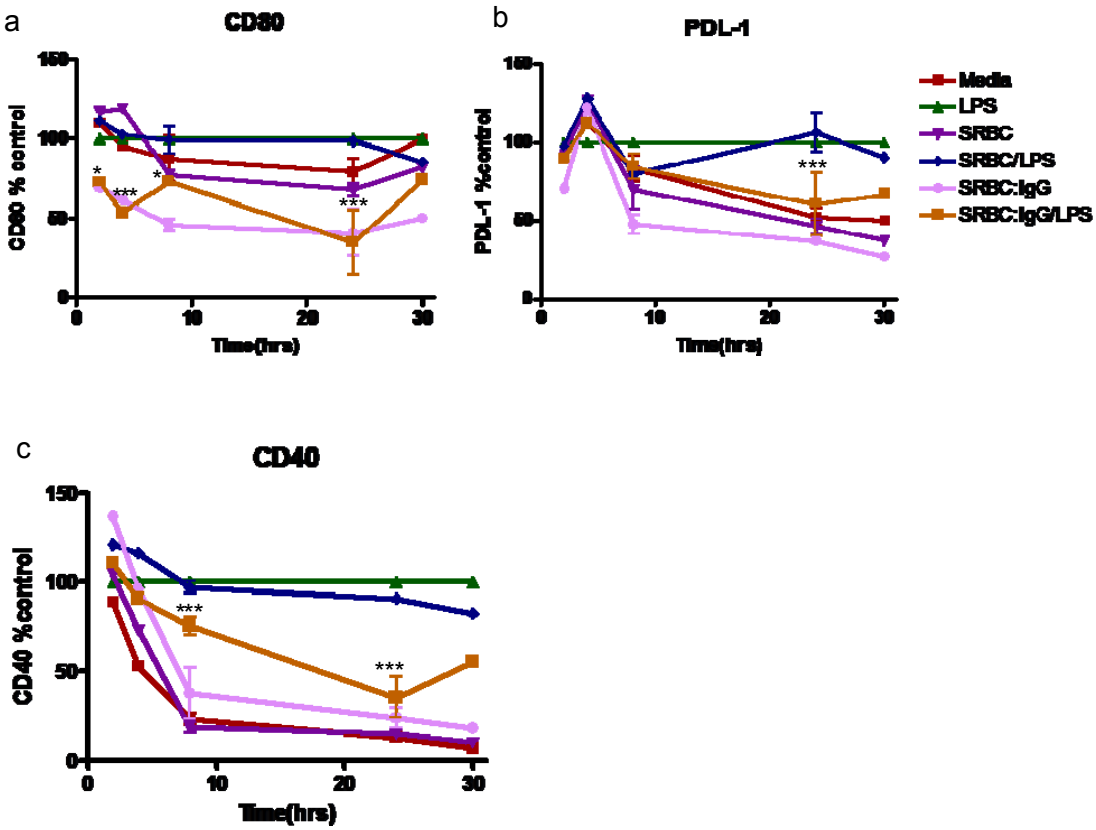
Primary Antibody	Company	Dilution
Anti-tubulin-Rabbit	Abcam	1:700
Anti-SPHK1-Rabbit	Abcam	1:700
Anti-RELMα-Rabbit	Abcam	1:700
Anti-iNOS-Mouse	Transduction Laboratories	1:700

Dilutions of primary antibodies made in 1.5-2 ml of 5% blocking solution (5% non-fat skim milk in TTBS)

Secondary Antibody	Company	Dilution
Anti-Rabbit cy3	GE Healthcare	1:1000
Anti-Mouse cy5	GE Healthcare	1:660

Dilutions of secondary antibodies made in 1.5-2mls of TTBS (appendix B)

Kinetic graphs for type II activation, with additional controls for the absence of LPS



FigA1 Macrophages exposed to type II-inducing stimuli exhibit reductions in co-signaling molecules maximally at 24 h post stimulation: Macrophages derived from C57BL/6 mice were stimulated with 200 ng/ml LPS alone or LPS in the presence of SRBC or opsonised SRBC (SRBC:IgG). Cells were also left non-stimulated in media or cultured with SRBC or SRBC-IgG in the absence of LPS. After 2, 4, 8, 24, or 30 h of culture, CD80 (a), PDL-1 (b), and CD40 (c) were assessed via flow cytometry. * P<0.05 ,**P<0.01 ,***P<0.001: Two way ANOVA with Bonferoni post test : SRBC:IgG+LPS vs LPS. Data points represent the mean +S EM of two experiments.

Effects of CD40 signaling on macrophage phenotype- 24 hour time point (as opposed to the presented 8 hour time point).

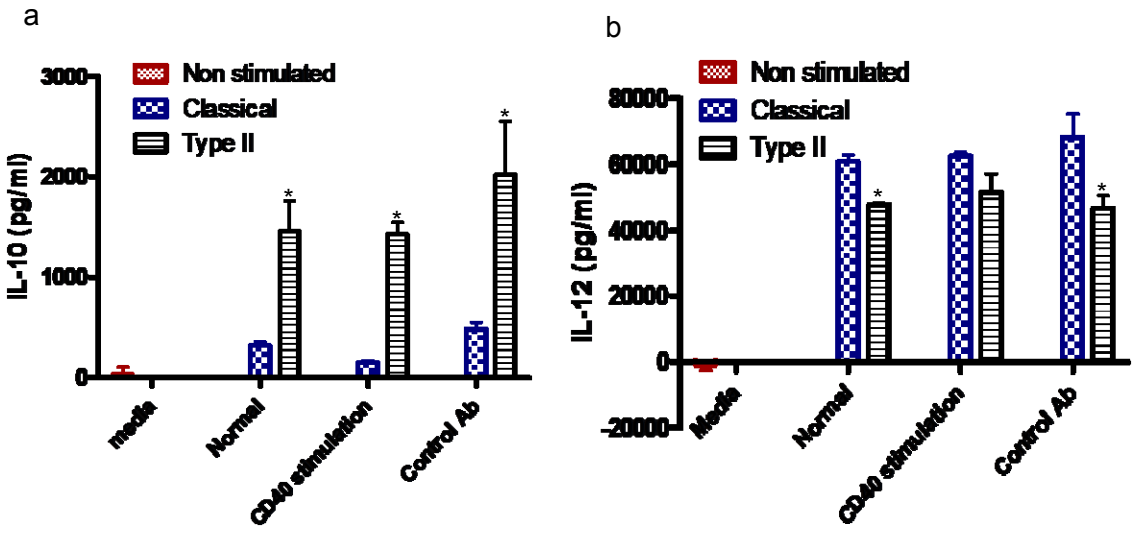
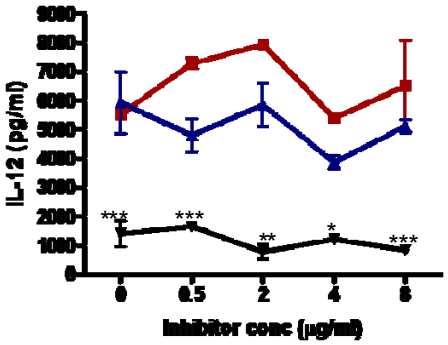


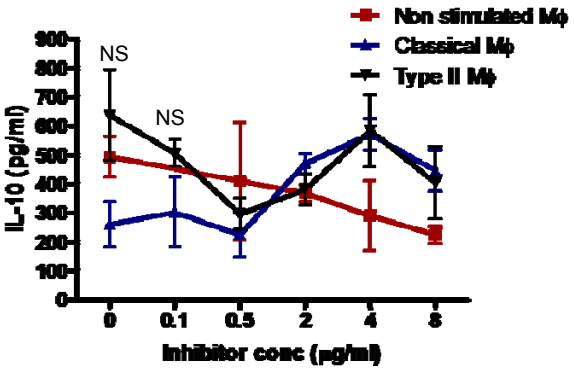
Figure A2 CD40 stimulation does not effect the IL-10/IL-12 ratio between classical and type II macrophages at 24 h after stimulation Macrophages (10^6 cells/ml) were exposed to classical or type II activating stimuli. Stimulatory rat-anti-mouse CD40 antibody (3/23) and IgG isotype control (rat IgG2) (8 µg/ml) (BD Bioscience) were added to appropriate cultures at the time of primary stimulation. After 24 h culture IL-10 and IL-12 were assessed via ELISA. *P<0.05 two way ANOVA with Bonferroni post-test; Type II versus Classical. Bars represent datapoints (mean+SEM) of duplicate wells.

Graphs on CD40/CD40L alterations, with data from co-cultures with non-activated macrophages: Supports the notion that remaining IFNγ (From overnight priming) in co-cultures elicited T cell responses in the absence of stimulants.

a



b



c

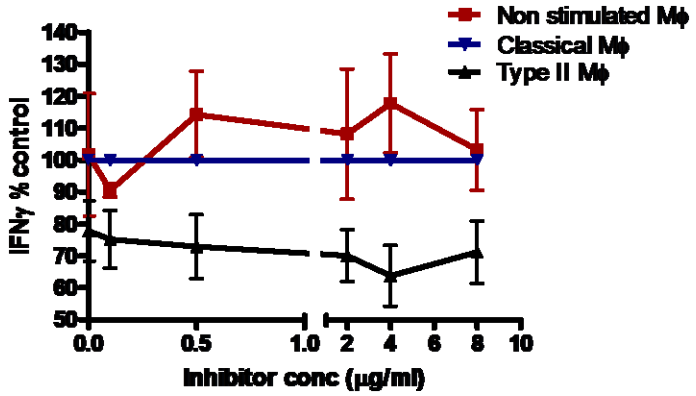


Figure A3 CD40/CD40L inhibition has no effect on IL-12 or IFNγ suppression by type II macrophages but alters IL-10 production Macrophages (10^6 cells/ml) were exposed to classical or type II activating stimuli. CD4 T cells were isolated from OTII mice and stained with CFSE. CD4 T cells ($2.5x10^6$ cells/ml) plus 1 µM $OVA_{323-339}$ peptide was added to macrophage cultures, along with varying concentrations (0.1-8 µg/ml) of anti-mouse CD40 ligand antibody (MR1) 3 h after classical or type II activation. After 72 h culture IL-12 (a), IL-10 (b), and IFNγ (c) were assessed via ELISA. *P<0.05 **P<0.01 ***P<0.001: Two way ANOVA with Bonferroni post test ; responses to Type II vs Classical MØ. Graphs represent datapoints (mean + SEM) from two experiments. (c) is presented as % of control compared to cultures with classical macrophages.

Kinetic graphs for schistosome egg exposure with additional controls in the absence of LPS

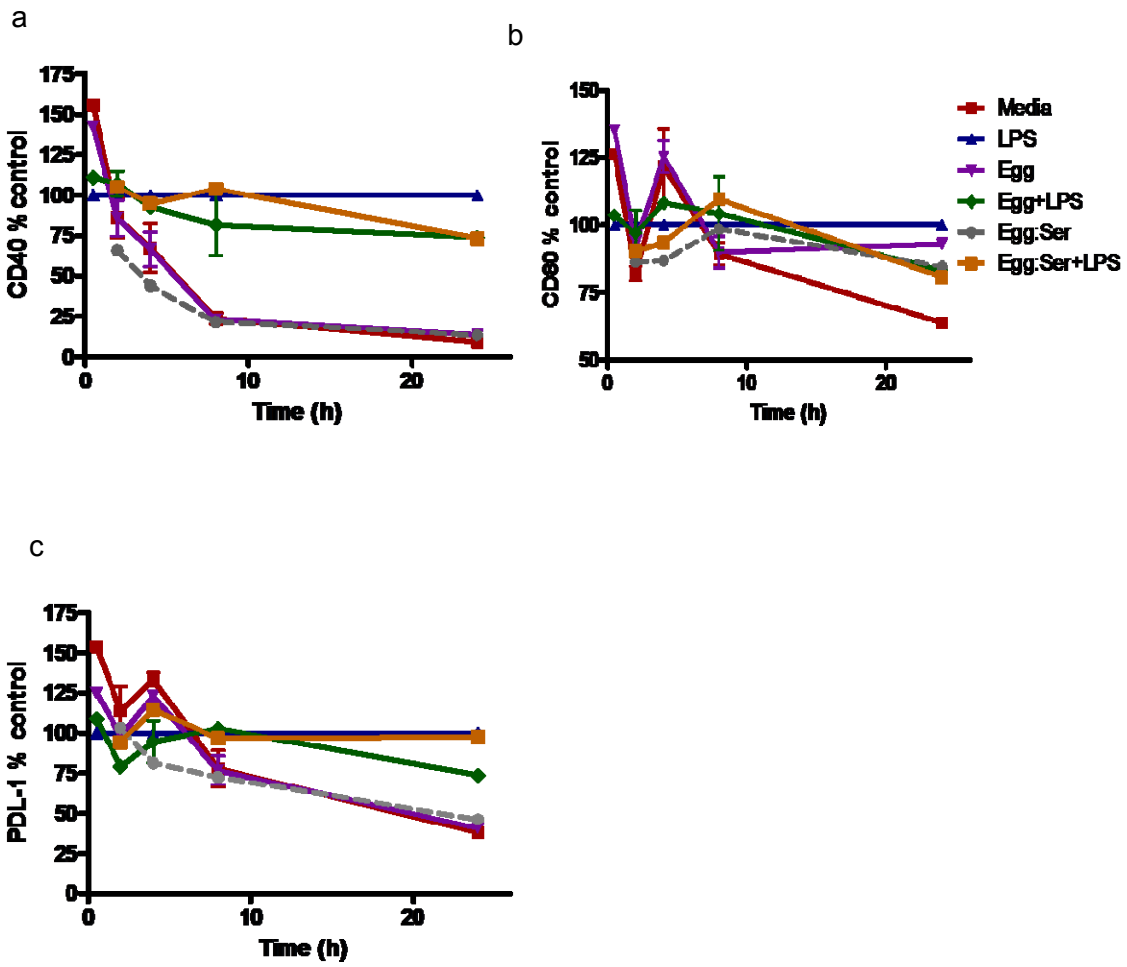


Figure A4 Schistosome egg and egg complex do not induce significant alterations in macrophage surface markers. Primary BMMØ (10^6 cells/ml) derived from C57BL/6 mice were primed with 20 U/ml IFNγ overnight followed by stimulation with Media, LPS (200 ng/ml), Schistosome egg (1000/well), Egg+LPS, opsonised Egg (Egg:ser), or opsonised egg + LPS (Egg:Ser+LPS). Cells were also exposed to Egg of Egg:ser in the absence of LPS and incubated at 37°C in 5%CO_2. After 0.5, 2, 4, 8 and 24 h culture CD40 (a), CD80 (b) and PDL-1 (c) were assessed via flow cytometry. Data represents the mean +SEM of two experiments, except for the 24 h time point which represents one experiment. Data is presented as % of control compared to cultures with LPS.

Graph illustrating the similar effect 1000 and 2000 schistosome eggs per well have on macrophage activation

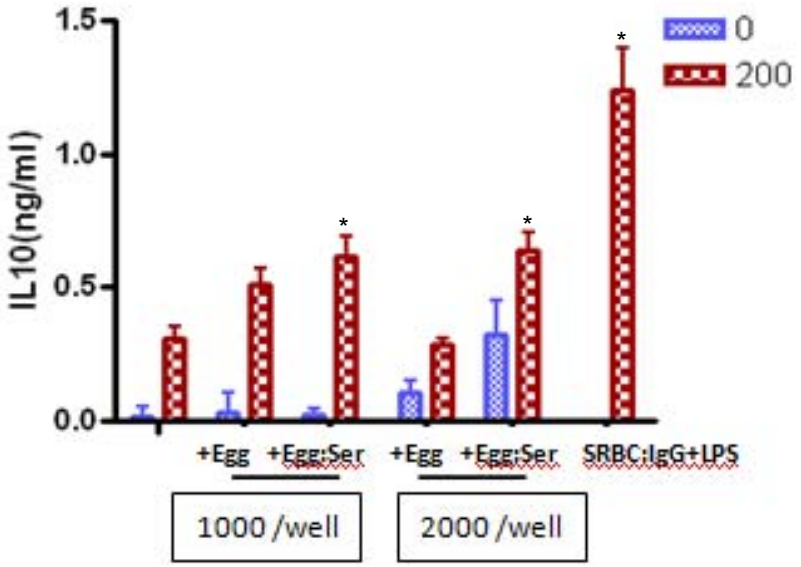


Figure A5 Schistosome eggs at either 1000/well or 2000/well elicit similar responses Primary BMMØ (10^6 cells/ml) derived from C57BL/6 mice were primed with 20 U/ml IFNγ overnight followed by stimulation with Media, LPS (200 ng/ml), Schistosome egg either at 1000/well or 2000/well in the absence of presence of LPS, or Egg complexed to serum (Egg:Ser) in the presence or absence of LPS. After 8 h culture IL-12 was assessed via ELISA. *P<0.05. One way ANOVA followed by Bonferroni post test; treatments vs LPS. Graphs represent the mean + SEM from duplicate wells.

Optimisation of IFNγ concentration for priming macrophages prior to stimulation- identifying which concentration provides the greatest inflammatory response

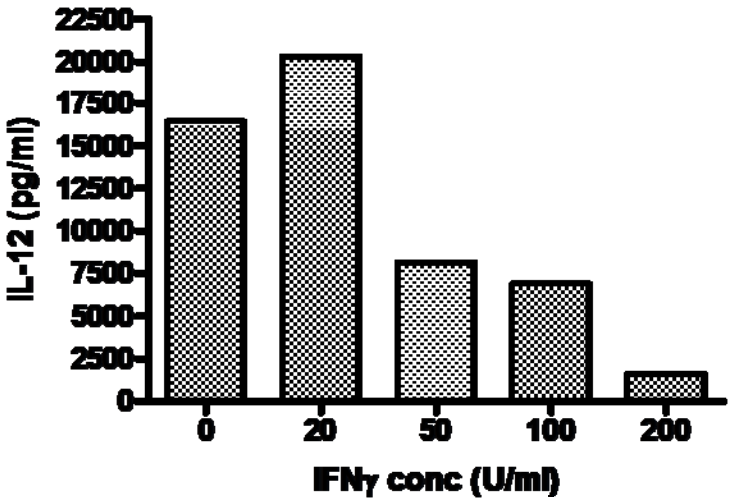


Figure A6 Priming macrophages with 20 U/ml IFNγ generates the most inflammatory conditions for further investigations. Primary BMMØ (10^6 cells/ml) derived from C57BL/6 mice were primed with 20, 50, 100 or 200 U/ml IFNγ overnight followed by stimulation with LPS (200 ng/ml), and incubated at 37^{o}C in 5%CO_2. After 8 h culture, IL-12 was assessed via ELISA.

Appendix B-Recipes

Isolation buffer (For CD4 T cell isolation)

2% FCS (ICP biologicals, Auckland)

2 mM EDTA (Merk)

1 x dPBS (Invitrogen, Auckland,NZ)

Phosphate buffered saline (PBS) (10x)

For 2 L of 10 X PBS:

170 g NaCl (Merck Ltd, Palmerston North, New Zealand)

62.32 g $Na_2HPO_4.12H_2O$ (Merck)

4.04 g $NaH_2pPO_4.2H_2O$ (Merck)

2 L ddH_2O

Facs Buffer (For flow cytometry)

0.1% sodium azide

97.9% PBS

2% FCS (ICP biologicals, Auckland)

Complete T cell medium (CTCM)

10% FCS

1% Hepes, Penstrep, Non-essential amino acids, L-Glutamine (Invitrogen)

0.1% β-Mercaptoethanol (Invitrogen)

85.9% Dulbecco's modified eagle medium (dMEM) (Invitrogen)

Wash buffer

96% Dulbecco's modified eagle medium (dMEM) (Invitrogen)

3% Hepes

1% Penstrep

Flow cytometry antibodies

Antibody	Company
Anti- CD40- PE (3/23)	(BD Bioscience)
Anti-CD80- FITC (16-10A1)	(BD Biosciences)
Anti-IgG2a –PE (eBR2a)	(BD Bioscience)
Anti- I-A^b- PE (AF6-120.1)	(BD Biosciences)
Anti-F4/80 bio (C1:A3-1)	(Serotec, Oxford, UK)
Anti- CD124-PE (mIL4-M1)	(BD Biosciences)
Anti-PDL-1- PE (M1H5)	(eBioscience, CA, USA)
Anti-PDL-2- PE (TY25)	(eBiosciences)
Anti-mouse-CD16/32 (2.4G2)	(BD Biosciences)
SaV CyC	(BD Biosciences)
Anti-CD25-FITC (3C7)	(BD Biosciences)
Anti-CD4-Cyc (RM4-5)	(BD Biosciences)
Anti-IgG2a- FITC (R35-95)	(BD Biosciences)

Tris buffered saline 10X (TBS) (500ml)

(Tris 200 mM, Nacl 1.36 M, pH 7.6)

2.1 g Tris, 40 g NaCl, 12 ml HCl to give pH7.6, dd$H_2$0 up to 500 ml

Tween Tris buffered saline (TTBS)

995 ml TBS (1X), 5 ml Tween-20

Tris glycine buffer X5

(Tris 125 mM, Glycine 950 mM)

Glycine 72.1 g, Tris 15.1 g, ddH_2O up to 1 L

Working transfer buffer

(Tris 25 mM, Glycine 192 mM , methanol 10% (v/v))

600 ml ddH_2O, 200 ml Tris Glycine buffer 5 X, 200 ml methanol

Running buffer SDS

(Tris 25 mM, Glycine 192 mM, SDS 0.1%)

1.0 g SDS, 200 ml Tris Glycine buffer 5 X, ddH_2O up to 1 L

RIPA Buffer

Tris-HCl: 1.2110 ,NaCl: 8.766 ,EDTA: 0.3722 ,TritonX-100: 10 ml ,SDS: 1.0 ,sodium deoxycholate: 10.0 (in g/L) .

Western Blott Gels:

<u>10% separating gel:</u>

ddH20 - 8ml

1.5 M tris 8.8 - 5ml

10% SDS - 200 µl (USB, Cleveland,Ohio)

Acrylamide - 6.66 ml (Biorad)

10% ammonium persulfate (APS) - 100 µl

TEMED - 10 µl (Amersham, England)

<u>4% stacking gel:</u>

ddH20 - 6.1 ml

1.5 M tris pH 6.8 - 2.5 ml

10% SDS - 100 µl

acrylamide - 1.33 ml

10% APS - 50 µl

TEMED - 10 µl

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