# Type II Activation of Macrophages and Microglia:
# Roles in T cell biasing and experimental autoimmune encephalomyelitis

**By**

**Sarrabeth Marie Stone**

**A thesis**
**submitted to the Victoria University of Wellington**
**in fulfilment of the requirements for the degree of**
**Doctor of Philosophy**

**Victoria University of Wellington**
**2013**

## Abstract

Multiple Sclerosis (MS) is an immune-mediated disease of the central nervous system (CNS) which causes demyelination and damage to the neuronal axons. MS is a significant health problem in New Zealand, affecting 1 in 1400 people. One of the major cell types involved in MS and its animal model, experimental autoimmune encephalomyelitis (EAE), are the proinflammatory subsets of T helper (Th) cells. However, many other cell types are also involved, including macrophages (MΦ) and microglia (MG). MΦ and MG are considered to be important drivers of inflammation during MS; however there is also evidence that indicates these cells can also play a protective role. In the periphery, MΦ can be induced into several activation states. One of these activation states, type II activation, is a regulatory phenotype, producing increased IL-10 and decreased IL-12 compared to classical activation. *In vitro* type II activation is induced by ligating FcγR with immune complexes (IC) with concurrent stimulation with lipopolysaccharide. Previous research has shown that type II activated MΦ and type II-inducing treatments are protective in EAE.

MΦ activation state affects the way the Th response develops, with classically activated MΦ promoting a Th1 response and type II MΦ promoting a Th2 response. In the current study, the role of MΦ in T cell biasing is investigated further. Type II activated MΦ altered the Th1/Th2 dichotomy away from a Th1 response and towards a Th2 response, as demonstrated by decreased production of IFN-γ and increased levels of CD124. Also, in a novel finding, type II activated MΦ also increased the production of IL-17A from T cells. This study also aimed to elucidate the pathways involved in biasing of the T cell response by classical and type II MΦ by blocking or enhancing specific pathways. It was found that, while the level of IFN-γ (the prototypical Th1 cytokine) was largely dependent on the levels of IL-10 and IL-12, IL-17A and CD124 expression appeared to be independent of these two cytokines. Type II MΦ have decreased expression of CD40 and PD-L1, it was found that these pathways are not strongly involved in T cell biasing by type II MΦ.

While it is acknowledged that MG can be pathogenic and protective, the direct effect type II activating treatments have on MG is unknown. In order to investigate whether MG can also be type II activated, MG were isolated from the CNS of adult mice their phenotype under different activating condition was assessed. Under type II activating conditions MG produced less IL-12 and more IL-10, suggesting type II activation is occurring. In addition, in

MG:T cell co-cultures, T cells cultured with classical or type II activated MG have similar, but not identical, profiles to T cells cultured with MΦ. Type II activated MΦ induced increases in CD124 and IL-17A from T cells, however, all MG activation states induced similar levels of IFN-γ.

To determine the effect of type II activating treatments *in vivo* bone marrow chimeric mice were created using mice congenic for CD45, which allow MG to be distinguished from invading cells. Treatment of mice with the IC before and during EAE results in decrease the incidence and severity of disease. IC treatments altered both the peripheral and the CNS immune environments. Despite inducing protection, IC treatments induced an increase in IL-17A in the peripheral immune system. In the brain, a population of resident cells that are $CD45^{int}$CD11b positive, and are likely to be CNS associated MΦ express decreased levels of MHC class II, suggesting a decreased ability to interact with T cells. Furthermore, parenchymal MG from the brains of IC treated animals have different levels of Iba1 compared to those from untreated animals, suggesting differential activation. Overall the data suggests that IC treatment induces a change in the activation state of CNS resident immune cells, that is likely to be protective in EAE.

## Acknowledgements

Firstly, I would like to thank my supervisor Anne La Flamme for her support and advice over the last few years and for giving me the opportunity to do my PhD. Her guidance and knowledge was invaluable throughout the course of my PhD. I would also like to thank my secondary supervisor Diane Kenwright for her support and encouragement.

To all the members of LAF past and present: Amanda, Bhagyashree, Dahna, Danielle, Dave, Degee, Hanane, Hannah, Jenni, Jonnel, Laura, Lisa, Maddie, Marie, Nikki, Pirooz, Praneta, Ryan, and Sara. I would like to thank you all for your help and support, coffee breaks and science talk. I could not have asked for a better group of people to work with. I would also like to thank all the members of the JHM group, and the proteomic group for helping to make the lab happy place to be. And also Sarah and Hannah from proteomics, for filling the lab with music.

I would particularly like to thank Marie, Maddie and Laura, who helped me with some of my big experiments and also for editing and formatting help. There is no possible way I could have done those experiments without your help. In addition a huge thanks to Marie for helping me out and taking me to the doctors when my foot broke its self in the lab, and for getting me safely home again. Thanks also for helping to get around in the first couple of weeks in the cast when I couldn't get down my stairs alone, I would have missed vital points in my experiments if it wasn't for your very kind assistance. I would also like to thank George; you're doing great, keep it up!

A big thank you to Alfonso and Brigitta from and MIMR, who isolated MG on the FACS Vantage DiVa cell sorter for me. And also to the BRU staff at the MIMR for assistance in making bone marrow chimeras. I would also like to thank Taryn Osmond for teaching me how to do i.v. injections, I don't think I will ever be as good at them as you are.

I would also like to thank the people who let me pick their brains on a variety of topics and techniques, particularly Ryan and Arun, whose advice regarding qPCR was invaluable.

To Ken for Taylor Preston's for the kind donation of fresh sheep blood that allow me to undertake this research.

A big thank you also goes to all the people who work and study on the third floor of AM, for sharing the highs and lows of doing a PhD with me, you guys certainly keep things interesting. I would especially like to thank Sarah, Clare, and Dylan, who were always close by when I needed to talk to someone, or someone to go forage for chocolate with.

I would like to thank my brothers Daniel and Galen, and his partner Theo, for all their support and encouragement throughout my studies, and for providing me with a much needed link to the outside world. And also to my parents who have been a constant source of support and have always shown enduring optimism and faith in by abilities. I would also like to thank my Uncle Mike, who has always been a big supporter of my choice to pursue a career in science. And also to the five furry four footed fiends, who didn't care if my experiments were working, as long as they got cheese.

I would also like to thank the Lotteries foundation for the PhD scholarship that allowed me to complete my PhD.

# Contents

## List of Figures

## List of Tables

## List of abbreviations

AG- aminoguanidine hemisulfate
APC- Antigen presenting cell
BBB- Blood brain barrier
BMMΦ- Bone marrow derived macrophage
BRU- Biomedical research unit
BSA- Bovine serum albumin
CBA- Cyometric bead array
CFSE- Carboxyfluorescein succinimidyl ester
CNS- Central nervous system
ConA- Concanavalin A
CTLA-4- Cytotoxic T lymphocyte-associated antigen
DAPI- 4',6-diamidino-2-phenylindole
DC- Dendritic cell
EAE- Experimental autoimmune encephalomyelitis
EBV- Epstein-barr virus
ECM- Extracellular matrix
EDTA- Ethylenediaminetetraacetic acid
ELISA- Enzyme linked immunosorbent assay
FACS- Fluorescence activated cell sorting
FcR- Fc receptor, binds constant region of antibodies
FCS- Foetal calf serum
FOXP3- Forkhead box P3
GFP- Green fluorescent protein
HLA- Human leukocyte antigen
Iba1- Ionised calcium binding adaptor molecule 1
IC- Immune complex, in this work IC refers to sheep red blood cells opsonised with anti-sheep red blood cell IgG
IFN- Interferon
Ig- Immunoglobulin
IL- Interleukin
IL-4Rα- Interlukin-4 receptor alpha chain, CD124
iNOS- Inducible nitric oxide synthase
i.p.- intra peritoneal
IVIG- intravenous immunoglobulin
LPS- Lipopolysaccharide
MBP- Myelin basic protein
MDSC- Myeloid derived suppressor cell
MG- Microglia
MHC- Major histocompatibility complex
MIMR- Malaghan institute of medical research
MOG- Myelin oligodendrocyte glycoprotein
MS- Multiple sclerosis
MΦ- Macrophage

NK- Natural Killer
NO- Nitric oxide
OVA- Ovalbumin
PAMP- Pathogen associated molecular patterns
PBS- Phosphate buffered saline
PCR- Polymerase chain reaction
PD-1- Programmed death 1
PD-L1- Programmed death ligand 1
PD-L2- Programmed death ligand 2
PE- Phycoerythrin
PFA- Paraformaldehyde
PLP- Proteolipid protein
PML- Progressive multifocal leukoencephalopathy
PRR- Pattern recognition receptors
RBC- Red blood cell
RORγt- Retinoic acid-related orphan receptor
RT- Reverse transcription
qPCR- Quantitative polymerase chain reaction
S1P- Sphingosine-1-phosphate
s.c.- Sub-cutanious
SPHK- Sphingosine kinase
SRBC- Sheep red blood cell
STAT- Signal transducer and activator of transcription
T-bet- T-box expressed in T cells
TCR- T cell receptor
TEC- Thymic endothelial cell
TGF- Transforming growth factor
Th- Helper T cell, $CD4^+$ T cell
TLR- Toll-like receptor
TMB- Tetramethylbenzidine
TNF- Tumour necrosis factor
Treg- Regulatory $CD4^+$ T cell
U- Units
VUW- Victoria University of Wellington

# Chapter 1: General Introduction

## 1.1 The Immune System

The mammalian immune system consists of two main branches, known as the innate and the adaptive immune systems. These two different branches co-operate and complement each other to produce a whole system which is very effective at the major goal of an immune system, protecting the host from invasion of pathogenic microbial organisms. The innate immune system consists of several cell types including neutrophils, macrophages (MΦ), monocytes, dendritic cells (DC), basophils and eosinophils. In terms of host protection, the innate immune system serves as the first line of defence against invading pathogens. These cells are able to respond quickly and many express receptors that allow them to recognise a broad range of pathogens. In addition, the innate immune system communicates the presence of a threat to the adaptive immune system, allowing the immune response to be propagated.

The adaptive immune system consists of lymphocytes (B and T cells). The adaptive immune system has two characteristics that make it functionally different from the innate immune system, specificity and memory. Lymphocytes form immune responses that are specific for the individual pathogens. During the generation of B and T cells in the bone marrow and the thymus, respectively, an extremely vast repertoire of receptors is formed. Each receptor recognises a specific antigen (a molecule against which an immune response is generated). Due to the large number of receptors, there are very few lymphocytes expressing each type of receptor. Thus, at the beginning of the adaptive immune response proliferation of the original cell must occur in a process known as clonal expansion. For this reason, the response of the adaptive immune system is much slower than that of the innate immune system. However, this is only the case with primary exposure to an antigen; upon secondary exposure to an antigen, memory cells which were generated during the first exposure are able to rapidly expand to fight infections more efficiently. Memory cells are thus key for providing immunity to reinfection.

In dealing with pathogens, it is essential that the immune system be able to distinguish self from non-self. The immune system has developed many ways to do this, including central and peripheral tolerance mechanisms; however, these methods are not absolute and self reactive immune responses can occur. Such self reactive responses can lead to autoimmune diseases such as multiple sclerosis (MS), which is an inflammatory disease of the central nervous system (CNS) that is mediated by the immune system. This general introduction will provide an overview of the immune system as well as discussing MS and one of its major

animal models, experimental autoimmune encephalomyelitis (EAE), and the immunological components of this disease.

## 1.2 Innate Immune System

The innate immune system consists of a variety of components; these include the cells of the innate immune system, the complement system, and mucosal barriers. Together these work to fight infection and aid the adaptive immune system, as well as performing a variety of housekeeping functions.

### 1.2.1 Pattern recognition receptors

Unlike the cells of the adaptive immune system that have receptors specific for antigens, the cells of the innate immune system express a range of germline encoded receptors, known collectively as pathogen recognition receptors (PRR). PRR are constitutively expressed and allow innate immune cells to recognise motifs that are common to pathogens (Akira, Uematsu, & Takeuchi, 2006; Mukhopadhyay, Plüddemann, & Gordon, 2009). These motifs are known as pathogen associated molecular patterns (PAMPs) and tend to be molecules that are essential for the survival of the pathogen, making it more difficult for pathogens to avoid identification by PRR, as the essential nature of the PAMPs makes them difficult to alter (Akira et al., 2006). Examples include lipopolysaccharide (LPS), a component of the gram negative bacterial cell wall, and viral and bacterial nucleic acids. There are several groups of PRRs including the toll-like receptors (TLR), nucleotide binding and oligomerisation domain (NOD)-like receptors and scavenger receptors (Akira et al., 2006; Jeannin, Jaillon, & Delneste, 2008; Mukhopadhyay et al., 2009). In addition to recognising pathogens, PRR can also recognise apoptotic and necrotic cells and facilitate their clearance (Jeannin et al., 2008).

### 1.2.2 Cells of the innate immune system

#### *1.2.2.1 Monocytes*

Monocytes, an innate immune cell type, are found in the blood and are derived from bone marrow precursor cells of the myeloid lineage (Mosser & Edwards, 2008). Monocytes can migrate into tissue where they differentiate into MΦ or DC. Migration of monocytes into tissue can be due to inflammatory signals or as a normal process to replenish the resident MΦ (Mosser & Edwards, 2008). In both mice and humans, there are two different subsets of

monocytes which are often thought of as having pro or anti-inflammatory properties. In humans, these cells are separated into different subsets based on their expression of CD16 and CD14 (Ingersoll et al., 2010). In mice monocytes are separated into subsets based on the expression of two chemokine receptors; the fractalkine receptor ($CX_3CR1$) and CCR2, and the expression of Ly6C (Geissmann, Jung, & Littman, 2003; Ingersoll et al., 2010). The $CX_3CR1^+Ly6C^-$ cells migrate into tissues under steady state while $CCR2^+Ly6C^+$ cells migrate in response to inflammatory conditions (Geissmann et al., 2003).

#### ***1.2.2.2 Macrophages***

MΦ are a cell type of the innate immune system found in tissues throughout the body (Mosser & Edwards, 2008). MΦ have two main functions, acting as part of the immune response and also as a housekeeping cell. The housekeeping functions of MΦ are related to homeostatic maintenance and do not elicit an immune response. Examples of housekeeping functions include the clearance of debris and apoptotic cells, and tissue remodelling (Mosser & Edwards, 2008). Immune functions of MΦ include antigen presentation, and the phagocytosis and destruction of pathogens. Traditionally, MΦ have been viewed as proinflammatory cells in the context of disease and infection. MΦ in this "proinflammatory" state are known as classically activated MΦ, and they secrete proinflammatory cytokines such as interleukin (IL)-12, IL-23 and tumour necrosis factor (TNF)-α (Mosser & Edwards, 2008; Sutterwala, Noel, Salgame, & Mosser, 1998). While MΦ are found in almost every organ of the body, not all MΦ are exactly the same; their function is slightly different in different organs, based on the milieu they are in. Some examples of different MΦ subsets include the kupffer cells of the liver, osteoclasts found in the bone, alveolar MΦ of the lung, and the microglia (MG) from the central nervous system (CNS) (Gordon & Taylor, 2005; Stout & Suttles, 2004).

#### ***1.2.2.3 Dendritic cells***

The major role of DC in the immune system is phagocytosis and presentation of antigens. By presenting antigens to T cells, DC (and other antigen presenting cells; APC) link the adaptive and innate arms of the immune system, initiating an adaptive immune response. DCs have a small cell body and a large number of processes which extend from them, these processes to survey their environment, looking for antigens and signs of infection (R. M. Steinman, 2007). DC have been shown to be capable of driving different T cell response pathways under different conditions (Anderson, Lucas, Gutiérrez-Kobeh, Field, & Mosser, 2004).

#### *1.2.2.4 Neutrophils*

Neutrophils are an extremely short lived cell type, having a life span of 6-8 hours in humans (Amulic, Cazalet, Hayes, Metzler, & Zychlinsky, 2012). Despite this, neutrophils have very important roles in both host defence and autoimmune diseases, and are often the first immune cells to reach a site of infection. Neutrophils are primarily classified as a granulocyte; however, they are also capable of phagocytosis (Amulic et al., 2012). Chemokines released at the site of inflammation can result in the recruitment of neutrophils to help fight infection. Neutrophils are capable of producing an oxidative burst, which involves the release of reactive oxygen species, which can be damaging to pathogens (Remer, Brcic, & Jungi, 2003). In addition to the oxidative burst, neutrophilic granules contain a variety of antimicrobial peptides which, when released into the phagosome or external environment, create an inhospitable environment for pathogens (Amulic et al., 2012). Furthermore, neutrophils are also important in the recruitment of other immune cells to inflammatory sites (Amulic et al., 2012).

#### *1.2.2.5 Basophils and eosinophils*

Both basophils and eosinophils are associated with parasitic infections and allergic disease (Min, 2008; Min et al., 2004; Shamri, Xenakis, & Spencer, 2011). In parasitic infections, eosinophils help to fight parasites through the release of granules which contain a variety of cytotoxic proteins, as well as cytokines and chemokines. However, the cytotoxic granules can also cause collateral damage to host cells, as seen in allergic disease (Shamri et al., 2011). Basophils produce cytokines and release granules which contain proteins such as histamine. In addition, basophils have also been implicated in the initiation of Th2 responses (Min, 2008; Sokol & Medzhitov, 2010).

#### *1.2.2.6 Natural killer cells*

Natural killer (NK) cells are considered part of the innate immune system, due to the fact that they do not have the antigen specificity associated with the adaptive immune system (Vivier et al., 2011). However, during haematopoiesis NK cells develop from the common lymphocyte progenitor, not from the common myeloid progenitor (Janeway, Travers, Walport, & Shlomchik, 2004). NK cells are cytotoxic, and are capable of killing tumour cells and virus infected cells by the release of perforin and granzymes which lyse the target cell (Moretta, Marcenaro, Parolini, Ferlazzo, & Moretta, 2008; Vivier et al., 2011).

## 1.3 Adaptive immune system

The adaptive immune system consists of two major types of cells, the B and T cells. These cells are collectively known as lymphocytes and provide antigen specific and memory responses important in host defence.

### 1.3.1 B cells

B cells are part of the adaptive immune system that develops in the bone marrow and reside mainly in secondary lymphoid tissues, such as the spleen and lymph nodes. The major role of B cells is the production of antibodies (immunoglobulin; Ig), which are central to humoral immunity. The B cell population of an individual contains an extremely vast array of B cell receptors. This variation is generated by rearrangement of immunoglobulin gene sequences, resulting in a B cell population that can identify an immense amount of different antigens (approximately $5x10^{13}$) (Pieper, Grimbacher, & Eibel, 2013). The B cell receptor consists of a cell surface bound antibody. Following activation and recognition of an antigen, B cells undergo somatic hyper maturation, which allows the B cell receptor to achieve a higher affinity for its antigen, and thus produce a stronger response (Janeway et al., 2004; Pieper et al., 2013). B cells can produce several different classes of antibody including $IgG_1$, $IgG_{2a}$, IgM, IgA, and IgE, which each have roles in different types of immune responses (e.g. bacterial vs. parasitic infections). Class switching to produce different antibodies is dependent on interactions with T cells; in the absence of these interactions B cells only produce IgM (Uronen & Callard, 2000). B cells are important in a wide range of conditions including infection, autoimmunity and allergy (Pieper et al., 2013).

### 1.3.2 Antigen presenting cells

B cells are capable of recognising antigens and can propagate an immune response in the absence of other cells, although interactions with T cells help to make the B cell response more efficient. In contrast, T cells can only respond to an antigen that is presented to it by another cell, known as an APC. APC are therefore essential for the development of T cell responses. Antigen is presented to T cells on major histocompatibility complex (MHC) molecules. There are two classes of MHC molecules; MHC class I, which is expressed by all cell types, and MHC class II, which is limited to DC, MΦ and B cells (Janeway et al., 2004); the term APC generally refers to these 3 cell types. Antigen presented on MHC class I molecules are generally endogenous or cytostolic proteins, that is proteins from inside the cell, such as viral proteins from an infected cell (Harty, Tvinnereim, & White, 2000).

However, APC can also express exogenous proteins on MHC class I; this is known as cross-presentation (Shen & Rock, 2006). Antigen presented on MHC II is exogenous, meaning the antigen has been taken up by the cell, for example by phagocytosis or endocytosis, and has then been processed for presentation to the T cells (Robinson & Delvig, 2002). APC also express co-stimulatory molecules that interact with receptors on T cells, and these interactions are essential for initiating the appropriate T cell responses (Smith-Garvin, Koretzky, & Jordan, 2009).

### 1.3.3 T cells

The term T cell represents a varied population of cells which are derived from the bone marrow but mature in the thymus. Broadly, these cell types can be divided into two separate groups based on their expression of αβ or γδ T cell receptors (TCR). γδ T cells represent a small number of the total T cell population. They are mainly found in epithelial and mucosal tissue and have both innate and adaptive characteristics. For example they express both TCR and other receptors, such as TLR, that can recognise molecules produced by stressed cells (Bonneville, O'Brien, & Born, 2010; Ciofani & Zúñiga-Pflücker, 2010). The αβ T cells can be further divided into two groups based on the expression of the CD8 or CD4 co-receptor, which recognises the conserved regions of MHC class I or class II, respectively. The T cell repertoire of an organism is generated in the thymus, where recombination of the VDJ gene segments generates the variable regions in the α and β chains of the TCR, leading to an incredibly vast array of TCRs that are specific for different antigens. This rearrangement of genes is responsible for the ability of T cells to responed to specific antigens (Janeway et al., 2004).

#### *1.3.3.1 $CD8^+$ T cells*

$CD8^+$ T cells (also known as cytotoxic T lymphocytes) are cytotoxic cells that recognise endogenous (or cytostolic) antigens presented on MHC class I molecules; however, they are also capable of recognising exogenous antigens presented on MHC I in a process known as cross presentation (Harty et al., 2000; Shen & Rock, 2006). The major role of $CD8^+$ T cells is to kill abnormal cells, such as damaged or virus infected cells (Hoves, Trapani, & Voskoboinik, 2010). $CD8^+$ T cells mediate cell death in several ways, including through the release of perforin and granzyme molecules, and stimulation of the FAS-FAS ligand (CD95-CD95L) pathway, which induces apoptosis (Hoves et al., 2010; Krammer, 2000). In

addition, $CD8^+$ T cells produce cytokines such as TNF-α and interferon (IFN)-γ, which are also capable of inducing apoptosis (Harty et al., 2000).

#### *1.3.3.2 $CD4^+$ T cells*

$CD4^+$ T cells (also known as T helper cells, Th) function to activate and direct other immune cells. $CD4^+$ T cells recognise antigen presented on MHC class II molecules (Robinson & Delvig, 2002) and their responses are essential for many immunological processes, such as class switching of antibodies by B cells. $CD4^+$ cells were originally thought to be activated to two distinct subsets, Th1 cells and Th2 cells (Mosmann, Cherwinski, Bond, Giedlin, & Coffman, 1986); however, more recently the Th1/Th2 dichotomy has been reviewed after the discovery of other Th lineages. The Th paradigm has been expanded to recognise at least 4 different Th cell types, Th1, Th2, Th17 and Tregs (Zhu & Paul, 2009).

***Th1***

Th1 cells produce several cytokines including IFN-γ, IL-2, and TNF-α. In addition to the cytokine profile, Th1 cells can be distinguished from other T cells by the expression of T-box transcription factor (T-bet) considered the master regulator of the Th1 lineage (Zhu & Paul, 2009). Th1 cells are induced through interaction with APCs presenting antigen and expressing IL-12. IL-12 acts through a transcription factor, signal transducer and activator of transcription (STAT)4, which can induce IFN-γ expression (Kaplan, Sun, Hoey, & Grusby, 1996). STAT1 is also important in Th1 differentiation; STAT1 is activated in response to IFN-γ and is essential for Th1 differentiation in vitro. It is thought that IFN-γ acts in an autocrine feedback loop through STAT1 to upregulate the IL-12 receptor (IL-12R) and T-bet expression (Lighvani et al., 2001); however, *in vivo* Th1 responses can develop in the absence of STAT1. STAT1 deficient mice produce IFN-γ in response to EAE induction or *Toxoplasma gondii* infection (Bettelli et al., 2004; Lieberman, Banica, Reiner, & Hunter, 2004). T-bet is also upregulated in these mice, but not to the same extent as wild type mice, suggesting that STAT1 and responsiveness to IFN-γ are important for optimal Th1 activation, but not necessarily differentiation.

Th1 cells are important in host defence against intracellular pathogens, and are primarily involved in cell mediated immune responses and induce isotype switching in B cells to produce IgG isotypes (Abbas, Murphy, & Sher, 1996). IFN-γ is the major cytokine secreted by Th1 cells and acts on MΦ to increase their microbicidal activity. The effects of IFN-γ on

MΦ include increased expression of MHC class I and II, and increased production of IL-12, nitric oxide (NO) and superoxide (Szabo, Sullivan, Peng, & Glimcher, 2003). In addition to roles in host defence, Th1 cells are often implicated in autoimmune conditions, such as MS and EAE.

***Th2***

Th2 cells can produce many cytokines including IL-4, IL-5, IL-10 and IL-13 (Zhu, Yamane, & Paul, 2010). The master regulator of the Th2 lineage is GATA-3, which can be used to identify this cell type; without expression of GATA3, Th2 differentiation does not occur (Zhu & Paul, 2008). It has been shown *in vitro* that both IL-2 and IL-4 are important for Th2 differentiation (Le Gros, Ben-Sasson, Seder, Finkelman, & Paul, 1990). IL-2 can induce upregulation of the IL-4 receptor alpha (IL-4Rα, CD124) chain, which is not expressed or is expressed only at low levels on resting T cells (Liao et al., 2008). IL-4 drives Th2 responses through the IL-4 receptor via STAT6 signalling (Le Gros et al., 1990; Zhu & Paul, 2008). However, the role of cytokines in Th2 differentiation *in vivo* remains controversial as mice that lack either IL-4 or STAT6 are still capable of developing Th2 immune responses to classic Th2 inducing stimuli such as *Nippostrongylus brasiliensis* and *Schistosoma mansoni* (Jankovic et al., 2000; van Panhuys et al., 2008). Th2 cells are important for defence against extracellular parasites, have roles in allergy and asthma, and play a key role in humoral immunity by inducing class switching of B cells to produce specific classes of antibodies including IgE and some IgG1 isotypes (Abbas et al., 1996; Zhu & Paul, 2008). In EAE, Th2 responses are associated with protection from disease (Chitnis et al., 2001).

***Th17***

Th17 cells are a relatively recently identified subset of T cells and are named after their ability to express IL-17A, the prototypical cytokine of this subset. In addition to IL-17A, Th17 cells can produce several other cytokines including IL-17F, IL-22 and IL-21 (Zhu & Paul, 2008). Murine Th17 cells differentiate in the presence of IL-6 and TGF-β (Bettelli et al., 2006). IL-23 is also important for the Th17 response, but unlike IL-6 and transforming growth factor (TGF)-β, IL-23 is important in the stabilisation and pathogenic function of the Th17 responses, and not for differentiation (Bettelli et al., 2006; McGeachy et al., 2007, 2009). The master regulator of the Th17 response is retinoic acid-related orphan receptor (ROR)γt, which is necessary for cells to respond to IL-6 and TGF-β (Ivanov et al., 2006). STAT3 is also very important in Th17 differentiation and stabilisation, being activated by IL-6 and IL-23 (Yang

et al., 2007). Th17 cells are associated with protection from extracellular bacteria (e.g. *Klebsiella pneumonia*) and fungi (e.g. *Candida albicans*). An important component of the protection from these pathogens is the promotion of neutrophil mediated inflammation by IL-17A (Louten, Boniface, & de Waal Malefyt, 2009). In addition to roles in host defence, Th17 cells can also be pathogenic and are associated with several autoimmune conditions including EAE (Bettelli et al., 2006).

***Tregs***

T regulatory cells or Tregs are a suppressive subset of Th cells. Tregs express high levels of the IL-2 receptor, CD25; however, as this marker is also upregulated on activated T cells from other lineages, it cannot be used on its own as a marker for the Treg lineage. In addition to CD25, Forkhead box P3 (FOXP3) is highly expressed in Treg cells and is considered to be the master regulator of this lineage (Bettelli et al., 2006; Zhu & Paul, 2008). TGF-β is important for the formation of Treg cells and the expression of FOXP3. TGF-β is also important in Th17 differentiation and the absence of IL-6 is necessary for Treg development, suggesting these cell types are mutually exclusive (Bettelli et al., 2006). Tregs are thought to be able to regulate other cells in both cell-cell contact dependent and independent methods. For example, Treg cells produce immunosuppressive cytokines such as IL-10 and TGF-β, and the high level of CD25 expressed by Tregs can act to sequester IL-2 and prevent it from stimulating other T cell lineages (Campbell & Koch, 2011; Sakaguchi, Wing, Onishi, Prieto-Martin, & Yamaguchi, 2009). *In vivo,* 2 types of Tregs exist; natural Tregs differentiate in the thymus, and inducible Tregs are induced in the periphery (Zhu & Paul, 2009). The balance between Treg cells and pathogenic subsets of other Th lineages is considered important in the regulation of autoimmune inflammation and maintenance of self tolerance. In EAE, Tregs are important regulators of disease, and their *in vivo* deletion leads to exacerbation of disease (Zhang et al., 2004).

### 1.3.4 Plasticity in the T cell response

It has long been considered that the different subsets of Th cells represent terminal differentiation of lineages, meaning that once a commitment has been made to a lineage the cell cannot be changed into a different Th lineage. Current evidence suggest that, for at least some Th subsets, terminal differentiation does not occur and the cells can differentiate further or "switch" phenotypes; a particularly good example of this is the Th17 lineage. In the absence of continual TGF-β signalling *in vitro* differentiated Th17 cells upregulate the

expression of IFN-γ in response to IL-23 stimulation, producing both IFN-γ positive and IFN-γ/IL-17A double positive cells (Lee et al., 2009). These cells also responded in a similar way to IL-12, although IL-12 could induce IFN-γ up regulation even in the presence of TGF-β (Lee et al., 2009). *In vivo* differentiated Th17 cells have also been shown to have a plastic phenotype (Kurschus et al., 2010). In transfer models of both colitis and EAE, transfer of *in vitro* polarised Th17 cell induces disease; however, the transferred cells alter their cytokine expression after transfer, showing increased number of IFN-γ producing cells and decreased numbers of IL-17A producing cells (Kurschus et al., 2010; Lee et al., 2009).

### 1.3.5 T cell activation

There are two signals necessary for T cell activation, stimulation of the TCR and activation of co-stimulatory pathways. In addition, a third signal involving soluble factors (e.g. cytokines) drives the differentiation of the T cells (Janeway et al., 2004). The first signal required for T cell activation is the stimulation of the TCR and for this to happen several requirements must be met. The TCR must recognise the antigen being presented to it and this is determined by the variable regions of the αβ chain that are expressed by the individual T cell (Smith-Garvin et al., 2009). A second requirement is that the antigen is being presented on an MHC molecule that is recognised by the CD4 or CD8 co-receptors (Janeway et al., 2004). The αβ chains of the TCR and the CD4 or CD8 co-receptors are important for the recognition of the antigen, but for activation, signalling through the CD3, which is co-expressed with the αβ chains of the TCR, must occur. The CD3 signalling pathways necessary for T cell activation, which does not transpire in the absence of CD3 signalling (Smith-Garvin et al., 2009).

The second signal required for T cell activation is the activation of co-stimulatory pathways. There are several known co-stimulatory pathways, which provide a stimulatory signal to the T cell, thus aiding the activation of the T cell. Other pathways provide an inhibitory signal, which can serve to suppress the T cell response and prevent damage to the host caused by an overzealous immune response. In the absence of adequate excitatory co-stimulation, T cells can undergo anergy and become unresponsive (Saibil, Deenick, & Ohashi, 2007). Several of these co-stimulatory and co-inhibitory pathways will be discussed in more detail below. In addition to the first two signals, there is a third signal for T cell activation which is involved in directing the T cell response and consists of the production of soluble factors, such as cytokines, by the APC (Janeway et al., 2004; Kaliński, Hilkens, Wierenga, & Kapsenberg, 1999). Indeed, DC that have been activated to express cell surface markers necessary for

T cell activation such as MHC, CD40 and CD86, but do not express biasing cytokines such as IL-12, can stimulate proliferation but are ineffective at directing the T cell to develop into an effector cell (Spörri & Reis e Sousa, 2005). Some of the cytokines involved in T cell differentiation have been described above.

#### *1.3.5.1 CD28-CTLA-4/CD80-CD86*

One of the first co-stimulatory molecules to be identified on T cells was CD28. CD28 is expressed on naïve T cells and interacts with CD80 (B7.1) and CD86 (B7.2) on APC to provide a stimulatory signal to T cells (Krummel & Allison, 1995). The difference in function between CD80 and CD86 is not well understood, and Manzotti et al. (2006) suggested this is likely in part due to different levels of expression of these markers (CD86 is often more highly expressed), making the interpretation of data obtained from CD80 and CD86 deficient animals difficult to interpret. There is some evidence to suggest that CD80 may be a more potent activator of CD28, inducing more rapid proliferation compared to CD86 (Manzotti et al., 2006). CD28 is an important molecule in EAE. CD28 deficient mice are protected from disease; however, disease can be induced following a second immunisation in a pathway that appears to rely on CD40/CD40L interactions (Girvin, Dal Canto, & Miller, 2002). This suggests that co-stimulation through other pathways can induce T cell activation; however, it appears to be less efficient than CD28 stimulation.

In addition to CD28, CD80 and CD86 also interact with the related molecule, cytotoxic T lymphocyte-associated antigen (CTLA)-4, which provides an inhibitory signal to the T cell. CTLA-4 is not expressed on naïve T cells but is upregulated following activation, and can then bind to CD80 and CD86, which it does with a much higher affinity than CD28, to inhibit the T cell response (Krummel & Allison, 1995). Stimulating T cells via CTLA-4 causes a decrease in their proliferation and IL-2 production (Walunas, Bakker, & Bluestone, 1996). This interaction is thought to be very important in the regulation of T cell responses, as CTLA-4 deficient mice suffer from a severe lymphoproliferative disorder that results in early death due to uncontrolled T cell responses (Waterhouse et al., 1995). In addition CTLA-4 is highly expressed by Tregs and is important in their ability to control immune responses (Jain, Nguyen, Chambers, & Kang, 2010).

***1.3.5.2 CD40/CD40L***

CD40L (CD154) is expressed on the surface of T cells and is upregulated following TCR stimulation. Interactions between CD40L and CD40 on APC are important for both the T cell and the APC. CD40L deficient mice have a deficiency in T cell priming, as seen by lower levels of proliferation and cytokine production in these T cells (Grewal & Flavell, 1998; Grewal, Xu, & Flavell, 1995). CD40L binding to CD40 on APC activates the APC to produce cytokines such as IL-12 and TNF-α (Grewal & Flavell, 1998). The CD40/CD40L interactions of B and T cells are essential for isotype switching by B cells and germinal vesicle formation (Grewal & Flavell, 1998). In people with hyper IgM syndrome, mutations in the CD40L gene prevent interactions between CD40 on B cells and CD40L on T cells, leading to an inhibition of isotype switching and an increased susceptibility to infection (Uronen & Callard, 2000). The interaction between CD40L and CD40 is very important in a wide range of conditions including infections, such as *Leishmania major* and EAE (Campbell et al., 1996; Grewal et al., 1996).

CD40L deficient mice on normally resistant backgrounds are susceptible to *L. major* and succumb to infection in a manner similar to that seen in susceptible strains. The susceptibility to infection in CD40L deficient mice is associated with decreased production of IFN-γ by T cells and IL-12 by MΦ (Campbell et al., 1996). CD40L deficient mice succumb to a standard dose of *L. major* but are capable of mounting an effective response against low level infection (associated with increased IFN-γ and IL-12 production), and are protected from a second high level infection (Padigel & Farrell, 2003). This suggests that anti-*L. major* responses can be mounted in the absence of CD40/CD40L interaction, but that they are less efficiently produced. In EAE, CD40L deficiency is associated with resistance to disease. This protection correlates with low IFN-γ production and little CD80/CD86 expression on APC in the lymph nodes; transfer of APC that transgenically express CD80 restores the susceptibility to disease (Grewal et al., 1996). This suggests that resistance to disease may be due to failure of APC to be activated and co-stimulate T cells in the absence of CD40/CD40L interactions. CD40/CD40L appear to be important in the induction of EAE in CD28 deficient mice following a second priming of myelin peptide (Girvin et al., 2002).

CD40/CD40L interactions not only have roles in driving the Th1 responses involved in *L. major* infection and EAE, but are also important in Th2 responses. Mice deficient in CD40L have exacerbated disease in *S. mansoni* infection, which is associated with an inability to mount a Th2 response (MacDonald et al., 2002). In addition, deficiency in CD40/CD40L

interactions decreases the production of IFN-γ and IL-4 by T cells, supporting the role of this pathway for both Th1 and Th2 responses (Poudrier et al., 1998). Studies suggest that CD40/CD40L interactions have dichotomous roles in directing APC and T cell activation. Different levels of CD40 stimulation have been shown to affect the activation of APC. Mathur *et al*. (2004) demonstrated that low level CD40 stimulation of MΦ induced the production of regulatory IL-10, while high level stimulation induced the production of proinflammatory IL-12 (Mathur, Awasthi, Wadhone, Ramanamurthy, & Saha, 2004). A similar result was seen in DC, where low level CD40 stimulation resulted in increased IL-10, and high level CD40 stimulation resulted in increased IL-12 (Murugaiyan, Agrawal, Mishra, Mitra, & Saha, 2006). This same pattern of responses is also seen in DC from $CD40^{+/+}$ or $CD40^{+/-}$ mice. $CD40^{+/+}$ DCs express higher CD40 and when stimulated with an anti-CD40 antibody, produce high levels of IL-12. In contrast, the $CD40^{+/-}$ DC express lower levels of CD40 and produce high levels of IL-10 upon stimulation. $CD40^{+/+}$ DC stimulate T cells to produce IFN-γ (prototypic Th1 cytokine), whereas $CD40^{+/-}$ DCs drive T cells to produce IL-10 and TGF-β (Treg associated cytokines), and $CD40^{-/-}$ DC promote production of IL-4 (prototypic Th2 cytokine) in T cells (Martin, Agarwal, Murugaiyan, & Saha, 2010). These data support the role of CD40 signalling in directing the T cell response.

#### ***1.3.5.3 PD-1/PD-L1-PD-L2***

Programmed death (PD)-1 and it's ligands, programmed death ligand (PD-L)1 and PD-L2, form an important regulatory pathway with a significant role in peripheral tolerance (Francisco, Sage, & Sharpe, 2010; Probst, McCoy, Okazaki, Honjo, & van den Broek, 2005). In mice, deficiencies in PD-1 lead to spontaneous strain specific autoimmune diseases. PD-1 deficiency in BALB/c mice results in autoimmune dilated cardiomyopathy (Nishimura et al., 2001), whereas PD-1 deficient C57BL/6 mice develop a lupus like syndrome characterised by glomerulonephritis and arthritis (Nishimura, Nose, Hiai, Minato, & Honjo, 1999). In support of the inhibitory role of this pathway, studies have shown that PD-L1 and PD-L2 can inhibit T cell proliferation *in vitro* and *in vivo* (Carter et al., 2002; Konkel et al., 2010), and induce anergy (Tsushima et al., 2007). Furthermore, PD-1 and PD-L1 deficient mice develop exacerbated EAE (Carter et al., 2007), whilst over expression of PD-L1 on DC is protective in EAE (Hirata et al., 2005).

PD-L1 and PD-L2 have vastly different expression profiles. PD-L1 is expressed on a wide range of both haematopoietic and non-haematopoietic cells, while PD-L2 is largely restricted

to MΦ and DC (Francisco et al., 2010). The exact role PD-L1 and PD-L2 is not known; however, studies using mice deficient in PD-L1 or PD-L2 suggest that they have different roles in controlling disease. For example, PD-L1 deficient mice have exacerbated disease in EAE, while PD-L2 deficient mice do not (Carter et al., 2007). The opposite response is seen in *Leishmania mexicana*, where PD-L1 deficient mice are protected but PD-L2 mice have exacerbated disease (Liang et al., 2006). Different T cell responses can regulate the expression of PD-L1 and PD-L2 on MΦ, with IFN-γ and LPS inducing PD-L1 activation and IL-4 inducing PD-L2 activation. This suggests that PD-L1 and PD-L2 could potentially have roles in regulating specific immune responses (Loke & Allison, 2003). In addition to the effects on T cells, PD-L1 and PD-L2 ligation likely affects the APC in a process known as "reverse signalling". DC treated with a soluble PD-1 protein take on a suppressive phenotype, expressing lower levels of CD40, CD80 and CD86 and increased IL-10 (Kuipers et al., 2006). This suggests that the role of The PD-1/PD-L1-PD-L2 pathway may have important roles in regulating immune responses on both sides of the immunological synapse.

## 1.4 Tolerance

The aim of an immune response is to fight infections to protect the host from pathogens; however, many of the processes involved can cause collateral damage to the host, therefore it is essential that immune responses are tightly regulated. In addition, the ability to distinguish self from non-self is paramount to protecting the host from damaging autoreactive immune responses. Several mechanisms have evolved to regulate the ability of the immune system to recognise and propagate responses against host proteins. These mechanisms are split into two groups known as central and peripheral tolerance. Failure of tolerance mechanisms can result in autoimmune disease which may manifest as organ specific (e.g. MS) or systemic (e.g. systemic lupus erythematosus) diseases.

### 1.4.1 Central tolerance

Lymphocyte receptors are randomly generated through the rearrangement of gene segments and are thus not created for specific antigens. For this reason, TCR can be generated that strongly recognise self peptides, and are therefore a risk factor for initiating autoimmune disease. Conversely, TCR can be generated that have little affinity for MHC-antigen complexes. Central tolerance occurs in primary lymphoid organs during development. In the thymus, processes of positive and negative selection remove both auto-reactive and

unresponsive T cells through interactions with antigen presenting cells such as thymic epithelial cells (TEC) and thymic DC (Kyewski & Klein, 2006). TEC express a large array of proteins, including proteins that are usually organ specific, with a repertoire that spans most if not all organs (Kyewski & Klein, 2006). Positive selection removes T cells that do not have a high enough affinity for the MHC-antigen complex. These cells do not receive survival signals and undergo "death by neglect" (Sprent & Kishimoto, 2001). The process of negative selection removes cells that have a high affinity for self antigens. Strong recognition of a self antigen during negative selection often leads to clonal deletion, whereby the reactive T cell undergoes apoptosis; however, some of these cells develop into Tregs (Hogquist, Baldwin, & Jameson, 2005; Kyewski & Klein, 2006). Despite the rigorous mechanisms of central tolerance, some self reactive immune cells escape into the periphery.

### 1.4.2 Peripheral tolerance

Peripheral tolerance regulates immune responses outside of the primary lymphoid organs. There are several mechanisms of peripheral tolerance. Inhibitory signals to the T cell, such as CTLA-4 and PD-L1/2, are involved in regulating T cell responses in the periphery (Francisco et al., 2010; Waterhouse et al., 1995). Indeed, disruption of these pathways leads to autoimmune and lymphoproliferative pathologies due to dysregulation of immune responses (Nishimura et al., 2001, 1999; Waterhouse et al., 1995). Treg cells are also important in the maintenance of peripheral tolerance due to their inhibitory effects on other immune cells (Sakaguchi et al., 2009). In addition, there are certain subsets of myeloid cells that are capable of mediating suppressive effects, including myeloid derived suppressor cells (MDCS) and certain subsets of MΦ and monocytes, such as type II MΦ (Anderson, Gerber, & Mosser, 2002; Gabrilovich & Nagaraj, 2009; Tierney, Kharkrang, & La Flamme, 2009).

The induction of anergy, a state of long-term hyporesponsiveness, is also a mechanism of tolerance. The induction of T cell activation requires at least two signals when antigen is presented to naïve T cells, and in the absence of these necessary signals, the T cell takes on an anergic state and becomes unresponsive to stimuli (Chappert & Schwartz, 2010; Saibil et al., 2007). Specific cell surface interactions, such as the PD-1/PD-L1 pathway can be important in the maintenance of anergy in the periphery (Saibil et al., 2007). Anergy most likely happens under non-inflammatory conditions *in vivo,* as inflammatory signals, such as PAMP, induce antigen presenting cells to up regulate co-stimulatory molecules (Mosser, 2003; Saibil et al., 2007).

## 1.5 Macrophages

Whilst MΦ have traditionally been associated with inflammatory responses, they exhibit remarkable plasticity in their activation state. It is now considered that MΦ activation represents a spectrum in which different but potentially overlapping responses are induced by different stimuli. Even though MΦ activation is thought of as a spectrum, several specific activation states have been identified. MΦ can be activated to take on proinflammatory (classical MΦ), regulatory (type II MΦ), wound healing (alternatively activated (aa)MΦ), and acquired deactivating phenotypes under certain conditions (Mosser & Edwards, 2008).

MΦ are often separated into 2 different subsets in the literature M1 and M2, with M1 MΦ being classically activated MΦ. M2 was originally used to describe the alternatively activated MΦ, but has since been used to broadly refer to any MΦ that is not a classically activated MΦ. This system of naming is therefore very limited and can become confusing, with MΦ subsets that are not truly alternatively activated being referred to as M2 MΦ. The M2 subset is often broken down into M2a (alternative), M2b (type II/regulatory) and M2c (acquired deactivating) (Martinez, Sica, Mantovani, & Locati, 2008). This can also be misleading, as it implies that there is a stronger relationship between all of the MΦ subsets classed as M2 than any of these subsets have with the classically activated or M1 MΦ, which is not necessarily true. For example, both classically activated MΦ and type II can drive T cell responses by presenting antigen; however, aaMΦ cannot (Anderson & Mosser, 2002; Edwards et al., 2006). Furthermore, classically and type II activated MΦ have high levels of inducible nitric oxide synthase (iNOS) and produce NO, whereas aaMΦ do not and have increased arginase which shunts arginine towards the production of extracellular matrix (ECM) components instead of NO (Edwards et al., 2006). Due to the confusing and inappropriate nature of the M1 and M2 naming system, it will not be used in this thesis. Instead, MΦ will be referred to as classically, type II, or alternatively activated.

### 1.5.1 Classical activation

Classically activated MΦ are a proinflammatory subset of MΦ that are typically associated with cell mediated immunity (Mosser & Edwards, 2008). Classical activation requires two signals. Initially both IFN-γ, which primes MΦ to become microbicidal, and TNF-α, were identified as being essential for MΦ activation. However, TLR stimulation can also induce a classical activation state due to the production of endogenous TNF-α that feeds back on the MΦ. Therefore, TLR stimuli can induce classical activation of MΦ in conjunction with IFN-γ

(Martinez et al., 2008; Mosser & Edwards, 2008). MΦ activated with TLR stimuli in the absence of IFN-γ are often termed innately activated MΦ. These MΦ are similar, but not identical, to classically activated MΦ, supporting the idea that MΦ activation occurs on a spectrum (Martinez et al., 2008). Certain pathogens (such as *Mycobacterium tuberculosis*) can inhibit MΦ ability to respond to IFN-γ and therefore prevent the MΦ from taking on a classical activation state, resulting in inefficient pathogen clearance (Kincaid & Ernst, 2003). MΦ have important roles in host defence against pathogens. When activated by classical stimuli, MΦ have an increased ability to kill intracellular pathogens. Killing of pathogens by classically activated MΦ is mediated by several pathways including, starvation by restriction of nutrients (such as iron), production of reactive oxygen species and NO, and acidification of the phagosome (Martinez et al., 2008; Mosser, 2003). Classically activated MΦ are often identified based on the production of high levels of proinflammatory cytokines such as IL-12, but also have increased expression levels of co-stimulatory molecules and produce high levels of NO. In the context of this thesis, classically activated MΦ will be identified mainly on the basis of high IL-12 and low IL-10 production (Edwards et al., 2006).

### 1.5.2 Alternative activation

Alternative activation occurs when MΦ are exposed to Th2 cytokines, namely IL-4 or IL-13. Production of these cytokines *in vivo,* in response to parasitic infection for example, can result in the activation of MΦ to aaMΦ (Mosser & Edwards, 2008). aaMΦ upregulate expression of the mannose receptor and arginase (Edwards et al., 2006; Stein, Keshav, Harris, & Gordon, 1992; Varin & Gordon, 2009). Arginase converts arginine to ornithine, which is used in the formation of components the ECM, such as collagen, that are secreted by aaMΦ and may function in resolving immune responses and promoting wound healing (Mosser & Edwards, 2008; Varin & Gordon, 2009). Unlike other MΦ activation states, aaMΦ do not promote T cell responses, suggesting they do not drive adaptive immune responses (Edwards et al., 2006). As aaMΦ do not express iNOS, they do not produce NO and thus have an impaired ability to kill intracellular pathogens, suggesting inappropriate activation of MΦ (e.g. in infections such as *L. major*) to an aaMΦ could be detrimental (Edwards et al., 2006; Martinez et al., 2008; Mosser & Edwards, 2008).

### 1.5.3 Type II activation

Type II activated MΦ were first described by Sutterwala et al., (1998). This MΦ activation state was induced through the binding of antibody receptors (FcγR) with concurrent

stimulation of TLR-4 by LPS (Anderson & Mosser, 2002). However, several other stimuli have been shown to induce similar, but not identical, activation states, supporting the idea that MΦ activation exists on a continuum (Mosser & Edwards, 2008). For example, monocytes treated with glatiramer acetate (GA), a commonly used treatment for MS, have a similar activation state to type II MΦ (Kim et al., 2004; Weber et al., 2007). Similarly to classically activated MΦ, type II activation of MΦ requires 2 signals. Binding the FcγR with immune complexes (IC) alone has little observable effect on the MΦ, but modifies the MΦ response when a second inflammatory signal, such as LPS, is present (Tierney et al., 2009).

The effect of type II activating conditions *in vitro* has been shown to alter the activation state of MΦ to an immunoregulatory phenotype. These MΦ produce increased levels of IL-10 (an immunosuppressive cytokine) and decreased levels of IL-12 (a proinflammatory cytokine), thus promoting an anti-inflammatory environment (Sutterwala et al., 1998). In addition, type II MΦ can present antigen to T cells; however, they have been shown to support a Th2 activation state, unlike classical activation which support a Th1 state (Anderson & Mosser, 2002). Several studies have documented the *in vivo* effect of type II MΦ. These studies show type II activated MΦ can affect T cell responses (promoting a Th2 response) and were found to be protective in a model of sepsis (Anderson, Gerber, & Mosser, 2002; Anderson & Mosser, 2002; Gerber & Mosser, 2001). In addition, in studies of EAE, both activating type II MΦ *in vivo* via treatment with IC, and adoptive transfer of type II MΦ were protective and showed an enhanced Th2 response (La Flamme et al., 2006; Tierney et al., 2009).

In addition to altered cytokine expression, type II MΦ also show altered expression of many proteins. Some examples include reduced expression of CD40, PD-L1, and CD80 (Tierney et al., 2009). Type II MΦ have also been shown to upregulate sphingosine kinase (SPHK), MHC class II, LIGHT, and activation of the PKB (AKT) pathway (Edwards et al., 2006; Polumuri, Toshchakov, & Vogel, 2007). As described above, the PD-1/PD-L1 pathway is inhibitory and ligation of PD-1 results in reduced proliferation and reduced expression of IL-2 by T cells (Carter et al., 2002). SPHK is an enzyme which metabolises the sphingosine derived from a membrane sphingolipid, into sphingosine-1-phosphate (S1P). S1P can act as an extracellular ligand or as an intracellular second messenger. S1P and SPHK are active in a wide array of immune cells. They have been implicated in assisting T cell egress from lymphoid organs, neutrophil activation and chemotaxis, and may aid wound healing when expressed in MΦ (Melendez, 2008). LIGHT is a member of the TNF super family that can act as a costimulatory molecule and promote T cell proliferation (Tamada et al., 2000). The

PKB pathway is thought to be involved in IL-12 downregulation in response to concurrent FcγR and toll-like receptor ligation (Polumuri et al., 2007).

## 1.6 Microglia

MG are the resident MΦ like cells of the CNS. MG are, as their name suggests, one of the glial cells, which are cells in the nervous system that function to support the neurons and the neuronal environment. MG are generally considered to be the only cell type in the CNS that is part of the immune system and for this reason it is believed that they have a strong influence on the way that immune responses develop in the CNS.

Although the origin of MG has long been debated, it is now widely accepted that MG are of haematopoietic origin. While MG are of haematopoietic origin, the majority of MG arise from the primitive MΦ which differentiate in the yolk sac (during primitive haematopoiesis) and migrate in to the brain, not from the definitive haematopoiesis through which the peripheral leukocytes develop (Ginhoux et al., 2010). MG have been observed in the brain of mouse embryos as early as 8 days post-fertilisation (Alliot, Godin, & Pessac, 1999). The adult pool of MG has been shown to be maintained by local self renewal, as opposed to migration of new cells into the tissue from the blood such as is seen in most other organs (Ajami, Bennett, Krieger, Tetzlaff, & Rossi, 2007).

There are two forms morphological of MG, the amoeboid and ramified forms. Neonatal MG have an amoeboid shape and are motile. In contrast, ramified MG are only found in the brains of older animals and are non-motile, with the exception of their processes which move to survey their environment (Schell, Crane, Smith, & Roberts, 2007). In mice, all MG have switched their phenotype from amoeboid to ramified by 14 days *postpartum* (Hirasawa et al., 2005), and the different morphologies likely reflect the function of microglia at different life stages. In the adult brain, ramified MG are often referred to as resting MG; however, these cells are actually continuously monitoring their micro-environment and are capable of eliciting immune responses and clearing cellular debris (Nimmerjahn, Kirchhoff, & Helmchen, 2005; Schlegelmilch, Henke, & Peri, 2011). The main role of the amoeboid MG of neonatal mice is believed to be the phagocytosis of neurons that are produced in surplus in the developing brain. Previous work has shown that many of these new neurons die and are phagocytised by microglia (Schlegelmilch et al., 2011). Amoeboid microglia also have important roles in synaptic pruning during development (Paolicelli et al., 2011).

MG are the resident MΦ like cells of the CNS (Mosser & Edwards, 2008); however, they are not the same as MΦ found in other areas of the body. MG are not continually renewed from the blood, unlike other populations of MΦ which are constantly being replenished by monocytes (Ajami et al., 2007; Mosser & Edwards, 2008; Ponomarev, Shriver, & Dittel, 2006; Schmid et al., 2009). In addition, the microenvironment of the CNS, which is considered an "immune privileged" environment, likely affects the response of MG. For example, the presence of neurons in glial cultures from neonatal rats decreased the reaction of the glial cultures to LPS, based on TNF-α and NO production, suggesting neurons may have an inhibitory effect on MG function (Chang et al., 2001). Thus, the CNS microenvironment may have a role in inhibiting MG to prevent overzealous immune responses initiated by MG. Due to the conditions in the CNS, it is important to consider that MG may react differently to other MΦ subsets found in other tissues and therefore need to be treated as a separate cell type from MΦ.

When MG are unactivated they have a ramified structure and express lower levels of CD45, but upregulate this marker on activation to a level similar as MΦ (Sedgwick et al., 1991). Therefore, under inflammatory conditions, MG and MΦ cannot be distinguished from each other as activated MG and MΦ are morphologically similar (amoeboid) and express the same surface markers, such as CD45, F4/80, MHC class II, and CD11b (Melchior, Puntambekar, & Carson, 2006; Ponomarev et al., 2006). It is thought that unactivated MG may have housekeeping functions, similar to those seen in MΦ populations in other tissues (Nimmerjahn et al., 2005).

## 1.7 Multiple Sclerosis

MS is an inflammatory disease of the central nervous system which is mediated by the immune system. MS most commonly presents itself in the third and forth decades of life and is around 2 times as common in women as it is in men (Kumar, Abbas, Fausto, & Mitchell, 2007; Lins, 2005). Clinical manifestations of MS include fatigue, decreased motor control, ataxia, optical neuritis, cognitive impairment and paralysis (Kumar et al., 2007; Lins, 2005). Three main forms of MS have been characterised based on the pattern of disease progression. The most common form of MS (85-90% of cases) is relapsing-remitting (RR-MS), where patients experience periods in which their symptoms are worsened which are intervened by

periods of reduced disease severity. Many patients with RR-MS will eventually develop a secondary progressive form. 10-15% of patients present with a primary progressive form. These progressive forms demonstrate a gradual worsening of symptoms, as opposed to the distinct attacks seen in RR-MS (Sospedra & Martin, 2005).

Multiple sclerosis literally means "multiple scars", which refers to the lesions that develop in the CNS (Weiner, 2009). MS lesions in the CNS are characterised by demyelination of neuronal axons and subsequent damage to the axons themselves (Compston, 2004; Frohman, Racke, & Raine, 2006). The axons can be damaged by inflammatory mediators such as NO, and it has also been suggested that NO may be able to block the transmission of signals down axons, thus increasing the impairments associated with active lesions (Compston, 2004). Remyelination can occur; however, over the course of the disease progressive loss of axons and myelin results in loss of function of neurons and scaring of the CNS (Bjartmar, Wujek, & Trapp, 2003; Frohman et al., 2006; Patrikios et al., 2006). It is believed that this axonal loss is involved in the transition of MS from relapsing remitting to progressive disease (Greenstein, 2007).

### 1.7.1 Aetiology

While the aetiology of MS is not known, it is believed to be caused by a combination of both environmental and genetic factors (Goodin, 2009; Hoffjan & Akkad, 2010; Lins, 2005). Several genes have been identified as having a role in MS. Many studies have consistently identified human leukocyte antigen (HLA) alleles as a risk factor for MS, with the DRB1*1501 allele consistently showing a strong association; however, other genes including cytokines and cytokine receptors (e.g. IL-2Rα and IL-7) and co-stimulatory molecules (e.g. CD40 and CD80) have been suggested to also have roles (Goodin, 2009; Hoffjan & Akkad, 2010; International Multiple Sclerosis Genetics Consortium et al., 2011). Studies in twins show a concordance rate of approximately 25% for monozygotic twins and 3-5% for dizygotic twins (Hawkes & Macgregor, 2009). While showing a genetic link, these concordance rates are relatively low, suggesting a strong role for other factors (such as environmental factors) in the aetiology of MS.

Many environmental factors have been attributed to increased risk of developing MS including geographical location, vitamin $D_3$ and exposure to sunlight, stress, and certain infections such as Epstein-barr virus (EBV) (Goodin, 2009; Haahr, Plesner, Vestergaard, &

Höllsberg, 2004; Munger, Levin, Hollis, Howard, & Ascherio, 2006). In support of the role of environmental factors, it has been observed that people who live in geographical locations where there is a low or high rate of MS, that then move to an area with opposing MS rates when they are older than 15, maintain the relative risk of developing MS of the area they grew up in. However, if they are younger than 15, their risk of developing MS will be that of the area they moved to, suggesting environmental effects are particularly important in childhood (Goodin, 2009; Kumar et al., 2007). Geographic location has a strong relationship with MS, which has higher incidences further away from the equator (Kumar et al., 2007; Lins, 2005). This effect can be seen not only globally but also locally. New Zealand has an overall incidence of around 1:1400, however, the rate is higher in the southern parts of the country compared to northern areas (Taylor et al., 2010).

### 1.7.2 Immunology

The role for the immune system as central mediators of disease is supported by several findings, including the presence of inflammatory infiltrates in demyelinating lesions in MS patients, and the efficacy of immunomodulatory and immunosuppressive drugs in the treatment of MS (Frohman et al., 2006; Morris & Yiannikas, 2012). In addition, several large genetic studies have found associations between genes involved in the immune system, such as certain HLA alleles, and MS (Goodin, 2009; Hoffjan & Akkad, 2010; International Multiple Sclerosis Genetics Consortium et al., 2011). However, many of the treatments that are effective in relapsing-remitting MS are not effective or are less effective in treating the progressive forms, which also show differing histopathological patterns in the CNS (Bradl & Lassmann, 2009). Therefore, it is important to remember that MS is a heterogeneous disease and that relapsing-remitting and progressive forms may be regulated by different, but possibly overlapping, processes.

MS is considered an immune mediated disease in which myelin specific T cells become activated and invade the CNS where they mediate disease processes. However, as studies have found that T cells specific for myelin antigens can be found in both the MS patients and healthy controls, the presence of myelin specific T cells is not sufficient for the development of MS (Martin et al., 1990; Pender, Csurhes, Houghten, McCombe, & Good, 1996). It has been shown that myelin reactive T cells in MS patients are phenotypically different from healthy controls, showing an activated or memory phenotype, whereas myelin reactive T cells from healthy controls appear naïve (Frohman et al., 2006; Lovett-Racke et al., 1998).

Traditionally MS has been viewed as being mediated by $CD4^+$ T cells, nevertheless, many other immune cells have been shown to have important roles in disease pathogenesis, including $CD8^+$ T cells, MΦ, MG, and B cells.

The event that triggers the initiation of MS is unknown. It has been proposed that the initial step in MS may be related to viral or bacterial infections (such as EBV), potentially by molecular mimicry in which lymphocytes are cross reactive, recognising both self and pathogen peptide, thus a response against a pathogen could potentially trigger the autoimmune disease. A second theory is that initiation occurs by bystander activation, in which an inflammatory environment can either enhance the co-stimulatory properties of APC, or enhance the activation of T cells by cytokines and other proinflammatory molecules, independently of TCR interaction (Sospedra & Martin, 2005). However, neither of these processes has been proven, and the initial step in MS pathogenesis remains elusive. It is thought that the initial activation of T cells occurs in the periphery. For disease to occur, the pathogenic T cell subsets then need to migrate into the CNS. In MS patients, breakdown of the blood brain barrier (BBB), a physical barrier that limits the access of cells and blood borne molecules into the brain, is apparent in magnetic resonance imaging scans (Carson, Doose, Melchior, Schmid, & Ploix, 2006). It is not known whether this breakdown occurs before CNS invasion or is a result of CNS inflammation; however, following CNS inflammation, movement across the BBB is considered important to disease pathogenesis as blocking this movement with the drug natalizumab (see 1.7.3) reduces disease severity (Polman et al., 2006). It has been postulated that the site of initial invasion into the CNS may be the choroid plexus or the subarachnoid space, although direct movement across the BBB is possible (Kivisäkk et al., 2009; Ransohoff, Kivisäkk, & Kidd, 2003).

Following T cell infiltration and recognition of antigen in the CNS, inflammatory lesions develop. This process is thought to involve recruitment of further peripheral cells, activation of the resident MG, and production of proinflammatory molecules (Sospedra & Martin, 2005). This process results in demyelination and loss of oligodendrocytes, with additional axonal damage (Frohman et al., 2006). While $CD4^+$ T cells are considered important to disease pathogenesis, they tend to be situated on the edge of inflammatory lesions (McFarland & Martin, 2007). It is thought that the majority of tissue damage is mediated by $CD8^+$ T cells, MΦ, and MG. MΦ and MG are capable of producing various toxic components, such as NO, glutamate, and proinflammatory cytokines, that are likely to be involved in CNS damage, and

can also present antigens to T cells (Lassmann, 2008; McQualter & Bernard, 2007; Raivich & Banati, 2004).

### 1.7.3 Current treatments

There are several treatments available for MS. The first treatment to be introduced specifically for MS was the cytokine IFN-β. IFN-β is a first line drug and is effective at reducing the relapse rate in patients with RR-MS by 30% (Khan et al., 2001). However 3-30% of patients taking IFN-β therapies develop neutralising antibodies to the drug, rendering the treatment ineffective (Bertolotto et al., 2002). GA is also considered a first line drug and has similar efficacy to IFN-β therapies (Khan et al., 2001). The low efficacy of these treatments demonstrates a need for more effective therapies.

There are other treatments for MS but these are often considered second line and only used if IFN-β and GA are ineffective. These drugs include immunosuppressive drugs such as mitoxantrone, which can only be used for a limited amount of time due to the negative effects of long term immunosuppressive drugs on the patient's health (Morris & Yiannikas, 2012). Another second line drug is natalizumab, which is a monoclonal antibody that binds to an important molecule for the migration of lymphocytes into the CNS in MS, alpha-4 integrin, and thus reduces the invasion of inflammatory cells into the CNS (Polman et al., 2006; Yadav & Bourdette, 2012). Natalizumab is more effective than GA or IFN-β, and has been shown to reduce relapse rates by up to 68% (Hutchinson, 2007; Polman et al., 2006). However, natalizumab was briefly taken off the market as it was found that it caused an increased risk of progressive multifocal leukoencephalopathy (PML) due to the reactivation of the JC (John Cunningham) virus in the CNS. The risk of developing PML whilst on natalizumab is around 1-2:1000; however, this varies depending on whether the patient is seropositive for the JC virus. Due to the increased risk of PML, natalizumab is only used if other treatments are ineffective (Yadav & Bourdette, 2012).

Fingolimod (Gilenya) is a more recent drug for the treatment of MS and is the first drug for MS to be taken orally. Fingolimod modulates the function of a S1P receptor which is important for the egress of lymphocytes from the lymph nodes. It is thought that fingolimod mediates its effects by preventing egress of lymphocytes from the lymph nodes and thus reducing the number of lymphocytes that invade the CNS. It appears that fingolimod is more effective at reducing relapse rates than IFN-β, showing decreases in relapse rate of 52-60%

(Cohen et al., 2010; Kappos et al., 2010); however, fingolimod treatment does have several safety concerns. Most notably, fingolimod can alter cardiac function, with cardiac side effects seen relatively frequently in patients on fingolimod due to the expression of S1P on cardiac tissues (Cohen et al., 2010; Yadav & Bourdette, 2012). In conclusion, there is a lack of highly effective drugs with good safety profiles for the treatment of MS, therefore it is imperative that new therapies are developed to help treat this disease.

## 1.8 Experimental autoimmune encephalomyelitis

EAE is one of the most studied models of inflammatory disease and has been used for decades as a model for MS. EAE is the most commonly used animal model for MS although other models do exist, including virally mediated models and models in which demyelination in induced by chemicals. EAE was used to develop several drugs including GA, mitoxantrone, and natalizumab for the treatment of MS (Baxter, 2007). EAE is induced by introducing myelin peptides or proteins to the peripheral immune system of animals in the presence of complete Freund's adjuvant (CFA), which boosts the immune response against the myelin components including myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP) and myelin basic protein (MBP) (Baxter, 2007; Merrill et al., 1992). This exposure activates myelin specific T cells, which then proceed to infiltrate the CNS and mediate destruction of the myelin sheath in a manner that is believed to be similar to MS (Mor & Cohen, 1992). EAE can also be induced by adoptive transfer in which transferring encephalitogenic T cells from an animal immunised with myelin peptide or protein to a naïve animal results in disease (Ponomarev et al., 2006).

EAE has been induced in a wide variety of species since its original induction in rhesus monkeys and rabbits, including rats, mice, guinea pigs, goats, hamsters, and sheep. Each of these species displays different symptoms and different patterns of disease (Baxter, 2007). Physically, in most mouse strains, EAE manifests as progressive paralysis starting in the tail and moving up the body (Baxter, 2007; Janeway et al., 2004). Mice are the most commonly used model for studying EAE. Different strains of mice have different patterns of disease, for example C57BL/6 mice display a progressive form of disease following MOG immunisation, while SJL/J mice have a relapsing remitting disease. In addition, different mouse strains with different genetic backgrounds have differing susceptibilities to the myelin peptides that induce disease (Baxter, 2007; Gold, Linington, & Lassmann, 2006).

There are many common features between EAE and MS such as demyelination, the perivascular location of lesions, and myelin as the major target of the immune response. However, there are also several differences, such as the fact that EAE is an induced disease while MS occurs spontaneously (Gold et al., 2006). There has been much criticism of EAE as a model for MS in recent years. Several treatments that showed promise in EAE failed to have a protective effect in MS. In some cases treatments caused detrimental effects in patients that could not be picked up in animal models, such as the reactivation of the JC virus causing PML in a subset of patients on nataluzimab (Baxter, 2007; Gold et al., 2006). However, EAE has successfully been used to model other drugs, such as GA, for treatment in MS. In addition, the lack of other suitable models for the development of treatments for MS necessitates the continued use of animal models in MS drug development.

### 1.8.1 Immunological components

EAE studies have shown proinflammatory autoreactive $CD4^+$ T cells are essential for the development of EAE. Although self-reactive T cells direct damage in the CNS and are believed to be the major cell type involved in the pathogenesis of disease, T cells are not the only cell type involved in EAE/MS. B cells, MΦ, MG, and DC are all believed to play important roles in disease pathogenesis.

Early studies into the immune response involved in EAE implicated Th1 cells in the pathogenesis of EAE. Th1 cells express the cytokines IFN-γ and IL-2. The implication of Th1 cells was in part due to the high number of $CD4^+$ cells in the brain and spinal cord of mice with EAE, which were found to express Th1 related cytokines such as IFN-γ (Merrill et al., 1992). In addition, mice lacking the p40 subunit of IL-12 are resistant to EAE (Cua et al., 2003). Given that IL-12 is essential for the formation of Th1 cells (Kaplan et al., 1996), this was a reasonable conclusion; however, several pieces of evidence contradicted the role of Th1 cells as primary mediators of disease. For example, deletion of IFN-γ, the prototypical Th1 cytokine results in more severe disease, as does deletion of STAT1 through which it signals (Bettelli et al., 2004; M. Krakowski & Owens, 1996). Also, it was found that the IL-12p40 subunit was shared by IL-23, and that it was this cytokine that was essential to the pathogenesis of EAE, not the IL-12 (Cua et al., 2003). It was later found that IL-23 is important in the maintenance of Th17 cells, which are now considered an important cell population in EAE (Bettelli et al., 2006; McGeachy et al., 2007, 2009).

The discovery of Th17 cells suggested that it may only be the Th17 cells and not the Th1 cells that are pathogenic in EAE. However, further studies suggested a role for both cell types. For example, in an adoptive transfer model, Th17 cells were unable to efficiently induce disease in the absence of Th1 cells, which were pathogenic alone. In the presence of Th1 cell, Th17 cells were able to induce a more severe disease than Th1 alone, suggesting that in the absence of Th1 cells, Th17 cannot enter the CNS (O'Connor et al., 2008). However it has also been shown that Th17 cells alone produce an atypical disease characterised by ataxia, as opposed to paralysis, and the presence of both Th1 and Th17 cells produced a more severe form of classical EAE (Domingues, Mues, Lassmann, Wekerle, & Krishnamoorthy, 2010). Thus, the data suggests both Th1 and Th17 are important cell types in EAE.

In addition to proinflammatory T cell subsets there are also T cells types that are associated with protection from EAE. One such T cell population are the Th2 cells. Th2 cells are not generally associated with the induction of EAE, but instead, Th2 responses are associated with protection from EAE (Chitnis et al., 2001; Gimsa, Wolf, Haas, Bechmann, & Nitsch, 2001; La Flamme et al., 2006). IL-4, the prototypical Th2 cytokine, signals via STAT6 and deletion of STAT6 results in more severe EAE and a decrease in Th2 responses (Chitnis et al., 2001). In addition to Th2 cells, Tregs have also been shown to be important in EAE. Protection from EAE by Tregs involves the production of IL-10, and the deletion of these cells has been shown to exacerbate disease (Zhang et al., 2004).

Despite the well documented role of T cells in EAE, they are not the only immune cell subset involved in the disease process. Studies have shown that both DC and MΦ are important for the induction of EAE. Both of these cell types are capable of presenting antigen to T cells and initiating an adaptive response. While MΦ can present antigens, the role of initiating adaptive immune responses in EAE is thought to be primarily performed by DC. It has been demonstrated that DC that have been loaded with MOG *in vitro* are able to induce EAE when introduced to naïve animals (Weir, Nicolson, & Bäckström, 2002). MΦ/monocytes have been shown to be essential for EAE, and MG have also been shown to have an important role. The role of MΦ and MG in EAE will be discussed in more detail below.

### 1.8.2 Role of MΦ and MG in EAE

MΦ have been shown to be essential to disease pathogenesis in EAE. The deletion of MΦ/monocytes from the periphery markedly reduced disease severity. In these MΦ depleted mice, leukocytes were not able to invade the CNS parenchyma, instead accumulating in the leptomeninges, suggesting MΦ are essential for entry to the CNS (Tran, Hoekstra, Rooijen, Dijkstra, & Owens, 1998). In addition, infiltrates of MΦ correlate with disease severity, regardless of the number of lymphocytes present (Gold et al., 2006).

MΦ and MG have many potential roles in tissue damage in EAE. MΦ/MG may act in inflammatory lesions to recruit immune cells into the CNS by secreting chemokines (Gold et al., 2006). In addition, they are capable of secreting compounds that can cause tissue damage, such as NO, which interferes with mitochondria and axonal transmission. MΦ/MG can also produce proinflammatory cytokines, such as IFN-γ and TNF-α, and are capable of antigen presentation (Gold et al., 2006; Lassmann, 2008; Melchior et al., 2006; Raivich & Banati, 2004). These proinflammatory functions likely contribute to the damage done to the CNS and to neurological impairment.

MΦ/MG may also have beneficial roles in EAE, such as the clearance of debris after resolution of the immune response. Both MΦ and MG are involved in phagocytosis of apoptotic T cells in lesions, and in the phagocytosis of myelin sheath components (Jack, Ruffini, Bar-Or, & Antel, 2005). MΦ have also been shown to be important in remyelination. In a study using lysolecithin, an oligodendrocyte toxin which causes demyelination, the depletion of MΦ severely impeded the remeylination of neurons. This study suggests that MΦ may be important in remyelination, possibly by removing myelin debris (Kotter, Setzu, Sim, Van Rooijen, & Franklin, 2001).

There is evidence that MG may have the ability to be either pathological or protective in EAE. There is a lot *in vitro* of data to suggest that MG are capable of a proinflammatory activation state that has the potential to be neurotoxic (Melchior et al., 2006). Many *in vivo* studies also support the pathogenic role of MG in EAE. Importantly, inhibition of MG activation *in vivo* of MG in EAE resulted in decreased disease severity, supporting a pathogenic role for MG in EAE (Heppner et al., 2005). As there is evidence for both pathological and protective roles for MG in EAE, it may be that the MG play different roles depending on the stage of disease.

Evidence to support the proinflammatory role of MG in EAE can be found in studies of bone marrow chimeric mice in which IL-23/IL-12 were not present in the CNS, but were in the peripheral immune system (Becher, Durell, & Noelle, 2003). These studies allow the proinflammatory effects of MG *in vivo* to be observed, albeit in an altered state. MG which are able to produce IL-23 and/or IL-12 are important in the maintenance of EAE. It was shown that mice in which the p40 subunit common to both IL-12 and IL-23 had been specifically deleted from the CNS, but not from the systemic immune system, reduced disease but not the degree of CNS inflammation. In the CNS, higher levels of Th2 cytokines (IL-4) and lower levels of Th1 cytokines (IFN-γ) were found. This suggests that the p40 subunit expression by CNS cells is important for maintenance of a Th1 response in the CNS (Becher et al., 2003). In addition, specific deletion of CD40 from the CNS but not the periphery also protected from disease, suggesting that activation of MG via CD40/CD40L interactions are important in EAE pathogenesis (Becher, Durell, Miga, Hickey, & Noelle, 2001). Interestingly, Th1 cells have been shown to induce a higher level of MG activation compared to Th2 cells, which could contribute to the limited disease seen in the absence of IL-12/IL-23, in which Th2 infiltrates are detected in the CNS (Gimsa et al., 2001).

In contrary to the evidence to suggest MG have a proinflammatory role in EAE, there is some evidence that MG may be activated to a protective activation state. *In vitro,* MG exposed to IL-4 or low levels of IFN-γ promoted neuronal survival, compared to unstimulated MG, suggesting that under controlled conditions MG may be able to promote repair and cell renewal. Repair is usually impaired by inflammatory immune responses, but a well controlled immune response may be protective. While the activation state of MG was not addressed in this study, it is possible that treatment with IL-4 induces an alternatively activated phenotype which may aid in the recovery from CNS insult (Butovsky et al., 2006).

The production of Ym1 (a marker of aaMΦ activation) mRNA by MG *in vivo* suggests that they can become alternatively activated. Some evidence for alternative activation is that IL-4 deficient mice show more severe disease, but when IL-4 is delivered to the CNS directly, the disease is lessened. IL-4 and Ym1 were not found at high levels in the MG of the normal CNS, but were increased in activated MG in the diseased CNS. This study suggests that the majority of MG in active EAE at peak disease are aaMG, as they express Ym1 protein. IL-4 appears to be especially important in the CNS, as IL-4$^{-/-}$ mice that express IL-4 in the periphery have exacerbated disease. In these mice the MG do not express Ym1, suggesting that they are not alternatively activated (Ponomarev, Maresz, Tan, & Dittel, 2007).

## 1.9 Aims and objectives

Broadly, this thesis has several aims which centre around investigating the type II activation of MΦ and MG. Type II MΦ have an immunoregulatory phenotype and have been shown to provide protection in EAE (Tierney et al., 2009). It has been shown that MG are important for regulating immune responses in the CNS. As MG are located at the site of inflammation in EAE and MS, it is likely that type II activated MG will be capable of regulating CNS immune responses and reducing disease severity. To date, there has been no conclusive evidence to show if MG are capable of achieving a type II activation state. This thesis aimed to determine if MG are capable of achieving type II activation *in vitro*. In addition, the interaction between type II MΦ and MG with T cells were assessed, and pathways involved in the biasing of the T cell response were investigated. Finally, this thesis aimed to determine if MG can be type II activated *in vivo* and the mechanisms involved in protection from EAE by type II inducing treatments.

### 1.9.1 Specific aims

1. To investigate in further detail the phenotype of the T cell response induced by activation of T cells by type II MΦ.
2. To elucidate the pathways involved in the biasing of the T cell response by type II MΦ.
3. To optimise a protocol for the isolation and culture of adult murine MG *in vitro*.
4. To determine the ability of MG to achieve a type II activation state *in vitro*.
5. To establish the ability of type II activated MG to bias the T cell response and to investigate possible pathways involved.
6. To investigate the role of type II activating treatments on the regulation of EAE, and the activation state of MG in mice treated with type II inducing treatments *in vivo*.

# Chapter 2:
# General Methods

## 2.1 Mice strains

All mice were housed at the Vertebrate Containment Facility of Victoria University of Wellington (VUW), Wellington, New Zealand, and in the Biomedical research unit (BRU) at the Malaghan Institute of Medical Research (MIMR), Wellington, New Zealand. In both locations, mice were housed in filter-topped cages in a temperature regulated environment with a 12 hour light/dark cycle, and supplied with food and water *ad libitum* (unless experiment required temporary food removal). All experimental work was approved by the Animal Ethics Committee of VUW (ethics approval numbers 2011R21 and 2011R30).

C57BL/6 mice were bred at the BRU at the MIMR (Wellington New Zealand). The breeding stock was originally obtained from Jackson Laboratory (Bar Harbour, ME, USA).

B6.SJL-ptprc$_a$mice were bred at the BRU at the MIMR (Wellington, New Zealand). These are congenic mice on the C57/BL/6 background, expressing CD45.1 as opposed to the CD45.2 expressed by C57BL/6 and 2D2 mice. The breeding stock was originally obtained from the Animal Resource Centre (Canning Vale, WA, Australia).

2D2 mice were bred at the Vertebrate Containment Facility of VUW (Wellington, New Zealand). 2D2 mice are on the C57BL/6 background and are transgenic, expressing a TCR specific for the $MOG_{35-55}$ peptide (Bettelli et al., 2003). The breeding stock was from the MIMR (Wellington, New Zealand) and was originally obtained from Prof. Vijay K. Kuchroo (Harvard Medical School, Boston, MA, USA).

BALB/c mice were bred at the Vertebrate Containment Facility of VUW (Wellington, New Zealand). The breeding stock was originally obtained from Frank Brombacher (University of Cape Town, Cape Town, South Africa).

IL-4R$\alpha^{-/-}$ mice were bred at the Vertebrate Containment Facility of VUW (Wellington, New Zealand). The breeding stock was originally obtained from Frank Brombacher (University of Cape Town, Cape Town, South Africa).

## 2.2 Peptides

$MOG_{35-55}$ peptides (MEVGWYRSPFSRVVHLYRNGK) and $PLP_{180-199}$ peptides (WTTCQSIAFPSKTSASIGSL) were purchased from GenScript Corporation (Piscataway, NJ, USA)

Ovalbumin $(OVA)_{323-339}$ peptide (ISQAVHAAHAEINEAGR) was purchased from Mimotopes (Clayton, VIC, Australia).

## 2.3 *In vivo* techniques

### 2.3.1 EAE induction

#### *2.3.1.1 BALB/c*

8-16 week old mice were immunised by subcutaneous (s.c.) injection in the hind flanks with an emulsion containing $PLP_{180-199}$ (100 µg per mouse; GenScript Corporation, USA) and complete Freunds adjuvant (500 µg heat-inactivated *M. tuberculosis* per mouse, Difco Laboratories, Michigan, USA; in incomplete Freund's adjuvant, Sigma St. Louis, MO, USA; Keating et al., 2009). The emulsion was created by passing the mixture through an 18 gauge needle until a thick consistency had been obtained. The mice also received intraperitoneal (i.p.) injections of pertussis toxin (400 ng per mouse; Sapphire Bioscience, Redfern, NSW, Australia) in 200 µl pertussis buffer (see appendix A) on days 0 and 2.

#### *2.3.1.2 C57BL/6*

16-22 week old bone marrow chimeric mice (C57BL/6→C57BL/6 or B6.SJL-$ptprc_a$→C57BL/6) were immunised by s.c. injection in the hind flanks (100 µl/hind flank) with an emulsion containing $MOG_{35-55}$ (50 µg per mouse; GenScript Corporation, USA) and complete Freunds adjuvant (500 µg heat-inactivated *M. tuberculosis* per mouse, Difco Laboratories, USA; in incomplete Freund's adjuvant, Sigma, USA). The emulsion was created by passing the mixture through an 18 gauge needle until a thick consistency had been obtained. The mice also received i.p. injections of pertussis toxin (200 ng per mouse; Sapphire Bioscience, Australia) in 200 µl pertussis buffer on days 0 and 2 (La Flamme et al., 2006; Tierney et al., 2009).

#### *2.3.1.3 Scoring of diseased mice*

Mice were weighed (using a Mettler PB6000 balance; Mettler Tolodo; Columbus, OH, USA) and scored daily after day 7 post-immunisation. The disease score was assigned using the following criteria; 0=unaffected, 1=tail tone lost, 2=flaccid tail, 3=flaccid tail and affected hind limbs, 4=paralysis of hind limbs, 5=moribund. Mice were euthanised if their disease score reached 5.

### 2.3.2 Immune complex (IC) treatment

IC were prepared as described below (2.5.1). Mice were treated by i.p. injection with $2x10^5$, $2x10^6$, or $2x10^7$ IC in 1x phosphate buffered saline (PBS, see appendix A) 7 days prior to immunisation, on the day of immunisation, and every $7^{th}$ day for the duration of the experiment (La Flamme et al., 2006).

### 2.3.3 GA(Copaxone®) treatment

BALB/c mice were treated with GA (Teva Pharmaceutical Industries Ltd. Petach Tikva, Israel) by adding 500 µg GA per mouse to the emulsion mixture. The emulsion was administered as described above (2.3.1.1). Thus, mice were treated with GA once, on the day of immunisation (Toker, Slaney, Bäckström, & Harper, 2011).

### 2.3.4 Taking tail bleeds for phenotyping

Mice were briefly placed under a heat lamp to encourage vasodilatation and then placed in a restraint. The tip of the mouse's tail was nicked with sharp scissors and blood drops were collected into a sterile microcentrifuge tube that contained 500 µl 1xPBS. The blood was processed as described below (2.4.2.6) for phenotyping mice.

### 2.3.5 Creation of bone marrow chimeric mice

6-8 week old C57BL/6 mice were given antibiotic treated water for 2 weeks (neomycin trisulfate 2 mg/ml, Sigma, USA). Mice were irradiated with 900 cGy following overnight fasting. Bone marrow was harvested from B6.SJL-ptprca and C57BL/6 mice by flushing the tibias and femurs with 1xdPBS (Invitrogen, USA) containing 1% PenStrep (Invitrogen, Carlsbad, CA, USA) with a 23 gauge needle (BD Biosciences, Franklin Lakes, NJ, USA).

The cell solution was homogenised by rapid pipetting with a 10 ml pipette and transferred to a clean tube. Cells were centrifuged at 760xg for 5 minutes, the supernatant was discarded, and cells were resuspended in 1xPBS and counted using a trypan blue exclusion assay (see 2.4.3). The cells were centrifuged at 760xg and resuspended in 1xPBS at $5x10^7$ cells/ml. Irradiated mice were injected intravenously with 200 µl of B6.SJL-ptprc$_a$ or C57BL/6 bone marrow cells ($1x10^7$ cells/mouse). Mice were maintained on antibiotic treated water for 2 weeks to reduce the risk of infection as mice are immunodeficient following irradiation. Mice were left to recover for 6-10 weeks and to allow full reconstitution of the bone marrow. After 6 weeks, blood samples were taken via tail bleed (see 2.3.4) to assess the expression of CD45.1 and CD45.2 on the peripheral leukocytes, and to assess the extent of reconstitution of the bone marrow. Mice were used for EAE experiments 6-10 weeks following irradiation.

## 2.4 *Ex vivo* techniques

### 2.4.1 Dissection and organ removal

Mice were sacrificed by $CO_2$ asphyxiation or by a lethal overdose of sodium pentobarbital (6 mg/mouse). To remove the spleen, the peritoneal cavity was opened and the spleen removed using scissors and forceps sterilised with ethanol. Bone marrow was harvested by carefully removing the skeletal tissue from the hind limbs with sterile scissors and forceps, and disjointing the hip. The tibias and femurs were severed at both ends and flushed with 1x dPBS (Invitrogen, USA) containing 1% PenStrep (Invitrogen, USA) via a 23 gauge needle (BD Biosciences, USA). For the removal of brains and spinal cords, the mice were first perfused to remove contaminating blood cells. To perfuse, the portal vein was severed and a 23 gauge needle inserted into the left ventricle of the heart to slowly perfuse the mouse with 10-20 ml 1x PBS. Following perfusion, the skin covering the upper back and head was removed, the skull cap carefully cut off, and the brain removed using sterile forceps. To isolate the spinal cord, the spinal column was severed at the lower back and just above the shoulders. A 19 gauge needle (BD Biosciences, USA) was inserted into the lumbar end of the severed spinal column and 1x PBS was used to push the spinal cord out of the cervical end of the spinal column.

## 2.4.2 Preparation of single cell suspensions

### *2.4.2.1 Spleens*

Following isolation, spleens were placed in wash buffer (see appendix A) or dynabead isolation buffer (see appendix A) for use in splenocyte restimulation assays (see 2.5.8) or T cell isolation (see 2.5.5), respectively. Spleens were dissociated by passing them through a 70 μM cell strainer (BD Biosciences, USA) into a 50 ml conical tube with wash buffer or Dynabead isolation buffer (see appendix A). Cells were centrifuged at 760xg for 5 minutes. To remove red blood cells (RBC), splenocytes were resuspended in 2 ml RBC lysis buffer (Sigma, USA) for 2 minutes at room temperature. The splenocytes were washed with 8 ml of wash or dynabead isolation buffer and centrifuged at 760xg for 5 minutes. Cells were resuspended in fresh wash or dynabead isolation buffer as appropriate, and counted using a trypan blue exclusion assay (see 2.4.3).

### *2.4.2.2 Isolation of CNS cells by Percoll*

Following isolation, brains and spinal cords were placed in 1xPBS. Brains and spinal cords were dissociated by passing them through a 70 μm cell strainer (BD Biosciences, USA) into a 50 ml conical tube with 1x PBS. Cells were centrifuged at 760xg for 5 minutes. For isolation for culture or for fluorescent activated cell sorting (FACS), cells were resuspended in 10 ml of 70% Percoll (Sigma, USA; see appendix A) and gently overlaid with 37% and 30% Percoll (see appendix A). The discontinuous Percoll gradient was centrifuged at 760xg for 30 minutes with slow acceleration and no brake. Mononuclear cells were removed from the 70:37% interface, then washed by diluting the solution at least 3 fold with 1xPBS and centrifuging at 760xg for 5 minutes. Cells were resuspended in MG media or FACS buffer (see appendix A), as appropriate, and counted using a trypan blue exclusion assay, prior to centrifugation at 760xg for 5 minutes and resuspention at the desired concentration in MG media or FACS buffer. For flow cytometric analysis of brains alone, dissociated brains were resuspended in 10 ml of 37% Percoll and centrifuged at 760xg for 30 minutes with slow acceleration and no brake. Myelin was removed from the surface of the Percoll and the excess Percoll was decanted. Cells were then resuspended in 1x PBS, washed by centrifugation at 760xg for 5 minutes and resuspended in FACS buffer.

***2.4.2.3 Microglia isolation by adhesion***

Isolation of MG by adhesion was adapted from a protocol by Yip, Kaan, Fenesan, & Malcangio (2009). Spinal cords were removed as described above (2.4.1) and placed in Hibernate media supplemented with 1xB27 supplement and 0.5mM L-glutamine (all from Invitrogen, USA). Spinal cords were minced with a blade and placed in Hibernate media containing 2 mg/ml papain (Sigma, USA) and incubated at 37ºC for 30 minutes. The tissue was left to settle and supernatant was discarded. 2 ml of supplemented Hibernate media was added, the tissue broken up by repeated pipetting, the tissue was left to settle, and then the supernatant was collected. This step was repeated twice more. The cell solution was centrifuged at 400xg for 5 minutes and the supernatant was discarded. Cells were resuspended in DFP media (see appendix A) and filtered through a 70 µm cell strainer (BD Biosciences, USA). 10 µl of cells were mixed with 10 µl of 20 µg/ml DAPI (Sigma, USA) and 10 µl was loaded onto an improved Neubauer haemocytometer (Hawksley, UK). The number of live cells was counted using an Olympus IX51 inverted microscope (Olympus, USA) using UV light generated by a mercury burner (Olumpus, USA). Cells were plated at 10,000, 20,000, or 40,000, cells/well in 96 or 24 well plates, or cells from a whole spinal cord were plated onto a non-tissue culture treated petri dish and incubated at 37ºC/5% $CO_2$ for 2 hours. The media was changed, and cells were removed from the plates at 2 or 24 hours after plating to be used for flow cytometry, as described (see 2.9).

***2.4.2.4 MG isolation with CD11b beads***

Two different bead kits were use to isolate MG. For isolation using the CELLection biotin binder kit (Invitrogen, USA), single cell solutions of spinal cords were obtained as described in 2.4.2.3, and resuspended at $1x10^7$ cells/ml in buffer (1xdPBS with 1% bovine serum albumin (BSA) and 2mM Ethylenediaminetetraacetic acid (EDTA)). Isolation was performed as described by the manufacturer. Briefly, 25 µl of beads were washed by adding buffer and placing on a magnet and removing the supernatant. Beads were resuspended and mixed with 2 µg biotinylated anti-$CD11b^+$ antibody. This solution was incubated for 30 minutes at room temperature. The mixture was washed three times by placing the solution on a magnet and removing the supernatant, and fresh buffer was added once the solution had been removed from the magnet. The beads were mixed with cells and incubated at 4ºC for 20 minutes. Unbound cells were removed by washing the solution on the magnet 3 times, as previously described. Cells were released from the beads by incubation with 260 units (U) of DNase I (Invitrogen, USA) in releasing buffer (RPMI 1640 with 1% FCS, 1 mM $CaCl_2$ and 5 mM

$MgCl_2$) for 15 minutes at room temperature, followed by vigorous pipetting. Cells were removed by placing the tube on the magnet and colleting the supernatant. The cells were then processed for flow cytometry as described (see 2.9).

For isolation with (microglia) Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) single cell suspensions of brain and spinal cord were generated by passing through a 70 μM cell strainer (BD Biosciences, USA). Myelin was either not separated, or else removed by resuspending cells in 37% Percoll followed by centrifugation at 760xg for 30 minutes, and collecting the myelin from the surface. Cells were then washed with 1xPBS. The (microglia) Microbeads kit (Miltenyi Biotec, Germany) was used according to manufacturer's instructions. Briefly, cells were resuspended in 90 μl dynabead isolation buffer (see appendix A), 10 μl of beads were added, and the solution was incubated for 15 minutes at 4ºC. The dynabead buffer was added and the solution centrifuged at 300xg for 10 minutes. The supernatant was then discarded and the cell-bead mix was resupended in 500 μl dynabead isolation buffer. An LS column (Miltenyi Biotec, Germany) was placed in a MidiMACs magnet (Miltenyi Biotec, Germany) and rinsed with 3 ml of dynabead isolation buffer. The cell-bead mix was added to the column and allowed to flow through before the column was washed 3 times with 3 ml of dynabead isolation buffer. The column was removed from the magnet and 5 ml of dynabead isolation buffer added to the top of the column. Cells were isolated by depressing the plunger of the column down. Cells were processed for flow cytometry as described (see 2.9).

#### ***2.4.2.5 Isolation of microglia from chimeric mice by fluorescent activated cell sorting (FACS)***

Chimeric mice were generated as described in 2.3.5. Immunised mice (and non-immunised controls, see 2.3.1.2) were sacrificed and central nervous tissue was removed as described (2.4.1). Tissue from both brains and spinal cord were dissociated and mononuclear cells were isolated on a discontinuous Percoll gradient (see 2.4.2.2). Isolated cells were stained for FACS as described (see 2.9) using CD45.2, CD11b and 4',6-diamidino-2-phenylindole (DAPI) to isolate live MG cells. Cells were isolated using a FACS Vantage DiVa cell sorter at MIMR (Wellington, New Zealand). RNA was isolated as described below (see 2.10).

#### *2.4.2.6 Blood*

Blood was isolated by tail bleeds (see 2.3.4 and 2.4.1). For phenotyping, blood (2-3 drops) was transferred to 15 ml conical tubes and centrifuged at 400xg for 5 minutes. The supernatant was removed and the cells were resuspended in 3 ml RBC lysis buffer (Sigma, USA) and incubated for 20 minutes at 37ºC. 1x PBS was added and the cells were centrifuged at 400xg for 5 minutes. The lysis step was repeated if required. Cells were resuspended in FACS buffer (see appendix A) and processed for flow cytometry as described in 2.9.

### 2.4.3 Trypan blue exclusion assay

Cells were mixed with 0.4% Trypan blue dye (Sigma, USA) at a desired dilution (generally either 1:2 or 1:10). 10 µl of the cell/trypan solution was loaded into an improved Neubauer haemocytometer (Hawksley, Lancing, UK). The number of live cells was counted using a compound microscope (CX41: Olympus, PA, USA).

### 2.4.4 Fixation and sectioning of tissues

Perfused brains and spinal cords were incubated in 4% paraformaldehyde (PFA, see appendix A) at room temperature for 48 hours. Tissues were transferred to 30% sucrose solution (with 1 mM sodium azide, see appendix A) and incubated at room temperature for 24 hours. The tissues were transferred to fresh 30% sucrose solution and stored at 4ºC. To section, fixed tissues were frozen, mounted with Jung tissue freezing medium (Leica Biosystems, Nussloch, Germany) and 30 µM sections were cut using a cryostat (Leica CM3050 S, Leica Biosystems, Germany) and transferred to a 24 well plate (approximately 6-10 section/well) containing 1x PBS with 1mM sodium azide. Tissue sections were stored at 4ºC.

### 2.4.5 Immunohistochemistry (IHC).

Sections were transferred to a clean 24 well plate containing 1xPBS (3 sections per well). 1x PBS was removed and sections were incubated with 500 µl/well peroxidise quenching solution for 20 minutes. Peroxidase quenching solution was removed and sections were washed 3 times for 5 minutes each with TPBS (see appendix A). After the 3$^{rd}$ wash, sections were incubated in IHC block (see appendix A) for 20 minutes. IHC block was removed from the wells, and 200 µl of primary antibody (1:300; ionised calcium binding adaptor molecule 1 (Iba1) Abcam, Cambridge, England) diluted in IHC block was added, and the sections were

incubated overnight at 4ºC. The primary antibody solution was removed and sections were washed 3 times for 5 minutes each with TPBS. After the 3rd wash, sections were incubated in 200 µl of secondary antibody (1:300, biotinylated donkey-anti-goat; Jackson Laboratories, Bar Harbor, Maine, United States) diluted in IHC block, and incubated at room temperature for 2 hours. The secondary antibody solution was removed and sections were washed 3 times for 5 minutes each with TPBS. After the 3rd wash sections were incubated in 200 µl/well of avidin-biotin ABC kit (Vector Labs, Burlingame, California, USA) for 1 hour at room temperature. The avidin-biotin ABC solution was removed, and sections were washed 3 times for 5 minutes each with TPBS. After the 3rd wash sections were developed using 200 µl/well of VIP vector substrate (Vector Labs, USA). When the colour had adequately developed the solution was removed, and sections were washed 3 times for 5 minutes each with TPBS and then mounted onto poly-L-lysine coated slides. After 48 hours, the slides were counterstained with haematoxylin (see 2.4.6).

### 2.4.6 Counterstaining with haematoxylin

Slides were rinsed with water and the sections were covered with haematoxylin solution (Sigma, USA) for 20 seconds, then rinsed and placed in water. Slides were placed in acid alcohol (see appendix A) for 1 minute, then transferred back to water briefly before being placed in 0.01% lithium carbonate (see appendix A) for 30 seconds. Finally, the slides were transferred back into water. Sections were dehydrated by successive 5 minute incubations in 70%, 95% and 100% ethanol, before being place in xylene (Sigma, USA) for at least 5 minutes. Coverslips were then placed on the slides using Depex (BDH laboratory Supplies, Poole, England). Sections were analysed on an Olympus IX51 inverted microscope (Olympus, USA), and photos were taken using CellA software (Olympus, USA).

### 2.4.7 Scoring of Iba1 stained sections

The intensity of the Iba1 stained sections was assessed visually. Four brain regions, the cerebellum, hippocampus, olfactory bulb and brain stem, were assessed. The sections were blinded for treatment group and mouse number at the time of analysis, and then un-blinded once the data entry had been completed. Brain regions were given a score between 0 and 3 based on the intensity of Iba1 staining (by comparison to pre-prepared example photos). As this procedure is subjective, sections were scored individually by three separate people.

## 2.5 *In vitro* techniques

### 2.5.1 Generation of IC

ICs were prepared by opsonising sheep red blood cells (SRBC, isolated from a healthy sheep, Taylor Prestons Ltd. Wellington, New Zealand) with rabbit anti-SRBC IgG polyclonal antibody (Sigma, USA). To opsonise the SRBC, $1x10^8$ SRBC/ml in 1xPBS were incubated with the highest non-agglutinating concentration of anti-SRBC IgG (Sigma, USA) as determined by an agglutination assay (see 2.5.9) for 30 minutes at room temperature on a rotator. The IC solution was centrifuged at 300xg for 5 minutes to remove non-bound antibody and resuspended in either 1xdPBS or complete T cell media (CTCM) for *in vivo* or *in vitro* use, respectively.

### 2.5.2 Bone Marrow Macrophage (BMMΦ) Culture

Bone marrow was harvested from the femurs and tibias of 8-16 week old mice (see 2.4.1). Bone marrow was homogenised by repeat pipetting with a 10 ml serological pipette. RBCs were lysed by incubation with 2 ml RBC lysis buffer for 2 minutes at room temperature. Cells were then washed in wash buffer, and resuspended at $1x10^6$ cells/ml in CTCM. Cells were cultured overnight in T75 $mm^2$ tissue culture flasks (BD Biosciences, USA), before non-adherent cells were transferred to 90 mm petri dishes (Techno Plas, St Marys, Australia) and cultured in the presence of GM-CSF and IL-3 (both at 5 ng/ml, Peprotech, New Jersey, USA). Fresh media containing GM-CSF and IL-3 (final concentration of both 2.5 ng/ml) was added on the $5^{th}$ day in culture. After 9 days in culture, media was removed and BMMΦ were isolated by blasting (using ice cold 1xdPBS (Invitrogen, USA) and rapid pipetting to detach the MΦ from the petri dish). BMMΦ were replated in CTCM at $1x10^5$ cells/well in a 96 well plate (BD Biosciences, USA), and cultured overnight in the presence of IFN-γ (20 U/ml). Following IFN-γ stimulation, BMMΦ were incubated alone or with LPS (200 ng/ml, Sigma, USA), in the presence or absence of IC (10 IC per MΦ, $1x10^6$/well) for 24 hours. For experiments investigating the pathways in MΦ responses and interaction with T cells, neutralising antibodies and recombinant cytokines were added at the time of MΦ stimulation or 4 hours post stimulation (for a list of antibodies and cytokines see appendix B).

### 2.5.3 RAW-264.7 Cell Culture

The established macrophage-like cell line, RAW-264.7, was a generous gift from the MIMR (Wellington, NZ). The cell cultures were maintained every 72 hours by seeding a T75 $mm^2$ cell culture flask (BD Biosciences, USA) at $1x10^6$ cells per 15 ml of CTCM and were then incubated for 72 hours at 37°C, 5% $CO_2$. When at 80% confluency, the monolayers were rinsed once in 1x dPBS (Invitrogen, USA) and then mechanically disrupted. Cell suspensions were transferred to a sterile 15 ml conical tube, centrifuged at 300x g for 5 min, and assessed for cell viability using the Trypan blue exclusion assay (section 2.4.3). RAW-264.7 cells were stimulated as described in 2.10.1.

### 2.5.4 MG Culture

Mice were euthanised, perfused and CNS tissue was removed as described (see 2.4.1). MG were isolated for culture under sterile conditions from the brains and spinal cords of naïve mice. For immediate culture, a single cell suspension of CNS tissue using a 70 μm cell strainer and MG were isolated using a 70:37:30% discontinuous Percoll gradient as described in 2.4.2.2. MG purity was assessed by flow cytometry. MG were seeded at $5x10^5$ cells/well in CTCM in a 6 well plate and cultured at 37ºC/5% $CO_2$ overnight. Cells were isolated from 6 well plates using ice cold 1x dPBS and cell viability was assessed using a trypan blue exclusion assay (see 2.4.3). Alternatively, MG were seeded at $1x10^5$ cells/well in a flat bottomed 96 well plate (BD Biosciences, USA) and stimulated with 20 U/ml IFN-γ overnight, before culture in the presence or absence of LPS (200 ng/ml, Sigma, USA) and IC ($1x10^6$/well).

For growth in GM-CSF, brain and spinal cord was digested with papain (as described in 2.4.3.2) and MG were isolated on a discontinuous Percoll gradient as described (2.4.2.2). MG purity was assessed by flow cytometry. All MG isolated from one mouse were seeded in CTCM in a T75 $mm^2$ flask (BD Biosciences, USA) and cultured for 4-8 weeks in the presence of 5 ng/ml GM-CSF. Media was changed every 3-4 days (Moussaud & Draheim, 2010).

For growth in M-CSF a single cell suspension of CNS tissue using a 70 μm cell strainer and MG were isolated using a 70:37:30% discontinuous Percoll gradient as described in 2.4.2.2. MG purity was assessed by flow cytometry. MG were resuspended in MG media supplemented with 10 ng/ml M-CSF and seeded at $5x10^5$ cells/well in a 24 well plate or at $5x10^4$ cells/well in a flat bottomed 96 well plate, and cultured for 4 weeks, with media

(supplemented with 10 ng/ml M-CSF) changed every 3-4 days (Ponomarev, Novikova, Maresz, Shriver, & Dittel, 2005). MG cultured in a 24 well plate were isolated using ice cold 1x dPBS and cell viability was assessed using a trypan blue exclusion assay (see 2.4.3). Following 4 weeks of culture, MG cultured in 96 well plates were stimulated with IFN-γ overnight, before culture in the presence or absence of LPS (200 ng/ml, Sigma, USA) and IC ($1x10^6$/well). In some cases, M-CSF was removed from the media 3 days prior to IFN-γ stimulation.

### 2.5.5 $CD4^+$T cell isolation

Single cell solutions were generated from 2D2 mice (see 2.4.2.1) and $10x10^7$ splenocytes were resuspended in 10 ml Dynabead isolation buffer in a 15 ml conical tube. 25 µl of washed mouse CD4 (L3T4) beads (placed on a magnet for 2 minutes in 1 ml dynabead isolation buffer, and resuspended in Dynabead isolation buffer at the original volume; Invitrogen, USA) were added per $1x10^7$ splenocytes. The splenocytes/bead solution was incubated for 20 minutes at 4ºC on a rotator. The solution was place on a DynaMag magnet (Invitrogen, USA) and the non-bead bound cells were discarded. The CD4 T cells/beads were washed 3 times by placing tube on the magnet and discarding the supernatant, and then the CD4 T cells/beads were resuspended at $10x10^7$ cells/ml in CTCM. To separate the bead bound cells from the beads, 10 µl of mouse CD4 DETACHaBEAD (Invitrogen, USA) per $1x10^7$ cells was added, and the solution was incubated on a rotator for 45 minutes at room temperature. The tube was placed on a magnet and the supernatant containing $CD4^+$ T cells was transferred to a fresh tube. The beads were washed 4 more times by placing the tube on the magnet and harvesting the supernatant to ensure a maximal yield of cells, and counted using a trypan blue exclusion assay (see 2.4.3). $CD4^+$ T cells were washed once and used for MΦ or MG:T cell co-culture.

### 2.5.6 T cell co-culture with MΦ or MG

MΦ and MG were stimulated as described above (see 2.5.2 and 2.5.4-grown in M-CSF), with the exception that the IFN-γ was washed out of the cultures with warm media following overnight stimulation, before the next stimuli were added. 4 hours post stimulation, $2.5x10^5$ $CD4^+$ T cells/well and MOG (25 µg/ml unless otherwise specified) or OVA (1 µM) was added to the cultures and incubated for 72 hours, at which time the supernatant was harvested and the cells were processed for flow cytometry. For experiments investigating the pathways involved in MΦ/MG T cell interactions, neutralising/inhibitory antibodies, recombinant

cytokines, and chimeric proteins were added at the time of MΦ stimulation or 4 hours post stimulation (for a list of antibodies, cytokines and proteins see appendix B).

### 2.5.7 CFSE cell proliferation assay

$CD4^+$T cells were isolated as in 2.5.5 and resuspended at $2x10^7$ cells/ml in 1xdPBS (Invitrogen, USA). 625 nM carboxyfluorescein succinimidyl ester (CFSE; Sigma, USA) was added and incubated at room temperature for 8 minutes in the dark. The reaction was quenched by adding an equal volume of 100% foetal calf serum (FCS). Cells were washed once with dPBS (Invitrogen, USA) and twice with CTCM. Cells were used in T cell co-cultures in the presence or absence or 1 mM aminoguanidine hemisulfate (AG, Sigma, USA).

### 2.5.8 Splenocyte restimulation assay

Splenocytes were isolated as described above and plated in CTCM at $1x10^6$ cells/well in a U-bottomed 96 well plate (BD Biosciences, USA). Splenocytes were cultured alone, or in the presence of 3 μg/ml ConA for 48 hours, or 27 μg/ml MOG for 72 hours.

### 2.5.9 Agglutination assay

An agglutination assay was performed on every fresh batch of sheep blood to determine the optimum concentration of anti-SRBC IgG to use. Serial two-fold dilutions of anti-SRBC IgG (Sigma, USA) in dPBS (starting at 1:50) were plated at 50 μl/well in a U bottomed 96 well plate in duplicate. SRBC were added to the wells at a final concentration of $1x10^8$ cells/well and incubated at room temperature for 2 hours. The highest non-agglutinating concentration of anti-SRBC IgG was determined by visualisation.

## 2.6 Cytometric bead array (CBA)

Mouse Th1/Th2/Th17 cytokine and mouse inflammation CBA kits (BD Biosciences, USA) were used to assess cytokine production by cultured cells. Briefly, 25 μl of culture supernatant or standards (in a serial dilution to form the standard curve) were mixed with 25 μl mixed capture beads and 25 μl of phycoerythrin (PE) detection reagent in a V-bottomed plate (BD Biosciences, USA) and incubated, protected from light, at room temperature for 2 hours. The samples were washed twice by the addition of wash buffer and centrifugation at 400xg for 5 minutes, then discarding the supernatant. The samples were resuspended and

collected using a FACs Canto II flow cytometer (BD Biosciences, USA) and analysed using BD FACSDiva and FCAP Array software (BD Biosciences, USA). The theoretical limit for detection ranged from 0.03-16.8 pg/ml for the mouse Th1/Th2/Th17 kit, and 2.5-52.7 pg/ml for the mouse inflammation kit, as described in the manufacturer's instruction booklets.

## 2.7 Enzyme linked immunosorbent assay (ELISA)

Purified "capture" antibodies were diluted in either ELISA capture buffer (see appendix A) or 1x PBS (depending on the cytokine in question), coated onto the bottom of 96 well ELISA plates (BD Biosciences, USA or Thermo Fisher, Massachusetts, USA) and incubated overnight at 4ºC. The capture solution was removed and blocking solution (5 or 10% FCS in 1x PBS) was used to block plates for 2 hours at room temperature. The plates were washed 4 times in ELISA wash buffer (0.05% Tween 20 (Sigma, USA) in PBS), 50 µl of standards and samples were added (standards and, if necessary, samples were diluted in block) and the plates were incubated overnight at 4ºC. The standards and samples were removed and the plates were washed 4 times. 50 µl of biotinylated "secondary" antibody, diluted in block, was added. After 1 hour incubation at room temperature the secondary antibody was removed and the plates were washed 6 times. 50 µl of Streptavidin conjugated horseradish peroxidase (BD Biosciences, USA) was added to the plates. After 1 hour incubation at room temperature the solution was removed and the plates were washed 8 times. 100 µl/well of Tetramethyl benzidine (TMB) solution (equal parts OptEIA™ TMB A and TMB B solutions, BD Biosciences, USA) was used to develop the wells. After sufficient colour had developed, the reaction was terminated by adding 100 µl/well stop solution (see appendix A). Absorbance was measured at 450 nm on an EnSpire 2300 multilabel plate reader (PerkinElmer, Massachusetts, USA) and a standard curve was generated to obtain concentration values for the samples using the EnSpire software (PerkinElmer, USA). For complete list of antibody combinations for ELISA see appendix B.

## 2.8 Griess reaction

Since NO is rapidly degraded into nitrite and nitrate, the Griess reaction measures nitrite as a indicator of NO production (Sun, Zhang, Broderick, & Fein, 2003). Nitrate standards were serially diluted in CTCM. Griess reagents A and B (see appendix A) were combined in equal volumes and 50 µl was added to 50 µl of the standards ($NaNO_2$, serially diluted) and samples

(culture supernatant) in a flat bottomed 96 well plate. Absorbance was measured at 570 nm on an EnSpire 2300 multilabel plate reader (PerkinElmer, USA) and a standard curve was generated to obtain concentration values of samples using the EnSpire software (PerkinElmer, USA).

## 2.9 Flow cytometry

All antibodies used in flow cytometry had been optimised to ensure both maximal signal and minimal background staining. Samples were washed in FACS buffer and incubated in 50 µl of a mixture containing both primary antibody and 2.4G2 blocking antibody (BD Biosciences, USA). Cells were stained for 20 minutes at 4ºC, then excess FACS buffer was added and the cells were centrifuged at 400xg for 5 minutes. If a biotinylated antibody was used, cells were incubated with 50 µl of streptavidin conjugated to an appropriate fluorophore, then excess FACS buffer was added and the cells were centrifuged and resuspended in FACS buffer. Cells were analysed on a Canto II flow cytometer or a FACS Vantage DiVa cell sorter (BD Biosciences, USA) and data was analysed using FLOWJO 7.6.1 software (Tree Star, Ashland, OR, USA). See appendix B for list of antibodies and secondary conjugates used.

## 2.10 Isolation of mRNA from MG isolated by FACS

Complete mRNA was isolated from sorted MG (stimulated as described for BMMΦ, see 2.5.2) using a Dynabeads mRNA DIRECT Micro kit (Invitrogen, USA) according to manufacturer's instructions. Briefly, cells were washed in 1xPBS and resupended in 100 µl lysis/binding buffer. Repeat pipetting was performed to ensure complete lysis of cells, and the lysates were stored at -80ºC for processing at a later date. 20 µl/sample Dynabeads Oligo $(dT)_{25}$ were washed with 1 ml lysis/binding buffer by placing the bead/buffer solution on a DynaMag™-Spin magnet (Invitrogen, USA) for 1 minute. The beads were removed from the magnet and resuspended in their original volume with lysis/binding buffer. 20 µl of washed beads per sample was mixed with the lysed cell solutions and incubated for 5 minutes at room temperature with rotation. The samples were washed by placing the sample on the magnet for 1 minute and discarding supernatant, then the tube was removed from the magnet and 100 µl of wash buffer A was added. The sample was washed once more with wash buffer A, and resuspended in wash buffer B, then transferred to a new tube and washed once with wash buffer B. After the final wash, the bead-mRNA solution was resuspended in 20 µl of ice

cold 10 mM Tris-HCL. mRNA was eluted by heating the solution to70ºC for 2 minutes and then immediately placing the tube on the magnet and collecting the mRNA containing supernatant. mRNA was used immediately for cDNA generation or stored at -80ºC until required.

### 2.10.1 Isolation of mRNA from RAW-264.7 Cell Lines for PCR Optimisation

RAW-264.7 cells were seeded at $5.25x10^5$ cells per 4 mL of CTCM supplemented with 20 U/ml of IFN-γ in a 60 mm diameter petri dish and incubated at 37 $^oC$, 5% $CO_2$ for 18 hours. Cells were then cultured with or without 200 ng/ml LPS stimulation for a further 24 hours. The confluent monolayer of approximately $5x10^6$ cells was rinsed once in 1x dPBS (Invitrogen, USA) before applying 1 ml of Trizol® (Invitrogen). The Trizol®-treated sample was transferred to a 1.5 ml microfuge tube and shaken with 0.2 mL of chloroform, incubated at room temperature for 3 minutes, and centrifuged at 4000x g for 15 minutes. The aqueous layer was collected to a new RNase-free tube and 2 volumes of room temperature absolute ethanol was added. The ethanol mixture was then applied to a High Pure RNA Isolation Kit spin column (Roche; Basel, Switzerland) by centrifugation at 8,000xg in a 2 ml collection tube. Samples were then prepared according the manufacturer's instructions. mRNA was used immediately for cDNA generation or stored at -80ºC until required.

## 2.11 Generation of cDNA

cDNA was generated by a reverse transcription (RT) reaction using a SuperScript III First-Strand Synthesis SuperMix kit (Invitrogen, USA) according to manufacturer's instructions. Briefly, 2 µl of RT master mix 1 (Table 2.1) was added to 6 µl of RNA. The solution was incubated at 65ºC for 5 minutes and then immediately placed on ice for at least 1 minute. 12 µl of master mix 2 (Table 2.1) was added to the solution. The solution was incubated at 50ºC for 50 minutes followed by 85ºC for 5 minutes to terminate the reaction. cDNA was used immediately or stored at -80ºC until required.

**Table 2.1 RT master mixes**

| | Reagent | Volume per reaction (20 µl final) | Volume added per reaction |
|---|---|---|---|
| RT master mix 1 | Oligo $(dT)_{20}$ | 1 µl | 2 µl |
| | Annealing buffer | 1 µl | |
| RT master mix 2 | 2x First-Strand Reaction mix | 10 µl | 12 µl |
| | SuperScript III/RNaseOUT Enzyme mix | 2 µl | |

## 2.12 Quantitative Polymerase Chain Reaction (qPCR)

### 2.12.1 Primers

Primers used in this study (Table 2.2) were synthesised by Invitrogen (USA). Lyophilised primers were reconstituted at 250 µM with DEPC treated $H_2O$ and stored at -80ºC. 10 µM working stocks were generated by dilution of primers in DEPC treated water.

**Table 2.2 Gene specific primers**

| Gene | Protein | Product length | | Primer sequence (3'→5') |
|---|---|---|---|---|
| *Il12b* | IL-12p40 | 255 base pairs | Forward | ATGGCCATGTGGGAGCTGGAGAAAG |
| | | | Reverse | GTGGAGCAGCAGATGTGAGTGGCT |
| *Ppia* | Cyclophillian A | 98 base pairs | Forward | GTCTCCTTCGAGCTGTTTGC |
| | | | Reverse | GAGGAACCCTTATAGCCAAATCC |

### 2.12.2 qPCR reaction

qPCR was performed using a Platinum SYBR Green qPCR SuperMIX-UDG kit (Invitrogen, USA). 4.5 µl of cDNA template was added to duplicate wells of a 96 well PCR plate (Thermofisher, USA) for each gene. 20.5 µl of qPCR master mix containing the appropriate gene specific primers were added to the appropriate wells. The PCR plate was briefly mixed and centrifuged at 400xg for 2 minutes. qPCR was performed on a CFX Connect™ Real-Time PCR Detection System, using the PCR program described in Table 2.4. Following amplification, a melt curve was performed between 65ºC and 95ºC at 0.5ºC increments.

**Table 2.3 qPCR master mix**

| | Reagent | Volume per reaction (25 µl final) |
|---|---|---|
| qPCR master mix | Platinum SYBR Green qPCR SuperMIX-UDG | 12.5 µl |
| | Forward primer (10 µM) | 0.5 µl |
| | Reverse primer (10 µM) | 0.5 µl |
| | DEPC treated $H_2O$ | 7.0 µl |

**Table 2.4 PCR cycling conditions**

| Temperature (ºC) | Time | Process | Cycles |
|---|---|---|---|
| 50 | 2 minutes | | 1 |
| 95 | 2 minutes | | 1 |
| 5 | 15 seconds | | 40 |
| 55 | 10 seconds | | |
| 72 | 30 seconds | Plate read | |
| 95 | 10 seconds | | 1 |
| 65→ 95 | | Melt curve | |

### 2.12.3 Gel electrophoresis

The size of the PCR products was assessed by gel electrophoresis using a 2% agarose gel (Bioline, London, UK) v/w in 1x TAE buffer (see appendix A) with 1 µg/ml ethidium bromide. Gels were run at 100V for 45-100 minutes. To determine product size, a 1 Kb plus ladder (Invitrogen, USA) was utilised. The gel was visualised under UV light.

## 2.13 Graphs and statistics

All graphs and statistical analyses were generated using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). To to find and remove outliers present in sample data, the ROUT test in Graphpad Prism was applied, when the ROUT test was applied, the most stringent test paramaters available in Graphpad Prism (Q=0.1%) were used to ensure only the most definitive outliers were removed. Comparisons between two groups were performed using an unpaired Student's t test, and for non-parametric comparison of two groups a Mann Whitney test was applied. For comparison of more than two treatment groups, a one way ANOVA with a Tukey's multiple comparison post test was performed. To compare groups based on two variables (e.g. multiple groups of immunised mice over disease course), statistics were

calculated using a two way ANOVA with a Sidak's multiple comparisons post test. p values below 0.05 were considered significant.

# Chapter 3:
# Biasing of the T cell Response by Type II MΦ

## 3.3 Results

### 3.3.1 Type II activation of MΦ with IC

Exposure of IFN-γ-primed MΦ to LPS causes classical activation and leads to a significant upregulation of IL-12 compared to unstimulated MΦ (i.e. medium alone; Figure 3.1b). Consistent with previous studies (Anderson & Mosser, 2002; Tierney et al., 2009), this production of IL-12 was significantly attenuated in IFN-γ-primed MΦ that were cultured with LPS in the presence of IC (LPS+IC) compared to LPS alone, although levels were still significantly increased compared to medium alone (Figure 3.1b). In addition, MΦ incubated with LPS+IC had significantly increased levels of IL-10 compared to all other culture conditions (Figure 3.1a). In contrast to MΦ cultured with LPS+IC, MΦ that were cultured with IC in the absence of LPS produced only low levels of IL-12 or IL-10 and were similar to MΦ cultured with medium alone (Figure 3.1a and b).

As expected, classically activated MΦ upregulated the expression of PD-L1 and CD40 compared to unstimulated MΦ. In comparison, MΦ cultured with LPS+IC significantly down regulated their expression of PD-L1 and showed a trend towards decreased levels of CD40 (Figure 3.1c and d) as reported previously (Tierney et al., 2009). Although the decrease in CD40 did not reach statistical significance in this study, previous work has demonstrated that this decrease is both significant and consistent (Tierney et al., 2009). As with cytokine production, MΦ cultured with IC in the absence of LPS had a similar expression of PD-L1 and CD40 as unstimulated MΦ (Figure 3.1c and d). Taken together, the reduced expression of IL-12, CD40, and PD-L1 along with the increased IL-10 found when IFN-γ-primed MΦ were cultured with LPS+IC compared to LPS alone are indicative of type II MΦ activation (Figure 3.1).

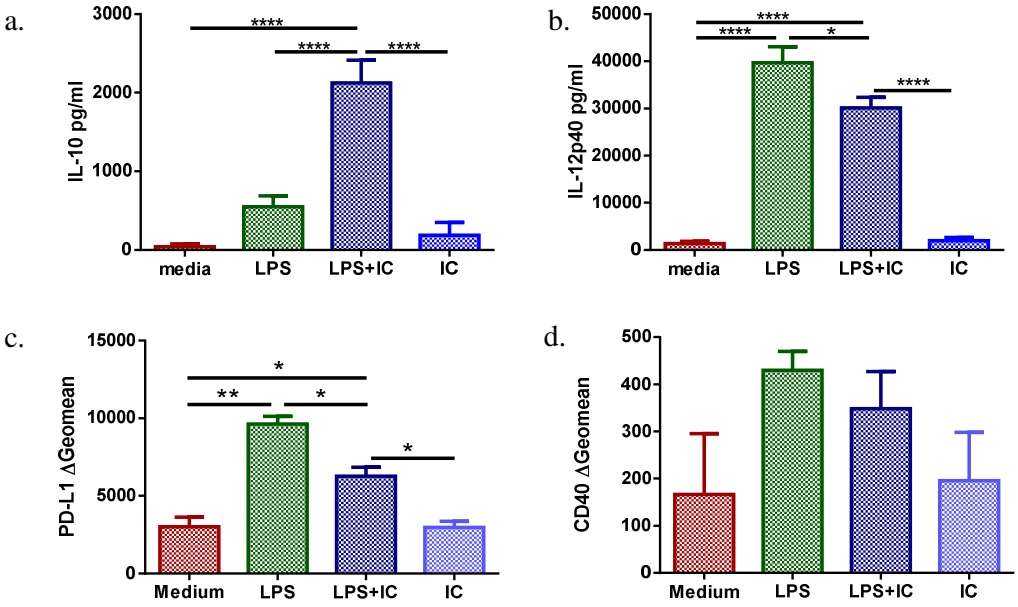



**Figure 3.1. Type II activated MΦ (LPS+IC) express less IL-12, CD40, and PD-L1 and more IL-10 than classically activated MΦ (LPS).** BMMΦ from C57BL/6 mice were plated at $1x10^5$ and primed with 20 U/ml IFN-γ overnight before stimulation with 200 ng/ml LPS, LPS+IC (10 IC per MΦ) or IC alone for 24 hours. IL-10 (a) and IL-12p40 (b) levels were measured by ELISA. Expression of PD-L1 (c) and CD40 (d) was assessed by flow cytometry. Shown are the means and SEM of triplicate wells from 13 experiments (a and b) or of flow cytometry data from 2 combined experiments (c and d). *p<0.05, **p<0.01, ****p<0.0001 by a one-way ANOVA with Tukey's multiple comparison post test.

### 3.3.2 Optimisation of co-culture conditions

2D2 transgenic mice express T cell receptors that are specific for the myelin protein $MOG_{35-55}$ ($V\beta_{11}$ and $V\alpha_{3.2}$; Bettelli et al., 2003). This strain spontaneously develops optic neuritis in approximately 30% of animals and EAE in around 4% due to impaired tolerance (Bettelli et al., 2003). To ensure that each 2D2 mouse expresses the MOG-specific TCR, T cells were routinely isolated via tail bleeds and confirmed to be positive for both $V\alpha_{3.2}$ (Figure 3.2a) and $V\beta_{11}$ (Figure 3.2b). $CD4^+$ T cells isolated from the spleens of 2D2 mice were also consistently found to be suitably pure (94.8±0.67; mean ± SEM from 11 experiments). Consistent with previous reports 2D2 $CD4^+$ T cells produced IL-2 in response to $MOG_{35-55}$, but not OVA peptide and did not produce IL-2 in the absence of peptide (Figure 3.2c; (Bettelli et al., 2003).

In order to optimise the culture system for presentation of antigen by MΦ, the response of T cells to $MOG_{35-55}$ presented by classically activated MΦ was evaluated using IL-2 as a read out of T cell activity. As shown in Figure 3.3, 2D2 $CD4^+$ T cells responded to $MOG_{35-55}$ in a concentration dependent fashion. Because previous studies have used 25 μg/ml $MOG_{35-55}$ and this peptide concentration led to high level IL-2 production in our T cell culture system, this peptide concentration was used for all subsequent experiments (Li et al., 2009).

Previous work has shown that type II activated MΦ produce decreased levels of IL-12 and increased levels of IL-10 compared to classically activated MΦ when cultured alone (Figure 3.1a and b; Anderson & Mosser, 2002). Interestingly, this altered cytokine profile is maintained when MΦ are cultured with T cells (Figure 3.4a and b). This finding shows that the presence of naïve T cells does not cause the MΦ to alter their phenotype and suggests that this phenotype is maintained over the culture time. While it is possible that some of the IL-10 is being produced by T cells that have been activated to a Treg or Th2 phenotype, it is likely that most of the IL-10 is being produced by MΦ, which are known to produce high levels of IL-10 upon type II activation.

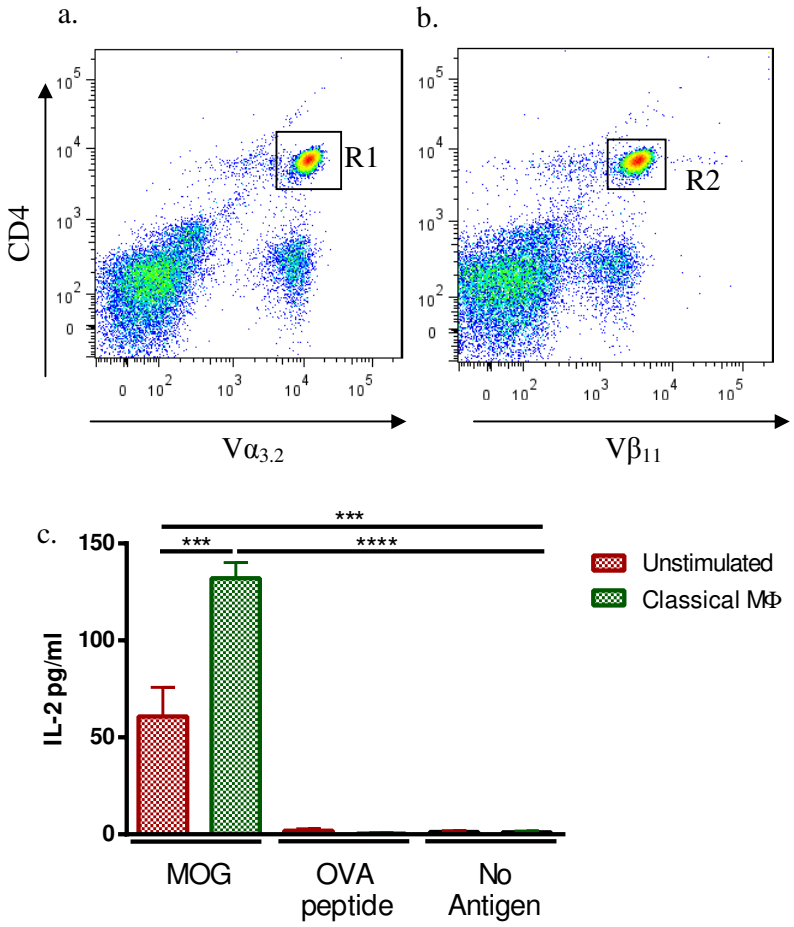



**Figure 3.2. CD4+ cells isolated from 2D2 mice express Vα3.2 and Vβ11 and are specific for MOG.** (a and b) Blood was obtained by tail bleed and the expression of $V\alpha_{3.2}$ (a) and $V\beta_{11}$ (b) were assessed by flow cytometry. R1 (a) contains $CD4^+V\alpha_{3.2}^+$ cells and R2 (b) contains $CD4^+V\beta_{11}^+$ cells. Shown are representative plots from 1 mouse. (c) BMMΦ were stimulated with 20 U/ml IFN-γ overnight and then cultured with or without LPS (200 ng/ml). Four hours post-stimulation, purified $CD4^+$2D2 T cells were added and cultured in the presence or absence of $MOG_{35-55}$ (25 μg/ml) or OVA peptide (1 μM). After 72 hours, IL-2 was measured using a CBA. Shown are the means and SEM of triplicate wells from one experiment. ***p<0.001 and ****p<0.0001 by one-way ANOVA with Tukey's multiple comparison post test.

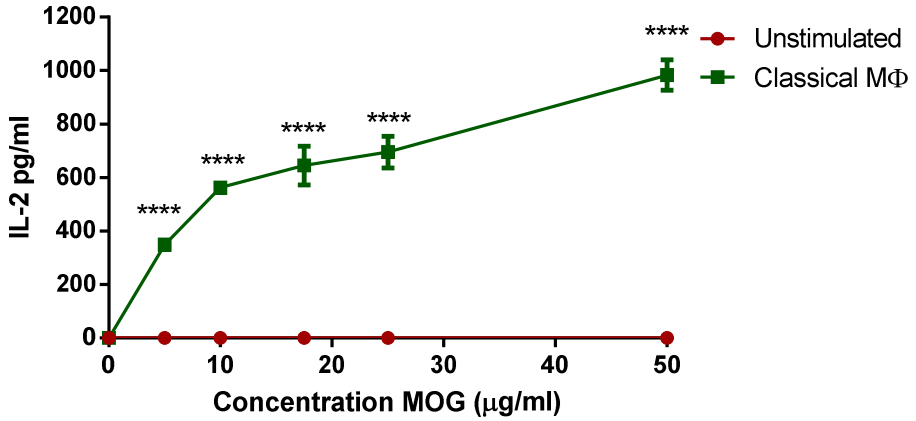



**Figure 3.3. CD4+ T cells respond to MOG in a concentration-dependent fashion.** BMMΦ were stimulated with 20 U/ml IFN-γ overnight and then cultured with or without LPS (200 ng/ml). After four hours, purified $CD4^{+}2D2$ T cells were added to the MΦ cultures in the presence or absence of $MOG_{35-55}$. After 72 hours, IL-2 was measured in the culture supernatants by ELISA. Shown are the means and SEM of triplicate wells from one experiment. ****$p<0.0001$ by two-way ANOVA with Sidak's multiple comparison post test.

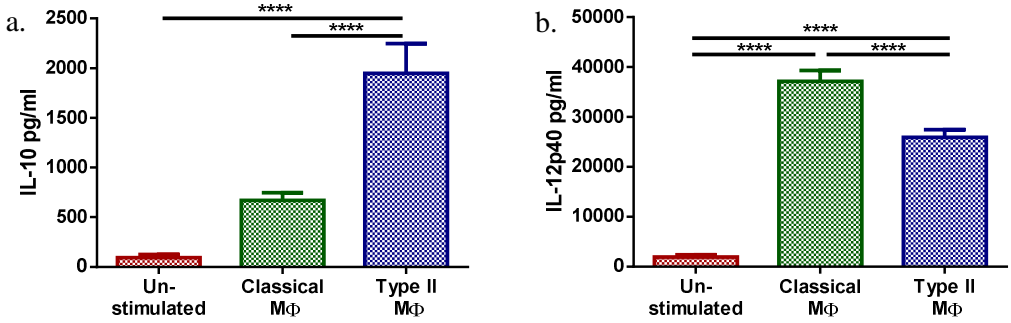



**Figure 3.4. Type II activated MΦ retain their cytokine profile in MΦ:T cell co-cultures.** BMMΦ were stimulated with 20 U/ml IFN-γ overnight and then cultured alone or with LPS (200 ng/ml) with or without IC (10 per MΦ). After four hours, purified $CD4^+2D2$ T cells and $MOG_{35-55}$(25 μg/ml) were added to the MΦ cultures for 72 hours. IL-10 (a) was measured in the culture supernatant by ELISA and CBA, and IL-12p40 (b) was measured by ELISA. Shown are the means and SEM of values combined from 16 (IL-10) or 17 (IL-12) independent experiments. ****$p<0.0001$ by one-way ANOVA with Tukey's multiple comparison post test.

### 3.3.3 Classically activated MΦ bias the T cell response to Th1

Upon activation, T cells alter the expression of several cell surface markers. These changes include upregulation of CD44 and down regulation of CD62L; thus, activated T cells are considered to be $CD44^{hi}CD62L^{lo}$. In MΦ:T cell co-cultures, T cells cultured with classically activated MΦ in the presence of $MOG_{35-55}$ express lower levels of CD62L (Figure 3.5a) and higher levels of CD44 (Figure 3.5b) compared to T cells cultured with unstimulated MΦ or in the absence of $MOG_{35-55}$. In addition, T cells cultured with classically activated MΦ also express higher levels of CD25 (Figure 3.5c), which is known to be upregulated on activated T cells. The level of IL-2 produced by T cells cultured with classically activated MΦ is greater than that produced by T cells cultured with unstimulated MΦ demonstrating that classically activated MΦ are more efficient at activating T cells in an antigen-specific manner (Figure 3.5d). Similarly, the expression of CD25 on T cells cultured with classically activated MΦ is also enhanced compared to co-cultures with unstimulated MΦ (Figure 3.5c). Overall, the alteration in the levels of CD62L, CD44, CD25 and IL-2 demonstrate that T cells cultured with classically activated MΦ achieve a more activated state than those cultured with unstimulated MΦ.

To determine how antigen presentation by classically activated MΦ affected T cell biasing, the production of IFN-γ (prototypical Th1 cytokine), IL-17A (Th17 cytokine), and the expression of CD124 (IL-4Rα; upregulated on Th2 cells) was determined. T cells cultured with classically activated MΦ produced higher levels of IFN-γ compared to T cells cultured with unstimulated MΦ (Figure 3.6a). Moreover, the decreased expression of CD124 on T cells cultured with classically activated MΦ suggests the T cells are becoming less responsive to IL-4 and thus are deviating away from a Th2 type response (Figure 3.6d). Given that classically activated MΦ have been shown to drive Th1 responses (Anderson and Mosser, 2002) it is likely that this decrease in CD124 is due to the T cells differentiating into Th1 cells. Interestingly, IL-6 was significantly increased in cultures containing classically activated MΦ (Figure 3.6c) and while this cytokine along with TGF-β drives Th17 development, no difference in IL-17A was detected (Figure 3.6b). Overall the decreased expression of CD124 and increased production IFN-γ supports the conclusion that classically activated MΦ bias the T cell response towards a Th1 phenotype.

Because proliferation is the hallmark of an antigen-specific T cell response, MOG-specific T cell proliferation was assessed in the co-cultured using the CFSE dye dilution assay. This

method demonstrated that despite significant cytokine production by the T cells, $CD4^+$ T cells cultured with classically activated MΦ did not proliferate at all while those cultured with unstimulated MΦ did (Figure 3.7a, c, and e). Since the proliferation of T cells can be inhibited by NO, which inhibits proliferation, reportedly without affecting the cytokine profile (van der Veen, Dietlin, Dixon Gray, & Gilmore, 2000), the presence of NO was measured by Griess reaction. In contrast to co-cultures of T cells and unstimulated MΦ, which produced only very low levels of NO, high levels of NO were detected in co-cultures containing classically activated MΦ (Figure 3.7e). In order to verify that this high level of NO can inhibit the proliferation of the $CD4^+$ T cells in this co-culture system, 1 mM AG was added to the cultures to specifically inhibit iNOS and thus prevent NO production (Figure 3.7e). The addition of AG had no effect on the proliferation of T cells cultured with unstimulated MΦ, which was expected as there was very little NO in the absence of AG (Figure 3.7c-f). However, when AG was added to cultures with classically activated MΦ, there was an increase in the proliferation of T cells indicating that the high NO levels prevented T cell proliferation (Figure 3.7a, d, e, and f).

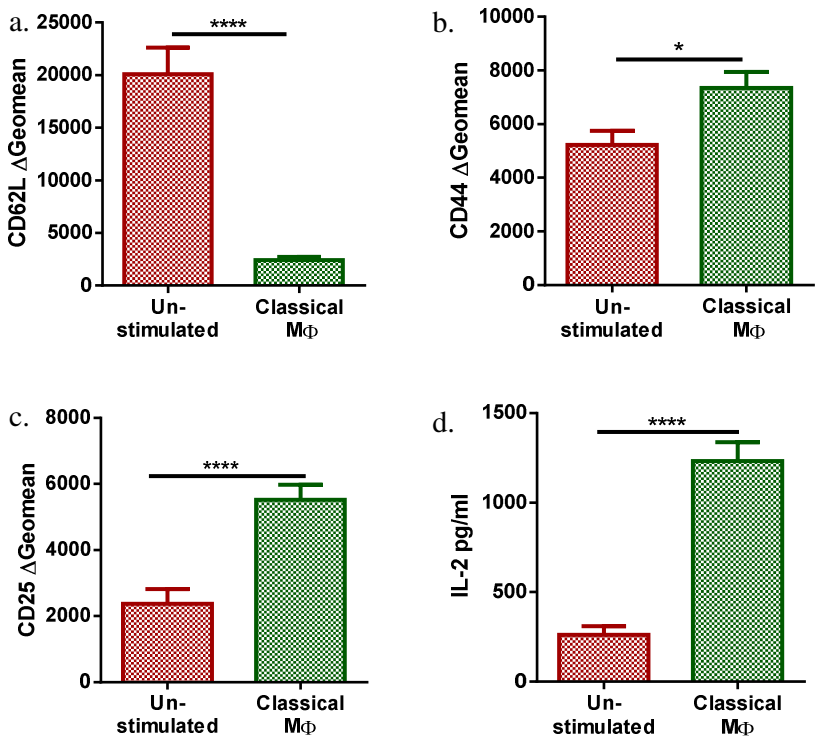



**Figure 3.5. Classically activated MΦ activate T cells more efficiently than un-stimulated MΦ.** BMMΦ were stimulated with 20 U/ml IFN-γ overnight and then cultured alone or with 200 ng/ml LPS. After four hours, purified $CD4^{+}2D2$ T cells were added to the cultures with 25 μg/ml MOG. Cells were cultured for 72 hours and the expression of CD62L (a), CD44 (b), and CD25 (c) was assessed by flow cytometry and IL-2 levels in the culture supernatants by ELISA (d). Data was subjected to the ROUT test which removed 1 outlier from CD25 (un-stimulated), before statistics were calculated. Shown are the means and SEM of triplicate wells from 19 (CD62L, CD44 and CD25) or 15 (IL-2) independent experiments. *$p<0.05$, ****$p<0.0001$ by unpaired Student's t test.

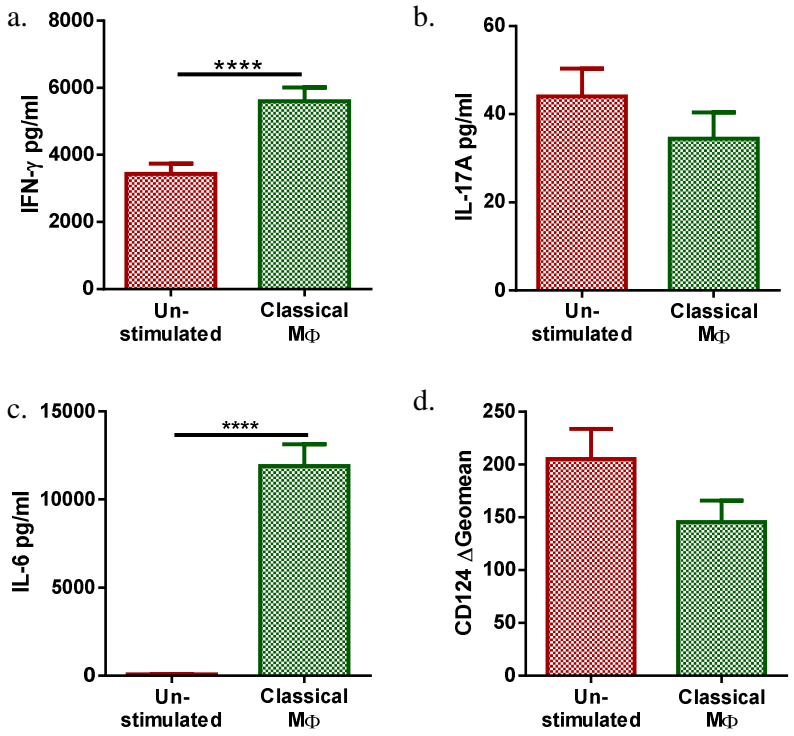



**Figure 3.6. Classically activated MΦ induce Th1 T cell biasing.** BMMΦ were stimulated with 20 U/ml IFN-γ overnight and then cultured alone or with LPS (200 ng/ml). After four hours, purified $CD4^{+}2D2$ T cells were added to the cultures with 25 μg/ml MOG. Cells were cultured for 72 hours and IFN-γ (a) and IL-17A (b) levels were measured by ELISA. IL-6 (c) was measured by CBA and CD124 (d) by flow cytometry. Data was subjected to the ROUT test which removed 1 outlier from IFN-γ (unstimulated) and 1 from CD124 (unstimulated), before statistics were calculated. Shown are the means and SEM of triplicate wells from 15 (IFN-γ), 12 (IL-17A), 3 (IL-6) or 19 (CD124) independent experiments. ****$p<0.0001$ by unpaired Student's t test.

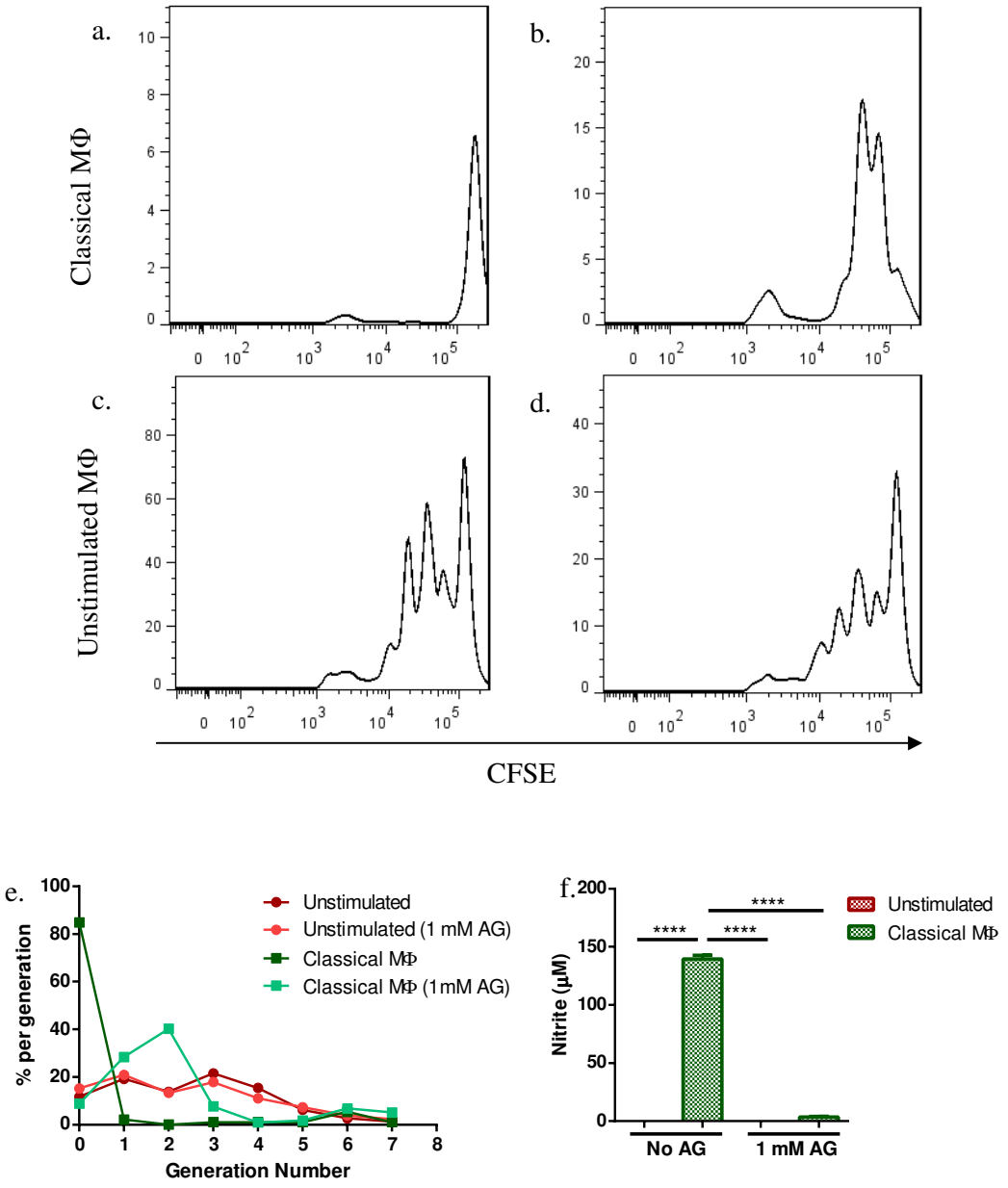



**Figure 3.7. Classically activated MΦ produce high levels of NO which inhibits the proliferation of T cells.** BMMΦ were stimulated with 20 U/ml IFN-γ overnight and then cultured with or without LPS (200 ng/ml) in the presence or absence of 1 mM AG. After four hours, purified CFSE-labelled CD4+2D2 T cells and MOG (25 μg/ml) were added to the cultures. Cells were cultured for 72 hours. Cells were analysed by flow cytometry to assess proliferation without (a, c) or with (b, d) 1 mM AG, proliferation is expressed as percentage per generation (e). Nitrite levels in the culture supernatants were measured by Griess reaction (f). Shown are representative plots and data from one of two representative experiment. ****p<0.0001 by one-way ANOVA with Tukey's multiple comparison post test.

### 3.3.4 Type II activated MΦ induce biasing of the T cell response

Both type II and classically activated MΦ produced similarly high levels of NO, which can be inhibited by the addition of 1 mM AG to the cultures (Figure 3.8a). In the absence of AG, no antigen-specific proliferation was measured in co-cultures with either type II or classically activated MΦ. The addition of 1 mM AG to the cultures to inhibit NO production allowed MOG-driven T cell proliferation to be evaluated (Figure 3.8b). In the presence of AG, type II activated MΦ induced slightly greater T cell proliferation than classically activated MΦ, however, this did not reach statistical significance (Figure 3.8b).

To assess how type II activated MΦ altered the activation state of T cells in addition to slightly enhancing proliferation, the expression of markers associated with activation were assessed including CD62L, CD44, and CD25. T cells cultured with type II activated MΦ expressed higher levels of CD62L, which is down-regulated upon activation, and lower levels of CD44, which is upregulated on activated T cells, compared to T cells cultured with classically activated MΦ (Figure 3.9a and b). In comparison, CD25, which is expressed on activated lymphocytes, and is also strongly associated with the Treg and Th2 lineages, was not significantly altered on T cells cultured with type II compared to classically activated MΦ (Figure 3.9c). Additionally, despite the slight increase in T cell proliferation induced by type II activated MΦ, there was no difference in the amount of IL-2 produced by T cells cultured with type II and classically activated MΦ (Figure 3.9 d). Overall, these changes in cell surface marker expression suggests that the T cells exposed to type II MΦ are not as activated as those exposed to classically activated MΦ, despite having equal or higher proliferative potential (Figure 3.8).

As reported previously, IFN-γ production by T cells cultured with type II activated MΦ was decreased compared to those co-cultured with classically activated MΦ (Figure 3.10a, Anderson & Mosser, 2002), and this decrease in IFN-γ is suggestive of a phenotypic switch away from the Th1 lineage. However, the production of IL-17A, the prototypical Th17 cytokine, was increased by T cells cultured with type II activated MΦ compared to classically activated MΦ (Figure 3.10b). IL-6 is one of the essential cytokines involved in Th17 differentiation (Bettelli et al., 2006), yet surprisingly, IL-6 levels were modestly but significantly lower in cultures containing type II MΦ compared to cultures containing classically activated MΦ (Figure 3.10c) suggesting that it is not an increase in IL-6 that is driving the IL-17A production. Additionally, T cells cultured with type II MΦ expressed

higher levels of CD124 compared to T cells cultured with classically activated MΦ (Figure 3.10d). Because CD124 is upregulated on Th2 cells in order to increase their responsiveness to IL-4, this receptor can be used as a surrogate for IL-4 production, as IL-4 is rapidly taken up in an autocrine loop making detection in culture supernatants difficult. IL-4 levels were measured by CBA or ELISA in several independent experiments; however, the levels of IL-4 were below detection (data not shown). Together these results indicate that T cells are being biased away from a Th1 phenotype and toward a mixed Th17/Th2 type response.

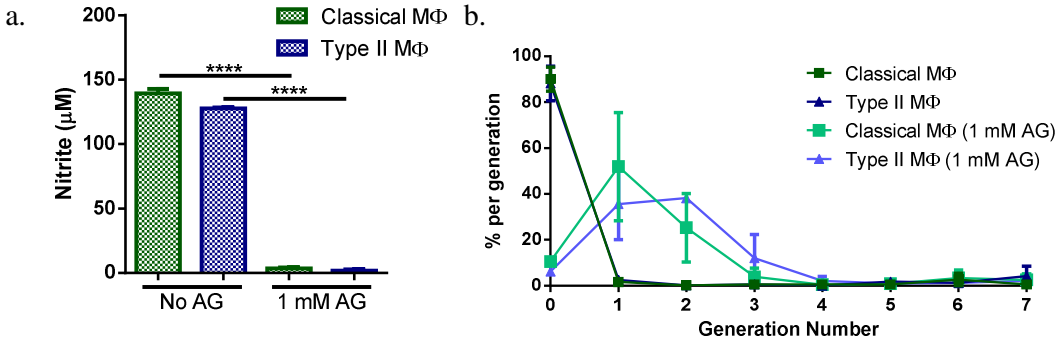



**Figure 3.8. Type II activated MΦ slightly enhanced proliferation of T cells in the absence of NO.** BMMΦ were stimulated with 20 U/ml IFN-γ overnight and then cultured with LPS (200 ng/ml) alone or with IC (10 per MΦ) in the presence or absence of 1 mM AG. After four hours, purified CFSE-labelled $CD4^{+}2D2$ T cells and MOG (25 μg/ml) were added to the cultures and cultured for 72 hours. Nitrite levels in the culture supernatants were measured using a Griess reaction (a), and proliferation was analysed by flow cytometry (b). Shown are the means and SEM combined from 2 independent experiments. ****$p<0.0001$ by one-way ANOVA with Tukey's multiple comparison post test (a).

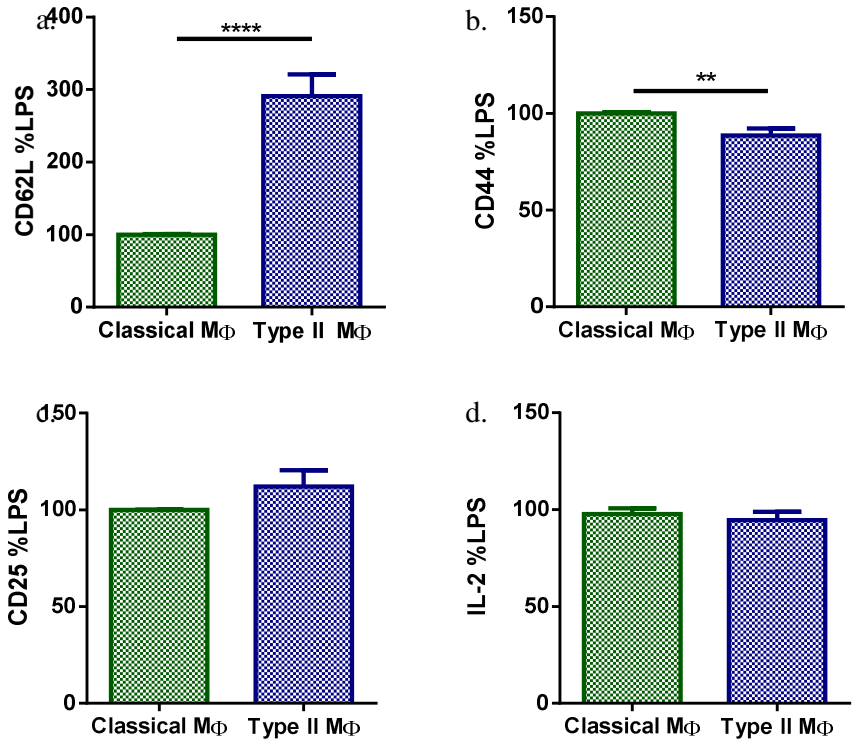



**Figure 3.9. Type II activated MΦ differentially activated T cells.** BMMΦ were stimulated with 20 U/ml IFN-γ overnight and then cultured with LPS (200 ng/ml) alone or with IC (10 per MΦ). After four hours, purified $CD4^+2D2$ T cells and MOG (25 μg/ml) were added to the cultures and cultured for 72 hours. CD62L (a), CD44 (b), and CD25 expression (c) were analysed by flow cytometry, and IL-2 levels by ELISA (d). Shown are the means and SEM of triplicate wells combined from 19 (CD62L, CD44 and CD25) or 15 (IL-2) independent experiments, and the results are expressed as percentage of LPS stimulated. **p<0.01 and ****p<0.0001 by unpaired Student's t test.

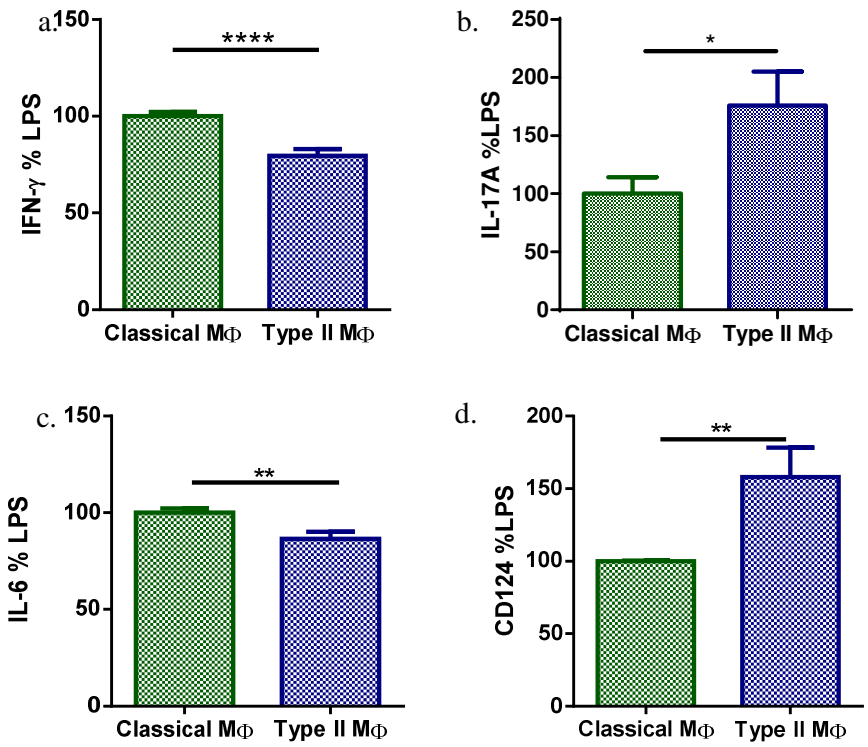



**Figure 3.10. In MΦ:T cell co-culture conditions, type II activated MΦ induce biasing of the T cell response away from Th1 phenotype.** BMMΦ were stimulated with 20 U/ml IFN-γ overnight and then cultured with LPS (200 ng/ml) alone or with IC (10 per MΦ). After four hours, purified $CD4^{+}2D2$ T cells and MOG (25 μg/ml) were added to the cultures and cultured for 72 hours. IFN-γ (a), IL-17A (b) levels were measured by ELISA, IL-6 (c) levels were measured by CBA and CD124 (d) expression was assessed by flow cytometry. Data was subjected to the ROUT test which removed 4 outliers from IFN-γ (type II MΦ), before statistics were calculated. Shown are the means and SEM of triplicate wells combined from 15 (IFN-γ), 12 (IL-17A), 3 (IL-6) or 19 (CD124) independent experiments, and the results are expressed as percentage of LPS stimulated. **p<0.01, ***p<0.001, and ****p<0.0001 by unpaired Student's t test.

## 3.4 Discussion

The studies in this chapter aimed to evaluate and compare the ability of type II and classically activated MΦ to induce biasing of the T cell response. Consistent with previous reports (Anderson & Mosser, 2002) classically activated MΦ were able to induce a Th1 response from naïve T cells characterised by the production of high levels of IFN-γ. In contrast, T cells activated by type II activated MΦ produced lower levels of IFN-γ, consistent with the T cell response being directed away from a Th1 type response. Although no IL-4 was detected in these cultures, this does not mean that IL-4 was not produced as it can be taken up quickly in an autocrine manner. Indeed, the increased level of CD124 (the α chain of the IL-4 receptor) on T cells cultured with type II MΦ suggests an increased ability to respond to IL-4, which could contribute to low or undetectable levels of IL-4 in the culture supernatant. In addition, increases in CD124 levels suggest that type II MΦ are biasing the T cells towards a Th2 phenotype. However, a surprising finding of this study is that type II activated MΦ also induce a robust and consistent increase in IL-17A; a finding that has not been reported elsewhere to date.

The novel finding that type II activated MΦ induced IL-17A production from T cells is surprising given that IL-10, which is produced in high levels by type II MΦ, suppresses IL-17A production (Gu et al., 2008). Induction of IL-17A by regulatory/suppressive cells of the myeloid lineage has been seen previously (Chatterjee et al., 2013). Myeloid derived suppressor cells have been shown to be capable of inducing IL-17A production from naïve T cells and was associated with increased IL-6 and TGF-β production (Chatterjee et al., 2013). Thus, there could be a number of potential explanations for the increase in IL-17A in this study. The most obvious is that type II activated MΦ are inducing a shift towards Th17 responses; however, several other findings make this possibility unlikely. Firstly, CD124 is up regulated in type II MΦ:T cell cultures and to date it has not been documented that Th17 cells up regulate CD124; and furthermore given that IL-4 is counter regulatory for Th17 cells (Harrington et al., 2005), an up regulation of CD124 on Th17 cells would be unlikely. Secondly, while IL-6 is present in both classical and type II MΦ:T cell cultures, it is slightly but significantly decreased in cultures with type II MΦ demonstrating that it is not an increase in IL-6 that is driving IL-17A production and thus Th17 biasing. However, GA activates MΦ/monocytes to an activation state that strongly resembles type II activation and have been shown increase production of TGF-β (Weber et al., 2007). TGF-β is often considered immunoregulatory and is involved in the formation of Tregs. However, in the presence of IL-6, TGF-β induced Th17 development (Bettelli et al., 2006). Although it is decreased, IL-6

is still present in the co-cultures containing type II MΦ and this level may be high enough to drive Th17 production in the presence of TGF-β. To our knowledge it has not been documented that MΦ stimulated with LPS+IC up-regulate TGF-β. However, the similarity between GA and LPS+IC stimulated MΦ suggests that increased TGF-β production by type II MΦ is a possibility. An increase in TGF-β production provides a potential pathway for increased IL-17A levels in this system. It has also been demonstrated that under some conditions Th17 cells can also produce IL-10, and that these cells have regulatory properties and are protective in EAE (McGeachy et al., 2007). Therefore it is possible that type II MΦ are inducing a regulatory Th17 subset as opposed to an inflammatory Th17 subset.

Another possible explanation for the increased IL-17A production by T cells activated by type II MΦ is that the increase is a reaction to the reduced level of IL-12 and IFN-γ in cultures containing type II MΦ. As both IL-12 and IFN-γ inhibit IL-17A production (Harrington et al., 2005; Komiyama et al., 2006) it is possible that the decreases in IL-12 and IFN-γ have allowed for an increase in IL-17A. Additionally, there is evidence for the transient nature of Th17 cells (Kurschus et al., 2010; Lee et al., 2009) and it is therefore possible that the IL-17A producing cells in these cultures may not represent a fully differentiated subset but may instead be a transient population that has arisen due to the lack of inhibitory cytokines. To determine if the population is in fact a distinct subset, the T cells could be restimulated after 7 days to see if the expression of IL-17A is maintained over time. In addition, determining if the T cells cultured with type II MΦ expressed RORγt, the master regulator of the Th17 cell lineage (Ivanov et al., 2006), and were exclusive producers of IL-17A, as IL-17A has been seen to be co-expressed with other cytokines such as IFN-γ (Kurschus et al., 2010; Lee et al., 2009) would also confirm if these T cells were biased to become Th17 cells.

While the decrease in IFN-γ and the increase in CD124 certainly demonstrate that biasing of the T cell response by type II is occurring and that the T cell response is likely being biased towards a Th2 response, the biasing is not as marked as that which has been observed in previous studies (Anderson & Mosser, 2002; Edwards et al., 2006). While significant the decrease in IFN-γ is slight and may not on its own be biologically relevant, however, this change in combination with other effects of type II MΦ on T cells may have an effect over all. There are several factors which could explain this difference including mouse strain and T cell clone. One important factor to consider is the strain of mice used for the experiments. In previous work MΦ were derived from BALB/c mice (Anderson & Mosser, 2002; Edwards et al., 2006), whereas in the current study the MΦ were derived from C57BL/6 mice as the 2D2

mouse strain is on a C57BL/6 background (Bettelli et al., 2003). The strain of mouse is relevant here as C57BL/6 mice preferentially produce a Th1 type immune response, whereas BALB/c mice preferentially produce a Th2 type response (Mills, Kincaid, Alt, Heilman, & Hill, 2000). It therefore may be that the T cells from mice with a BALB/c background, as used in previous studies, were more susceptible to Th2 biasing conditions than the T cells from a C57BL/6 background used in this study.

In addition to the mouse background, the T cell clone and TCR may also be involved in biasing though altered signalling pathways post TCR activation. Weak signalling through the TCR, such as with antigen that the TCR has a weak affinity for or low level of antigen which the TCR has a high affinity for, biases the T cell response towards a Th2 response, whereas high affinity antigen at a sufficient concentration will drive the T cell response to a Th1 response (Tao, Constant, Jorritsma, & Bottomly, 1997; Yamane & Paul, 2013). It is likely that the OVA specific T cell clone used previously (Anderson & Mosser, 2002) and the MOG specific T cell clone used in the current study have different affinities for their respective ligands, which could affect the biasing of the T cell response in co-culture with classical or type II activated MΦ.

In addition, it is possible that the time point of 72 hours used to assess T cell phenotype was too early to be able to accurately assess IL-4 production. It generally takes longer for IL-4 to be upregulated compared to IFN-γ (Bird et al., 1998); therefore, this early time point may not truly demonstrate the level of IL-4 production and T cell biasing that is occurring. Restimulation of the T cells at 7 days would possibly give a better idea of the level of IL-4 production as Anderson *et al.* (2002) and Edwards *et al.* (2006) found in their studies (Anderson & Mosser, 2002; Edwards et al., 2006). Finally, because IL-4 is rapidly taken up by CD124-expressing cells, assessing intracellular IL-4 by flow cytometry may yield a more accurate picture of T cell biasing in this study.

The MΦ used in this study were type II activated in response to LPS+IC and produced more IL-10 and less IL-12 compared to classically activated MΦ when cultured alone and with T cells. However, the level of IL-12 produced was comparatively high compared to previous studies (Anderson & Mosser, 2002; Edwards et al., 2006). Therefore, it is possible that this elevated level of IL-12 may also be contributing to the less pronounced biasing of T cells in this study given the importance of IL-12 in Th1 biasing, (Kaplan et al., 1996) and its ability to negatively regulate Th2 biasing by inhibiting GATA3 (Ouyang et al., 1998). This difference

in IL-12 production may be due to the higher concentration of LPS used in this study (200 ng/ml) compared to previous work (10 ng/ml) (Anderson & Mosser, 2002; Edwards et al., 2006).

In this study, T cells cultured with type II activated MΦ had a less activated phenotype, characterised by lower expression of CD44 and higher expression of CD62L, compared to T cell cultured with classically activated MΦ. This finding is in contrast to previous work in which T cells cultured with type II activated MΦ had the more activated profile (Edwards et al., 2006). The difference here may again come down to mouse strain and the T cell clone. Edwards et al., (2006) used an OVA specific T cell clone from a BALB/c background. It would not be unexpected for T cells from a BALB/c background to be more easily activated towards the Th2 phenotype and display markers of activation more strongly. Whereas in the current study, 2D2 T cells from a C57BL/6 background may be more easily activated under Th1 inducing conditions. Therefore, it is possible that the degree of activation of T cells by classical or type II MΦ is strain and TCR dependent. While the degree of T cell activation is significantly different between cultures containing classical and type II activation, this difference is less marked when compared to the levels seen on T cells cultured with unstimulated MΦ. However, the exact role the change in activation has in driving the T cell response and T cell biasing is not known.

PD-L1 is expressed on macrophages along with many other cells and binds to PD-1 on T cells to negatively regulate T cell proliferation (Carter et al., 2002; Konkel et al., 2010). Type II activated MΦ were found to express lower levels of PD-L1 compared to classically activated MΦ, and it is possible that this decrease in PD-L1 is partially responsible for the increased activation state of T cells cultured with type II activated MΦ. Due to the decrease in PD-L1, it was hypothesised that T cells cultured with type II activated MΦ would have a higher rate of proliferation compared to those cultured with classically activated MΦ. However, because the overall increase represented only a modest increase in proliferation rate which did not reach significance, the functional consequences of the decreased level of PD-L1 on T cell responses remains unclear.

## 3.5 Summary

In summary, classically activated MΦ biased T cells toward a Th1 phenotype, while type II activated MΦ biased T cells away from a Th1 and towards a Th2-like phenotype. However, in a novel finding, type II activated MΦ also promoted the production of IL-17A from T cells by a mechanism that has not yet been established. Furthermore, T cells activated by type II activated MΦ had a less activated phenotype but had a higher proliferative capacity compared to classically activated MΦ.

# Chapter 4:
# Elucidating the mechanisms involved in T cell biasing by Type II MΦ

## 4.1 Introduction

It has been proposed by Anderson & Mosser, (2002) that the biasing of T cell responses by classical and type II activated MΦ is caused solely by the differential production of IL-12 and IL-10. In the Anderson and Mosser study, IL-$12^{-/-}$ and IL-$10^{-/-}$ MΦ were classically or type II activated and it was found that they were unable to induce Th1 or Th2 biasing, respectively (Anderson & Mosser, 2002). Additionally, while the deletion of either IL-10 or IL-12 maintained the cytokine profile of classically and type II activated MΦ, the absolute values appeared to be altered compared to wild type MΦ with IL-10-deficient MΦ producing more IL-12 and IL-12-deficient mice producing less IL-10, suggesting a possible effect of gene deletion on the MΦ beyond the deleted gene (Anderson & Mosser, 2002). While this study certainly supports the role for IL-10 and IL-12 in T cell biasing, it does not allow for developmental effects of gene deletion or the temporal effects of altering IL-10 and IL-12 over the course of a MΦ:T cell co-culture which could account for some of the changes seen in this study.

Genetic deletion has been shown to alter the development of bone marrow derived MΦ. For example, a recent study has shown that MΦ isolated from the bone marrow of IL-4Rα (CD124) deficient mice produce higher levels of both IL-12 and IL-10 compared to wild type controls and this difference was due to a different environment *in vivo* and not to *in vitro* culture conditions (La Flamme et al., 2012). Overall this finding indicates that exposure to cytokines during development in the bone marrow may affect the final phenotype of the MΦ. Therefore, it is possible that deletion of IL-10 or IL-12 will alter the cytokine environment during MΦ development and cause developmental differences in the MΦ. Due the possible effects of gene deletion, the current study aimed to investigate the role of IL-10 and IL-12 in T cell biasing using blocking antibodies and recombinant proteins. As the IL-10 and IL-12 will only be blocked upon activation it can be assumed that these MΦ were functionally normal upon stimulation, and that any effect seen is a direct result of blocking or enhancing IL-10 and IL-12 and not a functional change that is an artefact of gene deletion. The cytokines can also be blocked or added either at the time of MΦ stimulation or at the time T cells are added to the culture, to help elucidate any temporal effects that IL-12 or IL-10 may have on the MΦ themselves versus the T cells. In addition, this method has the potential to assess the effect of different levels of cytokine by titrating the neutralising antibodies and the recombinant cytokines.

In addition to IL-12 and IL-10 levels, there are other molecules that are differentially expressed on type II MΦ compared to classically activated MΦ. These include CD40 and PD-L1, which are both significantly down-regulated after type II activation (Tierney et al., 2009). CD40 and PD-L1 are members of the co-stimulatory or co-inhibitory family of receptors involved in T cell:APC interactions and due to their differential expression on type II activated MΦ, were investigated for their involvement in T cell biasing by classically and type II activated MΦ. It has previously been demonstrated that different levels of CD40 signalling to the APC results in differential activation, with lower levels of CD40 stimulation resulting in increased IL-10 and higher levels of stimulation increasing IL-12 production (Mathur et al., 2004; Murugaiyan et al., 2006). Furthermore, $CD40^{+/+}$ DC drive Th1 responses while $CD40^{+/-}$ have been shown to activate T cells to a phenotype that resembles Tregs (S. Martin et al., 2010). As CD40 has been consistently found to be decreased on type II MΦ (Tierney et al., 2009), the possible role of CD40 levels in T cell biasing by classically and type II MΦ was investigated.

A study by Loke and Allison (2003) found that PD-L1 and PD-L2 are differentially regulated by T cell cytokines, with PD-L1 being up regulated on MΦ by Th1 cytokines and PD-L2 by Th2 cytokines (Loke & Allison, 2003). This finding suggests PD-L1 and PD-L2 may have roles in inhibiting different subsets of T cells. This idea is further supported by data from PD-L1 deficient mice with EAE and *L. mexicana* infection. In EAE, where Th1 responses are detrimental, PD-L1 deficiency results in increased disease severity, whereas in *L. mexicana* infection where Th1 responses are protective PD-L1 deficiency is not detrimental (Carter et al., 2007; Liang et al., 2006). Together these studies suggest that without PD-L1, Th1 responses may be enhanced due to a lack of the inhibitory effect that PD-L1 provides; therefore PD-L1 may have a role in regulating Th1 responses over Th2. The decrease in PD-L1 on type II MΦ does not have a large effect on the proliferation of T cells *in vitro* as seen in chapter 3, however, it is possible that the decrease in PD-L1 may be involved in T cell biasing. Although the association of PD-L1 with T cell responses is to inhibit Th1 responses, there is some evidence that it is also capable of promoting T cell responses (Wang et al., 2003). Therefore, it is possible that the level of signalling through PD-1 by PD-L1, or the environment in which the T cell is stimulated may be relevant. To this end, the potential of PD-L1 to influence T cell biasing was investigated.

Finally, previous results from this thesis have shown that in addition to affecting the Th1/Th2 dichotomy, type II activated MΦ also induce an increase in IL-17A production. As the

IL-17A producing Th17 subsets had not been identified at the time of the initial study into T cell biasing by type II MΦ (Anderson & Mosser, 2002), IL-17A production was not investigated in this study. Therefore, the pathways involved in IL-17A production by T cells cultured with type II MΦ and the factors involved in the modulation of the level of IL-17A have not previously been investigated.

## 4.2 Aims

The aim of this study was to investigate potential pathways involved in T cell biasing by type II MΦ compared to classically activated MΦ. These pathways include both soluble factors and cell surface markers that are differentially expressed on type II activated MΦ (i.e. IL-12, IL-10, CD40, and PD-L1).

### 4.2.1 Specific aims

1. To investigate the effect of blocking or enhancing IL-10 and IL-12 on MΦ alone.
2. To investigate the effect of blocking or enhancing IL-10 and IL-12 on T cell biasing by classically compared to type II activated MΦ.
3. To investigate the effect of inhibiting the CD40/CD40L pathway on T cell biasing by classically compared to type II activated MΦ.
4. To investigate the effect of enhancing or inhibiting the PD-1/PD-L1 pathway on T cell biasing by classically compared to type II activated MΦ.

## 4.3 Results

In order to investigate possible pathways involved in T cell biasing, recombinant cytokines were employed and in addition, cytokine blocking antibodies were used to neutralise IL-10 and IL-12 or inhibit CD40:CD40L (MΦ:T cell) or PD-L1:PD-1 (MΦ:T cell) interactions. Because MΦ express FcR, which bind the constant region of antibodies, it is possible that non-specific ligation by antibodies could alter the function or phenotype of the MΦ and this possibility must be addressed first. Indeed, the IC used in this study works by interacting with FcγR (Sutterwala et al., 1998), and the high density of antibodies on the IC crosslinks the FcγR which along with TLR-4 ligation, results in type II activation (Gallo, Gonçalves, & Mosser, 2010). As IC are washed before use to remove any unbound antibody, it is unlikely that there are free antibodies present in cultures containing IC. While individual antibodies (i.e. not IC) are unlikely to cross link the receptors as strongly, they may have a weaker effect on MΦ. Therefore, isotype control antibodies were employed to assess the non-specific effect of antibodies on MΦ:T cell interactions. Different isotype control antibodies were employed based on the isotype of the blocking antibody in question.

### 4.3.1 Effect of blocking or enhancing IL-10 and IL-12 on MΦ activation states

In order to assess how IL-10 and IL-12 are involved in T cell biasing by type II and classically activated MΦ, the direct effect of these cytokines on MΦ activation was assessed using blocking antibodies for IL-10 and IL-12, or recombinant cytokines. Additionally, the temporal involvement of these cytokines was investigated by comparing the effects of altering the cytokine balance at the time of stimulation or 4 hours post stimulation (i.e. when the T cells will be added in co-culture experiments). Because the comparative phenotype of classically activated and type II activated MΦ, and their effects on T cells, was specifically evaluated in Chapter 3, for the following experiments, which investigate the effect of inhibiting or activating specific pathways of classical or type II activated MΦ and their interactions with T cells, these groups will be analysed independently. This approach was selected because it is likely that these treatments will have different effects on these two distinct MΦ populations (i.e. there will be an interaction between the type of MΦ and the treatments).

The addition of recombinant IL-10 (rIL-10) to MΦ cultures decreased the production of IL-12 when added at the time of MΦ stimulation but had no effect if added 4 hours post stimulation (Figure 4.1b). The almost complete inhibition of IL-12 production by the addition of rIL-10 was observed in both type II and classically activated cultures. As expected, IL-10 was readily detected in cultures containing rIL-10 (Figure 4.1a). These results confirm that the presence of high levels of IL-10 at the time of MΦ stimulation can abolish IL-12 production in both classically and type II activated MΦ and suggests that an early production of IL-10 by type II MΦ may be involved in the decreased level of IL-12 produced by type II MΦ. While the addition of excess IL-10 at the time of stimulation abolished IL-12 production, the effect of IL-10 induced by type II activation on IL-12 needed to be assessed. In cultures containing a blocking antibody against IL-10 (αIL-10), no IL-10 was detected in the culture supernatants (Figure 4.1a). As the blocking antibody and the antibody used for detection of IL-10 by ELISA are the same, it is likely that there is still IL-10 in present in the supernatant; however, this is unlikely to be biologically active as it is antibody bound. The addition of αIL-10 did not alter the production of IL-12 by either classically activated or type II MΦ (Figure 4.1b). This is consistent with previous work in our laboratory that demonstrated that blocking IL-10 at the time of stimulation does not alter the production of IL-12 by classically or type II activated MΦ (Kharkrang, 2010).

In contrast to the decreased IL-12 production by the addition of rIL-10, supplementing the cultures with recombinant IL-12 protein (rIL-12p70) did not have a significant effect on IL-10 production when added with the stimuli or 4 hours post stimulation (Figure 4.2a). By 24 hours post-stimulation, the level of IL-12 in LPS-stimulated cultures with and without the additional rIL-12 were similar (Figure 4.2a). The addition of a blocking antibody against IL-12 (αIL-12) significantly reduced the amount of IL-12 detectable in the culture supernatant of both classically and type II activated MΦ (Figure 4.2b). However, the addition of the isotype control antibody did cause a slight but significant decrease in IL-12 in cultures containing type II MΦ, demonstrating a non-specific effect may account for a small amount of the change, this effect was small compared to the effect of αIL-12 (Figure 4.2b). Furthermore, there was no significant change in the level of IL-10 in the presence of the αIL-12 (Figure 4.2a). As with αIL-10, the antibody used for blocking and detection of IL-12 by ELISA are the same, thus, while it is likely that IL-12 is still present in the culture supernatant it will be antibody bound and will therefore likely be inactive. Together these results suggest that the reduced IL-12 produced by type II activated MΦ is not responsible for the enhanced IL-10 production. Together these results suggest that any effect of blocking IL-12 in MΦ:T cell co-cultures would be due to the absence of IL-12 alone and not due to a downstream enhancement in IL-10 by the MΦ.

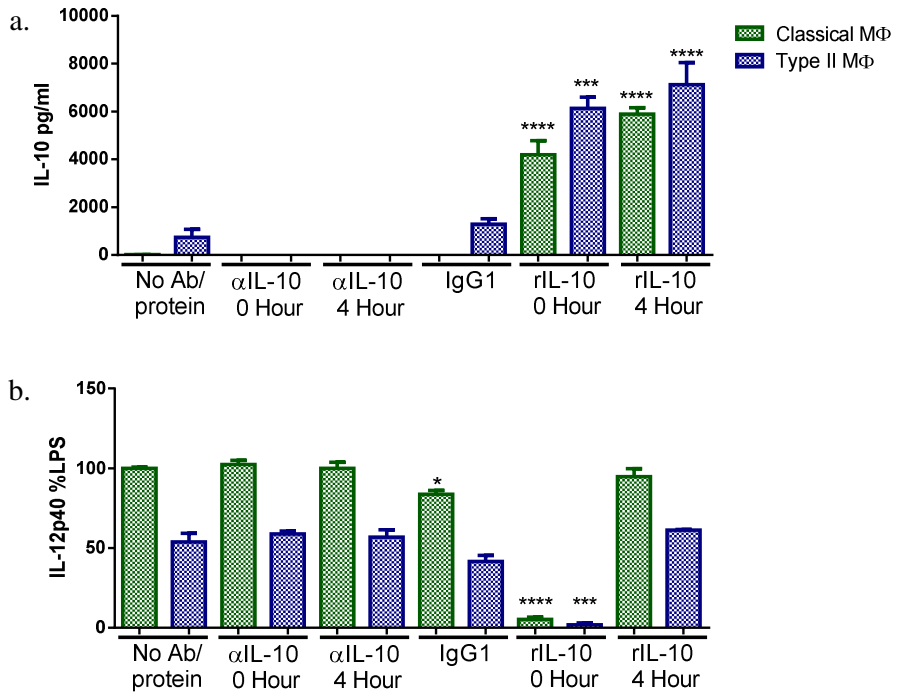



**Figure 4.1. Increasing the level of IL-10 in BMMΦ cultures at the time of stimulation decreased IL-12 production, however addition of a blocking antibody for IL-10 did not affect the levels of IL-12 in culture supernatants.** BMMΦ from C57BL/6 mice were plated at $1x10^5$ and primed with 20 U/ml IFN-γ overnight before stimulation with LPS (200 ng/ml) alone or with IC (10 IC per MΦ) for 24 hours. rIL-10 (5 ng/ml), αIL-10 (JES5-2A5, 2 μg/ml) or rat isotype ($IgG_1$, 2 μg/ml) was added to cultures either with the stimuli (0 hour) or 4 hours post stimulation. IL-10 (a) and IL-12p40 (b) levels were measured by ELISA. Shown are the means and SEM of triplicate wells from one (rIL-10) or two (aIL-10) experiment. *$p<0.05$, ****$p<0.0001$ by a one-way ANOVA with Tukey's multiple comparison post test, stars indicate comparison to no Ab/protein.

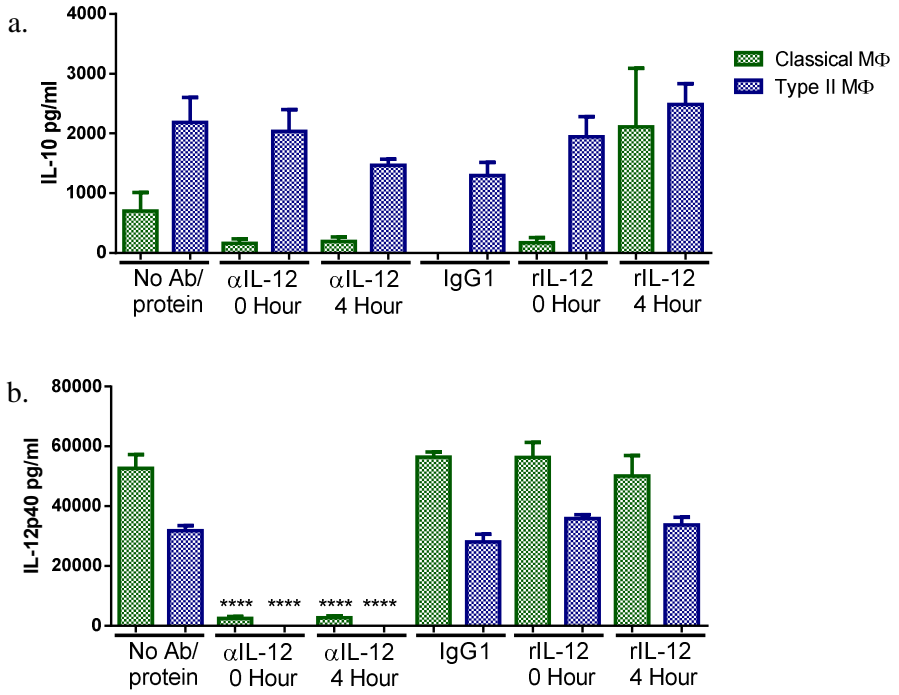



**Figure 4.2. Altering the level of IL-12 in BMMΦ cultures did not have a significant effect on IL-10 levels in culture supernatants.** BMMΦ from C57BL/6 mice were plated at $1x10^5$ and primed with 20 U/ml IFN-γ overnight before stimulation with LPS (200 ng/ml) alone or with IC (10 IC per MΦ) for 24 hours. rIL-12p70 (5 ng/ml), αIL-12 (C15.6, 2 μg/ml) or rat isotype ($IgG_1$, 2 μg/ml) was added to cultures either with the stimuli (0 hour) or 4 hours post stimulation. IL-10 (a) and IL-12p40 (b) levels were measured by ELISA. Shown are the means and SEM of triplicate wells from two or more experiments. ****p<0.0001 by a one-way ANOVA with Tukey's multiple comparison post test, stars indicate comparison to IgG1.

## 4.3.2 Effect of blocking or enhancing IL-10 and IL-12 on the ability of classically and type II MΦ to bias the T cell response

### *4.3.2.1 Altering IL-10 levels in MΦ:T cell co-cultures*

Similar to the neutralisation of IL-10 in MΦ only cultures (Figure 4.1 and Kharkrang, 2010), the addition of αIL-10 to MΦ:T cell co-cultures significantly decreased the level of detectable, and likely bioactive, IL-10 (Figure 4.3a). The addition of αIL-10 did not have a significant effect on the production of IL-12 (Figure 4.3b). This result demonstrates that the effect of the blocking antibody was similar in MΦ:T cell co-cultures compared to cultures with MΦ alone. The levels of IL-10 in culture supernatant where higher when rIL-10 was added with the T cells compared to addition with the MΦ stimuli (Figure 4.3a) possibly due to higher uptake when rIL-10 was added with the MΦ.

Consistent with the results seen in Figure 4.1, the addition of rIL-10 decreased the production of IL-12 by both classical and type II activated MΦ when added at the time of MΦ stimulation (Figure 4.3b). This result indicates that the MΦ phenotype is maintained during the MΦ:T cell co-culture. The addition of αIL-10 or rIL-10 did not change the expression of the activation markers CD44 and CD62L significantly nor did the addition of αIL-10 or rIL-10 significantly alter the levels of CD25 and IL-2 (see appendix C).

Although no difference was detected in T cell activation parameters, the addition of αIL-10 resulted in increased IFN-γ levels produced by T cells cultured with type II and classically activated MΦ, this increase was significant compared to the isotype control (Figure 4.4a). This elevation suggests that an increased production of IL-10 by type II MΦ may be at least partly responsible for the decreased production of IFN-γ by T cells when cultured with type II MΦ. In the same manner that the neutralisation of IL-10 led to increased IFN-γ production, the addition of rIL-10 to MΦ:T cell co-cultures resulted in decreased the production of IFN-γ from T cells stimulated by both classically and type II activated MΦ (Figure 4.4a). However, this decrease only occurred when rIL-10 was added to the co-cultures at the time of MΦ stimulation and not when the rIL-10 was added at the same time as the T cells (Figure 4.4a). This early effect suggests that the IL-10 may be acting primarily on the MΦ, as opposed to on the T cells directly, and that the effect of the IL-10 occurs in the first 4 hours of stimulation.

Additionally, despite the changes in IFN-γ production, αIL-10 did not have a significant effect on the level of IL-17A in supernatants from T cells cultured with either classical and type II activated MΦ, although a non-significant trend toward increased levels exists in the presence of αIL-10, suggesting that the increased production of IL-17A by T cells cultured with type II MΦ is not a result of increased IL-10 in these cultures (Figure 4.4b). Furthermore, the addition of rIL-10 at the time of MΦ stimulation reduced the level of IL-17A production in cultures containing classically activated MΦ but had no significant effect in cultures containing type II MΦ (Figure 4.4b). This suggests that the increased IL-10 production by type II MΦ was not likely to be responsible for the increase IL-17A in these cultures. Finally, the level of CD124 was not altered by the addition of αIL-10 or rIL-10 (Figure 4.4c), suggesting that if IL-10 contributes to Th2 biasing by type II MΦ, it is not by increasing the sensitivity of T cells to IL-4.

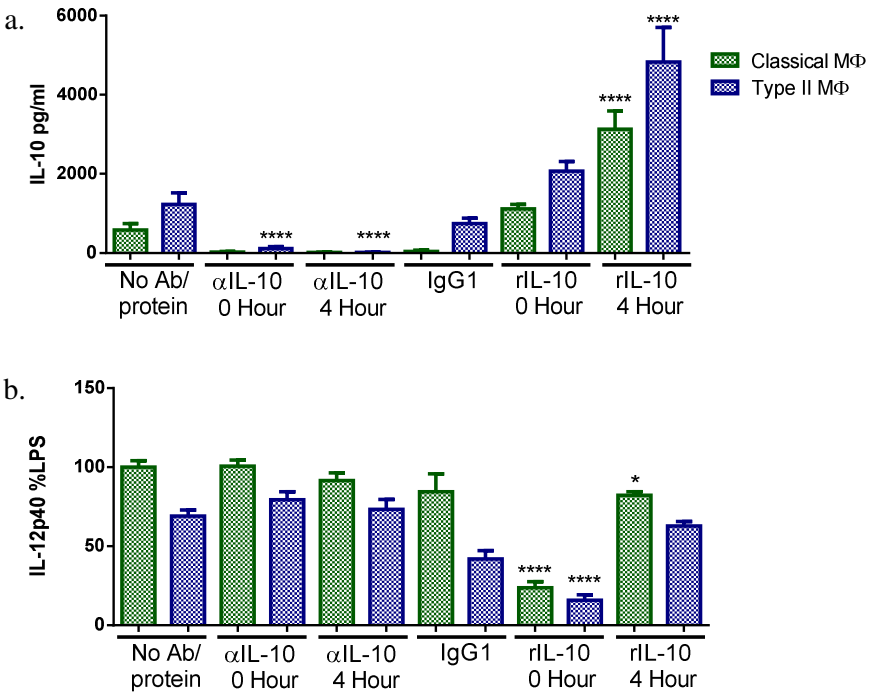



**Figure 4.3. The effect of αIL-10 and rIL-10 on IL-10 and IL-12 production by MΦ was maintained in MΦ:T cell co-culture.** BMMΦ were stimulated with IFN-γ (20 U/ml) overnight and then cultured with LPS (200 ng/ml) with or without IC (10 per MΦ). After four hours, purified $CD4^+2D2$ T cells and $MOG_{35-55}$ (25 μg/ml) were added to the MΦ cultures for 72 hours. rIL-10 (5 ng/ml), αIL-10 (JES5-2A5, 2 μg/ml) or rat isotype ($IgG_1$, 2 μg/ml) was added to cultures either with the stimuli (0 hour) or with the T cells (4 hour). IL-10 (a) and IL-12 (b) were measured by ELISA. Shown are the means and SEM of triplicate wells from three or more experiments, IL-12 data is presented as a percentage of LPS with no antibody/protein. *$p<0.05$, and ****$p<0.0001$ by one-way ANOVA with Tukey's multiple comparison post test, stars indicate comparison to no Ab/protein for recombinant proteins and to IgG1 for blocking antibodies.

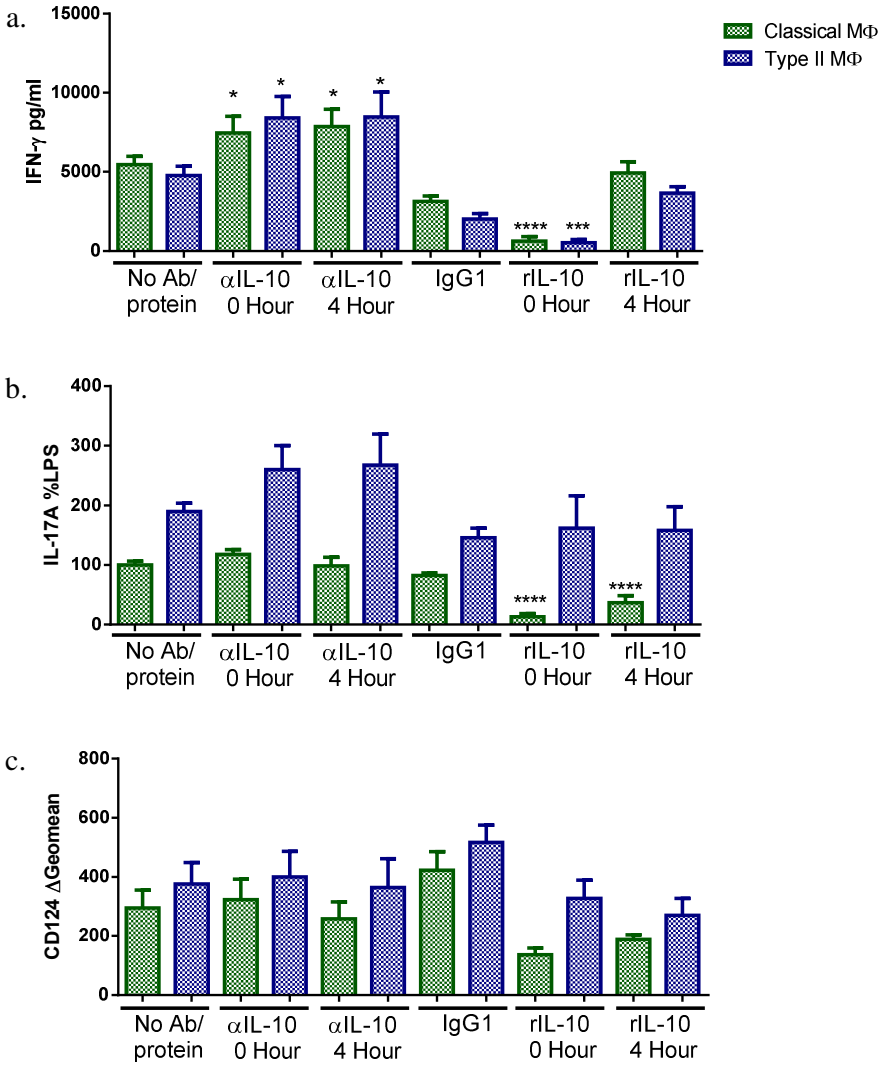



**Figure 4.4. Altering IL-10 levels in BMMΦ:T cell co-cultures affected IFN-γ levels but not IL-17A levels or CD124 expression on T cells.** BMMΦ were stimulated with IFN-γ (20 U/ml) overnight and then cultured with LPS (200 ng/ml) with or without IC (10 per MΦ). After four hours, purified $CD4^{+}2D2$ T cells and $MOG_{35-55}$ (25 µg/ml) were added to the MΦ cultures for 72 hours. rIL-10 (5 ng/ml), αIL-10 (JES5-2A5, 2 µg/ml) or rat isotype ($IgG_1$, 2 µg/ml) was added to cultures either with the stimuli (0 hour) or with the T cells (4 hour). IFN-γ (a) and IL-17A (c) were measured in the culture supernatant by ELISA, and CD124 (b) expression was measured by flow cytometry. Shown are the means and SEM of triplicate wells from three or more experiments, IL-17A data is presented as a percentage of LPS with no antibody/protein. *$p<0.05$, ****$p<0.0001$ by one-way ANOVA with Tukey's multiple comparison post test, stars indicate comparison to no Ab/protein for recombinant proteins and to IgG1 for blocking antibodies.

#### *4.3.2.2 Altering IL-12 levels in MΦ:T cell co-cultures*

To further investigate the role of MΦ cytokines in T cell biasing, the IL-12 pathway was assessed. The addition of αIL-12 to MΦ:T cell cultures resulted in a significant decrease in the detectable levels of IL-12 in cultures containing classical or type II activated MΦ when added both with the MΦ stimuli and with the T cells (Figure 4.5b). This change was consistent with adding αIL-12 to MΦ alone (Figure 4.2b), although the level of IL-12 remaining in MΦ:T cell cultures containing αIL-12 suggests that the cytokine was not completely neutralised (Figure 4.5b). Although the IL-12 was not completed blocked, the levels in the MΦ:T cell co-cultures were significantly decreased and resulted in altered responses such as the decrease in IFN-γ described bellow (Figure 4.6a). Neutralisation of IL-12 in MΦ:T cell cultures did not significantly alter the production of IL-10. There was a non-significant trend towards reduced levels of IL-10 when added at the time of MΦ stimulation, however, there was also a change seen with the isotype control antibody in cultures containing classically activated MΦ, suggesting the possibility of some non-specific effects with the IL-10 level (Figure 4.5a). To further study the IL-12 pathway, rIL-12 was used. The addition of rIL-12 to MΦ:T cell co-cultures did not alter the levels of IL-10 produced (Figure 4.5a). There was a trend towards increased levels of IL-12 which was significant in cultures containing classically activated MΦ, when rIL-12 was added with the T cells (Figure 4.5b). This relatively small change in IL-12 levels may have been be due to the high levels of IL-12 present in these cultures even in the absence of rIL-12, and this small increase could easily have been absorbed by the level of IL-12 produced after 72 hours in culture.

While the addition of αIL-12 or rIL-12 in MΦ:T cell co-cultures did not affect the activation state of the T cells cultured with either classically or type II activated MΦ as evidenced by similar expression of CD44, CD62L, CD25, and IL-2 (See appendix C), T cell biasing was altered. In particular, IFN-γ production was significantly decreased in co-cultures containing either classically and type II activated MΦ. This decrease was evident when αIL-12 was added with the MΦ stimuli or the T cells (Figure 4.6a). Moreover, this change in IFN-γ production correlated with the decreased levels of IL-12 seen with the addition of αIL-12 (Figure 4.5b). The addition of rIL-12 caused a significant increase in the level of IFN-γ produced by T cells cultured with type II activated MΦ. This increase was not observed in co-cultures with classically activated MΦ and was evident when the rIL-12 was added either at

the time of MΦ stimulation or at the time of T cell addition (Figure 4.6a). Furthermore, the increase in IFN-γ production only in type II MΦ co-cultures after rIL-12 addition suggests that the decreased level of IL-12 may be at least partly responsible for the decreased level of IFN-γ produced by T cells cultured with type II MΦ.

IL-17A levels were not altered by the addition of rIL-12, nor were they altered by the addition of αIL-12 (Figure 4.6b). IL-17A is increased in MΦ:T cell co-cultures containing type II MΦ compared to classically activated MΦ and these type II MΦ:T cell co-cultures also have decreased levels of IL-12. These data suggest that the decreased level of IL-12 produced by type II MΦ is not responsible for the increased IL-17A. Additionally, neither adding αIL-12 or rIL-12 reduced the level of CD124 on T cells (Figure 4.6c), suggesting the increased sensitivity of T cell cultured with type II MΦ is not due to the decreased IL-12 found in these cultures.

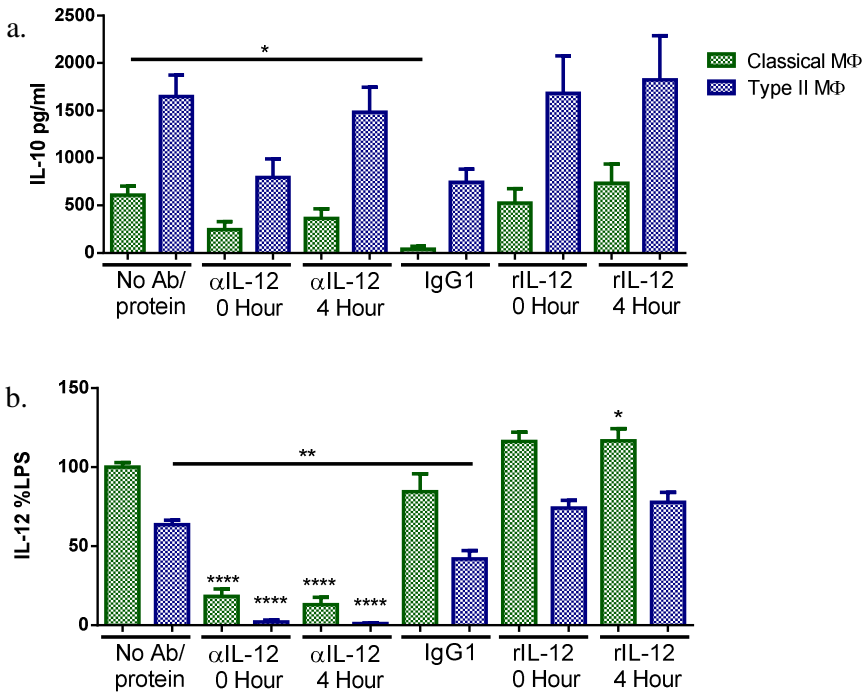



**Figure 4.5. The effect of αIL-12 and rIL-12 on IL-10 and IL-12 production by MΦ was maintained in MΦ:T cell co-culture.** BMMΦ were stimulated with IFN-γ (20 U/ml) overnight and then cultured with LPS (200 ng/ml) with or without IC (10 per MΦ). After four hours, purified $CD4^+2D2$ T cells and $MOG_{35-55}$ (25 μg/ml) were added to the MΦ cultures for 72 hours. rIL-12p70 (5 ng/ml), αIL-12 (C15.6, 2 μg/ml) or rat isotype ($IgG_1$, 2 μg/ml) was added to cultures either with the stimuli (0 hour) or with the T cells (4 hour). IL-10 (a) and IL-12 (b) was measured by ELISA. Shown are the means and SEM of triplicate wells from two or more experiments, IL-12 data is presented as a percentage of LPS with no antibody/protein. *$p<0.05$, **$p<0.01$ and ****$p<0.0001$ by one-way ANOVA with Tukey's multiple comparison post test, stars indicate comparison to no Ab/protein for recombinant proteins and to IgG1 for blocking antibodies.

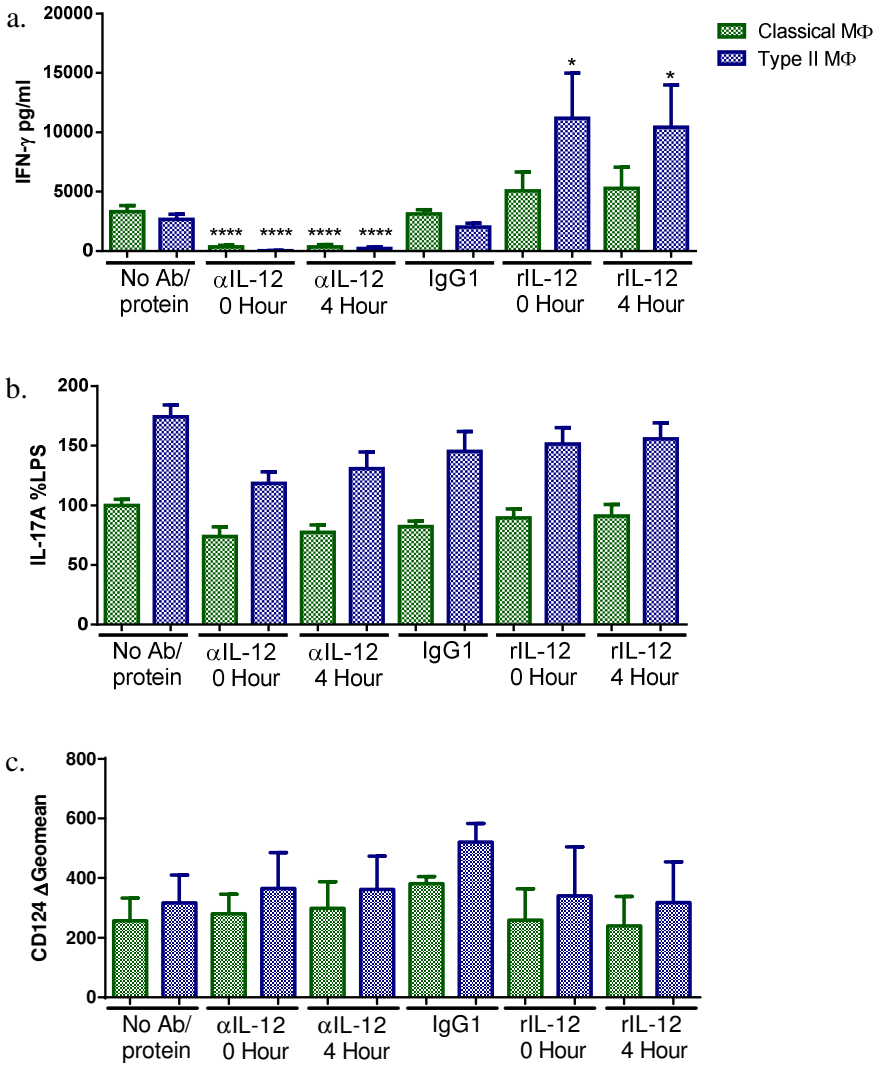



**Figure 4.6. Altering IL-12 levels in BMMΦ:T cell co-cultures affected IFN-γ levels but not IL-17A levels or CD124 expression on T cells.** BMMΦ were stimulated with IFN-γ (20 U/ml) overnight and then cultured with LPS (200 ng/ml) with or without IC (10 per MΦ). After four hours, purified $CD4^{+}2D2$ T cells and $MOG_{35-55}$ (25 μg/ml) were added to the MΦ cultures for 72 hours. rIL-12p70 (5 ng/ml), αIL-12 (C15.6, 2 μg/ml) or rat isotype ($IgG_1$, 2 μg/ml) was added to cultures either with the stimuli (0 hour) or with the T cells (4 hour). IFN-γ (a) and IL-17A (c) were measured in the culture supernatant by ELISA, and CD124 (b) expression was measured by flow cytometry. Shown are the means and SEM of triplicate wells from three or more experiments, IL-17A data is presented as a percentage of LPS with no antibody/protein. *$p<0.05$ and ****$p<0.0001$ by one-way ANOVA with Tukey's multiple comparison post test, stars indicate comparison to no Ab/protein for recombinant proteins and to IgG1 for blocking antibodies.

### 4.3.3 Effects of alterations to the PD-1/PD-L1 pathway on the ability of classically and type II MΦ to bias the T cell response

Because the changes in IL-10 and IL-12 production by type II and classically activated MΦ could account primarily for changes in IFN-γ production and not other markers of Th biasing such as CD124 and IL-17A, other pathways known to be altered in type II activation were investigated. Specifically, previous work has shown that the expression of both CD40 and PD-L1 is significantly reduced on type II activated MΦ compared to classically activated MΦ (Tierney et al., 2009) and these molecules play key roles in MΦ:T cell interactions. To study the PD-1/PD-L1 pathway, a blocking antibody for PD-1 (αPD-1) and a PD-L1 chimeric protein (PD-L1ch) were used. The αPD-1 antibody binds to the PD-1 receptor on the T cells and prevents interaction with molecules on the MΦ. As αPD-1 targets the PD-1 receptor on the T cells, it will inhibit interaction with both PD-L1 and PD-L2. However, type II and classically activated MΦ express only very low levels of PD-L2 (Tierney et al., 2009), and so it is likely that any effects of blocking PD-1 will be due to changes in interaction with PD-L1. The PD-L1ch protein is a chimera of murine PD-L1 and Fc fragment of human $IgG_1$ connected via a peptide linker (IEGRMD). The PD-L1ch binds to and stimulates PD-1, mimicking the effect of PD-L1 on the MΦ cell surface.

To find the optimum concentration of αPD-1 a titration of the antibody was performed. The addition of the αPD-1 antibody did not appear to have a large effect on IFN-γ, IL-17A or IL-2 production or CD124 expression (see appendix C), however, the greatest change appeared to occur at 2 μg/ml, therefore this concentration was chosen for further study. To help determine that the effects observed were due to the blocking of the PD-1/PD-L1 pathway, and not a result of the antibodies binding to the FcγR on the MΦ, an isotype control was also used. Similar to αPD-1, the effect of the PD-L1ch was titrated in MΦ:T cell co-culture conditions and was compared to the control protein. Addition of the PD-L1ch appeared to have little effect on IFN-γ, IL-17A and CD124, while IL-2 showed a trend towards decreased production at higher concentrations (see appendix C). Due to this decrease in IL-2 production, 8 μg/ml was selected for the subsequent experiments.

As expected, addition of αPD-1 to block PD-1 signalling on T cells did not affect the production of IL-10 and IL-12 by MΦ in the co-cultures (Figure 4.7). There was also no

significant difference in the expression of CD44, CD62L and CD25 following the addition of αPD-1 or the PD-L1ch (see appendix C). Addition of the αPD-1 antibody significantly reduced the production of IL-2, and there was a trend towards decreased levels of IL-2 in the presents of the PD-L1ch (Figure 4.8c). However, these changes were also seen with the isotypes, suggesting this is a non-specific effect. Despite the decrease in IL-2 production, the αPD-1 and PD-L1ch did not appear to alter the production of other cytokines or markers associated with Th biasing significantly. IFN-γ, CD124 and IL-17A were all unaltered by the presence of αPD-1, and additionally, IL-17 was not affected by the PD-L1ch (Figure 4.8a, b and d). Both IFN-γ and CD124 decreased in the presence of the PD-L1ch, however, this change was not significant, and changes in the control protein make these results difficult to interpret. Additionally, as there is no change in the parameters measured, a second possibility is that the αPD-1 and the PD-L1ch are not active in this system. Overall, these data suggest that altering the PD-1/PD-L1 pathway with the addition of αPD-1 does not have a strong affect on the activation state of T cells nor their biasing in this MΦ:T cell co-culture system, this result should be confirmed using another method, such as RNAi and positive controls.

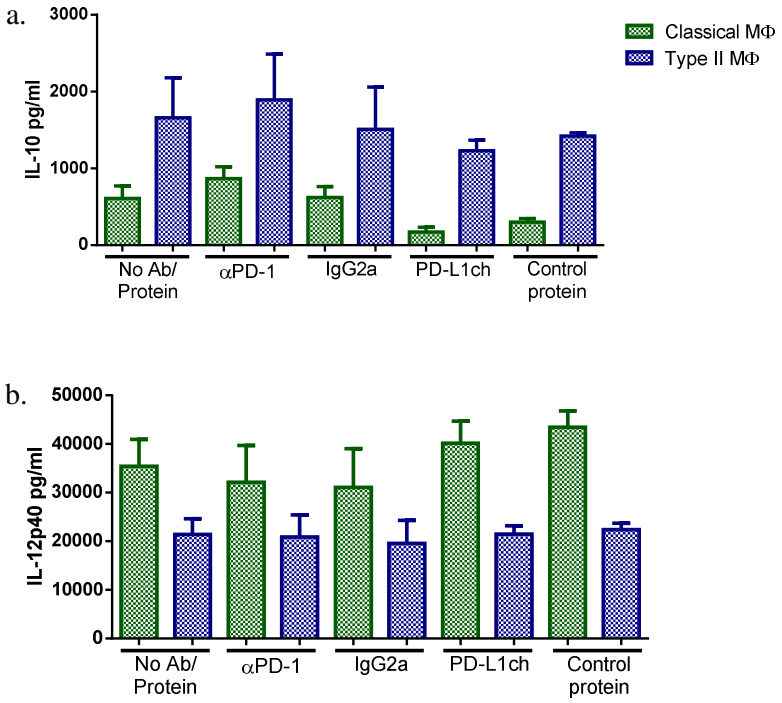



**Figure 4.7. Blocking or stimulating the PD-1/PD-L1 pathway in MΦ:T cell co-culture did not affect IL-10 or IL-12 production by MΦ.** BMMΦ were stimulated with IFN-γ (20 U/ml) overnight and then cultured with LPS (200 ng/ml) with or without IC (10 per MΦ). After four hours, purified $CD4^{+}2D2$ T cells and $MOG_{35-55}$ (25 μg/ml) were added to the MΦ cultures for 72 hours. αPD-1 (2 μg/ml, RMP1-14), rat isotype (2 μg/ml, $IgG_{2a}$), PD-L1ch (8 μg/ml) or control protein (8 μg/ml) was added. IL-10 (a) and IL-12 (b) were measured by ELISA. Shown are the means and SEM of triplicate wells from one-four (IL-10) or three-six (IL-12) combined experiments.

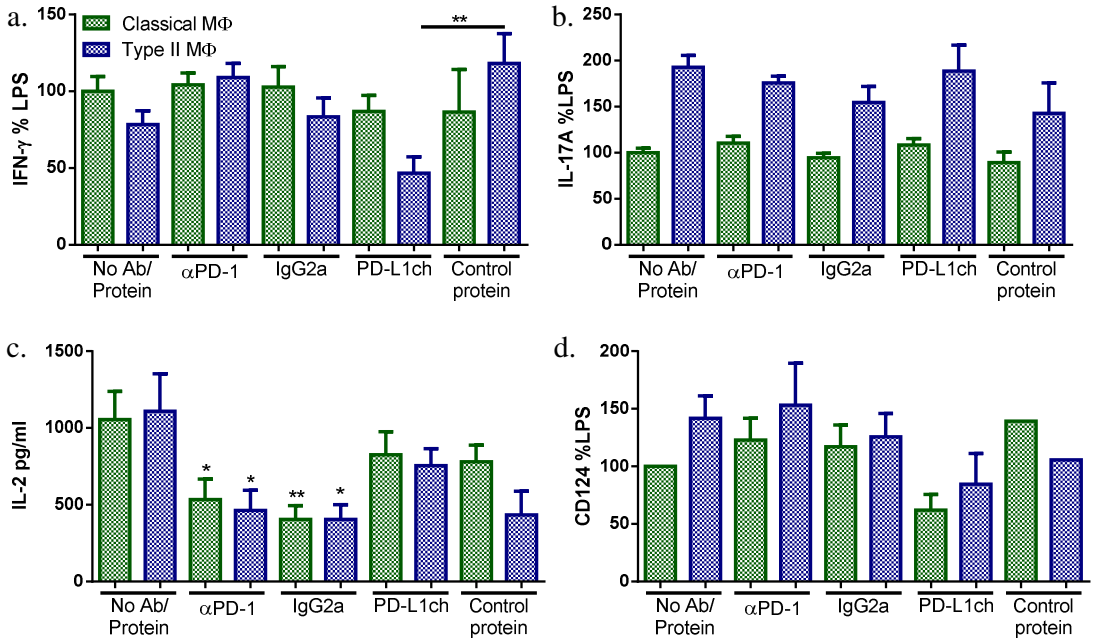



**Figure 4.8. Blocking or stimulating the PD-1/PD-L1 pathway did not affect T cell biasing in MΦ T cell co-culture.** BMMΦ were stimulated with IFN-γ (20 U/ml) overnight and then cultured with LPS (200 ng/ml) with or without IC (10 per MΦ). After four hours, purified $CD4^{+}2D2$ T cells and $MOG_{35-55}$ (25 μg/ml) were added to the MΦ cultures for 72 hours. αPD-1 (2 μg/ml, RMP1-14), rat isotype (2 μg/ml, $IgG_{2a}$), PD-L1ch (8 μg/ml) or control protein (8 μg/ml) was added. IFN-γ (a), IL-17A (b) and IL-2 (c) were measured in the culture supernatant by ELISA, and CD124 (d) expression was measured by flow cytometry. Shown are the means and SEM of triplicate wells from two-six (IFN-γ), two-five (IL-17A) or one-five (CD124 and IL-2) combined experiments. *$p<0.05$, **$p<0.01$ by one-way ANOVA with Tukey's multiple comparison post test. stars indicate comparison to no Ab/protein, unless otherwise shown.

**4.3.5 Blocking the CD40/CD40L pathway in MΦ:T cell co-cultures**

Due to the decreased expression of CD40 seen on type II MΦ (Tierney et al., 2009), as well as the proposed link between the strength of the CD40 signal and T cell biasing, the role of CD40 in T cell biasing by type II MΦ was investigated. To explore the role of CD40, an antibody specific for the CD40L (αCD40L) was added to the cultures, this antibody binds to CD40L and prevents interaction with CD40, effectively inhibiting signalling through the CD40 pathway. To find the optimum antibody concentration to inhibit CD40/CD40L interaction the effect of the antibody was titrated from 0.5-8 μg/ml. Titration of the αCD40L demonstrated that production of several cytokines (IFN-γ, IL-17A and IL-2) as well as the expression of CD124 was maintained across all doses of αCD40L (see appendix C). In contrast, the isotype control hamster, which controls for non-specific antibody effects, demonstrated decreased levels of all of these markers (see appendix C). A concentration of 4 μg/ml of αCD40L was chosen for further investigation because this concentration demonstrated a large difference between the αCD40L antibody and the isotype control, without the complete abrogation in IL-2 production seen when using 8 μg/ml isotype control (see appendix C).

The addition of αCD40L to MΦ T cell co-cultures did not alter the levels of IL-12 produced, and there was a significant increase in IL-10 production in cultures containing classically activated MΦ. However, there was considerable variability in the data, with some changes also evident in the isotype control (Figure 4.9). The presence of αCD40L did not induce a change in the expression of CD44, CD62L and CD25 (see appendix C). Since these markers are all associated with T cell activation, this finding suggests that decreasing CD40 signalling does not affect the activation state of the T cells. While there was a trend towards lower production of IL-2 in the presence of αCD40L, a similar decrease was seen with the isotype control, suggesting that this change was a non-specific effect caused by the presence of antibody (Figure 4.10c).

Reduced levels of CD40 signalling have been associated with Th2 responses and in addition the absence of CD40 signalling has been shown to abolish Th2 responses. Due to the decreased expression of CD40 on type II MΦ, this signalling pathway is likely to be reduced compared to signalling on classically activated MΦ. It was therefore hypothesised that the greatest effect of blocking CD40 signalling in this system would be on Th biasing and specifically Th2 responses. In contrast to expectations, while IL-17A and CD124 expression

were unaltered by the addition of αCD40L (Figure 4.10b and c), IFN-γ production was altered. MΦ:T cell co-cultures with αCD40L showed a trend towards increased IFN-γ and this increase was observed in co-cultures with either classically and type II activated MΦ (Figure 4.10a). In the experiment shown, there was a significant effect of using the isotype control antibody, which reduced IFN-γ production. Because this effect was opposite to that of adding αCD40L, this effect is likely to be specific to αCD40L and not due to the presence of antibody in general. However, this result needs to be cautiously interpreted (Figure 4.10a). These experiments suggest that inhibiting CD40 signalling may enhance IFN-γ but has little effect on IL-17A or CD124. However, the possibility that the antibody is not active should be taken into account when assessing this data. Taken together, these findings suggest that the decrease of CD40 on type II MΦ is not involved in the increase in IL-17A production or the increased sensitivity of these cells to IL-4 nor can it explain the reduced IFN-γ produced by T cells stimulated by type II activated macrophages.

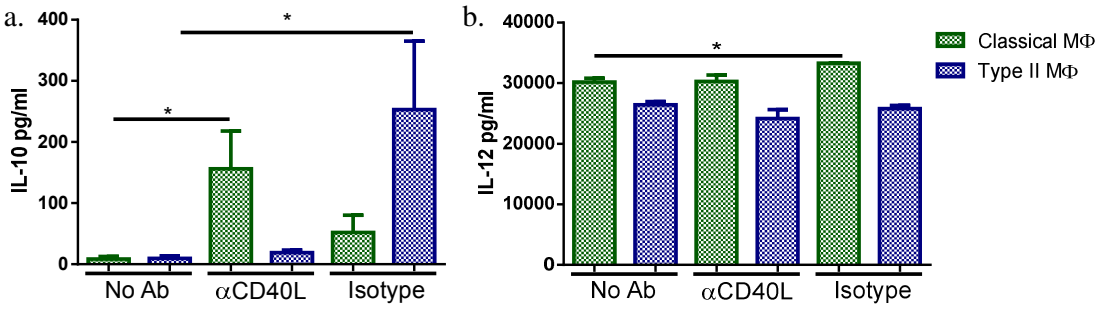



**Figure 4.9. Blocking the CD40/CD40L pathway did not significantly affect the production of IL-10 and Il-12 by MΦ in MΦ:T cell co-cultures.** BMMΦ were stimulated with IFN-γ (20 U/ml) overnight and then cultured with LPS (200 ng/ml) with or without IC (10 per MΦ). After four hours, purified $CD4^{+}2D2$ T cells and $MOG_{35-55}$ (25 μg/ml) were added to the MΦ cultures for 72 hours. 4 μg/ml of αCD40L (MR1) or hamster isotype control antibody ($IgG_1$) was added. IL-10 (a) and IL-12 (b) was measured by CBA and ELISA. Shown are the means and SEM of triplicate wells from one of two experiments (IL-12) or 2 combined IL-10. *$p<0.05$ by one-way ANOVA with Tukey's multiple comparison post test.

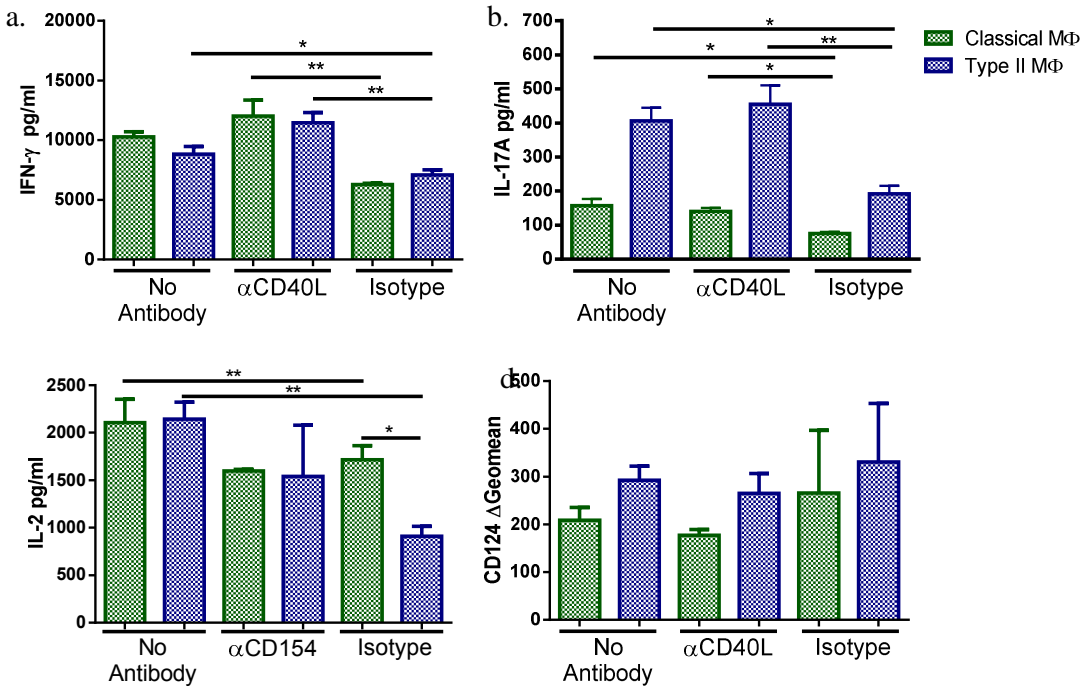



**Figure 4.10. Blocking the CD40/CD40L pathway induced a non-significant increase in IFN-γ production by T cells cultured with classically and type II activated MΦ but did not alter IL-17A production or CD124 expression.** BMMΦ were stimulated with IFN-γ (20 U/ml) overnight and then cultured alone or with LPS (200 ng/ml) with or without IC (10 per MΦ). After four hours, purified $CD4^{+}2D2$ T cells and $MOG_{35-55}$ (25 μg/ml) were added to the MΦ cultures for 72 hours. 4 μg/ml of αCD40L or hamster isotype control antibody ($IgG_1$) was added. IFN-γ (a), IL-17A (b) and IL-2 (c) were measured in the culture supernatant by CBA and ELISA, and CD124 (d) expression was measured by flow cytometry. Shown are the means and SEM of triplicate wells from one of two (IFN-γ, IL-17A and IL-2) or data from one of two (CD124) experiments. *$p<0.05$ and **$p<0.01$ by one-way ANOVA with Tukey's multiple comparison post test.

## 4.4 Discussion

This chapter focused on elucidating the pathways involved in the biasing of T cells by type II and classically activated MΦ. Anderson and Mosser (2002) initially attributed the T cell biasing by type II MΦ to their altered level of IL-10 and IL-12 production. This conclusion was based on experiments using IL-12 and IL-10 deficient MΦ, however, the phenotype of these MΦ was altered by the deletion of these genes, which complicates the results and makes their interpretation challenging (Anderson & Mosser, 2002). In the current study, blocking antibodies and recombinant proteins were used to assess the effect of IL-10 and IL-12 on T cell biasing by classically and type II activated MΦ. As stated in the results section, the blocking antibodies for IL-10 and IL-12 are also used in detection, thus it is likely that IL-10 and IL-12 still exist in the cultures but are antibody bound, this could be confirmed using an antibody that binds a different epitope of the cytokines

IL-10 is well known to have inhibitory effects on MΦ, and has been shown to reduce the production of several proinflammatory cytokines produced by MΦ including IL-12 (Kobayashi et al., 2012; Moore, de Waal Malefyt, Coffman, & O'Garra, 2001). IL-10 produced by MΦ can act in an autocrine manner to reduce IL-12 production (Sica et al., 2000). Consistent with previous results from our laboratory, blocking IL-10 did not alter the level of IL-12 production by MΦ alone in this system (Kharkrang, 2010). These results suggest that IL-10 production by type II MΦ is not responsible for the down regulation of IL-12 seen in this activation state. However further increase in IL-10 levels by the addition of rIL-10 decreased the production of IL-12 when added at the time of MΦ stimulation, but not when added 4 hours post stimulation. The absence of a change in IL-12 when the rIL-10 was added at 4 hours could be due to the IL-10 being ineffective or not at a high enough concentration to alter an already established response, alternatively this may potentially be due to the IL-12 not being taken up by the MΦ. Therefore, the addition of rIL-10 may have effectively stopped further IL-12 production by the MΦ, but the IL-12 produced by MΦ in the 4 hours before rIL-10 addition may still be present in the supernatant. However, previous work in our laboratory has demonstrated that IL-12 production by classically and type II activated MΦ is very low up to 4 hours post stimulation (Kharkrang, 2010). This suggests the IL-12 present when rIL-10 is added to cultures at 4 hours is a result of the rIL-10 being ineffective at this time point and not IL-12 left over from early production.

It has previously been shown that MΦ can become less responsive to IL-10 following LPS stimulation. Alveolar MΦ stimulated with LPS exhibit decreased responsiveness to IL-10 due to inhibited signalling through the IL-10 receptor. Also, pre-treating peritoneal MΦ with LPS decreased the ability of IL-10 to inhibit ceramide-induced TNF-α production (Fernandez, Jose, Avdiushko, Kaplan, & Cohen, 2004). Therefore it is likely that the MΦ in the current study had reduced responsiveness to IL-10 after 4 hours in culture, and therefore, addition of rIL-10 after 4 hours in culture did not induce down regulation of IL-12.

Addition of αIL-12 to MΦ cultures successfully reduced detectable IL-12 levels without having a significant effect on IL-10 production, although there was a non-significant trend towards decreased levels of IL-10. rIL-12 did not induce a significant change in either IL-10 or IL-12. While MΦ express the receptor for IL-12 (Grohmann et al., 2001; Kataoka, Komazawa, Oboki, Morii, & Nakano, 2004) and signalling through the IL-12 receptor can further enhance IL-12 production (Grohmann et al., 2001), the amount of rIL-12 (5 ng/ml) may not have been enough to alter the levels of IL-10 and IL-12 in this culture, given the high level of IL-12 produced by the MΦ themselves.

In MΦ:T cell co-culture conditions, altering the levels of IL-10 and IL-12 through blocking antibodies and recombinant cytokines had little effect on the activation state of the T cells. Earlier studies in this thesis (Chapter 3) identified 3 molecules that were differentially expressed on T cells cultured with classically or type II MΦ; these were IFN-γ, IL-17A and CD124. IFN-γ levels were significantly affected by increasing or decreasing the amount of IL-10 or IL-12 in culture, however, IL-17A and CD124 were not significantly affected in most cases (Table 4.1). The PD-1/PD-L1 and CD40/CD40L pathways did not have strong effects on the biasing of T cell responses by type II and classically activated MΦ (Table 4.1). The effects seen with αIL-10 and αIL-12 were in line with the current literature For example, αIL-12 neutralised IL-12 efficiently and inhibited IFN-γ production. Since as IL-12 is known to be essential for Th1 development, this result is in line with previous reports (Kaplan et al., 1996; Zhu et al., 2010). This data supports the set up of the current assay and suggests it is working as expected.

IL-10 is an anti-inflammatory cytokine known to reduce the production of IFN-γ by T cells and has been implicated in T cell biasing by type II activated MΦ (Anderson & Mosser, 2002). IL-10 mediates the majority of its effects by inducing down regulation of inflammatory cytokines and co-stimulatory molecules on APC (Ding, Linsley, Huang,

Germain, & Shevach, 1993; Fiorentino, Zlotnik, Mosmann, Howard, & O'Garra, 1991; Fiorentino, Zlotnik, Vieira, et al., 1991). This includes inhibiting production of IL-12 by APC (Kobayashi et al., 2012). As IL-12 is important in Th1 development, this decrease in IL-12 inhibits Th1 biasing and thus IFN-γ by T cells (Kaplan et al., 1996; Moore et al., 2001). However, T cells do express receptors for IL-10, and IL-10 is capable directly acting on T cells to inhibit the production of cytokines (Couper, Blount, & Riley, 2008; Naundorf et al., 2009).

When αIL-10 was added to MΦ:T cell co-cultures at the time of MΦ stimulation, T cells cultured with type II and classically activated MΦ significantly increased production of IFN-γ. Despite a similar decrease in IL-10 levels when the αIL-10 was added with the T cells, there was no increase in IFN-γ in these cultures. This suggests that in the 4 hours between the stimulation of the MΦ and the addition of T cells, the IL-10 present in the cultures may have acted on the MΦ in an autocrine manner to induce a change in phenotype, and that the effect of the decrease in the IL-10 is due to direct effects on the MΦ and not on the T cells. IL-12 is not altered when IL-10 is blocked however, IL-10 affects other molecules produced by MΦ including IL-1, TNF and CD80/CD86 (Ding et al., 1993; Moore et al., 2001). It is possible that autocrine signalling of IL-10 is affecting a pathway other than IL-10 such as co-stimulation which have not been measured in the current study.

Increasing the level of IL-10 in MΦ:T cell co-culture supports the role of IL-10 in T cell biasing by type II MΦ. The addition of rIL-10 to cultures resulted in a significant decrease in IFN-γ production, which correlated with decreased production of IL-12. Similar to the results seen in MΦ cultured alone, and in MΦ:T cell cultures with the addition of αIL-10, this change was only observed when the rIL-10 was added at the time of MΦ stimulation and not when it was added with the T cells. IL-10 is known to inhibit IL-12 production (Kobayashi et al., 2012; Moore et al., 2001), and as IL-12 is a key cytokine in the induction of Th1 cells, the reduction of IFN-γ is to be expected. These results suggest that the IL-10 may be involved in the regulation of IFN-γ by type II MΦ. The amount of rIL-10 added to the cultures was 5 ng/ml. This amount of IL-10 is higher by approximately 2-3 fold than that which is detected in the supernatant of cultures containing type II MΦ, which may explain why the rIL-10 has a much more dramatic effect than IL-10 produced endogenously by the MΦ in culture. In addition, adding the rIL-10 provides an instant high level of IL-10 that does not require production by MΦ, as the rIL-10 can act earlier it may be contributing to the increased effectiveness of the exogenous IL-10, compared to that which is produced by MΦ. The

decrease in IFN-γ occurred when the rIL-10 was added when the MΦ were stimulated but not when it was added with the T cells, despite the levels of IL-10 being higher when rIL-10 is added with the T cells. This supports the major effect of the IL-10 being on the MΦ, not on the T cells directly and that the IL-10 acts on the MΦ soon after stimulation.

Another key cytokine implicated in biasing of T cell responses by classical or type II MΦ is IL-12 (Anderson & Mosser, 2002). When αIL-12 was added at the time of MΦ stimulation a trend towards decreased the production of IL-10 was observed, this trend was not seen at 4 hours, however, the isotype control antibody had some effect in these experiments and thus it is possible that this change was non-specific. However, MΦ have receptors for IL-12 (Grohmann et al., 2001) and it is possible that a certain level of feedback though these receptors is necessary to stimulate IL-10 production, which also may explain this downward trend. IL-12 is a key cytokine in the development of Th1 cells and IFN-γ production (Kaplan et al., 1996). Therefore, it is possible that the decreased IL-12 production by type II MΦ may be involved in T cell biasing by reducing signalling through the IL-12 receptor and STAT-4 activation. Detectable IL-12 levels were similarly reduced by αIL-12 in conditions containing both classical and type II MΦ. Consistent with the role of IL-12 in Th1 development and IFN-γ production; in the current study, blocking IL-12 significantly inhibited the production of IFN-γ by T cells cultured with both classically and type II activated MΦ. This demonstrates that in the absence of IL-12 signalling, MΦ were unable to drive Th1 responses and is consistent with previous reports (Anderson & Mosser, 2002). The effects of αIL-12 were evident when added with the MΦ stimuli and with the T cells, this suggests that the IL-12 could potentially be acting on the T cells as opposed to directly on the MΦ. Due to the well known effect of IL-12 on T cells to induce Th1 responses, it is likely that this effect is due to a reduction in the signalling to T cells and not to an indirect effect on MΦ.

In the current study, addition of rIL-12 did not cause an increase in the levels of IL-12 detected in the culture supernatants. However, this does not mean that the rIL-12 is not being added at a sufficient level to be having an effect. Previous work in our laboratory has demonstrated that IL-12 production by classical or type II activated MΦ is comparably low at 2-4 hours of culture (Kharkrang, 2010), this early period of culture corresponds closely to the time points rIL-12 is added in the current study. Therefore, while the level of rIL-12 added to cultures is moderate over the entire course of the culture, it is likely that it is causing a significant increase in the early time points of the culture. The rIL-12 could therefore be looked upon as an increase in early IL-12. Given the role of IL-12 in Th1 development an

increase in IL-12 may be thought to cause an increase in IFN-γ production. In the current study, addition of rIL-12 did not cause an increase in IFN-γ production in cultures containing classically activated MΦ, however, the levels of IFN-γ were significantly increased in type II MΦ containing cultures. This increase in IFN-γ was not only significantly increased compared that produced by T cells cultured with type II MΦ in the absence of rIL-12, but also increased above the levels produced by cultures containing classically activated MΦ and rIL-12. This suggests that in the presence of exogenous IL-12, type II MΦ may be more effective at biasing T cells towards a Th1 response than classically activated MΦ.

The large increase in IFN-γ production occurs only in T cells cultured with type II activated MΦ suggesting that there is an intrinsic difference between classically and type II activated MΦ in presenting to T cells that extends beyond the IL-12/IL-10 dichotomy. There are several possibilities as to what this difference could be. Type II activated MΦ are known to express lower levels of PD-L1 compared to classically activated MΦ (Tierney et al., 2009). PD-L1 is known to have an inhibitory effect on the production of IFN-γ (Schreiner et al., 2004; Schreiner, Bailey, Shin, Chen, & Miller, 2008; Yamazaki et al., 2005), and there is evidence that PD-L1 is particularly important in the regulation of Th1 cells. The reduction in PD-L1 on type II MΦ may result in less of an inhibitory signal to the T cells, and consequently a higher production of IFN-γ. It has also been reported that type II activated MΦ increase expression of both MHC class II and CD86 (Edwards et al., 2006), thus it is possible that these T cells are receiving an increased stimulatory signal which is leading to increased levels of IFN-γ production.

IL-12 has a strong role in inducing T cell biasing, and the results of this study suggest that the decreased level of IL-12 produced by type II activated MΦ may play a role in the biasing of T cell responses. In addition to IL-12, it has been proposed that IL-10 may play a role in T cell biasing. IL-10 inhibits IL-12 production by MΦ (Kobayashi et al., 2012); however, in this study, blocking IL-10 production did not affect the level of IL-12 produced by MΦ cultured alone, suggesting that although IL-10 can act in an autocrine manner (Sica et al., 2000), increased IL-10 production by type II MΦ is not responsible for the decreased IL-12. In MΦ:T cell co-cultures there is no significant change in IL-10 production after IL-12 blocking. This result suggests that the effects that blocking IL-10 have on MΦ T cell co-cultures are independent of changes in IL-12 production.

Given the inhibitory role IL-12 and IFN-γ have on IL-17 production (Harrington et al., 2005; Komiyama et al., 2006), it was postulated that the decrease in these cytokines in cultures containing type II MΦ may be responsible for the increased levels of IL-17A in the same cultures. However, reduction in IL-12 levels using αIL-12, caused a decrease in IFN-γ without affecting IL-17A production. IL-17A production was also not altered by increased level of IFN-γ in this system. This finding suggests that the decreased levels of IL-12 and IFN-γ in cultures containing type II activated MΦ are not responsible for the increased levels of IL-17A. There was a significant reduction of IL-17A following the addition of rIL-10 to cultures containing classically activated MΦ at the time of MΦ stimulation; this same alteration was not seen in cultures containing type II activated MΦ. This suggest the increased IL-10 produced by type II MΦ is not responsible for the increased IL-17A and suggests the pathways controlling IL-17A production may be different when T cell are stimulated with classical or type II MΦ. Furthermore, the absence of a change in CD124 expression demonstrate that the change in IL-10 and IL-12 expression by type II MΦ is not responsible for the increased responsiveness of T cells cultured with type II activated MΦ to IL-4. Over all this data shows that the increases in IL-17A and CD124 by T cells cultured with type II MΦ occurs independently of the changes in IL-10 and IL-12, suggesting that another, as yet unidentified pathway, contributes to the biasing of T cells by type II MΦ.

Together these data suggest that the major role of IL-10 and IL-12 in regulating the T cell response is in controlling IFN-γ production. The data supports the hypothesis that the decreased level of IL-12 produced by type II activated MΦ is involved in T cell biasing, at least as far as IFN-γ production. In addition, IL-10 and IL-12 levels appear to be only involved in the regulation of IFN-γ production and not of other markers of T cell biasing such as IL-17A and CD124.

IL-4 production was not assessed in this model due to the difficulty in detecting it in culture supernatants. Assessment of IL-4 production, for example by flow cytometry, will be important in confirming the roles of these cytokines in T cell biasing. The major role of the IL-12 appears to be on the T cells themselves as altering the IL-12 level does not alter MΦ cytokines that were measured, but alters the T cells when IL-12 levels are altered at 0 or 4 hours, however it is possible the effect is at least partly on the MΦ. Alternatively, the major role of the IL-10 appears to be on the MΦ as altering IL-10 affects the MΦ cytokines themselves, T cells are also affected but both MΦ and T cells are only affected when the IL-10 level is altered at stimulation. If the major effect of IL-10 was on T cell a similar effect

would be expected when IL-10 levels are altered at stimulation and the time the T cells were added, however, the IL-10 may be in part acting on T cells directly. It is possible that there is another aspect of the MΦ which drives the Th1 response that is altered on type II MΦ compared to classically activated MΦ. This factor may be a cytokine or a cell surface molecule. For example, type II activated MΦ have recently been shown to produce IL-4 (La Flamme et al., 2012), which may have a role in altering T cell responses. Two potential molecules that are altered on type II MΦ compared to classically activated MΦ are CD40 and PD-L1.

As the changes in IL-12 and IL-10 production by type II MΦ appeared to only be involved in the level of IFN-γ production by T cells, other pathways were investigated. PD-L1 is significantly decreased on type II activated MΦ compared to classically activated MΦ (Tierney et al., 2009). PD-L1 is generally considered to provide inhibitory signals to T cells (Carter et al., 2002; Konkel et al., 2010; Tsushima et al., 2007), although stimulatory roles for PD-L1 have been reported (Wang et al., 2003). The reports of PD-L1 having both stimulatory and inhibitory effects on T cells suggest that the action of PD-L1 may be conditional. It is possible that the strength of the PD-L1 signal may be involved in biasing T cell responses. Due to the decreased expression of PD-L1 on type II MΦ, the PD-1/PD-L1 pathway was investigated for a possible role in T cell biasing. In this study, the interaction between PD-1 and PD-L1 were interrupted using a blocking antibody that binds PD-1. As neither classically or type II activated MΦ express high levels of PD-L2 (Tierney et al., 2009), the effect of the αPD-1 antibody can be considered to be a result of disrupted PD-1/PD-L1 interactions and not PD-1/PD-L2 interactions. In addition, a PD-L1 chimeric protein was used to mimic stimulation of the PD-1 receptor to investigate the effects of increased expression of PD-L1 on MΦ, such as on classical activated MΦ.

The effect of the PD-1/PD-L1 signalling pathway is thought to be mainly on the T cell. However, there is some evidence that ligation of PD-L1 and PD-L2 on DC can lead to phenotypic changes in a process known as “reverse signalling” (Kuipers et al., 2006). It is therefore possible that changing the level of PD-1/PD-L1 interaction may have an effect on the MΦ. However, the production of IL-10 and IL-12 were not disrupted by the presence of αPD-1 in culture, suggesting that reverse signalling does not have a role in MΦ function under the current conditions. In addition the level of IL-12 and IL-10 production by classical and type II activated MΦ was not altered by the addition of the PD-L1ch.

As PD-1 pathways are inhibitory to T cells, decreasing the interaction between PD-1 and PD-L1 would likely increase the activation state of T cells. Therefore, the activation state of T cells in the presence of αPD-1 was assessed, however, blocking the PD-1/PD-L1 pathway did not affect the activation state on MΦ. This result may suggest that in these cultures PD-L1 is not providing an inhibitory signal to T cells. The MΦ in this study are highly activated and effective APC which drive T cell activation efficiently. Therefore, inhibition of the PD-L1 pathway may not further increase the ability of MΦ to induce activation of T cells. The αPD-1 was used at 2 μg/ml in this study, however, αPD-1 has been used previously in proliferation assays at 5 μg/ml and been shown to support proliferation (Yamazaki et al., 2005). It is therefore possible that the antibody was not used at a high enough concentration. 2 μg/ml was chosen as it appeared to have the greatest effect in titrations. In addition, the decrease in PD-L1 on type II activated MΦ did not significantly alter T cell proliferation (Chapter 3). Therefore, a slightly lower concentration of αPD-1 than one which alters proliferation may potentially be more accurate at mimicking the change in PD-L1 expression on type II activated MΦ, in other conditions. However, given the absence of an effect of the αPD-1, it is also possible that the αPD-1 was not active in this assay, thus further experiments are required to confirm these data.

The activation state of T cells was also assessed in the presence of PD-L1ch, to increase the signalling through the PD-1 receptor on T cells. Inclusion of the PD-L1ch in MΦ:T cell co-cultures did not significantly alter the activation state of the T cells. However, as blocking this pathway does not have an effect on T cell activation it is unlikely that the altered PD-L1 expression on type II MΦ is involved in altered levels of T cell activation by type II MΦ. In this study the PD-L1ch was used at 8 μg/ml. This protein has been used previously to inhibit the proliferation of T cells at 5 μg/ml (Vogt et al., 2006), therefore, it is likely that 8 μg/ml is a sufficient concentration to be effective in these culture conditions, however, the it is also possible that the PD-L1ch was not active in the current study, thus these results should be confirmed through further experimentation.

In addition to the effects on T cell activation, the effect of the PD-L1 pathway on biasing was also assessed. Neither adding αPD-1 or PD-L1ch affected the production of IL-17A. This suggests that the decreased expression of PD-L1 on type II MΦ is not responsible for the increase in IL-17A production by T cells cultured with type II MΦ. Furthermore, blocking the PD-1/PD-L1 pathway did not affect CD124 production. There was a trend towards lower expression of CD124 on T cells cultured in the presence of the PD-L1ch; however, as this

decrease was also seen in on T cells cultured with the control protein, it is most likely non-specific. The production of IFN-γ was not significantly affected by the addition of αPD-1. The addition of PD-L1ch resulted in a decrease in IFN-γ in cultures containing type II MΦ, however; there were also changes seen with the control protein which makes interpretation of these results difficult. Overall these results suggest that the decreased expression of PD-L1 by type II activated MΦ does not alter T cell biasing.

In addition to PD-L1, type II MΦ also express lower levels of CD40 (Tierney et al., 2009). CD40 signalling is important in activation of MΦ and has a dual role in the production of cytokines such as IL-10 and IL-12 in MΦ and DC (Mathur et al., 2004; Murugaiyan et al., 2006). In addition, different levels of CD40 expression or stimulation have been associated with biasing of T cell responses by DC (S. Martin et al., 2010; Murugaiyan et al., 2006). Furthermore, previous studies have shown that CD40 is essential for both Th1 and Th2 type responses (K. A. Campbell et al., 1996; MacDonald et al., 2002; Poudrier et al., 1998). Due to the dichotomous role of CD40, this study aimed to investigate the role of the CD40/CD40L pathway in T cell biasing in type II MΦ. Low level stimulation of CD40 leads to increased production of IL-10, whereas high level stimulation leads to increased IL-12 (Mathur et al., 2004). It is therefore possible that in interacting with T cells, type II activated MΦ, with their lower expression of CD40, would be stimulated to produce more IL-10, which could promote inhibition of IL-12 production by MΦ, and thus less IFN-γ production by T cells. Conversely, classically activated MΦ which have a much higher expression of CD40 will receive higher stimulation when cultured with T cells which may induce them to up regulate IL-12, potentially driving Th1 responses.

The role of CD40 in T cell biasing by type II activated MΦ was investigated using an antibody against CD40L. This antibody binds to CD40L and inhibits interactions with CD40, reducing the signalling to CD40 on the T cell. Previous studies suggest that this reduction in CD40 signalling should result in decreased IL-12 production (Mathur et al., 2004). However, addition of αCD40L to cultures did not affect the production of IL-12 by either classically or type II activated MΦ. Furthermore, there was no significant change in the levels of CD124 expressed or the production of IL-17A or IFN-γ, although there was a non-significant trend towards an increase in IFN-γ production. Previous studies have used higher concentrations of αCD40L antibodies (e.g. 10 μg/ml, DeKruyff, Gieni, & Umetsu, 1997) compared to the 4 μg/ml used in the current study. The concentration used in here was chosen as the strong effect of the isotype control antibody at higher concentration would make distinguishing

specific effects difficult. It is therefore possible that this antibody in this study was not used at a high enough concentration; however, there are alternative possibilities. For example, CD40 interactions have previously been shown to be essential to the induction of Th1 responses under certain conditions; however, this is associated with deficiency in IL-12 production in the absence of CD40 stimulation (K. A. Campbell et al., 1996). However, in the current study MΦ were already expressing high levels of IL-12 in response to LPS, a process which does not require CD40 ligation (DeKruyff et al., 1997). Therefore, further stimulation of the MΦ via CD40 may be unable to induce higher levels of IL-12 production. Indeed, human monocytes treated with LPS show decreased responsiveness to CD40 stimulation (Sinistro et al., 2007). Therefore interaction with CD40 on T cells may not further stimulate IL-12 production, thus removal of this signal does not result in a change in IL-12 production.

While CD40 may not stimulate classically activated MΦ to produce IL-12 under the current conditions, it is still possible that the reduced CD40 interactions between T cells and type II activated MΦ may be involved in T cell biasing by type II MΦ. IFN-γ did show a slight increase in IFN-γ production in the presence of αCD40L. It is possible that low CD40/CD40L interactions between type II MΦ and T cells induce an inhibitory response (that is inhibitory to IFN-γ production by the T cells) and that further lowering CD40 stimulation prevents CD40/CD40L interactions completely, resulting in the removal of this inhibitory signal. The result of low level CD40 stimulation may be a change in IL-10 production as IL-10 is associated with low level CD40 stimulation (Mathur et al., 2004). It is also possible that this low level of CD40 stimulation alters another pathway in type II MΦ. However, these results are preliminary, and while interesting, merit further investigation. Altering the level of CD40 signalling suggests that this pathway does not alter IL-17A production nor does it affect IL-4 responsiveness (as measured by CD124 expression), implying another pathway is involved in these changes.

Alteration in the level of CD40 signalling also did not alter the activation state of T cells in culture with either classically or type II activated MΦ. IL-2 did show a non-specific decrease in production in the presence of αCD40L, however, as this was also seen in the isotype control, it may not be a real change due to lower CD40 signally, instead being simply a result of antibody in the culture. Isotype control antibodies account for the effects caused by antibodies binding non-specifically to the FcR on cells. However, it should also be considered that the effect of antibodies binding to FcR may be exaggerated when using

isotype controls. It is possible that, as these antibodies do not bind to anything they are more likely to end up attached to an FcR than a specific antibody which will bind to its target. Therefore, care must be taken from both sides when interpreting data using specific antibodies and non-specific controls. Due to that absence of any significant affect on T cell biasing by MΦ in the presence of aCD40L, this suggests that the decreased expression of CD40 on type II MΦ is not a key factor involved in T cell biasing by type II MΦ, however, it is possible that the αCD154 antibody was not active in the current culture as there was not much change in the markers measure, thus this data should be confirmed through further experimentation.

## 4.5 Summary

The results of the current study demonstrate that the level of IL-12 production by MΦ has a strong role in T cells biasing by classically and type II activated MΦ, as seen in its role in IFN-γ production, by directing acting on the T cell themselves. Furthermore the level of IL-10 production by MΦ also has an effect of IFN-γ production by T cells. Unlike IL-12, the IL-10 appeared to exert its effects by acting on the MΦ, not on the T cell directly. This study also suggested that the reduction of CD40 and PD-L1 on type II MΦ do not have a strong role in biasing the T cell response. None of the pathways investigated in this study had a significant effect on the level of IL-17A production or CD124 expression by T cells, which are expressed more highly on T cells cultured with type II MΦ compared to those cultured with classically activated MΦ. This data contradicts the report by Anderson & Mosser (2002), which suggested that the IL-10/IL-12 dichotomy was the only important factor involved in differential T cell biasing by classically and type II activated MΦ. Instead, while IL-10 and IL-12 are involved in the regulation of IFN-γ, this study suggests that there will be other pathways affecting the ability of type II and classically activated MΦ to bias the T cell response. In order to further investigate the pathway of T cell biasing, other markers of T cell responses, such as IL-4, should be assessed. IL-4 is difficult to detect in culture, partly due to its rapid reuptake by T cells. Therefore, further studies should include assessment of IL-4 by flow cytometry. It is highly likely that many pathways are involved in the biasing of T cell responses by type II MΦ, and while the alterations in individual pathways do not have an effect, there may be a cumulative effect by altering multiple pathways at once. The necessity of using control proteins can make experimentation of the results quite difficult and in addition, some of the pathways, particularly the CD40/CD154 and PD-1/PD-L1 did not show any change in the markers measured, and thus need to be treated with caution. Further

investigation of the current pathways, for example using genetically deficient mice or RNAi, would provide further information on any roles these pathways might play in T cell biasing.

# Chapter 5:
# Optimisation of protocols for isolation and culture of adult murine microglia

## 5.1 Introduction

In the past, much of the research conducted in the study of MG *in vitro* has employed cultures derived from neonatal or embryonic animals (Gingras, Gagnon, Minotti, Durham, & Berthod, 2007; Giulian & Baker, 1986). These cultures are often grown as mixed glial cultures with astrocytes present, and sometimes growth factors such as GM-CSF are added (Gingras et al., 2007; Giulian & Baker, 1986). Under these conditions neonatal/embryonic microglia survive and proliferate (Schell et al., 2007). This method has been used for many years and is considered a valid way of looking at MG *in vitro* (Giulian & Baker, 1986; Schell et al., 2007). However, it does have certain drawbacks, namely that neonatal MG are functionally different to adult MG due to the environment in which they are found, and the different roles the MG play at different stages of development (Moussaud & Draheim, 2010; Schell et al., 2007).

There are also several MG cell lines developed from neonatal brains available (Schell et al., 2007). These cell lines include Ra-2, 6-3, N9, N13 and BV-2 (Henn et al., 2009; Righi et al., 1989; Sonobe et al., 2005). There are limitations with the use of cell lines for the study of MG *in vitro*, as they have been shown to be functionally different to primary microglia under several conditions and are therefore unlikely to accurately model adult MG *in vivo* (Henn et al., 2009; Sonobe et al., 2005). Although, they are useful for performing large scale experiments where the number of cells required would be prohibitive if using primary MG. Cell lines are a valid model, but as with the neonatal MG, the differences they have from adult MG need to be taken into account. Due to these differences a protocol for the isolation and culture of adult murine MG was optimised in this chapter.

Several methods for the isolation of MG from adult animals have been described in the literature. Adult MG were first isolated from the brains of naïve rats by Sedgwick *et al.* in 1991 (Sedgwick et al., 1991). This method employed the use of a Percoll gradient to isolate the cells on the basis of specific gravity and is a commonly used method for the isolation of microglia. Percoll is a semi aqueous solution composed of polyvinyl-pyrrolidone-coated colloidal silica-gel particles (Wakefield, Gale, Berridge, Jordan, & Ford, 1982). In some studies, MG have been isolated using Percoll and expanded in culture with the use of growth factors such as M-CSF and GM-CSF (Moussaud & Draheim, 2010; Ponomarev et al., 2005). Cell sorting techniques (both magnetic beads and FACS) are also a common method for MG isolation and are often used in combination with Percoll. Another method for the isolation of

adult MG makes use of the adherent properties of MG to isolate them from other cell types in the CNS (Yip et al., 2009).

## 5.2 Aims

The aim of this chapter was to compare several of these methods in order to develop a protocol for the isolation and culture of adult murine microglia in our laboratory for their *in vitro* analysis.

### 5.2.1 Specific aims

1. To assess adherence, Percoll gradients and magnetic bead sorting as possible methods for the isolation of adult murine MG.
2. To assess the survivability and *in vitro* responsiveness of MG that had been isolated with optimised techniques.
3. To assess the effect that expansion with M-CSF or GM-CSF has on the survivability and responsiveness of MG *in vitro*.

## 5.3 Results

### 5.3.1 Isolation of MG from adult mice

In order to evaluate *ex vivo* responses of MG from adult mice, several methods of isolation were compared and optimised.

#### *5.3.1.1 Adhesion*

The first method of isolation of MG from the spinal cord used a protocol described by Yip et al., (2009) which relied on adhesion. This method involved mincing the spinal cord and enzymatically digesting it using papain. In Yip et al. MG were plated onto glass cover slips for immunocytochemical analysis; however, because for these studies the MG needed to be isolated for FACs analysis, the MG were instead plated in a variety of plastic tissue culture equipment. The cells were plated at 10,000, 20,000, or 40,000, cells/well in 96 or 24 well plates, or cells from a whole spinal cord were plated onto a non-tissue culture treated petri dish. The medium was replaced after 2 hours, during which time the MG should have adhered to the plastic surface. MG were removed from the plates at 2 or 24 hours after plating and assessed by flow cytometry for purity (Figure 5.1). This protocol resulted in extremely low yields with insufficient purity and was therefore abandoned (Figure 5.1).

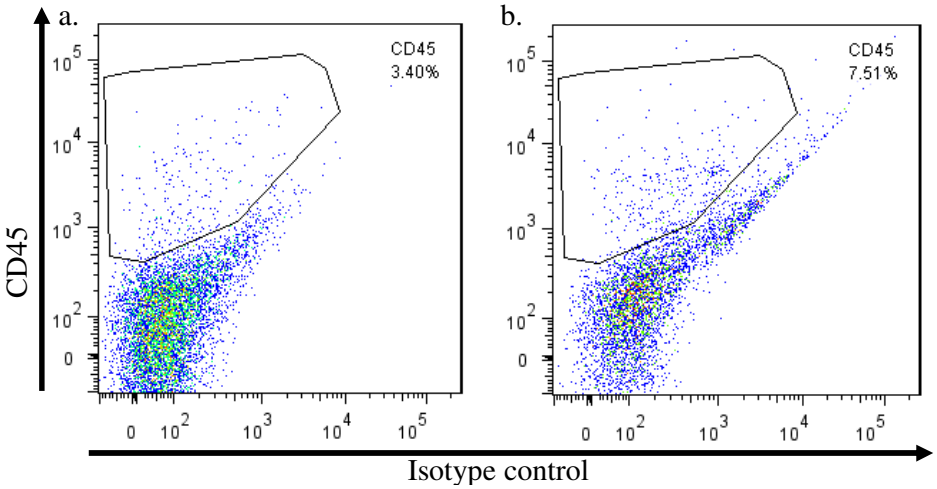



**Figure 5.1. Isolation of MG by adhesion results in insufficient yield and purity for *in vitro* culture conditions.** A spinal cord a from BALB/c mouse (n=1) was digested with papain before being washed and plated in a flat bottomed 96 well plate at 40,000 cells/well. Cells were incubated at 37ºC/5% CO2 for 2 hours at which time the medium was changed. Cells were isolated from culture using ice cold PBS at this time (a) and at 24 hours (a) after culture and assessed by flow cytometry. Shown are plots from one experiment.

***5.3.1.2 Beads***

A second technique used magnetic bead kits to isolate MG from adult mice. Two different magnetic bead setups were used, the CELLection™ Biotin Binder Kit (Invitrogen, USA) and CD11b (microglia) Microbeads (Miltenyi biotec, Germany). The CELLection™ Biotin Binder Kit employs magnetic beads which are attached to streptavidin via a DNA linker. Thus, any biotinylated antibody will bind to these beads and be used to isolate the target cells. As MG are the only cells in a normal CNS to express CD11b, this marker was chosen for MG isolation. Once the MG are bound to the beads, the CD11b negative cells can be removed by placing the tube on a magnet and removing the supernatant, thus removing anything not bound to the magnetic beads. Bead-bound CD11b positive cells can then be released using DNase I which severs the link between the bead and the streptavidin. After incubation with DNase I, the solution is placed back on the magnet and the supernatant containing released MG cells can be isolated.

Spinal cords were used to test the isolation of MG using this kit; however, few cells were obtained with this method. It appears that the reason for the low yield of cells is that the MG did not detach from the beads as anticipated (Figure 5.2). Very few $CD45^+$ cells were seen in the supernatant that was isolated following DNase I treatment, which theoretically should have contained the MG, based on the principles of the isolation (Figure 5.2b). However, a large number of $CD45^+$ events was found in the "bead-bound" fraction following DNase I treatment (Figure 5.2a). R1 may represent bead-bound cells and R2 beads that are not bound to cells (as the beads may have different auto fluorescence when bound to cells). Conversely, the cells may have detached during the process of staining for flow cytometry, thus R1 would represent MG and R2 beads (Figure 5.2a). These data suggest that MG do not efficiently detach from beads, making this an undesirable method for the isolation of adult MG.

The second bead method, CD11b Microbeads, was tested on both brains and spinal cords. Unlike the first bead method, this procedure was successful at isolating MG; however, the yield was still low. In addition, there appeared to be large amounts of debris in the "purified" cells. Figure 5.3 (a and d) shows that while $CD45^+$ cells were isolated, only a very small percentage of the total events were $CD45^+$ suggesting that the sample was impure, containing a large amount of debris. Removing the myelin from the CNS preparation using 37% Percoll before using the beads did not produce a significant reduction in the amount of debris seen by flow cytometry (Figure 5.3b and e).

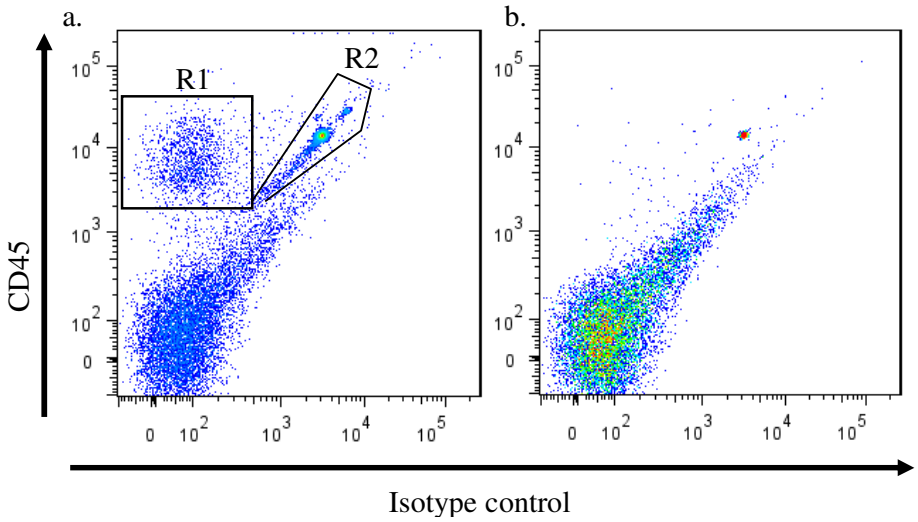



**Figure 5.2. Microglia attach to beads labelled with CD11b but do not detach.** Spinal cords from BALB/c mice were digested with papain to create a single cell suspension before incubation with magnetic beads labelled with CD11b for 20 minutes at 4ºC. Beads were washed to remove negative cells and then incubated with 250 U of DNase for 15 minutes at room temperature. Cells that were released from beads were collected by placing the solution on a magnet and removing the supernatant. Both bead bound (a) and released cells (b) were analysed by flow cytometry. Shown are plots from one experiment.

#### ***5.3.1.3 Percoll***

The most successful technique for the isolation of adult MG was to use a Percoll gradient composed of 3 layers of Percoll in decreasing concentrations (70%, 37% and 30%). This technique was compared to the isolation of MG using the CD11b Microbeads method and was found to produce a higher yield of cells with less debris (Figure 5.3c and f). To help ensure the highest possible yield, both the brains and the spinal cords were used to isolate MG. The cells isolated from the CNS using Percoll were analysed by flow cytometry to verify the identity and purity, and MG were routinely found to be a distinct population $CD45^{lo}CD11b^{+}$ (Figure 5.4). MG cells isolated using a Percoll gradient were routinely found to be suitably pure (83.16±1.585 %; mean±SEM from 26 experiments).

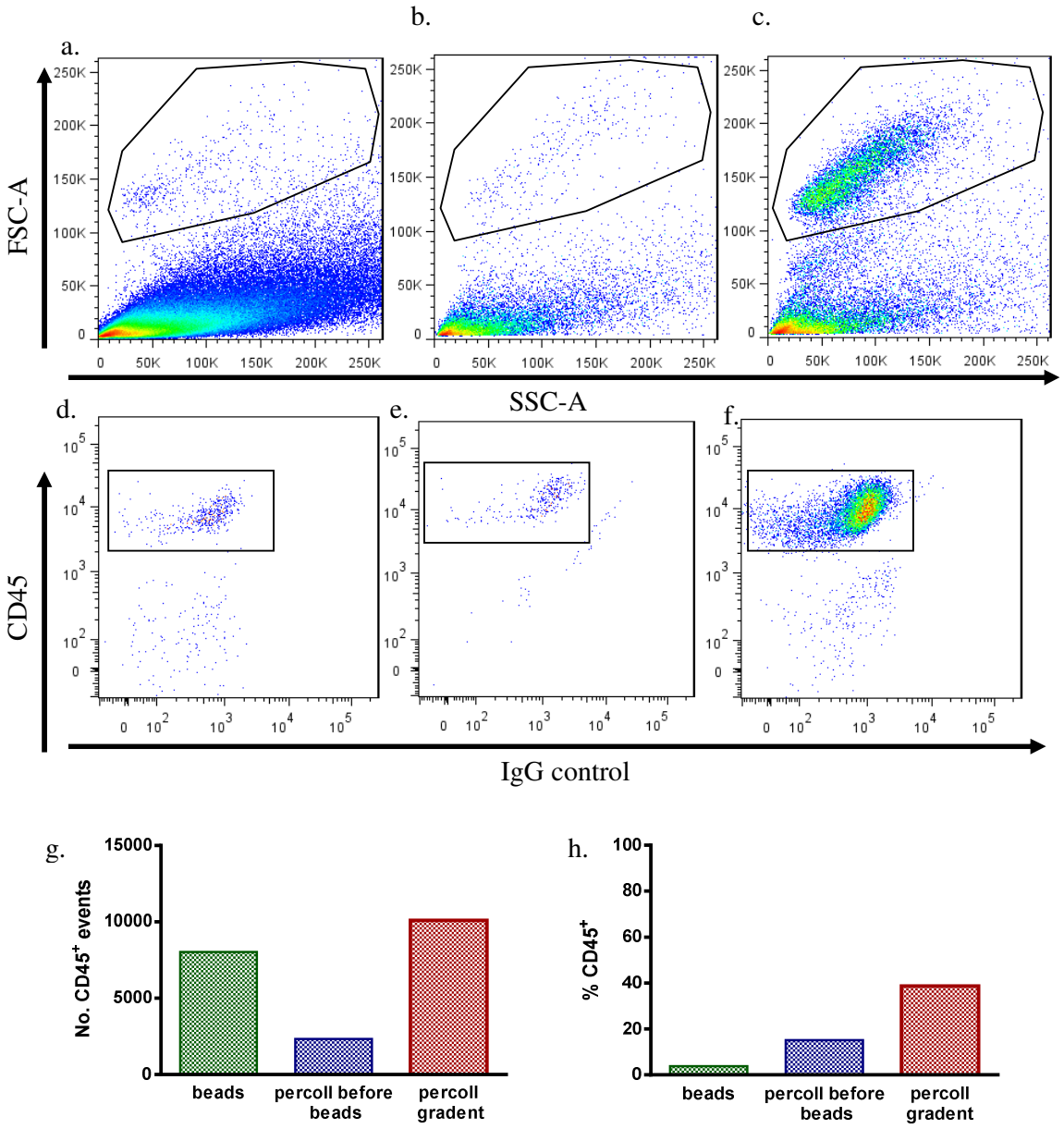



**Figure 5.3. Percoll results in a cleaner population with more cells than magnetic beads.** The brains and spinal cords of BALB/c mice were digested with accutase and MG were isolated with either CD11b Microbeads directly (a, d; n=1 mouse) or following the removal of myelin with 37% Percoll (b, e; n=1 mouse), or by Percoll gradient (c, f; n=1 mouse). Cells were stained with CD45 and analysed by flow cytometry. Live cells were gated using the FSC-SSC gate shown (a, b, c), and then $CD45^+$ cells were gated as shown (d, e, f). The number of CD45 positive cells (g), and the percentage of total events that were CD45 positive (h) were assessed. Data is from one experiment.

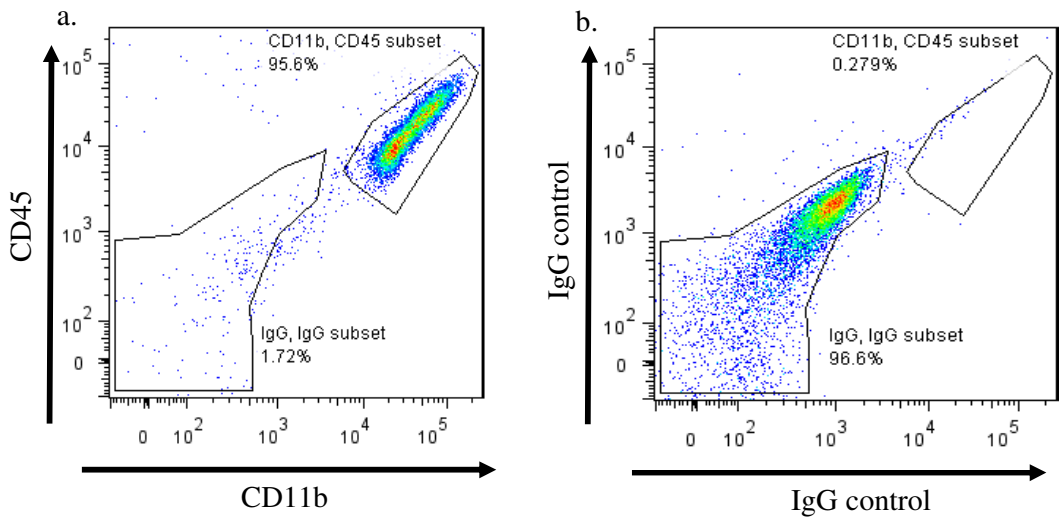



**Figure 5.4. Cells isolated using a Percoll gradient are CD45lo CD11b+, which are consistent with MG, and are of a sufficient purity for culture.** The brains and spinal cords of BALB/c mice were digested with accutase, and MG were isolated by Percoll gradient. Cells were stained with CD45and CD11b (a) or corresponding isotype control antibodies (b). Shown are representative plots from one experiment.

### 5.3.2 Culture of MG isolated from adult mice

To assess the *in vitro* responsiveness of MG freshly isolated from adult mice, MG from the CNS of adult BALB/c and C57BL/6 mice isolated using Percoll gradients were cultured overnight in the presence or absence of IFN-γ, followed by stimulation with LPS for 24 hours. MG were shown to produce the proinflammatory cytokine IL-12 in a concentration dependent fashion in response to LPS challenge (Figure 5.5). MG from C57BL/6 mice produced higher levels of IL-12 compared to those from BALB/c, and IFN-γ increased the level of IL-12 produced by MG in response to LPS (Figure 5.5). As C57BL/6 mice had a stronger response, this strain was chosen for all experiments after optimisation was complete.

The low level of cytokines produced by the MG suggests that MG produce very low levels of cytokines; however, an alternative explanation is that there are too few cells to produce a high concentration of cytokines. It is well known that extensive cell death often occurs in the early stages of adult MG culture (Aloisi, Simone, Columba-Cabezas, Penna, & Adorini, 2000; Brannan & Roberts, 2004); therefore, cell death resulting in low cell numbers in the cultures may be responsible for the low levels of cytokines detected. To assess this possibility, 24 hours after plating cells for culture (without any stimulation) MG were removed from culture plates by blasting with ice cold PBS. After blasting, cells isolated from the culture plate were counted using a trypan blue exclusion assay. However, in all experiments in which cells were plated at $1x10^5$ cells/well in a 96 well plate, no live cells were observed. This finding was confirmed using flow cytometry, where no CD45 or CD11b positive events were detected.

Other authors have reported between 42-80% viability after 24 hours in culture (Aloisi et al., 2000; Brannan & Roberts, 2004). To test the number of cells surviving after 24 hours in culture, cells were plated at $5x10^5$ in a 6 well plate. Cells harvested from plates after 24 hours in culture were assessed by flow cytometry, using CD45 and CD11b to identify the MG. After 24 hours in culture, cells remained $CD45^+$ $CD11b^+$ (Figure 5.6). Cells taken from plates after 24 hours in culture were re-plated at $1x10^5$ cells/well in a 96 well plate. These cells were stimulated with 200 ng/ml of LPS after overnight stimulation with IFN-γ; however no detectable levels of IL-12 were produced, indicating either that further cell death had occurred or that the remaining cells were not competent IL-12 producing cells.

The average yield of live microglia from 6 well plates was 31.1% (Table 5.1). To attempt to increase the yield of MG after immediate isolation and culture the protocol was altered.

These adjustments included different types of Percoll gradients, which have been reported to isolate larger numbers of MG (De Haas, Boddeke, Brouwer, & Biber, 2007), and other methods for dissociation of the tissue which may be gentler than the mechanical dissociation using a 70 μm nylon mesh cell strainer used previously in this study, and included glass homogenisers, wire mesh and enzymes. However, none of these methods increased the yield or survival of the MG. Due to the low number of cells that can be isolated from mice and the low number of MG that survive in culture, very large numbers of mice would be needed for each experiment. Because of these large numbers, other methods for MG culture were pursued.

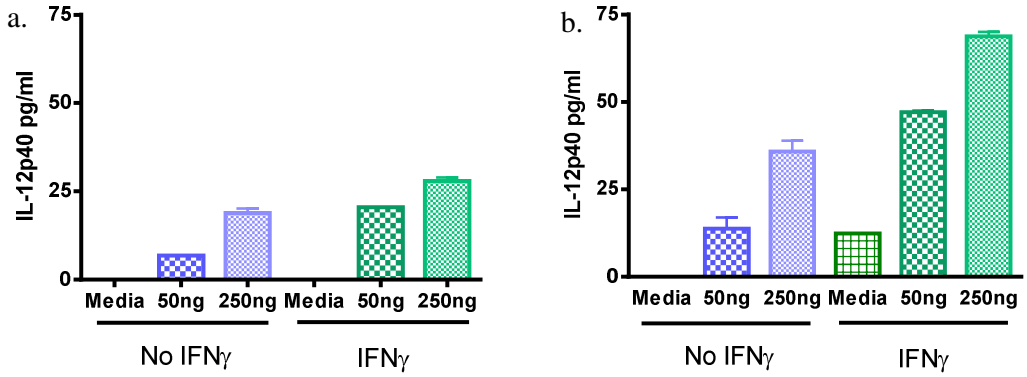



**Figure 5.5. MG isolated from the CNS of adult mice produce IL-12 in response to LPS in a concentration dependent fashion.** MG isolated from 19 week old BALB/c (n=3, a) and 18 week old C57BL/6 (n=4, b) mice were plated at a density of $1x10^5$ cells/well in a flat bottom 96 well plate and cultured overnight in the presence or absence of IFN-γ. MG from BALB/c mice (a) or C57BL/6 mice (b) were stimulated with 50ng/ml or 250ng/ml LPS for 24 hours. IL-12p40 production was measured by ELISA. Shown are the means and SEM from duplicate wells, or single wells where no error bar is present, from one experiment.

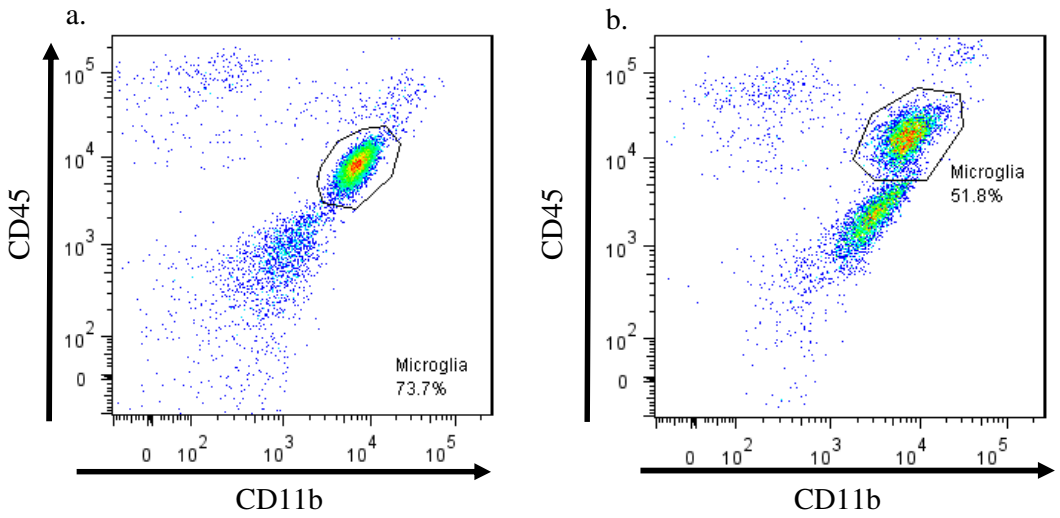



**Figure 5.6. MG retain their expression of CD45 and CD11b following 24 hours in culture.** MG isolated from the CNS of IL-4Rα$^{-/-}$ mice (n=4) using a Percoll gradient were plated at 5x10$^5$ cells per well in CTCM. After 24 hours in culture, media was removed and MG were removed from the wells with ice cold PBS. Cells were stained with CD45 and CD11b (freshly isolated, a; 24 hours, b). Plots are from one experiment.

**Table 5.1. MG have low survival following 24 hours in culture.** MG isolated from the CNS of IL-4Rα$^{-/-}$ (n=4 mice in experiment 1; and n=4 mice in experiment 3) or BALB/c (n=4 mice in experiment 2) using a Percoll gradient were plated at 5x10$^5$ cells per well in CTCM in a 6 well plate. After 24 hours in culture, media was removed and MG were removed from the wells with ice cold PBS and counted using a trypan blue exclusion assay.

| Mouse strain | Experiment | Number of cells recovered (24 hours) | Percentage viable |
|---|---|---|---|
| IL-4Rα$^{-/-}$ | 1 | 175,555 | 35.1 |
| BALB/c | 2 | 184,000 | 36.8 |
| | 2 | 140,000 | 28.0 |
| IL-4Rα$^{-/-}$ | 3 | 133,333 | 26.7 |
| | 3 | 144,444 | 28.9 |

### 5.3.3 Expansion of MG *in vitro*

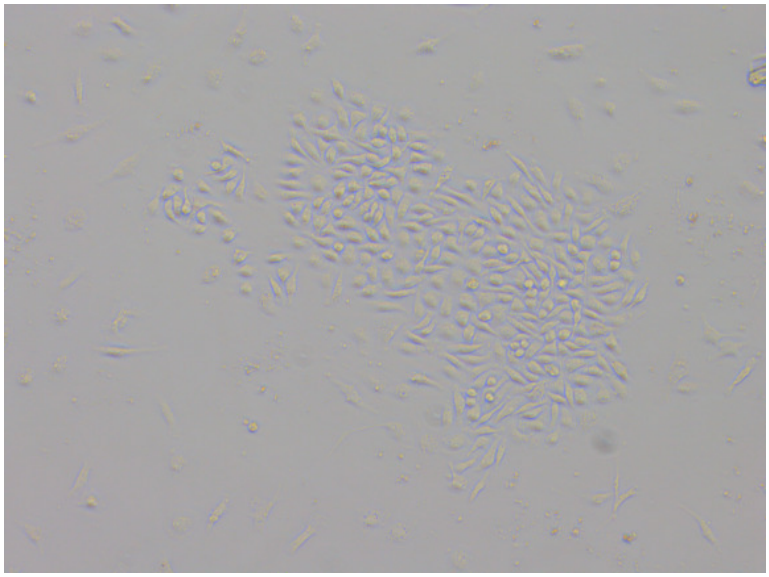
MG are capable of self renewal and can expand when given growth factors such as M-CSF or GM-CSF. Moussaud & Draheim (2010) described a protocol for the isolation and culture of MG from adult mice using GM-CSF. This protocol grows MG from mixed glial cells, isolated from adult mice, using GM-CSF until the MG reach confluence and detach from the surface of the culture flask. This protocol was attempted twice; neither time did the MG reach confluence, even after twice the length of time in culture as described previously (Moussaud & Draheim, 2010). In addition, when MG were harvested from these flasks with ice cold PBS (as they did not detach) and the suspension was counted with trypan blue, only a very small number of live cells could be isolated.

Ponomarev *et al.* (2005) described a similar protocol to the one described by Moussaud & Draheim (2010), but isolated MG using a 70:40% Percoll gradient and grew MG for 4 weeks in the presence of M-CSF, as opposed to GM-CSF. In this study MG were isolated using a 70:37:30% Percoll gradient and plated at 449,334 and 384,000/cells a well (in 2 separate experiments, n=5 and 4 mice respectively) in a 12 well plate in the presence of 10 ng/ml M-CSF. The media was changed every 3-4 days for 4 weeks. MG showed focal areas of proliferation in culture as early as 11 days and maintained a ramified morphology after 4 weeks in culture (Figure 5.7). After 4 weeks in culture MG were harvested by blasting wells with ice cold PBS with the intent to reseed the cells for stimulation. However in both experiments only 18,000 cells were recovered from each well (as determined by a trypan blue exclusion assay) following blasting.

Because it is likely that the MG did not survive the harvesting procedure, MG were plated in a 96 well, flat bottomed, tissue culture plate at 50,000 cells/well and grown in the presence of M-CSF for 4 weeks. The MG were then cultured for 3 days without M-CSF, before overnight incubation with or without IFN-γ and stimulation with LPS. IL-12 cytokine production was then assessed in supernatants after 24 hours of stimulation, IL-12 demonstrated the MG are capable of responding to LPS with the production of a proinflammatory cytokine (Figure 8a). However, as the cells became less healthy following culture without M-CSF for 3 days, the protocol was altered and the M-CSF was removed at the time of IFN-γ stimulation, so it was not present during stimulation (Figure 8b). MG which had the M-CSF removed 3 days before IFN-γ stimulation produced lower levels of IL-12 than those which were left with M-CSF

until the day of stimulation (Figure 8). These results suggest the MG do not survive well without the M-CSF.

a.



b.

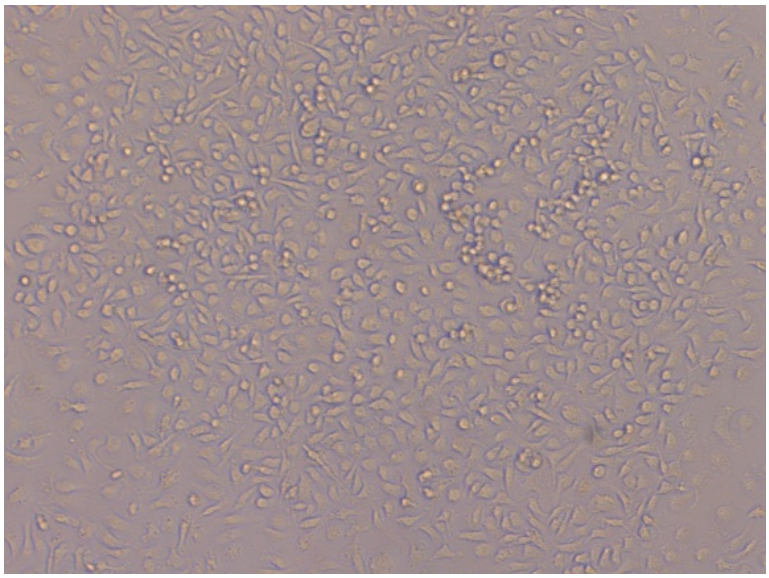


**Figure 5.7. MG proliferate in culture containing M-CSF.** MG were isolated from the CNS of C57BL/6 mice (n=5) using a Percoll gradient and grown in the presence of 10 ng/ml M-CSF for 4 weeks. After 11 days in culture, clusters of proliferation were clearly visible (a), and surive at least 4 weeks in *in vitro* culture (b). Pictures are from one representative experiment.

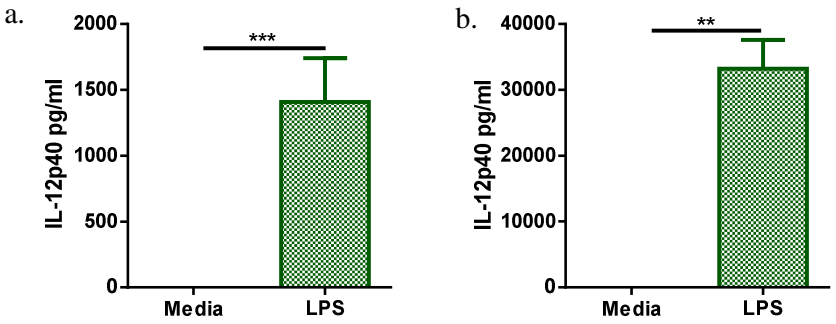



**Figure 5.8. MG produce higher levels of IL-12 when M-CSF is retained in culture until the day of stimulation**. MG isolated from adult C57BL/6 mice (n=5) were plated at a density of $5x10^4$ cells/well in a flat bottom 96 well plate and cultured for 4 weeks in media containing 10 ng/ml M-CSF. M-CSF was removed either 3 days (a) or immediately prior to overnight stimulation with IFN-γ. Cells were stimulated with 200 ng/ml LPS for 24 hours. IL-12 production was measured by ELISA. Shown are means and SEM of triplicate wells from three combined experiments (a) or one representative experiment (b). **p<0.01, ***p<0.001 by unpaired Student's t test.

## 5.4 Discussion

In this chapter a protocol for isolating and culturing adult MG was optimised. Several different protocols were attempted for the isolation of adult MG based on reports in the literature, including isolation on the basis of adhesive properties, CD11b expression using magnetic beads, and isolation based on their specific gravity using a Percoll gradient (Aloisi et al., 2000; Sedgwick, Schwender, Gregersen, Dörries, & Meulen, 1993; Yip et al., 2009). It was determined that only Percoll gradients gave both sufficient yield and purity for *in vitro* culture systems. Furthermore, it was shown that MG experience a high rate of cell death in the first 24 hours of culture, making direct culture of these cells impossible without using prohibitively large numbers of mice.

Several papers have used freshly isolated MG without culturing the cells long term with growth factors (Aloisi et al., 2000; Brannan & Roberts, 2004; Schell et al., 2007). These papers report between 42-80% survival of MG after 24 hours in culture and would therefore be likely to require large numbers of animals (Aloisi et al., 2000; Brannan & Roberts, 2004; Ponomarev et al., 2005). MG are notoriously difficult to maintain in long term culture, and even in short term culture it can be extremely difficult to avoid widespread cell death (Moussaud & Draheim, 2010). Indeed, in the current study, MG only had a viability of 31.1±2.03% (mean±SEM) following 24 hour culture. Due to the low numbers of cells that were surviving the first 24 hours in culture, a different method was employed which involved culturing MG in the presence of growth factors such as GM-CSF or M-CSF (Moussaud & Draheim, 2010; Ponomarev et al., 2005). MG grew in both GM-CSF and M-CSF; however, M-CSF was used in this protocol instead of GM-CSF because M-CSF is thought to be ubiquitously expressed in the CNS and maintains MG in a resting/ramified phenotype, while GM-CSF alters MG to take on a phenotype that is more similar to DC (Ponomarev et al., 2005).

This study found that growing MG in the presence of M-CSF resulted in the MG maintaining the ramified morphology of resting MG and express similar surface markers to *ex vivo* isolated MG, with no expression of MHC II, CD86 or CD40, which is consistent with resting MG (Ponomarev et al., 2005). Using this protocol MG were grown successfully and shown to produce IL-12 after LPS stimulation. Initially, M-CSF was removed from the cells 3 days before priming with IFN-γ, as this was suggested when using GM-CSF, to prevent any effect that the M-CSF may have on the responses of the MG (Moussaud & Draheim, 2010);

however, removing the M-CSF appeared to have a detrimental effect on MG. As M-CSF is ubiquitously expressed in the CNS it may be required for their survival long term (Ponomarev et al., 2005), therefore, the protocol was altered so M-CSF was left in the media until IFN-γ priming. This resulted in higher levels of cytokine production, likely due to healthier cells.

The isolation and culture of adult MG for functional *in vitro* assays is desirable due to the documented differenced between adult MG, neonatal MG, and MG cell lines. Neonatal MG are functionally different from those isolated from adult mice in a number of ways. For example, the production of NO by neonatal MG cultures has been shown to be higher than that of *ex vivo* isolated adult microglia. Also, the cytokine production by adult MG and neonatal MG is not identical and adult MG have also been show to be less effective at cross presenting antigens than neonatal MG (Beauvillain et al., 2008; Schell et al., 2007). Due to these differences between adult and neonatal MG, the data obtained in these studies, while providing an interesting glimpse into the function of MG, must be interpreted with an understanding that the system may not model the CNS perfectly. In addition, study of neonatal MG does not allow for the study of MG *ex vivo* following treatment, nor is the data obtained directly comparable to *in vivo* studies.

Cell lines are also commonly used to study MG; however, there are differences between the cell lines and primary MG, although some of these cell lines more closely model primary MG than others. For example, Ra-2 and 6-3 do not produce detectable levels of IL-12 or IL-23 under conditions that induce the same cytokines from a neonatal MG culture (Sonobe et al., 2005). BV-2 is the most frequently used and most well characterised of all the MG cell lines. In a study comparing the LPS response of BV-2 cells to neonatal MG cultures and *in vivo* MG responses, BV-2 cells were shown to respond similarly but not identically. The authors of this paper highlighted the fact that long term use of cell lines by individual laboratories can result in selection of the cell lines, and therefore skewed results are possible (Henn et al., 2009). The major advantage of these cells is that the number of cells available means that large experiments can be undertaken without the need to sacrifice large numbers of animals. Consequently, cell lines may be of particular use in early proof of concept work, or in situations where the number of cells required would make doing the experiment with primary cells difficult. Due to the differences in both neonatal MG and cell lines compared to primary MG, it was considered necessary to optimise a protocol for the isolation of adult murine microglia.

## 5.5 Summary

In conclusion, a wide variety of different techniques were attempted for the culture of adult MG. It was found that isolation of MG using a Percoll gradient gave the purest yield with the highest number of cells. MG do not survive well *in vitro* and it was found that culturing the MG with M-CSF was the most productive way of maintaining the cells *in vitro* and increasing cell survival.

# Chapter 6:
# Type II Activation of Adult Microglia

## 6.1 Introduction

Type II activation of MΦ has been well described, but while several studies have suggested that MG are capable of type II activation (Kim et al. 2004), the phenotype of these MG has not been as well characterised. Specifically, Kim et al. (2004) reported that human MG exposed to supernatants from T cells isolated from GA-treated patients expressed higher levels of IL-10 and lower levels of TNF-α and CD40 compared to MG exposed to supernatants of T cells isolated from untreated patients. This finding suggests GA can indirectly induce a type II like phenotype in MG (Kim et al., 2004). GA has been shown to increase the production of IL-10 and decrease TNF-α in cultured neonatal MG isolated from rats (Pul et al., 2011). Furthermore, in a Theiler's encephalomyelitis virus model of MS, treatment with anandamide (an endocannabinoid) causes MG to up regulate IL-10 and down regulate IL-12p70 and IL-23, a phenotype which is suggestive of type II activation (Correa et al., 2011). Because these studies have focused primarily on the IL-12/IL-10 balance, the ability of MG to express the full spectrum of type II activation, has not been determined nor has the ability of MG to be type II activated by IC been studied previously.

The local environment of a tissue can affect the way in which the immune response develops in that organ. For example, over expression of proinflammatory molecules can alter the clinical course of EAE. Over expression of IL-6 specifically in the cerebellum of C57BL/6 mice conditions the local milieu and results in a shift from a primarily spinal cord located disease (with the classic symptoms of ascending paralysis) to one in which lesion development is targeted to the cerebellum (these mice display altered disease signs such as ataxia) (Quintana et al., 2009). This phenotype was not the same for all inflammatory cytokines, as increases in IL-12 in the cerebellum did not result in atypical EAE (Quintana et al., 2009). Alteration in anti-inflammatory cytokines also alters disease progression. Delivery of fibroblasts retrovirally transformed to express IL-10 directly into the CNS results in lowered severity of EAE and decreased expression of MHC II on resident MG (Croxford, Feldmann, Chernajovsky, & Baker, 2001). In a similar manner MG can regulate the local immune environment altering neurological disease expression.

MG are known to be competent APC and are considered the major APC in the CNS in both normal and inflammatory conditions. Several *in vitro* studies have demonstrated the ability of MG to activate both naïve and memory T cells (Krakowski & Owens, 1997; Li, Wang, Guo, Bai, & Yu, 2007). Thus one mechanism by which MG may regulate CNS immune responses

involves interactions with T cells. MG are known to produce IL-12 *in vivo* which is drives Th1 type responses (Krakowski & Owens, 1997), and inhibition of MG activation *in vivo* results in reduced disease severity in EAE (Heppner et al., 2005). Also, specific deletion of IL-23, which stabilises pathogenic Th17 cells, in the CNS of mice but not in the periphery, reduces disease severity in EAE and skews the Th response in the CNS towards a Th2 profile (Becher, Durell, & Noelle, 2003). Conversely, MG have also been shown to have potential regulatory properties *in vivo*, for example the production of IL-4 by MG has been suggested as a regulatory mechanism in EAE (Ponomarev et al., 2007). Additionally MG have been shown to express increased levels of PD-L1 at peak disease (Magnus et al., 2005)., and since PD-L1 provides inhibitory signals to T cells, it is likely that this increase is a regulatory mechanism to reduce CNS inflammation. Therefore, the activation of MG to a more regulatory phenotype (i.e. type II activation) may be protective in EAE by modifying the T cell response phenotype in the CNS.

## 6.2 Aims and objectives

The primary aim of this chapter was to investigate the effects of prototypical classical and type II activating stimuli on primary adult murine MG to determine if MG could be type II activated like MΦ. Activation of MG was assessed on the basis of cytokine production, as well as the expression of CD40 and PD-L1, which are known to be altered in type II activation. Finally, given the ability of type II activated MΦ to bias Th responses, this chapter also aimed to establish if MG are capable of inducing T cell biasing in a similar fashion to type II activated MΦ.

### 6.2.1 Specific aims:

1. To compare cytokine production by MG stimulated under classical or type II activating conditions to assess the phenotypic change of the MG.
2. To evaluate changes in expression of surface markers, such as CD40 and PD-L1 on MG induced by classical or type II activating conditions.
3. To investigate the ability of classically and type II activated MG to activate T cell and bias the T cell response.
4. To determine if the PD-L1 pathway is involved in biasing of the T cell response by classically and type II activated MG.

## 6.3 Results

### 6.3.1Type II activation of MG with IC as demonstrated by changes in cytokine production

As shown previously (Aloisi, Penna, Cerase, Menéndez Iglesias, & Adorini, 1997; Moussaud & Draheim, 2010), when cultured with LPS, MG significantly up regulated production of IL-12 compared to medium alone (Figure 6.1b). In contrast, when MG were cultured with LPS in the presence of IC (i.e. type II activated), the MG produced significantly less IL-12 compared to those cultured with LPS alone (Figure 6.1a). MG cultured with LPS+IC also produced higher levels of IL-10 compared to LPS stimulated MG, however, this did not reach statistical significance (Figure 6.1a). MG cultured in medium alone or IC alone produced only low levels of IL-12 and IL-10 (Figure 6.1a and b). This pattern of cytokine expression in LPS+IC-treated MG is suggestive of type II activation and the changes are consistent with those seen when MΦ are type II activated.

In addition to IL-10 and IL-12, the production of several other proinflammatory molecules was assessed (Figure 6.2). Under type II activating conditions the level of IL-6 produced by MG was significantly lower compared production by LPS stimulated MG. Despite the decrease in IL-6 and IL-12, Monocyte chemoattractant protein 1 (MCP-1) and TNF-α production was not significantly altered by type II activating compared to classical activating conditions (Figure 6.2). Unstimulated MG produced very low levels of these cytokines (Figure 6.2). Although MCP-1 and TNF-α production were not significantly different when the M-CSF was removed just prior to IFN-γ stimulation, when the M-CSF was removed from the cultures 3 days prior to IFN-γ stimulation, a different pattern was observed. By removing M-CSF earlier, a significant down regulation of IL-6, MCP-1 and TNF-α under type II activating conditions was observed (see appendix C). Under these conditions, IL-12 levels had the same pattern of expression as when M-CSF was maintained for longer (see appendix C). Therefore the decreases in IL-12 and IL-6 under type II activating conditions were highly consistent and may represent phenotypical alternations in the MG while the changes to TNF-α and MCP-1 will require further elucidation. Finally, this reduction in IL-6 as well as IL-12 further supports the evidence that LPS+IC activated MG to a regulatory or type II activated state.

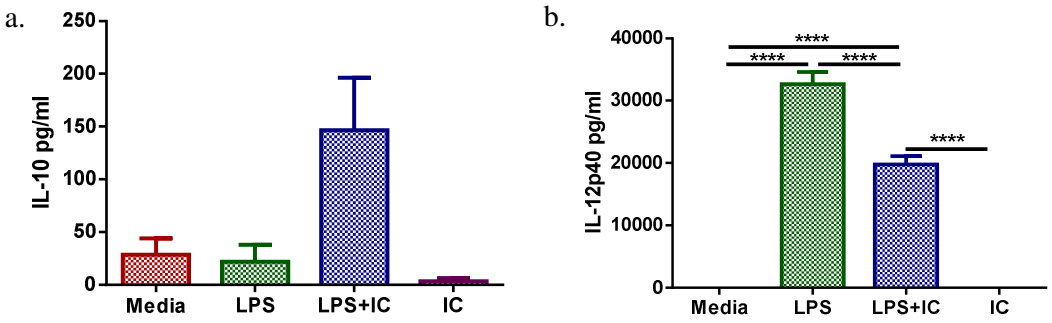



**Figure 6.1. MG are type II activated when cultured with LPS+IC.** MG were isolated from the CNS of adult mice (n=5) and plated at $5x10^4$ cells/well in a flat bottomed 96 well plate in the presence of M-CSF (5 ng/ml). After 4 weeks in culture M-CSF was removed and MG were primed with 20 U/ml IFN-γ overnight before stimulation with LPS (200 ng/ml) with or without IC ($10^6$/well) for 24 hours. IL-10 (a) and IL-12p40 (b) levels were measured by ELISA. Shown are the means and SEM from triplicate wells from two (IL-12p40, b) or three (IL-10, a) combined experiments. IL-10 data was subjected to the ROUT test which removed outliers, before statistics were calculated. ****$p<0.0001$ by one way ANOVA with a Tukey's multiple comparison post test.

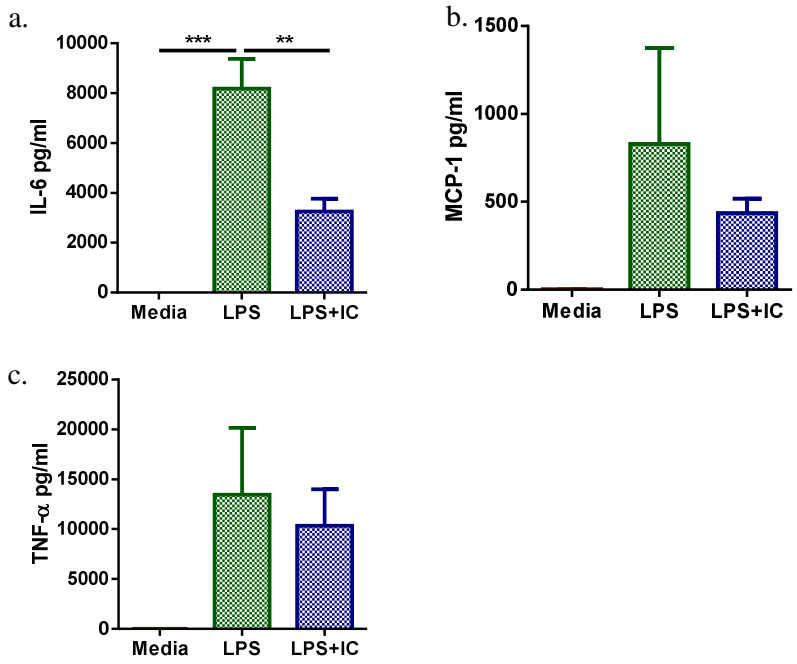



**Figure 6.2. MG cultured with LPS+IC produce less inflammatory cytokines and chemokines compared to LPS alone.** MG were isolated from the CNS of adult mice (n=5) and plated at $5x10^4$ cells/well in a flat bottomed 96 well plate in the presence of M-CSF (5 ng/ml). After 4 weeks in culture M-CSF was removed and MG were primed with 20 U/ml IFN-γ overnight followed by stimulation with LPS (200 ng/ml) with or without IC ($10^6$/well) for 24 hours. IL-6 (a), MCP-1 (b) and TNF-α (c) levels were measured by CBA. Shown are the means and SEM of triplicate wells from one experiment. **p<0.01, ***p<0.001 by one way ANOVA with a Tukey's multiple comparison post test.

### 6.3.2 Alterations in the expression in cell surface markers on type II activated MG

Type II MΦ are most commonly identified on the basis of their cytokine profile; however, previous work indicates that the expression of several cell surface markers is also of importance (Tierney et al., 2009). For example, type II MΦ are known to express lower levels of several co-stimulatory markers including CD40 and PD-L1 (Tierney et al., 2009). To assess whether type II activated MG follow this same pattern of cell surface expression, MG were harvested 24 hours post stimulation and analysed by flow cytometry. When stimulated with LPS alone, MG expressed higher levels of both CD40 and PD-L1 compared to media alone (Figure 6.3a and b). This change is consistent with classical activation of macrophages (Tierney et al., 2009). When MG were cultured with LPS+IC, the levels of CD40 expression was not different to MG stimulated with LPS alone (Figure 6.3a). In contrast, MG cultured with LPS+IC had a decreased expression of PD-L1 compared to MG cultured with LPS alone, which is reminiscent of type II activated MΦ (Figure 6.3b). Due to the low viability of MG once they have been removed from culture, flow cytometric analysis of MG was only successfully performed once. In all other experiments, not enough MG survived the process of preparation for flow cytometry to provide robust results.

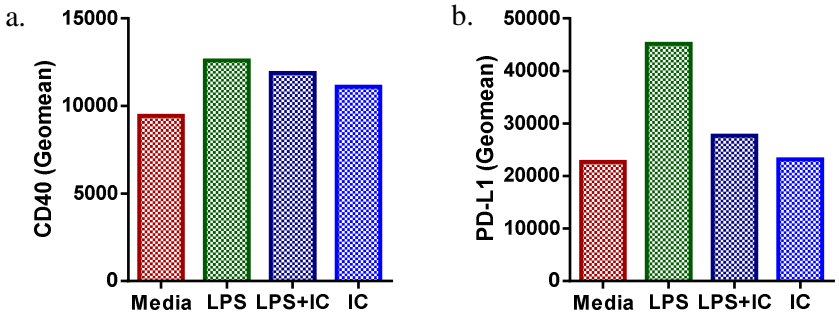



**Figure 6.3. MG cultured with LPS+IC have altered expression of co-stimulatory markers compared to culture with LPS alone.** MG were isolated from the CNS of adult mice (n=5) and plated at $5x10^4$ cells/well in a flat bottomed 96 well plate in the presence of M-CSF (5 ng/ml). After 4 weeks in culture M-CSF was removed 3 days before MG were primed with 20 U/ml IFN-γ overnight followed by stimulation with LPS (200 ng/ml) with or without IC ($10^6$/well) for 24 hours. MG were removed from culture plates by blasting with ice cold PBS after 24 hours in culture. CD40 (a) and PD-L1 (b) levels were assessed by flow cytometry. Data is from one experiment, each data point represents 3 combined wells.

### 6.3.3 T cell biasing by type II activated MG

In order to evaluate the ability of MG to activate T cells, a co-culture system was developed based on the co-culture system used to study MΦ:T cell interactions in Chapters 3 and 4. Using this co-culture system, it was found that MG could drive antigen specific T cell responses under non-biasing conditions (i.e. unstimulated MG). Specifically, in the presence of MOG, increased IFN-γ and IL-2 were detected compared to cultures in which MOG was absent (Figure 6.4a and b). These results indicate that the T cell response was antigen-specific and that this model is amenable to assessing antigen-specific MG:T cell interactions.

In this culture system, classically activated MG produced higher levels of IL-12 compared to type II activated MG (Figure 6.5b). In addition, co-cultures containing type II activated MG showed a trend towards increased production of IL-10 compared to those containing classically activated MG (Figure 6.5a). These data demonstrate that the respective activation states of the MG are maintained in T cell co-culture conditions.

In MG:T cell co-cultures, T cells cultured with unstimulated MG and T cell cultured with classically or type II activated MG did not have significantly different expression of the activation markers, CD44, CD62L and CD25. In particular, similar expression of CD44 and by T cells co-cultured with unstimulated, classical or type II activated MG was observed (Figure 6.6a). While CD62L and CD25 expression appeared to be higher on T cells co-cultured with type II activated MG, this increase did not reach significance (Figure 6.6b and c). However, IL-2 production was significantly elevated in co-cultures with type II activated MG compared to classical activation (Figure 6.6d). The expression of these markers was antigen specific as demonstrated by the low level of IL-2 production in the absence of MOG (Figure 6.4b); therefore, the similarity in activation marker expression on T cells from these MG co-cultures is not due to the absence of antigen-recognition by the T cells. Because T cells cultured with unstimulated MΦ were significantly less activated compared to those cultured with classically activated MΦ (as seen in Chapter 3), these data suggest that these primary MG may have an enhanced ability to activate T cells compared to MΦ despite showing similar activation states.

Similar to the activation markers, T cells cultured with MG produced a high level of IFN-γ regardless of the MG activation state (Figure 6.7a). This finding is in stark contrast to MΦ:T cell co-cultures in which T cells cultured with classically activated MΦ produced

significantly higher levels of IFN-γ compared to those with unstimulated MΦ (see Chapter 3). T cells cultured with type II activated MG showed a trend towards an increased expression of CD124 suggesting that the T cells cultured with type II MG may have an increased sensitivity to IL-4 similar to that of T cells in unstimulated MG co-cultures (Figure 6.7c). Finally, as observed with the MΦ:T cell co-cultures, T cells cultured with type II MG produced significantly higher levels of IL-17A compared to T cells cultured with either classical or unstimulated MG (Figure 6.7b). Overall, these data suggest that type II activated MG activate T cells in a distinct manner to type II activated MΦ despite having similar activation profiles.

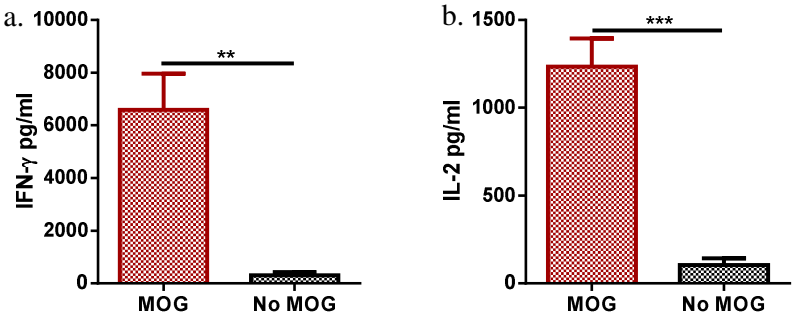



**Figure 6.4. Under non-biasing conditions MG can present to T cells and drive an antigen specific response.** MG were isolated from the CNS of adult mice (n=5) and plated at $5x10^4$ cells/well in a flat bottomed 96 well plate in the presence of M-CSF (5 ng/ml). After 4 weeks in culture M-CSF was removed and MG were primed with 20 U/ml IFN-γ overnight before purified $CD4^+2D2$ T cells and $MOG_{35-55}$ (25 μg/ml) were added to the MG cultures for 72 hours. IFN-γ (a) and IL-2 (b) levels were measured by ELISA. Shown are the mean and SEM of at least duplicate wells from two combined experiments. **p<0.01, ***p<0.001 by unpaired Student's t test.

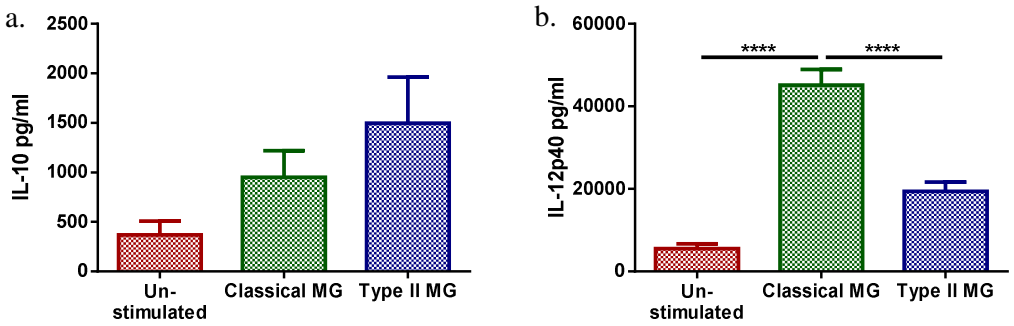



**Figure 6.5. MG in T cell co-culture retain their cytokine profiles.** MG were isolated from the CNS of adult mice (n=5) and plated at $5x10^4$ cells/well in a flat bottomed 96 well plate in the presence of M-CSF (5 ng/ml). After 4 weeks in culture M-CSF was removed and MG were primed with 20 U/ml IFN-γ overnight before stimulation with LPS (200 ng/ml) with or without IC ($10^6$/well). After four hours, purified $CD4^+2D2$ T cells and $MOG_{35-55}$ (25 µg/ml) were added to the MG cultures for 72 hours. IL-10 (a) and IL-12 (b) levels were measured by ELISA. Shown are means SEM of triplicate wells from seven combined experiments. ****$p<0.0001$ by one way ANOVA with a Tukey's multiple comparison post test.

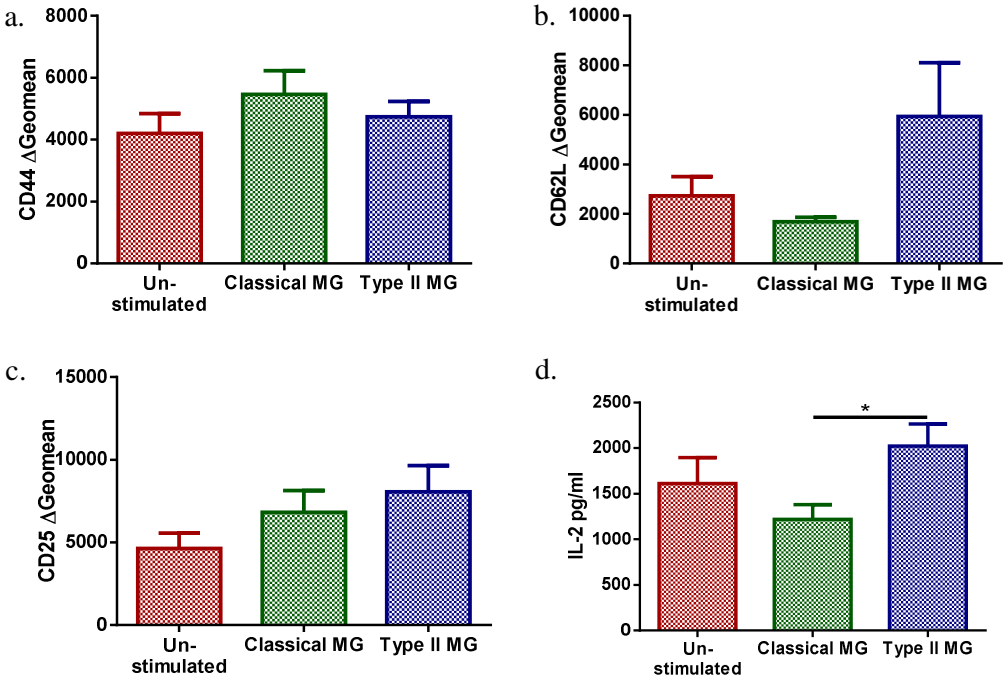



**Figure 6.6. Classical or type II activation of MΦ does not significantly alter the activation of T cells in co-culture compared to un-stimulated MG.** MG were isolated from the CNS of adult mice (n=5) and plated at $5x10^4$ cells/well in a flat bottomed 96 well plate in the presence of M-CSF (5 ng/ml). After 4 weeks in culture M-CSF was removed and MG were primed with 20 U/ml IFN-γ overnight before stimulation with LPS (200 ng/ml) with or without IC ($10^6$/well). After four hours, purified $CD4^+2D2$ T cells and $MOG_{35-55}$ (25 µg/ml) were added to the MG cultures for 72 hours. CD44 (a) CD62L (b) and CD25 (c) expression was measured by flow cytometry, and IL-2 (d) levels ELISA. Shown are the means and SEM of triplicate wells from seven combined experiments. *$p<0.05$ by one-way ANOVA with a Tukey's multiple comparison post test.

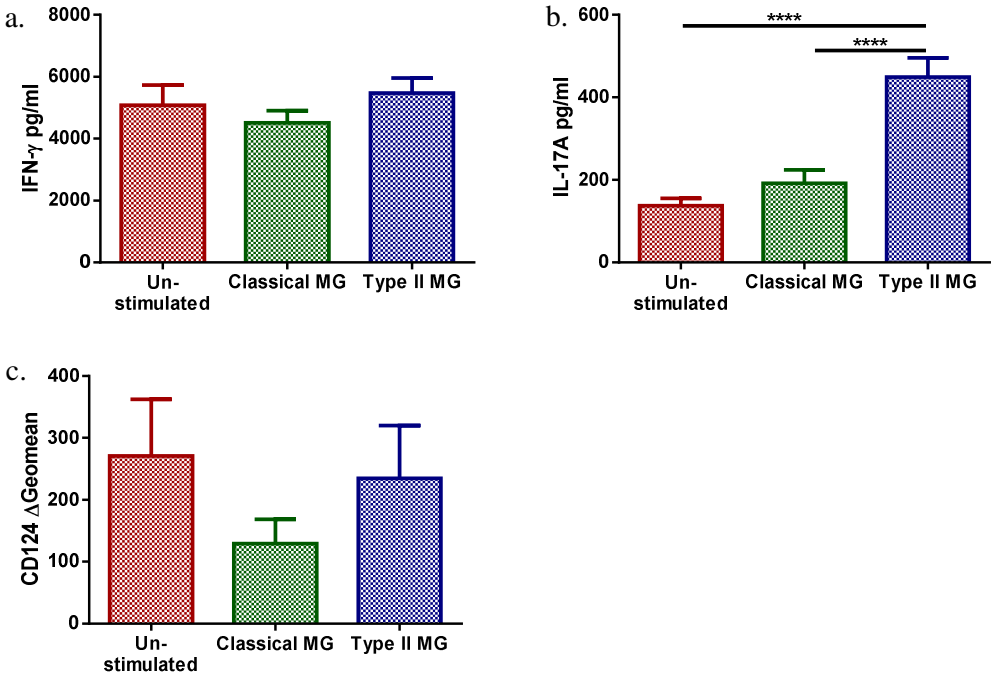



**Figure 6.7. Biasing of the T cell response by MG is not identical to the biasing induced by MΦ.** MG were isolated from the CNS of adult mice (n=5) and plated at $5x10^4$ cells/well in a flat bottomed 96 well plate in the presence of M-CSF (5 ng/ml). After 4 weeks in culture M-CSF was removed and MG were primed with 20 U/ml IFN-γ overnight before stimulation with LPS (200 ng/ml) with or without IC ($10^6$/well). After four hours, purified $CD4^+2D2$ T cells and $MOG_{35-55}$ (25 µg/ml) were added to the MG cultures for 72 hours. IFN-γ (a) and IL-17A (b) levels were measured by ELISA, and CD124 (c) expression was measured by flow cytometry. Shown are the means and SEM of at triplicate wells from seven combined experiments. ****p<0.0001 by one-way ANOVA with a Tukey's multiple comparison post test.

### 6.3.4 The PD-1/PD-L1/2 pathway in biasing of the T cell response by adult MG

Similarly to the investigations into the pathways involved in T cell biasing by classically activated and type II activated MΦ presented in chapter 4, the abitlity of classically and type II activated MG to bias T cell responses was specifically evaluated in above. Therefore, for the following experiments, which investigate the effect of inhibiting or activating specific pathways in classical or type II activated MG, these groups will be analysed independently, as was done for the pathways investigated in Chapter 4. This approach was selected because it is likely that these treatments will have different effects on these two distinct MG populations.

#### *6.3.4.1 Blocking the PD-1/PD-L1/2 pathway in MG:T cell co-cultures*

To understand if the reduced expression of PD-L1 on MG could affect T cell activation and biasing in MG:T cell co-cultures, the ability of PD-L1 and PD-L2 to ligate PD-1 on the T cell was blocked by the addition of αPD-1 antibody. As observed with MΦ:T cell co-cultures, the addition of αPD-1 did not alter the cytokine profile of the MG (Figure 6.8). MG in T cell co-cultures which contained αPD-1 expressed similar levels of IL-12 (Figure 6.8b) to MG cultured in the absence of αPD-1. There was a trend towards decreased IL-10 with the addition of the αPD-1 in all culture conditions, and this reached significance in cultures containing classically activated MG (Figure 6.8a). However, it should be noted that IL-10 production was also significantly lower in all cultures in the presence of the isotype control making it difficult to draw any solid conclusions from these findings (Figure 6.8a). No difference in the expression of CD44, CD62L and CD25, or IL-2 was detected in co-cultures to which αPD-1 was added (Figure 6.9) which suggests that blocking the PD-1 pathway does not affect the activation state of T cells when antigen has been presented by classical or type II activated MG it this system. Furthermore, the addition of αPD-1 did not affect the production of IFN-γ or IL-17A, or the level of CD124 expression on T cells in co-cultures containing unstimulated, classically, or type II activated MG (Figure 6.10). These data suggest that in this system, blocking the PD-1/PD-L1 pathway does not alter these indicators of antigen-specific T cell responses and suggest that a reduced expression of PD-L1 is not likely to be involved in T cell biasing in this system, however, as no effect is seen it is also possible that the molecule is not active in this system.

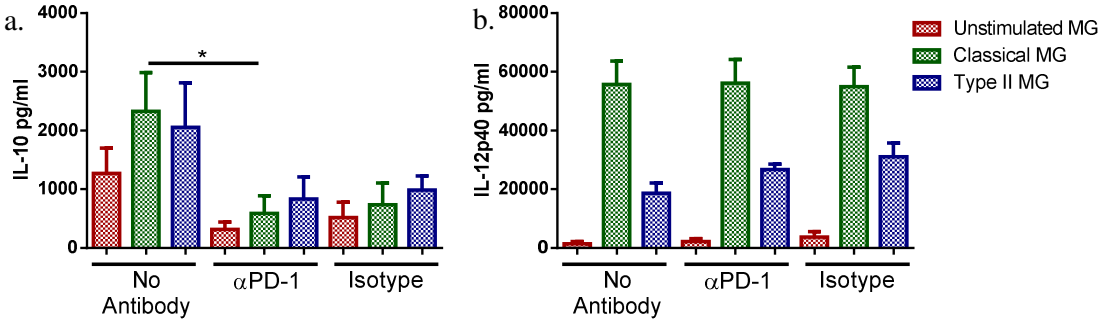



**Figure 6.8. Blocking the PD-1/PD-L1/2 pathway did not significantly alter IL-10 or IL-12 production by MG.** MG were isolated from the CNS of adult mice (n=5) and plated at $5x10^4$ cells/well in a flat bottomed 96 well plate in the presence of M-CSF (5 ng/ml). After 4 weeks in culture M-CSF was removed and MG were primed with 20 U/ml IFN-γ overnight before stimulation with LPS (200 ng/ml) with or without IC ($10^6$/well). After four hours, purified $CD4^+2D2$ T cells and $MOG_{35-55}$ (25 µg/ml) were added to the MG cultures for 72 hours. To block PD-1/PD-L1 interactions, 2 µg/ml of anti PD-1 (RMP1-14) or rat isotype was added to cultures with the stimuli. IL-10 (a) and IL-12 (b) levels were measured by ELISA. Shown are the means and SEM of triplicate wells from two combined experiments. *$p<0.5$, by one way ANOVA with a Tukey's multiple comparison post test.

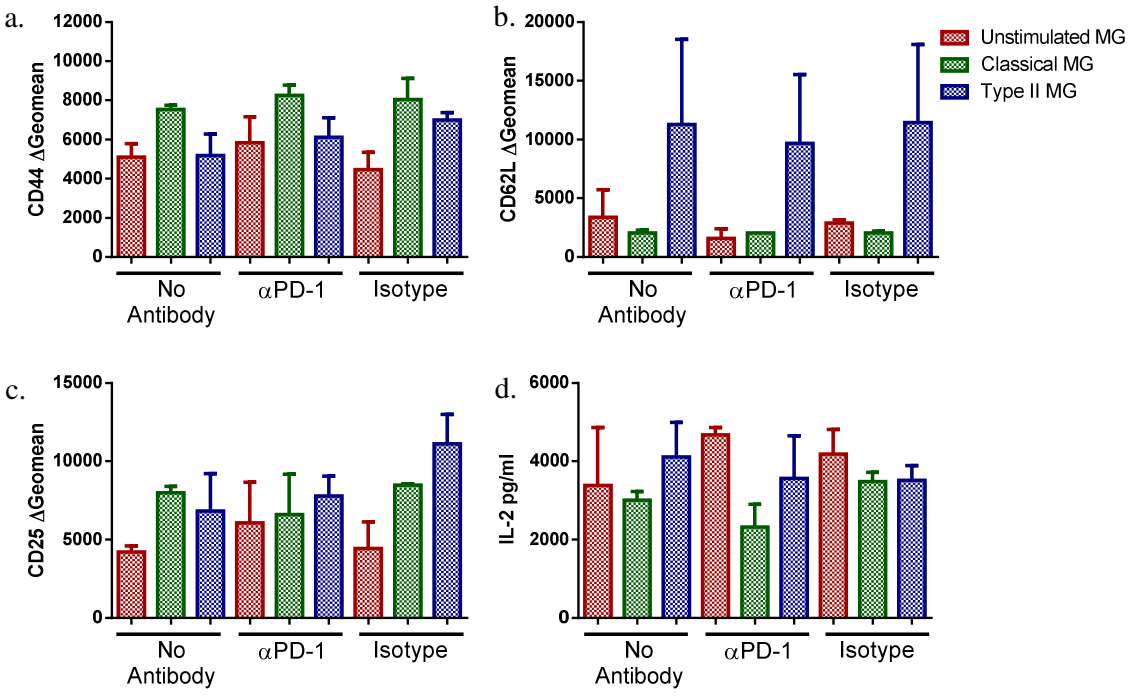



**Figure 6.9. Blocking the PD-1/PD-L1/2 pathway did not alter T cell activation by MG.** MG were isolated from the CNS of adult mice (n=5) and plated at $5x10^4$ cells/well in a flat bottomed 96 well plate in the presence of M-CSF (5 ng/ml). After 4 weeks in culture M-CSF was removed and MG were primed with 20 U/ml IFN-γ overnight before stimulation with LPS (200 ng/ml) with or without IC ($10^6$/well). After four hours, purified $CD4^+2D2$ T cells and $MOG_{35-55}$ (25 μg/ml) were added to the MG cultures for 72 hours. To block PD-1/PD-L1 interactions, 2 μg/ml of anti PD-1 (RMP1-14) or rat isotype was added to cultures with the stimuli. CD44 (a) CD62L (b) and CD25 (c) expression was measured by flow cytometry, and IL-2 (d) levels were measured by ELISA. Shown are the means and SEM of triplicate wells from two combined experiments.

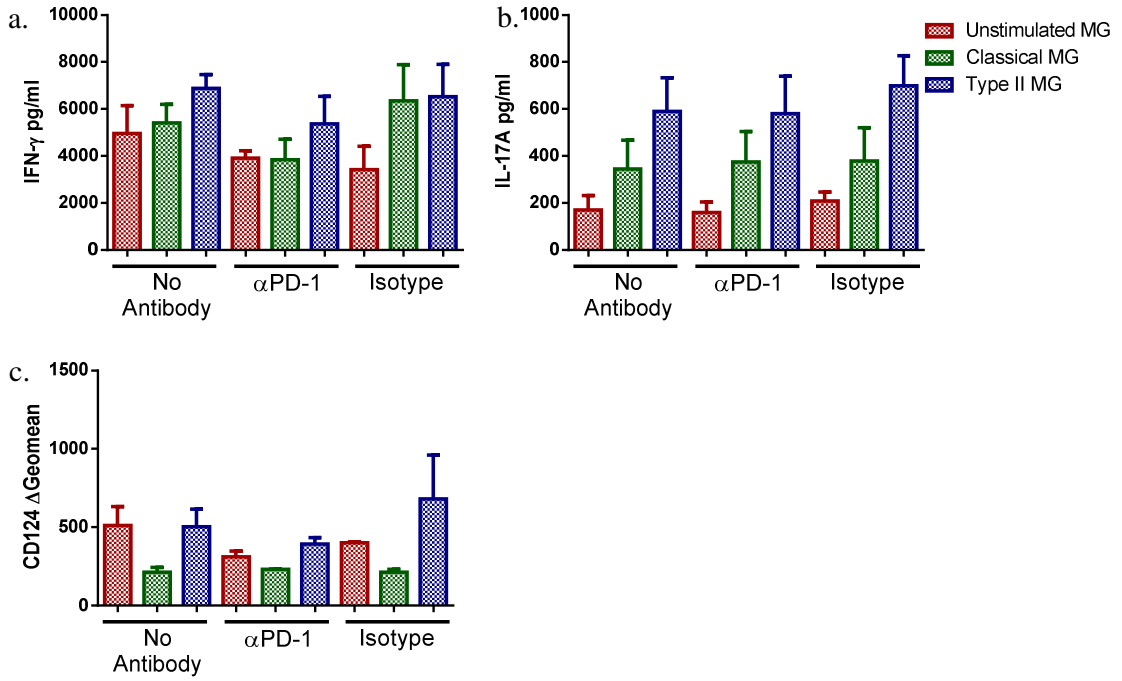



**Figure 6.10. Blocking the PD-1/PD-L1/2 pathway did not significantly alter T cell biasing by MG.** MG were isolated from the CNS of adult mice (n=5) and plated at $5x10^4$ cells/well in a flat bottomed 96 well plate in the presence of M-CSF (5 ng/ml). After 4 weeks in culture M-CSF was removed and MG were primed with 20 U/ml IFN-γ overnight before stimulation with LPS (200 ng/ml) with or without IC ($10^6$/well). After four hours, purified $CD4^+2D2$ T cells and $MOG_{35-55}$ (25 μg/ml) were added to the MG cultures for 72 hours. To block PD-1/PD-L1 interactions, 2 μg/ml of anti PD-1 (RMP1-14) or rat isotype was added to cultures with the stimuli. IFN-γ (a) and IL-17A (b) levels were measured by ELISA, and CD124 (c) expression was measured by flow cytometry. Shown are the means and SEM of triplicate wells from two combined experiments.

***6.3.4.2 Stimulating the PD-1/PD-L1 pathway in MG:T cell co-cultures***

To further assess whether the decreased expression of PD-L1 on type II MG was involved in T cell biasing, PD-L1ch was used to stimulate the PD-1 receptor, which results in increased signalling through PD-1. Similar to the addition of αPD-1 (Figure 6.8), the addition of the PD-L1ch did not have any effect on IL-10 or IL-12 production by MG (Figure 6.11). Although there did appear to be a trend towards decreased IL-10 with the addition of the PD-L1ch, this trend was also seen in the presence of the control protein. In addition, the PD-L1ch did not alter the activation state of the T cell in the co-cultures as assessed by CD44, CD62L and CD25 expression (Figure 6.12a, b and c). Furthermore, the PD-L1ch did not change in the level of IL-2 production under any of the MG stimulation conditions (Figure 6.12d). As found with the addition of αPD-1, the addition of the PD-L1 chimera to the co-cultures did not alter the level of IFN-γ or IL-17A (Figure 6.13a and b). Moreover, CD124 expression on T cells was not altered by PD-L1ch (Figure 6.13c). Taken together, these data suggest that the PD-1/PD-L1 pathway is not involved in the induction of T cell biasing in this MG:T cell co-culture system despite the decreased expression of PD-L1 on type II activated MG, however, an alternate possibility is that the molecule is not active in this system, based on the absence of a change in any marker.

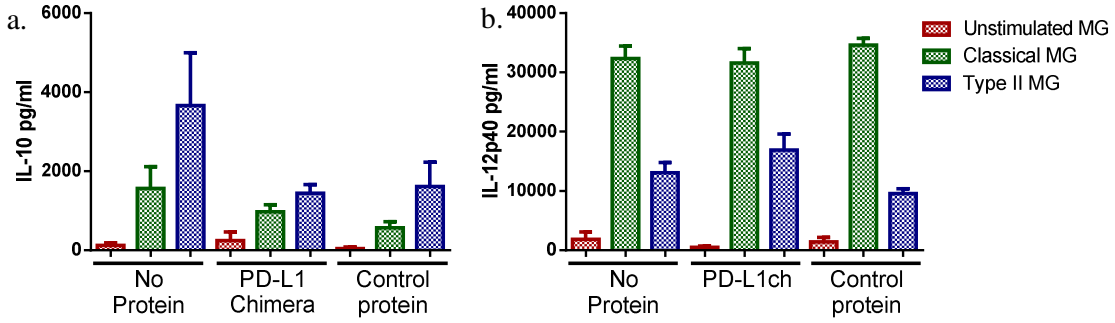



**Figure 6.11. Stimulating the PD-1/PD-L1 pathway did not significantly alter IL-10 or IL-12 production by MG.** MG were isolated from the CNS of adult mice (n=5) and plated at $5x10^4$ cells/well in a flat bottomed 96 well plate in the presence of M-CSF (5 ng/ml). After 4 weeks in culture M-CSF was removed and MG were primed with 20 U/ml IFN-γ overnight before stimulation with LPS (200 ng/ml) with or without IC ($10^6$/well). After four hours, purified $CD4^+2D2$ T cells and $MOG_{35-55}$ (25 μg/ml) were added to the MG cultures for 72 hours. To stimulated PD-1, 8 μg/ml of PD-L1ch or a control IgGch was added to cultures with the stimuli. IL-10 (a) and IL-12 (b) levels were measured by ELISA. Shown are the means and SEM of triplicate wells from two combined experiments.

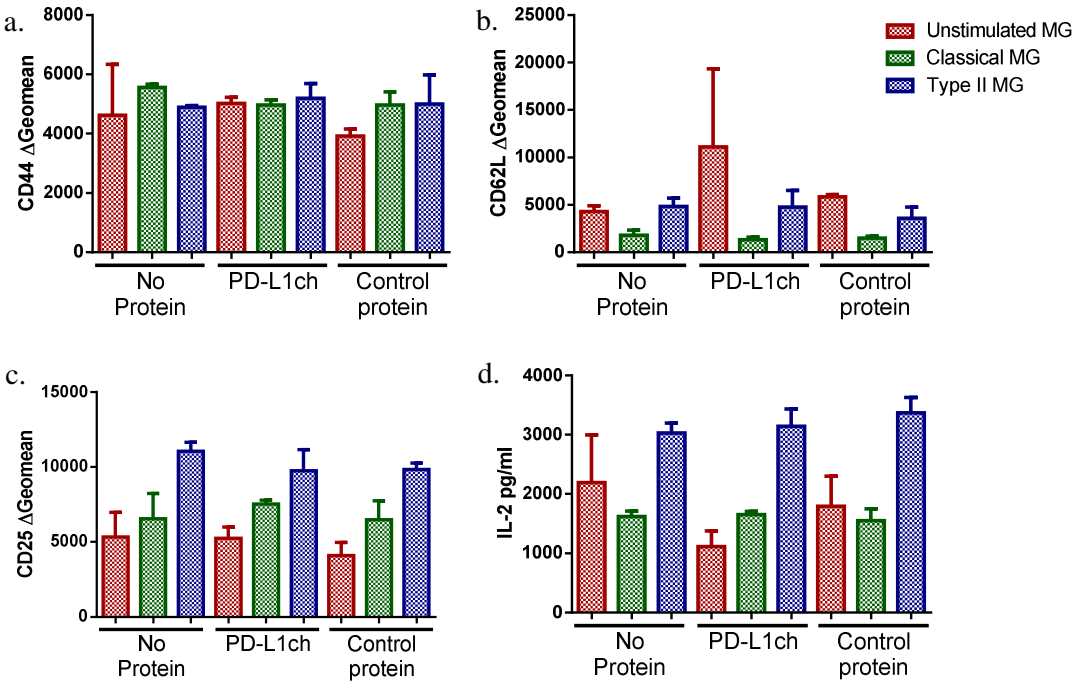



**Figure 6.12. Stimulating the PD-1/PD-L1 pathway did not alter T cell activation by MG.** MG were isolated from the CNS of adult mice (n=5) and plated at $5x10^4$ cells/well in a flat bottomed 96 well plate in the presence of M-CSF (5 ng/ml). After 4 weeks in culture M-CSF was removed and MG were primed with 20 U/ml IFN-γ overnight before stimulation with LPS (200 ng/ml) with or without IC ($10^6$/well). After four hours, purified $CD4^+2D2$ T cells and $MOG_{35-55}$ (25 µg/ml) were added to the MG cultures for 72 hours. To stimulated PD-1, 8 µg/ml of PD-L1ch or a control IgGch was added to cultures with the stimuli. CD44 (a) CD62L (b) and CD25 (c) expression was measured by flow cytometry, and IL-2 (d) levels were measured by ELISA. Shown are the means and SEM of triplicate wells from two combined experiments.

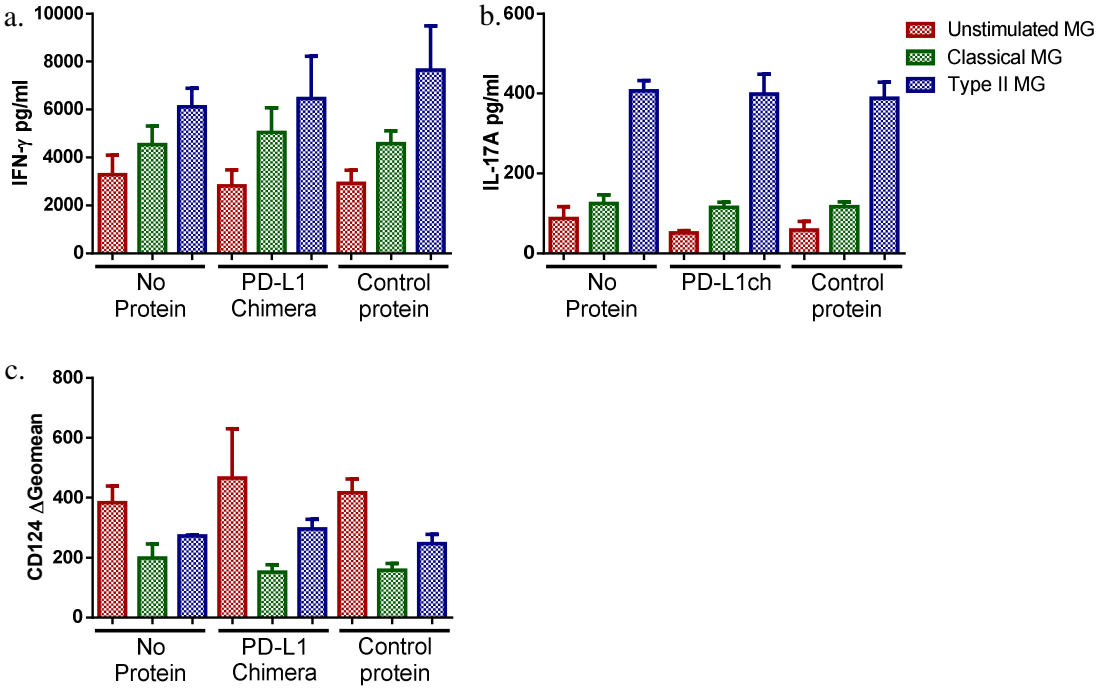



**Figure 6.13. Blocking the PD-1/PD-L1 pathway does not significantly alter T cell biasing by MG.** MG were isolated from the CNS of adult mice (n=5) and plated at $5x10^4$ cells/well in a flat bottomed 96 well plate in the presence of M-CSF (5 ng/ml). After 4 weeks in culture M-CSF was removed and MG were primed with 20 U/ml IFN-γ overnight before stimulation with LPS (200 ng/ml) with or without IC ($10^6$/well). After four hours, purified $CD4^+2D2$ T cells and $MOG_{35-55}$ (25 μg/ml) were added to the MG cultures for 72 hours. To stimulated PD-1, 8 μg/ml of PD-L1ch or a control IgGch was added to cultures with the stimuli. IFN-γ (a) and IL-17A (c) levels were measured by ELISA, and CD124 (b) expression was measured by flow cytometry. Shown are the means and SEM of at triplicate wells from two combined experiments.

## 6.4 Discussion

Type II activated MΦ have anti-inflammatory properties as seen by their altered cytokine profile and express lower levels of IL-12 and higher levels of IL-10 compared to classically activated MΦ (Anderson & Mosser, 2002). It has also been shown that type II inducing treatments have an ability to protect mice from proinflammatory conditions such as EAE and sepsis, suggesting type II MΦ can alter an inflammatory environment to provide protection (Anderson & Mosser, 2002; Tierney et al., 2009). It was found that MG are capable of being activated to a type II phenotype *in vitro*, and that this activation state is capable of inducing differential activation of T cells. MG are thought to be very important in controlling the immune microenvironment in the CNS. Activation of MG to an anti-inflammatory or regulatory state may thus provide protection against neuroinflammatory conditions such as EAE. The aim of this work was to assess whether MG are capable of type II activation by IC, a treatment that has been shown to protect from neuroinflammation during EAE (La Flamme et al., 2006).

The phenotype of type II activated MΦ has been well documented with the altered cytokine profile being the most commonly associated feature. MG cultured in similar conditions to type II MΦ are also capable of type II activation as demonstrated by the trend towards an increase in IL-10 production and the decrease in IL-12. Non-stimulated MG did not produce IL-12, which is consistent with previous reports showing that IFN-γ stimulation was not sufficient to induce IL-12 production in the absence of LPS (Aloisi et al., 1997). Type II MG also produce lower levels of IL-6. In addition, when, M-CSF is removed 3 days prior to IFN-γ stimulation type II MΦ produce less TNF-α and MCP-1. This change may be a phenotypic alteration or possibly a result of decreased viability in the absence of M-CSF. Whether these changes are retained when M-CSF in left in culture longer, which would enhance MG viability, needs to be investigated.

MG appear to die soon after they are removed from culture conditions making the assessment of cell surface molecules difficult. Nevertheless, flow cytometric analysis of cultured MG suggested that type II activated MΦ expressed lower levels of PD-L1 compared to classically activated MG, and this finding is consistent with type II activation. While CD40 levels were increased on classically and type II activated MG compared to non-activated MG, type II activated MG did not express the decreased levels of CD40 as expected from previous MΦ studies (Tierney et al., 2009). Overall, the phenotype of MG under type II activating

conditions, while not being completely identical to type II MΦ, is consistent with type II activation. This type II activation of MG represents an immunoregulatory phenotype which, if induced *in vivo* would likely be capable of modulating the CNS microenvironment to protect from the inflammation seen in EAE and MS.

One possible draw back with this particular model is that the exact number of MG per well in a culture plate cannot be quantified precisely. Because the MG die soon after they are removed from the culture, the reseeding of cells at a specific density is unfeasibly difficult. However, because similar numbers of MG are seeded in the original cultures for expansion by M-CSF, the number of MG per well in each experiment is relatively similar. Because ten IC are used for every MΦ to induce type II activation and the number of MG per well is not exactly known, it is possible that the number of immune complexes added ($10^6$/well, the same number that would be added to a well containing $10^5$ MΦ) may not be equivalent to the ratio used for MΦ activation. Since it has been documented that the amount of IC used and the amount of cross linking of the FcγR has an effect on the production of IL-10 (Gallo et al., 2010), a variable MG:IC ratio may explain the variable levels of IL-10 produced by type II activated MG. However, it has been shown previously that only 1-2 optimally opsonised IC are necessary to induce the switch from classical to type II activation in MΦ (Gallo et al., 2010). It is therefore unlikely the variable results stem from too many IC but instead from too many MG. One way to further optimise this protocol may be to quantify the average number of MG per well. This could be achieved by staining the MG in each well with a dye that can be measured by optical density or fluorescence and comparing this value to different concentrations of MΦ. This approach could be used to approximate the number of MG that grow in each well, and allow for normalisation of the experiment.

IL-12 is one of the most commonly used marker for classical activation of MΦ. This cytokine is important for driving the development of Th1 cells and in this way has a negative impact on the development of other cell types including Th2 and Th17 (Komiyama et al., 2006; Zhu et al., 2010). IL-12 is known to be produced by MG in EAE (Krakowski & Owens, 1997). In the earlier phases of the disease, proinflammatory cytokines are very important; indeed without the expression of IL-12p40 by resident CNS cells, EAE is less severe and the CNS microenvironment is biased towards Th2 (Becher et al., 2003). Early studies using IL-12p40-deficient mice or blocking antibodies suggested that IL-12 was an essential cytokine in EAE. However later studies showed that IL-23, which shares the p40 subunit of IL-12, was the essential cytokine in EAE (Cua et al., 2003). Nevertheless, studies have shown that Th1 cells,

along with Th17 cells, are important in the development of EAE (Domingues et al., 2010; O'Connor et al., 2008). The decrease in the level of IL-12 produced by type II MG suggests that they would be less supportive of an inflammatory environment. If this activation state were induced in EAE it may be protective as it would not be supportive of encephalitogenic Th1 cells and would potentially support a Th2 environment instead.

IL-10 is essential for protection from EAE (Bettelli et al., 1998). The effects of IL-10 on the course of EAE as a whole can be seen using IL-$10^{-/-}$ mice, which have increased disease severity, and IL-10 transgenic mice, which are protected from disease (Bettelli et al., 1998). IL-10 is important in regulating pathogenic T cell responses by being able to suppress both Th1 and Th17 cells and is a key cytokine produced by the Tregs involved in suppressing EAE (Fiorentino, Bond, & Mosmann, 1989; Gu et al., 2008; Zhang et al., 2004). In addition, IL-10 delivered directly to the CNS using fibroblasts induced to express IL-10 by retroviruses resulted in significant protection from EAE and suggests that local expression of IL-10 can be very effective at controlling inflammatory damage (Croxford et al., 2001). If the production of IL-10 could be induced earlier it may promote disease resolution or even prevent the development of the inflammatory response. Therefore, the increased production of IL-10 by the type II activated MG could be very beneficial in EAE.

In addition to IL-12/IL-23, IL-6 is essential for the induction of EAE. IL-6 deficient mice are resistant to active EAE induction, and have also been shown to be resistant to EAE induction by adoptive transfer using T cells from wild type animals (Mendel, Katz, Kozak, Ben-Nun, & Revel, 1998). The exact role that IL-6 plays in EAE is multifaceted. While IL-6 is important for the development of Th17 cells, adoptive transfer experiments suggest that the expression of IL-6 by resident CNS cells is also important (Bettelli et al., 2006; Mendel et al., 1998). Experiments using IL-6 deficient mice have demonstrated that in the absence of IL-6 VCAM-1, which is important for T cell trafficking into the CNS, is not upregulated on the endothelium as it is in wild type controls, thus preventing infiltration into the CNS (Eugster, Frei, Kopf, Lassmann, & Fontana, 1998). Furthermore, over expression of IL-6 by astrocytes specifically in the cerebellum switched the progression of C57BL/6 EAE from a primarily spinal cord based disease to one in the cerebellum (Quintana et al., 2009). This switch suggests that the expression of IL-6 may alter the microenvironment to encourage inflammation and immune cell trafficking. In this study, type II activated MG expressed lower levels of IL-6 compared to classical activation. As IL-6 has such an important role in EAE, it is possible that decreased expression of IL-6 by microglia could have a protective

effect by reducing the expression of VCAM-1 on the endothelium. Thus though a reduction in IL-6, type II activation of MG may provide a protective phenotype if induced *in vivo*. However, the overall reduction in IL-6 would need to be balanced with production by other cells as IL-6 can be produced by other cells including astrocytes (Ma et al., 2010).

MCP-1 (also known as chemokine ligand 2; CCL-2) is a CC type chemokine, and is released by both immune and non-immune cells. In the CNS, astrocytes, microglia and invading immune cells have been shown to produce MCP-1 (Hayashi, Luo, Laning, Strieter, & Dorf, 1995). MCP-1 is often described as having dual roles. MCP-1 is a chemotactic protein that is involved in the recruitment of immune cells, such as monocytes and T cells, into sites of inflammation, thus promoting inflammatory responses. Conversely, MCP-1 is also important in the development of Th2 type responses, which are associated with protection in EAE (Gu et al., 2000).

While type II activation of MG did not result in a decrease in MCP-1 when M-CSF was left in culture until IFN-γ stimulation, the decrease in MCP-1 when the M-CSF was removed 3 days prior to IFN-γ stimulation warrants further investigation. In EAE, elevated MCP-1 in the CNS is associated with increased disease severity in progressive and relapsing disease models (Huang, Wang, Kivisakk, Rollins, & Ransohoff, 2001). In addition, specific deletion of MCP-1 from the CNS but not the periphery demonstrated that production of MCP-1 by the resident cell is important for the development of disease, and this deletion of MCP-1 in the CNS provides protection form EAE (Dogan, Elhofy, & Karpus, 2008). Due to the well established role of MCP-1 in EAE, a decrease in production by MG *in vivo*, would likely be protective.

Similarly to MCP-1, TNF-α was not significantly altered by type II activation when the M-CSF was removed at the time of IFN-γ stimulation, but was significantly decreased when the M-CSF 3 days prior to IFN-γ stimulation; this interesting finding merits further investigation. TNF-α is a proinflammatory cytokine, which is important in host defence against pathogens but also has vital roles in the other conditions such as injury, burns and ischemia (Cairns, Panacek, Harken, & Banerjee, 2000). TNF-α is produced by many cell types including MΦ and MG and has been shown to be produced by both these cell types in EAE (Renno, Krakowski, Piccirillo, Lin, & Owens, 1995). In addition to its proinflammatory properties, TNF-α is capable of altering the permeability of the vascular endothelium, both in the peripheral circulation and in the BBB (Mayhan, 2002; Yang, Gong, Qin, Liu, & Lorris

Betz, 1999). A decrease in the effects of TNF-α on the BBB would help to prevent the movement of inflammatory cells from the blood into the CNS parenchyma. Therefore, if type II MG are shown to decrease TNF-α, this reduction in TNF-α may be a potential mechanism to decrease CNS inflammation in EAE.

CD40 expression in the periphery is essential for the development of EAE; however, interruption of the interaction between MG and T cells by specifically deleting CD40 from the CNS also results in decreased disease severity in EAE (Becher, Durell, Miga, Hickey, & Noelle, 2001), and in addition, ligation of CD40 on human MG is known to induce IL-12 production (Becher, Blain, & Antel, 2000). Further reports demonstrated that mice that lacked CD40 expression in the CNS had impaired MG activation. This lack of CD40 was not only associated with decreased MG activation, but also with decreased proliferation of T cells in the CNS (Ponomarev et al., 2006). If type II activated MG also showed a similar level of CD40 down regulation then it would be conceivable that they may be less responsive to CD154 stimulation by activated T cells that migrate into the CNS, which could result in lowered disease burden.

Due to the lowered level of CD40 seen on type II activated MΦ (Tierney et al., 2009), it was hypothesised that type II activation of MG may produce a similar effect on CD40 levels. If this activation state could be achieved *in vivo* it could provide a potential pathway for protection from disease. However, despite type II activated MG displaying a cytokine profile similar to type II activate MΦ, the expression of CD40 on type II activated MG was comparable to classical activation. This finding suggests that lowered CD40 expression is unlikely to be the method by which type II MG could protect from EAE.

PD-1/PD-L1 interactions are involved in peripheral tolerance and have been shown to be important in many autoimmune conditions, including EAE. Both PD-1 and PD-L1 knockout mice exhibit an increased severity of EAE compared to wild type strains (Carter et al., 2007; Ortler et al., 2008; Salama et al., 2003). PD-L1 knockout mice have also been shown to have increased T cell infiltrates into the CNS at peak disease (Ortler et al., 2008). In addition the expression of PD-L1 is markedly increased on MG in EAE, particularly during late stage of disease, suggesting PD-L1 may be important in the regulation of T cell responses in the CNS and the resolution of inflammation (Magnus et al., 2005). T cells cultured with neonatal microglia that express higher levels of PD-L1 (in response to high level IFN-γ stimulation) produce lower levels of proinflammatory cytokines (Magnus et al., 2005).

The results of this study suggest that type II activated MG express lower levels of PD-L1, compared to classically activated MG. Due to the inhibitory nature of PD-L1 and the protective role it plays in EAE, the decreased expression of PD-L1 on type II activated MG may not be advantageous. However, it is possible that the decreased expression of PD-L1, in combination with the other changes seen in type II MG (such as increased IL-10 and decreased IL-12) would create an overall environment that could support the expansion of protective T cell responses while inhibiting detrimental proinflammatory T cell responses. Thus, it needs to be determined whether MG can be type II activated *in vivo* in response to IC treatment, whether a decrease in PD-L1 also occurs *in vivo*, and if this change is associated with protection from EAE.

A large part of the role MG play in inducing or protecting from disease likely lies in their ability to interact with T cells. Other forms of activated MΦ, such as the alternatively activated MΦ, do not efficiently present antigen to T cells and are therefore unable to drive a T cell response (Edwards et al., 2006). Given the inability to induce antigen specific T cell responses, were MG activated to an alternatively activated MG should not be effective at regulating the T cells directly. Conversely, type II activated MΦ are capable of presenting to and driving the activation of T cells (Anderson & Mosser, 2002). Therefore, type II activation of MG may not simply be able to induce an immunoregulatory environment, but may also be capable of interacting with T cells to down regulate pathogenic T cell responses.

In the current study it was found that IFN-γ primed MG were capable of inducing a T cell response, inducing T cells to produce large amounts of both IFN-γ and IL-2. Interestingly, MG primed with IFN-γ alone or MG primed with IFN-γ and subsequently stimulated with LPS induced similar levels of IFN-γ and IL-2 from T cells. This increase in IFN-γ production in co-cultures occurs despite there being significantly different levels of IL-12, a cytokine known to be important in Th1 development (Kaplan et al., 1996), in cultures with LPS stimulated and non-stimulated MG cultures. This finding is contrary to the results seen in MΦ co-cultures, where IFN-γ-primed, LPS-stimulated MΦ induced T cells to produce significantly more IFN-γ than MΦ primed with IFN-γ but without LPS stimulation. As with MΦ cultures, IFN-γ was removed from the MG culture before the stimuli (e.g. LPS) were added. Therefore, the IFN-γ present in the culture supernatants is due to *in vitro* production not the result of residual IFN-γ left over from the priming process. This result may suggest that MG have an increased ability to activate T cells and, given that the MG vastly increased

IFN-γ production under non-biasing conditions, may suggest that MG preferentially bias towards Th1.

IFN-γ is used to prime MG in many studies, and it has been found that IFN-γ can induce activation of MG and the upregulation of several cell surface molecules such as CD40 (Li et al., 2007; Ponomarev et al., 2006). IFN-γ also stimulates MΦ, and is known to induce microbicidal activity (Szabo et al., 2003). In the current study, relatively low concentrations of IFN-γ (20 U/ml) were used to prime both MΦ and MG. This low level of IFN-γ was chosen because IL-10 production by MΦ has been shown to be highest at this concentration, and therefore this level of stimulation provides optimal conditions for type II activation of MΦ (Tierney et al., 2009). Although type II activation of MΦ does still occur using higher levels of IFN-γ (Gerber & Mosser, 2001; Sutterwala et al., 1998; Tierney et al., 2009). Previous reports using neonatal cultures have shown that MG stimulated with 100 U/ml IFN-γ are able to stimulate T cell proliferation and IFN-γ production (Li et al., 2007). In addition, IFN-γ doses as low as 10 U/ml have been shown to stimulate MG to become efficient APC to pre-activated Th1 and Th2 cell lines (Aloisi, Simone, Columba-Cabezas, Penna, & Adorini, 2000). This suggests the low level of IFN-γ used in the current study should be sufficient to induce MG to become efficient APC cells, despite the T cells used in this study being naïve, not pre-activated. Unstimulated MG (IFN-γ primed) produce low to undetectable levels of IL-12. Given that it has been shown that IFN-γ in combination with CD40/CD154 interactions drives IL-12 production (Aloisi, Penna, Polazzi, Minghetti, & Adorini, 1999), one could speculate that these MG may be producing low levels of IL-12, which is driving IFN-γ production, but as the IL-12 is being taken up by the T cells, it is therefore undetectable in the culture supernatant.

Unstimulated, IFN-γ primed MΦ do not drive an adaptive immune response in the same way that IFN-γ primed MG do. Possibly, this difference is because MG are more sensitive to IFN-γ and due to MG existing in an immune privileged environment where they would not be exposed to IFN-γ. MΦ in the periphery, which are more commonly exposed to IFN-γ may have a higher threshold for IFN-γ stimulation to prevent aberrant activation and collateral tissue damage. However, activation of T cells with MG that have not been primed with IFN-γ was not assessed in this study. IFN-γ priming was employed as early results in this study demonstrated that IFN-γ enhanced LPS driven IL-12 production by MG (Chapter 5). The relatively high level of CD124 expression on T cells cultured with unstimulated, IFN-γ-primed MG compared to those cultured with classically activated MG could suggest that these

IFN-γ primed MG, while activating T cells, are not as efficient at biasing the T cell response towards a Th1 type response.

MG in the current study were grown for 4 weeks in the presence of M-CSF in order to obtain high enough cell numbers for experimentation. It is possible that the exposure to M-CSF may alter the way the MG respond and affect the way in which the MG interact with T cells as MΦ were not differentiated using M-CSF in this work. M-CSF was chosen for the current study over other proliferative factors such as GM-CSF due to the fact that GM-CSF induces MG to differentiate into a DC like phenotype whereas MG exposed to M-CSF maintain a phenotype consistent with resting MG (Ponomarev et al., 2005). Indeed, treatment of adult MG with GM-CSF enhanced Th1 responses *in vitro* while M-CSF was not reported to have the same effect (Aloisi et al., 2000). Expansion of rat neonatal microglia with M-CSF did not affect their response to LPS (Vidyadaran et al., 2009). Furthermore, culture of adult MG for 4 weeks has been shown to have a similar phenotype to freshly isolated adult MG cells (Ponomarev et al., 2005). Thus, while it is possible the M-CSF is responsible for the differences in the MG responses compared to MΦ, the finding that the M-CSF expanded MG are similar to freshly isolated MG, suggests that this M-CSF culture method does not have a large effect on MG responses.

Type II activated MG bias the T cell response in a distinct way from type II MΦ, although some features are shared. For example, there is a strong trend towards an increase in CD124 expression on T cells cultured with type II activated MG compared to those activated by classically activated MG. This increase in CD124 suggests that the T cells may be more responsive to IL-4. This could be potentially protective in EAE as it has been shown that MG produce IL-4 during EAE and that disease is more severe if the production of IL-4 is prevented (Ponomarev et al., 2007). In addition, type II activated MG induced production of high levels of IL-17A, compared to T cells cultured with classically activated MG. The exact pathway leading to increased IL-17A production by type II activated MG as not been elucidated; however, it is possible that some of the potential mechanisms described in chapter 3 may be involved. For example, it is possible that these type II MG are producing increased levels of TGF-β. In conjunction with IL-6, which although significantly decreased in type II MG is still present in cultures, could potentially drive IL-17A production (Bettelli et al., 2006). This particular facet of T cell biasing of type II MG would potentially be detrimental in EAE. Th17 responses are considered pathogenic in EAE and are associated with the recruitment of neutrophils that are capable of causing significant tissue damage (Louten et al.,

2009). It should be noted that the level of IL-17A production, while significantly increased in T cells cultures with type II activated MG compared to other conditions, is relatively low compared to IFN-γ, therefore the ability of this cytokine to dominate an adaptive response *in vivo* remains to be determined.

Due to the differential effect that type II and classically activated MG have on T cells, the pathways involved in the biasing of the T cell response were investigated. Flow cytometry data suggested that PD-L1 was down regulated on type II MG and has been found on type II MΦ, and so this pathway was investigated. As stated previously, PD-L1 is generally considered an inhibitory pathway involved in suppression of T cell responses (Carter et al., 2002). In the CNS, PD-L1 is upregulated in late stages of EAE and may be involved in resolution of the immune response (Magnus et al., 2005). However, in the context of type II activation, PD-L1 may be involved in the biasing of T cell responses. Therefore, this decrease in PD-L1 may be protective by biasing the T cell response towards less pathogenic phenotype. In the current study, αPD-1 and the PD-L1ch were used to block or stimulate the PD-1/PD-L1 pathway to T cells respectively. Despite the well known role of the PD-1/PD-L1 pathway in the inhibition of T cell responses (Carter et al., 2002), neither the αPD-1 nor the PD-L1ch had any further effect on the activation state of T cells beyond what was induced by the different activation states of the MG. In addition, the alteration in the PD-1/PD-L1 pathway also did not alter the biasing of the T cell response. This data is in line with those from Chapter 4 which demonstrate that this pathway is also not strongly involved in T cell biasing by classically or type II activated MΦ and suggests that the reduced expression of PD-L1 on type II MG is not involved in T cell biasing.

It is possible that αPD-1 and the PD-L1 chimera were not used at high enough concentration to see an effect. Due to the limited numbers of MG available per experiment, it was not feasible to perform a titration experiment using MG, therefore the αPD-1 and the PD-L1ch were used at the same concentrations as in the MΦ:T cell experiments (Chapter 4). As explained Chapter 4, it is likely that these molecules are being used at sufficient concentration to elicit a response based on the concentrations from previous studies (Vogt et al., 2006; Yamazaki et al., 2005). As there was no change in any markers, an alternate possibility is that the αPD-1 and the PD-L1ch were not active in this system, PD-L1. The PD-1/PD-L1 pathway is known to be important in inhibiting proliferation (Carter et al., 2002; Konkel et al., 2010), which was not measured in this study, it is possible this could be used as a positive control. Additionally, other methods for altering PD-1/PD-L1 interaction may provide further

information, such as RNAi experiments. As the role of PD-L1 in EAE may be to restrain the proliferation of encephalitogenic T cells in the CNS, the decreased PD-L1 on type II MG may be important *in vivo* for the inhibition of T cell responses and not the biasing of T cell responses in the CNS.

## 6.5 Summary

This chapter conclusively demonstrates for the first time that in response to prototypical classical (LPS) and type II (LPS+IC) activating conditions, MG develop phenotypes that clearly resemble classical and type II activation respectively. As type II MΦ are immunoregulatory, and have been demonstrated to be protective in EAE (Tierney et al., 2009), these type II activated MG may also be protective in EAE. Their ability to regulated T cell resposnes is particularly importantas these type II activated MG would be acting at the site of inflammation to help regulate the local response.

Overall these data suggest that MG do not activate T cells in the same manner as MΦ, despite having similar activation profiles. Of note is that the T cells cultured with unstimulated MG have a similar activation state and IFN-γ production compared to T cells cultured with classically or type II activated MG. This finding suggests that the ability of MG to induce T cell responses is increased compared MΦ since unstimulated MΦ are poor activators of T cell responses. One of the major features of T cell biasing by type II MΦ (i.e. the decreased IFN-γ) is not present in the MG cultures suggesting the MG are not capable of biasing away from a Th1 type response. However, the T cells cultured with type II activated MG produced higher levels of IL-17A as well as a tendency towards increased levels of CD124 suggesting that some of the features of T cell biasing by type II MΦ and conserved by activation of T cells by type II MG. Finally, this work showed that the PD-1/PD-L1 pathway does not appear to have a role in the biasing of T cell responses by classically and type II activated MG.

# Chapter 7:
# Effects of type II inducing treatments on experimental autoimmune encephalomyelitis

## 7.1 Introduction

The previous results from this thesis indicate that *in vitro* stimulation of MG under type II activating conditions (LPS+IC) results in a type II activated phenotype similar to that seen with MΦ activated under the same conditions. In addition to this phenotypic similarity, the ability of type II activated MG to induce and bias an antigen-specific T cell response in co-culture was also similarly modified. As these results demonstrate that in *in vitro* cultures type II activated MG display a similar phenotype and function as type II activated MΦ, the effect of type II activating treatments on the MG phenotype *in vivo* was assessed.

Initially, for the study of MG activation *in vivo* it had been intended that the BALB/c model of EAE would be used because, although this model is used less frequently than the C57BL/6 model, it displays a different histopathological phenotype. Unlike C57BL/6 mice in which CNS lesions are primarily located in the spinal cord (Pham et al., 2011), lesions in BALB/c mice develop principally in the upper CNS and are more similar to those in MS patients (Personal communication, Dr Jacqueline Orian; La Trobe University, Melbourne, Australia; manuscript in preparation). However, despite the protection seen when C57BL/6 mice are treated with IC in EAE, the treatment of BALB/c mice did not provide protection (see appendix C). The IC treatment was compared to glatiramer acetate (GA), a common treatment for MS which has also been shown to induce a MΦ activation state similar to type II activation (Weber et al., 2007). However, GA provided minimal protection in BALB/c mice even at a concentration that provides almost complete protection in C57BL/6 mice (Toker et al., 2011; see appendix C). As IC treatment is not effective in BALB/c mice, C57BL/6 mice were used for the assessment of MG *in vivo*. MG in the CNS of C57BL/6 mice were assessed, thus the changes observed in this study are changes in the activation state of MG under inflammatory conditions as a whole, as opposed to a change specifically at the site of inflammation.

In the CNS of a naïve mouse, MG can be easily identified on the basis of $CD45^{lo}$ staining as they are the only CNS cell type that expresses this marker. However, *in vivo* study of MG in an inflamed CNS is hampered due to the fact that activated MG take on a phenotype that is indistinguishable from peripheral MΦ/monocytes that invade the CNS in EAE (Melchior et al., 2006; Ponomarev et al., 2006). In order to study MG *in vivo* bone marrow chimeric mice were generated. As described in methods, C57BL/6 mice, which express the CD45.2 allele,

were lethally irradiated and their bone marrow was replaced with that from B6.SJL-ptprc$^a$ mice, which express the CD45.1 allele but are otherwise considered genetically identical to C57BL/6 mice. As the CNS is not sensitive to irradiation, the MG do not get ablated in the same way the peripheral immune system does. Therefore, following bone marrow reconstitution, the peripheral immune system will express CD45.1 and the MG in the CNS will express CD45.2. Thus under inflammatory conditions, MG can be distinguished from invading cells on the basis of CD45.2 expression.

Treatment of mice with IC, which induce type II activation, has been shown to provide protection from EAE in C57BL/6 mice (La Flamme et al., 2006). IC treatment of unimmunised mice increased the levels of IL-10 and IL-5 produced by ConA stimulated splenocytes, suggesting an overall shift in the immune response towards a Th2 response. In addition protection from disease in immunised mice was associated with increased Th2 responses in the periphery. Furthermore, increases in IL-10 and IL-4, and a decrease in IL-12 was observed in the spinal cords of IC treated immunised mice (La Flamme et al., 2006). This study shows that IC treatment *in vivo* has the ability to alter both the peripheral and the CNS immune responses.

*In vitro*, type II activated MG display a phenotype that is highly likely to be protective *in vivo*. The proinflammatory molecules IL-12 and IL-6 that are detrimental in EAE (Becher, Durell, & Noelle, 2003; Quintana et al., 2009) are down regulated by type II activated MG (chapter 6). The reduction in these cytokines could potentially help to reduce disease by decreasing recruitment of immune cells and altering interactions between MG and T cells. Previous studies have shown that a reduction in pathological CNS inflammation can be induced by altering the ability of MG to produce proinflammatory cytokines or interact with T cells (Becher, Durell, Miga, Hickey, & Noelle, 2001; Becher et al., 2003).

## 7.2 Aims

The aim of this chapter was to optimise a protocol for the creation of bone marrow chimeras and to use this protocol to investigate the ability of type II activating treatments to affect the activation state of MG *in vivo.* In addition, effects of IC treatment on the peripheral immune system were investigated to look at any potential correlations between the peripheral immune system and the immune response and the CNS.

### 7.2.1 Specific aims

1. To assess the effects of type II activating treatments on the peripheral immune system, based on the changes in T cell cytokines seen in MΦ:T cell co-cultures.
2. To use flow cytometry to assess invasion of peripheral immune cells as well as the phenotype of both peripheral and resident immune cells by assessing cell surface expression.
3. To assess the pattern of MG activation in the CNS by immunohistochemistry.
4. To optimise a protocol for the generation of bone marrow chimers and the isolation of MG cells.
5. To use bone marrow chimeras to isolate MG from the CNS of naïve mice and mice with EAE to assess the effect of IC treatment on the MG by qPCR.

## 7.3 Results

### 7.3.1 Treatment with IC protects from EAE

Because the ultimate aim of this work was to determine if and how type II activation *in vivo* affected the resident MG using bone marrow chimeras, the ability of IC to induce protection in this model was first verified. Bone marrow chimeric mice were generated by irradiation of C57BL/6 mice and transfer of either B6.SJL-ptprc$_a$ bone marrow (B6→C57) or C57BL/6 bone marrow (C57→C57). The C57→C57 group served as a control for the B6→C57 group and allowed evaluation of any possible effects due to irradiation and reconstitution of the bone marrow. Six-ten weeks following irradiation, the phenotype of peripheral immune cells was assessed to determine the extent of reconstitution bone marrow. As expected, the level of CD45.2$^+$ cells in blood samples from B6→C57 mice was high with 95.66% ± 0.1897 cells positive for CD45.2 (mean ± SEM from 73 mice over 5 experiments) demonstrating high levels of reconstitution of the bone marrow. Treatment of B6→C57 mice with IC reduced the severity of disease in mice immunised for EAE as seen by reduced disease scores compared to untreated mice (Figure 7.1a, c and e). This reduction in severity reached statistical significance in some experiments, however, the severity of the disease course was quite variable between experiments. In contrast, the incidence of disease was more consistently decreased in IC-treated mice in these experiments than the disease score (Figure 7.1b, d and f), and IC-treated mice tended to have a lower cumulative disease (Figure 7.2). C57→C57 mice showed similar patterns of disease as B6→C57 mice indicating the irradiation and bone marrow reconstitution did not affect disease expression (see appendix C). Together these data show that IC treatment has a beneficial effect in EAE by reducing the severity and overall incidence of disease and that this chimeric model can be used to explore the involvement of the radiation-resistant, resident CNS immune cells (i.e. MG).

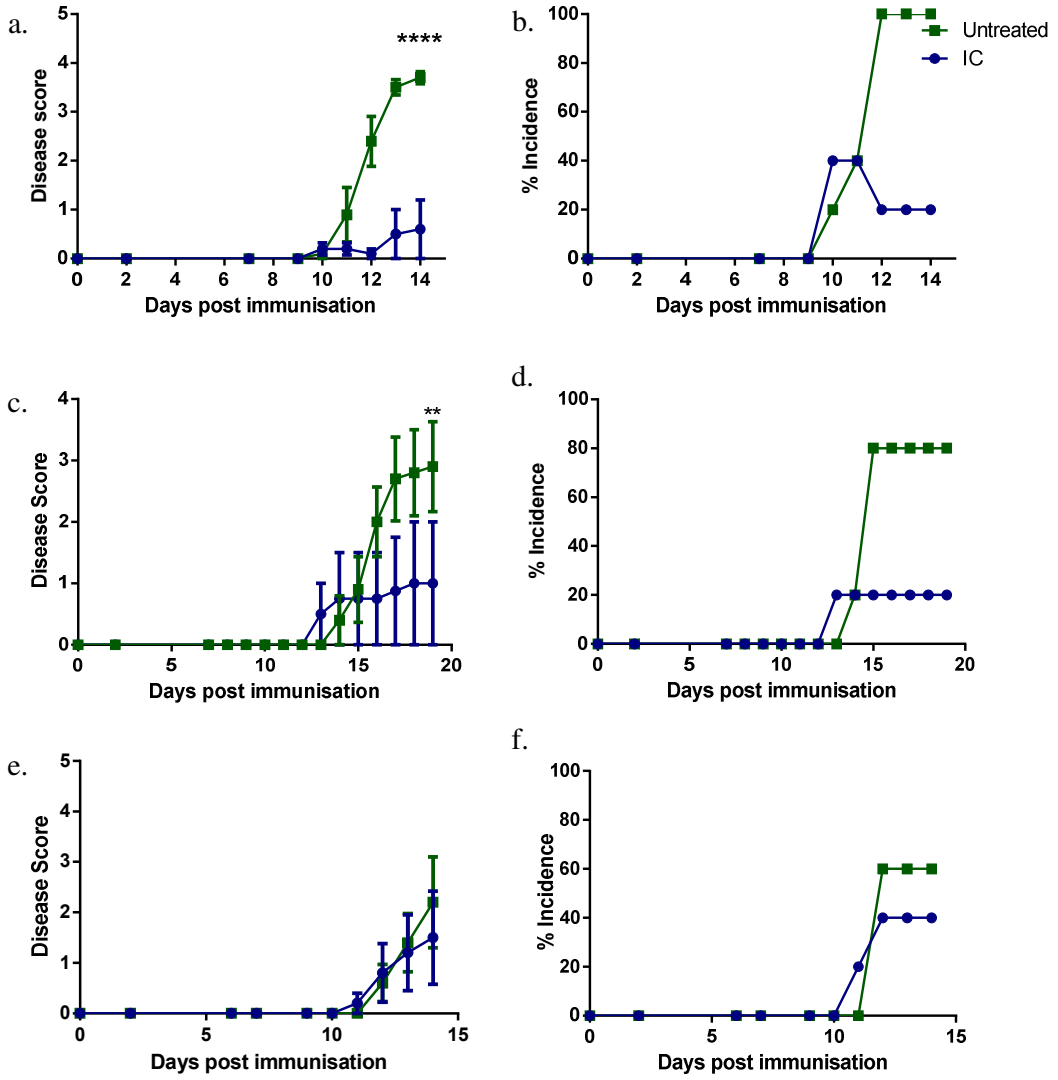



**Figure 7.1. Treatment of mice with IC reduced the severity and incidence of EAE.** Seven weeks following irradiation and reconstitution of B6→C57 animals, mice were pre-treated with $2x10^6$ IC by i.p. injection one week before immunisation and weekly thereafter. Mice were immunised to induce EAE as described in the methods. Disease scores (a, c, e) and incidence (b, d, f) for 3 of 5 experiments are shown (n=5/group). **p<0.01; ****p<0.0001 by two way ANOVA with Sidak's multiple comparison post test.

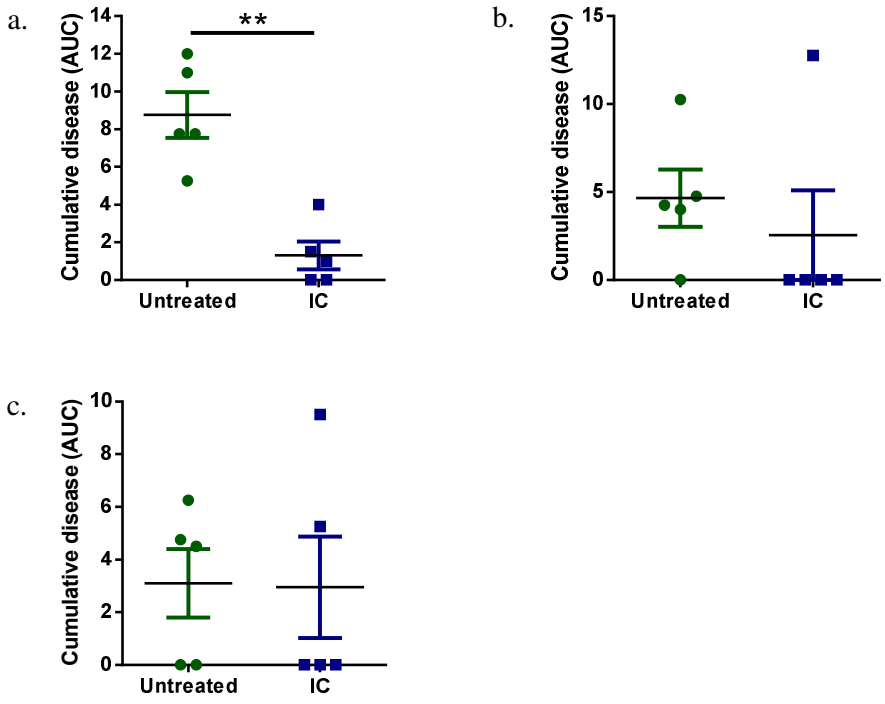



**Figure 7.2. Treatment of mice with IC reduced the cumulative disease in EAE.** Seven weeks following irradiation and reconstitution of B6→C57 animals, mice were pre treated with $2x10^6$ IC by i.p. injection one week before immunisation and weekly thereafter. Mice were immunised to induce EAE as described in the methods. Shown are the means and SEM of cumulative disease for individual mice (n=5/group) and calculated as the area under the curve or AUC. Shown are 3 of 5 individual experiments. **p<0.01 by Mann Whitney U test.

### 7.3.2 IC treatment alters the peripheral immune response

Previous studies have demonstrated the abilities of both IC and type II MΦ to induce protective changes in the peripheral immune responses (La Flamme et al., 2006; Tierney et al., 2009). The peripheral immune responses were assessed using restimulation assays in which splenocytes are stimulated with ConA (polyclonal stimulation) and MOG (antigen specific stimulation). In the current study, low levels of IL-4 were detected in supernatants from splenocyte cultures at peak disease stimulated with ConA, and there was a trend towards increased levels of IL-4 in ConA-stimulated splenocytes from IC-treated mice compared to untreated. This trend was seen in both immunised and unimmunised groups (Figure 7.3b). Only very low levels were detected without stimulation or in response to MOG (Figure 7.3b). Taken together, the increase in polyclonally-stimulated IL-4 suggests a shift in the overall immune environment in the periphery supporting the idea that IC treatment promotes a systemic Th2 environment *in vivo*.

In contrast to IL-4, ConA strongly induced the production of IFN-γ by all treatment groups (Figure 7.3a). In cultures from unimmunised mice, IC treatment resulted in a significant reduction in IFN-γ compared to untreated mice (Figure 7.3a). This effect was not mirrored in the cultures from immunised mice, in which equivalent levels of IFN-γ were produced (Figure 7.3a). Additionally, IFN-γ production by cultures from untreated immunised mice was modestly but significantly lower than that seen in cultures from untreated, unimmunised mice. Similar to polyclonal responses, antigen-specific IFN-γ production was equivalent between IC and untreated immunised mice suggesting that although immunisation induces antigen specific production of IFN-γ, IC treatment does not alter this response (Figure 7.3a). In contrast to IFN-γ, IL-17A production in response to both ConA and MOG was significantly higher in immunised mice compared to unimmunised for both treatment groups (Figure 7.3c). In addition, while immunisation induced significant production of IL-17A by both treatment groups, it was significantly higher in cultures from IC-treated mice compared to untreated mice suggesting that as found in *in vitro* cultures, IC treatment enhances antigen-specific IL-17A responses *in vivo* (Figure 7.3c).

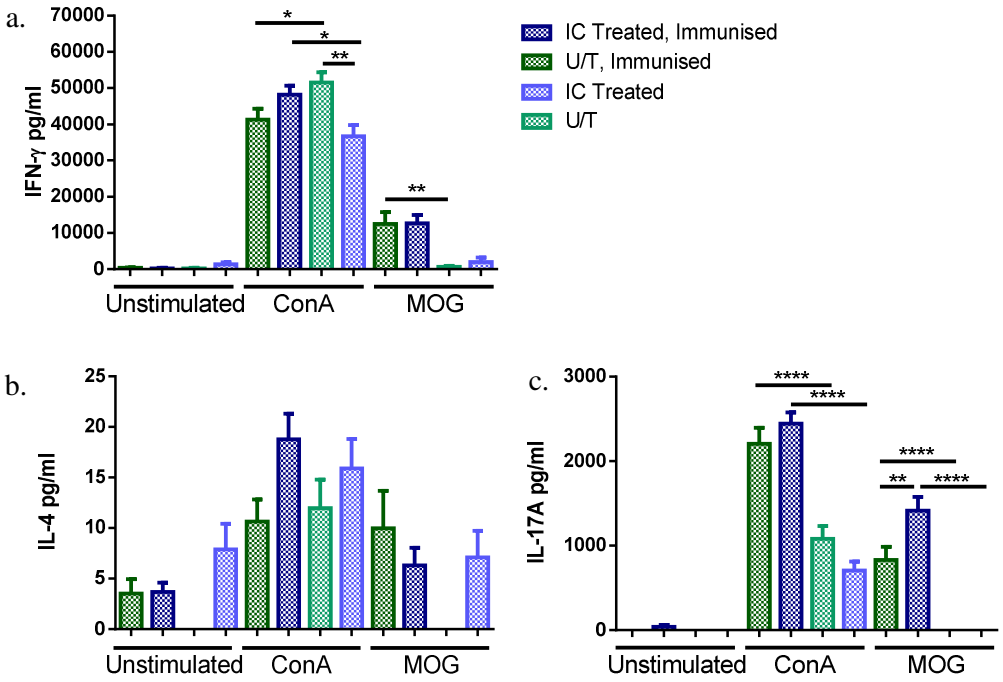



**Figure 7.3. Treatment of mice with IC alters the production of cytokines by splenocytes.** Seven weeks following irradiation and reconstitution of B6→C57 animals, mice were pre-treated with $2x10^6$ IC by i.p. injection one week before immunisation and weekly thereafter. Mice were immunised to induce EAE as described in the methods. At peak disease, splenocytes ($1x10^6$/well) were isolated and cultured alone or with ConA (3 μg/ml) or MOG (27 μg/ml) for 48 hours (ConA) or 72 hours (MOG). IFN-γ (a), IL-4 (b) and IL-17A (c) levels were measured by ELISA. Data shown are means and SEM of triplicate wells from two combined experiments (n=10 mice/group, for immunised and 4-6 mice/group for unimmunised). *p<0.05, **p<0.01, ****p<0.0001 by one way ANOVA with Tukey's multiple comparison post test.

### 7.3.3 IC treatment altered the phenotype of myeloid cells in the brain

To further investigate the *in vivo* activation state of MG from mice that had received a type II activating stimulus, the brains of C57→C57 mice were processed by flow cytometry. As the mice used for flow cytometry are C57→C57 and not B6→C57, the resident cells cannot be identified on the basis of expression of a specific CD45 isotype. However, given that most lesions develop in the lower CNS in this model, it is likely that the majority of the cells isolated from the brains of these animals are CNS resident and not a result of peripheral infiltration. Using the haematopoietic marker CD45 and the myeloid marker CD11b (i.e. complement receptor 2), 3 distinct populations of $CD45^{+}CD11b^{+}$ ($CD45^{hi}CD11b^{++}$, $CD45^{int}CD11b^{+}$, $CD45^{lo}CD11b^{+}$) cells can be found in the brains of C57→C57 mice, which represent 3 distinct populations of cells (Figure 7.4, see appendix D for gating strategy). These 3 different myeloid populations were present at different levels in the brains of immunised mice compared to naïve mice.

In immunised mice there is a comparably large population of $CD45^{hi}CD11b^{++}$ cells (Figure 7.4a). This same population is reduced in naïve mice, with only a small number present (Figure 7.5b). This population is composed of peripheral immune cells, which are known to be $CD45^{hi}$ (Ponomarev et al., 2006; Sedgwick et al., 1991; Zhang, Li, Ventura, & Rostami, 2002). However, it is possible that some activated MG may also be present in the $CD45^{hi}CD11b^{++}$ population, as MG increase CD45 on activation (Ponomarev et al., 2006). $CD45^{hi}CD11b^{++}$ cells are significantly increased in the brains of IC-treated immunised mice compared to IC-treated unimmunised mice (Figure 7.4c), and a strong trend towards increased $CD45^{hi}CD11b^{++}$ was seen between immunised and unimmunised mice from the untreated groups (Figure 7.4c). This result suggests that despite the absence of evident lesions in the brains of C57BL/6 mice, myeloid cell infiltration into the brain is induced by immunisation in this model.

In all treatment groups the most abundant myeloid population within the brain was the $CD45^{lo}CD11b^{+}$ population (Figure 7.4e). This $CD45^{lo}CD11b^{+}$ population represents the resident, parenchymal MG cells of the brain. As MG upregulate expression of CD45 following activation (Ponomarev et al., 2006; Sedgwick et al., 1991), this population may represent MG that are either unactivated or are not highly activated. Immunisation induced a trend towards decreased percentage of $CD45^{lo}CD11b^{+}$ cells in the myeloid compartment of the brain, compared to unimmunised mice, and this difference was also significant between

the IC-treated groups (Figure 7.4e). However, as both immunised groups also saw an increase in $CD45^{hi}CD11b^{+}$ cells, this proportional decrease could potentially be the result of an increase in the infiltrating cells. Alternatively, immunisation could induce a decrease in resting or unactivated MG.

The third population of $CD45^{+}CD11b^{+}$ cells was $CD45^{int}CD11b^{+}$ and the proportion of myeloid these cells was similar among all treatment groups (Figure 7.4d). As seen in the flow cytometry plots, this population is distinct although the level of CD45 expression on these cells overlaps with that of the resident MG population (Figure 7.4a and b). There is a general consensus in the literature that the $CD45^{hi}CD11b^{++}$ cells are peripheral immune cells and that $CD45^{lo}CD11b^{+}$ are resident MG (Melchior et al., 2006; Ponomarev et al., 2006; Sedgwick et al., 1991; Zhang et al., 2002). However, the literature is less clear on the exact cell type represented by the $CD45^{int}CD11b^{+}$ cells. These cells were found in both immunised and unimmunised mice and therefore, they are likely to be resident cells and represent a population of CNS-associated MΦ, such as perivascular MΦ (Zhang et al., 2002).

To understand if the activation state of these myeloid cells was altered, the expression of MHC class II, CD40, and PD-L1 was assessed. Immunisation did not alter MHC class II expression on resident MG cells ($CD45^{lo}CD11b^{+}$) nor did treatment with IC (Figure 7.6a). Similarly, expression of CD40 was consistent among all treatment groups (Figure 7.6c). PD-L1 was significantly increased on MG from untreated, immunised mice compared to untreated unimmunised mice. A similar trend in PD-L1 expression was seen in the IC-treated groups; however, this trend did not reach significance (Figure 7.6b). Overall, these data suggest that MG from immunised mice do not upregulate markers involved in T cell activation. Alternatively, the increase in PD-L1 expression on MG may indicate a switch to a suppressive phenotype that may regulate T cell-mediated inflammation in both treatment groups.

In contrast to MG ($CD45^{lo}CD11b^{+}$) cells, which did not have altered expression of MHC class II, MHC class II expression was upregulated on CNS-associated MΦ from immunised mice and this increase was significantly attenuated by IC treatment (Figure 7.5b). In addition, the percentage of MHC class II-positive cells in this CNS associated MΦ population was significantly higher in untreated compared to IC-treated, immunised mice. Neither the expression of CD40 nor the expression of PD-L1 was altered on CNS-associated MΦ (Figure

7.5a and c), and there was no difference in any of these activation markers between the two unimmunised groups (Figure 7.5). The increased expression of MHC class II on CNS-associated MΦ from untreated compared to IC-treated, immunised mice suggests that the MΦ population in the untreated mice may be more capable of presenting antigens and activating T cells.

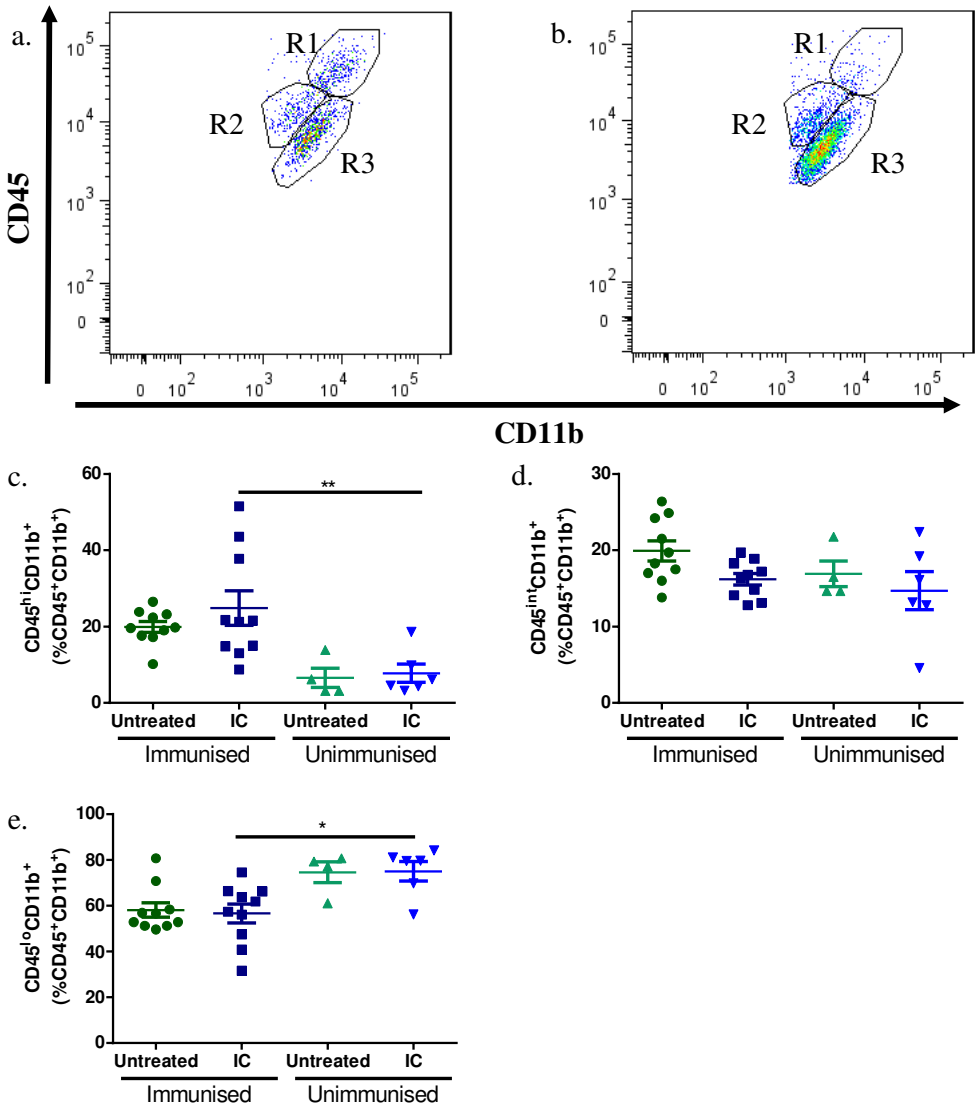



**Figure 7.4. EAE altered the composition of the myeloid compartment in the brain.** Six-seven weeks following irradiation and reconstitution of C57→C57 animals, mice were pre-treated with $2x10^6$ IC by i.p. injection one week before immunisation and weekly thereafter. Mice were immunised to induce EAE as described in the methods. At peak disease, haematopoietic cells were isolated from brains and analysed by flow cytometry. $CD45^+CD11b^+$ live, single cells were gated as shown in appendix. Representative plots from an immunised (a) or an unimmunised mouse (b) showing the gates: R1 ($CD45^{hi}CD11b^+$), R2 ($CD45^{int}CD11b^+$), and R3 ($CD45^{lo}CD11b^+$). $CD45^{hi}CD11b^+$ (c), $CD45^{int}CD11b^+$ (d), and $CD45^{lo}CD11b^+$ (e) are expressed as the percentage of total $CD45^+CD11b^+$ cells and shown are the means and SEM from two combined experiments (n=10/immunised groups, 6/IC treated, unimmunised group, and 4/untreated, unimmunised group). *p<0.5, **p<0.01, by one way ANOVA with Tukey's multiple comparisons post test.

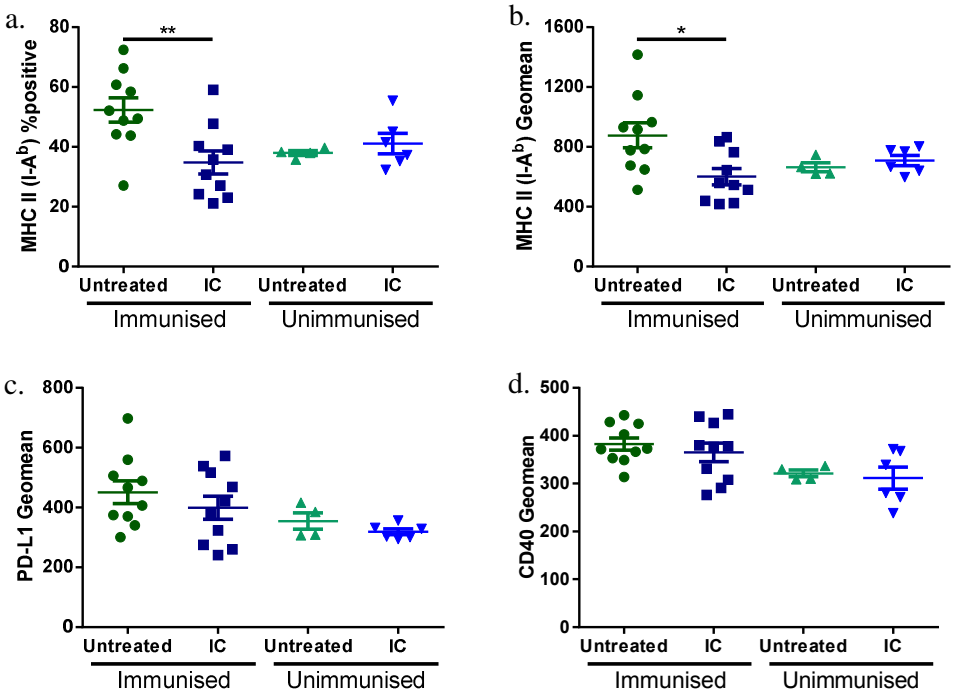



**Figure 7.5. IC treatment altered the expression of MHC class II on CNS-associated MΦ.** Six-seven weeks following irradiation and reconstitution of C57→C57 animals, mice were pre-treated with $2x10^6$ IC by i.p. injection one week before immunisation and weekly thereafter. Mice were immunised to induce EAE as described in the methods. At peak disease, haematopoietic cells were isolated from brains and analysed by flow cytometry. $CD45^{int}CD11b^+$ live, single cells were gated as shown in Figure 7.5. MHC class II (I-$A^b$) (percent positive, a; geomean, b), PD-L1 (c) and CD40 (d) expression were assessed on CNS associated MΦ ($CD45^{int}CD11b^+$) cells. Shown are the means and SEM from two combined experiments (n=10/immunised groups, n=6/IC treated, unimmunised group, and n=4/untreated, unimmunised group). *p<0.5, **p<0.01, by one way ANOVA with Tukey's multiple comparisons post test.

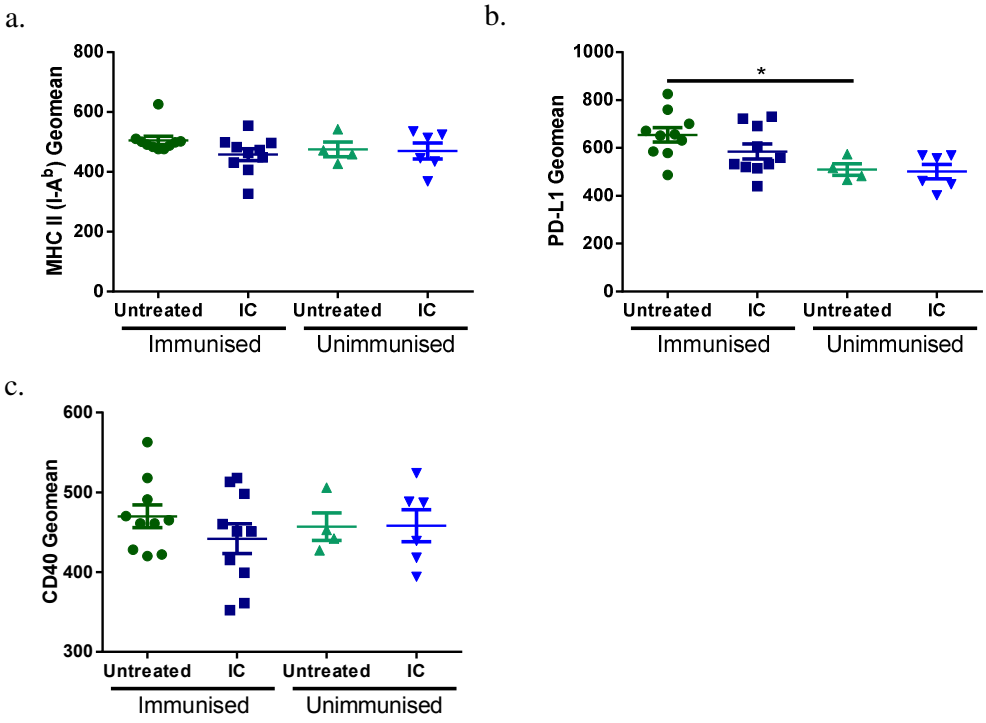



**Figure 7.6. IC treatment did not significantly alter activation markers on resident microglia.** Six-seven weeks following irradiation and reconstitution of C57→C57 animals, mice were pre-treated with $2x10^6$ IC by i.p. injection one week before immunisation and weekly thereafter. Mice were immunised to induce EAE as described in the methods. At peak disease, haematopoietic cells were isolated from brains and analysed by flow cytometry. $CD45^{low}CD11b^+$ live, single cells were gated as shown in Figure 7.5. MHC class II (I-$A^b$) (a), PD-L1 (b) and CD40 (c) expression were assessed on resident microglia ($CD45^{lo}CD11b^+$). Shown are the means and SEM from two combined experiments (n=10/immunised groups, n=6/IC treated, unimmunised group, and n=4/untreated, unimmunised group).

### 7.3.4 IC treatment altered the expression of the MG-associated marker, Iba1, compared to untreated mice during EAE.

To further investigate phenotypic changes in MG of the brain, the expression of Iba1 was observed by immunohistochemistry. Iba1 is a calcium binding protein that is expressed by MG and upregulated by activated MG, including MG in EAE (de Lago, Moreno-Martet, Cabranes, Ramos, & Fernández-Ruiz, 2012; Ito, Tanaka, Suzuki, Dembo, & Fukuuchi, 2001; Staniland et al., 2010). The level of Iba1 in these sections was graded on a scale of 0-3, with 3 being the largest amount of staining. As this test is subjective, the sections were blinded and scored independently by 3 individuals. Following activation MG undergo a morphological change, switching from a ramified to an amoeboid morphology (Melchior et al., 2006). MG in the brains of unimmunised mice stain weakly for Iba1 and have a distinct ramified morphology (Figure 7.7, normal). The MG in the brain of immunised mice (both IC and untreated groups) have an increased intensity of Iba1 staining compared to unimmunised mice. Purely from an observational point of view, large numbers of ramified MG are seen in these mice, supporting the identification of these cells as parenchymal MG. The proportions of ramified vs. amoeboid cells has not been assessed in this study.

Scoring of Iba1 stained sections show that there is strong trend towards increased Iba1 staining in cerebellum and hippocampus of untreated immunised mice which was attenuated by IC treatment and while these values did not reach significance, a strong trend was evident (Figure 7.7). Minimal change with IC treatment was seen in the olfactory bulb and the brain stem (Figure 7.8). The decrease in staining is characterised by decreased numbers and decreased intensity of MG based on visual comparison. These results suggest that IC treatment may be inducing differential activation of MG *in vivo*. In C57BL/6 mice lesions are thought to occur primarily in the spinal cord, while these data suggest that the local changes occurring in the spinal cord may have the ability to affect the overall activation state of the CNS as well.

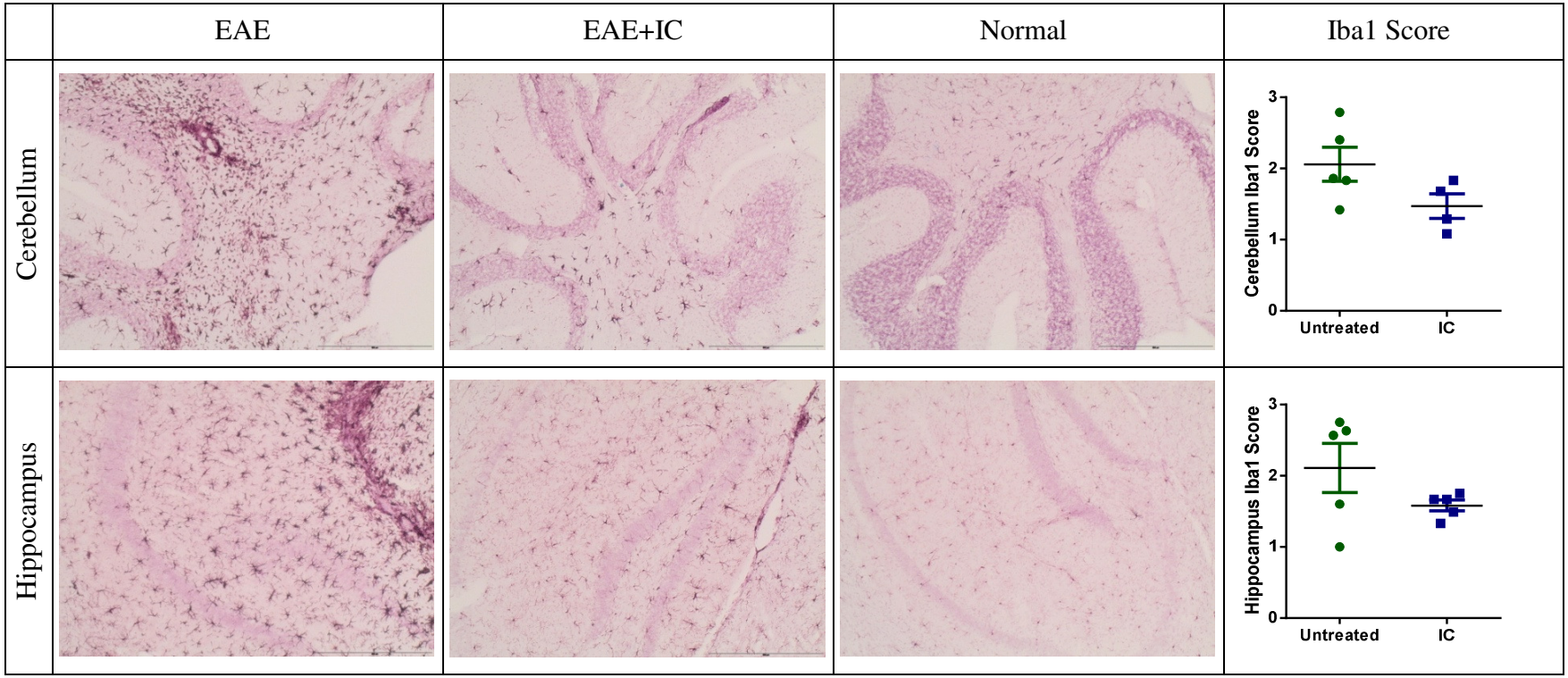



**Figure 7.7. IC treatment induced trend towards differential activation of resident MG in the cerebellum and the hippocampus during EAE.** Seven weeks following irradiation and reconstitution of B6→C57 animals, mice were pre-treated with $2x10^6$ IC by i.p. injection one week before immunisation and weekly thereafter. Mice were immunised to induce EAE as described in the methods. At peak disease, isolated brains were fixed in PFA and 30 µm tissue sections were stained for Iba1 expression by immunohistochemistry. Sections were counter stained with haematoxylin, and Iba1 expression assessed in the cerebellum and hippocampus. Sections (n=5/immunised groups, and n=4/untreated, unimmunised group) were scored blindly as follows: 0 = low, 1 = mildly elevated, 2 = moderately elevated, and 3 = high expression. Shown are representative sections and the means and SEM of averaged scores from 3 blinded assessors and are from one experiment.

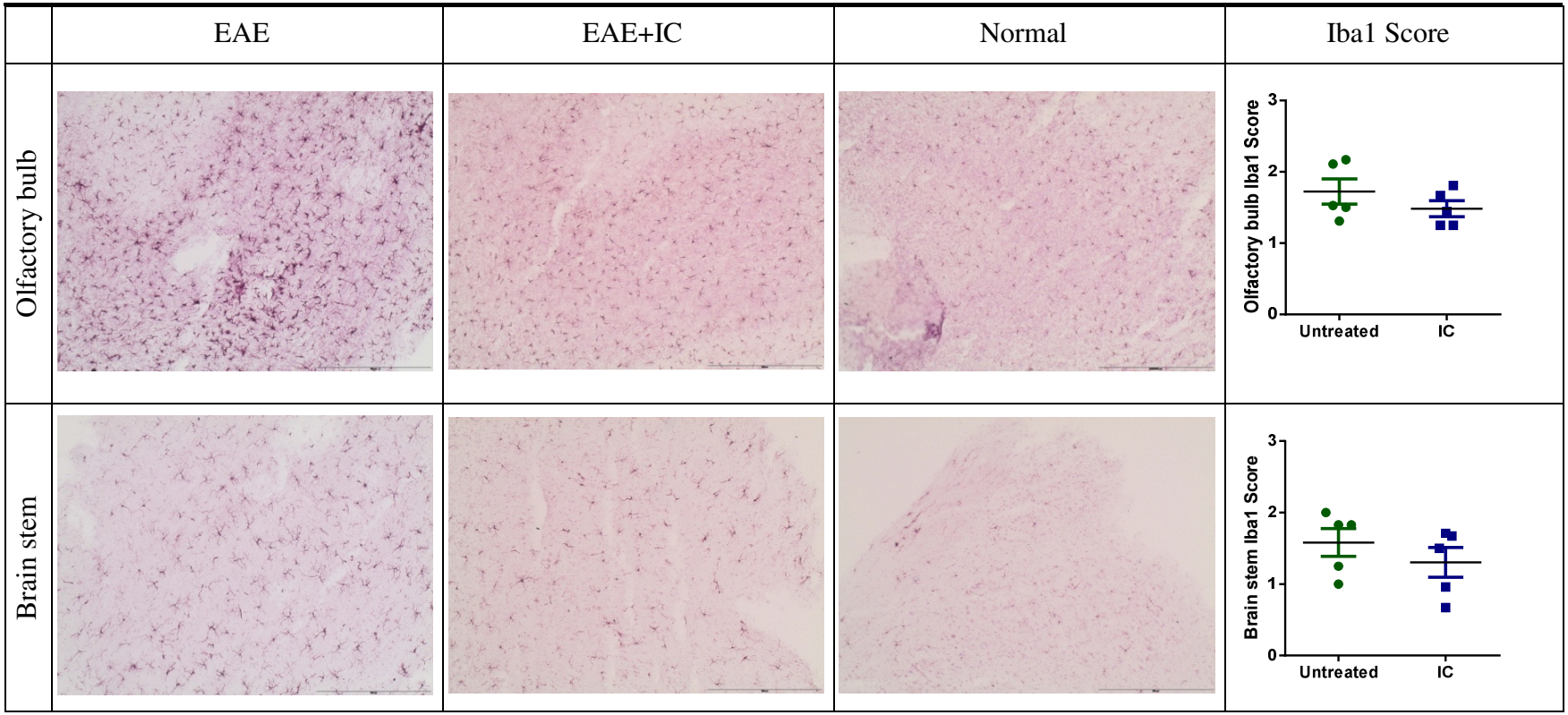



**Figure 7.8. IC treatment did not alter Iba1 expression on resident MG in the olfactory bulb and the brain stem during EAE.** Seven weeks following irradiation and reconstitution of B6→C57 animals, mice were pre-treated with $2x10^6$ IC by i.p. injection one week before immunisation and weekly thereafter. Mice were immunised to induce EAE as described in the methods. At peak disease, isolated brains were fixed in PFA and 30 µm tissue sections were stained for Iba1 expression by immunohistochemistry. Sections were counter stained with haematoxylin, and Iba1 expression assessed in the cerebellum and hippocampus. Sections (n=5/immunised groups, and n=4/untreated, unimmunised group) were scored blindly as follows: 0 = low, 1 = mildly elevated, 2 = moderately elevated, and 3 = high expression. Shown are representative sections and the means and SEM of averaged scores from 3 blinded assessors and are from one experiment.

### 7.3.5 Changes in IL-12 expression by MG

IC treatment during EAE has been shown to alter the immune response that develops in the spinal cord; however, this study was not able to distinguish between the contribution of resident immune cells in the CNS and infiltrating, peripheral immune cells (La Flamme et al., 2006). Therefore B6→C57 mice were generated to specifically enable the isolation and characterisation of the resident MG during EAE. B6→C57 mice were treated weekly with or without IC starting 7 days before immunisation. At peak disease (day 14-15), the resident, radiation-resistant MG (i.e. $CD45.2^+CD11b^+$ cells) were sorted by FACS (Figure 7.9), and these samples were processed for qPCR as described in methods. Because only a limited number of MG could be sorted from individual mice, the established macrophage-like RAW-264.7 cell line was used to generate template mRNA for the purposes of optimising the qPCR test parameters and confirmation of primer product size by gel electrophoresis (see section 2.5.3 and appendix C). Following optimisation using RAW-264.7 cells, the expression of IL-12p40 was evaluated in MG isolated from the brains of mice, and the melt temperature of the products was the same as seen with the RAW-267.4 cells. However, due to the low yield of cells in this experiment, little mRNA was obtained from each individual mouse such that in some treatment groups (untreated, immunised and unimmunised), there was insufficient RNA for qPCR. Preliminary results were obtained from the IC-treated groups and suggest that IL-12p40 is upregulated in sick mice compared to not sick mice (Figure 7.10). However, this result should be interpreted cautiously due to large variability in the unimmunised group, which was used to calculate the $\Delta(\Delta Ct)$ value. Despite this variability, IL-12p40 has previously been shown to be increased in the spinal cords of sick mice compared to mice in relapse (Issazadeh, Navikas, Schaub, Sayegh, & Khoury, 1998), and therefore the increased expression of IL-12p40 in the CNS of sick mice compared to not sick mice in this study is consistent with previous work. Overall, these preliminary qPCR findings support the use of bone marrow chimeras as a useful method to study the effects of IC treatment on the resident MG *in vivo*, and future work using this method can investigate the specific effects of IC treatment on other markers and cytokines such as CD40, PD-L1, MHC class II, and IL-10.

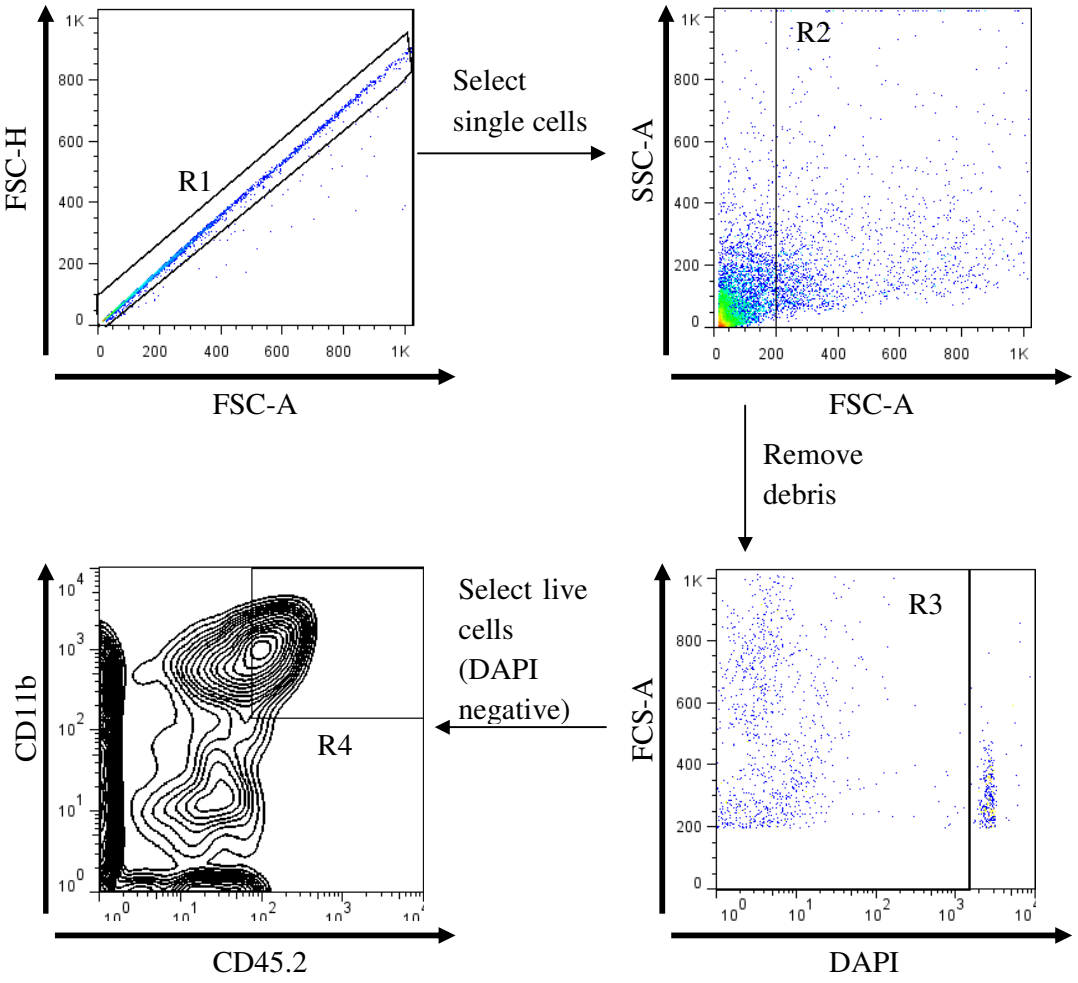



**Figure 7.9. Gating strategy for isolation of MG from B6→C57 mice by FACS.** Seven weeks following irradiation and reconstitution of B6→C57 animals, mice were pre-treated with $2x10^6$ IC by i.p. injection one week before immunisation and weekly thereafter. Mice were immunised to induce EAE as described in the methods. At peak disease brains and spinal cords were processed for FACS analysis as described in methods and isolated using the gaiting strategy described. R1, single cells; R2, cells (without debris); R3, live cells (DAPI negative); R4, $CD45.2^+CD11b^+$. Shown is data from one immunised mouse.

a.

| Immunisation state | Treatment | Disease state | n | ΔCt* (mean ± SEM) | Δ(ΔCt)# (mean ± SEM) | Fold change ($2^{-\Delta\Delta Ct}$) |
|---|---|---|---|---|---|---|
| Unimmunised | IC treated | Not sick | 2 | 9.37±2.69 | 0.0±2.69 | 3.3±3.14 |
| Immunised | IC treated | Not sick | 3 | 7.33±0.32 | -2.03±0.32 | 4.2±0.9 |
| Immunised | IC treated | Sick | 2 | 8.9±0.69 | -0.47±0.69 | 1.7±0.71 |

*Ct(IL-12p40)-Ct(Cyclophilin A)

#ΔCt sample- average ΔCt of unimmunised (physiological control)

b.

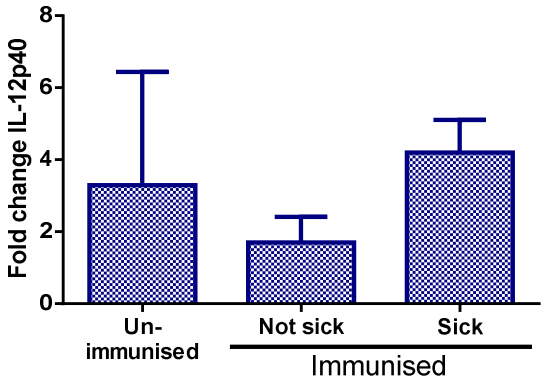



**Figure 7.10. Expression of IL-12p40by resident MG after IC treatment.** Seven weeks following irradiation and reconstitution of B6→C57 animals, mice were pre-treated with $2x10^6$ IC by i.p. injection one week before immunisation and weekly thereafter. Mice were immunised to induce EAE as described in the methods. At peak disease mice were euthanised and MG ($CD45.2^+CD11b^+$) were sorted by FACS from individual mice (Figure 7.9). (a) Total mRNA was isolated from MG, cDNA was generated, and IL-12p40 cDNA amplified with gene-specific primers using qPCR. IL-12p40 was normalised to the control gene cyclophilin A (ΔCt) and change relative to a physiological control (IC treated, unimmunised mice) was calculated (i.e. Δ(ΔCt)). Fold change relative to control was generated using the formula: fold change IL-12p40 = $2^{-\Delta(\Delta Ct)}$. (b) Shown are the mean ± SEM of expression relative to IC treated, unimmunised mice and are from one experiment.

## 7.4 Discussion

In this study, the role of type II activating treatments on MG was assessed. This treatment involves the intra-peritoneal injection of IC 7 days before the induction of EAE and every 7 days for the duration of the experiment and has previously been shown to be highly effective at suppressing EAE (La Flamme et al., 2006). The work in this chapter demonstrated that IC treatment was protective in EAE and induced an increase in the production of IL-17A in the peripheral immune system. In addition, IC treatment resulted in decreased expression of MHC class II expression on CNS associated MΦ and a trend towards decreased levels of Iba1 in certain brain regions such as the cerebellum of immunised mice. The use of bone marrow chimeras to allow the isolation and characterisation of MG for assessment MG activation *in vivo* was optimised. Together this work indicates that IC treatment not only alters peripheral immune responses but also has distinct effects on the phenotype of CNS-associated MΦ and MG.

In the current study IC treatment was protective in EAE, as seen by reduced disease scores and decreased incidence of disease. However, the disease course displayed a high degree of variability between experiments, and there are several possibilities that may explain this variability. First, it is possible that the age of the mice affected the disease course since previous studies in SJL/J mice have shown that mice under the age of 6 weeks are resistant to EAE (Smith, Eller, McFarland, Racke, & Raine, 1999). In addition, aged BALB/c mice are resistant to EAE when immunised at 18 and 24 months (Endoh, Rapoport, & Tabira, 1990) suggesting that EAE is not optimally induced in young or old mice. In the current study the age of mice at the time of immunisation was 17.93±0.822 weeks (mean±SEM, from 6 experiments). In contrast, C57BL/6 mice are most commonly used for EAE studies between 8 and 12 weeks. Because chimeras require 6-8 weeks following irradiation to ensure full reconstitution of the bone marrow, the use of older mice could not be avoided. While the use of mice of this age were not resistant to disease, it is likely that the age may be responsible for the highly variable disease course.

In a previous study from our laboratory, it was shown that mice treated with IC were resistant to EAE and had enhanced basal Th2 responses (La Flamme et al., 2006); however, Th17 and IL-17A production, in particular, was not assessed. Because the results from this thesis indicate that IL-17A production by T cells is enhanced by type II activation, the peripheral

immune response was investigated in the current study. While IC treatment showed a trend towards increased production of polyclonally stimulated IL-4, there was no augmentation in antigen-specific IL-4 production. Additionally, IC treatment did not alter either antigen specific or polyclonally stimulated IFN-γ production by splenocytes isolated from immunised mice, and this result is consistent with previous reports (La Flamme et al., 2006). As expected, immunisation of mice led to an increase in both antigen-specific and polyclonal production of IL-17A. However, in agreement with the *in vitro* co-culture results, antigen specific production of IL-17A was increased in the culture supernatant of splenocytes from IC treated, immunised mice compared to untreated, immunised mice. This study is the first to report that IL-17A is increased under type II activating conditions *in vivo*.

The increased production of IL-17A is interesting given that IL-17A is considered highly pathogenic in EAE (Bettelli et al., 2006; Domingues et al., 2010; O'Connor et al., 2008), and yet this pathogenic cytokine is induced by a treatment that provides protection in this model. The phenotype of circulating and CNS infiltrating T cells were not assessed in the current study; therefore, it is not known if the IL-17A is being produced by Th17 cells or another cell type such as IL-17A expressing Th1 cells. It has previously been shown that in some circumstances Th17 cells are capable of producing both IL-10 and IL-17A, and that the production of IL-10 is dependent on IL-6 and TGF-β (Esplugues et al., 2011; McGeachy et al., 2007; Xu et al., 2009). In addition to being synergistic, both IL-6 and TGF-β are capable of increasing IL-10 alone (Xu et al., 2009). Furthermore, these IL-10 producing Th17 cells have been shown to have immunosuppressive properties in both EAE and a model of intestinal inflammation (Esplugues et al., 2011; McGeachy et al., 2007), and it was demonstrated that the protection provided by antigen specific IL-10 producing Th17 cells was dependent on the IL-10 production (McGeachy et al., 2007). Thus, an increase in IL-17A does not necessarily correlate with an increase in disease severity.

While IL-10 was not measured in the current *in vivo* IC treatment study, it has been shown previously that IC treatment increases the production of IL-10 by splenocytes and in the spinal cord of mice with EAE (La Flamme et al., 2006). The IL-10 produced in IC-treated mice may be derived from many sources including MΦ and T cells, and it is possible that some of this cytokine is being produced by IL-10 producing Th17 cells. Furthermore GA, which produces a similar MΦ activation state as IC induces up regulation of TGF-β by monocyte/MΦ (Weber et al., 2007). Therefore, the type II activated MΦ, which also express

TGF-β, could induce IL-10 producing Th17 cells. Therefore, an analysis of the peripheral immune cells in IC treated immunised mice, compared to untreated immunised mice will be important to further establish the cellular sources of IL-17A and determine if this cell type also produces other cytokines, such as IL-10. The exact mechanism by which IC treatment mediates protection, even in the presence of increased IL-17A has not yet been elucidated, but is an extremely interesting feature of this treatment.

In this model, where IC is given by intraperitoneal injection, it is highly likely that peripheral MΦ/monocytes will come into direct contact with IC. For the same reasons, it is much less likely that MG will be directly activated after an intraperitoneal injection of IC. Movement of IC into the CNS would likely be inhibited by the BBB even considering that the BBB breaks down in EAE (Carson et al., 2006). Moreover, the delivery of the IC by intraperitoneal injection would only have limited entry into the blood compared to intravenous injection. Given the unlikelihood of MG being directly exposed to IC, it is more probable that IC treatment affects MG through an alteration in the peripheral immune response. For example, type II-activated MΦ/monocytes invading into the CNS may expose the MG to suppressive cytokines such as IL-10, which through a bystander effect could prevent harmful activation of MG in EAE and possibly promote a protective phenotype. A similar bystander effect was reported by Sica et al who showed that MΦ are able to suppress neighbouring cells through the production of IL-10 (Sica et al., 2000). Alternatively, MG activation could be altered by humoral factors such as serum cytokines. Indeed, it has been shown that changes in the systemic immune environment can affect MG, for example, induction of peripheral inflammation has been shown to increase MG activation in the CNS (Cardona et al., 2006; Thompson, Karpus, & Eldik, 2008). This suggests that a shift in the peripheral immune response induced by IC treatment may be a pathway by which resident MG activation could be altered.

To further assess the effect that IC treatment has on the activation state of MG *in vivo* brains from C57→C57 mice were assessed by flow cytometry. It was intended that the study of MG *in vivo* would be conducted in BALB/c mice due to the increased number of lesions found in the brains of this strain compared to C57BL/6 mice (Personal communication, Dr Jacqueline Orian). Analysis of MG activation in the brain of BALB/c mice would have provided a larger pool of MG located at the site of inflammation as well as a more relevant MG population. However, it was found that neither IC treatment, nor GA, a common treatment for MS was

strongly protective in EAE (see appendix C). Both GA and IC affect MΦ and promote Th2 responses (Aharoni, Teitelbaum, Sela, & Arnon, 1997; Anderson & Mosser, 2002; La Flamme et al., 2006; Weber et al., 2007). This suggests that these kinds of treatments have lower efficacy in BALB/c mice, therefore the C57BL/6 model was employed instead. Although the majority of CNS lesions are found in the spinal cord (Pham et al., 2011, Personal communication, Dr Jacqueline Orian) and comparatively fewer infiltrates are seen in the brains in the C57BL/6 model of EAE, infiltration and microglial activation still occurs. Thus, the brain was chosen for flow cytometric analysis as it is likely that the majority of the MG cells isolated for qPCR were from the brain, and MG activation in the brain was assessed by immunohistochemical staining. Moreover, because fewer lesions form in the brains during peak disease (d15), the majority of myeloid cells would be expected to be from resident populations (MG or CNS-associated MΦ).

In the myeloid compartment of the CNS, 3 distinct populations were identified based upon CD45 and CD11b co-expression. The $CD45^{hi}CD11b^{++}$ cells are representative of peripheral MΦ but may also contain activated MG which are known to upregulate CD45 upon activation (Ponomarev et al., 2006; Sedgwick et al., 1991). The possibility that MG may contribute to the $CD45^{hi}CD11b^{++}$ population is supported by the decrease in the percentage of MG cells in the brains of immunised mice. However, it is possible that this decrease is due to alterations in the proportions of cells in the myeloid compartment following infiltration; it is difficult to say conclusively which situation has occurred in C57→C57 chimeric mice where resident and peripheral cells express the same CD45 allele. The $CD45^{hi}CD11^{++}$ population contains both a $Gr1^{+}$ and a $Gr1^{-}$ population (see gating strategy in the appendix D). Gr1 is commonly used to identify neutrophils but actually is specific for two markers, Ly-6C on monocytes and Ly-6G on neutrophils (Slaney, Toker, La Flamme, Bäckström, & Harper, 2011). As it is not possible to differentiate between neutrophils and monocytes in the current study, and as the presence of another population would likely have confounding effects on the analysis of cell surface molecules on monocytes, this population was not analysed further.

Unactivated MG have previously been described in the literature as having either a low or low-intermediate expression of CD45 (Juedes & Ruddle, 2001; Ponomarev et al., 2006; Sedgwick et al., 1991). In the current study, two resident population were identified; $CD45^{int}CD11b^{+}$ and $CD45^{lo}CD11b^{+}$. While the $CD45^{lo}CD11b^{+}$ population is certainly a MG population, the cells which constitute the $CD45^{int}CD11b^{+}$ population are less clear in the

literature. A study by de Haas, Boddeke, & Biber, (2008) suggests that MG from different CNS regions display different levels of CD45 expression. This study showed that MG isolated from the spinal cord of adult C57BL/6 mice express higher levels of CD45 than MG isolated from the hippocampus (de Haas et al., 2008). It is possible that this different may also exist between other parts of the brain (e.g. MG from the cerebellum, cortex and hippocampus may have different resting levels of CD45). This finding raises the possibility that the $CD45^{int}CD11b^{+}$ cells may represent a subset of MG. If this is the case care must be taken when using whole brain analysis as different populations of MG may respond differently under inflammatory conditions.

An alternative explanation for the $CD45^{int}CD11b^{+}$ population is that they represent a population of CNS-associated MΦ, such as perivascular MΦ, as opposed to a resident MG cell found in the CNS parenchyma (Zhang et al., 2002). There are several populations of CNS-associated MΦ, including perivascular MΦ, and MΦ found in the choroid plexus and the meninges. These sites are located between the CNS parenchyma and the periphery and are all considered potential sites for entry of encephalitogenic T cells into the CNS (Ford, Goodsall, Hickey, & Sedgwick, 1995; Ransohoff et al., 2003). It has been shown in rats that these CNS-associated MΦ are more efficient at presenting to T cells *ex vivo* than MG cells (Ford et al., 1995). T cell require reactivation in the CNS for EAE to develop (Kivisäkk et al., 2009). As CNS-associated MΦ are found at locations that are associated with CNS infiltration, it is possible that they have important roles in antigen presentation and the reactivation of T cells. Indeed, MHC class II positive cells have been shown to interact with T cells in the leptomeningies and these cells are thought to play an important role in EAE (Kivisäkk et al., 2009). Therefore, it is possible that changes to this subset may alter either the invasion or reactivation of T cells entering the CNS.

In the current study, CNS-associated MΦ ($CD45^{int}CD11b^{+}$) from IC-treated, immunised mice expressed lower levels of MHC class II, and had fewer MHC class II positive cells compared to untreated, immunised mice. It is possible that this change is associated with the protection from EAE provided by type II treatments, as lower MHC class II could potentially reduce T cell reactivation and thus disease severity. The cause of the IC mediated decrease in MHC class II is currently unknown, it is possible that IC treatment induces a less inflammatory environment, resulting in decreased activation of the CNS-associated MΦ and thus decreased MHC class II expression. Alternatively, the MHC class II molecules may be being actively

sequestered by regulatory molecules such as MARCH-1. MARCH-1 is an E3-ubiquitin ligase which is known to be involved in the regulation of cell surface levels of MHC class II (Ohmura-Hoshino et al., 2009; Thibodeau et al., 2008). IL-10 has been shown to decrease MHC class II expression on human monocytes via a MARCH-1 dependent mechanism (Thibodeau et al., 2008). As increases in IL-10 have been shown to occur in the spinal cords of immunised mice treated with IC (La Flamme et al., 2006), it is possible that a similar mechanism may be involved in regulating cell surface levels of MHC class II on CNS-associated MΦ in IC treated mice. It has previously been shown that type II MΦ upregulate expression of MHC class II compared to classically activated MΦ (Edwards et al., 2006). Therefore the phenotype of the CNS-associated MΦ in IC treated immunised mice may not mirror type II activation completely. As MΦ activation occurs in a spectrum, these different populations of MΦ may share some immunoregulatory properties while maintaining some distinct characteristics.

In the current study, the $CD45^{lo}CD11b^{+}$ resident MG cells did not alter their expression of CD40 or MHC class II molecules. However, in both immunised groups there was a strong trend towards increased expression of PD-L1 compared to immunised mice, and this difference was significant between the IC treatment groups. It has previously been shown that PD-L1, which is inhibitory to T cells, is upregulated on MG at peak disease (Carter et al., 2002; Ortler et al., 2008). As the samples in this study were isolated at peak disease, these data are consistent with previous results. Although other important co-stimulatory molecules such as CD80 were not assessed, the absence of an increase in CD40 and MHC class II, together with the increase in PD-L1 suggest that the MG have been induced to a state that may be suppressive to T cells. Since both immunised groups expressed the same overall pattern on MG cells, it is unlikely that these changes are associated with the mechanism by which IC protect from EAE.

It has previously been demonstrated that the expression of CD40 on MG is important for the development of EAE (Becher et al., 2001). Based upon this study it was expected that MG cells would up regulate CD40 to induce disease, however no change in CD40 expression was seen in our study when unimmunised and immunised mice were compared. One possible explanation is that the MG cells may not express the same phenotype throughout the course of disease. As the previous study suggests a role of CD40 in the initial stages of EAE (Becher et al., 2001), peak disease may be too late a time point to pick up changes in CD40 expression.

Additionally, MG have been shown to up-regulate expression of CD45 following activation (Melchior et al., 2006; Ponomarev et al., 2006; Sedgwick et al., 1991), and therefore it may be that the $CD45^{lo}CD11b^{+}$ cell population is composed primarily of unactivated or not fully activated MG, which would not have altered CD40 expression.

Although flow cytometry data did not show that IC treatment altered the expression of common immune activation markers on resident MG in the CNS, immunohistochemical analysis demonstrated that in at least some areas of the brain, such as the cerebellum and the hippocampus there was a trend towards increased Iba1 staining, suggesting differential activation of MG occurred between the IC treated and the untreated immunised groups. These MG have a ramified phenotype, suggesting they are parenchymal MG, and the level of Iba1 staining in the cerebellum showed a strong trend towards decreased staining in immunised mice with IC treatment. Because the cerebellum is a commonly affected area in MS, this finding may be particularly relevant to disease (Frohman et al., 2006; Lassmann, Brück, & Lucchinetti, 2007). It is likely that the discordance between the flow cytometry data and the immunohistochmistry data is due to the exact markers investigated. The change in Iba1 expression on resident MG cells between the two immunised groups is most marked in the cerebellum, and a trend towards altered expression in the hippocampus was also apparent. However, these region specific changes suggest that the MG in all parts of the brain may not express the same level of certain markers and so the effect of a reduction in only one or two brain regions may be lost when looking at MG isolated from the whole brain (de Haas et al., 2008). Furthermore, the region specific changes in Iba1 may also suggest that different parts of the brain are exposed to different levels of stimulation in EAE. Taken together, these data suggest that a differential activation state of MG exists in IC treated immunised mice although the functional consequence of this change is not yet known.

Bone marrow chimeric mice were generated so that MG could be isolated from the brains of mice with EAE, without the infiltrating cells interfering with the purity of the MG sample. This technique is commonly used for the study of MG; however, as it had not been used previously in our laboratory, the method required optimisation. While irradiation and reconstitution of mice with bone marrow from a congenic mouse strain consistently worked well, several other technical aspects including the FACS sorting and the RNA isolation required more intensive optimisation. In particular, although the $CD45.2^{+}$MG were easily detected on the FACS Canto II, they were difficult to identify using the FACS Vantage DiVa

cell sorter on the basis of CD45.2 expression alone. However, the addition of CD11b staining aided the identification of MG on this machine, and MG were isolated with this method.

To isolate the RNA from the purified $CD45.2^+$ MG Trizol® was initially used but the resulting RNA was too dilute to be useful for qPCR and so the Dynabeads mRNA DIRECT Micro kit was utilised with greater success for the last experiment. Thus, the results for the level of IL-12p40 transcript were obtained from a single experiment, they provide support for the use of this model to answer questions about how MG change during neuroinflammation. Additionally, while these data are preliminary, they show that the level of IL-12p40 increased in mice that had established disease compared to mice that had been immunised but had no overt disease expression. This finding is consistent with reports that show increased IL-12 in the CNS of mice that have EAE (Issazadeh et al., 1998; La Flamme et al., 2006). Finally, as IL-12p40 is the common subunit of both IL-12 and IL-23 (Cua et al., 2003), the possibility that the elevated IL-12p40 represents an increase in IL-23 as opposed to IL-12 must be considered.

## 7.5 Summary

The overall aim of the current chapter was to assess the effect IC treatment had on MG *in vivo*, and to determine if any changes in the peripheral immune environment correlated with that seen in the MG. Although the disease course was variable between experiments, IC treatment was shown to provide protection from EAE. The protection afforded by IC treatment was not associated with an alteration in IFN-γ but instead with an increase in antigen specific IL-17A. Because GA, which induces a similar MΦ activation state as IC, leads to an increase in TGF-β, future research should assess the involvement of IL-10 and TGF-β in protection as well as the possibility that an IL-10/IL-17A Treg population may be induced.

Immunohistochemical analysis of brain sections suggested that MG from IC treated mice are differentially activated in EAE, and although a change in this population was not seen by flow cytometry, only a small pool of markers was assessed. These markers were chosen as they are relevant to type II MΦ activation, yet it is possible there were changes in expression markers that were not assessed. A significant change in the expression of MHC class II on CNS associated MΦ was seen by flow cytometry. As these MΦ are located at sites associated

with CNS infiltration, it is possible that these cells are involved in re-activation of T cells. The decrease in MHC class II may therefore reduce the interaction of T cells and CNS associated MΦ, and provide a potential pathway for protection from disease. Finally, to more comprehensively assess the effect of type II activation by IC treatment on CNS resident MG, a bone marrow chimera model was successfully established and validated for future work.

# Chapter 8:
# General Discussion

## 8.1 Overview

MS is an immune-mediated disease of the CNS, in which the myelin sheath and the neuronal axons which they surround, are damaged leading to a variety of disabilities in MS patients (Sospedra & Martin, 2005). The pathology of both MS and EAE, one of the animal models of MS, involve proinflammatory processes (Baxter, 2007; Sospedra & Martin, 2005), and it has been shown that treatments which induce deviation of the immune response, such as GA, are protective in MS and EAE (Khan et al., 2001; La Flamme et al., 2006; Tierney et al, 2009; Weber et al., 2007). The administration of IC is another such immunomodulatory treatment, which has been shown to be protective in EAE (La Flamme et al., 2006; Tierney et al., 2009). IC (in conjunction with LPS) generate immunoregulatory type II MΦ, which have the capacity to bias the T cell response in an antigen-specific manner (Anderson & Mosser, 2002). Thus the cellular pathways by which IC and type II MΦ modulate T cell responses and protect during EAE were investigated in depth.

Classically activated MΦ have been shown to bias the T cell response towards a Th1 response, and type II MΦ towards a Th2 response (Anderson & Mosser, 2002; Edwards, Zhang, Frauwirth, & Mosser, 2006). However, the effects these activation states have on other Th subsets had not previously been assessed. Chapter 3 investigated further the effects of type II MΦ on T cells. Consistent with previous studies, T cells cultured with type II activated MΦ produce decreased levels of IFN-γ compared to those cultured with classically activated MΦ (Anderson & Mosser, 2002). T cells cultured with type II MΦ also expressed increased levels of CD124, suggesting an increased ability to respond to IL-4. While IL-4 could not be measured in this study, possibly due to its rapid uptake following production, CD124 was used as a surrogate for IL-4 production. The change in CD124, together with the decrease in IFN-γ is consistent with a switch from a Th1 to a Th2 response. However, it was also found that T cells cultured with type II MΦ also produce increased levels of IL-17A, compared to those cultured with classically activated MΦ. The increase in both CD124 and IL-17A suggests the presence of a mixed Th2/Th17 phenotype, and is a novel finding of this study. Investigations into possible pathways involved in T cell biasing by MΦ (Chapter 4) revealed that IL-10 and IL-12, which have significantly altered profiles between classical and type II MΦ, are important in regulating IFN-γ production but do not have a significant role in regulating CD124 or IL-17A levels. Additionally, results suggest that the effect of IL-10 is primarily on the MΦ itself while the effect of IL-12 is primarily on the T cell. This is

consistent with previous reports that IL-10 and IL-12 are known to have strong effects on MΦ and T cells respectively (Kaplan et al., 1996; Kobayashi et al., 2012).

Further investigation into the pathways involved in T cell biasing by type II activated MΦ investigated the roles of PD-L1 and CD40, which have decreased expression on the surface of type II MΦ. These studies suggested that these individual pathways do not have a strong individual role in T cell biasing in this system although the possibility remains that a combined alteration in these two pathways may induce a much greater effect than each pathway alone. Further experiments into these markers are required to fully understand any role they may play as the absence of any effect could be due to the αPD-1 and the PD-L1ch being in active. In order to address this, and also remove the potentially confounding effects of the control antibodies/proteins, other approaches could be used, such as using genetically deficient mice or RNAi to block pathways.

As type II MΦ express lower levels of PD-L1 (Tierney et al., 2009), it was hypothesised that T cells cultured with these MΦ would have higher rates of proliferation, compared to those cultured with classical MΦ, due to a decrease in inhibitory factors. However, results for Chapter 3 demonstrated that, while type II and classically activated MΦ did activate T cells, neither of these MΦ activation states were able to induce T cell proliferation. Both classically activated and type II MΦ produce high levels of NO and inhibition of NO synthesis using AG restored the ability of MΦ to induced T cell proliferation, however, no significant difference in proliferation was seen between T cells cultured with classically or type II activated MΦ. Proliferation is the hallmark of antigen specific response, however, as classically and type II MΦ do not typically induce T cell proliferation in this study, proliferation was not routinely used as a readout of T cell activation.

As MG are the MΦ like cell of the CNS and are considered an important cell type in regulating immune responses in the CNS, the ability of MG to be type II activated was investigated. In chapter 6, the activation state of adult MG was assessed under type II and classical activating conditions *in vitro*. It was found that MG were capable of a type II like activation state, producing increased levels of IL-10 and decreased levels of IL-12 compared to classically activating conditions. In addition, type II MG produced decreased levels of IL-6. Like type II MΦ, these type II MG were able to bias T cell response, yet, the biasing by MG did not result in the same T cell phenotypes as those induced by MΦ. For example, MG

were able to stimulate T cells to produce IL-2 regardless of the stimuli they were given while MΦ required activation (with LPS or LPS+IC) to be able to stimulate IL-2 production by T cells. This result suggests that a fundamental difference in the way MG and MΦ interact with T cells may exist. Some features of type II MΦ:T cell interactions were similar in MG:T cell co-cultures, namely T cells cultured with type II MG produce higher levels of IL-17A and CD124. It is therefore difficult to predict how type II MG would affect T cell responses *in vivo*. However, the reduction in the levels of proinflammatory cytokines produced by type II activated MG, such as IL-6 and IL-12, suggests that type II activation of MG may be beneficial in neuroinflammatory conditions.

The role of type II inducing treatments *in vivo* was investigated using B6→C57 and C57→C57 chimeric mice. IC treatment has previously been shown to be protective in EAE and the results of the current study are consistent with previous data (La Flamme et al., 2006). Analysis of peripheral immune responses demonstrated that IC treated immunised mice have higher levels of antigen specific IL-17A, which is consistent with *in vitro* results where type II MΦ and MG also induced IL-17A production when in co-culture with T cells. It is interesting that an increase in IL-17A has been seen consistently throughout this thesis, under conditions that are considered to be anti-inflammatory or regulatory. Therefore, it is likely that the same mechanism is inducing the increase in IL-17A in all of these different type II activating situations. The increase in antigen specific IL-17A is seen in IC treated mice, which are protected from EAE; this suggests the increase in IL-17A may not be pathogenic, despite the well-known detrimental effect IL-17A can have in EAE (Bettelli et al., 2006; Kurschus et al., 2010; McGeachy et al., 2007; O'Connor et al., 2008). There is a subset of Th17 cells, sometimes referred to as "regulatory Th17 cells" which produce both IL-10 and IL-17A and have been shown to have immunoregulatory effects in EAE and a model of inflammatory bowel disease (Esplugues et al., 2011; McGeachy et al., 2007). It is possible that IC treatment and type II MΦ/MG are capable of inducing these "regulatory Th17 cells", and this possibility merits further investigation.

Further *in vivo* data demonstrated an alteration in the phenotype of resident cells in the CNS of IC-treated, immunised mice compared to untreated immunised mice. These included decreased levels of Iba1 staining on parenchymal MG, which suggest differential activation state of MG from IC-treated, immunised mice compared to untreated, immunised mice. Furthermore, resident CNS-associated MΦ ($CD45^{int}CD11b^{+}$) from IC-treated, immunised

mice expressed decreased levels of MHC class II, suggesting that these cells, which are likely located in areas associated with T cell infiltration into the CNS, may have a decreased ability to interact with and re-activate encephalitogenic T cells. At this point the current data does not demonstrate that MG from IC-treated mice take on a phenotype that is identical to the type II MΦ activation state *in vitro*; however, there is evidence to suggest that resident CNS cells may be activated to a regulatory phenotype *in vivo*. As MΦ activation occurs on a spectrum, it is possible that the CNS resident cells from IC-treated, immunised mice may share some characteristics of type II activation.

## 8.2 Use of animal models for the study of MS

MS is an inflammatory disease of the CNS in which demyelination occurs around the neuronal axons. MS has many forms, including the relapsing-remitting and the progressive forms; and it is possible that MS is a group of related disorders that share symptoms and pathophysiological characteristics, rather than a single disease (Heard, 2007). Furthermore, even amongst a particular disease form such as relapsing-remitting, there is wide variability in primary symptoms, disease progression, relapse rate, and even response to the current treatment options (e.g. IFN-β) (Axtell et al., 2010; Heard, 2007). The heterogeneous nature of the MS thus complicates investigations into its nature as well as potential treatments (Weiner, 2009). Finally, because MS is a disease of the CNS, a detailed, molecular study of this disease is difficult, with many studies relying on access to brain sections from autopsy or biopsy. Therefore, a suitable animal model that will allow a mechanistic dissection of the pathogenesis of MS is extremely desirable, and the current animal models of MS have been useful for developing treatments for MS as well as better understanding the underlying disease mechanisms.

EAE is the most commonly used animal model of MS. EAE has been induced in a wide variety of animals, ranging from mice to non-human primates (Baxter, 2007). The disease (EAE) which develops is not identical between species and varies dependent on the species as well as strain used. Different models of EAE model different aspects of MS pathology, for example, the BALB/c mouse model produce a more faithful model of human CNS lesions compared to other mouse strains, while the SJL/J mouse model has a relapsing remitting disease course. EAE has been used successfully to develop three treatments for MS: glatiramer acetate, natalizumab, and mitoxantrone (Baxter, 2007; Steinman & Zamvil, 2006; personal communication Dr Jacqueline Orian).

In addition to roles in developing treatments, EAE also has other uses. EAE studies can be employed to understand the roles molecules and pathways that have been identified in studies on MS patients, and which of these molecules may be potential therapeutic targets (Steinman & Zamvil, 2006). For example, IL-6 and IL-17A (both important in the Th17 response) were identified in MS lesions; studies in EAE later identified Th17 cells as an important cell type in EAE, and EAE was used to investigate this pathway (Bettelli et al., 2006; Lock et al., 2002). Additionally, osteopontin was identified in MS lesions, following this discovery osteopontin deficient mice were then found to have decreased EAE severity (Steinman & Zamvil, 2006). Serum levels of ostepontin is being investigated as a potential biomarker for MS (Shimizu et al., 2013). EAE has also been used to better understand the mode of action of current treatments such as GA (Weber et al., 2007).

In recent years EAE has been criticised due to its inability to predict possible side effects, and also because EAE has not produced many new treatments for MS in recent years. Additionally the current treatments are not viewed as effective enough and not all patients are responsive to the current treatments. PML is the most notable example of preclinical studies in EAE failing to predict a potentially life threatening side effects. Due to species barriers, the possibility of PML occurring could not have been tested (Steinman & Zamvil, 2006). Like many animal models, EAE has limitations in the area of opportunistic infections following treatment, particularly where species barriers are involved.

Part of the inefficacy of the current treatments may be related to the heterogeneity of MS. For the development of further MS treatments, it will be important to understand these inter-patient differences, and for this patient studies will be a vital source of information. However, EAE is also proving useful in understanding how patient differences can lead to different treatment outcomes, as it can be used to ask functional questions based on patient data. For example, current studies in EAE, that have been based on patient data, suggest that relapsing remitting patients with a Th17 dominate Th cell response are much less susceptible to IFN-β treatment than those with a Th1 dominant response (Axtell et al., 2010). Additionally, employing multiple animal models, which model different disease aspects, may help to address the patient heterogeneity and provide a better idea of which treatments are likely to translate into effective treatments for MS.

It is likely that the mechanisms are different between relapsing-remitting and progressive forms of the disease ('t Hart, Gran, & Weissert, 2011; Weiner, 2009). As such, different models may need to be employed to study the different disease subsets. EAE has been useful for studying relapsing remitting MS, but is not a good model for progressive forms of MS, however there are other models (Ransohoff, 2012). An example of a toxin based model of MS is cuprizone (a copper chelator given in chow), which induces ogliodendrocyte death (demyelination) and may prove to be an effective model of progressive disease (Ransohoff, 2012). Cuprizones mode of action is independent of T and B cells (Hiremath, Chen, Suzuki, Ting, & Matsushima, 2008), allowing the processes of demyelination and remyelination to be examined independent of adaptive immune involvement. MS involves multiple processes, while treatment strategies to prevent further disease development are important, it is also important to investigated ways to reverse the damage in the CNS to aid recovery (Weiner, 2009). After removal of cuprizone from the diet remyelination occurs thus the cuprizone model may be applied, not only to progressive disease, but also to study remyelination, and potentially develop treatments to increase remyelination, which may aid patient recovery and complement disease modifying therapies (Ransohoff, 2012; Weiner, 2009).

Both EAE and the cuprizone models have limitations (as all animal models do). EAE has proved effective in the development of treatments for MS, and is likely to be capable of producing more. Further refinement of the way EAE is used; applying the different models to answer different questions may improve its efficiency. In addition, the combination of studies in humans with studies in EAE has real potential to further the understanding of how MS works and may provide new treatment strategies.

## 8.3 Clinical applications

While there are several drugs available for the treatment of MS only two, GA and IFN-β, are considered first line drugs based on their safety profiles (Morris & Yiannikas, 2012). The second line treatments such as natalizumab and mitoxantrone have more limited use due to their potential side effects (Morris & Yiannikas, 2012). However, GA and IFN-β have limited efficacy, and are only capable of reducing the relapse rate of relapsing remitting MS by approximately 30% (Khan et al., 2001). Therefore, there is a need for both safer and more effective treatments for MS patients. While there are a range of new MS treatments that have recently become available to treat relapsing-remitting MS such as Gilenya (Fingolimod or FTY720) and Lemtrada (Alemtuzmab), the full impact of these treatments on disease and their side-effect profiles still need to be fully confirmed (Morris & Yiannikas, 2012).

GA, a common drug for the treatment of MS induces a change in the phenotype of MΦ/monocytes that is similar to type II activation, with GA-treated monocytes expressing a similar altered cytokine profile and an ability to bias T cells towards both Th2 and Treg responses (Weber et al., 2007). This study suggests that this type II activation of MΦ may be responsible for the protection mediated by GA and supports the idea that this approach is a viable one for MS therapies. However, IC treatment and GA treatment, while inducing similar MΦ activation states, may work through different mechanisms to protect from EAE. For example, in the current study, IC treatment was shown to consistently cause an increase in IL-17A in both *in vivo* and *in vitro* experiments. However, to our knowledge, GA has never been shown to upregulate IL-17A, and has been reported to decrease the production of IL-17A, in the periphery and in the CNS of mice with EAE (Rina Aharoni et al., 2010; Weber et al., 2007). Additionally, preliminary work in our lab has shown that in the same MΦ:T cell co-culture system, T cells co-cultured with GA treated MΦ did not produce IL-17A (see appendix C). Thus, while similar to GA, IC does not appear to completely mimic the effects of GA *in vivo* and therefore may represent an alternate treatment option.

IFN-β is one of the most commonly used drugs for the treatment of MS. However, in addition to only reducing the relapse rate by 30%, it is not successful in all patients and up to 30% of patients on IFN-β treatments generate neutralising antibodies which render the treatment ineffective (Bertolotto et al., 2002; Capobianco et al., 2008; Khan et al., 2001). Therefore, IC treatment could potentially provide a treatment for patients who do not respond or GA or IFN-β. In addition, work performed in our laboratory on human monocytes suggests that using IC in conjunction with GA may provide further benefit, raising the possibility of combined GA+IC therapy for the treatment of MS (Personal communication, Dr Delgertsetseg Chuluundorj, M.D. PhD; Victoria University of Wellington; manuscript in preparation). In addition to increasing the efficacy of treatment, using combined therapies may also allow the doses of the individual drugs to be reduced, which could potentially result in a reduction in side effects without a reduction in the efficacy of the treatments.

Intravenous immunoglobulin (IVIG), which is generated from pooled IgG serum from a large number of donors, was originally used to treat hypogammaglobulinaemia but has since been used for several other conditions including Guillain–Barré syndrome, rheumatoid arthritis, and MS (Schwab & Nimmerjahn, 2013). In clinical trials, IVIG has been found to be

effective in RRMS. These trials showed that IVIG treatment reduced relapse rate and had a beneficial effect on patient disability (Sorensen, Fazekas, & Lee, 2002). In EAE, IVIG treatment has been shown to protect from disease when given prophylactically, and protection is associated with induction of Treg cells (Ephrem et al., 2008; Humle Jorgensen & Sorensen, 2005).

The mechanism of action of IVIG is unclear and likely to involve many processes (Schwab & Nimmerjahn, 2013). As IC consist of an antibody-coated cell or molecule, it is possible that IVIG and IC may share some overlapping pathways. IC are not likely to perform some of the proposed functions such as cytokine and autoantibody neutralisation, nor are they likely to act through the inhibitory FcR, FcγRII, which is a proposed pathway for IVIG but is not involved in type II activation of MΦ (Schwab & Nimmerjahn, 2013; Sutterwala et al., 1998). It has been suggested that some of the effects of IVIG are due to effects on innate cells such as DC, as when DC are treated with the IVIG *in vitro* and then transferred to mice they are protected from immune thrombocytopenic purpura (Siragam et al., 2006). IC treatments are thought to act mainly on innate immune cells, and as IC have many antibodies bound to them, they are able to induce cross linking of receptors, which can potentially provide a stronger signal than individual antibodies, suggesting IC may act more strongly through these pathways (Gallo et al., 2010). The potential overlapping pathways between IVIG and IC support the idea that IC may provide a potential treatment for MS.

## 8.4 Future Directions

The work in this thesis further investigated the biasing of the T cell response by type II MΦ as well as the effect that a type II activating treatment, IC, had on EAE. Furthermore, the ability of MG to achieve type II activation and to induce biasing of the T cell response was investigated. While this work produced several interesting findings, it has also raised several more questions, particularly surrounding the biasing of the T cell and the effects of IC treatment *in vivo*.

Consistent with previous studies, MΦ:T cell co-cultures with type II activated MΦ have been shown to induce Th2 responses and while IL-4 was not detected in the current study, the decreased production of IFN-γ and the increased expression of CD124 do suggest a shift towards a Th2 type phenotype. However, a novel finding of this study is the increased production of IL-17A in these cultures, which was consistently increased throughout much of

the *in vitro* and *in vivo* work involved in this thesis. Further studies should investigate the phenotype of the cells producing the IL-17A as it seems counterintuitive given the protective and pathogenic roles type II MΦ and IL-17A have in EAE, respectively (Bettelli et al., 2006; Tierney et al., 2009).

To further investigate the phenotype of these IL-17A producing cells, a more in-depth characterisation of T cells cultured with classically and type II activated MΦ would be recommended. This study should include intracellular staining of cytokines to determine not only the proportions of T cells producing specific cytokines but also determine of populations of cells that are double positive for specific cytokines such as IL-17A and IL-10. This method would also allow for detection of IL-4-producing cells as IL-4 can be difficult to detect due to its rapid uptake. Furthermore verifying the T cell subsets by assessing the expression of transcription factors associated with specific lineages, including GATA-3, T-bet and RORγt would aid phenotypic analysis of T cells co-cultured with type II activated MΦ. Restimulation assays of T cells cultured with type II or classically activated MΦ could also be used to determine if the IL-17A population in these cultures represents a transient population of T cells that may further differentiate into another cell type. Once the phenotype of the T cells in culture with MΦ has been fully characterised, it should be investigated in MG:T cell cultures and also *in vivo*, as in both of these systems, consistent increases in IL-17A in the presence of IC was seen.

This thesis aimed to determine the pathways involved in the biasing of T cell responses by type II and classical MΦ. While this study strongly supports a role for the altered levels of IL-10 and IL-12 produced by type II and classically activated MΦ in biasing T cell responses, the data particularly implicates these cytokines as being involved in the regulation of IFN-γ levels. Some of this data, particularly the data involving increasing the level of IL-12 in culture requires further verification. In addition, none of the pathways investigated provided a potential mechanism for the increased IL-17A and CD124 in T cells cultured with type II activated MΦ. However, further pathways remain to be investigated, for example, type II MΦ have recently been shown to produce IL-4 which could be investigated as a possible mechanism involved in T cell biasing (La Flamme et al., 2012), and it has also been reported that type II MΦ have altered levels of cells surface receptors such as MHC class II, CD80 and CD86 (Edwards et al., 2006; Tierney et al., 2009). These pathways could be investigated for

potential roles in T cell biasing with the use of blocking antibodies, knockout mice, or potentially RNA interference.

Studies in MG *in vitro* demonstrated that MG are capable of achieving a regulatory activation state that strongly resembles, but is not identical to type II activated MΦ. For example, type II MG produced decreased levels of IL-6, whereas type II MΦ do not (Kharkrang, 2010). Furthermore MG appear to have an increased ability to activate T cells, this ability is particularly evident in the equivalent production of IFN-γ and IL-2 in all cultures containing IFN-γ primed MG, regardless of further stimuli provided to the MG. Further studies to investigate the inherent ability of MG to stimulate T cells could be performed. For example, the ability of MG that have or have not been primed with IFN-γ to stimulate T cells could be assessed. Also, the phenotype of MΦ and MG in the presence or absence of IFN-γ could be compared to assess pathways involved in T cell activation, such as co-stimulatory mechanisms, which have the potential be differentially expressed between MΦ and MG.

In addition to the interesting finding that IC treatment results in increased antigen specific IL-17A production in immunised mice, effects of IC treatment on CNS cells was also investigated. Although chimeric mice were generated to study the effects of IC treatment on resident MG, this protocol required extensive optimization and thus while the method is now established and validated, few experimental results were obtained. It was shown using qPCR that MG from IC-treated mice produced higher levels of IL-12 in sick mice compared to not sick mice. This result is consistent with previous reports and supports these chimeras as a method to study the effects of IC treatment on MG. However, due to the low yield of cells, the differences in MG activation between IC-treated and untreated mice were not able to be assessed. These experiments should be repeated to fully explore the effects of IC treatment on MG *in vivo*.

Further data suggested that IC treatment *in vivo* alters the phenotype of resident CNS associated MΦ in EAE. Further work to determine the location of these $CD45^{int}CD11b^{+}$ cells in the brain using immunohistochemistry to observe the cells *in situ* should be undertaken. It is likely that this cell type is a CNS-associated MΦ population located at perivascular locations, and in the meninges and choroid plexus. As the $CD45^{int}CD11b^{+}$ cells from normal mice but not IC treated mice showed increase expression of MHC II, it may be possible to assess MHC class II staining of CNS-associated MΦ by immunohistochemistry, to determine

if it is upregulated on CNS-associated MΦ in untreated immunised mice compared to immunised mice, and how IC treatment also affects the expression.

Overall these data form an interesting picture of how IC and type II activated MΦ regulate T cell responses and EAE, with regulatory mechanisms in EAE possibly involving both changes to the T cell response and the CNS environment. Further studies into the pathways involved will allow an even more comprehensive understanding of the type II MΦ phenotype and may support IC-mediated type II activation as a potential pathway for treatment of MS.

# References

## References

't Hart, B. A., Gran, B., & Weissert, R. (2011). EAE: imperfect but useful models of multiple sclerosis. *Trends in molecular medicine*, *17*(3), 119–125. doi:10.1016/j.molmed.2010.11.006

Abbas, A. K., Murphy, K. M., & Sher, A. (1996). Functional diversity of helper T lymphocytes. *Nature*, *383*(6603), 787–793. doi:10.1038/383787a0

Aharoni, R, Teitelbaum, D., Sela, M., & Arnon, R. (1997). Copolymer 1 induces T cells of the T helper type 2 that crossreact with myelin basic protein and suppress experimental autoimmune encephalomyelitis. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(20), 10821–10826.

Aharoni, Rina, Eilam, R., Stock, A., Vainshtein, A., Shezen, E., Gal, H., … Arnon, R. (2010). Glatiramer acetate reduces Th-17 inflammation and induces regulatory T-cells in the CNS of mice with relapsing–remitting or chronic EAE. *Journal of Neuroimmunology*, *225*(1–2), 100–111. doi:10.1016/j.jneuroim.2010.04.022

Ajami, B., Bennett, J. L., Krieger, C., Tetzlaff, W., & Rossi, F. M. V. (2007). Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nature neuroscience*, *10*(12), 1538–1543. doi:10.1038/nn2014

Akira, S., Uematsu, S., & Takeuchi, O. (2006). Pathogen Recognition and Innate Immunity. *Cell*, *124*(4), 783–801. doi:10.1016/j.cell.2006.02.015

Alliot, F., Godin, I., & Pessac, B. (1999). Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain. *Developmental Brain Research*, *117*(2), 145–152. doi:10.1016/S0165-3806(99)00113-3

Aloisi, F, Penna, G., Cerase, J., Menéndez Iglesias, B., & Adorini, L. (1997). IL-12 production by central nervous system microglia is inhibited by astrocytes. *Journal of immunology (Baltimore, Md.: 1950)*, *159*(4), 1604–1612.

Aloisi, F, Penna, G., Polazzi, E., Minghetti, L., & Adorini, L. (1999). CD40-CD154 interaction and IFN-gamma are required for IL-12 but not prostaglandin E2 secretion by microglia during antigen presentation to Th1 cells. *Journal of immunology (Baltimore, Md.: 1950)*, *162*(3), 1384–1391.

Aloisi, Francesca, Simone, R. D., Columba-Cabezas, S., Penna, G., & Adorini, L. (2000). Functional Maturation of Adult Mouse Resting Microglia into an APC Is Promoted by Granulocyte-Macrophage Colony-Stimulating Factor and Interaction with Th1 Cells. *The Journal of Immunology*, *164*(4), 1705–1712.

Amulic, B., Cazalet, C., Hayes, G. L., Metzler, K. D., & Zychlinsky, A. (2012). Neutrophil Function: From Mechanisms to Disease. *Annual Review of Immunology*, *30*(1), 459–489. doi:10.1146/annurev-immunol-020711-074942

Anderson, C.F., Gerber, J. S., & Mosser, D. M. (2002). Modulating macrophage function with IgG immune complexes. *Journal of Endotoxin Research*, *8*(6), 477–481. doi:10.1179/096805102125001118

Anderson, C.F., Lucas, M., Gutiérrez-Kobeh, L., Field, A. E., & Mosser, D. M. (2004). T cell biasing by activated dendritic cells. *Journal of Immunology*, *173*(2), 955–961.

Anderson, C.F., & Mosser, D. M. (2002). A novel phenotype for an activated macrophage: The type 2 activated macrophage. *Journal of Leukocyte Biology*, *72*(1), 101–106.

Anderson, Charles F., & Mosser, D. M. (2002). Cutting Edge: Biasing Immune Responses by Directing Antigen to Macrophage Fcγ Receptors. *The Journal of Immunology*, *168*(8), 3697–3701.

Axtell, R. C., de Jong, B. A., Boniface, K., van der Voort, L. F., Bhat, R., De Sarno, P., … Raman, C. (2010). T helper type 1 and 17 cells determine efficacy of interferon-β in multiple sclerosis and experimental encephalomyelitis. *Nature Medicine*, *16*(4), 406–412. doi:10.1038/nm.2110

Baxter, A. G. (2007). The origin and application of experimental autoimmune encephalomyelitis. *Nature reviews. Immunology*, *7*(11), 904–912. doi:10.1038/nri2190

Beauvillain, C., Donnou, S., Jarry, U., Scotet, M., Gascan, H., Delneste, Y., … Couez, D. (2008). Neonatal and adult microglia cross-present exogenous antigens. *Glia*, *56*(1), 69–77. doi:10.1002/glia.20565

Becher, B, Blain, M., & Antel, J. P. (2000). CD40 engagement stimulates IL-12 p70 production by human microglial cells: basis for Th1 polarization in the CNS. *Journal of neuroimmunology*, *102*(1), 44–50.

Becher, B, Durell, B. G., Miga, A. V., Hickey, W. F., & Noelle, R. J. (2001). The clinical course of experimental autoimmune encephalomyelitis and inflammation is controlled by the expression of CD40 within the central nervous system. *The Journal of experimental medicine*, *193*(8), 967–974.

Becher, Burkhard, Durell, B. G., & Noelle, R. J. (2003). IL-23 produced by CNS-resident cells controls T cell encephalitogenicity during the effector phase of experimental autoimmune encephalomyelitis. *The Journal of clinical investigation*, *112*(8), 1186–1191. doi:10.1172/JCI19079

Bertolotto, A., Malucchi, S., Sala, A., Orefice, G., Carrieri, P. B., Capobianco, M., … Giordana, M. T. (2002). Differential effects of three interferon betas on neutralising antibodies in patients with multiple sclerosis: a follow up study in an independent laboratory. *Journal of Neurology, Neurosurgery & Psychiatry*, *73*(2), 148–153. doi:10.1136/jnnp.73.2.148

Bettelli, E, Das, M. P., Howard, E. D., Weiner, H. L., Sobel, R. A., & Kuchroo, V. K. (1998). IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice. *Journal of immunology (Baltimore, Md.: 1950)*, *161*(7), 3299–3306.

Bettelli, Estelle, Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., … Kuchroo, V. K. (2006). Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*, *441*(7090), 235–238. doi:10.1038/nature04753

Bettelli, Estelle, Pagany, M., Weiner, H. L., Linington, C., Sobel, R. A., & Kuchroo, V. K. (2003). Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis. *The Journal of experimental medicine*, *197*(9), 1073–1081. doi:10.1084/jem.20021603

Bettelli, Estelle, Sullivan, B., Szabo, S. J., Sobel, R. A., Glimcher, L. H., & Kuchroo, V. K. (2004). Loss of T-bet, But Not STAT1, Prevents the Development of Experimental Autoimmune Encephalomyelitis. *The Journal of Experimental Medicine*, *200*(1), 79–87. doi:10.1084/jem.20031819

Bird, J. J., Brown, D. R., Mullen, A. C., Moskowitz, N. H., Mahowald, M. A., Sider, J. R., … Reiner, S. L. (1998). Helper T cell differentiation is controlled by the cell cycle. *Immunity*, *9*(2), 229–237.

Bjartmar, C., Wujek, J. R., & Trapp, B. D. (2003). Axonal loss in the pathology of MS: consequences for understanding the progressive phase of the disease. *Journal of the neurological sciences*, *206*(2), 165–171.

Bonneville, M., O'Brien, R. L., & Born, W. K. (2010). γδ T cell effector functions: a blend of innate programming and acquired plasticity. *Nature Reviews Immunology*, *10*(7), 467–478. doi:10.1038/nri2781

Bradl, M., & Lassmann, H. (2009). Progressive multiple sclerosis. *Seminars in immunopathology*, *31*(4), 455–465. doi:10.1007/s00281-009-0182-3

Brannan, C. A., & Roberts, M. R. (2004). Resident microglia from adult mice are refractory to nitric oxide-inducing stimuli due to impaired NOS2 gene expression. *Glia*, *48*(2), 120–131. doi:10.1002/glia.20066

Butovsky, O., Ziv, Y., Schwartz, A., Landa, G., Talpalar, A. E., Pluchino, S., … Schwartz, M. (2006). Microglia activated by IL-4 or IFN-γ differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells. *Molecular and Cellular Neuroscience*, *31*(1), 149–160. doi:10.1016/j.mcn.2005.10.006

Cairns, C. B., Panacek, E. A., Harken, A. H., & Banerjee, A. (2000). Bench to bedside: tumor necrosis factor-alpha: from inflammation to resuscitation. *Academic emergency medicine: official journal of the Society for Academic Emergency Medicine*, *7*(8), 930–941.

Campbell, D. J., & Koch, M. A. (2011). Phenotypical and functional specialization of FOXP3+ regulatory T cells. *Nature Reviews Immunology*, *11*(2), 119–130. doi:10.1038/nri2916

Campbell, K. A., Ovendale, P. J., Kennedy, M. K., Fanslow, W. C., Reed, S. G., & Maliszewski, C. R. (1996). CD40 Ligand Is Required for Protective Cell-Mediated Immunity to Leishmania major. *Immunity*, *4*(3), 283–289. doi:10.1016/S1074-7613(00)80436-7

Capobianco, M., Rizzo, A., Malucchi, S., Sperli, F., Di Sapio, A., Oggero, A., … Bertolotto, A. (2008). Glatiramer acetate is a treatment option in neutralising antibodies to interferon-beta-positive patients. *Neurological sciences: official journal of the Italian Neurological Society and of the Italian Society of Clinical Neurophysiology*, *29 Suppl 2*, S227–229. doi:10.1007/s10072-008-0945-y

Cardona, A. E., Pioro, E. P., Sasse, M. E., Kostenko, V., Cardona, S. M., Dijkstra, I. M., … Ransohoff, R. M. (2006). Control of microglial neurotoxicity by the fractalkine receptor. *Nature neuroscience*, *9*(7), 917–924. doi:10.1038/nn1715

Carson, M. J., Doose, J. M., Melchior, B., Schmid, C. D., & Ploix, C. C. (2006). CNS immune privilege: hiding in plain sight. *Immunological reviews*, *213*, 48–65. doi:10.1111/j.1600-065X.2006.00441.x

Carter, L., Fouser, L. A., Jussif, J., Fitz, L., Deng, B., Wood, C. R., … Carreno, B. M. (2002). PD-1:PD-L inhibitory pathway affects both CD4(+) and CD8(+) T cells and is overcome by IL-2. *European journal of immunology*, *32*(3), 634–643. doi:10.1002/1521-4141(200203)32:3<634::AID-IMMU634>3.0.CO;2-9

Carter, L. L., Leach, M. W., Azoitei, M. L., Cui, J., Pelker, J. W., Jussif, J., … Marusic, S. (2007). PD-1/PD-L1, but not PD-1/PD-L2, interactions regulate the severity of experimental autoimmune encephalomyelitis. *Journal of neuroimmunology*, *182*(1-2), 124–134. doi:10.1016/j.jneuroim.2006.10.006

Chang, R. C., Chen, W., Hudson, P., Wilson, B., Han, D. S., & Hong, J. S. (2001). Neurons reduce glial responses to lipopolysaccharide (LPS) and prevent injury of microglial cells from over-activation by LPS. *Journal of neurochemistry*, *76*(4), 1042–1049.

Chappert, P., & Schwartz, R. H. (2010). Induction of T cell anergy: integration of environmental cues and infectious tolerance. *Current Opinion in Immunology*, *22*(5), 552–559. doi:10.1016/j.coi.2010.08.005

Chatterjee, S., Das, S., Chakraborty, P., Manna, A., Chatterjee, M., & Choudhuri, S. K. (2013). Myeloid derived suppressor cells (MDSCs) can induce the generation of Th17 response from naïve CD4+ T cells. *Immunobiology*, *218*(5), 718–724. doi:10.1016/j.imbio.2012.08.271

Chitnis, T., Najafian, N., Benou, C., Salama, A. D., Grusby, M. J., Sayegh, M. H., & Khoury, S. J. (2001). Effect of targeted disruption of STAT4 and STAT6 on the induction of experimental autoimmune encephalomyelitis. *Journal of Clinical Investigation*, *108*(5), 739–747. doi:10.1172/JCI12563

Ciofani, M., & Zúñiga-Pflücker, J. C. (2010). Determining γδ versus αβ T cell development. *Nature Reviews Immunology*, *10*(9), 657–663. doi:10.1038/nri2820

Cohen, J. A., Barkhof, F., Comi, G., Hartung, H.-P., Khatri, B. O., Montalban, X., … Kappos, L. (2010). Oral Fingolimod or Intramuscular Interferon for Relapsing Multiple

Sclerosis. *New England Journal of Medicine*, *362*(5), 402–415. doi:10.1056/NEJMoa0907839

Compston, A. (2004). The pathogenesis and basis for treatment in multiple sclerosis. *Clinical neurology and neurosurgery*, *106*(3), 246–248. doi:10.1016/j.clineuro.2004.02.007

Correa, F., Hernangómez-Herrero, M., Mestre, L., Loría, F., Docagne, F., & Guaza, C. (2011). The endocannabinoid anandamide downregulates IL-23 and IL-12 subunits in a viral model of multiple sclerosis: evidence for a cross-talk between IL-12p70/IL-23 axis and IL-10 in microglial cells. *Brain, behavior, and immunity*, *25*(4), 736–749. doi:10.1016/j.bbi.2011.01.020

Couper, K. N., Blount, D. G., & Riley, E. M. (2008). IL-10: The Master Regulator of Immunity to Infection. *The Journal of Immunology*, *180*(9), 5771–5777.

Croxford, J. L., Feldmann, M., Chernajovsky, Y., & Baker, D. (2001). Different therapeutic outcomes in experimental allergic encephalomyelitis dependent upon the mode of delivery of IL-10: a comparison of the effects of protein, adenoviral or retroviral IL-10 delivery into the central nervous system. *Journal of immunology (Baltimore, Md.: 1950)*, *166*(6), 4124–4130.

Cua, D. J., Sherlock, J., Chen, Y., Murphy, C. A., Joyce, B., Seymour, B., … Sedgwick, J. D. (2003). Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature*, *421*(6924), 744–748. doi:10.1038/nature01355

De Haas, A. H., Boddeke, H. W. G. M., & Biber, K. (2008). Region-specific expression of immunoregulatory proteins on microglia in the healthy CNS. *Glia*, *56*(8), 888–894. doi:10.1002/glia.20663

De Haas, A. H., Boddeke, H. W. G. M., Brouwer, N., & Biber, K. (2007). Optimized isolation enables ex vivo analysis of microglia from various central nervous system regions. *Glia*, *55*(13), 1374–1384. doi:10.1002/glia.20554

De Lago, E., Moreno-Martet, M., Cabranes, A., Ramos, J. A., & Fernández-Ruiz, J. (2012). Cannabinoids ameliorate disease progression in a model of multiple sclerosis in mice, acting preferentially through CB1 receptor-mediated anti-inflammatory effects. *Neuropharmacology*, *62*(7), 2299–2308. doi:10.1016/j.neuropharm.2012.01.030

DeKruyff, R. H., Gieni, R. S., & Umetsu, D. T. (1997). Antigen-driven but not lipopolysaccharide-driven IL-12 production in macrophages requires triggering of CD40. *The Journal of Immunology*, *158*(1), 359–366.

Ding, L., Linsley, P. S., Huang, L. Y., Germain, R. N., & Shevach, E. M. (1993). IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. *The Journal of Immunology*, *151*(3), 1224–1234.

Dogan, R.-N. E., Elhofy, A., & Karpus, W. J. (2008). Production of CCL2 by central nervous system cells regulates development of murine experimental autoimmune encephalomyelitis through the recruitment of TNF- and iNOS-expressing macrophages and myeloid dendritic cells. *Journal of immunology (Baltimore, Md.: 1950)*, *180*(11), 7376–7384.

Domingues, H. S., Mues, M., Lassmann, H., Wekerle, H., & Krishnamoorthy, G. (2010). Functional and Pathogenic Differences of Th1 and Th17 Cells in Experimental Autoimmune Encephalomyelitis. *PLoS ONE*, *5*(11), e15531. doi:10.1371/journal.pone.0015531

Edwards, J. P., Zhang, X., Frauwirth, K. A., & Mosser, D. M. (2006). Biochemical and functional characterization of three activated macrophage populations. *Journal of Leukocyte Biology*, *80*(6), 1298–1307. doi:10.1189/jlb.0406249

Endoh, M., Rapoport, S. I., & Tabira, T. (1990). Studies of experimental allergic encephalomyelitis in old mice. *Journal of neuroimmunology*, *29*(1-3), 21–31.

Ephrem, A., Chamat, S., Miquel, C., Fisson, S., Mouthon, L., Caligiuri, G., … Misra, N. (2008). Expansion of CD4+CD25+ regulatory T cells by intravenous immunoglobulin:

a critical factor in controlling experimental autoimmune encephalomyelitis. *Blood*, *111*(2), 715–722. doi:10.1182/blood-2007-03-079947

Esplugues, E., Huber, S., Gagliani, N., Hauser, A. E., Town, T., Wan, Y. Y., … Flavell, R. A. (2011). Control of TH17 cells occurs in the small intestine. *Nature*, *475*(7357), 514–518. doi:10.1038/nature10228

Eugster, H. P., Frei, K., Kopf, M., Lassmann, H., & Fontana, A. (1998). IL-6-deficient mice resist myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. *European journal of immunology*, *28*(7), 2178–2187. doi:10.1002/(SICI)1521-4141(199807)28:07<2178::AID-IMMU2178>3.0.CO;2-D

Fernandez, S., Jose, P., Avdiushko, M. G., Kaplan, A. M., & Cohen, D. A. (2004). Inhibition of IL-10 Receptor Function in Alveolar Macrophages by Toll-Like Receptor Agonists. *The Journal of Immunology*, *172*(4), 2613–2620.

Fiorentino, D. F., Bond, M. W., & Mosmann, T. R. (1989). Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *The Journal of experimental medicine*, *170*(6), 2081–2095.

Fiorentino, D. F., Zlotnik, A., Mosmann, T. R., Howard, M., & O'Garra, A. (1991). IL-10 inhibits cytokine production by activated macrophages. *The Journal of Immunology*, *147*(11), 3815–3822.

Fiorentino, D. F., Zlotnik, A., Vieira, P., Mosmann, T. R., Howard, M., Moore, K. W., & O'Garra, A. (1991). IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *The Journal of Immunology*, *146*(10), 3444–3451.

Ford, A. L., Goodsall, A. L., Hickey, W. F., & Sedgwick, J. D. (1995). Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting. Phenotypic differences defined and direct ex vivo antigen presentation to myelin basic protein-reactive CD4+ T cells compared. *The Journal of Immunology*, *154*(9), 4309–4321.

Francisco, L. M., Sage, P. T., & Sharpe, A. H. (2010). The PD-1 pathway in tolerance and autoimmunity. *Immunological Reviews*, *236*(1), 219–242. doi:10.1111/j.1600-065X.2010.00923.x

Frohman, E. M., Racke, M. K., & Raine, C. S. (2006). Multiple Sclerosis — The Plaque and Its Pathogenesis. *New England Journal of Medicine*, *354*(9), 942–955. doi:10.1056/NEJMra052130

Gabrilovich, D. I., & Nagaraj, S. (2009). Myeloid-derived-suppressor cells as regulators of the immune system. *Nature reviews. Immunology*, *9*(3), 162–174. doi:10.1038/nri2506

Gallo, P., Gonçalves, R., & Mosser, D. M. (2010). The influence of IgG density and macrophage Fc (gamma) receptor cross-linking on phagocytosis and IL-10 production. *Immunology Letters*, *133*(2), 70–77. doi:10.1016/j.imlet.2010.07.004

Geissmann, F., Jung, S., & Littman, D. R. (2003). Blood Monocytes Consist of Two Principal Subsets with Distinct Migratory Properties. *Immunity*, *19*(1), 71–82. doi:10.1016/S1074-7613(03)00174-2

Gerber, J. S., & Mosser, D. M. (2001). Reversing Lipopolysaccharide Toxicity by Ligating the Macrophage Fcγ Receptors. *The Journal of Immunology*, *166*(11), 6861–6868.

Gimsa, U., Wolf, S. A., Haas, D., Bechmann, I., & Nitsch, R. (2001). Th2 cells support intrinsic anti-inflammatory properties of the brain. *Journal of Neuroimmunology*, *119*(1), 73–80. doi:10.1016/S0165-5728(01)00343-5

Gingras, M., Gagnon, V., Minotti, S., Durham, H. D., & Berthod, F. (2007). Optimized protocols for isolation of primary motor neurons, astrocytes and microglia from embryonic mouse spinal cord. *Journal of neuroscience methods*, *163*(1), 111–118. doi:10.1016/j.jneumeth.2007.02.024

Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., … Merad, M. (2010). Fate Mapping Analysis Reveals That Adult Microglia Derive from Primitive Macrophages. *Science*, *330*(6005), 841–845. doi:10.1126/science.1194637

Girvin, A. M., Dal Canto, M. C., & Miller, S. D. (2002). CD40/CD40L Interaction is Essential for the Induction of EAE in the Absence of CD28-Mediated Co-stimulation. *Journal of Autoimmunity*, *18*(2), 83–94. doi:10.1006/jaut.2001.0573

Giulian, D., & Baker, T. J. (1986). Characterization of ameboid microglia isolated from developing mammalian brain. *The Journal of Neuroscience*, *6*(8), 2163–2178.

Gold, R., Linington, C., & Lassmann, H. (2006). Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research. *Brain*, *129*(8), 1953–1971. doi:10.1093/brain/awl075

Goodin, D. S. (2009). The Causal Cascade to Multiple Sclerosis: A Model for MS Pathogenesis. *PLoS ONE*, *4*(2), e4565. doi:10.1371/journal.pone.0004565

Gordon, S., & Taylor, P. R. (2005). Monocyte and macrophage heterogeneity. *Nature Reviews Immunology*, *5*(12), 953–964. doi:10.1038/nri1733

Greenstein, J. I. (2007). Current concepts of the cellular and molecular pathophysiology of multiple sclerosis. *Developmental neurobiology*, *67*(9), 1248–1265. doi:10.1002/dneu.20387

Grewal, I. S., & Flavell, R. A. (1998). Cd40 and Cd154 in Cell-Mediated Immunity. *Annual Review of Immunology*, *16*(1), 111–135. doi:10.1146/annurev.immunol.16.1.111

Grewal, I. S., Foellmer, H. G., Grewal, K. D., Xu, J., Hardardottir, F., Baron, J. L., … Flavell, R. A. (1996). Requirement for CD40 Ligand in Costimulation Induction, T Cell Activation, and Experimental Allergic Encephalomyelitis. *Science*, *273*(5283), 1864–1867. doi:10.1126/science.273.5283.1864

Grewal, I. S., Xu, J., & Flavell, R. A. (1995). Impairment of antigen-specific T-cell priming in mice lacking CD40 ligand. *Nature*, *378*(6557), 617–620. doi:10.1038/378617a0

Grohmann, U., Belladonna, M. L., Vacca, C., Bianchi, R., Fallarino, F., Orabona, C., … Puccetti, P. (2001). Positive Regulatory Role of IL-12 in Macrophages and Modulation by IFN-γ. *The Journal of Immunology*, *167*(1), 221–227.

Gu, L., Tseng, S., Horner, R. M., Tam, C., Loda, M., & Rollins, B. J. (2000). Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1. *Nature*, *404*(6776), 407–411. doi:10.1038/35006097

Gu, Y., Yang, J., Ouyang, X., Liu, W., Li, H., Yang, J., … Xiong, H. (2008). Interleukin 10 suppresses Th17 cytokines secreted by macrophages and T cells. *European journal of immunology*, *38*(7), 1807–1813. doi:10.1002/eji.200838331

Haahr, S., Plesner, A. M., Vestergaard, B. F., & Höllsberg, P. (2004). A role of late Epstein–Barr virus infection in multiple sclerosis. *Acta Neurologica Scandinavica*, *109*(4), 270–275. doi:10.1046/j.1600-0404.2003.00221.x

Harrington, L. E., Hatton, R. D., Mangan, P. R., Turner, H., Murphy, T. L., Murphy, K. M., & Weaver, C. T. (2005). Interleukin 17–producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nature Immunology*, *6*(11), 1123–1132. doi:10.1038/ni1254

Harty, J. T., Tvinnereim, A. R., & White, D. W. (2000). CD8+ T Cell Effector Mechanisms in Resistance to Infection. *Annual Review of Immunology*, *18*(1), 275–308. doi:10.1146/annurev.immunol.18.1.275

Hawkes, C. H., & Macgregor, A. J. (2009). Twin studies and the heritability of MS: a conclusion. *Multiple Sclerosis*, *15*(6), 661–667. doi:10.1177/1352458509104592

Hayashi, M., Luo, Y., Laning, J., Strieter, R. M., & Dorf, M. E. (1995). Production and function of monocyte chemoattractant protein-1 and other beta-chemokines in murine glial cells. *Journal of neuroimmunology*, *60*(1-2), 143–150.

Heard, R. N. S. (2007). The spectrum of multiple sclerosis. *Current Allergy and Asthma Reports*, *7*(4), 280–284. doi:10.1007/s11882-007-0042-y

Henn, A., Lund, S., Hedtjärn, M., Schrattenholz, A., Pörzgen, P., & Leist, M. (2009). The suitability of BV2 cells as alternative model system for primary microglia cultures or for animal experiments examining brain inflammation. *ALTEX*, *26*(2), 83–94.

Heppner, F. L., Greter, M., Marino, D., Falsig, J., Raivich, G., Hövelmeyer, N., … Aguzzi, A. (2005). Experimental autoimmune encephalomyelitis repressed by microglial paralysis. *Nature Medicine*, *11*(2), 146–152. doi:10.1038/nm1177

Hirasawa, T., Ohsawa, K., Imai, Y., Ondo, Y., Akazawa, C., Uchino, S., & Kohsaka, S. (2005). Visualization of microglia in living tissues using Iba1-EGFP transgenic mice. *Journal of Neuroscience Research*, *81*(3), 357–362. doi:10.1002/jnr.20480

Hirata, S., Senju, S., Matsuyoshi, H., Fukuma, D., Uemura, Y., & Nishimura, Y. (2005). Prevention of Experimental Autoimmune Encephalomyelitis by Transfer of Embryonic Stem Cell-Derived Dendritic Cells Expressing Myelin Oligodendrocyte Glycoprotein Peptide along with TRAIL or Programmed Death-1 Ligand. *The Journal of Immunology*, *174*(4), 1888–1897.

Hiremath, M. M., Chen, V. S., Suzuki, K., Ting, J. P.-Y., & Matsushima, G. K. (2008). MHC class II exacerbates demyelination in vivo independently of T cells. *Journal of Neuroimmunology*, *203*(1), 23–32. doi:10.1016/j.jneuroim.2008.06.034

Hoffjan, S., & Akkad, D. A. (2010). The genetics of multiple sclerosis: an update 2010. *Molecular and cellular probes*, *24*(5), 237–243. doi:10.1016/j.mcp.2010.04.006

Hogquist, K. A., Baldwin, T. A., & Jameson, S. C. (2005). Central tolerance: learning self-control in the thymus. *Nature reviews. Immunology*, *5*(10), 772–782. doi:10.1038/nri1707

Hoves, S., Trapani, J. A., & Voskoboinik, I. (2010). The battlefield of perforin/granzyme cell death pathways. *Journal of Leukocyte Biology*, *87*(2), 237–243. doi:10.1189/jlb.0909608

Huang, D. R., Wang, J., Kivisakk, P., Rollins, B. J., & Ransohoff, R. M. (2001). Absence of monocyte chemoattractant protein 1 in mice leads to decreased local macrophage recruitment and antigen-specific T helper cell type 1 immune response in experimental autoimmune encephalomyelitis. *The Journal of experimental medicine*, *193*(6), 713–726.

Humle Jorgensen, S., & Sorensen, P. S. (2005). Intravenous immunoglobulin treatment of multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis. *Journal of the Neurological Sciences*, *233*(1–2), 61–65. doi:10.1016/j.jns.2005.03.005

Hutchinson, M. (2007). Natalizumab: A new treatment for relapsing remitting multiple sclerosis. *Therapeutics and Clinical Risk Management*, *3*(2), 259–268.

Ingersoll, M. A., Spanbroek, R., Lottaz, C., Gautier, E. L., Frankenberger, M., Hoffmann, R., … Randolph, G. J. (2010). Comparison of gene expression profiles between human and mouse monocyte subsets. *Blood*, *115*(3), e10–e19. doi:10.1182/blood-2009-07-235028

International Multiple Sclerosis Genetics Consortium, Wellcome Trust Case Control Consortium 2, Sawcer, S., Hellenthal, G., Pirinen, M., Spencer, C. C. A., … Su, Z. (2011). Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature*, *476*(7359), 214–219. doi:10.1038/nature10251

Issazadeh, S., Navikas, V., Schaub, M., Sayegh, M., & Khoury, S. (1998). Kinetics of expression of costimulatory molecules and their ligands in murine relapsing experimental autoimmune encephalomyelitis in vivo. *Journal of immunology (Baltimore, Md.: 1950)*, *161*(3), 1104–1112.

Ito, D., Tanaka, K., Suzuki, S., Dembo, T., & Fukuuchi, Y. (2001). Enhanced Expression of Iba1, Ionized Calcium-Binding Adapter Molecule 1, After Transient Focal Cerebral Ischemia In Rat Brain. *Stroke*, *32*(5), 1208–1215. doi:10.1161/01.STR.32.5.1208

Ivanov, I. I., McKenzie, B. S., Zhou, L., Tadokoro, C. E., Lepelley, A., Lafaille, J. J., … Littman, D. R. (2006). The Orphan Nuclear Receptor RORγt Directs the Differentiation Program of Proinflammatory IL-17+ T Helper Cells. *Cell*, *126*(6), 1121–1133. doi:10.1016/j.cell.2006.07.035

Jack, C., Ruffini, F., Bar-Or, A., & Antel, J. P. (2005). Microglia and multiple sclerosis. *Journal of Neuroscience Research*, *81*(3), 363–373. doi:10.1002/jnr.20482

Jain, N., Nguyen, H., Chambers, C., & Kang, J. (2010). Dual function of CTLA-4 in regulatory T cells and conventional T cells to prevent multiorgan autoimmunity. *Proceedings of the National Academy of Sciences*, *107*(4), 1524–1528. doi:10.1073/pnas.0910341107

Janeway, C., Travers, P., Walport, M., & Shlomchik, M. (2004). *Immunobiology* (6th ed.). Garland Science.

Jankovic, D., Kullberg, M. C., Noben-Trauth, N., Caspar, P., Paul, W. E., & Sher, A. (2000). Single Cell Analysis Reveals That IL-4 Receptor/Stat6 Signaling Is Not Required for the In Vivo or In Vitro Development of CD4+ Lymphocytes with a Th2 Cytokine Profile. *The Journal of Immunology*, *164*(6), 3047–3055.

Jeannin, P., Jaillon, S., & Delneste, Y. (2008). Pattern recognition receptors in the immune response against dying cells. *Current Opinion in Immunology*, *20*(5), 530–537. doi:10.1016/j.coi.2008.04.013

Juedes, A. E., & Ruddle, N. H. (2001). Resident and Infiltrating Central Nervous System APCs Regulate the Emergence and Resolution of Experimental Autoimmune Encephalomyelitis. *The Journal of Immunology*, *166*(8), 5168–5175.

Kaliński, P., Hilkens, C. M. ., Wierenga, E. A., & Kapsenberg, M. L. (1999). T-cell priming by type-1and type-2 polarized dendritic cells: the concept of a third signal. *Immunology Today*, *20*(12), 561–567. doi:10.1016/S0167-5699(99)01547-9

Kaplan, M. H., Sun, Y.-L., Hoey, T., & Grusby, M. J. (1996). Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature*, *382*(6587), 174–177. doi:10.1038/382174a0

Kappos, L., Radue, E.-W., O'Connor, P., Polman, C., Hohlfeld, R., Calabresi, P., … Burtin, P. (2010). A Placebo-Controlled Trial of Oral Fingolimod in Relapsing Multiple Sclerosis. *New England Journal of Medicine*, *362*(5), 387–401. doi:10.1056/NEJMoa0909494

Kataoka, T. R., Komazawa, N., Oboki, K., Morii, E., & Nakano, T. (2004). Reduced expression of IL-12 receptor β2 and IL-18 receptor α genes in natural killer cells and macrophages derived from B6-mi/mi mice. *Laboratory Investigation*, *85*(1), 146–153. doi:10.1038/labinvest.3700188

Keating, P., O'Sullivan, D., Tierney, J. B., Kenwright, D., Miromoeini, S., Mawasse, L., … La Flamme, A. C. (2009). Protection from EAE by IL-4Rα|[minus]|/|[minus]| macrophages depends upon T regulatory cell involvement. *Immunology and Cell Biology*, *87*(7), 534–545. doi:10.1038/icb.2009.37

Khan, O. A., Tselis, A. C., Kamholz, J. A., Garbern, J. Y., Lewis, R. A., & Lisak, R. P. (2001). A prospective, open-label treatment trial to compare the effect of IFNβ-1a (Avonex®), IFNβ-1b (Betaseron®), and glatiramer acetate (Copaxone®) on the relapse rate in relapsing-remitting multiple sclerosis: results after 18 months of therapy. *Multiple Sclerosis*, *7*(6), 349–353. doi:10.1177/135245850100700601

Kharkrang, M. C. L. (2010). *Mechanisms involved in type II macrophage activation and effector functions: Masters thesis*. Victoria University of Wellington.

Kim, H. J., Ifergan, I., Antel, J. P., Seguin, R., Duddy, M., Lapierre, Y., … Bar-Or, A. (2004). Type 2 Monocyte and Microglia Differentiation Mediated by Glatiramer Acetate Therapy in Patients with Multiple Sclerosis. *The Journal of Immunology*, *172*(11), 7144–7153.

Kincaid, E. Z., & Ernst, J. D. (2003). Mycobacterium tuberculosis Exerts Gene-Selective Inhibition of Transcriptional Responses to IFN-γ Without Inhibiting STAT1 Function. *The Journal of Immunology*, *171*(4), 2042–2049.

Kivisäkk, P., Imitola, J., Rasmussen, S., Elyaman, W., Zhu, B., Ransohoff, R. M., & Khoury, S. J. (2009). Localizing central nervous system immune surveillance: Meningeal antigen-presenting cells activate T cells during experimental autoimmune encephalomyelitis. *Annals of Neurology*, *65*(4), 457–469. doi:10.1002/ana.21379

Kobayashi, T., Matsuoka, K., Sheikh, S. Z., Russo, S. M., Mishima, Y., Collins, C., … Plevy, S. E. (2012). IL-10 Regulates Il12b Expression via Histone Deacetylation: Implications for Intestinal Macrophage Homeostasis. *The Journal of Immunology*, *189*(4), 1792–1799. doi:10.4049/jimmunol.1200042

Komiyama, Y., Nakae, S., Matsuki, T., Nambu, A., Ishigame, H., Kakuta, S., … Iwakura, Y. (2006). IL-17 Plays an Important Role in the Development of Experimental Autoimmune Encephalomyelitis. *The Journal of Immunology*, *177*(1), 566–573.

Konkel, J. E., Frommer, F., Leech, M. D., Yagita, H., Waisman, A., & Anderton, S. M. (2010). PD-1 signalling in CD4+ T cells restrains their clonal expansion to an immunogenic stimulus, but is not critically required for peptide-induced tolerance. *Immunology*, *130*(1), 92–102. doi:10.1111/j.1365-2567.2009.03216.x

Kotter, M. R., Setzu, A., Sim, F. J., Van Rooijen, N., & Franklin, R. J. M. (2001). Macrophage depletion impairs oligodendrocyte remyelination following lysolecithin-induced demyelination. *Glia*, *35*(3), 204–212. doi:10.1002/glia.1085

Krakowski, M. L., & Owens, T. (1997). The central nervous system environment controls effector CD4+ T cell cytokine profile in experimental allergic encephalomyelitis. *European journal of immunology*, *27*(11), 2840–2847. doi:10.1002/eji.1830271115

Krakowski, M., & Owens, T. (1996). Interferon-γ confers resistance to experimental allergic encephalomyelitis. *European Journal of Immunology*, *26*(7), 1641–1646. doi:10.1002/eji.1830260735

Krammer, P. H. (2000). CD95's deadly mission in the immune system. *Nature*, *407*(6805), 789–795. doi:10.1038/35037728

Krummel, M. F., & Allison, J. P. (1995). CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *The Journal of Experimental Medicine*, *182*(2), 459–465. doi:10.1084/jem.182.2.459

Kuipers, H., Muskens, F., Willart, M., Hijdra, D., van Assema, F. B. J., Coyle, A. J., … Lambrecht, B. N. (2006). Contribution of the PD-1 ligands/PD-1 signaling pathway to dendritic cell-mediated CD4+ T cell activation. *European Journal of Immunology*, *36*(9), 2472–2482. doi:10.1002/eji.200635978

Kumar, V., Abbas, A. K., Fausto, N., & Mitchell, R. (2007). *Robbins Basic Pathology, Eighth Edition* (8th ed.). Saunders/Elsevier.

Kurschus, F. C., Croxford, A. L., P. Heinen, A., Wörtge, S., Ielo, D., & Waisman, A. (2010). Genetic proof for the transient nature of the Th17 phenotype. *European Journal of Immunology*, *40*(12), 3336–3346. doi:10.1002/eji.201040755

Kyewski, B., & Klein, L. (2006). A Central Role for Central Tolerance. *Annual Review of Immunology*, *24*(1), 571–606. doi:10.1146/annurev.immunol.23.021704.115601

La Flamme, Anne C., Harvie, M., McNeill, A., Goldsack, L., Tierney, J. B., & Bäckström, B. T. (2006). Fcγ receptor-ligating complexes improve the course of experimental autoimmune encephalomyelitis by enhancing basal Th2 responses. *Immunology and Cell Biology*, *84*(6), 522–529. doi:10.1111/j.1440-1711.2006.01464.x

La Flamme, Anne Camille, Kharkrang, M., Stone, S., Mirmoeini, S., Chuluundorj, D., & Kyle, R. (2012). Type II-Activated Murine Macrophages Produce IL-4. *PLoS ONE*, *7*(10), e46989. doi:10.1371/journal.pone.0046989

Lassmann, H. (2008). Mechanisms of inflammation induced tissue injury in multiple sclerosis. *Journal of the neurological sciences*, *274*(1-2), 45–47. doi:10.1016/j.jns.2008.04.003

Lassmann, H., Brück, W., & Lucchinetti, C. F. (2007). The Immunopathology of Multiple Sclerosis: An Overview. *Brain Pathology*, *17*(2), 210–218. doi:10.1111/j.1750-3639.2007.00064.x

Le Gros, G., Ben-Sasson, S. Z., Seder, R., Finkelman, F. D., & Paul, W. E. (1990). Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. *The Journal of Experimental Medicine*, *172*(3), 921–929. doi:10.1084/jem.172.3.921

Lee, Y. K., Turner, H., Maynard, C. L., Oliver, J. R., Chen, D., Elson, C. O., & Weaver, C. T. (2009). Late Developmental Plasticity in the T Helper 17 Lineage. *Immunity*, *30*(1), 92–107. doi:10.1016/j.immuni.2008.11.005

Li, H., Ciric, B., Yang, J., Xu, H., Fitzgerald, D. C., Elbehi, M., … Rostami, A. (2009). Intravenous tolerance modulates macrophage classical activation and antigen presentation in experimental autoimmune encephalomyelitis. *Journal of Neuroimmunology*, *208*(1–2), 54–60. doi:10.1016/j.jneuroim.2009.01.002

Li, M., Wang, Y., Guo, R., Bai, Y., & Yu, Z. (2007). Glucocorticoids impair microglia ability to induce T cell proliferation and Th1 polarization. *Immunology letters*, *109*(2), 129–137. doi:10.1016/j.imlet.2007.02.002

Liang, S. C., Greenwald, R. J., Latchman, Y. E., Rosas, L., Satoskar, A., Freeman, G. J., & Sharpe, A. H. (2006). PD-L1 and PD-L2 have distinct roles in regulating host immunity to cutaneous leishmaniasis. *European Journal of Immunology*, *36*(1), 58–64. doi:10.1002/eji.200535458

Liao, W., Schones, D. E., Oh, J., Cui, Y., Cui, K., Roh, T.-Y., … Leonard, W. J. (2008). Priming for T helper type 2 differentiation by interleukin 2–mediated induction of

interleukin 4 receptor α-chain expression. *Nature Immunology*, *9*(11), 1288–1296. doi:10.1038/ni.1656

Lieberman, L. A., Banica, M., Reiner, S. L., & Hunter, C. A. (2004). STAT1 Plays a Critical Role in the Regulation of Antimicrobial Effector Mechanisms, but Not in the Development of Th1-Type Responses during Toxoplasmosis. *The Journal of Immunology*, *172*(1), 457–463.

Lighvani, A. A., Frucht, D. M., Jankovic, D., Yamane, H., Aliberti, J., Hissong, B. D., … O'Shea, J. J. (2001). T-bet is rapidly induced by interferon-γ in lymphoid and myeloid cells. *Proceedings of the National Academy of Sciences*, *98*(26), 15137–15142. doi:10.1073/pnas.261570598

Lins, H. (2005). Clinical Add-on: Multiple sclerosis – clinical picture and therapeutic strategies. *Signal Transduction*, *5*(5), 266–272. doi:10.1002/sita.200400056

Lock, C., Hermans, G., Pedotti, R., Brendolan, A., Schadt, E., Garren, H., … Steinman, L. (2002). Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nature Medicine*, *8*(5), 500–508. doi:10.1038/nm0502-500

Loke, P., & Allison, J. P. (2003). PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(9), 5336–5341. doi:10.1073/pnas.0931259100

Louten, J., Boniface, K., & de Waal Malefyt, R. (2009). Development and function of TH17 cells in health and disease. *Journal of Allergy and Clinical Immunology*, *123*(5), 1004–1011. doi:10.1016/j.jaci.2009.04.003

Lovett-Racke, A. E., Trotter, J. L., Lauber, J., Perrin, P. J., June, C. H., & Racke, M. K. (1998). Decreased dependence of myelin basic protein-reactive T cells on CD28-mediated costimulation in multiple sclerosis patients. A marker of activated/memory T cells. *Journal of Clinical Investigation*, *101*(4), 725–730.

Ma, X., Reynolds, S. L., Baker, B. J., Li, X., Benveniste, E. N., & Qin, H. (2010). IL-17 enhancement of the IL-6 signaling cascade in astrocytes. *Journal of immunology (Baltimore, Md.: 1950)*, *184*(9), 4898–4906. doi:10.4049/jimmunol.1000142

MacDonald, A. S., Patton, E. A., Flamme, A. C. L., Araujo, M. I., Huxtable, C. R., Bauman, B., & Pearce, E. J. (2002). Impaired Th2 Development and Increased Mortality During Schistosoma mansoni Infection in the Absence of CD40/CD154 Interaction. *The Journal of Immunology*, *168*(9), 4643–4649.

Magnus, T., Schreiner, B., Korn, T., Jack, C., Guo, H., Antel, J., … Wiendl, H. (2005). Microglial expression of the B7 family member B7 homolog 1 confers strong immune inhibition: implications for immune responses and autoimmunity in the CNS. *The Journal of neuroscience: the official journal of the Society for Neuroscience*, *25*(10), 2537–2546. doi:10.1523/JNEUROSCI.4794-04.2005

Manzotti, C. N., Liu, M. K. P., Burke, F., Dussably, L., Zheng, Y., & Sansom, D. M. (2006). Integration of CD28 and CTLA-4 function results in differential responses of T cells to CD80 and CD86. *European journal of immunology*, *36*(6), 1413–1422. doi:10.1002/eji.200535170

Martin, R., Jaraquemada, D., Flerlage, M., Richert, J., Whitaker, J., Long, E. O., … McFarland, H. F. (1990). Fine specificity and HLA restriction of myelin basic protein-specific cytotoxic T cell lines from multiple sclerosis patients and healthy individuals. *The Journal of Immunology*, *145*(2), 540–548.

Martin, S., Agarwal, R., Murugaiyan, G., & Saha, B. (2010). CD40 Expression Levels Modulate Regulatory T Cells in Leishmania donovani Infection. *The Journal of Immunology*, *185*(1), 551–559. doi:10.4049/jimmunol.0902206

Martinez, F. O., Sica, A., Mantovani, A., & Locati, M. (2008). Macrophage activation and polarization. *Frontiers in bioscience: a journal and virtual library*, *13*, 453–461.

Mathur, R. K., Awasthi, A., Wadhone, P., Ramanamurthy, B., & Saha, B. (2004). Reciprocal CD40 signals through p38MAPK and ERK-1/2 induce counteracting immune responses. *Nature Medicine*, *10*(5), 540–544. doi:10.1038/nm1045

Mayhan, W. G. (2002). Cellular mechanisms by which tumor necrosis factor-alpha produces disruption of the blood-brain barrier. *Brain research*, *927*(2), 144–152.

McFarland, H. F., & Martin, R. (2007). Multiple sclerosis: a complicated picture of autoimmunity. *Nature immunology*, *8*(9), 913–919. doi:10.1038/ni1507

McGeachy, M. J., Bak-Jensen, K. S., Chen, Y., Tato, C. M., Blumenschein, W., McClanahan, T., & Cua, D. J. (2007). TGF-β and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain TH-17 cell–mediated pathology. *Nature Immunology*, *8*(12), 1390–1397. doi:10.1038/ni1539

McGeachy, M. J., Chen, Y., Tato, C. M., Laurence, A., Joyce-Shaikh, B., Blumenschein, W. M., … Cua, D. J. (2009). The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17–producing effector T helper cells in vivo. *Nature Immunology*, *10*(3), 314–324. doi:10.1038/ni.1698

McQualter, J. L., & Bernard, C. C. A. (2007). Multiple sclerosis: a battle between destruction and repair. *Journal of Neurochemistry*, *100*(2), 295–306. doi:10.1111/j.1471-4159.2006.04232.x

Melchior, B., Puntambekar, S. S., & Carson, M. J. (2006). Microglia and the control of autoreactive T cell responses. *Neurochemistry international*, *49*(2), 145–153. doi:10.1016/j.neuint.2006.04.002

Melendez, A. J. (2008). Sphingosine kinase signalling in immune cells: Potential as novel therapeutic targets. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, *1784*(1), 66–75. doi:10.1016/j.bbapap.2007.07.013

Mendel, I., Katz, A., Kozak, N., Ben-Nun, A., & Revel, M. (1998). Interleukin-6 functions in autoimmune encephalomyelitis: a study in gene-targeted mice. *European journal of*

*immunology*, *28*(5), 1727–1737. doi:10.1002/(SICI)1521-4141(199805)28:05<1727::AID-IMMU1727>3.0.CO;2-#

Merrill, J. E., Kono, D. H., Clayton, J., Ando, D. G., Hinton, D. R., & Hofman, F. M. (1992). Inflammatory leukocytes and cytokines in the peptide-induced disease of experimental allergic encephalomyelitis in SJL and B10.PL mice. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(2), 574–578.

Mills, C. D., Kincaid, K., Alt, J. M., Heilman, M. J., & Hill, A. M. (2000). M-1/M-2 Macrophages and the Th1/Th2 Paradigm. *The Journal of Immunology*, *164*(12), 6166–6173.

Min, B. (2008). Basophils: what they "can do" versus what they "actually do." *Nature Immunology*, *9*(12), 1333–1339. doi:10.1038/ni.f.217

Min, B., Prout, M., Hu-Li, J., Zhu, J., Jankovic, D., Morgan, E. S., … Paul, W. E. (2004). Basophils Produce IL-4 and Accumulate in Tissues after Infection with a Th2-inducing Parasite. *The Journal of Experimental Medicine*, *200*(4), 507–517. doi:10.1084/jem.20040590

Moore, K. W., de Waal Malefyt, R., Coffman, R. L., & O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. *Annual review of immunology*, *19*, 683–765. doi:10.1146/annurev.immunol.19.1.683

Mor, F., & Cohen, I. R. (1992). T cells in the lesion of experimental autoimmune encephalomyelitis. Enrichment for reactivities to myelin basic protein and to heat shock proteins. *The Journal of clinical investigation*, *90*(6), 2447–2455. doi:10.1172/JCI116136

Moretta, A., Marcenaro, E., Parolini, S., Ferlazzo, G., & Moretta, L. (2008). NK cells at the interface between innate and adaptive immunity. *Cell death and differentiation*, *15*(2), 226–233. doi:10.1038/sj.cdd.4402170

Morris, K., & Yiannikas, C. (2012). Treatment Update in Multiple Sclerosis. *Current Allergy and Asthma Reports*, *12*(3), 246–254. doi:10.1007/s11882-012-0256-5

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., & Coffman, R. L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *The Journal of Immunology*, *136*(7), 2348–2357.

Mosser, D. M. (2003). The many faces of macrophage activation. *Journal of Leukocyte Biology*, *73*(2), 209–212. doi:10.1189/jlb.0602325

Mosser, D. M., & Edwards, J. P. (2008). Exploring the full spectrum of macrophage activation. *Nature Reviews Immunology*, *8*(12), 958–969. doi:10.1038/nri2448

Moussaud, S., & Draheim, H. J. (2010). A new method to isolate microglia from adult mice and culture them for an extended period of time. *Journal of Neuroscience Methods*, *187*(2), 243–253. doi:10.1016/j.jneumeth.2010.01.017

Mukhopadhyay, S., Plüddemann, A., & Gordon, S. (2009). Macrophage Pattern Recognition Receptors in Immunity, Homeostasis and Self Tolerance. In U. Kishore (Ed.), *Target Pattern Recognition in Innate Immunity* (pp. 1–14). Springer New York. Retrieved from http://link.springer.com.helicon.vuw.ac.nz/chapter/10.1007/978-1-4419-0901-5_1

Munger, K. L., Levin, L. I., Hollis, B. W., Howard, N. S., & Ascherio, A. (2006). Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. *JAMA: the journal of the American Medical Association*, *296*(23), 2832–2838. doi:10.1001/jama.296.23.2832

Murugaiyan, G., Agrawal, R., Mishra, G. C., Mitra, D., & Saha, B. (2006). Functional Dichotomy in CD40 Reciprocally Regulates Effector T Cell Functions. *The Journal of Immunology*, *177*(10), 6642–6649.

Naundorf, S., Schröder, M., Höflich, C., Suman, N., Volk, H.-D., & Grütz, G. (2009). IL-10 interferes directly with TCR-induced IFN-γ but not IL-17 production in memory T

cells. *European Journal of Immunology*, *39*(4), 1066–1077. doi:10.1002/eji.200838773

Nimmerjahn, A., Kirchhoff, F., & Helmchen, F. (2005). Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science (New York, N.Y.)*, *308*(5726), 1314–1318. doi:10.1126/science.1110647

Nishimura, H., Nose, M., Hiai, H., Minato, N., & Honjo, T. (1999). Development of Lupus-like Autoimmune Diseases by Disruption of the PD-1 Gene Encoding an ITIM Motif-Carrying Immunoreceptor. *Immunity*, *11*(2), 141–151. doi:10.1016/S1074-7613(00)80089-8

Nishimura, H., Okazaki, T., Tanaka, Y., Nakatani, K., Hara, M., Matsumori, A., … Honjo, T. (2001). Autoimmune Dilated Cardiomyopathy in PD-1 Receptor-Deficient Mice. *Science*, *291*(5502), 319–322. doi:10.1126/science.291.5502.319

O'Connor, R. A., Prendergast, C. T., Sabatos, C. A., Lau, C. W. Z., Leech, M. D., Wraith, D. C., & Anderton, S. M. (2008). Cutting Edge: Th1 Cells Facilitate the Entry of Th17 Cells to the Central Nervous System during Experimental Autoimmune Encephalomyelitis. *Journal of immunology (Baltimore, Md.□: 1950)*, *181*(6), 3750–3754.

Ohmura-Hoshino, M., Matsuki, Y., Mito-Yoshida, M., Goto, E., Aoki-Kawasumi, M., Nakayama, M., … Ishido, S. (2009). Cutting edge: requirement of MARCH-I-mediated MHC II ubiquitination for the maintenance of conventional dendritic cells. *Journal of immunology (Baltimore, Md.: 1950)*, *183*(11), 6893–6897. doi:10.4049/jimmunol.0902178

Ortler, S., Leder, C., Mittelbronn, M., Zozulya, A. L., Knolle, P. A., Chen, L., … Wiendl, H. (2008). B7-H1 restricts neuroantigen-specific T cell responses and confines inflammatory CNS damage: implications for the lesion pathogenesis of multiple

sclerosis. *European journal of immunology*, *38*(6), 1734–1744. doi:10.1002/eji.200738071

Ouyang, W., Ranganath, S. H., Weindel, K., Bhattacharya, D., Murphy, T. L., Sha, W. C., & Murphy, K. M. (1998). Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. *Immunity*, *9*(5), 745–755.

Padigel, U. M., & Farrell, J. P. (2003). CD40-CD40 Ligand Costimulation Is Not Required for Initiation and Maintenance of a Th1-Type Response to Leishmania major Infection. *Infection and Immunity*, *71*(3), 1389–1395. doi:10.1128/IAI.71.3.1389-1395.2003

Paolicelli, R. C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., … Gross, C. T. (2011). Synaptic pruning by microglia is necessary for normal brain development. *Science (New York, N.Y.)*, *333*(6048), 1456–1458. doi:10.1126/science.1202529

Patrikios, P., Stadelmann, C., Kutzelnigg, A., Rauschka, H., Schmidbauer, M., Laursen, H., … Lassmann, H. (2006). Remyelination is extensive in a subset of multiple sclerosis patients. *Brain: a journal of neurology*, *129*(Pt 12), 3165–3172. doi:10.1093/brain/awl217

Pender, M. P., Csurhes, P. ., Houghten, R. A., McCombe, P. A., & Good, M. F. (1996). A study of human T-cell lines generated from multiple sclerosis patients and controls by stimulation with peptides of myelin basic protein. *Journal of Neuroimmunology*, *70*(1), 65–74. doi:10.1016/S0165-5728(96)00105-1

Pham, H., Doerrbecker, J., Ramp, A. A., D'Souza, C. S., Gorasia, D. G., Purcell, A. W., … Orian, J. M. (2011). Experimental autoimmune encephalomyelitis (EAE) IN C57Bl/6 mice is not associated with astrogliosis. *Journal of Neuroimmunology*, *232*(1–2), 51–62. doi:10.1016/j.jneuroim.2010.10.006

Pieper, K., Grimbacher, B., & Eibel, H. (2013). B-cell biology and development. *Journal of Allergy and Clinical Immunology*, *131*(4), 959–971. doi:10.1016/j.jaci.2013.01.046

Polman, C. H., O'Connor, P. W., Havrdova, E., Hutchinson, M., Kappos, L., Miller, D. H., … Sandrock, A. W. (2006). A Randomized, Placebo-Controlled Trial of Natalizumab for Relapsing Multiple Sclerosis. *New England Journal of Medicine*, *354*(9), 899–910. doi:10.1056/NEJMoa044397

Polumuri, S. K., Toshchakov, V. Y., & Vogel, S. N. (2007). Role of Phosphatidylinositol-3 Kinase in Transcriptional Regulation of TLR-Induced IL-12 and IL-10 by Fcγ Receptor Ligation in Murine Macrophages. *The Journal of Immunology*, *179*(1), 236–246.

Ponomarev, E. D., Maresz, K., Tan, Y., & Dittel, B. N. (2007). CNS-derived interleukin-4 is essential for the regulation of autoimmune inflammation and induces a state of alternative activation in microglial cells. *The Journal of neuroscience: the official journal of the Society for Neuroscience*, *27*(40), 10714–10721. doi:10.1523/JNEUROSCI.1922-07.2007

Ponomarev, E. D., Novikova, M., Maresz, K., Shriver, L. P., & Dittel, B. N. (2005). Development of a culture system that supports adult microglial cell proliferation and maintenance in the resting state. *Journal of Immunological Methods*, *300*(1–2), 32–46. doi:10.1016/j.jim.2005.02.011

Ponomarev, E. D., Shriver, L. P., & Dittel, B. N. (2006). CD40 expression by microglial cells is required for their completion of a two-step activation process during central nervous system autoimmune inflammation. *Journal of immunology (Baltimore, Md.: 1950)*, *176*(3), 1402–1410.

Poudrier, J., van Essen, D., Morales-Alcelay, S., Leanderson, T., Bergthorsdottir, S., & Gray, D. (1998). CD40 ligand signals optimize T helper cell cytokine production: role in Th2 development and induction of germinal centers. *European Journal of Immunology*, *28*(10), 3371–3383. doi:10.1002/(SICI)1521-4141(199810)28:10<3371::AID-IMMU3371>3.0.CO;2-C

Probst, H. C., McCoy, K., Okazaki, T., Honjo, T., & van den Broek, M. (2005). Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. *Nature Immunology*, *6*(3), 280–286. doi:10.1038/ni1165

Pul, R., Moharregh-Khiabani, D., Škuljec, J., Skripuletz, T., Garde, N., Voss, E. V., & Stangel, M. (2011). Glatiramer acetate modulates TNF-α and IL-10 secretion in microglia and promotes their phagocytic activity. *Journal of neuroimmune pharmacology: the official journal of the Society on NeuroImmune Pharmacology*, *6*(3), 381–388. doi:10.1007/s11481-010-9248-1

Quintana, A., Müller, M., Frausto, R. F., Ramos, R., Getts, D. R., Sanz, E., … Campbell, I. L. (2009). Site-Specific Production of IL-6 in the Central Nervous System Retargets and Enhances the Inflammatory Response in Experimental Autoimmune Encephalomyelitis. *The Journal of Immunology*. doi:10.4049/jimmunol.0900242

Raivich, G., & Banati, R. (2004). Brain microglia and blood-derived macrophages: molecular profiles and functional roles in multiple sclerosis and animal models of autoimmune demyelinating disease. *Brain research. Brain research reviews*, *46*(3), 261–281. doi:10.1016/j.brainresrev.2004.06.006

Ransohoff, R. M. (2012). Animal models of multiple sclerosis: the good, the bad and the bottom line. *Nature neuroscience*, *15*(8), 1074–1077. doi:10.1038/nn.3168

Ransohoff, R. M., Kivisäkk, P., & Kidd, G. (2003). Three or more routes for leukocyte migration into the central nervous system. *Nature Reviews Immunology*, *3*(7), 569–581. doi:10.1038/nri1130

Remer, K. A., Brcic, M., & Jungi, T. W. (2003). Toll-like receptor-4 is involved in eliciting an LPS-induced oxidative burst in neutrophils. *Immunology Letters*, *85*(1), 75–80. doi:10.1016/S0165-2478(02)00210-9

Renno, T., Krakowski, M., Piccirillo, C., Lin, J. Y., & Owens, T. (1995). TNF-alpha expression by resident microglia and infiltrating leukocytes in the central nervous

system of mice with experimental allergic encephalomyelitis. Regulation by Th1 cytokines. *Journal of immunology (Baltimore, Md.: 1950)*, *154*(2), 944–953.

Righi, M., Mori, L., De Libero, G., Sironi, M., Biondi, A., Mantovani, A., … Ricciardi-Castagnoli, P. (1989). Monokine production by microglial cell clones. *European journal of immunology*, *19*(8), 1443–1448. doi:10.1002/eji.1830190815

Robinson, J. H., & Delvig, A. A. (2002). Diversity in MHC class II antigen presentation. *Immunology*, *105*(3), 252–262. doi:10.1046/j.0019-2805.2001.01358.x

Saibil, S. D., Deenick, E. K., & Ohashi, P. S. (2007). The sound of silence: modulating anergy in T lymphocytes. *Current opinion in immunology*, *19*(6), 658–664. doi:10.1016/j.coi.2007.08.005

Sakaguchi, S., Wing, K., Onishi, Y., Prieto-Martin, P., & Yamaguchi, T. (2009). Regulatory T cells: how do they suppress immune responses? *International Immunology*, *21*(10), 1105–1111. doi:10.1093/intimm/dxp095

Salama, A. D., Chitnis, T., Imitola, J., Ansari, M. J. I., Akiba, H., Tushima, F., … Khoury, S. J. (2003). Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis. *The Journal of experimental medicine*, *198*(1), 71–78. doi:10.1084/jem.20022119

Schell, J. B., Crane, C. A., Smith, M. F., Jr, & Roberts, M. R. (2007). Differential ex vivo nitric oxide production by acutely isolated neonatal and adult microglia. *Journal of neuroimmunology*, *189*(1-2), 75–87. doi:10.1016/j.jneuroim.2007.07.004

Schlegelmilch, T., Henke, K., & Peri, F. (2011). Microglia in the developing brain: from immunity to behaviour. *Current opinion in neurobiology*, *21*(1), 5–10. doi:10.1016/j.conb.2010.08.004

Schmid, C. D., Melchior, B., Masek, K., Puntambekar, S. S., Danielson, P. E., Lo, D. D., … Carson, M. J. (2009). Differential gene expression in LPS/IFN? activated microglia

and macrophages: in vitro versus in vivo. *Journal of Neurochemistry*, *109*(Suppl. 1), 117–125. doi:10.1111/j.1471-4159.2009.05984.x

Schreiner, B., Bailey, S. L., Shin, T., Chen, L., & Miller, S. D. (2008). PD-1 ligands expressed on myeloid-derived APC in the CNS regulate T-cell responses in EAE. *European journal of immunology*, *38*(10), 2706–2717. doi:10.1002/eji.200838137

Schreiner, B., Mitsdoerffer, M., Kieseier, B. C., Chen, L., Hartung, H.-P., Weller, M., & Wiendl, H. (2004). Interferon-β enhances monocyte and dendritic cell expression of B7-H1 (PD-L1), a strong inhibitor of autologous T-cell activation: relevance for the immune modulatory effect in multiple sclerosis. *Journal of Neuroimmunology*, *155*(1–2), 172–182. doi:10.1016/j.jneuroim.2004.06.013

Schwab, I., & Nimmerjahn, F. (2013). Intravenous immunoglobulin therapy: how does IgG modulate the immune system? *Nature Reviews Immunology*, *13*(3), 176–189. doi:10.1038/nri3401

Sedgwick, J. D., Schwender, S., Gregersen, R., Dörries, R., & Meulen, V. ter. (1993). Resident macrophages (ramified microglia) of the adult brown Norway rat central nervous system are constitutively major histocompatibility complex class II positive. *The Journal of Experimental Medicine*, *177*(4), 1145–1152. doi:10.1084/jem.177.4.1145

Sedgwick, J. D., Schwender, S., Imrich, H., Dörries, R., Butcher, G. W., & ter Meulen, V. (1991). Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system. *Proceedings of the National Academy of Sciences of the United States of America*, *88*(16), 7438–7442.

Shamri, R., Xenakis, J. J., & Spencer, L. A. (2011). Eosinophils in innate immunity: an evolving story. *Cell and Tissue Research*, *343*(1), 57–83. doi:10.1007/s00441-010-1049-6

Shen, L., & Rock, K. L. (2006). Priming of T cells by exogenous antigen cross-presented on MHC class I molecules. *Current Opinion in Immunology*, *18*(1), 85–91. doi:10.1016/j.coi.2005.11.003

Shimizu, Y., Ota, K., Ikeguchi, R., Kubo, S., Kabasawa, C., & Uchiyama, S. (2013). Plasma osteopontin levels are associated with disease activity in the patients with multiple sclerosis and neuromyelitis optica. *Journal of Neuroimmunology*, *263*(1–2), 148–151. doi:10.1016/j.jneuroim.2013.07.005

Sica, A., Saccani, A., Bottazzi, B., Polentarutti, N., Vecchi, A., Damme, J. V., & Mantovani, A. (2000). Autocrine Production of IL-10 Mediates Defective IL-12 Production and NF-κB Activation in Tumor-Associated Macrophages. *The Journal of Immunology*, *164*(2), 762–767.

Sinistro, A., Ciaprini, C., Natoli, S., Sussarello, E., Calo Carducci, F., Almerighi, C., … Bergamini, A. (2007). Lipopolysaccharide desensitizes monocytes-macrophages to CD40 ligand stimulation. *Immunology*, *122*(3), 362–370. doi:10.1111/j.1365-2567.2007.02648.x

Siragam, V., Crow, A. R., Brinc, D., Song, S., Freedman, J., & Lazarus, A. H. (2006). Intravenous immunoglobulin ameliorates ITP via activating Fcγ receptors on dendritic cells. *Nature Medicine*, *12*(6), 688–692. doi:10.1038/nm1416

Slaney, C. Y., Toker, A., La Flamme, A., Bäckström, B. T., & Harper, J. L. (2011). Naïve blood monocytes suppress T-cell function. A possible mechanism for protection from autoimmunity. *Immunology and Cell Biology*, *89*(1), 7–13. doi:10.1038/icb.2010.110

Smith, M. E., Eller, N. L., McFarland, H. F., Racke, M. K., & Raine, C. S. (1999). Age Dependence of Clinical and Pathological Manifestations of Autoimmune Demyelination. *The American Journal of Pathology*, *155*(4), 1147–1161.

Smith-Garvin, J. E., Koretzky, G. A., & Jordan, M. S. (2009). T Cell Activation. *Annual Review of Immunology*, *27*(1), 591–619. doi:10.1146/annurev.immunol.021908.132706

Sokol, C. L., & Medzhitov, R. (2010). Role of basophils in the initiation of Th2 responses. *Current Opinion in Immunology*, *22*(1), 73–77. doi:10.1016/j.coi.2010.01.012

Sonobe, Y., Yawata, I., Kawanokuchi, J., Takeuchi, H., Mizuno, T., & Suzumura, A. (2005). Production of IL-27 and other IL-12 family cytokines by microglia and their subpopulations. *Brain Research*, *1040*(1–2), 202–207. doi:10.1016/j.brainres.2005.01.100

Sorensen, P. S., Fazekas, F., & Lee, M. (2002). Intravenous immunoglobulin G for the treatment of relapsing–remitting multiple sclerosis: a meta-analysis. *European Journal of Neurology*, *9*(6), 557–563. doi:10.1046/j.1468-1331.2002.00501.x

Sospedra, M., & Martin, R. (2005). Immunology of Multiple Sclerosis*. *Annual Review of Immunology*, *23*(1), 683–747. doi:10.1146/annurev.immunol.23.021704.115707

Spörri, R., & Reis e Sousa, C. (2005). Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. *Nature Immunology*, *6*(2), 163–170. doi:10.1038/ni1162

Sprent, J., & Kishimoto, H. (2001). The thymus and central tolerance. *Philosophical Transactions of the Royal Society of London. Series B*, *356*(1409), 609–616. doi:10.1098/rstb.2001.0846

Staniland, A. A., Clark, A. K., Wodarski, R., Sasso, O., Maione, F., D'Acquisto, F., & Malcangio, M. (2010). Reduced inflammatory and neuropathic pain and decreased spinal microglial response in fractalkine receptor (CX3CR1) knockout mice. *Journal of Neurochemistry*, *114*(4), 1143–1157. doi:10.1111/j.1471-4159.2010.06837.x

Stein, M., Keshav, S., Harris, N., & Gordon, S. (1992). Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *The Journal of experimental medicine*, *176*(1), 287–292.

Steinman, L., & Zamvil, S. S. (2006). How to successfully apply animal studies in experimental allergic encephalomyelitis to research on multiple sclerosis. *Annals of neurology*, *60*(1), 12–21. doi:10.1002/ana.20913

Steinman, R. M. (2007). Dendritic cells: Understanding immunogenicity. *European Journal of Immunology*, *37*(S1), S53–S60. doi:10.1002/eji.200737400

Stout, R. D., & Suttles, J. (2004). Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *Journal of Leukocyte Biology*, *76*(3), 509–513. doi:10.1189/jlb.0504272

Sun, J., Zhang, X., Broderick, M., & Fein, H. (2003). Measurement of Nitric Oxide Production in Biological Systems by Using Griess Reaction Assay. *Sensors*, *3*(8), 276–284. doi:10.3390/s30800276

Sutterwala, F. S., Noel, G. J., Salgame, P., & Mosser, D. M. (1998). Reversal of Proinflammatory Responses by Ligating the Macrophage Fcγ Receptor Type I. *The Journal of Experimental Medicine*, *188*(1), 217–222. doi:10.1084/jem.188.1.217

Szabo, S. J., Sullivan, B. M., Peng, S. L., & Glimcher, L. H. (2003). Molecular Mechanisms Regulating Th1 Immune Responses. *Annual Review of Immunology*, *21*(1), 713–758. doi:10.1146/annurev.immunol.21.120601.140942

Tamada, K., Shimozaki, K., Chapoval, A. I., Zhai, Y., Su, J., Chen, S.-F., … Chen, L. (2000). LIGHT, a TNF-Like Molecule, Costimulates T Cell Proliferation and Is Required for Dendritic Cell-Mediated Allogeneic T Cell Response. *The Journal of Immunology*, *164*(8), 4105–4110.

Tao, X., Constant, S., Jorritsma, P., & Bottomly, K. (1997). Strength of TCR signal determines the costimulatory requirements for Th1 and Th2 CD4+ T cell differentiation. *The Journal of Immunology*, *159*(12), 5956–5963.

Taylor, B. V., Pearson, J. F., Clarke, G., Mason, D. F., Abernethy, D. A., Willoughby, E., & Sabel, C. (2010). MS prevalence in New Zealand, an ethnically and latitudinally diverse country. *Multiple sclerosis (Houndmills, Basingstoke, England)*, *16*(12), 1422–1431. doi:10.1177/1352458510379614

Thibodeau, J., Bourgeois-Daigneault, M.-C., Huppé, G., Tremblay, J., Aumont, A., Houde, M., … Steimle, V. (2008). Interleukin-10-induced MARCH1 mediates intracellular sequestration of MHC class II in monocytes. *European journal of immunology*, *38*(5), 1225–1230. doi:10.1002/eji.200737902

Thompson, W. L., Karpus, W. J., & Eldik, L. J. V. (2008). MCP-1-deficient mice show reduced neuroinflammatory responses and increased peripheral inflammatory responses to peripheral endotoxin insult. *Journal of Neuroinflammation*, *5*(1), 35. doi:10.1186/1742-2094-5-35

Tierney, J. B., Kharkrang, M., & La Flamme, A. C. (2009). Type II-activated macrophages suppress the development of experimental autoimmune encephalomyelitis. *Immunology and Cell Biology*, *87*(3), 235–240. doi:10.1038/icb.2008.99

Toker, A., Slaney, C. Y., Bäckström, B. T., & Harper, J. L. (2011). Glatiramer Acetate Treatment Directly Targets CD11b+Ly6G− Monocytes and Enhances the Suppression of Autoreactive T cells in Experimental Autoimmune Encephalomyelitis. *Scandinavian Journal of Immunology*, *74*(3), 235–243. doi:10.1111/j.1365-3083.2011.02575.x

Tran, E. H., Hoekstra, K., Rooijen, N. van, Dijkstra, C. D., & Owens, T. (1998). Immune Invasion of the Central Nervous System Parenchyma and Experimental Allergic

Encephalomyelitis, But Not Leukocyte Extravasation from Blood, Are Prevented in Macrophage-Depleted Mice. *The Journal of Immunology*, *161*(7), 3767–3775.

Tsushima, F., Yao, S., Shin, T., Flies, A., Flies, S., Xu, H., … Chen, L. (2007). Interaction between B7-H1 and PD-1 determines initiation and reversal of T-cell anergy. *Blood*, *110*(1), 180–185. doi:10.1182/blood-2006-11-060087

Uronen, H., & Callard, R. E. (2000). Absence of CD40–CD40 ligand interactions in X-linked hyper-IgM syndrome does not affect differentiation of T helper cell subsets. *Clinical & Experimental Immunology*, *121*(2), 346–352. doi:10.1046/j.1365-2249.2000.01307.x

Van der Veen, R. C., Dietlin, T. A., Dixon Gray, J., & Gilmore, W. (2000). Macrophage-Derived Nitric Oxide Inhibits the Proliferation of Activated T Helper Cells and Is Induced during Antigenic Stimulation of Resting T Cells. *Cellular Immunology*, *199*(1), 43–49. doi:10.1006/cimm.1999.1597

Van Panhuys, N., Tang, S.-C., Prout, M., Camberis, M., Scarlett, D., Roberts, J., … Le Gros, G. (2008). In vivo studies fail to reveal a role for IL-4 or STAT6 signaling in Th2 lymphocyte differentiation. *Proceedings of the National Academy of Sciences*, *105*(34), 12423–12428. doi:10.1073/pnas.0806372105

Varin, A., & Gordon, S. (2009). Alternative activation of macrophages: immune function and cellular biology. *Immunobiology*, *214*(7), 630–641. doi:10.1016/j.imbio.2008.11.009

Vidyadaran, S., Ooi, Y. Y., Subramaiam, H., Badiei, A., Abdullah, M., Ramasamy, R., & Seow, H. F. (2009). Effects of macrophage colony-stimulating factor on microglial responses to lipopolysaccharide and beta amyloid. *Cellular Immunology*, *259*(1), 105–110. doi:10.1016/j.cellimm.2009.06.005

Vivier, E., Raulet, D. H., Moretta, A., Caligiuri, M. A., Zitvogel, L., Lanier, L. L., … Ugolini, S. (2011). Innate or Adaptive Immunity? The Example of Natural Killer Cells. *Science*, *331*(6013), 44–49. doi:10.1126/science.1198687

Vogt, L., Schmitz, N., Kurrer, M. O., Bauer, M., Hinton, H. I., Behnke, S., … Bachmann, M. F. (2006). VSIG4, a B7 family-related protein, is a negative regulator of T cell activation. *Journal of Clinical Investigation*, *116*(10), 2817–2826. doi:10.1172/JCI25673

Wakefield, J. S., Gale, J. S., Berridge, M. V., Jordan, T. W., & Ford, H. C. (1982). Is Percoll innocuous to cells? *Biochemical Journal*, *202*(3), 795–797.

Walunas, T. L., Bakker, C. Y., & Bluestone, J. A. (1996). CTLA-4 ligation blocks CD28-dependent T cell activation. *The Journal of Experimental Medicine*, *183*(6), 2541–2550. doi:10.1084/jem.183.6.2541

Wang, S., Bajorath, J., Flies, D. B., Dong, H., Honjo, T., & Chen, L. (2003). Molecular Modeling and Functional Mapping of B7-H1 and B7-DC Uncouple Costimulatory Function from PD-1 Interaction. *The Journal of Experimental Medicine*, *197*(9), 1083–1091. doi:10.1084/jem.20021752

Waterhouse, P., Penninger, J. M., Timms, E., Wakeham, A., Shahinian, A., Lee, K. P., … Mak, T. W. (1995). Lymphoproliferative Disorders with Early Lethality in Mice Deficient in Ctla-4. *Science*, *270*(5238), 985–988. doi:10.1126/science.270.5238.985

Weber, M. S., Prod'homme, T., Youssef, S., Dunn, S. E., Rundle, C. D., Lee, L., … Zamvil, S. S. (2007). Type II monocytes modulate T cell–mediated central nervous system autoimmune disease. *Nature Medicine*, *13*(8), 935–943. doi:10.1038/nm1620

Weiner, H. L. (2009). The challenge of multiple sclerosis: how do we cure a chronic heterogeneous disease? *Annals of neurology*, *65*(3), 239–248. doi:10.1002/ana.21640

Weir, C. R., Nicolson, K., & Bäckström, B. T. (2002). Experimental autoimmune encephalomyelitis induction in naive mice by dendritic cells presenting a self-peptide. *Immunology and cell biology*, *80*(1), 14–20. doi:10.1046/j.1440-1711.2002.01056.x

Xu, J., Yang, Y., Qiu, G., Lal, G., Wu, Z., Levy, D. E., … Ding, Y. (2009). c-Maf regulates IL-10 expression during Th17 polarization. *Journal of immunology (Baltimore, Md.: 1950)*, *182*(10), 6226–6236. doi:10.4049/jimmunol.0900123

Yadav, V., & Bourdette, D. (2012). New disease-modifying therapies and new challenges for MS. *Current neurology and neuroscience reports*, *12*(5), 489–491. doi:10.1007/s11910-012-0295-2

Yamane, H., & Paul, W. E. (2013). Early signaling events that underlie fate decisions of naive CD4(+) T cells toward distinct T-helper cell subsets. *Immunological reviews*, *252*(1), 12–23. doi:10.1111/imr.12032

Yamazaki, T., Akiba, H., Koyanagi, A., Azuma, M., Yagita, H., & Okumura, K. (2005). Blockade of B7-H1 on Macrophages Suppresses CD4+ T Cell Proliferation by Augmenting IFN-γ-Induced Nitric Oxide Production. *The Journal of Immunology*, *175*(3), 1586–1592.

Yang, G. Y., Gong, C., Qin, Z., Liu, X. H., & Lorris Betz, A. (1999). Tumor necrosis factor alpha expression produces increased blood-brain barrier permeability following temporary focal cerebral ischemia in mice. *Brain research. Molecular brain research*, *69*(1), 135–143.

Yang, X. O., Panopoulos, A. D., Nurieva, R., Chang, S. H., Wang, D., Watowich, S. S., & Dong, C. (2007). STAT3 Regulates Cytokine-mediated Generation of Inflammatory Helper T Cells. *Journal of Biological Chemistry*, *282*(13), 9358–9363. doi:10.1074/jbc.C600321200

Yip, P. K., Kaan, T. K. Y., Fenesan, D., & Malcangio, M. (2009). Rapid isolation and culture of primary microglia from adult mouse spinal cord. *Journal of Neuroscience Methods*, *183*(2), 223–237. doi:10.1016/j.jneumeth.2009.07.002

Zhang, G.-X., Li, J., Ventura, E., & Rostami, A. (2002). Parenchymal Microglia of Naïve Adult C57BL/6J Mice Express High Levels of B7.1, B7.2, and MHC Class II. *Experimental and Molecular Pathology*, *73*(1), 35–45. doi:10.1006/exmp.2002.2441

Zhang, X., Koldzic, D. N., Izikson, L., Reddy, J., Nazareno, R. F., Sakaguchi, S., … Weiner, H. L. (2004). IL-10 is involved in the suppression of experimental autoimmune encephalomyelitis by CD25+CD4+ regulatory T cells. *International Immunology*, *16*(2), 249–256. doi:10.1093/intimm/dxh029

Zhu, J., & Paul, W. E. (2008). CD4 T cells: fates, functions, and faults. *Blood*, *112*(5), 1557–1569. doi:10.1182/blood-2008-05-078154

Zhu, J., & Paul, W. E. (2009). Heterogeneity and plasticity of T helper cells. *Cell Research*, *20*(1), 4–12. doi:10.1038/cr.2009.138

Zhu, J., Yamane, H., & Paul, W. E. (2010). Differentiation of Effector CD4 T Cell Populations*. *Annual Review of Immunology*, *28*(1), 445–489. doi:10.1146/annurev-immunol-030409-101212

# Appendices

# A- Recipes

## Phosphate buffered saline (PBS)

***10xPBS***

| | |
|---|---|
| NaCl | 170 g |
| $Na_2HPO_4.12H_2O$ | 62.32 g |
| $NaH_2PO_4.2H_2O$ | 4.04 g |
| 2 litres $ddH_2O$ | |

***1xPBS***

Dilute 10:1 $ddH_2O$ for working solution of 1X PBS.

## Percoll Diluent (v)

| | |
|---|---|
| 10X PBS | 45 ml |
| 0.6M HCl | 3 ml |
| $ddH_2O$ | 132ml |
| Sterilise with a syringe filter | |

## 70% Percoll (v/v)

| | |
|---|---|
| Percoll (stock) | 63% |
| Percoll diluent | 37% |

## 37% Percoll (v/v)

| | |
|---|---|
| Percoll (70%) | 53% |
| 1xPBS | 47% |

## 30% Percoll (v/v)

| | |
|---|---|
| Percoll (70%) | 43% |
| 1xPBS | 57% |

## FACs buffer (v/v)

| | |
|---|---|
| FCS | 2% |
| Sodium azide (1 M) | 0.1% |
| 1xPBS | 97.9% |

## Pertussis toxin buffer

| | |
|---|---|
| Tris | 15mM |
| Sodium chloride | 0.5M |
| Triton X-100 | 0.017% (v/v) |

In ddH2O, filter sterilised using 0.22μm syringe filter

**Complete T cell media (CTCM) (v/v)**

| | |
|---|---|
| Dulbecco's Modified Eagle Medium | 85.9% |
| FCS | 10% |
| L-glutamate (200 mM) | 1% |
| Penicillin/Streptomycin (100 U/ml/10 mg/ml) | 1% |
| HEPES buffer (1 M) | 1% |
| β-Mecaptoethanol (55 mM) | 0.1% |
| Non-essential amino acids (10nM) | 1% |

**Wash buffer (v/v)**

| | |
|---|---|
| Dulbecco's Modified Eagle Medium | 96% |
| HEPES buffer (1M) | 3% |
| Penicillin/Streptomycin (100 U/ml/10 mg/ml) | 1% |

**DFP media (v/v)**

| | |
|---|---|
| Dulbecco's Modified Eagle Medium | 83% |
| FCS | 15% |
| L-glutamate (200 mM) | 1% |
| Penicillin/Streptomycin (100 U/ml/10 mg/ml) | 1% |

**MG media (v/v)**

| | |
|---|---|
| Dulbecco's Modified Eagle Medium | 87.9% |
| FCS | 10% |
| L-glutamate (200 mM) | 1% |
| Penicillin/Streptomycin (100 U/ml/10 mg/ml) | 1% |
| β-Mecaptoethanol (55 mM) | 0.09% |

**ELISA capture buffer**

| | |
|---|---|
| $Na_2HPO_4$ | 0.1M |
| In $ddH_2O$, adjusted to pH 9. | |

**ELISA stop solution**

| | |
|---|---|
| $H_2SO_4$ | 0.18M |
| In $ddH_2O$ | |

**Dynabead isolation buffer (v/v)**

| | |
|---|---|
| FCS | 2% |
| EDTA (0.5 mM) | 0.004% |
| 1xdPBS | 47.006% |

**IHC block**

| | |
|---|---|
| Donkey serum (Sigma, USA) | 4% |
| 1xPBS | |

**Griess solution A (w/v)**

| | |
|---|---|
| Sulphanilamide | 1% |
| In 2.5% Phosphoric acid | |

**Griess solution B (w/v)**

| | |
|---|---|
| N-(1-napthyl) ethylenediamine | 1% |
| In 2.5% Phosphoric acid | |

**Peroxidase quenching solution (v/v)**

| | |
|---|---|
| Methanol | 50% |
| $H_2O_2$ | 1% |
| $ddH_2O$ | 49% |

**Acid alcohol (v/v)**

| | |
|---|---|
| HCl | 0.3% |
| Ethanol | 70% |
| $ddH_2O$ | 29.7% |

**0.01% Lithium Carbonate (w/v)**

| | |
|---|---|
| Lithium Carbonate | 0.01% |
| $ddH_2O$ | 99.99 |

**4% Paraformaldehyde (w/v)**

| | |
|---|---|
| Paraformaldehyde | 4% |
| 1xPBS | 96% |
| ph adjusted to 7.4 | |

**TPBS (v/v)**

| | |
|---|---|
| TritonX-100 | 0.2% |
| 1xPBS | 99.8% |

**PBST (v/v)**

| | |
|---|---|
| Tween 20 | 0.05% |
| 1xPBS | 99.5% |

**30% Sucrose**

| | |
|---|---|
| Sucrose (w/v) | 30% |
| Sodium Azide (1 M) (v/v) | 0.1% |
| $ddH_2O$ | 69.9% |

**TAE buffer**

***50x TAE buffer***

| | |
|---|---|
| Tris base | 242g |
| EDTA (0.5M stock) | 100ml |
| Glacial acetic acid | 5.71 ml |

In 1 litre of $ddH_2O$

***1xTAE buffer***

Dilute 50:1 with $ddH_2O$ for working solution of 1x TAE.

# B- Antibodies and proteins

## B-1.1 Flow cytometry antibodies

| Specificity | Label | Species and Isotype | Manufacturer | Optimal dilution |
|---|---|---|---|---|
| B220 | FITC | Rat IgG2a | BD (PharMingen) | 1:1000 |
| CD4 | V500 | Rat IgG2a | BD (PharMingen) | 1:1000 |
| CD8 | PerCP-Cy5.5 | Rat IgG2a | eBioscience | 1:400 |
| CD11b (MAC-1) | FITC | Rat IgG2b | BD (PharMingen) | 1:1000 |
| | PE | Rat IgG2b | eBioscience | 1:2000 |
| CD25 | APC | Rat IgG1 | BD (PharMingen) | 1:600 |
| CD40 | PE | Rat IgG2a | BD (PharMingen) | 1:200 |
| CD44 | PE | Rat IgG2a | eBioscience | 1:2000 |
| CD45 | Cyc | Rat IgG2b | BD (PharMingen) | 1:500 |
| CD45.1 | PE | Mouse IgG2a | Biolegend | 1:400 |
| CD45.2 | APC | Mouse IgG2, k | BD (PharMingen) | 1:200 (blood)<br>1:100 (CNS) |
| CD62L | APC | Rat IgG2a | BD (PharMingen) | 1:3000 |
| CD124 (IL-4Rα) | PE | Rat IgG2a | BD (PharMingen) | 1:400 |
| F4/80 | Biotin | Rat IgG | Serotec | 1:2000 |
| Gr-1 | PE | Rat IgG2b | BD (PharMingen) | 1:2000 |
| | APC-Cy7 | Rat IgG2b | BD (PharMingen) | 1:800 |
| I-Ab | PE | Rat IgG2a | BD (PharMingen) | 1:800 |
| PD-L1 | PE | Rat IgG2a | eBioscience | 1:1500 |
| $V\alpha_{3.2}$ | FITC | Rat IgG2b, k | BD (PharMingen) | 1:400 |
| $V\beta_{11}$ | PE | Rat IgG2b | BD (PharMingen) | 1:400 |
| Isotype controls | FITC | Rat IgG2a | BD (PharMingen) | 1:800 |
| | PE | Rat IgG1 | BD (PharMingen) | 1:800 |
| | | Rat IgG2a | BD (PharMingen) | 1:800 |
| | CyC | Rat IgG2a | BD (PharMingen) | 1:800 |
| | APC | Rat IgG2a | BD (PharMingen) | 1:800 |
| | APC-Cy7 | Rat IgG2b | BD (PharMingen) | 1:800 |
| | V500 | Rat IgG2a | BD (PharMingen) | 1:800 |

## B-1.2 Secondary reagents for flow cytometry

| Label | Manufacturer | Optimal dilution |
|---|---|---|
| FITC | BD (PharMingen) | 1:2000 |
| PE | BD (PharMingen) | 1:2000 |
| PE-Cy5 | BD (PharMingen) | 1:2000 |
| APC | BD (PharMingen) | 1:800 |
| APC-Cy7 | BD (PharMingen) | 1:400 |
| V450 | BD (PharMingen) | 1:2000 |
| V500 | BD (PharMingen) | 1:2000 |

## B-1.3. ELISA Antibodies

All standard curves were generated by 2-fold serial dilutions and contained 2 blank wells.

IL-12p40 (BD Biosciences)

| Reagent | Concentration/Dilution | Diluent |
|---|---|---|
| Capture | 1:1000 | 0.1 M Na2HPO4, pH=9.0 |
| Blocking solution | 10% FCS | 1x PBS pH=7.4 |
| Top standard | 4 ng/ml | 5% FCS in 1x PBS pH=7.4 |
| Detection | 1:1000 | 5% FCS in 1x PBS pH=7.4 |
| Streptavidin horseradish peroxidase | 1:2000 | 5% FCS in 1x PBS pH=7.4 |

IL-10 (BD Biosciences)

| Reagent | Concentration/Dilution | Diluent |
|---|---|---|
| Capture | 1:500 | 0.1 M Na2HPO4, pH=9.0 |
| Blocking solution | 10% FCS | 1x PBS pH=7.4 |
| Top standard | 25 ng/ml | 10% FCS in 1x PBS pH=7.4 |
| Detection | 1:2500 | 10% FCS in 1x PBS pH=7.4 |
| Streptavidin horseradish peroxidase | 1:1000 | 10% FCS in 1x PBS pH=7.4 |

IFN-γ (BD Biosciences)

| Reagent | Concentration/Dilution | Diluent |
|---|---|---|
| Capture | 1:1000 | 0.1 M Na2HPO4, pH=9.0 |
| Blocking solution | 5% FCS | 1x PBS pH=7.4 |
| Top standard | 4 ng/ml | 5% FCS in 1x PBS pH=7.4 |
| Detection | 1:4000 | 5% FCS in 1x PBS pH=7.4 |
| Streptavidin horseradish peroxidase | 1:2000 | 5% FCS in 1x PBS pH=7.4 |

IL-2 (BD Biosciences)

| Reagent | Concentration/Dilution | Diluent |
|---|---|---|
| Capture | 1:1000 | 0.1 M Na2HPO4, pH=9.0 |
| Blocking solution | 5% FCS | 1x PBS pH=7.4 |
| Top standard | 500 pg/ml | 5% FCS in 1x PBS pH=7.4 |
| Detection | 1:1000 | 5% FCS in 1x PBS pH=7.4 |
| Streptavidin horseradish peroxidase | 1:2000 | 5% FCS in 1x PBS pH=7.4 |

IL-17A (BD Biosciences)

| Reagent | Concentration/Dilution | Diluent |
|---|---|---|
| Capture | 1:500 | 0.1 M Na2HPO4, pH=9.0 |
| Blocking solution | 10% FCS | 1x PBS pH=7.4 |
| Top standard | 1 ng/ml | 5% FCS in 1x PBS pH=7.4 |
| Detection | 1:500 | 5% FCS in 1x PBS pH=7.4 |
| Streptavidin horseradish peroxidase | 1:1000 | 5% FCS in 1x PBS pH=7.4 |

IL-4 (BD Biosciences)

| Reagent | Concentration/Dilution | Diluent |
|---|---|---|
| Capture | 1:2000 | 1x PBS pH=7.4 |
| Blocking solution | 5% FCS | 1x PBS pH=7.4 |
| Top standard | 2350 pg/ml | 5% FCS in 1x PBS pH=7.4 |
| Detection | 1:4000 | 5% FCS in 1x PBS pH=7.4 |
| Streptavidin horseradish peroxidase | 1:2000 | 5% FCS in 1x PBS pH=7.4 |

**B-1.4. Antibodies and proteins for MΦ/MG:T cell co-culture**

Antibodies

| Specificity | Species and Isotype | Clone | Company |
|---|---|---|---|
| αIL-12p40 | Rat $IgG_1$ | C15.6 | BD Bioscience |
| αIL-10 | Rat $IgG_1$, κ | JES5-2A5 | BD Bioscience |
| αPD-1/CD279 | Rat $IgG_{2a}$, κ | RMP1-14 | Biolegend |
| αCD40L/CD154 | Ar Ham $IgG_3$, κ | MR1 | BD Bioscience |
| Isotype | Ar Ham $IgG_3$, κ | E36-239 | BD Bioscience |
| | Rat $IgG_{2a}$, κ | R35-95 | BD Bioscience |

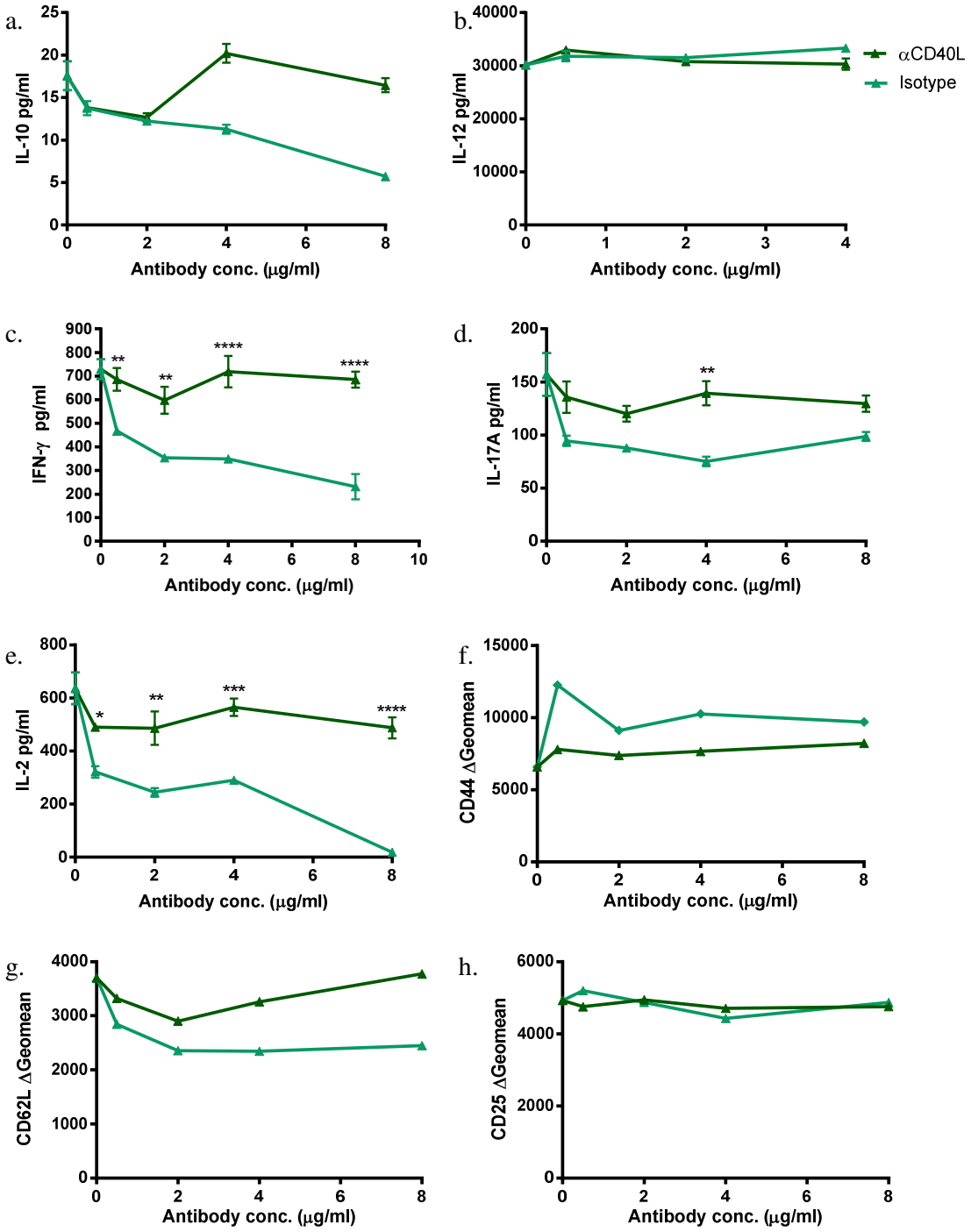
Protein

| Protein | Protein type | Company |
|---|---|---|
| IL-12p70 | Recombinant cytokine | BD Bioscience |
| IL-10 | Recombinant cytokine | BD Bioscience |
| Recombinant mouse B7-H1/PD-L1 Fc chimera | Chimeric protein | R&D systems |
| Recombinant human $IgG_1$ Fc | Chimeric protein | R&D systems |

# C. Supplementary Figures

## C-1 Supplementary figures for chapter 4

### C-1.1 Titration of the αCD40L antibody



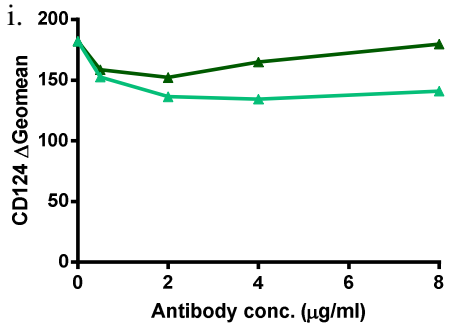
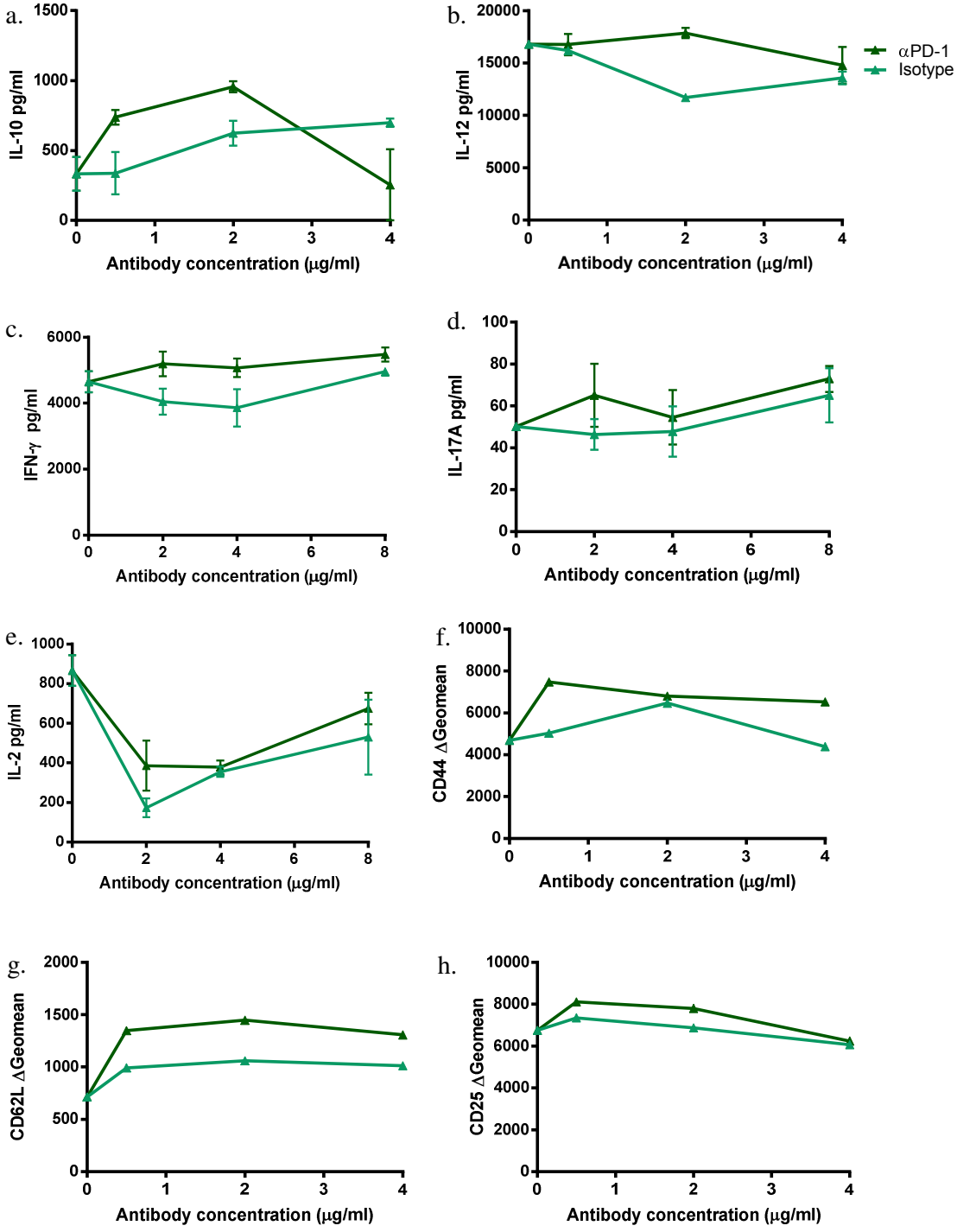



**Figure C1. T cells cultured with classically activated MΦ in the presence of αCD40L maintained cytokine production and expression of cell surface markers.** BMMΦ were stimulated with IFN-γ (20 U/ml) overnight and then cultured alone or with LPS (200 ng/ml) with or without IC (10 per MΦ). After four hours, purified $CD4^+2D2$ T cells and $MOG_{35-55}$ (25 μg/ml) were added to the MΦ cultures for 72 hours. αCD40L (MR1; 0.5-8 μg/ml) or hamster isotype control antibody ($IgG_1$) was added to the cultures. IL-10 (a) and IL-12 (b), was measured by ELISA, IFN-γ (c), IL-17A (d), and IL-2 (e) were measured in the culture supernatant by CBA, CD44 (f), CD62L (g), CD25 (h), and CD124 (i) expression was measured by flow cytometry. Shown are the means and SEM of triplicate wells from one of two experiments (IL-10, IL-12 IFN-γ, IL-17A, and IL-2) and data from one of two experiments (CD44, CD62L, CD25, and CD124). *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 by two-way ANOVA with Sidak's post test.

## C-1.2 Titration of the αPD-1 antibody



h.

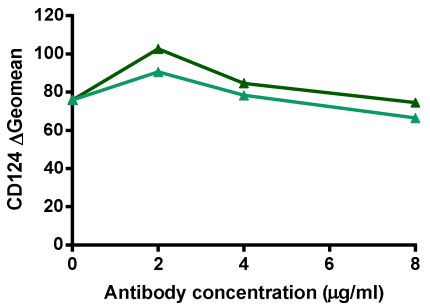



**Figure C2. Blocking the PD-1/PD-L1 pathway had only a small effect of T cells and MΦ.** BMMΦ were stimulated with IFN-γ (20 U/ml) overnight and then cultured alone or with LPS (200 ng/ml) with or without IC (10 per MΦ). After four hours, purified $CD4^+2D2$ T cells and $MOG_{35-55}$ (25 μg/ml) were added to the MΦ cultures for 72 hours. Various concentrations of αPD-1 (0.5-4 μg/ml) were added to cultures with the T cells. . IL-10 (a), IL-12 (b), IFN-γ (c), IL-17A (d), and IL-2 (e) was measured by ELISA, CD44 (f), CD62L (g), CD25 (h), and CD124 (i) expression was measured by flow cytometry. Shown are the means and SEM of triplicate wells from one of two experiments (IL-10, IL-12 IFN-γ, IL-17A and IL-2) and data from one of two experiments (CD44, CD62L, CD25 and CD124).

## C-1.3 Titration of the PD-L1 chimera

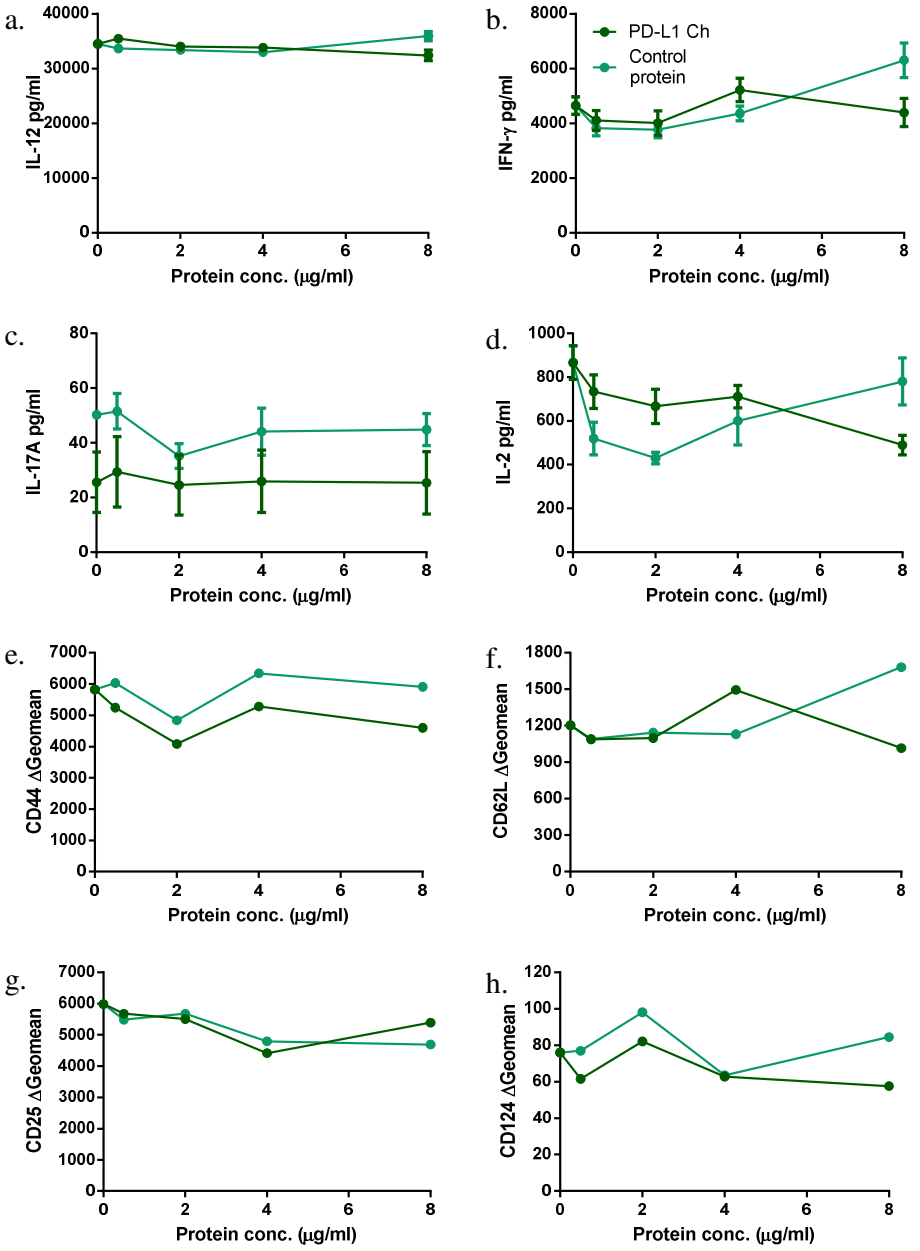



**Figure C3. Stimulating the PD-1/PD-L1 pathway may decrease the activation of T cells slightly, but has no effect on IL-12 production by MΦ.** BMMΦ were stimulated with IFN-γ (20 U/ml)overnight and then cultured alone or with LPS (200 ng/ml) with or without IC (10 per MΦ). After four hours, purified $CD4^{+}2D2$ T cells and $MOG_{35-55}$ (25 µg/ml) were added to the MΦ cultures for 72 hours. Various concentrations of PD-L1ch (0.5-8 µg/ml) were added to cultures with the T cells. IL-12 (a), IFN-γ (b), IL-17A c), and IL-2 (d) was measured by ELISA, CD44 (e), CD62L (f), CD25 (g) and CD124 (h) expression was measured by flow cytometry. Shown are the means and SEM of triplicate wells from one of two experiments (IL-12, IFN-γ IL-17A, and IL-2) and data from one of two experiments (CD44, CD62L, CD25 and CD124).

## C-1.4 Supplementary figures for IL-10, IL-12, PD-1/PDL-1 and CD154 pathways

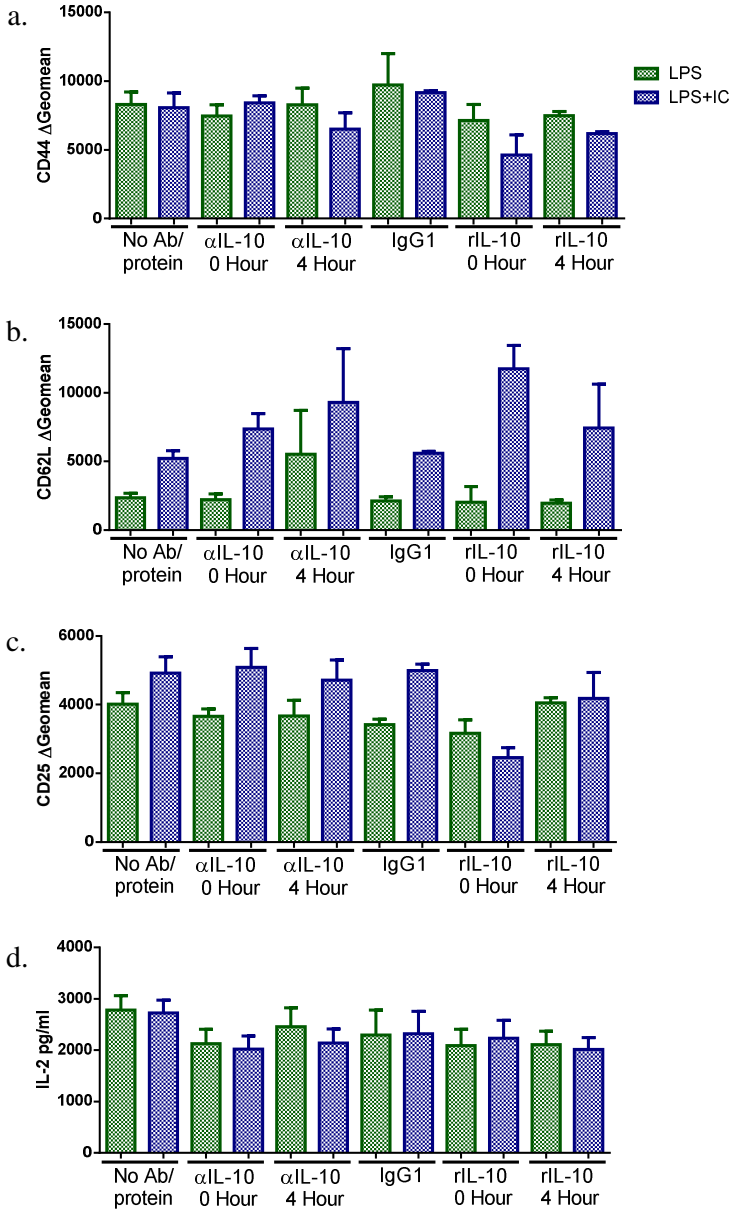



**Figure C4. Altering IL-10 levels in BMMΦ:T cell co-cultures did not significantly affect expression of markers of activation on T cells.** BMMΦ were stimulated with IFN-γ (20 U/ml) overnight and then cultured with LPS (200 ng/ml) with or without IC (10 per MΦ). After four hours, purified $CD4^{+}2D2$ T cells and $MOG_{35-55}$ (25 μg/ml) were added to the MΦ cultures for 72 hours. rIL-10 (5 ng/ml), αIL-10 (JES5-2A5, 2 μg/ml) or rat isotype ($IgG_1$, 2 μg/ml) was added to cultures either with the stimuli (0 hour) or with the T cells (4 hour). CD44 (a) CA62L (b), and CD25 (c) expression was measured by flow cytometry, IL-2 (d) was measured in the culture supernatant by ELISA. Shown are the means and SEM of triplicate wells from three or more experiments.

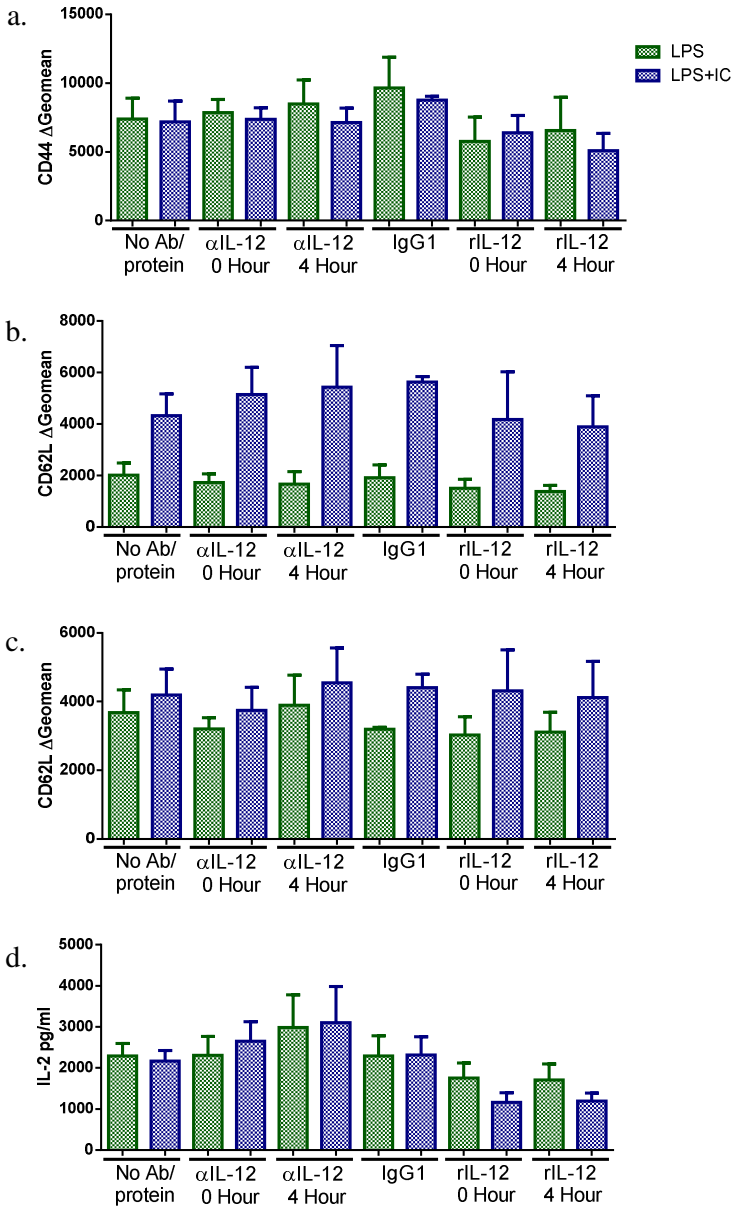



**Figure C5. Altering IL-12 levels in BMMΦ:T cell co-cultures did not significantly affect expression of markers of activation on T cells.** BMMΦ were stimulated with IFN-γ (20 U/ml) overnight and then cultured with LPS (200 ng/ml) with or without IC (10 per MΦ). After four hours, purified $CD4^+2D2$ T cells and $MOG_{35-55}$ (25 μg/ml) were added to the MΦ cultures for 72 hours. rIL-12p70 (5 ng/ml), αIL-12 (C15.6, 2 μg/ml) or rat isotype ($IgG_1$, 2 μg/ml) was added to cultures either with the stimuli (0 hour) or with the T cells (4 hour). CD44 (a) CA62L (b), and CD25 (c) expression was measured by flow cytometry, IL-2 (d) was measured in the culture supernatant by ELISA. Shown are the means and SEM of triplicate wells from three or more experiments.

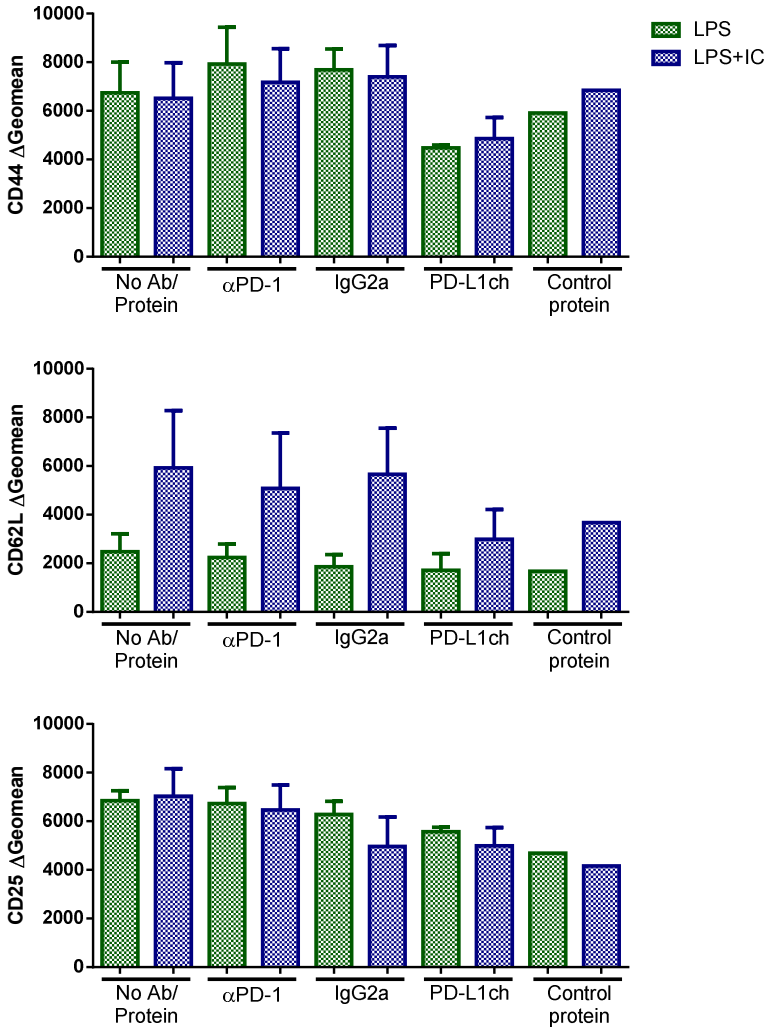



**Figure C6. Blocking or stimulating the PD-1/PD-L1 pathway did not affect expression of cell surface activation markers on T cells.** BMMΦ were stimulated with IFN-γ (20 U/ml) overnight and then cultured with LPS (200 ng/ml) with or without IC (10 per MΦ). After four hours, purified $CD4^{+}2D2$ T cells and $MOG_{35-55}$ (25 μg/ml) were added to the MΦ cultures for 72 hours. αPD-1 (2 μg/ml, RMP1-14), rat isotype (2 μg/ml, $IgG_{2a}$), PD-L1ch (8 μg/ml) or control protein (8 μg/ml) was added. CD44 (a), CD62L (b) and CD25 (c) expression was measured by flow cytometry. Shown are the means and SEM of triplicate wells from one-five combined experiments.

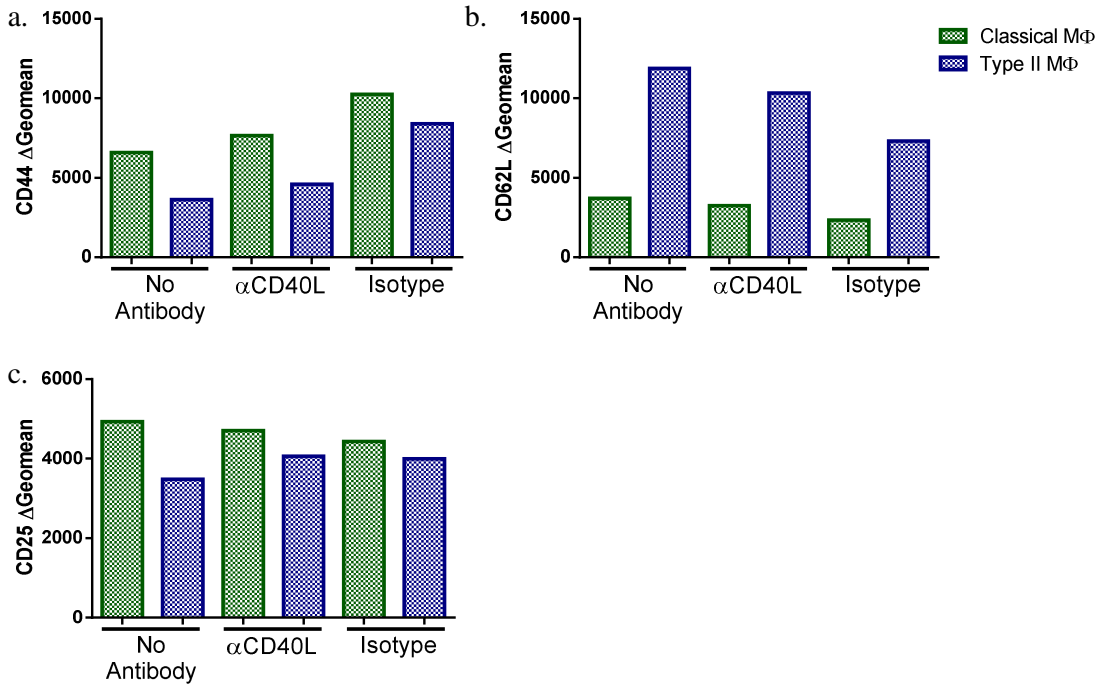



**Figure C7. Blocking the CD40/CD40L pathway did not significantly affect expression of cell surface activation markers on T cells.** BMMΦ were stimulated with IFN-γ (20 U/ml) overnight and then cultured with LPS (200 ng/ml) with or without IC (10 per MΦ). After four hours, purified $CD4^+2D2$ T cells and $MOG_{35-55}$ (25 μg/ml) were added to the MΦ cultures for 72 hours. 4 μg/ml of αCD40L or hamster isotype control antibody ($IgG_1$) was added. CD44 (a), CD62L and CD25 (c) expression was measured by flow cytometry. Shown is data from one of two experiments.

## C-2. Supplementary figures for chapter 6

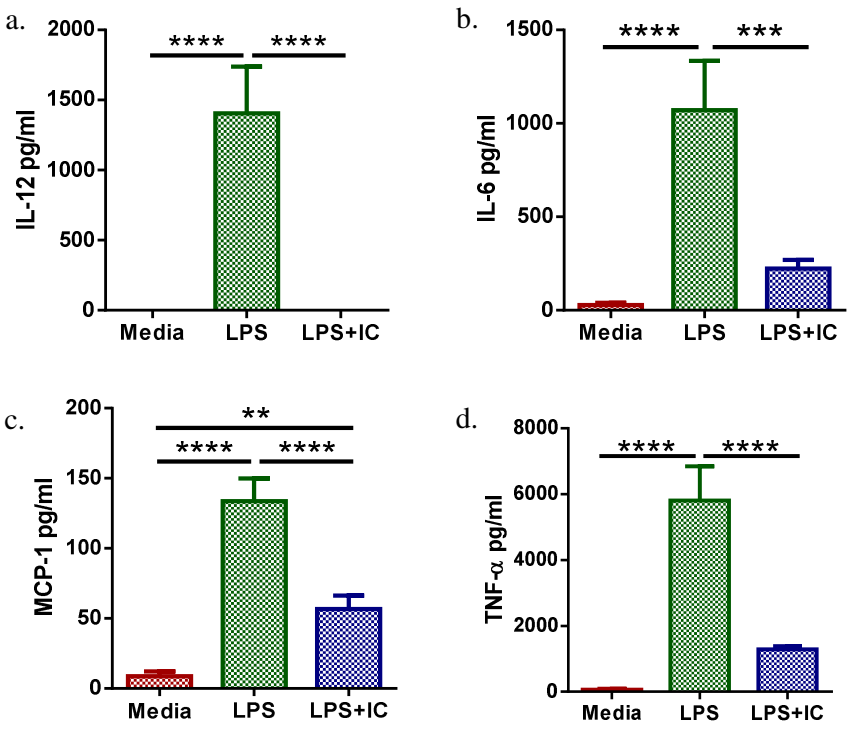



**Figure C8. MG cultured with LPS+IC produce less inflammatory cytokines and chemokines compared to LPS alone when M-CSF is removed 3 days before IFN-γ stimulation.** MG were isolated from the CNS of adult mice (n=5) and plated at $5x10^4$ cells/well in a flat bottomed 96 well plate in the presence of M-CSF (5 ng/ml). After 4 weeks in culture M-CSF was removed 3 days before MG were primed with 20 U/ml IFN-γ overnight followed by stimulation with LPS (200 ng/ml) with or without IC ($10^6$/well) for 24 hours. IL-12 (a) levels were measured by ELISA and IL-6 (b), MCP-1 (c) and TNF-α (d) levels were measured by CBA. Shown are the means and SEM of at least duplicate wells from four combined experiments. Raw data was subjected to the ROUT test which removed outliers, before statistics were calculated. **p<0.01, ***p<0.001, ****p<0.0001 by one way ANOVA with a Tukey's multiple comparison post test.

## C-3 Supplementary figures for chapter 7

### C-3.1 Disease course of BALB/c mice treated with IC or GA

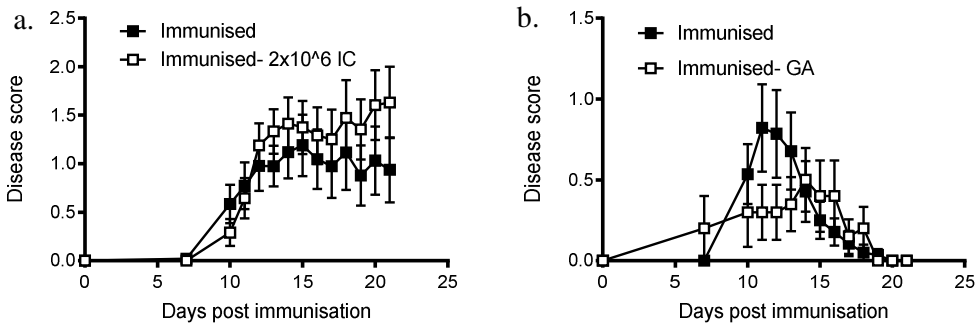



**Figure C9. Neither GA or IC treatment is strongly protective in a BALB/c model of EAE.** 8-16 week old BALB/c mice were pre-treated with $2x10^6$ IC by i.p. injection, this treatment was repeated every 7 days during the course of the disease. Mice were immunised for EAE seven days after the first IC treatment as described in methods (a). GA treatment was 500 μg/ml in the EAE emulation, and was thus given once at the time of immunisation (b). Disease scores presented in each graph are representative of five (a) or two (b) combined experiments.

## C-3.2 C57→C57 control treatments for experiments shown in Figure 7.1

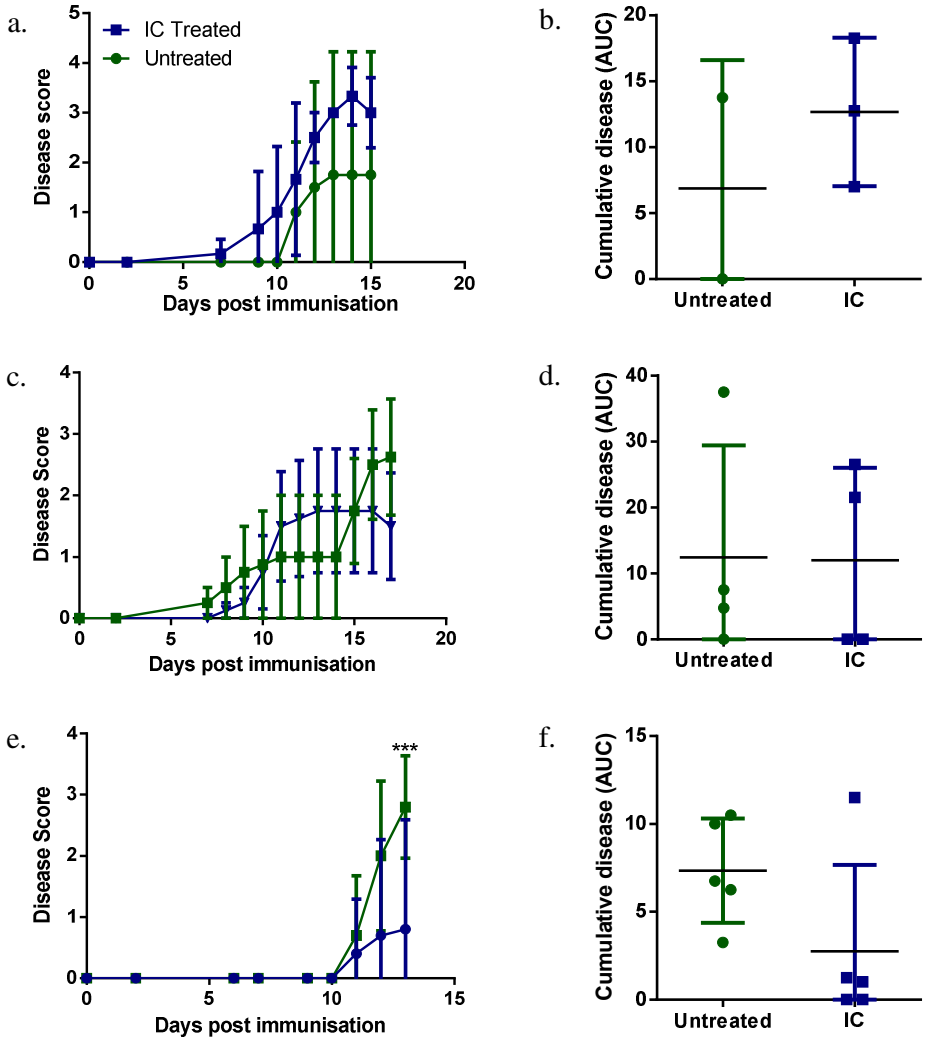
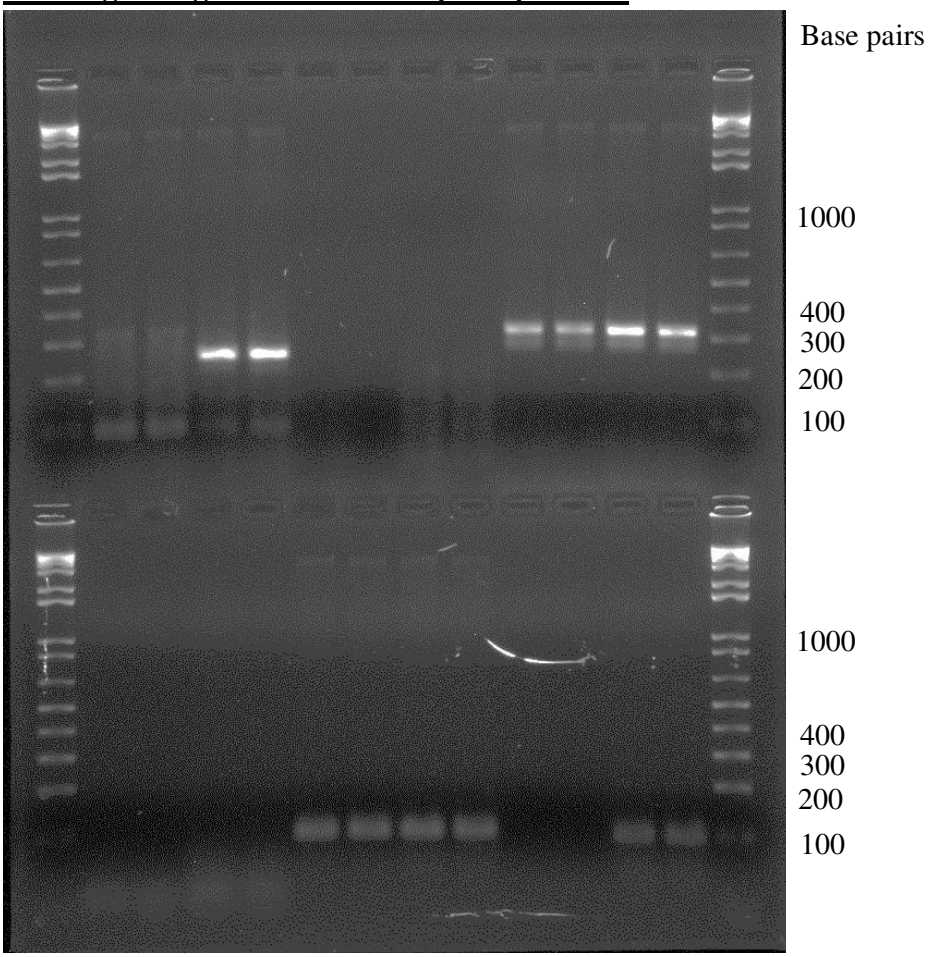



**Figure C10. Treatment of mice with IC reduces the severity and incidence of EAE in C57→C57 control chimers to a similar extent as B6→C57, graphs are controls for Figure 6.1.** Seven weeks following irradiation and reconstitution of C57→ animals, mice were pre-treated with $2x10^6$ IC by i.p. injection one week before immunisation and weekly thereafter. Mice were immunised to induce EAE as described in the methods. Disease scores (a, c, e) and disease burden (b, d, f) for 3 of 5 experiments are shown, number of mice/treatment group= 2 (untreated) and 3 (IC treated) (a and b); 4 (c and d) or 5 (e and f). ***p<0.001 by two way ANOVA with Sidak's post test.

**C-3.3 Agarose gel of RAW-264.7 qPCR products**




| Lane | Sample type (top section) | Sample type (bottom section) |
|---|---|---|
| 1 | 1 Kb plus ladder | 1 Kb plus ladder |
| 2 | IL-12, unstimulated RAW cells | IL-10, no template |
| 3 | IL-12, unstimulated RAW cells | IL-10, no template |
| 4 | IL-12, LPS stimulated RAW cells | IL-10, no reverse transcription |
| 5 | IL-12, LPS stimulated RAW cells | IL-10, no reverse transcription |
| 6 | IL-12, no template | Cyclophilian A, unstimulated RAW cells |
| 7 | IL-12, no template | Cyclophilian A, unstimulated RAW cells |
| 8 | IL-12, no reverse transcription | Cyclophilian A, LPS stimulated RAW cells |
| 9 | IL-12, no reverse transcription | Cyclophilian A, LPS stimulated RAW cells |
| 10 | IL-10, unstimulated RAW cells | Cyclophilian A, no template |
| 11 | IL-10, unstimulated RAW cells | Cyclophilian A, no template |
| 12 | IL-10, LPS stimulated RAW cells | Cyclophilian A, no reverse transcription |
| 13 | IL-10, LPS stimulated RAW cells | Cyclophilian A, no reverse transcription |
| 14 | 1 Kb plus ladder | 1 Kb plus ladder |

**Figure C11. Primers specific for IL-12 and Cyclophilin A amplify products of the expected size.**RAW-264.7 cells were cultured alone or stimulated with 200 ng/ml LPS for 24 hours and total RNA was isolated. cDNA was generated and IL-12p40, IL-10and Cyclophilin A cDNA were amplified with gene specific primers using SYBR green to detect amplification. The size of the PCR products was assessed by gel electrophoresis using a 2% agarose gel and were found to be of the correct size (IL-12p40, 255 base pairs and Cyclophilin A, 98 base pairs). Data shown is from one experiment.

**Type II activation using GA**

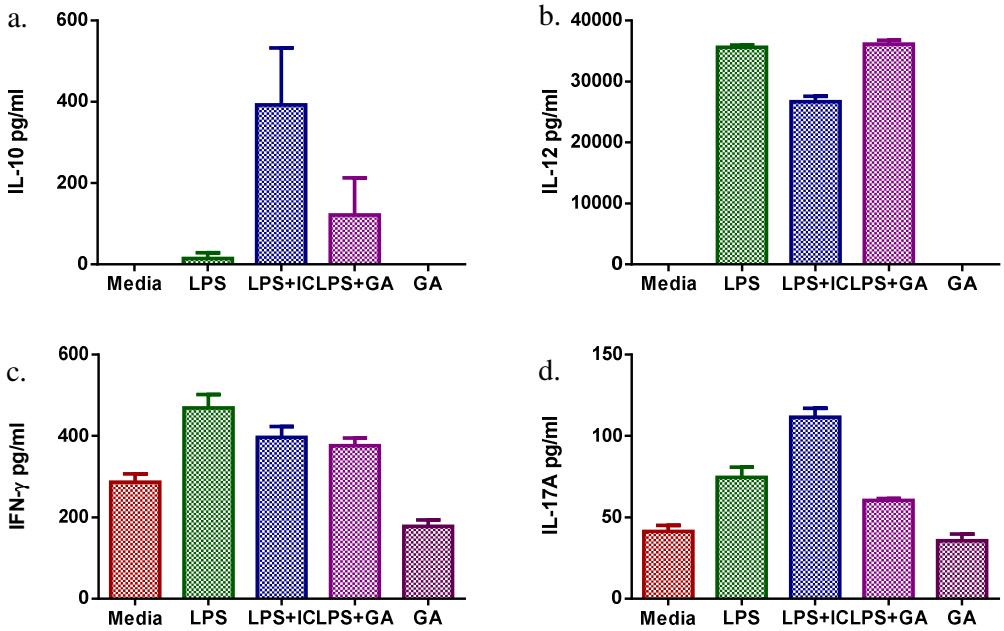



**Figure C12. GA induces a type II like phenotype in BMMΦ (a and b), which have a different effect on T cell biasing (c and d).** BMMΦ were stimulated with 20 U/ml IFN-γ overnight and then cultured with LPS (200 ng/ml) alone or with IC (10 per MΦ) or GA (100 µg/ml). (a and b) Supernatants were harvested after 24 hours, IL-10 (a) and IL-12p40 (b) levels were measured by ELISA. (c and d) Four hours following stimulation, purified $CD4^+$2D2 T cells and MOG (25 µg/ml) were added to the cultures and cultured for 72 hours. IFN-γ (c) and IL-17A (d) were measured by CBA. Shown are the means and SEM of triplicate wells from one experiment.

## D1 Gating strategy for flow cytometric analysis of brains

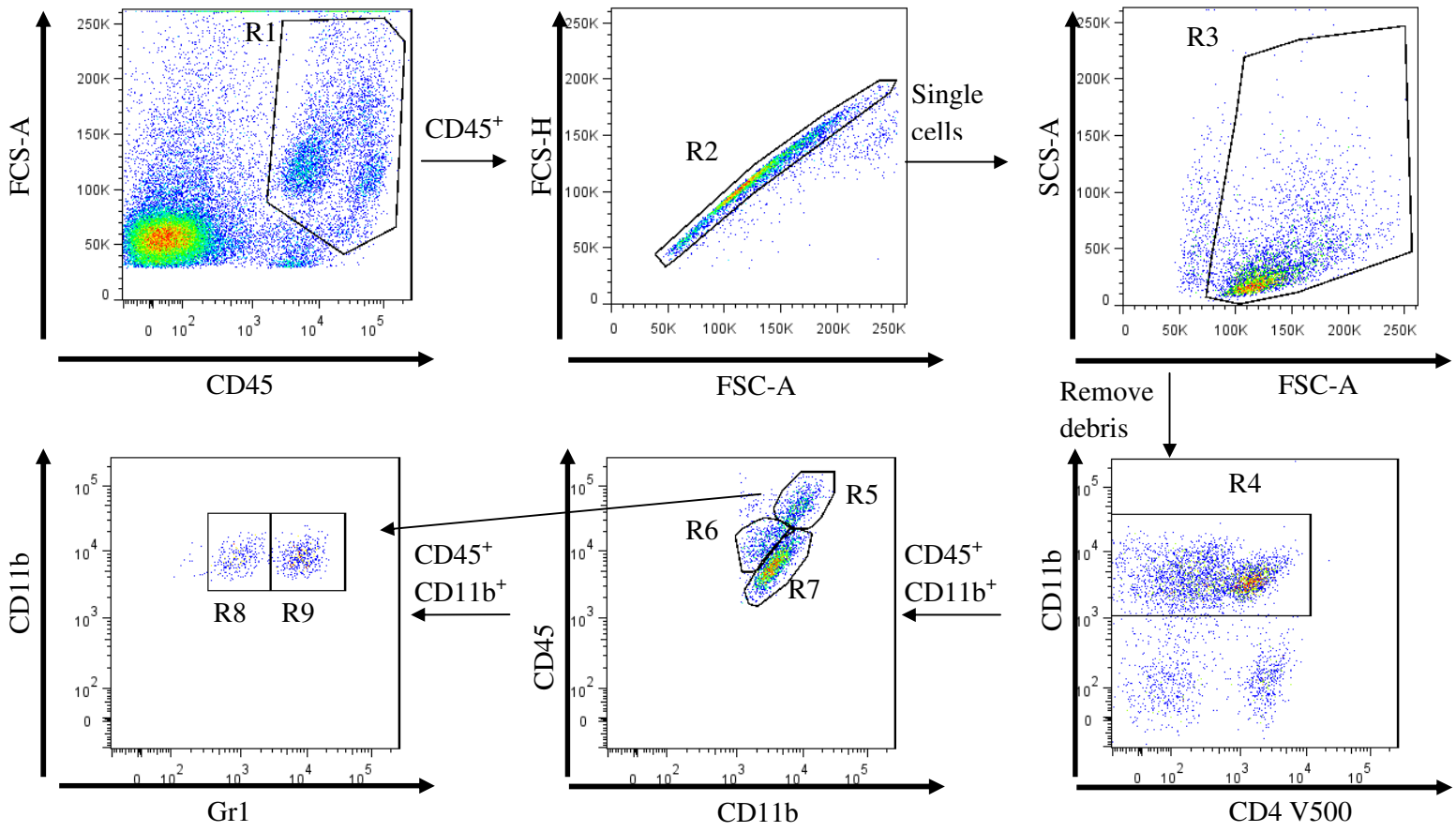


**Figure D1. Gating strategy for flow cytometry on brains from C57→C57 mice.** Seven weeks following irradiation and reconstitution of C57→C57 mice were pre-treated with $2x10^6$ IC by i.p. injection, this treatment was repeated every 7 days during the course of the disease. Mice were immunised for EAE 7 days after the first IC treatment as described in methods. At peak disease mice were euthanised, and brains were analysed by flow cytometry. $CD45^+CD11b^+$ were selected as shown. R1, $CD45^+$ cells; R2, single cells; R3, cells (without debris); R4, $CD11b^+$ cells; R5, $CD45^{hi}CD11b^+$; R6, $CD45^{int}CD11b^+$; R7, $CD45^{lo}CD11b^+$; R8, $CD45^{hi}CD11b^+Gr1^-$; R8, $CD45^{hi}CD11b^+Gr1^+$. Shown are representative plots from one immunised mouse