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Exploring the use of high-dose simvastatin as therapy for oxidative stress in disease models of neuroinflammation

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Declaration

I, Chantelle Elizabeth Bowers, confirm that the research presented throughout this thesis is my own unless otherwise stated. Studies on EAE (experimental autoimmune encephalomyelitis) were carried out in collaboration with Dr Roshni Desai, Ph.D based at the Institute of Neurology, University College London.

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Abstract

Background: There is growing evidence that HMG-CoA reductase inhibitors, otherwise known as the statin family, can exert pleiotropic effects in many areas. Of these, potential neuroprotective effects have gained significant attention. It is well established that microglia, the brain's resident phagocytes, play a pivotal role in the pathogenesis of neuroinflammation and neurodegeneration. This process is thought to be, in part, due to the presence of chronically activated microglia. In this study, we investigated the potential neuroprotective properties of HMG-CoA reductase using an *in vitro* model of inflammatory cell activation and *in vivo* models of posterior uveitis and multiple sclerosis. The focus of this study was to investigate the effect of simvastatin on the microglial cell and its activation products. Emphasis was placed on the production of reactive species released by this cell type and the subsequent damage these cause to biological macromolecules.

Methods: The microglial cell line BV2 were treated with simvastatin (1 μ M; 2 to 120 h), *in vitro* before being activated for 48 hours with a pro-inflammatory mix of LPS, TNF α and IFN γ . Supernatants were taken and nitric oxide levels measured using the Griess assay. The animal model of posterior uveitis, experimental autoimmune uveoretinitis (EAU), was established in wild type C57BL/6 mice through subcutaneous injection of IRBP₁₋₂₀. Mice were treated orally with simvastatin at 50, 75 or 100 mg/kg. Fundus images were taken before and after treatment administration for evaluation of clinical ocular pathology. Retinal flat mounts were prepared from simvastatin treated mice to assess cellular infiltrates. Experimental autoimmune encephalomyelitis (EAE), an *in vivo* model of MS, was induced by rMOG subcutaneous immunisation. The effect of simvastatin treatment was assessed clinically and by immunohistochemical analysis of tissue sections to determine cellular infiltrates and levels of oxidative damage to biological macromolecules, consistent with those assessed in EAU.

Results: The levels of nitric oxide produced by microglial cells were significantly reduced when exposed to a pre-incubation of simvastatin for ≥ 48 hours, compared to cells receiving the pro-inflammatory mix alone. In EAU, fundoscopic analysis revealed that high-dose simvastatin halts clinical disease progression in IRBP₁₋₂₀ induced posterior uveitis. Retinal flat mounts prepared from these cohorts showed a significant decrease in the expression of the innate immune cell surface receptor CD11b. Additionally, histological examination of eye sections displayed a significant reduction in lipid peroxidation as revealed by the marker 4-Hydroxynonenal (4HNE), nitrosylated proteins, as measured by 3-nitrotyrosine and oxidised DNA/RNA as determined by 8-OHdG. In line with this study, results from our EAE model demonstrated an important role for microglial cell number in disease, whilst also providing evidence of simvastatin decreasing oxidative damage to macromolecules in areas of extensive pathology.

Discussion: These data provide evidence to support the notion that microglial cell activation may contribute to the pathogenesis of neuroinflammatory disease and that statins may attenuate damage through their ability to inhibit the production of reactive species. Further to this, we provide therapeutic, chemical and physical evidence that simvastatin can provide protection against 1) nitric oxide production in an inflammatory environment 2) clinical disease attenuation in EAU and EAE and 3) reduction in peroxynitrite levels *in vivo*. Collectively, these data provide evidence that statins may attenuate microglial cell activation by ways of inhibiting the production of reactive species. Thus, the evidence presented in this thesis points to the importance and potential use of simvastatin therapy as a neuroprotective therapeutic agent.

Impact statement

HMG-CoA reductase inhibitors, commonly referred to as statins are potent inhibitors of HMG Co-A reductase, a rate-limiting enzyme in cholesterol biosynthesis. Statins have proven to significantly reduce the rates of cardiovascular events and general mortality in patients with coronary artery disease and continue to be widely prescribed in the clinics' today. However, in recent years, statins have garnered significant attention as an attractive potential tool for treating specific neurodegenerative and neuroinflammatory disorders. Of recent studies, one of the most compelling pieces of evidence to date, is the use of simvastatin as a tool for the treatment of neurodegenerative disease, specifically SPMS which was demonstrated in a randomised, placebo-controlled phase II trial MS-STAT (Chataway et al., 2014). The neuroprotective properties of high-dose simvastatin translated to an overall 43% reduction of annualised rate whole-brain atrophy compared with placebo. The specific mechanisms through which simvastatin operates to achieve these results is thought to be independent of the well-characterised lipid-lowering capabilities. One proposed alternative mechanism of action would depend on simvastatin unveiling itself as a potent attenuator of oxidative and nitrative stress in a pathogenic environment. An association made in recent years, highlighted the importance of a strong and credible link between reactive nitrogen and oxygen species and neuronal cell death, making this subject an attractive area for therapeutic targeting. To explore this, here, we investigated the use of chronic simvastatin treatment, using two experimental animal models of disease in addition to an *in vitro* inflammatory model system. EAE and EAU were used as tools to investigate the effectiveness of simvastatin therapy on clinical disease outcomes and the capability to modulate readouts of oxidative damage to macromolecules under these conditions. It is hoped that growth in this area will inform the development of future therapeutic strategies and support the current Phase III clinical trial in SPMS.

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Abbreviations

ANOVA	Analysis of Variance
ATP	Adenosine Diphosphate
BBB	Blood Brain Barrier
BRB	Blood Retinal Barrier
BV2	Cellosaurus BV-2
CBF	Cerebral Blood Flow
CNS	Central Nervous System
CO ₂	Carbon Dioxide
CSF	Cerebral Spinal Fluid
CFA	Complete Freund's Adjuvant
DA	Dark Agouti
DAB	Diaminobenzidine
DAMPs	Damage-Associated Molecular Patterns
DMSO	Dimethylsulphoxide
eNOS	Endothelial Nitric Oxide Synthase
EAE	Experimental Autoimmune Encephalomyelitis
EAU	Experimental Autoimmune Uveitis
FA	Fluorescein Angiography
H ₂ O ₂	Hydrogen Peroxide
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
4-HNE	4-Hydroxynonenal
HIF-1 α	Hypoxia Inducible Factor alpha
IFN- γ	Interferon- γ
iNOS	Inducible Nitric Oxide Synthase
IRBP	Interphotoreceptor retinoid-binding protein
LPS	Lipopolysaccharide
MBP	Myelin Basic Protein
MHC	Major Compatibility Complex
MS	Multiple Sclerosis
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAWM	Normal Appearing White Matter
Na ⁺ /K ⁺	Sodium/Potassium
NF- κ B	Nuclear Factor Kappa-Light-Chain-Enhancer of activated B cells
NO	Nitric Oxide
O ₂ ⁻	Superoxide
ONOO ⁻	Peroxynitrite
PAMPs	Pathogen-Associated Molecular Patterns

PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PPMS	Primary Progressive MS
PRRs	Pattern Recognition Receptors
rMOG	Recombinant Myelin Oligodendrocyte Glycoprotein
ROCK	Rho-associated Kinase
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RRMS	Relapsing Remitting MS
RT	Room Temperature
S.D.	Standard Deviation
S.E.M	Standard Error of Mean
SPMS	Secondary Progressive MS
Th-1	T-helper 1 cell
Th-2	T-helper 2 cell
TNF- α	Tumour Necrosis Factor-alpha
VEGF	Vascular Endothelial Growth Factor
3-NT	3-Nitrotyrosine
8-Oxo-dG	8-Oxo-2'-deoxyguanosine

Aims of the project

The primary research outcome for this body of work is to contribute in part, towards elucidating the mechanism of statin action, more specifically, simvastatin, in the context of secondary progressive multiple sclerosis (SPMS). It is hoped that growth in this area will inform the development of future therapeutic strategies and support the current Phase III clinical trial in SPMS.

We aim to investigate the neuroprotective effects of simvastatin in the context of oxidative stress, through employment of *in vitro* and *in vivo* chronic inflammatory model systems for measuring biomarkers and reaction products generated from an oxidative microenvironment.

Hypothesis

Chronic, high-dose simvastatin therapy can attenuate disease progression and improve clinical outcomes in IRBP₁₋₂₀ experimental autoimmune uveitis (EAU) and rMOG experimental autoimmune encephalomyelitis (EAE). Activated microglial cells capable of inducing reactive oxygen and reactive nitrogen species-mediated damage can be modulated by chronic simvastatin treatment.

Chapter 1

Introduction

1.1 The Immune System

The vertebrate immune system can be defined as a complex collection of components, each cooperating to generate an organised, accurate and appropriate response to infection and/or disease. These individual units of cells and molecules represent an extremely diverse and versatile repertoire, the coordination and intimate interaction of which is referred to as immunity and perceived as a state of protection. Cooperation within this system is not only essential to protect the body from infectious agents, but also provides a surveillance system to monitor the integrity of tissues. Thus, the ability of the immune system to recognise and respond to foreign entities is central to its operation (Janeway, 2005). The type of response elicited can be broadly separated into those provided by the innate or the adaptive immune system; two independent but interconnected mechanisms that cooperate to provide protection. The term inflammation is generally used to collectively define a biological series of events that surround an immune response. The factors capable of inducing a response vary from harmful stimuli (i.e. invading pathogens and toxins) to damaged host cells, with the ultimate aim being tissue healing and a return to homeostasis (Owen, 2013).

In the most general sense, immune responses and their components can be broadly separated into innate or “non-specific” immunity and adaptive, or “specific” immunity. Innate immune responses are described as those present at birth and intended to provide the first line of defence. Protection offered by this system is preoccupied with killing via macrophages, neutrophils and complement induced mechanisms (Punt, 2019). Conversely, the adaptive response is a highly sophisticated and elaborate subsystem with strict specificity of cells and processes for effective elimination of infectious agents within the body. These responses are potent and destructive, directed only towards the precise molecule that induced them. Perhaps the most powerful feature of this response is that it affords long-lasting protection, a principle more commonly known as immunological memory (Delves et al., 2017). Due to the inherent, destructive nature of this process, the body’s ability to distinguish between foreign and self is paramount to its function. Occasionally, the system fails to recognise these differences leading to destruction against self, subsequently giving rise to a collection of autoimmune diseases, which can often be fatal.

1.1.1 The central role of cytokines and chemokines in inflammation

1.1.1.1 CYTOKINES

A vitally important feature of the immune system is the ability to communicate via chemical cues. More commonly referred to as cytokines, these biologically active signalling proteins are designed to instruct intracellular and intercellular communication. The processes involved in cytokine-mediated communication are complex, however, in the simplest form, these molecules operate to influence the behaviour of cells that bear receptors for them – both immune and non-immune alike. Cytokines can either

act locally or systemically to mount, assist, sustain or orchestrate immune responses. Their mode of action can vary according to the target cell, resulting in autocrine, paracrine or endocrine signalling. Typically, cytokines are not stored as preformed proteins, rather, synthesised on demand through gene transcript initiation. The culmination of assorted signalling pathways initiated by cytokine-receptor binding control cell proliferation, death and differentiation as the variety of mediators can also stimulate, enhance or suppress other cytokines.

Cytokine receptor-ligand interactions are strong and once tightly bound transduce a cascade of downstream signalling through master transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and signal transduction and activator of transcription (STAT) (Darnell et al., 1994). These transcription factors represent prototypical proinflammatory signalling pathways and are amongst the most commonly triggered cytokine-ligand interactions which are known to play fundamental roles in immunoregulation and host defence systems (Stritesky et al., 2011) (Chen et al., 2017).

Generally speaking, cytokine activity can be categorised into two main sub-groups according to the target cell response, being either pro-inflammatory or anti-inflammatory. The archetypical pro-inflammatory cytokines, produced predominantly by activated macrophages and microglia, include TNF, IL-6, IL-1 and IFN- γ . TNF α is one of the dominant cytokines present in MS and has long been recognised as a potent systemic inflammatory inducer involved in the acute phase reaction and expressed in a great variety of cells. Capabilities of this cytokine include; monocyte/macrophage differentiation (Trinchieri et al., 1986), neutrophil activation (Moore et al., 1991) and synergistic cooperation with IL-1b (Dinarello et al., 1986). It is quite common for cytokines to display both or overlapping functional activities, a phenomenon described as promiscuity, whereby one cytokine can bind and activate more than one receptor across multiple different cell types. Similarly, one receptor can recognise and bind more than one cytokine-ligand. An example relevant to this thesis are the IL-1 family of cytokines, which are well-established contributing factors in MS pathogenesis and widely recognised for their promiscuous nature and prolific contributions to pathogenicity in both MS and its animal model equivalent experimental autoimmune encephalomyelitis (EAE).

1.1.1.2 CHEMOKINES

Cytokine proteins can be further divided into a number of distinct subsets as follows; chemokines, interleukins, lymphokines, interferons and tumour necrosis factors (TNF). These classifications are assigned according to their presumed function, target action, structure, and cell of secretion. Chemokines are described as 8-12kD polypeptides, expressed by both leukocytes and endothelial cells. The two most extensively studied families are CC and CXC. The former is structurally identifiable by its two adjacent cysteine residues and known to act primarily on monocytes, eosinophils and lymphocytes (Baggiolini,

1998). The latter is arranged such that the two cystine residues are separated by other amino acids and classically recognised for their tendency to act on neutrophils.

Chemokine signalling is especially important in the early stages of immune system engagement. Indeed, a hallmark of early inflammation is the presence of recruited leukocytes at the site of injury. The term leukocyte extravasation is used to describe the mobilisation of cells such as monocytes and neutrophils from the bloodstream – down a chemical gradient – to the area of tissue damage. Chemokine signals responsible for this guided migration exert their effects by direct activation of transmembrane G-coupled protein receptors (GPCRs) positioned on the cell surface. Once bound, this initiates downstream activation, cellular polarization, and cytoskeletal reorganisation.

Through sampling of patient serum, (Wilkinson et al., 1969) demonstrated the importance of chemokine specificity. CCL2 is one of the most potent chemotactic molecules, with a preference to induce the activation and accumulation of monocytes, microglia, and T-cells. On the other hand, CXCL8 is common to early inflammation and provides directional cues for rapidly trafficking neutrophils to the site of injury (Baggiolini and Clark-Lewis, 1992) (Yoshimura et al., 1987).

1.1.2 Innate immunity

There are two main types of innate immunity; immediate and induced. The former is commonly referred to as the first line of host defence and relies heavily on the presence of physical barriers to act as blockade towards potential pathogens. A clear example of this, is acidity of the skin on the outer surfaces along with mucosal linings that cover epidermal layers of the digestive, reproductive and respiratory tract. These immediate responses are non-specific and act rapidly (0–4 hours) for effective removal of any infectious agent attempting to gain entry to the body. In order for the second phase of innate immunity to awaken, these surfaces must firstly be breached (Al-Izki et al., 2012).

Indeed, not all types of innate responses are non-specific, as was originally thought. Induced innate immunity, akin to immediate, is broad-acting involving receptor engagement by infection-induced structures before activation, hence, able to discriminate between self and non-self. Detecting the presence of particular pattern-associated molecular patterns (PAMPs) is essential for mounting a response here. To understand the basic concepts of this type of recognition and engagement, attention must firstly be brought to the antigen presenting cell (APC), (i.e., macrophage, dendritic cell, B cell) which are major players in activating induced innate immune responses. APCs are covered with germ-line encoded pattern recognition receptors (PRRs) (Hoffmann, 2003). Several class of PRRs exist within the vertebrate immune system and amongst the most extensively studied are toll-like receptors (TLRs). Briefly, APC's through PRR's recognise epitopes positioned on the plasma membrane, as well as some intracellular compartments, of bacteria, viruses, fungi, parasites and yeast (Akira and Takeda, 2004) (Janeway Jr and Medzhitov, 2002).

In a quiescent state, the phagocytic machinery in APC's allows sampling of the microenvironment through ingestion and digestion of debris. However, epitope recognition and engagement through PRR's switches on a battery of genes for transcription that enable inflammatory capabilities via signalling pathways such as NF- κ B, JAK-STAT and MAPK (Kawai and Akira, 2007) (Hayden and Ghosh, 2004) (Guha et al., 2001). Perhaps one of the most widely studied examples of this process is lipopolysaccharide (LPS), a protein localised on the outer segment of gram-negative membranes. Recognition of this evolutionary conserved product swiftly induces the activation and subsequent secretion of cytokines. The critical role and responsibility of the APC to detect, engulf and inform the adaptive immune response lies at the centre of innate immunity, a connective, communicative bridge between two independent but interconnected apparatus (Delves et al., 2017).

The main cell types seen in these early stages of immune initiation are neutrophils, recruited in large numbers to the area of inflamed or affected tissue. The influx of neutrophils is later followed by mass monocytes that rapidly differentiate into macrophages at the site of injury (Lewis and McGee, 1992). Later, these events lead to a cascade of recruitment and activation employing a variety of cells and serum factors such as mast cells, macrophages, natural killer, eosinophils, and basophils. The purpose of such responses at this stage is to mount an immediate attack, through ingestion and intracellular destructive enzymes for instance, membrane attack proteins or proteases. Neutrophils and macrophages, complement factors and lysosomes within these cell types are principally responsible for such actions. This recruitment orchestrates a unique inflammatory response which depends entirely on the precise nature of the initial stimulus and location in the body (Abbas et al., 2020).

1.1.3 Adaptive immunity

In contrast to the innate response, adaptive immune responses are highly sophisticated and heavily regulated processes. Although distinct, the two systems are so closely interlinked that without information fed from the innate response, adaptive immunity could not be mobilised effectively. This part of the immune system becomes activated when innate responses are insufficient to control the invasion of pathogens. Responses initiated can take variable amounts of time to develop (from days to weeks) but are typically mobilised 4-5 days after the initial innate response. A major benefit of adaptive immunity is immunological memory, meaning subsequent reinfection with the same agent will be met with a rapidly acting and highly specific response. The adaptive system is principally mediated by two main types of lymphocytes, commonly referred to as T- and B-lymphocytes. Together, they drive the adaptive response via two central mechanisms; cell-mediated and humoral immunity, controlled by T-lymphocytes and B-lymphocytes, respectively. These cell types are unique in that both display individual receptors on their plasma membranes, tailored to recognise an almost limitless range of structures (Janeway Jr and Medzhitov, 2002).

While B-lymphocytes demonstrate a more direct path for activation and initiation of responses, T-lymphocytes encompass a heterogeneous population of cells with extremely diverse functions, including but not limited to cytokine release, response to APC's and stimulation of B-lymphocytes.

The order of events involved in T-lymphocyte initiation and sustained activation depend on antigen sequence recognition within their T-cell receptor (TCR). As previously mentioned, APCs function primarily to recognise antigen, engulf, digest, and inform the adaptive response. Briefly, digested antigen is packaged and expressed on the APC surface via its major histocompatibility complex (MHC), with the purpose of delivering antigen for presentation to the TCR. Not only does this process depend on TCR recognition and binding to cognate antigen, but co-stimulatory ligand receptor interaction is an absolute requirement to drive the naïve T-lymphocyte towards a specific lineage and full proliferative potential (Abbas et al., 2020).

1.1.3.1 ANTIGEN PRESENTATION

Antigen presentation is an absolute requirement for activation of CD4⁺ T cells (Fearon and Locksley, 1996). In the absence of APC's, CD4⁺ T-lymphocytes remain inactive and unable to mount an immune response. APC's fall into two main categories; the professional APC's and the non-professional APC's. The three main types of professional APC's are dendritic cells, macrophages, and B cells. Of these, dendritic cells are the most potent and have the broadest range. Identifiable in practically all peripheral tissues as well as in the circulation, APC's are tasked with the uptake of self and non-self-antigens. A fundamental process of the APC is to load proteins, that have been converted into peptides, onto MHC class-II molecules for presentation to the naïve T-lymphocyte reservoir. Antigen presentation are typically restricted to areas of lymph nodes and spleen where the cellular environment is conducive for crosstalk to take place. In contrast to the significant role APC's play in triggering adaptive responses, there is also considerable evidence to support their responsibilities in maintenance of immune tolerance and homeostasis (Abbas et al., 2020) (Flaherty, 2012).

Despite both macrophages and DC's originally being recognised for their APC attributes, they also perform several other disparate yet complementary immunological functions. The substantial overlap in surface markers, in addition to their established roles in maintaining homeostasis and mobilisation of adaptive immunity, made initial functional characterisation between these cells difficult to establish. However, it is now widely recognised that macrophages behave in a manner that more suitably reflects innate effector cells, as this population is heavily concerned with phagocytosis, digestion, and clearance of bacteria. DCs on the other hand, play a major role in naïve T-lymphocyte priming (Banchereau and Steinman, 1998) (Lanzavecchia and Sallusto, 2001).

Dendritic cell maturation is driven by antigen recognition and is characterised by several features. Classically, maturation has been defined as MHC class-II surface receptor upregulation, increased co-

stimulatory molecule expression, decreased phagocytic capacity and pro-inflammatory cytokines secretion. In addition, increased CCR7 expression is mobilised to assist in migration to lymph tissue for presentation to naïve CD4+ and CD8+ T-lymphocytes (Campbell et al., 1998) (Campbell and Butcher, 2000) (Sallusto et al., 2000). Inside the brain parenchyma, the microglial cell represents the main APC during neurodegeneration and adopts roles that reflect DC function (Perry et al., 2010).

MHC II molecules situated on APC's have critically important roles in antigen presentation. In addition, they serve equally important roles in homeostasis, complement activation and regulation of inflammation. The HLA complex is a human analogue to MHC II and constitutes all the functions of its respective murine equivalent. The HLA-DR complex-peptide combination constitutes a ligand for the TCR and is expressed at high levels on the APC surface. The role of HLA in disease has been studied extensively and has long been reported a major risk factor for complex diseases. The HLA allele HLA-DR2 is now commonly recognised for its increased genetic susceptibility towards the development of MS (Haines et al., 1998, Haines et al., 1996) (Barcellos et al., 2003) (Hollenbach and Oksenberg, 2015).

1.1.3.2 IMMUNOLOGICAL SYNAPSE

An immunological synapse is defined as the nano-scale gap where complicated interactional processes take place at the interface between an APC or target cell and lymphocyte (Bromley et al., 2001). Although granular details of this phenomena are beyond the scope of this thesis, a basic acknowledgement of existence is essential for understanding the communicative bridge between the effector cells of the innate immune system and the adaptive response. Simplistically speaking, an immunological synapse is a site of active signalling whereby interactions taking place in the synaptic cleft initiates the transformation of naïve T/B-lymphocytes towards full lineage commitment. An absolute requirement for full CD4+ T-lymphocyte activation is the combination of two prototypical receptor-ligand interactions for signal transduction. First, is antigen recognition in the form of MHC peptide (MHCp), mediated by the TCR (signal 1) and the second, is delivered by a co-stimulatory molecule (signal 2). The most prominent co-stimulatory signalling molecule is CD28 (Lenschow et al., 1996). There exists an assortment of other co-stimulatory molecules including intracellular adhesion molecule 1 (ICAM-1) (Van Seventer et al., 1990) and lymphocyte function-associated antigen 1 (LFA-1) (Springer et al., 1987) that carry out similar pivotal roles. Figure 1.1 illustrates the type of signals essential for forming an immunological synapse.

A functional immunological synapse depends on the cohesion of several cellular and molecular interactions. For instance, the CD4+ T-lymphocyte is capable of only recognising linear peptide fragments, around 9 amino acids in length, presented by the APC and nestled within the MHC class II groove. The synaptic cleft between these two cells is relatively small (13 nm), which explains the need for non-polymorphic co-stimulatory molecules to provide cell-to-cell junctional support. Adhesion molecules in this space classically enhance physical interaction, whereas co-stimulation enhances TCR signalling.

However, significant overlap does exist. Co-stimulation not only provides physical benefits but also great functional advantages in the form of signalling. For instance, adhesion molecules situated in the immunological synapse have been found to enhance T-lymphocyte sensitivity, in some cases up to 100-fold more (Bachmann et al., 1997) (Springer, 1990). Similarly, further spatial support is provided by the cytoskeleton, more specifically, the T-lymphocyte actin dynamics, a large network that drives the organisation of many of the molecules needed for signalling such as adhesion receptors, phosphatases, the TCR in addition to other intermediates. Of particular importance is the ability of actin to orchestrate the centralisation of TCR micro clusters, forming the canonical bullseye-like structure that consists of concentric rings with distinct receptor-ligand pairs, referred to as the mature immunological synapse (Monks et al., 1998) (Dustin et al., 1997) (Dustin and Long, 2010).

Active engagement between the TCR and APC can have an immediate impact on cell behaviour such as cell cycle control and proliferation. The type of receptor-ligand interactions present within the synapse dictates the amplification of internal signalling networks. Classic examples of functional outcomes include the polarised release of stimulatory cytokines or cytotoxic granules (Anikeeva and Sykulev, 2011) (Dustin and Long, 2010)

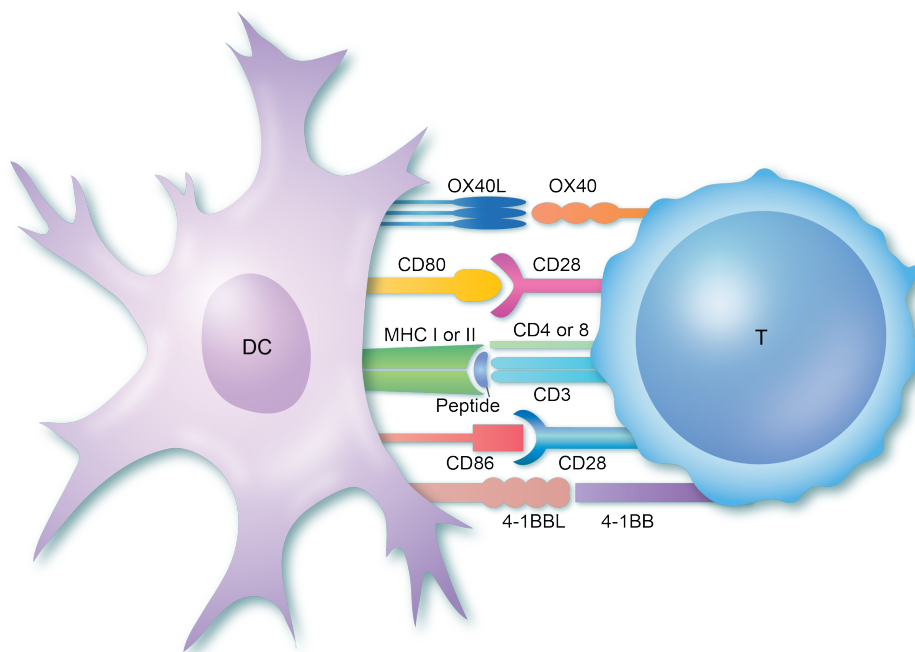


Figure 1.1 Immunological Synapse. Schematic representation of a stable immunological synapse forming between a T-lymphocyte and an APC. Cross talk during synapse formation depends on several molecular interactions including membrane receptors and cytosolic membranes, paired accordingly (from top to bottom) OX40L-OX40; CD80-CD28; MHC-CD3, 4 or 8; CD86-CD28. 4-1BBL-4-1BB. Image from (Cavanagh, 2021).

1.1.3.3 CD4+ T-LYMPHOCYTE SUBSET ACTIVATION

T-lymphocytes can be identified by the protein complex cluster of differentiation-3 (CD3), a co-receptor situated on the plasma membrane and a component of the TCR complex. The distinguishing features that set apart two major subgroups within this family is the expression of either CD4+ or CD8+ membrane proteins, classically referred to as T helper cells and cytotoxic T cells, respectively. Binding of these surface co-stimulatory signalling molecules is a prerequisite for complete T-lymphocyte activation and subsequent lineage commitment (Germain, 2002).

A major distinction between the two surface molecules is one of cognate antigen recognition, whereby, CD4+ T cells can only recognise antigenic peptide derived from exogenous proteins that have been fragmented and processed for presentation by MHC class II molecules on the surface of APC's. CD4+ T cells are classically known to operate through what is called the exogenous pathway. CD8+ T-lymphocytes on the other hand, are restricted to peptide recognition presented via the MHC class I molecule (Seder and Ahmed, 2003).

Commonly referred to as T helper (Th) cells, the CD4+ T cell population is a group of lymphocytes comprising several effector subsets including: Th1, Th2, Th17, Th22 and T regulatory (Tregs). Each subset can be uniquely identified for roles in defence against intracellular microorganisms, antibody production, inflammation, and immunosuppression, respectively. Th cell subset differentiation is determined according to the types of signal driven through the TCR and accessory molecules of a naïve T-cell. These signals derive from unique patterns of cytokine expression profiles and activation of master transcriptional factors. To begin with, the naïve T cells are uncommitted but as they develop, become polarised and restricted to a specific lineage. At this stage, the T cell is capable of synthesising and secreting different patterns and quantities of cytokines which shapes the functional attributes of T-cell subsets. The Th1 cell subset is typically characterised by the production and release of IFN- γ and IL-12 whilst under the regulatory control of T-bet and STAT4 transcription factors, which have proven to be essential for Th1 polarisation (T R Mosmann and Coffman, 1989). Th1 cells have classically been recognised in response to intracellular pathogens such as viral, bacterial and tumour antigens and serve as potent activators of macrophages, enabling phagocytosis for pathogen clearance. Whilst production of IFN- γ promotes Th1 cells, it also exerts inhibitory effects on Th2 cell proliferation (Gajewski and Fitch, 1988).

The Th2 cell subset can be distinguished by the expression of cytokines IL-5, IL-4 and IL-13 and under regulatory control of Gata-3 and STAT-6 transcription factors (Zhu et al., 2006) (Kaplan et al., 1996) (Ouyang et al., 2000). Unlike Th1, Th2 cells orchestrate cellular responses that target extracellular pathogens, whilst also supporting B cells to develop and produce antibodies (McGuirk and Mills, 2002). Historically, Th1 and Th2 were viewed as the dominant Th cell populations implicated in adaptive immune responses. However, in recent years other distinct subsets have been discovered such as the Th17 population. Th17 cells have attracted significant attention for their roles in the pathogenicity of many

autoimmune diseases, and in particular MS (Aranami and Yamamura, 2008) (Jadidi-Niaragh and Mirshafiey, 2011) (Kebir et al., 2009). It is important to note that although lineage commitment is dominant, plasticity and differentiation pathways are, to some extent, dynamic. The T regulatory cell subset is an example of how the immune response balances between the pro-inflammatory and anti-inflammatory activation pathways.

1.1.3.4 CD8+ T-LYMPHOCYTE SUBSET ACTIVATION

A distinguishing feature of T-lymphocytes expressing CD8+ co-receptor is their ability to recognise antigen presented by MHC class-I molecules, a pathway commonly known for its endogenous activity. Cells containing MHC class-I load antigen found within its cytosol derived from intracellular viruses, bacteria, and tumour antigens (Zhang and Bevan, 2011). This subpopulation is widely referred to as the cytotoxic T cell subset and gets its name from the ability to kill target cells (Geiger et al., 1982). This subset can further be divided into Tc1 and Tc2 groups (Sad et al., 1995). Upon cytotoxic T cell engagement, clonal expansion of daughter cells will equip the immune system with sufficient amounts of cells for target cell destruction. Deployment of cytotoxic T cells induces death of target cells through either necrosis or apoptosis mechanisms. The first, requires fas-fas ligand binding, which is a transmembrane protein belonging to the TNF α death-inducing signalling pathway. The second mechanism causes exocytotic liberation of cytotoxic granules containing granzymes and perforin that forms transmembrane pores in the target cell, leading to lysis (Golstein et al., 1972).

To achieve maximal expansion, the CD8+ population must receive the signature inflammatory cytokines IL-2 and IFN γ , which will then induce naïve cytokine-driven phenotypic changes (Mescher et al., 2006) (Parish and Kaech, 2009). Many important costimulatory signals such as TNF/TNFR family members, CD27 and OX-40 support these transformations to undergo permanent lineage commitment (Croft, 2009).

1.1.4 Inflammation

1.1.4.1 ACUTE INFLAMMATION

Acute inflammation is an immediate, innate immunovascular response. A distinguishing feature of this type of response is the relatively short time frame, lasting from minutes to hours with the extent of injury largely determining duration. At the tissue level, there are five cardinal features of acute inflammation; redness, swelling, pain, immobility and heat (Rather, 1971). Most microvascular changes occur within a few minutes following tissue injury and result in marked, activated vasodilation of capillaries, venules, and arterioles. An increase in permeability can also be noted as part of these changes. Early entry of inflammatory cells and their mediators from the circulatory system is a fundamental part of this process.

Neutrophils are recruited in response to chemotactic agents such as CXCL-8 and C5a and are perceived as the hallmark cell type defining acute inflammation. These cells are capable of phagocytosis and activation of the respiratory burst for the abundant release of superoxide, a free radical that possess bactericidal properties. When no longer required, this inflammatory response must be actively terminated; failure to do so results in chronic inflammation and subsequent cellular destruction (Segal, 1989) (Segal and Abo, 1993).

1.1.4.2 CHRONIC INFLAMMATION

In contrast to acute inflammation, chronic inflammation is perceived as a state of prolonged inflammatory response, involving a slow progressive switch in the type of cells present and activated at the site of inflammation. Hallmarks of this type of inflammation are characteristic cycling of tissue destruction and repair, dominated by macrophages and, within the CNS, microglia. These cell types contain powerful destructive properties, mediated by proteases and the release of large quantities of reactive nitrogen and oxygen species. These inflammatory cascades can persist for months or years. Neuroinflammation is widely regarded as chronic inflammation, the features of which include sustained activation of glial cells and recruitment of monocytes from the circulation. Such processes involving chronic inflammation are typically associated with neurodegeneration (Streit et al., 2004) (Guzman-Martinez et al., 2019) (Chen et al., 2016).

1.1.4.3 AUTOIMMUNITY

At the turn of the twentieth century, consideration on what is now widely regarded as autoimmunity, first emerged (Ehrlich and Morgenroth, 1957) (Ehrlich, 1902). The essence of autoimmunity lies in the inherent ability of the immune system to develop autoantigens, coupled with an inability of the adaptive response to distinguish between self and non-self (Pressman and Grossberg, 1968) (Brent, 1997). A failure of these basic regulatory checks gives rise to a broad range of illness, collectively known as autoimmune diseases (Silverstein, 2000).

A general understanding of the paradigm underpinning T cell autoimmunity initially described by Mossman and colleagues (Fiorentino et al., 1989), when a clear distinction was made between two main CD4⁺ effector subsets. Th1 and Th2 cells, identifiable by their signature cytokines, perform individual but complementary functions. IL-4, IL-5 and IL-13 producing Th2 cells are classically involved in the clearance of extracellular pathogens and allergic responses. Whilst IFN γ producing Th1 cells are classically recognised in cell-mediated immune responses, hypersensitivity, and elimination of intracellular pathogens. Historically, Th1 cells were perceived as the primary effector cell of autoimmunity.

Clinical manifestations of disease vary greatly according to the target tissue, which can either be organ specific (i.e., uveitis) or systemic (i.e., systemic lupus erythematosus). Despite these variations, all types of autoimmune disease are characterised by the destruction of healthy host tissues and follow a pattern

of initiation, propagation, and resolution. This sequence of events is typically followed by cycling exacerbations, which are evidenced by symptomatic flares. Fundamental to the underlying mechanism of these diseases is defective elimination and/or control of self-reactive lymphocytes which creates an imbalance between effector and regulatory immune responses. Although, exact aetiology of these diseases remains elusive, they are largely idiopathic in nature and often multifactorial involving both environmental and genetic factors.

1.2 Neuroinflammation

The term neuroinflammation is largely reserved for the cellular, inflammatory responses of vascularised neuronal tissue. Resident CNS glial cells (astrocytes and microglia), peripherally derived immune cells and endothelial cells mediate inflammation through the production of cytokines, chemokines, and reactive oxygen species. The degree to which neuroinflammation exists heavily depends on the primary stimulus, disease context and duration. Microglial activation is a hallmark feature of neuroinflammation and remains the focal point of much discussion. These cells are principally responsible for a large portion of the innate immune response within the CNS where the intended outcome is protection. However, exaggerated, amplified or chronic microglial activation can induce robust pathological effects. A high degree of chronic neuroinflammation is characteristic of many neurodegenerative disorders and currently one of the most active areas of research. Indeed, microglia with an activated phenotype are present in large numbers at the site of damage in tissues sampled from patients with MS, Parkinson's, Alzheimer's, and amyotrophic sclerosis.

1.2.1 Immune privilege

The CNS was once considered an immune-privileged site, meaning that immune responses throughout these regions are suppressed. It is believed that immune privilege is a protective adaptation to reduce damage to cells with a limited capacity to regenerate. However, over the past few decades, it has become apparent that the CNS is a hub of complex neuroimmune and glial-lymphatic interactions. Such processes support homeostasis and have found to be dysregulated in neuroinflammatory and neurodegenerative disease.

1.2.2 Blood-brain barrier

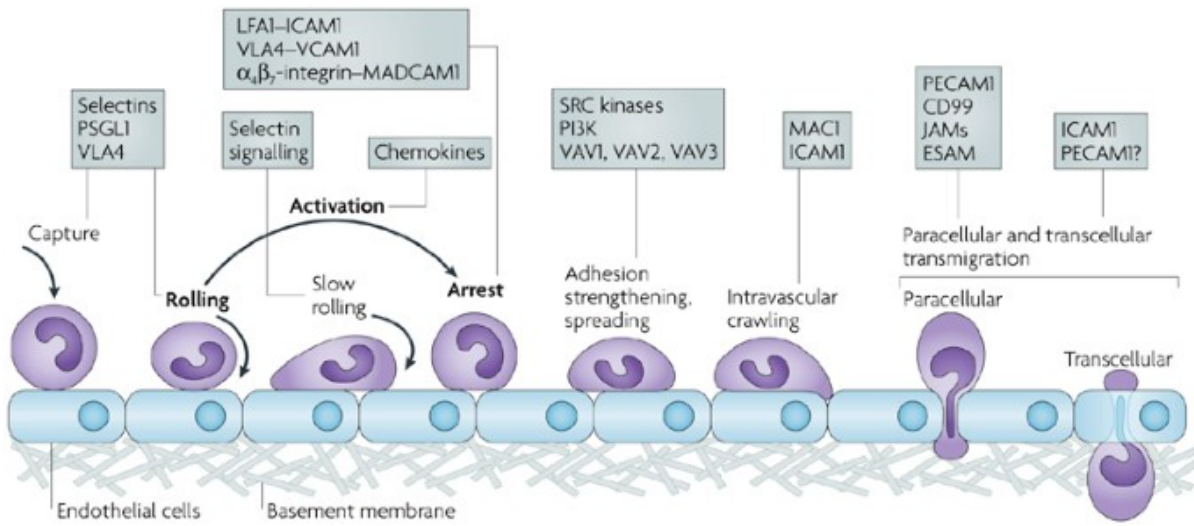
The blood-brain barrier (BBB) is a term used to describe the unique anatomical and physiological characteristics of a highly selective, physical interface in the form of a semi-permeable membrane (Reese and Karnovsky, 1967). Made up of endothelial cells forming tight junctional complexes the primary purpose of the BBB is to restrict migration and diffusion of molecules and cells from the peripheral circulation to the CNS as a form of protection from invading pathogens and potentially fatal neurotoxic

substances. Stable homeostatic control across the CNS is an essential requisite for preservation of normal brain activity and is achieved through a series of tightly regulated mechanisms for the selective transportation of cells, molecules and ions from the periphery to the brain (Goldmann, 1913). Those molecules able to passively diffuse across the BBB are small, lipid soluble substances imperative for life: O₂, CO₂ and H₂O (Serlin et al., 2015).

1.2.3 Leukocyte trafficking across the BBB during CNS disease

Under physiological conditions, an intact healthy BBB permits little movement across its interface (Hickey, 1999) (Hickey, 1991). Immunological reactions in the CNS occur particularly in association with infectious microorganisms and diseases that have an autoimmune component. Recruitment of leukocytes from the circulation to CNS tissue are key events in the development of inflammatory diseases such as MS, posterior uveitis and the *in vivo* models used herein. Immune cell migration across specialised barriers depends on interactions between activated leukocytes and actively participating endothelial cells that form the BBB and BRB. Leukocytes gain access to the CNS parenchyma via a multistep paradigm, first described by Springer and colleagues (1994), that results in transendothelial migration, termed extravasation or diapedesis. Occurring mostly in post-capillary venules, this process is characterised by four sequential tightly controlled stages, these are: chemoattraction, rolling adhesion, tight adhesion, and transmigration. The mechanisms underpinning these processes have been extensively reviewed elsewhere (Nieder Korn and Wang, 2005) (Hunt, 2006) (Nourshargh and Alon, 2014) (Ransohoff et al., 2003).

While resting T-lymphocytes have a limited capacity to invade the brain or spinal cord, activated T-lymphocytes express numerous molecules that promote migration into the CNS. These include adhesion molecules, chemokine receptors, cytokines, integrins, matrix metalloproteinases and reactive oxygen species. During the initial rolling phase, weak adhesion of leukocytes to the endothelial surface occur through low-affinity binding selectins such as P-selectin and E-selectin resulting in a characteristic slowing down of circulating leukocytes (Lawrence and Springer, 1991). At this stage, inflammatory stimuli (cytokines and chemokines) play an essential role in orchestrating the enhanced expression of endothelial cell adhesion molecules (CAMs) to increase cell affinity/avidity. The signalling interactions between CAMs expressed on the surface of endothelial cells and their cognate ligands (integrins) present on activated leukocytes are a crucial step in transmigration. Tight leukocyte adhesion is achieved through the paired interactions between LFA-1/ICAM-1, VLA-4/VCAM, leading to leukocyte arrest (Wong and Dorovini-Zis, 1992) (Wong and Dorovini-Zis, 1995) (Cayrol et al., 2008) (Larochelle et al., 2012) (Steiner et al., 2010). These interactions are vital for facilitating the migration of leukocytes across both the BBB and BRB barrier *in vivo* (Figure 1.2).



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Figure 1.2 Leukocyte trafficking across the BBB. Schematic representation of leukocyte trafficking across the BBB depicting the primary steps of capture, rolling, arrest, adhesion, and transmigration. The key adhesion and integrin couples are highlighted, including chemokines. PSGL, P-selectin glycoprotein ligand-1; VLA, very late antigen; LFA, Lymphocyte function-associated antigen; VCAM, vascular cell adhesion molecule; MADCAM, mucosal addressin cell adhesion molecule; PECAM, Platelet endothelial cell adhesion molecule; JAM, Junctional adhesion molecules; ESAM, endothelial cell-specific adhesion molecule. Image taken from Ley et al., (2007).

1.3 The central nervous system

1.3.1 CNS anatomy and physiology

The nervous system is an immensely complex network of specialised signalling molecules, cell types and nerves, with a basic purpose to facilitate actions via signal transmission to and from different parts of the body. These collaborative processes depend on a variety of sensory information gathered from stimuli in our external environment (Squire, 2012). In the most general sense, the nervous system can be structurally divided into two major regions; the central and peripheral systems, otherwise referred to as the CNS and PNS, respectively. For simplicity, anything contained within the cranial cavity such as the brain, and/or the vertebral cavity such as the spinal cord, is typically classified as the CNS. Anything that sits outside these regions comprises the PNS. It should be noted, however, that this view is a drastic oversimplification and should not always be interpreted as binary. Indeed, significant overlap exists between the two structures. The retina for instance, despite its peripheral location, belongs to the CNS (Haines and Ard, 2002).

Although neurobiological systems in mammals are extraordinarily complex, current understanding of nervous tissue can be reduced on a cellular level, to two fundamental cell types: glial cells and neurons. The neuron or nerve cell represents a basic unit of communication within the nervous system. Due to its electrical excitability, perceivably this cell is the more functionally important of the two and makes up most of the nervous tissue. The primary components of a neuron are the soma (cell body) and processes that project from it (dendrites and axon). Dendrites are branch-like structures that extend from the soma and have functions in receiving and processing incoming information. The axon extends from the cell body and has primary roles in carrying and propagating action potentials to the synaptic cleft. Transmission of signals across specialised synaptic connections is essential for neuron-neuron and neuron-muscle cell communication. Synaptic signals received by the neuron may be excitatory or inhibitory in origin and mechanisms for transmission occur within all regions of the nervous system. An assortment of chemical and electrical components work in tandem to facilitate and direct signal transmission across the synaptic or neuronal junctions. Neurotransmitters are endogenous chemical signalling molecules that relay signals between neurons by crossing the synapse. These chemicals include, but are not limited to, serotonin, dopamine, acetylcholine and a variety of hormones. Successful and efficient transfer of neurotransmitters across this gap are crucial to communication and maintaining homeostasis (Purves et al., 2012).

The other major cell type of the CNS are glia. Glial populations demonstrate great heterogeneity and comprise four major subsets: astrocytes, microglia, oligodendrocytes and their progenitors NG2-glial. Collectively, these populations constitute 33-66% of the total brain mass and were first described as support structures that enable neuronal activities. However, it is clear from recent findings that glial populations are functionally diverse and many aspects are yet to be characterised (Haines and Mihailoff, 2018). To date,

their roles have been described in protection (Glezer et al., 2007) (Nguyen et al., 2002) (Medzhitov and Janeway, 2002), maintenance of homeostasis (D'Ambrosio et al., 1999) (Simard and Nedergaard, 2004), and myelin formation (Tripathi et al., 2017) (Nave, 2010) (Bunge, 1968).

Due to the delicate and intimate nature of communication mechanisms in the CNS, disturbances, or interferences to the regular patterns of conductivity can have devastating effects on CNS physiology and often lead to disease. These disruptions can differ dramatically in scope, impact and ensuing cellular and molecular characteristics, according to a multitude of factors such as severity, duration, and nature of the interruption. For example, in patients with SPMS, the focal point of this thesis, characteristic axonal loss leads to severe disruptions in signal transmission and a breakdown of communication at the tissue level. A phenomenon that has long been recognised as a major determinant of permanent disability.

1.3.2 Spinal cord

The spinal cord is a long, tubular structure that extends caudally from the medulla oblongata. This tissue continues from the lower part of the brain stem down towards the lumbar region of the vertebral column and serves as a communication highway between the brain and the rest of the body. Anatomically, the spinal cord can be divided into three segments: cervical, thoracic, and lumbar regions, each serving different functional purposes. Briefly, the cervical region is connected to various motor and sensory functions that control movement from the neck and arms, whereas the thoracic region serves the thorax and abdomen. Both lumbar and sacral regions collect sensory stimuli and direct functional responses to the pelvic region, legs, and feet. In addition, the spinal cord can be structurally divided according to concentrated regions of afferent (sensory neurons) or efferent (motor neurons) neuronal tracts that cooperate to transmit signalling to and from the CNS, respectively (Cramer, 2014) (Fuller, 2013).

The spinal cord is made up of grey and white matter, two distinctly different tissues in terms of colour and function. Anatomically, grey matter forms the interior portion of the spinal cord and is classically recognised for its “butterfly appearance”. This tissue is densely populated with neuronal cell bodies (unmyelinated axons, dendrites), neuroglia (microglia, astrocytes, oligodendrocytes), small capillaries and synapses. White matter, on the other hand, surrounds the grey matter and is lipid-rich, pale in colour and mainly composed of myelin surrounding axons. Colours ascribed to these regions reflect the appearance of tissues with the unaided eye and represent specific compositions that translate to function (FitzGerald, 1992).

In transverse sections, the grey matter is divided into dorsal (posterior) and ventral (anterior) horns. The dorsal horns comprise neuronal synapses, ready to receive sensory information into the spinal cord such as vibrations, fine touch and proprioception. The ventral horns contain tracts of motor neurons that are responsible for facilitating information via axons to the ventral roots of the spinal column and control striated muscle responses. Similarly, the white matter can be subdivided into dorsal and ventral

columns each containing axonal tracts with precise functions. The white matter dorsal column is designed to process and relay ascending sensory information. The white matter ventral column carries both ascending and descending pathways containing information about pain, temperature, and motor information, respectively. These motor components provide cardiac, skeletal, smooth muscle control in addition to glandular secretion (Purves et al., 2012).

1.3.3 Retina

The retina is a delicate transparent piece of tissue, approximately 0.5 mm in width, that lines the inside of the optic cup. Formed during development as part of the neuroectoderm, the retina is considered part of the CNS and constitutes sensory neuronal circuits that form part of the visual pathway. Its primary function depends on light penetrating the many layers and striking the light-sensitive photoreceptors, in turn, this initiates a cascade of biochemical and electrical activities which are then used to inform the brain for visual recognition (Croner and Kaplan, 1995) (Kolb, 1995).

The five main types of neurons situated in the retina are: ganglion cells, bipolar cells, amacrine cells and photoreceptors, as illustrated in Figure 1.3 (Vecino et al., 2016) (Masland, 2012) (Van Buren, 1963). The light-sensing photoreceptors, rods, and cones, operate by transferring electrical signals to the ganglion cells, whose axons form the optic nerve, and are situated at the front of the retina. Together these components facilitate the organisation and subsequent interpretation of the visual image (Strauss, 2005).

Microscopically, the vertebrate retina can be divided into a number of distinct function-specific layers: internal limiting membrane, nerve layer, ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer, the photoreceptors and finally, the retinal pigment epithelial layer (RPE) (Hoon et al., 2014). The RPE is a specialised monolayer that forms the outer blood retinal barrier and is concerned with specific functions of transportation including exchange of essential nutrients and water. The retina also constitutes three layers of nerve cells and two layers of synapses; the optic nerve is responsible for carrying ganglion cell axons to the brain. Each of these layers comprise specific cellular compartments and cell types with independent metabolic and nutritional requirements. To satisfy these requirements, there are two distinct vascular networks that supply the retina: central retinal artery and the choroidal blood vessels (Pournaras et al., 2008). The central retinal vasculature constitutes four main branches and receives 20-30% blood flow which provides a rich oxygen supply and nourishment for the retina's inner layers. Whereas the latter, receives a majority share of blood flow (65-85%) and has a critical role in maintaining the outer retinal layer, in particular the photoreceptors (Hayreh, 1975).

1.3.3.1 A WINDOW TO THE BRAIN

The retina, by direct comparison to the brain and spinal cord, contains fewer classes of neurons arranged in a relatively simplistic manner. Mechanistically speaking, this model is less difficult for

researchers to disentangle as the laminar highly organised structure of the retina makes it easier to interpret inflammatory events. Furthermore, the retina is the only part of the CNS that can be accessed and visualised non-invasively (London et al., 2013). Together, these advantages make retinal disease models highly attractive and valuable investigative tools for research. In the context of this body of work, these benefits provided a strong platform from which we were able to study details of drug mechanism of action in CNS tissue (Remington, 2012).

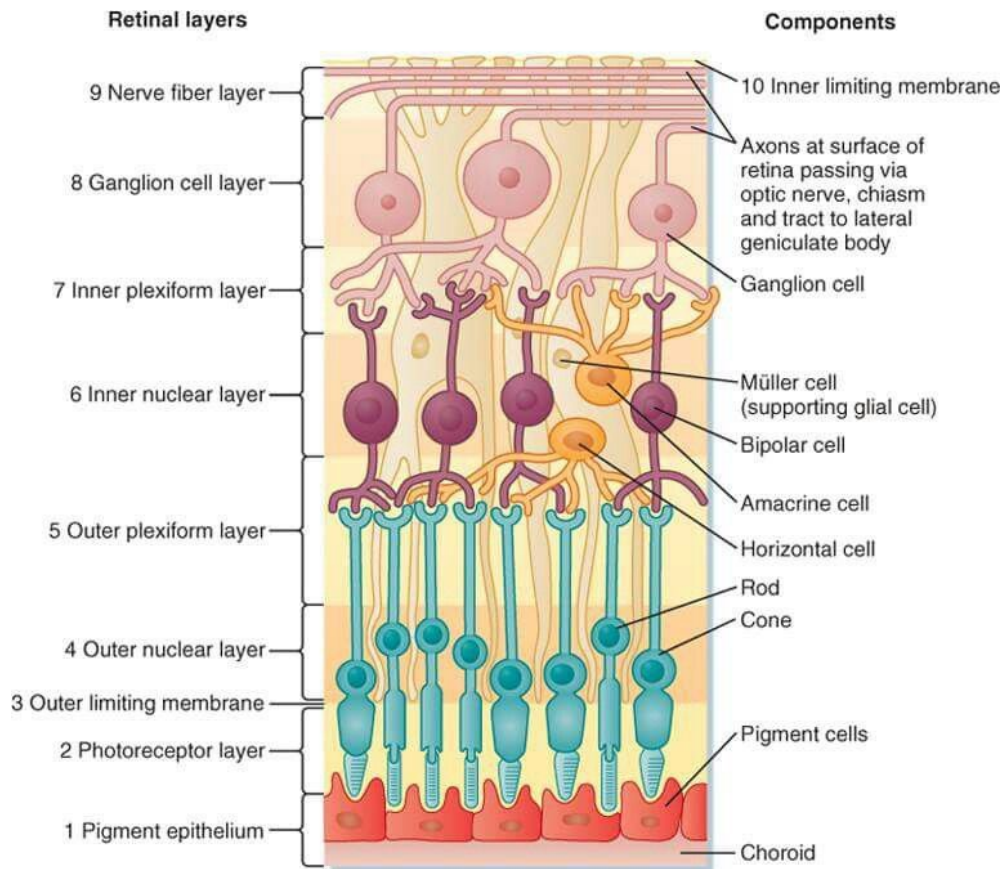


Figure 1.3 Illustration of the organisation of retinal layers. The retinal layers outlined and numbered from (1) pigment epithelium (2) photoreceptor layer (3) outer limiting membrane (4) outer nuclear layer (5) outer plexiform layer (6) inner nuclear layer (7) inner plexiform layer (8) ganglion cell layer (9) nerve fibre layer (10) inner limiting membrane. Image from (Koeppen and Stanton, 2009)

1.4 The role of microglia under physiological and pathophysiological conditions

Microglial cells represent a specialised population of the innate immune system. Originating from the yolk-sac during embryogenesis, these myeloid precursors commence their migratory journey towards the CNS in early life, where they begin to disperse and populate primitive nervous tissue. Accounting for approximately 10-20% of the total cell population, microglia are the most abundantly studied macrophage of the CNS. Others that are less well-characterised include meningeal and perivascular macrophages. In a series of papers published by Del Rio-Hortega (1919) microglia were originally described as resident immune competent phagocytes (Sierra et al., 2016). Intensive research over the past century has since confirmed beyond doubt, their primary role as an intrinsic effector cell of the CNS, possessing diverse and distinct roles in development, maintenance of homeostasis, pathology, neurogenesis, vascular repair and synaptic remodelling in the adult brain (Rezaie and Male, 2002) (Li and Barres, 2018) (Sierra et al., 2010) (Schafer et al., 2012) (Fourgeaud et al., 2016) (Tremblay et al., 2010) (Halder and Milner, 2019) (Low and Ginhoux, 2018) (Schwarz et al., 2011) (York et al., 2018).

1.4.1 Microglia plasticity

Microglia are a single-cell type with complex phenotypic heterogeneity and are highly plastic in nature. In the healthy adult brain, microglia adopt a ramified morphology, characterised by a small cell body (± 4 to $7 \mu\text{m}$ in diameter *in vivo*) and multiple branched processes that can extend up to $50 \mu\text{m}$ from the soma (Nimmerjahn et al., 2005) (Arnoux et al., 2013). The morphological phenotype displayed under physiological conditions here, are typically referred to as “resting microglia”. While this name implies inactivity, recent *in vivo* imaging studies have shown microglia contain dynamic processes that continually alternate between extension and retraction to enable efficient sampling and monitoring of their local microenvironment a feature typical of surveillance macrophages (Nimmerjahn et al., 2005). Indeed, resting microglia are equipped with highly sensitive signalling apparatus and characterised by multiple-branched, fine processes that form an orderly network and tile the three-dimensional space of the brain parenchyma for subtle disturbances to the local microenvironment (Kreutzberg, 1996). Accordingly, phenotypic regulation is largely determined by tissue alterations detected from the environment, rather than developmental origin. It is the “resting” microglia that play an essential role in maintaining healthy CNS tissue. Indeed, microglia are amongst the first cell type to respond to insult or injury and swiftly switch their phenotype towards activation and begin migration towards the site of injury (Davalos et al., 2005).

In contrast to the resting microglia, the activated forms are morphologically different – identified by their ameboid-like structure – and undergo immense transformations that represent a proinflammatory M1 macrophage (Sica and Mantovani, 2012) (Perego et al., 2011) (Imai et al., 2006). The ameboid-like

morphology has a characteristically large soma (± 10 to $12 \mu\text{m}$ in diameter *in vivo*) with fewer, thicker processes (Mosser et al., 2017).

Functional activity of the microglial cell correlates well with its structural organisation. Therefore, alterations in the shape, size and organisation of their processes are indicative of function. Suzumura et al., (1991) were amongst the first group to report striking morphological changes in response to proinflammatory stimuli that are now described as ameboid, intermediate and ramified. It is important to note that whilst it is common to refer to microglia in a binary morphological and hence, functional state, this terminology is inadequate to describe the continuum of phases by which microglia can be found *in vivo*. Morphological transformations between the ramified and ameboid microglia can be seen in Figure 1.4.

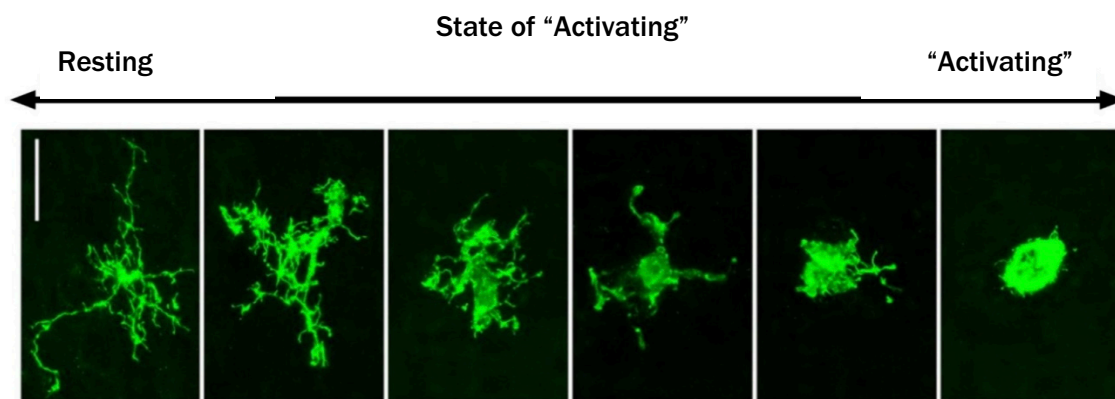


Figure 1.4 Cell morphology of activated microglia. Morphological transformations of the microglial cell shifting from a ramified resting state (left) towards an ameboid activated state (right). Image from (Rawlinson et al., 2020)

1.4.1.1 MICROGLIA ACTIVATION

Microglia are complex and dynamic mediators of neuroinflammation. Surface membrane proteins such as PRRs, scavenger receptors and chemokine receptors recognise pro-inflammatory mediators from the surrounding area and react. Consequently, rapid activation of the resting microglia into a motile effector cell contributes towards ongoing inflammation, resolution, and neuroprotection. Once activated during disease, microglial cells have the capacity to present antigen, proliferate and migrate towards the site of active injury (Remington et al., 2007) (Babcock et al., 2003) (Tran et al., 2000) (Ladeby et al., 2005) (Ponomarev et al., 2005) (Frei et al., 1987).

1.4.1.2 TLR SIGNALLING NETWORKS

TLRs are amongst a class of PRRs most relevant to microglia and recognised for their ability to detect DAMP and PAMP signals (Hanisch and Kettenmann, 2007) (Boivin et al., 2007) (Pineau and Lacroix, 2009). TLR engagement triggers the activation of key transcription factors such as NF κ B. Indeed, upregulation of TLRs have been reported in response to bacterial LPS, viral stimuli and CNS autoimmunity (Laflamme et al., 2001) (Zekki et al., 2002) (Bsibsi et al., 2002). TLRs can be found on astrocytes, neurons and vascular endothelial cells (Carty and Bowie, 2011).

Recent studies have provided overwhelming evidence that microglia constitutively express TLR2 (Bsibsi et al., 2002) (Olson and Miller, 2004) (Laflamme et al., 2001) (Kielian et al., 2005). TLR2 signalling in the CNS has been described as a bridge to neuroinflammation and in particular, is implicated in the pathogenesis of both MS and EAE (Babcock et al., 2006) (Zekki et al., 2002). TLR2 responds to a wide range of endogenous DAMPs and exogenous ligands (Shi et al., 2013). Indeed animal studies of EAE have demonstrated that loss of TLR2 provides protection against the development of disease (Reynolds et al., 2010) (Rocca et al., 2017). Moreover, increased levels of TLR2 agonists have been observed in patients with SPMS (Andersson et al., 2008) (Sweeney et al., 2011) (Nyirenda et al., 2015) and found to be expressed in glial cells of MS patients (Bsibsi et al., 2002) (Guo et al., 2010).

Further to this, TLR-MyD88 signalling transduction cascades promote the translocation of NF κ B to the nucleus which in turn mediates secretion of IL-6, promoting the differentiation of Th17 lymphocytes (Shi et al., 2013). This cell type is a critical driver of pathogenesis in MS (Jadidi-Niaragh and Mirshafiey, 2011) (Mellanby et al., 2012) (Kawai and Akira, 2007). Moreover, NF κ B activation through TLR signalling in microglia induces the transcription of proinflammatory related cytokines and chemotactic agents leading to mass release of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which can cause glia-mediated neuronal vulnerability and neurotoxicity (Saito et al., 2006) (Karin and Wildbaum, 2015) (Gill and Windebank, 2000) (Schneider et al., 1999) (Ho et al., 2005). Indeed, NF κ B has proven to induce nitric oxide synthase (Akama et al., 1998) (John et al., 2003).

1.4.1.3 M1/M2 POLARISATION

The classification of activated microglial cell immunophenotypes is somewhat arbitrary as nomenclature well studied for *in vitro* macrophages is often extrapolated for this cell type (Martinez and Gordon, 2014) (Mackaness, 1962) (Nathan et al., 1983). One of the central tenets used to describe the two main activation phenotypes is the M1/M2 polarisation hypothesis. Traditionally, depending on the type of signal received, activated macrophages and microglia are categorised according to two primary functional modes “classical” (IFN γ) or “alternative” (IL-4), corresponding to M1 and M2, respectively. The classically activated macrophages express iNOS and take on phagocytic roles, whereas the alternatively activated contribute towards neuroprotection. Figure 1.5 illustrates typical features of an M1 and M2 polarised microglia.

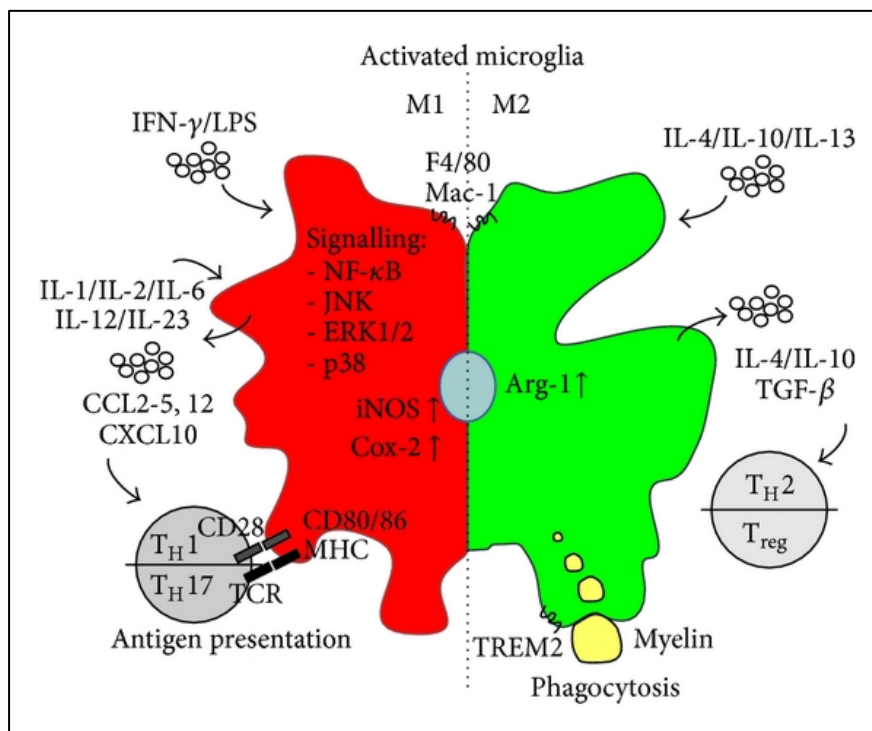


Figure 1.5 Microglia polarisation. Illustration showing the typical features of classical M1(left) and alternative M2 (right) polarised microglia. Image taken from (Goldmann and Prinz, 2013).

1.4.1.3.1 M1 microglia

M1 activation is perceived as an aggressive and robust immediate inflammatory response that often leads to chemokine and cytokine (IL-6, TNF α , IL-1 β) related cytotoxicity. M1 polarised microglia are often characterised by the expression of iNOS, NADPH oxidase, MHC-II, MMP-12, Fc receptors and integrins. F4/80 and Mac-1 surface markers are typically upregulated in response to IFN γ released by Th cells and bacterial LPS stimulation. The upregulated expression of proinflammatory cytokines such as TNF α , IL-1 family, IL-6, IL-12, IL-17, IL-18 and IL-23 (Nathan, 1983) (Nathan et al., 1983) (Biswas and Mantovani, 2010) (Orecchioni et al., 2019) as well as chemokines for immune cell recruitment (CXCL1, CXCL9, CXCL10, CCL5, CCL20) are characteristic features of M1 polarisation. Collectively, these expression profiles are used to signify activation status (Boche et al., 2013) (Lively and Schlichter, 2018). In the M1 state, TLR and IFN γ mediated signalling is controlled by STAT1, NF- κ B and transcription factor interferon regulatory factor 5 (IRF5) which in response, typically stimulate the production of TNF α , IL-12 and IL-23 (Krausgruber et al., 2011).

Pertinent to this thesis are the enhanced inflammatory responses from the M1 phenotype that lead to reactive species production such as NO, superoxide and hydrogen peroxide. Release of neurotoxic molecules such as proinflammatory cytokine and reactive oxygen and nitrogen radicals from M1 microglia can directly cause neuronal apoptosis (Wilkinson and Landreth, 2006) (Bal-Price et al., 2002) (Gao et al., 2003a) (Hickman and El Khoury, 2014). NADPH oxidase catalyses the production of superoxide radicals and plays a pivotal role in inflammation-mediated neurotoxicity following microglia stimulation (Qin et al., 2004). More specifically, activation of the gp91phox complex in activated microglia has shown to induce free radical-mediated neuronal injury (Hur et al., 2010).

M1 polarised microglia can initiate BBB disruption by secreting matrix metalloproteinases (Gottschall et al., 1995) (Lukes et al., 1999). In MS patients, BBB breakdown precedes the signs of new lesion formation as detected by MRI (KERMODE et al., 1990). Moreover, BBB disruptions leading to increased permeability often facilitates the infiltration of peripheral blood derived monocytes and neutrophils into the CNS (Song et al., 2015) (Gerwien et al., 2016) (Rosell et al., 2008).

1.4.1.3.2 M2 microglia

M2 microglia typically secrete anti-inflammatory cytokines, identical to those commonly used to describe M2-like macrophages, including TGF β , IL-4, IL-10 and IL-13. In addition, they secrete chemokines CCL22, CCL17 and CCL24 that elicit anti-inflammatory responses. iNOS and ARG1 cellular expression are perceived as representatives of the M1 and M2 response, respectively, as these factors both compete for the same substrate – arginine. In order to distinguish between M1/M2, the ratio of iNOS and ARG1 is often compared as the overexpression of ARG1. ARG1 is known to downregulate the production of NO and iNOS (Corraliza et al., 1995). JAK-STAT pathway activation classically represents M2

polarisation, which has been reported to engage the STAT6 pathway via IL-13 and IL-4 induction, a process which is mediated by STAT3 via IL-10 (Lang et al., 2002). These signalling cascades prompt the upregulation of M2-like genes including, FIZZ1, or Ym1, ARG1 (Pauleau et al., 2004) in addition to CD206 or mannose receptor (Stein et al., 1992). Further, recent evidence suggests a dynamic role for M2 microglia in facilitating tissue remodelling and repair.

The high degree of overlap existing between microglia subtypes suggests that phenotypes represent a spectrum. Recent substantial profiling studies have reordered current thinking behind the functional significance of microglia. Some findings have unambiguously demonstrated transcriptome differences between resident microglia and peripheral macrophages (Wes et al., 2016) (Murray et al., 2014) (Xue et al., 2014) in response to identical stimuli (Yamasaki et al., 2014) (Ajami et al., 2011) (Gautier et al., 2012). Accordingly, the M1/M2 paradigm is now perceived as an oversimplified and a somewhat outdated model, insufficient to accurately capture the range of microglia observed *in vivo*. Nevertheless, this traditional model continues to be used as a crude template to determine microglia activation status whilst major investigations are ongoing. Indeed, many research groups are currently focused on deciphering more accurate methods to describe the spectrum, or continuum, by which microglia react from M1 state to M2.

1.4.1.4 SURFACE EXPRESSION HETEROGENEITY

Characteristic features of microglia heterogeneity can be demonstrated using surface marker expression patterns, which they broadly share with monocytes and macrophages. The plethora of combinations reflects the spectrum of cellular phenotypes and biological functions. When compared with other CNS macrophages, the downregulated microglia phenotype would typically include low expression of MHC class II, CD45 and Fc receptors. Recently, the cell surface markers transmembrane protein 119 (TREM119) and purinergic receptor P2Y₁₂ have emerged as novel, more reliable and specific markers for the detecting resting state microglia (Bennett et al., 2016) (Satoh et al., 2016). On the other hand, the classically activated M1-like phenotype has demonstrated upregulated patterns of MHC class II, CD86, CD40 and CD68 across multiple *in vivo* neuroinflammatory scenarios. Table 1.1 highlights the surface markers commonly used to distinguish between resting and activated microglia.

Marker	Status	References
CD11b	Both resting and activated M/M	(Perego et al., 2011) (Li et al., 2016)
CD45	Nucleated hematopoietic cell surface	(Campanella et al., 2002) (Penninger et al., 2001)
CD68 (ED1)*	Active phagocytosis M/M	(Perego et al., 2011) (Ito et al., 2001) (Damoiseaux et al., 1994)
Iba-1	Both resting and activated M/M	(Imai et al., 1996) (Ito et al., 1998) (Taylor and Sansing, 2013)
F4/80	Both resting and activated M/M	(Taylor and Sansing, 2013) (Ginhoux et al., 2010)
IB4	Both resting and activated M/M	(Patel et al., 2013)
Ym-1	Activated M/M (M2)	(Perego et al., 2011)
Iba-1+, CD206+	Activated M/M (M2)	(Perego et al., 2011)
Iba-1+, CD16/32+	Activated M/M (M1)	(Hu et al., 2012)

Table 1.1. Microglial cell markers classically used to distinguish between the resting and activated phenotype. Iba, Ionized calcium binding adaptor molecule 1; Ym-1, chitinase-like 3 protein; Table adopted from Lee et al., (2014b).

1.4.1.5 MICROGLIA AS AN APC

While the microglial cell is not a professional APC like the DC, nevertheless, under certain circumstances microglia can take on similar roles. While their primary role remains to counteract any disturbances to immunological homeostasis behind the BBB, microglia can behave as an intermediary to enable efficient communication with the adaptive system. In disease, especially that of neurodegenerative origin such as SPMS, microglia represent the main APC in the brain parenchyma and localise to regions of neuronal damage. Here, microglia activation results in the upregulated expression of MHC-II and costimulatory molecules such as CD40 and CD80/CD86 for effective formation of an immunological synapse ready to present antigen to T-lymphocytes (Schetters et al., 2018)

1.4.2 The downregulated microglia phenotype

Microglia function is tightly regulated within the CNS and plays critical roles in CNS immune physiology. By direct comparison to other CNS macrophages, under physiological conditions, microglia display a strikingly downregulated phenotype. Active suppression of this phenotype depends on proficient communication pathways between microglia and neighbouring cells, in particular neurons and astrocytes. Accordingly, several intrinsic (Pu.1, Irf8, Runx-1) and extrinsic (TREM2, CX3CR1, CD200) mechanisms exist to maintain microglia in a resting state.

1.4.2.1 SOLUBLE FACTORS

Structurally, in the brain and spinal cord, microglia are in close proximity to oligodendrocytes, astrocytes and neurons. Neurotransmitter surface receptors receive a signal, either excitatory or inhibitory in origin (Pocock and Kettenmann, 2007). Astrocytes are the most abundant CNS glial type. Forming direct contacts with neurons, astrocytes are active generators of soluble factors for modulating synaptic transmission (Perea et al., 2009). Previous studies on adult human brain tissue identified all three types of GABA and GABA receptor expression on both astrocytes and microglia (Lee et al., 2011) (Kuhn et al., 2004). GABA is widely recognised as the main inhibitory neurotransmitter in the brain and has established roles as a neuroprotective agent. Activating the GABA receptors actively suppresses IFN γ and LPS mediated responses that sit upstream of NF- κ B and P38 MAP kinase signalling pathways. ATP was recently identified as a soluble factor released from astrocytes and reported to induce microglia chemotaxis (Wu et al., 2007). *In vivo* imaging studies on living mice demonstrated a rapid attraction of resting microglia processes to local damage. These studies can be simulated by ATP due to activation of P2Y₁₂ receptor expressed on the resting microglia. Relevant to this thesis are the identified cell signalling pathways involved in these processes. Studies on microglia migration demonstrate the need for P13K activation via P2X receptors that results in F-actin polymerisation. F-actin is regulated by the isoprenoid Rac GTPase, and together, the two mediate the location of PI3K to the cell membrane (Ohsawa et al., 2007) (Sasaki and Firtel, 2006).

1.4.2.2 RECEPTORS

Aside from soluble mediators, direct cell-to-cell modulation of microglia has also proven to contribute to the remarkably downregulated microglia phenotype observed in normal tissue. The membrane-bound proteins CD200 and CX3CL1, located on neurons carry cognate receptors that bind microglia and macrophages only (CD200R and CX3CR1, respectively (Cardona et al., 2006) (Billadeau and Leibson, 2002). Both CD200R and CX3CR1 are transmembrane glycoproteins embedded in the cell membrane and carry ITIM motifs (immunoreceptor-tyrosine-based inhibitory motif) in their intracellular domain. Upon ligand-binding, these domains recruit SHP1 and SHP2 phosphatases which in turn can inhibit various downstream immune signalling pathways (Billadeau and Leibson, 2002) (Crocker et al., 2007). Whilst microglia bound CD200R interacts with neuronal derived CD200, the resting state is maintained. However, dysregulated or defective CD200 signalling has been implicated in a number of neuroinflammatory disorders including multiple sclerosis (Hernangómez et al., 2012) (Varnum et al., 2015) (Xie et al., 2017). Similarly, in experimental animal models of MS, mice deficient in CD200 display an accelerated disease onset and progression (Hoek et al., 2000).

The chemokine CX3CL1, commonly referred to as fractalkine, is constitutively expressed on healthy neurons and its target receptor is found predominantly on microglia (Tarozzo et al., 2003) (Harrison et al., 1998) (Paolicelli et al., 2014) (Hatori et al., 2002) (Meucci et al., 2000). Stable signalling between the two have been recognised as a fundamental part in maintaining the microglia surveillance phenotype (Nishiyori et al., 1998) (Zujovic et al., 2000) (Mizuno et al., 2003) (Biber et al., 2007). CX3CL1 also exists in a soluble, cleaved form, and behaves as a chemoattractant for inflammatory cells. Once bound to CXCR1 situated on microglia, several important intracellular messengers are triggered within the cell such as, NF- κ B, AKT and P13K, all of which are recognised to play important roles in migratory functions of microglia, apoptosis, proliferation, and transcription. It is important to note that the fractalkine receptor is not exclusive to microglia but also expressed on dendritic cells, monocytes, and natural killer cells. Since these cell types seldom cross a healthy, intact blood brain barrier, microglia are perceived as the main recipient of CX3CL1 under physiological conditions.

In the context of this thesis, neurodegenerative processes observed in SPMS are characterised by loss of neurons. Microglia released from the tonic inhibitors provided by neurons removes stable communication and the CNS scaffold supporting a downregulate phenotype. Thus, microglia become active, mobilise, and follow chemotactic signalling towards the site of injury.

1.4.3 Locations and functions of microglia

Microglia distribution in the CNS is not uniform, densities vary considerably between regions and model species examined (Lawson et al., 1990). Adding to the diversity, microglia display phenotypic heterogeneity within as well as across anatomical regions. Under physiological conditions in humans, the

number of microglial cells present in the brain at any one-time account for 0.5-16.6% of the total cell population (Mittelbronn et al., 2001). In mice, this number increases to 5-12% (Lawson et al., 1992). These numbers are under tight regulatory control and replaced at a steady-state turnover. They embody multifaceted roles important in both building and defending the CNS (Tay et al., 2017). Transcriptome profiling of microglia established differences in surface expression proteins between different regions of the brain and spinal cord, suggesting that microglia responses to pathological insults may differ according to area. Indeed, animal studies of LPS or IFN γ injected in several CNS regions led to distinct and diverse microglia responses (De Biase and Bonci, 2019). Further adding to the complexity, these responses show differences between grey and white matter regions (Kim et al., 2000) (Phillips et al., 1999) (McKay et al., 2007). As the field of microglia profiling is beginning to develop, it is becoming clear that this cell population is exceedingly diverse (Silvin and Ginhoux, 2018) (Olah et al., 2011) (E Hirbec et al., 2017) (Eggen et al., 2019) (Smolders et al., 2019).

1.4.3.1 SPINAL CORD

Microglia colonise the spinal cord during early development as myeloid derived precursors. Akin to observations in the brain, the route of migration typically follows the direction of white to grey matter, with the associated influx of this population intimately linked to ICAM-2 expression on blood vessels (Rezaie et al., 1997). Studies reported on microglia density in rat spinal cord revealed grey matter constitutes 7% of the cell population, while this number increases to 11% in white matter (Ling, 1976). In EAE, animal models have shown microglial cell densities to rise with highest numbers detected in the lumbar spinal cord. These populations have been well characterised by the classically activated marker MHC-II (Vass and Lassmann, 1990). Spinal microglia can acquire various immunophenotypes in response to a variety of stimuli (Kobayashi et al., 2013) (Nikodemova et al., 2014) (Zhou et al., 2014) of which largely subscribe to the M1 and M2 functional categories.

Spinal cord microglia are distinct from other tissue macrophages in that, they express high levels of the receptor CXCR1 (Yona et al., 2013). Iba-1 is commonly used for studying spinal microglia as even distribution of this protein within the cytoplasm and microglia processes makes it a useful tool for identification and analysis of known morphological types (Kolos and Korzhevskii, 2016) (Korzhevskii and Kirik, 2016) (Kongsui et al., 2014). Indeed, activated microglia are typically amoeboid and are known to be highly motile phagocytic cells. However, unlike spinal cord white matter activated microglia, for those residing in grey matter spinal cord, activation often occurs in the absence of morphological change (Vela et al., 1995) (McKay et al., 2007).

By direct comparison to the brain, little has been established regarding the physiological and pathophysiological function of microglia in the spinal cord. Despite this, there are commonalities in reactivity. Generally speaking, in response to short-term moderate injury, microglia change their

morphology and project their processes towards the site of injury to begin migration (Avignone et al., 2015) (Zhang et al., 2008). On the other hand, chronically activated microglia promote transition towards a neurotoxic phenotype – quintessentially representative of an M1-like macrophage. At the later stages of chronic inflammation, microglia begin to work in tandem with local glia to form astrocyte-microglia scars (Stoll et al., 2006).

1.4.3.2 RETINA

Under physiological conditions, the organisation of microglia in the retina have been well mapped. These locations are spatially and functionally distinct niches, largely confined to the ganglion cell layer, inner plexiform layer and outer plexiform layer they are evenly distributed and extensively ramified to enhance surveillance of their microenvironment (Ashwell et al., 1989) (Kohno et al., 1982). Their role in these regions is to maintain neurotransmission and neuronal synaptic architecture (Wang et al., 2016). Here, reciprocal signals between microglia and neurons support the maintenance of homeostasis and include CX3CL-CX3CR1 and CD200-CD200R interactions as well as sialic acid-binding immunoglobulin-like lectin-11 (SIGLEC-11).

In retinal neurodegenerative disease where chronic inflammation is common, resident microglia populations migrate to the subretinal space to take on a neuroprotective role. Often persistent inflammation can lead to pathological activation and disease (Langmann, 2007) (Geissmann et al., 2010) (Prinz et al., 2011). In experimental autoimmune uveitis (EAU), peroxidised docosahexaenoic acid (22:6HP) was recently recognised as a potent chemoattractant for microglial migration towards the photoreceptor layer (Saraswathy et al., 2006).

1.4.4 Pathological microglia in SPMS

Of particular relevance to this thesis are the pathogenic roles microglia play in SPMS. In this context, microglia have characteristic roles in orchestrating bidirectional inflammatory responses between resident and infiltrating peripheral cells (ESIRI and READING, 1987) (Ferguson et al., 1997). Recent data suggests that activated microglia and their by-products mediate mechanisms of neurodegeneration and axonal dysfunction.

There is a strong correlation between immune activation and oxidative damage. The term microgliosis refers to an intense chronic activation of microglia within the CNS. Accumulating evidence points to prolonged microglia activation as a persistent and potent source of neurotoxic factors that include ROS and RNS, $TNF\alpha$ and $IL-1\beta$. Further, prolonged microglia activation leads to the generation of pathological levels of ROS and RNS (Gonsette, 2008) (Lucas et al., 2006) (Block et al., 2007) that is harmful to neurons and oligodendrocytes. It has been postulated that dysregulated overproduction of ROS and RNS can cause direct harm to mitochondria function and mediate processes of demyelination and

axonal damage (Nikić et al., 2011) (Bhat et al., 2015) (Rohowetz et al., 2018) (Rojo et al., 2014) (Davalos et al., 2012) (Block and Hong, 2007). These and other data demonstrate that free radicals and their products are a major cause of neuronal dysfunction and death (Block et al., 2006) (Gao et al., 2003a) (Wu et al., 2006).

ROS and RNS generation represent a pro-inflammatory immune response and are increasingly implicated in the pathogenesis of many neurodegenerative disease such as Parkinson's disease, Alzheimer's, ALS and MS (Block and Hong, 2005). Over the past decade, research centred around the importance of microglia in this group of diseases has been extensively reviewed (Ransohoff, 2016) (Cai et al., 2014) (Sanchez-Guajardo et al., 2015) (Cooper-Knock et al., 2017) (Lall and Baloh, 2017) (Gao et al., 2011) (Gao et al., 2003b) (Gao et al., 2002) and continues to be an active area of intense study.

In the MS brain, both microglia and macrophages accumulate at the site of activate demyelination and neuronal injury and together, constitute the most abundant cell types (Bogie et al., 2014) (Trapp et al., 1998) (Ferguson et al., 1997). The CSF of MS patients exhibit elevated levels of microglia markers that correlate with clinical disease and is often used to map severity (Prineas et al., 2001) (Masvekar et al., 2019). Differentiating between macrophages and resident microglia has been notoriously challenging. However, a recently characterised TMEM119 microglia-specific marker in rodent CNS and the human brain has given researchers new-found confidence in their ability to identify resident microglia pools. There is now good agreement that microglia phenotypes dominate the initial areas of new lesions and the leading edge of slowly expanding lesions (Zrzavy et al., 2017) (Brück et al., 1995) (Trebst et al., 2001). Under these circumstances, Marik et al., (2007) claim microglia activation precedes and encourages the formation of hypoxia-like plaques. On the other hand, macrophage phenotypes populate the lesions after initial destruction of myelin and adopt important roles in phagocytosis of debris (Barnett and Prineas, 2004).

Although an in-depth understanding of the functional microglia phenotypes at different stages during lesion formation is limited, it is generally accepted they are overwhelmingly proinflammatory in origin – particularly at the progressive stages. An abundance of data supports that microglia clusters in MS closely associate with stressed oligodendrocytes and the expression of proinflammatory mediators including, but not limited to, $\text{TNF}\alpha$, $\text{IL-1}\beta$ and neurotoxic substances such as proteolytic enzymes, nitric oxide and oxygen radicals (Van noort, 2010) (van Horssen et al., 2012). Moreover, monocyte and macrophage numbers in early MS lesions correlate well with demyelination and microglial cell silencing in EAE has been shown to delay and reduce the severity of clinical disease.

1.5 Neuroinflammatory diseases of the CNS and Retina

1.5.1 Multiple sclerosis

1.5.1.1 CLINICAL OVERVIEW

More than two hundred years since it was first described by Jean-Martin Charcot in the 1800's; more than two hundred years later, MS remains the prototypical immune-associated, chronic demyelinating disease of the CNS. Over time, cascades of neuroinflammation progress towards profound neurodegeneration within the brain and spinal cord leading to the development of sensory disturbances, visual problems, limb weakness, gait instability and ataxia (Compston, 2006) (Peterson et al., 2001) (Kidd et al., 1999). In advanced disease, cognitive deficits have been reported in addition to fatigue, bladder dysfunction and heat insensitivity. Although largely idiopathic in nature, an interplay of genetic, environmental and virulent factors have been implicated in the aetiology. The earliest manifestations of MS typically begin in adulthood, with increased incidence rates amongst the female population (2:1). MS is clinically characterised by a relapsing-remitting disease course (RRMS), in which periodic attacks of debilitating neurological deficit cycle between partial recovery of neurological function. These phases of relapse are extremely variable in nature and duration. Often, the RRMS stage is followed by progressive and persistent accumulation of neurological deficit in the absence of recovery. This phase, defined as secondary progressive MS (SPMS) is neurodegenerative in origin. In some instances, patients with MS (~20%) experience a progressive disease course from diagnosis, commonly known as primary progressive MS (PPMS). Evidence gathered so far indicate major pathological differences between RRMS and the two progressive forms of MS, but no essential differences between SPMS and PPMS (Barnett and Prineas, 2004) (Kassmann et al., 2007). A testament to these differences are the mainstay immunosuppressive and immunomodulatory therapies commonly prescribed for the effective treatment of RRMS yet they remain ineffective for SPMS. It is for these reasons, more than 15 years later, IFN β therapy persists as the first-line treatment for RRMS.

The most widely used and highly accredited diagnostic procedure for MS is application of the McDonald criteria (Thompson et al., 2018). Recently revised in 2017, assessment techniques are based on the combined evaluation of neurological signs, symptoms, and neuroimaging, in addition to laboratory testing. Typically, early-stage diagnosis of MS is difficult to achieve due to overlapping similarities with other neurological disorders. Whilst MRI imaging is used to localise demyelinating lesions disseminated in space and time, the expanded disability status scale (EDSS) is used to assess the magnitude of disability and monitor severity changes to neurological deficit. The EDSS is also commonly used to assess the efficacy of novel therapies in clinical trials. To achieve a confident and uniform diagnostic procedure, CSF and evoked potential is often used in parallel.

While CNS inflammation, observed primarily during the RRMS phase, can be well controlled with modern therapies, the current challenge is progressive disease, during which most irreversible disability occurs (Correale et al., 2016) (Abdelhak et al., 2017). Currently, we have no effective treatment options for SPMS patients, and a limited understanding of the pathogenic mechanisms dominating this phase of disease.

1.5.1.2 OPTIC NEURITIS

Clinically, MS patients display a large variety of symptoms. As part of the CNS fabric, the retina is frequently involved. Roughly 60-70% of MS patients will experience a form of optic neuritis throughout the course of disease, presenting as either an initial symptom or at the later stages (Said, 2013) (Lampert et al., 2015). Manifestations of optic nerve and retinal involvement include impairment or complete loss of vision, decreased contrast sensitivity, diminished colour vision and pain during eye movement (Ziemssen et al., 2006) (Kale, 2016). Similar to findings in human studies, pathological alterations in the retina have been reported in animal models of MS. Amongst the numerous target autoantigens for developing EAE, MOG-induced disease appears to have predilections for optic tract and retinal pathology which more often results in optic neuritis-like defects (Shao et al., 2004) (Quinn et al., 2011). Consequently, this model is often used to mimic optic-neuritis. Compared with MBP-induced EAE for instance, MOG specific T-lymphocytes induce a greater density of lesions in the optic nerve (Kezuka et al., 2011). Common eye-related pathologies observed during EAE include optic nerve demyelination, increased inflammatory cell infiltration and gliosis in the retina these pathological features recapitulate classic hallmarks of MS (Horstmann et al., 2016) (Tian et al., 2010) (Guy, 2008) (Horstmann et al., 2013). Moreover, the features of optic neuritis observed in EAE resembles the complex neurobiological tissue reactions observed in typical MS-related lesions elsewhere in the CNS, such as the brain and spinal white matter (Calabresi et al., 2010).

1.5.1.3 AETIOLOGY OF MULTIPLE SCLEROSIS

Over the past century the causes of MS have been the subject of intense debate. In recent years, epidemiological data identified strong, credible links between several environmental and genetic factors, purportedly involved in the aetiology of MS (Handel et al., 2010). One of the most compelling pieces of evidence to date, reported a strong genetic association between specific types of human leukocyte antigen class-II (HLA-II) and increased risk of developing MS. Genome-wide association studies have confirmed that variation within the HLA-II molecule exerts the greatest individual effect on risk (Consortium, 2005) (2007) (Burton et al., 2007) (Baranzini et al., 2009) (Australia and Consortium, 2009) (Consortium, 2010) (Sanna et al., 2010) (De Jager et al., 2009). These studies revealed over 100 MS risk loci, many of which overlap with other autoimmune diseases. It has been suggested that the HLA-II molecules associated with MS might bind preferentially to specific CNS epitopes and subsequently prime an antigenic T-lymphocyte

response. However, despite considerable effort, a single CNS antigen has not yet been identified and the controversy surrounding this topic has raged unabated for several decades (Ramagopalan et al., 2010) (McFarland and Martin, 2007) (Dendrou et al., 2015).

Environmental risk factors of MS include low vitamin D status, cigarette smoking and Epstein bar viral (EBV) infection. EBV is considered the most relevant environmental risk factor of MS with past exposure, as evidenced by seropositivity, almost regarded a prerequisite for developing the disease (Haahr and Höllsberg, 2006) (Munger et al., 2011). Despite strong epidemiological evidence to support an influential role for EBV, this notion remains controversial as some research groups report an absence of infection in the MS brain (Levin et al., 2010) (Willis et al., 2009) (Magliozzi et al., 2013) (Guan et al., 2019). Furthermore, given the high global prevalence of EBV, only a relatively small proportion develop MS, indicating that while viral exposure may play an important role, it is not necessarily sufficient to trigger the disease alone. Although clear links to the pathogenesis of MS have not yet been elucidated, many researchers speculate that infection-mediated molecular mimicry is at play and/or bystander activation of APCs and autoreactive T-lymphocytes (Lünemann et al., 2007) (Palle et al., 2017).

1.5.1.4 “OUTSIDE-IN” OR “INSIDE-OUT?” - THE TWO THEORIES OF AETIOPATHOGENESIS

Due to the overwhelming inflammatory nature of MS, particularly at the early stages of disease, it was traditionally assumed that an aberrant CD4+ T cell subpopulation was the primary trigger. This hypothesis is referred to as “outside-in” and used to describe a situation whereby autoreactive peripheral T-lymphocytes recognise CNS antigen(s) and traverse the CNS parenchyma to induce the recruitment of other leukocytes. Together, along with B cells and macrophages these immune responses elicit an autoimmune reaction causing tissue damage, where myelin is a prominent target. Clinical features of MS such as a relapsing-remitting disease course combined with an increased incidence amongst the female population, are typical features of human autoimmune diseases, although the PPMS course does not follow this classic pattern (Trapp and Nave, 2008). Whilst the traditional “outside-in” model remains the central focus for many research groups, this hypothesis continues to be challenged by an alternative “inside-out” theory, an opposing view suggesting that initial malfunction occurs within the CNS. In line with other neurodegenerative disorders such as Parkinson’s and Alzheimer’s disease, this hypothesis argues MS is primarily neurodegenerative in origin (Hauser and Oksenberg, 2006) (Stys et al., 2012). Accompanying inflammation is thought to be responsible for chronic shedding of autoantigens, thus, autoimmune activation represents a secondary reaction to a primary degenerative process. Mass immunological activation, aimed at clearing up debris, purportedly initiates the release of antigenic peptides originating from cellular components such as; myelin basic protein, myelin oligodendrocyte glycoprotein and proteolipid protein are amongst the most commonly studied (Davies et al., 2005) (Maña et al., 2009).

The majority of pathological observations can be equally described using both theories due to the highly heterogeneous nature of clinical manifestations and the technical difficulties in dissecting triggering mechanisms. Despite uncertainties surrounding the lack of specific T cell targets, however, the current prevailing view is that MS can generally be considered autoimmune in origin. Indeed, several lines of evidence indicate myelin antigens are central to disease pathogenesis. Additionally, inflammation within the CNS can be adequately controlled with modern therapies at the RRMS stages.

1.5.1.5 THE INFLAMMATORY CASCADE IN MS

Although uncertainties remain regarding the specific T-lymphocyte target, MS is widely accepted to be an autoimmune-mediated T-lymphocyte driven disease predominating during the early stages of MS lesion formation. At present, the prevailing view implicates a more critical role for the Th17 cell. The experimental model, EAE, first elucidated a pathogenic role for Th17 cells in autoimmunity. Although precise pathogenic mechanisms in MS are still under rigorous interrogation, the cascade leading to EAE has been well studied and reviewed in great detail (Constantinescu et al., 2011) (Lassmann and Bradl, 2017) (McCarthy et al., 2012). Briefly, autoreactive CD4+ T-lymphocytes differentiate into pathogenic Th1 and Th17 and enter the CNS at the choroid plexus. In the subarachnoid and perivascular space, these T-lymphocytes interact with APCs resulting in expansion of autoreactive T-lymphocytes. Following this, mass production of pro-inflammatory cytokines by infiltrated immune cells ultimately leads to the destruction of BBB integrity, further inflammatory signalling cascades and eventual tissue damage. Transcriptome analysis recently identified shared gene signatures with myelin-specific CCR6+ T-lymphocytes from MS patients and EAE driven pathogenic Th17 cells (Cao et al., 2015) suggesting the above paradigm is to some extent applicable to the pathogenesis of MS. Today, it is widely accepted that myelin reactive T-lymphocytes play a key role in the pathogenesis of MS (Zozulya and Wiendl, 2008) (Hohlfeld and Wekerle, 2004) (Frohman et al., 2006) (Hafler et al., 2005).

1.5.1.6 LEUKOCYTE TRAFFICKING IN MS

Leukocyte trafficking across the BBB in MS has been well documented. An important number of studies have identified that mass infiltration of pro-inflammatory leukocytes into the CNS represent an early step in lesions development (Babbe et al., 2000) (Ransohoff et al., 2003) (Larochelle et al., 2011). It remains under debate whether disruption to the BBB precedes immune infiltration or is a consequence of leukocyte accumulation. A large proportion of work elucidating mechanisms for crossing the BBB during MS were first carried out using the animal model EAE and since confirmed in human disease. Adhesion molecules ICAM-1, VCAM-1 and E-selectin have found to be upregulated on activated brain micro vessels in active lesions of MS patient samples (Sobel et al., 1990) (Peterson et al., 2002), quantitative

immunohistochemical analysis of these adhesion molecules have since demonstrated that upregulation parallels disease onset (Doerck et al., 2010).

In addition, the expression of chemotactic agents released from CNS resident cells encourages blood circulating monocytes to leave the periphery and transform into macrophages at the site of injury (Cannella and Raine, 1995) (Dopp et al., 1994) (Steffen et al., 1994). Once transformed, these cells are known to exacerbate disease by providing a potent source of inflammatory cytokines. Although naïve T-lymphocytes cannot readily penetrate the BBB, those that have received activation signals are permitted entry, irrespective of their antigen specificity (Goverman, 2009). However, it is worth noting that only T-lymphocytes recognising CNS antigen will persist.

1.5.1.6.1 CD4+ involvement

A considerable amount of published literature pays special attention to the CD4+ populations and related cytokines that dominate tissues in active and chronic lesions in addition to CSF and serum samples from both MS patients and EAE models alike (Hafler et al., 1987) (Ota et al., 1990) (Zamvil et al., 1985) (Zhang et al., 1994). Discovery of T-lymphocytes in the CSF of MS patients provided the initial rationale to support an autoimmune hypothesis (Hafler et al., 1985) (Hafler et al., 1987). While HLA associations encoding functionally relevant proteins for initiating CD4+ cellular responses provided further evidence (Lincoln et al., 2005) (Oksenberg et al., 2004). Pathogenic T cells were first identified by experiments on animal model adoptive transfer in 1980s (Ben-Nun and Lando, 1983). From these studies, a strong and credible pathogenic link was made between MHC-II alleles and an increase propensity for developing EAE (Sawcer et al., 2011). Since, CD4+ T cells have long been recognised as key players in the pathogenic mechanisms of MS disease. Therefore, in MS, it was initially proposed that aberrant Th1 cell populations producing IFN γ were principally responsible for driving disease development. The ability to induce EAE by adoptive transfer techniques of CD4+ T cells, coupled with findings of elevated IFN γ in MS patients provided compelling evidence to support this notion (Mendel et al., 1995) (Madsen et al., 1999) (McRae et al., 1992). However, discovery of Th17 cells in EAE broke the traditional Th1/Th2 dichotomy, as these cells were found widespread throughout autoimmune diseases. Since this, Th17 cells have been described as the main pathogenic contributor to EAE (Langrish et al., 2005) (Park et al., 2005). Furthermore, Th17 have been observed in MS brain lesions (Tzartos et al., 2008).

Compared with classically recognised Th1 and Th2 CD4+ T-lymphocytes, the Th17 lineage represents an additional effector arm, characterised by distinct cytokine expression profiles including: IL-17, IL-21 and IL-22. The current assumption is that Th17 targeted pathogens are distinct from those implicated in Th1 and Th2 responses (Weaver et al., 2006) (Elyaman et al., 2009) (Bettelli et al., 2007) (Wilson et al., 2007). Whilst significant support exists for Th17 involvement, the role of Th1 cells has not been overlooked, but instead, speculated to work collaboratively to mediate MS disease (Pittock and

Lucchinetti, 2007) (Tzartos et al., 2008). Indeed, elevated levels of IL-17 have been identified in circulating leukocytes of MS patients with active disease, when compared to individuals with inactive disease and healthy controls (Durelli et al., 2009) (Ramgolam et al., 2009). Recently, elevated levels of IL-17 have been shown to correlate with MS disease severity (Matusevicius et al., 1999). Furthermore, IL-17 expression in active MS is associated with CNS infiltrating T-lymphocytes and glial cells (Tzartos et al., 2008). Important findings in EAE demonstrate IL-17 deficient mice present with delayed disease onset, decreased severity scores and decreased histopathological changes (Komiyama et al., 2006). Moreover, focused neuroinflammatory studies discovered a direct and sustained interaction between Th17 cells and induced neuronal dysfunction (Siffrin et al., 2010). A testament to the importance of IL-17 are the findings that, IL-17 neutralisation antibodies have found to attenuate disease in EAE.

1.5.1.6.2 CD8+ involvement

Much of the early immunopathological studies focused on the contributions CD4+ populations make towards the development of MS, however, since therapies successful in EAE failed to translate to MS in the clinic, current focus has paved the way for investigations into the role CD8+ play in pathogenesis (Salou et al., 2015). A broad range of evidence now suggests that the pathological role of this T-lymphocyte subset may have been grossly underestimated and now appear as potential major effector cells, especially when studied in human samples. Since CD8+ T-lymphocytes carry cytotoxic potential, this population is conceivably more likely to mediate CNS damage (Annibali et al., 2011) (Ifergan et al., 2011). Recent evidence points towards a more active role for CD8+ T-lymphocytes in the development of disease (Crawford et al., 2004) (York et al., 2010). (Salou et al., 2015) demonstrated that lesion infiltrating CD8+ T cells express granzyme B and IFN γ which provides potential mechanistic insight. Furthermore, *in vitro* BBB migration assays show increased migratory potential of MS T-lymphocytes producing IL-17, granzyme B and IFN γ . *In vivo* EAE studies later confirmed this finding (Ifergan et al., 2011) (Skulina et al., 2004). Single-cell analysis of infiltrating T-lymphocytes subpopulations extracted from MS patients show clonal expansion of the CD8+ population (Junker et al., 2007). Whilst CD8+ may behave as potent effectors of CNS damage, both CD4+ and CD8+ populations produce IL-17 at the same frequency in brain lesions, therefore, both subsets are likely to act synergistically to trigger and perpetuate disease (Friese and Fugger, 2005). Consequently, many research groups are currently working towards developing new therapeutic approaches targeting this previously ignored subpopulation (Sinha et al., 2014) (Friese and Fugger, 2005) (Traugott et al., 1983) (Machado-Santos et al., 2018).

1.5.1.7 IMMUNE CELL MIGRATORY MECHANISMS IN MS

A hallmark of MS is recruited blood-derived monocyte/macrophages into CNS tissues. In this autoimmune setting, microglia and monocytes/macrophages are considered crucial in promoting demyelination and axonal damage as they migrate to areas of damage (Lampron et al., 2013). In MS, imbalanced chemokine expression levels are implicated in disease pathogenesis, often, increased amounts are found on autopsy tissues from both active and chronic lesions (Simpson et al., 1998b) (McManus et al., 1998). Several sets of chemokine receptors and their ligands have been identified as key pathogenic players in orchestrating migratory processes. Expressed on both immune derived and CNS resident cells, these include: CCL21, CCL22, CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL11, CCL17, CCL19, CXCL1, CXCL8, CXCL9, CXCL10, CXCL11 and CXCL16. The current and ubiquitous application of chemokine immunomodulatory therapies for MS and EAE are a testament to their significant involvement in disease. Moreover, chemokine expression profiles are reportedly linked to disease activity in MS (Bartosik-Psujek and Stelmasiak, 2005) and in recent years evaluated as a biomarker to discriminate between RRMS and SPMS forms (Tejera-Alhambra et al., 2015). Table 1.2 has been designed to give an overview of cytokine expression profiles in MS.

1.5.1.7.1 CCL2/MCP-1

CCR2 is one of the most important receptors for recruitment of peripheral monocytes to lesion areas. The ligand for CCR2 binding is expressed on microglia, monocyte chemoattractant peptide-1 (MCP-1/CCL2), and has found to be increased in animal models of demyelination as well as in MS lesions (Berman et al., 1996) (Simpson et al., 1998b). In culture, bound CCL2 triggers the activation of chemotaxis. The presence of CCR2 is crucial for the development of acute EAE. Indeed, animal models lacking CCR2 on peripheral monocytes were unable to infiltrate CNS tissue. Monocyte infiltration in EAE was seen to correlated with disease progression (Ajami et al., 2011). Moreover, one study observed that CCL2 production by microglia and astrocytes is responsible for directing leukocytes to sites of axonal injury (Babcock et al., 2003).

The CCR2 receptor and its ligand CCL2 are likely to be essential in the pathogenesis of MS. In active MS lesions, CCR2 has been detected on activated microglia and macrophages and CCL2 chemokine by macrophages and astrocytes in chronic MS lesions. Moreover, the expression of CCR2 has found to be higher on T cells from SPMS patients when compared to RRMS. Further, CCL2 expression is lower in the serum and CSF sample of RRMS patients during relapse and in healthy controls. Moreover, CCL2-blocking antibodies have proven to protect against EAE (Youssef et al., 1999).

1.5.1.7.2 CCR6

CCR6 is implicated in the migration of CD4⁺ T helper subsets (Th1, Th17 and Th22) and accompanying signature cytokines (IFN γ , IL-17, IL-22, respectively). In MS, quantified expression levels revealed significant differences between the forms of MS, RRMS and SPMS. A considerable amount of research has focused on CCR6⁺ directed migrations of IL-17 Th producing cells, with identified mechanisms across the choroid plexus in EAE said to be a prerequisite for disease initiation (Reboldi et al., 2009). These findings have been supported by *in vitro* studies on human brain endothelial cell layers in MS brain tissue (Restorick et al., 2017). Recent studies concerned with subtypes of CCR6⁺ memory Th cells identified a distinct Th17 subset, Th17.1 (CCR6⁺, CXCR3⁺, CCR4⁻). These cells have found to be capable of infiltrating the CNS, both *in vitro* transmigration assays and *ex vivo* autopsied brain tissues. Th17.1 cells have cytotoxic potential and strongly co-express GM-CSF and IFN γ , suggesting they may also be involved in disrupting BBB permeability in MS. However, IL-17 KO mice still develop disease, albeit a much milder form. Similarly, CCR6 KO mice develop a less severe form of EAE (Liston et al., 2009) (Moriguchi et al., 2013)

1.5.1.7.3 CCR5

CCL5, commonly referred to as RANTES, is often used as a marker for M1-polarised macrophages (Shukaliak and Dorovini-Zis, 2000). Mainly expressed by astrocytes and vascular endothelial cells, CCL5 has been detected in active demyelinated MS lesions as a strong monocyte chemoattractant (Park et al., 2009) (Boven et al., 2000). The homing receptor for this chemokine, CCR5, can mostly be found on circulating monocytes and dendritic cells (Subileau et al., 2009). Further, the CCR5 receptor has recently been utilised for its role as a marker of MS severity (Pittaluga, 2017) (Conti and DiGiacchino, 2001).

Chemokine	Chemokine Receptor	Target Cells	CSF	Blood	Brain Lesion	References
CCL2/MCP-1	CCR1; CCR2	Monocytes, T Lymphocytes, NK cells, B cells, dendritic cells	Decreased	Decreased	Expressed by astrocytes and macrophages within acute and chronic MS lesions	(Sørensen et al., 2001) (Simpson et al., 1998a) (McManus et al., 1998) (Sindern et al., 2001) (Bartosik-Psujek and Stelmasiak, 2005) (Van Der Voorn et al., 1999) (Narikawa et al., 2004) (Scarpini et al., 2002)
CCL3/MIP1-a	CCR1; CCR5	Monocytes, T and B lymphocytes, Neutrophils, eosinophils	Increased	-	Expressed by macrophages and microglia, detected in actively demyelinating plaques	(Balashov et al., 1999) (Miyagishi et al., 1995) (Simpson et al., 1998a)
CCL4/MIP1-b	CCR5	CD8+ lymphocytes	-	-	Expressed by macrophages and microglia, detected in actively demyelinating plaques	(Simpson et al., 1998a)
CCL5/RANTES	CCR1; CCR3; CCR5	T lymphocytes, monocytes, eosinophils, dendritic cells	Increased	Increased	On macrophages and microglia, detected in actively demyelinating plaques; expressed by perivascular cells	(Simpson et al., 1998a) (Martinez-Caceres et al., 2002) (Sørensen et al., 1999) (Sindern et al., 2001) (Bartosik-Psujek and Stelmasiak, 2005)
CCL7/MCP-3	CCR1; CCR2; CCR3; CCR5	Monocytes, T and B lymphocytes, NK, eosinophils and dendritic cells	-	-	On astrocytes and inflammatory cells, expressed within acute and chronic MS lesions	(Simpson et al., 1998a) (McManus et al., 1998)
CCL8/MCP-2	CCR1; CCR2; CCR3; CCR5	Monocytes, T lymphocytes, eosinophils and dendritic cells	-	-	On astrocytes and inflammatory cells, expressed within acute and chronic MS lesions	(Simpson et al., 1998a) (McManus et al., 1998)
CCL17/TARC	CCR4	T lymphocytes	Increased	Decreased		(Narikawa et al., 2004)
CCL19/ELC	CCR7	Dendritic cells, T lymphocytes and activated B cells	Increased	-		(Pashenkov et al., 2003) (Kivisäkk et al., 2004)

CCL21/SLC	CCR7	Dendritic cells, T lymphocytes, activated B cells, NK cells	Increased	-		(Pashenkov et al., 2003) (Kivisäkk et al., 2004)
CXCL1/GRO-a	CXCR2	Neutrophils, monocytes			Detected at high levels on hypertrophic astrocytes around active MS lesions	(Omari et al., 2005)
CXCL8/IL-8	CXCR1; CXCR2	Neutrophils, monocytes	Increased	Increased	Detected at high levels on hypertrophic astrocytes around active MS lesions	(Bartosik-Psujek and Stelmasiak, 2005) (Omari et al., 2005) (Bartosik-Psujek et al., 2004) (Lund et al., 2004)
CXCL9/MIG	CXCR3	T lymphocytes, NK cells	Increased		Expressed by astrocytes in actively demyelinating lesions	(Sørensen et al., 1999) (Simpson et al., 2000)
CXCL10/IP-10	CXCR3	T lymphocytes, NK cells				(Sørensen et al., 2001) (Sørensen et al., 1999) Simpson et al., 2000) (Balashov et al., 1999) (Franciotta et al., 2001) (Scarpini et al., 2002) (Martinez-Caceres et al., 2002)
CXCL12/SDF-1	CXCR4	T and B lymphocytes, monocytes, plasma cells, dendritic cells	Increased		Expressed on astrocytes and blood vessels in both active and chronic inactive MS lesions	(Krumbholz et al., 2006)
CXCL13/BCA-1	CXCR5	T and B lymphocytes, monocytes and dendritic cells	Increased	-	Expressed within perivascular infiltrates in actively demyelinating lesions	(Krumbholz et al., 2006)
CX3CL-1/fractalkine	CX3CR1	Monocytes, T lymphocytes, NK cells and microglia	Increased	Increased		(Kastenbauer et al., 2003)

Table 1.2 Overview of chemokine profile expression in MS. MCP, monocyte chemoattractant protein; MIP1, macrophage inflammatory protein; RANTES, Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted; TARC, thymus- and activation-regulated chemokine; ELC, Epstein-Barr virus-induced-molecule-1-ligand-chemokine; SLC, Secondary lymphoid tissue chemokine; GRO, growth-related oncogene; IL, Interleukin; MIG, monokine induced by interferon- γ ; IP, Interferon-gamma-inducible protein; SDF, stromal cell-derived factor; BCA, B cell-attracting chemokine. Table taken from (Szczeniński and Losy, 2007)

1.5.1.8 KEY CYTOKINES INVOLVED IN THE PATHOGENESIS OF MS

Cytokines are critically involved in several pathogenic mechanisms associated with the clinical course of MS. The plethora of therapies for manipulation of cytokine availability and/or signalling demonstrates that targeting these pathways is an attractive strategy for MS treatment.

For decades, clinical studies have presented strong evidence for striking differences in cytokine quantities and expression profiles at the different stages of MS disease. Progressive MS when compared with RRMS forms, for example, exhibit increased levels of IL-1 β and IFN γ coupled with decreased levels of IL-12 and IL-4. Similarly, patients with progressive forms show higher levels of IFN γ , IL-10 and TNF α than healthy individuals. Moreover, inverse correlations have been observed between IL-10 and EDSS scores in progressive MS patients. Such findings underscore the complexity and interplay of cytokine networking cascades in addition to the imbalances present during progressive MS (Kallaur et al., 2017). Table 1.2 summaries several prototypical cytokines and their roles in both MS and EAE.

Cytokine	Main Producers	Levels in MS patients	Role in EAE	Potential treatments of MS
GM-CSF	T cells	Elevated	GM-CSF-deficient mice are completely resistant to EAE (McQualter et al., 2001)	Phase 1b trial of humanized anti-GM-CSF mAb MOR103 in MS is completed (Constantinescu et al., 2015)
IFN- β	pDCs	Not reported	<i>Ifnb^{-/-}</i> mice exhibit increased EAE severity (Teige et al., 2003)	First line treatment of RRMS (Limmroth et al., 2011)
IFN- γ	Th1 cells, NK cells, NKT cells	Elevated	<i>Ifng^{-/-}</i> mice exhibit increased EAE severity (Ferber et al., 1996)	Intravenous infusion of IFN- γ exacerbates disease in MS patients (Panitch et al., 1987)
IL-1 β	Monocytes, macrophages	Elevated	<i>Il1r1^{-/-}</i> mice are resistant to EAE (Sutton et al., 2006)	Not reported
IL-10	Tregs, macrophages, DCs, B cells	Reduced	<i>Il10^{-/-}</i> mice exhibit increased EAE severity (Bettelli et al., 1998)	Not reported
IL-12	DCs, macrophages	Elevated	IL-12 <i>p35^{-/-}</i> exhibit increased EAE severity (Becher et al., 2002)	Anti-IL-12/IL-23 p40 mAb Ustekinumab does not show efficacy in treating RRMS in phase II trial (Segal et al., 2008)
IL-17	Th17 cells, $\gamma\delta$ T cells, NKT cells	Elevated	<i>Il17a^{-/-}</i> mice are partially resistant to EAE (Komiyama et al., 2006)	Anti-17A mAb Secukinumab reduces disease severity in RRMS patients (Havrdová et al., 2016)
IL-23	DCs, macrophages	Elevated	<i>Il23r^{-/-}</i> mice are completely resistant to EAE (Cua et al., 2003)	Anti-IL-12/IL-23 p40 mAb Ustekinumab does not show efficacy in treating RRMS in phase II trial (Segal et al., 2008)
TNF- α	Macrophages	Elevated	<i>Tnfrsf1a^{-/-}</i> mice are partially resistant to EAE (Wheeler et al., 2006)	Treatment of MS patients with anti-TNF- α exacerbates disease in MS patients (Titelbaum et al., 2005)

Table 1.3. Cytokine expression profiles in EAE and MS. GM-CSF, Granulocyte-macrophage colony-stimulating factor; EAE, Experimental autoimmune encephalomyelitis; mAb MOR103, human monoclonal antibody to granulocyte-macrophage colony-stimulating factor; IFN- β , Interferon-beta; RRMS, relapsing-remitting multiple sclerosis; IFN- γ , interferon-gamma. IL-, Interleukin; TNF- α , tumour necrosis factor-alpha. Table adapted from (Palle et al., 2017)

1.5.1.9 MS PATHOLOGY

MS is typically described as a multifactorial, autoimmune-driven chronic demyelinating and neurodegenerative disorder with several components proposed to contribute to the pathophysiology. These include inflammatory and neurodegenerative factors as well as aberrant vascular and redox signalling (Lassmann, 2014) (Lassmann et al., 1991) (Lucchinetti et al., 1996) (Gilgun-Sherki et al., 2004). Despite the impressive pace by which data on MS has accumulated in recent years, the aetiopathogenesis remains largely unknown and pathological mechanisms elusive. Although clinical manifestations of MS are heterogeneous, they are primarily thought to be a consequence of aberrant electrical properties of axons due to the processes of demyelination. Much of our present-day understanding derives from early animal studies with many mechanistic similarities described in both acute MS lesions and EAE alike.

The clinical variability of MS has been documented in many pathological studies and the spectrum of clinical phenotypes correlate well with differences in cellular and inflammatory responses (De Stefano et al., 1998) (Furby et al., 2008) (Nijeholt et al., 1998). Inflammation is present at all stages of MS, however, the degree to which it exists, and composition, both differ between the different types of MS lesion. Classically, inflammation is more pronounced in RRMS (Frischer et al., 2009) (Lassmann, 2013). MRI examination of new MS lesions indicates BBB damage and gadolinium-enhancement are driving forces behind these observations which become rare when patients enter the progressive stage (Grossman et al., 1988) (Miller et al., 1993). By direct comparison, at the later phases of SPMS, inflammation largely subsides and tissue pathology instead consists of aggregated microglia and blood monocytes (Lassmann et al., 1994) (Ozawa et al., 1994) (Kouwenhoven et al., 2001). The extent to which inflammatory activity exists in the early stages, varies between patients and over time; a phenomenon that largely dictates the spectrum of MS phenotypes.

At the tissue level, MS is characterised by perivascular inflammation and focal destruction of myelin. It is considered a heterogeneous disease involving different patterns and mechanisms of injury (Lucchinetti et al., 1996) (Lassmann et al., 2007) (Lassmann et al., 2001). Typically, MS lesions feature BBB breakdown, multifocal inflammation, reactive gliosis, demyelination, and oligodendrocyte loss. Over time, a continuation of inflammatory “attacks” leads to well-demarcated areas of myelin loss and subsequent formation of astrocytic scars, distinguishable between the relapsing and progressive phases (Lassmann, 2014) (Lassmann et al., 2012) (Thompson et al., 1991) (Huijbregts et al., 2004) (Ge et al., 2000). As the disease progresses, demyelination and inflammation can lead to irreversible axonal destruction and eventual degeneration (Noseworthy et al., 2000). Notably, severe demyelination often leads to functional changes detrimental to axons, such as swelling and transection (Trapp et al., 1998) (Nikić et al., 2011). It is widely accepted that myelin loss leads to impaired axonal function, due to this, disruptions to proper neurological function have been attributed to demyelination (McDonald and Sears, 1970) (Waxman, 1977) (Waxman et al., 1980) (Dziedzic et al., 2010) (Bitsch et al., 2000) (Haines et al., 2011). Indeed, the irreversible

disability observed in progressive MS patients is believed to derive from neuronal death and tissue atrophy (Noseworthy et al., 2000) (Friese et al., 2014).

MS lesions can develop throughout the CNS, however, there is a predilection for optic nerves, periventricular white matter tissue, cerebellum, brain stem and spinal cord white matter (Noseworthy et al., 2000) (Popescu and Lucchinetti, 2012) (Calabrese et al., 2010) (Calabresi et al., 2010). Depending on the anatomical location of the lesion, they can give rise to a variety of neurological signs and symptoms. Lesion development within the optic nerves for instance, would lead to visual problems (Hauser and Oksenberg, 2006).

MS was traditionally considered a white matter phenomenon; however, in recent years, it has become increasingly clear that grey matter pathology is an important aspect of this disease (Calabrese et al., 2010) (Geurts and Barkhof, 2008) (Pirko et al., 2007) (Bö et al., 2006). Evidence of grey matter damage has been described during the early stages of RRMS and PPMS through to late stage SPMS, with observations noted more frequently in the latter (Kutzelnigg et al., 2005) (Fisniku et al., 2008) (Geurts and Barkhof, 2008). Different molecular mechanisms are known to drive damage in different regions of the same MS lesion, making unpicking the sequence of events that initiate tissue damage difficult to understand.

1.5.1.10 LESION PATHOLOGY

Based on observed immunological differences amongst patients, (Lucchinetti et al., 1996) classified active MS lesions into four subtypes: patterns I-IV (Lucchinetti et al., 2000). Key differences in lesion pathology were identified between patients, whilst lesions in a given individual were found to conform to one pattern. Lassmann et al., (2001) described the major differences based on morphological characteristics and mechanisms of tissue injury.

1.5.1.10.1 Slowly expanding lesions of SPMS

Progressive MS is characterised by slowly expanding lesions (Popescu and Lucchinetti, 2012) (Elliott et al., 2019). Classic features of this type include microglia clustering at the lesion edge with T-lymphocytes being infrequent and mostly restricted in the perivascular region. BBB damage is no longer evident and ongoing myelin destruction and atrophy occurs in the absence of overt inflammation (Hochmeister et al., 2006). Moreover, activated microglia and complement deposition are found in close concert with myelin damage and are major features of progressive lesions (Jäckle et al., 2020). The concept of silent progression is consistent with an underlying degenerative disease process, which proceeds largely independently of autoimmune inflammation. Progressive MS also presents with major histological features of grey matter demyelination in cerebellar cortex and deep grey matter nuclei (Cree et al., 2019). It has been postulated that inflammatory infiltrates become “trapped” behind an intact barrier and perpetuate disease, rather than peripheral cells driving the expansion of lesions in progressive MS.

1.5.1.10.2 Optic neuritis in MS

Recent post-mortem studies of MS patients reported extensive neurodegeneration of the ganglion and inner nuclear cell layers and the retina that correspond to severe localised inflammatory clusters (Green et al., 2010). While mechanisms underlying these pathological changes remain unresolved, activated microglia have emerged as a likely candidate. Indeed, this cell type plays an important role in a diverse number of retinal pathologies with degenerative and chronic immune reactions as key pathophysiological components (Rashid et al., 2019). Evidence for microgliosis observed in optic neuritis is suspected to play a role in neurodegeneration while therapies aimed at reducing microglia activation have shown promising results (Fairless et al., 2012)(Bosco et al., 2008).

1.5.2 Posterior uveitis

1.5.2.1 CLINICAL OVERVIEW

Uveitis is a general term used to describe intraocular inflammation and encompasses a wide variety of disorders of the uveal tract composed of the ciliary body, iris, and choroid. Uveitis can be classified according to the anatomically affected areas as anterior, intermediate, and posterior, in addition to panuveitis, which encompasses all areas. Posterior uveitis is believed to be autoimmune in origin and driven by aberrant immune recognition to self. If untreated, uveitis can result in significant visual deficit and blindness which can individually account for 5-20% of blindness in developed countries and 25% in developing countries (Gritz and Wong, 2004). Clinical manifestations of this disease include redness, pain, photophobia, and decreased vision. Further, inflammation typically presents in the retina, choroid or blood vessels as retinitis, choroiditis, or optic neuritis. Posterior uveitis is often associated with several other conditions such as Behcet's disease, toxoplasmosis, sarcoidosis and MS. In most cases, the aetiology of inflammation in uveitis is non-infectious and idiopathic. It has been known for some time that activated T-lymphocytes play an integral immunopathogenic role in the development of disease as evidenced by mass CD4+ T-lymphocyte infiltration and the upregulation of HLA-DR on ocular tissues (Deschenes et al., 1988) (Charteris and Lee, 1990). Indeed, immunopathologic analysis shows that CD4+ T-lymphocytes are the major infiltrating cell in the immediate perivascular area and blood vessel walls. A proportion of these are activated and express IL-2 receptor. By comparison to CD4+ cells, CD8+ T-lymphocytes are relatively rare (Charteris et al., 1992). Patients frequently demonstrate immune responses targeted to ocular antigens such as retinal soluble antigen (S-Ag), inter-photoreceptor retinoid-binding protein (IRBP) and recoverin (Gery et al., 1986).

1.5.2.2 EXPERIMENTAL AUTOIMMUNE UVEITIS

Experimental autoimmune uveitis (EAU) is the animal model equivalent of human posterior uveitis and is used for studying T-lymphocyte mediated, organ-specific autoimmune neuroinflammatory diseases. Our current understanding of the immune mechanisms involved in non-infectious uveitis largely derive from EAU studies. Induced by immunisation with retinal antigens this model was originally developed in guinea pigs and later translated to mice in 1988 through the use of retinal soluble antigen (SAg) and interphotoreceptor retinoid-binding protein (IRBP) (Caspi et al., 1988) (Caspi et al., 1990) (Forrester et al., 1992) (Agarwal and Caspi, 2004). IRBP is the selected antigen used for experimental investigation in this thesis. A 148-kDa protein located within the inter-photoreceptor matrix, the primary role of this protein is transportation of vitamin A derivatives between the retinal pigment epithelium RPE and photoreceptor layer (Borst et al., 1989). Following subcutaneous immunisation of this retinal antigen, animals typically develop uveitis within 2 weeks and the evolving disease targets neural retina and related tissues. The main clinical features of EAU are persistent vasculitis and retinal inflammation which if left to progress, lead to photoreceptor destruction and loss of visual function (Chan et al., 1990).

While no animal model can recapitulate the entire spectrum of human uveitis, the rodent form closely mimics classical hallmarks and has been characterised extensively. Accordingly, a number of non-invasive clinicopathological grading systems have become commonplace for assessing disease severity (Copland et al., 2008) (Agarwal and Caspi, 2004) (Xu et al., 2008) (Harimoto et al., 2014). Over the years, this model has served as an invaluable tool to help researchers understand the basic molecular mechanisms of tolerance and autoimmunity. Moreover, since the FDA approval of several immunotherapies developed using this model, EAU became a desirable method for evaluating novel agents (Nussenblatt, 2002) (Dick et al., 2004) (Sharma et al., 2009) (Whitcup et al., 1993b) (Whitcup et al., 1993a) (Abdelhak et al., 2017) (Martín et al., 2005).

1.5.2.3 PATHOGENESIS OF EAU

It is likely that EAU possess many pathological similarities to human uveitis. The importance of Th1 subsets with self-reactivity has long been implicated as a critical feature of this disease. Of late, as with MS, Th17 autoreactive cells have also received much attention for their pathogenic roles in disease initiation and progression. This T cell subtype has a specific molecular signature and can be identified by the expression of ROR γ t and CCR6 (Pène et al., 2008). Proinflammatory cytokine products secreted from the Th1 and Th17 subset, IFN γ and IL-17, support disease pathogenesis. Early steps in EAU lead to a breach in the BRB which later permits mass infiltration of CD4+ T lymphocytes and monocytes moving into the retina via the retinal endothelium and RPE (Liversidge et al., 1990). The underlying mechanisms facilitating this movement is thought to operate in a LFA-1 and ICAM-1 dependent manner (Greenwood et al., 2003a) (Greenwood and Calder, 1993) (Greenwood et al., 1994) (Greenwood et al., 1995) (Mesri et al., 1996).

Upon arrival, these immune infiltrates cause extensive tissue damage (Dick et al., 1996) (Liversidge and Forrester, 1988). In particular, monocyte recruitment is necessary for full disease expression. While endothelial cells activated by local cytokines IL-1 β and TNF α support movement across the BRB, the chemokine receptors CCR2 and CX3CR1 have been shown as integral to leukocyte recruitment (Xu et al., 2005) (Dagkalis et al., 2009).

1.6 Generation of reactive species in the CNS

1.6.1 Redox biology

Biological tissues within aerobic multicellular organisms require molecular oxygen to meet their energetic demands. A normal physiological outcome of these metabolic processes is the production of free radical formation. Free radicals can be defined as short-lived, highly reactive, unstable atoms or molecules with unpaired electrons. A basic chemical notion that is redox biology involves the oxidation-reduction reaction, including electron transfer, free radical formation, and oxygen metabolite production. These processes are tightly regulated throughout the body for the maintenance of a balanced homeostatic state (Ursini et al., 2016). ROS and RNS are routinely generated by a variety of tissues and play critical roles in cell growth, immunity, and cell signalling. ROS collectively refers to oxygen-derived free radicals, such as superoxide (O₂⁻), hydrogen peroxide (H₂O₂), hydroxyl (•OH) and peroxy (RO₂•). Similarly, nitrogen-containing oxidants, such as peroxynitrite (ONOO), nitrogen dioxide (NO₂) and nitric oxide (NO) are commonly referred to as RNS (Gabbita et al., 2000). Although both species are highly reactive and can be inherently toxic, in low or moderate amounts they are considered essential for neuronal development and function, posing very little threat to their environment (Ray et al., 2012). In excess, however, ROS and RNS are lethal to cells and dysregulated overproduction can cause irreversible modifications to biologically relevant macromolecules, such as lipids, proteins, carbohydrates, and DNA. These irreversible alterations often lead to the inhibition of normal cellular functions and promotion of apoptosis via intracellular and extracellular signalling pathways (Benedetti et al., 1980) (Ichihashi et al., 2001) (Berlett and Stadtman, 1997) (Halliwell, 1999) (Dean et al., 1997).

1.6.2 Oxidative stress

The phenomenon commonly referred to as oxidative stress describes a situation whereby ROS and/or RNS production exceeds the capacity of endogenous enzymatic and non-enzymatic antioxidant defence mechanisms. The imbalance between free radical production and inherent antioxidant defence is often described as self-perpetuating. Observations to support this notion were formed on the basis that oxidative stress-induced excessive ROS release triggers cellular damage to aforementioned macromolecules which themselves may behave or become ROS. Indeed, a number of studies have identified unregulated

production of RNS and ROS species as casual factors of several pathologies including, but not limited to, cancer (Diehn et al., 2009) (Waris and Ahsan, 2006) (Liou and Storz, 2010), rheumatoid arthritis (Mateen et al., 2017), cardiovascular disease (Sugamura and Keane Jr, 2011), neuroinflammatory and in particular neurodegenerative disorders (Akbar et al., 2016). The CNS is particularly susceptible to oxidative stress due to modest antioxidant defences, elevated production of ROS and RNS and limited capacity for regeneration. Accordingly, oxidative stress is perceived to be a major driving force for tissue injury in chronic inflammatory, vascular and neurodegenerative disorders of the CNS (Lassmann and van Horssen, 2016).

1.6.3 Mechanisms of microglia-induced reactive species

Amongst the endogenous sources of ROS and RNS, activated microglia represent major contributors due to their high rates of oxygen consumption. While surveillance microglia display relatively low levels of ROS and RNS, during M1 conversion, free radical production is dramatically elevated owing to the activation of NADPH (NOX) and nitric oxide synthase (iNOS) (Qin et al., 2004) (Saha and Pahan, 2006).

1.6.3.1 NADPH OXIDASE DERIVED ROS PRODUCTION

Key producers of ROS in microglia are the NOX family of NADPH oxidases, which comprise several members. Embedded in the cell membrane, NADPH oxidase is a multi-protein electron transport system that constitutes cytosolic and membrane components. The subunits localised to the membrane include p22phox and gp91phox, while p40phox, p47phox and p67phox are found in the cytoplasm. NOX activation requires assembly of multiple subunits and with support from small G proteins (Rac1 and Rac2), phosphorylation of p47 initiates migration of the cytosolic elements to the plasma membrane for activation. For the purpose of these set of experiments described in this thesis, it is important to emphasise that activation of NADPH oxidase is controlled by Rho GTPases, and specifically Rac2 has shown to regulate this (Diebold and Bokoch, 2001) (DerMardirossian et al., 2004). Both human and rodent microglia alike, express high levels of NADPH oxidase, in particular NOX2 (also referred to as gp91phox) (Sorce et al., 2014).

The biological function and sole purpose of NOX is to generate superoxide radicals via the catalysed reduction of molecular oxygen. Superoxide has known roles in several physiological functions ranging from gene regulation, cellular signalling and innate defence. Careful regulation of NADPH oxidase is critical to maintaining homeostasis and healthy levels of intracellular ROS. Under physiological conditions, NADPH oxidase lies dormant and inactive but quickly becomes activated in response to several cytokine or bacteria-related stimuli (Bedard and Krause, 2007) (Qin et al., 2004) (Shahraz et al., 2021). Superoxide has important roles in CNS host defence, while redox signalling has also proven to shape different activation microglia phenotypes (Nayernia et al., 2014) (Lambeth and Neish, 2014).

The primary role of NOX in microglia is to support the process of phagocytosis for degradation of internalised material (Shahraz et al., 2021). The term respiratory or oxidative burst describes a scenario where reactive oxygen species, hydrogen peroxide and superoxide radical are rapidly released from the cell, often as a direct immunological response to infection and/or injury. This vital process in the innate defence system can be carried out by myeloid and lymphoid cells (Slauch, 2011).

1.6.3.2 EVIDENCE OF NOX ACTIVITY IN MS

Many members of the NOX family have been identified in active brain lesions of rodent EAE studies and MS patient samples alike (Kandagaddala et al., 2012b) (Kandagaddala et al., 2012a) (Di Filippo et al., 2016). This family of enzymes are primarily found in the progressive forms of MS and are regionally associated with chronically activated lesions (Fischer et al., 2012) (van Horsen et al., 2012). While activated p47phox has shown to promote EAE, (van der Veen et al., 2000) demonstrated that genetic deletion or pharmacological inhibition of this functional subunit leads to a phenotypic switch from M1 to M2 (Liu et al., 2006) (Becanovic et al., 2006) and a significant reduction of the pro-inflammatory factors TNF α , CCR2 and CCL2.

1.6.3.3 INOS DERIVED RNS PRODUCTION

Nitric oxide is a small, free radical, short-lived intermediate. Depending on the levels of NO present, this molecule may act on the one hand as a master physiological regulator, or on the other as capable of inducing devastating cellular effects. This paradoxical phenomenon exhibits both cytoprotective and cytotoxic properties in different contexts. At picomolar concentrations NO behaves as a signalling molecule and plays significant roles in several aspects of CNS physiology, including neurological transmission, vasodilation, immunity and cell death regulation (Calabrese et al., 2007). In contrast, the persistent presence of micromolar NO has proven to be pathological and linked to brain dysfunction.

NO is synthesised by the enzyme nitric oxide synthase (NOS) and diffuses from its site of origin, across the cell membrane to its target. Additionally, NO can be released locally in bursts. Here, NO forms noncovalent and covalent linkages with nonprotein and protein targets to elicit biological effects. S-nitrosylation is the process whereby NO covalently links to protein targets causing a change in function. A hallmark of microglial cell activation is the elevated production of NO that may contribute to neuronal death by inhibition of mitochondrial respiratory function, inhibition of mitochondrial cytochrome oxidase and the release of exocytotoxic concentrations of glutamate via N-methyl-D-aspartate receptor (Brown and Cooper, 1994) (Stewart et al., 2002) (Ghasemi and Fatemi, 2014).

Microglia NO is mostly synthesised from L-Arginine, the rate limiting step, by the enzyme inducible nitric oxide synthase (iNOS). Unlike other isoforms of NOS (eNOS and nNOS), iNOS functions independently from intracellular Ca²⁺ levels and is transcriptionally induced by several proinflammatory

mediators such as $\text{TNF}\alpha$, $\text{IFN}\gamma$ and bacterial LPS (Possel et al., 2000). Hence, microglia upregulation of iNOS correlates with the acquisition of an M1 macrophage-like phenotype and is utilised as a potent immune defence mechanism for the effective removal of microorganisms. Induction of iNOS is dependent on $\text{NF}\kappa\text{B}$ signalling which in turn, supports inflammation-mediated stimulation of the transcript. Once produced, soluble iNOS collects in the cell's cytoplasm and serves as a hub of NO (Dell'Albani et al., 2001). Structurally, iNOS is unusual from other isoforms in that it requires five cofactors to function: flavin mononucleotide (FMN), flavin adenine, haeme, calmodulin, tetrahydrobiopterin (BH_4) and flavin adenine dinucleotide (FAD) (Crane et al., 1998) (Förstermann et al., 1994).

1.6.3.4 EVIDENCE OF INOS ACTIVITY IN MS

In recent years, the role of NO in MS has been studied in great detail. NO has been identified in higher-than-normal concentrations within inflammatory lesions and CSF of active MS patients (Giovannoni, 1998). Tissue elevated NO levels are purportedly due to the presence of iNOS generated by cells with phagocytic capacity. While it is generally understood that iNOS plays both a pathogenic and regulatory role in biological systems, in MS and EAE alike, clinical associations have been documented between this enzyme and tissue pathology (Danilov et al., 2003) (Cross et al., 1997) (Okuda et al., 1995). In rats, direct exposure to NO reportedly induces irreversible conduction blockade in axons, a mechanism believed to perpetuate spinal cord degeneration (Smith et al., 2001). Moreover, high concentrations of NO, peroxynitrite and other reactive nitrogen species have found to correlate with greater severity and chances of clinical relapse (Calabrese et al., 2002a) (Okuda et al., 1995). Identified mechanisms underpinning this process involves T-lymphocyte driven $\text{IFN}\gamma$ induced iNOS expression in macrophages and microglia which are capable of generating high levels of peroxynitrite and NO productions documented to cause tissue destruction in the CNS (Danilov et al., 2003). Indeed, iNOS inhibition at defined stages of EAE have proven to be clinically beneficial (Cross et al., 2000). Similarly, RNS and ROS scavengers are capable of attenuating focal axonal degeneration, without altering the number of immune cells in EAE lesions.

1.6.3.5 CROSSTALK BETWEEN ROS AND RNS: THE FORMATION OF PEROXYNITRITE

There are several mechanisms that can lead to neurotoxicity, sustained elevated levels of NO are one. However, *in vivo* it is likely NO will react with O_2 generated via NOX to produce peroxynitrite (ONOO^-), which is potentially more toxic than either NO or O_2 alone (Bal-Price et al., 2002) (Beckman and Crow, 1993). Indeed, *in vivo* studies focused on Alzheimer's models regard interplay between NOX and iNOS to produce O_2 , NO and ONOO^- as essential for neurodegeneration (Mander and Brown, 2005) (Barua et al., 2019). Peroxynitrite initiates DNA strand breaks and subsequently activates poly (ADP-ribose) synthetase. Poly ADP synthase is known to deplete local ATP stores to repair damaged DNA. This process is extremely metabolically expensive, therefore, often results in cell death. Additionally, peroxynitrite

is believed to cause axonal damage, characteristics of which are similar to acute axonopathy (Smith et al., 1999) (Touil et al., 2001). Moreover, NO can react with nitrogen trioxide (N₂O₃) and molecular oxygen to nitrosylated amines and thiols. Thus, NO has the capacity to actively imbalance redox homeostasis due to its reactivity with ROS (Mander and Brown, 2005) (Bal-Price et al., 2002) (Li et al., 2005) (Cross et al., 1997).

1.6.4 Oxidative stress detection in MS patients

Free radicals and their products can damage biological molecules including carbohydrates, lipids, proteins, and nucleic acids. The new compounds formed as a consequence of these reactions have been detected and quantified in MS patients. Biomarkers of oxidative stress detectable in serum, erythrocytes, CSF, saliva, and urine are collectively presented in Table 1.4.

Type of Damage	Detection Product	Evidence in MS
Lipid	MDA	(Bö et al., 1994) (Plemel et al., 2015) (Haider et al., 2011) (Wang et al., 2014)
Protein	Nitrosylation	(Hooper et al., 1997) (Brundin et al., 1999) (Calabrese et al., 2002b) (Bizzozero et al., 2005)
DNA/RNA	8-OHdG	(Haider et al., 2011) (Tasset et al., 2012) (Ljubisavljevic et al., 2016) (Kharel et al., 2016)

Table 1.4. Biomarkers of oxidative stress detected in MS patients. MDA, malondialdehyde; 8-OHdG, 8-Hydroxy 2-deoxy Guanosine. Adapted from (Adamczyk and Adamczyk-Sowa, 2016)

1.7 The pharmacological action of statins on immune responses

1.7.1 HMG-CoA reductase inhibitors (statins)

HMG-CoA (3-hydroxy-3-methyl-glutaryl-coenzyme A) reductase inhibitors, generically referred to as statins, belong to the class of cholesterol-lowering agents that transformed cardiovascular medicine. Their application to cardiovascular disease led to a remarkable decline in cardiovascular death and disability in patients with, or at risk of developing coronary heart disease (CHD) (Chou et al., 2016) (Mills et al., 2010). Originating from fungi, statins exert their biological effect upstream of the cholesterol synthesis pathway through competitive inhibition of the rate-limiting enzyme HMG-CoA reductase. The consequent reduction in mevalonate, a precursor to cholesterol, lowers circulating LDL levels, providing the original rationale for therapeutic use (Brown et al., 1978) (Endo et al., 1976) (Endo et al., 1977) (KANeko et al., 1978). Indeed, hypercholesterolaemia is one of the main risk factors for developing atherosclerosis. Today, statins are amongst the most prescribed drugs in the UK with proven excellent safety and efficacy profiles. A testament to the drugs success and use as a reliable mainstay treatment are the UK's National Institute of Clinical Excellence (NICE) recently updated guidelines on widening inclusion criteria for patient prescriptions. Relevant to this thesis, is the fact that this family of drugs are now off patent and low cost, making them desirable candidate agents for repurposing.

1.7.2 Pharmacological properties of HMG-CoA reductase inhibitors

Lovastatin was the first statin to receive FDA approval for commercial availability in 1987. Outstanding results were achieved in the prevention of acute cardiovascular events and reduced mortality rates in ischemic heart disease upon introduction. The great success of lovastatin inspired intensive pharmaceutical development. Research groups in both academia and industry ran in parallel to produce lovastatin analogues, synthetic and semi-synthetic derivatives alike. The products generated from such a revolution gave rise to a heterogeneous class of drugs (Endo, 2008) (Endo et al., 1976). Simvastatin was the first semi-synthetic lovastatin derivative to launch and the second to receive clinical approval; all other approved HMG-CoA reductase inhibitors are completely synthetic (Shepherd et al., 1995). Aside from sharing the same mode of action, simvastatin and lovastatin differ in a number of ways (Vega et al., 1991) (Alberts, 1990). The tailored pharmacokinetic properties absent from naturally isolated lovastatin gives rise to a plethora of pharmacological changes according to chemical structure, polarity, half-life and potency which in turn, governs absorption, distribution, metabolism and excretion (Ward et al., 2019) (Fong, 2014) (Schachter, 2005). However, these two compounds share one crucially important property, the ability to readily cross the BBB. Since the BBB and BRB demonstrate similar transport and permeation

characteristics, these compounds are perceived as ideal candidates for explorative CNS disease therapy. Irrespective of their structural and biokinetic differences, all statins competitively displace HMG-CoA at nanomolar concentrations. Considering their different pharmacokinetic properties, it is unsurprising that the degree of inhibition and potency can vary considerably. Indeed, such stark differences in pharmacokinetic and pharmacodynamic properties highlights the importance and necessity for rigorous investigation, particularly when seeking to repurpose agents within this family (Table 1.5) (Brown and Goldstein, 1986) (Dietschy and Wilson, 1970).

Name	IC ₅₀ HMG-CoA reductase (nM)	Oral Absorption (%)	Bioavailability (%)	Liver Extraction (%)	Protein Binding (%)	Metabolism CYP450	Elimination t 0.5 (h)
Pravastatin	4	35	18	45	50	(3A4)	1.8
Lovastatin	2-4	30	5	>70	>98	3A4 (2C8)	3
Simvastatin	1-2 (active metabolite)	60-85	<5	>80	>95	3A4 (2C8, 2D6)	2
Fluvastatin	3-10	98	30	>70	>98	2C9	1.2
Atorvastatin	1.16	30	12	70	>98	3A4 (2C8)	14
Rosuvastatin	0.16	50	20	63	90	2C9 (2C19)	19
Pitavastatin	0.1	80	60	?	96	(2C9)	12

Table 1.5. General and clinical pharmacology of statin heterogeneity from (Amly and Karaman, 2015).

1.7.3 Statin pleiotropic mechanisms

Until recently, reduction in circulating LDL cholesterol was believed to be the main mechanism of action for the statin family of therapeutics. However, in recent years, it has become increasingly clear that the breadth of observed benefits cannot be ascribed solely to their cholesterol-lowering properties (Packard et al., 1998) (Buchwald et al., 1995). Indeed, complex immunological interplay takes place during the formative stages of atherosclerotic plaque development. After further study in this field, it quickly became apparent that statins hold the ability to modulate inflammation. From these observations, considerable interest arose in their potential to treat autoimmune diseases though modulation of upstream isoprenoids within this pathway (Greenwood et al., 2006) (Bloch, 1965). Among the many isoprenoid intermediates present, the most widely studied are farnesyl pyrophosphate (FPP) and geranylgeranyl

pyrophosphate (GGPP), found downstream of L-mevalonic acid. Both isoprenoids can bind the C terminal of small G-proteins and together, perform adjunctive roles in the posttranslational modification of a variety of signalling molecules (Xu et al., 2015). These signalling pathways can be seen in Figure 1.6.

1.7.4 Prenylation

In simple terms, prenylation is the covalent attachment of lipids to proteins. More specifically, isoprenoid moieties such as FPP and GGPP irreversibly attach to cysteine residues on the C-terminal of target proteins containing a carboxy-terminal CaaX (where C is cystines, a is an aliphatic amino acid and X is any amino acid) motif. This post-translational process is essential for membrane targeting and appropriate subcellular localisation of proteins for proper function. To give an idea of scalability, prenylated CaaX-containing proteins have been estimated more than 100 encoded proteins in the human genome.

Pertinent to this thesis, is the ability of FPP and GGPP to serve as lipid attachments for small G-proteins that comprise the GTPase family and nuclear lamins, including but not limited to Rho, Ras, Rac and cell-division cycle 42 (CD42). Cycling between an active GTP-bound state and inactive GDP-bound configuration, these small proteins have critical roles in controlling multiple cellular signalling pathways related to myelination, cell differentiation, proliferation, trafficking and cytoskeletal dynamics (Cox and Der, 1992). Manipulation of these otherwise tightly regulated isoprenoids can introduce significant changes to vital cellular processes. Indeed, it has been proven that blockade of small G-protein lipid modifications acts to functionally inhibit essential elements involved in the inflammatory signalling cascade. A consequence of statin-induced isoprenoid inhibition points towards this class of therapeutics having multiple pharmacological activities (Roberts et al., 2008) (Etienne-Manneville and Hall, 2002) (Cox and Der, 1992). Research over the past three decades shows statins have diverse effects on the different pathways involved in inflammation, angiogenesis (Greenwood et al., 2006) (Weis et al., 2002) (Lee et al., 2014a), apoptosis (Dirks and Jones, 2006) as well as anti-oxidative properties (Wassmann et al., 2002) leading to new therapeutic possibilities.

The collective impact statins have on these alternatively modified pathways is commonly referred to as cholesterol independent. Statin pleiotropy is broad reaching and to date, has identified roles in improving endothelial function (Halcox and Deanfield, 2004) (John et al., 2005) (Wolfrum et al., 2003), reducing inflammation (Robinson et al., 2005) (Shishehbor et al., 2003b) (Schönbeck and Libby, 2004) and oxidative stress (Yilmaz et al., 2004) (Beltowski, 2005) (Ortego et al., 2005), augmenting atherosclerotic plaque stability and inhibiting thrombogenic responses (Werner et al., 2002). A standard mechanism underlying the pleiotropic effects of statins is centred around Rho inhibition and its downstream target, Rho-associated coiled-coil containing protein kinase (ROCK). Relevant to this body of work are the Rho/ROCK signalling pathways with important regulatory functions in actin cytoskeleton remodelling, nitric oxide bioavailability (Laufs et al., 1997) (Di Napoli et al., 2005) (Takemoto et al., 2002),

migration and adhesion (Li et al., 2007) and generation of reactive oxygen species (Maack et al., 2003) (Jones et al., 2003).

1.7.5 The effect of statins on ROS and RNS production

Since inflammation is closely related to the production of ROS and RNS, recent investigations have demonstrated a regulatory role for statins via the cholesterol independent pathway. In cardiac myocytes, statins demonstrated a downregulation in iNOS transcript and protein (Madonna et al., 2005) (Domínguez et al., 2011). Moreover, statins have shown to reduce protein levels of active subunits with the NADPH complex (Delbosc et al., 2002) (Maack et al., 2003) (Wassmann et al., 2001) (Antoniades et al., 2010) (Antoniades et al., 2011).

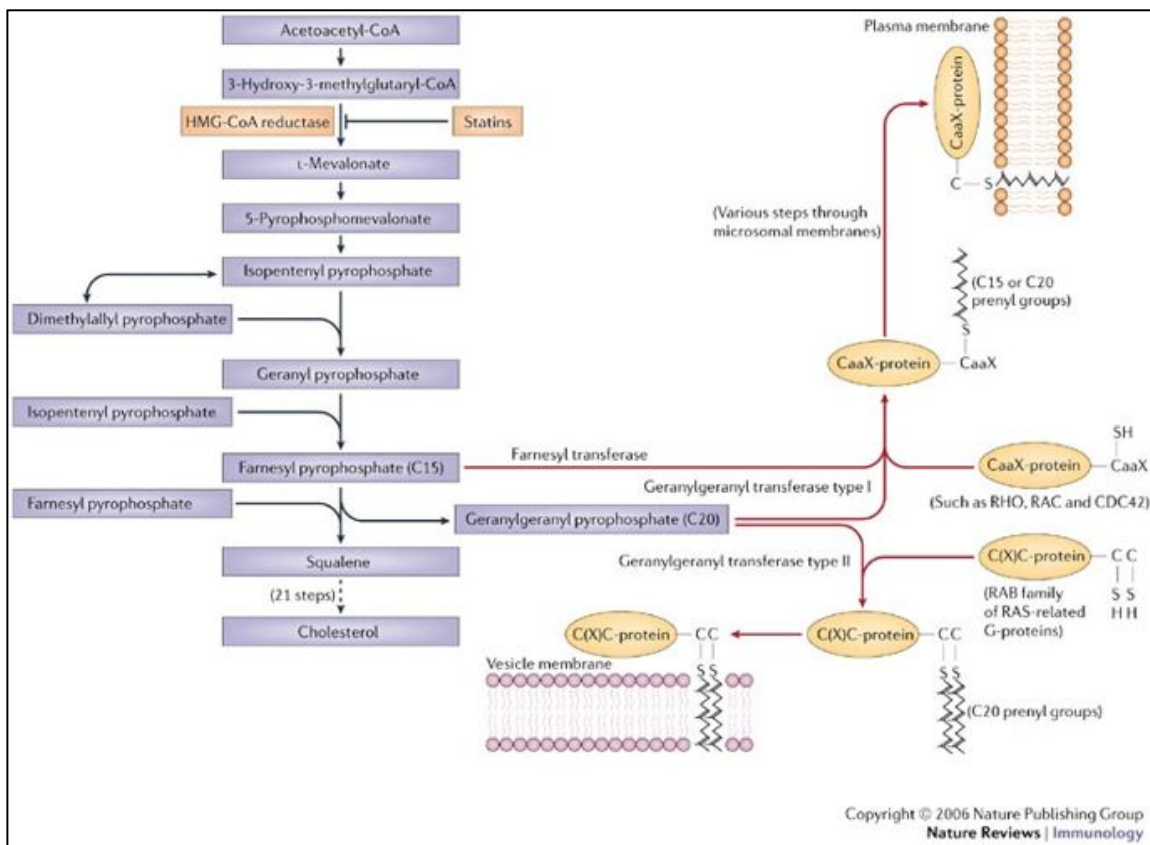


Figure 1.6. Mechanistic insight into HMG-CoA reductase inhibition. Schematic representation of the cholesterol biosynthesis pathway including all relevant isoprenoid intermediates influenced by the upstream effects of HMG-CoA reductase inhibition. From Greenwood et al., (2006)

1.8 Statin therapy in neuroinflammatory diseases of the CNS

Since discovery of their pleiotropic potential, statins have attracted considerable attention for their unique ability to modulate many facets of the immune response. *In vitro*, *in vivo*, and clinical data have demonstrated that statins are able to inhibit T cell proliferation, reduce co-stimulatory molecule expression, inhibit migratory pathways for leukocytes crossing the CNS, reduce antigen presentation via inhibition of IFN γ , necessary for the induction of MHC-II and pertinent to this thesis, reduce oxidative stress (Laufs et al., 1998) (Laufs and Liao, 1998) (Rikitake et al., 2005a). The uncovered immunomodulatory effects appear to be largely driven by inhibiting the isoprenylation of small GTP-binding proteins, independent of their cholesterol-lowering effects. These named pleiotropic effects make statins an attractive treatment option for immune-mediated disorders such as uveitis and MS.

1.8.1 Statin therapy in uveitis

Aberrant immune processes play a pivotal role in the pathogenesis of several autoimmune disorders, including uveitis. The statin class of drugs have shown to reduce local and systemic inflammation, independent of their cholesterol-lowering ability and that they may be beneficial in several retinal disorders (Kwak et al., 2000). A central process in the pathogenesis of uveitis is the migration of systemic immune cells to the target tissue. The mechanism underlying this process depends on leukocyte migration and expression of endothelial cell adhesion molecules. In uveitis, this process is aided by integrin lymphocyte function associated antigen-1 (LFA-1) which binds ICAM-1 (Weitz-Schmidt et al., 2001). It has been known for some time that statins alter the expression of ICAM, leading to inhibition of lymphocyte adhesion and activation (Kwak and Mach, 2001). In statin-treated uveitis, reduced leukocyte infiltration of the eye is a key and consistent finding (Greenwood et al., 2006). Indeed, a number of pre-clinical and clinical studies have demonstrated therapeutic efficacy in uveitis and its animal model equivalent, EAU (Shirinsky et al., 2017) (Lightman, 1997) (Gilbert et al., 2017), outlined in Table 1.6. Further to this, retrospective studies support a protective use for simvastatin against the development of uveitis (Borkar et al., 2015). Consequently, a clinical trial NCT02252328 is currently underway at Moorfields Eye Hospital to investigate the effectiveness of simvastatin in reducing the immune burden that is often observed in uveitis. This is the first study conducted to investigate the potential effects of statins, specifically simvastatin, in uveitis.

Statin	Dose and treatment protocol	Animal model	Results	References
Lovastatin	20 mg per kg, given intraperitoneally each day	B10RIII mice (antigen: IRBP)	Attenuation of disease; decrease in leukocyte infiltration; modulation of T _H 1-cell responses	(Gegg et al., 2005)
Atorvastatin	10 mg per kg, given orally each day	B10RIII mice (antigen: IRBP)	Mild attenuation of disease	(Thomas et al., 2005) (Kohnno et al., 2006)
Atorvastatin	1 or 10 mg per kg, given orally each day	B10RIII mice (antigen: IRBP)	No effect on disease	(Thomas et al., 2005)

Table 1.6. A summary of published pre-clinical data on the clinical outcome of statin use in models of uveitis. Original data references included. IRBP, interphotoreceptor retinoid-binding protein.

1.8.2 Statin therapy in SPMS

There is major unmet clinical need for disease modifying treatments in secondary progressive MS. For these patients, currently no licenced treatment exists to target accumulating disability at the neurodegenerative stages. The idea of repurposing statins for this disease is not new, recent observations suggest that statins may possess neuroprotective and potentially neuroregenerative properties in addition to the well-studied and reviewed immunomodulatory activities (Markovic-Plese et al., 2008) (Greenwood et al., 2006) (Zeiser, 2018) (Kwak et al., 2000). This concept was first introduced last decade and saw a surge in research interest which gave rise to an abundance of supportive *in vitro*, *in vivo* and clinical data (Zeiser et al., 2009) (Youssef et al., 2002) (McFarland et al., 2014) (Chataway et al., 2014) (Paintlia et al., 2010) (Pan et al., 2010). The first *in vivo* proof-of-concept experiment was performed by Singh and colleagues who demonstrated the effectiveness of lovastatin in attenuating the clinical signs of EAE (Stanislaus et al., 2001b). Greenwood et al., developed this idea further by showing that lovastatin treatment administered concomitantly with EAE induction, prevented the onset of disease (Walters et al., 2002). While exact mechanisms underpinning these findings remain to be elucidated, the observed immunomodulatory and neuroprotective discoveries incentivised a wave of clinical trials in MS. Accordingly, multiple randomised, placebo-controlled, double-blind prospective trials were undertaken to evaluate the efficacy of statins in RRMS. Despite the strong pre-clinical data, results from these clinical trials were mixed and did not provide decisive evidence for efficacy of statin use, either as monotherapy or in combination with the mainstay

treatment IFN β (Birnbaum et al., 2008) (Paul et al., 2008) (Togha et al., 2010) (Lanzillo et al., 2010) (Sorensen et al., 2011).

Table 1.7 summarises the heterogenous outcome of a number of landmark *in vivo* and clinical studies for the treatment of EAE and MS, respectively. Suggested mechanisms are purported to operate in a number of ways, although, most relevant to this body of work are the findings by (Stanislaus et al., 1999) (Stanislaus et al., 2001b) who attributed EAE disease attenuation to inhibition of iNOS expression and proinflammatory cytokine production.

Despite the observed inconsistencies when combining statin therapy with RRMS, more promising results were reported in the double-blind controlled MS-STAT1 trial in SPMS patients. Over the course of 2 years, treatment with high dose simvastatin the study showed a 43% reduction in annualised brain atrophy rate. Moreover, simvastatin had a positive effect by delaying disability progression, as assessed by the EDSS score (Chataway et al., 2014). The mechanisms underpinning these benefits have not yet been uncovered, although scientist speculate overarching vascular function and neuronal cell protection may be at play. Findings from this landmark trial forms the basis of this thesis today and prompts further exploration into the alternative, cholesterol-independent capabilities statins may have on this multifactorial disease.

Following the great success of MS-STAT1, was the launch of MS-STAT2. This phase III study is set to be a pivotal trial for SPMS. Patient recruitment is near completion and is a much eagerly awaited trial for a number of reasons; this study is paving the way for the area of drug repurposing and is entirely academically led. To support progression of this trial, a mechanism of action for simvastatin in the context of SPMS will be immensely valuable.

Statin	Dose and treatment protocol	Animal model and patients	Results	References
Atorvastatin	1 and 10 mg per kg, given orally each day	SJL/J mice (antigen: PLP) C57BL/6 mice (antigen: MOG) B10. PL mice (antigen: MBP)	Attenuation of disease; decrease in leukocyte infiltration; shift from T _H 1- to T _H 2-type cytokine profile; inhibition of T cell proliferation; inhibition of co-stimulatory molecule expression	(Youssef et al., 2002)
Atorvastatin	10 mg per kg, given orally or subcutaneously each day	SJL/J mice (antigen: PLP)	Attenuation of disease; decrease in leukocyte infiltration; blockade of T _H 1-cell responses with increase in T _H 2-type cytokines; inhibition of T cell proliferation	(Aktas et al., 2003)
Atorvastatin	10 mg per kg, given orally each day	C57BL/6 mice (antigen: MOG)	Attenuation of disease; decrease in leukocyte infiltration; blockade of T _H 1-cell responses	(Gegg et al., 2005)
Lovastatin	10 mg per kg per day, given intraperitoneally	Biozzi ABH mice (spinal-cord homogenate)	Attenuation of disease; inhibition of leukocyte migration across the blood-brain barrier	(Greenwood et al., 2003c)
Lovastatin	2 and 5 mg per kg per day, given intraperitoneally	SJL/J mice (antigen: PLP)	Attenuation of disease; inhibition of pro-inflammatory cytokine biosynthesis; shift towards T _H 2-biased T cell responses	(Nath et al., 2004)
Lovastatin	2 mg per kg per day, given intraperitoneally	Lewis rats (antigen: MBP)	Attenuation of disease; inhibition of iNOS expression and proinflammatory cytokine production	(Stanislaus et al., 2001b, Stanislaus et al., 1999)
Lovastatin	2 mg per kg per day, given intraperitoneally	Lewis rats (antigen: MBP)	Attenuation of disease; shift from T _H 1- to T _H 2-type cytokine profile	(Stanislaus et al., 2002)
Lovastatin	2 mg per kg per day, given intraperitoneally	Lewis rats (antigen: MBP)	Attenuation of disease; reduction in mononuclear-cell infiltration; reduction in inflammatory cytokine production and LFA1 expression	(Stanislaus et al., 2001b)
Lovastatin	2 mg per kg per day, given intraperitoneally	Lewis rats (antigen: MBP)	Attenuation of disease; decrease in leukocyte infiltration; increase in T _H 2-type cytokine transcription	(Stanislaus et al., 1999)
Simvastatin	20 mg per kg per day, given orally	Brown Norway rats (antigen: MOG)	No effect on disease	(Sättler et al., 2005)
Simvastatin	80 mg/d, given orally	RRMS (n = 30)	Decrease in the number and volume of Gd-enhancing lesions	(Vollmer et al., 2004)
Atorvastatin	80 mg/d, given orally	RRMS (n = 80)	Decreased in the number and volume of Gd-enhancing lesions	(Paul et al., 2008)
Simvastatin	80 mg/d, given orally	SPMS (n = 140)	Reduced the annualised rate of whole brain atrophy	(Chataway et al., 2014)

Table 1.7. An overview of published pre-clinical and clinical studies evaluating the efficacy of statin therapy in EAE and MS, respectively. SJL/J mice, Swiss Jim Lambert; PLP, myelin proteolipid protein; MOG, myelin oligodendrocyte glycoprotein; MBP, myelin basic protein; RRMS, relapsing-remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis. Table adapted from (Greenwood et al., 2006).

1.9 Aims

While many observations have been made and attributed to the pleiotropic nature of statins, not all cellular and molecular mechanisms have been elucidated, particularly those relating to attenuated cellular oxidative stress. It is therefore our aim to investigate the potential effect of simvastatin on ROS and RNS production in the context of SPMS. For this, we rely on well-established, predictable models as investigative tools to gain further insights into the pathways that afforded the observed clinical benefits in MS-STAT1.

Chapter 2

Materials and methods

2.1 Laboratory equipment

The following equipment was used throughout the experimental procedures.

Equipment	Model	Manufacturer
Centrifuge	Centrifuge 5415D, 5415R	Eppendorf (Hamburg, Germany)
	Function line Labofuge 400R	Heraeus (Hanau, Germany)
Confocal microscope	Zeiss LSM 710 confocal	Zeiss (Oberkochen, Germany)
Fume Cupboard	Enterprise DynamicFlow™ Fume Cupboard	Premier Laboratory Systems (Cambridge, UK)
Hood	Hera Safe	Kendro Laboratory Products (Asheville, USA)
Incubator	Hera Cell	Heraeus (Hanau, Germany)
Microscope (confocal)	LSM710/LSM700	Zeiss (Oberkochen, Germany)
Microscope (optical)	Leica DM IL	Leica (Milton Keynes, UK)
Nanodrop spectrophotometer	Nanodrop ND100™	ThermoFisher (Wilmington, USA)
qPCR device	7900HT Fast Real-Time PCR system	Applied Biosystems (Foster City, USA)
Retinal imaging system for mice and rat	Micron III	Phoenix Research Labs (Pleasanton, USA)
RT-PCR/cDNA synthesis device	T100™ Thermal Cycler	Bio-Rad (Hemel Hempstead, UK)
Waterbath	Shake Temp SW22	Julabo (Seelbach, Germany)

Table 2.1. Equipment used throughout experimental procedures

2.2 General reagents

Product	Manufacturer
2-metacaptoethanol (50 mm)	Gibco® Life Technologies (Carlsbad, USA)
4% Paraformaldehyde (PFA)	Electron Microscopy Sciences (Pennsylvania, USA)
Phosphate-buffered saline (DPBS) (1X), no calcium, no magnesium	Gibco® Life Technologies (Carlsbad, USA)

Table 2.2. General reagents used throughout experimental procedures

2.3 Tissue culture reagents

Product	Manufacturer
Foetal Calf Serum	Invitrogen (Paisley, UK)
BSA 22% w/v in PBS	First Link UK (Wolverhampton, UK)
Ascorbic Acid	Sigma Aldrich (Poole, UK)
DMEM/F-12	Life Technologies (Loughborough, UK)
DDPBS (1X), no calcium, no magnesium	Gibco® Life Technologies (Carlsbad, USA)
EGM™-2 Basal Medium	Lonza (Basel, Switzerland)
EGM™-2 BulletKit™	Lonza (Basel, Switzerland)
F-10 with Glutmax	Invitrogen (Paisley, UK)
Hematoxylin solution modified acc. to Gill II	Sigma Aldrich (Poole, UK)
HBSS (10X), no calcium, no magnesium	Gibco® Life Technologies (Carlsbad, USA)
HBSS (1X), calcium, magnesium	Gibco® Life Technologies (Carlsbad, USA)
RPMI 1640 Medium, GlutaMAX™	Gibco® Life Technologies (Carlsbad, USA)
Collagenase/Dispase	Lorne Laboratories (Reading, UK)
1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES)	Invitrogen (Paisley, UK)
Penicillin/Streptomycin	Invitrogen (Paisley, UK)
Puromycin	Sigma Aldrich (Poole, UK)
Basic fibroblast growth factor (bFGF)	Sigma Aldrich (Poole, UK)
Trypsin	Sigma Aldrich (Poole, UK)
Collagen type I	BD Biosciences (San Jose, USA)
Fibronectin	Sigma Aldrich (Poole, UK)
Recombinant Human Interferon- γ (IFN- γ)	ThermoFisher Scientific (Waltham, Massachusetts, USA)
LPS <i>Escherichia coli</i> O111:B4	Sigma Aldrich (Poole, UK)
Recombinant Human Tumor Necrosis Factor- α (TNF)	ThermoFisher Scientific (Waltham, Massachusetts, USA)

Table 2.3. Reagents used in tissue culture experimental procedures Stocks of cells, when applicable, were frozen in cryovials in media supplemented with 10% DMSO and stored at -80°C for short term storage or in liquid nitrogen for long-term storage.

2.4 Antibodies table

Antibody	Immunogen	Species	Isotype	Clone	Reactivity	Conjugation	Source
Anti-Nitrotyrosine	Nitrated KLH (Keyhole Limpet Hemocyanin)	Rabbit	IgG ₁		Multiple	Unconjugated	Millipore
Anti-Nitrotyrosine	nitrated KLH (Keyhole Limpet Hemocyanin)	Rabbit	IgG ₁		Multiple	Unconjugated	Sigma
Anti-Iba1	C-terminal of Iba-1	Rabbit	IgG ₁		Mouse, Rat, Human	Unconjugated	WAKO
Anti-NCF1/p47-phox	Human NCF1/p47-phox aa 378-390 (C terminal)	Goat	IgG ₁		Mouse, Rat, Human, Rabbit	Unconjugated	Abcam
Anti-8-oxo-dG	8-oxo-dG-conjugated-KLH	Mouse	IgG _{2b}	15A3	Multiple	Unconjugated	Trevigen
Anti-ED1	ED-1	Mouse	IgG ₁	ED1	Rat	Unconjugated	BioRad
Anti-CD68	CD68	Rat	IgG ₁	FA-11	Mouse	Unconjugated	BioRad
Anti-CD11b	Mac-1 alpha	Rat	IgG _{2b}	M1/70	Mouse, Human	Alexa Flour-647	BD Pharmingen
Anti-4HNE	4-hydroxynonenal	Rabbit	IgG	HNE13-M	Mouse, Rat, Human	Unconjugated	Alpha Diagnostics International
Anti-pimonidazole	Pimonidazole adducts	Rabbit	IgG ₁	MAb1	Rat, Mouse	Unconjugated	Hypoxyprobe
Anti-eNOS/NOS Type III	Endothelial nitric oxide synthase	Mouse	IgG ₁	Clone 3	Mouse, Human	Unconjugated	BD Pharmigen
Anti-iNOS	Inducible nitric oxide synthase	Mouse	IgG ₁	Clone 2	Mouse	Unconjugated	BD Pharmigen
Anti-HSC-70			IgG ₁	N27F3-4	Mouse, Human	Unconjugated	Sigma

Table 2.4. A list of antibodies used throughout all experimental applications

2.5 Animal model: IRBP₁₋₂₀ induced EAU

Adult female C57BL/6 mice (6-8 weeks old, Charles River, UK) were randomised into cages at the beginning of each experiment with food and water *ad libitum* and left to acclimatise for one week. Human IRBP₁₋₂₀ (LAQGAYRTAVDLESASQLT) was used to induce EAU (Caspi et al., 1988). Mice received two subcutaneous injections either side of the flank (100 µL), comprising 400 µg IRBP₁₋₂₀ peptide emulsified 1:1 v/v in Complete Freund's Adjuvant (CFA: Sigma-Aldrich, USA) and supplemented with 1.5 mg/ml *Mycobacterium tuberculosis* (DIFCO Laboratories, Detroit, Michigan). In all immunisations, Pertussis toxin at 1.5 µg (PTX: Tocris, Oxford, UK) was added to RPMI 1640 media (100 µL; Thermofisher, UK), and supplemented with 0.1% mouse serum (Sigma-Aldrich, USA) then injected intraperitoneally (IP) on the day of immunisation. Control animals received 100 µL of an emulsion comprising CFA and PBS alone. All experiments were performed in accordance to the UK Animals Act of 1986 (Scientific Procedures) and the ARRIVE guidelines.

2.5.1 Clinical evaluation: Mouse fundus examination

Clinical disease score was assessed at regular intervals by fundus examination, via bright-field live imaging under a binocular microscope (Micron III, Phoenix Research Labs, USA). Briefly, the mice were lightly sedated under general anaesthesia using a combination of both Ketamine (Orion Pharma, Finland) to a final concentration of 6 mg/ml in combination with Medetomidine (Vetoquinol UK Limited) final concentration of 6 mg/ml, after which, 1% Tropicamide (Bausch & Lomb UK Limited, Surrey) was applied as an ophthalmic dilating solution. Once the pupil had fully dilated after several minutes, mice were placed on a purpose-built stage for imaging and the microscope positioned to surround the cornea. It should also be noted that 2.0 mg/g Carbomer (Novartis, Switzerland) was generously applied throughout the imaging process to keep the eye lubricated. The fundus of a mouse is the interior surface of the eye and includes the retina and optic disc. A number of retinal images were taken, and microscope lens positioned accordingly to cover the entirety of the retinal area for later clinical evaluation.

2.5.2 Clinical disease scoring

Clinical assessment criteria was based on previously published data (Xu et al., 2008) and focused on severity of the following parameters: optic disc inflammation, retinal vessel cuffing, retinal tissue infiltrate and structural damage (Table 2.5). Each of these parameters were awarded a score on a scale from 0 to 5 and the collective total was taken to represent the whole eye. A maximum score of 20 could be obtained per eye.

Score	Optic Disc	Retinal Vessels	Retinal Tissue Infiltrate	Structural Damage
1	Minimal Inflammation	1-4 mild leukocyte cuffing	1-4 small lesions or 1 linear lesion	Retinal lesions or retinal atrophy involving $\frac{1}{4}$ to $\frac{3}{4}$ retina area
2	Mild inflammation	>4 mild cuffing or 1-3 moderate leukocyte cuffing	5-10 small lesions or 2-3 linear lesions	Pan retinal atrophy with multiple small lesions (scars) or <3 linear lesions (scars)
3	Moderate inflammation	>3 moderate leukocyte cuffing	>10 small lesions or >3 linear lesions	Pan retinal atrophy with >3 linear lesions or confluent lesions (scars)
4	Severe inflammation	>1 severe leukocyte cuffing	Linear lesion confluent	Retinal detachment with folding
5	Not visible (white out or extreme detachment)	Not visible (white out or extreme detachment)	Not visible (white out or extreme detachment)	Not visible

Table 2.5. Conventional clinical scoring scale for evaluating EAU disease severity. Table outlining criteria used to evaluate the extent of disease severity in mice immunised with IRBP₁₋₂₀. Scores were allocated according to the above hallmarks visible on the fundus images, each eye was given a total score out of 20. Table adopted from (Xu et al., 2008).

2.5.3 Fluorescein angiography

Fluorescein angiography is an imaging technique that allows for visualisation of the intact vasculature of the retina and optic disc. The dye, sodium fluorescein, is a small organic molecule that when injected appropriately is restricted to the blood compartment of the normal retina. Leakage out into the tissues is restricted by the BRB. Once bright field images had been collected from each mouse, a single bolus (100 μ L) of 2% Fluorescein was administered subcutaneously. The microscope was set to collect data with an excitation filter placed on the camera so that only fluorescent, yellow-green light at 530 nm was recorded. The microscope lens was positioned such that the optic disc was centralised and after 1.5 minutes post injection, the first image was captured. This is ample time to allow the fluorescein to circulate throughout the vasculature of the retina where it can later be visualised. A second and final image was then taken at 7 minutes post-injection, which allows fluorescein to diffuse through leaky vessels into the surrounding tissue allowing for a leakage ratio to be calculated between the two timepoints.

2.5.4 Simvastatin treatment in IRBP₁₋₂₀ model

Simvastatin therapy was administered via oral gavage, daily for a total period of 8 days. Treatment began on day 14 at the onset of infiltration – as determined by fundoscopic analysis – and continued until peak disease at day 21, after which eyes were enucleated. Treatment concentrations used were 50 mg/kg, 75 mg/kg and 100 mg/kg or vehicle (0.5% Methylcellulose) all emulsified in 0.5% methylcellulose. Only animals that showed positive for disease on day 14 post-induction (PI) through fundoscopy were selected to continue through the study and received either treatment or vehicle, those that did not show signs of disease development at these stages were discounted and not continued through study.

2.5.5 IRBP₁₋₂₀ model exclusion criteria

Eyes were assessed on day 14 PI using fundoscopy and clinical disease was scored according to the parameters outlined in Table 2.5. Based on pooled scores from both eyes, mice that did not reach a combined total of ≥ 1.5 at day 14 PI were removed from the study as these animals are less likely to develop disease.

2.5.6 Histology

2.5.6.1 NEURORETINA FLAT MOUNT PREPARATION AND IMMUNOFLOURESCENCE

Enucleated eyes were immediately placed in 4% paraformaldehyde diluted in 2X phosphate buffered saline (PBS) for 2 minutes to pre-fix the outside, before transferring to 2X PBS for 5 minutes. The neuroretina was then dissected and post-fixed in 100% Methanol for 20 minutes and either stained immediately or placed at -20°C for long-term storage. Methanol was aspirated and replaced with 2X PBS for 3 x 5 minutes. The tissue was then incubated in blocking buffer to prevent non-specific binding [3%

Triton X-100, 1% Tween-20, 0.5% BSA, 0.1% in 2X PBS], for a total of 30 minutes at room temperature. Primary antibodies were diluted to the appropriate concentration in blocking buffer and incubated overnight at 4°C. Negative controls were incubated with blocking buffer alone.

Once the overnight incubation was complete, primary antibody was removed and replaced with blocking buffer for washing with a total of 3 x 15-minute changes. Primary antibodies were detected with species-specific secondary antibodies, diluted in blocking buffer to the appropriate concentration and incubated at room temperature for a total of 2 hours. The secondary antibody incubation period was followed by 3 x 15 minutes washes in blocking buffer with one final wash in 2X PBS, before mounting in fluorescent mounting medium (DAKO) and coverslipped.

2.5.6.2 HISTOLOGY PREPARATION OF RETINAL SECTIONS

At peak disease, day 21 PI, eyes were enucleated and immediately placed in 10% formalin for a 24-hour period, after which, whole eyes were embedded in paraffin. Eyes were serially cross sectioned at 12 µm, mounted and used to grade disease based on cell infiltration and retinal structure, as described by Copland *et al.*, 2008 (Table 2.6).

Immunofluorescent techniques were adopted to visualise markers. Briefly, sections were deparaffined using HistoChoice (Sigma, UK) for 5 mins. After this, slides were carried through a process of rehydration, sequentially incubating in; 100% Ethanol [2 x 1-minute changes], 70% Ethanol [1-minute] and finally ddH₂O for 5 minutes. To expose the epitope for antibody recognition, sections were next introduced to an antigen retrieval method, involving a 25-minute incubation at 98°C immersed in retrieval buffer [10mM Sodium Citrate, 0.05% Tween-20, pH 6.0 in ddH₂O]. Once sections had undergone the process of antigen retrieval, tissue was permeabilised using PBT [1X PBS, 0.1% Tween-50] for 2 x 4 minutes. In order to prevent non-specific binding, sections were blocked using 0.5% Bovine Serum Albumin (BSA) in 1X PBS for a minimum of 30 minutes at room temperature. All antibodies were diluted to the appropriate concentration in block solution and left to incubate overnight at 4°C. Following the overnight incubation, primary antibodies were aspirated and washed with PBT for 2 x 4 minutes. Secondary antibodies were also diluted in block solution and left for 1 hour at room temperature. Washes were performed after this stage with PBT, 2 x 4 minutes and one final wash performed in PBS alone, before mounting with fluorescence mounting medium (DAKO).

2.5.6.3 HISTOPATHOLOGICAL ANALYSIS

Retinal sections (12 µm) were taken from normal naïve, vehicle treated (0.5% methylcellulose) and simvastatin treated (50 mg/kg, 75 mg/kg and 100 mg/kg) animals. Haematoxylin and Eosin staining was carried out, according to the manufacturer's instructions (Sigma-Aldrich, UK), so that tissue architecture could be easily visualised for assessment. Images of each sections were obtained using

Invitrogen's EVOS FL auto 2 cell imaging system, operating in transmission mode. The retinal sections were then graded according to the criteria outlined in Table 2.6, taken from those previously described by Copland *et al* (2008).

2.5.6.4 CONFOCAL LASER MICROSCOPY

Fluorescent images from immunohistochemically labelled sections were obtained using Zeiss LSM710 confocal microscope with a x40 objective. Excitation wavelengths of 488 nm, 543 nm and 647 nm were provided by argon and helium-neon gas lasers, respectively. Emission filters were utilised for detection of fluorescent signal. Control tissues exposed to species-specific Ig were used to set parameters for laser power, once established, these were fixed throughout the acquisition protocol for later comparison of the average intensities between treatment groups. Information was gathered covering the thickness of the section, which was later compared for average fluorescence intensity. Briefly, captured images were imported to Image J (NIH, Bethesda, MD), the brightest images of the stack were averaged for intensity, categorised by marker and compared across treatment groups.

Neuroretina flat mount acquisition involved obtaining staining data throughout the entire tissue thickness, spanning; ganglion cell layer, inner plexiform layer, outer plexiform layer, outer nuclear layer, external limiting membrane and finally photoreceptor. Additionally, in order to be truly representative of the entire neuroretina, it was also important to capture the area of positive staining by use of the tile scan function. Data gathered was imported to Image J (NIH, Bethesda, MD) and separated according to fluorophore, the images were then stitched, and maximum intensity projection created; from which positive cell count could be determined by eye.

Location	Finding	Score
Cell Infiltration		
Ciliary body	Cell infiltrate < 5 cells	1
	Mild thickening	2
	Moderate thickening	3
	Gross thickening	4
Vitreous	Cells < 5	1
	Cells 5-25	2
	Cells 25-50	3
	Cells 50-100	4
	Cells > 100	5
Vasculitis (mural or extravascular cells)	< 10% vessels involved	1
	10%-25%	2
	25%-50%	3
	50%-75%	4
	> 75%	5
	Cells in or around wall	1
	Mild perivascular cuffing	2
	Moderate cuffing	3
	Gross cuffing	4
Rod outer segment	Cell infiltrate	1
	Partial loss	2
	Moderate loss	3
	Subtotal loss	4
	Total loss	5
Choroid	Cell infiltrate	1
	Mild thickening	2
	Moderate thickening	3
	Gross thickening	4
	Granulomas 1	1
	Granuloma 2-5	2
Structural/Morphological Changes		
Rod outer segments	Cell infiltrate	1
	Partial loss	2
	Moderate loss	3
	Subtotal loss	4

Neuronal layers	Cell infiltrate	1
	Partial loss	2
	Moderate loss	3
	Subtotal loss	4
	Total loss	5
Retinal morphology	Folds < 10%	1
	Folds 10%-50%	2
	Folds > 50%	3
* Maximum possible total score: infiltration, 30; structural, 12	Folds > 50%	3

Table 2.6. Criteria used to evaluate histopathological features in EAU. Histological analysis was performed upon Haematoxylin and Eosin staining by two independent observers following the above criteria for grading, adopted from Copland et al (2008).

2.6 Statistical analysis

Pearson correlation coefficients were used to assess association between variables where indicated. An ordinary one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test was used to compare clinical score, clinical grading and angiographic data between all treatment groups. An independent, two-tailed t-test was used to assess differences in mean staining intensity between vehicle treated and Simvastatin treated groups across all markers used for reactive oxygen species detection. A p value for each dataset represented as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

2.7 Animal model: rMOG induced EAE

Female Dark Agouti rats (DA; 8-9 weeks old, ~150 g, Harlan, UK) were housed in cages of two and randomised into treatment groups, prior to the onset of experiments. All animals were given water *ad libitum* and exposed to a twelve-hour light-dark cycle. The acclimatisation period between arrival of the animals and induction of EAE was one week. EAE was induced by a single subcutaneous injection in the rear flank near the base of the tail; comprising 100 µg rMOG (MOG₁₋₁₂₄; synthesised in the laboratory of Prof Christopher Linington, University of Glasgow) immersed in incomplete Freund's adjuvant (IFA; Sigma-Aldrich, USA) and administered under general anaesthesia (2% isoflurane in room air) as perviously described (Davies *et al.*, 2013). All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act of 1986, and the ARRIVE guidelines.

2.7.1 Neurological evaluation: Daily scoring system

All behavioural assessments and analyses were performed masked and examined through the course of the experiment. Animals were weighed daily to give an indication of the overall health, in addition to daily examinations of neurological deficit. Disease progression was assessed according to a 10-point scoring system in order to determine the degree of neurological deficit. Briefly, one point was assigned to each of the following neurological signs: tail tip weakness, tail weakness, tail paralysis, absence of toe spreading reflex, abnormal gait, left and right hind limb paresis, left and right hind limb paralysis and moribund (Davies *et al.*, 2013). A final total score between 0 and 10 would thereby be representative of the neurological function of an individual animal (Table 2.7).

2.7.2 Neurological evaluation: Pre-perfusion scoring system

A more detailed 25-point scale (developed by the Smith Lab, Institute of Neurology, UCL) was used to assess neurological deficit before perfusion fixation of the animals on the final day of experiments. Zero indicates normal function, whereas ascending scores indicate increasing disabilities. Subjecting animals to a more rigorous neurological assessment before perfusion allow for a more detailed description of

dysfunction, hence, each hind limb was assessed for stretch withdrawal, pinch withdrawal, toe spreading, spasticity and plantar placement, one point being awarded for each characteristic present per limb. Thus, a maximum of 10-points could be obtained for each; a score of zero indicating normal function. Additionally, tail function (0-3 points) and hip flexion (0-2) were also assessed for a total maximum score of 25-points per animal. Both the aforementioned 10-point system and a more detailed 25-point scoring system are used routinely in the laboratory of K.Smith and have previously demonstrated robust clinical outcomes suitable for publication (Davies et al., 2013a) (Table 2.8).

2.7.3 Disease parameters

Parameters taken into consideration when assessing the contribution of various pathological markers, were evaluated based on the total cumulative neurological scores on the final day of experiments, together with the per-perfusion score (Tables 2.7 and 2.8).

2.7.4 Animal inclusion criteria

After immunisation, animals were recruited for a total period of 1 week. During this time, any animals that presented with extremely severe (score above 8) or extremely mild (score 1) disease at onset were excluded from the study. In addition, animals with a score of ≥ 4 were not recruited as disease at this stage is too severe to respond to therapy. For the inclusion of animals, we aimed to recruit those demonstrating tail weakness, hence, a daily neurological deficit score between 2-3, identified by the criteria outlined in Table 2.7.

Neurological Deficit	
Clinical Sign:	Tail tip weakness
	Tail weakness
	Tail paralysis
	Abnormal gait
	Abnormal toe spreading
	Unilateral hind limb weakness
	Bilateral hind limb weakness
	Unilateral hind limb paralysis
	Bilateral hind limb paralysis
	Moribund

Table 2.7. Conventional scoring scale used for evaluating the extent of neurological deficit in animals immunised with rMOG. A single point was given for each of the clinical signs present to give a total score out of 10.

Neurological Deficit	Clinical Symptoms	Score
Hind Limbs		
Stretch withdrawal	Delayed, weak, partial	1
	No response	1
Pinch Withdrawal	Delayed, weak, partial	1
	No response	1
Toe Spreading	Incomplete, Asymmetrical foot splaying or toe spreading	1
	No response	1
Spasticity	Partial rigidity or immobilization, some residual movement	1
	Complete rigid spasticity	1
Plantar Placement	Attempted but unsuccessful or incomplete plantar placement during walking	1
Tail		
	Tail tip weakness	1
	Complete tail weakness	1
	Complete tail paralysis	1
<i>Hips</i>		
	Asymmetrical, some flexion	1
	Complete absence of flexion or dragging	1

Table 2.8. 25-point scoring scale for the evaluation of neurological deficit, pre-perfusion.

Table outlining criteria used to evaluate the extent of neurological dysfunction in animals immunised with rMOG, prior to and following treatment with Simvastatin. A score of one was given for each deficit, resulting in a total score out of 25.

2.7.5 Administration of simvastatin treatment in rMOG-EAE

At the onset of neurological deficit, rMOG animals were paired based on the similarity of timing and neurological signs, as determined by their 10-point daily neurological score (Table 2.7), each animal of the pair was then randomly assigned to receive either simvastatin (25 mg/kg) or vehicle.

Simvastatin was administered orally via the feed. Briefly, specialised chow was produced (ENVIGO, Madison, USA) at a simvastatin concentration of 0.6 g/kg and calculated to deliver a daily dose of 25 mg/kg based on the weight of animals and average daily food consumption. The following formula and assumptions were used to calculate simvastatin concentration in the chow:

$$\text{Single Daily Dose (SD)} = 25 \text{ mg/kg (simvastatin)}$$

$$\text{Body Weight (BW)} = 350 \text{ g average body weight of animal}$$

$$\text{Daily Food Intake (FI)} = 15 \text{ g diet per day}$$

$$\text{Diet Dose (DD)} = 0.6 \text{ g of Simvastatin/Kg of chow}$$

$$\text{FORMULA: } DD = (SD \times BW) \div FI$$

The animals were then allocated either vehicle chow or simvastatin containing chow ad libitum.

2.7.6 Tissue processing

To evaluate tissue hypoxia, four hours prior to perfusion fixation intravenous injections of pimonidazole (60 mg/kg in sterile saline; HPI Inc, USA) were administered into the saphenous veins of all animals, under light anaesthesia (2% isoflurane in room air). Once the four-hour period has elapsed, animals were perfused transcardially, initially with 1 X PBS (Sigma Aldrich, UK) followed by 4% paraformaldehyde (PFA) for fixation, under general anaesthesia (2% isoflurane in room air).

The spinal cord was harvested in its entirety and further post-fixed overnight at 4°C in 4% PFA, with subsequent cryoprotection in 30% sucrose (BDH International, UK) in PBS. The spinal cords were dissected according to anatomical positioning, the lumbar region utilised and embedded in cryomolds containing OCT medium (Leica, UK). The tissue was then frozen by immersion in pre-cooled isopentane (VWR International, UK) and submerged in liquid nitrogen. Sections were cut to a thickness of 12 µm using a cryostat instrument (Leica, Microsystems, Germany) at -20°C, and thaw-mounted onto glass slides (Superfrost, WVR, USA). All sections were stored frozen at -20°C until use. The cords were embedded up to 6 in the same block, to allow parallel assessment on the same slide of control and EAE tissue at different severities and/or stages of disease.

2.7.6.1 HISTOLOGY: IMMUNOHISTOCHEMISTRY AND IMMUNOFLOUORESCENCE (IHC/IF)

All cryosections were air dried prior to use for 10 minutes. Firstly, sections were rehydrated in 1 X PBS for a total of 5 minutes. If detection systems involved the use of DAB they were then pre-treated with 0.3% hydrogen peroxide (Sigma, UK) in neat methanol (VWR International, UK). Alternatively, for immunofluorescence imaging methanol alone was used. For antigen retrieval 1 mg/ml of Sodium Borohydride was used diluted in PBS for 2 x 5 minutes, followed by 3 x PBS washes. The blocking stage was immediately carried out with 5% donkey serum in PBS and left to incubate for at least 15 minutes at room temperature. Primary antibodies were then immediately placed in the appropriate blocking buffer and left to incubate overnight at 4°C. The following day, primary antibody was aspirated and the secondary added, incubated again in blocking buffer and left to bind at room temperature for 2 hours. After this point, 3 x washes were performed with PBS before the slides were mounted using fluorescence mounting medium (DAKO).

2.7.7 Microscopy

2.7.7.1 LIGHT MICROSCOPY AND QUANTIFICATION

Tissues stained and developed for visualisation using DAB (3, 3'-diaminobenzidine) HRP substrate were viewed and photographed with Invitrogen's EVOS 2 FL auto 2 cell imaging system. The illumination remained at a constant intensity throughout the image acquisition process. All analysis and quantification were performed blind and carried out as previously described (Davies *et al.*, 2013). Briefly, captured micrographs were imported into Image J (NIH, Bethesda, MD) and converted from RGB 24 bits per pixel (bpp) to grey scale 8 bpp images. Once the images had been converted to grey scale, staining intensity for pimonidazole could be measured by manually demarcating the edges of the whole spinal cord section, including both the grey and white matter. Next, analysis of the staining was carried out by counting the number of pixels above a set threshold (expressed as percentage of cord area) and compared between treatment groups. Quantification was expressed as the whole percentage coverage when performed on the whole spinal cord area, or dorsal column regional area only. The naïve, non-immunised animals were absent of spinal cord hypoxia thus, these sections were used to set the threshold as this would act as a negative control. Any pixels detected above this threshold would be classed as positive and levels quantified. An average pixel count was collected from all naïve sections to set the baseline threshold.

2.7.7.2 CONFOCAL LASER MICROSCOPY AND QUANTIFICATION

Fluorescent images were acquired using a Zeiss LSM 710 spectral confocal laser scanning microscope, with a x40 objective. Excitation wavelengths of 488 nm, 594 nm and 647 nm were provided

by argon and helium-neon gas lasers, respectively. All samples contained DAPI staining with an excitation 405 diode laser for detection.

Data acquisition was mainly focused on the white matter, dorsal column region of the spinal cord sections, where most of the pathology was observed. Normal, non-immunised animals were used to set the acquisition parameters, for laser power and fluorescence detection. Once parameters had been established in the non-immunised, normal spinal cord sections these were kept constant throughout the acquisition process. Information was also gathered regarding the length of the section, which was later compared for average fluorescence intensity. Briefly, captured micrographs were imported to Image J (NIH, Bethesda, MD), the brightest images of the stack were averaged for intensity, categorised by marker and compared across treatment groups.

2.7.8 Statistical analysis

Pearson correlation coefficients were used to assess the association between variables where indicated. A two-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons was used to compare the daily 10-point neurological deficit score between treatment groups.

An independent, two-tailed, t-test was used to assess differences in mean clinical neurological deficit score obtained using the 25-point point grading system pre-perfusion (Table 2.8). A p value for each dataset represented as *, $P < 0.05$; **, < 0.01 ; ***, $P < 0.001$.

2.8 Tissue culture

2.8.1 Thawing cells

Cells cultured from frozen stocks were recovered by submerging in a water bath at 37°C for a quick thawing process. Next, cells were washed with 10 ml of warmed media and centrifuged at 260 g for 10 mins in an MSE Harrier 18/80 centrifuge (Sanyo Gallenkamp PLC, UK). The cells were then counted using a haemocytometer and viability determined by a Trypan Blue exclusion assay. From this, the cell density was calculated and seeded at the appropriate quantity according to cell type. Once the cells had been transferred to a T75 cm² vented tissue culture flasks, they were maintained in culture at 37°C, 5% CO₂ humidified incubator (Phillip Harris, UK).

2.8.2 Freezing cells

A set density of cells in their respective media were transferred to cryotubes, incubated on ice for 10 minutes and supplemented with DMSO [10% v/v], which was added incrementally to a final volume of 1 ml. Cryotubes were immediately transferred to a polypropylene freezer box and submerged in liquid nitrogen for long term storage.

2.8.3 Cell Lines

2.8.3.1 HUMAN CEREBRAL MICROVASCULAR ENDOTHELIAL (hCMEC/D3)

Immortalised hCMEC/D3 cells, kindly donated by Prof. Ignacio Romero (Open University), were used between P26-P29 of expansion and cultured in complete culture media consisting of the following: EGM™-2 Basal Medium supplemented with 5% Foetal Bovine Serum, 1% Penicillin/Streptomycin [10,000 units/ml Penicillin, 10,000 µg/ml Streptomycin] (ThermoFisher, UK), 1 mg/ml Hydrocortisone, 2.5 mg/ml Acid ascorbic, 5 mL and 10 µg/ml bFGF. Cells were seeded at a density of 8×10^4 to 1×10^5 on Collagen I [1:60 in DPBD] (ThermoFisher, UK), coated six-well plates with complete media changes every two days until absolute confluency. Thereafter, complete cell media was substituted for a starvation (EGM) media in preparation for experimental manipulation.

2.8.3.2 MICROGLIAL CELL LINE (BV2)

The BV2 immortalised murine microglia cell line was provided by Prof. Kenneth Smith (Department of Neuroinflammation, Queen Square Multiple Sclerosis Centre, Institute of Neurology, University College London, UK) and cultured in Dulbecco's Modified Eagle Medium-GlutMax (DMEM, Gibco, Life Technologies) supplemented with 10% Foetal Bovine Serum (Gibco), 1% Penicillin/Streptomycin [10,000 units/ml Penicillin, 10,000 µg/ml Streptomycin] (ThermoFisher, UK) and cultured at 37°C in a fully humidified chamber with 5% CO₂. Unless otherwise specified, all cultured cells throughout the study were incubated under the atmospheric and temperature regulated conditions as stated above. Cells were seeded on twelve-well plates at a density of 0.3×10^6 with media changes every two days. An optimal confluency of 80-90% was reached before the media was replenishment and incubation with selected treatment began. All stimulation and treatment variants were carried out on cultures between P19-P25.

2.8.4 Simvastatin stock preparation

Cells were treated with Simvastatin (1 µM) where specified. Briefly, Simvastatin was dissolved in DMSO (100%) before diluting further in PBS to achieve the final concentration. All control samples were treated with vehicle containing PBS and 0.01% DMSO. Doses used in the study are closely related to blood levels of Simvastatin found in patients treated with up to 80 mg daily (Bonn et al., 2002). This assumes complete absorption of Simvastatin in a 70 kg man with a blood volume of 5 L.

2.8.5 In vitro cell stimulation and simvastatin treatment

Simvastatin was added to the respective culture medium (1 µM) with or without the simultaneous addition of LPS, TNFα or IFNγ, either together or individually for various lengths of time (2-120 hours).

For Simvastatin incubation periods spanning four or five days, cell line specific media was replenished daily to restore optimal growth conditions, alongside any treatment included in the original medium.

2.8.6 SDS-PAGE and Western Blot

Cells were lysed in 80 μ L (12-well plates) or 160 μ L (6-well plates) RIPA buffer containing protease and phosphatase inhibitors (Sigma, UK), centrifuged for 5 min at 13.1 g. 20 μ L of supernatant was then collected and mixed with 80 μ L of sample buffer containing DTT, boiled for 5 min and centrifuged again before loading. If samples were not used immediately, they were frozen at -20°C for short-term storage or -80°C for long term. BCA assay was used to determine protein concentration and 15 μ g loaded onto an 8-10% resolving/ 5% stacking SDS-PAGE gel or 4-12% NuPAGE™ Novex pre-cast gel and run in NuPAGE™ MOPS running buffer for 90 min at 160 V.

After which, gels were ran overnight at 4°C for wet transfer of proteins onto a nitrocellulose membrane. Membranes were blocked using 5% milk in PBS-Tween (0.1%). Primary antibodies (iba-1, WAKO; CD68, BD; HSC-70, Sigma) were incubated at 4°C overnight in 5% BSA. Membranes were washed several times then secondary antibodies added and incubated in 5% BSA for 1 hour at room temperature. Membranes were developed using ECL (Abcam) as per manufacturer's instructions. All western blots were probed for the housekeeping gene HSC-70, which was used in normalisation for protein quantification. Protein concentrations were determined using a standard Bradford assay.

2.8.7 Nitrite + nitrate measurements.

To measure nitric oxide concentrations in the culture medium, supernatants were assayed for NO₂⁻ + NO₃⁻ levels using a commercially available Griess reagent detection kit (Promega, Wisconsin, USA), as per the manufactures' instructions and measured at 550nm using a spectrophotometer.

2.8.8 Bradford assay

Protein concentrations were quantified using the Bradford Assay (Bradford, 1976). Using a commercially available kit with Coomassie blue dye (Quick start Bradford protein assay, Bio-rad), a standard curve of known bovine serum albumin concentrations were used to compare test samples. Briefly, protein binding induces a colour change from red to blue that can be quantified using a spectrophotometer at wavelength 595 nm.

2.8.9 *In vitro* RNA isolation

To analyse differences in mRNA transcript expression, RNA was isolated from BV2 cells using RNeasy mini kit (Qiagen, Venlo, Netherlands) following the manufacturers protocol. First, adherent BV2 cells were homogenised in culture plates using RLT lysis buffer. Each sample was then passed through a Qiagen spin column and equal volume of 70% ethanol was added to the lysate to provide ideal binding

conditions. After this, a series of buffers and centrifugation steps were carried out, according to instructions followed in the commercial kit, to purify the RNA. Finally, the RNA was filtered and eluted with RNase free water. All RNA concentrations of the freshly isolated RNA were taken immediately to a Nanodrop Spectrophotometer (Thermo Scientific, MA, USA) to determine the concentration at 260 nm absorbance.

2.8.9.1 QUANTITATIVE REVERSE-TRANSCRIPTION PCR (RT-QPCR)

Relative mRNA expression was analysed using two-step real time qPCR techniques. First, freshly isolate RNA must be converted to cDNA by reverse transcription. Here, the cDNA synthesis kit (Bioline, London, UK) was used according to the manufactures' instructions with cycling conditions set as follows: PCR protocol (x1 cycle); Reverse transcription 30 min at 42°C RTase inactivation 5 min at 85°C. Following this, the transcribed cDNA (10 ng) was added to a master mix consisting: SYBER green (2x SYBR Green Power Up Select Master Mix, ThermoFisher), dNTP's, DNA polymerase, primers (200 nM) (Table 2.9). After this, PCR plates were run on Applied Biosystems (Life Technologies, CA, USA) QuantStudio 7 Flex Real-Time PCR System. Amplification curves were generated through the detection of SYBR green fluorescence and a fluorescence threshold (Ct) was set during the exponential phase of the curve. Experimental gene Ct values were calculated according to the $\Delta\Delta C_t$ method. Briefly, gene of interest Ct values are normalised against our GAPDH housekeeper. Relative gene expression was then compared to the expression in the control sample. The full equation can be found below:

$$\text{Relative Expression} = 2 - ((C_t \text{ Test Gene} - C_t \text{ Housekeeper}) - \Delta C_t \text{ Control Sample})$$

House-keeper genes were selected after testing in the biological samples for stability and treatment differences. The house-keeper selected for these set of experiments was GAPDH.

2.8.9.2 RT-QPCR PRIMERS

Primers	Sequence
iNOS F:	CAGCTGGGCTGTACAAACG
iNOS R	ATGTGATGTTTGCTTCGGA
GAPDH F	AACTTTGGCATTGTGGAAGG
GAPDH R	ACACATTGGGGGTAGGAACA

Table 2.9. Primers used for RT-qPCR protocol

Chapter 3

Simvastatin therapy: Microglia and excessive reactive nitrogen species production

3.1 Aims

1. To establish a suitable *in vitro* inflammatory model for the generation of reactive oxygen and nitrogen species in sufficient levels to cause cellular damage.
2. To determine whether chronic simvastatin therapy can provide protection against the production of excessive amounts of reactive nitrogen species.

3.2 Hypothesis

Chronic simvastatin pre-treatment is capable of attenuating reactive nitrogen species production in an activated microglial cell line.

3.3 Results

3.3.1 Nitric oxide production in BV2 microglial cell line

It is well-established that pathologically elevated levels of nitric oxide lead to the development of oxidative stress. In tissues under continuous pathological strain, abundant levels of nitric oxide can react with the free radical superoxide anion to form peroxynitrite. This molecule contains powerful oxidizing properties that are capable of damaging an array of macromolecules within cells, including DNA, lipids and proteins (Burney et al., 1999) (Murphy et al., 1998) (Beckman and Koppenol, 1996) (BECKMAN, 1994).

To establish an *in vitro* model system, we stimulated the immortalized murine microglial cell line, BV2, with our selected proinflammatory mix of LPS (5 $\mu\text{g}/\text{ml}$), IFN γ (100 units/ml) and TNF α (10 ng/ml) and measured the production of NO. The presence of nitric oxide in supernatant, harvested at the time points indicated, was determined using a commercialised Griess assay kit which converts all stable by-products of nitric oxide in the sample to nitrite (Griess, 1879). Following treatment with the mix of inflammatory molecules we observed a striking upregulation of NO. Control cells incubated with normal media generated small amounts of nitric oxide. By comparison, BV2 cells exposed to our proinflammatory cocktail demonstrated a striking upregulation of NO production when measured at 24-, 48-, 72-, 96- and 120-hours post incubation. The production of nitrites peaked at 48- hours, yielding a maximum concentration of 50 μM before reaching a plateau. At all subsequent timepoints thereafter, similar levels of nitrites were maintained (Figure 3.1B).

Associated with the increased level of NO we also observed morphological changes to the cells. Prior to receiving a stimulus, the BV2 cells exhibited a more ramified morphology, consistent with a non-activated state. After exposure to the proinflammatory mix, there was notable change to BV2 cell morphology. The cells rounded and appeared more amoeboid overall, displaying a large granular and foamy cytoplasm that is indicative of ingested products. These features are characteristic of (Reese and Karnovsky, 1967) classically activated phagocytic microglia (Figure 3.1A) (Suzumura et al., 1991) (Smith, 2001, Suzumura et al., 1990) (Nimmerjahn et al., 2005). Quantitative analysis of these cell changes are shown in Figure 3.5.

To corroborate these findings, the most prominent morphological changes occurred at 48 hours post-incubation. Consequently, all incubation periods going forwards were carried out up to 48-hours. Indeed, the 24-hour incubation period did yield a considerable amount of nitric oxide when compared to the control cells, although negligible differences were observed regarding cell morphology.

LPS is known to induce cell death above certain concentrations. Therefore, it was important to test whether our selected stimulation mix could initiate such responses (Sarih et al., 1993). To investigate this, the trypan blue exclusion assay was employed for identification of dead cells within the treated

population (Figure 3.1C). When quantified, no significant differences in cell death were observed between control and inflammatory stimulated cells at the 48h timepoint (Figure 3.1D).

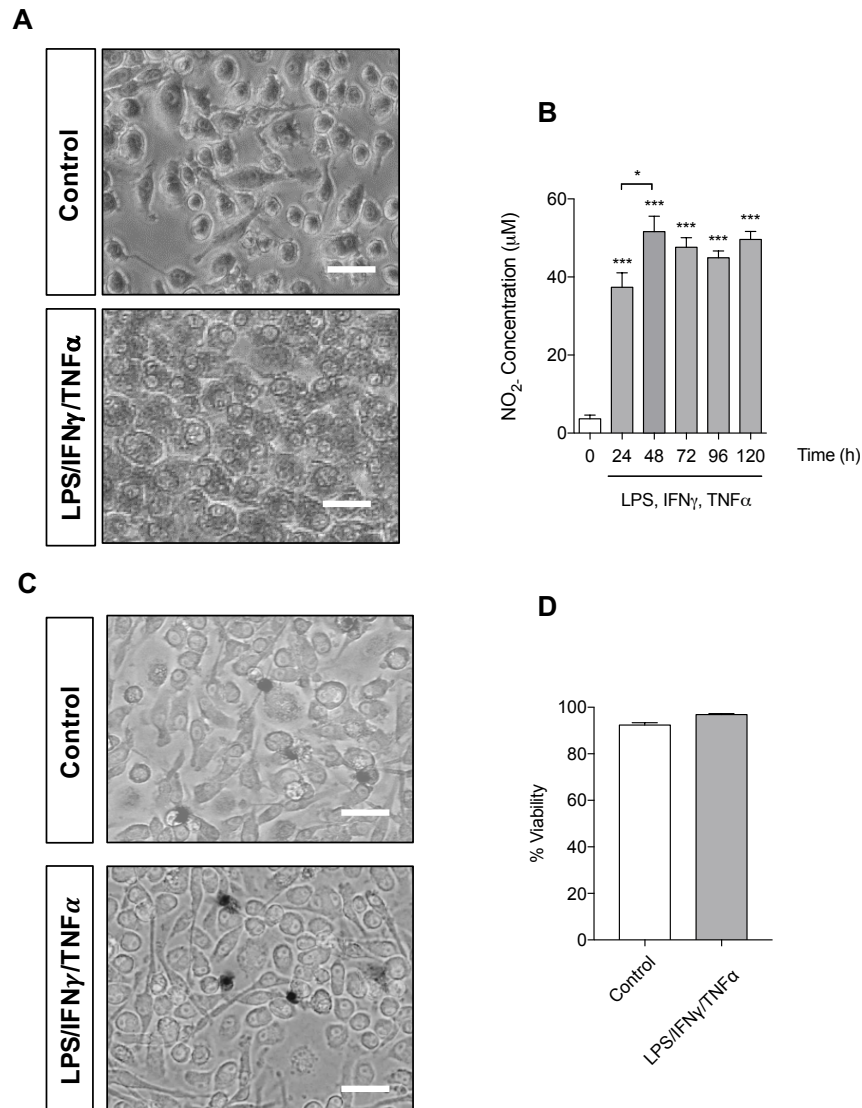


Figure 3.1. Nitric oxide induction in the BV2 cell line. Cells were stimulated with a mix of LPS (5 μ g/ml), IFN γ (100 units/ml) and TNF α (10ng/ml) for various lengths of time, 0, 24, 48, 72, 96 and 120 hours. After incubation, Griess assay was used to measure the concentration of nitrites in the supernatant. (A) Representative images of BV2 cells after 48 hours incubation with culture media or proinflammatory mix. Nitrites concentration (μ M) in the supernatant after 24, 48, 72- and 96-hours incubation with the proinflammatory mix (B) n=4 per treatment group. (C) Representative micrographs of trypan blue exclusion assay taken 48 hours, incubated in either normal culture medium or stimulation medium. (D) Quantification of viable BV2 cells present *in situ* after 48 hours normal culture or stimulation medium. Mean \pm SEM (*, P<0.05) (***, P<0.0001). Statistical significance determined using one-way ANOVA, coupled with Dunnet's post analysis. Scale bar = 10 μ M.

3.3.2 *In vitro* characterization of an activated microglial cell

Microglia are highly heterogenous, plastic in nature and capable of displaying a variety of phenotypes. Detection of cytokine expression profiles, surface receptors and cellular morphology can be used collectively to characterize a particular phenotype at any one time (Hanisch and Kettenmann, 2007) (Hanisch, 2013).

A surface marker commonly used to define microglia is the Ionized calcium-binding adapter molecule 1 (iba1). This highly expressed protein can identify both macrophage and microglial cells (Ito et al., 1998). Here, we used a western blot technique to probe protein lysate for the presence of iba1 in our BV2 cells. Under stimulated and non-stimulated conditions, semi-quantitative analysis showed similar levels across groups (Fig 3.2A and B).

A classic hallmark of activated microglia is expression of the cell surface receptor CD68, which in higher levels is typically associated with polarization towards the proinflammatory M1 phenotype (Waller et al., 2019). To establish the levels of expression of CD68 in BV2 cells under different conditions, protein lysates were prepared for western blot analysis. Unlike Iba1, a significant difference was observed in the stimulated cell population when compared to the control group (Figure 3.2C).

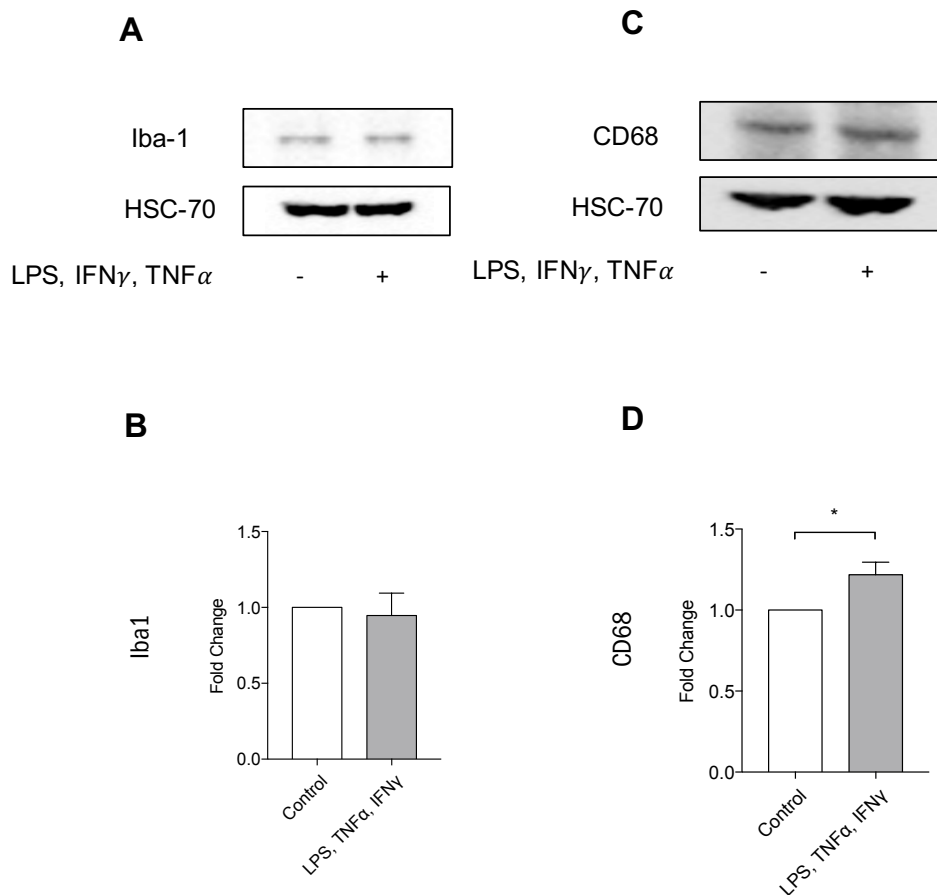


Figure 3.2. In vitro characterisation of an activated microglial cell line. BV2 cells were incubated with normal control media or a pro-inflammatory mix of LPS (5 μ g/ml), IFN γ (100 units/ml) and TNF α (10ng/ml) for 48 hours. Protein lysate for each condition were prepared for western blot and probed using anti-CD68 and anti-Iba1. (A) Representative immunoblot of prepared protein lysate derived from BV2 cells, probed for Iba1 and housekeeping HSC-70 with and without exposure to the stimulatory mix. Densitometric quantification performed from at least three independent experiments. (B) Quantitative analysis of BV2 cells expressing levels of Iba-1 in control media and pro-inflammatory mix. (C) Representative immunoblot of BV2 lysate with or without exposure to pro-inflammatory mix probed for CD68 levels and housekeeping HSC-70. (D) Densitometric quantification derived from at least three independent experiments. A significant upregulation observable in the stimulated group when compared with control. Results expressed as mean \pm SEM. Statistical analysis determined using uncoupled, students t-test. (*, $P < 0.05$).

3.3.3 Effect of simvastatin treatment on BV2 cell viability in culture

In previous studies, the statin family of drugs have demonstrated a concentration dependent effect on cell death. Here, the trypan blue assay was used to evaluate BV2 cell viability *in situ* after exposure to simvastatin, either in isolation or with the combined effect of LPS, IFN γ and TNF α . At the maximum incubation period of 120 hours with simvastatin only (1 μ M), no differences in BV2 cell viability were observed when compared to the untreated control (Figure 3.3A and B).

Next, the trypan blue assay was used to determine the combined effects of simvastatin (1 μ M) and our selected proinflammatory mix on BV2 cell viability. Incubations with simvastatin were carried out at 2h, 48h and 120 hours before exposure to stimulus for an additional 48 hours. Quantitative analysis revealed no significant differences between treatment groups. Therefore, none of the stipulated combinatory treatment strategies had an effect on cell viability. It is for these reasons that the stated experimental parameters were kept at a constant going forwards to allow for further interrogation (Figure 3.3C and D).

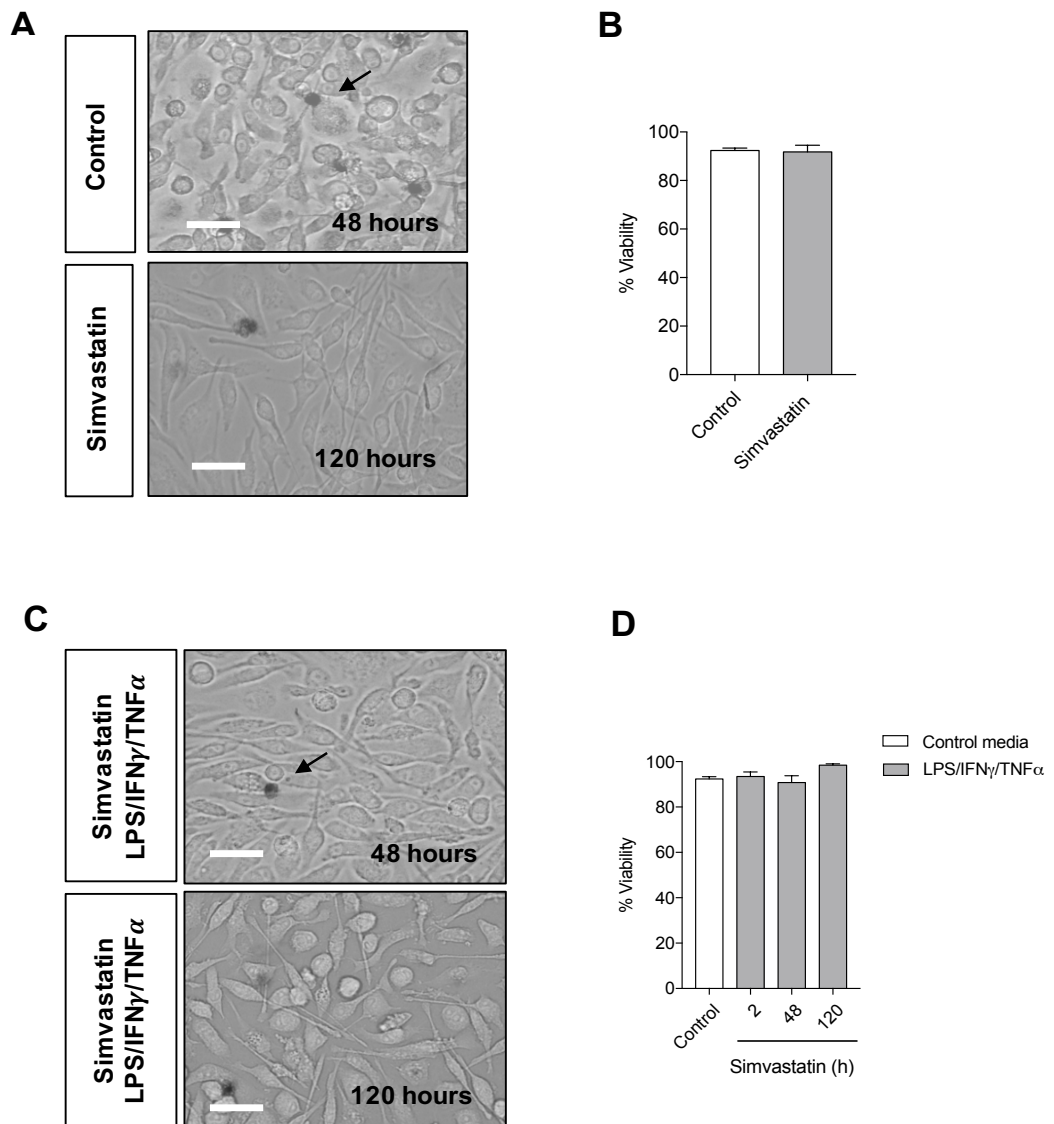


Figure 3.3. BV2 cell viability in situ. BV2 cells grown in culture and exposed to control media or a mix of LPS, IFN γ , and TNF α with or without Simvastatin (1 μ M) pre-treatment were assayed with trypan blue for percentage cell viability. (A–C) Control, LPS, IFN γ and TNF α incubation for 48 hours, Simvastatin (1 μ M) alone for 120 hours, Simvastatin (1 μ M) pre-treatment for 48 hours before incubation with LPS, IFN γ and TNF α for 48 hours and Simvastatin (1 μ M) pre-treatment for 48 hours before exposure to LPS, IFN γ and TNF α for 48 hours. Values presented as mean \pm SEM, n=3 per group. Statistical significance determined using students t-test. Black arrows depicting trypan blue cell uptake. Scale bar = 10 μ M.

3.3.4 Chronic simvastatin pretreatment attenuates nitric oxide production in a microglial cell line

To investigate the capability of simvastatin to attenuate nitric oxide production in a neuroinflammatory environment, BV2 cells were pre-treated with simvastatin ($1\mu M$) for various lengths of time (2, 6, 24, 48 and 72 hours) before exposure to the proinflammatory cocktail comprising; LPS ($5\mu g/ml$), IFN γ (100 units/ml) and TNF α (10 ng/ml) for a total of 48 hours. To evaluate the effect of simvastatin pre-treatment on nitric oxide production in this model system, supernatant was harvested from cultured cells and nitrite levels, as an indirect marker of nitric oxide production were quantified using the Griess assay.

As we have previously shown (Figure 3.1B), the maximum levels of nitric oxide produced using this protocol was found to occur at 48 hours. Therefore, we treated BV2 cells with the inflammatory mix for 48 hours which resulted in a similar significant induction of nitric oxide formation (Figure 3.4). With prior simvastatin treatment at 2-, 6- and 24-hours the levels of nitric oxide did not differ from those cells that were treated with the inflammatory mix alone. However, with prior simvastatin incubation at the 48- and 72-hour timepoints, a significant inhibition of production was observed. Production of nitrate-nitrite levels were normalised to total protein in the sample using the Bradford assay for quantification.

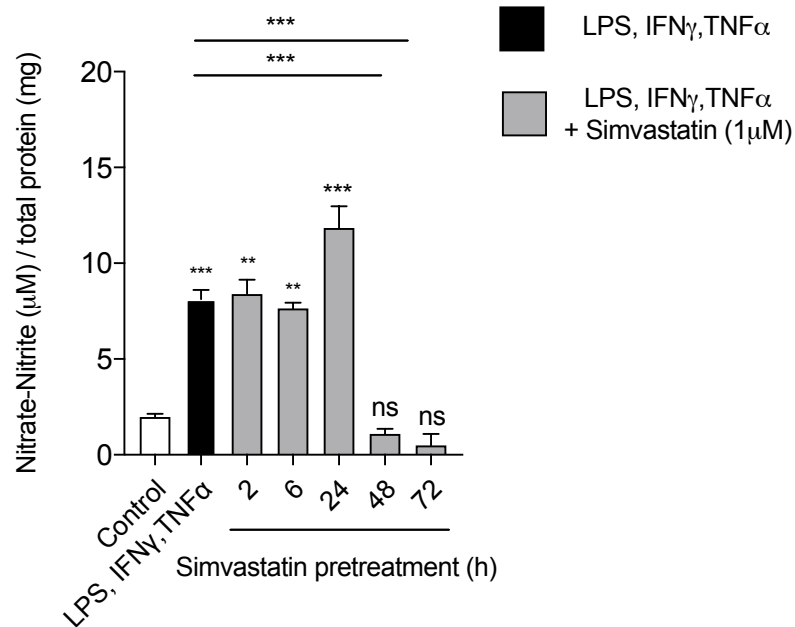


Figure 3.4. Simvastatin pretreatment attenuates nitric oxide production in BV2 cell line at incubations above 24 hours. BV2 cells in culture were grown in either normal control medium, stimulatory mix of (LPS, IFN γ and TNF α) alone for 48 hours or in combination with simvastatin pretreatment at 2h, 6h, 24h, 48h and 72 hours prior to receiving stimulation. Supernatant harvested and screened for nitric oxide production using the Griess assay. Protein levels (mg) were determined for each sample and used to normalize to nitrite-nitrate levels. Values presented as mean \pm S.E.M, n=4 per group. Statistical significance determined using one-way ANOVA coupled with Dunnett's post test. * $p < 0.05$; **, $P \leq 0.01$; *** $p < 0.0001$. ns = no significance.

3.3.5 BV2 microglial cell response to a proinflammatory mix

After exposure to our selected proinflammatory mix, BV2 microglia cells not only demonstrated striking biochemical changes (Figure 3.6) but also morphological transformations (Figure 3.5A). These induced changes represented a move away from the ramified, resting state of microglia and towards a rounded, activated phenotype. Typically, the classically resting microglia displays many branched processes that project from the cell body. When activated, microglia take the form of an amoeboid-like structure. Visually, the main features of activation are mostly round in shape with a large cell body and short, thick or often absent processes. These changes were quantified according to the presence/absence of processes present in the sampled field of view and categorised into amoeboid (rounded) or bipolar/ramified (containing two or more processes).

Control BV2 cells grown under normal culture conditions mainly exhibited a rounded morphology with few or absent processes characteristic of an activated phenotype. Only the occasional ramified or bipolar cell were observed amongst this population and accounted for less than 40% of the total sample (Figure 3.5C). This suggests that BV2 cells cultured under normal conditions exhibit a morphology similar to those observed in a typically activated state. However, after 48 hours exposure to our proinflammatory mix, the percentage of amoeboid cells present in the sampled fields, increased to over 80% (Figure 3.5B). Hence, the majority of cells sampled appeared rounded, large and amoeboid in shape. To corroborate these morphological changes, a striking 10-fold increase in nitrite-nitrate concentration over control conditions were observed in the same cell population, indicating that the activation state – as determined morphologically – often reflects its biochemical status. Whilst at first glance control cells appear round in morphology, the lack of nitrite-nitrate in the supernatant does not validate these observations (Figure 3.1B). Furthermore, upon closer inspection of the stimulated BV2 cultures, a foamy or granular cytoplasm can be seen. This type of microglia structure generally reflects ingested material from the local microenvironment which is suggestive of phagocytic microglia.

After a 120-hour incubation with simvastatin alone, another marked change in morphology was observed. Statin treatment resulted in a shift towards a classically resting and ramified morphology, with many prominent processes. Bipolar cell types were a dominant feature of this population, as demonstrated by two major branching projections. Consistent with this resting state, we also detected low levels of nitrite-nitrate production (Figure 3.1B).

Having demonstrated that our proinflammatory mix dramatically increases nitric oxide production in the BV2 cell line and maintains an activated amoeboid morphology, next, we set out to ascertain whether simvastatin treatment was able to reverse this phenotype. BV2 cells that had been pre-treated for 120-hours with simvastatin were exposed to our proinflammatory mix for a further 48 hours. Simvastatin pre-treatment not only induced a resting microglia phenotype but critically, was also able to restore nitrite-nitrate production back to control levels (Figure 3.5; 3.1B).

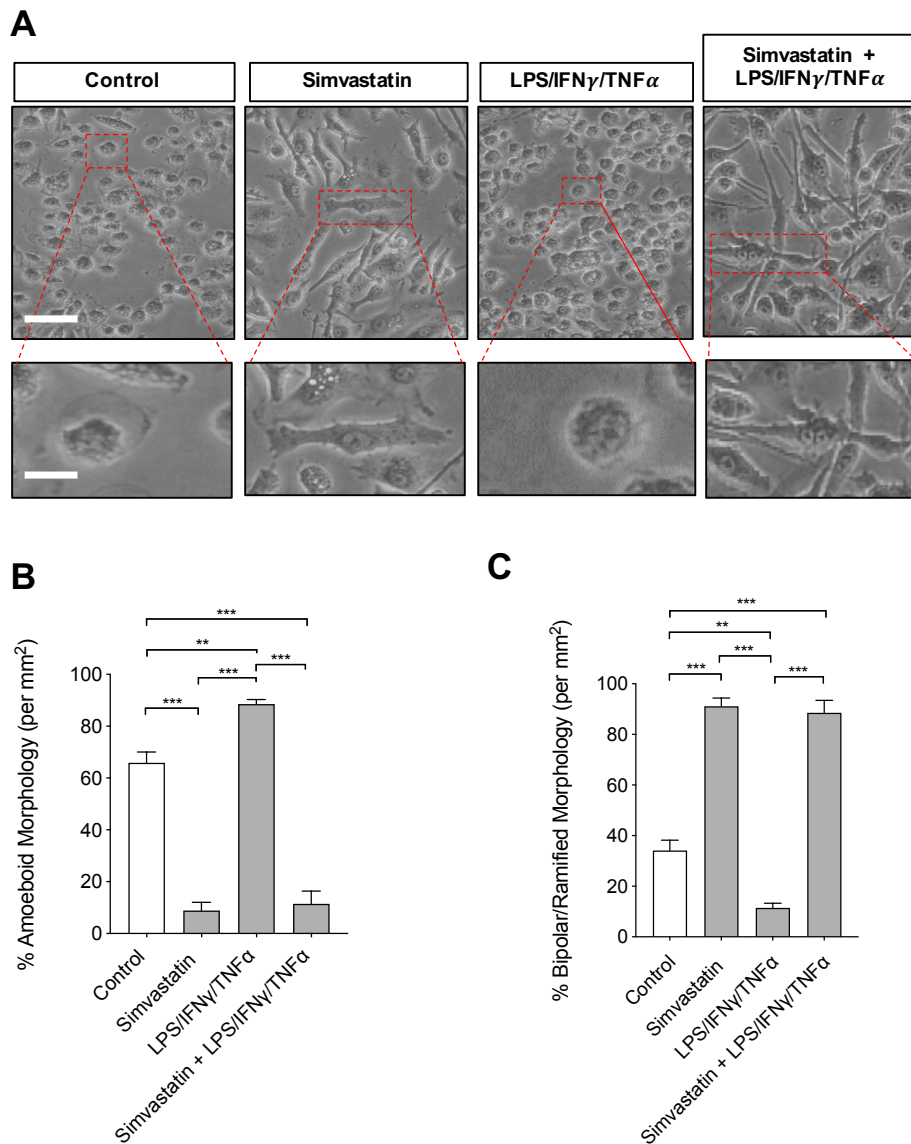


Figure 3.5. Simvastatin treatment at 120 hours alters cell morphology in a microglial cell line.

BV2 cells were incubated in normal media or pre-exposed to Simvastatin ($1\mu\text{M}$) for 120 hours before receiving a mix of pro-inflammatory cytokines; LPS, IFN γ and TNF α for 48 hours. (A) Representative micrographs of cells in culture, receiving normal media, Simvastatin alone for 120 hours, pro-inflammatory mix alone for 48 hours, or Simvastatin and pro-inflammatory mix in combination. (B) Quantification of amoeboid shaped cells, percentage of total cell population per mm². Treatment conditions compared between control media, Simvastatin alone ($1\mu\text{M}$), pro-inflammatory mix alone and both conditions in combination. (C) Quantified bipolar and ramified morphological changes, between treatment groups. Mean values presented \pm SEM. Statistical significance determined using a one-way ANOVA and Tukey's post-test. $n=3$ per group. (**, $P\leq 0.01$), (***, $P<0.0001$). Scale bar (top) = $50\mu\text{M}$. Scale bar (bottom) = $20\mu\text{M}$.

3.3.6 Preliminary data: The effects of simvastatin pretreatment on inducible nitric oxide synthase (iNOS) in microglial cells

The data obtained below was generated from a single experiment, therefore, extra caution should be taken when interpreting the results.

The inducible nitric oxide synthase protein (iNOS) is responsible for generating large amounts of nitric oxide in microglial cells (Colton et al., 1994) (Possel et al., 2000). The purpose of this experiment was to determine whether simvastatin pre-treatment influences the regulation of iNOS through modulation of either mRNA and/or protein. The microglial cell line, BV2, was exposed to our proinflammatory cocktail comprising: LPS, IFN γ and TNF α for 48 hours, after receiving a pre-treatment of simvastatin for various periods of time. Quantitative PCR was used to examine levels of iNOS mRNA in the cells after these treatments. The proinflammatory mix alone upregulated iNOS mRNA levels, which is evidenced by a 25-fold increase over control cells. However, pre-treating BV2 cells with simvastatin did not attenuate the increase in gene expression (Figure 3.6A).

The amount of iNOS protein present in BV2 microglial cells under different conditions was measured, using western blot analysis. Results from this semi-quantitative technique revealed that iNOS protein levels appeared reduced with pre-treatment of simvastatin for 2, 48 and 120 hours when compared to the stimulated group (Figure 3.6B).

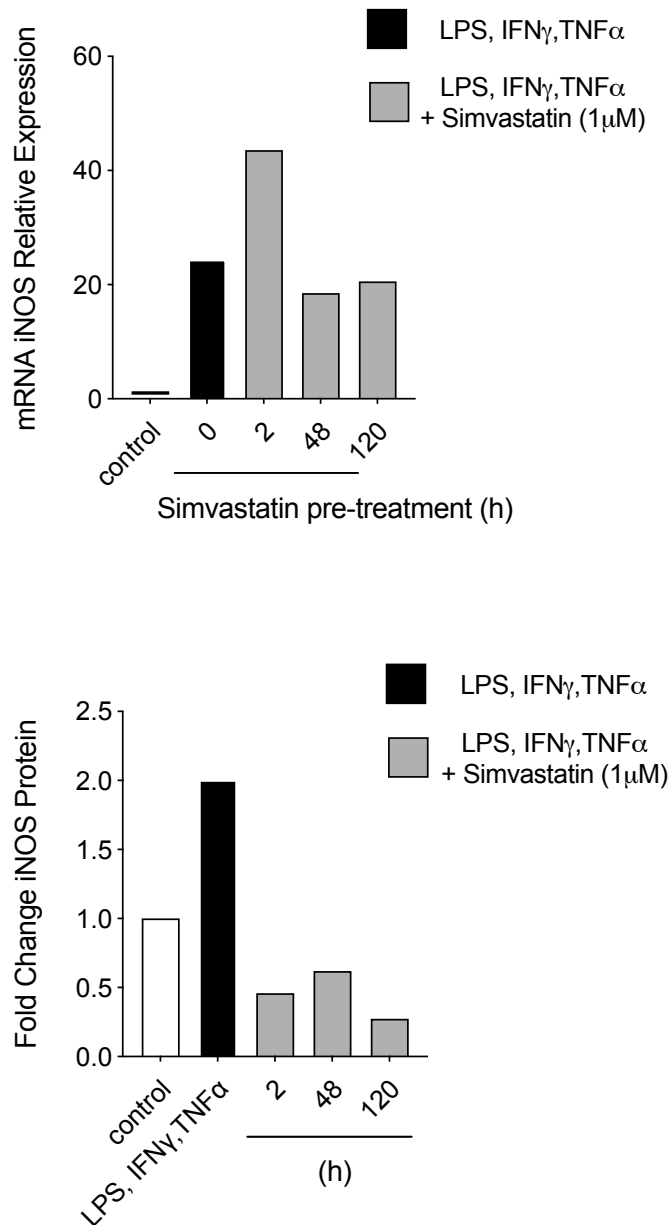


Figure 3.6. mRNA and protein levels of iNOS in simvastatin pre-treated BV2 cells.

(A) iNOS mRNA levels following simvastatin (1 μ M) pretreatment at 0, 2, 48 and 120 hours, before exposure to LPS, IFN γ and TNF α for 48 hours. n = 1-2 per group. (B) iNOS protein levels following simvastatin (1 μ M) pretreatment for 0, 2, 48, 120 hours, before exposure LPS, IFN γ and TNF α for 48 hours. n = 1 per group.

3.4 Discussion

The aim of this current study was to design a reliable and robust *in vitro* model of inflammation. One that would simulate an *in vivo* scenario pertinent to those observed in SPMS. For this, an activated microglial cell phenotype – representing prototypical proinflammatory signalling responses – was developed to allow us to interrogate the effects of simvastatin on microglial cell function, with a particular emphasis on NO responses. The findings revealed a unique ability of simvastatin to modulate microglial activation via reduction of produced NO. Additionally, simvastatin modified cell morphology to represent ramified resting microglia. Furthermore, the study provides supportive evidence that chronic, but not acute treatment with simvastatin is efficacious. The potential relevance of this finding to repurposing simvastatin for use in SPMS is discussed below.

3.4.1 *in vitro* production of nitric oxide by classically activated microglia

Sources of NO in the brain are multiple and vary across nervous tissues. However (Boje and Arora, 1992) and others identified iNOS in microglia as a major source of chronic exposure capable of inducing neurotoxicity (Bö et al., 1994) (Chao et al., 1996) (Brown et al., 1995). These destructive mechanisms have mostly been attributed to the NO reactive derivative, peroxynitrite. A substantial amount of evidence exists to support the cytotoxic potential of this powerful molecule toward oligodendrocytes and neurons (Dawson and Dawson, 1995).

The proinflammatory cocktail used here to initiate microglia activation, and subsequent polarisation to the M1 phenotype, comprised LPS, IFN γ and TNF α . This combination has previously been shown to induce the expression of iNOS in macrophages (Lambden, 2015). In selecting these proinflammatory stimuli, it was important to recapitulate a holistic immune response that would represent an *in vivo* scenario pertaining to MS. Typical proinflammatory responses are reflected as multi-faceted and characterised by elevated levels of cytokines and reactive species.

CNS pathologies are often subject to exaggerated TNF α responses (Mogi et al., 1994) (Fillit et al., 1991) (Feuerstein et al., 1994) (McCoy and Tansey, 2008) (Muhammad, 2019), with pleiotropic roles identified in stimulating vascular endothelium, proliferation of immune cells and tissue damage. TNF α is a potent promoter of the transcription factor NF κ B, often in MS patients, TNF α protein expression levels are reportedly higher in circulating serum samples, particularly at peak relapse and advanced disease as observed in SPMS (Obradović et al., 2012) (Vladić et al., 2002) (Selmaj et al., 1991) (Domingues et al., 2017). The importance of TNF α in MS disease pathogenesis is further underscored by the development of several generations of multiple blockade therapies, such as certolizumab, golimumab, etanercept and infliximab, which have been trialled for clinical use (Skurkovich et al., 2001) (Magliozzi et al., 2018) (Kemanetzoglou and Andreadou, 2017) (1999) (Van Oosten et al., 1996) (Mausner-Fainberg et al., 2015).

The microglial cell bears receptors for both TNF α (Bruce et al., 1996) and IFN γ . While it has been reported that IFN γ alone is capable of inducing iNOS expression, when used together, these two proinflammatory mediators have been shown to act synergistically to activate microglial cells via the CD40 pathway, a key cellular event in MS pathology (Chester et al., 1998) (Koide et al., 1994) (Tan et al., 1999). In addition to this, aggregation of CD4+ and CD8+ cell infiltrates at the site of active lesions have been shown to localise with expression of both TNF α and IFN γ (Gardner et al., 2013) (Cannella and Raine, 1995). These elevations are reflected in the protein expression profiles of SPMS cases using serum screened samples. These patients also demonstrate higher frequency of lesions in MRI, correlating with higher TNF α and IFN γ (Magliozzi et al., 2018), which underscores their important involvement in the pathophysiology and progression of disease (Kallaur et al., 2017). Stimulation of microglia by IFN γ and TNF α *in vitro* induces the production of proinflammatory mediators and a change in functional behaviour. Accordingly, there is an increase in MHC presentation (Frei et al., 1987), adhesion molecule expression (Sébire et al., 1993), phagocytosis and cytokine production (Merrill and Benveniste, 1996). Of particular relevance to our study is NO upregulation, a concept that has remained a central theme throughout this thesis (Merrill et al., 1993). Moreover, these two cytokines have been recognised during the initiation and maintenance of glial activation in Parkinson's disease – a neurodegenerative disorder sharing many pathological aspects with SPMS (Le et al., 2001).

Whilst aiming to simulate a pathological CNS environment, it is equally important to achieve full activation potential of the mechanisms responsible for reactive oxygen and nitrogen species production within microglia. (Murphy et al., 1993) demonstrated that LPS alone can reliably and rapidly induce iNOS upregulation in glial cultures (Ransohoff and Perry, 2009). However, it has been shown that a combinatory effect with TNF α and IFN γ results in strong potentiation of NO production and more accurately captures neurotoxic effects (Papageorgiou et al., 2016), particularly in the BV2 cell line as used here (Gresa-Arribas et al., 2012) (Nayak et al., 2014). Therefore, LPS was included in the proinflammatory mix in our study. This bacterial cell wall component is known for its ability to potently shift microglia towards the classical state of activation and representative of an M1 phenotype.

LPS is typically known to bind to TLR4 and activate NF κ B signalling (Hoshino et al., 1999). The TLR-4 receptor is a pivotal contributor to microglial cell responses to endotoxins, endogenous HSP and endogenous ligands HMGB1, amongst others. LPS induced-TLR4 activation is frequently used to model aspects of brain conditions where TLR4 stimulation occurs. Indeed, TLR4 expression has been documented in CSF mononuclear cells of MS patients (Andersson et al., 2008). In addition, LPS has been used previously as a powerful tool for modelling an array of *in vitro* neurodegenerative disorders such as ALS, Parkinson's, Alzheimer's, to name a few (Lehnardt et al., 2003) (Lehnardt et al., 2002). Due to this widespread use, many groups have gathered transcriptional profiling data according to the combinations of

proinflammatory molecules use here (Martinez et al., 2006) (Martinez et al., 2008). For us, this will make future mechanistic experiments easier to map and unpick. It is important to note that signalling pathways common to all three proinflammatory molecules work independently to drive the induction of NFκB translation to the nucleus. This process is said to be essential for inducing iNOS expression. Collectively, the selected mediators operate to increase the likelihood of successfully achieving high levels of iNOS stimulation (Eberhardt et al., 1998) (Spink et al., 1995) (Taylor et al., 1998) (Teng et al., 2000) (Xie et al., 1994).

After stimulation, our *in vitro* inflammatory model yielded over 50μM NO production at the 48-hour time point (Figure 3.1B). Much of the current literature surrounding BV2 modelling of neuroinflammation is in line with this, as others achieved similar stimulatory potential (Gresa-Arribas et al., 2012) (Sheng et al., 2011). The Griess assay – used here – is routinely adopted as an indirect measure of NO production levels. Specifically, the technique quantifies stable end products of NO degradation, nitrite and nitrate, that can be measured in a variety of biological samples as an indication of NO status. There are obvious limitations when sampling specific *in vivo* tissues only, as this may not be an accurate representation of whole-body NO composition. Nevertheless, the Griess assay continues to be used routinely both *in vitro* and *in vivo* as an index of NO production whilst newer, more sophisticated methods remain under development.

In addition to its undeniable roles in pathophysiology, it is important to reiterate that over the past two decades, NO has been recognised for its pivotal roles as a biological messenger in a multitude of physiological processes (Whittle, 1995) (Picón-Pagès et al., 2019). The dual nature of iNOS-induced NO has been examined extensively across countless *in vitro* and *in vivo* platforms, resulting in a unanimous agreement that the harmful vs. beneficial outcomes observed with NO is unquestionably concentration-dependent (Colasanti and Suzuki, 2000). However, NO quantities are notoriously difficult to accurately measure for a number of reasons. This free radical has a very short half-life and several natural scavengers exist within the circulation that render concentrations variable. Hence, *in vitro* demonstrations are difficult to extrapolate *in vivo*, further to this, is the debate on whether it is reasonable to attempt to extrapolate at all, given the rather limited repertoire of measurement tools available at present. Despite these difficulties, a significant breakthrough by Smith et al., (Smith et al., 2001) reported axonal conduction block *in vivo* with as little as 1μM NO, in addition to a strong concentration dependent effect (1-10μM). This is particularly relevant to SPMS as axonal degeneration is a key cause of permanent disability. Studies such as these, have provided benchmark concentrations that allow us to determine what might be biologically relevant to physiological or pathophysiological processes. As such, it has been postulated anything above 15μM is expected to be outside of the physiological range. This finding supports the notion that our model of inflammation yields sufficiently large quantities of NO to cause oxidative tissue damage *in vivo*.

When exposed to the proinflammatory mix for 48 hours, the BV2 microglia cells demonstrated increased levels of CD86 activation marker (Sato et al., 1995). A considerable amount of literature has reported on the characteristic features of classically activated M1 microglial cell phenotypes. The upregulated expression of both CD86 combined with elevated NO production confirms this activation state. Taken together, these data points towards the development of an *in vitro* inflammatory model system that recapitulates many aspects of an *in vivo* scenario in SPMS. Furthermore, stimulation of a NO response in the microglial cell line allows us to directly investigate the effects of simvastatin on this free radical.

3.4.2 Simvastatin attenuates nitric oxide production of proinflammatory microglia *in vitro*

Much of the current literature investigating the therapeutic properties of statins and their potential in attenuating iNOS-induced NO production is mixed and alternative mechanisms of action have been postulated according to disease context. Pahan and colleagues (Pahan et al., 1997) were amongst the first group to demonstrate a 90% inhibition in LPS-induced upregulation of iNOS with lovastatin treatment at 24 hours, assessed in rat primary macrophages and astrocytes. In contrast to this, an increase in iNOS mRNA and protein levels in a smooth muscle culture treated with Fluvastatin has been reported by (Chen et al., 2000). Similarly, in endothelial cells, Wagner (Wagner et al., 2002) found a significant reduction in iNOS gene expression, attenuated by atorvastatin, cerivastatin and pravastatin; when assessed independently. These differences were attributed to the lipophilic status of the statin selected. (Ikeda et al., 2001) and (Hamelin and Turgeon, 1998) provided evidence to support this hypothesis. Accordingly, the lipophilic statins – lovastatin and fluvastatin – were said to induce an upregulation in iNOS expression and subsequent NO increase in treated cardiac myocytes, whereas hydrophilic pravastatin did not. However, these findings directly contradict other studies (Madonna et al., 2005) where iNOS transcription was attenuated with lovastatin treatment in an identical cell type. The studies discussed here are merely to provide the reader with an overview of recent developments in the search for breakthroughs regarding the effects of the statin class of drugs on NO production. The large variability in findings is testament to the pleiotropic diversity of this family of therapeutics and to possible context-dependent effects.

Debate surrounding the ability of statins to behave as an antioxidant therapy continues. A lack of comprehensive studies published in this field, particularly those pertaining to statin metabolism and distribution in the brain, makes it difficult to draw definitive conclusions. Furthermore, most published data is centred around the beneficial effects of statins in the vasculature and their ability to rescue aspects of dysfunctional endothelial cells that would typically display reduced NO bioavailability (O'Driscoll et al., 1997) (Laufs et al., 1998) (Kureishi et al., 2000) (Lefer et al., 2001) (Wagner et al., 2002). Indeed, the ability of statins to increase NO levels in the context of atherosclerosis and cardiovascular diseases at large, has provided immensely valuable insights into statin cholesterol independent mechanisms. The beneficial effects of statins on the eNOS isoform include increasing localised NO concentrations through inhibition

of isoprenoid synthesis which are essential posttranslational lipid attachments important for GTPase signalling.

GTPases such as Rho are fundamental for initiating Rho/Rho-kinase (ROCK) mediated pathways that, under physiological conditions, lead to eNOS mRNA instability. Blocking this pathway results in an increase of eNOS derived NO and eNOS mRNA is stabilised for longer (Sawada and Liao, 2014) (Rikitake and Liao, 2005) (Yao et al., 2010) (Nohria et al., 2009) (Laufs and Liao, 1998) (Endres et al., 1998) (Yamada et al., 2000). It should be noted that increased concentrations of eNOS produced NO sit within the physiological range to improve blood flow. In direct contrast to this, is the iNOS isoform derived NO. This protein can stimulate the production of damaging levels of NO, sufficient to induce neurotoxicity.

Whilst there is an abundance of data to support the use of statins to provide vascular protection by increasing eNOS derived NO, statins are only just beginning to emerge for their putative neuroprotective effects. This can be explained by the dual role they seemingly exhibit on the different NOS isoforms, where transcription is said to be blocked through the isoprenoid pathway for iNOS (Lefer et al., 1999). The variability of study outcomes has heightened the need for further understanding in this field.

Our observation that there was a temporal effect of simvastatin on LPS, IFN γ and TNF α induced-NO production (Figure 3.6) corroborates a previous *in vitro* study which similarly demonstrated reduced levels of NO production with simvastatin treatment in a similar microglial cell line (McFarland et al., 2017). The strength of observed inhibition at the ≥ 48 h timepoints demonstrated a potent reduction; so much so that NO production levels returned to near-baseline control.

In our model system, reduced levels of NO were not observed until microglial cells had been exposed to simvastatin for 48 hours or more. This finding indicates that effective blocking of NO is unlikely due to inhibition of iNOS translation as this protein turnover has a half-life of 2-3 hours (Kolodziejcki et al., 2004) (Kolodziejcka et al., 2005) (Panda et al., 2005). It is conceivable, however, that RhoA dependent signalling may be influenced by simvastatin. The Rho family comprises three main subtypes: Rac1, RhoA and Cdc42. Previous studies have reported a downstream role for RhoA in TNF α signalling which precedes the activation of NF κ B (Shih et al., 2011) (Perona et al., 1997).

A key post-translational modification regulating the proper function of small GTPases is prenylation (McTaggart, 2006). This process involves the covalent attachment of either GGPP (for Rho, Rac and Cdc42) or FPP (for Ras) which is required for targeting and embedding in the cell membrane (Hooff et al., 2010) (Pechlivanis and Kuhlmann, 2006) (Ishibashi, 2011). Both GGPP and FPP are downstream products of the cholesterol biosynthesis pathway, hence, prenylation process can also be blocked by statins (Cordle et al., 2005). Moreover, the activation of RhoA by GGPP embedding in the membrane effectively signals NF κ B induction (Perona et al., 1997). Indeed, the RhoA- NF κ B interaction has shown to be important in cytokine-activated NF κ B processes. Activation of this transcription factor we know is

essential for iNOS induction. Further to this, NFκB has a half-life of 31 hours, which could go some way towards explaining the 48-hour timeframe required for NO blockade (Backlund, 1997), although experiments would need to be carried out using our model system in order to confirm this. It is important to note that NO quantities in all cases, were normalised to the amount of total protein present in the sample, as measured by the Bradford assay.

3.4.3 Simvastatin induces a resting microglia phenotype *in vitro*

It is important to recognise that our stimulated *in vitro* microglia represent a classically activated M1 macrophage-like phenotype (Figure 3.7A). Morphological features displayed an ameboid-like structure resembling end stage phagocytic cells or “glitter cells” that have been described in the literature for over a century (Merzbacher, 1907). When treating microglia with proinflammatory cytokines, they consistently induce cytoskeletal changes that visually represent large, round flat-shaped cells (Abd-El-Basset and Fedoroff, 1995) a finding that was replicated here. This activated phenotype fits with our desired outcome as it closely aligns with the predominant morphology type that clusters around the lesion edge in EAE (Bauer et al., 1994b).

Here, we uncovered a unique role for simvastatin in its ability to alter morphology in the microglial cell. Previously, simvastatin was reported to induce actin and intermediate filament cytoskeletal changes (Chubinskiy-Nadezhdin et al., 2017) (Trodden et al., 2016). The Rho family of GTPases (RhoA, Rac and Cdc42) have identified roles in regulating actin and microtubule dynamics. Rho effectors are implicated in the formation of stress fibers and focal adhesion which are composed of actin associated proteins such as myosin II, a molecule with a critical role in controlling actomyosin contractility (Etienne-Manneville and Hall, 2002) (Pellegrin and Mellor, 2007). In endothelial cells, stress fiber formation is indicative of activated phenotypes and encourages contraction mechanisms that lead to cell rounding. These events precede increased vascular permeability responses, paracellular gap formation and are key determinants of barrier dysfunction (Dudek and Garcia, 2001) (Hirata et al., 1995) (Garcia and Schaphorst, 1995). Similar to the endothelial cells, microglia display a rounded morphology when activated. While resting, ramified microglia are known to acutely move their processes without translocation of the cell body. On the other hand, ameboid cells move their entire cell body and require dramatic changes to cytoskeletal dynamics to enable migration through tissues. Previous studies performed on endothelial cells have identified statin-induced passive relaxation of the cytoskeleton. Myosin light chain (MLC) phosphorylation is a well-established key event in cell contraction and a process that depends on the activity of Rho GTPase (Amerongen et al., 2000) (Hernández-Perera et al., 2000). Indeed, statins have previously demonstrated potent inhibition of MLC phosphorylation, via the RhoA/RhoA kinase pathway (Ma and Ma, 2011) (Zeng et al., 2005). It is likely that similar mechanisms are at play in the microglial cell, although further study is needed to confirm this.

3.4.4 Regulation of NO production through iNOS gene and protein expression - preliminary data

Our results in Figure 3.6 demonstrated a significant reduction in microglia-produced NO with simvastatin treatment at ≥ 48 h. In this study, we attempt to delve into potential mechanisms underpinning this response. However, it must be reiterated that the data presented herein is preliminary (n=1) and so extra caution should be taken when interpreting the results.

To block the neurotoxicity observed in diseased tissues under threat of neurodegeneration and ensuing microgliosis, iNOS inhibitors have received much attention for their potential application in the treatment of MS. Indeed, iNOS inhibitors have proven to reduce neurological deficiency in models of EAE, particularly at the later stages (Okuda et al., 1998) (Zhao et al., 1996). iNOS targeting therapies continue to be an active area of research and many potent inhibitors have been discovered with high selectivity over related NOS isoforms. Despite the progress made, iNOS inhibition in EAE has generated variable and sometimes inconsistent results, depending on several factors such as the model system used and treatment regime, therefore, thorough investigation is imperative.

In microglial cells, iNOS expression is chiefly governed by transcriptional regulators. Due to this, we next investigated the mRNA levels of iNOS present in our model system. First, it was important to establish the mRNA iNOS response with our selected proinflammatory mix (LPS, TNF α and IFN γ). When comparing to the non-stimulated control cells we identified a sharp increase in iNOS mRNA after exposure to the proinflammatory mix at 48 hours. The detected upregulation of iNOS mRNA gave us confidence in that iNOS transcription had been switched on in this *in vitro* model system. When our BV2 cells were pre-treated with simvastatin, at all timepoints tested iNOS mRNA was still detectable at levels in line with the proinflammatory mix. Conversely, western blot analyses of iNOS BV2 cell lysates with proinflammatory stimulation produced detectable levels of iNOS, while simvastatin pre-treatment at 2, 48 and 120h appeared to reduce these levels.

Amongst the most recent data published on this topic, (Madonna et al., 2005) proposed a mechanism for simvastatin inhibition of iNOS. Treatment with simvastatin using embryonic cardiac myoblasts led to inhibition at the transcription level of the master regulator NF κ B. This mechanism was proposed to operate through the isoprenoid Rho kinase and I κ B/NF κ B. Similar cholesterol-independent effects were observed in macrophages using lovastatin, atorvastatin, fluvastatin and pravastatin (Huang et al., 2003). This mechanism was also supported by the work of (Hilgendorff et al., 2003) using human monocytes and a selection of the statin family, that displayed different levels of potency.

Generally, cytokines bind to receptors on the microglia surface and activate kinases that later initiate intracellular cascades of phosphorylation. These phosphorylation networks subsequently elicit biological effects through the activation of transcription factors such as NF κ B and STAT-1a. The activated domains of these transcription factors translocate to the nucleus and bind to the promoter region of the

iNOS gene to trigger transcription and subsequent iNOS protein synthesis. Responses such as these have been well characterised in the macrophage and different proinflammatory triggers are known to induce distinct signalling pathways. Major activators of NF κ B are LPS, and TNF α . Taking LPS binding to TLR-4 as an example, this complex comprises the TNF-receptor associated factor 6 (TRAF6) and upon activation leads to the induction of a kinase cascade involving mitogen-activated protein kinase kinase, (MEKK). This in turn, causes phosphorylation of both I κ B kinase (IKK) and subsequent phosphorylation of I κ B. I κ B is a ubiquitin that is degraded in the proteasome, this action causes the release of dimerised NF κ B. The NF κ B is then free to translocate to the nucleus and bind to the promoter region on the iNOS gene (Zhou et al., 2006).

By contrast, IFN γ dependent signalling activates the JAK/STAT-1 α pathways and the iNOS promoter region is occupied by the interferon regulatory factor 1 (IRF-1) for induced iNOS expression (Ganster et al., 2001) (Kleinert et al., 2003) (Dell'Albani et al., 2001). In mice, IRF-1 gene knock out studies showed altering the iNOS promoter led to reduced activity (Kamijo et al., 1994) (Martin et al., 1994). Besides these, the transcription factor CCAAT box/enhancer binding protein (C/EBP) has also shown to influence iNOS promoter induction (Eberhardt et al., 1998). Similar distinct signalling pathways for iNOS induction have recently been confirmed in the BV2 cell line (Shen et al., 2005). Understanding and delineating these distinct pathways adds scope for further exploration into the mechanism underpinning our observation of reduced NO levels in the microglial cell line with simvastatin treatment.

Furthermore, it is important to acknowledge that whilst iNOS transcriptional regulation has been distinguished for some time in macrophages. iNOS activity and function is also governed by a number of less characterised co-factors, namely: NADPH, FAD, FMN, and co-factor BH $_4$. Therefore, it is probable that, in this model system, simvastatin could potentially be reducing the activity of the protein.

3.5 Conclusion

Together, these results provide strong evidence that BV2 cell stimulation by our selected proinflammatory mix, has proven to be a suitable *in vitro* model system to further investigate the potential mechanism of simvastatin action on microglial cell activation. Exposing microglial-like cells to a combination of LPS (5 μ g/ml) IFN γ (100 units/ml) and TNF α (10 ng/ml) resulted in pathologically elevated levels of NO production. After pre-treatment with simvastatin at 48 hours and above, effective production of NO was blocked. The mechanism through which this occurs remains unknown, although, speculations involve interference with iNOS transcription and protein synthesis. Coupled with these findings, we discovered the unique ability of simvastatin to induce morphological changes in microglia to represent a resting phenotype. Thorough investigation is essential to understanding the distinct signalling

pathways involved in these transformations. Taken together, these results provide important insights into the potential application of simvastatin in dampening a chronically activated microglia response.

Chapter 4

Simvastatin therapy: Attenuating neurological deficit and oxidative stress markers in rMOG model of EAE

4.1 Aims

1. To determine the efficacy of chronic simvastatin therapy on the disease course of rMOG EAE.
2. To establish the effects of simvastatin therapy on known hypoxic markers in the inflamed spinal cord in rMOG EAE
3. To determine whether simvastatin therapy alters the morphological and/or functional state of microglia in EAE.

4.2 Hypothesis

Chronic simvastatin therapy in a model of multiple sclerosis is capable of disease attenuation and reduction in oxidative stress markers.

4.3 Results

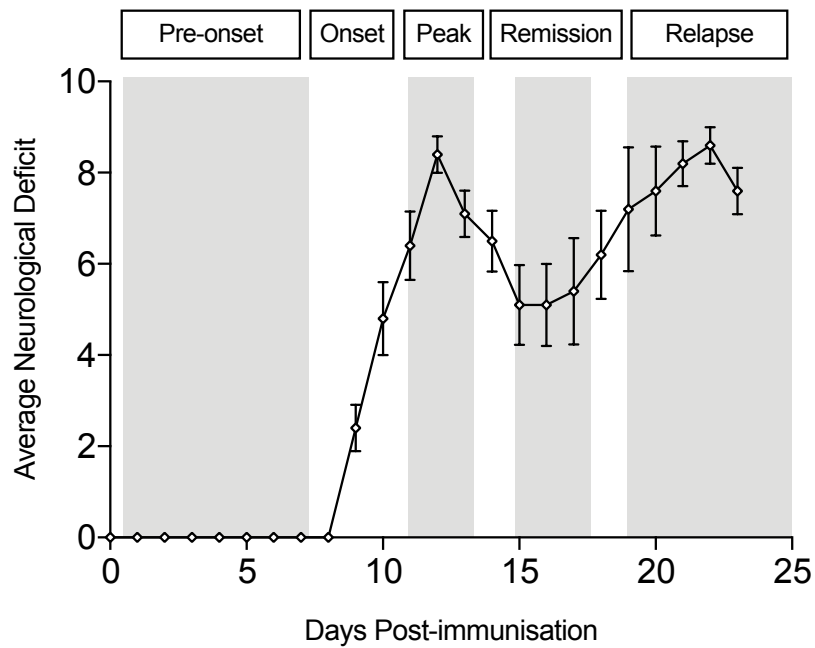
4.3.1 Clinical course of rMOG EAE and simvastatin administration

Subcutaneous injection of 100 µg rMOG in female Dark Agouti rats induced a relapsing-remitting disease, characterised by ascending motor paralysis. Typical disease onset was observed 9-11 days post-immunisation with an average incidence rate of 96%. The onset of clinical disease was coupled with a loss in body weight. The first week of disease manifestation varied in severity, with characteristics ranging from tail weakness to a complete flaccid paralysis of the lower limb extremities. The first peak of disease was followed by remission (15-17 days post-immunisation), presenting as improved motor function, either partial or complete. This remission phase was closely followed by an aggressive disease relapse (19 days post-immunisation). Spasticity and paralysis were dominant throughout which can be seen in our collated data (Figure 4.1A). Control animals exhibited no neurological disability or loss in body weight.

The first peak of disease has previously been well documented for having an extensive inflammatory component, characterised histologically by the infiltration of a large number of inflammatory cells, particularly concentrated within the white matter that at this stage is devoid of any obvious demyelination. In contrast, the relapse phase typically presents with extensive demyelination upon histological analysis (Davies et al., 2013a).

Simvastatin (~25 mg/kg) therapy was administered orally in the chow to a randomised cohort of subjects for a total of 15 days post-disease onset (days 9-11), as outlined in the materials and methods section. This chronic treatment strategy preceded the first peak of disease before continuing into the aggressive relapse phase. The experimental endpoint at 23 days post-immunisation represented a chronic diseased state (Figure 4.1B.)

A



B

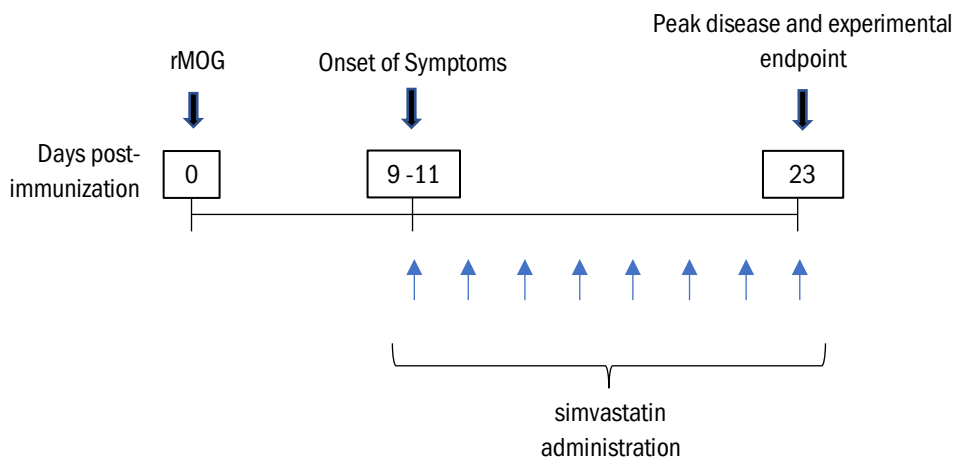


Figure 4.1. Clinical disease course of rMOG experimental autoimmune encephalitis.

(A) rMOG-EAE induced disease presented with first symptoms at day 9-11 post-immunisation, which were clinically characterised by criteria outlined in chapter 2. Disease course progressed with ascending neurological deficit until day 15-17 where the phenotype entered a remission phase. After which, increasing neurological deficit was observed in a relapse phase. (B) Schematic representation of the treatment strategy used throughout experiment.

4.3.2 Chronic simvastatin therapy in rMOG-EAE disease – assessing neurological function

4.3.2.1 SIMVASTATIN THERAPY DOES NOT INFLUENCE WEIGHT OF ANIMALS

The daily weight of each animal was recorded as an additional, overall indicator of health. Simvastatin treatment was administered orally, *ad libitum*, via the feed. Specialised chow was manufactured to incorporate treatment at a known concentration (~25 mg/kg) and control chow, exposed to identical manufacturing procedures, was provided to a separate cohort of animals at the same time. The weight of the animals was monitored not only as an indicator of health but also as a surrogate estimate for food intake across treatment groups for palatability and consumption amounts.

In the early stages of disease, average weight began to climb gradually and uniformly across treatment groups. Throughout the time-course of rMOG-EAE, and in particular during neurological deficit, a sharp decline in weight occurred at disease onset. This overall decline was based on the average weight per treatment group and appeared to reach a plateau towards the chronic, late disease stages. No differences were seen between the simvastatin and vehicle treated groups (Figure 4.2A).

4.3.2.2 SIMVASTATIN THERAPY ATTENUATES CLINICAL DISEASE COURSE OF EAE

Animals were monitored daily for signs of neurological deficit, based on a conventional 10-point scoring system (materials and methods). The first symptoms typically began with tail tip weakness before progressing to ascending motor paralysis and reaching peak disease with complete hind limb paralysis (score 9). Vehicle treated rMOG animals followed the standard course of EAE, entering the first peak of disease with an average neurological deficit score of 8.4 ± 0.4 (mean \pm SEM) within 4 days of disease onset and presenting clinically with bilateral hind limb paralysis. The remission phase in this cohort of animals, expectedly, showed neurological improvement with an average clinical score of 5.1 ± 0.9 (mean \pm SEM). Signs of disease at this stage were mostly characterised by abnormal gait and the absence of plantar placement. The chronic, relapse phase presented within 3 days of remission, marked with declining neurological function. The average neurological deficit score at this termination end point was 7.6 ± 0.9 (mean \pm SEM).

Animals treated with simvastatin (~25 mg/kg) followed the expected disease course of EAE, displaying peak disease within 4 days of the onset of symptoms. Although, not significant, the neurological deficit score at this time point appeared to be lower on average 6.3 ± 1.2 (mean \pm SEM) when compared with the vehicle receiving group 8.4 ± 0.4 (mean \pm SEM, Figure 4.2B). More importantly, whilst the disease onset was identical, temporal differences were observed in the remission phase of the treated group, appearing to be accelerated. The period of remission in the treated group was longer overall, with signs of recovery starting to emerge day 3 post-disease onset and start to relapse at day 10, whereas control animals started to enter the remission stage day 5 post-disease onset and began to relapse day 9. Hence, 7 days of

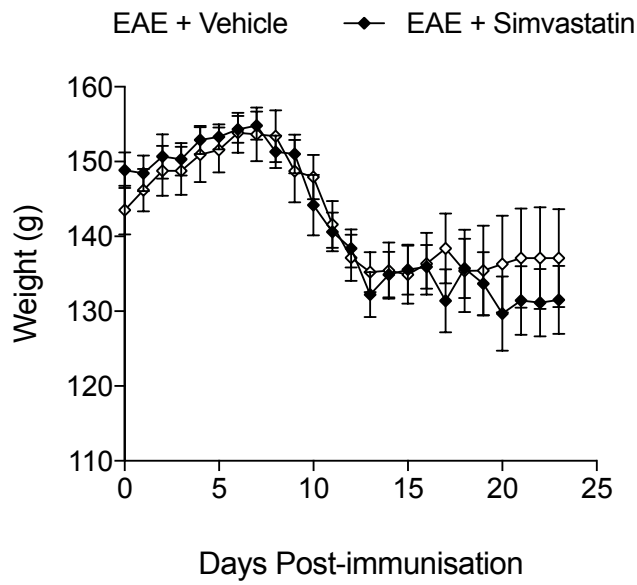
remission observed in the treated group but only 4 days in the untreated group. At the experimental end point the treated group had a significantly lower clinical score than the untreated animals ($P < 0.05$).

Overall, simvastatin therapy attenuated disease across time, resulting in a significant difference in neurological deficit score between the two treatment groups at termination, and hence, a general improvement in neurological function. The simvastatin treated group displayed an average deficit score of 5.3 ± 0.4 (mean \pm SEM; $n=6$), significantly lower than the control group 7.6 ± 0.5 (mean \pm SEM; $n=5$). In the cohort receiving simvastatin, reversal of hind limb paralysis was frequently observed, moreover, neurological function was partially restored.

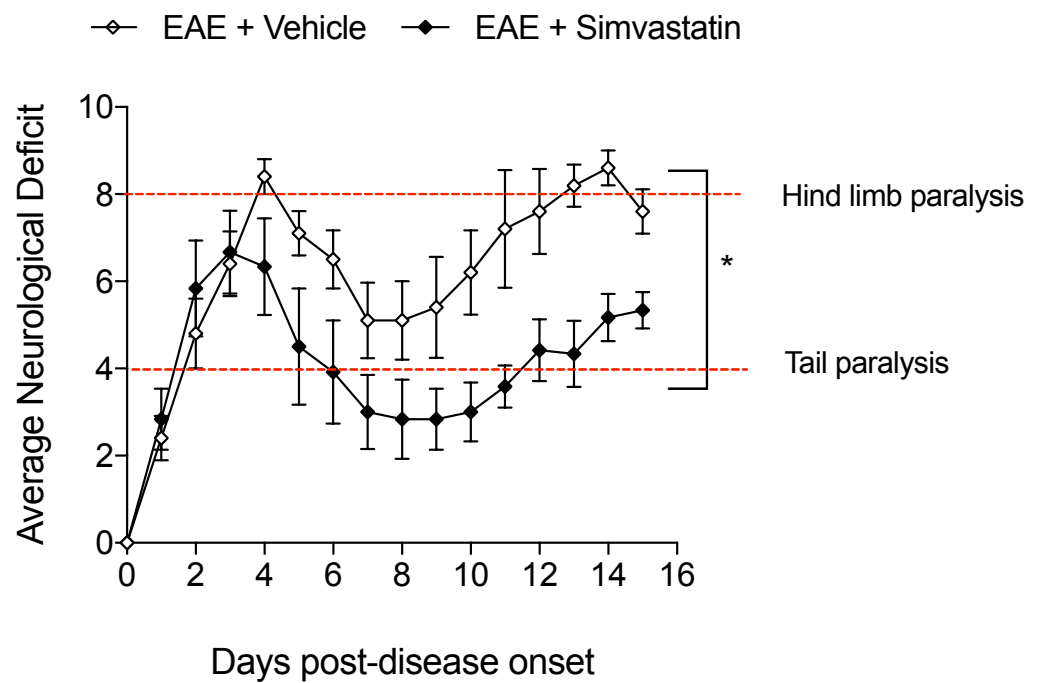
It is worth noting that the conventional 10-point scoring system used to evaluate neurological deficit is not graduated equally across scores. Thus, animals with a cumulative score of 7 display complete bilateral hindlimb weakness with absent or incomplete plantar placement, and therefore, unable to walk. An animal displaying a score 5 presents with complete tail paralysis, in the absence of hind limb weakness, but can walk albeit with an abnormal gait. Differences in the final number of animals per treatment group reflects variability in the model. Animals that followed an abnormal disease course were removed from the study due to ethical reasons.

To assess the extent of neurological deficit in greater detail, a 25-point scoring system (materials and methods) was applied to all animals pre-perfusion, at the experimental end point. The evaluation of such parameters allows for a more precise description of the subtypes of neurological improvement and functional outcomes, in addition to the efficacy of treatment on asymmetrical or symmetrical motor output. When assessments were made with a more detailed grading criteria the rMOG cohort that received vehicle ($n=5$) presented with an average neurological deficit score of 20.3 ± 2.6 (mean \pm SEM) compared with significantly lower average with those receiving simvastatin therapy (11.75 ± 3.0 ; mean \pm SEM) ($n=6$). Descriptively, an average neurological deficit score of 11 would present with more severe bilateral hindlimb deficits including varying degrees of responsiveness to; stretch withdrawal, pinch withdrawal, toe spreading, spasticity and plantar placement. Additional measurable outcomes include severity of weakness/paralysis found in tail, hip and vestibular changes (materials and methods). Hence, a score of 20 using this set of criteria would present with an overall increase in severity between each of the parameters with particular emphasis on symmetrical hind limb paralysis (Figure 4.2C).

A



B



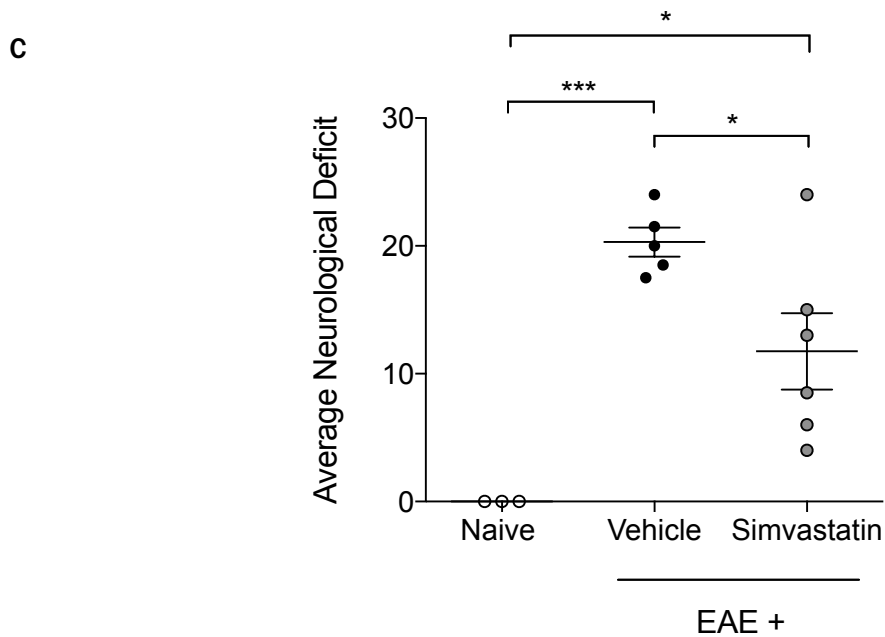


Figure 4.2. The effect of simvastatin therapy on neurological deficit in rMOG EAE.

(A) Graph displays the average weight of animals from both treatment groups throughout the course of disease, data recorded from the day of immunisation until experimental end point at termination. (B) Graph showing the average changes in neurological deficit (non-linear 10-point scoring system) throughout disease duration, beginning at the onset of symptoms and progressing through to first peak, remission phase and relapse. simvastatin treatment began at the onset of neurological deficit and was maintained for the next 15 days until experimental endpoint (*, $P < 0.05$). (C) The average neurological deficit displayed at experimental endpoint, pre-perfusion, assessed according to a 25-point scoring scale (materials and methods). Groups compared were non-immunised, rMOG-EAE receiving vehicle and rMOG-EAE receiving simvastatin (~25 mg/kg) daily (*, $P < 0.05$) (***, $P < 0.0001$). One-way ANOVA with Tukey's post-test. Values represented as the mean \pm SEM.

4.3.3 Neuroretinal inflammation in EAE

A rodent model of multiple sclerosis induced by rMOG peptide illustrates the intimate relationship between CNS tissue and the retina by demonstrating an increase in the number of activated microglial cells. EAE induced by rMOG initiates a relapsing-remitting phenotype, principally dependent on phasic immune cell recruitment. Striking similarities between tissues of affected spinal cord and retinal tissue can be observed. Our experimental approach throughout this study focused on the late-stage, chronic relapsing phase of disease with statin treatment introduced at the onset of symptoms.

The calcium binding protein (Iba1) is a pan identifier of the microglia and macrophage lineage. It was used throughout this study for the detection of spinal cord inflammation. ED1 (cluster of differentiation 68 – CD68) marker was also employed for its specific recognition of activated microglia within rat CNS tissues. Together, these markers allowed us to investigate specific myeloid cell involvement in spinal cord inflammation.

Immunofluorescent labelling of the neuroretina from naïve, non-immunised control animals and rMOG-induced EAE were performed using the pan microglia and macrophage marker Iba-1 along with the activated microglial cell marker ED1, cell numbers were then quantified as indicated in Figure 4.3A. The number of Iba-1 positive cells in the neuroretina of naïve animals, and in the rMOG immunised mice treated with both vehicle and simvastatin (~25 mg/kg) were similar (Figure 4.3B), with no statistical differences between the groups (Figure 4.3C).

Unlike Iba-1, however, the number of ED1 positively stained cells in the neuroretina of rMOG immunised animals, irrespective of treatment, were significantly elevated compared to naïve, non-immunised mice. Simvastatin treatment did not appear to have an impact on ED1 cell number and similar number to the diseased, vehicle treated cohort (Figure 4.3D).

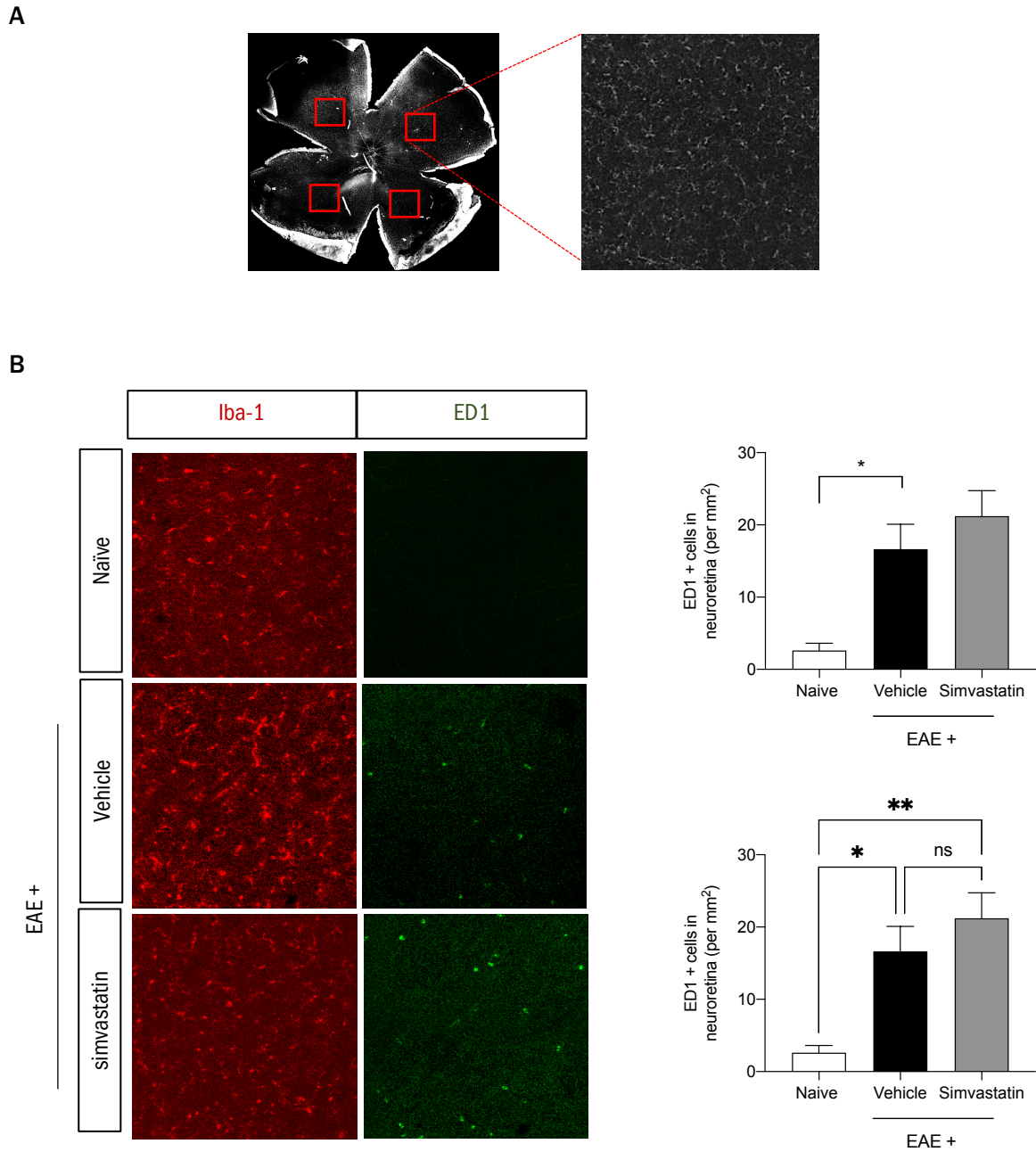


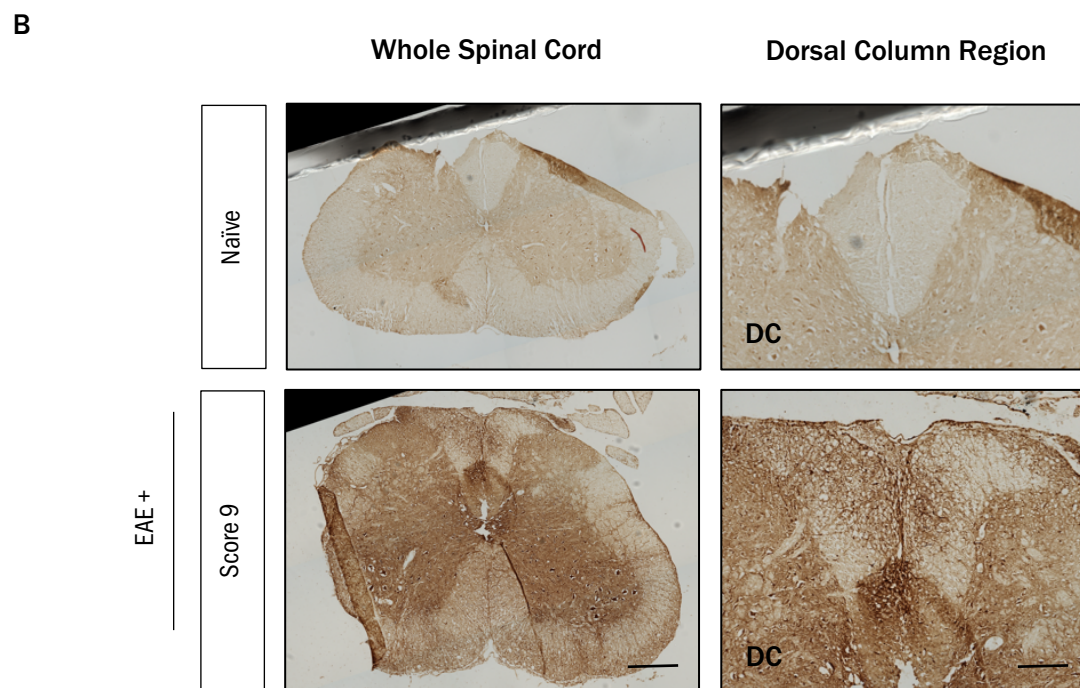
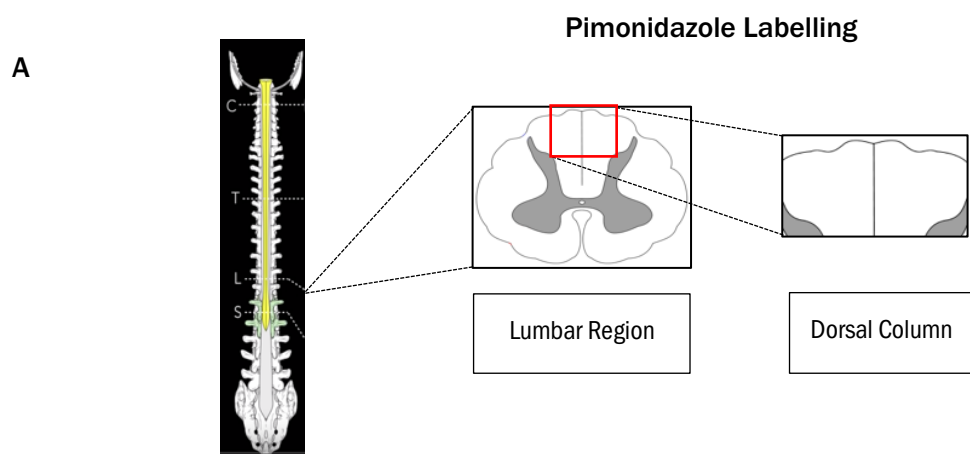
Figure 4.3. Immune cell involvement in the neuroretina of a model of multiple sclerosis. Rats were exposed to rMOG peptide and neuroretinas harvested at day 23 p.i. after receiving either vehicle or simvastatin (~25 mg/kg) over a 12-day period. (A) Schematic representation of data acquisition strategy used for cell quantification. (B) Representative micrographs of neuroretinas, naïve, non-immunised and rMOG immunised receiving either vehicle or simvastatin (~25 mg/kg). Immunofluorescent labelling for iba-1 (red) and ED1 (green). (C) Quantified levels of iba-1 positive cells per mm² of neuroretina. (D) Quantified levels of ED1 positive cells per mm² of neuroretina. n = 5-6 per group. Mean ± SEM. (*, P<0.05). One-way ANOVA used for statistical analysis.

4.3.4 The effects of simvastatin therapy on hypoxia in rMOG EAE

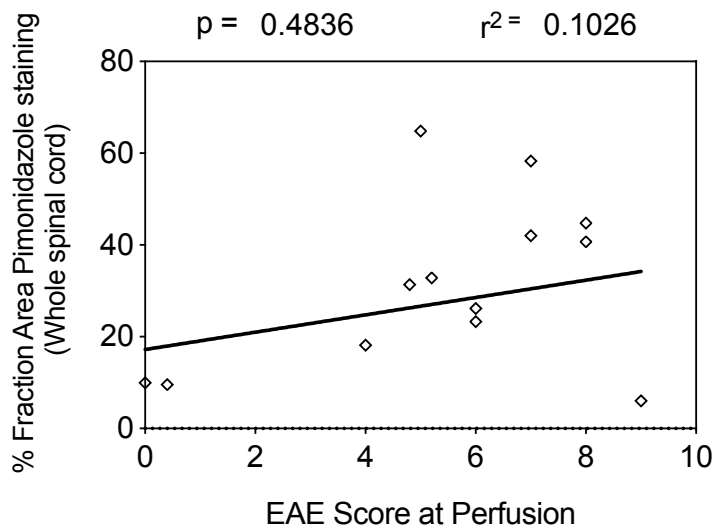
4.3.4.1 HYPOXIA IS A FEATURE OF EAE

Hypoxia is a feature of EAE, and its presence can be determined by the intensity of pimonidazole (Hypoxyprobe, Inc.) staining in the lumbar spinal tissue of EAE animals (Figure 4.4A). Pimonidazole, an intravenous probe used for its ability to detect low tissue oxygen concentrations was administered to all animals prior to perfusion and left to circulate for 4 hours before termination. The product pimonidazole forms adducts in cells under hypoxic ($pO_2 < 10$ mmHg) conditions only. This measurement technique is highly specific and allows for the detection of hypoxia using immunohistochemical methods. All animals were carried through to the late stages of relapsing EAE disease before termination. Naïve animals were those not immunised with rMOG peptide, and therefore, normal. The staining patterns observed in this cohort were faint and punctate (Figure 4.4). On the contrary, immunohistochemistry revealed intense positive staining in both grey and white matter regions of lumbar spinal tissue in rMOG immunised animals. White matter labelling was focused in particular around regions extending out of the grey matter and punctate in pattern. The most prominent staining within the white matter was concentrated to the posterior, dorsal column region along with evidence of clustering in the most central part and penetrating deep towards the grey matter. The grey matter pimonidazole labelling, was ubiquitous and mostly uniform throughout (Figure 4.4B).

The extent of positive pimonidazole labelling as determined by percentage coverage across the entire surface of lumbar, transverse sections, did not correlate with neurological deficit ($p = 0.4836$, $r^2 = 0.1026$, $n = 12$, Figure 4.4C). However, when focus was placed on the white matter dorsal column region only, pimonidazole labelling correlated strongly with the pre-perfusion (25-point scoring system, materials and methods) neurological deficit score ($p = 0.0156$, $r^2 = 0.7214$, $n = 12$, Figure: 4.4D).



C



D

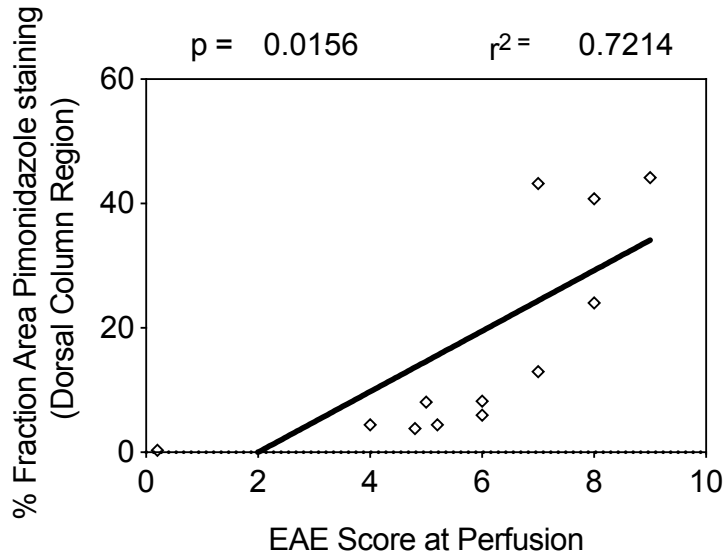


Figure 4.4. Immunolabelling of tissue hypoxia in rMOG-EAE. (A) Schematic of spinal cord section and focus to represent the tissues examined throughout study. (B) Transverse spinal tissue sections of lumbar region, immunolabelled using anti-pimonidazole at late-stage disease. Naïve, non-immunised animals. Intense staining seen in both grey and white matter of rMOG-EAE with a neurological disease score of 9 (materials and methods). Scale bar 500 μm whole spinal cord; 100 μm dorsal column region (Bii). Note the differences in staining intensity concentrated in the dorsal column region (DC). (C) Linear regression showing the correlation between whole cord pimonidazole staining intensity and disease severity. (D) Linear regression to demonstrate the correlation between dorsal column hypoxia and disease severity. All micrographs are representative.

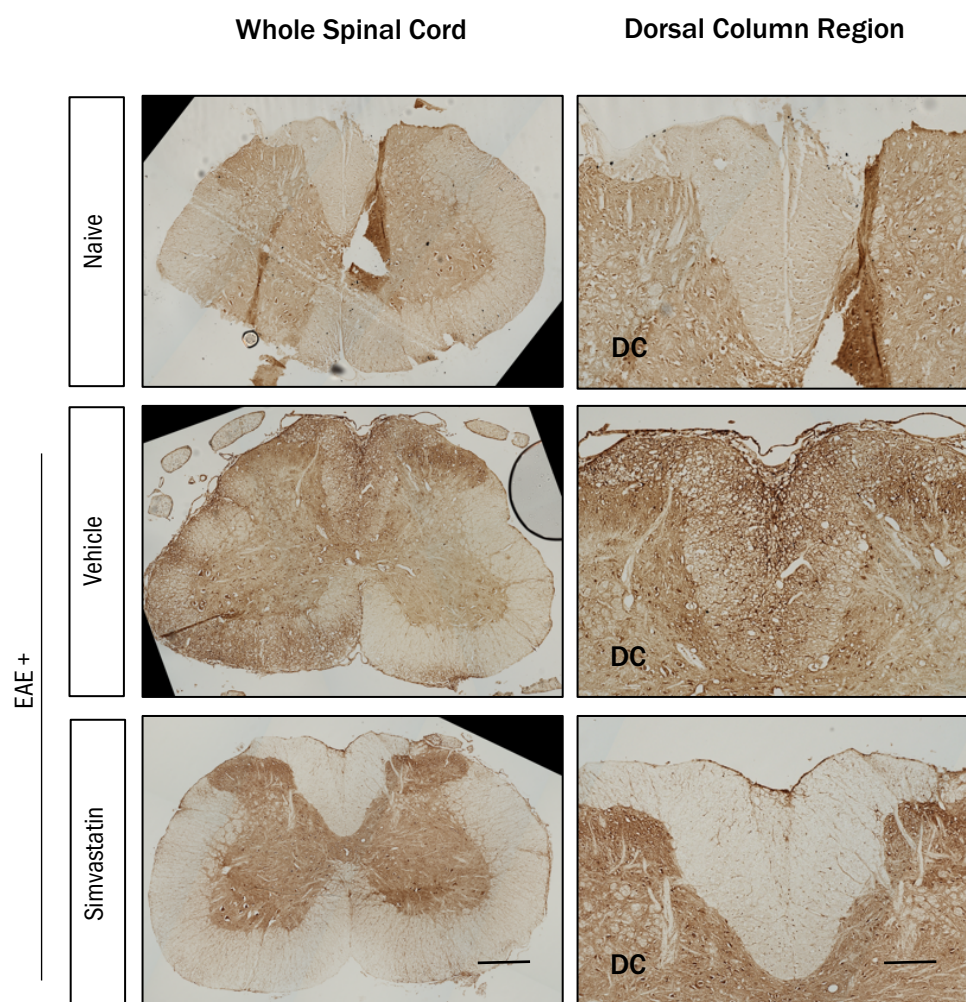
4.3.5 Simvastatin treatment reduces hypoxic marker staining intensity in the dorsal column of EAE spinal tissue

Simvastatin (~25 mg/kg), administered daily via the feed, was maintained for a total of 15 days starting from the onset of disease and terminating at the late stages of disease relapse (day 23). Pimonidazole was used to detect tissue hypoxia and compared between the simvastatin treated cohort and vehicle receiving cohort in addition to naïve, asymptomatic subjects. The naïve animals were those not immunised with rMOG peptide, and therefore, normal.

When comparing between EAE groups, the presence of hypoxic staining in simvastatin treated mice was universally and markedly reduced. When measuring the intensity of staining across the entire transverse spinal section, significant hypoxia could be detected in rMOG animals compared to non-immunised. However, whilst there was a reduction in signal in the simvastatin treated group this was not significant (Figure 4.5B). Nevertheless, the simvastatin treated group were not significant from the control group indicating that statin treatment was having a global effect on hypoxia. In the dorsal column region however, where most inflammation and pathology are recorded, a highly significant difference was evident between vehicle and simvastatin treated groups ($p = 0.0005$, Figure 4.5C), highlighting the importance of simvastatin's role in the prevention of tissue hypoxia in this region.

A

Pimonidazole Labelling



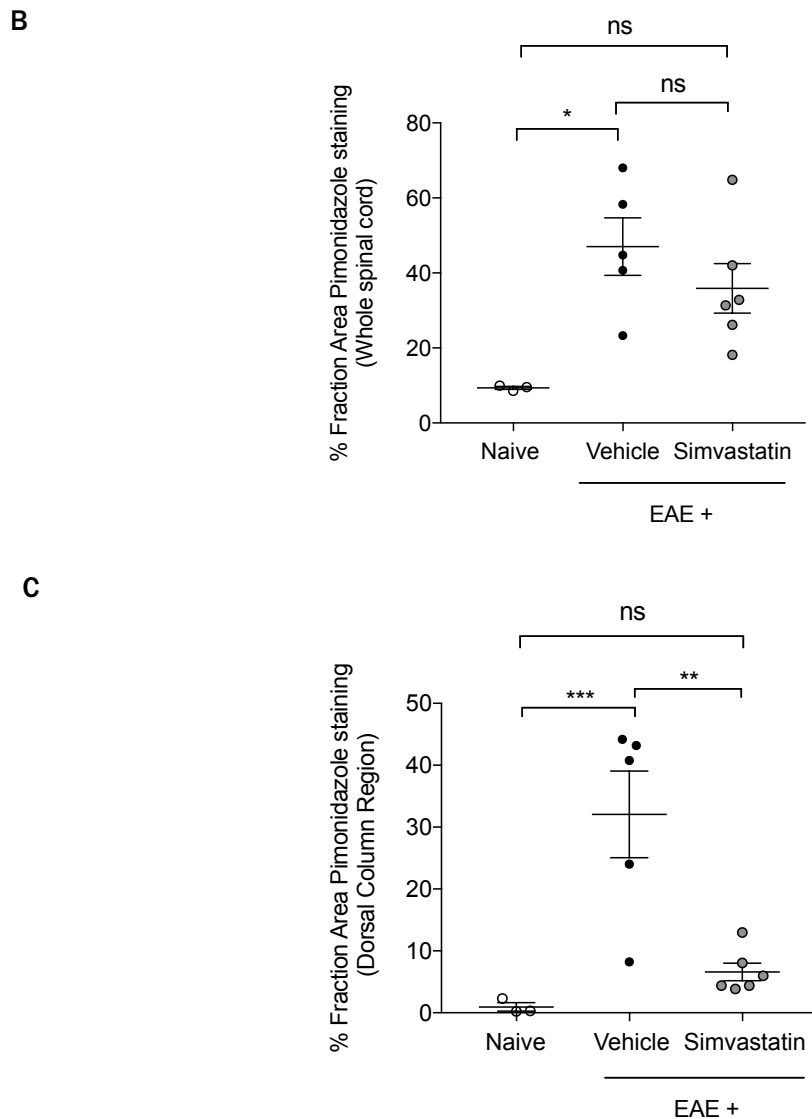


Figure 4.5: Simvastatin therapy reduces tissue hypoxia staining in dorsal column rMOG-EAE. (A) Representative micrographs of anti-pimonidazole detection in non-immunised, transverse lumbar spinal comparing non-immunised control and rMOG induced EAE treated with vehicle or simvastatin (~25 mg/kg) during late relapsing phase of the disease, day 23 p.i. Scale bar 500 μ m whole spinal cord; 100 μ m dorsal column region. (B) Graph showing the percentage of transverse, lumbar whole spinal cord section coverage with positive staining for pimonidazole. The vehicle cohort expressed a significant increase in tissue hypoxia over non-immunised control. (C) Quantification of immunohistochemical staining for hypoxia, in the lumbar spinal cord, derived from transverse sections, and specifically focused on staining intensity within the dorsal column region. Comparisons were made between non-immunised control, rMOG-EAE vehicle and simvastatin (~25 mg/kg) treated. Mean \pm SEM (*, $P < 0.05$) (**, $P \leq 0.01$;) (***, $P < 0.0001$). ns = no significance. Statistical significance determined using one-way ANOVA with Tukey's post-test.

4.3.6 Cell infiltration assessment in EAE treated with simvastatin

Pathological changes in the spinal cord of rMOG-EAE animals were observed by light microscopic evaluation of H&E-stained tissue on day 23 p.i. after receiving daily treatment with either vehicle or simvastatin (~25 mg/kg). Treatment began at the onset of symptoms and continued until experimental endpoint. A large number of inflammatory cells were observed in spinal sections of vehicle receiving animals. In those animals with mild disease, labelling was mostly limited to the white matter and situated towards lateral regions of the spinal cord section. Whereas, in the more severe forms, cell infiltrates appeared deeper in the parenchyma, penetrating medially towards the centre of the tissues. The extent of cellular infiltration – as measured following the methods outlined by (Chan et al., 2008) – increased with the magnitude of neurological deficit, revealing a strong and significant positive correlation ($r^2 = 0.8188$, $p = 0.0131$, $n = 11$) (Figure 4.6C).

The most striking differences were observed in the dorsal column region (Figure 4.6A). This area has classically been recognised for containing most pathological changes throughout the course of this disease. Quantitative analysis revealed a significant difference in histopathological scoring when rats were treated with simvastatin resulting in a reduction in the histological score (1.2 ± 0.3 , $n=6$) when compared with control (2.5 ± 0.2 , $n=5$) (Figure 4.6B).

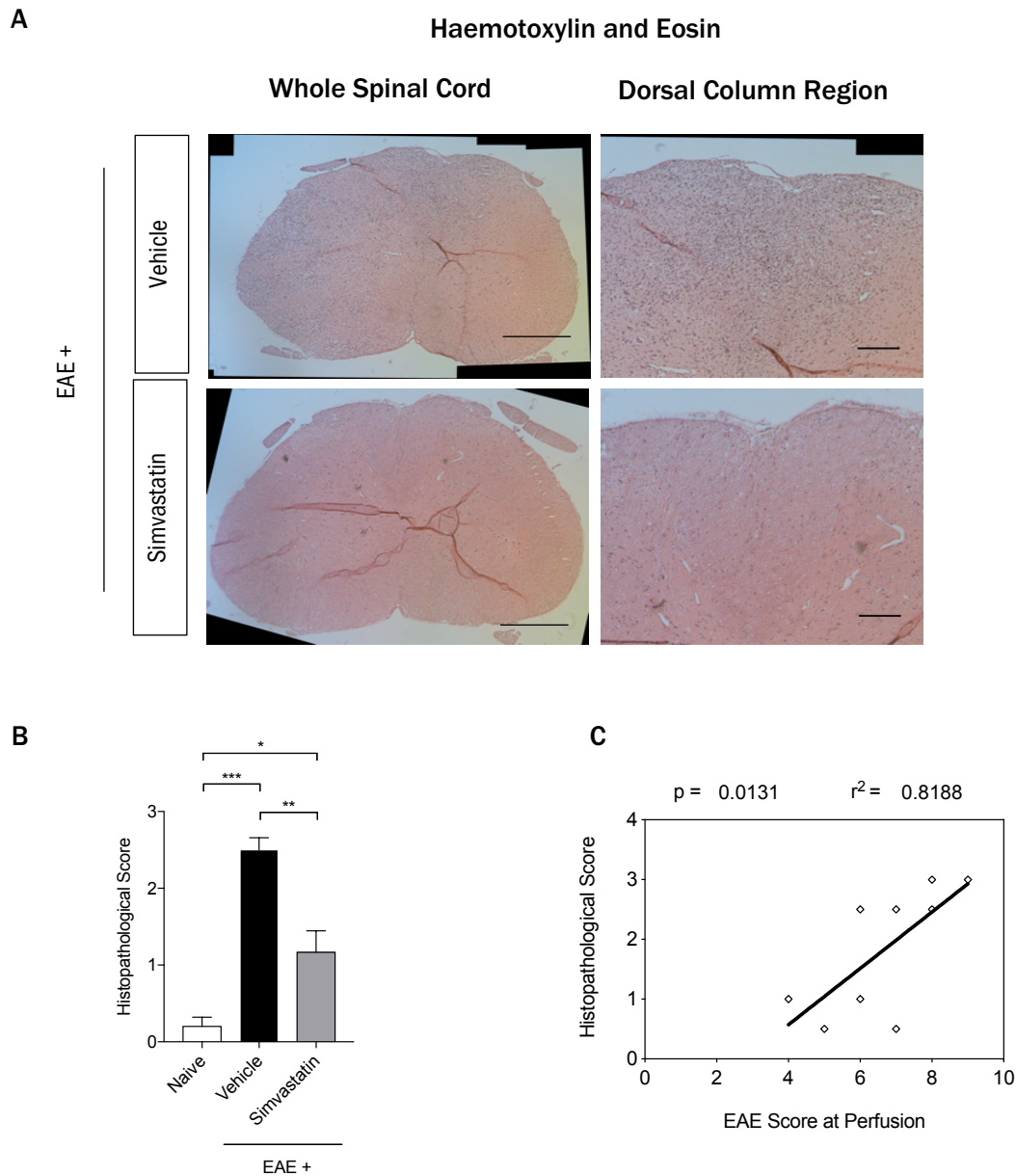


Figure 4.6. Observation of pathological changes in the spinal cord of rMOG EAE rats.

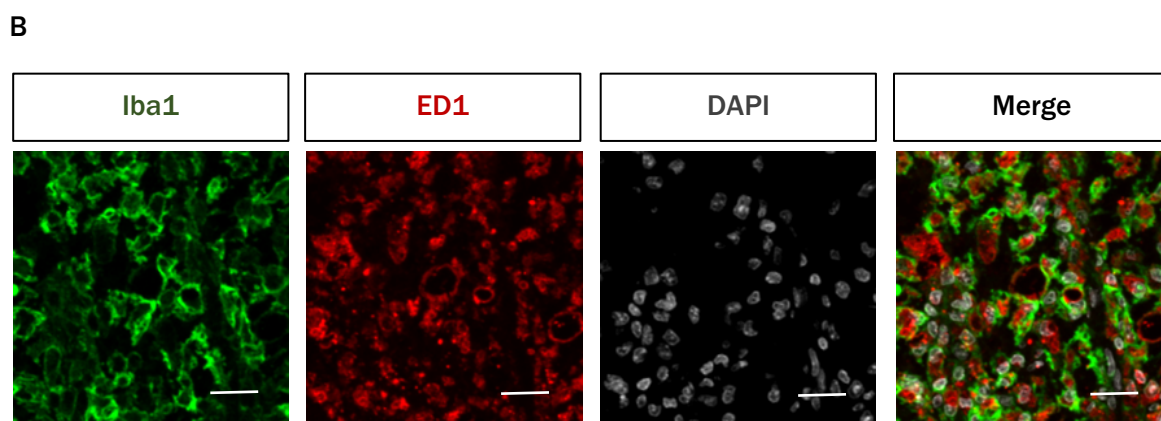
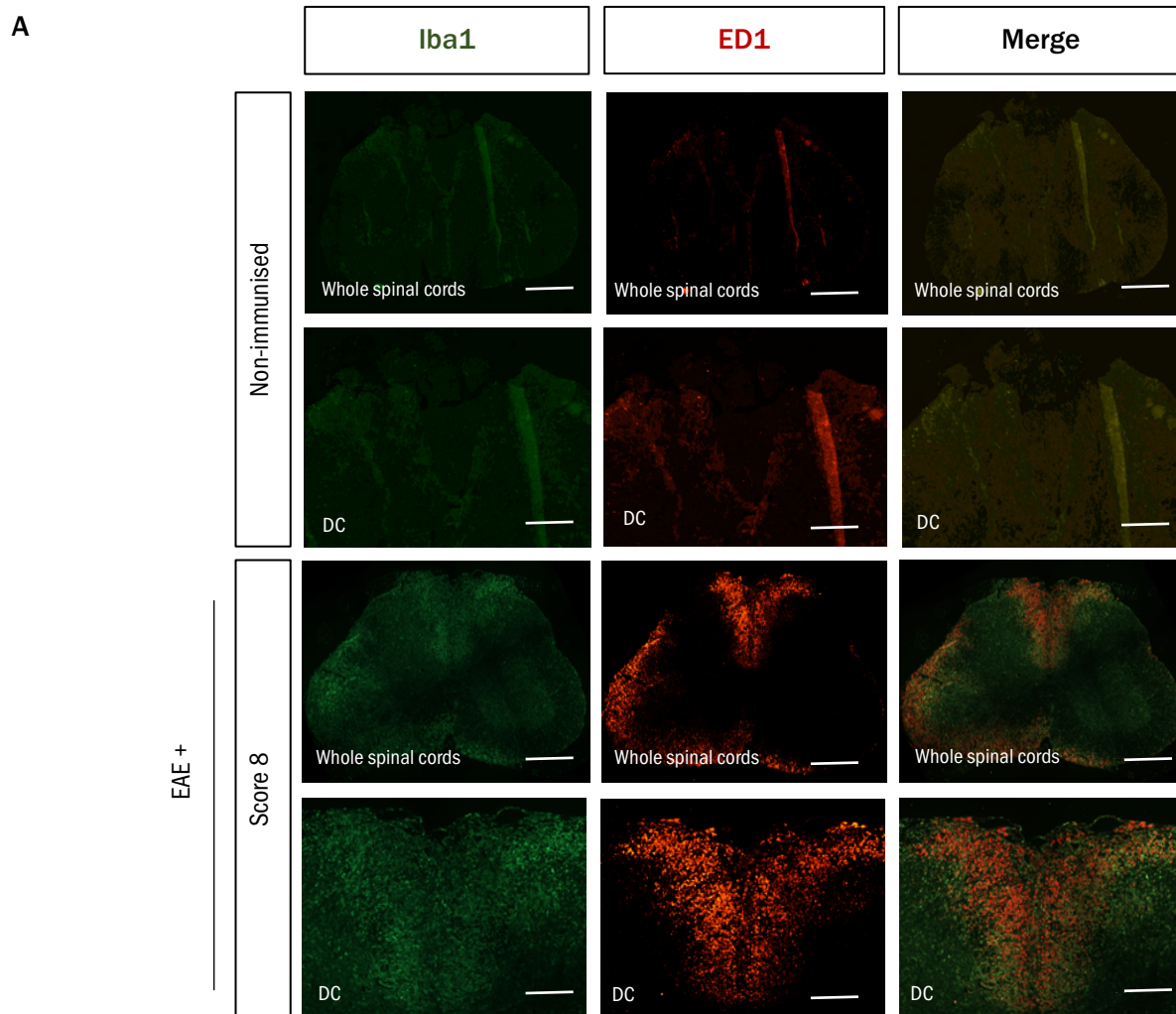
(A) H&E-stained transverse spinal cord sections in late phase EAE, day 23 p.i. rMOG immunised animals were treated with either vehicle or simvastatin (~25 mg/kg) at the onset of symptoms and continued daily doses until experimental end point. Large amounts of cell infiltration evidenced in the dorsal column region (DC) of vehicle when compared with simvastatin treatment. (B) Quantitative analysis of cellular infiltration into the whole transverse spinal section (materials & methods), compared between treatment groups and non-immunised, naïve control. (C) Linear regression showing a positive relationship between EAE neurological deficit score at experimental endpoint and histopathological score. $n = 5-6$ per group. Mean \pm SEM. (*, $P < 0.05$) (**, $P \leq 0.01$); (***, $P < 0.0001$). One-way ANOVA with Tukey's post-test.

4.3.7 Assessing inflammatory markers at the late stages of rMOG-EAE disease

4.3.7.1 MYELOID CELL EXPRESSION AS A CORRELATE OF DISEASE SEVERITY

Microscopically, the spinal cord of asymptomatic animals showed little sign of immunoreactivity when stained for Iba-1 and ED1, by contrast, symptomatic rMOG animals exhibited striking positivity characterised by varying amounts (Figure 4.7A.). At peak disease (neurological deficit score 7-9), inflammatory cells were found to penetrate deep into the parenchyma, with evidence of co-expression (Figure 4.7B).

When assessing iba1 percentage coverage within the dorsal column region of lumbar, transverse spinal sections (Figure 4.7C), the extent of inflammation was not directly related to the neurological deficit score at perfusion ($r^2 = 0.6818$, $p = 0.0850$, $n = 11$). However, ED1 immunoreactivity was also found to positively correlate with neurological deficit scores at the experimental endpoint ($r^2 = 0.5083$, $p = 0.0138$, $n = 11$) (Figure 4.7D).



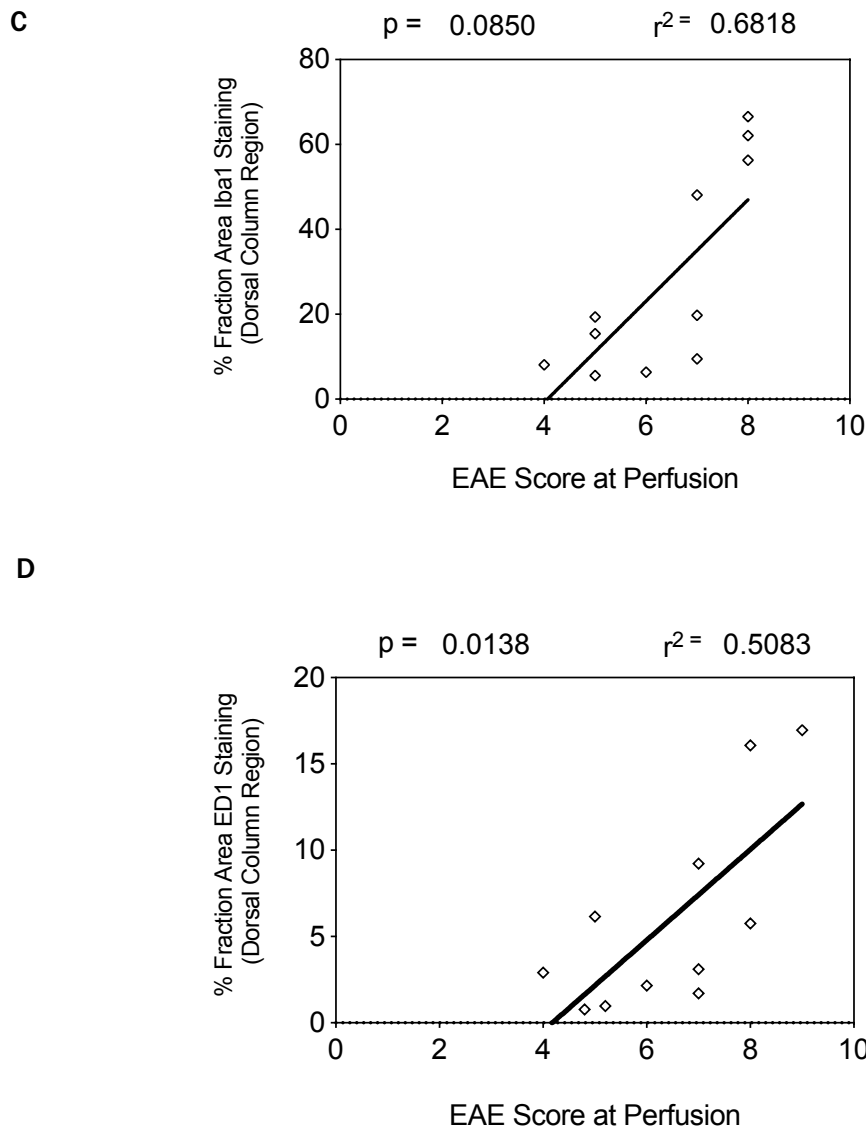


Figure 4.7. Myeloid cell infiltration in rMOG model of multiple sclerosis

Naïve, non-immunised animals and rMOG induced animals were harvested day 23 p.i. at late-stage relapsing disease. Lumbar region spinal cord sections were immunolabelled for myeloid derived cells. (A) Representative micrographs of anti-iba1 antibody (green), ED1 (red) immunostained lumbar spinal cord, transverse sections. Non-immunised animals shown compared with age-matched rMOG immunised with a specific focus on the dorsal column region (DC). (B) Dorsal column white matter immunolabelling of rMOG immunised animals with anti-iba1 (green), ED1 (red) and DAPI (grey) demonstrating co-expression. (C) Percentage area of Iba1 staining in the dorsal column of spinal cord sections. Linear regression comparing the staining intensity to EAE neurological dysfunction score at perfusion ($n = 11$). (D) Linear regression showing relationship between percentage ED1 immunostaining in the dorsal column region of spinal cord sections compared with EAE neurological deficit score before experimental endpoint ($n = 11$).

4.3.8 Simvastatin therapy administered during rMOG-EAE reduces the number of Iba1 and ED1 cells present in inflamed spinal tissue

Ample evidence exists to support the anti-inflammatory and immunomodulatory properties of simvastatin. The focus of this study, however, was to investigate the involvement of the microglial cell in rMOG disease pathology and assess the impact, if any, of simvastatin on this process. Thus, the pan macrophage/microglial marker (Iba1) and the specifically targeted activated microglial cell marker (ED1) were used in combination to assess the levels of cell involvement at late-stage relapsing disease.

The dorsal column region of lumbar spinal cord exhibits most of the disease pathology in this model. Microscopically, in non-immunised, asymptomatic animals, immunoreactivity of Iba1 and ED1 was negligible. However, this was greatly increased, predominantly in the white matter of the dorsal column region, in vehicle receiving rMOG animals (50.6 ± 8.3 , $n=5$ and 10.2 ± 2.7 , $n=5$, percentage positive stained area respectively) (Figure 4.8A). Iba1 staining patterns presented differently to ED1 in that Iba1 coverage not only concentrated in the dorsal white matter region but also spread proximally, towards the grey matter. Conversely, ED1 labelling was largely restricted to the white matter tissue. Furthermore, treatment with simvastatin resulted in a significant decrease in the percentage density coverage of both Iba1 and ED1 immunomarkers when compared with vehicle receiving counterparts (10.7 ± 2.2 , $n=6$ and 2.4 ± 0.8 , $n=6$, respectively), quantitatively reaching near-control levels (Figures 4.8 B,C).

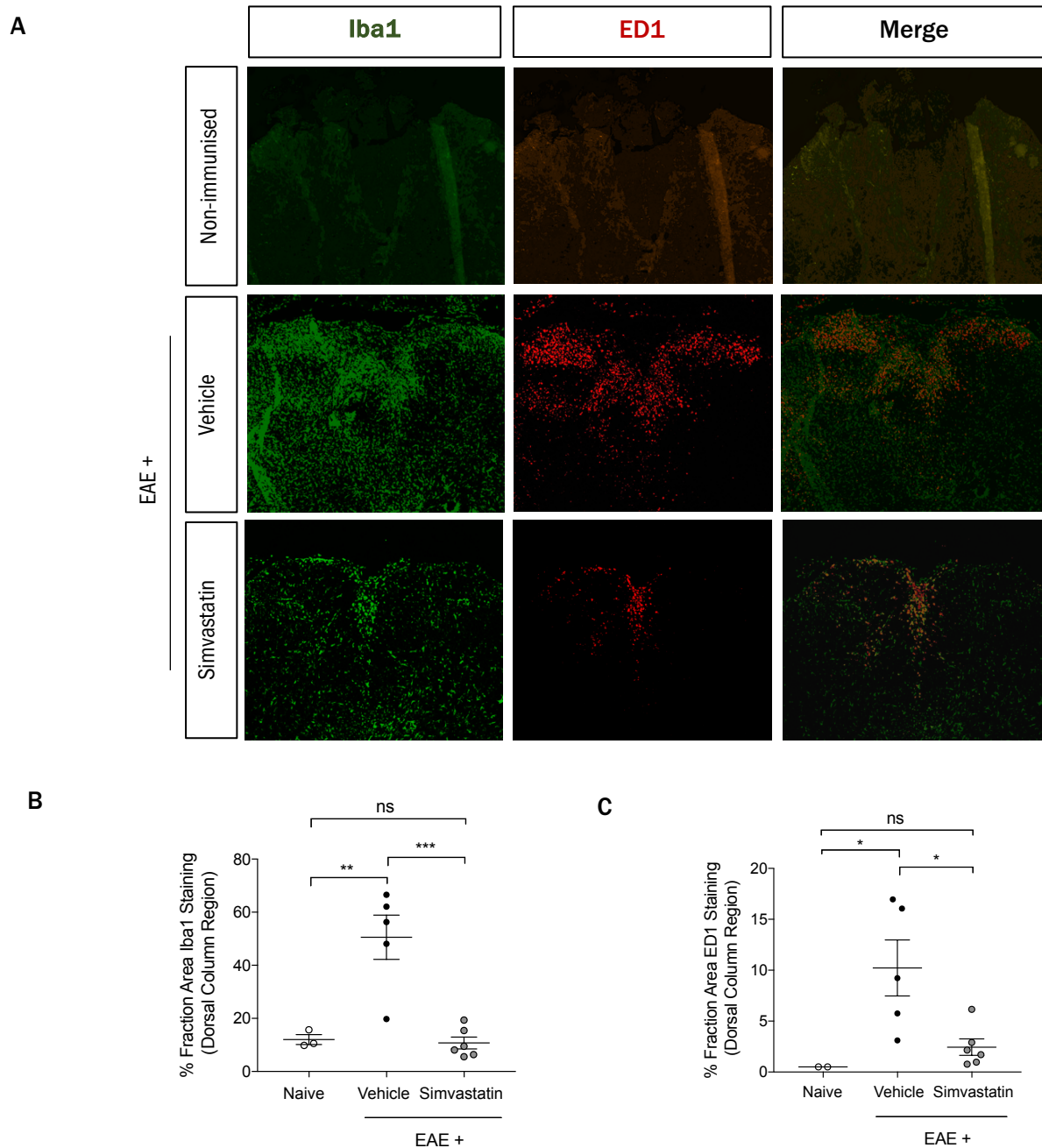


Figure 4.8 Simvastatin therapy reduces levels of Iba1 and ED1 cell expression.

Naïve, non-immunised animals and rMOG induced animals were harvested day 23 p.i. in the late stages of relapsing disease. Dorsal column of lumbar region spinal cord sections were immunolabelled for myeloid derived cells. (A) Representative micrographs of non-immunised animals compared with age-matched rMOG immunised animals were immunostained for anti-Iba1 antibody (green), ED-1 (red). (B) Graph to show percentage staining intensity of Iba1 expression in dorsal column region of naïve and rMOG animals treated with either vehicle or simvastatin (~25 mg/kg). (C) Plot to demonstrate the levels of ED1 immunolabelling in the spinal cord of naïve and rMOG challenged animals receiving either vehicle or simvastatin (~25 mg/kg). Graphs presented as average ± SEM. (n=3-6) Mean ± SEM. *, P≤0.05; **, P≤0.01; ***, P≤0.001; One-way ANOVA performed with Tukey’s post-test.

4.3.9 Assessing levels of Nitrosylated protein in rMOG EAE spinal cord

Anti-3-nitrotyrosine was employed throughout this study as a readout for the presence of peroxynitrite formation and nitrosylated proteins. Dorsal column lumbar spinal cord in non-immunologically challenged, asymptomatic animals, displayed only non-specific immunofluorescent background labelling (Figure 4.9A). However, strong evidence of 3-nitrotyrosine immunoreactivity was found in identical regions of the control rMOG challenged cohort, presenting with a fairly homogenous distribution and robust signal. This demonstrates that in EAE there is significant peroxynitrite formation resulting in nitrosylation of cellular proteins and is thus a feature of this disease.

Quantitative assessment, as determined by the percentage density coverage of immunofluorescent signal, revealed that in the vehicle receiving cohort 3-nitrotyrosine was increased over naïve counterparts but not significantly. Instead, the simvastatin treated group is significantly increased over naïve levels when intensity is measured in the lumbar spinal cord of diseased animals (Figure 4.9B). However, the simvastatin treated group demonstrated equal levels of 3-nitrotyrosine staining and similar patterns of distribution when directly compared with the vehicle receiving group (8.0 ± 1.4 , $n=5$ and 5.7 ± 1.0 , $n=4$, respectively). This suggests that simvastatin does not impact on the protein nitrosylation.

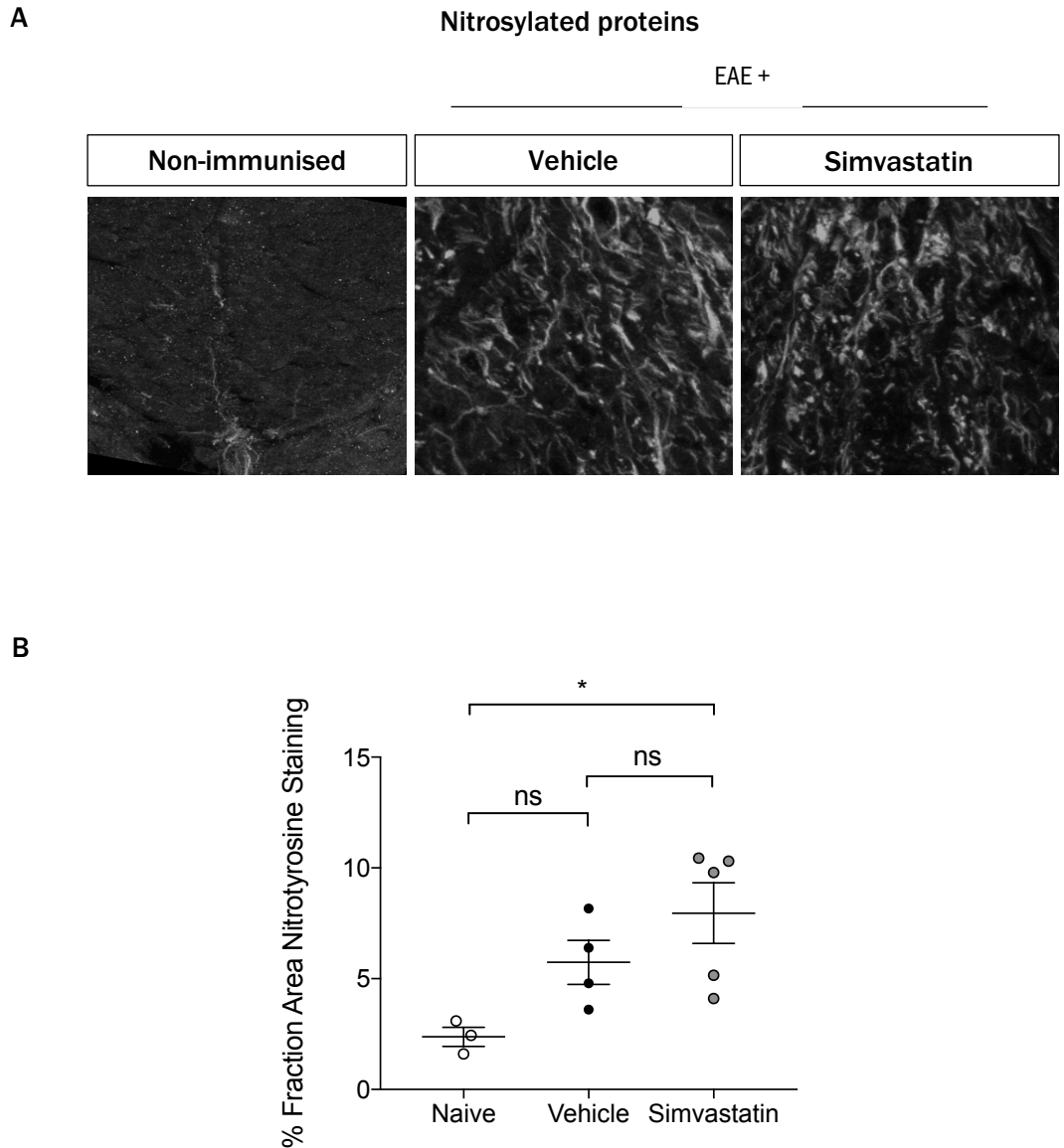


Figure 4.9. Peroxynitrite expression in rMOG-EAE. Naïve and rMOG induced lumbar spinal cords compared at day 23 p.i. after receiving either vehicle or simvastatin (~25 mg/kg) for a total of 19 days. (A) Representative confocal micrographs labelled for anti-3-nitrotyrosine expression in the dorsal column region of naïve and rMOG induced animals, staining intensity compared between treatment groups. (B) Quantitative analysis of dorsal column anti-3-nitrotyrosine staining, displayed as percentage density coverage across cross-sectioned spinal cord. Mean ± SEM. *, $P \leq 0.05$; (n= 3-5 per group). One-way ANOVA performed with Tukey's post-test. ns = no significance.

4.3.10 Simvastatin therapy in rMOG EAE reduces levels of oxidative DNA damage in the inflamed spinal cord

8-Oxo-2'-deoxyguanosine (8-Oxo-dG) is widely accepted as one of the major products of nuclear and mitochondrial DNA damage. In this study we assessed the levels of oxidative DNA damage using an anti-8-OHdG labelled antibody on inflamed spinal tissue. Upon close observation using confocal microscopy, clear and obvious differences in staining intensity were noted between the naïve, non-immunised population and rMOG-induced animals. The former exhibited low, non-specific labelling, whereas the latter generated clusters of intense fluorescent signal throughout the dorsal region white matter. Importantly, differences were noted between the rMOG treatment groups, with simvastatin therapy demonstrating a consistent reduction in staining intensity, evenly distributed across the dorsal segments (Figure 4.10A). Quantitative analysis confirmed these observations, revealing a significant increase in labelling in EAE disease over the non-immunologically challenged, supporting the notion of oxidative DNA damage as a common feature of EAE disease. Another important finding in this experiment, was revealed when comparisons between the simvastatin receiving population and vehicle demonstrated a consistent and significant decrease in 8-OHdG signal after sustained simvastatin therapy (Figure 4.10B).

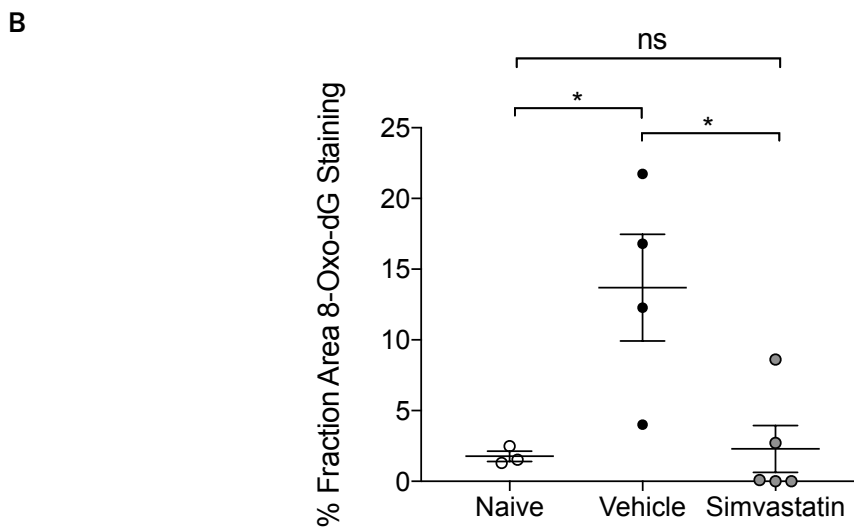
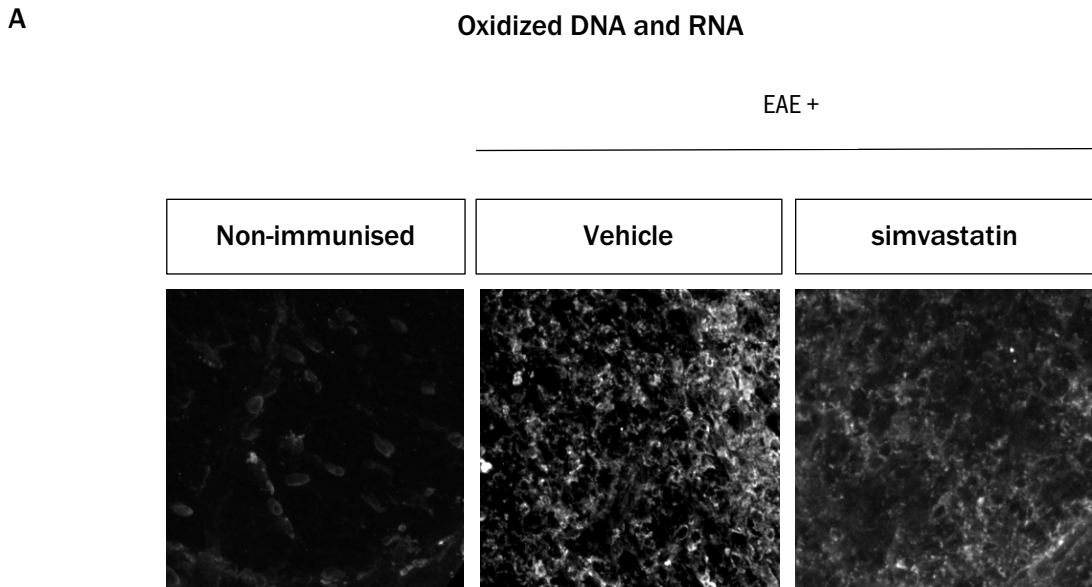


Figure 4.10. Oxidative damage detection in EAE using 8-OHdG immunohistochemistry.

Naïve and rMOG induced lumbar spinal cords compared at day 23 p.i. after receiving either vehicle or simvastatin (~25 mg/kg) for a total of 19 days. (A) Representative confocal micrographs labelled for anti-8-OHdG expression in the dorsal column region of naïve and rMOG induced animals, staining intensity compared between treatment groups. (B) Quantitative analysis of dorsal column anti-8-OHdG staining displayed as percentage density coverage across cross-sectioned spinal cord. Mean \pm SEM. *, $P \leq 0.05$; (n= 3-5 per group). One-way ANOVA performed with Tukey’s post-test.

4.4 Discussion

The present study uses a well-established model of MS to interrogate the potential neuroprotective effects of simvastatin by assessment of 1) clinical outcome 2) regional CNS inflammatory cell infiltration and 3) tissue specific oxidative and nitrative damage biomarkers. The evidence presented herein suggests that high-dose simvastatin therapy is well-tolerated by oral administration in the dark agouti rat. Treating EAE animals with a chronic dosing regimen of simvastatin significantly ameliorated clinical signs of disease. A reduction in myeloid cell infiltration in the diseased spinal tissue alluded to underlying anti-inflammatory mechanisms. Furthermore, simvastatin therapy demonstrated neuroprotective potential by reducing oxidative and nitrative markers in the inflamed spinal cord. The relevance of these findings to the pleiotropic nature of statins and potential application in the context of SPMS will be discussed below.

4.4.1 rMOG induced active EAE

EAE is an artificially induced inflammatory demyelinating disease of the CNS and the most commonly used animal model for the human disease MS (Lassmann, 1983) (Lassmann and Bradl, 2017). First developed by Rivers in the 1930s (Rivers et al., 1933) (Schwentker and Rivers, 1934) (Rivers and Schwentker, 1935) this prototypical autoimmune driven disease remains one of the most widely used methods adopted for the induction of MS-like phenotypic disease in rodents. The degree of heterogeneity and susceptibility to EAE is great and multiple factors are known to influence disease outcome, including peptide antigen, strain and sex of animal (Yasuda et al., 1975) (Smilek et al., 1991) (Okuda et al., 2002) (Maron et al., 1999) (Lyons et al., 1999) (Sinha et al., 2008). EAE can be induced in a number of species including mice, rats, guinea pigs, rabbits and primates (Mehta et al., 1981, Pender and Sears, 1984) (Brok et al., 2001). Depending on the antigen used and genetic make-up of the animal, these parameters reportedly influence the level of responsiveness to various immunological and neuropharmacological therapies. While no single model recapitulates exactly all aspects of MS, animal models are essential to understanding the induction and pathogenesis of disease, in addition to providing a platform for efficacy testing of novel therapies. Indeed, most of our current understanding regarding MS pathogenesis was formed on the basis of findings generated from this model. Of significance, all current FDA-approved immunomodulatory drugs are effective to some extent in treating EAE. Thus, this model has become a strong indicator for the efficacy of potential treatment outcomes in MS (Robinson et al., 2014). Several variations of MS models exist and the most widely used and better understood is the rodent rMOG model of EAE, as used here. The pathological mechanisms have shown to closely reflect the spectrum of those observed in MS and characterisation studies have given researchers in the field confidence using this model system to study drug efficacy (Storch et al., 1998).

rMOG induced EAE generates a predominantly relapsing-remitting disease phenotype at the early stages, while progressive-like features manifest at the later timepoints (Zeis et al., 2008). Our findings

demonstrate clear clinical disease stages defined by periods of neurological deficit that are in line with MOG-EAE characterisation studies in the field (Stosic-Grujicic et al., 2004) (Khorshid Ahmad et al., 2017). This model typically exhibits clinical signs of disease activity, determined by the degree of neurological deficit, around day 11 after initial immunisation. The disease onset phase is described as the first inflammatory “attack” period and is accompanied by mass mononuclear cell infiltration into the spinal cord. Thereafter, the disease progresses towards a relapsing phase, whereby relapses are superimposed upon a background of slowly increasing neurological deficit. The first relapse commonly takes place 12-14 days post injection, a finding that was consistent in our study (Figure 4.1). The initial relapsing/remitting phase is known to be associated with synchronised periods of neurological dysfunction which, according to (Davies et al., 2013b), relates to sites of observable pathology. In our study, once the first peak of remission had passed, an abrupt relapse phase ensued and maintained in pathology and severity. These later stages of chronic inflammation more closely represent a progressive phenotype with worsening pathology (Tanuma et al., 2000). Due to this, all tissue collection and analysis for our study, was carried out at the later stages.

It is important to note that technical challenges remain when developing animal models that fully represent the progressive forms of MS. To date, available EAE models resembling this type of pathology are lacking. (Tsunoda et al., 2005) claimed to have developed a method for converting RRMS disease to a SPMS phenotype using ultraviolet radiation in the mouse. While another study used Biozzi ABH mice to generate a slowly progressive disease course following an initial stage of relapse-remission (Al-Izki et al., 2012). The described pathological features of this model were gliosis, marked demyelination and accompanying neuronal loss (Baker et al., 1990) (Burrows et al., 2019) (Hampton et al., 2008). Seemingly, these pathological characteristics more accurately capture a SPMS disease course, more so than the selected EAE model used herein. However, it should be cautioned that our current understanding of the pathogenic mechanisms underpinning progressive disease are less well characterised and understood. At present, these models are perceived as unreliable and under researched, accordingly, they provide little insight into efficacy and mechanisms of novel therapies. For now, studying the responsiveness of therapies in RRMS at the later stages of EAE is an acceptable and commonly applied approach.

4.4.2 Simvastatin therapy improves neurological deficit in EAE: A role for microglia and macrophages

In EAE, neurological deficit is used to indicate disease severity. Here, we used a conventional scoring system (material and methods) to assess daily, the clinical signs of disease. While there is no international scoring system for EAE there are commonly applied assessment methods that have proven robust and reliable. The 10-point scoring method used here primarily focuses on radiating paralysis with unilateral or bilateral involvement. A more extensive 25-point scoring system developed in the laboratory of K.J. Smith (University College London) was used alongside to accurately capture the magnitude of

neurological damage (Davies et al., 2013b). A common and important clinical sign often missed with the conventional 10-system, particularly at the later stages of disease, is spasticity.

The findings from our current study support a strong correlation between the quantity of ED-1 positive cells and neurological deficit. Adoptive transfer studies have demonstrated on numerous accounts that T cells are necessary for the induction of EAE (Engelhardt, 2006) (Carson et al., 2006). However, the cellular mechanisms governing EAE development are still under debate. Both resident microglia and peripheral-derived monocytes have been implicated in disease progression due to their ability to secrete proinflammatory cytokines (Benveniste, 1997), present antigen and partake in demyelination by phagocytosis of degraded myelin (Bauer et al., 1994a). Research groups focused on quantifying the levels of macrophages and microglia in active MS lesions found a six to twelve times higher rate than T cells (Lucchinetti et al., 2000). In EAE, characterisation studies confirmed T-lymphocyte populations present in high levels at peak disease, and decline thereafter, while the number of microglia and macrophages by comparison, remain higher. Other studies on EAE have shown that mass infiltration of peripheral monocytes localise to the meninges surrounding the CNS and perivascular space and positively correlate with tissue damage (Ajami et al., 2011) (Douset et al., 1999) (Shemer and Jung, 2015) (Rausch et al., 2003) (Ponomarev et al., 2005) (Floris et al., 2004) (Rasmussen et al., 2007). These clustered monocytes also express higher levels of cellular activation makers, such as the one used here, ED1. Increased levels of ED1 labelled monocytes have been found in the diseased spinal cord of EAE at peak disease compared with the early stages (Davies et al., 2013b). In line with this, MRI imaging techniques revealed an increased infiltration of magnetic-labelled monocytes (Engberink et al., 2010).

The general dogma is for EAE pathogenesis is, once the acute inflammatory attack subsides during the remission phase, widespread cellular activation would return to a state of homeostasis (Bauer et al., 1994a) (Bradl and Lassmann, 2009) (Stadelmann et al., 2011) (Rawji and Yong, 2013) (Yamasaki, 2014). However, there is now more data to support the hypothesis that a subpopulation of microglia, retain an activated function during remission. This idea has been supported by the discovery of large quantities of ameboid shaped microglia concurrent with enhanced NOX activity. The findings of such studies support the notion of unresolved inflammation trapped within the CNS, provides a pathological explanation for the chronic features observed in progressive MS (Radbruch et al., 2016). At this phase of the disease, microglia are seen to be the source of persistent oxidative stress, locally correlating with the ongoing subclinical neuronal dysfunction. Taken together, our findings along with others in the field implicate a major role for macrophages and microglia in the pathogenesis of EAE, which mimics human disease.

When assessed using two individual scoring methods, we found treatment with simvastatin proved to effectively reduce the average neurological deficit score by the experimental end point. For the experiential design, we forged a therapeutic approach by administering simvastatin at the onset of clinical signs, thus, first allowing the disease to establish. When administered treatment at this stage, we found

simvastatin reduced the duration of peak disease. Furthermore, from peak disease onwards, the simvastatin group consistently showed a lower average neurological deficit score for the duration of disease, when compared with vehicle receiving animals. In addition to this, we found simvastatin treatment accelerated the onset of the remission phase and continued to be significantly reduced throughout the second relapse phase until the end point. These findings are in line with others in the field. The statin family of drugs have repeatedly demonstrated efficacy in reducing neurological deficit across a number of EAE models (Youssef et al., 2002) (Stanislaus et al., 2001b) (Walters et al., 2002, Peng et al., 2006) (Greenwood et al., 2003c).

Due to the observed improvement in neurological deficit with simvastatin therapy, next, we aimed to monitor the recruitment of infiltrating macrophages and resident microglia whilst at the same time assess their activation status. By identification of the surface marker Iba-1, which recognise all macrophages and microglia, we confirm that fewer macrophages and microglia are present in the healthy CNS. In late stage EAE, however, we saw high levels of expression in the inflamed spinal cord. More importantly, both macrophages and microglia (Iba-1), and their activated forms, ED-1, were significantly reduced following administration of simvastatin.

In our study, simvastatin treatment was delivered at the onset of symptoms which is in line with microglia activation and peripheral-derived monocyte infiltration into the CNS during EAE (Kierdorf et al., 2019). Here, we demonstrate that oral administration of simvastatin in the EAE dark agouti rat dramatically attenuated infiltration of leukocytes into the spinal cord, as evidence by a reduction in Iba-1 and ED-1 staining, and substantially alleviated clinical signs of disease in both the first and second peak of relapse. Over the past decade, several members of the statin family have proven to affect BBB function and inhibit the infiltration of mononuclear cells into the CNS. Indeed, infiltration of inflammatory cells into the CNS have been described as key events in MS pathogenesis resulting in BBB disruption that precedes damage to target tissues (Sospedra and Martin, 2005). Substantial evidence exists to support the hypothesis that statins disrupt leukocyte-endothelial signalling and prevent successful migration across the BBB. The first reported *in vivo* study to demonstrate leukocyte inhibition in EAE was performed by Singh and colleagues (Stanislaus et al., 2001a). These findings were subsequently confirmed by a number of other research groups and implemented in a variety of EAE models (Youssef et al., 2002) (Ifergan et al., 2006a) (Stanislaus et al., 2001a). Although exact details of this inhibition are still under review, several theories exist for how it might occur and point towards a cholesterol-independent pleiotropic mechanism. First, statins have proven to modify endothelial cell activity and function by interference with ICAM-1 mediated signalling (Walters et al., 2002) (Prasad et al., 2005) (Greenwood et al., 2003c). This molecule is essential for the adhesion and engagement of endothelial cells to begin active facilitation of transendothelial migration mechanisms (Etienne et al., 1998) (Adamson et al., 1999). Our lab were amongst the first to describe an endothelial cell ICAM-1/Rho dependent signalling mechanism to permit the movement of leukocytes through a specialised BBB (Etienne-Manneville et al., 2000) (Adamson et al., 2002) (Greenwood

et al., 1995) (Male et al., 1994). Briefly, ICAM-1 binding stimulates a reorganisation of the endothelial actin cytoskeleton which in turn forms stress fibres and activates the small GTP-binding protein Rho.

The two intracellular signalling molecules principally responsible for isoprenylation within the cholesterol biosynthesis pathway are FPP and GGPP. Binding of these molecules to small GTPases such as Rho, is an absolute requirement for activation. In the cytoplasm, Rho is present in an inactive GDP-bound form. The hydrophobic chains of FPP and GGPP are essential for tethering Rho to the cell membrane, enabling proper function and localisation (Hall, 1998) (Wang and Casey, 2016). Other small GTPases that are known to depend on c-terminal anchoring to the cell membrane for activation include Rac, and Ras. Protein prenylation can be manipulated with various pharmacological inhibitors, the Greenwood lab were amongst the first to identify an inhibitory role for statins in this process (Greenwood et al., 2003b). It is now widely accepted that statins inhibit isoprenoid pyrophosphate synthesis, precursors required for the prenylation and posttranslational activation of Rho GTPase. Thus, statin-induced inhibition of functional Rho prevents effective ICAM-1 signalling and restricts leukocyte migration into the CNS (Konstantinopoulos et al., 2007). This suggests that observed beneficial clinical outcomes of simvastatin on EAE here, are at least in part due to decreased inflammatory cell infiltration.

While these findings proved to be a major breakthrough for understanding statin-induced immunomodulation and clinical improvement in EAE models, it likely that a number of pleiotropic mechanisms are be working in tandem to ameliorate the clinical signs of disease *in vivo*. Other key steps involved in leukocyte transmigration are production of chemokines by the endothelial cells to encourage chemotaxis. Simvastatin has been reported to reduce BBB migration of monocytes extracted from MS patients by directly decreasing the BBB endothelial cell secretion of CXCL10 and CCL2 (Ifergan et al., 2006b). Additionally, in cultured human and murine microglia it has been observed that simvastatin directly effects the surface expression of key chemokines involved in cell motility, both CCR5 and CXCR3 were reduced in response to simvastatin treatment (Kuipers et al., 2006). The same study also identified alternations in cytoskeletal distributions, which may be essential for responding to damage *in vivo*. Further to this, statins have shown to inhibit the chemokine MCP-1 in mononuclear cells. It has also been proposed that statin-induced alterations in matrix metalloprotease secretion may disrupt leukocyte-endothelial cell signalling in an isoprenoid-dependent mechanism (Turner et al., 2005) (Wong et al., 2001). Mehte *et al.* (2005) reported that statins inhibit TLR-4 expression in monocytes in an isoprenylation-dependent manner. Indeed, failure to engage the TLR receptor prevents downstream signalling that would otherwise coordinate the activation of MAPK, JAK/STAT and $\text{NK}\kappa\text{B}$ signalling pathways. Consequently, the expression of cytokines and costimulatory molecules will be altered.

While the majority of beneficial effects in EAE were observed and described in response to lovastatin treatment, previous studies have reported conflicting results with different members of the statin family in EAE, some reported a functional benefit, while others reported worsening of neurological

dysfunction (Birnbaum et al., 2008) (Togha et al., 2010) (Lanzillo et al., 2010) (Orefice et al., 2007). These mixed results can be explained by the highly variable pharmacokinetic and pharmacodynamic properties of the statin family. However, evidence exists to support the hypothesis that simvastatin, in this context, may behave in a similar way. First, comparative *in vitro* permeability assays found lovastatin and simvastatin to be equipotent (Ifergan et al., 2006b). In mice, lipophilic statins (simvastatin) have reportedly crossed the BBB (Johnson-Anuna et al., 2005). Similar to lovastatin, simvastatin was chosen here for its powerful BBB penetration profile. Further, simvastatin treatment *in vitro* has shown to directly reduce the expression of adhesion molecules in circulating monocytes, via reduction of CD45 and CD11a on PBMCs by inhibiting the activation of RhoA (Rezaie-Majd et al., 2003) (Yoshida et al., 2001). Moreover, critical chemokines and cognate receptors required for the process of transendothelial migration showed reduced expression by simvastatin in a isoprenoid-dependent manner (Veillard et al., 2006) (Romano et al., 2000).

An alternative explanation for reduced cellular activation markers, points to recently accumulated *in vivo* and clinical data that describes a statin-induced macrophage-like phenotypic paradigm switch, from M1 to M2 (van der Meij et al., 2013) (Zhang et al., 2018) (Yang et al., 2016) (Fujita et al., 2010). Here, our results compliment these findings, providing evidence that simvastatin reduces the number of ED1+ cells in the spinal cord of EAE. To corroborate this, our *in vitro* data also suggests that simvastatin reduces the activation status of microglia. Indeed, other research groups have reported on a similar role for lovastatin in reducing the activation status of microglia in culture (Pahan et al., 1997). However, further investigations involving in-depth macrophage and microglia phenotypic analysis are required to confirm this.

Taken together, we provide evidence that simvastatin monotherapy may represent a suitable treatment for improved neurological deficit and has the potential to be beneficial in MS. We also provide additional insights to support an effective treatment regime, namely, the duration, concentration, and administration method of simvastatin treatment. It is worth noting that dosing calculations for this experiment reflect the maximum permitted dose for humans (equivalent to 80mg) set out by the national institute for health and care excellence.

4.4.3 Experimental Optic Neuritis – A role for simvastatin?

Due to the assumed neuroprotective and well-documented immunomodulatory pleiotropic properties of statins, here, the potential role of simvastatin to reduce either microglial cell recruitment into the retina or activation status, was assessed. ED1, the murine equivalent of CD68, is a surface marker upregulated during microglia/macrophage activation and has classically been used to detect this status by immunofluorescence and immunohistochemical staining procedures (Dijkstra et al., 1985). In line with others, our findings demonstrate a pronounced response in microglia reactivity in the neuroretina during EAE, as demonstrated by increased numbers of ED1 stained cells when compared to naïve age-matched controls. Using the microglia specific TMEM119 marker, recent study findings have confirmed that these

populations are resident microglia (Manogaran et al., 2019). In addition to activation status, we assessed cell numbers of Iba-1 stained retinal populations, classically used to detect monocytes/macrophages and microglia, independent of their activation status. This marker allowed us to measure recruited cells into the retina. The levels of Iba1 cells were found to be equal between normal healthy control animals and rMOG EAE. At this late stage of disease, it is likely that monocytic migration is not promoted, as previously documented cases of optic neuritis suggest. Instead, local inflammatory responses are likely to be driven by activation of resident microglia, a finding possibly reflected in our current study. Using a model of EAE to investigate ON (Hu et al., 1998) found macrophage infiltration and redistribution towards the blood vessels began at day 7 to 8 post-immunisation. Microglia and macrophages are major sources of TNF α . This proinflammatory signalling protein is known to effectively increase BBB permeability and induce the expression of ICAM-1, an essential endothelial cell adhesion molecule instrumental in the process of leukocyte migration across the BRB (Bamforth et al., 1996) (Claudio et al., 1994) (Engelhardt et al., 1994) (Devine et al., 1996) (Greenwood and Calder, 1993) (Greenwood et al., 2003a). Moreover, it has been suggested that BBB breakdown at these early stages of disease allow inflammatory mediators from the periphery to reach microglia and stimulate their activation (Gehrmann et al., 1993).

In vivo studies with statin treatment have shown to reduce the number of ED1 stained infiltrating cells from the circulation into diseased CNS tissues (Van Linthout et al., 2007). These findings were attributed to a combination of statin-mediated pleiotropic effects on endothelial cell adhesion molecules. In this study, the authors reported inhibition of isoprenylated processes that led to the inactivation of RhoA, which is an essential molecule for actin cytoskeletal modelling, a key step for successful cellular migration across the vascular endothelium (Yoshida et al., 2001). These findings propose just one potential mechanism through which simvastatin may be reducing ED-1 levels, others suggest a M1 to M2 phenotypic switch (Zhang et al., 2018) (Fu et al., 2019).

4.4.4 Simvastatin treatment reduces the extent of hypoxia in the inflamed spinal cord.

Hypoxia is a common feature of MS. Recent studies provide compelling evidence for hypoxia-like white matter lesions in progressive MS (Aboul-Enein et al., 2003). Important links have been identified between brain metabolism and neurodegeneration, correlating cognitive defects to a reduction in cerebral metabolic rates of oxygen metabolism (Brooks et al., 1984). Spectroscopy studies in MS patients indicate around 50% may have hypoxia in the cortex (Yang and Dunn, 2015). The prevalence of hypoxia in MS lesions is further supported by EAE studies showing deoxyhaemoglobin via MRI measurements in the spinal cord (Nathoo et al., 2013). In these models, hypoxia (low PO₂) was found to promote demyelination (Davies et al., 2013a) (Desai et al., 2016).

In recent years, several studies have focused on the strong cellular expression of HIF-1 α (Aboul-Enein and Lassmann, 2005) (Graumann et al., 2003) and other hypoxia-related antigens (Lassmann et al.,

2003) (Mh ille et al., 2008) (McMahon et al., 2012) (Regenold et al., 2008) (Davie et al., 1994) in MS lesions. HIF1 α is a master regulator of the hypoxic response and is upregulated in response to low oxygen levels. HIF1 α is known to activate NF- κ B, a process that is particularly relevant in MS due to the strong inflammatory component. Pimonidazole was used in this study to detect low oxygen levels. This molecule readily penetrates all tissues, including those within the CNS. In the presence of low oxygen tension, pimonidazole is enzymatically reduced to a form that encourages permanent tissue binding, the product of which can be detected via immunohistochemical techniques (Arteel et al., 1995). It is worth noting that at physiological concentrations of oxygen pimonidazole is not reduced and does not bind to tissue (Liu et al., 2004). Here, immunohistochemical labelling for pimonidazole adducts disclosed striking positivity for tissue hypoxia in the spinal cord of rMOG-EAE diseased animals when compared with healthy controls. These findings are in line with other EAE studies where hypoxia staining intensity was markedly increased in diseased animals using the same antigenic rMOG trigger and detection methods with pimonidazole (Davies et al., 2013a). The same research group also demonstrated that spinal hypoxia in EAE closely correlates with the spatial and temporal progression of neurological deficits, beginning with the sacral portion of the spinal cord before reaching the lumbar regions (Desai et al., 2016). In our study, we confirmed that the spinal cord of rMOG-EAE rats featured hypoxia. Furthermore, our study confirmed the observation that the degree of hypoxia correlates well with disease severity.

Here, we show that simvastatin treatment significantly reduces the level of hypoxia detected in the spinal cord of EAE animals. There are several possible explanations for this observation. First, simvastatin could be sequestering HIF-1 α responses. Under hypoxic conditions and in an NF κ B-dependant manner, HIF-1 α accumulates in cells and orchestrates transcriptional activation of target genes involved in cell survival and proliferation. Previous studies have made associations between the transcription factor HIF-1 α and statins. (Hisada et al., 2012) described a pleotropic mechanism for fluvastatin in attenuating the expression of HIF-1 α in vascular smooth muscle cells. This response was attributed to the inhibition of isoprenylation and subsequent post-translational modification essential for a functional Rho/Rho-associated kinase pathway, a signalling cascade that has been found to induce HIF-1 α degradation (Takata et al., 2008). Similarly, (Wilson et al., 2002) identified an inhibitory role for simvastatin on HIF1 α regulated gene expression by ways of inhibiting small GTP-binding proteins via down regulation of isoprenoid intermediates. In other studies, Ras inhibition has shown to downregulate HIF-1 α (Blum et al., 2005). The pleotropic effects of statins in these studies, collectively, points towards statin-induced inhibition of isoprenylation of Ras, RhoA, Rac which are downstream of FPP and GGPP. Therefore, it is possible that simvastatin may be exerting similar effects in this model system to reduce the expression of HIF-1 α and related hypoxic response events. Indeed, widespread iNOS expression can be detected in progressive forms of MS and is concurrent with the presence of hypoxia. The biological importance of these discoveries relates to an *in vivo* scenario whereby NO competes with oxygen for the same binding site on mitochondrial

cytochrome c oxidase (Smith and Lassmann, 2002) (Jekabsone et al., 2007), reducing oxygen availability to the tissue which leads to hypoxia.

An alternative explanation for the observed beneficial effects of simvastatin on hypoxia could be due to improved mechanisms of perfusion. MS lesions are commonly associated with watershed areas, which have significantly lower rates of perfusion. Lack of sufficient blood flow, particularly in the microvasculature, starves tissues of oxygen. It is well documented that simvastatin can increase the expression of the functional protein eNOS and subsequently NO (Laufs et al., 1998) (Laufs and Liao, 1998) (Takemoto et al., 2002) (Ming et al., 2002). Locally produced NO is known to induce vessel relaxation and widening, which improves flow and oxygen delivery. Thus, simvastatin in this context could operate by restoring normal tissue oxygen levels.

Another possible explanation for a reduction in hypoxia levels with simvastatin treatment may be due to a reduction in the accumulation of immune cells at the site of injury. It is widely accepted that inflammation results in dramatic shifts in metabolic and increases oxygen consumption (Kominsky et al., 2010). In Figure 4.8 of this thesis, we provide supportive evidence to shows reduced cellular infiltration into the inflamed spinal cord with simvastatin treatment.

Positive labelling for hypoxia was observed across the entire spinal cord cross-section, however, staining was most obvious in the white matter. The white matter of spinal tissue is enriched with axons and myelin, by contrast, the grey matter mostly consists of high-density neuronal cell bodies. Since pimonidazole does not label myelin a probable explanation for our observed staining pattern places oligodendrocytes as a likely candidate. Indeed, the work carried out by our collaborators (Kenneth J Smith, University College London) demonstrated colocalization of pimonidazole staining with oligodendrocyte cell bodies (Davies et al., 2013a). Other cell-specific markers including β -tubulin, neuronal cell bodies and astrocytes also showed signs of colocalization patterns with pimonidazole. However, an important discovery by (Lyons and Kettenmann, 1998) placed emphasis on hypoxia-induced oligodendrocyte death when exposing these cells to low oxygen conditions for as little as 6 hours resulted in a staggering 90% death. Here, we found intense pimonidazole staining corresponded to clusters of ED1+ cellular infiltration suggesting a role for activated microglia and macrophages.

4.4.5 Nitrosylated protein: A feature of rMOG-EAE

Under inflammatory conditions, excessive superoxide is generated either enzymatically via NADPH oxidases located on the surface of macrophages, microglia and polymorphonuclear cells, or non-enzymatically by dysfunctional mitochondria (Vignais, 2002). Superoxide is inherently unstable and therefore spontaneously dismutates to form hydrogen peroxide and oxygen (Fridovich, 1978). During inflammation, NO levels are elevated, giving rise to the inevitability of a superoxide-NO reaction (Huie and Padmaja, 1993). A diffusion-limited action that almost always results in the irreversible formation of

peroxynitrite (Beckman and Koppenol, 1996). Peroxynitrite is a highly reactive free radical, with a short half-life, and therefore locally interacts with proteins in the vicinity of its generation. 3-Nitrotyrosine is a putative footprint of tyrosine residues that have been nitrated as a consequence of peroxynitrite, a molecule considered to be a useful and accurate measure of NO-mediated damage.

In our study, we observed nitrotyrosine positive cells in the inflamed spinal cord of EAE. The majority of positive labelling was located within the white matter of the dorsal column and diffusely spread in pattern. Of late, nitrotyrosine has become increasingly recognised to play an active and important role in the pathogenesis of MS. Indeed, elevated levels of CSF nitrotyrosine concentrations have been detected in patients with MS (Calabrese et al., 2002b). In addition, a clinical association has been made between high concentrations of NO and greater disease severity with chances of relapse. Moreover, both iNOS and nitrotyrosine expression have previously been detected in chronic lesions of MS patients (Liu et al., 2001). In these studies, the cytotoxic effects were attributed to persistent high-level production of NO generated from the inducible form of nitric oxide synthase (iNOS), findings such as these paved the way for investigations into iNOS inhibition in an MS-like setting. (Ding et al., 1998) and colleagues were amongst the first to describe the protective benefits of iNOS inhibition in an EAE setting, along with many others that followed (Sonar and Lal, 2019) (Teixeira et al., 2002) (Okuda et al., 1997). However, the role of iNOS inhibition in EAE has not always been uniform but instead offered conflicting results which have led to ongoing investigations (Zielasek et al., 1995) (Fenyk-Melody et al., 1998) (Niedbala et al., 2011) (Brenner et al., 1997). In view of the discrepancies, it is now generally understood that inhibition of iNOS at different stages of the immune response can differentially alter brain and spinal cord pathology. Despite the plethora of literature available, the effects of iNOS on EAE function remain controversial. The variability of findings is also heightened when taking into account the diverse stages and subsequent pathologies presented in EAE. Thus, understanding the cellular and molecular mechanisms through which iNOS functions in a tailored manner, could help to design better strategies for the clinical management of neuroinflammation and neuronal autoimmunity.

The role of simvastatin to act as a candidate iNOS inhibitor, has been demonstrated throughout the literature both *in vitro* and *in vivo*. (Trocha et al., 2010) identified a protective role for simvastatin in regulating aberrant elevated iNOS levels *in vivo*. Furthermore, (Madonna et al., 2005) showed that simvastatin attenuated the expression of cytokine-induced iNOS in embryonic cardiac monocytes, a mechanism believed to involve blockade of small GTPases responsible for downstream transcriptional signalling of NF- κ B. Despite these claims, simvastatin treatment in rMOG-EAE did not seem to impact the magnitude of nitrative damage present at the late stages of disease, suggesting simvastatin may not be impacting specific drivers of nitrotyrosine production in this particular model system.

4.4.6 DNA/RNA damage is reduced in rMOG- EAE spinal tissue simvastatin treatment

Often, one of the earliest detectable events observed at the tissue level of neurodegenerative conditions is increased oxidative DNA damage, a process that can occur in both nuclear and mitochondrial DNA. Oxidative DNA damage has been hypothesised to contribute to motor-neuron degeneration in an array of disorders well documented and reviewed throughout the literature (Martin et al., 1999) (Lovell and Markesbery, 2007) (Chang et al., 2008) (Warita et al., 2001) (Markesbery and Carney, 1999) (Abolhassani et al., 2017). Induced by the hydroxyl radical, 8-OHdG is an oxidized derivative of deoxyguanosine that serves as a cellular marker for oxidative stress. Recently, 8-OHdG has emerged as a potential diagnostic and disease biomarker in MS (Rasche et al., 2016). Previous in-depth studies on active MS brain lesion showed severe signs of oxidative stress characterised by extensive accumulation of oxidised phospholipids and oxidised DNA/RNA in addition to enhanced expression of antioxidant factors (Haider et al., 2011). In this study, we performed immune histological analysis on rMOG-EAE spinal cord tissue to assess the expression levels of biomarkers indicative of such damage and to determine whether simvastatin is capable of alleviating or reversing these processes.

Animal models of stroke (Nagotani et al., 2005) and asthma (Ahmad et al., 2011) have demonstrated efficacy of simvastatin treatment in reducing tissue levels of 8-OHdG. To support this, (Franzoni et al., 2003) showed antioxidant activity against free radicals increased with simvastatin treatment *in vitro*. Moreover, in patients with cardiovascular disease, simvastatin was found to reduce circulating plasma levels of 8-OHdG (Moon et al., 2014). In the context of SPMS, the potential impact of simvastatin on this type of oxidative damage is less obvious. Indeed, 8-OHdG has been detected at significantly elevated levels in EAE spinal tissue; our findings are in line with this observation. However, (Hasseldam et al., 2016) described 8-OHdG positivity in spinal cord grey matter sections at the early stages of rodent EAE. Here, we observe positive staining for DNA/RNA damage at the late stages of EAE and with localisation to the white matter. Importantly, our treatment with simvastatin had a noticeable impact on the amount of damage present, causing a significant reduction in the presence of this marker in the diseased dorsal column. A similar finding was previously reported, the first of many studies to demonstrate statins' brain-protective property against ischemic damage *in vivo*, whereby the authors attributed the neuroprotective properties of statins to their ability to increase vascular eNOS (Rikitake et al., 2005b) (O'Driscoll et al., 1997) (Endres et al., 1998). A well-documented pathway in the cholesterol-independent signalling cascade involving inhibition of isoprenoid intermediates, is the ability of statins to increase the expression of the functional protein eNOS. Downstream ROCK-Rho signalling inhibition extends eNOS mRNA half-life, thereby allowing eNOS to accumulate and produce NO (Laufs et al., 1998) (Laufs and Liao, 1998) (Takemoto et al., 2002) (Ming et al., 2002). Unlike iNOS, that can produce excessive toxic levels of NO, highly localised NO concentration generated by eNOS are known to have a protective role and improve vascular perfusion. It may be the case here that simvastatin is increasing localised levels of NO and thereby, alleviating an

environment under oxidative stress through improved perfusion. Indeed, in both MS and EAE alike are characterised by poor vessel perfusion. Further, other groups have described a more direct neuroprotective mechanism for statins through modulation of excitotoxicity (Zacco et al., 2003). Moreover, (Hayashi et al., 2005) made an *in vivo* observation that statins reduce neuronal stress in ischemic brain injury by demonstrating a reduction in 8-OHdG and HNE tissue immunoreactivity, a similar finding and approach to what was used in our current study.

4.5 Conclusions

Collectively, the data generated from this chapter provide original insight to support a neuroprotective role for simvastatin in EAE. Taken together, simvastatin therapy has shown to ameliorate neurological deficit and attenuate the expression of biomarkers that represent tissue damage caused by oxidative stress.

Chapter 5

Simvastatin therapy: Attenuating ocular pathology and oxidative stress markers in EAU

5.1 Aims

1. To determine the efficacy of chronic simvastatin therapy on the disease course of IRBP₁₋₂₀ induced EAU.
2. To determine whether retinal inflammation by EAU can be attenuated with chronic simvastatin therapy
3. To determine whether simvastatin can reduce vascular permeability in EAU.
4. To establish the effects of simvastatin therapy on microglial activation through structural and functional alterations.
5. To determine whether simvastatin therapy can reduce the presence of oxidative stress markers in the inflamed EAU retina.

5.2 Hypothesis

Chronic simvastatin therapy can attenuate EAU disease progression and reduce the amount of oxidative stress.

5.3 Results

5.3.1 Characterising clinical disease course in IRBP₁₋₂₀ induced experimental autoimmune uveitis

5.3.1.1 ASSESSING OCULAR PATHOLOGY IN IRBP₁₋₂₀ EAU

Subcutaneous injection of IRBP₁₋₂₀ peptide (300-500 µg), in female C57BL/6 mice induced a monophasic and chronic posterior inflammatory disease, characterized by ascending infiltration and structural disturbances. Typical disease onset presented 14 days post-immunization (p.i.) with an incidence of 70%, before reaching peak incidence of 90% at day 21 p.i.

At the onset of disease, severity varied considerably. The early indicators of disease, identified by fundoscopic retinal analysis, were characterized by the presence of optic disc swelling, which would then later mature to infiltrating inflammatory cells through processes of extravasation. Severe disease forms, seen at the later, chronic stages of disease or peak of incidence (day 21 p.i.), would present with multiple inflammatory lesions distributed throughout the retina, coupled with intense vasculitis and a more resolved optic disc swelling, but the presence of tissue damage. The measurement parameters for disease assessment were as follows: optic disc swelling, retinal vessel involvement, tissue infiltrate and structural damage. The degree of clinical disease was measured using a 20-point scoring system (materials and methods). Briefly, scores were assigned on a scale of 1 to 5, based on the severity of each parameter outlined above.

At day 7 p.i. there was little retinal change when compared with complete Freud's adjuvant (CFA) controls only. Changes began to present at day 11 p.i. with the earliest signs of disease mainly manifesting as optic disc swelling. Although these differences could be identified in 50% of the population, they were very mild and representative of the early stages of disease.

Disease severity at the early stages of induction (day 7, 10 and 14) was significantly lower than the later stages, including selected experimental endpoint (day 21), beginning with 6% disease incidence combined with an average severity of 6.1 ± 0.39 . Progression towards a maximum of 75% was achieved by day 21, accompanied with an average clinical pathology of 7.6 ± 0.33 (Figure 5.1B). This progressive disease trajectory permitted a therapeutic window for simvastatin administration, between the days of 14 and 21, the aim being to examine the therapeutic effects of simvastatin in this chronic model.

Day 14 was typically where the onset of immune cell infiltration began, coupled with severe optic disc swelling and vasculitis. Day 21 was where disease generally reached its peak being characterized fundoscopically by aggressive infiltration, with punctate lesions visible on the surface of the retina. What was also found to be prevalent at this stage was severe vasculitis, or vessel cuffing, often involving multiple vessels, both vein and main arteries in addition to extensive tissue damage in areas (Figure 5.1A), at this point eyes were harvested for further analysis. After peak disease, severity plateaus and was maintained until day 32 (Figure 5.1B).

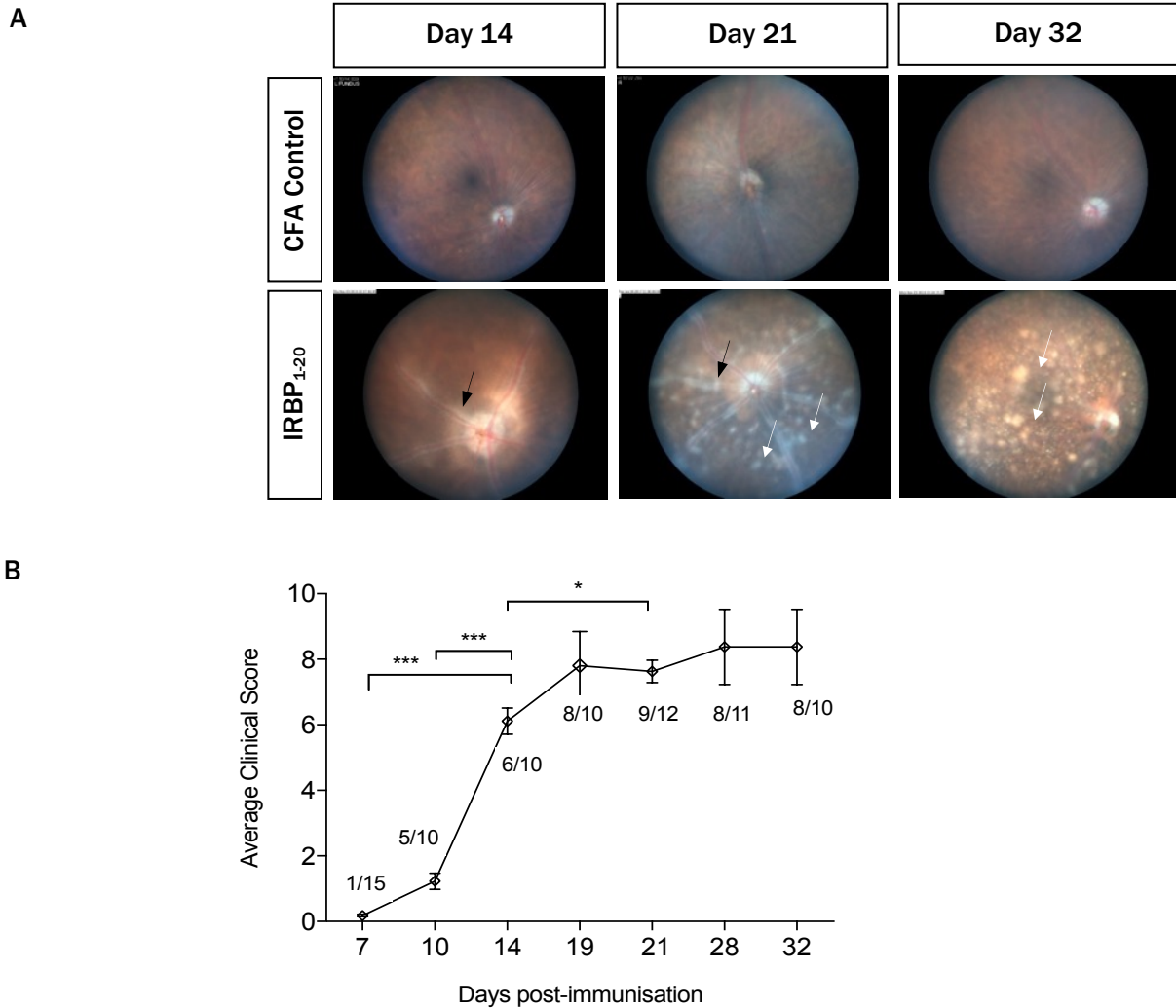


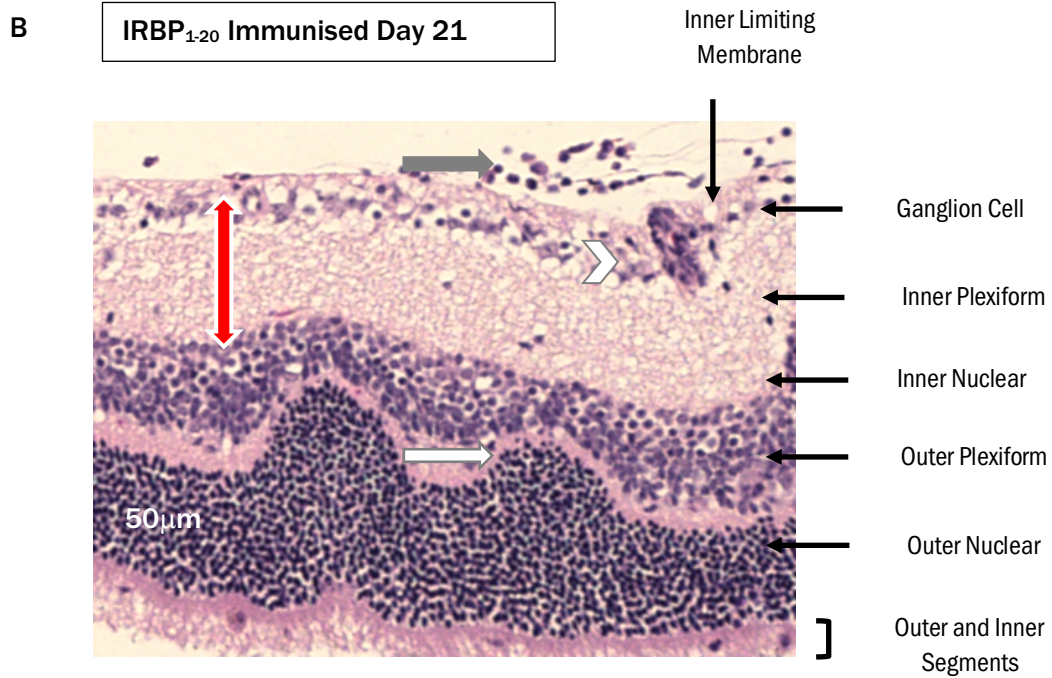
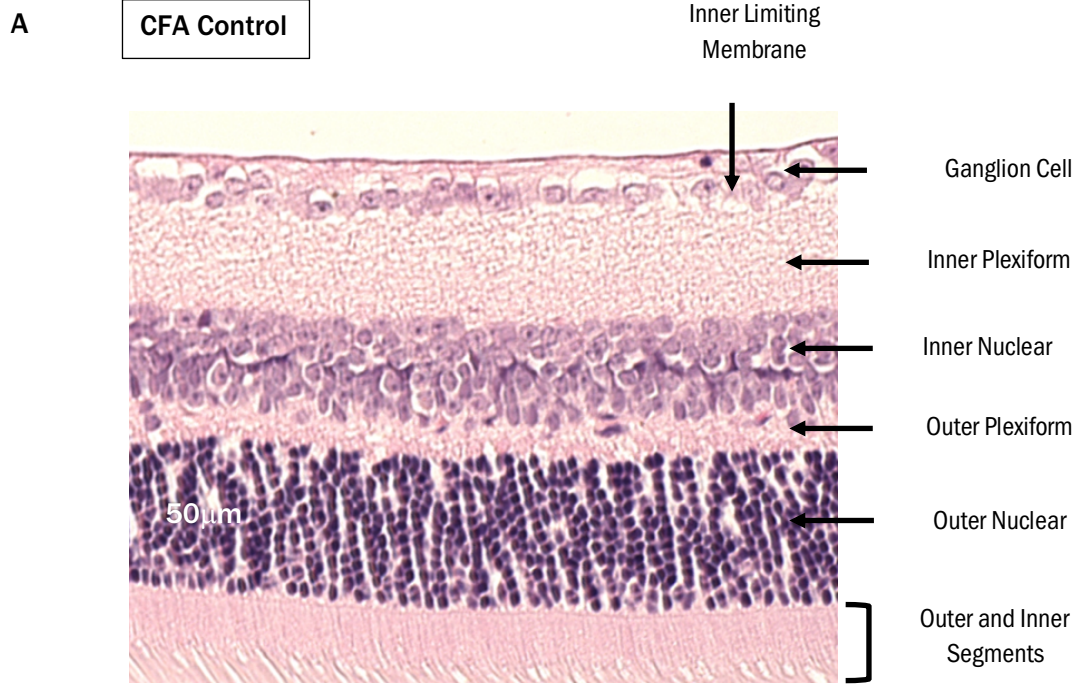
Figure 5.1. Clinical evaluation of retinal disease at different stages of IRBP1-20 induced EAU. C57BL/6 were immunised with IRBP1-20 peptide and clinical evaluation via fundoscopy performed. (A) Representative images of mouse fundus and CFA control retina at days 14, 21 and 32 p.i. respectively, (top row). OD, optic disk. Representative fundus images of mouse retina IRBP1-20 immunised Day 14 p.i. mouse fundus showing severe optic disk inflammation, onset of vasculitis (black arrows). Day 21 p.i. showing mild optic disk inflammation, vascular cuffing (black arrows) and multiple populations of retinal infiltrates (white arrows). Day 32 p.i. mouse fundus showing multiple retinal inflammatory lesions (white arrows). Bottom row. (B) Clinical time course of IRBP 1-20 immunised C57BL/6 mice. Clinical evaluation (materials and methods) based on fundoscopy images at timepoints 7, 10, 14, 19, 21, 28 and 32 p.i. Disease incidence rates indicated by numerical overheads. One-way ANOVA performed for statistical analysis with Tukey's post-test. \pm S.E.M. * $P < 0.05$; *** $P \leq 0.001$.

5.3.1.2 HISTOPATHOLOGICAL ASSESSMENT

In addition to the clinical scoring system provided by digital fundus imaging, disease was also evaluated by conventional histopathology of retinal cross sections. Typical grading scores followed the methods provided by previously published criteria (Copland et al, 2008) (materials and methods).

H&E staining of CFA control animals (Figure 5.2A) demonstrate well-organised and uniform retinal layers, spanning; ganglion cell layer, inner plexiform, inner nuclear, outer plexiform, outer nuclear and outer and inner segments. Characteristic of healthy tissues, this serves as a comparison for all other IRBP₁₋₂₀ immunised mouse tissues. Figure 5.2B, is a representative micrograph of the severity of EAU achieved in this study at peak disease, 21 days post-immunisation. The most striking changes were observed in the vitreous (grey arrow), evidenced by populations of infiltrating cells, along with moderate levels of vasculitis (white arrowhead) as seen in the ganglion cell layer. This neuronal cell layer also presented with a moderate loss of tissue integrity and a notable amount of oedema (red arrow). Other features of disease involved retinal folds, inflammatory infiltrates (white arrow).

Comparisons were made between the clinical scoring system and histological grading. Figure 5.2C represents funduscopy images for control and clinical score of 15 at peak disease, alongside the corresponding H&E-stained tissue sections. All observations were performed sequentially on the same eye. Quantitative assessment of both grading systems shows an interdependence between the two criteria, Figure 5.2D demonstrating a strong positive and significant correlation ($p=0.0002$, $r^2=0.539$). When comparing the histopathological scoring between the CFA only control and the IRBP₁₋₂₀ induced mice, we can confirm sufficient disease induction from assessments using the conventional histopathological grading criteria. IRBP disease resulted in a culminated average score of 14.38 ± 1.141 , in contrast to all control samples 0.25 ± 0.1708 (Figure 5.2E).



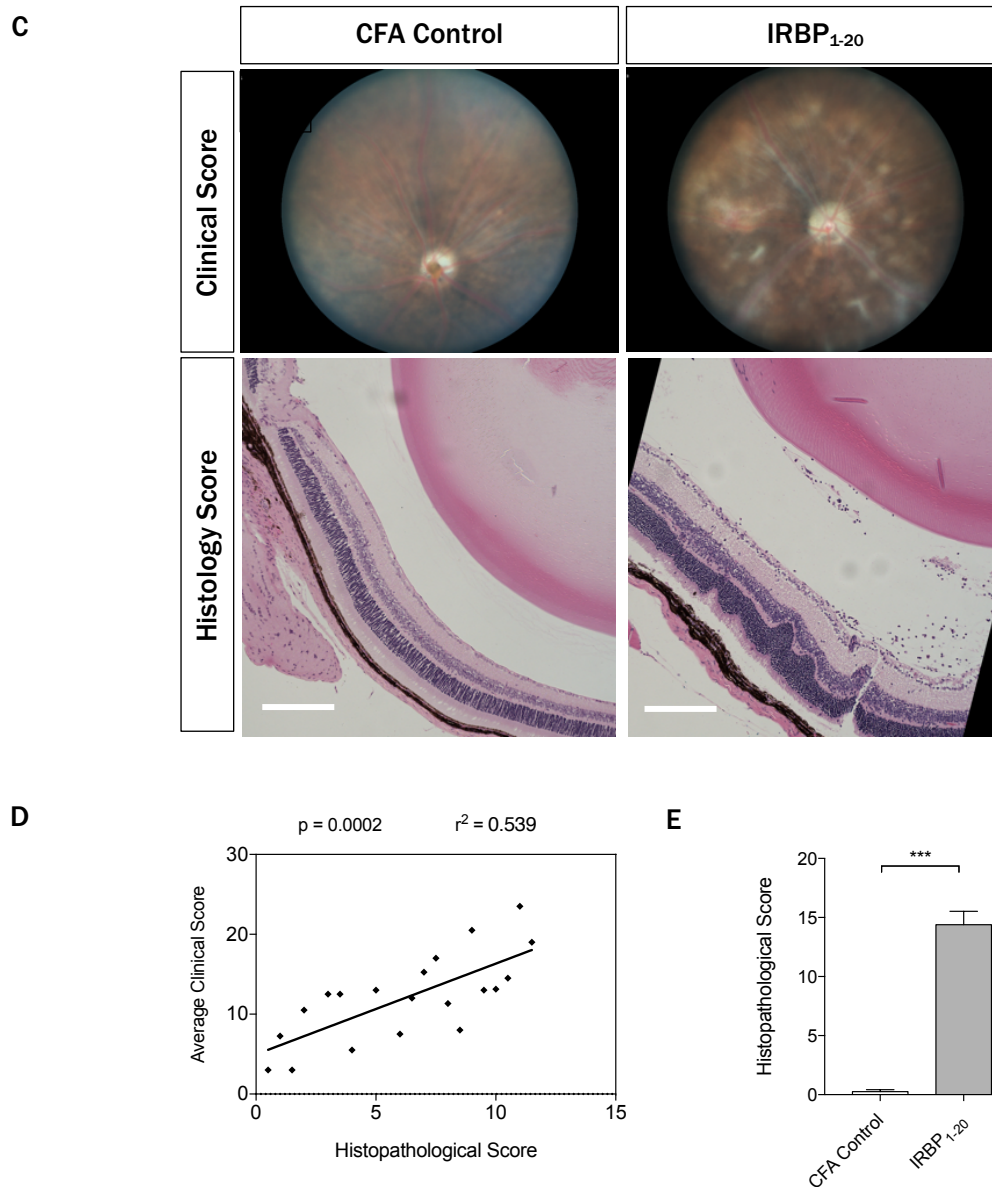


Figure 5.2. Histopathological evaluation of IRBP₁₋₂₀ induced EAU.

(A) Representative histological findings from IRBP₁₋₂₀ and CFA control (B) mice at day 21 post-immunisation. Sections (12 μm) were stained with haematoxylin and eosin. Micrographs captured at X20 magnification, outlining retinal layers; ganglion cell layer, inner plexiform, inner nuclear, outer plexiform, outer nuclear and inner and outer segments. Grey arrow; infiltrating cells, White arrowhead; vasculitis. Red arrow; oedema. White arrow; retinal folds. Scale bar = 50 μm (C) Representative funduscopy images taken at day 21 post IRBP immunisation and CFA control. Corresponding H&E-stained retinal tissues at X5 magnification. IRBP immunised displayed at a clinical disease score of 15 (materials and methods). Scale bars: 100 μm . (D) Linear regression showing the correlation between clinical score acquired by funduscopy and histopathological score. $n = 20$. (E) Quantification of average histopathological scores for EAU mice compared with CFA control, analysis histopathological changes, based on a 42-point scoring system. Statistical analysis, student's t-test. \pm S.E.M. ***, $P \leq 0.001$. $n = 10$ per group.

5.3.2 Chronic high-dose simvastatin therapy attenuates clinical ocular pathology in IRBP₁₋₂₀ EAU

Subcutaneous injection of IRBP₁₋₂₀ in female C57BL/6 mice induced a chronic posterior uveitic disease, characterised by ascending ocular infiltration, targeting the neural retina and uveal tissue (iris, ciliary body and choroid). In order to represent a more closely resembling therapeutic approach, treatment was administered at the first signs of clinical disease and continued daily until peak disease, where maximum cellular and structural damage had been reported to occur (Figure 5.3A).

Fundoscopic retinal analysis was employed to determine the clinical disease score of IRBP₁₋₂₀ induced experimental autoimmune uveitis (EAU) animals after simvastatin therapy. All asymptomatic, CFA control animals displayed healthy retinas under fundoscopic analysis. Symptomatic IRBP₁₋₂₀ animals, however, exhibited striking differences with varying amounts of retinal disease identified based on the presence of inflammatory lesions, vasculitis, optic disc swelling and structural damage, (Figure 5.3B). At peak disease, day 21 p.i., animals receiving vehicle (0.5% methylcellulose) were characterized by severe signs of inflammatory cell infiltration evidenced by multiple small retinal and linear lesions, either punctate and/or confluent in pattern. These features were also accompanied by vasculitis involving multiple retinal vessels and severe optic disc swelling in addition to tissue damage (Figure 5.3B).

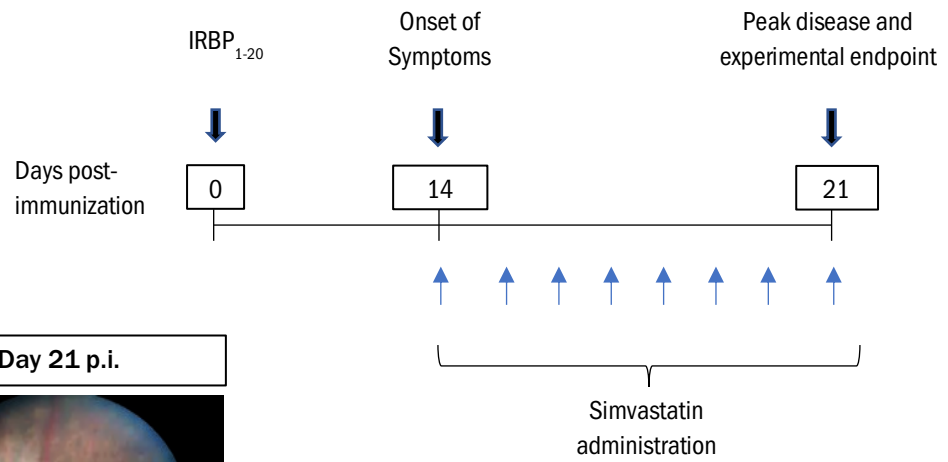
Mice were treated with different doses of simvastatin (50 mg/kg, 75 mg/kg and 100 mg/kg). According to the aforementioned treatment strategy, therapy began at day 14 p.i. and was administered daily, via oral gavage, until the experimental endpoint at day 21 when fundus imaging was carried out. Animal selection criteria took place at day 14 p.i. and those without definitive signs of disease were not selected to continue through the study.

It is evident from fundoscopic analysis at day 21 p.i. that both neither receiving animals and simvastatin therapy at 50 mg/kg, had any impact on disease progression, and instead followed the expected clinical time course for this model, being, a progressive increase across all measurement parameters: optic disc swelling, inflammatory lesions, vasculitis, and structural damage (Figure 5.3C). However, after chronic simvastatin treatment (8 days) at the higher doses of 75mg/kg and 100mg/kg, fundoscopic quantification revealed significantly milder disease compared to vehicle treated mice (Figure 5.3C), indicating reduced ocular pathology and disease attenuation. Accordingly, the average clinical score, determined by fundus analysis, for vehicle treated IRBP₁₋₂₀ animals on day 21 was 8.53, similar to the average score seen with 50 mg/kg simvastatin, 8.76. However, when treated with simvastatin at 75 mg/kg a significant reduction in clinical score was noted, 6.12, when compared with vehicle control. At the higher dose of 100 mg/kg a significant reduction when compared with control, was also noted.

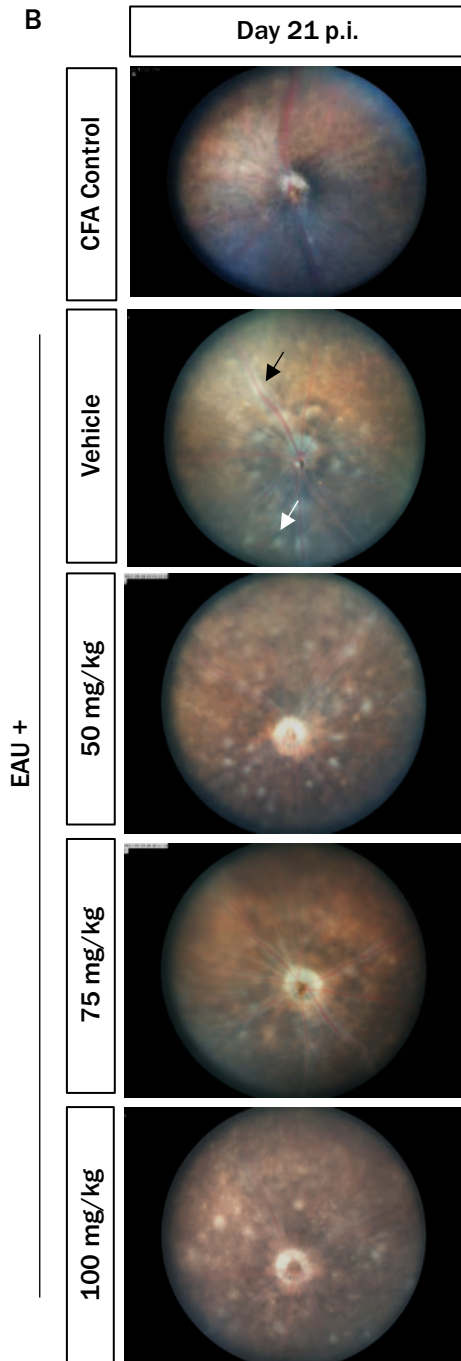
Representative fundus images at day 21 provides details of the characteristic features of disease with high dose simvastatin therapy (75 mg/kg and 100 mg/kg), with often milder optic disc swelling, mild to moderate vasculitis and fewer/smaller inflammatory cell lesions with mostly absent linear lesions when

compared with vehicle (Figure 5.3B). This, therefore, showed that higher doses of statin resulted in a milder disease overall as determined by funduscopy.

A



B



C

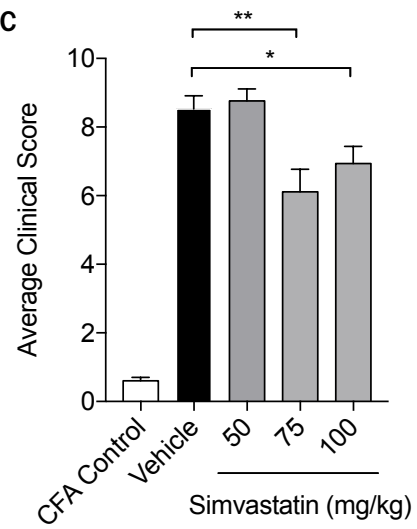


Figure 5.3. Simvastatin therapy attenuates clinical disease in IRBP₁₋₂₀

Animals were immunised at day 0 with a single subcutaneous injection of IRBP₁₋₂₀ and imaged at day 14 p.i. to positively select for those demonstrating signs of disease. Treatment vehicle (0.5% methylcellulose) or Simvastatin (50 mg/kg, 75 mg/kg or 100 mg/kg) was administered orally on day 14 and continued daily until peak disease at day 21. Fundoscopic images taken at both timepoints, after which, eyes were enucleated and prepared for histological analysis. (A) Schematic representation of treatment strategy used for Simvastatin or vehicle administration, beginning at the onset of infiltration and continuing until peak disease. (B) Representative fundoscopic images of CFA control retinas and IRBP-EAU treated with either vehicle, or Simvastatin at stipulated concentrations. White arrows indicating inflammatory lesions, black arrows to demonstrate vasculitis. (C) Graph showing quantitative analysis of the average clinical score per treatment group and presented at day 21 p.i. One-Way ANOVA. \pm S.E.M. *, $P < 0.05$; **, $P \leq 0.01$; $n = 20-25$ per group.

5.3.3 Histological examination of IRBP-EAU with simvastatin treatment

Histological disease was evaluated, across the whole eye section, at peak disease day 21 p.i. according to previously published criteria (materials and methods) based on the presence of ocular pathological features such as: mass cellular infiltration into the vitreous; retinal folds, oedema and infiltrates; choroiditis, vasculitis, rod outer segment and neuronal layer degeneration. The CFA control group did not express any of the aforementioned pathological features. Vehicle treated IRBP-EAU mice exhibited a variety of histological changes, namely; vasculitis, oedema and neuronal layer degradation all of which were frequently observed and seen to be hallmark features of this disease (Figure 5.4A). Quantification of tissue disturbances in the vehicle treated confirmed such observations, exhibiting changes significantly above CFA control levels and revealing an overall score of 18.71 ± 2.63 (Figure 5.4B). When assessing the presence and severity of each parameter across all simvastatin treatments, although scores appeared reduced, changes were not significant (50 mg/kg; 13.5 ± 2.57 , 75 mg/kg, 17.13 ± 2.95 and 100 mg/kg; 15.25 ± 1.87).

The above histopathological grading criteria used to assess EAU disease can be subcategorised into two distinct and separate arms, according to the type of damage present. Since a reduction was observed, albeit not significant, in histology findings between simvastatin treated groups and vehicle, we next dissected out the grading criteria in an effort to maximise those differences in specific parameters that may otherwise have been masked. The two main assessment categories used in the above analysis comprise either inflammatory or structural/morphological changes, which we went on to examine in isolation. Inflammatory features included: quantified number of cellular infiltrates present within the multiple anatomical features (vasculitis, choroiditis, ciliary body and vitreous cell infiltration) throughout a cross-section of the eye, scores ascending with accumulative infiltration, and a maximum score for this component totaling 30 (Figure 5.4C). Structural differences were classified as; rod outer segment immune infiltration, neuronal layer loss and retinal folds (Figure 5.4D). Taken in isolation we see similar trends in both assessment approaches, a sharp and significant increase above CFA control when disease is induced, with less disease amongst the treated groups, but still not significant to confirm attenuation using these methods.

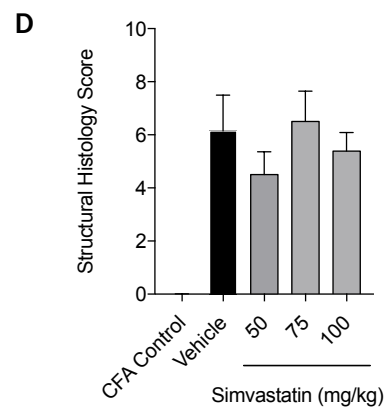
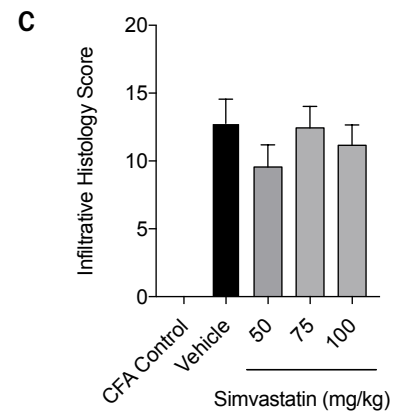
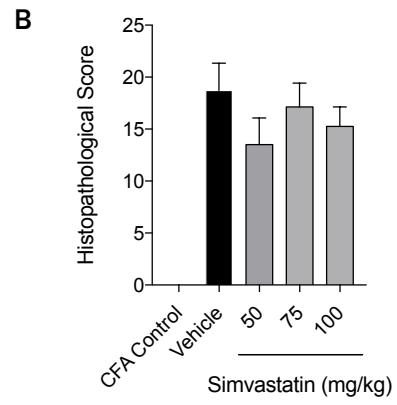
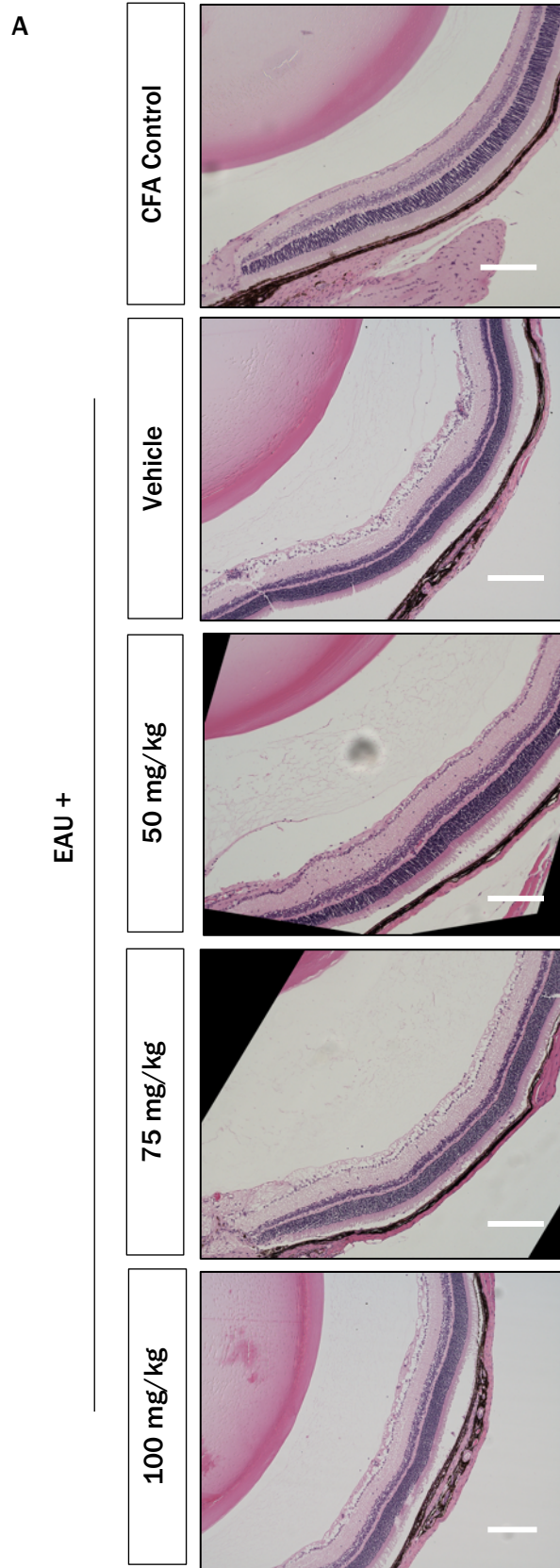


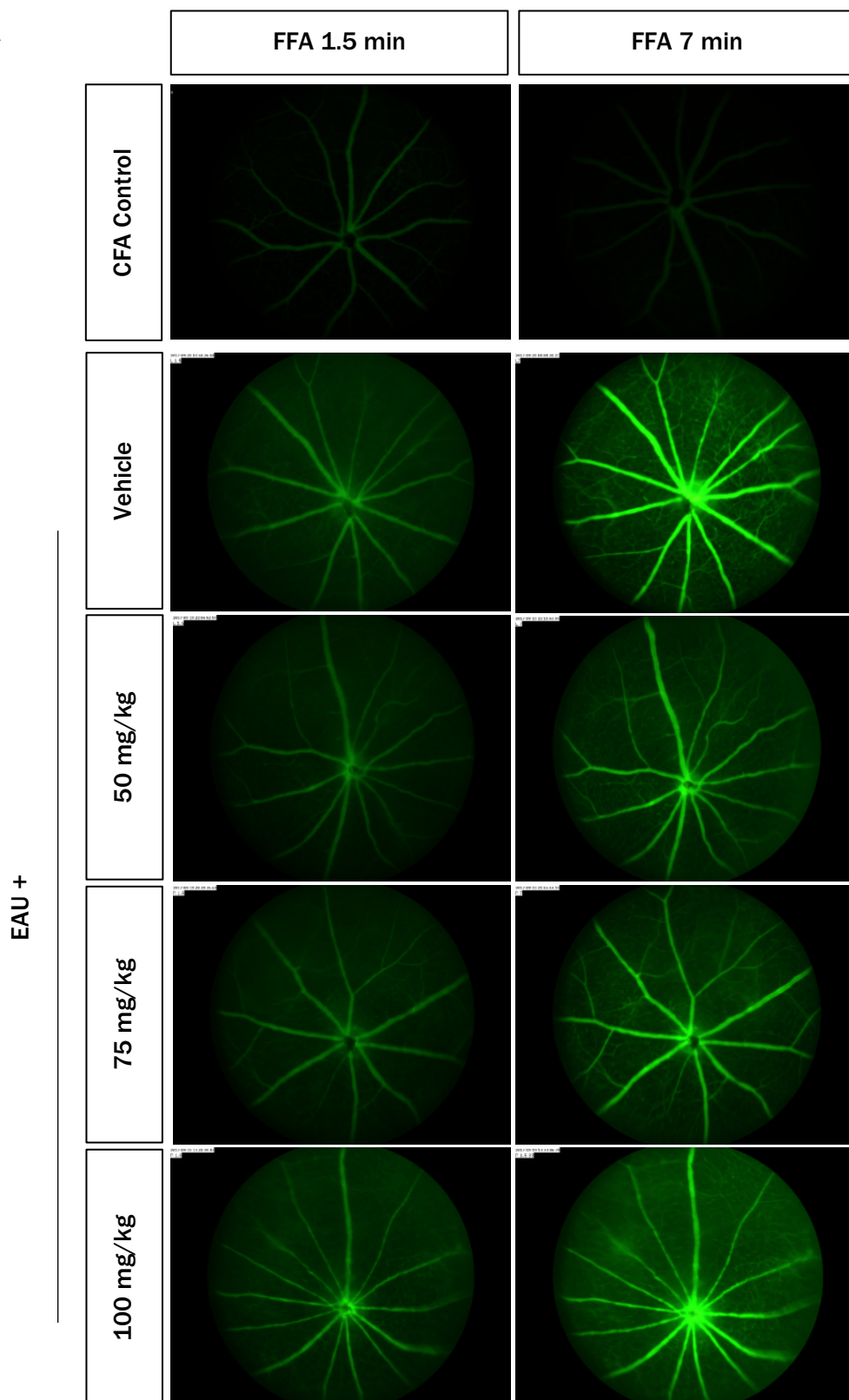
Figure 5.4: Simvastatin therapy on histopathological grading of EAU disease. Eyes were harvested at peak disease day 21 p.i. following treatment; vehicle (0.5% methylcellulose) or Simvastatin over an 8-day period and prepared for histological analysis. Formalin-fixed, paraffin embedded techniques were used to prepare the eyes and cross-sections mounted onto slides and stained for H&E. (A) Representative micrograph at X20 magnification, demonstrating a CFA control, IRBP₁₋₂₀ immunised alone and IRBP-EAU with simvastatin treatment at 50 mg//kg, 75 mg/kg and 100 mg/kg. (B) Graph showing average histopathological scores per treatment group based on grading criteria (materials and methods). (C) Graph showing average infiltrative histology score based on inflammatory components of the disease, sub categorised from total scoring (A). (D) Graph showing average structural damage quantified per treatment group, sub categorized from total scoring (B). One-way ANOVA performed for statistical analysis. n =10-15 per group.

5.3.4 Low-dose simvastatin therapy in IRBP-EAU decreases vascular leakage

Live imaging techniques were utilised at day 21 p.i. before mice were sacrificed to evaluate the amount, if any, of leakage present in this model and whether simvastatin had any impact on this. Briefly, intraperitoneal injections of fluorescein (2%) were used to assess alterations in vascular permeability with simvastatin therapy. Following the administration of fluorescein, images were taken at 1.5 minutes and again at 7 minutes, then leakage ratio data calculated and compared across treatments, representative images can be found in Figure 5.5A. After receiving chronic therapy for eight days, at either 50 mg/kg, 75 mg/kg or 100 mg/kg pooled data revealed a significant decrease in leakage ratio between vehicle and simvastatin receiving animals at the experimental endpoint of day 21 (Figure 5.5B).

Next, the calculated leakage ratio was plotted against average clinical scores using day 21 data for any corresponding trends. A positive and significant correlation was reported in the vehicle treated EAU linking increased vessel leakage with high clinical disease scores ($p = 0.0092$, $r^2 = 0.41$, $n = 15$, Figure 5.5C). Strikingly, with simvastatin treatment at each of the listed concentrations, this relationship is lost 50 mg/kg ($p = 0.265$, $r^2 = 0.111$, $n = 14$, Figure 5.5D), 75 mg/kg ($p = 0.796$, $r^2 = 0.0042$, $n = 18$, Figure 4.5E) and 100 mg/kg ($p = 0.272$, $r^2 = 0.091$, $n = 15$, Figure 5.5F).

A



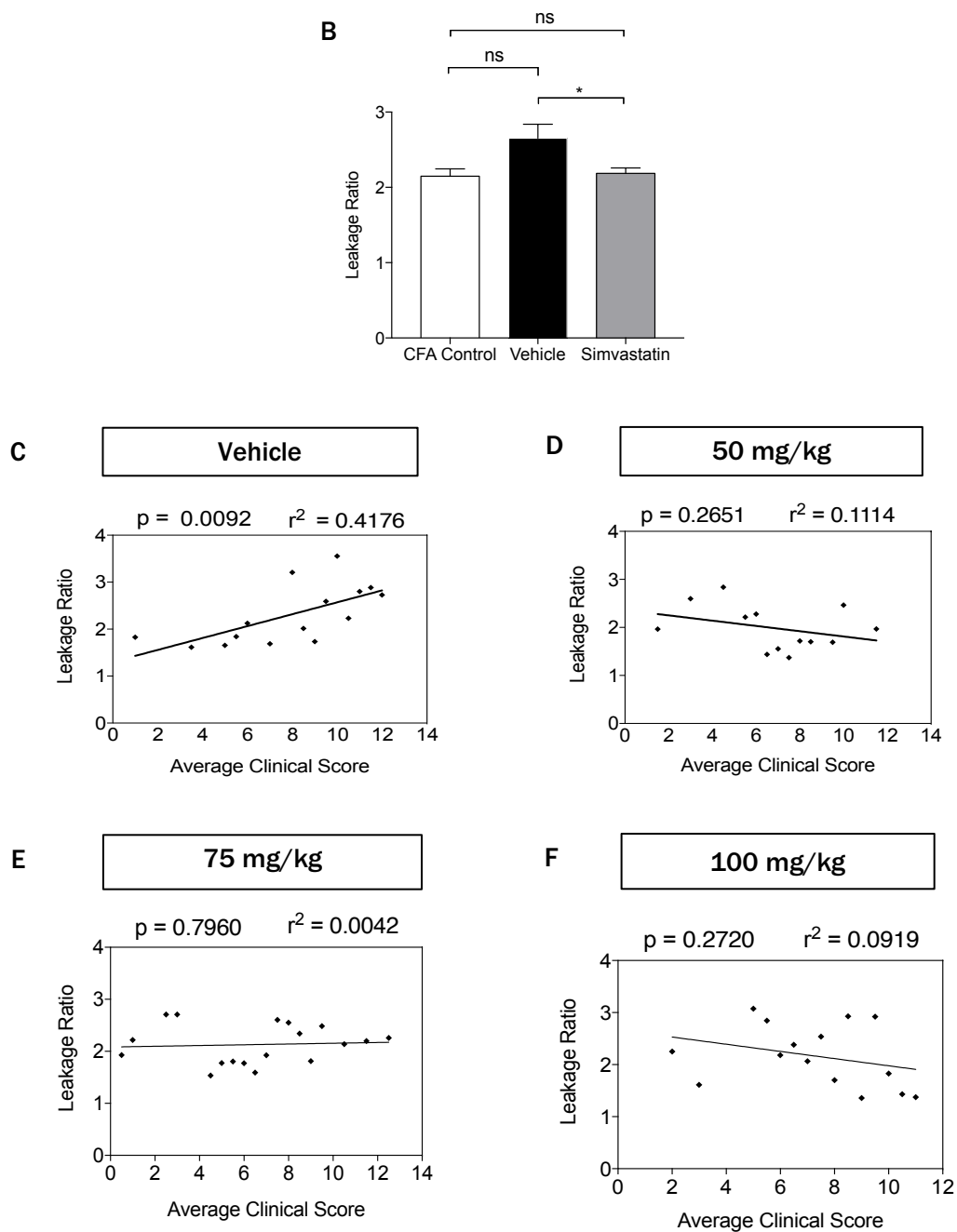


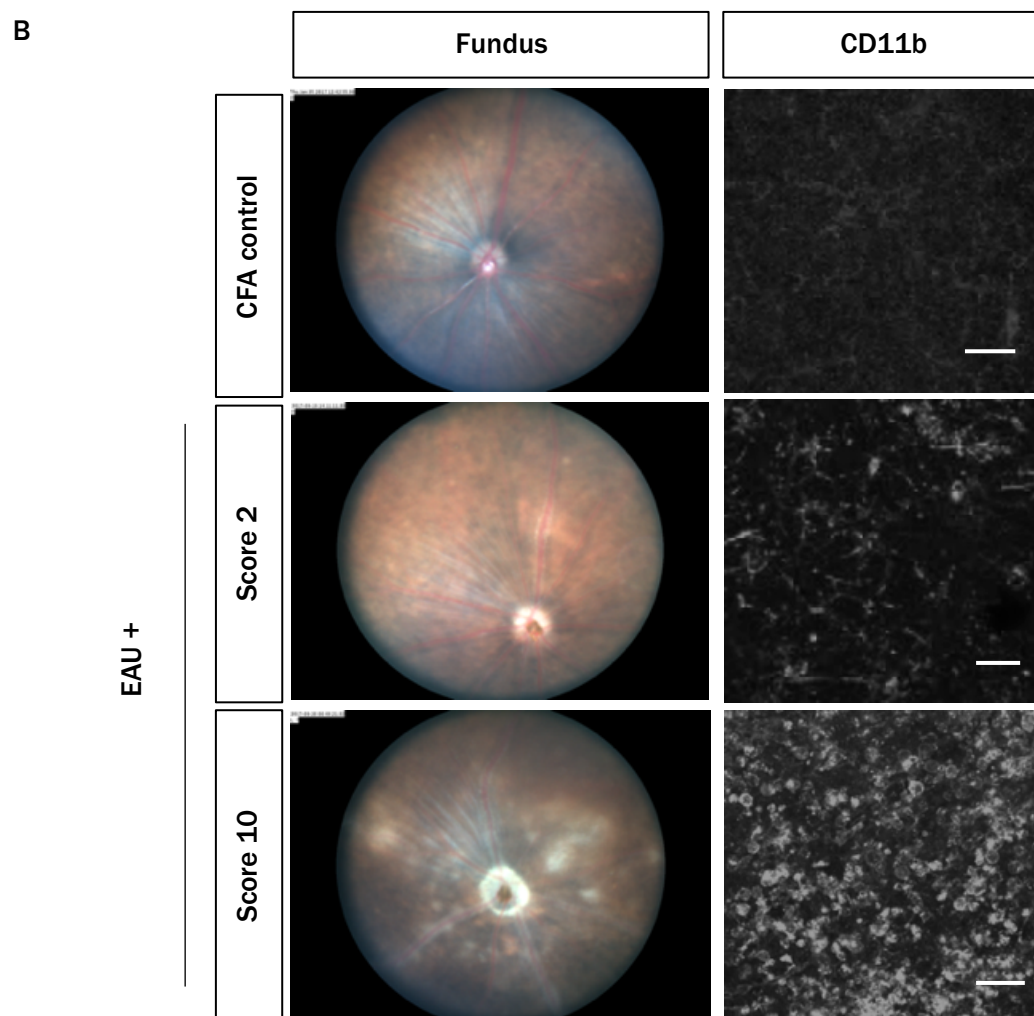
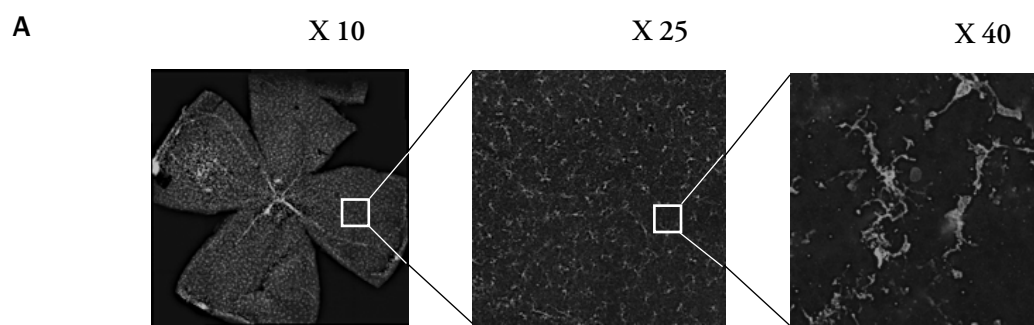
Figure 5.5. Low dose simvastatin shows reduced vascular leakage in a model of EAU measured by fluorescein angiography. IRBP1-20 Immunised C57BL/6 mice day 21 p.i. treated with either vehicle, 50 mg/kg, 75 mg/kg or 100 mg/kg simvastatin. (A) Representative images of day 21 p.i. using bright field (BF) or fluorescein (FFA) at 1.5 min and 7 min (B) Leakage ratio analysed from fluorescein angiography images day 21 p.i. Mice were compared according to CFA control, vehicle-treated and all pooled Simvastatin treated data (50 mg/kg, 75 mg/kg and 100 mg/kg). $n = 5-46$ per group. One-way ANOVA performed. (C-F) Linear regression of leakage ratio data alongside clinical score data at day 21 p.i. for vehicle, 50 mg/kg, 75 mg/kg and 100 mg/kg treated groups, (F) T-test with Welch's correction. *, $P < 0.05$.

5.3.5 CD11b as a correlate of disease severity in IRBP₁₋₂₀ EAU

Integrin alpha M (ITGAM), otherwise known as cluster of differentiation molecule CD11b, is expressed on the surface of many myeloid cell populations, predominantly those of the innate immune system and include labelling for monocytes/macrophages, neutrophils, natural killer cells and granulocytes. In addition, this marker also identifies CNS tissue resident microglia. On circulating immune cells, it is widely recognised as being involved in the regulation of transendothelial adhesion and migration, as such was utilised in this study to measure neuroretinal inflammation.

Representative images of neuroretina stained for the myeloid cell marker CD11b were captured at different magnifications to demonstrate the data acquisition methods used for cell quantification and subsequent analysis (Figure 5.6A). Microscopically, symptomatic IRBP₁₋₂₀ animals exhibited varying amounts of retinal inflammation, characterised by mass infiltrative CD11b immunoreactivity, which was largely absent in asymptomatic, control animals, as seen in neuroretinal micrographs (Figure 5.6B). CD11b positive cells were predominantly found around the major veins and arteries of the retina. As disease severity increased, the inflammatory cells were found to penetrate the deeper retinal layers migrating from the superficial plexus into the outer plexiform, whereas control, non-immunised animals displayed little CD11b positive cell count in the superficial plexus and an absolute absence in subsequent layers.

The extent of inflammation, as defined by the number of CD11b positive cells, during these chronic stages of disease was directly related to ocular pathology as assessed by fundoscopy, ($p= 0.0009$, $r^2 = 0.55$, $n= 24$, Figure 5.6C). Thus, the neuroretinas of those animals with a higher clinical fundus score presented with an increased number of CD11b expressing myeloid cells.



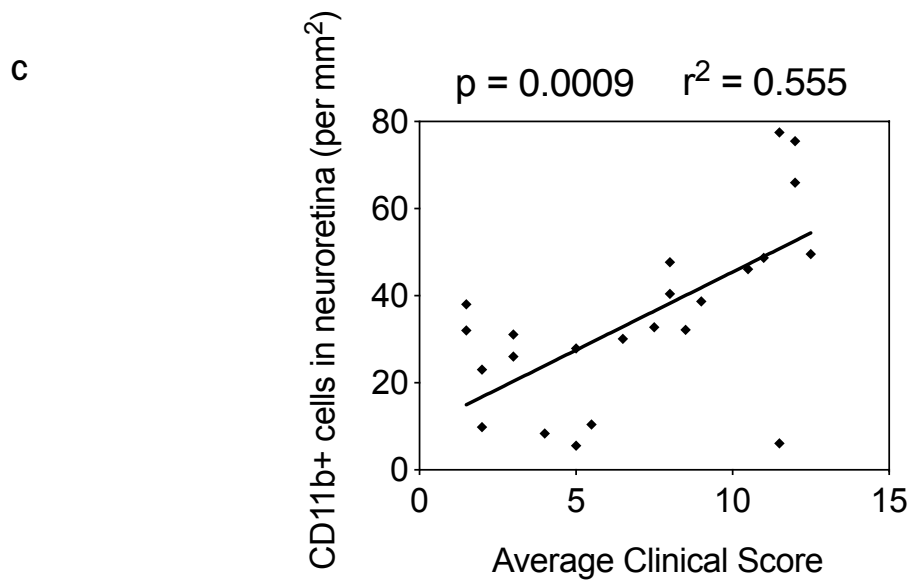


Figure 5.6. CD11b cell expression correlates with disease severity in EAU.

(A) Representative micrographs of strategy used for neuroretinal flat mount quantitative analysis. Micrographs representing the various fields of view at 10X, 25X and 40X magnification, respectively. (B) Representative fundoscopic micrographs presented in bright field (fundus) alongside their corresponding neuroretina flat mounts immunolabelled with anti-CD11b prepared at day 21 p.i. Complete Freund's Adjuvant (CFA) control, together with IRBP1-20 immunised animals displaying a clinical score 2 and 10. No such staining identified in the CFA control animals. Scale bar = 50 μ m. (C) Linear regression showing a strong positive relationship between fundoscopic derived ocular pathology (materials and methods) and CD11b expressing myeloid cell count in the neuroretina (per mm²).

5.3.6 Simvastatin therapy reduces myeloid cell marker expression in a concentration dependent manner

To explore the effects of simvastatin on CD11b expressing myeloid cells in IRBP₁₋₂₀ EAU model animals were treated with a range of concentrations (50 mg/kg, 75 mg/kg and 100 mg/kg) over an 8-day period. At peak disease, day 21 p.i, eyes were enucleated, and quantification of retinal cell populations were compared in flat-mounted retinas, representative images of the findings are displayed in Figure 5.7A.

IRBP-EAU induction gave rise to mass retinal cell infiltration, when quantified CD11b+ positive cells within the superficial plexus were compared with CFA control counterparts, as indicated by an average score of 53.09 ± 7.9 and 7.16 ± 1.59 respectively across the neuroretinal surface. These levels were not altered at the lower concentration of 50 mg/kg (54.49 ± 3.99) but became markedly reduced as simvastatin concentration was elevated further. At 75 mg/kg there was a reduction in CD11b expression of approximately 50% to 28.6 ± 8.06 but with no further decrease at 100 mg/kg (31.5 ± 2.449) (Figure 5.7 B).

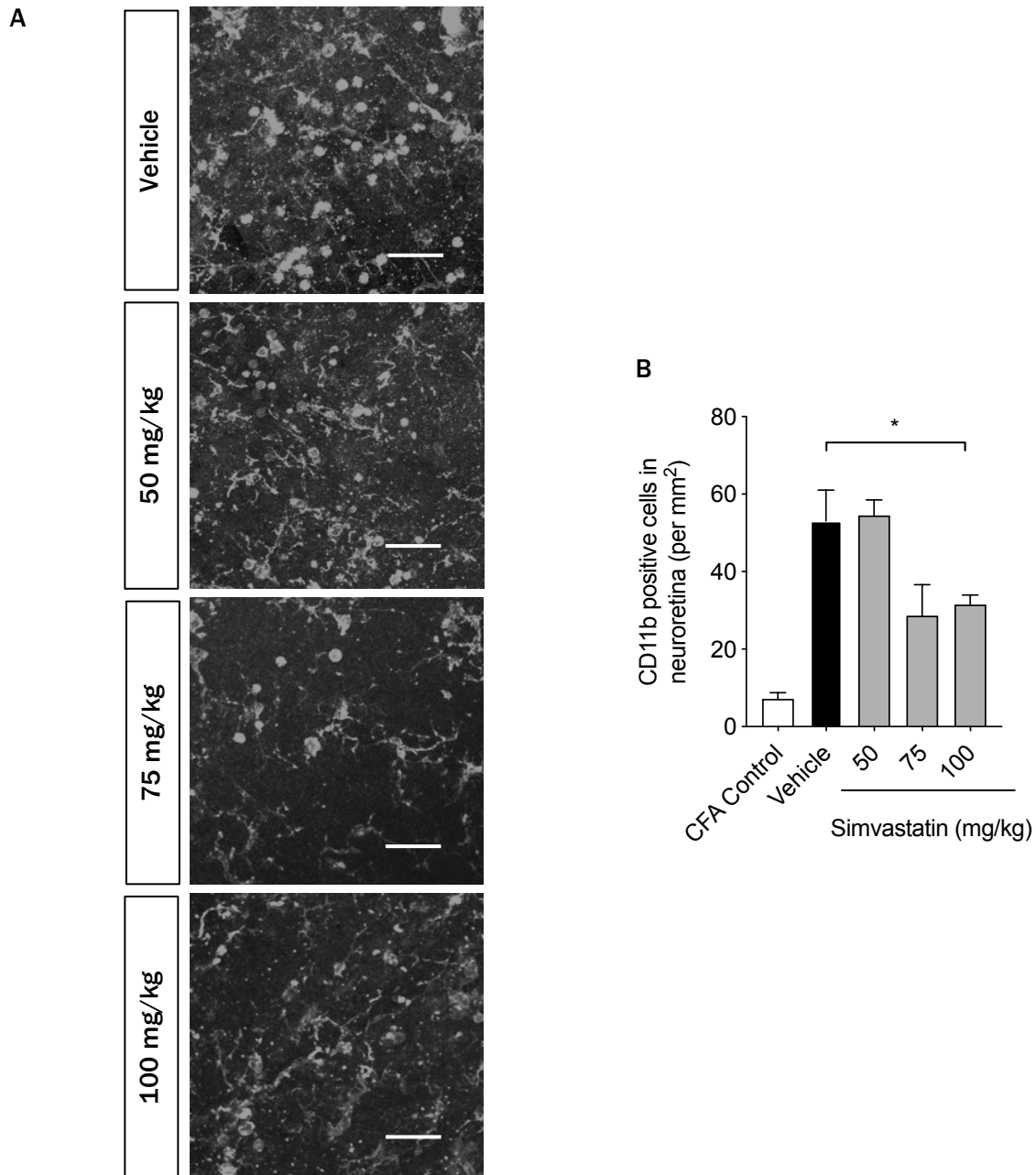


Figure 5.7. Simvastatin therapy reduces CD11b expression in a concentration dependent manner.

CD11b expression immunolabelling in CFA control neuroretinas, and IRBP1-20 induced EAU treated with either vehicle (0.5% methylcellulose), 50 mg/kg, 75 mg/kg or 100 mg/kg. All eyes were enucleated at day 21 post-immunisation and neuroretinas prepared for immunolabelling. (A) Representative confocal images at X40 magnification of neuroretinas, immunolabeled with anti-CD11b surface marker. CD11b expression present in the superficial plexus in diseased animals, no such staining was observed in the CFA control animals. (B) Graph showing quantified levels of CD11b (grey) cell expression in neuroretina (per mm²), comparing CFA control and all treatment groups. n= 7-11 per group. Scale bar = 10µm. Averages presented ± S.E.M. One-way ANOVA demonstrates significant differences. *, P<0.05.

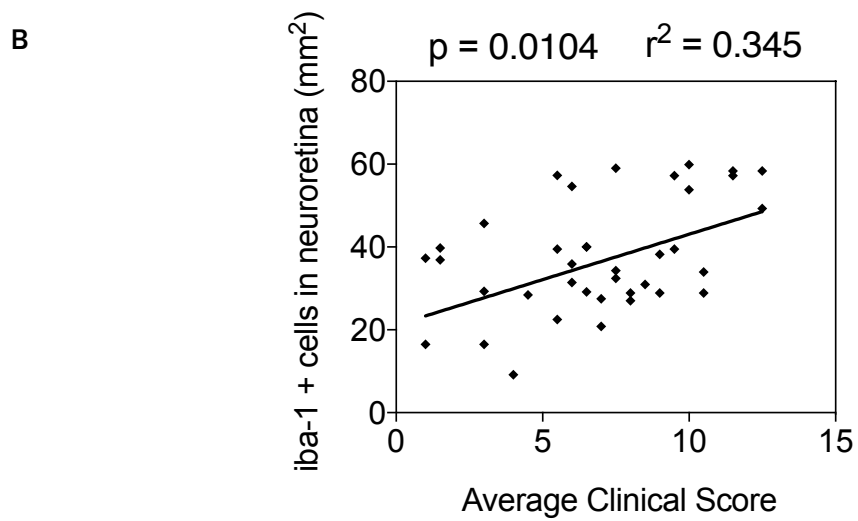
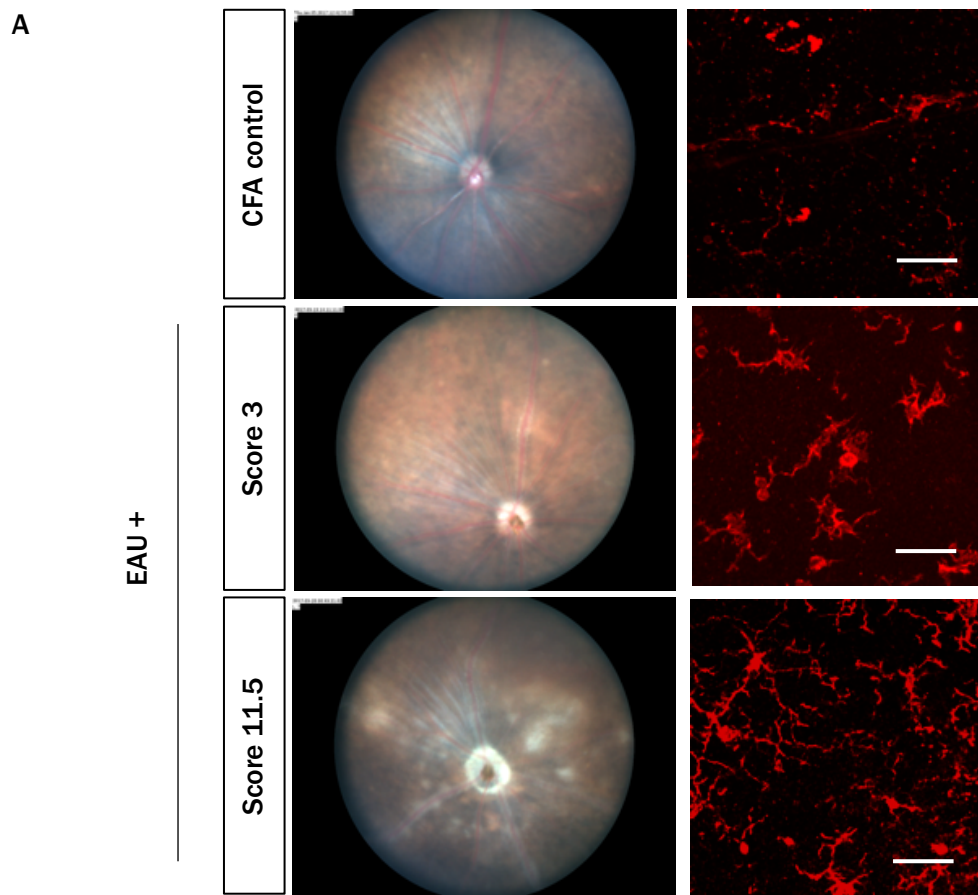
5.3.7 Macrophage and microglial Expression in IRBP-EAU

There are many publications describing the aetiological implications of the macrophage and microglial cell in chronic retinal inflammation. One of the most widely recognised and ubiquitously expressed surface markers used to define these populations is the Ionized calcium-binding adapter molecule 1 (Iba1). This study set out to investigate firstly, whether Iba1 expressing macrophages and tissue resident microglial cell number are influenced by IRBP disease, and secondly, the impact, if any, simvastatin may have on these cells. It is important to note that the Iba1 cell surface marker recognises tissue resident microglia and macrophages, independent of their activation state.

We previously demonstrated a concentration-dependent simvastatin effect on the number of CD11b expressing myeloid cells present in the diseased retina (Figure 5.7). Next, the levels of Iba1 expressing macrophages and microglial cells, a subset of the total CD11b expressing myeloid population, were analysed as a means of differentiating between the specific populations influenced in chronic retinal disease with simvastatin therapy. Similarly, the Iba1 immunolabeled population were analysed at day 21 p.i. to establish the impact on chronic, late-stage disease.

When examining and quantifying immunofluorescent staining in the neuroretina of non-immunologically challenged mice, we detected levels of Iba1+ cells in significantly lower quantities than that of the IRBP-induced group, with an average of 13.25 and 37.79, respectively. Representative micrographs of which can be seen in Figure 5.8A. Indeed, the number of Iba1 expressing cells correlated positively with the clinical disease score in EAU, thus, the higher the clinical severity the more quantified Iba1+ cells present in the superficial plexus of the neuroretina. Linear regression analysis revealed a strong and positive correlation ($p = 0.010$, $r^2 = 0.345$, $n = 35$, Figure 5.8B).

Administration of simvastatin at all concentrations used throughout this study (50 mg/kg, 75 mg/kg and 100 mg/kg), did not influence the levels of Iba1 positive cells present in the superficial plexus layer when compared with vehicle receiving counterparts (Figure 5.8C). Representative confocal micrographs of Iba1 immunolabelling in the neuroretina at the highest treated concentration (100mg/kg), gives an indication by eye, of cell expression (Figure 5.8D). Hence, Simvastatin treatment in this model did not impact the number of macrophage/microglial cell populations at end stage disease.



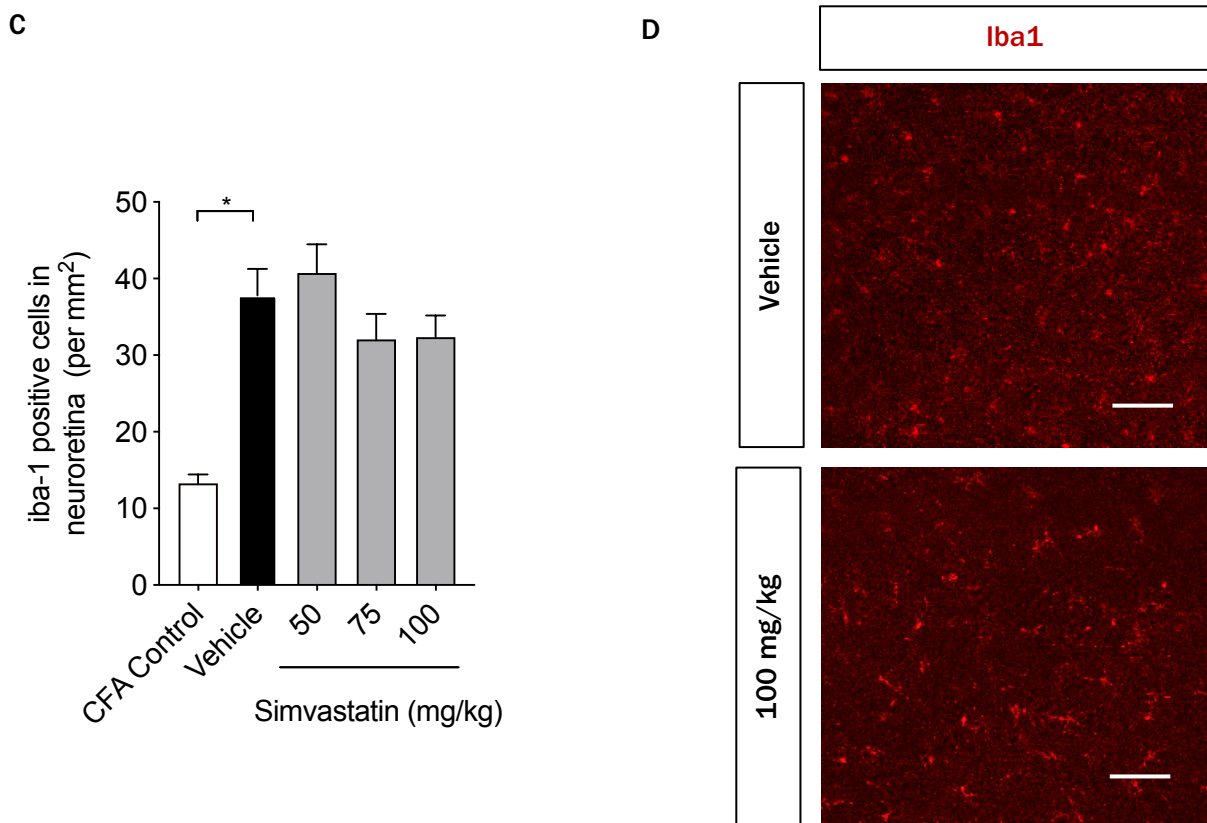


Figure 5.8: Simvastatin does not alter Iba1 expression in IRBP-EAU disease

CD11b expression immunolabelling in CFA control neuroretinas, and IRBP₁₋₂₀ induced EAU treated with either vehicle (0.5% methylcellulose), 50 mg/kg, 75 mg/kg or 100 mg/kg. All eyes were enucleated at day 21 post-immunisation and neuroretinas prepared for immunolabelling. (A) Representative confocal images at X40 magnification of neuroretinas, immunolabeled with anti-Iba1 surface marker. Iba1 (red) expression present in the superficial plexus in diseased animals, present in lower amounts in the CFA control population. (B) Linear regression of Iba1 cells (per mm²) against average clinical disease score n = 35. (C) Graph showing quantified levels of Iba1 cell expression in superficial plexus of neuroretina comparing CFA control and all treatment groups, n = 7-11 per group. (D) Representative micrographs of Iba1 staining in the superficial plexus of vehicle treated compared with the highest concentration of simvastatin (100 mg/kg). Scale bar = 20µm. Means ± S.E.M. One-way ANOVA demonstrates significant differences. *, P<0.05.

5.3.8 Simvastatin therapy on markers of oxidative stress in IRBP₁₋₂₀ EAU

Aberrant, net accumulation of reactive oxygen species in cells and tissues, through adduct formation, causes irreparable damage to DNA, proteins and lipids. Determination of increased oxidative stress levels within tissues can be measured by detection of ROS and RNS-mediated alterations to these macromolecules through immunohistochemical techniques. These methods are currently amongst the most reliable means for assessing free radical damage and are fast becoming a key instrument in redox biology. Hence, our choice for using these techniques in the following experiments.

5.3.8.1 NITROSYLATED PROTEIN, A HALLMARK OF OXIDATIVE DAMAGE, IS DETECTABLE IN IRBP₁₋₂₀ EAU

Nitrosylation is a pathophysiological post-translational modification that affects a wide variety of proteins involved in a number of cellular processes. Nitrotyrosine is a product of tyrosine nitration mediated by local, overburdening of reactive oxygen and nitrogen species within tissues. Nitrotyrosine is typically identified as being an indicator of cell damage and inflammation in addition to being an indirect marker of nitric oxide production.

The presence of nitrotyrosine has been reported previously in many studies of retinal disease. Here we examined IRBP₁₋₂₀ tissue to determine the magnitude of such damage in our model of uveitis and to ascertain the effect of statin treatment. Oxidative protein damage, as indicated by anti-nitrotyrosine immunostaining, was found to be a common feature of EAU (Figure 5.9A) in IRBP₁₋₂₀ immunised mice, with strong and significant staining throughout the retina being observable in those animals presenting with ocular pathology, whilst remaining virtually absent in the naïve animals. Positive immunostaining was most pronounced in the outer nuclear layer of the vehicle treated (0.5% methylcellulose) animals, with a weaker, punctate staining pattern present in the inner nuclear layers. The simvastatin treated (100 mg/kg) cohort presented with significantly lower levels of staining intensity overall, when compared with the vehicle treated (Figure 5.9B). Low levels of non-specific staining can be seen in the naïve, non-immunologically challenged animals, appearing mostly in the rod outer segment layer.

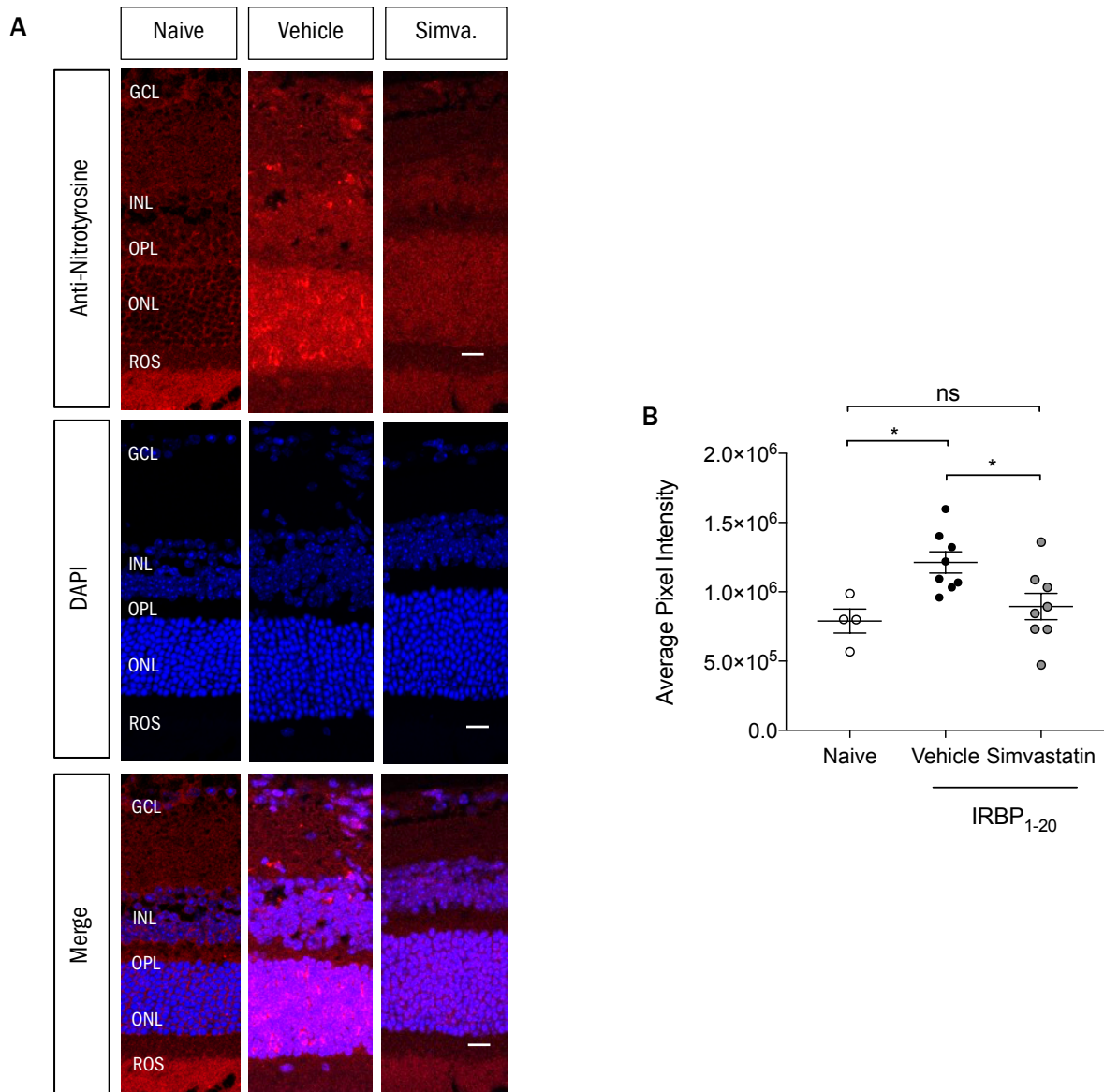


Figure 5.9. Simvastatin therapy reduces levels of nitrosylated protein in the retina of EAU.

Nitrosylated protein expression in retinal cross-sections of naïve, non-immunised animals and IRBP₁₋₂₀ EAU, treated with either vehicle (0.5% methylcellulose) or simvastatin (100 mg/kg). At 21 days post-immunisation, eyes were enucleated and used for immunostaining. (A) Representative microscopy images immunostained for anti-nitrotyrosine (red) and DAPI (blue). Immunofluorescent staining indicates anti-nitrotyrosine labelling in the ONL, and weak staining in the INL of vehicle receiving animals. When comparing simvastatin treatment reduced anti-nitrotyrosine staining intensity was seen in both the ONL and INL. No such positive labelling was observed in the naïve animals. Scale bar = 20µm (B) Average pixel intensity for anti-nitrosylated protein staining, per treatment group comparing non-immunised, naïve animals with IRBP₁₋₂₀ vehicle or simvastatin receiving, (n=8 per group). A significant difference was found between the vehicle receiving and simvastatin treated groups. GCL, ganglion cell layer; INL inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. ± S.E.M. *, P<0.05; One-way ANOVA used with Tukey's post-test. Simva. (simvastatin).

5.3.8.2 OXIDATIVE DNA DAMAGE AS A MARKER OF STRESS IN EAU

In nuclear and mitochondrial DNA, 8-hydroxy-2'-deoxyguanosine (8-OHdG) is one of the most predominant forms of free radical-induced oxidative lesions. It is a major product of DNA oxidation and the most representative modification to date. Therefore, this study sought to establish whether this type of free radical damage exists in IRBP₁₋₂₀ induced EAU, to further allow interrogation of the effectiveness of simvastatin in reducing oxidative damage.

In control animals anti-8OHdG immunoreactivity was negligible but in IRBP-EAU animals intense, significant staining was evident throughout the retina. The most striking labelling was found in the inner nuclear and outer nuclear layers of the retina (Figure 5.10A), with a scattered pattern also presenting in the ganglion cell layer. When comparing the vehicle cohort with those that received simvastatin, the staining intensity as assessed by eye appeared somewhat less throughout the entirety of the tissue in the treated mice. However, whilst quantitative assessment of the signal intensity revealed a lower value in the statin treated animals this proved not to be significant (Figure 5.10B).

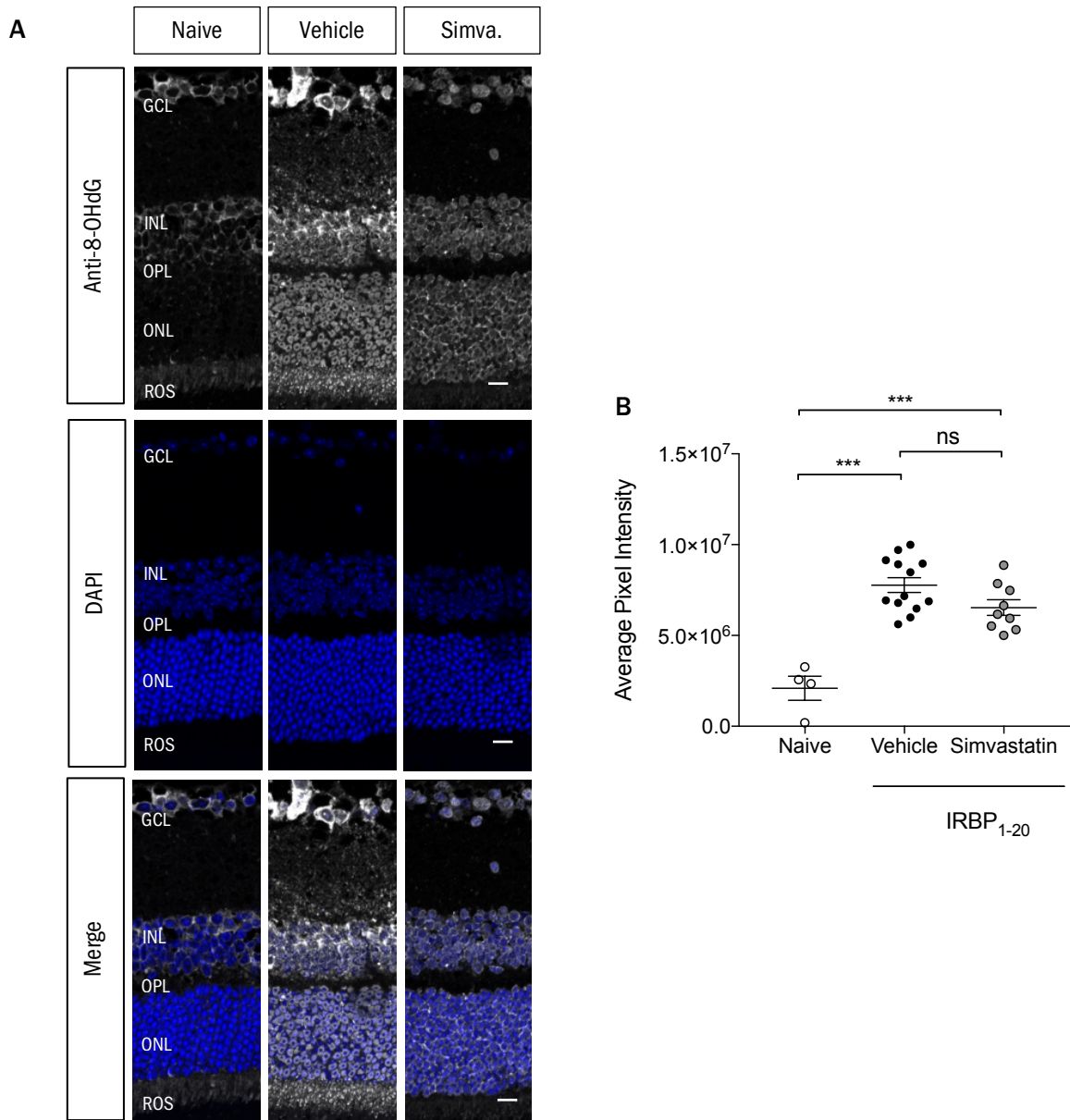


Figure 5.10. 8-OHdG expression in EAU retina. Anti-8-OHdG expression in retinal cross-sections of IRBP-EAU at day 21 p.i. treated with either vehicle (0.5% methylcellulose) or simvastatin (100 mg/kg). (A) Representative micrographs of 8-OHdG (grey) expression in naïve, non-immunised animals and IRBP-120 treated with either vehicle or simvastatin for a total period of 8 days post-disease onset. Counterstained for DAPI (blue). Positive staining revealed in the GCL, INL and ONL of both vehicle and simvastatin treated IRBP-EAU, whilst absent in the non-immunised animals. (B) Mean pixel staining intensity of retinal tissues immunostained with anti-8-OHdG and DAPI. Group comparison includes naïve, non-immunised, vehicle treated, and simvastatin treated. Values are mean \pm S.E.M. ***, $P \leq 0.001$, $n = 9-13$ per group. GSL, ganglion cell layer; INL inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. One-way ANOVA used with Tukey’s post-test. Simva. (Simvastatin).

5.3.8.3 TISSUE LIPID PEROXIDATION MARKER IS REDUCED IN RESPONSE TO SIMVASTATIN TREATMENT

Having investigated free radical damage to proteins and DNA we next looked at damage to lipids. The immunomarker 4-Hydroxynonenal (4HNE) is produced by lipid peroxidation in cells and tissues with elevated levels of oxidative stress and has been widely used as a reliable biomarker for oxidative lipid damage. In the first instance, we aimed to determine whether oxidative stress processes in IRBP-EAU resulted in detectable levels of 4HNE. If increased levels were detected our objective was then to assess the capacity of simvastatin to reduce these levels.

Following induction of IRBP-mediated EAU we observed significantly marked levels of 4HNE immunoreactivity present in the retina (Figure 5.11A) when compared with non-immunologically challenged mice. This was mostly concentrated in the inner and outer plexiform layer being more uniform in the former and more punctate in the latter. Variable degrees of staining were also evident in the ganglion cell layer. Positive labelling was virtually absent in the non-immunised control group.

Quantification methods were then adopted to measure the signal intensity across the whole retina. The data confirmed our subjective observations as strong 4HNE positive staining was found in the vehicle treated IRBP-EAU animals compared to naïve controls and that in diseased mice this was significantly attenuated by simvastatin (Figure 5.11B). Positive staining was also found in the rod outer segment layer, but unchanged between treatments. It is evident from these findings that simvastatin therapy holds the capability to reduce 4HNE staining to near-control levels.

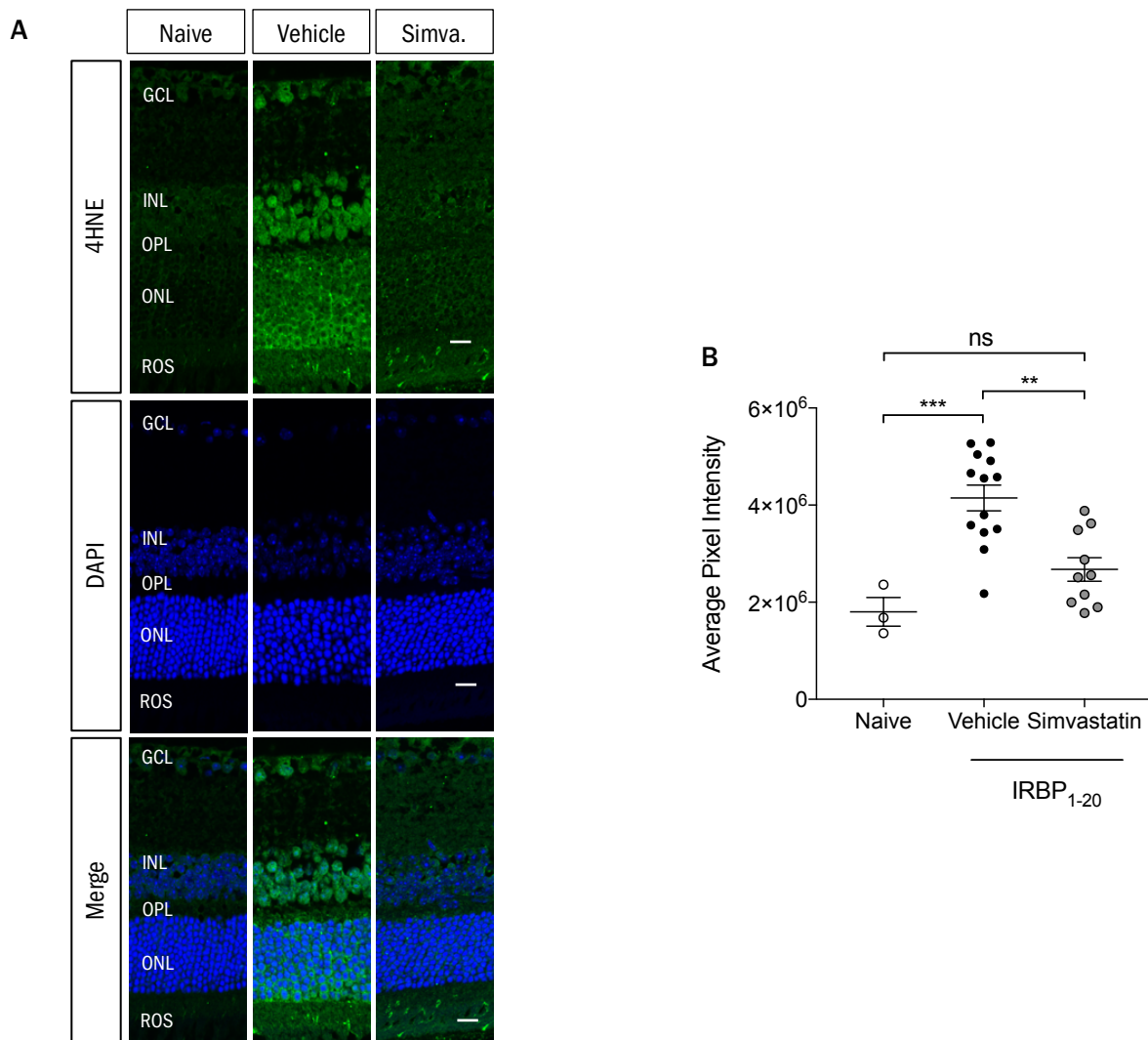


Figure 5.11. Simvastatin therapy reduces levels of lipid peroxidation in retina of EAU disease.

Anti-4HNE expression in retinal cross-sections of IRBP-EAU at day 21 p.i. treated with either vehicle (0.5% methylcellulose) or simvastatin (100 mg/kg). Eyes were enucleated at peak disease and prepared for immunohistochemistry analysis. (A) Representative microscopy images of naïve, non-immunised animals and IRBP1-20 EAU disease, receiving either vehicle or simvastatin treatment. Immunostained for 4HNE (green) and counterstained with DAPI (blue). Positive 4HNE staining seen faintly in the GCL and more intensified in the INL and ONL of IRBP-EAU with vehicle, all of which are reduced with treatment of simvastatin and absent in non-disease retinas. Scale bar = 20 μ m. (B) Average pixel staining intensity for lipid peroxidation (4HNE) across the entire retina. Groups comparing naïve, with IRBP-EAU, vehicle and simvastatin treatment, the intensity of which is significantly reduced with simvastatin treatment. GCL, ganglion cell layer; INL inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Values are mean \pm S.E.M. ***, $P \leq 0.001$, **, $P \leq 0.01$, ns = not significant. n= 10-14 per group.

5.3.8.4 NADPH OXIDASE SUBUNIT EXPRESSION DURING LATE-STAGE IRBP-EAU

NADPH oxidase activation depends on phosphorylation of the principle cytosolic subunit p47phox. Using immunofluorescent techniques, here, we assess the activation of this subunit in the retinal tissue of IRBP-EAU mice. p47 phox staining in naïve mice was recognisable in the inner nuclear layers with spots of immunoreactivity in the ganglion cell layer, although, this was negligible and representative non-specific staining, distinctly different from that observed in the EAU induced samples (Figure 5.12A). Upon induction of EAU, p47-phox signal was increased overall in the retina, with most staining residing within the ganglion cell layer population. In addition, we also observed a somewhat homogenous staining pattern in the inner and outer nuclear layers, patterns of which were absent in the naïve retinas. In mice receiving simvastatin, a noticeable reduction in p47phox staining was observed, particularly in the outer nuclear layer. A reduction in staining intensity was also noted in the inner nuclear layer and ganglion cell layers. Overall, in EAU mice there was a significant decrease in the strength of staining with simvastatin treatment (Figure 5.12B).

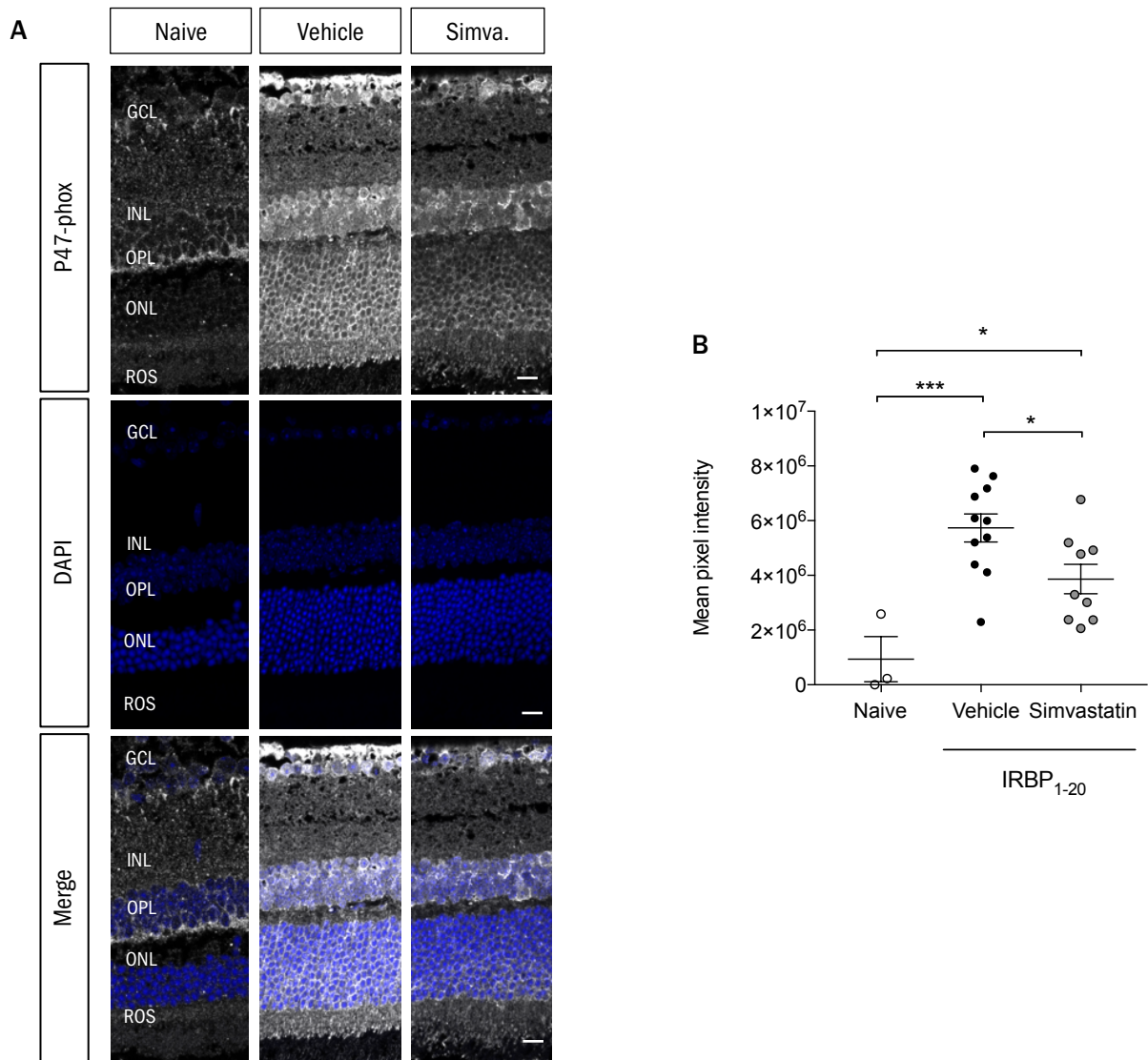


Figure 5.12. Simvastatin therapy reduces levels of NADPH oxidase subunit labelling in EAU.

Anti-p47phox expression in retinal cross-sections of IRBP-EAU at day 21 p.i. treated with either vehicle (0.5% methylcellulose) or simvastatin (100 mg/kg). Eyes were enucleated at peak disease and prepared for immunohistochemistry analysis. (A) Representative microscopy images of naïve, non-immunised animals and IRBP₁₋₂₀ EAU disease, receiving either vehicle or simvastatin treatment. Immunostained for p47-phox (grey) and counterstained with DAPI (blue). Intense positive p47-phox staining can be seen mostly in the regions of GCL, INL and ONL, of which, simvastatin treatment appears to moderate these staining patterns. P47-phox labelling was absent in non-disease retinas. Scale bar = 20µm (B) Average pixel staining intensity for NADPH oxidase expression (p47-phox) across the entire retina. Groups comparing naïve, with IRBP-EAU, vehicle and simvastatin treatment; intensity of which is significantly reduced with simvastatin treatment. Original magnification X40. GSL, ganglion cell layer; INL inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Values are mean \pm S.E.M. n= 9-11 mice per group. *, $P \leq 0.05$; ***, $P \leq 0.001$.

5.4 Discussion

The present study uses a well-established model of uveitis to investigate the potential neuroprotective effects of simvastatin. IRBP₁₋₂₀ induced EAU mimics chronic posterior uveitis in the mouse. Here, we administer simvastatin at several concentrations to determine the outcome on clinical ocular pathology, immune cell infiltration and markers of reactive species damage in the inflamed retina. Collectively, our data provides evidence that high-dose simvastatin substantially alleviates clinical signs of progression in EAU, whilst significantly reducing the level of immune infiltrating cells. Moreover, our findings support a neuroprotective role for simvastatin by ways of reducing reactive species biomarker damage in diseased retinal tissue. The implications of these findings and potential use for simvastatin as a neuroprotective agent appropriate for repurposing in SPMS, will be discussed below.

5.4.1 IRBP₁₋₂₀ as a model of uveitis

Uveitis, or uveoretinitis, is a general term referring to inflammation of the retina and uvea. The interphotoreceptor retinoid-binding protein (IRBP), human peptide₁₋₂₀ is a major component of retinal photoreceptor outer segments and one of the most common immunogens known to induction EAU in mice. The primary structural target of inflammation is the photoreceptor layer. Indeed, autoreactive CD4⁺ T cells are found within this layer and have identified roles in disease initiation and photoreceptor destruction (Sanui et al., 1989) (Rizzo et al., 1996). Immunisation with retinal antigens often requires additional adjuvants for CD4⁺ T cell priming and polarization in peripheral lymphoid organs into pathogenic Th1 and Th17 cells. Upon activation, Th cells begin migration towards the eye and orchestrate signalling pathways for BRB breakdown and leukocyte extravasation into the retina (Caspi et al., 1988).

While no single animal model represents the complete spectrum of human uveitis, IRBP₁₋₂₀ EAU is a well-established chronic model used mostly in translational research. This model, however, is considered to be both phenotypically and mechanistically more representative of human disease than others in the field (Caspi et al., 1988). Indeed, the central features identified in mice, parallel many clinical characteristics seen in human disease. Consequently, EAU can act as a powerful method to assess the effectiveness of novel therapies. The cardinal clinical features most representative of human disease in IRBP₁₀₋₂₀ are: posterior retinal and choroidal lesions, vasculitis, granuloma formation, photoreceptor damage in addition to varying degrees of anterior chamber infiltration (Lipski et al., 2017). The induced phenotype used throughout our study did indeed display all of these typical disease features (Caspi, 2003) (Figure 5.1A).

In this study, clinical disease scores used for monitoring severity and tissue damage were based on visual assessment of the retina using fundoscopic imaging techniques. Accompanying histological scoring was also performed at the experimental endpoint as an additional metric to measure clinical outcomes. In line with previous studies, clinical signs of infiltration began at day 10 post immunisation. These initial stages were later followed by a progressive phenotype with worsening disease until reaching a peak at day

21; thereafter severity plateaued (Figure 5.1 A, B). For the purpose of these set of experiments it was not necessary for the mice to continue beyond peak disease (day 21), however, numerous characterisation studies for this model have reported that the natural progression after this stage would eventually lead to clinical resolution around day 65. Although the resolution phase, previously defined between day 28 and day 65 post-immunisation, is perceived as a period of clinical disease score recovery, found evidence of structural damage and persistent lymphocytic marker CD45+ cellular infiltration throughout this phase has been reported (Copland et al., 2008). The presence of such persistent infiltration demonstrated persistent ongoing, chronic inflammation.

In our study, clinical fundoscopic scores aligned closely with histological changes and the two were found to correlate significantly. This is a common finding and consistent with current published data (Caspi et al., 1988) (Liyanage et al., 2016). In line with previous reports, our study also highlighted cellular inflammation as the most prominent feature of disease, particularly at the onset (Caspi et al., 1990) (Copland et al., 2008). In addition to this, we observed marked pan retinal structural changes which correlated well with disease progression and was particularly associated with the later stages of EAU. These changes are typically recognised as a consequence of mass inflammatory cell infiltration (Chan et al., 1990).

5.4.2 Simvastatin reduces vascular leakage in a model of uveitis

The concept of using statins to reduce vascular permeability is not new, an important number of studies have supported this idea including those performed by (Gegg et al., 2005) who demonstrated lovastatin efficacy in an acute model of EAU. Similar to lovastatin, simvastatin has shown beneficial outcomes on vascular endothelial cells *in vitro*, but as yet, has not been examined *in vivo* in uveitis. Current evidence supports the idea that simvastatin exhibits many vasculoprotective properties such as the ability to upregulate basement membrane proteins, alter tight junctions (Wilkinson et al., 2018) (Chen et al., 2012) and modify cytoskeletal rearrangements (Jacobson et al., 2004) in disease models. Thus, the major objective for this study was to confirm a role for simvastatin in attenuating vascular leakage, which would further support our hypothesis that simvastatin possesses vasculoprotective as well as neuroprotective properties.

To test the effect of simvastatin on permeability, we employed fluorescein angiography to evaluate retinal vascular leakage in mice at day 21 post EAU induction. Upon examination, vehicle treated EAU mice demonstrated significant levels of fluorescent permeation from the major retinal vessels, which is indicative of breakdown of the anterior (vascular) BRB. Here, simvastatin treated animals provided data to support the protective properties against vascular leakage. Leakage localised to the tissue parenchyma were distinguishable from those without obvious signs of leakage by well-defined, sharp outlines of imaged vessels. This observation is due to dye retention within the vascular lumen which has not permeated local tissues. To support these findings, vascular leakage correlated well with EAU clinical disease score (Figure

5.5C). Following simvastatin administration at all concentrations tested, this relationship was lost, and disease severity no longer paralleled the magnitude of leakage (Figure 5.5D, E, F).

Previous EAU characterisation studies confirmed that BRB breakdown is a fundamental aspect of disease development. (Xu et al., 2003) colleagues discovered that cell adhesion molecule expression increases with disease progression. In particular, ICAM-1, P-selectin and E-selectin were found predominantly in retinal venules, at the site of BRB breakdown, cell adhesion and extravasation. Moreover, following high dose (80mg) simvastatin therapy, clinical studies have shown a significant reduction in sVCAM assessed plasma levels (Vladimirova-Kitova et al., 2011). In line with these findings, lovastatin treatment in cultured retinal endothelial cells attenuated tight junctional proteins occluding, VE-cadherin and TNF α (Li et al., 2009). Mechanistically, these changes were described as lovastatin-mediated amelioration of ICAM-1 expression-induced by TNF α and in operated in part, through the inhibition of NF- κ B.

As mentioned in previous chapters, statins can attenuate RhoA activation through the inhibition of prenylated processes. This mechanism is particularly interesting as we (Figure 3.2) and others have shown that simvastatin is a potent regulator of cell actin-cytoskeleton remodelling (Fürst et al., 2002) (Dick et al., 2015) (Jacobson et al., 2004) (Chubinskiy-Nadezhdin et al., 2017) (Han and Kim, 2018). Indeed, it is well established that endothelial cytoskeletal reorganisation enables early blood-brain barrier disruption (Shi et al., 2016).

A number of studies have shown that early BRB permeability may be partially reversible making this area of research a target for therapeutic intervention (Simpkins, 2016). This discovery informed our experimental design, with the decision made to introduce simvastatin therapy at the onset of symptoms, where only mild BRB had taken place. This study highlights the pleiotropic nature of simvastatin and suggests that the therapeutic relevance of different effector mechanisms may change according to cell type, model used and the concentration of therapy. Here, we present novel data to demonstrated that simvastatin prevents vascular leakage in a well-established model of uveitis. Further in-depth study would be necessary to identify the exact mechanism involved.

5.4.3 Simvastatin attenuates clinical disease progression in a model of uveitis: A role for immune cells

EAU pathogenesis is driven by mass infiltration of leukocytes into the retina, these cells have identified roles in tissue destruction via the release of reactive oxygen and nitrogen species. The well-studied immunomodulatory properties of statins have been used extensively in an attempt to combat the potent autoimmune targeted damage in uveitis. (Stanislaus et al., 1999) were amongst the first to demonstrate clinical attenuation of EAU with the application of lovastatin. This discovery paved the way for further efficacy studies using the statin family of therapies in the context of uveitis. Since these findings, other

members of this family have been explored with pravastatin and atorvastatin being the most recent. The heterogenous nature of statins including chemical structure, polarity, half-life and potency governs their absorption, destruction, metabolism and excretion in different model systems (Hunninghake, 1992).

Evidenced by visual analysis of live fundoscopic retinal images, here, we reveal a unique role for simvastatin in attenuating clinical ocular pathology in mice with posterior uveitis. Typically, at the early stages of IRBP-induced disease, acute inflammation dominates the retina. This type of inflammation features severe optic disc swelling. As the disease progresses towards the chronic stages of inflammation swelling in this region resolves (Copland et al., 2008). Severe disease is characterised by mass inflammatory cell infiltration that obscures blood vessels. Representative fundoscopic images taken from the cohort of mice treated with 50 mg/kg demonstrate such patterns of disease at day 21. However, when we examined retinal images taken from mice receiving 75 mg/kg and 100 mg/kg simvastatin an increase in optic disc swelling coupled with a reduction in tissue damage provided sufficient evidence to suggest that the disease has not yet progressed from the acute phase. The assessment method employed for fundoscopic analysis mostly reflects vasogenic oedema, thus, any observed differences with simvastatin treatment represent these parameters.

It is important to note that the upper dose limit of simvastatin for humans is currently 80 mg/day. The dosage used throughout this study are comparable to those used previously in murine studies and are necessarily higher than human concentrations due to significant up-regulation of HMG-CoA reductase feedback mechanism with statin treatment in rodents (Kita et al., 1980).

Ample *in vitro* and *in vivo* evidence exists to support the potent anti-inflammatory properties of simvastatin (Leung et al., 2003) (Franiak-Pietryga et al., 2009) (Panichi et al., 2006) (McKay et al., 2004). Previous *in vitro* studies (Jameel et al., 2013) found simvastatin has the ability to suppress CD4⁺ T cell activity, a pivotal player in EAU disease pathogenesis (Pahan et al., 1997) also observed that treatment of macrophages *in vitro*, and resident CNS microglia and astrocytes, with lovastatin prevented the expression of TNF α and IL-1 β . Further to this, it has been appreciated for some time that statins have antiproliferative effects on lymphocytes and other cell types (Chakrabarti and Engleman, 1991). Collectively, these well-established immunosuppressive properties could provide a plausible explanation for our observed effects. Indeed, similar clinical outcomes have been achieved in models of EAU when treated with other members of the statin family (Gegg et al., 2005) (Kohno et al., 2007) (Kohno et al., 2006). However, the inherent heterogeneity of statins introduces significant variation across many aspects of therapeutic repurposing, from drug dosage titration to efficacy of outcome and clinical response. Hence, not all statins have the same immunomodulatory activity in retinal disease. For example, lovastatin has proven more efficacious in retinal inflammatory disease than atorvastatin, whereas simvastatin has not yet been tested in this disease context (Jameel et al., 2013) (Gegg et al., 2005).

It should be noted that we did not observe differences in histological scoring when assessing the extent of infiltration and structural damage across all treatment groups. The lack of apparent histological changes could perhaps reflect the insensitivity of this measurement technique and more in-depth analysis is necessary.

Despite the many mechanisms of action described in the literature, a common feature of statin treatment in retinal neuroinflammation is the reduced accumulation of mononuclear cell infiltrates into the target tissue (Stanislaus et al., 2001b) (Stanislaus et al., 1999) (Youssef et al., 2002) (Greenwood et al., 2003b) (Aktas et al., 2003). In EAU, one of the largest infiltrating cell populations into the retina up to peak disease are CD11b+ myeloid cells, dominating over CD4+ T cell populations (Kerr et al., 2008). These myeloid cells rapidly increase in numbers from the onset of clinical signs up to peak disease and can comprise up to 79% of the total leukocyte population, as identified by flow cytometry techniques (Robertson et al., 2002) (Jiang et al., 1999). CD11b is a pan leukocyte surface integrin marker used to identify a mixture of cells including monocytes, macrophages, natural killer cells and granulocytes. It is important to note that the relative proportions of these cell types vary according to disease model and duration of pathology (Chan et al., 1990) (Kerr et al., 2008) (Dick et al., 1997). CD11b is known to mediate inflammation by regulating adhesion and migration related interactions between leukocytes and the stimulated endothelium. In line with others, here we observed a strong, positive correlation between clinical ocular pathology and CD11b+ expressing cellular infiltrates in the retina at peak disease (Kerr et al., 2008) (Copland et al., 2008). The importance of these interactions in retinal disease is underscored by the discovery that CD11b blockade improves clinical outcome measures in a variety of EAU models (Whitcup et al., 1993b). Moreover, statin therapy applied to previous *in vitro* and *in vivo* scenarios has proven to reduce migration and infiltration of CD11b+ cells (Bedi et al., 2017). In our study, we saw a substantial reduction in CD11b+ cellular infiltrate into the retina of simvastatin treated EAU mice, when compared with vehicle-treated controls at peak disease. These observed effects were dose-dependent with the highest concentration at 100 mg/kg more potent than 75 mg/kg. Similar findings were revealed in inflamed neuronal tissue when (Reis et al., 2012) showed lovastatin therapy decreased CD11b mRNA in response to treatment.

Initially, it was proposed that monocytes represent the primary cell type to significantly downregulate CD11b adhesion expression in response to statin treatment (Weber et al., 1997). However, EAU characterisation studies have since suggested that natural killer cells constitute a large proportion of the CD11b+ population and therefore, it is likely that any statin-induced changes may be influencing this cell type (Vivier et al., 2008) (Yu et al., 2013). Recently, CD11b signal disruption was directly linked to neutrophil extravasation (Hyun et al., 2019). Despite ongoing debate regarding the specific cell type at play during peak EAU, a consistent outcome of statin therapy is the reduced expression of CD11b+, in addition to other subunits associated with the heterodimer complex, infiltrating cells to the target tissue. A key step

in leukocyte migration from the periphery across the BRB involves cell-to-cell communication between the surface expression of glycoprotein ICAM-1 located on endothelial cells and LFA-1; CD11a/CD18, belonging to an integrin superfamily of adhesion molecules found on leukocytes and lymphocytes. These interactions are essential for stabilising and facilitating both adhesion and migration of leukocytes during CNS inflammation. (Gegg et al., 2005) were amongst the first to attribute a role for statins in impairing this interaction, by decreasing the expression of ICAM-1 on endothelial cells. The authors described a mechanism through which statins inhibit isoprenoid pyrophosphate synthesis, a precursor required for the prenylation and posttranslational activation of Rho GTPase. Similar to our findings here, they showed that clinical signs of EAU were significantly reduced with lovastatin treatment. Several reports have now revealed that statins are also capable of modulating the expression of adhesion molecules within the CD11 complex located on leukocytes (Weber et al., 1997). A study performed on PBMCs found simvastatin downregulated CD18 expression at both the mRNA and protein level, resulting in decreased binding to HUVEC (Rezaie-Majd et al., 2003).

Activation of retinal microglia is known to initiate neuroinflammation in ocular autoimmunity. It is generally accepted that during in the early phases of EAU, microglia migrate to the photoreceptor cell layer where they generate destructive amounts of TNF α and peroxynitrate. (Okunuki et al., 2019) and colleagues provided evidence that this cell type is essential for the induction of retinal immune response as microglial ablation completely blocks disease. Based on our observation that simvastatin reduces CD11b+ cell infiltration into the retina, within this population we scrutinized the potential impact simvastatin has specifically on monocyte infiltration specifically. Several studies report on the immunomodulatory effects of statins within this cell population. In monocytes, Mac-1 (CD11b/CD18) is a key molecule involved in adhesion to the endothelial cells for facilitated crossing of the BRB in an ICAM-1 dependent manner. In previous studies, lovastatin treatment *in vitro* has shown to reduce the expression of CD11b and consequently adhesion in these cells through the alteration of functional RhoA (Yoshida et al., 2001).

Next, we used the surface protein Iba-1, a well-established marker for identifying macrophages and microglial cells in both the activated and resting state on disease retinal treated tissues. This marker that has been found in increased quantities during EAU disease. In our study, quantified levels of Iba-1 positive cells significantly increased in the retina of EAU mice. This finding correlated well with clinical signs of disease. Simvastatin administration at 100 mg/kg did not alter the level of retinal Iba-1 positive cells, suggesting that simvastatin at this dosage, in this model, did not impact on monocyte and macrophage recruitment into the inflamed retina. These findings are in contrast with others in the field as significant evidence supports the notion that statin treatment decreases the levels of CCL2 and CXCL8, which are two main chemokines involved in monocyte recruitment across endothelial cells (Morikawa et al., 2002) (Romano et al., 2000) (Veillard et al., 2006). Indeed, many *in vitro* studies have demonstrated an essential role for CCR2 signalling in the recruitment of microglia to inflammatory sites of the retina (Kuipers et al.,

2006) (Ghittoni et al., 2005). It is important to reiterate that despite some evidence to the contrary of these findings, each statin member is heterogenous and their behaviour is context dependent.

A limitation of this study is that the Iba-1 marker does not differentiate between monocytes/macrophage and resident microglia pools. Recently, several research groups have directed efforts towards developing novel, resident microglia specific markers such as TMEM119 and P2RY12 (Butovsky et al., 2014) (Bennett et al., 2016) (Satoh et al., 2016). Further high-dimensional analysis studies have revealed significant overlap in cytokine expression profiles between resident microglia and peripheral myeloid cells, which adds to the complexity of disentangling the two populations (Ajami et al., 2018).

Given our findings that simvastatin does not impact the recruitment of Iba-1 infiltrating monocytes into the inflamed retina, we next wanted to test whether simvastatin is capable of reducing the amount of tissue specific ROS and RNS damage. Proposed not as a consequence of reduced cell number, but instead by modulation of microglia cell function. To do this, we measured outcomes of microglia-induced ROS and RNS with a panel of biomarkers.

5.4.4 Simvastatin reduces oxidative stress biomarker expression at the chronic inflammatory stages of EAU

We provided a rationale for examining reactive species biomarkers in response to simvastatin treatment in the previous chapter focused on EAE. While our aims remain the same, this model system was used to further underpin our hypothesis. Indeed, EAU shares many pathological mechanistic similarities with EAE. As both are T cell mediated autoimmune disease of the CNS, EAU is often perceived on a par with EAE but without debilitating neurological side effects and intensive animal monitoring.

The presence of oxidative damage in our IRBP model was evident after 21 days, detected by the increased expression of nitrosylated protein (3-NT), lipid peroxidation (4-HNE) and DNA oxidation (8-OHdG) in the retinal layers. This finding provided us with a suitable panel of tools to investigate the potential impact on these cellular biomarkers of reactive species damage.

5.4.5 Nitrosylated protein expression in EAU

In our study, intense nitrotyrosine staining was found most prominently in the retinal outer nuclear layer, a finding that is echoed in the literature (Komeima et al., 2008). When animals were treated with 100 mg/kg simvastatin we found a significant reduction in the quantified levels of nitrotyrosine staining. These findings are in line with others in the field. In statin-treated patients, significantly reduced levels of systemic protein-bound nitrotyrosine, independent of alterations in lipoprotein and C-reactive protein have been found (Shishehbor et al., 2003a). This data is further supported through the observation that simvastatin treatment also attenuated systemic nitrotyrosine (Ceriello et al., 2004).

While mechanisms underpinning these observations are still speculative, one possible explanation is that simvastatin may be attenuating the levels of iNOS generated NO and subsequent tissue-damaging levels of peroxynitrite. Indeed, previous work performed on *in vivo* inflammatory models describe a role for simvastatin in inhibiting iNOS protein transcript (Ceriello et al., 2002). An alternatively proposed explanation for these findings, proposed (Shishehbor et al., 2003b), suggest that statins promote systemic antioxidant effects by methods of suppressing distinct oxidation pathways such as myeloperoxidase-derived NO.

5.4.6 Lipid peroxidation in EAU

In line with others, we report an increased expression of the lipid peroxidation product 4-HNE in our model of EAU (Devarajan et al., 2007) (Dogru et al., 2018). Positive staining was dominated the inner and outer nuclear layers of the retina, surrounding, but not co-localising with, the nuclear stain DAPI. Intense staining was also observed at the photoreceptor level.

Lipid peroxidation is especially relevant to oxidative stress in MS. Lipids constitute nearly 70% of the net dry weight of myelin, compared with 33% seen in grey matter (Bosch-Morell et al., 1996) (Morell, 1993). Lipid peroxidation is the oxidative degradation of lipids which are especially vulnerable to oxidative stress insults and can alter the structure and dynamics of lipid membranes. Recently, a growing body of evidence has implicated products of lipid peroxidase as key mediators of many pathological states, including neurodegenerative diseases. This is largely due to their ability to serve as secondary messengers (Gaschler and Stockwell, 2017). Moreover, lipid peroxides can contribute to further ROS and RNS generation and initiate a chain reaction of lipid peroxidation.

Substantial evidence exists to support the notion that elevated lipid peroxidation products are prevalent in MS patients (Hunter et al., 1985) (Toshniwal and Zarling, 1992). The IRBP model of uveitis involves both adaptive and innate immune responses to mediate retinal damage. The target protein used for induction is located in the extracellular space between photoreceptors and RPE. Thus, these regions are localised to active inflammation and oxidative stress as monocytes are attracted to the site of damage. With simvastatin treatment we observed a striking and significant decrease in lipid peroxidation across the whole retina. Exact mechanisms by which these processes operate are yet to be elucidated.

Additional lipid peroxidation products found in MS lesions are associated with low-density lipoprotein is 4-hydroxynonenal (HNE). 4-HNE has shown to be toxic to neurons as well as having the ability to potently inhibit glutamate transporters. Evidence to support the notion that statins are capable of reducing these lipid peroxidation products has been reported (Wilson et al., 2001). Moreover, others have found statin therapy significantly lowered levels of lipid peroxidation, interestingly this was coupled with increased activity of antioxidant enzymes such as: catalase, glutathione peroxidase and superoxide dismutase (Broncel et al., 2006).

5.4.7 Oxidative DNA/RNA damage in EAU

Damaged or altered DNA is a reliable indication of tissue oxidative stress. This process can occur in both nuclear and mitochondrial DNA. 8-hydroxy-2'-deoxyguanosine (8-OHdG) – the oxidized form of deoxyguanosine – is a reliable biomarker of oxidative DNA damage. 8-OHdG has consistently proven to be elevated in the blood of MS patients and due to ongoing validation studies, is quickly becoming a reliable biomarker of disease (Ljubisavljevic et al., 2016) (Cokluk et al., 2017). In addition, it has recently been revealed that 8-OHdG is abundantly expressed in MS patient tissues (Haider et al., 2011) (Van Horsen et al., 2008). This marker has also been identified extensively in a number of other neurodegenerative disorders (Tasset et al., 2012) (Kikuchi et al., 2011), and used in these cases, to confirm disease presence and severity.

Evidenced by substantial 8-OHdG staining in the inflamed retina, our current study supports the notion that IRBP induced EAU generates pathological redox imbalances that lead to oxidative stress and subsequent DNA damage. 8-OHdG positive staining localised to the inner and outer nuclear layers, however, co-localisation was not seen with the nuclear stain DAPI, indicating that mitochondrial DNA and not nuclear DNA, is the primary target of oxidative damage at this chronic stage of disease. Generally, 8-OHdG levels increase preferentially in mitochondria as a result of the single-stranded nature of mtDNA which makes it more susceptible to damage (Mikhed et al., 2015). Mitochondrial DNA is also more susceptible to oxidative damage than nuclear DNA for several other reasons, firstly, mitochondrial DNA is in direct contact with the mitochondrial produced ROS, a process commonly observed in neuroinflammation. Increasing evidence also suggests that compromised mitochondria, structurally or functionally, perpetuates excessive ROS production. Furthermore, mitochondrial DNA is not protected by histones or other DNA-associated proteins (Yakes and Van Houten, 1997) (Santos et al., 2003). In addition to this, the DNA repair machinery inherent to mitochondria are less efficient than mtDNA (Croteau and Bohr, 1997) (Mandavilli et al., 2002). Therefore, tissues rich in mitochondria such as the retina are typically more sensitive to oxidative insults and altered mtDNA. Pertinent to this thesis are the findings that identified regions rich with 8-OHdG staining correlates with regions of high macrophage and microglial activity.

Relevant to this study are the findings that statins have demonstrably reduced levels of 8-OHdG in a variety of patient samples and across a number of disease models ranging from diabetes (Carrillo-Ibarra et al., 2018) to cerebral infarction, (Nagotani et al., 2005) and atherosclerosis (Moon et al., 2014). Here, our findings show similar 8-OHdG staining patterns across both the vehicle treated and simvastatin treated groups. Although, simvastatin treatment did not significantly alter 8-OHdG tissue expression levels in diseased retinal tissue, there was a downward trend with statin administration and perhaps with increased statistical power this may become more obvious.

5.4.8 NADPH oxidation in EAU

NADPH oxidases are a family of enzymes capable of catalysing the production of ROS. The prototypical NOX, NOX2 isoform, is composed of three cytosolic components (p47^{phox}, p67^{phox}, p40^{phox}) and two membrane bound subunits (p22^{phox}, gp91^{phox}). Upon stimulation, p47^{phox} becomes phosphorylated and all cytosolic components translocate to the membrane where they associate with membrane-bound units. This movement facilitates the formation of a functional NOX complex that uses reduction equivalents of NADP to reduce oxygen to superoxide. These electron transport membrane proteins are responsible for ROS generation, primarily superoxide anion but also hydrogen peroxide. Elevated ROS ultimately leads to oxidative stress which has been associated with a myriad of inflammatory and degenerative pathologies, as mentioned throughout this thesis (Hu et al., 2012).

Accumulating evidence has placed elevated levels of phosphorylated p47^{phox} subunit as an integral feature of pathogenesis in neurodegenerative disease, being well-characterised in pre-clinical models of Alzheimer's disease and Parkinson's disease (Wilkinson and Landreth, 2006) (Zhan et al., 2002). A direct correlation between increased p47^{phox} expression in diseases with a neurodegenerative origin have shown to negatively impact cognitive performance. In progressive MS, patient data has identified a link between high expression levels and active lesions which primarily localised with activated microglia and macrophages. A direct impact of localised increased activity are upregulated processes of inflammatory oxidative burst which has been linked to demyelination and free radical-mediated tissue injury in MS pathogenesis (Choi et al., 2015) (Fischer et al., 2012).

Here, we assessed tissue levels of p47^{phox} expression using immunohistochemical techniques and found a significant decrease with simvastatin treatment at the high concentrations of 100 mg/kg. Therefore, this dataset suggested an important role for simvastatin in mitigating harmful quantities of activated NADPH complexes.

The observed decrease in p47^{phox} expression with simvastatin treatment could be explained in part through inhibition of key protein isoprenoid intermediates in the cholesterol synthesis pathway. Specifically, Rac2 is a key component of the NADPH oxidase complex required for assembly and activation of NADPH. Rac2 is also part of the Rho subfamily and regulation of this protein depends on isoprenylation processes for appropriate translocation to the plasma membrane surface. Further, the Rac2 protein is found in both macrophages and microglia (Choi et al., 2012) (Yu et al., 1998).

Moreover, previous *in vitro* and *in vivo* studies have described statin antioxidant activity by way of decreasing ROS levels. It is believed that these mechanisms involve p21 Rac isoprenylation inhibition, which affects a key functional role for the NADPH oxidase system within endothelial cells. Additionally, it has previously been reported that integrin adhesion depends on Rho for activation. This finding provides some evidence to support a non-cholesterol dependent isoprenoid mechanism and potential intervention site for simvastatin in this context.

5.4.9 Conclusions

Collectively, the data generated from our current studies provide original insight to support a neuroprotective role for simvastatin in EAU. Taken together, simvastatin therapy has shown to reduce ocular pathology and attenuate the expression of biomarkers that represent protein nitrosylation, lipid peroxidation and NADPH oxidase activity in the inflamed retina.

Chapter 6

General summary

This thesis aimed to address the action of simvastatin in the context of effective treatment for secondary progressive MS. Previously reported phase II clinical trial data (MS-STAT1 NCT03387670) (Charaway et al., 2014) examined the effect of two year, high-dose simvastatin therapy on SPMS patients. The results revealed a strong influence of simvastatin on brain atrophy rates with a striking 43% reduction in annualised rate of whole-brain atrophy. Moreover, simvastatin demonstrated a notable decrease in the severity rates of a conventional clinical scoring system, typically used to measure disability. Perhaps one of the most important outcomes from this clinical study pertinent to the body of work present herein, was the unchanged levels of peripheral immune cell markers. These results sparked further investigation into an alternative, neuroprotective role for simvastatin. Findings from the MS-STAT phase II trial supported the advance of a phase III trial MS-STAT2 (NCT03387670) and MS-OPT (NCT03896217).

Simvastatin is currently widely prescribed for the treatment of vascular disease and has an excellent safety profile. Proven effective neuroprotective mechanisms could make this drug an appealing candidate for patients with SPMS. Although progressive MS accounts for most of the disability experienced in MS patients, there are still no satisfactory treatment available in the clinic today. This underscores the urgent need for developing effective therapies in this area. The RRMS and SPMS forms have very different clinical profiles, dominating RRMS disease is a large inflammatory component which over the years, has been the target of many successful therapies, such as IFN β and glatiramer acetate, anti-VLA4 and Fingolimod. In contrast to the RRMS phenotype, the PPMS and SPMS forms exhibit a progressive and neurodegenerative pattern (Lassmann et al., 2012) (Correale et al., 2016). By developing drugs with the target pathology of neurodegeneration in mind, there is a higher chance of finding effective therapies for these stages of disease.

While a substantial number of murine models have been used to demonstrate that statins have a neuroprotective function, different mechanisms of action have been proposed. In a model of Parkinson's disease studies state that statins function by protecting dopaminergic neurons (Hernández-Romero et al., 2008) while others suggest that neurological damage can be lowered by statins protecting striatal neurons and minimizing markers of oxidative stress (Selley, 2005). In EAE, statins have demonstrated improved survival of oligodendrocyte precursors and improved myelination (Paintlia et al., 2009). Despite the plethora of aetiologies, a common feature of neurodegenerative disease is chronic activation of immune cells within the CNS. The production of neurotoxic factors such as NO and O $_2^-$ release by these cells, have been increasingly implicated in the progressive processes of neurodegeneration. Here, our hypothesis supports a neuroprotective mechanism of action for simvastatin in SPMS by way of blocking microglia function.

In this thesis, we selected two models of chronic neuroinflammation with an autoimmune origin, as appropriate tools for investigating simvastatin efficacy. While most of the *in vivo* data generated serves to support proof-of-concept, an *in vitro* model system of inflammation was developed alongside to allow further interrogation into the mechanisms that underpin simvastatin function. The key findings of this

thesis are as follows: first, we revealed simvastatin pre-treatment prevents microglia produced NO in an *in vitro* model of inflammation. Second, in addition to attenuating clinical pathology, RNS and ROS biomarkers of damage were found to be reduced with treatment in the inflamed spinal cord of EAE. Third, whilst simvastatin treatment demonstrated a significant clinical amelioration of ocular pathology in a well-established model of uveitis, it also proved to reduce ROS and RNS marker damage in the chronically inflamed retina.

Simvastatin treatment administration at the onset of symptoms in our model of MS and uveitis, resulted in attenuation of clinical scores. Similar changes in neurological function and improvement have been observed in previous *in vivo* investigations of EAE and EAU and thought to operate by inhibiting T cell proliferation and migratory pathways as well as CNS infiltration of peripheral monocytes. However, those studies were designed as prophylactic therapy, with much focus on preventing disease development during the early stages. In our study, attention was given to downregulating or reversing pathological mechanisms once disease has commenced.

Here, we provide evidence that simvastatin treatment reduces tissue oxidative damage in a model of uveitis and MS. Additionally, we provide evidence that simvastatin directly inhibits the production of NO by activated microglia. These findings highlight the potential protective benefits of statin monotherapy in an environment under oxidative stress.

These findings give rise to several important questions that would form the basis of future projects. Future exploratory avenues would be directed towards underpinning the mechanisms involved in protecting macromolecules from damaging oxidative insults.

- 1) Despite the observation that microglial cells produce less cytokine-induced NO, the mechanism by which this operates is currently unknown. This can be further interrogated *in vitro* utilising the inflammatory model developed herein. iNOS is the likely culprit for simvastatin to have inhibitory effects through the transcriptional factor NF κ B signalling as demonstrated in previous studies and amongst different cell types.
- 2) The activation status of simvastatin treated microglia can be investigated through phenotypic profiling to determine whether this plays a central role to the observed changes in ROS and RNS markers.
- 3) The observed reduction in p47phox levels with simvastatin treatment during EAU could be further examined *in vitro*, as this data points towards a potential role for simvastatin to modulate Rac2 function. Accordingly, the effects of simvastatin on Rac2 signalling are essential for the assembly and activation of the NADPH oxidase complex involved in the respiratory burst within macrophages and microglia.

- 4) Finally, further *in vivo* investigations using alternative models MS that more accurately reflect the chronic progressive pathology would be beneficial to strengthen our proof-of-concept dataset regarding the capability of simvastatin to attenuate ROS and RNS damage.

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