Investigating the effects of novel heparan sulfate mimetic 'HS16-35' on immune cell migration in an *in vitro* model of the blood-CSF barrier.

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This thesis is dedicated in full to the late Annette Fairweather
(1928-2014)

If you only knew how often I thank you.

ABSTRACT

The central nervous system was traditionally considered an immune-privileged site, defined as being immunologically inactive. However, recent studies have elucidated that a number of immune cells traffic into and out of the brain in healthy humans to conduct routine immunosurveillance. A unique immunological interface, the choroid plexus, acts as a gatekeeper for the entry of these immune cells during homeostasis. Although the mechanisms are not well described, the choroid plexus also has the capacity to regulate the responses of migrating leukocytes during inflammation.

Multiple sclerosis is a complex neuroinflammatory disease characterized by demyelination in the CNS. Autoreactive immune cells invade the central nervous system and orchestrate an attack against myelin sheaths, the insulation layer that protects neurons. The disease affects nearly 1 in 1,000 New Zealanders, and currently has no cure. The most successful treatments for multiple sclerosis target the initial stages by inhibiting the entry of these cells into the central nervous system, however these are often associated with severe side and life-threatening effects and cannot prevent the progression of the disease.

Heparanase, the ubiquitously expresses heparan sulfate degrading enzyme has been thoroughly implicated in the disease processes of multiple sclerosis, and its animal model, EAE. Autoreactive lymphocytes exploit heparanase activity to degrade the extracellular matrix and destabilize the barriers that maintain the relative immune privileged status of the central nervous system. Exogenous heparan sulfate mimetics have previously been shown to ameliorate symptoms of EAE by interfering with heparanase activity. However, the commercialization and clinical translation of these inhibitors is currently inhibited by the complexity of their synthesis. 'HS16-35' is a novel heparan sulfate mimetic developed by the Ferrier Institute, comprised of a dendritic core with four heavily sulfated oligosaccharide arms. The synthesis of this compound is much shorter due to its smaller size; however, it has been shown to act similarly to native heparan sulfate molecules. We proposed that HS16-35 is protective in preventing the migration of autoreactive immune cells across the choroid plexus by inhibiting lymphocyte heparanase.

To investigate the efficacy of HS16-35 *in vitro*, we first established an experimental transwell model of the choroid plexus. This model incorporated core components of the choroid plexus, including fenestrated capillaries, the stromal matrix and epithelial monolayer. We first showed that the model was capable of mimicking homeostatic trafficking across the choroid plexus epithelium, which formed a selective but permeable barrier. Then, we induced T-cell specific inflammatory migration using Concanavalin A or T_H1-

type cytokines. This migration was found to be interferon- γ dependent and could be mitigated with anti-interferon- γ treatment.

Once this model was established, we next investigated whether HSI6-35 was effective in inhibiting inflammatory migration across this structure. To adapt HSI6-35 to an *in vitro* dose, we performed cell viability assays. This confirmed that the compound was mildly cytotoxic to epithelial choroid plexus cells but not murine splenocytes. Further experiments found that low-dose HSI6-35 did not impact monolayer permeability. Transwell migration assays showed that low-dose HSI6-35 was effective in reducing ConA and interferon- γ mediated inflammatory T-cell migration to a level comparable to homeostatic trafficking. Finally, we assessed cytokine profiles of leukocytes and epithelial choroid plexus cells treated with HSI6-35 and found that HSI6-35 reduced the expression of key cytokines involved in MS pathogenesis.

In summary, the work described in this thesis showed how HSI6-35 may be protective during EAE by suppressing the inflammatory response of autoreactive T-cells, in addition to regulating the infiltration of immune cells into the CNS through the choroid plexus. In a broader sense, these findings show that HSI6-36 may be effective in treating MS by regulating, not inhibiting lymphocyte migration into the CNS, mitigating some of the severe side effects that other migration-inhibitors face.

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Chapter I: General Introduction

Abbreviations

Ab	antibody	HSPG	heparan sulfate proteoglycans
AEC	animal ethics committee	ICAM-1	intercellular adhesion molecule-1
Ag	antigen	IFN γ	interferon gamma
ANOVA	analysis of variance	ILC	innate-lymphoid cell
APC	antigen presenting cell	IL-x	interleukin-x
BBB	blood-brain barrier	JAM	junctional adhesion molecule
BCSFB	blood-CSF barrier	JCV	John-Cunningham virus
CCL-	chemokine (c-c motif) ligand	M1	classically activated macrophage
CDP	confirmed disability progression	M2	alternatively activated macrophage
CDx	cluster of differentiation	MAG	myelin associated protein
CGM	complete growth media	MBP	myelin basic protein
ChP	choroid plexus	MHC I/II	major histocompatibility complex I/II
CNS	central nervous system	MOG	myelin oligodendrocyte protein
ConA	concanavalin A	MS	multiple sclerosis
CSF	cerebral spinal fluid	PBS	phosphate buffered saline
CTCM	complete tissue culture media	PML	progressive multifocal leukoencephalopathy
CXCL-	chemokine (c-x-c motif) ligand	PPMS	primary progressive MS
Da	Daltons	PRR	pattern-recognition receptor
DC	dendritic cell	RRMS	relapsing remitting MS
DMEM	Dulbecco's modified eagle medium	SPMS	secondary progressive MS
DMF	dimethyl fumarate	TCR	T cell receptor
DMSO	dimethyl sulfoxide	TLR	toll-like receptor
DMT	disease modifying therapy	TNF α	tumour necrosis factor alpha
dPBS	Dulbecco's phosphate buffered saline	Treg	T regulatory cell
EAE	experimental autoimmune encephalomyelitis	UVR	ultraviolet radiation
EBV	Epstein Barr virus	VCAM-1	vascular cell adhesion protein-1
ECPC	epithelial choroid plexus cell	VLA-4	$\alpha 4\beta 1$ integrin
EDSS	expanded disability status score	xg	relative centrifugal force
GWAS	genome wide association study	ZO-1	zona occludens protein-1
HS	heparan sulfate	α IFN γ	anti-interferon gamma

1.1. The immune system

To survive, we must be protected from a continual onslaught of organisms that are inhaled, swallowed, or inhabit the skin and mucosa. The immune system is the defence force of the body, a network of special organs, circulating effector cells and molecules that prevent serious infection causing harm to the host. The immune system has two arms, each defined by the speed and specificity of their reactions: the innate and adaptive immune responses. Both systems are populations of white blood cells called leukocytes, originating from the hemopoietic stem cells of the bone marrow that commit to different lineage fates. Mature leukocytes may reside in peripheral tissue, circulate in the blood or travel through the lymphatic system, depending on the cell type and inflammatory state of the body.

1.1.1. Innate Immunity

The innate immune system is recognizable by its rapid, non-specific response to foreign bodies. The innate system paves the way for the adaptive immune system by managing the infection in its early stages and providing essential information about the type and nature of the pathogen. The cells of the innate immune system include; phagocytes (neutrophils, macrophages, monocytes, or dendritic cells); granulocytes (eosinophils, basophils, or mast cells) or innate lymphocytes (natural killer lymphocytes or innate lymphoid cells). These cells populate most peripheral tissues, allowing them to respond within minutes to a potential threat. Cells of the innate immune system are unique in that they are capable of identifying a vast number of pathogens. The functionality of the innate immune response relies on pattern recognition receptors (PRR), which are capable of recognising a limited number of pathogen associated molecular patterns (PAMPs) at the site of infection.¹⁻³ The four classes of pathogen; viruses, bacteria, fungi and parasites that share class-specific molecular signatures detectable by PRRs.¹⁻³ Toll-like receptors (TLRs) are transmembrane proteins capable of recognising extracellular bacteria or bacteria internalised in vesicles during phagocytosis. Cytoplasmic PRR's, including NOD-like receptors (NLR's) recognise intracellular bacteria and mRNA from viral infections.¹⁻³ A key feature of the innate immune system is priming the adaptive immune response. The innate immune response lacks specificity, so on its own cannot effectively control an infection.² It must pass on information about the pathogen to the adaptive immune system, as the adaptive response cannot initiate a response against a pathogen without input from the innate system.⁴

1.1.2. Adaptive Immunity

The adaptive response is characterized by a much slower, but more specific response. A key feature of the adaptive immune response is its capacity to form immunological memory, which allows the host to

respond with greater speed and efficacy if it encounters the same pathogen at a later time. The cells of the adaptive immune system are B lymphocytes (B cells) and T lymphocytes (T cells). B cells and T cells express distinct antigen receptors and perform vastly different roles in the body. As described, this response lags behind the innate immune response, however these cells have the advantage of antigen-specific recognition of invading pathogens. They complete the immune arsenal to rid the body of infection and form immunological memory to protect the host if they encounter the same pathogen again.

1.2. B cells

B cells mature in the bone marrow and are known primarily for the production of antibodies and antigen presentation to T cells.^{2,4} The initial antibodies produced by a naïve B cell are not secreted, rather inserted into the membrane, functioning as a B-cell receptor (BCR).⁵ Each B cell produces a single, unique type of antibody, with specificity to only one antigen at its binding site as according to the clonal selection theory.⁵ The secondary lymphoid organs house naïve B cells in the body, where they receive a constant influx of antigen in the lymph.² When a B cell recognises antigen on the BCR, the receptor-antigen complex is internalised and digested to produce peptide fragments to present to T cells.⁶ B cell activation generally relies on 'help' from follicular helper T cells (T_{FH}) at the border of the T and B-cell zones.⁶ When B cells present antigen loaded onto major histocompatibility II complexes (MHC), T_{FH} cells conjugate the CD40 ligand to CD40 on the B-cell surface, followed by the release of stimulatory chemokines interleukin-4 (IL-4) and IL-21 which induces clonal expansion.^{2,6} Activated B cells migrate back into the interfollicular regions in response to chemokine gradients where they continue to proliferate and exert their effects. The majority of effector B cells will differentiate into short-lived plasma cells, with the rest becoming memory B cells, which can expand rapidly again upon antigen rechallenge.^{2,5} Antibodies produced by effector B cells aid in the overall immune response through neutralization, opsonisation and complement activation.²

1.3. T cells

Precursor T cells are derived from the bone marrow but migrate to the thymus where they develop further.² Like B cells, each mature T cell expresses a T-cell receptor (TCR) with unique specificity for a singular antigen.² Development of the TCR in the thymus leads to two classes of T cells, the majority expressing the $\alpha\beta$ T-cell receptor, and <5% bearing the $\gamma\delta$ receptor.⁷ The so called 'uniqueness' of the TCR relies on recombination of specific genes in the β -chain, tested and paired against an invariant α -

chain, which is subsequently rearranged to form a novel TCR.⁸ Newly formed T cells then undergo positive and negative selection to ensure that the cell can recognise the MHC complex, but with an acceptable affinity, as those that bind too strongly have the potential to develop autoimmunity.^{2,8} Positive selection determines the class of T cell; i.e. if they recognise MHC I, they become CD8⁺ T cells, and those that interact with MHC II will become CD4⁺ T cells.^{7,8} Mature T cells leave the thymus to circulate between the blood and lymphoid organs, scavenging their specific antigen.² To initiate an immune response T cells must be presented with a peptide antigen on the MHC complex of an APC, which induces clonal expansion and the differentiation into effector T cells.² These effector cells have specific weaponry that allow them to remove the pathogen from the body, and the ability to respond immediately to future infection with the same pathogen.⁴

1.3.1. CD8⁺ T cells

Naive T cells bearing the co-receptor 'CD8' differentiate into CD8⁺ cytotoxic lymphocytes based on their ability to recognize MHC class I. CD8⁺ T cells provide vital immune defence against cytosolic pathogens and tumour surveillance.^{2,9} These cells are most known for their 'killer function', in which infected target cells present fragmented antigen from endogenous pathogens on MHC I, and patrolling CD8⁺ lymphocytes recognise and induce apoptosis in these cells.⁹ Activated CD8⁺ T cells harbour several mechanisms to kill infected cells: the production of cytokines such as IL-2, IFN γ and TNF α , the release of cytotoxic granules or Fas/FasL interactions through the caspase pathway.^{2,10} These mechanisms are discreet in that they avoid non-specific damage to the surrounding tissue. However, because these effector functions are so destructive, CD8⁺ T cells can contribute to excessive amounts of damage when the immune response becomes dysregulated in diseases such as MS.¹¹

1.3.2. CD4⁺ T cells

CD4⁺ T cells recognize exogenous peptide presented on the MHC class II complex, and like CD8⁺ T cells, require antigen presentation for activation.^{2,12} In contrast to CD8⁺ cytotoxic lymphocytes, CD4⁺ T cells then enter further stages of differentiation into T helper cells (T_H) to direct the proper immune response against a specific antigen.¹² CD4⁺ T cells do not act directly on their target cells, rather orchestrating the effector functions of other immune cells.⁴ This allows the host to form a specialised and cooperative response to each class of pathogen, clearing the infection more efficiently. There are many defined CD4⁺ T helper cell subsets, but the major players are: T_H1, T_H2, T_H17 and regulatory T cells (Treg).¹³ These

subsets are distinct in their cytokine determinants, fate-specific transcription factors and the immune cells they target for recruitment.

T_H1 cells differentiate in response to the fate-specifying cytokines $IFN\gamma$ and IL-12 produced by APC's early in infection.¹⁴ These cells can be recognised by their expression of transcription factor T-bet, which is turned on in response to $IFN\gamma$ signalling through the JAK-STAT pathway.¹⁵ Differentiated T_H1 cells produce proinflammatory cytokines such as $IFN\gamma$, forming a positive feedback loop for further T_H1 differentiation.¹⁵ This subset is important in eradicating infections mediated by intracellular pathogens.¹⁶ The cytokines produced by T_H1 -type cells are also important in enhancing macrophage antimicrobial activity to kill intracellular pathogens.² T_H1 subsets have also been identified as key role players in autoimmune diseases, particularly MS.¹⁷

T_H2 type cells are the key subset in eliminating extracellular parasite infections through promoting eosinophil, basophil and mast cell function in addition to inducing B-cell class switching for IgE production.¹⁸ IL-4 determines T_H2 -type differentiation, signalling through the STAT6 and the transcription factor GATA-3.¹² The major cytokine responses associated with T_H2 immunity are IL-4, IL-5 and IL-13.¹⁶ As with T_H1 subset dysregulation, an inappropriate T_H2 response associated with IgE production can lead to the development of allergy and asthma.¹⁹

T_H17 cells are characterised by their production of IL-17 and IL-22, and are a more recent addition to the T helper cell subset.¹³ This population arises in response to $TGF\beta$, IL-6 and IL-23, which induce the transcription factor ROR γ T.¹⁸ T_H17 responses coordinate neutrophil function in destroying extracellular bacteria and fungi.¹⁸ In conjunction with T_H1 cells, T_H17 subsets have been implicated in the pathogenesis of MS.²⁰

1.3.3. Regulatory T cells

In contrast to effector T cell subsets, $CD4^+$ Treg cells regulate the immune reaction by preventing the development of autoreactive immune responses.² Tregs constitutively express CD25, the receptor for IL-2, and can be further identified by the transcription factor FoxP3.¹³ FoxP3 interferes with the NFAT:AP-1 transcriptional complex on the IL-2 gene promoter, preventing the transcription of IL-2.^{21,22} Tregs can be further divided by type: natural Tregs which arise in the thymus, and induced Tregs, which develop in the periphery. Regardless, both Treg subsets share many phenotypic and functional characteristics. ¹³

Tregs suppress other T cell functions through a number of mechanisms, primarily by interfering with interactions between APC and T cells. Through the production of IL-10, Tregs can dampen proinflammatory cytokine release by APC and inhibit antigen presentation on MHC class II molecules, preventing T cell differentiation and effector functions.²³ In addition Tregs have been shown to compete with naïve T cells, blocking CTLA-4:B7 receptor interactions which are required for co-stimulation and activation of T cell effector functions.²² Finally, Tregs have been shown to sequester IL-2, a cytokine responsible for T cell proliferation.²³ Because of their essential function in regulating the immune response, dysfunction of Treg responses is heavily implicated in the development of autoimmunity.²⁴

I.4. Central Nervous System Immune Privilege

The brain and spinal cord are vital organs, essential not only for survival, but proper functionality of the body's other organs, consciousness, higher thought, and our unique experience as humans. Although inflammation serves as an important component in protecting the body from pathogens and maintaining homeostasis, it acts as a double-edged sword within the central nervous system (CNS). The brain is a particularly sensitive organ with limited capacity to repair, as a large proportion of its cells exist in a post-mitotic state.²⁵ Blood-borne macrophages and microglia, in particular are the principal effector cells responding to CNS disruption.²⁶ Depending on their activation status and the pathogenic insult, macrophages that have infiltrated the brain release an array of pro-inflammatory mediators; cytokines, glutamate, free radicals and matrix metalloproteinases (MMP's).^{27,28} These factors promote apoptosis in the surrounding tissue, some of which cannot be repaired, resulting in scar tissue formation.²⁹ It is well understood that neuronal processing depends on a series of activity-dependent pathways through key neuron circuitry, and that damage to, or removal of neurons in the system may lead to cognitive deficits.³⁰

For its immediate protection, the brain is also encased in a rigid skull, which cannot accommodate excess cellular infiltrates or swelling, both of which are hallmark features of the inflammatory response.² Cerebral edema is generally associated with poor neurological function in the long-term and is a strong independent predictor of mortality.³¹ Hence, the incentive to minimise inflammation within the CNS is strong.

Historically, the concept of CNS immune privilege has been ill-defined. The term describes the experimentally defined phenomenon wherein tissue transplanted into the CNS does not elicit an immune response against the foreign antigens and subsequent rejection as it would when grafted into peripheral tissue such as the skin.^{32,33} The secure nature of the CNS from the peripheral immune system, due in part to the impermeability of the blood brain barrier (BBB), choroid plexus (ChP), absence of traditional lymphatics, and apparent quiescence of microglia, the brain's resident macrophage population led scientists to believe that the CNS was immunologically inert and distinct from the periphery.^{34,35}

It was not until some 30 years ago that a dramatic re-evaluation of the cellular responses within and surrounding the CNS began.³⁶ While we now know that the CNS is immune privileged in that immune responses in the CNS is restricted, continuous immune surveillance occurs to protect the brain and spinal cord from infection.³⁴ The immune responses in the CNS to injury or infection generally adopt a pro-resolving and pro-wound healing response to minimise tissue damage. A neuroprotective immune response is acute and self-limiting and results in pathogen elimination, tissue repair and the restoration of

homeostasis in the CNS. Chronic neuroinflammation in the CNS results from dysregulation of the immune response and is a prominent feature of neurodegenerative diseases such as MS. These responses are aggressive and pro-inflammatory, resulting in oxidative stress, neuronal death, tissue atrophy and cognitive decline. In healthy individuals, two unique structures, the (BBB) and the ChP maintain the immune privileged state of the CNS. The BBB is an endothelial barrier that encompasses the cerebral vasculature, and is considered an absolute barrier, in which leukocytes crossing this structure constitute a hazard to the neural parenchyma.³⁷ In diseases such as MS, BBB tight barrier function becomes dysregulated, and autoreactive lymphocytes exploit this structure to enter the CNS.³⁸ In contrast, the choroid plexus can be considered an 'epithelial gate' into the CNS, allowing selective immune cell entry into the CNS.³⁷ In contrast to the inert barrier function of the BBB, the ChP appears to actively participate in modulating the immune responses occurring in the CNS.^{39,40} The primary focus of this thesis is the choroid plexus, which will be expanded upon below.

1.5. Multiple Sclerosis

Multiple sclerosis (MS) is the most common cause of non-traumatic neurological disability in young adults, affecting approximately 2.8 million individuals worldwide, with twice as many women diagnosed versus men.^{41,42} MS is a chronic, T-cell mediated inflammatory disease of the CNS that presents as focal demyelination, neuronal loss, gliosis and an aberrant inflammatory response mounted against CNS antigens.² The most common form of the disease known as relapsing remitting multiple sclerosis (RRMS), is characterised by active immune-mediated lesion formation in the brain, followed by periods of subclinical disease where recovery occurs to baseline health levels.⁴³ Over time, most RRMS patients progress to a secondary progressive form of the disease (SPMS) in which remission does not occur.⁴³ More rarely, patients are immediately diagnosed with primary progressive MS (PPMS), in which isolated immune attacks do not occur but instead disease symptoms culminate and progress until eventual death of the patient.¹⁷

The current literature offers little to explain why a nonspecific inflammatory event leads cells to attack self-antigens in the CNS. In the majority of cases, MS is not an immediately life threatening condition, but continuous autoimmune assault towards the CNS can lead to substantial disability through numerous sensory, motor, autonomic and neurologic dysfunctions.⁴⁴ Disease onset generally occurs in the late twenties or early thirties, a key time for individuals to be establishing their independence, starting a family, becoming homeowners or excelling in their chosen career. MS robs these individuals of their quality of life, with up to 50% of patients requiring permanent mobility assistance just two decades after their diagnosis.¹⁷

1.5.1. Risk factors

Although the aetiology of MS is unknown, it is postulated that complex gene-environment interactions leads to the onset of disease pathology. The genetic components are likely responsible for the direct triggering of an autoimmune response, whereas environmental factors modulate activation thresholds and the overall inflammatory milieu.¹⁷ Genetic variation accounts for less than 30% of the overall risk factor with GWAS studies identifying little over 200 genes identified as 'risk regions', in which polymorphisms of these genes increases genetic predisposition in patients.^{17,45,46} Most of these genes code for molecules expressed on the immune system, with the most prominent gene identified as the human leukocyte antigen (HLA) on chromosome 6. The DRB1*1501 HLA class II allele variant has been consistently associated with increased susceptibility to MS in European/Pakeha, Asian and African American populations.⁴⁷ The class I allele, A*02:01 has also been shown to affect risk. It is also understood that polymorphisms in genes

that code for T-cell activation and proliferation, namely IL-2 and IL7R, alongside other co-stimulatory factors, such as TNF α are key components in determining overall disease onset in MS patients, and even other immune disorders.^{48–50}

Non-genetic factors have a larger contribution to overall MS incidence than genetic factors themselves, making up almost two-thirds of the risk.⁵¹ Without a common exogenous risk factor that links all MS patients, it is difficult to suggest whether MS is a disease that begins in the CNS or is a response triggered in the periphery that infiltrates the CNS. However there are numerous common risk factors that have repeatedly been shown to predispose individuals to MS; viral infection, vitamin D levels, smoking or obesity.^{17,46,51} Because there is no singular set of risk factors, there is no real way to prevent or lessen its socioeconomic impacts, making it a particularly difficult disease to manage.

1.5.2. Immunopathology

MS is widely believed to be a disease of autoimmune origin, evidenced by the main target of pathology; myelin-protein derived antigens in the brain and spinal cord. The presence of autoreactive CD4⁺ T cells in the CNS is detectable from the earliest stages of MS. These T-cells recognise and orchestrate an immune attack towards several candidate autoantigens; myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and myelin associated glycoprotein (MAG) to cause neuronal damage in concentrated regions of the brain termed 'lesions' and the emergence of symptoms.^{43,52} Interestingly, CD4⁺ T cells harbouring TCR against these antigens have been found circulating in healthy individuals, however at a lower frequency than MS patients.^{53,54} In addition, these cells show a much higher avidity and proliferative response to myelin antigens in MS patients versus healthy, suggesting disease onset is associated with dysregulation of regulatory function in peripheral tolerance mechanisms.^{53,54} Although MS is considered to be a primarily T-cell-mediated disease, defective B cells, regulatory cells and macrophage/microglia have all been implicated at different time-points in disease pathology.¹⁷

The initial event that triggers the breakdown of tolerance and activation of autoreactive lymphocytes into aggressive effector subsets is unknown. The literature suggests that molecular mimicry, bystander activation, novel autoantigen presentation or the release of previously sequestered CNS antigen into the peripheral circulation may drive activation of these effector cells.¹⁷ For the activated immune cells to elicit damage in the CNS, they must migrate into the CNS through two unique tissues, the BBB and ChP. Proinflammatory factors relating to metabolic stress or infection may upregulate the expression of endothelial adhesion molecules in these tissues, facilitating the extravasation of lymphocytes from the

systemic circulation into the CNS.⁵² In addition to CD4⁺ T cells, macrophages, and CD8⁺ T cells and dominate the early infiltrate.⁵⁵ These cells direct damage towards myelin sheaths, the insulating layer that surrounds neurons. These lesions are generally self-limiting and there is little damage to the surrounding white or grey matter.¹⁷

As the disease progresses, demyelination becomes more diffuse throughout the brain, with notable ventricular atrophy and the formation of tertiary lymphoid structures in the meninges.¹⁷ In these latter stages, infiltration into the CNS reduces as chronic inflammation is well established. B cells make up a greater proportion of the pathogenic population, with microglia and macrophages in a state of chronic inflammation.

1.5.3. Treatments

Because of the autoimmune and degenerative nature of MS, there is no cure. For the majority of patients, who are afflicted with RRMS, there is a broad range of disease modifying treatments (DMT's) available. These drugs are somewhat effective in managing relapses but will not prevent a transition to SPMS.⁵⁶ These drugs are difficult to design because they must be effective against MS pathology but have minimal impact on the normal protective function of the immune system. In NZ, both progressive forms of the disease have no existing treatments, options for these patients focus on the managing individual symptoms.⁵⁷ Frontline treatments for RRMS in New Zealand include ocrelizumab, fingolimod, teriflunomide, dimethyl fumarate and natalizumab.⁵⁷

1.5.3.1. Natalizumab

Natalizumab, sold under the brand name Tysabri® is a monoclonal antibody and one of the most successful treatments for MS. Natalizumab targets the migration of self-reactive lymphocytes into the CNS in the initial stages of disease. Effector lymphocytes extravasating into the CNS express integrin $\alpha_4\beta_1$, (VLA-4). VLA-4 is the cognate ligand for vascular cell adhesion protein 1 (V-CAM1), upregulated on the inflamed luminal surface of endothelial cells of the blood-brain barrier.^{2,58,59} This interaction is required for the firm adhesion, attachment and migration of leukocytes into white matter lesions. Natalizumab is a highly specific VLA-4 glycoprotein antagonist, preventing the lock and key ligand-receptor interaction from occurring, denying self-reactive lymphocytes entry to the CNS. It was approved for use in RRMS patients following a successful clinical trial in 2004,⁶⁰ but was pulled from the market briefly when a larger cohort study revealed serious and potentially fatal complications in a small percentage of patients.^{61,62} 1.5 to 2.8 in every 1000 patients treated with natalizumab develop progressive multifocal leukoencephalopathy (PML).⁶³ PML

is a devastating demyelinating disorder caused by the opportunistic infection of the John-Cunningham virus (JCV). An estimated 39 to 58 percent of the general population have antibodies against the JCV, indicating a present or historical infection.⁶³ Usually controlled by the immune system, in patients that are immunosuppressed, the JCV can undergo active replication in glial cells of the CNS. PML presents as a demyelinating disorder similar to MS, causing lytic damage to the oligodendrocytes of the CNS. The disease course is rapid and destructive, and primarily leads to death or severe long-term disability.^{62,63} Key risk factors for patients considering natalizumab are a seropositive result for JCV antibodies, previous use of immunosuppressants and the length of natalizumab therapy.⁶⁰ Because natalizumab significantly reduces relapse rates, expanded disability status scale (EDSS) score and the number of gadolinium-enhancing lesions,⁶³ these key risk factors are considered by neurologists before natalizumab treatment is initiated.

1.5.3.2. Ocrelizumab

Ocrelizumab, clinically known as Ocrevus® is a recombinant humanized anti-CD20 monoclonal antibody that is a promising new treatment option for patients with RRMS and progressive forms of the disease.^{64,65} The drug targets B cells, an underestimated but key player in the pathology of MS. B cells mediate the disease by producing autoantibodies, self-antigen presentation, pro-inflammatory cytokine production and the formation of ectopic lymphoid tissues in the meninges.^{64,66,67} When treated with ocrelizumab, CD20-expressing B cells are depleted through a number of cell-death pathways, although the mechanism by which this occurs is not well understood. Because CD20 is expressed on circulating immature, mature and memory B lymphocytes but not plasma and stem cells, B cells are not permanently depleted and will begin to self-replenish should the patient stop drug infusions.⁶⁵ Following the first and second infusions at day 1 and 15, the drug is administered intravenously by a clinician once every 24 weeks, negating the need for regular visits to a hospital. In two phase III clinical trials of ocrelizumab versus an older, gold standard treatment; interferon- β 1a in patients with RRMS, researchers found that in every aspect of clinical disease assessment, ocrelizumab outperformed the older treatment. At 12, and 24-weeks post treatment, ocrelizumab reduced relapse rate and confirmed disability progression (CDP) by up to 40 percent. Ocrelizumab is also presented as an alternative for patients on Tysabri® who have JCV positive CSF results, as there are currently no reported cases of PML with Ocrevus® treatment.⁶⁷

1.5.3.3. Fingolimod

Fingolimod (FTY720), sold under the brand name Gilenya® was the first approved oral-DMT for RRMS patients in 2010 and is still a first line treatment in NZ. The mechanism of action of fingolimod differs

greatly from other MS treatments, it has dual action by inducing lymphopenia and neuroprotection. Fingolimod is a synthetic immunomodulator, designed after the fungal metabolite, myriocin.⁶⁸ It is a structural analogue to lipid sphingosine, the native sphingosine-1-phosphate receptor (SIP_{1,3,4,5}-receptor) ligand. These receptors affect a number of differential signalling pathways associated with cell migration, chemotaxis, survival, proliferation and activation.^{69,70} In particular, the SIP₁-receptor is a G-protein coupled receptor expressed on CCR7+ immune cells, another key receptor involved in lymphocyte homing.⁷¹ A proposed model of lymphocyte circulation suggests that SIP₁-receptors are required at exit sites in lymph nodes.⁷² SIP₁-receptors allow lymphocytes to cycle between the blood and lymphoid organs, surveying organs for antigen before returning to the lymphatics and beginning again. SIP is found in the blood and lymph in higher concentrations than the lymph nodes, facilitating the egress of lymphocytes in response to a chemotactic gradient.^{68,72}

Fingolimod acts as a powerful functional antagonist for the SIP₁ receptor.⁷³ SIP₁-receptor lacking lymphocytes cannot respond to endogenous sphingosine-1-phosphate gradients and their egress from secondary lymphoid organs is prohibited.^{2,73,74} Pathogenic cells that would otherwise extravasate into the CNS can no longer drain into the efferent lymphatics and exert their effects. The most prominent populations that Fingolimod sequesters in the lymph nodes are naïve and central memory T cells, but the drug also exerts modest effects on B cells and NK cells.⁶⁹ Both CCR7 negative effector T cells and effector memory T cells are not affected by the drug and may continue to circulate in the blood.^{69,75} A major caveat of fingolimod treatment is the broad expression of SIP receptors, leading to a number of (usually dose-dependent) side effects which can be detrimental enough for patients to stop taking the therapy.⁷³ In addition, concerns over PML in patients treated with fingolimod were confirmed, although at a lower frequency than natalizumab, at <0.1 per 1,000 patients treated.

1.5.3.4. Teriflunomide

Teriflunomide, known clinically as Aubagio® is a once-daily oral disease modifying therapy approved for the treatment of RRMS. Teriflunomide is a repurposed drug and the active metabolite of leflunomide; an immunomodulator on the market for rheumatoid arthritis.⁷⁶ Teriflunomide targets proliferating lymphocytes by inhibiting pyrimidine synthesis. By specific, non-competitive and reversible inhibition of dihydro-orotate dehydrogenase (DHODH), a mitochondrial enzyme, teriflunomide prevents proliferation of activated lymphocytes which requires *de novo* pyrimidine synthesis.⁷⁷ Resting lymphocytes can still self-renew using the pyrimidine salvage pathway but cannot undergo proliferation in response to activation

signals.⁷⁷ This ensures that normal homeostatic proliferation and immune surveillance can continue uninterrupted.⁷⁸ Several other studies have described how teriflunomide acts through impairing the formation of immunological synapses, and decreases the release of IL-6, IL-8 and CCL2 from monocytes.^{79,80} In an open-label Phase III trial of teriflunomide in RRMS patients, researchers discovered that the drug reduced overall lymphocyte numbers, increased the ratio of CD4⁺/CD8⁺ T cells and skewed CD4⁺ T helper differentiation in favour of Tregs, with a significant reduction in clonal diversity.^{81,82} Overall, this suggested a shift from pro-inflammatory responses to regulatory. In addition, patients could still mount an appropriate immune response, indicative that this drug is immunomodulatory not suppressive.⁸¹ Two phase III clinical trials (TEMSO and TOWER) have demonstrated that teriflunomide has positive effects on relapse rate, disability progression and MRI-determined outcomes.^{83,84} Overall, teriflunomide is well tolerated by patients, however was found not to be appropriate in patients with hepatic dysfunction, or in patients that were switching from other MS drugs; who had potential for rapid disease worsening.⁸⁵

1.5.3.5. Dimethyl Fumarate

Tecfidera®, the MS drug with dimethyl fumarate (DMF) as an active ingredient is a twice-daily oral DMT approved for RRMS. DMF is a simple compound developed from fumaric acid.⁸⁶ It has been used to treat psoriasis since the early 1990's, which shares an immune-mediated basis with MS.⁸⁶ When taken orally, DMF is converted by gastrointestinal enzymes into its primary active principle, monomethyl fumarate where it exerts therapeutic effects.⁸⁷ Although its' mechanism of action is widely unknown, DMF has been shown to have a multitude of biological targets. One of the major effects, shown both *in vitro* and *in vivo* is the capacity to shift the total immune response to an anti-inflammatory, T_H2 response.⁸⁸ Although T cells are the major population targeted, modulation of many other cell types including dendritic cells, macrophages, microglia, neurons and astrocytes have shown phenotypic changes related to anti-oxidative responses.^{89–91} Shifting the state of the immune system to an anti-inflammatory state has clear benefits in MS treatment, as evidenced by the two phase III clinical trials, DEFINE and CONFIRM. Both studies showed that annualized relapse was significantly reduced in patients taking DMF versus placebo, with positive effects on secondary endpoints including reductions in the number lesions, new or enlarging on T2-weighted MRI images.^{92,93} In October 2014, one case of fatal PML was reported due to long-term lymphopenia on DMF treatment, which must be taken into consideration for clinicians prescribing DMF.^{92,93} There is also limited data to suggest that DMF may show some teratogenicity in humans, due to its ability to cross the placenta, so is not appropriate for pregnant women.⁸⁶

As previously described, disease modifying therapies for patients with RRMS are available, however their use may be limited by severe side effect profiles, namely PML associated with long term lymphopenia. No currently available treatments can cure the disease or consistently prevent the progression to secondary progressive MS.⁵⁶ Given that most MS patients will rely on DMT's for the remainder of their lives, the cost to efficacy ratio is low.⁹⁴ The market also lacks treatment options for patients with both progressive forms of MS, whose treatment focuses solely on managing symptoms. Thus, there is a clear need for novel, targeted therapies to help all of these patients.

I.6. EAE as a model of MS

Experimental autoimmune encephalomyelitis (EAE) has been a widely used experimental model for MS for decades. Indeed, several of the DMT's currently used for MS were developed in the EAE model first.⁹⁵ Although this thesis does not involve any *in vivo* animal studies using EAE mice, future chapters explore the use of leukocytes derived from mice with active EAE disease. EAE is induced in mice by priming the peripheral immune system with myelin-specific antigens in an emulsion with complete Freund's adjuvant, which exacerbates the immune response to the myelin peptides.^{95,96} Similar to MS, the disease is primarily mediated by T cells, orchestrating an immune response against myelin that results in focal areas of demyelination in the central nervous system.⁹⁷ Other immune components including B cells, macrophages, microglia and DC are also implicated in disease progression.¹⁷ EAE manifests as paralysis that begins in the tail, moving upward to encompass the hind limbs, which can be quantified on a disability scale from 0-5, (described in 2.2.3).⁹⁸

I.7. The Choroid Plexus

The ChP is a unique immunological interface that lies at the border between the blood and cerebral-spinal fluid (CSF). Nestled between the ventricles and folds of pia mater, the ChP is a convoluted structure comprised of specialised epithelial cells, called ependymal cells, and a stromal matrix underlying a dense network of fenestrated capillaries.^{37,99} The ChP is found in the lateral, third and fourth ventricles of both human and mice brains, following the corresponding choroidal fissure of its' respective ventricle.¹⁰⁰

The fenestrated capillaries of the ChP provide little to no barrier function, indeed they act like the proximal tubules of the kidney, allowing free passage to large solutes and metabolites into the stromal space.¹⁰¹ It is the tight junction molecules; occludins, claudins and cadherins at the apical surface of the ependymal cells that are tasked with forming the blood-CSF barrier (BCSFB).¹⁰²

The ChP carries out a variety of functions; arguably the most important of which is the production and maintenance of the CSF.¹⁰³ For many years, this was considered the primary and only function of the ChP.³³ Recent studies have elucidated the key role the ChP plays as an immunological interface of the CNS, governing homeostatic trafficking into and out of the CNS.⁹⁹ The stroma of the ChP houses a small population of tissue-resident APC's, poised for interaction with trafficking lymphocytes.^{39,104,105} Strominger et al. (2018), suggest that the ChP exists as an active immunomodulatory gate for bidirectional lymphocyte movement and as a niche for T cell stimulation, preconditioning the CNS for immune surveillance.³⁹

Fenestrated capillaries in the ChP are easily traversed by leukocytes, which enter the ChP stroma. They rarely cross into the CSF due to the strong barrier properties of the ChP epithelia.³⁹ Under normal conditions, the ChP epithelium produces an array of soluble mediators, that presumably govern which cells can translocate into the CSF. Like the CSF, the ChP adopts an immunosuppressive state under homeostatic conditions.³⁷ At rest, the ependymal cells of the ChP constitutively express various chemokines; CXCL10 and CCL20 in particular, are key mediators of chemoattraction and migration for cell-mediated immunity.¹⁰⁶ At rest, these cells also release a slew of T_H2 and M2-type cytokines, skewing any entering cells towards a pro-resolving milieu.¹⁰⁷

Figure I.I: The composition of intercellular molecules that maintain the blood-CSF barrier between polarized epithelial cells. Tight junction molecules: claudins, occludins and junctional adhesion molecules interact with zona occludens-1 to form the apical-side tight barrier function. Gap junctions are comprised of nectin, which localises with E-cadherin on the basolateral side. E-cadherin forms adherens junctions through interactions with the intracellular armadillo family proteins; β -catenin and p120, which bind to α -catenin, linking to the actin cytoskeleton. Image adapted from Tietz and Engelhardt., (2015).¹⁰⁸

During inflammation, resident dendritic cells in the ChP stroma will interact with the pooled lymphocytes, presenting CNS antigens from CSF sampling.¹⁰⁹ Indeed, one study showed that the majority of $CD4^+$ T cells in the ChP stroma express TCR against neuroantigens in healthy individuals.¹¹⁰ A small population of resident $CD4^+$ T cells live in the CP stroma, which primarily exhibit effector memory phenotypes and express T_H1 -type chemokine receptors, with high expression of IL-4, IFN γ and FoxP3 versus those in circulation.¹¹⁰ These cells are thought to conduct regular neurosurveillance, offering a protective, regulatory role with immediate effector functions. Other $CD4^+$ T cells transiently enter the ChP to pass through into the CSF, which adopt a central memory phenotype.¹¹¹ However, stromal dendritic cells have the capacity to skew the response of these $CD4^+$ T cells, based on the inflammatory or tolerogenic phenotype they adopt.³⁹

Interestingly, the ChP is newly implicated as a direct immunomodulatory gate in the pathogenesis of MS.^{112–115} It is well understood that a breakdown of the BBB leads to the infiltration of autoreactive lymphocytes into the CNS, however the ChP is now suggested as the initial site of lymphocyte entry and priming of the inflammatory response.¹¹⁶

In a two-way communication model, the ChP is also continuously exposed to T-cell-derived chemokines and cytokines.³⁹ Such crosstalk between the ChP and its resident T-cell population may have the potential to alter the chemokine and adhesion molecule profile of the ChP epithelium during inflammation.^{109,117,118} Kunis et al., (2013) demonstrate that primary ChP cells respond to proinflammatory T_H1 type cytokines IFN γ and TNF α by upregulating genes for chemokines associated with T-cell activation and trafficking, monocyte trafficking and increased expression of adhesion molecules.¹¹⁹ Following the immune activation and homing of T_H1 and T_H17 cells into the CNS through the ChP, CCL5 and CXCL9-CXCL11 are released by the epithelium and further propagate the lymphocyte recruitment process to this structure.³⁹ High levels of CD68⁺ staining in the ChP stroma suggest the presence of activated macrophages, which are not typically found in healthy tissue.^{113,120} T cells in the ChP interact with resident monocyte and dendritic cell subsets, undergoing proliferation and activation given that their cognate peptide is presented.³⁹ T_H1 activation in the ChP likely shifts the immune milieu, facilitating further invasion through this structure, and shifting the makeup of cell-mediated immunity in the CSF. Interestingly, adhesion molecules such as ICAM-1, VCAM-1, P-selectin and E-selectin are expressed apically on the ChP epithelium, rather than vasculature.^{114,120–122} These findings support the notion of bidirectional lymphocyte trafficking through this structure, and interactions between leukocytes homing to the ChP and resident immune cells that propagate peripheral inflammation throughout the CNS.^{39,119}

The ChP constitutively produces CCL20, a CCR6 ligand, and a strong chemoattractant for T_H17 CD4⁺ T cells.¹²³ It is reported that CCL20 expression in the ChP mediates the initial step of CNS infiltration by autoreactive T_H17 lymphocytes in EAE.¹²³ In a two-step model, Reboldi et al., (2009) describe how this initial infiltration triggers a CCL6-independent wave of parenchymal invasion by other immune cells.^{37,123} Although, notably this tissue produces CCL20, even in the absence of T_H17 cells in healthy individuals which acts a Treg chemoattractant during homeostasis, suggesting a role in neurosurveillance that becomes dysregulated in disease.³⁷ In addition, macrophages entering the CNS in response to spinal cord injury through the ChP adopt a functionally distinct, regulatory M2 phenotype versus those that enter through the leptomeninges closer to the site of injury which adopt a classic, proinflammatory M1 state.¹⁰⁷

This suggests that entry through the ChP can affect the fate of leukocytes by skewing them towards a more regulatory phenotype. In healthy individuals, the ChP may indeed act as a regulatory structure to promote an active, CNS-protective response despite the state of the peripheral inflammatory response.

Although it is now clear that the ChP acts as a permissive gate for leukocyte trafficking into the CNS, the mechanisms by which it becomes dysregulated in diseases such as MS are unclear. Certainly, the ChP plays a role in MS pathogenesis, it is an inherently leakier tissue than the BBB so many autoreactive lymphocytes penetrate this structure and pool in the CSF. MRI of patients with MS and neuromyelitis optica spectrum disorder show significant enhancement in thickness of the ChP.¹²⁴ Both pathogenic T and B cells have been shown to exploit the ChP to gain entry to the CNS during active MS.^{125,126} Because the ChP has not been a focal point of neuroimmunology, we fail to understand the mechanisms by which these cells gain entry to the CNS. Understanding of these mechanisms will provide opportunity to develop novel therapeutic interventions that target these processes, allowing us to control inflammatory responses in the CNS and promote repair.

1.8. Heparanase/heparan sulfate

Heparan sulfate proteoglycans (HSPG's) are the most abundant components of the extracellular matrix (ECM), playing a major role in maintaining tissue architecture and integrity.¹²⁷ Heparan sulfates (HS) are complex linear polysaccharide chains, bound heavily by sulfate.¹²⁷ They exist either as free unconjugated chains, or covalently bound to protein cores in the extracellular matrix. HSPG's are involved in a number of complex biological pathways that propagate cell signalling, adhesion, migration and can act as a depot for growth factors.¹²⁸

Mammalian heparanase is an endo- β -D-glucuronidase responsible for cleaving heparan sulfate chains. It is synthesized as a large latent precursor (65 kDa) and undergoes proteolytic processing in the acidic lysosome environment, yielding 8 kDa and 50 kDa subunits that associate to form a heterodimer.^{129,130} Because the processed enzyme becomes highly active, it is under strict regulation.^{128,131,132} Heparanase is constitutively inactive in most cell types, its expression is restricted to platelets, leukocytes and the placenta, with no expression in healthy connective tissues and epithelia.^{128,132} The heparanase-coding gene is under promoter methylation and the inhibitory activity of p53 in homeostatic conditions.

Heparanase has been implicated as a major player in the pathogenesis of cancer, regulating angiogenesis and metastasis. Cancers with elevated levels of heparanase expression are generally associated with an

aggressive phenotype and poor post-operative survival rate. Thus, heparanase has gained prominence in cancer biology as a potential anti-neoplastic drug. Research in this area is still relatively new, with promising results in a number of drug candidates as an adjuvant to chemotherapy. Emerging evidence also suggests a role for heparanase in other chronic inflammatory conditions, such as MS.^{133,134}

During inflammation, upregulated heparanase activity cleaves native heparan sulfate molecules present in the ECM, which breaks down the matrix integrity and destabilises the underlying endothelial cell barrier.¹³⁰ In cancer, this well-described role facilitates angiogenesis and metastasis.¹³⁰ In terms of cell signalling, this process modulates the interactions of chemokines, cytokines, and other signalling molecules with their receptors. Heparanase activity can release HS-sequestered proteins, leading to the formation of chemokine gradients at the BBB.¹³⁵ This can facilitate trafficking of inflammatory leukocytes through the disrupted endothelial monolayer.¹³⁵ In addition to changes in leukocyte trafficking into the CNS during inflammation, heparanase has also been shown to regulate the activation of microglia and astrocytes.¹³³ Its involvement in similar CNS disorders suggests that heparanase may govern some of the early neuroinflammatory events that occur in MS, including the trafficking of autoreactive immune cells across CNS barriers.

Indeed, heparanase has been a target of MS research for some time now, there is significant literature to support the role that heparanase contributes to disease pathology in EAE and MS. Willenborg and Parish., (1988) showed that administration of heparin and fucoidan, two highly sulfated polysaccharides completely inhibited the onset of adoptive transfer EAE, and the total absence of lesions in the CNS. Parish suggested that heparin and other sulfated polysaccharides strengthened the ECM scaffolding by occupying and blocking the active site of heparanase, expressed by lymphocytes emigrating into the CNS.^{136,137} Subsequent studies confirmed this by showing that low-dose modified heparins led to complete inhibition of adoptively transferred EAE in Lewis rats.¹³⁸ This was attributed to the specific inhibitory effect on T-cell heparanase expression in relation to cell trafficking, which provided rationale for its use as an immunomodulator.¹³⁸ In 1990, this group also established concanavalin (ConA) stimulation *in vitro* induces T cell heparanase expression, which is associated with the ability of these cells to penetrate vascular endothelium into target tissues.¹³⁹ This could be inhibited with low dose heparin at 0.1 µg/mL and *in vivo*, heparin completely inhibited the capacity of lymphocytes to migrate to the antigenic site to elicit an immune response.¹³⁹

Because heparanase expression in T cells has been shown to be upregulated in response to *in vitro* MBP stimulation, it can be hypothesized that heparanase expression is upregulated in lymphocytes from MS patients, contributing to an overall increase in T-cell aggressive phenotype.¹³⁶ de Mestre et al., (2007) confirm this using *in vivo* studies with a rat EAE model; heparanase mRNA expression was found to be induced in antigen-specific CD4⁺ T cells and its expression directly correlated to the cells' encephalitogenicity. Interestingly, while heparanase was highly expressed in the CD4⁺ cells infiltrating the CNS of these rats, little to no expression was measured in CD8⁺ T cells, macrophages/microglia and astrocytes during active disease.¹⁴⁰ In addition, T-cell heparanase, even devoid of its enzymatic activity has been shown to have pro-adhesion properties, mediating rolling and arrest during shear flow conditions.¹⁴¹ Together, this data suggests that heparanase plays a major role in establishing and maintaining disease progression in MS.

The current literature sets out a clear rationale for the role of heparanase in MS disease progression. In short, virtually all cerebral blood vessels express at least one class of HSPG's on their surface.¹⁴² Activated CNS-antigen specific CD4⁺ T cells and monocytes exploit the enzymatic and pro-adhesion properties of heparanase to migrate through the ECM and leaky BBB endothelium.^{139,141,142} *In vivo* animal work has shown that blocking ECM degradation is protective in EAE, and overexpressing heparin prevents innate immune cell and T-cell infiltration into the CNS.^{137,142,143} In addition, heparan sulfate mimetics have been shown to be powerful inhibitors of T_H1/T_H17 type effector functions, skewing instead towards a FoxP3⁺ Treg response.¹⁴⁴ Because of its well-described role, heparanase is an attractive target for drug development pathways

I.9. HSI6-35

Because heparan and heparan sulfate mimetics have previously been shown to inhibit migration across the barrier structures into the CNS, we propose that HSI6-35 will inhibit migration across an experimental model of the ChP. 'HSI6-35' is a single entity HS-glycomimetic cluster, which is part of a larger, targeted library of HS-mimetics conjugated to simple sulfated saccharides developed by the Zubkova lab at the Ferrier Research Institute. Many labs across the world have developed HS mimetics, which interfere with heparanase activity, but their development is often hindered by the size and complexity of the molecules.¹⁴⁵ These glycomimetics are smaller in size and have a much shorter synthesis, making them accessible and commercially viable. HSI6-35 shows potent heparanase inhibition with low IC_{50} values, comparable to competitors in development. In early cancer research, these compounds display stunning anti-cancer properties, inhibiting growth and metastasis of human myeloma tumour cells and angiogenesis.¹⁴⁵ More interestingly, HSI6-35 reportedly lacks anticoagulant activities and cytotoxicity, a major issue of other HS-mimetics in clinical development.¹⁴⁶

Figure I.2: The chemical structure of HSI6-35. HSI6-35 is comprised of a dendritic core with four identical sulfated oligosaccharide arms.

I.10. Research Aims

The aim of this research was to establish whether the novel HS-mimetic, HSI6-35 could inhibit the migration of immune cells across a model of the ChP. First, this project required the establishment of an experimental *in vitro* model of the ChP. This model was capable of mimicking both homeostatic and inflammatory trafficking into the CNS. Next, the cytotoxicity of HSI6-35 on murine splenocytes and ChP epithelium was assessed to determine the optimum concentration to treat cultures *in vitro*. Using this migration model, I assessed the efficacy of low-dose HSI6-35 to inhibit migration across the resting and inflamed ChP epithelium. Finally, to understand whether HSI6-35 modulates lymphocyte migration through other mechanisms, I investigated the cytokine profiles of murine splenocytes and ChP epithelium in response to HSI6-35 treatment under both homeostatic and inflammatory conditions. In summary, my specific research aims were:

1. To establish an experimental *in vitro* model of the ChP capable of mimicking both homeostatic and inflammatory trafficking.
2. To determine the optimum concentration of HSI6-35 for use in this model system.
3. To evaluate the ability of HSI6-35 to inhibit migration across resting and inflamed ChP epithelium.
4. To investigate the cytokine profiles of murine splenocytes and ChP epithelium in response to HSI6-35 treatment under both homeostatic and inflammatory conditions.

Chapter 2: Materials and Methods

2.1. Materials

2.1.1. Table of Materials

Item	Catalog Number	Company
2-Mercaptoethanol	21985-023	Life Technologies
6.5 mm Transwell® with 8.0 µm Pore Polyester Membrane Insert, Sterile	3464	Corning
Collagen, Type I solution from Rat Tail	C3867	Gibco
Concanavalin A from Canavalia ensiformis, Type IV-S, lyophilized powder,	C5275	Sigma Aldrich
Corning® Matrigel® Basement Membrane Matrix, LDEV-free,	354234	Corning
Dimethyl Sulfoxide (DMSO) Hybri-Max™, Sterile-filtered	D2650	Sigma Aldrich
DPBS (10X), no calcium, no magnesium	I4200-075	Gibco
Dulbecco's Modified Eagle Medium (DMEM), high glucose, pyruvate	I1995-073	ThermoFisher
Evans Blue Dye	E2129-100G	Sigma Aldrich
Fetal Bovine Serum, New Zealand Origin, Sterile Filtered	F8067, Lot 16A327	Sigma Aldrich
Freund's Adjuvant, Complete	F5881	Sigma Aldrich
HEPES (1M)	I5630-080	ThermoFisher
InVivoMAb anti-mouse IFN γ	BE0055	BioXCell
L-Glutamine (200mM)	25030-081	ThermoFisher
MEM Non-Essential Amino Acids Solution (100X)	I1140-050	ThermoFisher
Millex-GP Syringe Filter Unit, 0.22 µm, polyethersulfone, 33 mm, gamma sterilized	FPE-204-030	Interlab
MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)	M6494	ThermoFisher
Myelin Oligodendrocyte Glycoprotein Peptide (35-55), mouse, rat	RPI0245	Genescript
Penicillin-Streptomycin (10,000 U/mL)	I5140-122	ThermoFisher
Pertussis toxin from <i>B. pertussis</i>	I81	List Biochemicals
Recombinant Mouse Rantes (CCL5)	CHM342	Prospec
Recombinant Murine IFN γ	315-05	PeproTech
Red Blood Cell Lysing Buffer Hybri-Max™	R7757	Sigma Aldrich
Trypan Blue Solution, 0.4%	I5250-061	Gibco
TrypLE™ Express Enzyme (1X), no phenol red	I2604-021	Gibco

2.2. Animals and Tissue Harvest

2.2.1. Animals

8-week-old C57BL/6J mice were purchased from the Biomedical Research Unit (BRU) at the Malaghan Institute for Medical Research (Wellington, New Zealand). Animals were housed in a specific-pathogen-free facility on a 12-hour light/12-hour dark cycle, and *ad libitum* access to food and water for the duration of all experiments.

2.2.2. Ethics declaration

All experimental measures were approved by the Victoria University of Wellington Animal Ethics Committee under Ethics Application #25295 (20-09-2017). Experiments were performed in accordance with all relevant guidelines and procedures at the School of Biological Sciences Animal Facility (Victoria University of Wellington).

2.2.3. EAE induction in animals

Myelin Oligodendrocyte Glycoprotein (MOG₃₅₋₅₅ peptide) (Genescript) emulsions were prepared at a concentration of 50 µg/200 µL per mouse which included 500 µg heat-inactivated *Mycobacterium tuberculosis* in complete Freund's adjuvant. Pertussis toxin (List Biochemicals) was administered at a concentration of 200 ng per mouse, prepared in 200 µL Pertussis toxin buffer (2.14.9). 100 µL MOG emulsion was injected subcutaneously into each leg using a 23G needle, followed by 200 µL pertussis toxin injected intraperitoneally. Mice were weighed and scored daily for motor deficits and ascending paralysis using a numerical scale as follows: 0- no disease, 0.5- loss of tail tonicity, 1.0- half-tail paralysis, 1.5- three-quarter tail paralysis, 2.0- full tail paralysis, 2.5- full tail paralysis and hind limb weakness, 3.0 one hind limb paralysed or severe weakness in both hind limbs, 3.5- on hind limb paralysed and severe weakness in the other hind limb, 4.0- full hind limb paralysis and front limb weakness, 5.0- moribund/death. Mice losing >20% of their starting weight or surpassing a score of 4.5 with no active recovery were euthanised.

2.2.4. Tissue Isolation

Mice were euthanised by lethal carbon dioxide asphyxiation in a euthanasia chamber. Death was verified by establishing the loss of reflexes and the presence of fixed and dilated pupils. Following death, mice were thoroughly swabbed with 70% ethyl alcohol in ddH₂O. Splenic tissue was harvested and placed in 1 mL Wash Buffer (DMEM supplemented with 3% HEPES, 1% Penicillin-Streptomycin) and placed on ice until further use.

2.2.5. Splenocyte isolation

Spleens were mechanically digested into a single cell suspension via forced passage through a 70 μ m cell strainer using Wash Buffer, and the thumb-end of a plunger fitting a 1 mL syringe (BD). The cell suspension was centrifuged at 700 xg for three minutes. The cell pellet was re-suspended in 2 mL Red Blood Cell Lysis Buffer (Sigma Aldrich). Cells were incubated at room temperature for 2 minutes and lysis was terminated by the addition of 8 mL Wash Buffer, followed by centrifugation again at 700 xg for three minutes. Isolated splenocytes were then re-suspended in 10 mL CTCM (DMEM supplemented with 10% Fetal Bovine Serum (FBS), 1% Penicillin-Streptomycin, 1% HEPES, 1% Non-essential amino acids, 1% L-glutamine and 0.01% Beta-mercaptoethanol).

2.2.6. Splenocyte viability assessment

To assess the percentage of live splenocytes, cells were resuspended in CTCM as described in 2.2.5. Splenocytes were diluted in a 1:10 ratio in trypan blue dye (Sigma). 10 μ L of this cell-dye solution was added into a Neubauer haemocytometer with coverslip (Hawksley), and cells were counted using a compound microscope. Using this assessment, splenocytes were diluted in CTCM to a concentration of 1×10^7 cells/mL.

2.3. General Epithelial Cell Culture

2.3.1. ECPC-4 cell culture

The transformed C57BL/6 Mus *musculus* Epithelial Choroid Plexus Cell (ECPC-4) cell line (RRID: CVCL_4836) was purchased through Riken Cell bank from Fukuoka University, Fukuoka, Japan. ECPC-4 cells are immortalized via transfection with IgH enhancer-SV40 T fusion gene. All cell culture was conducted in a sterile, class II laminar flow hood. Cells were cultured as recommended by the manufacturer, at 1×10^6 cells/mL in T75 flasks (Corning) in 20 mL Complete Growth Media (DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin). Cultured cells were incubated at 37°C, 5%CO₂ RH.

2.3.2. Thawing Cells

Vials were removed from liquid nitrogen storage and placed into a 37°C water bath to thaw. Under a sterile laminar flow hood, cells were transferred to a falcon tube containing 9 mL Complete Growth Media. The vial was washed thoroughly with another 1 mL growth medium to recover the maximum number of cells. To remove the freezing medium, the tube was centrifuged at 400 xg for five minutes, and the pellet was re suspended in 10 mL growth media. The cells were then transferred to a T25 flask at a concentration of 2×10^6 cells/flask (in 10 mL complete growth media) and incubated for 24 hours at 37°/5% CO₂ RH.

2.3.3. Passaging Cells

At 80% confluency, as assessed visually, cells were subcultured with 0.25% Trypsin (Sigma) at a passage ratio of 1:4 into fresh T75 flasks. Cells were used up to and including Passage 20. Growth media was removed from the flask via vacuum aspiration, and cells were gently washed with 10 mL 1X dPBS (Gibco). The dPBS was aspirated, followed by the addition of 3 mL Tryple® (ThermoFisher) to the flask. Cells were incubated at 37°/5% CO₂ RH for 1 minute. Upon removal from the incubator, the flask was gently tapped to encourage cells to detach. 7 mL growth medium was then added to the flask using a serological pipette with a blasting technique to detach any remaining cells from the surface of the plastic. Cells were then centrifuged at 400 xg for 3 minutes and re-suspended in 10 mL growth media. Trypan-blue exclusion dye in a 1:10 ratio was used to assess viability and cell concentration.

2.3.4. Freezing Cells

Cells were centrifuged at 400 $\times g$ for 3 minutes, re-suspended in 10 mL freezing medium (10% DMSO in 90% FBS) and adjusted to a concentration of 1×10^6 cells/mL. The cell solution was transferred to cryovials in 2 mL aliquots. These vials were placed into an IPA-lagged freezing container and stored at -80°C for 24 hours. The vials were then transferred to liquid nitrogen for long term storage.

2.4. Establishing a Transwell Monolayer Culture

These methods discuss the optimised conditions that the Transwell experiment was run under. A major part of this thesis research was establishing the model, and in doing so, finding the best conditions to mimic *in vivo* migration. An appendix methods section can be found after this chapter, which includes methods used in setting up this experiment, but were not successful and/or not used in the final model. This will provide a concise methods section here, and extra information to support the results and discussion chapters.

2.4.1. Preparation of Transwells for Cell Culture

2.4.1.1. Collagen coating

Inverted Transwells were incubated with 75 μL 1 mg/mL Rat Tail Collagen Type I (Sigma Aldrich) under a tissue culture hood for 2 hours. Excess collagen was gently pipetted from surface, the Transwells were rinsed twice with 1X dPBS (Thermo Fisher) and left to dry under the hood. To avoid contamination, Transwells were sterilised under UV light for 30 minutes prior to seeding.

2.4.1.2. Seeding ECPC-4 Cells onto Transwells

Figure 2.1: Simplified schematic showing how we seed ECPC-3 cells onto a Transwell.

ECPC-4 cells were passaged and re-suspended in a 50 mL conical centrifuge tube (Falcon) as described in 2.3. The concentration of cells was adjusted to 6.6×10^5 cells/mL in warm CTCM. Cells were seeded at a volume of 75 μL to the inverted mesh of the Transwell (absolute cell number = 5×10^4). To promote adherence, cells were incubated in this inverted orientation for 12 h. At 12 h, the Transwells were gently oriented back into an upright position using sterilized tweezers. To the lower compartment of the

Transwell culture, 600 μ L complete growth media was added; enough to generously submerge the ECPC-4 monolayer.

2.4.2. Splenocyte Stimulation

2.4.2.1. Concanavalin (ConA)

Splenocytes were seeded at 1×10^7 cells/mL in 100 μ L aliquots into a 96 well plate. For stimulation with ConA, cells were treated at 1 μ g/mL in a total of 200 μ L, containing CTCM, splenocytes and ConA. For Transwell experiments, splenocytes were stimulated for a total of 24 h at 37°C/5%CO₂ RH. Media containing ConA was removed following centrifugation of the plate at 400 xg for 4 minutes. Splenocytes were re-suspended in 200 μ L fresh CTCM without ConA for 24h before addition to the Transwell culture.

2.4.2.2. Interferon- γ (IFN γ)

Splenocytes were seeded at 1×10^7 cells/mL in 100 μ L aliquots into a 96 well plate. For stimulation with IFN γ , cells were treated at 20 U/mL in a total of 200 μ L, containing CTCM, splenocytes and IFN γ . Cells were stimulated with IFN γ for a total of 48 h at 37°C/5%CO₂ RH. Supernatant containing IFN γ was not aspirated before adding splenocytes into the Transwells.

2.4.2.3. Anti-interferon- γ (α IFN γ)

Anti-IFN γ was added to splenocyte cultures following the removal of ConA, as described in 2.4.2.1. For designated wells, anti-IFN γ antibody was added at a concentration of 10 μ g/mL in a total volume of 200 μ L, containing CTCM, splenocytes and anti-IFN γ . Cells were then directly transferred into the Transwell culture. In the Transwells, splenocytes were treated with anti-IFN γ for a total of 24 h.

2.4.3. Treatment of the Transwell Culture with HSI5-35

2.4.3.1. Splenocyte Treatment

Splenocytes were seeded at 1×10^7 cells/mL in 100 μ L aliquots into a 96 well plate. Designated wells for HSI6-35 (Ferrier Research Institute, NZ) may also include ConA, IFN γ and/or anti-IFN γ . HSI6-35 was incubated in the splenocyte culture at 60 μ g/mL in a volume of 200 μ L for 48 h prior to addition to the Transwell. Media containing HSI6-35 was aspirated following centrifugation of the plate at 400 xg for 4 minutes. In wells pre-treated with ConA, splenocytes were re-suspended in fresh CTCM + HSI6-35, omitting ConA before addition to the Transwell culture. Wells treated with HSI6-35 minus ConA did

not receive replacement media. In total, all splenocyte wells were cultured with HSI6-35 for a total of 72 h.

2.4.3.2. Epithelial Monolayer Treatment

ECPC-4 cells that had successfully formed a monolayer culture on the Transwells (occurs approximately ~24 h after cells seeded) were treated with HSI6-35 at 60 µg/mL for 24 h prior to the addition of splenocytes to the upper well. Upon starting the Transwell culture, and the addition of the CCL5 cytokine, media containing fresh HSI6-35 was added. In total, the epithelial cells were treated with HSI6-35 for 48 h.

2.5. Transwell Migration Assay

Figure 2.2: Simplified schematic showing how the transwell migration assay is run.

2.5.1. Cytokine Stimulation: IFN γ

To simulate an inflammatory environment like that of EAE and MS, ECPC-4 cells were stimulated with IFN γ . IFN γ promotes the upregulation of adhesion molecules on the surface of epithelial cells, mimicking the pathogenic conditions that occur in the brain of MS patients. 36 hours after seeding ECPC-4 cells on Transwells as described in 2.4.1.2, IFN γ was supplied in warm CTCM to the lower compartment of IFN γ stimulated wells at a concentration of 20 U/mL for 24 h before the start of the migration assay.

2.5.2. Splenocyte Migration

This assay was used to assess the migration of splenocytes across an *in vitro* model of the choroid plexus. Splenocytes harvested and treated as described in 2.2.5 and 2.4.2, or untreated splenocytes were re-suspended vigorously in their respective wells of the 96-well round bottom plate (In Vitro). Cells were then transferred to the upper compartments of prepared Transwell monolayer cultures, as detailed in 3.1. To ensure all cells were collected, 10 μ L warm CTCM was added to each well, the plate was vortexed for 15 seconds, and remaining media was transferred into the Transwells. The total duration of the migration phase of this experiment was 24 h.

2.5.3. Chemokine Stimulation: CCL5

The migration of splenocytes in the assay was mediated by CCL5 (RANTES) (Prospec). To the lower compartment of cytokine-stimulated wells, CCL5 was added at a concentration of 100 ng/mL in 600 μ L warm CTCM. To unstimulated wells, only fresh CTCM was added. The culture was incubated for 24 hours at 37°C/5%CO₂ RH. To assess the capacity of migration, the fractions of the upper and lower wells were separated and prepared for Flow Cytometry.

2.6. Flow Cytometry

To investigate the extent of migration across the monolayer, flow cytometry was performed after staining with a full panel of CD4-BV421, CD45-BV510, B220-AF488, CD8-PerCP-Cy5.5, CD11b-PE-Cy7, CD62L-APC, CD44-PE and CD3-APC-Cy7.

2.6.1. Antibody List

Table 2: Materials for Flow Cytometry Staining

Product	Label	Clone	Isotype	Catalog Number	Company
Anti-mouse/human CD45R/B220	AF488	RA3-6B2	Rat IgG2a, κ	103228	BioLegend
Anti-mouse CD62L Antibody	APC	MEL-14	Rat IgG2a, κ	104411	BioLegend
Anti-mouse CD11c Antibody	APC	N418	Armenian Hamster IgG	117310	BioLegend
Anti-mouse CD3 Antibody	APC/Cy7	17A2	Rat IgG2b, κ	100221	BioLegend
Anti-mouse CD4	BV421	GK1.5	Rat IgG2b, κ	100438	BioLegend
Anti-mouse CD45	BV510	30-F11	Rat IgG2b, κ	103138	BioLegend
Anti-mouse/human CD11b	PE	M1/70	Rat IgG2b, κ	101208	BioLegend
Anti-mouse CD44	PE	IM7	Rat IgG2b, κ	103007	BioLegend
Anti-mouse CD8a	PerCP/Cy5.5	53-6.71	Rat IgG2a, κ	100734	BioLegend

2.6.2. Preparation of Solvents

2.6.2.1. FACS buffer

2% FBS and 0.1% IM sodium azide in 1X dPBS.

2.6.2.2. Fc Block

Purified Rat Anti-Mouse CD16/CD32 (BD) is diluted at 1:300 in FACS buffer.

2.6.2.3. Compensation Beads

One drop UltraComp eBeads™ Compensation Beads (ThermoFisher) in 100 μ L FACS buffer with 1 μ L antibody working stock.

2.6.2.4. Fluorescence Minus One (FMO) Control

50 μ L full panel stain in FACS buffer, omitting desired FMO control antibody. For these experiments, FMO controls included B220, CD3, CD11b, CD44 and CD62L.

2.6.3. Full Panel Stain

Table 3: Dilution of Antibodies in the full panel stain

Product	Final Dilution in FACS Buffer
Anti-mouse CD4	1:200
Anti-mouse CD45	1:400
Anti-mouse/human CD45R/B220	1:400
Anti-mouse CD8a	1:400
Anti-mouse/human CD11b	1:800
Anti-mouse CD62L Antibody	1:400
Anti-mouse CD3 Antibody	1:200
Anti-mouse CD44	1:400

2.6.4. Flow Cytometry Protocol

The full panel stain, Fc block and FMO controls were prepared and stored on ice, protected from light before the preparation of samples for Flow Cytometry. The upper and lower fractions of the Transwell culture were collected. To ensure all cells were collected, samples were vigorously re-suspended in their respective wells using a 1000 μ L pipette (Falcon) set to 2/3 of the total volume of each sample. The underside of each Transwell was gently blasted with media to encourage partially migrated cells to detach. Medium containing cells from the upper well was plated directly into a round bottom 96-well plate (In Vitro). Media from the lower well were placed into 1.2 mL microcentrifuge tubes and centrifuged at 700 $\times g$ for 5 minutes or until a visible pellet formed. 350 μ L supernatant was discarded, and the tubes were vortexed to resuspend cells in solution.

Cells were plated in a round bottom 96-well plate (In Vitro) in a checkerboard pattern. FACS buffer was added to each well to a final volume of 250 μ L. The plate was centrifuged at 400 $\times g$ for 4 minutes (4°C). Supernatant was removed and plated in a flat-bottom 96-well plate for utilization in ELISA assays. The

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plate was vortexed for 15 seconds to resuspend cells in the remnant media. 50 μ L Fc block was added to each well and the plate was incubated on ice, in the dark for 15 minutes. 150 μ L FACS buffer was then added to each well, the plate was centrifuged again at 400 $\times g$ for 4 minutes (4°C). Supernatant was discarded via gentle flicking. The plate was vortexed for 15 seconds to resuspend cells in the residual media. Each sample was re-suspended in 50 μ L full panel stain and placed on ice for 20 minutes, protected from light. Samples were centrifuged at 400 $\times g$ for 4 minutes (4°C) and re-suspended in 200 μ L FACS buffer. Samples were processed in the 96-well plate on a Canto II Flow Cytometer and BD FACS Diva Software. Results were analysed using FlowJo software (BD).

2.7. IFN γ ELISA

2.7.1. Standards

rmIFN γ (BD) standards were performed in duplicate rows of an ELISA plate, with the top standard set to 4 ng/mL in blocking buffer. 2-fold serial dilutions were performed to generate the standard curve, with two blank wells included for negative control.

2.7.2. ELISA Protocol

ELISA's were performed in accordance with the manufacturer's protocols, which will be summarised here (BD Biosciences). The capture antibody, rat anti-mIFN γ (BD) was diluted in capture buffer (2.14.7) to a final dilution of 1 μ g/mL and plated into a 96-well ELISA plate at 50 μ L/well. The coated plates were incubated overnight at 4°C, protected from light. The following day, plates were incubated with 100 μ L blocking solution (2.14.7) for two hours at room temperature. After this incubation, plates were washed 4 times in a wash buffer comprised of PBS with 0.05% Tween20 (Sigma) and excess moisture from the wells was stamped onto a paper towel with force until wells appeared dry. Standards and samples were plated in 50 μ L aliquots and left for 120 minutes at room temperature. After this incubation, the plate was washed 4 times in the washing buffer again. The biotinylated detection antibody (biotin rat anti-mIFN γ , BD) was diluted to 125 ng/mL in blocking buffer and added at a volume of 50 μ L to each well. The plate was again incubated at room temperature for one hour, then rinsed 6 times in the washing buffer and stamped to dry. Streptavidin horse-radish peroxidase (BD) was diluted in blocking buffer to 1:2000 and added at 50 μ L to each well. The plate was incubated in the dark at room temperature for one hour, then washed eight times in the washing buffer. Room temperature tetramethyl benzidine (TMB) substrate A and B were thoroughly mixed in equal volumes and 100 μ L of this mixture was dispensed into each well. The plate was observed for colour change, and once a sufficient colour developed, stop solution (2.14.7) was added at a volume of 100 μ L. The plate was read at 450 nm on the EnSpire 2300 multilabel plate reader (PerkinElmer). A linear standard curve was generated using the values generated from the standard rows, and the concentration of IFN γ in solution was quantified using the formula of the curve.

2.8. MTT Assay

2.8.1. Cell Specimens

2.8.1.1. Splenocytes

Splenocytes were harvested from healthy mice as described in section 2.2.5 and diluted to a single-cell suspension of 1×10^6 cells/mL in warm CTCM. Cells were seeded into a 96-well round-bottom plate in 100 μ L aliquots, with an absolute cell number of 1×10^5 cells/well.

2.8.1.2. ECPC-4 Cells

ECPC-4 cells were cultured and prepared as described in 2.3.1 into a single cell suspension of 1×10^5 cells/mL in warm CTCM. Cells were seeded into a 96-well round bottom plate in 100 μ L aliquots, with an absolute cell number of 1×10^4 per well.

2.8.1.3. HSI6-35 Treatment

The primary variable for this experiment was the concentration of HSI6-35. For the 48 hours prior to the addition of MTT solution splenocytes or ECPC-4 cells in a 96-well round bottom plate were treated with HSI6-35 at a concentration of 6 μ g/mL, 60 μ g/mL or 600 μ g/mL in warm CTCM at 37°C/5%CO₂ RH. 0 μ g/mL was used as a control for each condition. Each treatment was performed in triplicate.

2.8.1.4. Concanavalin A (ConA) Treatment

For stimulation with ConA, cells were treated at 1 μ g/mL in a total of 200 μ L, containing CTCM, +/- HSI6-35 and ConA. Splenocytes were stimulated for a total of 24 h at 37°C/5%CO₂ RH. Media containing ConA was removed following centrifugation of the plate at 400 xg for 4 minutes. Splenocytes were re-suspended in 200 μ L fresh CTCM without ConA and incubated for 24 hours prior to the addition of MTT solution.

2.8.1.5. Interferon- γ (IFN γ) Treatment

For stimulation with IFN γ , cells were treated at 20 U/mL in a total of 200 μ L, containing CTCM, +/- HSI6-35 and IFN γ . Cells were stimulated with IFN γ for a total of 48 hours at 37°C/5%CO₂ RH. Supernatant containing IFN γ was not replaced before the addition of MTT solution to the wells.

2.8.1.6. MTT Assay Protocol

The 96-well plate containing treated cells was centrifuged at 400 xg for 4 minutes to settle cells at the bottom of the wells. 170 μ L of supernatant was removed and set aside for further analysis. Samples were then re-suspended in 50 μ L warm CTCM. To each well, 20 μ L sterile-filtered MTT solution (2.14.8) was

added. The samples were incubated for 120 minutes at 37°C/5%CO₂ RH, protected from light. 50 µL MTT solubilizer (2.14.8) was added to each well. The plate was then incubated again at 37°C/5%CO₂ RH, protected from light for a further 14 hours. The plate was read at 470 nm on a 2300 EnSpire™ Multilabel Plate Reader (PerkinElmer).

2.9. Evans Blue Permeability Assay

2.9.1. Solvents

2.9.1.1. Evans Blue Dye

Evan's blue dye (Sigma-Aldrich) was diluted to a concentration of 0.67 mg/mL in warm CTCM. The dye was sterile filtered through a 0.22 µm syringe filter unit (Millex-GP).

2.9.2. Permeability Assay Protocol

2.9.2.1. Establishing a monolayer of ECPC-4 cells

ECPC-4 cells were seeded onto 8 µm pore-size Transwells and cultured as described in methods section 2.4.1.2. The presence of an intact monolayer was verified using simple light microscopy.

2.9.2.2. Treating the monolayer with HSI6-35

120 minutes prior to starting the permeability assay, HSI6-35 was added at concentrations of 6 µg/mL, 60 µg/mL, or 600 µg/mL in warm CTCM to the lower compartment of each Transwell culture. A 0 µg/mL culture was used as a control, and all treatment conditions were performed in triplicate.

2.9.2.3. Evans Blue Permeability Assessment

Sterile Evan's Blue dye at 0.67 mg/mL was added in 200 µL aliquots to the top compartment of each Transwell culture, taking care to dispense the dye slowly from the pipette tip. The cultures were incubated at 37°C/5%CO₂ RH for 90 minutes. Metal tweezers were used to remove the Transwells from each well of the 24-well plate and set aside for further analysis. The plate was swirled was gently swirled for 30 seconds to distribute the dye evenly, and 200 µL aliquots were removed from each sample. In a 96-well plate, these samples were read on a 2300 EnSpire™ Multilabel Plate Reader (PerkinElmer) at 470 nm to determine the extent of the dye that had crossed the monolayer.

Figure 2.3: Simplified schematic showing how the EBD permeability assay is run.

2.10.LEGENDPlex™ Mouse Inflammation Panel

This assay was conducted as according to the manufacturer's protocol which will be summarized below.

2.10.1. Preparation of Tissue Culture Supernatant

Live splenocyte cultures in a 96-well plate were centrifuged at 400 xg for 4 minutes. 170 µL supernatant from each well was removed and stored at -20°C until required.

2.10.2. Standard preparation

The Mouse Inflammation Panel Standard Cocktail is supplied at 10,000 pg/mL when reconstituted in 250 µL assay buffer (supplied in kit). For this assay, it was used undiluted as the top standard (C7). Serial 1:4 dilutions of the cocktail were performed 6 times in succession to obtain a standard curve. One sample of assay buffer alone was used as a negative control (C0).

2.10.3. Assay Procedure

All reagents were warmed to room temperature before starting the assay. In a 96-well V-bottom plate, 25 µL assay buffer was added to all wells. To the standard wells, 25 µL standard was added, and in the test wells, 25 µL cell-culture supernatant was added. Each standard and sample were performed in duplicate. The Mouse Inflammation Panel Premixed Beads were added in 8 µL aliquots to each well, making the total volume 58 µL in each well. The plate was sealed and covered in aluminium foil to protect from light. Samples were then shaken at 800 rpm on a plate shaker for 120 minutes at room temperature. Following this, the plate was centrifuged at 250 xg for 5 minutes to sediment the beads. The supernatant was discarded from the plate by forceful inversion. Each well was rinsed with 200 µL wash buffer, and the plate was again shaken at 800 rpm on a plate shaker. The centrifuge step was repeated, and supernatant discarded as previously described. 8 µL detection antibodies were added to each well, and the plate was sealed and covered in aluminium foil to protect from light. The samples were shaken at 800 rpm on a plate shaker for 60 minutes at room temperature. 8 µL SA-PE was added to each well and the plate was shaken for a further 30 minutes at 800 rpm. The plate was centrifuged again, and supernatant discarded as described above. 150 µL Wash Buffer was added to each well and samples were resuspended by gentle pipetting. The samples were read using a Canto II flow cytometer on the same day, and results analysed using the LEGENDPlex software (v8.0) provided in the kit.

2.1.1. Statistics

Graphs and statistics for this thesis were generated using GraphPad Prism (v8.0). Some of the figures in this thesis were generated by combining experimental replicates from up to three experiments to account for inter-experimental variability and provide more statistical power. In experiments where two or more variables are being compared, we conducted a one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-hoc test. For grouped analyses, (wherein two groups are compared) we conducted a two-way analysis of variance with Sisak's post-hoc multiple comparisons test. For all statistical analyses, significance was determined at $p < 0.05$ (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

Supplementary Methods

2.12. Establishing a Transwell Monolayer Culture

These methods discuss the trialled conditions that were used to establish the transwell assay. These were tested and ultimately not used in the final methods, however formed an important part of this thesis, and will be discussed in the results section of chapter 3.

2.12.1. Preparation of Transwells for Cell Culture

2.12.1.1. Matrigel as a basement membrane

Matrigel was thawed on ice overnight. All materials that Matrigel would contact (24-well plate, four 8 µm Transwell® inserts, pipette tips), were placed on ice for thirty minutes. Matrigel was diluted to a concentration of 3 mg/mL in ddH₂O. 30 µL diluted Matrigel was added to the inverted Transwell inserts, and the plate was incubated for 30 minutes at 37°C/5% CO₂ RH to allow the Matrigel to set. To ensure all excess Matrigel was removed, the inserts were washed twice with ice cold 1X dPBS (Thermo Fisher). To avoid contamination, the Transwells were sterilised under a UV light, on ice for 30 minutes prior to seeding.

2.12.1.2. Seeding ECPC-4 Cells onto Transwells (2 hours)

ECPC-4 cells were passaged and re-suspended in a 50 mL conical centrifuge tube (Falcon) as described in the general methods. The concentration of cells was adjusted to 6.6×10^5 cells/mL in warm CTCM. Cells were seeded at a volume of 75 µL to the inverted mesh of the Transwell (absolute cell number = 5×10^4). To promote adherence, cells were incubated in this inverted orientation for 2 h. At 2 h, the Transwells were gently oriented back into an upright position using sterilized tweezers. To the lower compartment of the Transwell culture, 600 µL complete growth media was added; enough to generously submerge the ECPC-4 monolayer.

2.12.2. Transwell Migration Assay

2.12.2.1. Chemokine Stimulation: Embedded CCL5

The migration of splenocytes in the assay was mediated by CCL5 (RANTES). Managing the chemokine gradient in experiment I, two variables were used; CCL5 embedded in low melting agarose or free-floating CCL5. This subsection describes how CCL5 in agarose was prepared, although ultimately this method was not used in the final Transwell model.

2.12.2.2. Low-melting agarose

Under a laminar flow hood, of low-melting agarose was dissolved in DMEM at a concentration of 15 mg/mL (1.5%). The solution was heated in a microwave oven until bubbles appeared. The flask was swirled gently to ensure all agarose particles had been dissolved and left to cool.

2.12.2.3. Dilution of soluble CCL5 chemokine

CCL5 was provided as a stock solution of 1×10^5 ng/mL and diluted in the cooled agarose to a final concentration of 100 ng/mL. The solution was resuspended gently to ensure the chemokine was evenly distributed before the agarose set.

2.12.2.4. Seeding agarose into 24 well plates

200 μ L agarose/CCL5 solution was added into designated wells of a 24-well plate. 200 μ L DMEM was added to each well containing the agarose, creating a 'slow release' of CCL5. The culture was incubated for 24 hours at 37°C/5%CO₂ RH. To assess the capacity of migration, the fractions of the upper and lower wells were separated and prepared for Flow Cytometry.

2.13.MTT Assay: CCL5 toxicity

2.13.1. Cell Specimens

2.13.1.1. Splenocytes

Splenocytes were harvested from three healthy mice as described in the 2.2.5, and diluted to a single-cell suspension of 1×10^6 cells/mL in warm CTCM. Cells were seeded into a 96-well round-bottom plate in 100 μ L aliquots, with an absolute cell number of 1×10^5 cells/well.

2.13.2. Treatment Conditions

2.13.2.1. CCL5 Treatment

The primary variable for this experiment was the concentration of CCL5. Designated wells of a 96-well round-bottom plate were seeded with 100 μ L splenocyte suspension from each of the spleens as described in 2.2.6 and stimulated with 100 μ L CCL5 at 100 ng/mL, 200 ng/mL, 400 ng/mL or 600 ng/mL. A control condition of 0 ng/mL in 200 μ L warm CTCM was used for each spleen. The plate was then incubated for 24 hours at 37°C/5%CO₂ RH.

2.13.2.2. Starvation

Our control included FCS starvation, wherein FCS was omitted from the CTCM.

2.13.3. MTT Assay Protocol

The 96-well plate containing treated cells was centrifuged at 400 $\times g$ for 4 minutes to settle cells at the bottom of the wells. 170 μ L of supernatant was removed and set aside for further analysis. Samples were then re-suspended in 50 μ L warm CTCM. To each well, 20 μ L sterile-filtered MTT solution (2.14.8) was added. The samples were incubated for 120 minutes at 37°C/5%CO₂ RH, protected from light. 50 μ L MTT solubilizer (2.14.8) was added to each well. The plate was then incubated again at 37°C/5%CO₂ RH, protected from light for a further 14 hours. The plate was read at 470 nm on a 2300 EnSpire™ Multilabel Plate Reader (PerkinElmer).

2.14. Reagent Formulas

All reagents used in tissue culture were sterile filtered through a 0.22 μm syringe filter unit (*Millex-GP Syringe Filter Unit, 0.22 μm , polyethersulfone, 33 mm, gamma sterilized*). All glassware used for tissue culture purpose was autoclaved prior to use.

2.14.1. Complete Growth Media (CGM)

Product	Composition	Company	Catalog Number
<i>Dulbecco's Modified Eagle Medium</i>	89%	Life Technologies	11995-073
<i>Fetal Bovine Serum (FBS/FCS)</i>	10%		
<i>Penicillin-Streptomycin</i>	1%	Life Technologies	15140-122

2.14.2. Complete Tissue Culture Media (CTCM)

Product	Composition	Company	Catalog Number
<i>Dulbecco's Modified Eagle Medium</i>	85.9%	Life Technologies	11995-073
<i>Fetal Bovine Serum (FBS/FCS)</i>	10%		
<i>Penicillin-Streptomycin</i>	1%	Life Technologies	15140-122
<i>HEPES</i>	1%	Thermo Fisher	15630106
<i>MEM Non-essential amino acids (NEAA)</i>	1%	In Vitro	11140050
<i>L-glutamine</i>	1%	Life Technologies	25030-081
<i>Beta-mercaptoethanol</i>	0.1%	Life Technologies	21985-023

2.14.3. Wash Buffer

Product	Composition	Company	Catalog Number
<i>Dulbecco's Modified Eagle Medium</i>	96%	Life Technologies	11995-073
<i>Penicillin-Streptomycin</i>	1%	Life Technologies	15140-122
<i>HEPES</i>	3%	Thermo Fisher	15630106

2.14.4. Dulbecco's Phosphate-Buffered Saline

Product	Composition	Company	Catalog Number
<i>dPBS (10X), no calcium, no magnesium</i>	10%	Thermo Fisher	11995-073
<i>ddH₂O</i>	90%	-	-

2.14.5. Fluorescence-Activated Cell Sorting (FACS) Buffer

Product	Composition	Company	Catalog Number
dPBS (10X), no calcium, no magnesium	9.8%	Thermo Fisher	11995-073
ddH ₂ O	88.2%	-	-
Fetal Calf Serum (FCS/FBS)	2%		

2.14.6. 10X Phosphate-Buffered Saline

Product	Composition
NaCl	170 g
Na ₂ HPO ₄ ·12H ₂ O	62.32 g
NaH ₂ PO ₄ ·2H ₂ O	4.04 g
ddH ₂ O	2 litres

2.14.7. ELISA Buffers

Product	Composition
Capture buffer	0.1M Na ₂ HPO ₄ in ddH ₂ O, balanced to pH 9.0.
Blocking buffer	5% FCS in 1X PBS.
Stop solution	0.18M H ₂ SO ₄ in ddH ₂ O.

2.14.8. MTT Buffers

Product	Composition
MTT Solution	MTT at 5 mg/mL in PBS (sterile filtered)
MTT Solubiliser	10% Sodium dodecyl sulfate (SDS) and 45% dimethylformamide (DMF) in ddH ₂ O. pH balance to 4.5 with AcOH

2.14.9. Pertussis Toxin Buffer

Product	Composition
Tris	15mM
Sodium Chloride	0.5M
Triton X-100	0.017% (v/v)
In ddH ₂ O	

Chapter 3: Developing an *in vitro* model of the choroid plexus for the study of leukocyte trafficking

3.1. Introduction

Migration is a fundamental cellular process for tissue development and homeostasis in multicellular organisms. Key biological processes rely on the capacity of cells to migrate in response to chemo-attractants, such as conception, early embryo patterning and wound healing. The immune system relies on recruitment of lymphocytes from the lymph nodes to inflamed tissues to develop a protective immune response to pathogen invasion.¹⁴⁷ Inversely, aberrant cellular migration can greatly contribute to disease pathology; for instance, in the development of tumour microenvironments and metastasis in cancer, or the invasion of autoimmune cells into an immune privileged site, in diseases such as MS.

The Transwell migration model and its associated migration assays are classical techniques that allow us to quantify the movement of various cell types through a microporous membrane. The model was first introduced by Dr. Stephen Boyden in 1961, who analysed the trafficking of leukocytes in response to chemotactic agents.¹⁴⁸ Thus, the model is often referred to as the Boyden-Chamber assay. The applications of this model are broad, and vary greatly between papers, however the general premise is quite simple. Two media-filled wells are separated by a microporous polyester membrane. Cells are added to the upper well, and a chemokine or other stimuli is applied to the lower well. The micropores of the Transwell membrane are selected so that cells must actively rearrange their cytoskeleton to migrate through the membrane.¹⁴⁷ This negates the idea of passive migration, in which cells simply fall through the membrane pores due to gravity, which is not considered true migration. Some migration assays, including the one presented in this thesis include a monolayer of cells across the membrane, which mimic a barrier structure. We incorporated epithelial ChP cells in this model, as cells entering the CSF through the ChP must navigate across the epithelium, a highly selective structure.

Figure 3.1: Schematic showing a Transwell model. The upper compartment of the Transwell cultures was considered the 'Peripheral Blood', and the lower chamber was considered the 'Cerebral Spinal Fluid', where a chemoattractant was added. A collagen coating was applied to the outer edge of the membrane to act as the ChP stromal matrix. Epithelial ChP cells were seeded onto the collagen layer.

3.2. Aims

This chapter explores the processes and protocols that were used to develop a functional model of the ChP *in vitro*. Previous literature has shown that *in vitro* transwell models can be helpful to characterise the cell and molecular mechanisms that mediate pathogenic migration of lymphocytes into the CNS. Certainly, the ChP's anatomical location makes it a difficult structure to study *in vivo*, so there is a definitive need in the field. To faithfully reproduce the ChP's barrier properties and *in vivo* features, we conducted an extensive review of the current literature to select the most appropriate model to begin with. Our initial model was based upon Monnot and Zheng's (2013) paper, which introduced a protocol for a transwell model of the ChP using primary rat-derived ChP cells.¹⁴⁹ Throughout the course of this thesis, we adapted aspects of this model where we saw opportunity to more accurately mimic characteristics and functionality of the ChP.

We investigate the use of chemo-attractants and proinflammatory molecules to create a platform for inflammatory and pathogenic migration, similar to that of MS. This section was also crucial to develop a functional model to test our heparan-sulfate mimetic 'HS16-35', which has a proposed mechanism of action that includes inhibition of migration into the CNS.

It is important to note that many of the experiments in this chapter were completed only once, that these assays served as 'checkpoint experiments' in which we confirmed whether techniques that were adapted from other papers were functional for use in this thesis.

3.2.1. Specific Aims

1. To develop an *in vitro* model of the choroid plexus.
2. To demonstrate that this experimental approach models migration across an epithelial monolayer in both homeostatic and inflammatory conditions.

3.3. Results

3.3.1. CCL5 is a non-toxic chemokine to splenocytes

The CCL5 (RANTES) receptor (CCR5) has been shown to be significantly upregulated on CD4⁺, CD8⁺ and CD19⁺ lymphocytes in active lesions of MS patients.¹⁵⁰ Its cognate ligand, CCL5 is a potent T cell chemoattractant that facilitates the homing process of T_H1 cells to lesion sites through the choroid plexus.³⁹ Animal models of MS have shown that CCL5 can be upregulated by 146-fold in the ChP stroma and capillaries during active disease.¹⁵¹ Previous work by this group has shown that clozapine-treated mice have delayed onset EAE and reduced disease severity versus vehicle treated controls, in part due to reduced infiltration of lymphocytes into the CNS. Clozapine significantly downregulates the expression of CCL5 and CCL2 in the CNS compartment, implicating these chemokines as responsible for the early infiltration of autoreactive lymphocytes in EAE.¹⁵² In our model of the ChP, we selected CCL5 as a chemoattractant, based upon other migration models that had found CCL5 to be the most potent inducer of migration versus a panel of other chemokines in cells isolated from MS patients.¹⁵³

Previous literature using CCL5 as an *in vitro* chemokine has ranged in dose from 20 ng/mL to 600 ng/mL to 600 ng/mL.^{154–157} To gauge the most effective yet cost-effective dosing regimen of CCL5 (Prospec) on leukocytes, we assessed the viability of isolated splenocytes from C57BL/6J mice in response to 24h stimulation with control CTCM media, or media supplemented with increasing concentrations of CCL5 (**Figure 3.2**). Stimulation with 100 ng/mL significantly increased viability from control across three experiments by up to 150%. It appears that both 200 and 400 ng/ml treatments also trended towards an increased viability, however these results are not significant. In contrast to these results, 600 ng/mL of CCL5 began to have a cytotoxic effect on splenocyte integrity, as viability was below control values, and this reduced viability was significant compared to the 100 ng/mL treatment. From these findings, we selected the dose with greatest increase in viability but lowest cost (i.e. 100 ng/mL) for our transwell model.

Figure 3.2: High concentrations of CCL5 decrease splenocyte viability. Shown are viability scores as a percentage of untreated control with mean $\pm$ SEM. Cytokine CCL5 is nontoxic to splenocyte cultures following 24h stimulation at 100, 200 and 400ng/mL in CTCM ($n = 3$ spleens, with 2 duplicates. Results were analysed using an ordinary two-way ANOVA (* = $p < 0.05$, ** = $p < 0.05$, *** = $p < 0.01$).

3.3.2. Matrigel is not a suitable stromal matrix component

The choroid plexus is a dynamic tissue structure, with three core components that are adaptable to an *in vitro* model. (1) the tight barrier of epithelial cells, with their apical side facing the CSF, (2) the underlying stromal matrix which houses a resident population of immune cells, and (3) the layer of fenestrated endothelium with direct contact to the peripheral blood. The 8 μm diameter micropores of the polyester (PET)-membrane mimic the leaky endothelium, which requires extravasating lymphocytes to actively rearrange their actin cytoskeleton to migrate across. Selection of an appropriate stromal matrix for this model requires immune cells to be able to migrate successfully through it. The matrix must also provide essential directional cues to the cultured epithelial monolayer as to which side is apical. Mature ChP epithelium is apically enriched with microvilli and transport machinery, whereas the nuclei are basally located.¹⁵⁸ Tight junction molecules form between cells and adhesion molecules are expressed on the apical face and alongside barrier molecules. Matrigel (Corning), is a common basement membrane component for the use of cell invasion assays. For cells to effectively navigate through an extracellular matrix *in vivo* or Matrigel *in vitro*, they must secrete appropriate matrix modifying proteases, such as MMP's.¹⁵⁹

We assessed the capacity of leukocytes isolated from the spleen of C57BL/6J mice to migrate through an 8 μm micropore transwell (Corning) coated with Matrigel solution (**Figure 3.4**). Matrigel was diluted to 3 mg/mL and aliquoted at 30 μL /well, then set at 37°C for 30 minutes before isolated splenocytes were added to the upper chamber of the transwell. After 24 hours, we isolated the upper and lower fractions of the transwell culture and used flow cytometry to assess the extent of migration across the basement membrane. Results from this assay showed that Matrigel almost completely inhibited the trafficking of CD45⁺ leukocytes across the transwell, versus the transwell alone, regardless of stimulation with CCL5 (**Figure 3.4**). This shows that cells did not express the appropriate matrix-modifying proteases associated with an invasion phenotype.

Figure 3.3: The gating strategy used to analyse flow cytometry data from the migration experiments in Chapter 3. Our gating strategy for flow cytometry in chapter 4. All flow cytometry data was assessed using FlowJo v10. Initially, CD45^{high} lymphocytes were gated out, then gated to exclude doublets. All CD45^{high} singlets were placed on a CD4 vs CD8 plot and gated for CD4⁺ and CD8⁺ expression. Cells that were non-T cells were then gated based on B220 vs CD11b expression, with B220^{high}CD11b⁻ as B cells, B220^{int}CD11b⁺ as dendritic cells and B220⁻CD11b⁺ as myeloid cells.

Figure 3.4: Matrigel is not a suitable basement membrane component for studying leukocyte trafficking. Shown are the means $\pm$ SEM of the percentage of CD45+ splenocytes that migrated through a Transwell culture either without a basement membrane or with Matrigel as a basement membrane component ($n = 2$). Matrigel significantly reduced the number of migrating leukocytes through the Transwell culture. Results were analysed using an ordinary two-way ANOVA, with significance determined at $p < 0.05$ (* = $p < 0.05$, ** = $p < 0.05$, *** = $p < 0.01$, **** = $p < 0.001$).

3.3.3. ECPC-4 cells are capable of forming a functional monolayer

The epithelial monolayer of the ChP is arguably the most important factor in forming an effective blood-CSF barrier and maintaining the immune privilege of the CNS. We investigated the capability of the commercially available epithelial choroid plexus cell line 'ECPC-4' to form a selective, functional monolayer for the study of *in vitro* trafficking across the ChP. ECPC-4 cells have been reported to maintain the characteristics of ChP, with expression of specific molecules such as transthyretin and α_2 -macroglobulin in long-term culture.¹⁶⁰ On Transwell inserts coated with 0.01% rat tail collagen (type II), ECPC-4 cells formed a confluent monolayer less than 24 hours after seeding at 1.66×10^5 cells/cm² (**Figure 3.5a, 3.5b**). Even with extended culture, we saw that ECPC-4 cells were capable of forming contact-inhibited monolayers on transwell inserts.

To measure the functionality of our ECPC-4 monolayer, we adapted an Evans blue dye assay for *in vitro* use, allowing us to test the permeability of a Transwell culture treated with dye in a more rudimentary sense. Evans blue is classically used *in vivo* to assess the barrier function of the BBB in healthy animals versus EAE. The dye easily passes through leaky, inflamed endothelium but not healthy vasculature. *In vitro*, we utilized the model to assess the barrier capacity of the epithelial monolayer. We saw that ECPC-4 cells that had formed a confluent monolayer were effective in preventing dye from diffusing into the lower chamber versus a collagen coated transwell alone, indicating the proper formation of tight barriers in our *in vitro* culture (**Figure 3.5c**). In a checkpoint experiment, we assessed whether isolated splenocytes from C57BL/6J mice were capable of migrating across an ECPC-4 monolayer. At 24 hours after splenocytes were added to the upper transwell chamber, we saw that CD45⁺ leukocytes were capable of migrating through the monolayer, however at a lower frequency than if there was no epithelium (**Figure 3.5d**). This is indicative that the ECPC-4 monolayer is governing selective migration of CD45⁺ leukocytes. In these cultures, it appears that CCL5 stimulation has a greater impact on cell migration than the transwell alone. We hypothesize that when no epithelial monolayer is present, CCL5 freely diffuses into the upper chamber of the transwell culture, and cells do not follow a chemotactic gradient into the lower well. Non-permeable tight junction molecules between ECPC-4 cells in our transwell culture likely inhibit the paracellular movement of CCL5 in large volumes, maintaining a chemotactic CCL5 gradient that CD45⁺ cells are responsive to.

Figure 3.5: ECPC-4 cells are capable of forming an intact but permeable barrier on Transwells after 24h (a). Shown are Epithelial Choroid Plexus Cells (ECPC-4) 12h after seeding onto a Transwell at 1.66×10^5 cells/cm². **(b)** The same Transwell culture at 24h post-seeding, now formed a confluent monolayer culture. The error bar represents 250 μ m. **(c)** Evan's blue dye was added to the top compartment of Transwell cultures $\pm$ epithelial layer. Shown is the extent of dye penetration into the lower well versus a control culture without dye means $\pm$ SEM. When only basement membrane matrix (collagen) was present, there was a significant increase in the concentration of dye that permeated into the lower well versus with an intact epithelial monolayer ($n = 3$). Results were analysed using an unpaired t test with significance set at $p < 0.05$ (* = $p < 0.05$, ** = $p < 0.05$, *** = $p < 0.01$, **** = $p < 0.001$). **(d)** Shown are the means $\pm$ SEM comparing CD45+ migration across a transwell culture lacking an epithelial monolayer or with an established ECPC-4 monolayer ($n = 2$). ECPC-4 cells significantly reduced migration into the lower well versus control. Results were analysed using an ordinary two-way ANOVA, with significance determined at $p < 0.05$ (* = $p < 0.05$, ** = $p < 0.05$, *** = $p < 0.01$, **** = $p < 0.001$).

3.3.4. Inflammatory migration can be induced with Concanavalin A stimulation

One of the specific aims for adapting this model for the study of leukocyte trafficking across the ChP was to show that this model can mimic inflammatory trafficking in the context of MS. It is clear that the neuroinflammation in models such as the EAE model alters the transcriptome of the ChP from healthy controls, heavily upregulating the expression of adhesion molecules, T cell activation markers, such as CD154 and proinflammatory cytokines and chemokines.^{151,161,162}

Because MS and EAE are primarily T-cell mediated diseases, we stimulated isolated splenocytes from healthy C57BL/6J mice with Concanavalin A for 24 hours prior to putting them in a Transwell culture. ConA is a lectin extracted from *Canavalia ensiformis* (jack beans), that irreversibly binds α -D-mannosyl and α -D-glucosyl residues to activate the immune system, induce cytokine production and the recruitment of lymphocytes.¹⁶³ ConA acts as a potent T cell mitogen by inducing cross linking of the TCR, leading to T cell activation and proliferation in an antigen-independent manner.¹⁶⁴

Because ConA has previously been shown to induce clustering of lymphocytes, we could not add our cell cultures stimulated with ConA directly into the Transwells and assume the clumped lymphocytes would fit through the pores.¹⁶⁵ After 24 hours of ConA stimulation, the media containing ConA was aspirated from the plate and splenocytes were resuspended in fresh media to reverse any agglutination of lymphocytes in the Transwell culture. The results from two independent experiments showed that splenocytes stimulated with ConA prior to the Transwell migration assay exhibited a significant increase in the number of cells that migrated across the ECPC-4 monolayer into the lower well versus the media-alone control (**Figure 3.6**). This suggests that ConA is an excellent pro-inflammatory stimulus for mimicking inflammatory trafficking across the ChP.

To further examine the effects of ConA stimulation on the Transwell migration model, we utilized flow cytometry to look at a breakdown of the individual cell types penetrating the ChP monolayer. As expected, we observed a significant increase in the number of CD4⁺ and CD8⁺ T cells migrating across the Transwell culture in response to ConA versus control (**Figure 3.7a**). These cells appeared to become more sensitized to CCL5 when ConA-stimulated, with a trend toward higher cell counts in the +CCL5 condition versus -CCL5. In contrast to T cells, B cells, B220^{int}CD11b⁺ dendritic cells and CD11b⁺ myeloid cells were not greatly affected by ConA stimulation, as there was no observable increase in cell number migration across the monolayer. (**Figure 3.7**) An appendix of experimental replicates can be found at the end of this chapter.

Figure 3.6: ConA induces inflammatory migration across the ECPC-4 monolayer. (a) Shown are the means $\pm$ SEM from 2 experiments ($n = 6$) in which ConA significantly increased the number of migrating CD45+ healthy splenocytes across an epithelial monolayer *in vitro*. Analysis was conducted using an ordinary 2-way ANOVA, with significance at $p < 0.05$ (* = $p < 0.05$, ** = $p < 0.05$, *** = $p < 0.01$, **** = $p < 0.001$).

Figure 3.7: ConA elicits a greater migratory response from T cells: (a) Shown are technical replicates from one experiment ($n = 3$). There was a clear increase in CD4+ T cell migration across the monolayer in response to 24h ConA stimulation. CD4+ T cells stimulated with ConA were more sensitive to and showed a stronger chemo-attractive response to CCL5. Analysis on these samples were performed using an ordinary two-way ANOVA with significance determined at $p < 0.05$. CD8+ T cells in this culture showed a similar pattern to CD4+ T cells, with a ConA-dependent increase in migration and sensitization to CCL5 chemokine stimulation. Data was analysed with an ordinary two-way ANOVA with significance determined at $p < 0.05$. (* = $p < 0.05$, ** = $p < 0.05$, *** = $p < 0.01$, **** = $p < 0.001$). **(b).** By contrast, in the same splenocyte culture, there is no observable change in B cell, Dendritic Cell or general myeloid cell migration across an epithelial monolayer after 24h in co-culture.

3.3.5. Inflammatory migration induced with ConA is partially IFN γ dependent

Because IFN γ plays such an essential role in mediating the autoimmune response in MS and EAE, alongside dysfunctional immune responses at the ChP in other disorders, we suggested that IFN γ produced as a result of ConA stimulation was the key cytokine responsible for enhanced migration across the ECPC-4 monolayer in our model.^{166,167} To confirm this, we proposed that treatment with an anti-IFN γ antibody following ConA stimulation would reduce the overall number of cells migrating across the monolayer if IFN γ was the primary cytokine responsible for enhancing migration in our model. Interestingly, in murine splenocyte cultures that were stimulated with ConA for 24 hours, which were then subsequently treated with an α IFN γ antibody for 24 hours, there was a trend towards significant reduction in the number of CD45⁺ lymphocytes that migrated to the lower chamber (**Figure 3.8a**). However, the reduction in cell count for these α IFN γ treated conditions was still significantly above the media-alone control, indicating that IFN γ plays a large role but is not fully responsible for inflammatory migration across the ECPC-4 monolayer (**Figure 3.8a**).

Analysis of the supernatant from these experiments revealed that ConA induced a strong IFN γ response flooding into the supernatant, which could be completely ablated by α IFN γ treatment (**Figure 3.8b**). Together, these results suggest that IFN γ is in-part responsible for increased migration of splenocytes across the epithelial monolayer, but other factors, including cytokine production and TCR activation through ConA are required for inflammatory migration similar to EAE or MS.

Figure 3.8: ConA induces IFN γ dependent migration, which can be reduced with IFN γ neutralising antibody (a). In splenocyte cultures initially stimulated with ConA for 24h, then an anti-IFN γ antibody for 24h, there was a reduction in the number of migrating CD45+ splenocytes across an epithelial monolayer *in vitro* (ns). Shown are technical replicates from one experiment (n = 3). The data was tested for significance using an ordinary two-way ANOVA with p = 0.0551 (b) An ELISA of supernatant from the samples in a-c reveals that ConA induces a strong IFN γ response in CD45+ cells, and the α IFN γ is effective in neutralising IFN γ in these cultures.

3.4. Discussion

The aim of this chapter was to develop an *in vitro* working model of the choroid plexus. Because of the ChP's anatomical location, it is difficult to study *in vivo* and requires a large volume of animals. Generally, ChP research relies on histology to study, which is low throughput, so our goal was to create an isolated system that provides a window into the ChP and allows for higher throughput research. The research focuses on adapting the model described in Monnot and Zheng's., (2013) paper, in which they utilised primary rat choroid plexus epithelial cells and the Z310 cell line to establish Transwell choroidal epithelial cultures for the use in blood-CSF barrier research. As of now, very few other research labs; Monnot and Zheng., (2013), Strazielle et al., (2016) and Vogel et al., (2015) have published working Transwell models of the ChP for the study of leukocyte trafficking, but there is little literature that supports a mouse model, (Lazarevic et al., (2016)) the primary research animal for MS; hence there is absolute need for further development in this field.

Indeed, the choroid plexus is an intrinsic site of leukocyte trafficking into the CNS, acting as an educational gate for immune surveillance under normal physiological conditions, as opposed to the blood-brain barrier which poses as an absolute barrier at homeostasis.^{37,149,168} For an extended period of time, the choroid plexus has been ignored as an important immunological interface despite its daily role in immune functioning; up to 500,000 cells traffic through the ventricles each day.¹⁶⁸ The key outcome of this chapter was to assess the capacity of this transwell model to mimic homeostatic and inflammatory immune cell trafficking through the choroid plexus, to provide a platform for the study of the new antimigratory drug 'HS16-35'.

The Transwell cultures relied on three key components to produce an accurate model of the blood-CSF barrier; **(1)** the layer of fenestrated endothelial cells that line the cerebral vascular walls, **(2)** the underlying ChP stromal matrix, in which migrating leukocytes may interact with a small collection of resident immune cells and, **(3)** the specialised epithelial layer, which forms a tight barrier and governs which cells enter the ventricles. The epithelium prevents free diffusion of blood molecules leaking from the fenestrated endothelium from entering the CSF, but also controls the transport of metabolites and migrating lymphocytes into and out of the CNS. Little is known of the cellular and molecular mechanisms that govern immune cell trafficking across this structure. However, it is suggested that the ChP plays a greater role in homeostatic migration and immunosurveillance than during neuroinflammation. **Figure 3.9** describes how this was set up in our model.

This research began with confirming that CCL5 was non-toxic to murine leukocytes in culture. Previous research by this group has shown that downregulation of CCL5 in EAE mice reduced the number of infiltrating lymphocytes into the CNS, consequently reducing the onset of the disease and its severity.¹⁵² Because of this work, CCL5 is heavily implicated as one of the primary cytokines mediating cellular migration into the CNS. Although Stanford et al., (2003) describe that CCR5 ligands induce much more potent migratory response from leukocytes *in vivo* versus *in vitro*, in keeping with this group's research goal, and the current literature that describes CCL5 as heavily upregulated in the CSF of MS patients, we chose this as our primary chemokine to mediate migration.^{153,169} This research proved that CCL5 was not cytotoxic to splenocytes isolated from healthy C57/BL6J mice up to concentrations of 400 ng/mL, even increasing viability at lower concentrations.

Figure 3.9: Schematic demonstrating how core components of the BCSFB were adapted to an *in vitro* model. We incorporated the 3 most prominent features of the ChP- the fenestrated endothelium, the stromal matrix, and the tight barrier of epithelial cells.

After selecting an appropriate mediator of chemotaxis for leukocytes, the next step was choosing an appropriate stromal matrix that facilitated an environment for immune cell interactions but also provided key directional cues for the polarised epithelial layer to be cultured upon. Matrigel is a common basement membrane component that has been used to study the invasive and metastatic capacity of cancer cells to disseminate to other sites.¹⁷⁰ Cells that adopt an aggressive metastatic phenotype can easily navigate the dense molecular network that forms the basement membrane (which normally hinders the movement of cells and other molecules). Although, during inflammation it is not uncommon for leukocytes to release

matrix degrading enzymes that make the basement membrane more permeable to migrating cells.¹⁷⁰ Even at its most dilute concentration, leukocytes were unable to permeate the Matrigel coating, and the Matrigel in the media made the samples difficult to safely run through the flow cytometer. Other research groups have incorporated laminin^{171,172} or collagen^{149,173} as their stromal matrix mimic. We opted for collagen, which proved to be no issue for all immune subsets to navigate through, but still provided ECPC-4 cells with a components of the basement membrane to grow on.¹⁷¹

The epithelial monolayer that formed the tight barrier on our Transwell cultures was one of the most important components of the model. The epithelium prevents free diffusion of blood molecules leaking from the fenestrated endothelium from entering the CSF, but also controls the transport of metabolites and migrating lymphocytes into and out of the CNS. Little is known of the cellular and molecular mechanisms that govern immune cell trafficking across this structure. However, it is suggested that the ChP plays a greater role in homeostatic migration and immunosurveillance than during neuroinflammation. ECPC-4 cells were chosen in this model as they have been reported to maintain the characteristics of primary choroid plexus epithelium, with the expression of cell-type specific molecules.¹⁷⁴ In stark contrast, Lazarevic et al., (2016) express concern regarding a comprehensive characterization of this model's expression of TJ markers, CP epithelial markers, functional barrier characteristics and their capacity to form contact-inhibited monolayers versus primary cell culture. In rebuttal to these points, we performed a number of tests to assess whether these are suitable for this research. Reiterating a benefit of this model that was described earlier, an immortalised cell line reduces the need for large numbers of mice through sourcing primary ChP epithelial cells. This is in keeping with principle of the Three 'R's' of animal research. For primary epithelial cultures, 8 to 16 mice are required per preparation, and by troubleshooting this model with over 25 experiments, it becomes hard to ethically justify the number of mice that would be required.¹⁷²

The capacity of epithelial cells to form a tight barrier with appropriate junctional complexes is typically assessed via trans-epithelial electrical resistance measurements (TEER).^{175,176} However, this method relies on expensive equipment that our lab did not have access to. We adapted the Evans blue dye assay protocol, which is classically used to study *in vivo* BBB permeability to measure the permeability and tight barrier function of our model.¹⁷⁷ Not only did this assay provide key information that our ECPC-4 cells could form an effective barrier and prevent dye penetration into the lower well, we were also able to

microscopically confirm that ECPC-4 cells seeded onto Transwells did not continue to divide past confluency, indicating the formation of contact inhibited-monolayers.

Once we had confirmed the functionality of our monolayer culture, we next demonstrated that the model was capable of mimicking homeostatic and inflammatory migration of leukocytes across the ChP. To induce an inflammatory response in splenocytes, we stimulated with ConA for 24 hours before putting them into the Transwell. ConA is a T cell activation stimulant, with preferential differentiation capacity toward T_H1 and T_H17 $CD4^+$ T cells, so we expected that proliferating T cells will flood the media with proinflammatory cytokines; primarily IFN γ , TNF α and IL-6.¹⁷⁸ *In vivo*, these responses are often associated with high macrophage and dendritic numbers, which become activated and respond to kill pathogens. Chapter four will investigate the cytokine responses of these cells in our model. Interestingly, between migration experiments, there was significant variability between the number of macrophages and dendritic cells migrating through the monolayer, and if these cells increased in migration in relation to ConA stimulation. Because ConA activates T cells in an antigen independent manner, dendritic cells and macrophages are not activated first and so their activation status directly corresponds to the costimulatory molecules released by T cells in culture. There may be variation that we have not yet investigated in these responses.

Lider et al., (1990) reported that ConA stimulation induced T cell heparanase expression, which is a key factor of the *in vivo* system that we wanted to preserve for testing our heparan sulfate mimetic. Because of the substantial research that supports T cell heparanase expression as a mechanism for lymphocyte extravasation into the CNS, we suggest that our ConA-induced increase in migration is at least partially dependent on T cell heparanase expression. This is further explored in later chapters.

ConA-stimulation appeared to sensitize lymphocytes, particularly effector and memory $CD4^+$ and $CD8^+$ T cells to CCL5 treatment, increasing the number of cells migrating into the lower chamber versus the non-CCL5 counterpart. Studies have shown that in response to ConA stimulation and TCR activation, naïve T cells differentiate into their effector/memory counterparts. As part of sweeping phenotypic changes, they down-regulate receptors for chemokines that are constitutively produced in the tissues, such as CXCR4 and CCR7.¹⁷⁹ Depending on the extracellular signals and $T_H1/T_H2/T_H17$ polarization, these lymphocytes acquire chemokine receptors for CCR3, CCR5 or CXCR3, allowing them to migrate to inflamed tissues.¹⁷⁹ In T cells stimulated with ConA, we hypothesized that the CCR5 receptor is

upregulated in response, allowing the cells to better respond to the CCL5 gradient in the Transwell culture.

Interestingly, we also demonstrated that ConA induced CD4⁺ and CD8⁺ T-cell specific inflammatory migration that was heavily dependent on IFN γ production, which could be partially inhibited with an anti-IFN γ antibody. These results recapitulate previous findings by this group that reducing the number of IFN γ ⁺ CD4⁺ T cells may have a positive impact on disease onset and symptom manifestation in EAE mice.¹⁸⁰ The two-faced role of IFN γ in EAE pathology is emphasized by considering initial studies that identified a positive correlation between IFN γ and MS/EAE symptomology against contrasting literature that suggests a beneficial role in disease resolution. In healthy mice, immunization with encephalitogenic IFN γ ⁺ T_H1 cells was capable of inducing EAE.¹⁸¹ Similarly, mice deficient in TH1 transcription factor 'T-bet' were immune to developing classic EAE.^{182,183} In humans, the pathogenic role of IFN γ has been shown in clinical studies that treated MS patients with IFN γ , severely exacerbating relapses and symptomology.¹⁸⁴ In contrast, SPMS patients treated with α IFN γ antibodies had showed mild reduction in disease severity.¹⁸⁵ Other studies have challenged the belief that IFN γ is purely pathogenic in MS/EAE. For instance, both IFN γ -/- and IFN γ R -/- mice have been shown to have earlier disease onset, more severe symptoms, and lethality of EAE versus control counterparts.^{186–190} In terms of IFN γ therapy, EAE mice treated with IFN γ both systemically and intraventricularly have shown positive effects on EAE symptoms.^{191,192} Given the pleiotropic role of IFN γ in MS pathology, described by Arellano et al., (2015), specifically regulating but not completely ablating the IFN γ response in the CNS may prove beneficial in dampening the pathogenic CD4 T_H1 cells that drive major inflammatory events in the CNS.¹⁹³

The benefit of further developing this model in the field of neuroimmunology is clear; the assay has a 'build-a-bear' principle, in which many of the components can be interchanged to study separate aspects of the ChP, even outside the scope of MS. For example, following stroke Martha et al., (2020) report that genes associated with chemokine production are upregulated in the infarct, promoting peripheral immune infiltration. By substituting CCL5 for stroke associated chemokines, CXCL3 and CCL4, this model can simply be adapted to study migration across the ChP in the context of stroke.¹⁹⁴ Transwell assays classically assess cellular migration through cell counting of the upper and lower chamber, then staining the transwell membrane to count the proportion of cells that are actively migrating.¹⁷¹ We have also adapted this assay for flow cytometric analysis which can be further optimised to look at individual subsets of rare cells of interest, or by isolating specific groups of immune cells to study their individual role in disease progression

in the model. This adaptation provides higher specificity and clarity to the assay than its previous applications have shown.

3.5. Summary

The findings from this chapter support that we were able to reproduce the ChP in an *in vitro* model. Previously, the ChP has been hard to research because of its anatomical location, and the requirement for large numbers of animals to assess its functions. Our model was capable of mimicking inflammatory trafficking using ConA stimulation, which was in part due to IFN γ released by cells. This trafficking was T-cell specific, similar to the encephalitogenic trafficking in MS and EAE. Upregulated migration in these conditions could be partially abolished with anti-IFN γ treatment.

3.6. Appendix

Figure 3.10: ConA induces increased migration across our transwell model: Shown are technical replicates from one experiment ($n = 3$). In healthy splenocytes cultured with ConA, there was a significant upregulation in T cell, myeloid cell, and dendritic cell, but not B cell trafficking. A one-way ANOVA was conducted using the cell counts from the bottom well. $p < 0.05$. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

Figure 3.11: ConA induces increased migration across our transwell model. (a). Shown are technical replicates from one experiment ($n = 3$). In healthy splenocytes cultured with ConA, there is a trend towards increased migration across the monolayer in CD45+ cells versus control.

**Chapter 4: Does the heparan sulfate mimetic ‘HS16 35’
affect leukocyte migration in an experimental model of
the choroid plexus?**

4.1. Introduction

The previous chapter established a functional model of the choroid plexus for the *in vitro* study of leukocyte trafficking. This chapter will use that *in vitro* model to investigate the mechanism of action of a novel heparan sulfate mimetic.

HSI6-35 is a heparan sulfate glycomimetic comprised of a dendritic core with four identical sulfated oligosaccharide arms. The commercialization and clinical use of other HS-mimetic competitors currently in development is hindered by the molecular size of these compounds, which have a complex synthesis.¹⁴⁵ The Zubkova lab at the Ferrier Research Institute developed a library of single entity glycomimetics with simpler synthesis, making the compounds accessible and commercially available. *In vitro*, they have been shown to employ clustering effects to mimic natural HS molecules in the ECM.¹⁹⁵ HSI 6-35 has previously been shown to inhibit heparanase with high potency, with the advantage that it lacks the anti-coagulant activity and cytotoxicity that other HS mimetics have faced.¹⁴⁵ *In vivo*, this would likely reduce the side effect profile of HSI6-35 compared to other HS mimetics.

4.2. Aims

We propose that HSI6-35 will inhibit migration across this model of the ChP. To test this hypothesis, we will first assess whether the compound is cytotoxic, and select an appropriate dosage for *in vitro* treatment. Finally, we will evaluate the compound in the transwell system to understand whether it is effective at reducing leukocyte migration across this structure.

4.2.1. Specific Aims

1. To determine whether HSI6-35 affects the viability of isolated splenocytes or the ECPC-4 cellular monolayer at doses that are therapeutic *in vivo*.
2. To evaluate the cytokines produced by splenocytes and ECPC4 cells treated with HSI6-35.
3. To assess the effect of HSI6-35 on immune cell migration in a transwell model during both homeostatic and inflammatory conditions.

4.3. Results

4.3.1. HSI6-35 is non-toxic to murine splenocytes, but exerts mildly cytotoxic effects on ECPC-4 cells

Previous unpublished work by this group showed that intraperitoneal administration of HSI6-35 at 600 µg/mouse daily was effective at reducing disease severity in EAE (unpublished results). To adapt this dosing regimen for *in vitro* use, we exposed splenocytes and ECPC-4 cells to a wide range of concentrations (6 µg/mL, 60 µg/mL, and 600 µg/mL) for a 24-hour treatment period to ensure that the compound was not directly cytotoxic to the cells. In this assay, we also treated splenocytes with ConA and IFN γ to assess whether these agents altered cell viability in our transwell migration model.

An MTT assay revealed that ECPC-4 cells were significantly impacted by increasing concentrations of HSI6-35, most notably at 600 µg/mL, which decreased cell viability to $70.6\% \pm 3.45$ of the untreated control (**Figure 4.1a**). As in the previous chapter, we performed an adapted Evans blue permeability assay to understand if the extent of cell death was severe enough to interrupt the monolayer after 24 hours of HSI6-35 exposure at 6 µg/mL, 60 µg/mL and 600 µg/mL. Across two experiments, there was no clear increase in dye penetration through the ECPC-4 monolayer into the lower chamber between any of the HSI6-35-treated groups and untreated control (**Figure 4.1b**). Contrary to the MTT results, it appears that at 600 µg/mL treatment trended towards less dye penetrance versus control. This suggests that the monolayer was becoming more strengthened with HSI6-35 treatment compared to control. Splenocytes showed a much more robust response to HSI6-35 exposure, and no cytotoxicity was detected within the selected dosing range, even when splenocytes were stimulated with ConA or IFN γ (**Figure 4.2a**). Interestingly, EAE splenocytes exhibited a much greater response to ConA stimulation versus healthy splenocytes, consistent with a greater initial inflammatory state of the cells (**Figure 4.2b**). Based upon these results, we selected 60 µg/mL (12 µM) as our optimal concentration in the transwell migration model.

a. **Epithelial Choroid Plexus Cell-4 MTT**

Figure 4.1: HSI6-35 is mildly cytotoxic to ECPC-4 cells but does not interrupt monolayer permeability. (a) Shown are viability scores as a percentage of untreated control with mean $\pm$ SEM from 2 separate experiments. Results show that increasing concentrations of HSI6-35 for 24h in culture significantly reduced ECPC-4 cell viability from control, regardless of stimulation with ConA or IFN γ . The data from this experiment was analysed using an ordinary two-way ANOVA with $\alpha = 0.05$ (* = $p < 0.05$, ** = $p < 0.05$, *** = $p < 0.01$). (b) An Evans blue dye assay was done on the epithelial monolayer after 24h treatment to assess whether increasing concentrations of HSI6-35 interrupted the monolayer. There was no significant difference in dye penetration from the untreated control in any of the treatment groups ($n = 2$).

b. **EBD Assay on ECPC-4 Monolayer**

Figure 4.2. HSI6-35 does not exert cytotoxic effects on healthy or encephalitogenic splenocytes. (a) Shown is viability as a percentage of untreated healthy control with mean $\pm$ SEM from 2 experiments (n=2). With the same treatment, HSI6-35 showed no cytotoxicity towards healthy splenocytes after 24 hours. As expected, ConA significantly increased viability from control. (b) When compared to the healthy control, splenocytes isolated from a mouse with active EAE had a greater response to ConA stimulation. However, in all conditions HSI6-35 had no cytotoxic effect on EAE splenocytes. The data from this experiment was analysed using an ordinary two-way ANOVA with $\alpha = 0.05$ (* = $p < 0.05$, ** = $p < 0.05$, *** = $p < 0.01$).

4.3.2. HSI6-35 inhibits migration in a Transwell model of the choroid plexus

As described in Chapter 3, we established a reproducible model of the ChP, adapted slightly from Monnot and Zheng's (2013) paper, and using this model, we evaluated the effect of exposure to HSI6-35 at the concentration identified in 4.3.1 on immune cell migration. Murine splenocytes were stimulated with or without ConA for 24 hours and then resuspended in fresh media containing HSI6-35 at 60 µg/mL for 24 hours. In parallel, ECPC-4 monolayers on transwell inserts were treated with HSI6-35 at the same concentration for 24 hours prior to the migration assay. The treated splenocytes were then added to the upper chamber and allowed to migrate for 24 hours in the presence or absence of CCL5 in the lower chamber. The gating strategy for flow cytometric analysis can be found in **Figure 4.3**. As seen in **Figure 4.4a**, ConA drastically increased the number of CD45⁺ cells translocating into the lower chamber. Treatment with HSI6-35 completely ablated this inflammatory trafficking, with the number of cells penetrating the monolayer comparable to the media alone and media + CCL5 conditions. **Figure 4.5** are representative flow plots, demonstrating the number of CD45⁺ cells migrating into the lower chamber when treated with or without HSI6-35. This is an excellent visual representation of the extent that HSI6-35 inhibits leukocyte migration in this model.

When the specific cellular populations were assessed separately, it was clear that CD4⁺ and CD8⁺ T cells were the most affected by HSI6-35 especially after ConA stimulation, which induced a dramatic increase in the number of T cells crossing the monolayer (**Figure 4.4b**). This enhanced migration, which is characteristic of MS, was completely ablated in CD4⁺ T cells and reduced greatly in CD8⁺ T cells when cells were pre-treated with HSI6-35 in culture (**Figure 4.4b**). In contrast, ConA stimulation did not enhance the migration of B cells, myeloid cells, and dendritic cells to the lower chamber (**Figure 4.4c**). However, there was still a notable reduction in DC migration when HSI6-35 was present compared to the control conditions. Taken together, these results strongly suggest that HSI6-35 may be effective in inhibiting migration of autoreactive T lymphocytes into the CNS through the ChP epithelium.

Figure 4.3: Our gating strategy for flow cytometry in chapter 4. All flow cytometry data was assessed using FlowJo v10. Initially, CD45^{high} lymphocytes were gated out, then gated to exclude doublets. All CD45^{high} singlets were placed on a CD4 vs CD8 plot and gated for CD4⁺ and CD8⁺ expression. Cells that were non-T cells were then gated based on B220 vs CD11b expression, with B220^{high}CD11b⁻ as B cells, B220^{int}CD11b⁺ as dendritic cells and B220-CD11b⁺ as myeloid cells. T cells were further gated on their expression of CD62L and CD44 with CD62L⁺CD44⁻ as naïve T cells, CD44⁺CD62L⁺ as effector/effector memory T cells and CD62L-CD44⁺ as central memory T cells.

Figure 4.4: HSI6-35 inhibits ConA-mediated migration of CD45⁺ leukocytes across an epithelial monolayer. HSI6-35 appears to be more effective in inhibiting CD4⁺ T cells, CD8⁺ T cells and DC (a) Shown are individual data points from one experiment $\pm$ SEM. When splenocytes are treated with ConA, there is a significant increase in migration across the epithelial monolayer. This inflammatory trafficking is completely ablated in cultures pre-treated with HSI6-35, which returns migration to control levels. The data from this experiment was analysed using an ordinary two-way ANOVA with $\alpha = 0.05$ (* = $p < 0.05$, ** = $p < 0.05$, *** = $p < 0.01$). (b) CD4⁺ and CD8⁺ T

cells show strong upregulation in trafficking in response to ConA stimulation, but this could be completely inhibited with HSI6-35 treatment. These results were analysed using an ordinary two-way ANOVA with $\alpha = 0.05$ (* = $p < 0.05$, ** = $p < 0.05$, *** = $p < 0.01$). (c) B220+, myeloid cells and dendritic cells show no response to ConA stimulation, but still respond to HSI6-35 treatment by reduced trafficking (n/s).

Figure 4.5: ConA induces an increase in migration across a Transwell culture which is mitigated by HSI6-35. Shown here are four representative flow plots from a migration experiment in which the effects of 48h HSI6-35 pre-treatment on migration into the lower well was examined. The black line shows the lymphocyte population, and the number in the corner indicates the number of cells in that population. What can be observed from these flow plots is that ConA stimulation increases the raw number of cells migrating through the Transwell. Pre-treatment with HSI6-35 shows a visual reduction in migration through the Transwell in both Unstimulated and ConA stimulated conditions.

The flow cytometry panel included CD44 and CD62L to allow a better understanding of the activation states of the CD4⁺ and CD8⁺ T cells migrating through the epithelial monolayer (**Figure 4.6a, 4.6b**). In mice, CD4 and CD8 T cells can be categorized into naïve, effector, or memory phenotypes based on their differential expression of CD44 and CD62L. CD44⁺CD62L⁺ cells are considered naïve (T_N), with a shift to CD44⁺CD62L⁻ considered effector or effector memory (T_{E/EM}). Finally, CD44⁺CD62L⁺ cells are considered central memory (T_{CM}).¹⁹⁶ The ChP stroma houses a population of resident CD4⁺ effector memory T cells, whereas CD4⁺ T cells in the CSF generally express central memory phenotypes.³⁹ We expected to see these populations represented more heavily than naïve populations.

4.3.2.1. CD4⁺ T cells

Using these markers, we found that naïve CD4⁺ T cell (CD62L⁺CD44⁺) migration was not enhanced by ConA stimulation, which is not surprising given that ConA induces T cell activation. ConA increased CD44⁺ (T_E or T_{CM}) but not naïve populations (**Figure 4.6a**). The average number of total (upper + lower chamber) CD4⁺ naïve T cells in the control, vehicle-treated condition was $5.4 \times 10^3 \pm 672$, whereas with ConA stimulation, this number decreased to $4.3 \times 10^3 \pm 640$. With HSI6-35 treatment, the total number of naïve CD4⁺ T cells sits at $6.8 \times 10^3 \pm 492$ and does not decrease with ConA treatment maintaining an average of $7.0 \times 10^3 \pm 538$ (**Figure 4.7a**). In addition, naïve CD4⁺ T cells did not respond to CCL5 stimulation as there was no change in migration across the ECPC-4 monolayer between $\pm$ CCL5 stimulated conditions (**Figure 4.6a**).

In contrast to naïve CD4⁺ T cells, CD4⁺ T_E cells displayed increased trafficking across the ECPC-4 monolayer in response to ConA stimulation. In the media-alone and media + CCL5-stimulated conditions, there was no difference in migration, however when stimulated with ConA there was an observable increase in the number of CD4⁺ T_E cells penetrating the monolayer in response to CCL5 stimulation (**Figure 4.6a**). HSI6-35-treated CD4⁺ T_E cells showed low-level trafficking, comparable to the media alone condition, and did not show increased trafficking in response to CCL5 stimulation (**Figure 4.6a**). Interestingly, HSI6-35 treatment of previously Con-A stimulated cultures clearly decreased the number of migrating CD4⁺ T_E cells from the ConA-alone stimulated condition, with these cells no longer showing a sensitized response to CCL5 stimulation (**Figure 4.6a**). **Figure 4.7a** shows the total number of CD4⁺ T_E cells in the transwell culture (upper + lower chambers). ConA stimulation clearly increased the number of CD4⁺ T_E, but this effect was amplified when cells were also stimulated with CCL5, which further

increased the number of effector cells (**Figure 4.7a**). This CCL5-mediated response was not seen in cultures treated with ConA and HSI6-36.

Finally, central memory CD4⁺ T cells show the most dramatic increase in migration across the monolayer in response to ConA stimulation. Similarly, to the trend seen with T_{EM} CD4⁺ cells, media + CCL5 stimulation was not sufficient to increase migration of CD4⁺ T_{CM} into the lower chamber compared to media alone (**Figure 4.6a**). When previously stimulated with ConA, CD4⁺ T_{CM} showed a sensitized, greater migratory response to CCL5 stimulation versus ConA alone. When stimulated with HSI6-35 alone, these cells showed a low migratory response, comparable to the media alone and media + CCL5. Interestingly, HSI6-35 treatment of ConA-stimulated cultures was also capable of reducing CD4⁺ T_{CM} migration to levels comparable to the media alone control; however, these cells still demonstrated the CCL5 sensitized-migration characteristic of ConA-stimulated migration (**Figure 4.6a**). In regard to the total number (upper + lower chambers) of CD4⁺ T_{CM} in each transwell culture, in wells not stimulated with ConA, the CD4⁺ T_{CM} population was virtually absent compared to ConA-stimulated wells (**Figure 4.7a**). In addition, in ConA-stimulated wells, CD4⁺ T_{CM} show increased cell counts in response to CCL5 similarly to CD4⁺ T_E cells (**Figure 4.7a**). There appears to be no difference in the overall cell count between ConA stimulated ± HSI6-35 conditions, however the CCL5-dependent increase in CD4⁺ T_{CM} cell number is not present in HSI6-35 treated wells (**Figure 4.7a**).

4.3.2.2. CD8⁺ T cells

With the same markers, we found that in contrast to CD4⁺ T_N cells, naïve CD8⁺ T cells greatly upregulated trafficking across the transwell culture in response to ConA stimulation (**Figure 4.6b**). This finding was unexpected, because generally naïve cells are not capable of entering the CNS. Unlike other T cell subsets, the number of CD8⁺ T_N cells trafficking into the lower chamber was not altered with CCL5 stimulation, regardless of stimulation with ConA or treatment with HSI6-35. When treated with HSI6-35 alone, CD8⁺ T_N trafficking decreased slightly from the media-alone controls (**Figure 4.6b**). When previously stimulated with ConA, then treated with HSI6-35, CD8⁺ T_N cells decreased dramatically in migration compared to ConA-stimulated, untreated wells (**Figure 4.6b**). Unexpectedly, the total number of CD8⁺ T_N cells increased in response to ConA stimulation, and this increase is not altered by HSI6-35 treatment (**Figure 4.7b**).

Both CD8⁺ T_E and T_{CM} showed a similar pattern in this model. With media alone or media + CCL5, there was low level migration across the monolayer, which was not specific to CCL5 stimulation (**Figure 4.6b**).

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With ConA stimulation, both groups expanded rapidly in total cell number, and the number of cells that migrated through the monolayer (**Figure 4.6b, Figure 4.7b**). The proliferation seen in ConA stimulated wells increased with CCL5 stimulation, similar to the CD4⁺ T_E and T_{CM} subgroups (**Figure 4.7b**). Both CD8⁺ T_E and T_{CM} subgroups became sensitized to CCL5 stimulation when costimulated with ConA, increasing migration across the transwell culture from ConA-stimulated wells alone (**Figure 4.6b**). Again, treatment with HSI6-35 caused complete ablation of Con-A-mediated trafficking in these subsets, returning migration across the monolayer to a level comparable to the media alone control (**Figure 4.6b**). Interestingly, HSI6-35 treatment also reduced the total number of T_E and T_{CM} in culture when previously stimulated with ConA. HSI6-35 treated samples also lack the CCL5-dependent increase in cell number seen when stimulated with ConA alone (**Figure 4.7b**).

Figure 4.6: HSI6-35 inhibits migration of on CD4⁺ T_{EM}, T_{CM} and CD8⁺ T_N, T_{EM}, T_{CM} but not CD4⁺ T_N subsets. (a) Shown are individual data points from one experiment $\pm$ SEM. When splenocytes are treated with ConA, there is an increase in central memory and effector/effector memory but not naïve CD4⁺ T cell migration across the epithelial monolayer. This inflammatory trafficking is completely inhibited in cultures pre-treated with HSI6-35, which returns migration to the level of media alone conditions. **(b)** Shown are individual data points from one experiment $\pm$ SEM. When splenocytes are treated with ConA, there is an increase in central memory, effector/effector memory and naïve CD8⁺ T cell migration across the epithelial monolayer. This inflammatory trafficking is completely inhibited in cultures pre-treated with HSI6-35, which returns migration to the level of media alone conditions.

Figure 4.7: ConA increases the total number of effector and central memory CD4⁺ T cells but decreases the total number of effector and central memory CD8⁺ T cells . (a) Shown are the total number (upper + lower wells) of CD4⁺ cells in the transwell culture following stimulation $\pm$ ConA and treatment $\pm$ HSI6-35. Each bar represents the mean of one experiment with triplicate values $\pm$ SEM. Naïve CD4⁺ T cells appear to increase in number following treatment with HSI6-35, regardless of treatment with or without ConA. Both effector CD4⁺ and central memory CD4⁺ T cells are increased in number following treatment with ConA. Central memory CD4⁺ T cells appear to be mildly upregulated in response to HSI6-35 treatment. **(b)** Shown are the total number of CD8⁺ T cells in the transwell culture following stimulation $\pm$ ConA and treatment $\pm$ HSI6-35. Each bar represents the mean of one experiment with triplicate values $\pm$ SEM. Interestingly, ConA stimulation increased the total number of Naïve CD8⁺ T cells, regardless of stimulation with or without HSI6-35. In contrast,

whilst ConA increases the number of effector and central memory CD8⁺ T cells, this increase is dampened slightly with HSI6-35 treatment.

Chapter 4: Does 'HSI 6 35' affect leukocyte migration

Chapter 3 outlined a clear role for IFN γ in MS pathogenesis and in our model, so we focused heavily on its involvement in HSI6-35's effects in this chapter. To determine whether IFN γ was involved in the ConA-induced immune cell migration in this model, healthy murine splenocytes and the ECPC-4 monolayer were stimulated with IFN γ for 24 hours. Following this, the media was aspirated, and the ECPC-4 cells treated with HSI6-35 for a further 24 hours before adding splenocytes to the upper chamber of the transwell. As α IFN γ treatment had previously been shown to reduce migration in this model, we used ConA stimulation and α IFN γ stimulated splenocytes as controls to assess the extent that HSI6-35 reduced migration in this model (3.3.5)

Figure 4.8. shows the cell counts of all CD45⁺ leukocytes that successfully migrated into the lower chamber. Although the cell-specific effects of each stimulatory condition is dampened here, there is a dramatic decrease in the total number of cells migrating across the monolayer when pre-treated with HSI6-35, despite prior treatment with or without IFN γ . This decrease in migration is below that of the homeostatic, media alone condition. When each immune cell subset was assessed separately, CD4⁺ and CD8⁺ T cells were again identified as most affected by HSI6-35 treatment, especially following prior stimulation with IFN γ (**Figure 4.9**). When CD4⁺ and CD8⁺ T cells were stimulated with IFN γ , there was mild increase in trafficking across the monolayer, consistent with findings from chapter 3 (**Figure 4.9**). These cells also appeared more sensitized to CCL5 stimulation than media alone stimulated wells, with increased migration between the -CCL5 and +CCL5 IFN γ stimulated wells. T cell migration enhanced by IFN γ was not as prominent as ConA-stimulated wells, which showed increased migration from the media alone and IFN γ -treated wells, especially notable with the CD8⁺ T cell population (**Figure 4.9**). This is not surprising, as we have previously demonstrated that ConA-induced T cell specific increase in migration. Here, the α IFN γ antibody exerted a mild effect on CD8⁺ T cell migration but not CD4⁺ cells (**Figure 4.9**). HSI6-35 was particularly effective in reducing T cell migration below baseline (media alone) and the α IFN γ control, additionally removing the CCL5-sensitization seen when stimulating the leukocytes with IFN γ alone (**Figure 4.9**).

When examining non-T cell populations, the effect of IFN γ on migration was completely absent. Whereas in B cells, IFN γ did not alter migration across the monolayer from the media alone control, in both myeloid cells and dendritic cells, IFN γ stimulation clearly decreased migration (**Figure 4.10**). As previously described, ConA did not affect the migration of non-T-cell subsets, a result that is consistent in these findings. Finally, although there was no increase in B cell, myeloid cell or dendritic cell migration in

response to these inflammatory stimuli, when treated with HSI 6-35, these subsets were all dramatically decreased in migration across the monolayer from the media alone control (**Figure 4.10**).

Figure 4.8: HSI6-35 reduced general leukocyte migration through a transwell model of the ChP. Shown are experimental values of the number of cells crossing into the lower chamber with the mean $\pm$ SEM. Although IFN_γ, ConA and αIFN_γ appear not to alter the migration of CD45+ cells across the ECPC-4 monolayer, 24h HSI6-35 treatment at 60 ug/mL is effective in reducing migration into the lower chamber from the media alone condition.

Figure 4.9: HSI6-35 reduced IFN γ -mediated CD4⁺ and CD8⁺ T cell migration through a transwell model of the ChP. Shown are experimental values of the number of cells crossing into the lower chamber with the mean $\pm$ SEM. An increase in migration is seen with IFN γ stimulation from the media-alone control, and an even greater increase with ConA stimulation. α IFN γ treatment partially reduced CD8⁺ but not CD4⁺ trafficking into the lower well. When cultures were treated with HSI6-35, there was a notable decrease in the number of cells penetrating the monolayer, below the media-alone control. This HSI6-35 mediated decrease is still seen when splenocytes had previously been stimulated with IFN γ .

Figure 4.10: HSI6 reduced B cell, myeloid and dendritic cell migration through a transwell model of the ChP. IFN γ stimulation did not increase migration of B cells across the ECPC-4 monolayer and decreased the number of migrating myeloid and dendritic cells. In addition, ConA stimulation failed to increase migration in any of the populations shown. Regardless, HSI6-35 treatment reduced migration across the ECPC-4 monolayer from the media alone control in B cell, myeloid cell, and dendritic cell population.

4.3.3. HSI6-35 may be effective as an antimigratory agent by inhibiting proinflammatory cytokine release

Because IFN γ has previously been shown to promote migration in this model of the ChP, the research required further investigation into how HSI6-35 may modulate immune cell responses. The supernatant from splenocytes treated with or without ConA in the presence or absence of HSI6-35 was tested for IFN γ using a sandwich ELISA, to understand if HSI6-35 is effective in this model by inhibiting proinflammatory cytokine release, in a similar mechanism to the α IFN γ antibody. When stimulated with ConA alone, splenocytes produced high levels of IFN γ (**Figure 4.1 Ia**). as expected, however, increasing concentrations of HSI6-35 led to a significant reduction in IFN γ in the culture supernatant. Given the known role for IFN γ in promoting migration, this reduction in IFN γ may be an alternative mechanism to heparanase inhibition by which HSI6-35 may affect immune cell migration.

To elucidate how HSI6-35 was effective at inhibiting migration of ConA-stimulated splenocytes in our model of the ChP, we evaluated the cytokines in the supernatant of the splenocyte and ECPC-4 cultures stimulated with or without ConA and/or HSI6-35 at 6 or 60 μ g/mL for 24 hours using a LEGENDPlex™ mouse inflammation assay. This assay allowed us to quantify 13 cytokines in culture supernatants. Additionally, we also investigated cytokine production by splenocytes from animals with EAE responded in the presence or absence of HSI6-35 to understand whether an on-going immune response altered the effect of HSI6-35 on ConA-stimulated or unstimulated splenocytes.

Four of the thirteen cytokines were upregulated by ConA in cultures from the healthy control in splenocytes: IFN γ , IL-17A, IL-6 and TNF α . In **Figure 4.1 Ib**, we compare the cytokine responses of EAE splenocytes to a healthy, ConA-stimulated, untreated control. In healthy splenocytes, IFN γ appeared to be reduced by up to 30% from the untreated healthy control with both 6 μ g/mL and 60 μ g/mL treatments. Interestingly, EAE splenocytes showed a more robust response, with untreated ConA-stimulated splenocytes upregulating IFN γ expression by more than 600% from control. This expression was diminished back to baseline with 6 μ g/mL treatment, and down by 400% with 60 μ g/mL treatment.

ConA-stimulated splenocytes from EAE mice showed increased expression of IL-17A (up to 1,500%) compared to splenocytes from healthy mice (**Figure 4.1 Ib**). While IL-17A levels were modestly affected by HSI6-35 in healthy splenocyte cultures, HSI6-35 reduced IL-17A production by up to 1,000% compared to the untreated EAE splenocytes. Additionally, although the effect of HSI6-35 on IL-6 was

highly variable in healthy splenocyte cultures, untreated EAE splenocytes elicited a strong IL-6 response (>275% increase compared to healthy), which was reduced to levels comparable to controls with both concentrations of HS16-35 (**Figure 4.11b**). Interestingly, healthy splenocytes upregulated their expression of TNF α in response to increasing concentrations of HS16-35 treatment. In contrast, EAE splenocytes expressed high levels of TNF α , which was reduced almost to healthy baseline by both 6 μ g/mL and 60 μ g/mL of HS16-35 (**Figure 4.11b**). In contrast to splenocytes, ConA did not induce cytokine production by ECPC-4 cells; however, there was constitutive expression of CCL2 and IFN γ by ECPC-4 cells, which were increased by HS16-35 treatment (**Figure 4.12**).

Figure 4.11: HSI6-35 mitigates proinflammatory cytokine responses from encephalitogenic splenocytes. (a) Shown are individual data points from one experiment $\pm$ SEM. When splenocytes are stimulated with ConA, there is a significant increase in the amount of IFN γ released into the media. With increasing concentrations of HSI6-35, the concentration of IFN γ in the supernatant significantly decreases. The data from this experiment was analysed using an ordinary two-way ANOVA with $\alpha = 0.05$ (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) **(b)** Shown are the means $\pm$

SEM from duplicate splenocyte cultures treated with ConA for 24 hours, then resuspended in media containing HSI6-35. EAE splenocytes upregulated the expression of four key proinflammatory cytokines; IFN γ , IL-17A, IL-6 and TNF α , which trended towards being downregulated with increasing concentrations of HSI6-35 treatment (n/s).

Figure 4.12: ECPC-4 cultures release increasing concentrations of IFN γ and CCL2 which correlates with treatment concentration of HSI6-35. Shown are the means $\pm$ SEM from duplicate ECPC-4 cultures treated with HSI6-35 for 48 hours. ECPC-4 cells expressed detectable levels of IFN γ and CCL2, which trended towards being upregulated with increasing concentrations of HSI6-35 treatment (n/s).

4.3.4. HSI6-35 may be effective in inhibiting encephalitogenic leukocyte entry through a model of the ChP

Finally, to elucidate if HSI6-35 is effective in inhibiting pathogenic migration into the CNS, we assessed the migration of leukocytes isolated from mice with EAE. EAE was induced in C57BL/6J mice as described in 2.2.3. Mice developed chronic EAE before sacrifice, and single cell leukocyte suspensions were cultured from splenic tissue as described in 2.2.5. Splenocytes were also isolated from healthy, untreated age and sex-matched controls (n =3 mice per group). Splenocyte cultures were stimulated with or without ConA for 24 hours and then resuspended in fresh media containing HSI6-35 at 60 µg/mL for 24 hours. In parallel, ECPC-4 monolayers on transwell inserts were treated with HSI6-35 at the same concentration for 24 hours prior to the migration assay. The treated splenocytes were then added to the upper chamber and allowed to migrate for 24 hours in the presence or absence of CCL5 in the lower chamber.

This experiment was not seen to completion because of an incubator failure. This experiment would have served as a major component for obtaining biological replicates of both healthy and EAE immune cell migration, supporting the results that have already been showcased in this chapter. This project had already faced major disruptions to the development of this chapter, as Transwell inserts took 8 months to be delivered to us during the COVID-19 pandemic. This experiment used all the remaining Transwell inserts, so it could not be replicated before the conclusion of this thesis due to further shipping delays. As this was not completed, I am vitally aware that strong conclusions regarding the efficacy of HSI6-35 on migration across the ECPC-4 monolayer can be drawn yet. As many of the experiments in this chapter could only be conducted a single time, no statistical analyses are presented. For the purpose of the discussion, I will theorize about the observed differences in migration and present supporting literature, although further experimental work is required to confirm the efficacy of this compound with statistical confidence.

4.4. Discussion

This chapter explores the use of novel heparan sulfate mimetic 'HSI6-35' on our previously established Transwell migration model of the ChP. We aimed to identify the effects of HSI6-35 on leukocytes isolated from healthy and EAE mice to determine how HSI6-35 is protective in sick mice and how it may modulate immune responses in healthy individuals. We first adapted the optimal *in vivo* dose to ensure that there was no negative impact on leukocyte and epithelia viability, or monolayer permeability. We found that 60 $\mu\text{g/mL}$ (12 mM) showed no adverse effects on cells in culture, whereas higher doses showed some cytotoxicity to the epithelial monolayer. The results from the MTT and Evans blue dye permeability assays provided essential information for adapting the effective *in vivo* dosing regimen to an *in vitro* model of the choroid plexus. Indeed, Zubkova et al., (2018) found that HSI6-35 was effective in inhibiting heparanase enzyme activity, with an IC_{50} in the low micromolar range. We do not yet know if the unpublished *in vivo* data from this group reduced disease severity as a result of heparanase inhibition at the BBB or due to some other biological mechanism that has not been investigated. Considering Parish et al., 1988 and Naparstek et al., (1990) findings, it seems likely that inhibition of T-cell produced heparanase activity is the primary mechanism of action of this drug and the rationale for its success *in vivo*. To account for the inevitable metabolic loss of drug in the plasma in mice, versus the direct application onto to the model, we selected 60 $\mu\text{g/mL}$ as the standard treatment for the transwell migration model.

Additionally, we found that at this dose, ConA and IFN γ -induced trafficking in our transwell cultures could be completely inhibited. Further investigation of the cell types impacted by HSI6-35 treatment showed that T cells and dendritic cells were particularly reduced however the drug showed efficacy on all major immune cell subsets including B lymphocytes and myeloid cells. Using $\alpha\text{IFN}\gamma$ as a control, which had previously been shown to reduce proinflammatory trafficking across the transwell culture, HSI6-35 was capable of reducing migration to a greater extent than this control.

Finally, we measured cytokine responses in isolated healthy and EAE leukocyte cultures to ConA stimulation and low/optimal doses. Many of the cytokines act as part of a tightly regulated network where the action of one cytokine is affected by the production and behaviour of other cytokines. In addition, many of these cytokines play a pathogenic or regulatory role in the development of inflammatory insult in MS. We identified four cytokines that were upregulated in response to ConA stimulation in EAE splenocytes: IFN γ , IL-6, IL-17A and TNF α . The expression of all of these cytokines was modulated in response to low and moderate dose HSI6-35 treatment, trending towards downregulation.

IL-17A is a cytokine that can be considered a ringmaster in the pathology of multiple sclerosis and EAE. IL-17A is produced by CD4⁺ T helper 17 (T_H17) cells, a prominent population that drives disease progression.¹²⁶ In active lesions, T_H17 cells, astrocytes and oligodendrocytes release IL-17A which synergizes with proinflammatory cytokines and other mediators of tissue damage to drive cellular responses within the lesion.^{197–199} Setiadi et al., (2019) report a positive correlation between serum IL-17A concentrations and the number of relapses in MS patients²⁰⁰. In addition, IL-17A has recently been shown to facilitate breakdown of the blood-brain barrier in RRMS. A breakthrough study by Huppert et al., (2010) showed that brain endothelial cultures co-treated with IL-17A and IL-6 downregulated genes coding for proteins associated with BBB integrity; occludin (OCLN), zonula occludens-1 (ZO-1), VE-cadherin and platelet endothelial cell adhesion molecule (PECAM-1), resulting increased cellular permeability of the *in vitro* cultures.²⁰¹ In addition, IL17A^{-/-} mice or mice treated with i.p. αIL-17A before disease onset show less disruption of BBB integrity and lower disease scores.^{201,202}

IL-6 is another cytokine with a vital role in EAE and MS. IL-6 is a key component of the acute phase response and is secreted by lymphocytes, macrophages, microglia and some types of non-immune cells such as endothelial cells and neurons.²⁰³ Hand-in-hand with IL-17A, IL-6 aggravates the clinical manifestations of MS through dual actions in the CNS and periphery. IL-6 promotes the generation of T_H17 type cells in the peripheral lymphoid tissues, which are known to play a major role in the immune assault against CNS antigens, perpetuating the neuroinflammatory response.²⁰⁴ In patients with active RRMS, IL-6 has been shown to promote the resistance of effector T cells to regulation by Tregs.²⁰⁵ Recently, Erta et al., (2016) showed that in female mice with astrocytic IL-6 deficiency, there was a modest amelioration of EAE pathology as seen through later onset of clinical symptoms, fewer inflammatory infiltrates, and reduced demyelination from controls.²⁰⁶ IL-6 responses in MS patients are upregulated in the sera and CSF by up to 17 times that of healthy controls. Because IL-6 drives immunoglobulin responses from B cells, systemic upregulation of IL-6 in MS supports a role for B cells in disease pathology.²⁰⁷ Overall, the literature highlights a definite role for IL-6 in the pathogenesis of EAE and MS.

TNFα has been a suggested target for MS therapies for decades now. TNFα is a pleiotropic cytokine with many homeostatic roles as well as known pathological roles in diseases such as rheumatoid arthritis, irritable bowel syndrome and psoriasis.²⁰⁸ Many of these diseases show modest responses to antiTNFα therapies. Because TNFα signals through dual pathways based on its receptor-ligand complex formation, either TNFR1 or TNFR2, a preference towards TNFR2 signalling may have a protective effect in MS.²⁰⁸

Indeed, an *in vivo* study has shown the dichotomy between TNFR1 and TNFR2's roles in EAE. TNFR1^{-/-} mice show reduced clinical manifestations of EAE symptoms, lower CD3⁺ T cell infiltrates and activated microglia/macrophages versus wildtype mice. In contrast, TNFR2^{-/-} mice have worsened disease course.²⁰⁹ TNFR2 also plays a neuroprotective role during remyelination by protecting oligodendrocyte precursor cells (OPC's) from oxidative stress and boosting OPC maturation through TNFR2-stimulation in astrocytes.^{210,211} Because of TNF α 's dual roles in MS, it is difficult to suggest if altering its expression will be protective or detrimental.

Previous literature has elucidated that ConA stimulation of murine splenocytes induces a wave of IFN γ signalling.²¹² IFN γ is a cytokine with dual roles in MS, it is heavily upregulated in lesion areas and is associated with a destructive T_H1 response in the CNS.²¹³ Indeed, in both MS and EAE models, IFN γ administration is associated with increased relapse rate and severity of symptoms.^{184,213} Skewing the cytokine milieu and immune response to T_H2 in EAE clearly ameliorates symptoms of the disease.²¹⁴ In stark contrast, IFN γ is also suggested to have a beneficial role by limiting the extent of disease, as seen by increased disease severity in IFN γ knockout mice.²¹⁵ In EAE, IFN γ may also enhance oligodendrocyte survival, which is associated with reduced disease severity and lesser demyelination than vehicle treated controls.²¹⁶

Because many of these cytokines have been identified as having dual roles in disease pathology of MS, it could be suggested that regulating, not completely inhibiting the T_H1 and T_H17 cytokine responses *in vitro* may be protective in EAE and MS.

Focusing on the choroid epithelium, we saw increasing concentrations of CCL2 and IFN γ expression which correlated with HSI6-35 concentrations in our ECPC-4 cell line. Interestingly, Robichon et al., (2020) showed that CCL2 secretion is associated with pathogenic macrophage migration into the CNS in EAE and when inhibited, protects against EAE by reducing cell infiltrates into the CNS.¹⁵² However, CCL2 gene is highly expressed in the basal state of the choroid plexus, compared to other CNS tissues, which may be reported in this assay, with some variability.²¹⁷ Shifts in IFN γ expression in the choroid plexus have been reported in aging-associated cognitive decline and Alzheimer's disease but expression is also important in regular immune surveillance and repair, reported by Kunis et al., (2013).^{119,161,218}

MS has classically been considered a T-cell mediated disease, with studies showing that relapses are predominantly mediated by an aberrant and unregulated pro-inflammatory effector T cell response,

encompassing both CD4⁺ and CD8⁺ T cells that traffic into the neural parenchyma.⁴⁶ These cells express receptors for CNS antigens, and complex interplay between them and CNS-resident cells lead to the production of inflammatory mediators that can recruit further inflammatory cells into the CNS to induce neuroaxonal damage and neurodegeneration. Reboldi et al., (2009) elucidated how the choroid plexus acts as the initial site of entry for T_H17 cells in EAE, which induced a secondary wave of inflammatory cell infiltration through the now-permeable cerebral vasculature.¹²³ Interestingly, we saw that with ConA stimulation, there was higher numbers of CD8⁺ T cells than CD4⁺ T cells, particularly of naïve phenotypes crossing the ChP monolayer. This is in line with supporting literature, which indicates that CD8⁺ T cells are the predominate lymphocyte present in the lesions of patients with MS.^{219,220} It was unusual however, that CD8⁺ T_N cells were actively penetrating the epithelial monolayer given that naïve cells generally do not express the correct chemokine receptors or adhesion molecules to do so *in vivo*. It could be postulated that this is a result of bystander activation, in which ConA stimulated CD4⁺ T cells undergo activation and clonal expansion in an Ag-independent manner, releasing high concentrations of proinflammatory cytokines into the supernatant. Although the mechanism behind this phenomenon is unknown, type I interferons have previously been reported to induce functional activation in CD8⁺ T cells which remain in a T_N-type phenotype.²²¹

We observed very low numbers of CD4⁺ naïve T cells penetrating the monolayer, in both unstimulated and ConA-stimulated cultures, as they likely do not express the correct adhesion molecules or activation markers to actively migrate through the monolayer. Because we know that similar trafficking patterns happen *in vivo*, this was indicative once again that the ECPC-4 monolayer was a selective, yet permeable barrier similar to the ChP in the brain. Both central memory T cell and effector memory T cell trafficking across the monolayer was upregulated with ConA stimulation, which was significantly reduced in both conditions when treated with HSI6-35. CD8⁺ T cells generally dominate the early MS infiltrates, and are more abundant in both forms of progressive MS in the CP itself.^{17,222} In our model of the ChP, we saw that HSI6-35 targeted these key pathogenic populations, namely CD4⁺ T_{EM} and T_{CM} and CD8⁺ T_N, T_{EM} and T_{CM} lymphocytes. By regulating entry of these cells across the ChP, some of the initial inflammatory events that establish MS disease progression could be arrested before symptom onset.

In addition, MS pathology occurs as the result of insufficient tolerance mechanisms, and the resistance of effector T cell groups to Treg-mediated regulation. Koliesnik et al., (2020) reported that heparan sulfate mimetic 'PG545', inhibits the Erk1/2 signalling pathway that drives T cell activation and polarization. They

also report that through this mechanism, effector T_{H1} and T_{H17} responses are suppressed in preference of FoxP3⁺ Treg subsets both *in vitro* and in an *in vivo* DHT model.¹⁴⁴ Similarly, if our heparan sulfate mimetic is capable of skewing the immune response in favour of Treg responses in the neural parenchyma, T_{H1} and T_{H17} responses in the ChP and CSF may be sufficiently regulated and unable to elicit CNS injury. This suppression may be evidenced through the reduction of key T_{H1}/T_{H17} cytokines in our Transwell system when treated with HSI6-35, although this requires more investigation. Finally, ConA-induced T cell heparanase expression has reportedly been inhibited with HS-like compounds, as described by Lider et al., (1990). Inhibiting heparanase *in vivo* has been reported to be protective in EAE, by mitigating the enzyme's capacity to destabilize the ECM and surrounding vasculature and inhibiting its proadhesion properties in sheer flow environments.^{138,223} The multiple effects of heparan sulfate mimetics on CNS infiltration, alongside our *in vitro* data show that HSI6-35 may exert a regulatory effect that is effective in protecting against disease in EAE and MS.

In MS and EAE, the infiltration of autoreactive T lymphocytes, macrophages and demyelinating antibodies into the CNS is one of the hallmark features of disease development.¹⁷ Inhibiting the trafficking of early peripheral cell infiltrates has proved to be one of the most successful treatment options for MS patients, evidenced by frontline drugs such as natalizumab.²²⁴ A major caveat of these drugs is they provide complete inhibition of immune cell trafficking, regardless of whether the cells are performing routine immune surveillance or pathogenic inflammation. This provides significant risk to patients, more so if they are CSF⁺ for the JC virus as these patients can develop life-threatening PML due to defective immune surveillance in the CNS.⁶² Interestingly, HSI6-35 did not completely suppress cell migration through the transwell culture, but reduced trafficking to near baseline healthy levels. This suggests that HSI6-35 may restrict but not indiscriminately suppress immune cell trafficking into the CNS in a pathogenic model. Together, this chapter provides major groundwork to elucidate how HSI6-35 acts *in vivo* and contributes to disease protection in EAE.

4.5. Summary

This chapter investigated the effect of novel HS-mimetic, HSI6-35, on the migration of immune cells through a model of the ChP. HSI6-35 exerted no cytotoxic effects on splenocytes isolated from healthy mice or mice with EAE. It showed limited toxicity on ECPC-4 cells and did not disrupt overall barrier function of the epithelial monolayer. Using the transwell migration model, we found that the HS mimetic significantly reduced the number of cells that migrated through the monolayer, in both homeostatic and proinflammatory conditions. Specifically, HSI6-35 appeared to be most effective in inhibiting ConA-stimulated CD4⁺ and CD8⁺ T cell migration but showed broad inhibition of homeostatic migration across all CD45⁺ leukocytes. Finally, we found that four key proinflammatory cytokines, IL-6, IFN γ , TNF α and IL-17A were downregulated in EAE splenocytes stimulated with ConA in the presence of HSI6-35 *in vitro*. Taken together these findings suggest that the mechanism, by which HSI6-35 inhibits migration in our ChP model, involves regulating cytokine (esp. IFN γ)-induced migratory signals and may not be solely by inhibiting heparanase-mediated ECM degradation.

Chapter 5: General Discussion

5.1. Overview

Multiple sclerosis is a chronic, neurodegenerative disease characterised by confluent areas of demyelination in the white and grey matter of the brain and spinal cord. These areas of sclerotic damage encompass the loss of myelin sheaths and death of oligodendrocytes and in the latter stages, neuronal death. Inflammatory damage to the neuronal parenchyma is considered to be of autoimmune origins, but no definitive aetiology of MS has been discovered. The inflammatory migration of leukocytes into the CNS is one of the key features of MS, with early lesions showing peripheral infiltrates and breakdown of the blood-brain barrier.¹⁷

Inhibiting the infiltration of these cells has been the main target of several frontline therapies. Although these drugs successfully inhibit immune cell trafficking into the CNS, decreasing disease severity and relapse rate, they are often associated with severe and sometimes life-threatening side effects.⁵⁹ Because of their broad-spectrum immunomodulatory actions, they do not distinguish between immune cells conducting homeostatic surveillance or pathogenic infiltration, leading to immune dysfunction in the CNS. Unchecked, pathogens in the CNS can freely exert their effects, completely unregulated to cause CNS injury. Hence, there is an unmet need for treatments that regulate, not inhibit trafficking into the CNS.

Although the CNS was traditionally considered an immune privileged site, recent studies have illuminated that homeostatic trafficking of leukocytes for immunosurveillance through the ventricles is governed by the choroid plexus. In contrast to the blood-brain barrier, in which trafficking through this structure constitutes a hazard to the tissue, the choroid plexus accommodates over 500,000 cells trafficking through the ventricles each day, each performing an important role in maintaining the immunosuppressed phenotype inside the CSF.¹⁶⁸ Interestingly, during homeostatic functions and even sterile injury, the choroid plexus releases a slew of immunomodulatory mediators which push the immune cells in its immediate vicinity and CSF into a pro-resolving, wound-healing phenotype.³⁷ In MS, there is emerging evidence to suggest that the choroid plexus contributes to disease pathology. Indeed, there are significant enhancements in choroid plexus anatomy of MS patients, documented on MRI by Kim et al., (2020).²²⁵ In addition, the ChP houses a small population of resident dendritic cells, who have been reported to conduct antigen sampling in the CNS.¹¹¹ Alongside these cells, CD4⁺ T cells with TCR against CNS antigens reside in the choroid plexus.^{166,226} Depending on the inflammatory or tolerogenic state that DC in the ChP adopt, the ChP has immense capacity to govern the type of response entering the CNS. These DC and other stromal macrophages express high levels of MHC II on their surface, indicating that the ChP acts as an

interphase for constitutive T cell stimulation and reactivation in CNS inflammatory disorders.^{120,125,168,227} Indeed, the ChP integrates and responds to various signals from the peripheral immune system, including inflammation. In response to peripheral cytokine stimulation, the ChP upregulates key adhesion molecules, indicating that it may facilitate inflammatory trafficking into the CNS.¹²¹ Inhibiting the expression of these molecules, has shown to be effective in protecting from EAE, as described in Breuer et al., (2018) where inhibiting the interaction of laminin 411:MCAM in the ChP restricted encephalitogenic transmigration in an *in vitro* model of ChP endothelium and in EAE mice.²²⁸

The requirement for a working *in vitro* model for the study of migration into the CNS is clear, and something the current study addresses. Only one other study reports the development of a mouse-derived ChP for the study of leukocyte trafficking, using primary mouse ChP cells.¹⁷² To reduce the number of animals whilst troubleshooting, we used an immortalised ChP epithelial cell line; ECPC-4. Although Munechika et al., (1996) report that this cell line maintains characteristic feature of the ChP epithelium such as transthyretin and α -macroglobulin, Lazarevic et al., (2016) raise concern over the capacity of this cell line to model functions of the ChP, namely the expression of adhesion molecules and its ability to form a tight barrier with contact-inhibition. This thesis demonstrated that in culture, ECPC-4 cells formed complete monolayers with tight barrier functions and were suitable for use in the Transwell migration assay.

In addition, we observed that inflammatory trafficking of leukocytes across the ECPC-4 monolayer could be induced by ConA or IFN γ stimulation. ConA has previously been shown to increase trans-endothelial T cell migration in a model of general vasculature.²²⁹ In addition, T_H1-type cytokines such as IFN γ or TNF α have been shown to increase CD4⁺ T cell migration across this model.²²⁹ Our findings remain consistent with this literature, in which we observed T-cell specific increases in migration with stimulation of both ConA and IFN γ .

The literature offers a clear role for heparanase, the ubiquitously expressed heparan sulfate degrading enzyme in many neuroinflammatory diseases, including MS. It has been extensively studied in cancer research for its ability to fragment HS residues from their proteoglycan core, leading to ECM remodelling, the propagation of cell signalling, metastasis, and leukocyte migration.¹³⁵ Some of the initial work focusing on the role of heparanase in MS investigated T cell heparanase release in the cerebral vasculature, which facilitated inflammatory trafficking across the destabilised BBB.¹³⁷ The current study focused on novel heparan sulfate mimetic HSI 6-35, part of a targeted library of novel compounds developed by the Zubkova

lab at the Ferrier Research Institute. Because HSI6-35 is comprised of a dendritic core and heavily sulfated maltose groups, its synthesis is much quicker, making it a commercially available cheaper alternative to other HS-like compounds currently in clinical development.¹⁴⁵ We investigated the capacity of HSI6-35 to mitigate ConA and IFN γ -mediated migration across our transwell model of the choroid plexus. With HSI6-35 treatment, we saw suppression of CD45⁺ leukocyte migration to baseline levels (chapter 4), which may provide a protective effect *in vivo*.

Controversially, there are a limited number of studies that suggest heparanase, or displacing interactions with its cognate ligand, HSPG's may provide a protective effect in the EAE model of MS. Harris et al., (2016) describe that displacing heparanase's endogenous interactions with HSPG's by small molecule inhibitors was protective in EAE. I.P. injection of these compounds was effective in reducing the appearance of symptoms and inflammation during disease progression. The authors ascribe this to the direct inhibition of binding to heparan sulfate, which blocked leukocyte rolling in the vasculature and subsequent migration into the CNS.²³⁰ This defends against the notion that heparanase activity, in and of itself is pathogenic in EAE. In addition, Muramtatsu and Kadomatsu., (2014) describe how midkine, a small cytokine that binds highly sulfated proteins can influence the disease course of EAE. By removing midkine using RNA aptamers, Takeuchi et al., (2014) found that EAE progression was suppressed by expansion of Tregs and DCregs and suppression of T_H1/T_H17 polarisation.²³¹ Because midkine so heavily associates with HSPG's, and it's removal has been shown to be beneficial, this suggests that heparanase activity, through cleaving HS residues would reduce binding sites for midkine, offering a similar advantage in reducing leukocyte trafficking and increasing Treg numbers.^{231,232}

Subsequently, studies have shown that the sulfated proteoglycans; chondroitin sulfates are heavily expressed by astrocytes and microglia. During brain injury, several studies have shown that chondroitin sulfates proved a hostile environment that impedes OPC mobilization and remyelination, as treatment with chondroitinase is beneficial on OPC recruitment *in vivo*.^{233–235} *In vitro*, highly sulfated proteoglycans have been shown to delay OPC maturation which is expected to delay the remyelination response *in vivo*.²³⁶ Inhibiting the synthesis of CSPG's was also found to accelerate OPC maturation and remyelination in a murine model of focal demyelination.²³⁷ This suggests in part, that the activity in heparan sulfate-degrading enzymes such as heparanase and chondroitinase may prove beneficial in providing a matrix for migrating OPC's to reach the lesion site and accelerate recovery. Only one study has administered heparanase to EAE animals as a therapeutic effort. Bitan et al., (2010) showed that I.P. heparanase

administration ameliorated clinical signs of EAE in a dose-dependent manner. This protection was associated with inhibition of splenocyte proliferation and the modulation of cytokine responses in favour of a T_H2 response.²³⁸ Although this study suggests that heparanase may be protective in EAE, the therapy administered was recombinant heparanase, with loss-of-function mutations so it was unclear if its protective effects were due to fragmenting HSPG's.

On the other hand, there is an extensive list of literature that discusses the potential detrimental role for heparanase in EAE and MS. Much of this discusses its role in breaking down the tight barrier function of the BBB and ChP, which we discussed further in chapter 4. Consistent with our findings *in vitro*, Irony-Tur-Sinai et al., (2003) showed that synthetic heparin mimetic RG-13577 ameliorated EAE, reducing clinical symptomology and reducing inflammation in the spinal cord. *In vitro*, RG-13577 impaired leukocyte trafficking mechanisms, reduced T cell heparanase expression and interestingly, astrocytic heparanase expression related to its activity against Erk1/2 phosphorylation.²³⁹ These findings are similar to Koliesnik et al's., (2020) experiment showing that another heparan sulfate mimetic, PG545 inhibits Erk1/2 phosphorylation, suppressing TH cell polarization and proliferation.¹⁴⁴ If our mimetic HSI6-35 acts through this pathway *in vivo*, it may be able to regulate T_H1/T_H17 polarisation in favour of Treg regulation, although this must be investigated further. Early evidence from our cytokine assays, in which HSI6-35 appears to downregulate key T_H1/T_H17 cytokines and promote a naïve T cell phenotype may indicate that HSI6-35 acts in a similar mechanism. In addition, the ConA mediated increase in total CD8⁺ T_E and T_{CM} cell numbers appears to be reduced when splenocyte cultures were treated with HSI6-35, evidence which further points to the suppression of T cell proliferation.

Heparanase has previously been shown to modulate the interactions between APC and T cells by digesting HSPG's on the surface of APC's. Two studies by Chung et al., (2007, 2014) using isolated murine splenocytes demonstrate that a specific receptor; the dendritic-cell associated HSPG dependent integrin ligand (DC-HIL) can inhibit T-cell activation by binding syndecan-4 on T_{EM} cells.^{240,241} If heparanase is present, it may inhibit the interaction between DC-HIL and its cognate ligand by fragmenting HS side chains on syndecan-4, allowing T cell activation and proliferation. By suppressing heparanase through this mechanism, T cell proliferation could be suppressed which may be protective in EAE and MS. Further work to investigate these interactions and how they may be altered by HSI6-35 stimulation is needed.

In addition to leukocyte-derived heparanase in the periphery, there are multiple roles for heparanase inside the CNS in both EAE and MS. Heparanase has been shown to be upregulated in the colonic

epithelium of patients with inflammatory bowel disease, a chronic inflammatory condition.²⁴² Because the mucosa responds to chronic inflammation by upregulating heparanase, it is suggested that in the ChP epithelium, similar expression of heparanase could occur during chronic inflammatory insult. Inside the CNS, van Horssen et al., (2006) identified that heparan sulfate distribution in active lesions of MS patients was discontinuous and irregular, which was not identified in the neural parenchyma of chronic inactive lesions or healthy controls. This suggests that in part, heparanase activity in the CNS may facilitate the development of lesions by digesting the ECM and facilitating further recruitment of pro-inflammatory leukocytes and mediators.²⁴³ Targeting heparanase within the CNS is a future goal of this project.

5.2. Limitations and future directions

The current study was limited in several areas, namely obtaining biological replicates for a number of experiments. One of the major impacts on research efficacy was a significant delay in shipping Transwell inserts due to the COVID-19 pandemic (8 months); so that we only had the time and materials to conduct a limited number of migration experiments with the HSI6-35 heparan sulfate mimetic before the conclusion of this thesis. In two separate models of proinflammatory trafficking (IFN γ mediated and ConA mediated), the HS-mimetic was capable of reducing migration, and although these are not replicates, the results show a general trend that this compound may be effective. In terms of interpreting the results of this study, this limits our capacity to draw any major conclusions about the efficacy of HSI6-35 on migration through a model of the choroid plexus, although this work will be continued farther in a doctorate degree.

A major caveat of this study was that we were not able to test HSI6-35 on encephalitogenic splenocytes isolated from animals with EAE. In our cytokine analysis, we saw that EAE splenocytes untreated with our heparan sulfate mimetic produced a much greater cytokine response, that could be ameliorated with HSI6-35 treatment. Because T cell heparanase response is also associated with the encephalitogenicity of invading lymphocytes and is upregulated during EAE, it is likely that we may see a greater impact on migrating leukocytes when heparanase expression is inhibited.¹⁴⁰ This experiment was planned and conducted in triplicate, but due to an incubator failure, this could not be seen to completion. This is the definitive next step for the continuation of this project. In addition to this, future experiments could involve transitioning to cultures of primary choroid plexus cells from healthy animals and EAE animals as our monolayer. This may provide a better replication of the *in vivo* state of this interface in both health and disease.

The Transwell cultures themselves were somewhat limited in mimicking the ChP *in vitro*. Primarily, this is due to the model being 'unifactorial', i.e., we only utilised one key chemokine identified as a major player in cell infiltration in MS and EAE: CCL5. In future experiments, the model could be upgraded to better reproduce the inflamed CNS by using other cytokines and chemokines upregulated in the CSF of MS patients. For example, CCL2 has been identified as another key chemokine that acts as a myeloid chemoattractant protein in EAE, whereas CCL5 does not. When CCL2 was suppressed, Robichon et al., (2020) found that there was decreased monocyte infiltration into the CNS of EAE animals.¹⁵² By including chemokines like CCL2, a population of cells not affected by CCL5 stimulation can be targeted. For these

cultures, we could establish an 'EAE media' of sorts, which provide leukocytes in culture with essential inflammatory mediators that more accurately mimic the environment inside the CNS of an EAE animal. In addition, a logical next step for this project would be to assess the expression of various chemokine receptors. During MS pathology; CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CXCL9 and CXCL10 have all been identified at lesion sites and reported in the CSF of patients experiencing active relapse at levels significantly higher than patients with non-inflammatory neurological disease.^{150,244,245} To assess which chemokines healthy versus encephalitogenic cells respond to, a panel of these cytokines could be applied, with flow cytometry to assess the expression or upregulation of the receptor complex.

In a similar manner, other immune cell stimulants could be utilized to study the migration of various subpopulations across our monolayer. For example, B cells have recently been identified as a pathogenic population in MS disease progression.⁶⁷ Although, it is not until the latter stages of disease that B cells enter the CNS, where they reside in tertiary lymphoid structures in the CNS and contribute to disease pathology. To study the effects of B cell migration into the CNS in the late-stage MS, a B-cell specific stimulus could be applied. For example, LPS stimulation of splenocyte cultures leads to polyclonal B cell activation, clonal expansion and the expression of inflammatory cytokines; IL-1, IL-6, IL-8, and TNF- α in a similar manner to ConA stimulation of T cells.²⁴⁶ This would allow us to assess the capacity of HSI6-35 to regulate inflammatory B cell trafficking across the choroid plexus in the context of EAE, which has not previously been elucidated.

One of the issues that we experienced with ConA or IFN γ stimulation of healthy splenocyte cultures was variability of the response. The appendix in chapter 3 elucidates how three different experimental replicates induce vastly different degrees of migration across our ECPC-4 monolayer. Although each experiment showed that ConA upregulated migration across the monolayer, the extent and significance varied greatly. This may be general variation due to external factors such as animal age or health, however it may also be representative of lab technique, as the difference in migration appeared to increase and become clearer with each replicate.

Interestingly, Stone et al., (2019) incorporate cultures of astrocytes, pericytes and microglia in the CNS compartment of their BBB transwell model. Future directions for this project include the co-culture of either resident stromal cells of the ChP or resident CNS immune cells such as microglia, which secrete soluble factors and provide key cell-cell interactions that will more accurately mimic this tissue and the

response of immune cells *in vitro*. Dysregulation of these cells has been documented in EAE and MS so will provide a more dynamic aspect to the system.²⁴⁷

It may prove beneficial to isolate supernatant from the upper and lower wells of our transwell assay following the experiment's completion to assess how the cytokine responses differ in cells that successfully navigate the ChP epithelium versus those who did not migrate. Similarly, creating a flow cytometry staining panel that allows us to look at T_H cell subsets will allow us to assess whether HSI6-35 skews T helper cell differentiation in favour of T_{reg} subsets versus T_H1/T_H17 responses as previously described in the literature.

To understand the mechanisms by which HSI6-35 is acting on both our ChP monolayer and leukocytes in culture, future experiments could investigate mRNA and protein expression. For the ECPC-4 or primary choroid epithelium, this would focus on whether the HS-mimetic downregulates expression of adhesion molecules or tight junction molecules, particularly in cell cultures that have been co-cultured with activated, proinflammatory leukocytes. This would assess the capacity of HSI6-35 to overcome the effects of proinflammatory cytokines in the media on the epithelial cells, which has previously been shown to upregulate adhesion molecules and increase the permeability of the ChP.²⁴⁸ For both immune cells and the epithelia, we will investigate whether heparanase expression is altered, to assess whether cells upregulate transcription when heparanase in the ECM is inhibited in a feedback loop. Additionally, the intracellular pathways that HSI6-35 may regulate have not been elucidated, but literature suggests that the MAP kinase pathway, specifically Erk1/2 phosphorylation may be at play. Using western blotting techniques, we aim to discover the intracellular mechanisms of HSI6-36.

We have not yet elucidated the mechanism by which HSI6-35 exerts its regulatory effects on migrating leukocytes. To assess if this regulation lies with inhibiting heparanase, or in suppressing proinflammatory cytokine release, we will use splenocytes from heparanase^{-/-} mice in the transwell model of the ChP and BBB. If there is a significant difference in migration across the monolayer with the knockout splenocytes, we can assess the amount that heparanase inhibition contributes to migration, and if this is a synergistic effect with inhibiting proinflammatory cytokine release. Gutter-Kapon et al., (2016) performed a similar experiment, mimicking the tumour microenvironment with heparanase^{-/-} macrophages. When heparanase expression was abolished, cell motility and phagocytosis was significantly reduced, and proinflammatory cytokine release was inhibited. This indicates that heparanase is a critical component of macrophage activation and recruitment.²⁴⁹

To understand if HSI6-35 will be clinically translatable, we will test the capacity of the mimetic against commercially available migration inhibitors for MS, particularly Natalizumab in the transwell migration model. Hopefully, at this time we will also have established an *in vitro* working model of the BBB, where we can additionally assess HSI6-35's activity against Natalizumab. A major research goal for this project is to show that HSI6-35 regulates, not completely ablates cellular migration into the CNS. This provides a platform for functional immunosurveillance to continue, while pathogenic lymphocytes and their mediators are unable to enter the CNS to exert their effects. A major factor contributing to the dangerous side effect profile of other migration inhibitors is the potential for PML due to the indiscriminate inhibition of lymphocyte entry into the CNS.⁶² We hope to show that HSI6-35 targets particular subsets of immune cells, suppressing their effector functions and capacity to migrate into the CNS. Indeed, preliminary evidence suggests that HSI6-35 exerts its effects primarily on T-lymphocytes. This can then be transitioned into animal research, using EAE mice to further develop the drug for clinical use.

Both progressive forms of MS are characterized by well established, chronic inflammation in the CNS, with a fading number of cells actively migrating in. At these stages, there is notable ventricular enlargement with brain atrophy and demyelination in the cortical matter as a result of tertiary lymphoid structure development.¹⁷ Diffuse demyelination throughout the brain is an excellent target for therapies that promote OPC recruitment and maturation, which in turn promotes remyelination and repair of the damaged tissue.²⁵⁰ Although our transwell model lacks the capacity to study remyelination capacity of HSI6-35, a transition to a mouse model will allow us to assess this. The cuprizone model is a diet-mediated inducer of demyelination in the corpus callosum, also leading to oligodendrocyte death. A recent study by Macchi et al., (2020) showed that the expression of genes related to HS-production in oligodendrocyte precursors were upregulated following demyelination, which was inversely correlated with lesion area and positively correlated with the remyelination potential. In addition, the study also reports that the expression of HSPG's by this population modulates the response of microglia in favour of an M2 phenotype, promoting healing responses. This suggests that HSPG's in the brain may perform important roles in the CNS following injury, and if HSI6-35 enters the CNS, it could exert positive effects on lesion resolution.²⁵¹ If this is possible, the capacity for repair could be applied to other neuroinflammatory conditions such as neuromyelitis optica spectrum disorders, in which demyelination and neuronal damage occurs as a consequence of astrocytic damage and is fatal in one third of patients.²⁵²

In conclusion, this research has made significant headway in developing a working transwell model of the choroid plexus using immortalised choroid plexus epithelial cells. We showed that the model could reproduce inflammatory leukocyte trafficking across the inflamed ChP. In addition, IFN γ was identified as a key factor that mediated trafficking across this structure. A potential mechanism for HSI6-35, a heparanase inhibitor was identified by cytokine analysis; suppressing the proinflammatory cytokine release associated with EAE leukocytes. Further work is required to assess if this is dependent on inhibition of the MAP kinase pathway associated with T cell activation and proliferation. Finally, the work in this thesis showed that both ConA-dependent and IFN γ dependent migration could be ameliorated with HSI6-35 treatment. This suggests that treatment with HSI6-35 may be effective at ameliorating disease in EAE splenocytes, and potential in MS patients. As with every scientist researching MS, it is our hope to one day provide patients with a safer, more effective treatment to manage their MS so that they feel like more confident, independent individuals in their daily lives.

LITERATURE

All figures in this thesis were created using **BioRender.com**, and all graph/chart elements were created using **Prism v8.0**.

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