IMMUNOMODULATORY EFFECTS OF INTERLEUKIN-4 INDUCED PROTEIN 1 (IL4I1) ON MULTIPLE SCLEROSIS PATIENT DERIVED LYMPHOCYTES

A Dissertation
Submitted to the Faculty of the
Graduate School of Arts and Sciences
Of Georgetown University
In partial fulfillment of the requirements for the
degree of
Doctor of Philosophy
in Neuroscience

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Washington, DC
June 8, 2018
ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory neurodegenerative disease of the central nervous system (CNS) that afflicts over 2.3 million individuals worldwide. The pathogenesis of MS involves autoimmune destruction of myelin but is poorly understood. Accurate biomarkers are yet to be identified and would provide valuable information to patients and their treating clinicians. Likewise, effective treatments are few and in high demand. Interleukin-4 induced protein 1 (IL4I1), a secreted enzyme expressed by immune cells, is a promising candidate for both roles.

Here we show that IL4I1 regulates the immune system via T-cell modulation, promoting CNS remyelination and improving motor symptoms in MS-model mice. Never before studied in human MS, IL4I1 is known to enhance immune evasion of some human cancers, suggesting that the immune-modulating properties of IL4I1 may extend beyond mouse models, to true MS. It has been proposed that IL4I1 targets TOB1, a Th17 antiproliferative protein inversely correlated with risk of developing MS. Therefore, it is possible that IL4I1 levels may also correlate with disease state.

To elucidate the role of IL4I1 on inflammation in humans and to identify its potential in patient remyelination, the inflammatory profiles of peripheral blood mononuclear cells (PBMCs) and blood plasma from healthy controls and MS patients are quantified at baseline, following T-cell stimulation, and after treatment with IL4I1. We find that endogenous IL4I1 expression is...
reduced in MS, and that IL4I skews T cells to a regulatory state in healthy controls. We confirm that IL4I1 promotes *TOB1* expression in healthy controls; however, we see no such effect in MS patients, who express reduced levels of *TOB1* at baseline. These results suggest that an IL4I1-TOB1 immunomodulatory pathway is perturbed in MS and may be a hallmark of disease.

Finally, we show that the *in vitro* immune-modulation of human lymphocytes by IL4I1 translates to enhanced remyelination *in vivo*. Focally demyelinated nude mice exhibit increased myelin staining following injection of human IL4I1-treated human lymphocytes into the lesion. We conclude that IL4I1 demonstrates potential as an immune-modulating and remyelination-enhancing therapeutic in MS, and that its relevance to MS warrants investigation in clinical studies.
This work is dedicated to my exceptionally supportive family.

To my mom, who taught me to be a scientist and who loves me unconditionally. Μανούλα μου γλυκιά, είναι αδύνατο να σου δείξω πόσο σ’αγαπώ. Μου έχεις δώσει όλα όσα έχω στη ζωή. To Soulie, who knows me better than I know myself, for being my advocate and giggle-fit partner since day one. To Bjoren, who has a bigger heart and more convoluted cerebral cortex than anyone I will ever know. To Naomi, whose mind and body inspired me to devote my PhD to studying MS, and to Teddy, who is very important to me.

I love you all,
Στεφανούλι
ACKNOWLEDGEMENTS

The work presented in this thesis could not have been completed without the collaborations upon which it was built. Thank you to the Georgetown University Multiple Sclerosis and Neuroimmunology Center for taking an interest in clinical research and identifying eligible patients for this study. Please relay my sincere appreciation to those de-identified individuals who donated their time, blood and hope for our mutual goal of improving the future of MS care. Thank you to my co-mentor, Dr. Anton Wellstein, so willingly extended his human sample expertise to me, as well as to the Georgetown University shared resources on which I relied for this work. Thank you to the Interdisciplinary Program in Neuroscience for surrounding me with talented teachers and friends, and to the Georgetown-Howard Universities Center for Clinical and Translational Science fellowship for two years of funding my research (NIH/NCAT, 1TL1TR001431). I’d also like to specifically thank Ms. Samantha Guerry and the TurnFirst Foundation for financially and intellectually supporting this work, in part through her contributions to the Georgetown University Medical Center Partners in Research grant.

I would be remiss if I did not acknowledge the incredible support and flexibility that I received from my thesis committee: Dr. Brent Harris, Dr. Faria Amjad, Dr. R. Scott Turner and Dr. Gregory Wu. I am proud to have assembled such a wonderful group of creative thinkers. Many thank yous to each and every member of the Huang Lab, specifically to Jingwen Hu, Helena Oft Mahesh Kumar and Dr. Sonia Nanescu who contributed most to my project. Thank you also to past lab members, Dr. Kelly Chamberlain for her continual support and Dr. Konstantina (Nada) Psachoulia for introducing me to the lab and inspiring me to pursue IL4I1. Finally, thank you to my advisor, Dr. Jeffrey Huang, for taking a chance and letting me embark on this journey away from the expertise of our lab, so that I could study patient samples. I hope that you will agree, it was a very fruitful endeavor.
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LIST OF ABREVIATIONS

AAM: Alternatively-activated macrophages

APC: Antigen presenting cell

aRRMS: Active relapsing-remitting multiple sclerosis

BBB: Blood-brain barrier

BCSFB: Blood-cerebrospinal fluid barrier

CAM: Classically-activated macrophages

CFI: Corrected immunofluorescence intensity

CIS: Clinically isolated syndrome

CNS: Central nervous system

DMD: Disease-modifying drug

Dpl: Days post-lesion

EAE: Experimental autoimmune encephalomyelitis

EBV: Epstein-Barr virus

EDSS: Kurtzke Expanded Disability Status Scale

ELISA: Enzyme-linked immunosorbent assay

EM: Electron microscopy

FDA: Food and Drug Administration

Gad+: Gadolinium enhancing

GMSNC: Georgetown University Multiple Sclerosis and Neuroimmunology Center

GU: Georgetown University

GU-CRU: Georgetown University Clinical Research Unit

GUH: Georgetown University Hospital
HC: Healthy control
HLA: Human leukocyte antigen
IHC: Immunohistochemistry
IL4I1: Interleukin-4 induced protein 1
JCV: John Cunningham virus
LAAO: L-amino acid oxidase
MBP: Myelin basic protein
MHC: Major histocompatibility complex
MOG: Myelin oligodendrocyte protein
MRI: Magnetic resonance imaging
MS: Multiple sclerosis
mTOR: Mammalian target of rapamycin
naRRMS: Non-active relapsing-remitting multiple sclerosis
NGC: Non-grafted control
OPC: Oligodendrocyte progenitor cell
PBMC: Peripheral blood mononuclear cell
PML: Progressive multifocal leukoencephalopathy
PNS: Peripheral nervous system
PPMS: Primary progressive phenotype
qRT-PCR: quantitative real-time polymerase chain reaction
RRMS: Relapsing-remitting multiple sclerosis
SLE: Systemic lupus erythematosus
SPMS: Secondary progressive multiple sclerosis
**TCSR:** Tissue Culture Shared Resource

**Th1:** Type 1 T helper cells

**Th2:** Type 2 helper cells

**Th17:** T helper 17 cells

**Treg:** Regulatory T cells

**TBS:** Translational Biomedical Sciences

**WT:** Wild-type
CHAPTER I: INTRODUCTION

A. THE HEALTHY CENTRAL NERVOUS SYSTEM: MYELINATION AND INFLAMMATION REGULATION

1. Basics of the Nervous System: Structure and Function

The nervous system functions through a network of cells capable of receiving and transmitting information. Nervous tissue is characterized by the presence of two distinct cell types: neurons, and glial cells, or glia. Neurons receive signals at their dendrites: short, branch-like extensions that are sensitive to changes in their environment. These signals are integrated at the cell body, and may be propagated as an electrical impulse down the length of the neuron’s primary extension, called an axon. At its destination, the axon splits into numerous terminal endings which are capable of producing a signal, in the form of a chemical neurotransmitter. This chemical message is received by the next receptive cell type(s) in the relay chain, whose identity depends on the function and target of the neuron innervating it. Myelin, an insulating layer wrapped tightly around the axon, allows for the quick and efficient propagation of this electrical signal (Castlefranco and Hartline, 2015; Nelson and Jenkins, 2017), which is necessary for the release of neurotransmitter messages to target cell(s). While critical to the efficacy—and even survival—of the neuron, this lipid-rich coating is synthesized and assembled by non-neural cells of the nervous system.

Glia are a heterogeneous group of cell types, including astrocytes, microglia and oligodendrocytes, with a wide range of important functions. Once thought to do little more than
hold neural cells together, glial cells are now known to play many critical roles in the nervous system. These roles include distribution of nutrients, removal of waste, protection against pathogens, and coordination and support of proper nervous system activity (Allen and Barres, 2009; Jäkel and Dimou, 2017; Newman, 2003). One such support mechanism, of particular interest to this thesis, is the production of myelin around the neuronal axon, a process called myelination.

The human nervous system is divided into peripheral and central components. A defining characteristic of all vertebrates, the central nervous system (CNS) is organized into a brain and dorsal spinal cord. These structures receive sensory input from the body, integrate this information, and in turn transmit signals that control the activity of the body. Branching out from the CNS, neural tissue comprising our peripheral nervous system (PNS) sends and receives signals between the CNS and the body that it influences. Collectively, these systems protect us from harmful deviations away from bodily homeostasis, and allow us to think, feel, move, perceive and interact with the world around us (Kandel, 2000). Both systems exert their effects through neuronal networks whose axons display varying (and dynamic) degrees of myelination, as accomplished by specialized glial cells within each system. The myelinating cells of the CNS are called oligodendrocytes. The CNS and its oligodendrocytes are of particular interest to this work.

2. Myelination of the Central Nervous System

Oligodendrocytes, the myelinating cells glial cells of the CNS, arise from oligodendrocyte progenitor cells (OPCs). Derived primarily from the spinal cord venricular zone, oligodendrocytes are the last to be generated in CNS development (Menn et al., 2006; Thomas et al., 2000). Driven by specific molecular cues, the differentiation of OPCs into mature oligodendrocytes and the subsequent myelination of CNS axons begins during embryogenesis (for review see Emery, 2010).
While the bulk of human CNS myelination is complete by 9 months of age, OPCs persist in abundance throughout much of life (Dimou et al., 2008; Rivers et al., 2008; Yeung et al., 2014). A rare instance of regeneration within the CNS, OPCs maintain their ability to proliferate and differentiate well into human adulthood; they do so in response to environmental cues and oligodendrocyte cell death (Hughes et al., 2013). Upon cellular maturation, oligodendrocytes attain myelinating capabilities. Myelin is composed of tightly wrapped layers of oligodendrocyte cell membrane (Fig. 1.1). Still a living appendage of the oligodendrocyte from which it extends, this membrane is spiraled tightly around an axonal segment, extruding cytoplasm from between its lipid-rich layers to form a compact, multi-layered myelin sheath (Snaidero et al., 2014). A single oligodendrocyte is capable of myelinating as many as 40 different axonal segments simultaneously, and thus exerts significant influence on the structure and function of the CNS (Michalski and Kothary, 2015).

**Figure 1.1.** Electron microgram of healthy myelinated axon. Cross-sectional view showing axon and surrounding myelin (modified from Siegel et al., 1999).

Oligodendrocytes serve the CNS in numerous ways. Through myelination, these cells heavily influence signal propagation along axons and between interacting neurons. Like the insulation that surrounds an electrical wire, myelin decreases the capacitance and increases the resistance of the axon. By reducing current loss over distance, myelin increases the rate at which electrical impulses propagate down an axon while keeping axonal diameter constant. This increases the speed and
fluency with which we can send, receive and respond to information about our environment without relying on larger-diameter axons (Castelfranco and Hartline, 2015). Effectively, the presence of myelin allows the CNS to meet the demands of our large and complex bodies in a space-efficient manner. Though best known for their role in neuronal signal propagation, oligodendrocytes are also important for neuronal health, releasing trophic factors and providing metabolic support through mechanisms independent of myelination (Nave and Trapp, 2008; Saab et al., 2013).

Neuronal tracts connecting highly communicative regions of the CNS are served by more robustly myelinated axons. Regions of the brain and spinal cord containing the myelinated axons of neuronal tracts are referred to as white matter, as their high lipid-density makes them light in color on gross anatomy. This is in contrast to gray matter, which contains a relatively high density of neuronal cell bodies (Fig. 1.2). Heavily myelinated white matter tracts include the dorsal column of the spinal cord, which carries ascending sensory information from the body to the brain; the cortico-spinal tract, carrying descending motor requests from the brain back down to the body; and the corpus callosum, forming a structural and functional connection between the left and right cerebral hemisphere (Purves et al., 2001).

Figure 1.2. Diagrammed representation of brain and spinal cord. Representative cross sections of human (left) and spinal cord (right) illustrating visible qualitative differences between gray matter and white matter. (Left image modified from Plum, 1988; right image modified from Drake et al., 2005)
3. **Remyelination of the Central Nervous System**

While regular myelin turnover is a dynamic process that persists through adulthood even in the absence of injury, myelin regeneration (remyelination) in response to trauma is a tightly-regulated process that is spontaneously initiated by the neuroimmune system. Injury to the CNS, such as by infection or stroke, may cause oligodendrocyte death and/or myelin degeneration (demyelination). Remyelination of a damaged area (a lesion) occurs readily and efficiently in response to acute damage (Jeffery and Blakemore, 1997; Lindner et al., 2008). This stepwise process is driven in part by the release of soluble protein messengers called cytokines. Inflammation resolution, followed by OPC recruitment and differentiation into oligodendrocytes, precedes successful remyelination in the healthy CNS (Patel et al., 2011; Mi et al., 2009). Though highly functional, remyelinated axons comport a thinner and denser myelin sheath as compared to the myelinated axon before injury (Hanafy and Sloan, 2011). This distinction is quantifiable using electron microscopy to visualize large axons such as those in sections of the spinal cord (Fig. I.3).

![Figure I.3. Electron micrograph of remyelinated axon.](image)

Cross sectional comparison between axon comporting original myelin (left) with a remyelinated CNS axon (right) (modified from Huang et al., 2011).

The interplay between inflammatory and regenerative processes in the CNS is tightly-controlled and time-specific. An initial spike in pro-inflammatory activity serves to draw resources to the damaged region, for example, by mobilizing and attracting the appropriate cell types necessary to
clear myelin debris, eliminate potential pathogens, and protect the area from subsequent insult. This aggressive pro-inflammatory response is short-lived; its timely resolution prevents excessive damage to healthy tissue, and creates a suitable environment for the repair process to proceed (Franklin and ffrench-Constant, 2008; Irvine and Blakemore, 2008). This resolution is accomplished by the release of cytokines, chemokines and growth-factors from astrocytes, microglia and other local cells. As inflammation subsides, the natural process of remyelination proceeds, with OPCs recruitment to the lesion and differentiation into myelin-producing oligodendrocytes. Healthy immune system activity is thus important to the successful remyelination of the CNS. Experimental focal demyelination performed in rodent CNS (described further in Section B.5) has allowed us to establish healthy timeframes for these hallmark events; OPC recruitment to the lesion is evident at 5 days post-lesion (dpl); differentiation of OPCs into oligodendrocytes/early remyelination is characteristic of 10dpl tissue, and remyelination is typically complete by 20 dpl (Jeffery and Blakemore, 1995).

4. Inflammation and the Healthy Immune Response

The immune system defends its host organism from potentially harmful invaders, or pathogens, by recognizing them as “non-self” and treating them as antigens, which trigger an immune response. A near prerequisite to life, systems of defense are present even in the most evolutionarily primitive lifeforms (Bickle and Kruger, 1993). Humans and other vertebrates have evolved innate (natural) and acquired (adaptive) forms of immunity, whose carefully orchestrated activities protect us from a host of potential pathogens including microorganisms, parasites, tumor cells and foreign tissue. Both innate and acquired immune responses are initiated, propagated and regulated by a spectrum of cytokines released by host immune cells (Ward and Rosenthal, 2014).
The innate immune system offers a first line of defense against infection by providing an immediate response to the chemical properties of antigens. Comprised of physical barriers (skin, mucous membranes) and specialized blood cells, the innate immune system does not require previous exposure in order to be activated (Turvey and Broide, 2010). Though they are derived from a common myeloid-progenitor, innate immune cells differentiate into distinct cell types with diverse and specified functions. Monocytes (termed macrophages in the CNS) consume and destroy bacteria, foreign cells and dead or damaged host cells. Monocytes also secrete chemokines, cytokines that direct fellow innate immune cells to act appropriately. These other cells include neutrophils and eosinophils that release bacteria-killing enzymes, basophils and mast cells that increase blood flow to the area though targeted histamine release and natural killer cells that destroy the membranes of infected host cells and cancer cells. The innate immune response can also activate the complement system, a complex cascade of over 30 regulatory proteins circulating in the plasma, that can destroy or neutralize pathogens through a variety of mechanisms (Clarke and Tenner, 2014).

Acquired immunity elicits a slower response in which specialized immune cells encounter a new antigen, “learn” how to attack it, and “memorize” it for a rapid and targeted response upon subsequent encounters a specific pathogen. The acquired immune response consists of four general steps: recognition of the antigen, activation and mobilization of resources, regulation and resolution. The specialized blood cells responsible for acquired immunity, called lymphocytes or white blood cells, consist of thyroid-derived T cells, which mediate cellular immunity, and bone-marrow derived B cells, which conduct the humoral immune response (Medzhitov and Janeway, 1998).
4.1 Recognition

Self-identification proteins, called major histocompatibility complex (MHC) proteins, are present in a host-specific combination on the surface of all host-cells. This personalized expression pattern is genetically determined at the human leukocyte antigen (HLA) gene locus. In humans, the MHC complex is also referred to as the HLA complex. Expressed on the surface of cell membranes, these glycoproteins allow the immune system to distinguish “self” from “non-self”. As such, cells lacking the appropriate molecular signature, that are encountered in circulation by the immune system, are identified as “non-self” and are attacked. Antigen presenting cells (APCs), such as macrophages, dendritic cells and B cells, recognize, engulf and disassemble the foreign body. APCs then display characteristic fragments of the intruder on their membrane-bound HLA proteins, and present them as antigens to circulating T cells. T-cell receptor binding to the antigen-displaying MHC protein on the surface of the APC results in T-cell activation (Viret and Janeway, 1999).

4.2 Activation and Mobilization

Once activated, T cells of various subtypes are able to produce a wide range of responses aimed at eliminating the pathogen. This T-cell mediated branch of the immune response, called the cellular immune response, involves activation, mobilization and proliferation of cytotoxic T cells and helper T cells. Antigen presentation to CD8+ “cytotoxic” T cells activates the cell and familiarizes it with the molecular signature of a given pathogen. Once activated, CD8+ T cells search for and ingest the pathogen or destroy it by releasing cytotoxic enzymes.

T cells possessing the CD4 surface protein, CD4+ T cells or T helper cells, are involved in a wide variety of immune processes. T helper cells release cytokines that induce proliferation and
differentiation of activated lymphocytes, and promotes their entry into circulation. This process, called clonal expansion, magnifies the acquired immune response. When an APC binds to a naïve CD4+ T cell, it activates the T cell and induces its differentiation into one of many function-specific subtypes as suited to treat the threat at hand. The four dominant CD4+ subtypes, which are also of interest to this thesis, are type 1 T helper cells (Th1), T helper 17 cells (Th17), type 2 helper cells (Th2) and regulatory T cells (Treg). Th1 and Th17 subtypes are pro-inflammatory. Th1 cells secrete immune-stimulating cytokines (e.g. IFNγ, TNFα) responsible for cell-mediated immunity against intracellular bacteria and viruses. Th17 cells produce pro-inflammatory cytokines (e.g. IL17, IL22) that are responsible for cell-mediated immunity to extracellular pathogens and fungi. Treg cells and, often, Th2 cells, are able to modulate and resolve the immune response. By secreting anti-inflammatory cytokines (e.g. IL4, IL13), Th2 cells protect us against extracellular parasites. Th2 cells are also responsible for B-cell activation, acting as a bridge between T-cell mediated cellular immunity and B-cell mediated humoral immunity (Luckheeram et al., 2012).

In humoral immunity, antigens encountered by B cells are engulfed and displayed on the B-cell surface. When this B cell contacts a helper T cell that has been activated by the same antigen, the B cell differentiates into effector B cells, or plasma cells, and memory B cells. Plasma cells produce free-floating antibodies that are specific to the antigen that triggered a given response. These antibodies are released into the general circulation, recognize the antigen against which they were produced, and assist in its destruction and removal. Meanwhile, memory B cells are dormant but activate quickly upon subsequent encounters with the same antigen. This secondary immune response is more rapid, robust, and highly specific to the antigen in question (Quintin et al., 2014).
4.3 Regulation and Resolution

The immune system’s reactivity to damage and infection is requisite to our survival. However, the body’s return to homeostasis is critical to readiness for subsequent attacks (Barton, 2008). Treg cells are responsible for tamping down inflammation after its job is done. By releasing immunosuppressive cytokines (e.g. IL10, TGFβ), these cells regulate and resolve the immune response. During resolution, activated immune cells stop receiving the pro-inflammatory cytokine signals that had kept them active. With the exception of memory cells that are reactivated upon subsequent antigen exposure, activated immune cells undergo apoptosis in the absence of stimulation, and are cleared from circulation. The immune system can then replenish its resources in preparation for a future threat, and the body can begin its reconstruction (Ayala et al., 2003).

5. Immune Regulation in the Central Nervous System

The structures and processes of the immune system, as they pertain to protection of the CNS, are referred to as the neuroimmune system. With limited ability to regenerate, the CNS interacts with the immune system in a tightly regulated manner. This relative “immunologic privilege” protects the brain and spinal cord from damage associated with inflammation (Gimsa et al., 2013). This protection is accomplished by specialized structures that act to separate the CNS from circulating blood and its contents, creating the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB).

With the exception of specialized zones and circumventricular organs, the CNS is physically isolated from peripheral blood by the BBB and BCSFB. These barriers serve to protect the CNS from damage by restricting passage of potentially harmful molecules and cell types while still promoting CNS health by assisting in the delivery of nutrients and removal of waste from the
brain and spinal cord. The regulatory functions of the BBB/BCSFB are made possible by the highly selective semipermeability of its specialized structural elements. Unlike elsewhere in the body, capillaries in the brain are surrounded by endothelial cells forming tight junctions with the basal lamina. These endothelial cells are surrounded by astrocytic protrusions, called “end-feet”, forming the thick basement membrane of the BBB. In this capacity, astrocytes provide structural support to this barrier, while also contributing to blood flow in the brain (Figley and Stroman, 2011). The BCSFB functions similarly to the BBB, acting through specialized epithelial cells of the choroid plexus and the surrounding arachnoid membrane (Johanson et al., 2011).

During healthy homeostasis, leukocyte penetration into the CNS is minimal, and immune responses within the CNS are not sustained (Muldoon et al., 2013). BBB integrity decreases naturally with aging and pathologically in some neurodegenerative disorders. Permeability of the BBB is enhanced by inflammatory mediators including cytokines, prostaglandins and bradykinin. T-cell infiltration occurs more readily when in an activated state, and is characteristic of chronic neuroinflammatory disease.

6. Immune Dysregulation and Autoimmunity

The health of our innate and adaptive immune systems are critical to our survival, and it exists in a delicate and well-timed balance between activation and quiescence. An under-responsive immune system puts us at increased risk of infection and disease. Failure to recognize and destroy tumor cells, for example, underlies oncogenesis and cancer progression (Hanahan and Weinberg, 2000). Meanwhile, excessive or chronic immune activity is also maladaptive, and underlies common allergies and autoimmune conditions. The relationship between cancer and autoimmune disease is further discussed later in this chapter (see Chapter I.C.1). Autoimmunity results from
the body’s inappropriate recognition of “self” as “non-self”. This aberrant inflammation causes rejection of healthy tissue and manifests clinically in accordance with the tissue being targeted. Chronic inflammation can cause widespread tissue damage though oxidative stress, subsequent molecular cascades and cell death (Trapp and Stys, 2009). Relevant to this thesis, targeted autoimmune destruction of myelin, breakdown of the BBB and chronic inflammation in the central nervous system underlie the development and clinical manifestations of multiple sclerosis (MS).
B. MULTIPLE SCLEROSIS: DYSREGULATED INFLAMMATION AND IMPAIRED REMYELINATION

1. Research Motivation

Multiple sclerosis is a heterogeneous and so far unpredictable disease. Virtually every aspect of MS—its etiology, clinical presentation, rate of progression and response to treatment, to name a few—varies from case to case (Compston and Coles, 2008; Reich et al., 2018). It has even been proposed that multiple sclerosis is not one single disease, but a combination of immune and neurodegenerative disorders (Louapre and Lubetzki, 2015; Steinman, 2001). We do not know what causes MS. Various clinical/radiological and epidemiological findings have fueled numerous theories of MS etiology, the most intriguing and relevant of which, are mentioned throughout this section. What is known, is that the MS experience is different for each person, which makes individualized care paramount to improving patient outcomes. While its heterogeneity and unpredictability makes MS difficult to understand, these qualities also make it a fascinating and important disease to study, treat, and cure.

2. Clinical Presentation, Diagnosis and Prognosis

The potential symptoms associated with multiple sclerosis are protean. Physical symptoms vary between individuals, but commonly include numbness, pain (Solaro, 2013; Gaby, 2013), difficulty walking (Kieseier and Pozzilli, 2012), urinary incontinence (Gallien and Robineau, 1999), fatigue, tremor, and other sensory-motor disturbances (Lublin et al., 2014). Because the optic nerve is a heavily myelinated sensory apparatus and target of MS pathogenesis, patients frequently present
with visual disturbance early in the course of the disease (Hickman et al., 2014). Clinical effects of MS are not confined to physical symptoms. Increased rates of cognitive decline in the MS population are well documented. Likewise, about 50% of individuals with MS experience comorbid depression, resulting in a devastating 7 to 8-fold increase in risk of suicide in the MS population compared to global rates (Compston and Coles, 2008).

2.1 Clinical Subtypes
Clinical presentation varies considerably across demographic groups and even between individuals; however, a generalized time-course of disability (Fig. I.4) delineates two distinct but overlapping clinical phases: relapsing-remitting MS (RRMS) and secondary progressive MS (SPMS) (Dutta and Trapp, 2014). Typically, MS is preceded by an apparently isolated instance of neurologic impairment, or clinically isolated syndrome (CIS). After this symptomatic period, or “attack”, CNS activity returns to normal and symptoms may not recur for years, if at all (Miller et al., 2012). At the first recurrence of symptoms, clinically definitive MS enters the differential diagnosis. It is estimated that 80% of CIS cases develop clinically definitive MS within 3 years of the initial attack (Compston and Coles, 2008). A combination of clinical, radiological and lab findings are considered in the diagnosis of MS (Thompson et al., 2018), which are further described in section 2.2.

Relapsing remitting MS is characterized by a repeating pattern of acute impairment flanked by prolonged asymptomatic periods that can last for months or years (Fig. I.4). At the time of diagnosis, 85% of patients present with this phenotype. Over time, the frequency of attacks may increase, shortening the interspersed periods of disease remission. With disease progression, remission also becomes less complete, as chronic disability accumulates. After years, or even
decades, of increasingly frequent relapses and decreasingly successful remission, 45-65% of RRMS patients gradually transition to a progressive form of the disease.

Secondary progressing MS is characterized by a linear accumulation of disability and irreversible decline (Fig. I.4). A smaller subset of individuals with clinically definitive MS experience a similarly steady accumulation of symptoms from disease onset, without first experiencing RRMS. (Compston and Coles, 2008; Thompson et al., 2018). This primary progressive phenotype (PPMS) is not further discussed in this thesis due to its low prevalence and resulting absence from the patient population studied in this work.

![Schematic representation of disability over time leading up to and including clinically definitive MS](image)

**Figure I.4.** Schematic representation of disability over time leading up to and including clinically definitive MS. Depicts symptomatic progression from relapsing remitting (RRMS) to secondary progressive phase (SPMS). CIS = clinically isolated syndrome (modified from Compston and Coles, 2004).

### 2.2 Diagnostic Criteria and Measurements

Currently regarded as the gold standard for MS diagnostics, the McDonald criteria include evidence of clinical disability, positive radiological findings, and, if needed, an elevated IgG index (indicating intrathecal production of IgG antibodies) (Thompson et al., 2018). Radiology, specifically magnetic resonance imaging (MRI), can also provide meaningful data throughout the course of disease. Due to changes in water content, lesioned areas may appear as dark holes (if T1-weighted) or bright spots (if T2-weighted) on imaging. (Fig. I.5). Common sites of lesion
distribution suggestive of MS include the optic nerve and chiasm, periventricular white matter, subpial cortex, brainstem, and cervical spinal cord (Popescu and Lucchinetti, 2012).

Figure I.5. Brain MRI of individual with MS. Lesions appear as bright spots on T2 (FLAIR)-weighted image (left). Those that represent chronic damage appear black on T1-weighted image (right) (Chen, 2012; courtesy of Daniel Reich, NINDS).

Changes in inflammatory activity, as well as the dissemination of lesions through anatomical space and across the disease time-course, all illustrate the often dynamic nature of MS. Specific visual characteristics vary with disease state. For example, the appearance of gadolinium, a peripherally administered tracer, within a CNS lesion indicates an active inflammation associated with a breach in the BBB. Such gadolinium enhancing ( gad+ ) lesions are characteristic of actively relapsing disease. This is in contrast to radiological findings associated with progressive stages of MS, which depict areas of chronic demyelination and cell neuronal death, with little to no inflammatory activity.

Standardized clinical disability scales, such as the Kurtzke Expanded Disability Status Scale (EDSS), may also be used to help assess symptom severity, disease progression and response to treatment (Kurtzke, 1983). Other standardized measures include the MS Functional Composite, which considers physical as well as cognitive function (Meyer-Moock et al., 2014).
3. Epidemiology and Risk Factors

MS affects over 2.3 million people worldwide (Vos et al., 2015). Since this official 2015 estimate, rates of MS diagnosis continue to increase. Recent preliminary data estimates nearly 1 million cases in the US alone (National Multiple Sclerosis Society [NMSS], 2017). MS is the most common cause of immune-mediated CNS dysfunction (Berer and Krishnamoorthy, 2014; NMSS, 2017). With diagnosis typically occurring between 20-40 years of age, symptoms debilitate individuals during what would otherwise be highly productive periods of their lives. While the etiology of MS is not well understood, it appears to be multifactorial in origin, with clear genetic and environmental influences.

Like most autoimmune diseases, MS prevalence is greater in females compared to males, occurring in a 3:1 ratio. There is a 1-3% chance of developing MS in individuals born to a parent or having a sibling with the disease, and a 30% concordance rate between monozygotic twins (Compston and Coles, 2008). An established association exists between MS and specific MHC allele genotypes, specifically DRB1*1501, DRB5*0101, DQA1*0102 and DQB2*0602 (Olerup and Hillert, 1991). Meanwhile, HLA-C5 (Yeo et al., 2007) and HLA-DRB1*11 (Dean et al., 2008) appear to confer a protective effect. While there is strong evidence for genetic predisposition to MS, most people with any given genetic risk factor do not develop the disease, suggesting that the etiology of MS is multifactorial.

There are several known environmental risk factors for developing MS. With some geographical exceptions, the incidence of MS increases with distance from the equator and decreased average exposure to sunlight, with MS prevalence peaking in northern European region (Compston and Coles, 2008). Furthermore, individuals with MS exhibit reduced serum vitamin D levels as compared to healthy controls (Ashcerio et al., 2010). Together, these findings support the
vitamin D hypothesis of MS etiology, which proposes that vitamin D is protective against MS while vitamin D deficiency underlies development to the disease (Ascherio and Munger, 2007). Infection with measles, mumps, rubella and/or Epstein-Barr virus (EBV) during young adulthood, as compared to infection during childhood, also correlates with greater subsequent risk of MS (Compston and Coles, 2008; Martyn et al., 1993). This, among other findings, supports the hygiene hypothesis of allergic disease, which posits that early exposure to antigens is critical to healthy immune system development and function later in life (Compston and Coles, 2008). Molecular similarities between viral proteins and myelin-associated proteins lends support to the idea that autoimmune disease etiology results from molecular mimicry: the cross-activation of immune cells by pathogen-derived peptide sequences that highly resemble “self”-derived peptide sequences (Kohm et al., 2003). For example, structural homology has been identified between EBV and myelin basic protein (MBP) as well as between the rubella virus and myelin oligodendrocyte protein (MOG) (Cong et al., 2011). It is thus theorized that MS pathogenesis involves the production of antibodies that recognize both EBV (or rubella virus) and, inadvertently, MBP (or MOG). MBP and MOG are important components of myelin and are frequently targeted in clinical MS and animal models of the disease.

4. Pathophysiology

Multiple sclerosis is a chronic inflammatory disease characterized by immune-mediated myelin degeneration and impaired remyelination. The relapsing remitting pattern of MS symptoms is thought to reflect the interplay between CNS inflammation/damage and CNS repair. During each attack, pathological levels of inflammation dominate the CNS environment, damaging critical components of this important system. Cytokines released by pro-inflammatory cells promote
inflammatory responses (e.g. Th1, Th17; IFNγ, TNFα, IL17). While controlled inflammation is critical for survival of cells and organisms, aberrant or excessive inflammation results in cytotoxicity, tissue injury, and appears to prevent natural mechanisms of repair (Compston and Coles, 2008).

Periods of symptom remission occur when inflammation subsides and repair/remyelination processes are allowed to progress. Anti-inflammatory responses are mediated by cytokine release from anti-inflammatory or immune regulatory cells (e.g. Treg, Th2; IL10, TGFβ). This cycle results in the relapsing-remitting pattern experienced by RRMS patients (Imitola et al., 2005). In these early stages of the disease, spontaneous OPC recruitment, differentiation, and remyelination of lesioned areas is evident. In more progressed stages, however, spontaneous remyelination fails (Fig. 1.6). What causes the progression from successful remyelination to progressive disease is poorly understood, and the subject of active research. In this thesis, I discuss the idea that overwhelming inflammation prevents spontaneous repair/remyelination and underlies the progression from RRMS to SPMS. In turn, a persistent deficit in myelin/oligodendrocyte-derived support causes irreversible neuronal damage and progressive cell death, producing the clinical phenotype that defines SPMS.

**Figure 1.6.** Postmortem cross-section of MS brain. Reveals areas of successful remyelination and failed remyelination (modified from Franklin, 2002).
Neurological disability, BBB compromise, and inflammation within the CNS are established characteristics of MS pathophysiology. However, the sequence and proposed causal relationship of these events as they pertain to MS pathogenesis, pathophysiology and disease progression, remains unclear. The outside-in hypothesis posits that peripherally-derived autoreactive T cells penetrate the CNS, initiating the inflammation that produces subsequent neurological damage (Mahad et al., 2015). This is in contrast to the inside-out hypothesis, which asserts that inflammation is secondary to an initial neuronal disturbance (Trapp and Nave, 2008). Regardless of sequence and causality, most treatment options currently approved for MS are immunosuppressive or immunomodulatory. Effective therapies focused on neuro-regeneration or neuroprotection are yet to be developed (Rice, 2014; Stangel, 2008).

5. **Experimental Rodent Models of Multiple Sclerosis**

Much of what clinicians and scientists understand about MS has been learned from animal models of disease. The work presented in this thesis was performed using two well-established mouse models of MS pathophysiology and behavior: toxin-induced focal demyelination and experimental autoimmune encephalomyelitis (EAE). The experimental focal demyelination rodent model allows researchers to study, characterize and manipulate the biological processes involved in CNS remyelination. In this model, demyelination is induced via injection of a myelin-specific toxin, such as lysolethicin, into white matter of the brain or spinal cord. By selectively targeting myelin, remaining cell types and activities are preserved; this allows us to trace the cellular and molecular repair processes that follow demyelination though specific analysis of lesioned areas (Denic et al., 2011).
The experimental autoimmune encephalomyelitis (EAE) mouse model, a commonly used preclinical model for MS research, is also used in this thesis. This model allows us to assess the behavioral outcomes of these processes on a living, whole-organism level and is thought to resemble some aspects of MS pathogenesis, including immune-mediated CNS demyelination and axonal injury (Kornek et al., 2000; Nikić et al., 2011). Autoimmunity against myelin is induced by inoculation with an abundant protein or amino acid sequence of myelin (typically MOG₃₅-₅₅) followed by pertussis toxin, to ramp up the immune response. The resulting motor impairment and scoring system for EAE are detailed in Chapter II.

6. Current Standards of Care

The current standard of care for MS treatment includes relapse treatment and prevention. Intravenous corticosteroid (e.g. methylprednisolone or prednisone) is typically administered to treat acute relapses, which acts to suppress immune activity. Plasma exchange and intravenous immunoglobulin therapy may also be indicated to treat discrete inflammatory attacks.

Numerous disease modifying drugs (DMDs) have been approved by the U.S. Food and Drug Administration (FDA) for chronic use in RRMS, some of which I describe here. These drugs act to decrease the frequency and severity of relapses by modifying how the immune system functions. First-line treatment currently includes Interferon-β (Avonex) and glatiramer acetate (Copaxone). Avonex, which is administered as a subcutaneous or intramuscular injection, suppresses antigen presentation by APCs as well as entry of lymphocytes into the CNS. Side effects include flu-like symptoms and the production of neutralizing antibodies that render the drug ineffective. Copaxone is an injected amino acid random polymer that mimics the amino acid sequence of MBP, acting as a decoy for the immune system. Copaxone also antagonizes APC
activity and increases the Th2-associated cytokine response. Other FDA approved drugs include intravenously infused monoclonal antibodies. Alemtuzumab (Lemtrada) is an anti-CD52 antibody that depletes the body’s source of mature lymphocytes, suppressing the immune activity that is thought to underlie disease relapse. Like all immunosuppressive drugs, side effects are widespread and include increased risk of infection. Natalusumab (Tysabri), an anti-α4β1 integrin antibody, prevents leucocyte adhesion to vascular endothelia. This prevents T-cell binding and penetration into the CNS (Rommer et al., 2014). Serious side effects include progressive multifocal leukoencephalopathy (PML), caused by activity of the John Cunningham virus (JCV), which is harbored and actively suppressed by 70-90% of humans. Four out of 1000 Tysabri treatment cases result in PML (Warnke et al., 2015). Physical rehabilitation and cognitive therapy are also frequently used tools to help improve comprehensive patient outcome.

While there are currently upwards of 15 FDA approved drugs indicated to treat RRMS, drug safety, efficacy and availability are often problematic. Furthermore, all pharmacological treatment options to date focus on relapse prevention and symptom mitigation in RRMS. No therapies capable of disease reversal have been identified, and none have been proven effective against progressive disease (Stangel, 2012). Understanding the pathophysiology of disease progression is requisite to developing therapies that can prevent, reverse, and ultimately cure MS.

Our findings demonstrate that controlling inflammation can reinstate an environment permissive of the body’s innate mechanisms of repair, preventing disease progression before irreversible damage occurs. In this thesis, I posit that interleukin-4 induced protein 1 (IL4I1) demonstrates potential in controlling CNS inflammation to this end. As an endogenously expressed protein, IL4I1 is likely to be better tolerated; this is an attractive feature in the context of current therapy safety profiles.
C. INTERLEUKIN-4 INDUCED PROTEIN 1 (IL4I1): INFLAMMATION REGULATION AND IMPLICATIONS IN MYELIN REGENERATION

1. Interleukin-4 Induced Protein 1

Il4I1 is a secreted L-amino acid oxidase (LAAO) expressed by immune cells. As an LAAO, IL4I1 oxidizes L-amino acids, namely phenylalanine, to their keto-acid forms, producing \( \text{H}_2\text{O}_2 \) and \( \text{NH}_3 \) in the process. This protein shares significant primary structural homology with a component of snake venom (Raibekas and Massey, 1998), and was originally discovered as a product of B cells (Chavan et al., 2002). However, IL4I1 is now known to be expressed predominantly by mature dendritic cells and macrophages upon stimulation by IL4 (Cousin et al., 2005; Lasourdis et al., 2011; Marquet et al., 2010). Particularly high expression has been detected in microglia, the resident immune cells of the CNS (Zhang et al., 2014: Fig. I.7). This has been confirmed by our lab, which detected high IL4I1 expression from a subtype of monocyte-derived macrophages (Psachoulia et al., 2016). Termed alternatively-activated macrophages (AAM), these IL4I1-expressing macrophages have an anti-inflammatory phenotype. This is in contrast to classically activated-macrophages (CAM), which are the other end of the phenotype spectrum, and tend to have a pro-inflammatory effect. In humans, \( IL4I1 \) has been mapped to chromosome 19q13.3-13.4. This chromosomal region has an established association with autoimmune susceptibility, already linking it to SLE, rheumatoid arthritis, diabetes type 1, and even MS (Becker et al., 1998; Chavan et al., 2002). Despite its suggestive location, IL4I1 had not yet been examined in the context of any autoimmune disease, including MS.
2. **IL4I1 in the Clinical Literature**

While our lab is the first to study this protein in the CNS, IL4I1 is referenced in scientific literature primarily in the context of oncology. Increased IL4I1 levels have been implicated in several B-cell lymphomas and lymphoid tumors (Lasourdis et al., 2011). Strikingly, *IL4I1*-expressing tumors exhibit an enhanced ability to evade the host immune system. By inhibiting CD8^+^ T cell antitumor responses, for example, IL4I1 increases the survival of cancer cells (Bod et al., 2017a). As mentioned previously in this chapter (see Chapter 1.A.6), the relationship between cancer and autoimmune diseases such as MS, is noteworthy. While autoimmunity is the result of an overactive immune system recognizing “self” as “non-self”; in essence, cancer is the exact opposite: the result of an underactive immune system that is recognizing “non-self” as “self”. While they are ‘opposite’ problems, they are in a way the *same*: the result of a dysfunctional immune response. An understanding of the same basic phenomenon is the crux of advancement in both fields. IL4I1’s proven *harm* in tumor biology makes it a possible *therapeutic* in autoimmune disease.

**Figure 1.7. Il4i1 is expressed by microglia.** Cellular expression of Il4i1 as reported in RNA-sequencing transcriptome and splicing database of glia, neurons and vascular cells of the cerebral cortex reveals. FPKM = Fragments Per Kilobase of transcript per Million mapped reads (Zhang et al., 2014).
3. **IL4I1 in the Central Nervous System**

To our knowledge, we are the first to study IL4I1 in the CNS. The potential involvement of IL4I1 in remyelination was identified upon analysis of a previously published rat remyelination transcriptome resulting from the post-doctoral work of my mentor, Dr. Jeffrey Huang (Huang et al., 2011). This work identified Il4i1 gene expression as being highly upregulated during late inflammation, preceding remyelination, in a rodent model of MS. The timing and reproducibility of this expression pattern suggests that Il4i1 expression may be a driving force behind the important cellular changes taking place during this time. Because IL4I1 has not been widely studied, much is left to be discovered including how it might improve medicine. The promise of IL4I1 in this domain will be discussed in later sections of this thesis (see Chapter III.A.1).
CHAPTER II: MATERIALS AND METHODS

A. MATERIALS AND METHODS: CHAPTER III

1. Mice

All experiments were performed in accordance with approved Institutional Animal Care and Use Committee (IACUC) protocols of Georgetown University. C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and Charles River. *Il4i1*–/– mice were purchased from MMRRC (UC Davis, CA).

2. Focal Spinal Cord Demyelination

Focal demyelination was induced by injecting 1.0% lysolecithin (Sigma-Aldrich Company Ltd, Dorset, UK) in saline into the spinal cord ventral funiculus of male or female wildtype C57BL/6 or *Il4i1*–/– mice at 10-12 weeks old. For treated animals, 200 ng/ml of recombinant mouse IL4I1 (R&D Systems) was co-injected along with 1.0% lysolecithin into the ventral spinal cord. The animals (n=3-5 in each group) were sacrificed at 3, 5, 10, 15 and 20 days after surgery for analysis.

3. Experimental Autoimmune Encephalomyelitis (EAE) and Recombinant IL4I1 Therapeutic Treatment

EAE INDUCTION: C57BL/6 female mice (Charles River) at age 10 weeks were acclimatized for 7 days. EAE was induced according to the Hooke Laboratories protocol1. Briefly, mice were immunized by subcutaneous (s.c.) injection of an emulsion of MOG35-55 in complete Freund's
adjuvant (CFA) above the cervical and lumbar spine (Day 0), followed by administration of pertussis toxin (PTX) in PBS intraperitoneally (i.p.), first on the day of immunization (Day 0), and then again the following day (Day 1). Pre-filled MOG_{35-55}/CFA Emulsion syringes and PTX were obtained from Hooke Laboratories (Cat. No: EK-2110). Each 1mL syringe contains ~1mg MOG_{35-55}/mL emulsion, ~2-5mg killed mycobacterium tuberculosis H37Ra/mL emulsion (all concentrations adjusted by lot for consistent EAE induction). Emulsion was administered s.c. at two sites, 0.1mL/site (0.2 mL/mouse total). PTX was administered i.p. at 0.13mL/dose, and repeated 24 hours later. Approximately 250ng PTX/dose or 2.5µg/mL for each of the two PTX administrations were used. The mice were scored blindly and daily from EAE Day 7 until EAE Day 35 according to the protocol from Hooke Laboratories.

EAE SCORING: The scoring system used was as follows: 0.0 No obvious changes in motor function; 0.5 Tip of tail is limp; 1.0 Limp tail; 1.5 Limp tail and hind leg paralysis; 2.0 Limp tail and weakness of hind legs OR signs of head tilting; 2.5 Limp tail and dragging of hind legs OR strong head tilting; 3.0 Limp tail and complete paralysis of hind legs OR Limp tail with paralysis of one front and one hind leg; 3.5 Limp tail and complete paralysis of hind legs plus mouse unable to right itself when placed on its side; 4.0 Limp tail, complete hind leg and partial front leg paralysis. Mouse is minimally moving but appears alert and feeding; 4.5 Complete hind and partial front leg paralysis, no movement around the cage. Mouse is not alert; 5.0 Mouse is found dead due to paralysis OR Mouse is euthanized due to severe paralysis.

IL4I1 TREATMENT IN EAE: For IL4I1 therapeutic treatment studies, mice were housed in groups of five per cage and identified by ear notches. The mice that developed EAE were then
randomly assigned into treatment or untreated group within each cage in a balanced manner to achieve groups with similar time of EAE onset and similar onset scores (as recommended by Hooke Laboratories). Treatment began at the time of EAE onset. Recombinant mouse IL4I1 (≈1.5µg/dose/mouse) was diluted in 1XPBS and injected intravenously (i.v.) into the tail vein at clinical score 2.0-2.5. A second dose was injected after three days of rest. The clinical scores and weight of mice were recorded daily until the end of experiment. Treated mice were anesthetized with isoflurane before IL4I1 injections to minimize stress, while untreated mice were not handled. For each experiment, ten mice were analyzed for IL4I1 treatment, and ten mice were analyzed as untreated control. The experiment was repeated three separate times.

4. RNA Extraction, cDNA Synthesis and qRT-PCR

For cells, total RNA was extracted by the RNeasy Micro kit (QIAGEN). For tissue, total RNA was isolated using the TRIzol™ Reagent protocol² (Life Technologies) and homogenizer (Argos). PCR primers were purchased from Bio-Rad. Sybr-Green RT-PCR was performed using the SsoAdvanced Universal SYBR® Green Supermix (BioRad) and analyzed by the CFX96 Touch™ Real-Time PCR Detection System (BioRad). Results were normalized against peptidylprolyl isomerase A (Ppia) and expressed as mean ± SEM. Ppia is a recommended normalization factor for gene expression studies (Gong et al., 2014).
5. **Antibodies and Cytokines**

Recombinant mouse IL4I1 was obtained from R&D Systems (Minneapolis, MN). The following antibodies were used for immunohistochemistry. Primary antibodies: rat anti-CD11b (1:100; AbD Serotec), rabbit anti-Ym1 (1:100; Stemcell Technologies), mouse anti-iNOS (1:50; BD Pharmingen), rabbit anti-Olig2 (1:300; Millipore), mouse anti-CC1 (1:300; Millipore), mouse anti-Nkx2.2 (1:100; DSHB), mouse anti-GFAP (1:400; Sigma-Aldrich). **Secondary antibodies:** Alexa Fluor® 488 Goat Anti-Rabbit IgG (1:1000), Alexa Fluor® 488 Goat Anti-Rat IgG (1:500), Alexa Fluor® 594 Goat Anti-Mouse IgG (1:1000), Alexa Fluor® 594 Chicken Anti-Goat IgG (1:500) and Alexa Fluor® 594 Goat Anti-Rat IgG (1:500). **Flow cytometry primary antibodies:** PE/Cy7 anti-CD4 (BioLegend), Brilliant Violet 711 anti-T-bet (BioLegend), PE anti-RORγt (BD Pharmingen) and PerCP/Cy5.5 anti-Gata3 (BioLegend). Live/Dead Fixable Yellow Dead Cell Stain Kit (Invitrogen) was used to monitor cell death.

6. **Immunohistochemistry (IHC)**

Mice were perfusion-fixed with 4% (w/v) paraformaldehyde (PFA; Sigma-Aldrich) in PBS. Spinal cord tissue was dissected and lightly postfixed in 4% PFA at room temperature (RT). Tissue was cryoprotected in 20% (w/v) sucrose (Sigma-Aldrich) in PBS before freezing in OCT on the surface of dry ice. 12µm spinal cord cryosections were collected directly on SuperFrost®Plus slides (VWR International) and were allowed to dry for 30 minutes before storing at -80°C.

For *in vitro* experiments, cells were fixed with 4% (w/v) PFA for 10 min and then washed with PBS. Sections/cells were then incubated in blocking solution (0.1% [v/v] Triton X-100 and 10% fetal bovine serum in PBS) for 1 hour at RT. Primary and secondary antibodies were diluted in PBS blocking solution and applied to sections/cells overnight at 4°C. Tris-buffered saline (TBS)
was substituted for PBS when immunolabeling with anti-CC1. For detection of Nkx2.2 and CC1, mouse-on-mouse antigen retrieval was performed before immunohistochemistry according to the manufacturer’s instructions (M.O.M.™ kit; Vector Laboratories). For immunohistochemistry of spinal cord sections from mice with EAE, roughly 1mm of the lower lumbar spinal cord (containing the most obvious pathology) was collected from each mouse, and n=3 sections (12um) were randomly chosen for immunostaining analysis.

7. **Electron Microscopy (EM)**

Electron microscopy was performed on lysolecithin demyelinated mouse spinal cord sections. Lesioned mice were transcardially perfused with a 0.9% NaCl solution for exsanguination followed by a 0.1M Millongs buffering solution containing 4% paraformaldehyde and 5% glutaraldehyde (pH 7.3). Whole animal carcasses were post-fixed in the same aldehyde fixative solution for 2 weeks at 4C. Lumbar spinal cords were harvested and stored in 0.1 M cacodylate buffer (pH 7.4) overnight to remove excess aldehyde. The following day the samples were post fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour with constant agitation, rinsed in 0.1 M cacodylate buffer, dehydrated by serial dilutions of ethanol and infiltrated with and embedded in Poly/Bed 812 resin (Polysciences, Inc., Warrington, PA). All samples were oriented for cross section analysis. One micron sections, stained with toluidine blue, were used to identify lesioned site before collection for EM analysis. Following site identification, 90 nm sections were stained with a combination of lead citrate and uranyl acetate and used for ultrastructural evaluation. All images were collected using a JEOL JEM 1230 transmission electron microscope equipped with a Gatan Orius SC1000 side mount CCD camera.
8. **g-ratio Analysis**

Axon and myelin circumference were measured with ImageJ software. The g-ratio was calculated as the axonal circumference (internal to the myelin layers) divided by the circumference of compact myelin (external to the myelin layers) at a given cross section of the axon. Myelin tongues and non-compacted myelin were excluded from this calculation.

9. **Flow Cytometry**

Spinal cords were collected from EAE mice at Day 35 in PBS and were mechanically dissociated. Tissues from 2 mice belonging to the same group were then combined and passed through 100nm filter. Density gradient-isolated cells were then incubated with the anti-CD4 Ab and Live/Dead. For intracellular staining, cells were incubated in Fix/Perm solution and Perm buffer (BioLegend) and were then incubated in primary Abs. Single staining tissue samples were used for both tissue types. Cells were gated at the Georgetown Lombardi Comprehensive Cancer Center Flow Cytometry & Cell Sorting Shared Resource (FCSR).

10. **Imaging and Quantification**

For quantification of immunohistochemical staining, cells were manually counted from low magnification (10x for tissues; 20x for cell cultures), non-overlapping images, using Adobe Photoshop. For tissues, lesioned areas were identified by increased nuclear (DAPI) staining density. Cell-types of interest were quantified within this area. Co-localization was determined either as a percentage (the ratio of cells expressing two markers divided by the number of cells expressing a single marker multiplied by 100) or per mm² (the number of cells expressing one or two markers divided by the area in µ² multiplied by 1,000,000). For all quantification a minimum
of 3 sections from n=3-5 mice were examined. The proportion or density of cells was determined per mouse. The average and standard error was then calculated for each group using Microsoft Excel.

11. Statistical Analysis

All statistics were performed using GraphPad Prism 6 (La Jolla, CA, USA). Data is represented as mean ± SEM. For all other data significance was determined using two-tailed Student's t-tests, or one- and two-way analysis of variance (ANOVA) with Tukey's range test for post-hoc analysis. EAE clinical score significance were computed using two-way ANOVA with Sidak’s multiple comparison test. Exact $P$ values are stated where appropriate. Statistical significance is reported as ns, $P > 0.05$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$.

(Modified from Psachoulia et al., 2016)

1 https://hookelabs.com/protocols/eaeAI_C57BL6.html
B. MATERIALS AND METHODS: CHAPTER IV

1. Study Subjects

Thirty-seven patients with clinically definitive multiple sclerosis were included in this study. Of these patients, 11 were characterized as having active relapsing-remitting MS (aRRMS), nine as having non-active relapsing-remitting disease (naRRMS), and six as having secondary progressive MS (SPMS). All patients were non-pregnant, DMD-naïve females aged 18-65 who were being seen by neurologists at Medstar Georgetown University Hospital (GUH), Georgetown Multiple Sclerosis and Neuroimmunology Center (GMSNC) and were residents of the Washington D.C. metropolitan area. MS diagnosis and clinical subtype was defined according to the revised McDonald criteria (2010 revisions; Polman et al., 2011) and Lublin and Reingold clinical course definitions (2013 revisions; Lublin et al., 2014) with practical modifications to fit GMSNC patient demographics. Specifically, aRRMS phenotype was defined as having clinically definitive RRMS with a radiological or clinical relapse within 12 months. Radiological relapse is defined by two or more new lesions, one enlarging T2 lesion, or one new T1 gad-enhancing lesion in the brain or spinal cord. Blood was drawn prior to potential administration of steroid treatment. Non-active subtype consisted of individuals with clinically definitive RRMS who had not experienced a relapse or radiological changes within the last 12 months. Patients diagnosed with SPMS had not experienced relapses in the past 12 months and exhibited accumulation of disability, defined by a worsening score on the Kurtzke Expanded Disability Status Scale (EDSS) (Kurtzke, 1983) that persisted for six months or more. Steadily increasing neurologic dysfunction without recovery was objectively documented by a treating neurologist. Healthy control (HC) subjects were defined as having no history of neurological pathology or autoimmune disease, and satisfied
the same inclusion/exclusion criteria as their patient counterparts. Commercially available HC samples were used in this study (Precision for Medicine). This study was approved by the Georgetown University Institutional Review Board (Assessing Interleukin-4 Induced Protein 1 (IL4I1) in Patients with Multiple Sclerosis: IRB #2015-1048), and all patients provided written informed consent in accordance with this approval.

2. **Collection and Handling of Human Samples**

**BLOOD COLLECTION:** Patient blood was drawn by trained phlebotomists at GMSNC or Georgetown University’s Clinical Research Unit (**GU-CRU**). Three 8ml CPT-citrate vacutainers (BD cat.#: 362761) and one 6ml lavender-top K$_2$EDTA tube (BD cat. # 1268061) was drawn per patient (tubes provided by GUH). As a Translational Biomedical (**TBS**) scholar, I received GU-CRU priority. Samples were de-identified by an alphanumerical number code, kept protected from light, and transferred to the neighboring Georgetown University Tissue Culture Shared Resource (**TCSR**) within two hours of blood draw or kept briefly at 4°C until transfer. Healthy control samples were purchased as previously isolated PBMCs and plasma (Precision for Medicine).

**BLOOD PROCESSING:** Whole blood was processed at the TCSR, where distinct protocols were followed for isolation of peripheral blood mononuclear cells (**PBMCs**) and for plasma. (A) PBMCs were isolated from CPT-citrate tubes by density gradient centrifugation at 1500 x g, for 30 min at room temperature (RT) with the break on. PBMC cell layer was transferred to a 15ml centrifuge tube and supplemented with 15ml PBS and wash buffer, then spun at 300 x g for 15 min at RT. Cell supernatant was removed and 5ml PBS was used to gather all pellets into one 15ml tube. Total volume was brought to 10ml in PBS and cell count was obtained. Cells were again
spun at 300 x g, for 10 min at RT. Supernatant was removed and supplemented with freezing medium (FBS with 10% DMSO) to make ~1x10^6 cells per 2ml cryovial aliquots (ThermoFisher cat.#: 02682558). Isolated PBMCs were immediately stored at -80° for 24h before transfer to -150° (gas phase LN2). (B) Plasma was isolated from lavender-top tubes by gradient centrifugation at 1750 x g for 10 min at RT. Plasma supernatant was aliquoted into 0.5ml cyrovials (ThermoFisher cat.#: 02682559) and immediately stored at -80°. Samples were transferred to the Huang Lab on dry ice immediately prior to experimental use.

3. **Cell Culture, Activation and Treatment**

THAWING AND PLATING: Frozen PBMCs were thawed and plated following relevant sections contained the Helmholtz Zentrum Munich Clinical Cooperation Group Immune Monitoring Protocol^1^ with specific parameters and procedures optimized for this experiment. Cells were thawed quickly in 37 °C water bath until last visible crystal, then supplemented with 1ml warmed C.T.L. Wash medium, added dropwise. C.T.L. Wash medium had been freshly prepared from of C.T.L. Wash (ImmunoSpot cat. #: CTLW-010) in RPMI 1640 (1:9) plus 1% GlutaMAX™ (Gibco cat #: 35050061), and brought to 37°. Thawed PMBCs were transferred to 15ml Eppendorf tube, and original cryovial was rinsed twice with C.T.L. Wash medium to maximize PBMC recovery. Wash medium was added to cells for a total volume of 10ml before centrifugation at 500 x g for 7 min, RT. Supernatant was discarded and cell pellet was resuspended in 10ml wash medium. Centrifugation step was repeated (500 x g for 7 min at RT) and supernatant was again discarded. Cell pellet was then resuspended in 10ml 37° C.T.L. Test medium (ImmunoSpot cat. #: CTLT-005; plus 1% GlutaMAX™ and 1% penicillin-streptomycin (Gibco cat. # 15070063). Total live cell count was extrapolated from a 5µl sample, analyzed in an equal volume of Trypan Blue
(Sigma-Aldrich cat. #: 302643). Cell density was brought to 1x10^6 cells/ml in test medium. Cells were plated at 900µl cell suspension per well of a 12-well culture plate.

STIMULATION: (A) For experiments utilizing anti-CD3/CD28 antibody complex for T-cell activation (primary method used): Plated cells were immediately supplemented with ImmunoCult™ human CD3/CD28/CD2 T cell activator tetrameric antibody complex (Stemcell Technologies cat. #: 10970) at 20µl/ml, or PBS. (B) For anti-CD3/28 plus IL2 stimulation (preliminary experiments): recombinant human interleukin-2 (Gibco cat. #: PHC0026) was added along with anti-CD3/CD28/CD2 antibody complex, at 10µl/ml in PBS. (C) For anti-CD3, anti-CD28 stimulation (preliminary experiments): 12-well culture plates were pre-coated with anti-human CD3 (ThermoFisher cat. #: 16-0037-85) at 10µl/ml in PBS, or PBS (control wells) and left to incubate overnight at 4°C. Wells were washed twice with 1ml PBS before cell plating. Immediately after plating, cells were supplemented with anti-human CD28 (ThermoFisher cat. #: 16-0037-85) at 10µl/ml in PBS, or PBS (control wells). (D) For anti-CD3, anti-CD28 plus IL2 stimulation (preliminary experiments): recombinant human interleukin-2 was added along with anti-CD28 solution, at 10µl/ml in PBS. (E) For anti-CD3/28 stimulation (preliminary experiments): Plated cells were immediately supplemented with ImmunoCult™ human CD3/CD28 T cell activator tetrameric antibody complex (Stemcell Technologies cat. #: 10971) at 20µl/ml or PBS. (F) For anti-CD3/28 plus IL2 stimulation (preliminary experiments): recombinant human interleukin-2 was added along with anti-CD3/CD28 antibody complex, at 10µl/ml in PBS. After stimulation by any method (A-F), culture plates were left to incubate at 37 °C and 5% CO₂ for 48h.
IL4I1 TREATMENT: Following 48h of stimulation, cells were supplemented with recombinant human IL4I1 protein (R&D cat. #: 5684-AO) or PBS (sham-treatment). For primary treatment method: IL4I1 was added at a concentration of 200ng/ml in PBS, and cells were left to incubate at 37 °C and 5% CO₂ for 24h. For treatment dose preliminary experiments: IL4I1 at concentrations of 100, 200, 500 and 1000ng/ml in PBS were added to cells stimulated by methods A-F. For time point preliminary experiments: cells stimulated by method C were treated with IL4I1 at 200ng/ml in PBS, or PBS (sham-treatment) and culture plates were left to incubate at 37 °C and 5% CO₂ for 6h, 24h or 72h.

4. RNA Extraction, cDNA Synthesis and qRT-PCR

RNA EXTRACTION: Messenger RNA was extracted from cells following the TRIzol™ Reagent protocol², with specific parameters and procedures optimized for this experiment. Before mRNA extraction, an additional live cell count was obtained. Cells suspended in test medium were transferred to one 15ml Eppendorf tube per experimental condition for per donor. Wells were scraped and rinsed with 1ml PBS which was added to the 15ml tube to maximize cellular yield. Tubes were spun at 500 x g for 5 min at RT and cell supernatant was discarded. Each cell pellet was homogenized in 750µl TRIzol™ Reagent (Invitrogen cat. #: 15596026) per 5x10⁶ cells and 1µl GlycoBlue coprecipitant (ThermoFisher cat. #: AM9515) was added to each homogenate. Tubes were incubated on ice for 5 min before adding 200µl chloroform per 1ml TRIzol™ Reagent. Tubes were vortexed, incubated for 3 min and centrifuged for 15 min at 12,000 x g at 4 °C. The aqueous phase for each sample was removed and supplemented with an equal volume of isopropanol (organic layer was frozen at -20 °C for subsequent protein analysis methods described in section 4). Tubes were vortexed, incubated for 10 min and spun for 15 min at 12,000 x g at 4
°C. Supernatant was discarded and cell pellet was washed twice in 200µl EtOH (75%), spinning at 12,000 x g for 15 min at 4 °C for each wash. Supernatant was discarded and cell pellets were resuspended in 30µl RNase-free water. mRNA concentration was quantified by NanoDrop (ThermoFisher) and tested for high integrity by Georgetown University Genomics and Epigenomics Shard Resource (GESR).

cDNA SYNTHESIS: High integrity mRNA (RIN > 7) was used to synthesize cDNA using the iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad cat. #: 172-5035) and following the associated protocol. PCR primers for human 18S, β-ACTIN, EF1A, GAPDH, HPRT, IFNγ, IL10, IL17A, IL4I1, TCRζ, TGFβ, TNFα, TOB1 were purchased from Bio-Rad (cat. #: 10025636). Sybr-Green RT-PCR was performed using the SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad cat. #: 1725271) and analyzed by the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). All samples were assayed in triplicate. Results were normalized against β-actin and expressed as mean ± SEM.

5. Enzyme-Linked Immunosorbent Assay (ELISA)

Freshly thawed plasma from healthy control and patient groups was used to quantify circulating levels of IFNγ, IL17, IL4I1, TGFβ and TOB1 proteins. IFNγ concentrations were detected using R&D Systems human IFNγ DuoSet® ELISA kit (cat. #: DY285B-05) and its associated protocol, at 1:5 and 1:10 plasma dilutions. For quantification of IL17, 1:1 and 1:2 plasma dilutions were run on R&D Systems human IL17 DuoSet® ELISA kit (cat. #: DY307-05) following its associated protocol. IL4I1 was detected on MyBioSource human IL4I1 ELISA plates (cat. #: MBS2515832), using plasma dilutions of 1:1 and 1:2 and following methodology developed by MyBioSource.
TGFβ concentrations were detected in activated samples at 1:80 and 1:160 dilutions using R&D Systems human TGF-β1 plates (cat. #: DY240-05) and its associated protocol. TOB1 was quantified on MyBioSource human TOB1 ELISA plates (cat. #: MSB282569) using 1:1 and 1:2 plasma dilutions and following MyBioSource protocol TOB1 ELISA protocol.

6. Flow Cytometry

Flow cytometric analysis was performed on healthy control PBMCs after 48h stimulation with ImmunoCult™ human CD3/CD28/CD2 T cell activator tetrameric antibody complex (Stemcell Technologies cat. #: 10970) at 20µl/ml, or PBS (sham-stimulation) and 24h treatment with recombinant human IL4I1 protein (R&D cat. #: 5684-AO) or PBS (sham-treatment). Four panels were designed for distinct cell population analyses. All protocols and reagents used were designed and purchased from BioLegend. Cells were gated at the Georgetown Lombardi Comprehensive Cancer Center Flow Cytometry & Cell Sorting Shared Resource (FCSR).

MEMORY/ACTIVATED Th1, Th2 AND Treg QUANTIFICATION: BioLegend methodology for intracellular transcription factor measurement using True-Nuclear™ was followed for sample preparation and staining. Primary antibodies used for this analysis were: GATA3, CD197/R7, FoxP3, T-bet, CD4, CD25, CD8a, CD56, CD14 and CD15. To identify T cells, samples were gated on Live/Dead and analyzed for CD56, CD14 and CD15. For CD4+ T cell subpopulations, cells were gated on Live/Dead, CD3+ and CD4+, and were analyzed for CD25 and T-bet (for Th1), CD25 and GATA3 (for Th2), CD25 and FoxP3 (for Treg) and CD45RA and CD197 (to distinguish memory vs activated cells).
Th17, CTL, Th1 AND Th2 QUANTIFICATION: Following the BioLegend intracellular cytokine staining protocol[^10^], cells were treated with Brefeldin A for 4 hours in order to block cytokine transportation before staining. Primary antibodies used for this were: GrnzB, CD197/R7, CD45RA, IL10, IFNγ, CD4, L17, CD56, CD14 and CD15. Baseline gating on Live/Dead were used on all of the following analyses: For T cell populations, cells were analyzed for CD56, CD14 and CD15. For T helper cell populations: gating on CD3⁺; analyzed for CD4 and CD8. For CD4 Effector/Memory T cells: gate on CD3⁺ and CD4⁺; analyzed for CD197 and CD45RA. For CD8 Effector/Memory T cells: gate on CD3⁺ and CD8⁺; analyzed for CD197 and CD45RA. For Th1/Th17: gating on CD3⁺ and CD4⁺; analyzed for IFNγ and IL17A. For Th1/Treg: gating on CD3⁺ and CD4⁺, IFNγ⁻ and IL17A⁻; analyzed for IL10. CD8: gating on CD3⁺ and CD8⁺; analyzed for IFNγ and GrnzB.

MEMORY/ACTIVATED NK, NkT, B-CELL QUANTIFICATION: Standard surface immunostaining with Fc block was performed following BioLegend surface staining protocol[^11^]. Primary antibodies used for this analysis were: IgD, CD95, CD25, CD19, CD38, CD56, CD27 and CD3. Baseline gating on Live/Dead were used on all of the following analyses: For T cell/B cell/NK cell identification, cells were analyzed for CD3 and CD19. To identify T-cell populations, samples were gated on CD19⁻ and CD3⁺, and analyzed for CD25 and CD56. For NK cells identification, cells were gated on CD19⁻ and CD3⁻ and analyzed for CD25 and CD56. Cells were gated on CD19⁺ and CD3⁻ with analysis for CD25 and CD27 (for B-cell populations) and for IgD and CD38 (for plasmablasts).
MONOCYTE/CAM/AAM/DC/pDC QUANTIFICATION: Standard surface immunostaining with Fc block\textsuperscript{11} and BioLegend monocyte blocker protocol\textsuperscript{12} were followed. Primary antibodies used for this analysis were: CD123, CD163, CD86, CD19, CD56, CD15, HLADR, CD206, CD14, CD11c, CD16 and CD11b. Live/Dead and CD19/CD56/CD15\textsuperscript{−} gatings were used on all of the following analyses: Monocyte/macrophage vs DC were analyzed for CD11b and CD11c. For monocyte vs macrophage: gating on CD11b\textsuperscript{+}; analyzed for CD14 and CD16. For macrophage subtypes: gating on CD11b\textsuperscript{+}, CD123\textsuperscript{−}, CD14\textsuperscript{−} and CD16\textsuperscript{+}; analyzed for CD163 and CD206 as well as CD86 and HLADR. For DC subtypes analysis was for CD123 and CD11c.

7. Statistical Analysis

All statistics were performed using GraphPad Prism 7 (La Jolla, CA, USA). Data is represented as mean ± SEM after identifying statistical outliers. Generally, student’s t-tests are conducted for paired, within-group analyses, and ordinary one-way analysis of variance (ANOVA) with Tukey's multivariable comparisons \textit{t post-hoc} analysis (alpha = 0.05) are used for unpaired, between-group comparisons. Exact metrics used in each experimental analysis are clearly described in associated figure legends. Exact \( P \) values are stated where appropriate. Statistical significance is reported as ns, \( P > 0.05 \), *\( P \leq 0.05 \), **\( P \leq 0.01 \), ***\( P \leq 0.001 \), ****\( P \leq 0.0001 \).

\textsuperscript{1}https://www.helmholtz-muenchen.de/fileadmin/Immunmonitoring/pdf/PBMC_Isolation_Cryo_Thaw.pdf
\textsuperscript{2}https://assets.thermofisher.com/TFS-Assets/LSG/manuals/trizol_reagent.pdf
\textsuperscript{3}http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10043078.pdf
\textsuperscript{4}https://resources.rndsystems.com/pdfs/datasheets/dy285b.pdf
\textsuperscript{5}https://resources.rndsystems.com/pdfs/datasheets/dy317.pdf
\textsuperscript{6}https://www.mybiosource.com/images/tds/protocol_manuals/800000-9999999/MBS2515832.pdf
\textsuperscript{7}https://resources.rndsystems.com/pdfs/datasheets/dy240.pdf
\textsuperscript{8}https://www.mybiosource.com/images/tds/protocol_manuals/000000-7999999/MBS282569.pdf
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\textsuperscript{10}https://www.biolegend.com/media_assets/support_protocol/Intracellular_Staining_Protocol_082615.pdf
\textsuperscript{11}https://www.biolegend.com/media_assets/support_protocol/BioLegend_Surface_Staining_Flow_Protocol_060215.pdf
C. MATERIALS AND METHODS: CHAPTER V

1. Mice

Female athymic, “nude” mice (RjOrl:NMR1-Foxn1\textsuperscript{nu}/Fox1\textsuperscript{nu}) were purchased from The Jackson Laboratory (Bar Harbor, ME). All experiments were performed in accordance with approved Institutional Animal Care and Use Committee (IACUC) protocols of Georgetown University.

2. Focal Spinal Cord Demyelination and Peripheral Blood Mononuclear Cell Grafting

Focal demyelination was induced by injecting 1.0% lysolecithin (Sigma-Aldrich Company Ltd, Dorset, UK) in saline into the spinal cord dorsal horn of female athymic mice at 8-10 weeks old. Forty-eight hours after lysolecithin injection, PBS-treated or IL4I1-treated lymphocytes from healthy control donors (10\textsuperscript{5} live cells suspended in 1µl PBS) were grafted into the lesion site, which was previously marked by charcoal. Non-grafted control mice (NGC) did not undergo the grafting procedure. Mice in each of 3 groups (NGC, PBS-grafted and IL4I1-grafted), with n=3-5 per group, were sacrificed 15 days after lysolecithin-injection was performed.

3. Human Cell Culture for Grafting

THAWING AND PLATING: Frozen healthy control PBMCs were thawed and plated following relevant sections contained the Helmholtz Zentrum Munich Clinical Cooperation Group Immune Monitoring Protocol\textsuperscript{1} with specific parameters and procedures optimized for this experiment. Cells were thawed quickly in 37 °C water bath until last visible crystal, then supplemented with 1ml warmed C.T.L. Wash medium, added dropwise. C.T.L. Wash medium had been freshly prepared from of C.T.L. Wash (ImmunoSpot cat. #: CTLW-010) in RPMI 1640 (1:9) plus 1%
GlutaMAX™ (Gibco cat #: 35050061), and brought to 37°. Thawed PMBCs were transferred to 15ml Eppendorf tube, and original cryovial was rinsed twice with C.T.L. Wash medium to maximize PBMC recovery. Wash medium was added to cells for a total volume of 10ml before centrifugation at 500 x g for 7 min, RT. Supernatant was discarded and cell pellet was resuspended in 10ml wash medium. Centrifugation step was repeated (500 x g for 7 min at RT) and supernatant was again discarded. Cell pellet was then resuspended in 10ml 37° C.T.L. Test medium (ImmunoSpot cat. #: CTLT-005; plus 1% GlutaMAX™ and 1% penicillin-streptomycin (Gibco cat. # 15070063). Total live cell count was extrapolated from a 5µl sample, analyzed in an equal volume of Trypan Blue (Sigma-Aldrich cat. #: 302643). Cell density was brought to 1x10⁶ cells/ml in test medium. Cells were plated as 900µl cell suspension per well of a 12-well culture plate.

STIMULATION AND TREATMENT: Plated cells were immediately supplemented with ImmunoCult™ human CD3/CD28/CD2 T cell activator tetrameric antibody complex (Stemcell Technologies cat. #: 10970) at 20µl/ml, or PBS. Plates were left to incubate at 37 °C and 5% CO₂ for 48h. Following 48h of stimulation, cells were supplemented with recombinant human IL4I1 protein (R&D cat. #: 5684-AO) or PBS (sham-treatment). IL4I1 was added at a concentration of 200ng/ml in PBS, and cells were left to incubate at 37 °C and 5% CO₂. After 24h incubation in IL4I1 or PBS, cells were prepared for engrafting into 2 dpl athymic mouse lesions. Lymphocytes were prepared by collecting them, with media, into a 15ml Eppendorf tube and spinning at 600 x g for 10min at 4 °C. Supernatant was removed and cells were resuspended in 1 ml media at 37 °C for live cell quantification. Suspended cells were again centrifuged at 600 x g for 10min at 4 °C, supernatant was removed and cells were resuspended in PBS to generate 10⁵/µl live cell suspension for grafting.
4. Spinal Cord Extraction and Immunohistochemistry (IHC)

PERFUSIONS: Mice were perfusion-fixed with 4% (w/v) PFA (Sigma-Aldrich) in PBS. Spinal cord tissue was dissected and postfixed for 45 min in 4% PFA at RT. Tissue was cryoprotected in 20% (w/v) sucrose (Sigma-Aldrich) in PBS at 4°C overnight before freezing in optimal cutting temperature medium on the surface of dry ice. Frozen spinal cord sections were cryosectioned at 12 µm thickness, collected on SuperFrostPlus slides (Stellar Scientific) and dried for 30 min before storage at −80 °C until IHC staining.

IMMUNOHISTOCHEMISTRY: Sections were thawed for 30 min at RT and incubated in blocking solution (0.1% [v/v] Triton X-100 and 10% fetal bovine serum in TBS) for 1 hour at RT. Rat anti-myelin basic protein (MBP) primary antibody (AbD Serotec) was diluted 1:400 in TBS blocking solution and applied to sections overnight at 4°C. AlexaFluor® 488 secondary antibody (ThermoFisher) was used at a concentration of 1:500 and applied for 45 min at RT. FluoroMyelin™ dye (ThermoFisher) at was applied for 45 min at 4°C, at a concentration of 1:75 in TBS blocking solution. To label nuclei, Hoechst stock (ThermoFisher cat. #: 33342) was diluted 1:20000 in TBS and applied with secondary antibodies for 45 min.

5. Imaging and Quantification

For quantification of ICH, stained areas were manually captured as 10x magnified, non-overlapping images, using Adobe Photoshop. For all experimental groups quantified, a minimum of 3 sections from n=3-5 mice were examined. ImageJ software was used to quantify MBP and FluoroMyelin™ stain intensity. Images were de-identified such that sections were analyzed with
blindness to experimental condition. Demyelinated lesions were identified as areas of high cellular density, as defined by nuclear (DAPI) staining. Images were split by channel and lesioned regions of interest (ROIs) were superimposed onto 488 (MBP) and 594 (FluoroMyelin™) channels for antibody-specific intensity quantification per unit area. Fluorescence intensity in non-lesioned areas was measured on each channel in order to correct for background fluorescence. Corrected Fluorescence Intensity (CFI) was calculated as: CFI = IntDensity of lesion – (area of lesion x mean fluorescence of background) per channel. CFIs were averaged per mouse per condition and transferred to GraphPad Prism 7 (La Jolla, CA, USA) for normalization and graphic representation.

6. **Statistical Analysis**

All statistics were performed using GraphPad Prism 7 (La Jolla, CA, USA). Data is represented as mean ± SEM after identifying statistical outliers. One-way analysis of variance (ANOVA) with Sidak’s multiple comparison test. Exact $P$ values are stated where appropriate. Statistical significance is reported as ns, $P > 0.05$, $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$.

1. [https://www.helmholtz-muenchen.de/fileadmin/Immunmonitoring/pdf/PBMC_Isolation_Cryo_Thaw.pdf](https://www.helmholtz-muenchen.de/fileadmin/Immunmonitoring/pdf/PBMC_Isolation_Cryo_Thaw.pdf)
CHAPTER III: IL4I1 SUPPRESSES INFLAMMATION AND PROMOTES REMYELINATION IN MOUSE MODELS OF MULTIPLE SCLEROSIS

A. INTRODUCTION

1. Background and Rationale

Multiple sclerosis is a chronic inflammatory disease characterized by myelin destruction in the CNS (Dutta and Trapp, 2014). Demyelinated lesions within the brain and/or spinal cord produce wide-ranging neurological deficits, whose pattern of presentation often shifts across the time-course of the disease (Compston and Coles, 2008). In the initial stages of MS, symptoms typically follow an “on-off” pattern as the body fluctuates between inflammatory relapses and subsequent remission of symptoms (RRMS clinical subtype). As inflammatory episodes accumulate over time, symptom presentation assumes a linear trend of increasing neurologic dysfunction, without discrete periods of relapse or remission (SPMS clinical subtype).

The progression from RRMS to SPMS mirrors the body’s declining ability to repair and recover. Spontaneous repair of demyelinated lesions is successful during early stages of MS; however, remyelination fails in progressive disease, accompanied by axonal dystrophy and irreversible neurological disability (Dutta and Trapp, 2011; Franklin et al., 2012). The underlying mechanisms that permit the transition from successful CNS remyelination to failed spontaneous repair are not well understood. In health, as in early stages of MS, demyelination is met by an acute influx of pro-inflammatory cells to the site of injury. The resulting uptick in inflammatory
activity is limited in duration and extent. Its timely resolution is followed by the recruitment of oligodendrocyte progenitor cells (OPCs) to the lesion, OPC differentiation into mature oligodendrocytes, and axon remyelination (Bramow et al., 2010; Franklin and ffrench-Constant, 2008). While the causal relationship of these progressive steps is hotly debated, it is clear that oligodendrocytes are vulnerable to damage and death associated with inflammation (Jablonska et al., 2012; Akassoglou et al., 1998).

It has been proposed that unmitigated inflammation is responsible for MS pathogenesis and progression (Fitzner and Simons, 2010; Franklin, 2002). Autoreactive T cells are thought to be a key driver of MS pathogenesis (Compston and Coles, 2008). Indeed, high concentrations of both CD4+ and CD8+ T-cell subtypes have been implicated in MS lesions (Reich et al., 2018). Likewise, increased activity of functionally pro-inflammatory macrophages (classically-activated macrophages; CAM) have been found to correlate with OPC death and failed remyelination (Miron and Franklin, 2014). This is in contrast to their anti-inflammatory counterparts on the opposite end of the macrophage activation-state spectrum (Murray and Wynn, 2011); alternatively-activated macrophages (AAM) have been implicated in successful oligodendrocyte differentiation and remyelination (Miron et al., 2013; Schonberg et al., 2007). Like pro-inflammatory T cells, CAM have been associated with increased disability in mouse models of MS (Ellwardt and Zipp, 2014; Mikita et al., 2011). The pro-inflammatory skew implicated in MS pathology makes immune-modulation an intriguing target for promoting natural repair processes that are deficient in progressive MS.

Interleukin-4 induced protein 1, a secreted enzyme primarily expressed by AAM (Psachoulia et al., 2016; Zhang et al., 2014), has been found to have immune-modulating effects in certain kinds of cancers (Boulland et al., 2007; Copie-Bergman et al., 2003; Lasourdis et al.,
as well as bacterial infections (Puiffe et al., 2013). As an L-amino acid oxidase (LAAO), IL4I1 is known to primarily convert L-phenylalanine (an L-amino acid) into phenylpyruvate (an \(\alpha\)-keto acid), producing ammonia (NH\(_3\)) and hydrogen peroxide (H\(_2\)O\(_2\)) in the process (Chavan et al., 2002).

The potential involvement of IL4I1 in spontaneous remyelination was first suggested upon analysis of a previously published rat remyelination transcriptome (Huang et al., 2011). Repeated microarray analysis was performed on experimentally demyelinated CNS tissue throughout the process of natural repair and remyelination. This work aimed to identify key players involved in the spontaneous remyelination process by tracking gene expression chronologically through increasing inflammation, inflammation resolution and successful remyelination. \textit{Interleukin-4 induced 1} was found to be the predominant gene expressed during inflammation resolution, preceding remyelination (\textbf{Fig. III.1}). \textit{Interleukin-4 induced 1} expression peaks after extracellular membrane breakdown-associated genes begin to decline (Matrix metalloproteinase-12; \textit{Mmp12}) and key genes of myelination rise in expression (Myelin basic protein; \textit{Mbp}). The timing and reproducibility of this expression pattern suggests that \textit{Il4i1} expression may be a driving force behind the important cellular changes taking place during this time, and that involvement of \textit{Il4i1} in remyelination merited further investigation.
Here, we find that IL4I1 increases AAM presence in focally demyelinated mouse lesions, while decreasing CAM density. Furthermore, IL4I1 treatment promotes oligodendrocyte infiltration of the lesion, which results in enhanced remyelination. Compared to untreated CNS lesions, treated tissue expresses reduced levels of pro-inflammatory cytokines ($\text{Ifng}$ and $\text{Il17}$). In contrast, lesions of IL4I1 knock-out mice ($\text{Il4il}^{-/-}$ mice) exhibited impaired remyelination, oligodendrocyte penetration, and persistent CAM presence. We also demonstrate that IL4I1 treatment reduces and even reverses MS-like paralysis in a behavioral mouse model of MS, suppressing pro-inflammatory T-cell density (Th1 and Th17) in spinal cords of treated mice. Our findings implicate IL4I1 as an important player in immune regulation and remyelination after injury. Capable of enhancing remyelination and reversing paralysis in mouse models of MS, IL4I1 shows promise as a potential therapeutic for controlling relapse and preventing progression in human disease.
The mouse model-based findings discussed in this chapter are seminal for my subsequent studies on human samples (detailed in Chapters IV and V). As a co-investigator under the guidance of Dr. Konstantina Psachoulia, here I focus on the aspects of her project to which I contributed most significantly, and which are most relevant to my work with patient cells. The data presented in this chapter are published in *Brain*:


2. Approach

The progression of MS from RRMS to SPMS is characterized by chronic inflammation and failed remyelination. Interleukin-4 induced protein 1, an enzyme secreted by immune cells including AAM, is known to induce immune-regulatory effects. The expression pattern of *Il4i1* over the course of recovery following demyelination in rodent models of MS—peaking during late inflammation preceding remyelination—suggests that IL4I1 is involved in immune-modulation and successful remyelination. We hypothesize that IL4I1 plays a critical role in inflammation resolution and remyelination, with potential therapeutic implications in MS. To recognize the potential significance of IL4I1 in CNS recovery after injury, this study set out to achieve the following aims:

**Aim 1:** Quantify the effect of IL4I1 on the processes of inflammation resolution and remyelination.

To study the cellular and molecular environment in MS-like lesions, the lysolethicin-lesion focal demyelination mouse model was used. This model allows us to assess the progress of inflammation
resolution and remyelination at discreet time points that span the recovery process. In order to determine the effect of IL4I1 on this process, two lesion conditions were induced in wild-type (WT) mice: lysolethicin-induced demyelination without IL4I1 co-injection into the lesion (control condition), and lysolethicin-induced demyelination with IL4I1 co-injection (treatment condition). Key time points of interest to this study are 5, 10 and 20 days post-lesion (dpl). These points in time typically correspond to OPC recruitment to the lesion (5dpl), differentiation of OPCs into oligodendrocytes/early remyelination (10dpl) and complete remyelination (20dpl) (Jeffery and Blakemore, 1995).

The effect of Il4I1 co-injection on inflammation was assessed by quantifying the presence of relevant cell types in the lesions of control and treated mice. Immunohistochemical (IHC) staining for pro-inflammatory CAM (CD11b⁺iNOS⁺) and anti-inflammatory AAM (CD11b⁺Ym1⁺) was performed on spinal cord sections derived from control and treated mice sacrificed at 5, 10 and 20 days post-lesion. The progress of remyelination was also visualized at these time points by IHC staining for OPCs (Nkx2.2⁺Olig2⁺) and mature oligodendrocytes (CC1⁺Olig2⁺). The degree of remyelination at 10 days post-lesion was further assessed by g-ratio analysis on electron micrographs of lesioned sections.

**Aim 2:** Characterize the role of endogenous IL4I1 expression in inflammation resolution and remyelination. To determine if loss of IL4I1 function affects inflammation or impairs remyelination efficiency, IL4I1 knock-out mice (Il4i1⁻/⁻ mice) were obtained and used for focal spinal cord demyelination. Lysolethicin-mediated demyelination was performed in WT and Il4i1⁻/⁻ mice. Perfused spinal cords were extracted at 5, 10 and 20 days post-lesion. To assess the role of Il4i1 gene expression in inflammation, IHC staining for CAM (CD11b⁺iNOS⁺) and AAM
(CD11b<sup>+</sup>Ym1<sup>+</sup>) in lesioned tissue was compared between Il4i1<sup>−/−</sup> and WT mice. To assess the role of Il4i1 gene expression in remyelination, IHC for mature oligodendrocyte (CC1<sup>+</sup> Olig2<sup>+</sup>) was performed and cell density was compared between conditions. g-ratio measurements of 10 days post-lesion tissue were also made to quantify the degree of remyelination in these sections.

**Aim 3:** Determine the potential of IL4I1 as a therapeutic agent. To identify and quantify any effects of IL4I1 treatment on behavior, the experimental autoimmune encephalomyelitis (EAE) behavioral mouse model of MS was employed. Unlike the lysolethicin-mediated focal demyelination model, which allows us take snap-shots of cellular and molecular processes taking place *in vivo*, the EAE model allows us to assess the behavioral outcomes of these processes on a living, whole-organism level. We induced EAE in WT mice by inoculation with MOG<sub>35-55</sub> followed by pertussis toxin. In order to identify IL4I1’s influence on EAE symptoms, mice received two tail injections of either IL4I1 or PBS (1µg/ml blood volume). By administering IL4I1 intravenously in EAE mice, this model allows us to determine if IL4I1 can modulate the peripheral immune response associated with immune-mediated demyelination. The first dose was administered upon reaching a clinical score of 2.0 (defined by partial hind limb paralysis), and a second dose was administered three days after the first. Motor impairment was scored for 33 days post-inoculation and compared between groups.

**Aim 4:** Elucidate the mechanism of IL4I1-mediated immune modulation. In order to understand what cell types IL4I1 targets to exert its effects, we looked for changes in immune cell-specific cytokine expression in 10dpl from treated and untreated WT mice. After mRNA extraction and cDNA synthesis, tissue was analyzed using qRT-PCR for MS-associated cytokines gene
expression. Quantified expression levels were compared between mice that did and did not receive co-injection with IL4I1. The effect of IL4I1 on specific immune cells was further investigated by performing flow cytometry on spinal cords extracted from EAE mice. The population densities of T-cell subtypes Th1 (T-bet⁺CD4⁺), Th2 (Gata3⁺CD4⁺) and Th17 (Rorγt⁺CD4⁺) in spinal cords extracted from EAE mice at 35 days post-inoculation were compared between mice that received tail injections of IL4I1 vs. those that received PBS control injections.
B. RESULTS

1. IL4I1 Modulates Inflammation in CNS Lesions

We found that co-injection with IL4I1 into lysolethicin-demyelinated spinal cord lesions had multiple effects across important post-lesion time points. Immunohistochemical staining at 10dpl, a time point associated with oligodendrocyte differentiation in the healthy CNS, revealed significantly decreased cell density of iNOS$^+$CD11b$^+$ (CAM; pro-inflammatory) cells in WT mice that received co-injection with IL4I1, as compared to those that did not (Fig. III.2.A). Likewise, IL4I1-treated mice exhibited reduced Ym1$^+$CD11b$^+$ (AAM; anti-inflammatory) cell density in lesions at 20dpl, at which point healthy remyelination is complete (Fig. III.2.B). Conversely, when compared to WT, lesions of Il4i1$^{-/-}$ mice contained increased iNOS$^+$CD11b$^+$ cell density at 10dpl (Fig. III.2.A) and decreased Ym1$^+$CD11b$^+$ cell density at 5dpl—at which OPC recruitment typically occurs (Fig. III.2.B). The persistently elevated intralesional levels of iNOS$^+$CD11b$^+$ CAM in Il4i1$^{-/-}$ mice indicates unresolved inflammation caused by lack of Il4i1 gene expression (Fig. III.2.A). Cumulatively, these findings demonstrate that IL4I1 is critical to, and able to enhance, inflammation mitigation following CNS injury.
Figure III.2. *Il4i1*−/− mice have increased iNOS⁺ and decreased Ym1⁺ cell density in CNS lesions while IL4I1-treatment decreases iNOS⁺ and increases Ym1⁺ cell density in WT lesions. Quantification of (A) iNOS⁺ CD11b⁺ cells, and (B) Ym1⁺ CD11b⁺ cells per mm² in lesions of WT, *Il4i1*−/− and IL4I1-treated mice at 5, 10 and 20dpi. Immunostaining of (C) iNOS (green), CD11b (red) and DAPI (blue) and (D) Ym1 (green), CD11b (red) and DAPI (blue) in WT and *Il4i1*−/− mice at 10dpi. For cell counts, n=3-5 mice per group were used and n=3 10x magnification images per mouse were analyzed. Scale bar, 100µm. Statistical significance reported as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; ANOVA followed by post-hoc analysis.
2. **IL4I1 Promotes Remyelination of CNS Lesions**

To determine the involvement and effect of IL4I1 on subsequent OPC recruitment and oligodendrocyte differentiation, IHC staining for OPCs (Nkx2.2\(^+\)Olig2\(^+\)) and mature oligodendrocyte (CC1\(^+\)Olig2\(^+\)) was performed in WT, Il4i1\(^{-/-}\) and WT-treated mouse lesions at 5, 10 and 20dpl. Quantification revealed increased Nkx2.2\(^+\)Olig2\(^+\) (Fig. III.3.A) and CC1\(^+\)Olig2\(^+\) (Fig. III.3.B) cell density in lesions from WT mice that received co-injection with IL4I1, as compared to those that did not. This finding suggests that IL4I1 promotes OPC recruitment and the mature oligodendrocyte presence in the 5dpl lesion. Wild-type IL4I1-treated mice continued to show increased CC1\(^+\)Olig2\(^+\) cell density at 10 and 20dpl as compared to controls (Fig. III.3.A), indicating sustained oligodendrocyte presence.

Lesions from Il4i1\(^{-/-}\) mice displayed significantly lower CC1\(^+\)Olig2\(^+\) cell density at 20dpl (Fig. III.3.B). The pattern of CC1\(^+\)Olig2\(^+\) cell distribution in Il4i1\(^{-/-}\) lesions at 10dpl is particularly intriguing. While intralesional areas are relatively devoid of CC1\(^+\)Olig2\(^+\) cells, Il4i1\(^{-/-}\) lesions are surrounded by increased CC1\(^+\)Olig2\(^+\) cell density (Fig. III.3.C). This distribution suggests that the absence of Il4i1 prevents penetration of oligodendrocytes to the site of demyelination. Together, these data indicate that IL4I1’s immunomodulatory effect promotes OPC recruitment to—and oligodendrocyte differentiation in—CNS lesions, and that IL4I1 is required for both processes to occur spontaneously in nature.

The increased presence of CC1\(^+\)Olig2\(^+\) cells in IL4I1-treated 10 days post-lesions tissue does not necessarily imply enhanced remyelination. To assess the success of these cells in remyelinating nude axons, g-ratio analysis was performed on electron micrographs of cross-sectioned tissue (Fig. III.4). The g-ratio, which compares an axon’s internal diameter to the axon’s myelinated diameter, allows us to quantify the degree of myelination around any individual axon.
Through this analysis, we found that IL4I1-treated mice displayed a lower average \( g \)-ratio (increased myelination) as compared to controls, while \( Il4i1^{--} \) lesions corresponded with an increased average \( g \)-ratio (fewer remyelinated axons, less efficient myelination). As was suggested by our previous finding that CC1\(^+\)Olig2\(^+\) oligodendrocytes are enhanced in IL4I1-treated WT lesions and reduced in \( Il4i1^{--} \) lesions (Fig. III.3), \( g \)-ratio analysis confirmed that IL4I1 is critical to—and able to enrich—remyelination in 10dpl mouse CNS tissue.

Figure III.3. Olig2\(^+\) cell density in CNS lesions decreased in \( Il4i1^{--} \) mice and increased in IL4I1-treated WT mice. Quantification of (A) Nkx2.2\(^+\)Olig2\(^+\) and (B) CC1\(^+\)Olig2\(^+\) cells per mm\(^2\) in lesions at 5, 10 and 20dpl in WT, \( Il4i1^{++} \) and IL4I1-treated mice. (C) Immunostaining of Olig2 (green), CC1 (red) and DAPI (blue) in lesions at 10dpl. Lesions are characterized as the cluster of DAPI nuclei in the spinal cord ventral funiculus. Mice were \( n=3-5 \) per group and \( n=3 \) 10x magnification images per mouse were analyzed. Scale bar, 100\( \mu \)m for immunofluorescence images. Statistical significance reported as \( *P<0.05 \), \( **P<0.01 \), \( ***P<0.001 \), \( ****P<0.0001 \); ANOVA followed by post-hoc analysis.
Figure III.4. Remyelination reduced in Il4i1−/− lesions and enhanced in IL4I1-treated WT lesions. A. g-ratio analysis of remyelinated axons and corresponding axonal diameter. B. Scatter plot showing the overall g-ratio in the mouse groups. C. Electron micrographs of CNS lesion tissue at 10dpl show reduced remyelination of axons in Il4i1−/− lesions, and greater remyelination of axons in IL4I1-treated lesions compared to WT Statistical significance reported as ****P<0.0001; Two-way ANOVA followed by post-hoc analysis.
3. IL4I1 Reverses Clinical Severity in EAE

Using a focal CNS demyelination mouse model of MS, we found that IL4I1 can modulate inflammation and enhance remyelination. To have potential as a therapeutic agent, however, its effect must translate from promising molecular findings to improved behavioral outcomes. We capitalized on the EAE mouse model of MS to observe the behavioral effects of IL4I1 treatment. A commonly accepted preclinical model of MS, EAE is thought to resemble some aspects of MS pathogenesis, including immune-mediated CNS demyelination and axonal injury (Kornek et al., 2000; Nikić et al., 2011). Here, we found that IL4I1-treated EAE mice experienced a reduction in peak symptom severity, began recovery from peak severity sooner, and recovered more effectively than did untreated EAE mice (Fig. III.5).

Whereas all mice displayed typical symptom accumulation until tail injections were administered, only those that received vehicle injections continued to progress across the typical EAE trajectory. These mice had peak motor impairment at clinical score 3.5 (defined by complete hind limb paralysis) and maintained high levels of paralysis for the duration of the experiment. This is in contrast to the IL4I1-treated mice, who progressed only to a score of 2.5-3.0 (limp tail and dragging of hind legs). Astonishingly, these mice exhibited reversal of EAE symptoms, ending the experiment at a clinical score of only 1.5 (defined by limp tail and minor inhibition of hind legs).
Figure III.5. IL4I1-treatment reduces peak symptom severity and reverses motor symptoms in EAE mice. Clinical scores of WT (n=10) and IL4I1-treated (n=10) mice for 33 days after EAE induction. IL4I1 was administered intravenously by tail injection at around score 2.0, and again three days later. Average injection dates for IL4I1-treated mice are indicated by red arrows. Statistical significance is reported as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; Two-way ANOVA followed by post-hoc analysis.
4. **IL4I1 Reduces Th1 and Th17 Expression and Population Density**

Finally, in order to identify how IL4I1 is exerting these effects, qRT-PCR for MS-related pro- and anti-inflammatory cytokines was performed on lesioned CNS tissue from WT and IL4I1-treated WT mice. Significance was found in pro-inflammatory cytokines, *Ifng* and *Il17*, whose expression was significantly reduced in IL4I1-treated samples. Expression of *Il4*, a Th2-associated anti-inflammatory cytokine, was not affected ([Fig. III.6.A](#)). These data suggest that IL4I1 acts to attenuate Th1 and Th17 cell activity, the pro-inflammatory T-cell subtypes associated with IFNγ and IL17, respectively. Furthering this suggestion, flow cytometric analysis of spinal cords retrieved from EAE mice at 35 days post-inoculation revealed reduced population density of T-bet⁺CD4⁺ (Th1 cells; 4.5-fold reduction) and Rorγt⁺CD4⁺ (Th17 cells; 3.1-fold decrease) in treated mice as compared to control. A 2.7-fold decrease was also seen in Gata3⁺CD4⁺ (Th2) cell population ([Fig. III.6.B](#)).
Figure III.6. IL4I1-treated mitigates *Ifng* and *Il17* expression in CNS lesions and reduces CD4+ cell density in EAE mouse spinal cords. A. Normalized expressions of *Ifng*, *Il17* and *Il4* in WT (n=4) and IL4I1-treated (n=6) spinal cord lesions at 10dpl by qRT-PCR. B. Flow cytometry analysis of spinal cord showing the percentage and total number of gated T-bet CD4+ (Th1), Rorγt CD4+ (Th17) and Gata3 CD4+ (Th2) cells in WT and IL4I1-treated mice at 35 days after EAE induction. Samples from n=2 mice were combined for each group and analyzed. Statistical significant reported as ***P<0.001; ****P<0.0001; Two-Way ANOVA followed by post-hoc analysis.
C. DISCUSSION

The progression from relapsing-remitting multiple sclerosis to secondary progressive multiple sclerosis is characterized by decreasing repair/remyelination and increasing neuronal dystrophy (Franklin, 2002). In early stages of the disease, injury is met with an appropriate inflammatory response and debris is cleared preceding tissue repair. Remyelinating oligodendrocytes serve the lesioned area after inflammation has subsided, but the causality of this stepwise progression has not been fully determined. What is known, is that progressive MS is characterized by failed remyelination (Dutta and Trapp, 2011; Franklin et al., 2012). The work presented in this chapter aimed to determine the mechanism of this failure, by testing the effects of a novel immunomodulatory protein, IL4I. Our results indicate that unresolved inflammation causes failed remyelination, as is seen in progressive MS.

Our data suggest that IL4I1 modulates inflammation and promotes remyelination in CNS lesions in mice. We also found that IL4I1 reverses paralysis in EAE model mice. Finally, our data indicate that IL4I1 reduces pro-inflammatory T-cell gene expression in demyelinated CNS lesions and reduces CD4+ T-cell population density in spinal cords from postmortem EAE mice. Therefore, we propose that IL4I acts by influencing T-cell activity and proliferation. With regard to inflammation, we found that IL4I1 co-injection into lesions of WT mice increases AAM (Ym1+ CD11b+) cell density in demyelinated lesions at 20dpl, and decreases CAM (iNOS+CD11b+) cell density in lesions at 10 and 20dpl. No significant reduction Ym1+ CD11b+ cells was seen at 5 or 10dpl, nor was a significant increase in iNOS+CD11b+ cell density seen at 5dpl in treated mice. Opposite effects were seen in Il4i1−/− mice. Regarding remyelination, we found that OPC (Nkx2.2+ Olig2+) cell density is increased at 5dpl in WT treated mice. Meanwhile, mature oligodendrocyte
(CC1$^+$ Olig2$^+$) cell density is persistently increased in IL4I1-treated WT mice across the time course of remyelination. These findings are in contrast to Il4i1$^{-/-}$ mice, which exhibit reduced CC1$^+$ Olig2$^+$ cell density in 20dpl tissue. Finally, we found that IL4I1-treatment of EAE mice resulted in reduction and reversal of MS-like symptoms, suggesting that the effects of IL4I1 on inflammation and remyelination translate into tangible behavioral benefits that may be applicable to human disease.

An important caveat to this work is its inability to identify the function of endogenous Il4i1 gene expression in macrophages, specifically. Repeating this study with a macrophage-specific Il4i1$^{-/-}$ mouse could address this distinction. Furthermore, while our EAE work demonstrates promising effects of IL4I1-treatment, it does not address the role of endogenous I4i1 gene expression in EAE. Loss-of-function experiments employing Il4i1$^{-/-}$ mice for EAE should be conducted to address this issue in the future.

The delay in AAM reduction and CAM increase suggests that IL4I1’s ultimate effect on the macrophage population is indirect. This hypothesis is supported by findings from our lab showing that iNos expression in purified astrocyte, microglia and RAW264.7 (macrophage cell line) cultures is not affected by IL4I1 treatment (Psachoulia et al., 2016). Therefore, these cell-types appear not to be the target through which IL4I1 exerts its effects, leaving other cell populations in CNS lesions as potential targets for IL4I1 and surrogates of its effects. Previous studies have suggested that IL4I1 regulates CD4$^+$ T-cell proliferation and activity (Cousin et al., 2015; Lasourdis et al., 2011). Pro-inflammatory CD4$^+$ T-cell subtypes, namely Th1 and Th17 cells, have been implicated in in CNS infiltration following BBB breakdown, driving inflammation and oligodendrocyte destruction (Ghasemlou et al., 2007; Ryu et al., 2015; Traka et al., 2015).
Therefore, we hypothesized that IL4I1 exerts its effects on CAM, AAM, OPCs and mature oligodendrocytes via T-cell modulation.

To test this hypothesis, we conducted qRT-PCR analysis of MS-associated gene expression in lysolethicin-induced lesions of WT mice. We found reduced expression of *Ifng* (a pro-inflammatory, Th1-associated cytokine) and *Il17* (a pro-inflammatory, Th17-associated cytokine). We also quantified CD4+ T-cell subtype population density in postmortem EAE spinal cords. This study revealed lowered Th1 (T-bet+CD4+), Th17 (Rorγt+CD4+) and Th2 (Gata3+CD4+) cell density in spinal cords from EAE mice that received intravenous tail-injections of IL4I1. While intriguing, we did not anticipate IL4I1’s suppressive effect on Th2 population. It is possible that IL4I1 acts through multiple, even contradictory mechanisms, ultimately resulting in a net immunomodulatory effect. Poor cell viability could also account for the perceived reduction in Th2. It should be noted that, due to the low cell counts, our flow cytometry findings are not robust and merit further investigation.

The idea that macrophages can directly influence T cells is not new. Functionally immunomodulatory macrophages induce Treg in human samples (Schmidt *et al.*, 2016). In fact, IL4I1 has also been suggested to increase the relative proportion of modulatory T cells (Treg, Th2) to pro-inflammatory T cells (Th1, Th17), shifting the CD4+ T-cell population to a net regulatory state (Boulland *et al.*, 2007; Cousin *et al.*, 2015). The mechanism through which IL4I1 induces these changes is not clear, but multiple possibilities have been proposed.

As an LAAO, IL4I1 catalyzes the conversion of phenylalanine to phenylpyruvate, NH₃, and H₂O₂ in the process. With significant structural homology snake-derived LAAOs, which induces apoptosis by H₂O₂ toxicity, it is possible that IL4I1 effects T cells through its production of H₂O₂ (Raibekas and Massey, 1998). While all T-cell subsets exhibit reduced proliferation from
H$_2$O$_2$ toxicity, the effects appear to be differentially distributed across subsets (Boulland, et al., 2007). IL4I1-generated H$_2$O$_2$ may disproportionately impair the proliferation and function of pro-inflammatory T-cells, reducing their abundance and/or effects relative to regulatory T-cells. It has been proposed that IL4I1-generated H$_2$O$_2$ affects T-cell signaling; while long-term exposure of T-cells to H$_2$O$_2$ results in apoptosis, brief exposure to H$_2$O$_2$ downregulates the T-cell receptor $\zeta$ sidechain ($\text{TCR}\zeta$) (Otsuji et al., 1996). This IL4I1-induced TCR$\zeta$ downregulation may suppress antigen-specific T-cell proliferation and cytokine secretion (Boulland et al., 2007).

It has also been proposed that IL4I1 affects T cells by maintaining high levels of TOB1, an antiproliferative protein of particular importance to Th17 proliferation (Santarlasci et al., 2014; Scarlata et al., 2015). High levels of TOB1 impair Th17 entry into the cell cycle by targeting critical cyclins and cyclin-dependent enzymes involved in this process (Santarlasci et al., 2014). Tob1 knock-out mice exhibit increased inflammation, more prominent infiltration of T-cells, increased myelin-reactive Th1 and Th17 cells, and reduced numbers of Treg cells (Schulze-Topphoff et al., 2013). Likewise, Tob1 knock-out EAE mice exhibit earlier and more aggressive motor impairment as opposed to controls. Intriguingly, human TOB1 expression correlates with progression from clinically isolated syndrome (CIS) to clinically definitive MS (CDMS) (Corvol et al., 2008). This correlation, coupled with the functional relationship between IL4I1 and TOB1, suggests that endogenous IL4I1 levels in human blood may correlate with MS diagnosis and progression.

The potential implications of IL4I1 in human disease is further strengthened by the IL4I1 gene location on human chromosome 19q13.3-13.4. This chromosomal region has an established association with autoimmune susceptibility, already linking it to systemic lupus erythematosus (SLE), rheumatoid arthritis, and diabetes type 1, and even MS (Chavan et al., 2002). Despite these
implications, IL4I1 has never been studied in humans in the context of autoimmune disease until now. The encouraging mouse model findings described in this chapter provide a solid foundation off which to extend the study of IL4I1 on inflammation and remyelination to human samples.
CHAPTER IV: IL4I1 EXPRESSION IS REDUCED IN MS PATIENTS AND IL4I1 TREATMENT EFFECT IS DISTINCT BETWEEN HEALTHY CONTROL AND MS PATIENT POPULATIONS

A. INTRODUCTION

1. Background and Rationale

   Over 2 million people worldwide are currently afflicted with MS, and the rate of diagnosis is on the rise (Feigin et al., 2017). Approximately 85% of MS patients present with RRMS, with an average age of diagnosis of 34 (McKay et al., 2015). About half of these individuals will go on to develop SPMS within 20 years of diagnosis (Tremlett et al., 2008). Reliable biomarkers, which could differentiate between disease subtypes and predict progression, have not yet been identified (Segal, 2014). However, differentiation between MS subtypes is not possible without understanding the underlying biology specific to different stages of disease progression.

   Past research aiming to address this issue has yielded contradictory results. For example, numerous studies conclude that RRMS is characterized by aberrant inflammation while SPMS is not (Antel et al., 2012; Iwanowski and Losy, 2015; Lassmann et al., 2007). However, others suggest that pro-inflammatory mechanisms are also at play in SPMS (Frischer et al., 2009; Lassmann, 2013; Weiner, 2008). Additional research is needed to settle this debate. Incongruous findings between studies may be attributed to the intrinsic heterogeneity of MS. To address this potential confound, this study delineates three phenotypic subtypes of MS: active relapsing-remitting MS (aRRMS), non-active relapsing MS (naRRMS) and secondary progressive MS.
(SPMS) (patient group definitions are detailed in Section 2.1). Furthermore, while significant progress has been made in drug development in recent decades, much is left to be desired. All MS therapeutics currently approved by the FDA are indicated only for RRMS symptom mitigation, at which they are moderately effective. To date, no pharmaceutical therapies are effective for SPMS (Stangel, 2012). These shortcomings reflect our limited understanding of MS pathogenesis and progression. As corroborated by mouse model findings from our lab, the mechanisms underlying MS progression appear to involve aberrant inflammation and its prevention of spontaneous remyelination (Psachoulia et al., 2016).

Interleukin-4 induced protein 1, an amino acid oxidase secreted primarily from alternatively activated macrophages, has been shown to modulate activity of immune cells in both mouse model and human sample studies (Boulland et al., 2007; Copie-Bergman et al., 2003; Cousin et al., 2016; Lasourdis et al., 2011). Mouse model work from our lab has demonstrated that, through immune-modulation, IL4I1 has implications in CNS remyelination. As discussed in Chapter III, we found that IL4I1 ameliorates MS-like pathology, symptomatology, and MS-associated pro-inflammatory cytokine expression in focally demyelinated CNS lesions and in experimental autoimmune encephalomyelitis (EAE) model mouse studies (Psachoulia et al., 2016). Though our mouse model findings are encouraging, MS is a human disease; the validity of model-derived findings in human pathology is not guaranteed.

The work presented in this chapter investigates the effects of IL4I1 on MS patient immune cells in order to determine its potential as a therapeutic agent in MS. Several previous findings suggest that IL4I1 has potential implications in human disease. The human IL4I1 gene has been mapped to chromosomal locus 19q13.3-13.4—a region associated with autoimmune susceptibility to lupus, rheumatoid arthritis, diabetes type 1, and MS (Becker et al., 1998; Chavan et al., 2002).
Furthermore, it has been proposed that IL4I1 acts through TOB1, an antiproliferative protein primarily affecting Th17 cells (Santarlasci *et al.*, 2014). Deficiency in *TOB1* expression has been linked to the development of MS from clinically isolated syndrome (CIS) (Corvol *et al.*, 2008). Therefore, a correlation between MS and IL4I1 levels may also exist. The research discussed in this chapter aims to establish a potential correlation between circulating levels of IL4I1 and disease status, and to test the effects of IL4I1 on human immune cells. We hope to elucidate underlying inflammatory mechanisms that define patient subtypes, through the study of IL4I1 in and on patient samples. Our central hypotheses are that IL4I1 mitigates inflammation in human lymphocytes and that endogenous circulating levels of IL4I1 correlate with MS disease status.

Ultimately, we found significantly reduced baseline expression of *IL4I1* in PBMCs from patients with non-active relapsing-remitting MS. Though not as dramatic, *IL4I1* expression was also reduced in both other patient groups (active relapsing-remitting MS and secondary progressive MS) as compared to healthy controls. Baseline concentrations of TGFβ in MS patient plasma was also reduced. Additionally, PBMCs from patients with progressive disease were less responsive to stimulation than other groups, as defined by increases in pro-inflammatory gene expression. Once stimulated, these PBMCs showed less inflammatory activity than those from other groups tested. With regards to the effect of IL4I1 on stimulated immune cells, healthy control T cells were skewed towards a regulatory state following IL4I1 treatment; these samples had reduced Th17 and effector T cell representation, with increased relative density of Treg and Th2 cells. In patient samples, IL4I1 increased the expression of TGFβ in non-active relapsing-remitting patient lymphocytes. We also found that, while IL4I1 reduces *IL17* expression by lymphocytes from healthy control and non-actively relapsing remitting patients, it has a positive effect on *IL17* expression in active relapsing-remitting and secondary progressive lymphocytes. Compared to
healthy control, lymphocytes derived from secondary progressive patients show a significant increase in $IL17$ expression in response to IL4I1. Finally, our data confirm that IL4I1 increases the expression of the antiproliferative gene, $Tob1$, in healthy control lymphocytes, while having no significant effect on $Tob1$ expression in patient-derived samples.

2. **Approach**

To determine the effect of IL4I1-treatment on inflammation and to identify the potential correlation between endogenous IL4I1 levels and disease status, peripheral blood samples were obtained from healthy controls and from individuals in specific subgroups across the MS phenotypic spectrum. Blood plasma was isolated for detection of MS-associated cytokines, IL4I1, and other proteins of interest using ELISA. Peripheral blood mononuclear cells (PBMCs)—a heterogeneous population of cells consisting of lymphocytes (T cells, B cells and NK cells; 70-90%), monocytes (10-20%) and dendritic cells (1-2%) in healthy human blood (Kleiveland, 2015)—were also isolated for gene expression quantification (using qRT-PCR) and cellular composition analysis (by flow cytometry) in the presence and absence of IL4I1. To better mimic the global MS population and to limit potentially confounding variables, only female samples were included in the analyses presented in this chapter.

Specific cell types and cytokines of interest were chosen based on current knowledge of MS development, progression and mitigation. Specifically, pro-inflammatory immune cells (Th1, Th17, CAM) and associated pro-inflammatory cytokines (IFN$\gamma$, IL17, TNF$\alpha$) are thought to be involved with progression, while the anti-inflammatory effects of regulatory immune cells (Treg, Th2, AAM) and cytokines (IL10, TGF$\beta$) have been implicated in symptom reduction and CNS repair (Becker et al., 2013; Rodgers and Miller, 2012; Rossi et al., 2015).
This study required approval by the Institutional Review Board of Georgetown University (IRB #2015-1048; see Appendix for relevant documentation), as well as outreach to multiple collaborators between Georgetown University (GU) and Georgetown University Hospital (GUH). Patients were identified, consented and enrolled through partnership with Georgetown Multiple Sclerosis and Neuroimmunology Center (GMSNC).

2.1 Human Subject Groups
Clinical aspects of this study were designed in collaboration with neurologists at GMSNC. Five subject groups were defined following standard McDonald diagnostic criteria (2010 revisions; Polman et al., 2011) and Lublin and Reingold clinical course definitions (2013 revisions; Lublin et al., 2014) with practical modifications to fit patient demographics (Fig. IV.1):

**Group 1:** Active relapsing-remitting MS (aRRMS). Patients diagnosed with RRMS who have experienced a radiological or clinical relapse within 1 year. Radiological relapse is defined by two or more new lesions, one enlarging T2 lesion, or one new T1 gad+ lesion in the brain or spinal cord. Blood was drawn prior to potential steroid treatment (n=11 females enrolled).

**Group 2:** Non-active relapsing-remitting MS (naRRMS). Patients with RRMS who have not relapsed or had radiological changes within the last 1 year (n=9 females, n=2 males enrolled).

**Group 3:** Secondary progressive MS (SPMS). Individuals diagnosed with SPMS and have therefore not experienced relapses (as defined above) in the past 12 months. Exhibit accumulation of disability, defined by a worsening EDSS score that persists for six months or more. Steadily
increasing neurologic dysfunction without recovery is objectively documented by a treating neurologist (n=6 females enrolled).

**Group 4:** Other autoimmune diseases (OAD). These individuals are diagnosed with an autoimmune disease that is definitively not MS, but has symptoms affecting the CNS. Such diseases include SLE, Sjögrens, Behcets, and rheumatoid arthritis. If taking immunosuppressive treatments, details will be noted for analysis (n=0 enrolled).

**Group 5:** Healthy controls (HC). Individuals have no history of neurological pathology or autoimmune disease, and are gender-matched to their MS counterparts. Commercially available HC plasma and PBMCs were purchased from commercial donors fulfilling these criteria. Sample isolation/handling procedures (Fig. IV. 2) were consistent between HC and patient blood (n=11 females, n=2 males\(^1\) enrolled).

In order for our study population to best mirror the general population most impacted by MS, enrollment was restricted to individuals between 18-65 years of age, and only female samples were analyzed in the work herein discussed. When possible, patients were naïve to disease modifying drugs (DMDs). For non-DMD naïve patients, past and current treatment regimens were clearly documented for consideration upon analysis. Pregnant women were excluded from this study in the interest of controlling for confounding variables.

\(^{1}\)Male samples were excluded from general analysis. \(^{2}\)OAD group was included to serve as an additional control group to distinguish MS-specific findings from nonspecific results that are also found in other autoimmune conditions. *No patients were enrolled in this group.*
Call for Patients to Participate in MS Research!

We are looking for patients between ages 18-65 who meet the criteria of one of our patient group (groups 1-4). Group criteria are defined as:

1. **Active RRMS**
   - Diagnosed with RRMS and have experienced a relapse* within the last 12 months. Patients are preferentially naïve to DMDs, when possible.

2. **Non-active RRMS**
   - Diagnosed with RRMS and have NOT experienced a relapse* within the last 12 months. Preferentially naïve to DMDs, when possible.

3. **Non-active SPMS**
   - Diagnosed with SPMS and have NOT experienced a relapse* within the last 12 months. Confirmed accumulation of disability** or steady increase in neurologic dysfunction without unequivocal recovery. Naïve to DMDs, when possible.

4. **Other Autoimmune**
   - Diagnosed with an autoimmune disease that is definitively not MS, but with symptoms affecting the CNS (e.g. SLE, SJögrens, Behcets, RA etc.) Naïve to immunosuppressive treatments, when possible.

5. **Healthy controls:** No history of neurological pathology or autoimmune disease.

**About the study:** In collaboration with the research laboratories of Dr. Jeffrey Huang and Dr. Anton Wellstein (GUMC), we are studying the levels and effects of a novel protein, called IL411, on patient blood samples. Extensive data from our lab suggest that IL411 ameliorates symptomatology and reduces MS-pathophysiology in mouse-models of MS. The current study aims to extend these findings to human samples, assessing IL411 as a potential biomarker and treatment option in MS.

Participation involves a one-time blood draw of ~30mL. Plasma and PBMCs from the blood will be isolated and analyzed for this study. To report eligible patients, please contact:

- **Dr. Faria Amjad**
  - GUH Neurology Department
  - 301-326-6398
  - Faria.S.Amjad@gunet.georgetown.edu
  - with clinical questions

- **Stephanie Davis**
  - MD/PhD Candidate in Neuroscience
  - 617-319-4625
  - sed51@georgetown.edu
  - with research-related questions

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*Relapses are defined clinically or radiologically. Radiological relapse is defined as at least two new lesions, one enlarging T2 lesion, or one new T1 gadolinium-enhancing lesion in the brain or spinal cord. **Accumulation of disability is defined by a worsening EDSS that persists over at least 6 months or steadily increasing neurologic dysfunction objectively documented by treating neurologist.

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Figure IV.1. Study flyer summarizing inclusion and exclusion criteria for patient enrollment. Patient groups and criteria were designed in collaboration with the Georgetown University Hospital MS Clinic. Flyers were hung across Georgetown University Hospital Department of Neurology and distributed to medical residents and attendings in neurology.
Figure IV.2. Integrated protocol flowchart detailing the procedures used for sample isolation and handling.

BD Vacutainer recommended procedures were modified and expanded upon to resemble commercially available samples and to accommodate the abilities and limitations of the collaborators involved: Georgetown University Hospital Neurology Department MS Clinic, Georgetown University Clinical Tissue Culture Shared Resource (TCSR).

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**PLASMA**

1. Centrifuge plasma supernatant
2. Store at -80°C
3. Use within 6 months

**PBMCs**

1. Place in -80°C freezer
2. Store within 6 months
3. Use by date

**Cytokine expression**

1. Place in -80°C freezer
2. Store within 6 months
3. Use by date

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**Tissue Culture Shared Resource**

- **Clinic/CRU:**
  - 100-200 ml brain tissue
  - 200-500 ml blood
- **Lab:**
  - 50-150 ml blood
  - 10-15 ml plasma
  - 10-20 ml PBMCs

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**Additional Information**

- **Assessing Interferon-gamma Induced Protein 1 (IFN-γ)**
- **Sampling:**
  - Blood samples from patients with multiple sclerosis
  - Brain tissue from deceased patients
  - Blood samples from healthy controls
- **Analysis:**
  - ELISA (Enzyme-Linked Immunosorbent Assay)
  - Flow cytometry
  - Western blotting

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**Footnotes:**

- 1. Interferon-gamma Induced Protein 1 (IFN-γ)
- 2. Multiple Sclerosis
- 3. Immune Response
2.2 Human Sample Acquisition, Handling and Processing

Because this project extends beyond the expertise of my primary mentor, Dr. Jeffrey Huang, whose lab specializes in the study of inflammation and remyelination in mouse models of MS, I established a co-mentorship with Dr. Anton Wellstein. Dr. Wellstein has significant experience handling patient-derived samples through his work in tumor biology and immunology. Blood draw from patients and isolation of plasma and PBMC components was performed in partnership with several GU shared resources: namely the Clinical Research Unit (GU-CRU) and Tissue Culture Shared Resources (GU-TCSR). Blood collection, isolation, storage and transfer procedures were designed and employed in collaboration with GUH, GU-CRU and GU-TCSR (Fig. IV.2).

2.3 Experimental Parameters and Preliminary Data

2.3.1 B-actin as Housekeeping Control Gene for qRT-PCR Analysis

RATIONALE: Endogenous reference genes, against which experimental measurements can be normalized, are an important part of accurate qRT-PCR data analysis. Identifying an appropriate endogenous control is often overlooked in science (Chapman and Waldenström, 2015), but comparison against a suboptimal reference gene yields biologically irrelevant data (Dheda et al., 2005; Nolan et al. 2006). An optimal reference gene exhibits relatively early detection within the range of genes being studied, and, most importantly, is expressed consistently across experimental variables (Kozera and Rapacz, 2013). Basic metabolism genes, or “housekeeping genes”, often fulfill these requirements because they are involved in vital processes and are thus constitutively active at high and stable levels (Thellin et al., 1999).

For the gene expression aspect of this study, an appropriate endogenous control must show relative stability across resting, stimulated, and IL4I1-treated conditions. Common endogenous
reference genes for vertebrate gene expression include \( \beta\)-actin, which codes important structural elements of the cell, \( GAPDH \), a glycolytic enzyme, and \( 18S \), a ribosomal RNA subunit active in protein synthesis (Chapman and Waldenström, 2015). Hypoxanthine guanine phosphoribosyltransferase (\( HPRT \)) and translation elongation factor \( EF1A \) are also commonly used in relevant literature as endogenous controls (Chapman and Waldenström, 2015; Kozera and Rapacz, 2013; Thell et al., 2016), and are thus included in this analysis.

METHODS: The expression of common endogenous control genes (\( \beta\)-actin, \( GAPDH \), \( HPRT \), \( EF1A \) and \( 18S \)) was measured by RT-qPCR in healthy human PBMCs under three conditions of interest: baseline, stimulated + PBS, and stimulated + IL4I1. To do this, cells were thawed and immediately plated onto anti-CD3-coated (10\( \mu \)l/ml) or PBS-coated culture plates, then supplemented with soluble anti-CD28 (10\( \mu \)l/ml) or PBS, respectively. After 48 hours of incubation, cells were treated with recombinant human IL4I1 (200ng/ml) or PBS, creating three culture conditions: unstimulated/sham-treated (“baseline”), stimulated/sham-treated (“+PBS”), and stimulated/IL4I1-treated (“+IL4I1”). After 24 hours, mRNA was extracted for cDNA synthesis and RT-qPCR analysis.
RESULTS:

**Figure IV.3.** *B-actin* expression is least affected by experimental parameters. Quantified raw Ct values detected by qRT-PCR for common housekeeping genes in cultured human PBMCs from healthy controls. Comparison across unstimulated (baseline), stimulated then vehicle-treated (+PBS) and stimulated then IL4I1-treated (+IL4I1) cells. Average raw Ct values for **A. B-actin** (n=6), **B. GAPDH** (n=4), **C. HPRT** (n=2), **D. EF1A** (n=2) and **E. 18S** (n=3) with corresponding P-values indicating differences between groups. Numbered dots indicate individual donors on each graph **F.** Combined data for visual comparison. Stimulation consisted of anti-CD3 pre-coat (10µl/ml) plus soluble anti-CD28 (10µl/ml) for 48h before PBS or IL4I1 treatment (200nl/ml) for 24h. Graphs show mean with SEM. Ordinary one-way ANOVA with Tukey’s multiple comparisons test, alpha = 0.05. Statistical significance reported as *P<0.05, ****P<0.0001.
INTERPRETATION AND CONCLUSIONS: Relative differences in expression of each gene across experimental conditions identified $\beta$-actin as the most appropriate reference for gene expression in this study (Fig. IV.3). As compared to GAPDH, 18S, HPRT and EF1A, $\beta$-actin exhibits minimal fluctuation (highest P-values) in expression levels between baseline, stimulated and treated conditions. Furthermore, $\beta$-actin demonstrates high expression levels (low Ct values) as compared to the other genes tested, and is thus the most suited candidate for this work moving forward.

DISCUSSION AND CAVEATS: These data show a significant spread in some conditions, especially upon stimulation and IL4I1 treatment, in which more experimental procedures have been performed. Those genes that have the least spread within conditions have only $n=2$. While sufficient for our purposes, the spread of these data could be reduced and their power increased if more patient samples were included. It may also be important to note that these experiments were performed on cells that were stimulated by anti-CD3 (pre-coat) and anti-CD28 (soluble), whereas subsequent experiments revealed optimal gene expression changes using a slightly different method of stimulation (see Section 2.2.iii). It is possible, though unlikely, that a different control gene would have been optimal under a different method of stimulation.

2.3.2 Twenty-Four-Hour Treatment with IL4I1

RATIONALE: IL4I1 appears to have a dynamic effect over time. Chronological assessment of recombinant murine IL4I1 administration to cultured tumor cells revealed an immediately increasing effect of treatment that plateaued around 35 hours post-treatment (Bod et al., 2017b). Previous studies from our lab that investigate IL4I1 in mouse-derived cells found optimal effects
on inflammatory gene expression after 24h treatment with IL4I1 (Psachoulia et al., 2016). However, treatment response is not always consistent between species. Human T cells cultured with IL4I1-transfected cells show no increased effect at 24 hours as compared to 3 hours of exposure (Boulland et al., 2007). However, this finding may be specific to IL4I1-administration via secretion by transfected cells in culture. The variability in reported treatment duration, plus the dynamic effects of IL4I1 over time, suggests that optimal timing is study-specific and an important issue to address. Though dissimilar, these previous findings offer a range around which to search for an optimal treatment duration for the study at hand.

METHODS: The expression of representative cytokines (TNFα, TGFβ, IFNγ, IL10 and IL17) was measured by RT-qPCR in healthy human PBMCs after stimulation (as above) and treatment with IL4I1 (200ng/ml) or PBS (control). mRNA was extracted from cells after 6, 24, and 72 hours of treatment. These time points span above and below IL4I1-treatment parameters reported by other studies (Bod et al., 2017b; Psachoulia et al., 2016). Fold change in expression between control (+PBS) and treated (+IL4I1) cells was quantified and compared between treatment durations.

RESULTS:

Figure IV.4. IL4I1 exerts greatest effect on cytokine gene expression at 24h post-treatment. Quantified qRT-PCR expression of representative MS-associated cytokines in PBMC-derived lymphocytes from healthy control. Cells were stimulated by anti-CD3 (pre-coat, 10µl/ml) and anti-CD28 (soluble, 10µl/ml) for 48h then treated with IL4I1 (200ng/ml) for 6h, 24h or 72h before mRNA extraction. Fold change in expression is normalized to β-actin and PBS-treated controls. Cells obtained from the same individual were used for all experiments (n=1).
INTERPRETATION AND CONCLUSIONS: The effect of IL4I1 on each gene of interest (*TNFα*, *TGFβ*, *IFNγ*, *IL10* and *IL17*) was greatest after 24 hours of treatment ([Fig. IV.4](#)). As compared to 6 and 72 hours of treatment at same dose (200ng/ml), 24 hours of IL4I1 treatment maximally reduces expression of *TNFα*, *IFNγ*, *IL10* and *IL17* in stimulated human cells. Maximal increase in *TGFβ* is also observed at this time point. As such, stimulated cells were treated with IL4I1 for 24 hours before mRNA extraction and gene expression analysis in all subsequent experiments.

DISCUSSION AND CAVEATS: It is clear from these data that IL4I1 exerts its greatest effect on the genes tested, under the parameters tested, at 24 hours. However, it was curious that this effect appears to be a decrease in *TNFα*, *IFNγ*, *IL10* and *IL17* (both pro- and anti-inflammatory cytokines) and an increase in *TGFβ* (immune modulatory). The polarity of these findings is not always reproduced in subsequent experiments (*see Section B. Results*). While it was critical that these experiments be performed on cells from the same individual for control purposes, these data were obtained from only one individual. Likewise, these preliminary experiments were performed using only one stimulation method (anti-CD3 then anti-CD28) and only one concentration of IL4I1 (200ng/ml). It is also noteworthy that, for some genes, the direction of IL4I1’s effect is different over time. This could be an artifact of small sample size, or it could indicate that IL4I1’s effect is dynamic, perhaps involving feedback mechanisms. This further strengthens the importance of controlling for time as a parameter in future studies. Analyzing gene expression at intermediate time points could also help to elucidate this concept.
2.3.3 Anti-CD3/CD28/CD2 Stimulation and IL4I1 at 200ng/ml

RATIONALE: In order to maximize physiological relevance, research investigating inflammation in MS is typically conducted on PBMCs that have been stimulated with T-cell activating antibodies such as anti-CD3, anti-CD28 and anti-CD2 (El Behi et al., 2017; Gimsa et al., 2013; Haines et al., 2015; Schulze-Topphoff et al., 2013). Just as the duration of IL4I1 treatment affects gene expression results, stimulation methods and treatment concentrations are also likely to influence outcome measurements. Suboptimal PBMC stimulation or IL4I1-treatment may prevent real effects from being observed. Meanwhile, overstimulation or excessive treatment may obscure results by producing a ceiling effect, or be toxic to the samples being studied. This study’s inclusion of clinical samples, which are often especially vulnerable to damage and death, further exemplifies the importance of optimal cell culture parameters.

Multiple stimulation protocols have been implicated in related literature pertaining to the stimulation of human PBMCs and/or the effects of IL4I1 in various organisms. Namely, common methods include stimulation by anti-CD3 and anti-CD28 (Deshmukh et al., 2013; Haines et al., 2015), anti-CD3/CD28 tetrameric antibody complexes (Gimsa et al., 2013; Perriard et al., 2015; Schulze-Topphoff et al., 2013) anti-CD3/CD28/CD3 tetrameric antibody complexes (El Behi et al., 2017). Other studies supplement these methods with soluble IL2 (Jin et al., 2012; Scarlata et al., 2015; Schmidt et al., 2017). Each combination of antibodies activates and expands T cells by crosslinking its corresponding ligand (CD3/CD28/CD2) on the T-cell membrane. While activated T cells produce IL2 which is secreted into the media, supplementing with exogenous IL2 can help maintain T cell proliferation, which may be recommended in certain experimental set-ups.

A wide range also exists for IL4I1 treatment concentrations cited in the literature. Mouse-model demyelination studies performed in our lab found significant changes induced by injection
of IL4I1 at 200ng/ml (Psachoulia et al., 2016). This is beyond the 1.5 to 150ng/ml range of IL4I1 used in murine tumor cell proliferation research (Bod et al., 2017b). Identifying an optimal stimulation method and treatment concentration combination particular to the parameters of this study is critical to obtaining robust and meaningful effects. Stimulated PBMCs are referred to as PBMC-derived lymphocytes in this thesis.

METHODS: To identify a stimulation method and treatment concentration appropriate for this study, healthy human PBMCs were stimulated by one of six stimulation methods and treated with one of four concentrations of IL4I1 (100, 200, 500, 1000ng/ml), or PBS. Stimulation methods tested were: (method #1a) anti-CD3 (pre-coat, 10µl/ml) and anti-CD28 (soluble, 10µl/ml), (method #2a) anti-CD3/CD28 tetrameric antibody complex (20µl/ml) and (method #3a) anti-CD3/CD28/CD2 tetrameric antibody complex (20µl/ml), each without or with addition of IL2 at 20µl/ml (methods #1b, #2b and #3b). Stimulation occurred for 48 hours, followed by 24 hours of treatment before mRNA extraction, cDNA synthesis and qRT-PCR analysis. The fold change in expression for five genes of interest (TNFα, TGFβ, IFNγ, IL10 and IL17) was compared between each experimental condition.
RESULTS:

Figure IV.5. MS-associated cytokine expression is optimally affected by 24h treatment with IL4I1 at 200ng/ml following stimulation by anti-CD3/CD28/CD2 for 48h. Quantified qRT-PCR detection of gene expression in healthy control PBMCs across six experimental stimulation methods (48h) and four IL4I1-treatment concentrations (24h). Stimulation methods tested were: anti-CD3 (pre-coat, 10µl/ml) with anti-CD28 (soluble, 10µl/ml), anti-CD3/CD28 tetrameric antibody complex (20µl/ml) and anti-CD3/CD28/CD2 tetrameric antibody complex (20µl/ml), each with and without addition of IL2 (20µl/ml). Fold change in expression of A. TNFα, B. TGFβ, C. IFNγ, D. IL10 and E. IL17 after 24h treatment with IL4I1 at 100, 200, 500 or 1000ng/ml. Fold change in expression is normalized to β-actin and PBS-treated controls. Cells obtained from the same individual were used for all experiments (n=1).
INTERPRETATION AND CONCLUSIONS: Of the parameters tested, stimulation by method #3a followed by IL4I1 treatment at 200ng/ml produced the most pronounced effects on the greatest number of targets. Three of the five genes analyzed (TGFβ, IFNγ and IL17) were optimally responsive to IL4I1 under these conditions, under which the expression of each was reduced (Fig. IV.5.B, C, E). TNFα was maximally affected by 100ng/ml after stimulation by method #2a (Fig. IV.5.A), while IL10 was most responsive to stimulation method #1b and 1000ng/ml L4I1 (Fig. IV.5.D). Though peak response for these genes was not achieved from stimulation method #3a with 200ng/ml IL4I1, TNFα and IL10 did still experience relatively significant decreases at this stimulation-dose pair. Therefore, subsequent experiments were conducted using these parameters.

DISCUSSION AND CAVEATS: While encouraging that a common treatment dose and stimulation method could be identified as being effective across genes of interest, the nature of these effects is sometimes surprising. It was unexpected that gene expression would change so drastically between IL4I1 doses, or by addition of IL2, for example. These unexpected inconsistencies could be explained by a complicated and highly sensitive relationship between IL4I1 and the immune cell signaling, or they could also be a byproduct of a low sample size (n=1). Analysis of multiple samples, rather than one representative sample, could help strengthen these data.
2.4 Overarching Goals and Hypotheses

2.4.1 Establish Baseline Inflammatory Profiles and Response to Stimulation in Human PBMCs from HC and MS Patients across Stages of Progression

In order to better understand the immune dysregulation characteristic of MS at baseline, and to interpret experimentally-induced perturbations of that baseline, inflammatory cytokine levels and gene expression were measured in blood samples from healthy controls (HC) and MS patients across stages of disease progression: active RRMS, non-active RRMS and SPMS. Pro-inflammatory (TNFα, IFNγ and IL17) and anti-inflammatory (TGFβ and IL10) cytokine gene expression was measured in resting PBMC cultures by quantitative real-time PCR (qRT-PCR), and circulating cytokine levels were measured in plasma by enzyme-linked immunosorbent assay (ELISA), for comparison between groups. Because it has been proposed that MS is a pro-inflammatory, Th1-driven disease (Ando et al. 1989), we hypothesized that pro-inflammatory gene expression and circulating cytokine levels would correlate with disease state; increased in MS than HC, with highest levels in actively-relapsing-remitting disease, following the current understanding of pathology underlying each subtype. In turn, we hypothesized that anti-inflammatory cytokines would follow an opposite pattern.

While the inflammatory status of patient samples at baseline can inform us about the characteristics of specific MS subtypes, the behavioral patterns of disease-state cells can elucidate underlying dynamics of disease activity and progression. Having developed a baseline inflammatory profile for healthy controls and patient sub-groups, we can quantify the effects of experimental manipulations within each group. The response of cultured PBMCs to stimulation by anti-CD3/CD28/CD3 (10µl/ml; 48 hours) was quantified by qRT-PCR and compared between healthy controls and MS patient groups. Because MS pathogenesis is thought to result from aberrant immune system activity, we hypothesized that patient cells would undergo an exaggerated
pro-inflammatory response to stimulation as compared to healthy controls, with maximal inflammation in active RRMS samples.

2.4.2 Determine the Potential of IL4I1 as an Immune-Modulating Treatment on Human Cells

The effects of IL4I1 on stimulated PBMCs (PBMC-derived lymphocytes) within each group was assessed by comparing PBMC-derived lymphocytes that were treated with IL4I1 to PBMC-derived lymphocytes that were sham-treated with PBS. In order to determine the IL4I1-induced fold change in expression of \( \text{TNF}_\alpha, \text{TGF}_\beta, \text{IFN}_\gamma, \text{IL10} \) and \( \text{IL17} \), qRT-PCR was used to quantify expression of each gene in (+PBS) versus (+IL4I1) samples for each group. With these data, gene-specific and disease-state-specific comparisons were made. This allows us to identify differences between patient groups in the response to stimulation of a specific gene, as well as differences between the effects of IL4I1 on specific genes as a function of patient group.

To elucidate the effect of IL4I1 on cell proliferation and differentiation, healthy control PBMC-derived lymphocytes were analyzed by flow cytometry. Antibody labeling for relevant pro-inflammatory (Th1, Th17 and classically-activated macrophages [CAM]) and anti-inflammatory cell types (Th2, Treg and alternatively-activated macrophages [AAM]) was performed and compared between conditions. Because past research, including work from our lab, has shown that IL4I1 has an immunomodulatory effect, we hypothesized that IL4I1 treatment of human PBMCs will reduce pro-inflammatory MS-associated gene expression (\( \text{TNF}_\alpha, \text{IFN}_\gamma \), and \( \text{IL17} \)) and enhance anti-inflammatory cytokine expression (\( \text{TGF}_\beta \) and \( \text{IL10} \)). Likewise, we hypothesized that IL4I1 would decrease the populations of pro-inflammatory cells (Th1, Th17, CAM) while increasing relative proportions of regulatory cells (Th2, Treg, AAM).
2.4.3 Assess IL4I1 as a Marker of Disease State

To identify a potential relationship between MS progression and IL4I1 status, endogenous IL4I1 was measured in healthy controls and MS patients across the disease spectrum. First, *IL4I1* expression in healthy human PBMCs was confirmed by qRT-PCR. Next, *IL4I1* expression in and circulating IL4I1 protein levels (captured by sandwich ELISA) were measured in all patient groups. Previously identified correlations between the rate of progression from CIS to CDMS and an individual’s circulating levels of TOB1, an anti-proliferative protein through which IL4I1 has been proposed to function, suggests that IL4I1 may also correlate to disease state. We therefore hypothesized that IL4I1 protein and gene expression levels will be reduced in MS as compared to HC, and that this reduction will be more pronounced with disease progression.

2.4.4 Elucidate the Mechanism of IL4I1-Mediated Immune Modulation of Human PBMCs

To better understand how IL4I1 elicits its effects, two prominent theories were tested: (1) that IL4I1 acts by maintaining high levels of the antiproliferative protein, TOB1, and (2) that IL4I1 downregulates T-cell receptor sidechain ζ (TCRζ) expression. Both mechanisms result in a skew towards immunomodulatory T cell populations. To determine the validity of these theories in human samples, qRT-PCR was used to quantify *TOB1* and *TCRζ* expression in healthy control (+PBS) and (+IL4I1) PBMC-derived lymphocytes. First, fold change in expression in response to treatment was compared between both TOB1 and TCRζ. This analysis was expanded to MS patient cells for TOB1, which showed potential in preliminary HC samples, where it was further investigated by sandwich ELISA detection of circulating TOB1 protein. In line with previous studies, we hypothesized that IL4I1 would reduce *TOB1* and *TCRζ* expression in HC samples, and that this effect would be maintained in patient cells.
B. RESULTS

The results presented in this section are organized by the experimental condition being assessed and the concepts being investigated as follows: baseline inflammatory cytokine analysis (subsection 1), response to stimulation and activity of stimulated cells (subsections 1 and 2), response to IL4I1-treatment (subsections 4 and 5), baseline analysis of IL4I1 (subsection 6), IL4I1 mechanism of action (subsections 7 and 8). Within subsections, significant findings are presented before null results; HC data are presented first, followed by aRRMS, naRRMS and SPMS. Effects seen on flow cytometry are summarized in Table IV.1 and all corrected P-values obtained from gene expression analysis are summarized in Table IV.2.

1. Plasma Concentration of TGFβ is Reduced in MS

In order to describe the inflammatory environment characteristic of MS-subtypes and healthy controls, cytokine protein levels were measured in plasma ELISA and cytokine gene expression was measured by qRT-PCR in unstimulated PBMCs. In agreement with the concept that MS is characterized by a relatively unmitigated inflammatory environment, we detected significantly reduced concentrations of TGFβ across all three patient groups as compared to HC. This effect was not reflected in TGFβ mRNA expression, however, which may be explained by translational modifications, differences in rates of transcription/translation or in vivo half-lives (Greenbaum, et al., 2003). The most pronounced reduction was in plasma derived from aRRMS patients, with small increases in naRRMS and again in SPMS (Fig. IV.6). The other cytokines tested were found to have concentration levels too low for between-group analyses. Quantified qRT-PCR detection of
TNFα, TGFβ, IFNγ, IL10 and IL17 in unstimulated PBMCs did not find any significant differences in baseline expression between groups (Fig. IV.7).

Figure IV.6. TGFβ plasma protein levels are reduced in all subtypes of MS. Quantitative ELISA data for TGFβ protein concentrations (pg/ml) measured in plasma collected from healthy control donors (HC; n=2) and patients diagnosed with active relapsing-remitting MS (aRRMS; n=2), non-active relapsing-remitting MS (naRRMS; n=2) and secondary progressive MS (SPMS; n=2). Graph shows mean with SEM. Ordinary one-way ANOVA with Tukey’s multiple comparisons test, alpha = 0.05. Statistical significance reported as **p<0.01, ***p<0.001.

Figure IV.7. Baseline expression of MS-associated genes is similar between healthy control and MS patient-derived PBMCs. Quantified average qRT-PCR detection of MS-associated cytokine gene expression in cultured PBMCs obtained from female donors with active relapsing-remitting MS (aRRMS), non-active relapsing-remitting MS (naRRMS), secondary progressive MS (SPMS) and healthy controls (HC). A. TNFα expression in HC (n=5), aRRMS (n=6), naRRMS (n=7), and SPMS (n=5). B. TGFβ expression in HC (n=4), aRRMS (n=6), naRRMS (n=7), and SPMS (n=5). C. IFNγ expression in HC (n=6), aRRMS (n=6), naRRMS (n=7), and SPMS (n=5). D. IL10 expression in HC (n=4), aRRMS (n=7), naRRMS (n=7), and SPMS (n=5). E. IL17 expression in HC (n=1), aRRMS (n=5), naRRMS (n=6). IL17 was not detectable in SPMS. Dashed line represents no difference from HC. Graphs show mean with SEM. Ordinary one-way ANOVA with Tukey’s multiple comparisons test, alpha = 0.05.
2. Pro-inflammatory Response to Stimulation is Diminished in SPMS Patient PBMCs

As a proof-of-concept, response to T-cell activation by anti-CD3/CD28/CD2 was determined by comparing gene expression in stimulated PBMCs (PBMC-derived lymphocytes) to sham-stimulated controls (baseline PBMCs) using qRT-PCR. Indeed, stimulation was found to increase pro-inflammatory cytokine expression ($\text{TNF}\alpha, \text{IFN}\gamma, \text{IL17}$) in all groups (Fig. IV. 8.A, C, E). Expression of regulatory cytokines ($\text{TGF}\beta, \text{IL10}$) were relatively unaffected (Fig. IV. 8.B, D).

To then characterize these changes as a function of disease state, the stimulation-induced fold change in expression for each gene was compared across groups. While the stimulation-induced increase in $\text{TNF}\alpha$ expression was comparable between HC-, aRRMS- and naRRMS-derived cells, stimulation had a smaller effect on $\text{TNF}\alpha$ expression in SPMS (Fig. IV. 9.A). The increase in $\text{TNF}\alpha$ expression by SPMS cells was significantly lower than it was in both HC and naRRMS groups. Likewise, the increase in $\text{IFN}\gamma$ expression induced by stimulation was suppressed in SPMS (Fig. IV. 9.B). This reduction was significant in comparison to aRRMS, whose $\text{IFN}\gamma$ response was similar to HC. Samples from naRRMS patients also showed reduced stimulation-induced increases in $\text{IFN}\gamma$ expression, but this was not a significant effect. Expression change was comparable between HC, aRRMS and naRRMS for these pro-inflammatory cytokines (Fig. IV.10). Stimulation-induced expression change for regulatory cytokines ($\text{TGF}\beta, \text{IL10}$) was not significantly different between groups (Fig. IV.10). These analyses were not possible for $\text{IL17}$ in SPMS due to undetectable $\text{IL17}$ expression by qRT-PCR at baseline. Ultimately, these findings support the notion that immune activity and reactivity is dynamic over the course of disease.
Figure IV.8. Expression of pro-inflammatory genes is increased by T-cell activation across disease states. Average change in qRT-PCR detection of gene expression between baseline and anti-CD3/CD28/CD2-stimulated PBMCs derived from healthy controls (HC), active relapsing-remitting MS (aRRMS), non-active relapsing-remitting MS (naRRMS) and secondary progressive (SPMS) patients. Fold change in expression of (A) TNFα in HC (n=3), aRRMS (n=7), naRRMS (n=6) and SPMS (n=5); (B) TGFβ in HC (n=2), aRRMS (n=6), naRRMS (n=6) and SPMS (n=5); (C) IFNγ in HC (n=4), aRRMS (n=6), naRRMS (n=6) and SPMS (n=5); (D) IL10 in HC (n=2), aRRMS (n=7), naRRMS (n=5) and SPMS (n=5); and (E) IL17 in HC (n=1), aRRMS (n=5) and naRRMS (n=5). Baseline expression of IL17 was not detected in SPMS. Dashed line represents no difference from baseline expression. Graph shows mean with SEM.
Figure IV.9. Change in TNFα and IFNγ expression induced by PBMC stimulation is reduced in SPMS. Average difference in pro-inflammatory gene expression between baseline and stimulated conditions normalized to change in HC. PBMCs in culture were supplemented with PBS (baseline condition) or anti-CD3/CD28/CD2 (stimulated condition) at 10µl/ml for 48h before mRNA extraction. A. Fold change in TNFα expression for HC (n=3), aRRMS (n=7), naRRMS (n=7) and SPMS (n=5). B. Fold change in IFNγ expression for HC (n=4), aRRMS (n=6), naRRMS (n=7) and SPMS (n=5). Dashed line indicates equal change in expression as HC. Unpaired t tests, two-tailed. Statistical significance reported as *P<0.05, **P<0.01.

Figure IV.10. PMBC stimulation affects TGFβ and IL10 expression similarly across groups. Average difference in regulatory gene expression between baseline and stimulated conditions normalized to change in HC. PBMCs in culture were supplemented with PBS (baseline condition) or anti-CD3/CD28/CD2 (stimulated condition) at 10µl/ml for 48h before mRNA extraction. A. Fold change in TGFβ expression for HC (n=3), aRRMS (n=6), naRRMS (n=7) and SPMS (n=5). B. Fold change in IL10 expression for HC (n=2), aRRMS (n=7), naRRMS (n=6) and SPMS (n=5). Dashed line indicates equal change in expression as HC. Unpaired t tests, two-tailed.
3.  *TNFα* expression is reduced in PBMC-derived lymphocytes from SPMS patients

Once stimulated, the aggressiveness of these activated lymphocytes was compared between groups. Cytokine gene expression quantification was used to identify differences in inflammatory activity as a function of disease state. Expression of MS-associated cytokines was measured by qRT-PCR in PBMC-derived lymphocytes from each group. Between-group comparison revealed a significant decrease in *TNFα* expression by PMBC-derived lymphocytes from SPMS patients, as compared to PBMC-derived lymphocytes from healthy control donors (Fig. IV.11). No significant differences were found in *TGFβ, IFNγ, IL10* or *IL17* expression by stimulated cells between groups (Fig. IV.12). This finding again supports the concept of reduced immune activity in SPMS.

![Figure IV.11. Reduced TNFα expression in PBMC-derived lymphocytes of progressive MS patients. Quantified average qRT-PCR detection of TNFα gene expression in cultured PBMCs obtained from female donors with active relapsing-remitting MS (aRRMS; n=9), non-active relapsing-remitting MS (naRRMS; n=8), secondary progressive MS (SPMS; n=5) and healthy controls (HC; n=7). T cells were activated by anti-CD3/CD28/CD2 stimulation (48h, 20µl/ml) and sham-treated with PBS (24h, 20µl/ml) before RNA extraction. Dashed line represents no difference from HC. Graph shows mean with SEM. Ordinary one-way ANOVA with Tukey’s multiple comparisons test, alpha = 0.05. Statistical significance reported as * P<0.05.](image)
Figure IV.12. TGFβ, IFNγ, IL10 and IL17 expression is similar in PBMC-derived lymphocytes across groups. Average qRT-PCR detection of MS-associated cytokine gene expression in stimulated PBMCs. Cells were obtained from female donors with active relapsing-remitting MS (aRRMS), non-active relapsing-remitting MS (naRRMS), secondary progressive MS (SPMS) and healthy controls (HC). T cells were activated by anti-CD3/CD28/CD2 stimulation (48h, 20µl/ml) and sham-treated with PBS (24h, 20µl/ml) before RNA extraction. A. TGFβ expression in HC (n=7), aRRMS (n=9), naRRMS (n=8), and SPMS (n=5). B. IFNγ expression in HC (n=7), aRRMS (n=9), naRRMS (n=8), and SPMS (n=5). C. IL10 expression in HC (n=6), aRRMS (n=6), naRRMS (n=7), and SPMS (n=5). D. IL17 expression in HC (n=6), aRRMS (n=9), naRRMS (n=8), and SPMS (n=5). Dashed line represents no difference from HC. Graphs show mean with SEM. Ordinary one-way ANOVA with Tukey’s multiple comparisons test, alpha = 0.05.
4. IL4I1 Skews CD4\(^+\) and CD8\(^+\) T Cell Populations Towards a Regulatory State in Healthy Controls

The effect of IL4I1 treatment on human PMBC composition was analyzed by flow cytometry. PBMC-derived lymphocytes from a representative healthy donor were analyzed after treatment with IL4I1 (+IL4I1) or vehicle (+PBS). IL4I1-treated cells had increased concentrations of modulatory T-cell subtypes. Specifically, both Treg (CD4\(^+\) IL10\(^+\) IFN\(\gamma\)) and Th2 (CD4\(^+\) IL17\(^-\) IFN\(\gamma\) IL10\(^-\)) subtypes had greater representation in treated samples (3.48\% vs. 0.66\% for Treg; 67.18\% vs. 37.16\% for Th2). Furthermore, pro-inflammatory Th17 (CD4\(^+\) IL17\(^+\) IFN\(\gamma\)) populations were reduced in IL4I1-treated samples (0.96\% vs. 30.80\%) (Fig. IV.13). Effector T-cell populations were also modulated by IL4I1-treatment, with both in CD4\(^+\) effector cells (CD4\(^+\) CD197\(^-\) CD45RA\(^+\)) and CD8\(^+\) effector cells (CD8\(^+\) CD197\(^-\) CD45RA\(^+\)) reduced in treated samples (6.48\% vs. 8.49\% for CD4\(^+\) and 24.26\% vs. 34.6\% for CD8\(^+\) effector cells) (Fig. IV.14). No significant effect of treatment was found in natural killer or B-cell populations (Fig. IV.15.A), nor monocytes, macrophage or dendritic cells (Fig. IV.15.B). This modulatory effect on human T cells is mirrored by our previous findings in MS model mice (Fig. III.6.B), suggesting that that IL4I1 induces T-cell regulation both in mouse models and human MS.
IL4I1 decreases Th17 and increases Treg and Th2 cell density in PBMC-derived lymphocytes. Flow cytometric analysis of live CD4\(^+\)-gated PBMC-derived lymphocytes from a representative healthy control donor under untreated (+PBS) and treated (+IL4I1) conditions. Analysis of IFN\(\gamma\) vs. IL10 expression (A) and IFN\(\gamma\) vs. IL17 expression (B) differentiates between CD4\(^+\) T-cell subtypes: regulatory T cell (Treg); type 2 helper T cell (Th2); T helper 17 cell (Th17).
Figure IV.14. **CD4⁺ and CD8⁺ effector cells are reduced by IL4I1.** Flow cytometric analysis treated (+IL4I1) and untreated (+PBS) PBMC-derived lymphocytes from a representative healthy control donor. Effector cell identification by CD197 vs. CD45RA expression with gating for live CD4⁺ (A) and live CD8⁺ (B) cells. CD8⁺ effector T cell (DC8⁺ET); CD4⁺ effector T cell (CD4⁺ET).
Monocyte/CAM/AAM/DC/pDC expression panel.

B. NK/NkT/B cell/memory/activation expression panel.

Flow cytometric analysis of derived lymphocytes from healthy controls.

Figure IV.15. IL4I1 does not influence NK, B cell, CAM, AAM nor DC population density of PBMC

Condition: A. NK/NK/T/B cell/monocyte/macrophage/DC expression panel. B. Monocyte/CAM/AAM/AVM/DC/pDC expression panel. C. Monocyte/macrophage or DC

 derived lymphocytes from a representative healthy control donor under untreated (+PBS) and treated (+IL4I1) conditions.

Flow cytometric analysis of PBMC-derived lymphocytes from a representative healthy control donor under untreated (+PBS) and treated (+IL4I1) conditions.

Figure IV.15. IL4I1 does not influence NK, B cell, CAM, AAM nor DC population density of PBMC-derived lymphocytes from healthy controls.
5. **IL4I1 has Distinct Effects on Cytokine Expression in PBMC-derived Lymphocytes Derived from Healthy Controls and MS Patients at Various Stages of Disease Activity**

In order to determine the effect of IL4I1 on human inflammatory cell activity in the context of MS and non-disease states, mRNA expression of key inflammatory genes previously associated with MS pathogenesis was compared between treated (+IL4I1) and untreated (+PBS) PBMC-derived lymphocytes. In healthy control cells, IL4I1 was found to reduce expression of *IL10* (Fig. IV.16). No other cytokines of interest were affected by IL4I1 treatment in this population (Fig. IV.18). In patient populations, increased *TGFβ* expression by naRRMS lymphocytes was the only significant IL4I1-treatment effect observed (Fig. IV. 17). No other changes in this population were noted (Fig. IV. 20). We did not detect any significant gene expression changes between treated and untreated lymphocytes in active-RRMS or SPMS samples (Fig. IV. 19; Fig. IV. 21). To identify differences in sensitivity to IL4I1 as a function of disease state, individual gene response was compared between groups. This analysis revealed that IL4I1 increases *IL17* expression in SPMS-derived lymphocytes as compared to HC (Fig. IV.22). The effect of IL4I1 on *TNFα*, *TGFβ*, *IFNγ* and *IL10* was similar between each groups (Fig. IV.23). The inconclusiveness of these data likely reflects the heterogeneous nature of MS at large (Arellano *et al.*, 2017; El Behi *et al.*, 2017; Hegen *et al.*, 2016).
**Figure IV.16.** *IL10* expression is reduced by IL4I1 in PBMC-derived lymphocytes from healthy control donors. Quantified average qRT-PCR detection of *IL10* gene expression in PBMC-derived lymphocytes from healthy control female donors (n=8). Cells were treated with IL4I1 (24h, 200ng/ml) or PBS (24h, 20µl/ml) after stimulation by anti-CD3/CD28/CD2 (48h, 20µl/ml). A. *IL10* expression presented as $2^{-ΔΔCt}$, normalized to β-actin control gene; numbers indicate individual donors. B. Fold change in *IL10* expression between control (+PBS) and treated (+IL4I1) conditions ($2^{-ΔΔCt}$); dashed line demarks no change between PBS and IL4I1 treatment. Graphs show mean with SEM. Paired t tests, two-tailed. Statistical significance reported as * P<0.05.

**Figure IV.17.** *TGFβ* expression is increased by IL4I1 in PBMC-derived lymphocytes from individuals with non-active relapsing remitting MS. Quantified average qRT-PCR detection of *TGFβ* gene expression in PBMC-derived lymphocytes from non-active relapsing remitting female donors (n=7). Cells were treated with IL4I1 (24h, 200ng/ml) or PBS (24h, 20µl/ml) after stimulation by anti-CD3/CD28/CD2 (48h, 20µl/ml). A. *TGFβ* expression presented as $2^{-ΔCt}$, normalized to β-actin control gene; numbers indicate individual donors. B. Fold change in *TGFβ* expression between control (+PBS) and treated (+IL4I1) conditions ($2^{-ΔΔCt}$); dashed line demarks no change between PBS and IL4I1 treatment. Graphs show mean with SEM. Paired t tests, two-tailed. Statistical significance reported as ** P<0.01.
Figure IV.18. *TNFα, TGFβ, IFNγ* and *IL17* expression is not significantly affected by IL4I1 in PBMC-derived lymphocytes from healthy control donors. Quantified average qRT-PCR detection of gene expression in PBMC-derived lymphocytes from healthy control female donors. Cells were treated with IL4I1 (24h, 200ng/ml) or PBS (24h, 20µl/ml) after stimulation by anti-CD3/CD28/CD2 (48h, 20µl/ml). Gene expression presented as $2^{-\Delta\Delta Ct}$, normalized to β-actin control for A. *TNFα* (n=7), C. *TGFβ* (n=5), E. *IFNγ* (n=6) and G. *IL17* (n=5); numbers indicate individual donors. Average fold change in gene expression between control (+PBS) and treated (+IL4I1) conditions ($2^{-\Delta\Delta Ct}$) for B. *TNFα* (n=7), D. *TGFβ* (n=5), F. *IFNγ* (n=6) and H. *IL17* (n=5); dashed line demarks no change between PBS and IL4I1 treatment. Graphs show mean with SEM. Paired t tests, two-tailed.
Figure IV.19. MS-related cytokine gene expression is not significantly affected by IL4I1 in PBMC-derived lymphocytes from individuals with active relapsing-remitting MS. Quantified average qRT-PCR detection of gene expression in PBMC-derived lymphocytes from active relapsing-remitting female donors. Cells were treated with IL4I1 (24h, 200ng/ml) or PBS (24h, 20µl/ml) after stimulation by anti-CD3/CD28/CD2 (48h, 20µl/ml). Gene expression presented as $2^{-\Delta\Delta Ct}$, normalized to β-actin control for A. TNFα (n=9), C. TGFβ (n=8), E. IFNγ (n=9), G. IL10 (n=8) and I. IL17 (n=9); numbers indicate individual donors. Average fold change in gene expression between control (+PBS) and treated (+IL4I1) conditions ($2^{-\Delta\Delta Ct}$) for B. TNFα (n=9), D. TGFβ (n=8), F. IFNγ (n=9), H. IL10 (n=8), and J. IL17 (n=9). Graphs show mean with SEM. Paired t tests, two-tailed.
Figure IV.20. *TNF*α, *IFN*γ, *IL10* and *IL17* expression is not significantly affected by IL4I1 in PBMC-derived lymphocytes from individuals with non-active relapsing remitting MS. Quantified average qRT-PCR detection of gene expression in PBMC-derived lymphocytes from non-active relapsing remitting female donors. Cells were treated with IL4I1 (24h, 200ng/ml) or PBS (24h, 20µl/ml) after stimulation by anti-CD3/CD28/CD2 (48h, 20µl/ml). Gene expression presented as $2^{-\Delta\Delta Ct}$, normalized to *β*-actin control for A. *TNF*α (n=8), C. *IFN*γ (n=8), E. *IL10* (n=8) and G. *IL17* (n=6); numbers indicate individual donors. Average fold change in gene expression between control (+PBS) and treated (+IL4I1) conditions ($2^{-\Delta\Delta Ct}$) for B. *TNF*α (n=8), D. *IFN*γ (n=8), F. *IL10* (n=8) and H. *IL17* (n=6); dashed line demarks no change between PBS and IL4I1 treatment. Graphs show mean with SEM. Paired t tests, two-tailed.
Figure IV.21. MS-related cytokine gene expression is not significantly affected by IL4I1 in PBMC-derived lymphocytes from individuals with secondary progressive MS. Quantified average qRT-PCR detection of gene expression in PBMC-derived lymphocytes from secondary progressive female donors (n=5). Cells were treated with IL4I1 (24h, 200ng/ml) or PBS (24h, 20µl/ml) after stimulation by anti-CD3/CD28/CD2 (48h, 20µl/ml). Gene expression presented as $2^{-\Delta\Delta Ct}$, normalized to β-actin control for A. TNFα, C. TGFβ, E. IFNγ, G. IL10 and I. IL17; numbers indicate individual donors. Average fold change in gene expression between control (+PBS) and treated (+IL4I1) conditions ($2^{-\Delta\Delta Ct}$) for B. TNFα, D. TGFβ, F. IFNγ, H. IL1 and J. IL17; dashed line demarks no change between PBS and IL4I1 treatment. Graphs show mean with SEM. Paired t tests, two-tailed.
Figure IV.22. IL4I1 increasing *IL17* expression in secondary progressive MS relative to its effect on healthy control cells. Fold change in qRT-PCR detection of MS-associated cytokine gene expression in IL4I1-treated PBMC-derived lymphocytes from female donors with active relapsing-remitting MS (aRRMS; n=9), non-active relapsing-remitting MS (naRRMS; n=6), secondary progressive MS (SPMS; n=5) and healthy controls (HC; n=6). Cells were stimulated by anti-CD3/CD28/CD2 (48h, 20µl/ml) then treated with IL4I1 (24h, 200ng/ml) and normalized to PBS-treated controls (24h, 20µl/ml). Dashed line demarks no difference between PBS and IL4I1 treatment. Graphs show mean with SEM. Ordinary one-way ANOVA with Tukey’s multiple comparisons test, alpha = 0.05. Statistical significance reported as *P<0.05.

Figure IV.23. Effect of IL4I1 on *TNFα*, *TGFβ*, *IFNγ* and *IL10* expression is similar across healthy and MS-patient donors. Fold change in qRT-PCR detection of MS-associated cytokine gene expression in IL4I1-treated PBMC-derived lymphocytes from female donors with active relapsing-remitting MS (aRRMS), non-active relapsing-remitting MS (naRRMS), secondary progressive MS (SPMS) and healthy controls (HC). A. *TNFα* expression in HC (n=7), aRRMS (n=9), naRRMS (n=8), and SPMS (n=5). B. *TGFβ* expression in HC (n=7), aRRMS (n=8), naRRMS (n=7), and SPMS (n=5). C. *IFNγ* expression in HC (n=7), aRRMS (n=9), naRRMS (n=8), and SPMS (n=5). D. *IL10* expression in HC (n=7), aRRMS (n=8), naRRMS (n=8), and SPMS (n=5). Cells were stimulated by anti-CD3/CD28/CD2 (48h, 20µl/ml) then treated with IL4I1 (24h, 200ng/ml) and normalized to PBS-treated controls (24h, 20µl/ml). Dashed line demarks no difference between PBS and IL4I1 treatment. Graphs show mean with SEM. Ordinary one-way ANOVA with Tukey’s multiple comparisons test, alpha = 0.05.
6. **IL4I1 Expression is Reduced in MS**

In order to assess the potential of *IL4I1* expression as a surrogate marker of disease state in humans, its expression in non-disease cells had first to be confirmed and quantified. Baseline qRT-PCR analysis of six HC-derived PMBCs confirmed that IL4I1 is expressed at detectable levels (Fig. IV.24). To identify potential correlations between disease state and *IL4I1* expression, HC baseline expression was quantified and compared to active-RRMS, non-active RRMS and SPMS subgroups. We detected significantly lower expression of *IL4I1* in naRRMS as compared to HC, with active-RRMS and SPMS tending towards reduced levels as well (Fig. IV.25). These findings suggest that IL4I1 gene expression is indeed unique across disease state. Follow-up quantification of IL4I1 protein levels in plasma from each patient group did not differ significantly between groups (Fig. IV.26).

![Figure IV.24. IL4I1 is expressed by healthy human PBMCs. Average qRT-PCR detection of expression for β-actin (reference) and IL4I1 in cultured (unstimulated) PBMCs from healthy control donors (n=8). Numbered dots represent average triplicate value for each individual donor. Graph shows mean with SEM.](image-url)
**Figure IV.25.** Baseline *IL4I1* expression is reduced in PBMCs from MS patients. Quantified average qRT-PCR detection of *IL4I1* gene expression in cultured PBMCs obtained from female donors with active relapsing-remitting MS (aRRMS; n=6), non-active relapsing-remitting MS (naRRMS; n=6), secondary progressive MS (SPMS; n=3) and healthy controls (HC; n=8). Dashed line demarks no difference from HC. Graph shows mean with SEM. Ordinary one-way ANOVA with Tukey’s multiple comparisons test, alpha = 0.05. Statistical significance reported as **P<0.01.

**Figure IV.26.** Baseline *IL4I1* plasma protein levels are similar between healthy control and MS patients. Quantitative ELISA data for IL4I1 protein concentrations (pg/ml) measured in plasma collected from healthy control donors (HC; n=2) and patients diagnosed with active relapsing-remitting MS (aRRMS; n=2), non-active relapsing-remitting MS (naRRMS; n=2) and secondary progressive MS (SPMS; n=2). Graph shows mean with SEM. Ordinary one-way ANOVA with Tukey’s multiple comparisons test, alpha = 0.05.
7. IL4I1 has a Distinct Effect on TOB1 Expression in Healthy Control Lymphocytes

To identify the mechanisms through which IL4I1 may affect human PBMCs, we quantified its effect on the gene expression of TOB1 and TCRζ, two previously suggested targets of IL4I1. In our hands, IL4I1 increased TOB1 expression in HC lymphocytes, while not significantly affecting TCRζ (Fig. IV.27). Though prominent in healthy lymphocytes, this increase in TOB1 expression did not occur in any disease samples (Fig. IV.28). Collectively, these data suggest that IL4I1 acts through TOB1, which is known to prevent Th17 proliferation, in eliciting its immunomodulatory effects; however, this pathway appears to be perturbed in MS.

Figure IV.27. IL4I1 treatment of PBMC-derived lymphocytes increases TOB1 and does not affect TCRζ gene expression. Quantified average qRT-PCR detection of gene expression in PBMC-derived lymphocytes from healthy control female donors (n=6). Cells were treated with IL4I1 (24h, 200ng/ml) or PBS (24h, 20µl/ml) after stimulation by anti-CD3/CD28/CD2 (48h, 20µl/ml). Gene expression presented as $2^{-\Delta\Delta C_t}$, normalized to β-actin control for A. TOB1 and C. TCRζ; numbers indicate individual donors. Average fold change in gene expression between control (+PBS) and treated (+IL4I1) conditions ($2^{-\Delta\Delta C_t}$) for B. TOB1 and D. TCRζ; dashed line demarks no change between PBS and IL4I1 treatment. Graphs show mean with SEM. Paired t tests, two-tailed. Statistical significance reported as *P<0.05.
8. Baseline Expression of TOB1 is Similar Between MS- and HC-derived PBMCs

To elucidate previous findings suggesting that TOB1 expression correlates with MS development, we compared baseline TOB1 gene expression and plasma concentrations between healthy controls and or three MS patient subgroups. qRT-PCR analysis of cultured PBMCs found no significant differences in expression between groups (Fig. IV.29). Quantified plasma protein concentration of TOB1 by ELISA was also not reliably different between groups (Fig. IV.30). This finding suggests that TOB1, neither its gene expression nor plasma protein concentration, does not serve as a marker of disease state.

Figure IV.28. Relative to the effect of IL4I1 on HC-derived lymphocytes, TOB1 expression is reduced by IL4I1 in all subtypes of MS. TOB1 expression in PBMC-derived lymphocytes in HC (n=6), aRRMS (n=9), naRRMS (n=8), and SPMS (n=5). Cells were stimulated by anti-CD3/CD28/CD2 (48h, 20µl/ml) then treated with IL4I1 (24h, 200ng/ml) and normalized to PBS-treated controls (24h, 20µl/ml). Dashed line demarks no difference between PBS and IL4I1 treatment. Graphs show mean with SEM. Ordinary one-way ANOVA with Tukey’s multiple comparisons test, alpha = 0.05. Statistical significance reported as *P<0.05; **P<0.01.
Figure IV.29. **TOB1 expression at baseline is similar between healthy control and patient samples.** Quantified average qRT-PCR detection of MS-associated cytokine gene expression in stimulated PBMCs. Cells were obtained from female donors with active relapsing-remitting MS (aRRMS; n=7), non-active relapsing-remitting MS (naRRMS; n=7), secondary progressive MS (SPMS; n=5) and healthy controls (HC; n=7). T cells were activated by anti-CD3/CD28/CD2 stimulation (48h, 20µl/ml) and sham-treated with PBS (24h, 20µl/ml) before RNA extraction. Dashed line represents no difference from HC. Graphs show mean with SEM. Ordinary one-way ANOVA with Tukey’s multiple comparisons test, alpha = 0.05.

Figure IV.30. **Baseline TOB1 plasma protein levels are not significantly different between healthy and patient groups.** Quantitative ELISA data for TOB1 protein concentrations (pg/ml) measured in plasma collected from healthy control donors (HC; n=2) and patients diagnosed with active relapsing-remitting MS (aRRMS; n=2), non-active relapsing-remitting MS (naRRMS; n=2) and secondary progressive MS (SPMS; n=2). Graph shows mean with SEM. Ordinary one-way ANOVA with Tukey’s multiple comparisons test, alpha = 0.05.

Table IV.1. **Summary of healthy control lymphocyte subtypes affected by IL4I1 treatment.** Lists relative percent populations of each cell type under untreated (stimulated; +PBS) and treated (+IL4I1) conditions.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Cell Type</th>
<th>Stimulated (+PBS)</th>
<th>Treated (+IL4I1)</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+IFNγ-IL10+</td>
<td>Treg</td>
<td>0.66%</td>
<td>3.48%</td>
<td>(-) 18.97</td>
</tr>
<tr>
<td>CD4+IFNγ-IL17+</td>
<td>Th17</td>
<td>30.80%</td>
<td>0.96%</td>
<td>(-) 32.08</td>
</tr>
<tr>
<td>CD4+CD10+IFNγ-IL17-</td>
<td>Th2</td>
<td>37.16%</td>
<td>67.18%</td>
<td>(+) 1.81</td>
</tr>
<tr>
<td>CD4+CD197-CD45RA+</td>
<td>CD4+ Effector</td>
<td>8.49%</td>
<td>6.48%</td>
<td>(-) 1.31</td>
</tr>
<tr>
<td>CD8+CD197-CD45RA+</td>
<td>CD8+ Effector</td>
<td>34.60%</td>
<td>26.24%</td>
<td>(-) 1.32</td>
</tr>
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</table>
Table IV.2. Summary of corrected P-values for cytokine profile data. Baseline gene expression (A) and plasma protein (B) profile between-group comparisons. Ordinary one-way ANOVA with Tukey’s multiple comparisons test. C. Gene expression response to stimulation between-group comparisons. Unpaired t-tests, who tailed. D. PBMC-derived lymphocyte gene expression between-group comparisons. Ordinary one-way ANOVA with Tukey’s multiple comparisons test. E. IL4I1 treatment effect on gene expression within-group comparisons. Paired t-tests, two tailed. F. IL4I1 treatment effect on gene expression between-group comparisons. Ordinary one-way ANOVA with Tukey’s multiple comparisons test. Yellow boxes indicate statistical significance, reported as *P<0.05, **P<0.01. Increased expression values are indicated in bold; italicized values indicate decreased expression.

### A. Baseline gene expression profile between-groups

<table>
<thead>
<tr>
<th>Condition</th>
<th>Comparison</th>
<th>TNFa</th>
<th>TGFβ</th>
<th>IFNγ</th>
<th>IL10</th>
<th>IL17</th>
<th>TOB1</th>
<th>IL4I1</th>
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<tbody>
<tr>
<td>Baseline</td>
<td>HC vs. aRRMS</td>
<td>0.9346</td>
<td>0.9747</td>
<td>0.9772</td>
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<td>0.9996</td>
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<td>0.2946</td>
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<tr>
<td></td>
<td>HC vs. naRRMS</td>
<td>0.98</td>
<td>0.8247</td>
<td>0.9988</td>
<td>0.9983</td>
<td>0.463</td>
<td>0.753</td>
<td><strong>0.0045</strong></td>
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<tr>
<td></td>
<td>aRRMS vs. SPMS</td>
<td>0.9953</td>
<td>0.9614</td>
<td>0.9413</td>
<td>0.907</td>
<td>0.1326</td>
<td>0.805</td>
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<td>0.7333</td>
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<td>0.7216</td>
<td>n/a</td>
<td>0.8898</td>
<td>0.987</td>
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### B. Baseline plasma protein profile between-groups

<table>
<thead>
<tr>
<th>Condition</th>
<th>Comparison</th>
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<th>IL4I1</th>
<th>TOB1</th>
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<tbody>
<tr>
<td>Baseline</td>
<td>HC vs. aRRMS</td>
<td>***0.0006</td>
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<td>HC vs. naRRMS</td>
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<td>aRRMS vs. naRRMS</td>
<td>0.8835</td>
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<td>aRRMS vs. SPMS</td>
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<tr>
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<td>naRRMS vs. SPMS</td>
<td>0.8848</td>
<td>0.9998</td>
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### C. Gene expression response to stimulation between-groups

<table>
<thead>
<tr>
<th>Condition</th>
<th>Comparison</th>
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<th>TGFβ</th>
<th>IFNγ</th>
<th>IL10</th>
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<tr>
<td>Δ Baseline vs. Stimulated (+PBS)</td>
<td>HC vs. aRRMS</td>
<td>0.2422</td>
<td>0.3045</td>
<td>0.9223</td>
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<tr>
<td></td>
<td>HC vs. naRRMS</td>
<td>0.2624</td>
<td>0.1249</td>
<td>0.2318</td>
<td>0.0818</td>
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<tr>
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<td>HC vs. SPMS</td>
<td>**0.0023</td>
<td>0.2938</td>
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<td>aRRMS vs. naRRMS</td>
<td>0.4616</td>
<td>0.6051</td>
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<tr>
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<td>aRRMS vs. SPMS</td>
<td>0.3539</td>
<td>0.6826</td>
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<tr>
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<td>naRRMS vs. SPMS</td>
<td>*0.0197</td>
<td>0.2528</td>
<td>0.2335</td>
<td>0.1138</td>
</tr>
</tbody>
</table>

### D. PBMC-derived lymphocyte gene expression profile between-groups

<table>
<thead>
<tr>
<th>Condition</th>
<th>Comparison</th>
<th>TNFa</th>
<th>TGFβ</th>
<th>IFNγ</th>
<th>IL10</th>
<th>IL17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulated (+PBS) vs. Treated (+IL4I1)</td>
<td>HC vs. aRRMS</td>
<td>0.5038</td>
<td>0.9778</td>
<td>0.7443</td>
<td>0.9731</td>
<td>0.9871</td>
</tr>
<tr>
<td></td>
<td>HC vs. naRRMS</td>
<td>0.8867</td>
<td>0.974</td>
<td>0.6298</td>
<td>0.9996</td>
<td>0.8381</td>
</tr>
<tr>
<td></td>
<td>HC vs. SPMS</td>
<td>**0.0032</td>
<td>0.2755</td>
<td>0.2419</td>
<td>0.9801</td>
<td>0.2863</td>
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<tr>
<td></td>
<td>aRRMS vs. naRRMS</td>
<td>0.8086</td>
<td>&gt;0.9999</td>
<td>0.9954</td>
<td>0.9844</td>
<td>0.9357</td>
</tr>
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<td></td>
<td>aRRMS vs. SPMS</td>
<td>0.2997</td>
<td>0.4086</td>
<td>0.6862</td>
<td>&gt;0.9999</td>
<td>0.361</td>
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<tr>
<td></td>
<td>naRRMS vs. SPMS</td>
<td>0.1444</td>
<td>0.4445</td>
<td>0.8113</td>
<td>0.9888</td>
<td>0.6386</td>
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### E. IL4I1 treatment effect on gene expression within-groups

<table>
<thead>
<tr>
<th>Condition</th>
<th>Comparison</th>
<th>TNFa</th>
<th>TGFβ</th>
<th>IFNγ</th>
<th>IL10</th>
<th>IL17</th>
<th>TOB1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ Stimulated (+PBS) vs. Treated (+IL4I1)</td>
<td>HC</td>
<td>0.1087</td>
<td>0.8042</td>
<td>0.8895</td>
<td>**0.0035</td>
<td>0.1202</td>
<td>**0.0363</td>
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<td></td>
<td>aRRMS</td>
<td>0.6358</td>
<td>0.0973</td>
<td>0.7656</td>
<td>0.2297</td>
<td>0.6152</td>
<td>0.8715</td>
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<tr>
<td></td>
<td>naRRMS</td>
<td>0.9347</td>
<td>**0.0052</td>
<td>0.2855</td>
<td>0.9659</td>
<td>0.4297</td>
<td>0.7626</td>
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<tr>
<td></td>
<td>SPMS</td>
<td>0.4897</td>
<td>0.4846</td>
<td>0.3856</td>
<td>0.0874</td>
<td>0.2843</td>
<td>0.8962</td>
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</table>

### F. IL4I1 treatment effect on gene expression between-groups

<table>
<thead>
<tr>
<th>Condition</th>
<th>Comparison</th>
<th>TNFa</th>
<th>TGFβ</th>
<th>IFNγ</th>
<th>IL10</th>
<th>IL17</th>
<th>TOB1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated (+IL4I1)</td>
<td>HC vs. aRRMS</td>
<td>0.7199</td>
<td>0.944</td>
<td>0.7306</td>
<td>0.1961</td>
<td>*0.1396</td>
<td>*0.0025</td>
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<tr>
<td></td>
<td>HC vs. naRRMS</td>
<td>0.8537</td>
<td>0.8529</td>
<td>0.375</td>
<td>0.2651</td>
<td>0.4983</td>
<td>*0.0026</td>
</tr>
<tr>
<td></td>
<td>HC vs. SPMS</td>
<td>0.3508</td>
<td>0.9986</td>
<td>0.3591</td>
<td>0.9229</td>
<td>0.0397</td>
<td>*0.0115</td>
</tr>
<tr>
<td></td>
<td>aRRMS vs. naRRMS</td>
<td>0.995</td>
<td>0.9928</td>
<td>0.9059</td>
<td>0.9976</td>
<td>0.8977</td>
<td>0.9997</td>
</tr>
<tr>
<td></td>
<td>aRRMS vs. SPMS</td>
<td>0.8442</td>
<td>0.9857</td>
<td>0.8448</td>
<td>0.6178</td>
<td>0.7497</td>
<td>0.9984</td>
</tr>
<tr>
<td></td>
<td>naRRMS vs. SPMS</td>
<td>0.7463</td>
<td>0.9386</td>
<td>0.9958</td>
<td>0.7167</td>
<td>0.4382</td>
<td>0.9794</td>
</tr>
</tbody>
</table>
As detailed in *Chapter III*, data generated previously by our lab show that IL4I1 reduces inflammation and promotes remyelination in MS mouse models (Psachoulia *et al.*, 2016). The work presented in this chapter investigates IL4I1 in the context of human MS. Plasma and peripheral blood mononuclear cells (PBMCs) were obtained from healthy controls (HC) and individuals from three patient groups: active relapsing-remitting MS (aRRMS), non-active relapsing remitting MS (naRRMS) and secondary progressive MS (SPMS), which were defined based on disease phenotype. Differences between groups at baseline, after stimulation, and following IL4I1 treatment, were determined by gene expression, plasma protein concentration and cellular composition analyses.

Cumulatively, we found reduced concentrations of TGFβ protein in plasma obtained from all MS-subtypes (*Fig. IV.6*), with no significant differences in baseline gene expression between groups (*Fig. IV.7*). We also found a suppressed response to stimulation by anti-CD3/CD28/CD2 in SPMS, with significantly less of an increase in TNFα and IFNγ as compared to naRRMS. Reduced TNFα response was also significant compared to HC (*Fig. IV.9*). The pro-inflammatory activity of these stimulated SPMS lymphocytes was reduced, as evidenced by significantly lower TNFα expression compared to HC (*Fig. IV.11*).

With regards to the effects of IL4I1 on human lymphocytes, flow cytometric analysis of healthy control cells shows a skew towards immune regulation (*Fig. IV.13 and 14*). Treated HC lymphocytes have reduced population density of pro-inflammatory T cells (Th17, CD4+ effector T cells and CD8+ effector T cells), as well as increased representation of regulatory cell types (Treg and Th2). While no significant changes in Th1 cell density were detected by T-bet+ CD4+ analysis...
as was seen in previous mouse work (see Chapter III), a significant reduction was observed in CD4+IFNγ+IL17+ cells (from 35.32% to 1.98%) following treatment (Fig. IV. 13.B). We hypothesize that this population represents a Th1-like subset of Th17 cells previously termed Th17-Th1. In the presence of IL12, Th17-Th1 exhibit plasticity towards the Th1 phenotype (Annuziato et al., 2007), thus accounting for a potential indirect suppression of Th1 by IL4I1 in humans. Flow cytometry was not performed in patient groups due to sample availability and should be a subject of future studies.

The within-group effect of IL4I1 on gene expression was limited to IL10 in HC and TGFβ in naRRMS lymphocytes, both of which were increased (Fig. IV.17). No significant effects on gene expression were identified in aRRMS or SPMS. However, normalization of within-group trends to the effect of IL4I1 on HC reveals significantly increased expression of IL17 in SPMS following treatment (Fig. IV.22). While IL4I1 reduces IL17 expression in HC lymphocytes (Fig. IV.18.H), it has the opposite effect on IL17 in SPMS (Fig. IV. 21.J). This suggests that the signaling pathway downstream of IL4I1 is significantly altered in this group. No other between-group differences in IL4I1 treatment effects were found (Fig. IV. 23). Correlational analyses aimed to assess the potential of IL4I1 as a disease biomarker identified significantly reduced baseline IL4I1 gene expression in naRRMS compared to HC (Fig. IV.25). IL4I1 expression was also lower in both other MS groups, but not to significance. Plasma concentrations did not mirror this finding, with no significant differences between groups (Fig. IV.26).

Ultimately, these data suggest that the effect of IL4I1 on human HC and MS samples is mixed. IL4I1-induced immune-modulation—as was found in our previous work in mouse models—is evidenced by several of our findings presented in this chapter, but not by others. In support of this notion, IL4I1 treatment increased the regulatory to pro-inflammatory T cell ratio in
treated HC lymphocytes. We also found that IL4I1 promotes TGFβ expression by naRRMS-derived lymphocytes; this increase may correct a baseline deficit in TGFβ, which was seen in all MS patient plasma. However, why this treatment effect was only seen in naRRMS is not clear. Furthermore, the IL4I1-induced skew towards regulatory T cell states in HC samples was not reflected by relative increases in regulatory gene expression in these samples. Rather, IL4I1’s effect on HC gene expression was negligible except for an observed increase in IL10.

In an effort to elucidate the mechanism through which IL4I1 produces the T cell modulation found by this and other studies, we determined the effect of IL4I1 on gene expression of two previously implicated effector proteins: TCRζ and TOB1. We found that IL4I1 significantly increased the expression of TOB1 in HC lymphocytes, while its effect was negligible on TCRζ expression in the same cells (Fig. IV.27). These data corroborate previous findings suggesting that IL4I1 acts through TOB1 (Santarlasci et al., 2014) and refute previous research implicating TCRζ downregulation in IL4I1 activity (Boulland et al., 2007). In following with TOB1 and its implications as biomarker in MS, baseline expression and plasma concentrations were compared between groups. No significant differences were found, suggesting that the predictive nature of TOB1 in the development of clinically isolated syndrome (CIS) to clinically definitive MS does not extend through subsequent disease progression. If both findings are correct, this indicates that disparate pathogenic mechanisms are responsible for MS development and MS progression.

Differences in baseline profiles, responses to stimulation and IL4I1 treatment effects between groups supports the notion that IL4I1 is a heterogeneous disease. Our data also reflect significant heterogeneity within patient groups. Within each group studied, individuals are similar along the diagnostic criteria employed in this study (detailed in Section 2.1). However, cytokine expression characteristics were often wide-ranging between individuals within the same group.
This inter-group variability is well documented (El Behi et al., 2017; Hegen et al., 2016), and is often true of work done on human samples. The variability inherent to all scientific findings, especially those regarding human samples (and even more so, MS patient cells), typically decreases with increasing sample size.

Incongruity in our data may be explained by a combination of multiple factors, including low sample size and inherent sample heterogeneity. For example, the discrepancy between IL4I1’s effect on healthy control T cell populations (which were skewed towards a regulatory state) and its effect on HC cytokine expression (which was unaffected except for an increase in IL10) may result from having performed flow cytometry on only one HC sample. Low sample size and high patient variability may also contribute to differences between gene expression and plasma protein concentrations within groups. However, several other explanations exist, such as post-translational modifications and differences in rates of transcription/translation or in vivo half-lives (Greenbaum, et al., 2003), which do not necessarily imply that such findings lack validity.

Undetected differences in IL4I1 molecular structure may explain these discrepancies. Two distinct isoforms of human IL4I1 have been confirmed in vivo, with several potential single-nucleotide polymorphism (SNPs) and mutations that effect IL4I1 enzymatic activity and 9 known splice variants (Molinier-Freknel et al., 2015). Furthermore, molecular sequence analysis reveals two potential tyrosine phosphorylation sites, 3-4 glycosylation sites, 5 FAD co-factor binding regions and one cleavage site, potentially important to enzyme activation (Chavan et al., 2002; Molinier-Frenkel et al., 2015). It is possible, for example, that net plasma IL4I1 levels are similar between HC and patient groups, but that the relative presence of a particular isoform or phosphorylation state differs significantly between groups. Deeper analysis of IL4I1 isolates will be necessary to identify potential correlations between protein variants and disease state.
Furthermore, differences in treatment history may account for many otherwise unexplained results. Future analyses should take DMD use into account as a variable of interest. Unintended inconsistencies between subject groups may also contribute to unexpected findings. For example, though HC donors were selected to match the gender, age range and races of patient samples, all patient donors were seen by GUH physicians and lived in the Washington D.C. area, while commercial HC donors may live anywhere in the country. This difference likely brings more demographic variability to HC than MS subgroups. Increased sample size, tighter control over patient treatment histories and HC demographics may reduce inter-subject variability in future work.
CHAPTER V: IL4I1 ENHANCES MOUSE MODEL REMYELINATION THROUGH REGULATION OF ENGRAFTED HUMAN LYMPHOCYTES

A. INTRODUCTION

1. Background and Rationale

Unregulated inflammation and CNS demyelination are hallmarks of multiple sclerosis (MS) pathology. In health, CNS demyelination is spontaneously repaired through a series of stepwise process including (i) recruitment of oligodendrocyte progenitor cells (OPCs) to the lesioned area, (ii) differentiation of OPCs into mature oligodendrocytes and, (iii) complete remyelination of denuded axons by mature oligodendrocytes. In lysolethicin-mediated rodent models of demyelination, these steps typically correspond to 5, 10 and 20 days-post lesion, respectively (Jeffery and Blakemore, 1995).

An abundance of past research suggests that the process of spontaneous remyelination is driven by key inflammatory events (Miron and Franklin, 2014; Moore et al., 2015). As such, presence of healthy and functional innate and adaptive immune cells is critical for successful remyelination (Bieber et al., 2003; Kotter et al., 2001). Furthermore, the activation and timing of immune cells present in the lesioned area is an important determinant of remyelination success. While pro-inflammatory stimuli from Th1, Th17 and classically-activated macrophages (CAM) appear to trigger early steps of the remyelination process, these cells and their associated cytokines have inhibitory effects on later steps of this process (Moore et al., 2015). Meanwhile, modulatory immune cells, such as Treg and alternatively-activated macrophages (AAM), seem to positively
influence later stages of remyelination (Becker et al., 2013; Butovky et al., 2005; Miron et al., 2013).

In this chapter, I propose that interleukin-4 induced protein 1 (IL4I1) may contribute to the maintained regenerative effects of AAM in demyelinated lesions in vivo. An L-amino acid oxidase (LAAO) predominantly secreted by AAM, IL4I1 has been shown to promote the relative abundance of regulatory T-cell subtypes, whose activity is correlated with remyelination and symptom reduction (Rogers and Miller, 2012; Rossi et al., 2015). In Chapter III of this thesis, I provide evidence that direct CNS injection of IL4I1 modulates inflammation and promotes remyelination in focally demyelinated lesions of mouse models of MS. However, species specific differences make it difficult to extrapolate human relevance from mouse models of disease. For this reason, my subsequent work investigates the effects of IL4I1 in human samples. As discussed in Chapter IV, this work demonstrates that IL4I1 modulates inflammatory lymphocyte activation state in cultured human lymphocytes. However, in vitro effects such as this are not necessarily conserved in the context of complex organismal biology. Following this line of research towards patient relevance, the work presented in this chapter combines in vitro human and in vivo mouse models. This paradigm allows us to study how IL4I1’s effect on human peripheral blood mononuclear cell (PBMC)-derived lymphocytes in vitro can influence endogenous remyelination in vivo. Our data indicate that IL4I1 enhances the ability of human lymphocytes to promote remyelination in nude mouse lesions.
2. **Approach**

To investigate how the cellular-level effects of IL4I1 may translate to systemic changes on an organismal level, we performed focal-demyelination in nude mice, prepared IL4I1- and sham-treated human lymphocyte cultures, and adopted a nude mouse grafting model recently developed by El Behi *et al.*, 2017. We hypothesize that remyelination will be enhanced in lesions that were grafted with IL4I1-treated lymphocytes.

**2.1 Human Cell Culture**

Peripheral mononuclear cells (PBMCs) were obtained from healthy control donors fulfilling the criteria defined previously (*see Chapter IV*). After thawing, cells were plated and immediately stimulated with CD3/CD28/CD2 (20µl/ml). Following 48h of stimulation, cells were supplemented with recombinant human IL4I1 protein (200ng/ml) or PBS (untreated control) for 24h. Live cells were brought to a concentration 10⁵/µl PBS for grafting.

**2.2 Focal Demyelination and Lymphocyte Grafting**

The feasibility and efficacy of engrafting human PBMC-derived lymphocyte into demyelinated nude mouse spinal cord lesions was recently established (El Behi *et al.*, 2017). Our study uses athymic “nude” mice, which lack T cells and cell-mediated immunity, in order to definitively assess the influence of exogenous immune cells on CNS repair. This experimental set-up allows us to study the effects of IL4I1-treated and untreated human lymphocytes on endogenous remyelination.

Focal demyelination was performed by lysolethicin injection (1.0%) into the spinal cord dorsal horn of female nude mice aged 8-10 weeks. Prior to injection, needle tips were coated in
charcoal to mark the lesion site for future identification. Two days post-lesion, human lymphocytes were engrafted into the lesion site by 1µl injection of $10^5$ live cells suspended in PBS. Mice (n=3-5 per group) either received IL4I1-treated lymphocytes or sham-treated cells. All lymphocytes were derived from healthy control PBMCs. A third group of lesioned mice were not grafted in order to serve as a control. Mice were euthanized and perfused with PFA (4.0%) at 15 days-post lesion, a time point corresponding with late oligodendrocyte progenitor cell differentiation and early remyelination (Jeffery and Blakemore, 1995).

### 2.3 Assessment of Remyelination

Spinal cords were post-fixed and lesioned cryosections were mounted onto microscope slides for immunohistochemical (IHC) analysis of remyelination. Myelinated areas were marked using antibodies against myelin basic protein (MBP) and by FluoroMyelin™ dye. Remyelination was quantified as the corrected immunofluorescence intensity (CFI) within each lesioned area, which was identified by high nuclear density on DAPI staining. The mean CFIs for BMP and FluoroMyelin™ were compared between lesions from mice grafted with IL4I1-treated lymphocytes (“IL4I1”), mice grafted with PBS-treated lymphocytes (“PBS”) and normalized to non-grafted control mice (“NGC”)
B. RESULTS

1. Remyelination is Enhanced in Mouse Lesions Engrafted by IL4I1-Treated Human Lymphocytes

To determine the relevance of IL4I1’s effect on human lymphocytes on remyelination in vivo, IL4I1-treated and sham-treated healthy control human PBMC-derived lymphocytes were engrafted into focally demyelinated lesions in nude mouse spinal cord. Intraleisional myelin was identified by FluoroMyelin™ and MBP immunostaining at 15 days post-lesion (Fig. V. 1.E), and lesions were imaged for quantification of stain intensity. Both markers of myelin were increased in lesions that received IL4I1-treated human cells.

FluoroMyelin™ fluorescence was significantly higher within lesions that were engrafted with IL4I1-treated lymphocytes, as compared to lesions that received sham-treated lymphocytes or non-grafted control lesions (Fig. V. 1.A and B). While PBS-grafted lesions and non-grafted lesions showed similar degrees of FluoroMyelin™ fluorescence, IL4I1-treated lesions saw a 2-fold increase in FluoroMyelin™ compared to PBS-grafted lesions, and a 2.7-fold increase from non-grafted lesions. Fluorescence intensity of MBP quantified from imaged lesions was also greater in lesions that received IL4I1-treated cells, as compared to PBS-treated cell grafts or non-grafted lesions (Fig. V. 1.C and D). Though PBS-treated lymphocyte grafts did show stronger MBP staining as compared to non-grafted lesions, this increase was not significant. Intensity of MBP fluorescence within IL4I1-lesions saw a 1.7-fold increase from PBS-lesions and a 2.8-fold increase from non-grafted control lesions.
Figure V.1. Remyelination is enhanced by Il4I1-treated human lymphocytes grafted into nude mouse demyelinated lesions. Immunohistochemical (IHC) staining and quantification of myelin within spinal cord dorsal horn lesions of nude mice at 15 days-post lesion (dpl). At 2dpl, lesions were engrafted with IL4I1-treated human lymphocytes (IL4I1; n=4), sham-treated human lymphocytes (PBS; n=5) or were used as non-grafted controls (NGC; n=3). Representative 20x images showing (A) FluoroMyelin dye (C) Myelin Basic Protein (MBP) stain and (E) merged myelin staining in NGC, PBS and IL4I1 lesions. Lesioned areas are outlined in yellow. Quantified fluorescence intensity of (B) FluoroMyelin and (D) MBP within NGC, PBS and IL4I1 lesions are normalized to background fluorescence and lesion size. Graph shows mean with SEM. Ordinary one-way ANOVA with Tukey’s multiple comparisons test, alpha = 0.05. Statistical significance reported as *P<0.05; **P<0.01; ***P<0.001.
C. DISCUSSION

This small-scale study provides intriguing evidence for the relevance of IL4I1 in MS remyelination. The novel experimental model used in this work, grafting human lymphocytes into nude mouse CNS lesions, allows us to differentiate between graft vs. host immune cell contribution while also observing the direct effect of human lymphocyte activity on the remyelination of CNS tissue. Our data indicate that mouse CNS remyelination, identified through two distinct IHC markers—FluoroMyelin™ and BMP—is enhanced by the presence of IL4I1-treated human lymphocytes. We did not see a significant difference in myelin staining between our two control groups: lesions grafted with sham-treated cells and non-grafted lesions. This is in line with previous findings comparing non-grafted nude mouse lesions to lesions grafted with healthy donor lymphocytes (El Behi et al., 2017).

Increased fluorescence in IL4I1-treated grafts was consistent between both myelin markers used, FluoroMyelin™ and MBP, strengthening our confidence in the validity of this effect. However, while FluoroMyelin™ staining intensity was significantly lower in PBS-grafts as compared to IL4I1-grafts, this was not seen in MBP staining of the same tissues (Fig. V. 1.B and D). This is likely due to small sample size, and should be increased in future studies.

Synthesizing the findings from previous studies on IL4I1, we hypothesize that IL4I1 is able to promote remyelination by modulating the inflammatory environment created by the engrafted lymphocytes. As suggested by flow cytometry data presented in Chapter IV, IL4I1 skews healthy control stimulated T cells towards a regulatory state (Fig. IV.13). The cytokine secretions associated with regulatory immune cells (Treg, Th2, AAM) are known to support the
remyelination progression in a lesioned environment (El Behi et al., 2017; Rogers and Miller, 2012). Our findings imply that IL4I1 instills a modulatory immune environment that is conducive to remyelination. It is not clear from these data, however, on what step in the remyelination process IL4I1-treated lymphocytes are exerting their effect. To identify if IL4I1-treated cells are influencing OPC recruitment and/or OPC differentiation into mature oligodendrocytes, IHC co-staining for intralesional OPCs (Olig2⁺Nkx2.2⁺) and mature oligodendrocytes (Olig2⁺CC1⁺) at 5 and 10 days-post lesion should be performed. Immunohistochemical identification and quantification of inflammatory markers, such as CAM (CD11b⁺iNOS⁺) or AAM (CD11b⁺Ym1⁺), could also help elucidate the mechanism through which IL4I1-treated lymphocytes enhance remyelination.
Multiple sclerosis (MS) is a chronic inflammatory disease characterized by autoimmune demyelination of the central nervous system, which presents with extensive neurological and neurocognitive deficits. Over 2 million individuals are currently diagnosed with MS, without a cure (Reich et al., 2018). Though pharmaceutical treatment options are available for early stages of the disease, their efficacy is limited and side effects can be severe. No disease modifying drugs (DMDs) have proven effective in progressive disease, and a reliable biomarker of progression is yet to be discovered (Segal, 2014). Interleukin-4 induced protein 1 (IL4I1), a secretory enzyme associated with alternatively-activated macrophages (AAM), was first implicated in CNS remyelination from microarray analyses of data previously obtained by my mentor, Dr. Jeffrey Huang. Prior to the work presented in this thesis and recently published by our lab, to our knowledge, IL4I1 had never been studied in the context of MS.

The effects of Il4i1 were first studied by our lab in mouse models of MS. As detailed in Chapter III, we found that Il4i1 reduces inflammation and promotes remyelination in focally demyelinated spinal cord lesions of wild-type (WT) mice; meanwhile, inflammation was unresolved and remyelination was impaired in Il4i1−/− mouse lesions. These improvements in MS-like pathology translated to amelioration of MS-like symptomatology, as demonstrated by reduced clinical scores in experimental autoimmune encephalomyelitis (EAE) model mice that received Il4i1 treatment. Our mouse model data are encouraging; however, true MS is a human disease, to
which model-derived benefits may not necessarily apply. Subsequent work, presented in *Chapter IV*, focuses on IL4I1 in human samples.

Despite the suggestive location of the human *IL4I1* gene, which maps to a chromosomal locus previously associated with autoimmune susceptibility (Chavan *et al.*, 2002), no work had previously been done on IL4I1 in human MS. A similar immunomodulatory effect of IL4I1 as we observed in mice had previously been described in human samples; however, this effect has been primarily studied in the context of tumor biology, where *IL4I1* expression negatively correlates with patient outcomes (Boulland *et al.*, 2007; Copie-Bergman *et al.*, 2003). The relationship between cancer and autoimmune disease, such as MS, is intriguing. While oncogenesis and tumor progression are enhanced by the immune system’s failure to discriminate between ‘self’ and ‘non-self’, autoimmunity is characterized by its failure to recognize ‘self’. IL4I1-mediated immune-evasion, which is detrimental in oncology, further supports our hypothesis that IL4I1 is beneficial in MS. In *Chapter IV* of this thesis, I investigate IL4I1 on human samples, in the novel context of multiple sclerosis. This work is novel also in that I am the first to bring human sample research to the Huang Lab. As such, significant preliminary work was required, to develop appropriate study designs and define optimal experimental parameters, making possible the research findings presented in this thesis.

We found that IL4I1 treatment of healthy control (HC) human lymphocytes reduces the relative population density of pro-inflammatory T cells, while increasing that of regulatory T cell subtypes. Furthermore, IL4I1 was found to increase the expression of *TGFβ* in PBMC-derived lymphocytes from non-active relapsing remitting (naRRMS) patients. These data suggest an immunomodulatory effect of IL4I1, and they corroborate our *Chapter III* mouse model findings. Other effects of IL4I1 on human samples, such as reduced *IL10* expression by HC lymphocytes,
conflict with those obtained in mice. As expected, we saw greater variability of data obtained from human samples as compared to genetically homogeneous mouse models. Cytokine expression profiles were often wide-ranging between individuals within the same experimental group. These discrepancies are important to recognize, as they may explain the seemingly unpredictable differences in patient outcomes between individuals with clinically identical presentations.

Several findings presented in Chapter IV are worthy of further discussion. Our data indicate that PBMCs obtained from individuals with secondary progressive MS (SPMS) are less responsive to T-cell activation than other groups. Meanwhile, stimulated SPMS-derived PBMCs display less pro-inflammatory activity as compared to other groups. Furthermore, both IL17 expression and plasma IL17 were undetectable in SPMS by qRT-PCR and ELISA. This reduced cytokine expression by SPMS samples has been documented (Frisullo et al., 2008), and may reflect an effective ‘inflammatory burnout’ resulting from the excessive pro-inflammatory activity of the preceding relapsing-remitting phase of MS. To test the hypothesis that reduced immune activity and reactivity in SPMS is caused by RRMS-induced inflammatory exhaustion, primary progressive MS (PPMS) patients, whose disease activity is not preceded by RRMS, should be included in future studies and compared to SPMS.

In accordance with previous research, our human sample data suggest that IL4I1 influences TOB1 expression (Santarlasci et al., 2014). We also found that Th17 cell prevalence and IL17 gene expression is reduced in HC lymphocytes following IL4I1 treatment. Given the previously established effect of TOB1 in preventing Th17 proliferation, it is possible that IL4I1 is working through TOB1 to exert these effects. Interestingly, we found that IL4I1 treatment has an opposite effect on SPMS cells, on which it stimulates IL17 expression. This may indicate that IL4I1 does not promote TOB1 expression in SPMS, or that TOB1 does not fulfill its antiproliferative effects
against Th17 cells in SPMS. Indeed, IL4I1 appears to reduce TOB1 expression in our SPMS samples, which could explain its relative failure to reduce IL17 expression upon IL4I1 treatment. Other patient groups tested (aRRMS and naRRMS) exhibited a similar reduction in TOB1 expression following IL4I1 treatment as compared to HC. These groups also underwent less significant reductions in IL17, or even demonstrated relative increases in IL17, in response to IL4I1. To distinguish if this failure occurs between IL4I1 and TOB1 or between TOB1 and Th17 proliferation/activity, future analyses should compare the effects of TOB1 treatment to those of IL4I1 treatment on MS samples. Furthermore, to differentiate between the effects of Th17 proliferation and Th17 activity, flow cytometric analysis of TOB1- and IL4I1-treated lymphocytes should be extended to MS patient samples.

On average, PBMCs obtained from individuals in the naRRMS group exhibited significantly reduced levels of IL4I1 expression at baseline. Lymphocytes from this subgroup also experienced the most significant immunomodulatory effect of IL4I1 treatment of the groups studied. Together this suggests that therapeutic IL4I1 supplementation may be maximally effective to patients in the naRRMS disease state. Because IL4I1 expression levels appear to be distinct between disease states, we are encouraged that IL4I1 gene expression may serve as a reliable biomarker of disease state. In order to determine the predictive power of IL4I1 expression on disease progression, longitudinal tracking of patient outcomes should be performed. Such a study would allow us to identify correlations between current expression and future outcomes.

As is true for most studies on patient samples, particularly those studying samples derived from patients with disease as heterogeneous as MS, our human sample data are characterized by significant inter-group variability. To the best of our ability, experimental parameters were optimized to compensate for this anticipated variability. When possible, experiments were
designed such that individual subjects served as their own controls, reducing interpersonal variability. Still, inherent variability between human subjects is best compensated by significant increases in sample size. This is the goal of all future studies. Specific to the data presented in Chapter IV, consideration of treatment regimen as a possible covariate may further clarify our findings. Furthermore, enrolling patients in the ‘other autoimmune disease’ (OAD) group (described in Chapter IV Section 2.1) would help to discriminate between autoimmune-specific and MS-specific findings.

Future studies on patient samples should also investigate other previously proposed mechanisms of IL4I1 activity not considered in the work of this thesis. For example, the effect of IL4I1 may result, in part, from its catabolism of phenylalanine, whose presence is required for the activation of the mammalian target of rapamycin (mTOR) (Grohmann and Bronte, 2010). This protein kinase regulates important cellular processes, such as differentiation of naïve CD4+ T-cells, as a function of nutrient status, such as amino acid levels (Chi, 2012). mTOR exists in two distinct protein complexes, mTOR complex 1 and mTOR complex 2. These complexes are essential in dictating pro-inflammatory and regulatory T-cell differentiation, respectively (Gordon and Martinez, 2010). It has been proposed that IL4I1 prevents the polarization of naïve CD4+ T cells to pro-inflammatory subtypes by depleting cellular phenylalanine, which is preferentially required for mTOR complex 1 activation (Bod et al., 2007a; Cousin et al., 2015). As an L-amino acid oxidase, IL4I1 catalyzes the conversion of phenylalanine to phenylpyruvate, producing NH3, and H2O2 in the process. This thesis research investigates the IL4I1-induced downregulation of T-cell receptor ζ sidechain (TCRζ), proposed as a consequence of H2O2 toxicity (Boulland et al., 2007). We found that IL4I1 did not affect TCRζ expression. However, the effects of H2O2 are extensive, and we did not pursue other mechanisms of IL4I1-induced H2O2 toxicity which may explain its
differentially distributed effects across T cell subtypes (Boulland, et al., 2007). It has been proposed that IL4I1-mediated H$_2$O$_2$ toxicity preferentially affects pro-inflammatory T cell subtypes for several reasons. Unlike their pro-inflammatory CD4$^+$ counterparts, Treg cells are equipped with high concentrations of thioredoxin-1, a protective enzyme whose antioxidant activity increases Treg resistance to H$_2$O$_2$ toxicity (Lasourdis et al., 2011). Anti-inflammatory Th2 cells are also suppressed by IL4I1-generated H$_2$O$_2$, but this effect is apparently counteracted by overwhelming suppression of pro-inflammatory T-cell subtypes. In order to identify additional mechanisms employed by IL4I1, future analyses of mTOR signaling and catalase-induced H$_2$O$_2$ neutralization in IL4I1-treated cells are required.

Though potentially informative, our human sample-derived in vitro data does not necessarily predict results on a whole organism-level. From what we understand, MS is a dynamic disease whose pathogenesis involves complicated interactions between the immune and central nervous systems. In order to confirm the effects of IL4I1 in vitro in the context of in vivo remyelination, IL4I1-treated human lymphocytes were grafted into nude mouse focally demyelinated CNS lesions for remyelination analysis. As detailed in Chapter V, we found that IL4I1-treated lymphocytes promoted remyelination in nude mouse CNS lesions. In consideration of our Chapter IV findings implicating TOB1 in IL4I1-induced treatment effects, the mechanism of IL4I1 action could be elucidated by repeating this experiment in tob1$^{−/−}$ mice. Furthermore, in the interest of bringing these findings closer still to patient relevance, nude mouse grafting experiments should be repeated using MS-derived lymphocytes.

Ultimately, the pursuit of my research interests—investigating the potential of IL4I1 as a biomarker and treatment option in MS—have thus far guided my work through the use of 3 mouse models and 2 human-derived biological samples. In each progressive step of my research
questions, we grow closer to understanding the relevance of IL4I1 in patient care. The studies described in this thesis will benefit most significantly by increasing sample size in order to counteract inherent patient variability. Having firmly laid the groundwork for optimal sample analysis, I look forward to increasingly robust conclusions based on future analysis of increasingly numerous samples.
APPENDIX: INSTITUTIONAL REVIEW BOARD APPROVALS

Georgetown University Institutional Review Board

Date: 3/21/2016
To: Jeffrey Huang
From: Amy Harchelroad
Institutional Review Board
IRB#: 2015-1048
Title: Assessing Interleukin-4 Induced Protein 1 (IL4i1) in Patients with Multiple Sclerosis
Approval Date: 3/11/2016
Expiration Date: 3/10/2017
Action: Final Approval
Initial Review

Attachments being reviewed: 5 documents were reviewed as part of this submission:

- Informed Consent for Clinical Research.docx
- Huang CITI training HIPAA
- Huang CITI training human subj
- Protocol for IL4i1 study on MS patient blood (1).docx

Stamped Documents: 2 documents were reviewed as part of this submission:

- Informed Consent for Clinical Research.docx.pdf

The revisions to your above referenced protocol and consent form were approved through expedited review by the IRB Chair or a designee on 3/21/2016. This is to inform you that you may commence your project.

Any investigator whose project is externally funded must submit the applicable sponsor grant or contract for review and approval by the appropriate sponsored research office of the recipient...
institution [GU or MRI]. The project cannot proceed without the approval of the sponsored research office.

Approval for this study is through 3/10/2017. The IRB requires that you submit an application for continuing review at the end of the approval period and/or at study completion. Please note that this office will automatically terminate the project on the date stated above, unless reviewed and re-approved by the IRB. **It is the PI’s responsibility to submit the application for continuing review and the appropriate IRB forms at least one month before the expiration date.**

Federal law requires registration with ClinicalTrials.gov of all clinical trials supported by federal funding. ClinicalTrials.gov is the National Library of Medicine’s clinical trials Protocol Registration System ("PRS"). Similar registration requirements apply for clinical trials subject to FDA regulation. In addition, the International Committee of Medical Journal Editors (ICMJE) requires registration of clinical trials in a public registry prior to enrollment as a condition for consideration for publication. Georgetown University has established a central ClinicalTrials.gov registration process. Please contact the Georgetown University PRS administrator, Patricia Mazar at mazarp@georgetown.edu to set up a PRS user account to register clinical trials. The e-mail should contain the principal investigator’s full name, department, phone number and e-mail address. Additional information, including an explanation of which clinical trials must be registered, may be found at http://ora.georgetown.edu, http://clinicaltrials.gov, http://prsinfo.clinicaltrials.gov and at http://www.icmje.org/clin_trialup.htm

For all DoD sponsored research please make note that you must obtain approval from the DoD human subjects committee as well as the local IRB approval before commencing research on this project.

** If promotional advertisements will be used for patient recruitment, they must be submitted for IRB review and approval prior to their use.

** Any incentives for participation in research are subject to IRB review and approval as well.

Please remember to:
1. Seek and obtain prior approval for any modifications to the approved protocol.
2. Promptly report any unexpected or otherwise significant adverse effects encountered in the course of this study to the Institutional Review Board within 7 calendar days. This includes information obtained from sources outside MedStar Health Research Institute and Georgetown University that reveals previously unknown risks from the procedures, drugs or devices used in this study.

Please refer to this date and the protocol number listed above when making inquiries concerning this study.

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Warning: If the reader of this message is not the intended recipient you are hereby notified that any dissemination, distribution or copying of this information is STRICTLY PROHIBITED.

Georgetown University IRB
Medical-Dental Building, SW104
3900 Reservoir Road NW
Washington, DC 20057
(202)687-1506 telephone
(202)687-4847 facsimile
Date: 2/26/2018

To: Jeffrey Huang

From: Amy Harchelroad
Institutional Review Board

IRB#: 2015-1048

Title: Assessing Interleukin-4 Induced Protein 1 (IL4i1) in Patients with Multiple Sclerosis

Approval Date: 2/24/2018

Expiration Date: 2/23/2019

Action: Continuing Review - Expedited

Attachments being reviewed:

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Your above referenced continuing review was approved through expedited review by the IRB Chair or a designee on 2/24/2018.

This is to inform you that you may continue your project.

Please note that this approval is granted until 2/23/2019. The IRB requires that you submit an application for annual renewal at the end of the approval period and/or at study completion. Please note that this office will automatically terminate the project on the date stated above, unless reviewed and re-approved by the IRB. **It is the PI’s responsibility to submit the**
application for annual renewal and the appropriate IRB forms at least one month before the expiration date.

Please remember to:
1. Seek and obtain prior approval for any modifications to the approved protocol.
2. Promptly report any unexpected or otherwise significant adverse effects encountered in the course of this study to the Institutional Review Board within 7 calendar days. This includes information obtained from sources outside MedStar Health Research Institute and Georgetown University that reveals previously unknown risks from the procedures, drugs or devices used in this study.

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REFERENCES


