ANALYSIS OF PATIENTS WITH SPMS: IDENTIFICATION OF PATIENT SUBGROUPS AND DIFFERENCES IN T CELL BIOLOGY

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Dedicated to my late father, Dr. Abdellaziz El Jaouhari
(1962-2015)

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LIST OF ABBREVIATIONS

APCs- antigen presenting cells

BBB- brain blood barrier

BSC- biological safety cabinet

CCR1- C-C chemokine receptor type 1

CIS- Clinically Isolated Syndrome

CNS- central nervous system

CSF- cerebral spinal fluid

DMTs- disease-modifying treatments

DIT- dissemination in time

DIS- dissemination in space

EAE- experimental autoimmune encephalomyelitis

EDSS- Expanded Disability Status Scale

Epstein-Barr virus (EBV)

FoxP3- Factor forkhead box P3

FMOs- Fluorescence minus ones

FVS 510- Fixable Viability Stain 510

GFAP- glial fibrillary acidic protein

GWAS- gene wide association studies

HCs- healthy controls

IFN-γ- inteferon-gamma

IL- interleukin

mAB- monoclonal antibody

MBP- myelin basic protein

MHC- major histocompatability complex

MMP- matrix metalloproteinases

MRI-Magnetic resonance imaging

MS- Multiple Sclerosis

MSFC- MS Functional Composite score

MSSS- multiple sclerosis severity score

NF-κB -nuclear factor kappa-light-chain-enhancer of activated B cells

Nfl- neurofilament light chain

OCBs- oligoclonal bands

OPN- osteopontin

PASAT- paced auditory serial addition task

PBS- phosphate buffer saline PBMC- peripheral blood mononuclear cell

PMA- Phorbol 12-myristate 13-acetate

PML- progressive multifocal leukoencephalopathy

PPMS- primary progressive multiple sclerosis

PRMS- progressive-relapsing multiple sclerosis

RA- retinoic acid

RRMS- relapsing-remitting multiple sclerosis

SD- standard deviation

SPMS- secondary progressive multiple sclerosis

TCM- central memory T cell

TCR- T cell receptor

TEM- effecotry memory T cell

TEMRA- terminally differentiated effector memory cells re-expressing CD45RA

Th-T helper

TLR-Toll-like receptor

T-regs-T regulatory cells

THESIS ABSTRACT

Multiple sclerosis (MS) is a chronic, inflammatory and neurodegenerative disease of the central nervous system (CNS). The development of progressive disability is a critical determinant of long term prognosis in multiple sclerosis. While the current opinion is that T cells play a role in CNS injury in relapsing-remitting MS (RRMS), their contribution to secondary progressive MS (SPMS) and the development of progressive disability is not clear. Previous work in our laboratory identified a putative biomarker in naïve CD4 T cells of patients with MS, which was associated with T cell activation and a rapid conversion from RRMS to SPMS. Therefore, in this project, our aim was to further investigate the potential of this putative biomarker and the expression of select cytokines in various CD4+ T cell subsets to differentiate between patients with SPMS with a slow vs. rapid conversion from RRMS to SPMS, i.e. slow progressors and fast progressors.

We report an increase of interferon-gamma (IFN-γ) expressing CD4 T cells in fast progressors compared to slow progressors, which strongly suggests a Th1, pro-inflammatory deviation in the peripheral immune system of fast progressors. As IFN-γ secreting T cells are pro-inflammatory and are associated with relapses in MS, the elevated IFN-γ levels in fast progressors may explain their relatively rapid disease course. Additionally, we report an age-associated decline in IFN-γ expressing CD4 T cells in slow progressors, but not in fast progressors. Possibly, non-immune disease mechanisms become increasingly important with age in the slow progressors, whereas peripheral immune mechanisms still persist in the fast progressors. We believe our results may shed light on the involvement of the peripheral immune system in disease progression in MS, particularly in the transition from RRMS to SPMS.

Importantly, our findings also suggest that fast progressors, a subset of patients with SPMS, may still benefit from anti-inflammatory therapies, which has important therapeutic implications.

RÉSUMÉ DE LA THÈSE

La sclérose en plaques (SP) est une maladie chronique, inflammatoire et neurodégénérative du système nerveux central. Le développement de l'incapacité progressive est un facteur déterminant du pronostic dans SP. Bien que l'opinion actuelle soit que les lymphocytes T jouent un rôle dans les exacerbations du système nerveux central dans la maladie en SP cyclique (poussées-rémissions), leur contribution à SP progressive secondaire et au développement de l'incapacité ne sont pas clairs. Des études précédentes dans notre laboratoire ont identifié un biomarqueur putatif dans des lymphocytes T CD4+ naïfs de patients atteints de SP, qui était associée à l'activation des lymphocytes T et à une conversion rapide du SP cyclique vers SP progressive secondaire. Par conséquent, dans ce projet, notre objectif était d'étudier la capacité de ce biomarqueur putatif et l'expression de cytokines sélectionnées dans différentes sous-populations de lymphocytes T CD4+, de différencier entre les patients avec SP avec une conversion lente ou une conversion rapide du SP cyclique à SP progressive secondaire, c'est-à-dire les progressistes lents et les progressistes rapides.

Nous rapportons une augmentation de l'expression du IFN-γ dans les lymphocytes T CD4+ dans des progressistes rapides par rapport aux progressistes lents, ce qui suggère une déviation Th1, pro-inflammatoire dans le système immunitaire périphérique des progressistes rapides. Comme les lymphocytes T sécrétrices d'IFN-γ sont pro-inflammatoires et sont associées à des exacerbations dans la SP, les niveaux élevés d'IFN-γ dans des progressistes rapides peuvent expliquer leur évolution rapide de la maladie. En outre, nous documentons un déclin associé à l'âge des lymphocytes T CD4+ qui expriment l'IFN-γ dans des progressistes lents, mais pas dans des progressistes rapides. Peut-être, les mécanismes de maladies non immunitaires deviennent de

plus en plus importants avec l'âge dans les progressistes lents, alors que les mécanismes immunitaires périphériques persistent encore dans les progressistes rapides. Nous croyons que nos résultats appuient l'implication du système immunitaire périphérique dans la progression de la maladie chez les SP, en particulier lors de la transition du SP cyclique au SP progressive secondaire. Il est important de noter que nos résultats suggèrent également que les progressistes rapides, un sous-groupe de patients atteints de SP progressive secondaire, peuvent encore bénéficier de traitements anti-inflammatoires, ce qui a d'importantes implications thérapeutiques.

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"And behold, with every hardship, comes ease.
Verily, with every hardship comes ease." Quran 94:5-6

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Miss you forever, love you always Baba.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Introduction

Multiple Sclerosis (MS) is a chronic, inflammatory and neurodegenerative disease of the central nervous system (CNS) that frequently leads to progressive neurological decline. Current opinion favors the view that MS is the consequence of an aberrant immunological attack against CNS elements, particularly myelin, the protective sheath of nerves, and is characterized by demyelination, perivascular inflammatory infiltrates, astrogliosis and axonal injury in the CNS. [1, 2] The majority of patients with MS have an initial relapsing-remitting disease course, termed relapsing-remitting MS (RRMS). A high percentage of these patients eventually develop a progressive disease course independent of relapses, termed secondary progressive MS (SPMS). The research discussed in this thesis focuses on SPMS and questions whether some markers expressed in immune cells have the potential to identify patients with rapid vs. slow progression to SPMS. The following comments and literature review provides the background upon which this research is based.

MS was first described in 1868 by Jean Martin Charcot, who noted the accumulation of inflammatory cells in the brain and spinal cord white matter of patients with intermittent episodes of neurological dysfunction. This led to the term *sclérose en plaques disséminées* or Multiple Sclerosis, referring to the scars (*sclerae*, better known as lesions or plaques) that Charcot initially observed in post-mortem tissue. [3-5] While MS was classically considered a disease of the white matter, it is now understood that MS affects grey matter as well; several studies demonstrate demyelination in grey matter structures such as the cortical mantle, grey-

white matter junctions, basal ganglia, and cerebellum. Additionally, cortical grey matter regions can exhibit diffuse neuronal, glial and synaptic loss, consistent with the findings of atrophy and cortical thinning in MS. Some link the pathology in grey matter to clinical manifestations of MS, such as seizures and cognitive dysfunction. [6-8]

Although MS can develop at any age, the onset occurs most commonly in adults between the ages of 20 and 40. MS can also have its onset in children and adolescents; this early onset occurs in up to 10% of cases. Symptoms include but are not limited to: extreme fatigue, tingling, problems with balance and coordination, double vision, cognitive impairment and mood changes. [9-11] Currently, MS affects approximately 2.5 million people worldwide and is the major cause of neurological disability in young adults in the Western hemisphere. The prevalence of MS varies throughout the world: the prevalence is low in regions close to the equator but increases as latitude increases in both the Northern and Southern hemispheres. The reason for the differences in geographical distribution depends, at least in part, upon both genetic and environmental differences between the populations in the different regions Indeed, some of the highest incidence rates occur in Canada and Northern Europe. The gender distribution is also imbalanced; with a 2:1 ratio between women and men affected with the disease. [12, 13]

At present, MS is not a curable disease. However, the past few decades in clinical research have led to significant advances in the understanding of the disease and an impressive expansion of approved treatments aimed at limiting or halting worsening disability. As of 2017, nine disease-modifying treatments (DMTs) are approved for MS by the Food and Drug Administration, USA, and the European Medicines Agency, including a few orally administrated drugs: fingolimod, dimethyl fumarate and teriflunomide. [4, 14, 15]

Subtypes of MS

The disease course of MS is unpredictable and its clinical presentation and evolution vary. Various expert international panels (e.g. Poser et al. 1983) developed criteria for the diagnosis of MS and for the identification of MS subtypes. The most recent approach led to the so-called McDonald Criteria in 2001. These criteria were subsequently modified several times to improve diagnostic accuracy and reliability. [16-19]

A diagnosis of MS, according to the McDonald Criteria, requires evidence of lesion dissemination in time (DIT) and space (DIS). DIS requires the presence of at least one T2 bright lesion (indicating inflammation) in two or more of the following locations of the CNS: periventricular, juxtacortical, infratentorial, or the spinal cord. DIT can be established by either a new T2 or a gadolinium-enhancing lesion (indication of breakdown of brain-blood barrier) not seen on a previous MRI scan or by the presence of both gadolinium-enhancing and non-enhancing lesions in any one scan. Diagnostic criteria also include clinical and paraclinical laboratory assessments and the exclusion of alternative diagnoses. Since its implementation, the McDonald Criteria have resulted in earlier diagnoses of MS with higher degrees of specificity and sensitivity. [16, 17, 20]

In accordance with the most recent McDonald Criteria, there are three subtypes of MS: relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), and primary progressive MS (PPMS).[16] Clinically isolated syndrome (CIS) is considered to be the first clinical manifestation of a demyelinating disease that could be MS. A diagnosis of CIS is made when a patient presents with clinical symptoms suggestive of MS but the findings do not satisfy the

clinical and radiological criteria of DIT and DIS. Approximately 45% of patients with CIS subsequently develop new symptoms or brain lesions that satisfy the diagnostic criteria for MS within two years. Some of the risk factors for conversion from CIS to definite MS include the presence of MRI lesions or oligoclonal bands (OCBs) in the cerebral spinal fluid (CSF), demographics, and recently, low vitamin D levels and Epstein–Barr virus (EBV) serology. [17, 21-23]

In the vast majority of patients (85-90%), MS begins with a relapsing-remitting course, termed relapsing-remitting MS (RRMS), whereas a minority of patients have progressive disease from onset, termed primary-progressive MS (PPMS). Patients with RRMS experience periods of 'attacks' that result in varying degrees of disability followed by periods of remission. Following an attack, a patient may recover completely or partially. [24] With PPMS, patients experience a gradual neurological decline without acute attacks. After 20-25 years, approximately 90% of untreated patients with RRMS stop having attacks and transition into a progressive disease course, termed secondary-progressive multiple sclerosis (SPMS). SPMS is usually diagnosed retrospectively when a patient shows gradual worsening after an initially relapsing disease course. [20, 21, 25, 26]

While MS subtypes can be classified as relapsing or progressive in terms of their current diagnostic status and history, these categories provide no information on the current, ongoing disease progress. For this reason, the MS Phenotype Group recently recommended the addition of disease modifiers to MS subtypes in order to incorporate ongoing disease activity and progression. The first disease modifier categorizes patients as 'active' or 'not active', as reflected by clinical relapses or MRI scans revealing gadolinium-enhancing lesions or new or enlarging

T2 lesions observed within a predetermined evaluation period, which has been arbitrarily determined to be one year.

The second disease modifier clarifies whether a patient with SPMS or PPMS is currently progressing, since progressive disease does not evolve in a uniform fashion and patients can remain stable over periods of time. Hence, a patient with SPMS who gradually worsened during the predetermined evaluation period would be classified as 'SPMS- with progression', whereas a patient who is relatively stable would be classified as 'SPMS- without progression'. These meaningful descriptors are thought to reflect ongoing inflammatory or neurodegenerative processes and can thus provide insight into prognosis and therapeutic decisions. [27]

The current diagnostic criteria for MS are still not perfect, and there will likely be more changes incorporated as more technological advancements are seen in laboratory tests and clinical imaging. Continuing to refine the criteria will improve the sensitivity and specificity of diagnoses, enhance communication between physicians and researchers, and increase the homogeneity of patient groups in clinical trials, all of which will hopefully contribute to better outcomes for patients with MS. [20]

Overview of the immune system

The main objective of this study is to examine selected differences in T cell biology in the peripheral blood of patients with MS. Thus, a brief overview of the components of the immune system relevant to this study is required.

The immune system is a diffuse, complex network of organs, tissues, cells and cell products that function together to protect the body from pathogens and foreign substances. This is achieved by the immune system's ability to discriminate 'self' from 'non-self' structures.The immune system has two lines of defense: innate immunity and adaptive immunity. The innate immune system is our body's first line of defense against infection; it is a non-specific, fast response that has no immunological memory. Some of the cells involved in the innate response are phagocytic cells, such as macrophages and dendritic cells, natural killer cells, and plasma proteins such as the complement system.

Adaptive immunity, in contrast to innate immunity, involves a slow, coordinated attack against a specific antigen, resulting in long-lasting defense and memory against that antigen. It consists of both a humoral response mediated by B cells and their antibodies, and a cell-mediated response carried out by two general types of T cells: CD4+ T cells, which mainly have 'helper' functions and CD8+ T cells, which mainly have cytotoxic functions. Both B cell and T cell precursors arise in the bone marrow, however, the thymus is where T cell progenitors undergo a complex process of maturation, selection and differentiation. [28]

As this research focuses on CD4 T cells and various markers expressed on these cells, the subsequent remarks focus on CD4 T cells. Each T cell expresses a unique antigen-binding receptor, the T cell receptor (TCR). T cells recognize antigens bound to major histocompatibility complex (MHC) proteins on antigen presenting cells (APCs). T cell activation requires two signals. For CD4 T cells, signal one involves binding of the cell surface TCR-CD3 complex and its CD4 co-receptor to antigen fragments presented on class II MHC molecules. The second signal involves binding of the T cell's co-receptor, CD28, to B7 molecules (CD80 and CD86) on

the same APC. Together, these signals are required for T cell activation, proliferation and differentiation. If only the first signal is given to the CD4 T cell, it becomes anergic and is no longer able to respond to antigens. [28]

CD4+ T cells can help B-cells differentiate into antibody secreting cells and they can also secrete cytokines, which are small proteins involved in cell signaling that influence many cell types. Thus, CD4 T cells play an important role in initiating and maximizing the immune response to an antigen. [28]

Pathogenesis of MS

Nearly a century after Charcot's initial description of multiple sclerosis, Elvin Kabat observed increases in oligoclonal immunoglobulin in the cerebrospinal fluid (CSF) of patients with MS. This finding supported Charcot's observations and lent further support to the inflammatory nature of the disease. [29]

In 1933, Thomas Rivers generated an autoimmune, sometimes demyelinating, disease in mammals by immunizing them with CNS myelin; this led to the current animal model of MS, experimental autoimmune encephalomyelitis (EAE). [30] These observations led to the hypothesis that MS is an autoimmune disease that targets one or more myelin antigens. Many think that autoimmune dysregulation plays an important role in demyelination. [1, 31]

It is widely accepted that both environmental and genetic factors contribute to autoimmune dysregulation in MS. In the past few decades, several large, population-based MS twin studies

demonstrated a concordance rate of 20-40% in monozygotic twins and 3-5% concordance in dizygotic, same-sex pairs. [9, 31-33] Furthermore, genome wide association studies (GWAS) found over 100 genetic variants associated with the disease, each variant conferring a small genetic risk, suggesting that the genetic variance is a complex interaction of many variants. Some of the environmental factors associated with disease risk include Epstein Barr Virus infection, smoking, childhood obesity and low levels of Vitamin D. [34] To date, the aetiology of MS remains uncertain, although it is known that several genetic, environmental and immunological factors contribute to disease susceptibility. [9, 31, 35]

Autoimmune dysregulation in MS

The currently favoured view in the MS field is that autoreactive pro-inflammatory T cells are the driving force behind CNS tissue injury.[2] The presence of activated myelin-reactive T cells in the periphery of individuals with MS initially led to the idea that there may be a disruption of tolerance to CNS antigens in the periphery. However, as myelin-reactive T cells were later discovered in the periphery of healthy individuals, this proved incorrect. [36]

One hypothesis to explain the initiation of MS in a genetically susceptible individual; is that T cells in the periphery encounter a common environmental antigen such as a viral antigen or other infectious antigen and become activated. According to the concept of “molecular mimicry”, a self-antigen may share epitopes with a foreign antigen: a T cell recognizing the foreign antigen may thus recognize and respond to the self-antigen. In MS, the hypothesis is that an environmental antigen and a CNS self-antigen, likely one derived from myelin, bear cross-reactive epitopes. [35]

Upon activation, the T cells are able to differentiate and produce pro-inflammatory cytokines. T cell activation also upregulates integrin expression such as VLA-4, which allows T cells to migrate across the brain-blood barrier (BBB) and enter into the CNS. In the CNS, they encounter the cognate antigen and are subsequently reactivated. The T cells engage in a pro-inflammatory response against myelin or other CNS epitopes, which activates nearby immune cells and attracts further inflammatory cells into the CNS. The migration of these immune cells across the BBB into the CNS requires adhesion and transmigration, which is likely facilitated by the selective expression of adhesion molecules, chemokines and chemokine receptors, and matrix metalloproteinases (MMP). [2, 35]

Of the activated inflammatory cells, it is believed that macrophages result in the most damage to the CNS and are directly involved in demyelination by phagocytosing myelin. The resulting damage to the myelin leads to the exposure of other myelin epitopes that further triggers inflammatory responses from infiltrating cells, a phenomenon known as 'epitope spreading'. In addition to phagocytosis by macrophages, myelin destruction can be mediated by direct injury by T cells, complement activation, oxygen free radicals, non-specific cytotoxicity, and apoptosis. After repeated injury, myelin regeneration and repair eventually cease, ultimately leading to the degeneration of axons and irreversible damage in the CNS. According to this 'outside-in' model of MS, the pathophysiology begins with immune dysregulation that targets the CNS. [2, 36]

Some challenge this outside-in model and propose an 'inside-out' model, which argues that the initial malfunction occurs in the CNS with cytodegeneration, perhaps of the oligodendrocyte-myelin complex. In a predisposed host, the consequent release of highly antigenic components

induces an autoimmune and inflammatory response which in turn leads to further damage. [36, 37]

While the outside-in model continues to be the predominantly held view, substantial evidence supports each model.[37] Autoimmunity and neurodegeneration are both important manifestations of MS, and there is little disagreement of their central role in the ongoing pathology and immunobiology of MS. The question that is yet to be answered is whether cytodegeneration (inside-out) or an autoimmune attack (outside-in) is the initial trigger in MS. Research should continue to investigate and therapeutically target both components of MS, ensuring a well-rounded approach for all stages of the disease. [37]

Inflammation and Degeneration in Progressive MS

Usually, SPMS is diagnosed retrospectively, after a patient has gradually worsened following an initially relapsing disease course. At present, no clinical, imaging or pathological criteria clearly indicate the point at which RRMS becomes SPMS, especially since the transition is usually gradual. Due to the lack of criteria, it is a challenge to study patients with RRMS and identify imaging or clinical biomarkers of patients undergoing a transition to SPMS. [27]

Unlike patients with RRMS, patients with SPMS and PPMS do not usually respond to various immunomodulatory agents in clinical trials. [38] Also, in both progressive subtypes, new T2 lesions on MRI scans (which reveal areas of demyelination) are unusual. These observations led some to believe that progressive MS is a distinct, non-inflammatory disease, in which neurodegeneration is responsible for the CNS damage. That being said, the majority of clinical,

imaging and genetic data suggest that SPMS and PPMS are indeed part of the spectrum of MS, with any differences being more relative than absolute. [27] While SPMS and PPMS differ in clinical manifestations, the age of onset of progression is similar in both, and both have grey as well as white matter lesions. Therefore, the overwhelming opinion is that RRMS and progressive MS represent two phenotypes of the same disease. [39-41]

Some suggest that the CNS damage in progressive MS is independent of peripheral immune activation and is primarily due to neurodegeneration. Others believe that inflammation drives CNS injury in all forms of MS but that the mechanisms by which this occurs differ between RRMS and progressive subtypes. [42, 43] One study supporting the latter view demonstrates CNS infiltration of T cells, B cells and plasma cells in all subtypes of MS, with RRMS having the highest levels of infiltration. Interestingly, patients with SPMS and PPMS that progressed rapidly have comparable levels of infiltration to patients with RRMS.[44] Thompson et al. (2004) also report higher levels of inflammation in patients with PPMS in the early phase of the disease compared to patients in the later phases of the disease; supporting the notion that early inflammation drives neurodegeneration in progressive MS.[45]

If inflammation is the driving force behind CNS damage in all forms of MS, the question remains as to why patients with progressive MS benefit little from anti-inflammatory drugs, unlike patients with RRMS. A common response is that once patients enter the progressive phase of MS, the disease becomes mainly neurodegenerative and is independent of any inflammatory processes.[43] An alternative theory proposes that these therapies are ineffective in progressive MS because the inflammation is confined to the CNS unlike in RRMS. It is possible that the ongoing inflammation and neurodegeneration in progressive MS is no longer influenced by the

infiltration of peripheral immune cells, as the inflammation is confined within the brain-blood barrier. Therefore, anti-inflammatory drugs that target the periphery and that are unable to cross the brain-blood barrier would be ineffective in modulating the local inflammatory environment in the CNS. [46]

Nevertheless, several studies report abnormalities in the peripheral immune system in patients with progressive MS.[47] Brown M et al. (2010) describe higher levels of T cell activation genes, an indication of T cell dysregulation, in all forms of MS.[48] Previous studies in our laboratory revealed abnormalities in the homeostatic balance and regulation of naive CD4 T cells in both RRMS and progressive MS subtypes.[49, 50] Another study demonstrates a correlation between changes in activated T cells and clinical and MRI measures of disease activity in both patients with RRMS and progressive MS. [51] Further, individuals with progressive MS that underwent autologous stem cell transplantation after immunoablation showed stabilization of their disease. [52, 53] Together, these studies indicate a potential role for the peripheral immune system in progressive MS.

CD4+ T subsets in MS

Recent advances in multiparametric flow cytometry enable the precise and detailed characterization of lymphocyte subsets in peripheral blood mononuclear cells (PBMCs). Thus, flow cytometry is a powerful tool in immunophenotyping patient samples and facilitates an understanding of the role that different lymphocyte subsets play in the pathogenesis of MS. [54, 55]

The immune-mediated destruction of myelin involves many types of immune cells. Autoreactive CD4+ T cells play a central role in initiating the immune response and contributing to the activation of CD8+ T cells and B cells in MS.[2, 56]

Once a naïve CD4+ T cell is activated, it undergoes a developmental pathway involving several phenotypic stages that can be identified by the differential expression of CD45 isoforms, integrins, chemokine/cytokine receptors and homing receptors. In addition, several biochemical pathways are activated such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway, which in turn regulates the proliferation and maturation of T cells. [28, 57]

Some propose a progressive T cell differentiation model from naïve to central memory (TCM), effector memory (TEM) and ultimately a terminally differentiated T cell subset (TEMRA), based on the differentiation state, proliferative capacity, telomere length and *in vitro* activation/conversion assays. The exact mechanisms by which TCM, TEM and TEMRA cells are generated after naïve T cell activation remain yet to be defined. [58]

The four main CD4+ T cell subsets are identified based on the coordinate expression of CCR7 and CD45RA. Naïve cells express both CCR7 and CD45RA. The lymph node homing receptor CCR7 distinguishes between TCM cells ($CD45RA^{-}CCR7^{+}$) and TEM cells ($CD45RA^{-}CCR7^{-}$). TEMRA cells are defined as CD45RA+CCR7- CD4+ T cells. [59] Naïve and central memory cells continuously circulate to secondary lymphatic organs, by means of their expression of CCR7 and CD62L homing receptors. Effector memory cells, on the other hand, do not recirculate through secondary lymphatic organs but instead patrol peripheral organs. Notably,

activation lowers the T cell's threshold for future activation. Thus, effector and memory cells do not require co-stimulation through CD28 for activation, unlike naïve cells. [28, 59] Upon stimulation, naïve T cells produce IL-2, while TCM, TEM and TEMRA cells produce both IFN-γ and TNF-α. Naïve and TCM cells also have a higher proliferative capacity than TEM and TEMRA cells. [58]

Several studies show a disruption of naïve CD4+ T cell homeostasis in both patients with RRMS and patients with PPMS. [50, 60, 61] Another study shows that myelin-reactive T cells stem mainly from CD4+CD45RA+ naïve and TEMRA cells and not from TCM or TEM cells, possibly suggesting a role for naïve and TEMRA cells in mediating demyelination. [62] In addition, Corvol et al. (2008) demonstrate reduced expression of the TOB1 gene, a critical regulator of T cell proliferation, in naïve CD4 T cells of patients with CIS that rapidly converted to MS. This suggests that naïve CD4 T cells may be involved in progression from CIS to RRMS. [63]

In patients with MS treated with Gilenya, an immunomodulatory drug, an overall decrease in CD4+ and CD8+ T cells in the peripheral blood is documented. In particular, a reduction in naïve and central memory T cells is seen but not in effector memory T cells. Gilenya functions by blocking the egress of lymphocytes from secondary lymphocyte organs. Thus, because naïve and TCM cells express CCR7, which allows them to circulate through secondary lymphoid organs, they become trapped there. [64] Song et al. (2014) assessed the proportions of different T cell subsets in patients with MS being treated by Gilenya, and demonstrated a correlation between increased $CD4^+$ TCM percentages at 3 and 6 months during therapy and relapses in

patients. [65] Another study documents an increase in TEM cells in untreated patients with RRMS and progressive MS compared to healthy donors. [66]

Regulatory T cells (T-regs) are another subset involved in autoimmune disorders including MS. T-regs can be natural or adaptive (or inducible) and regulate autoimmunity by mediating tolerance to self-antigens. Natural T-regs express high levels of CD25 and the transcription factor forkhead box P3 (FoxP3). The thymus generates natural T-regs when CD4 T cells with a high affinity for self-antigens are positively selected.[67] Adaptive T-regs are derived from uncommitted naïve cells in the periphery that are activated under the influence of immunosuppressive cytokines IL-10 and TGF-β, as well as retinoic acid (RA). It is thought that natural T-regs play a role in preventing autoimmunity, and adapative T-regs are likely generated in response to foreign antigens during infection. The distinction between natural and adaptive T-regs is not always clear, however, especially because the peripheral conversion of natural T-regs to adaptive T-regs can occur in the presence of TGF-Beta and retinoic acid. [68, 69]

T-regs play an important role in immune homeostasis; in healthy individuals, potentially autoreactive T cells specific for myelin antigens are present, suggesting that the regulatory mechanisms that normally keep them in check may be impaired in individuals with MS. Indeed, T regulatory responses, reflected by levels of IL-10 secretion, are reduced in patients with MS.[70] In another study, the transfer of CD4+CD25+ T reg cells reduced disease severity in EAE mice, presumably mediated by IL-10, as the transfer of T-reg cells from IL-10-deficient mice failed to reduce disease severity.[68, 71]

While many studies have examined alterations in CD4+ T cell biology in patients with MS, most studies have focused on RRMS and not progressive MS, and few have examined the role of specific CD4+ T cell subsets. Thus, more studies of this nature are required to enhance our understanding of the underlying pathogenesis in MS, particularly in progressive MS.

Cytokines in MS

T cells are typically categorized according to the cytokine profiles they produce upon activation. CD4+ T cells that produce IFN-γ, IL-2, and TNF-α are defined as inflammatory Th1 cells and are responsible for immunity against intracellular pathogens. Th1 cells can also be pathogenic in EAE. CD4+ T cells that produce IL-4, IL-5, IL-10, or IL-13 are classified as Th2 and are responsible for immunity against extracellular pathogens. Th2 cells are anti-inflammatory and have a protective role in EAE. [72-74]

In human CD4+ T cells, the Th1/Th2 paradigm is less stringent. Some report that myelin-reactive T cells in patients with RRMS have a Th1 bias, although it has become apparent that these T cells secrete both Th1 and Th2 cytokines, depending on the culture conditions. [75, 76] The observation that myelin-specific cytokine responses can be shifted to Th1 or Th2 depending on in vitro conditions has therapeutic implications. [72, 73] Indeed, one of the immunomodulatory treatments for patients with RRMS is glatiramer acetate, and its mechanism of action includes a cytokine shift towards Th2. [77]

In addition to Th1 and Th2 T cells, IL-17 producing T cells represent a distinct T cell subset termed Th17. [78] This T cell subset plays a critical role in MS in addition to other autoimmune

diseases. [68] Th17 cells are linked to the pro-inflammatory cytokine IL-23, which regulates the function and proliferation of Th17 cells. One study demonstrates that IL-23 deficient mice have few Th17 cells and are resistant to developing autoimmune diseases such as EAE or collagen-induced arthritis. Another study shows that IL-$17^{-/-}$ EAE mice have a milder disease with delayed onset, reduced disease scores and early recovery. [79] In addition, mice with collagen-induced arthritis that are treated with anti-IL 17 antibody show an improvement in joint damage. Recent experimental vaccine strategies against IL-17 show an ability to protect mice against myocarditis, EAE and arthritis. [80]

Some previous work led to the hypothesis that MS is a Th-1 mediated disease, based on extrapolation from mouse models in which IL-12p40-defective (IL-$12p40^{-/-}$) mice were resistant to EAE; IL-12 is necessary for the differentiation of Th1 cells. In addition, treating patients with MS with IFN-γ exacerbated the disease. Unexpectedly, mice lacking Th1 cells developed more severe EAE. These discrepant observations were resolved by the discovery of IL-23, which is structurally related to IL-12. Later, experiments showed that IL-23 is necessary for the expansion of IL-17 secreting CD4+T cells, or Th17 cells. [68]

The current opinion is that MS is both a Th1- and Th17-mediated disease. [68, 81] Of interest, some report an increase in Th17 cells in the peripheral blood of individuals with MS, raising the possibility of a role for Th17 cells in the pathogenesis of MS. Interestingly, some also document an increase of Th17 cells in progressive MS, supporting a role for Th17 cells in progressive forms of MS as well. [66, 81] Elevated frequencies of IL-17 and IFN-γ producing T cells in the peripheral blood is associated with disease activity in MS, and these cytokines are also expressed in brain lesions. [68]

In the MS field, there is an ongoing effort to characterize the differences in cytokine profiles induced in individuals in response to various therapies. The observed heterogeneity amongst patients with MS could explain the differences in clinical disease course and severity. Together, with the observation that interventions can shift cytokine responses away from Th1 to Th2 profiles, these observations may lead to more effective and personalized therapeutic approaches in MS.

Biomarkers in MS

A biomarker is a measurable parameter that can provide information on normal or pathological processes, as well as the response to therapeutic interventions. [82] In MS research, various investigators seek three main types of biomarkers: 1) those that can predict an individual's risk of developing MS; 2) those that can predict progression and the risk of developing severe attacks; 3) those that can predict response to specific treatments.[83]

Physicians frequently rely on clinical epidemiological studies to identify markers of good prognosis such as female sex, young age of onset, occurrence of optic neuritis, long intervals between initial and second relapse, normal MRI imaging at onset, etc. [82]

Some biomarkers that are currently in use in MS include the following: oligoclonal bands (OCBs) in the CSF of patients, MRI imaging, and JC viral titers. OCBs were first identified in the CSF of patients with MS in 1957. Almost 90% of patients with MS have OCBs in the CSF, a finding which led to their inclusion as a biomarker for diagnosing MS. Recent studies find that

the presence of OCBs predict the conversion from CIS to MS. [83, 84] MRI imaging is another biomarker currently in clinical use and is the primary tool for determining efficacy in phase II and III clinical trials. White matter lesions on MRI are used to identify CIS to MS conversion, and the number of baseline MRI lesions correlate with disease severity after 20 years. In addition to lesion load, lesion location has also proved important; lesions found infratentorially are associated with disease progression. [82, 83]

Natalizumab is currently one of the most effective treatments for multiple sclerosis. Unfortunately, a rare side effect that has emerged from its use is progressive multifocal leukoencephalopathy (PML). PML is caused by the reactivation of a latent JC virus that subsequently transports to the CNS, resulting in a debilitating encephalopathy that is fatal in up to 50% of cases. Now patients are screened for previous infection with JC prior to treatment with Natalizumab by determining seropositivity for JC viral antibodies. This led to the subsequent quantification of JC viral load, a laboratory test that serves as an extremely useful clinical biomarker in patients under consideration for treatment with Natalizumab. [85]

One potential biomarker that is showing promise in research is the presence of CSF neurofilaments, which are typically released after axonal damage. Neurofilaments consist of light, medium, and heavy chains and α-internexin. Neurofilament light chain (Nfl) levels in the CSF are increased in RRMS and in patients with progressive MS. In patients with RRMS, neurofilament levels correlate with active lesions on MRI scans. [86] In other studies, Nfl levels predict conversion from CIS to MS, conversion from RRMS to SPMS, and disease severity. [87, 88] Several other potential biomarkers of neuronal and glial cell damage are elevated in the CSF of patients with MS in comparison to healthy controls. These include glial fibrillary acidic

protein (GFAP), myelin basic protein (MBP), S100B (an astrocyte proliferation marker), tau, NCAM, NGF, CNTF and ferritin. [83]

Two other potentially promising markers are Chitinase-3-like 1 and Osteopontin. Chitinase-3-like 1, or YKL-40, is a glial activation marker that is elevated in the sera of patients with various inflammatory conditions. In MS, increased YKL-40 levels in the CSF predict conversion from CIS to MS. A similar study finds that YKL-40 levels are associated with rates of conversion to MS and the accumulation of rapid disability. [89, 90] Osteopontin (OPN), an early activation maker on T cells, is highly expressed in MS lesions, and is markedly increased in the blood and CSF of patients with MS compared to healthy controls. OPN correlated with disease severity and relapse rate in a 5-year follow up study. [83]

Several studies show that early therapeutic intervention can improve long-term progression. Therefore, a biomarker with the potential to identify individuals with CIS that have a high risk of developing MS, would allow for early intervention and possibly improve outcomes. [91, 92] One of the challenges associated with biomarker discovery in MS is that MS is highly variable from immunological, neuropathological, clinical, imaging and therapeutic perspectives. Moreover, many changes in MS are subclinical making it difficult to define therapeutic responses. [82, 83] Additionally, a useful clinical biomarker is one that can be measured easily and has a rapid turn-around time.

Measures of disability and progression in MS

A number of tools are available for the quantification of disease progression and severity in patients with MS. These measures are used to determine the level of disability a patient has reached and are often employed as endpoints in clinical trials as a means of assessing the efficacy of certain therapeutic interventions. [93]

The most frequently used primary outcome measure in clinical trials is relapse rates, a tangible indication of inflammatory disease activity. [94] Relapse rates are a useful endpoint in clinical trials; they are easy to quantify and their prevention immensely benefits patients. One limitation, however, is that the probability of relapses changes over time: the number of relapses seems to decrease as patients progress. Moreover, subclinical changes that contribute to disability progression in MS may not be reflected by relapse rates alone. Further, some argue that in order to measure a significant reduction in relapses, clinical trials need to last at least a year. [93, 94] These limitations led to an increased interest in the use of MRI as an alternate measure for disease activity in MS. MRI metrics such as lesion count, lesion volume or whole brain volume are now being used as surrogate endpoints in clinical trials, although the relationship between changes in MRI scans and long-term disability in MS remain unclear. [93, 94]

A widely-used index of disability is the Expanded Disability Status Scale (EDSS), developed by John Kurtzke in 1983. It is a physician-administered assessment scale that evaluates disability in 8 different functional systems of the CNS, resulting in a composite score. The scale ranges from 0 (normal neurological status) to 10 (death due to MS), increasing by 0.5 increments.

Patients with scores between 1.0 to 4.5 are fully ambulatory, while those with scores between 5.0 and 9.5 have impaired ambulation. [95]

Clinical trials that have used the EDSS score have typically defined progression as a sustained increase in the EDSS score by 0.5 if the baseline score is greater than 5.5, or an increase by 1.0 if the baseline score is less than 5.5. [93] The use of the EDSS score in clinical trials has been challenged as the score changes little over time and therefore may not be suitable for detecting therapeutic-related changes. In addition, the scale has been criticized for its non-linearity; the upper scale values are mainly based on walking ability and patient handicaps, while the lower scale values are based on abnormalities detected by the neurological exam of the functional systems. Therefore, the scale between 1.0 and 2.0 versus 6.0 and 7.0 carries a different significance. [93, 95]

The MS Functional Composite (MSFC) score is another instrument that was developed to address the limitations of the EDSS score. It is a composite score of three tests: a timed 25-foot walk, a 9-hole peg test, and a paced auditory serial addition task (PASAT), which assess ambulation, hand function, and speed of processing, respectively. Studies report that MSFC scores correlate with EDSS scores, relapse rates, changes in MRI lesion burden, as well as some patient-reported outcomes.[95] However, as the tests are equally weighted in the composite score, changes in individual components of the tests may not be reflected in the MSFC score. Further, improvements in the PASAT test results could be due to practice efforts rather than true improvements in cognitive dysfunction. Despite its anticipated advantages, the MSFC score has not been incorporated in many clinical trials and is mainly used as a secondary endpoint along with the EDSS score. [93, 95]

While EDSS and MSFC scores can be used to compare disability between groups of patients, they fail to take into consideration the disease duration of these patients. For instance, a patient with an EDSS score of 6.0 and a disease duration of 7 years has a different rate of disease progression compared to a patient with an EDSS score of 6.0 and a disease duration of 25 years, despite having the same EDSS score. One solution to this is to follow patients longitudinally and measure the time taken to reach a certain level of disability. The time taken for a patient to reach an EDSS score of 3 (defined as moderate disability) or an EDSS score of 6 (defined as unilateral assistance required to walk 100 meters) has often been used in several studies; both scores are well-defined milestones that can be reliably determined retrospectively. [96, 97]

An interesting approach to determining disease progression is the global multiple sclerosis severity score (MSSS), which is based on databases from 11 countries, and contains information on 9,892 patients. [98] The MSSS relates a patient's current EDSS score to the distribution of disability in patients with the same disease duration. Thus, a patient who accumulated disability over a relatively short period of time would be assigned a high MSSS score, and vice versa. One study shows that the MSSS predicts disease severity over time, and another study reports a correlation between the MSSS and axonal biomarkers. [99, 100] The idea of EDSS rankings at given disease durations, inspired the development of a new online tool called the MSBase Registry, which tracks long-term outcomes in MS. Inputting an EDSS score for a given disease duration provides a disability rank for an individual, based on comparisons to the EDSS scores databased from 19,500 individuals with MS internationally. By entering serial EDSS scores, the MSBase Registry can be used to chart relative disease progression over time for any patient. [101]

The heterogeneous and complex nature of MS presents a challenge to measuring and comparing disease progression in patients. The appraisal of new treatments in clinical trials requires sensitive measures that can reliably detect small changes in disability and disease progression, which in turn may reflect long-term changes in the disease course. The clinical course of MS will likely change in the coming years with the advent of new therapies, and the development of various outcome measures will be vital in assessing these anticipated changes.

Hypotheses and Objectives

Previous work in our laboratory examined the naïve CD4 T cell gene expression profiles of patients with SPMS and HCs using whole genome microarrays.[102] Unsupervised hierarchical clustering, based on the most variably expressed genes, segregated patients with SPMS into two subgroups. In the first subgroup, the most differentially expressed genes were upregulated compared to HCs, while in the second subgroup, the most differentially expressed genes were downregulated compared to HCs. A 5 gene T cell activation transcriptional signature was found specific to the first subgroup of patients. Interestingly, the first subgroup of patients had a shorter RRMS duration before conversion to SPMS compared to the second subgroup of patients, suggesting that the first subgroup of patients may have had a more rapid, aggressive disease course. Subsequent protein expression studies found that 3 of the 5 signature genes (TLR2, TLR4, and CCR1) had higher levels of membrane expression on naïve CD4 T cells in the first subgroup of patients with SPMS compared to the second subgroup. [102]

In MS, the development of progressive disability is a critical determinant of long term prognosis.[103] In this study, our primary objective was to further investigate the potential of the putative biomarker signature consisting of TLR2, TLR4 and CCR1, to differentiate between patients with SPMS with rapid vs. slow conversion from RRMS to SPMS. Hereinafter, patients with SPMS that had a relatively rapid conversion from RRMS to SPMS (i.e. shorter RRMS duration) will be referred to as *fast progressors*, while patients that had a relatively slow conversion from RRMS to SPMS (i.e. longer RRMS duration) will be referred to as *slow progressors*.

Studies report that even minor changes in lymphocyte subpopulations can serve as useful biomarkers. [65] Hence, in addition to naïve CD4+ cells, we sought to measure surface expression of these markers in TCM, TEM, TEMRA and regulatory CD4+ T cells. We also measured changes in TLR2, TLR4 and CCR1 surface expression following short term and long term stimulation. Our **hypothesis** was that the surface expression of these markers would be higher in the various T cell subsets in the fast progressors than in the slow progressors.

Furthermore, we chose to characterize the intracellular expression of cytokines IFN-γ, IL-17A, IL-10 and IL-4, in the various T cell subsets in the two patient groups. As some evidence indicates that IFN-γ and IL-17A are pro-inflammatory in MS and are associated with disease activity, we **hypothesized** that their expression would be higher in the fast progressors vs. the slow progressors.[68] We also **hypothesized** that IL-10 and IL-4 expression would be higher in the slow progressors than the fast progressors, as these cytokines are classically involved in immunosuppression and have a protective role in EAE. [2]

CHAPTER 2: METHODS

Criteria for Patient Selection

Our study proposal was based on results from previous work in our laboratory that found differences in naïve CD4 gene expression and surface protein expression between fast progressors and slow progressors (see *Hypothesis and Objectives*). Hence, we were interested in further investigating the differences in CD4 T cell biology between patients with SPMS that accumulated disability relatively rapidly (i.e. fast progressors) and those that accumulated disability relatively slowly over their disease course (i.e. slow progressors).

In determining how to define 'fast' and 'slow' progressors, and consequently our selection criteria for patients with MS in our study, we considered various approaches. As discussed earlier in (see *Measures of Disability and Progression*), one way of measuring relative disease progression is using the time taken to reach an EDSS score of 3 or 6. However, we found that this method limited patient recruitment as many patients had missing or no clinical information at these milestone EDSS scores. Next, we considered using MSSS scores, a measure that incorporates EDSS scores and disease duration at a single time point. The issue we encountered was that not all patients had been recently seen by their physician, and therefore their EDSS scores were not up to date.

A colleague at the Montreal Neurological Institute, Dr. Stanley Hum, used group-based trajectory modeling to identify longitudinal patterns of MS disease progression for women and

men, using EDSS scores. [104] We considered selecting patients from disease trajectories that indicated rapid disability accumulation vs. slow disability accumulation. However, many of these patients had RRMS or PPMS, or did not satisfy other criteria, e.g. some were being currently treated with disease-modifying therapies.

Ultimately, we decided to select patients with SPMS based on RRMS duration, similar to the findings that differentiated the two subgroups of patients with SPMS in the previous study in our laboratory. We felt this was a reliable approach as progression to SPMS is considered to be an irreversible milestone, and SPMS is associated with most of the severe neurological disabilities found in patients with MS. [105, 106]

Patients and Healthy Controls

In collaboration with Laboratoires CDL© (Montreal, QC) and the multiple sclerosis clinics at the Montreal Neurological Institute, we recruited thirteen patients with SPMS (mean age 56.3) with the assistance of Drs. J Antel, A Bar-Or, P Giacomini, and Y La Pierre. Our inclusion criteria were: patients between the ages of 18 to 70 with a clinical diagnosis of SPMS plus an RRMS duration of either: 1) $\leq$ 10 years, that we termed fast progressors, or 2) $\geq$ 20 years, that we termed slow progressors. Six of the patients were fast progressors (mean age 53.9) and 7 were slow progressors (mean age 58.3). Efforts were made to age-match the fast and slow progressors. (Table 1) All patients had been seen by a neurologist with expertise in MS diagnosis and management within the previous 2 years. Our exclusion criteria consisted of treatment with disease-modifying therapies within the 3 months prior to the study visit, relapse(s) within 3

months of study visit, known infection within 2 weeks of study visit, or a history of other autoimmune disorders.

In addition, we recruited six, age-matched healthy controls (mean age 56.3). The exclusion criteria for controls were a history of multiple sclerosis or autoimmune disorders, or a history of treatment with immunomodulatory drugs.

A total of 90ml of venous blood was drawn for the immunophenotypic studies. Ethical approval was obtained from the Institutional Review Board, Faculty of Medicine at McGill University, Montreal QC. Informed consent was obtained from all participants.

Table 1: Summary of Patients Recruited

	All Patients	**Fast Progressors**	**Slow Progressors**
Sample Size (n)	**13**	**6**	**7**
Participants	**5M:8F**	**2M:4F**	**3M:4F**
Mean Age	**56.3**	**53.9**	**58.3**
Mean RRMS Duration	**17.8**	**6.5**	**27.5**
Mean EDSS Score	**6.4**	**6.6**	**6.2**

PBMC Isolation and Cryopreservation

Blood samples collected in heparin-coated vacutainers were first diluted with phosphate buffer saline (PBS). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque™ PLUS (Amersham Biosciences, Uppsala, Sweden). All PBMC samples in this study were cryopreserved prior to experimentation in order to allow several samples to be run simultaneously, thus ensuring consistency across samples.

Following isolation, PBMCs were resuspended in PBS. A small sample was removed, stained with Trypan Blue (Sigma-Aldrich Co.©) and a cell count was then conducted using a hemocytometer and microscope.

The remaining PBMCs were centrifuged and suspended in the appropriate volume of freezing medium consisting of 90% Dimethyl Sulfoxide (Sigma-Aldrich Co.©) and 10% Human AB Serum (Sigma-Aldrich Co.©). A 1ml aliquot of PBMCs, not exceeding $21.5x10^6$ cells/ml, was placed in each cryovial.

Cryovials were immediately transferred to a Mr. Frosty Freezing container (Nalgene®) and placed in a -80°C freezer for 12-72 hours. Afterwards, they were stored in a liquid nitrogen storage tank for a minimum of one week prior to use in experiments.

Thawing

Following retrieval from the liquid nitrogen tank, the cryovials were quickly thawed in a 37°C water bath, disinfected with 70% ethanol, and transferred to a Biological Safety Cabinet (BSC) for ensuing work.

Cells were added in a gradual, drop-wise fashion to complete media (*Complete RPMI 1640 medium containing 10% ht. inactivated fetal bovine serum, 2mM L-glutamine, 100U/ml Penicillin/Streptomycin*) until completely resuspended and then centrifuged. Then, DNAse-complete media reagent was added to cells and incubated at room temperature for fifteen minutes in order to remove clumps and prevent consequent cell loss. The cells were then washed with complete media, centrifuged, and resuspended in PBS. A small volume was stained with Trypan Blue (Sigma-Aldrich Co.©), and cell recovery and viability is determined with a hemocytometer and microscope. The remaining cells were placed in a 37°C incubator (%5 CO_2) with the cap of the falcon tube loosened, and then left to rest for approximately one hour.

An appropriate number of cells were removed in order to assess surface protein expression and intracellular cytokine expression in freshly isolated, uncultured, unstimulated PBMCs, thus serving as a baseline expression for all markers. In the results section, this is deemed as timepoint 0 as they were stained immediately.

Stimulation and Staining

Pre-staining

Initially, we optimized our stimulation experiments. We identified two important issues related to stimulation. Firstly, we observed downregulation of CD4 surface expression after 24 and 48 hours stimulation with PMA/Ionomycin, with consequent progressive difficulty in identifying CD3+CD4+ cells. We discovered that phorbol esters such as PMA often result in the rapid internalization of CD4 molecules through coated vesicles.[107] For this reason, we decided to stain cells with anti-CD4 antibody before stimulation and then stain for the remaining markers in the panel after stimulation. Using this method, we were able to successfully stain for CD4 and accurately identify CD3+CD4+ T cells after stimulation for 24 and 48 hours, without any significant decreases in this population (*data not shown*).

The second issue was related to detection of regulatory T cells. In our study, we defined regulatory T cells as $CD3^{+}CD4^{+}CD25^{hi}CD127^{lo}$.[108] However, as activated T cells often upregulate their expression of CD25, we were not able to reliably identify regulatory T cells in stimulation experiments. Therefore, we pre-stained cells with anti-CD25 and anti-CD127 antibodies before stimulation to allow subsequent identification of the regulatory T cell population throughout stimulation experiments. We chose not to use FoxP3 as a marker for regulatory T cells, as it requires the use of the FoxP3 staining buffer set, which has been known to have potential deleterious effects on tandem fluorophores (APC-CY7, etc.) in a multi-color assay and would have thus required extensive testing and optimization for use in our panels.

Hence, before stimulation, all cells were stained with anti-CD4 antibodies for 30 minutes at 4°C. In addition, cells stained with the Panel #2 antibodies (Table 2), which includes the regulatory T cell markers, were pre-stained with anti-CD25 and anti-CD127 antibodies for 30 minutes at 4°C. After pre-staining, the cells were washed. Control cells that were cultured without being stimulated were similarly pre-stained.

Stimulation

Cells were plated in non-treated, sterile Corning® 96 well plates (Sigma-Aldrich Co.©) at a concentration of 500,000 cells per well. Phorbol 12-myristate 13-acetate (PMA) and Ionomycin (Sigma-Aldrich Co.©), at concentrations of 20ng/µl and 1ng/µl, respectively, were used to stimulate the cells for 4, 24, and 48 hours in a 37°C incubator (%5 CO_2). We determined the optimal concentrations of PMA and Ionomycin in preliminary experiments: we tested various concentrations and assessed surface expression of the T cell activation marker CD69 as well as expression of our markers and cytokines of interest.

To allow for the accumulation and detection of intracellular cytokines, BD GolgiStop™ (BD Biosciences, San Jose, USA) was added to the cells 4 hours before the end of each stimulation timepoint. As such, cells stimulated for 4 hours received GolgiStop™ concurrently with PMA/Ionomycin. Control cells without PMA/Ionomycin were included for each timepoint.

Immunostaining

At the appropriate timepoints, the cells were removed and washed with PBS. Cells were then stained with BD Horizon™ Fixable Viability Stain 510 (FVS 510) (BD Biosciences, San Jose,

USA) to allow us to discriminate between viable and non-viable cells. Afterwards, cells were incubated at room temperature with Human BD Fc Block™ (BD Biosciences, San Jose, USA) to minimize non-specific binding of monoclonal antibodies (mAbs). This was also done for freshly isolated, unstimulated PBMCs.

Extracellular (surface) protein and intracellular protein staining for all cells was conducted using the mAbs listed in the immunofluorescence panels shown in Table 2 below. All fluorescent-conjugated mAbs listed in Table 2 were purchased from BD Biosciences (San Jose, CA, USA).

Extracellular staining was conducted first with the appropriate mAbs of each panel for 30 minutes at 4°C. The cells were fixed using BD Cytofix/Cytoperm Kit for thirty minutes at 4°C. Then, the cells were stained intracellularly using the appropriate mAbs of each panel for thirty minutes at 4°C. Finally, the cells were resuspended in PBS/2% FBS and data acquired on a BD LSR Fortessa™ (BD Biosciences, San Jose, USA). Cells that were pre-stained with CD4 and/or CD25 and CD127, were only stained with the remaining mAbs in the respective panels.

Table 2: Multicolor Flow Cytometry Panels

Antibody Panel #1	
Marker	**Fluorochrome**
CD4	BUV395
CD3	PERCP-CY5.5
CD45RA	BV421
CCR7	PECF594
CD127	AF647
CD25	BB515
CD69	APC-CY7
IL-4	BV786
IL-10	BV650
TLR2	PE

Antibody Panel #2	
Marker	**Fluorochrome**
CD4	BUV395
CD3	PERCP-CY5.5
CD45RA	BV421
CCR7	PECF5940
CD69	PE
IFN-γ	AF700
IL-17A	BV786
TLR4	APC
CCR1	AF488

Antibody Panel #3	
Marker	**Fluorochrome**
CD4	BUV395
CD3	AF700
CD44	APC-Vio770
CD40L	PE
CD95	EFV10
CD45RA	BV421
CCR7	Texas Red

Controls

We used single immunostains of each marker in the immunofluorescence panels (Table 2) and unstained cells were used as compensation controls during data acquisition. In order to set gates, a fluorescence minus one (FMO) panel was used for the following markers and cytokines: TLR2, TLR4, CCR1, IFN-γ, IL-17A, IL-4 and IL-10.

Previous optimization experiments revealed the FMOs of one patient sample could be reliably used to set the gates for other samples. Thus, we opted to designate one patient sample from the fast group and one from the slow group for the FMOs.

Identifying T cell subsets and Gating Strategy

After we acquired samples through flow cytometry, we uploaded data on FlowJo workspaces for analysis. In order to identify our T cell subsets and determine the levels of marker surface expression and intracellular cytokine expression, we followed the gating strategy demonstrated in Figure 1. Single cells were gated based on forward and side scatter plots (Figure 1), in order to exclude doublet cells which can lead to false positives in marker expression. Then, we discriminated viable from non-viable cells so that surface marker expression was based on viable cells.

Next, we gated on lymphocytes from a forward and side scatter plot, and then a plot of CD4 vs. CD3 was selected, from which we selected our double-positive CD3+CD4+ T cell

population. From our CD3+CD4+ T cell population, we identified naïve, TCM, TEM and TEMRA cell based on a plot of CD45RA and CCR7. Additionally, from our CD3+CD4+ T cell population, we identified T reg cells as $CD25^{hi}CD127^{low}$, from a plot of CD25 and CD127 (Figure 2). After gating our T cell subset populations of interest, we determined the frequency of cells positively expressing each marker and cytokine of interest.

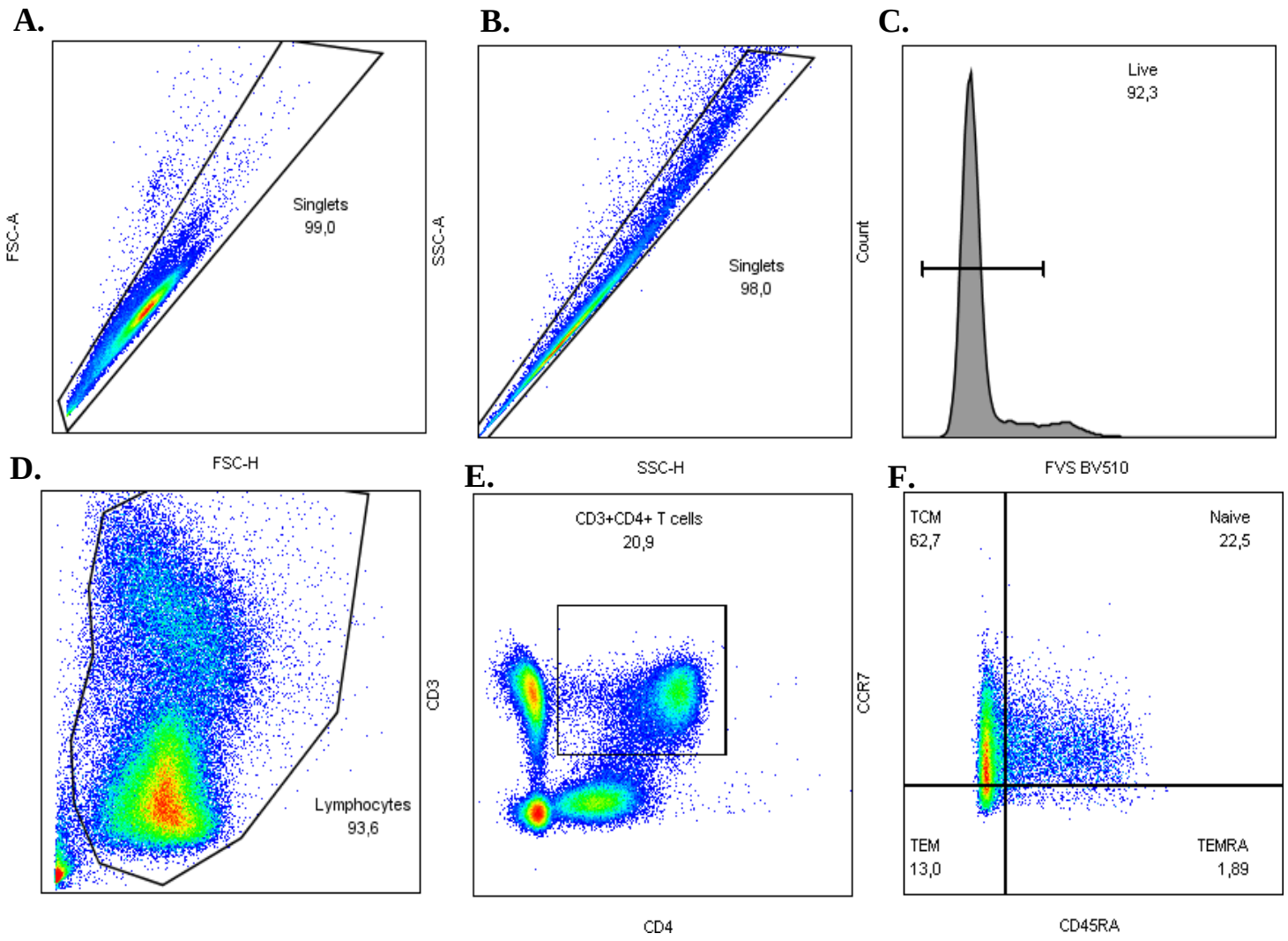

Figure 1: Gating strategy employed in FlowJo workspaces. A) and B) Singlet cells were selected from forward and side scatter plots. C) Viable cells were gated for further analysis. D) Gating of lymphocytes. E) CD3+CD4+ T cells were gated from a plot of CD3 and CD4 F) Naïve, TCM, TEM and TEMRA CD4+ T cell subsets were defined from a plot of CCR7 and CD45RA.

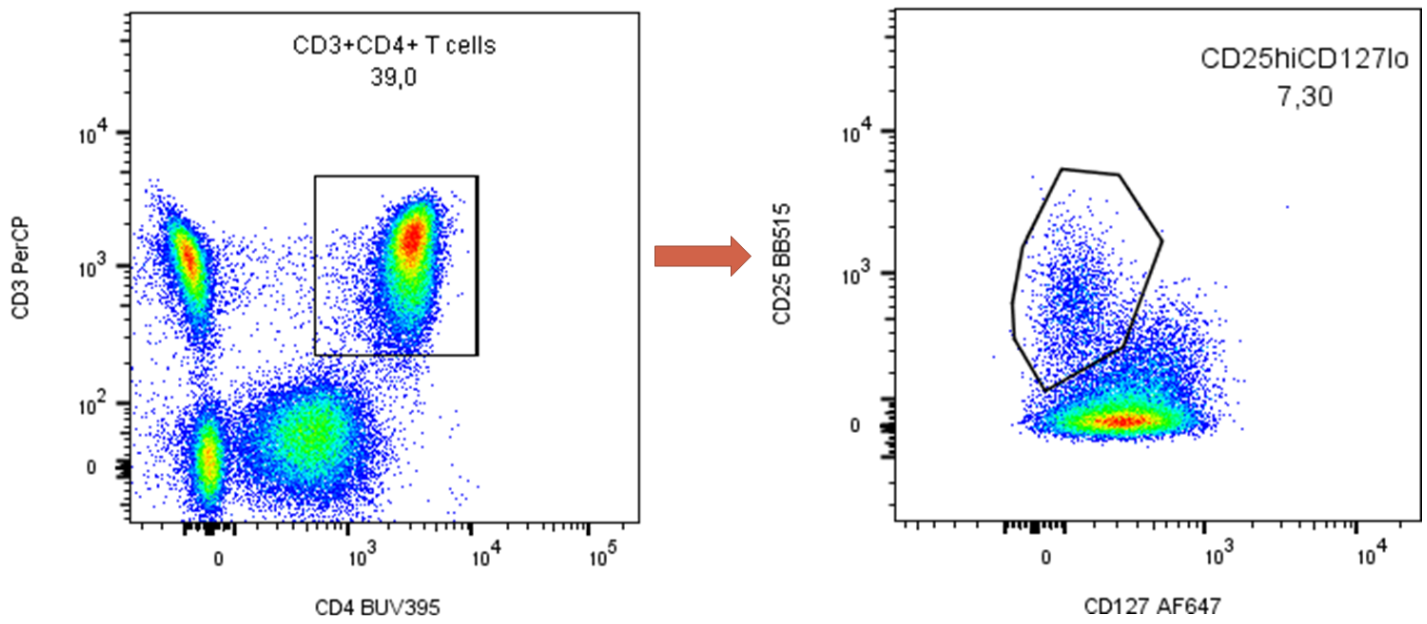

Figure 2: Gating strategy used to define CD4+ T-regs. CD4+ T reg cells were identified as $CD25^{hi}CD127^{low}$, from a plot of CD25 and CD127.

Statistics

All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software Inc, La Jolla, USA).

We compared mean surface protein expression of our putative biomarkers between fast and slow progressors using the unpaired Student's t-test. We compared the median expression levels of cytokines between fast and slow progressors using the Mann-Whitney test. For repeated measures of cytokine expression levels, we utilized Friedman's test followed by Dunn's multiple comparisons test.

Additionally, we compared cytokine expression levels between our three cohorts (fast progressors, slow progressors and HCs) using the Kruskal-Wallis test followed by Dunn's multiple comparisons test. To assess correlations between cytokine expression levels and age, RRMS duration and MS duration, we employed Spearman's correlation. Student's unpaired t test was used to compare normally distributed clinical parameters of each cohort, and the Mann-Whitney test was used otherwise.

A $P \leq 0.05$ was considered statistically significant.

CHAPTER 3: RESULTS AND STATISTICAL ANALYSES

The main objective of this study was to examine CD4+ T cell subsets in patients with SPMS in an attempt to identify possible biomarkers or cytokines that can distinguish between subgroups of patients that had rapid vs. slow progression from RRMS to SPMS i.e. fast vs. slow progressors.

As studies have shown that minor changes in lymphocyte subpopulations can serve as useful biomarkers, we determined the surface expression levels of putative biomarkers, TLR2, TLR4 and CCR1, in naïve cells, TCM, TEM, and TEMRA CD4+ T cells. [65]

Additionally, we measured the intracellular expression of cytokines IFN-γ, IL-17A, IL-10 and IL-4 in the various CD4+ T cell subsets, to determine whether fast progressors had higher levels of pro-inflammatory cytokines. 13 patients with SPMS, including 6 fast progressors (mean age 53.9) and 7 slow progressors (mean age 58.3) from the Montreal Neurological Institute MS clinics, and 6 HCs (mean age 56.3) were included in this study. Table 3 below shows the clinical and demographic characteristics of all participants.

Characteristics	HCs (n=6)	SPMS patients (n=13)	Fast progressors (n=6)	Slow progressors (n=7)	P-value
Age, y (SD)	56.3 (9.9)	56.3 (8.8)	53.9 (9.8)	58.3 (7.2)	0.4
Age at MS onset, y (SD)	-	26.4 (8.5)	32.3 (9.7)	22.2 (3.5)	0.04
RRMS duration, y (SD)	-	17.8 (11.1)	6.45 (2.5)	27.5 (4.5)	<0.0001
Total MS duration, y (SD)	-	29.6 (11.0)	20.5 (8.6)	36.1 (7.4)	0.01
SPMS duration, y (SD)	-	8.4 (4.3)	8.75 (3.9)	8.6 (3.9)	0.86
EDSS (median)	-	7	7.25	6.5	0.18
EDSS at SPMS onset (median)	-	6	7	5.5	0.09

Table 3: Clinical and demographic characteristics of patients with SPMS, including fast and slow progressors, and HCs. P values reflect comparisons between fast vs. slow progressors. P value of Age comparison of HCs vs. SPMS patients= 0.99. SD= standard deviation.

CD4+ T cell Biomarker and Intracellular Cytokine Expression in Fast vs. Slow Progressors

Putative Biomarker Expression in CD4+ T cell Subsets

We compared the surface expression of TLR2, TLR4 and CCR1 in fast vs. slow progressors in various T cell subsets at unstimulated (0h) and stimulated timepoints (4h, 24h and 48h). Our results show that there were no differences in surface expression of TLR2, TLR4, or CCR1 in the T cell subsets between fast and slow progressors at any timepoint (Appendix 1).

Intracellular Cytokine Expression in CD4+ T cell Subsets

In the fast vs. slow progressors, we compared the intracellular expression of IFN-γ, IL-17A, IL-10, IL-4 in the various T cell subsets at unstimulated (baseline/0h) and stimulated timepoints (4h,24h, and 48h).

IL-17A expression

In the four T cell subsets (Naïve, TCM, TEM, TEMRA), fast progressors do not show increased numbers of IL-17A producing T cells compared to slow progressors, both at baseline and after stimulation (Appendix 4).

IL-10 expression

Similarly, the proportion of IL-10+ cells does not differ significantly between fast and slow progressors in any of the T cell subsets, at baseline as well as after stimulation (Appendix 4).

IL-4 expression

Our results also indicate that the proportion of IL-4+ cells in T cell subsets does not differ significantly between fast and slow progressors, at baseline and after stimulation (Appendix 4).

Interferon-gamma (IFN-γ) Expression

Naïve CD4+ T cells

In slow progressors, the proportion of IFN-γ^+ cells in naïve cells is low at 0h and 4h, and peaks slightly at 24h, where percentages are significantly different from baseline values (0% vs. 0.4%, P=0.0009) (Figure 3). Interestingly, in this group of patients, the percentages correlate negatively with age (r=-0.61, P=0.028) and RRMS duration (r=-0.86, P=0.02) at 24 hours, but not with total MS duration (r=0.39, P>0.05) (Figure 4).

In the fast progressors, the percentage of IFN-γ^+ cells peaks at 24h and is, significantly higher than at baseline (2.1% vs. 0%, P=0.0009) (Figure 3). In this group, IFN-γ expression does not correlate with age, RRMS duration or total MS duration (Figure 4). In comparison to the slow progressors, fast progressors have significantly higher levels of IFN-γ^+ expressing cells at 4h (0.56 vs. 0.2%, P=0.031) (Figure 3).

TCM CD4+ T cells

The % IFN-γ^+ cells in TCM cells of slow progressors is low at baseline (0h), and increases significantly at 4h and 24h after stimulation (1.8% vs. 0%, P=0.002; 0.6% vs. 0%, P=0.008). (Figure 3) Similar to the findings in naïve cells, the percentages correlate negatively with age at 24h (r=-0.78, P=0.047), but not with RRMS duration or total MS duration (Figure 4).

In fast progressors, the % IFN-γ^+ cells peaks after 4h stimulation, and is significantly higher than baseline values (5.0% vs. 0%, P=0.0009). The % IFN-γ^+ cells remain high at 24h compared to baseline (3.6% vs. 0%, P=0.02) (Figure 3). In this patient group, the proportion of IFN-γ^+ cells does not correlate with age, RRMS duration, or total MS duration (Figure 4). The fast progressors have significantly higher levels of IFN-γ^+ expressing cells than slow progressors at 4h (5.0 % vs. 1.8%, P=0.032) and at 24h after stimulation (3.6% vs 0.6%, P=0.02) (Figure 3).

TEM CD4+ T cells

In slow progressors, the % IFN-γ^+ expressing cells is negligible at baseline (0h), and increases significantly at 4h compared to baseline values (7.93% vs. 0%, P=<0.0001) (Figure 3). Comparably, the percentages correlate with age at 48h (r=-0.79, P=0.048), but not with RRMS duration or total MS duration (Figure 4).

In fast progressors, the percentage of IFN-γ^+ cells dramatically increases at 4h stimulation, at which it is significantly higher than baseline values (21.5% vs. 0%, P=0.0003) (Figure 3). These values do not correlate with age, RRMS duration, or total MS duration (Figure 4). Moreover, at 4h, the % IFN-γ^+ expressing cells is significantly higher in fast progressors than in slow progressors (21.5 % vs. 7.93%, P=0.02) (Figure 3).

TEMRA CD4+ T cells

The proportion of IFN-γ^+ cells in TEMRA cells in slow progressors is negligible at baseline (0h), increases significantly at 4h compared to baseline (5.1% vs. 0%, P=0.001), and then decreases back to near-baseline values at 24h and 48h (Figure 3). Unlike in the other T cell

subsets, the proportion of IFN-γ^+ expressing cells does not correlate with age, RRMS duration or total MS duration.

In fast progressors, the % IFN-γ^+ expressing cells is significantly higher at 4h after stimulation than at baseline (10.1% vs. 0%, P=0.0009) (Figure 3). No correlations between the % IFN-γ^+ cells and age, RRMS duration, or total MS duration were found in the fast progressors. In addition, at 24h, the proportion of IFN-γ^+ cells is significantly higher than that seen in slow progressors (4.51 % vs. 0.7%, P=0.005) (Figure 3).

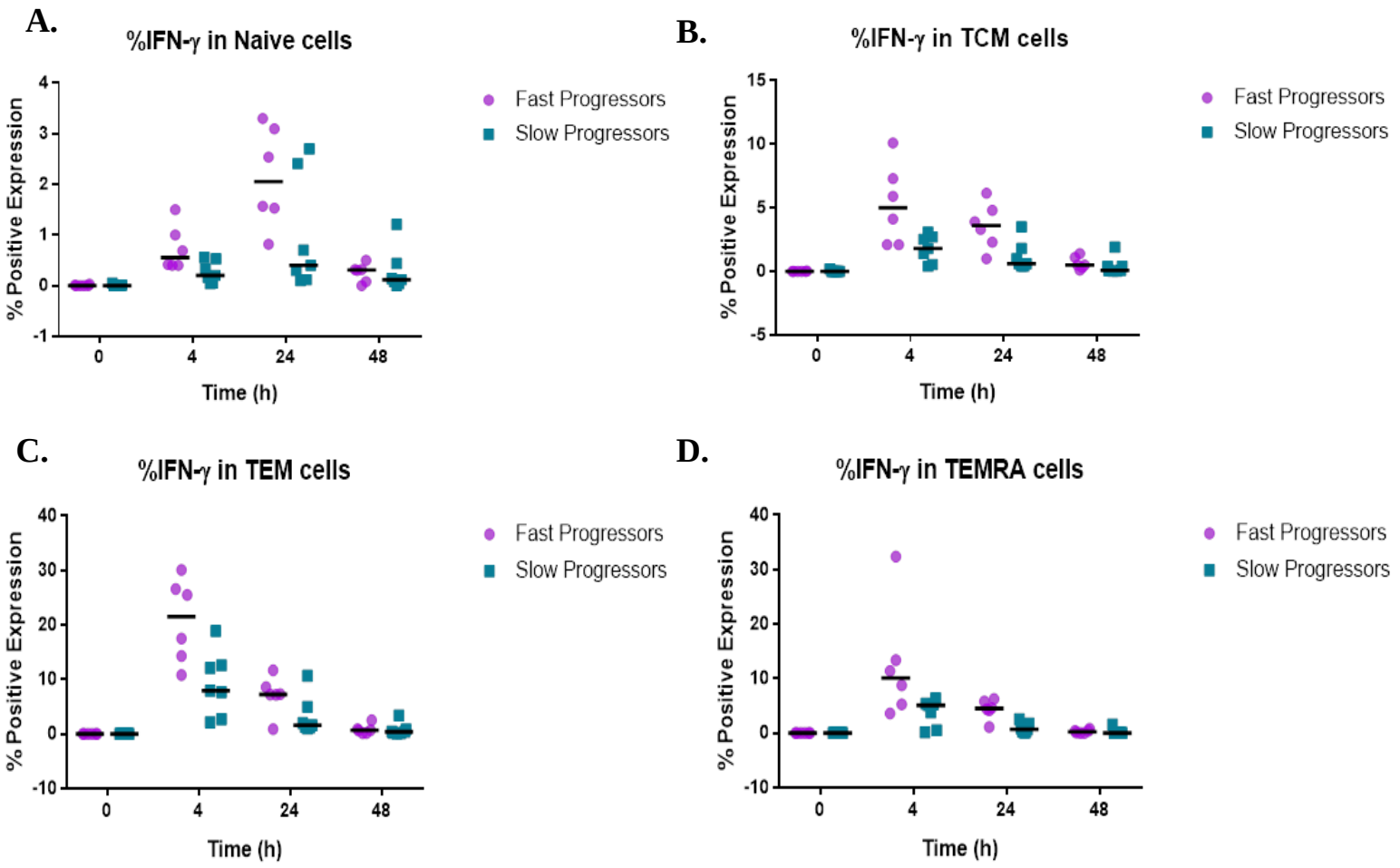

Figure 3: Interferon-gamma (IFN-γ) Expression in CD4+ T cells in Fast versus Slow Progressors. A) Naïve CD4+ T cells: Fast progressors show significantly higher levels of % IFN-γ+ expressing cells than slow Progressors at 4h after stimulation (0.56 vs. 0.2%, P=0.031). **B)** TCM CD4+ T cells: The fast progressors have significantly higher levels of IFN-γ+ expressing cells than slow progressors at 4h (5.0 % vs. 1.8%, P=0.032) and at 24h after stimulation (3.6% vs 0.6%, P=0.02) **C)** TEM CD4+ T cells: At 4h, the % IFN-γ+ expressing cells is significantly higher in fast progressors than in slow progressors (21.5 % vs. 7.93%, P=0.02). **D)** TEMRA CD4+ T cells: At 24h, the proportion of IFN-γ+ cells is significantly higher than that seen in slow progressors (4.51 % vs. 0.7%, P=0.005). Medians shown.

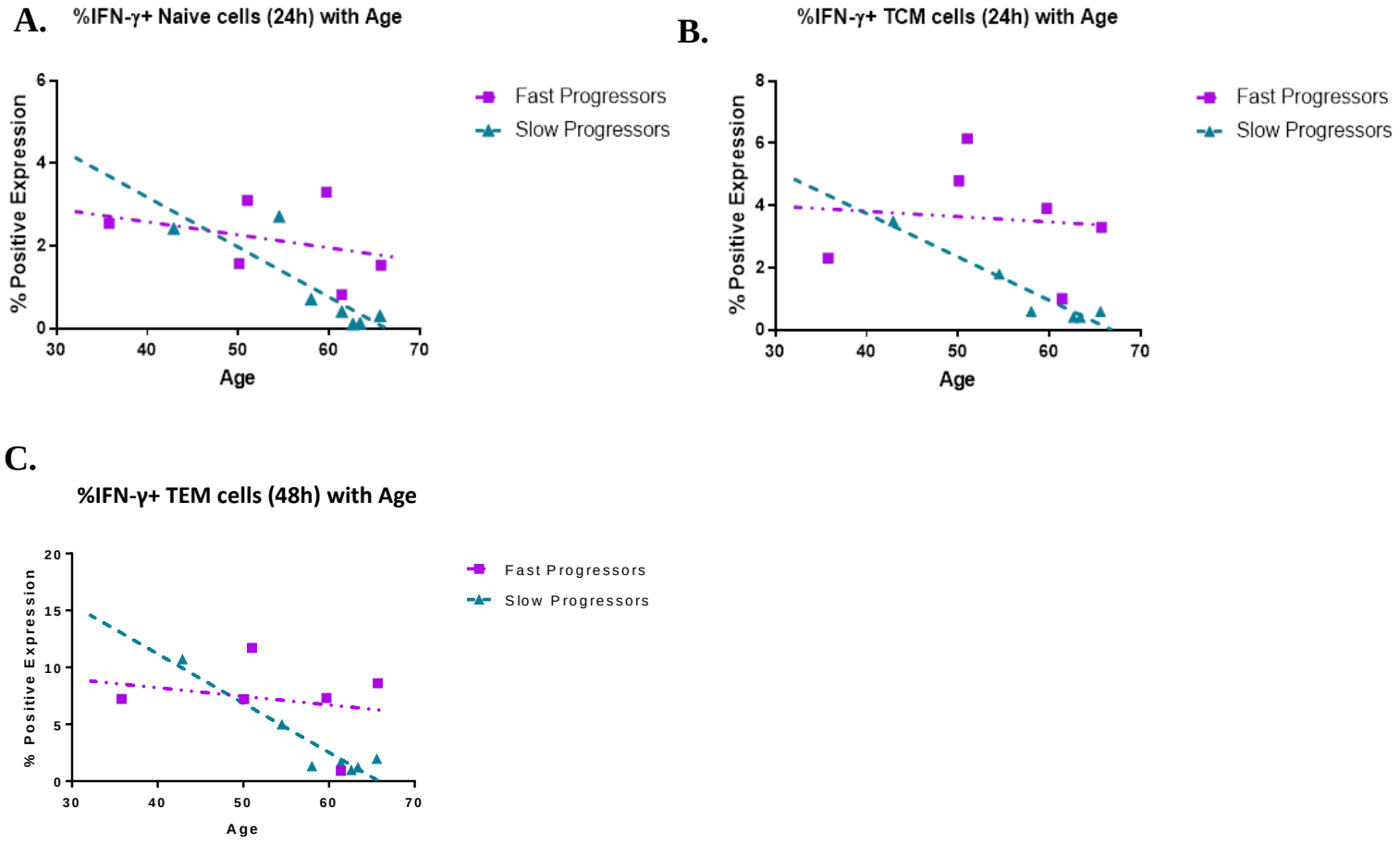

Figure 4: Age-associated correlations in intracellular IFN-γ expression in CD4+ T cells in Fast and Slow Progressors. A) Naïve CD4+ T cells: Slow progressors, but not fast progressors, show a significant negative correlation between IFN-γ expression and age at 24 hours (r=-0.61, P=0.028). **B)** TCM CD4+ T cells: Slow progressors, but not fast progressors, show a significant negative correlation between IFN-γ expression and age at 24 hours (r=-0.78, P=0.047). **C)** TEM CD4+ T cells: Slow progressors, but not fast progressors, show a significant negative correlation between IFN-γ expression and age at 48h (r=-0.79, P=0.048).

Comparison of intracellular IFN-γ expression in patient subgroups to healthy controls (HCs)

We investigated the proportion of IFN-γ^+ cells in healthy controls as means of examining what occurs in a healthy immune system and potentially clarifying our findings in the patient subgroups. In comparison to the two subgroups of patients, the proportion of IFN-γ^+ expressing naïve T cells peaks at 4hs in HCs and is significantly higher than in slow progressors (2.4% vs. 0.2%, P=0.0009), but not fast progressors (Figure 5).

Similarly, the % IFN-γ^+ expressing TCM cells peaks at 4 hours, is significantly higher in HCs than in slow progressors (8.4% vs. 1.8%, P=0.004), but not fast progressors. In TEM cells, the % IFN-γ^+ is significantly higher in HCs than slow progressors at 24 hours stimulation (17.3% vs. 1.6%, P=0.005), but not fast progressors. The proportion of %IFN-γ^+ expressing TEMRA cells is also significantly higher in HCs than slow progressors at 24 hours stimulation (12.7% vs. 0.7%, P=0.0003) (Figure 5).

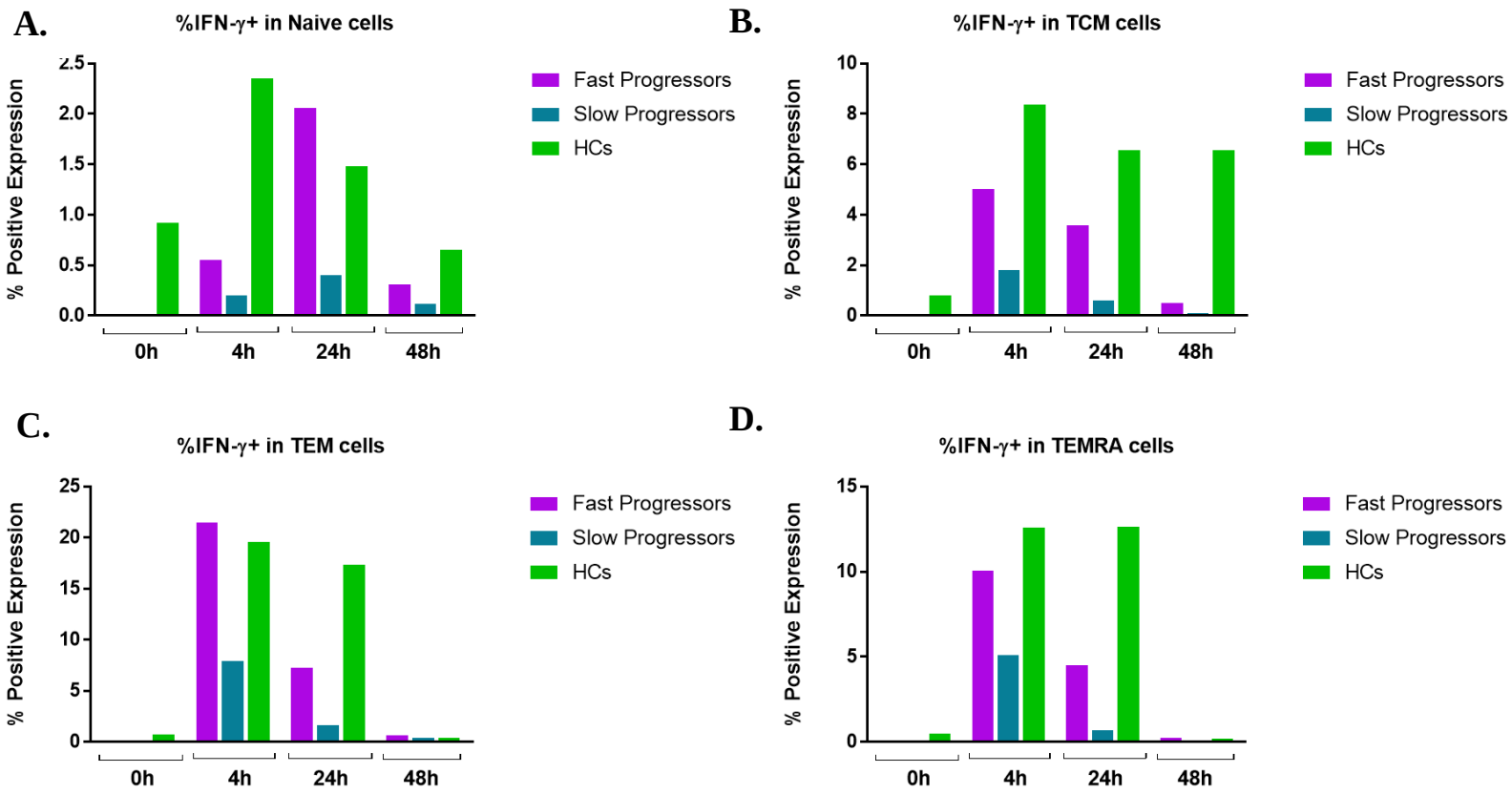

Figure 5: Interferon-gamma (IFN-γ) Expression in CD4+ T cells in patient subgroups compared to healthy controls. **A)** Naïve CD4+ T cells: The % IFN-γ+ expressing peaks at 4hs in HCs and is significantly higher than in slow progressors (2.4% vs. 0.2%, P=0.0009), but not fast progressors. **B)** TCM cells: The % IFN-γ+ expressing cells peaks at 4 hours, and is significantly higher in HCs than in slow progressors (8.4% vs. 1.8%, P=0.004), but not fast progressors. **C)** TEM cells: The % IFN-γ+ is significantly higher in HCs than slow progressors at 24 hours after stimulation (17.3% vs. 1.6%, P=0.005), but not than fast progressors. **D)** TEMRA cells: The % IFN-γ+ expressing cells is significantly higher in HCs than slow progressors at 24 hours after stimulation (12.7% vs. 0.7%, P=0.0003), but not fast progressors. Medians shown.

Intracellular IL-10 and IL-4 Expression in T regs in Fast vs. Slow Progressors

We analyzed intracellular expression of IL-4 and IL-10 in T reg cells, which were defined as CD3+CD4+CD25^{hi}CD127^{lo} in this study. Fast and slow progressors show no significant differences in the % IL-4+ expressing T reg cells at baseline and after stimulation. Similarly, no differences in the % IL-10+ expressing T reg cells were found at baseline and after stimulation (Appendix 2).

Comparison of CD4+ T cell subset proportions in Fast vs. Slow Progressors

We compared the proportions of various T cell subsets in fast vs. slow progressors at baseline and after stimulation. The proportion of Naïve, TCM, TEM, and TEMRA cells in CD3+CD4+ cells did not differ between fast and slow progressors, both at baseline and after stimulation (Appendix 3).

CHAPTER 4: DISCUSSION

Introductory Remarks

Multiple sclerosis (MS) is an inflammatory and neurodegenerative disease of the central nervous system (CNS) characterized by demyelination, perivascular inflammatory infiltrates, astrogliosis and axonal injury in the CNS.[3, 36] While the disease course of MS is unpredictable and its clinical evolution varies, the development of progressive disability is a known critical determinant of long term prognosis in MS. Currently, the favored opinion is that T cells play a role in CNS injury in RRMS, however, their contribution to SPMS and to the development of progressive disability is not clear. [2, 27, 103]

Previous work in our laboratory identified a putative biomarker signature expressed on naïve CD4+ T cells, consisting of TLR2, TLR4 and CCR1, which was associated with T cell activation and rapid conversion from RRMS to SPMS.[102] This thesis focuses on investigating the potential of the aforementioned putative biomarkers, as well as selected cytokines, to differentiate patients with SPMS with rapid vs. slow conversion from RRMS to SPMS i.e. fast and slow progressors.

Biomarker Studies

We compared the surface expression levels of TLR2, TLR4 and CCR1 on naïve, TCM, TEM and TEMRA cells between fast and slow progressors, in both stimulated and unstimulated conditions. Our results show no differences in TLR2, TLR4 or CCR1 surface expression between fast and slow progressors, in any of the T cell subsets. These findings contrast with

previous findings in our laboratory, which documented higher levels of TLR2, TLR4 and CCR1 expression in naïve CD4 T cells of fast progressors, and an increase in the expression of these markers with stimulation. [102]

The different results could potentially be attributed to differences in methods; the previous study in our laboratory isolated naïve cells prior to stimulation, whereas we stimulated peripheral blood mononuclear cells (PBMCs). Hence, the various cell types present in culture in our experiments may have influenced the surface expression of these markers. Further, our stimulant consisted of PMA/Ionomycin, while in the previous study anti-CD3/CD28 monoclonal antibodies were used. [102] The different stimulants may have activated different downstream pathways, with consequent differences in surface expression of various proteins. One study documented varying results in cytokine production depending on the stimuli used, the concentrations employed, and the time of assessment of cytokine production.[109] The findings in this study suggest that differences in stimulation protocols can significantly impact results, potentially offering an explanation of our confounding results.

Cytokine Studies

A widely-held view is that MS is a both a Th1- and Th17-mediated disease. Studies have shown that cytokines IFN-γ and IL-17A are pro-inflammatory in MS, while cytokines IL-10 and IL-4 are immunosuppressive and have a protective role in MS. [68, 72-74] Indeed, some report an increase of IL-17A-producing T cells in progressive MS. Further, studies document the presence of IL-17A and IFN-γ producing T cells in brain lesions in MS, as well as an association with disease activity in MS. [68, 81] In our study, we measured the intracellular expression of

IFN- γ, IL-17A, IL-10 and IL-4 in CD4+ T cell subsets in fast and slow progressors, in order to determine if these cytokines potentially play a role in disease progression and can distinguish between these two patient subgroups. In addition, our aim was to better understand the role of peripheral immune mechanisms in SPMS, which has not been studied extensively in the literature.

Our results show that the proportion of IL-10+, IL-4+ and IL-17A+ cells do not differ significantly between fast and slow progressors in any of the T cell subsets. This is in contrast to what we expected; we originally hypothesized that the proportion of IL-17A+ cells would be higher in fast progressors, and the proportion of IL-10+ and IL-4+ cells would be higher in slow progressors. Studies have reported that patients with RRMS have the highest levels of immune cell infiltration in the CNS compared to patients with progressive MS forms, suggesting lower levels of peripheral immune involvement in progressive MS.[44] Hence, because we are investigating patients with SPMS, we might expect less peripheral immune involvement in these patients, which might explain why we found no differences in IL-10, IL-4 and IL-17A producing T cells between the two subgroups. Another explanation could be our chosen method of stimulation, as studies document varying levels of cytokine detection depending on the stimulant used, the incubation time, and the type of immune cells placed in culture. [109]

In contrast to the above comments, we find that the proportion of IFN-γ^+ cells is significantly higher in fast than slow progressors in each of the CD4+ T cell subsets. As IFN-γ is a pro-inflammatory cytokine, the results strongly suggest a Th1, pro-inflammatory deviation in the peripheral immune system of fast progressors. Moreover, the relatively reduced levels of IFN-γ^+ expressing cells in slow progressors, indicates either an absence or a suppression of a Th1

deviation in this patient subgroup. Importantly, the fact that this difference was found in naïve T cells, a subset that is antigen-inexperienced, points to a possible pre-programming of CD4+ T cells in fast progressors towards a Th1 pro-inflammatory response. [57] Studies report that myelin-reactive T cells in patient with RRMS have a Th1 bias. Hence, it is possible that part of the IFN-γ secreting T cells in fast progressors are myelin-reactive and are directly involved in disease pathology. [76] Notably, T cell subset proportions do not differ between fast and slow progressors, therefore we believe the differences seen in IFN-γ+ T cells accurately reflect the levels of IFN-γ+ T cells in the peripheral blood (Appendix 3).

We speculated whether the elevated levels of IFN-γ+ T cells in fast progressors reflect an inflammatory response that has persisted since the RRMS phase. Studies have shown that treatment with IFN-γ resulted in exacerbations in patients with MS and increased disease activity in the CNS. [110] Moreover, IFN-γ+ secreting T cells are associated with relapses in patients with MS. [68] Therefore, the elevated IFN-γ levels could potentially explain the relatively rapid disease course that the fast progressors experienced since they quickly transitioned from RRMS to SPMS. Interestingly, patients with SPMS and PPMS that progressed rapidly show levels of immune cell infiltration in the CNS that are comparable to what occurs in patients with RRMS. [44] Given that fast and slow progressors differ solely by RRMS duration in their clinical characteristics, it is plausible that the elevated levels of IFN-γ played a role in the rapid conversion from RRMS to SPMS in fast progressors. Moreover, this supports the involvement of peripheral immune mechanisms in the transition from RRMS to SPMS.

Our results also show a negative correlation between age and the proportion of IFN-γ^+ cells in naïve, TCM and TEM subsets in slow progressors but not fast progressors. This age-associated

decline of IFN-γ^+ T cells suggests that in the slow group, there is a progressive movement away from the Th1 inflammatory response with age that is not seen in the fast group. Possibly, non-immune disease mechanisms become increasingly important in the slow group with increasing age, whereas immune mechanisms persist in the fast group. This finding further supports the involvement of the peripheral immune system in disease mechanisms in SPMS, a concept which has often been dismissed given that patients with SPMS benefit little from immunomodulatory therapies. [43] Importantly, our results suggest that fast progressors, a subgroup of patients with SPMS, may potentially benefit from anti-inflammatory therapies. Given the limited treatment options for patients with SPMS, these findings could have important therapeutic implications.

IFN-γ producing T cells in Healthy Controls

We compared our findings in the fast and slow progressors with healthy controls, in order to understand how patient cytokine responses differ from those in healthy immune systems. Our results show higher levels of IFN-γ^+ cells in healthy controls than in fast and slow progressors in nearly all of the CD4+ T cell subsets. This conflicts with what has been found in the literature: studies show elevated levels of IFN-γ^+ cells in patients with MS compared to healthy controls. [111-113] However, as mentioned earlier, the method of stimulation used, the cells examined, and the time of stimulation can drastically affect cytokine findings as has been shown in studies.[109]

Interestingly, while the proportion of IL-10+ cells did not differ in fast vs. slow progressors, this proportion was significantly higher in slow progressors compared to healthy controls and fast progressors in naïve cells and TEMRA cells (Figure 6, Appendix 5). As IL-10 has an anti-

inflammatory role in MS, this indicates a deviation towards a Th2, immunosuppressive response in the slow group. [71] Studies have shown that IL-10 suppresses Th1-mediated proliferation, hence the elevated levels of IL-10+ cells in slow progressors may have suppressed IFN-γ producing T cells. [114] The elevated levels of IL-10 producing T cells in slow progressors could have persisted from the RRMS phase, and may have contributed to the delayed transition to SPMS. Studies have shown that levels of IL-10 secretion are generally reduced in patients with MS. Therefore, it is possible that the higher levels of IL-10 in the slow progressors is partly responsible for their relatively milder disease course. [70] In fact, one of the immunomodulatory treatments for patients with RRMS is glatiramer acetate, and its mechanism of action includes a cytokine shift towards a Th2 immune response. [77]

Interestingly, the participant group that had the highest levels of IFN-γ^+ cells had the lowest levels of IL-10+ cells, and vice versa (Appendix 5). This further supports the notion that IL-10 may have suppressed IFN-γ^+ secreting T cells in slow progressors, providing a potential explanation for the relatively milder disease course compared to fast progressors.

Future Directions

While our results suggest that elevated levels of IFN-γ^+ expressing CD4+ T cells play a role in the transition from RRMS to SPMS, we can not definitively demonstrate that since we studied patients with SPMS after this transition has occurred. Thus, in the future, it would be valuable to examine patients with RRMS that appear to be progressing rapidly versus those that are relatively stable and measure the proportion of IFN-γ^+ expressing CD4+ T cells in each cohort. Then, we could follow up with the patients, some of whom will likely transition to SPMS, and determine if a correlation exists between the level of IFN-γ^+ expressing CD4+ T cells in patients

with RRMS and the time to conversion to SPMS. In addition, as our findings potentially distinguish a subset of patients with SPMS that may still benefit from anti-inflammatory therapies, it would be of great interest to repeat this study in patients with PPMS.

Concluding Remarks

Overall, our findings point to a Th1 immune deviation in fast progressors, and a Th2 immune deviation in slow progressors. Possibly, these differences explain the relatively severe disease course that fast progressors have undergone. Furthermore, we believe our results have identified a subset of patients with SPMS that may still benefit from anti-inflammatory therapies. In Finally, our results support the involvement of peripheral immune mechanisms in disease progression in MS, particularly in the transition from RRMS to SPMS.

APPENDIX 1:

Putative Biomarker Expression in CD4+ T cell Subsets

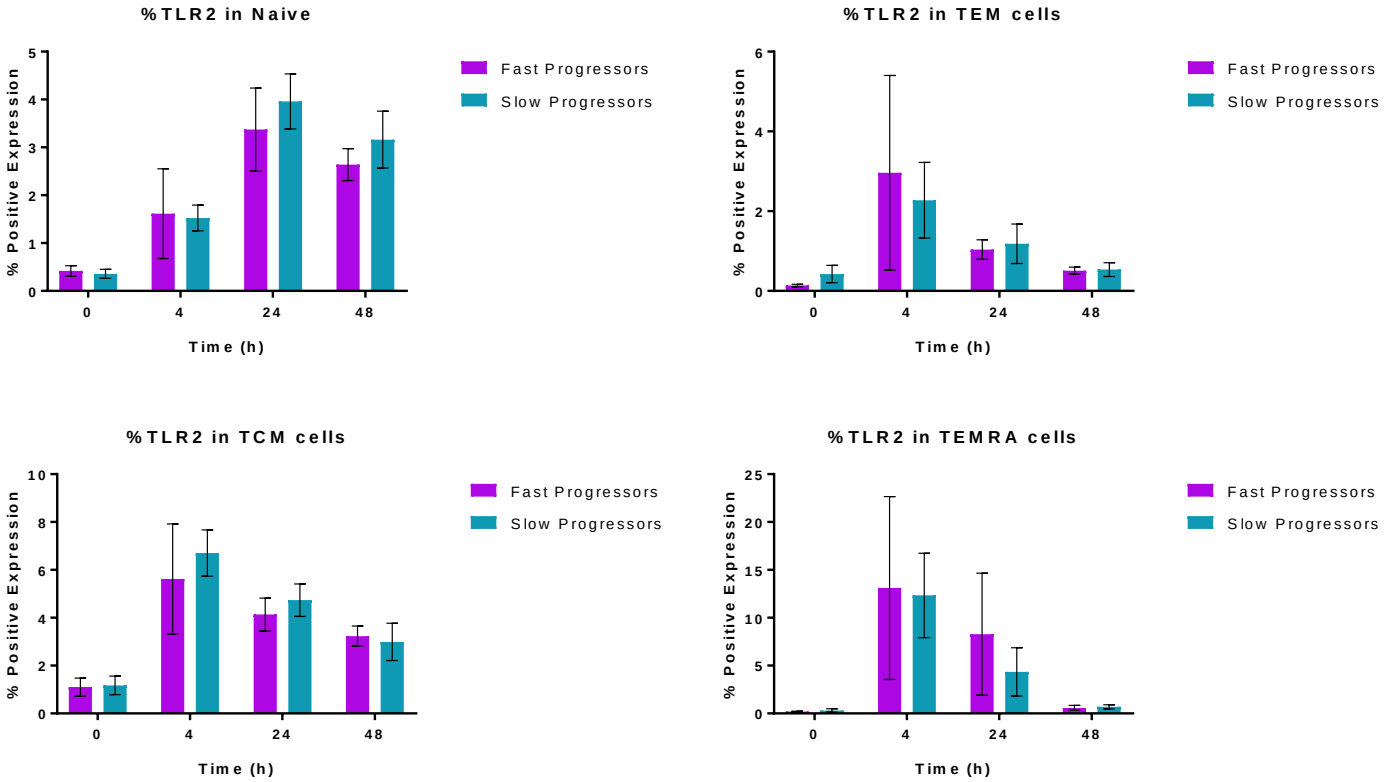

Figure 6: The % TLR2 expression does not differ between fast and slow progressors in CD3+CD4+, Naïve, TCM, TEM, TEMRA cells at any timepoint. Mean with SEM shown.

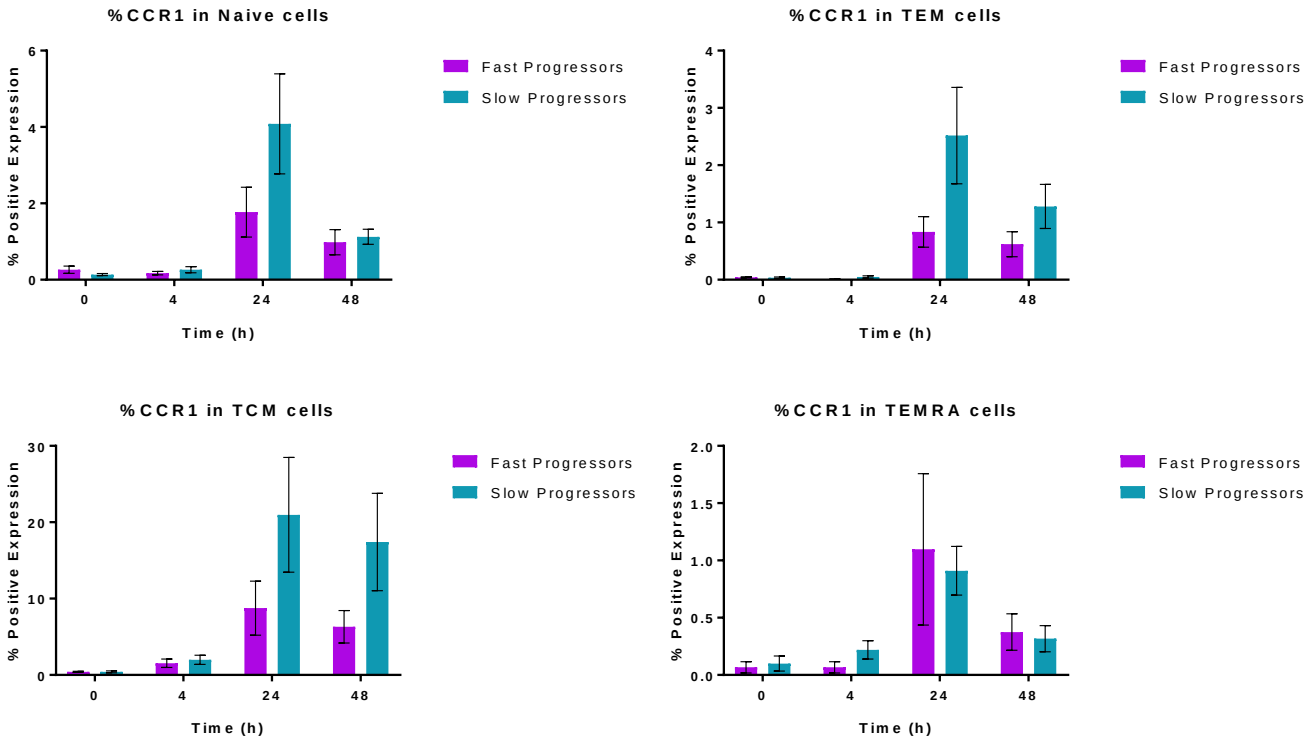

Figure 7: The % CCR1 expression does not differ between fast and slow progressors in CD3+CD4+, Naïve, TCM, TEM, TEMRA cells at any timepoint. Mean with SEM shown.

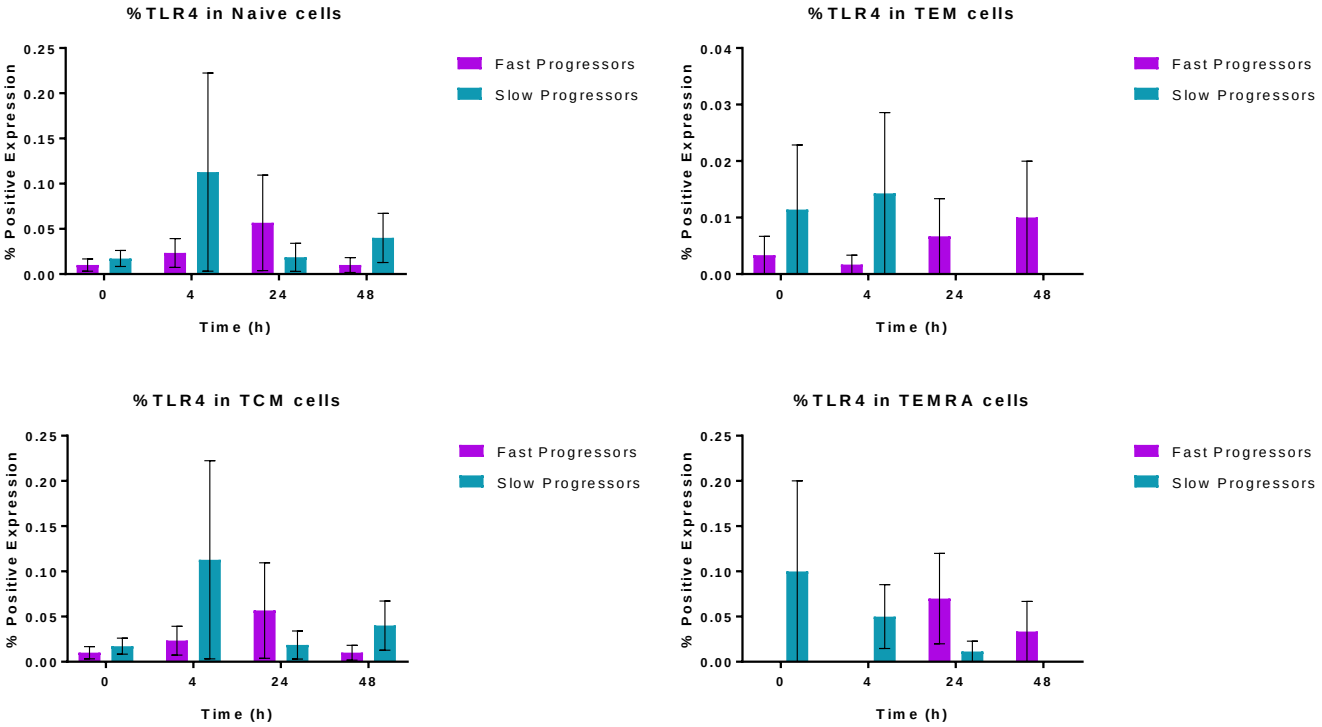

Figure 8: The % TLR4 expression does not differ between fast and slow progressors in CD3+CD4+, Naïve, TCM, TEM, TEMRA cells at any timepoint. Mean with SEM shown.

APPENDIX 2:
IL-4 and IL-10 Intracellular Expression in T regs

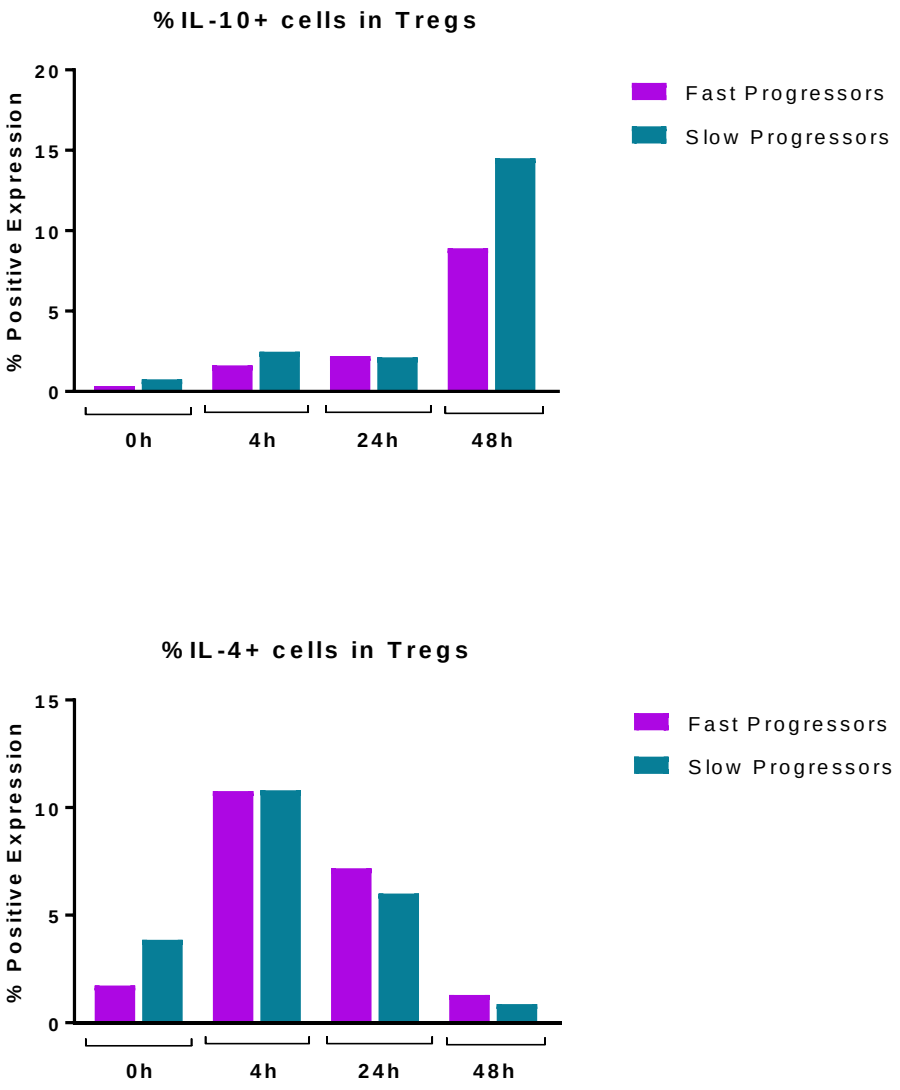

Figure 9: A) % IL-10 expression in T regs does not differ between fast and slow progressors at any timepoint. B) % IL-4 expression in T regs does not differ between fast and slow progressors at any timepoint. Medians shown.

APPENDIX 3:
Comparison of CD4+ T cell Subset Proportions in Fast vs. Slow Progressors

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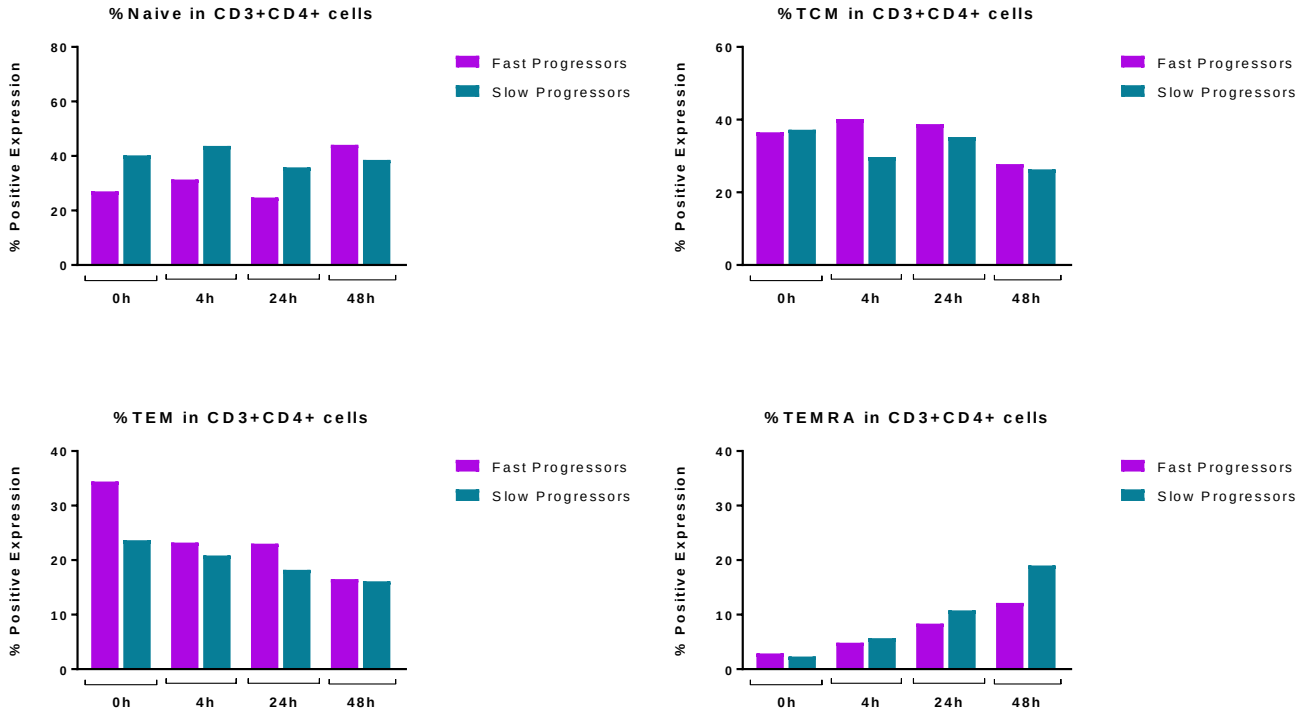

Figure 10: The proportion of Naïve, TCM, TEM, and TEMRA cells in CD3+CD4+ cells does not differ between fast and slow progressors, both at baseline and after stimulation. Medians shown.

APPENDIX 4:
Intracellular IL-4, IL-17A, and IL-10 Expression in CD4+ T cell Subsets in Fast vs. Slow Progressors

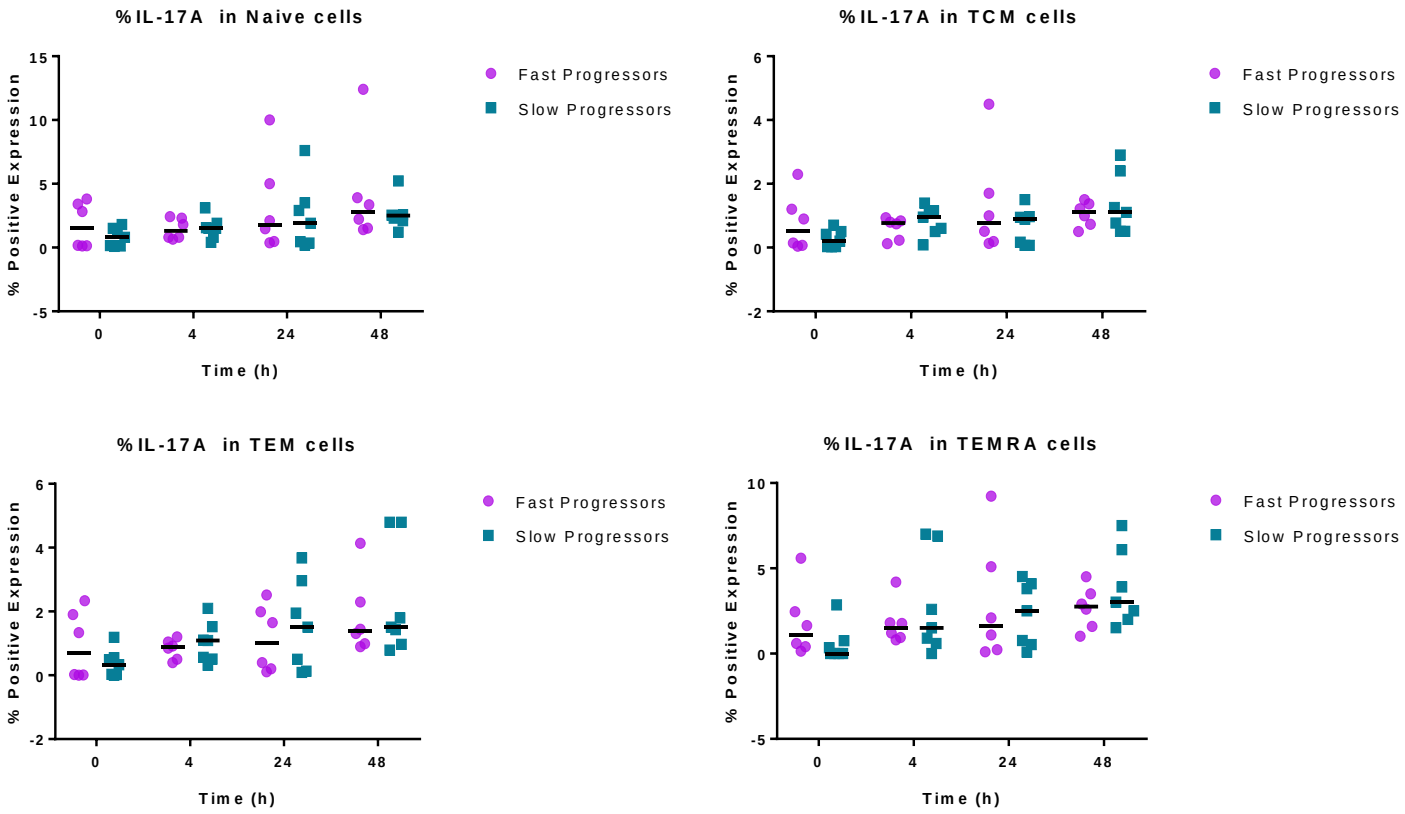

Figure 11: Fast progressors do not show increased numbers of IL-17A+ expressing T-cells compared to slow progressors, both at baseline and after stimulation. Medians shown.

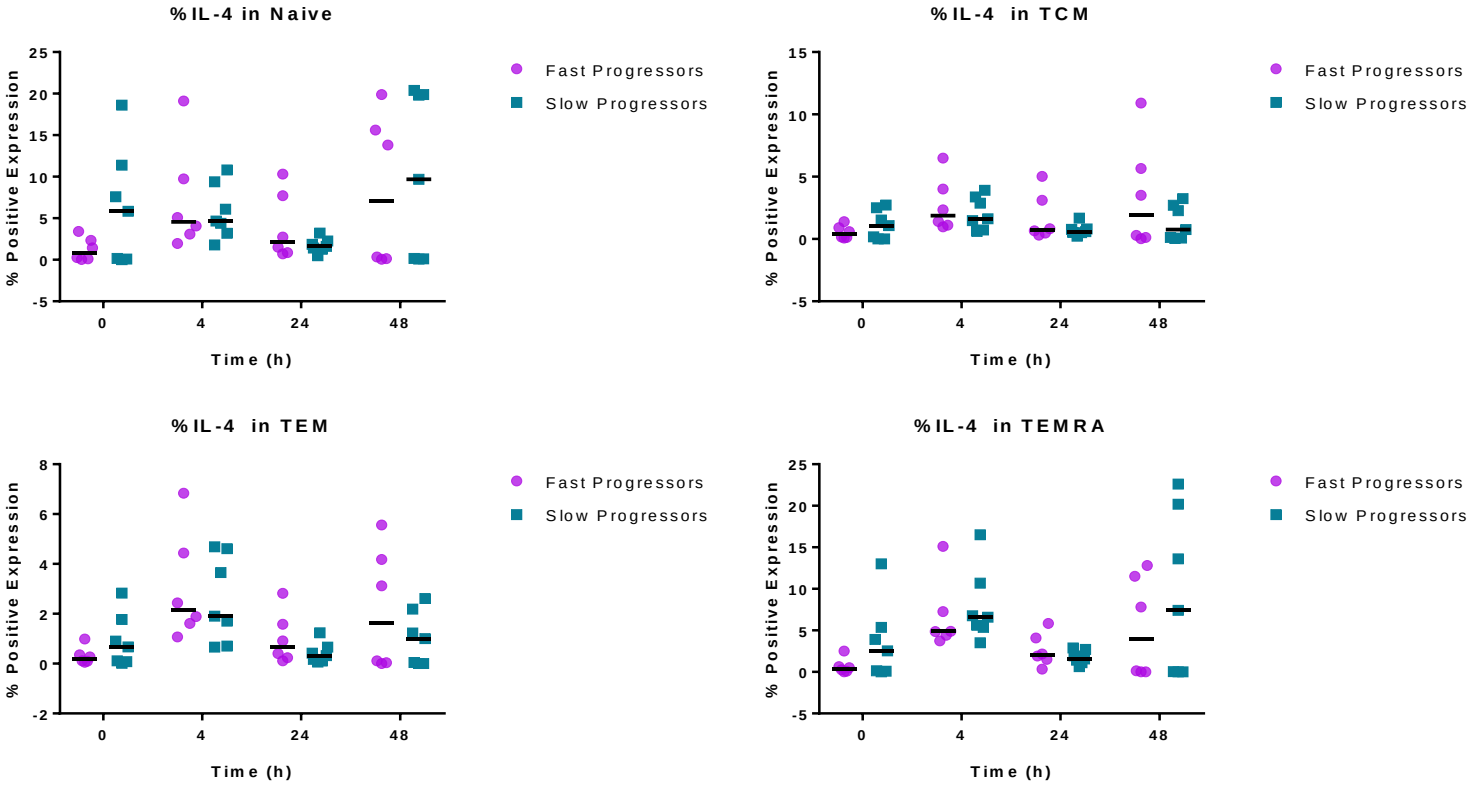

Figure 12: The proportion of IL-4+ expressing cells does not differ between fast and slow progressors in any of the CD4+ T cell subsets above, at any timepoint. Medians shown.

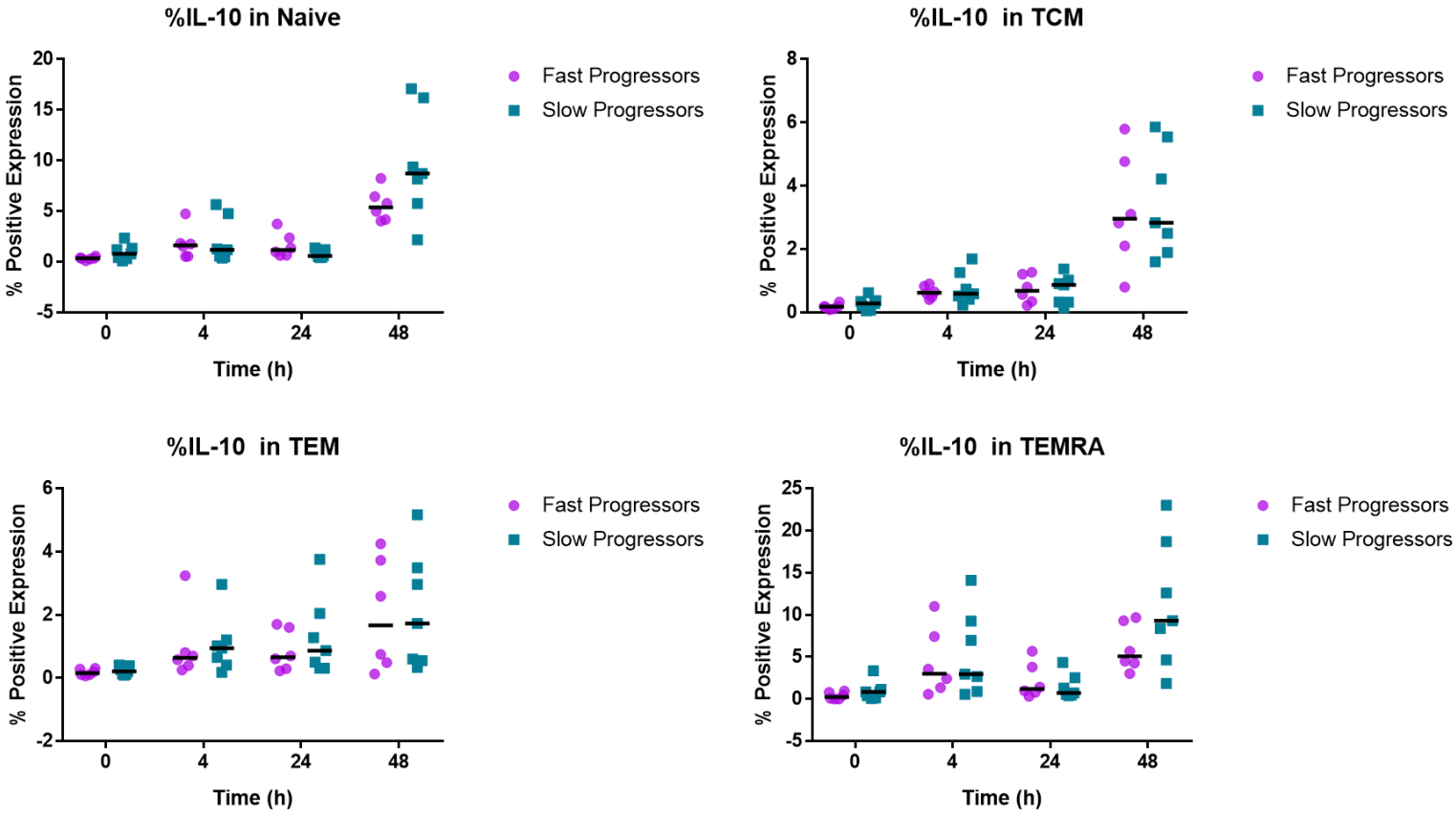

Figure 13: The proportion of IL-10+ expressing cells does not differ between fast and slow progressors in any of the CD4+ T cell subsets above, at any timepoint. Medians shown.

APPENDIX 5:
Intracellular IL-10 Expression in CD4+ T cell Subsets in Fast and Slow Progressors and Healthy Controls

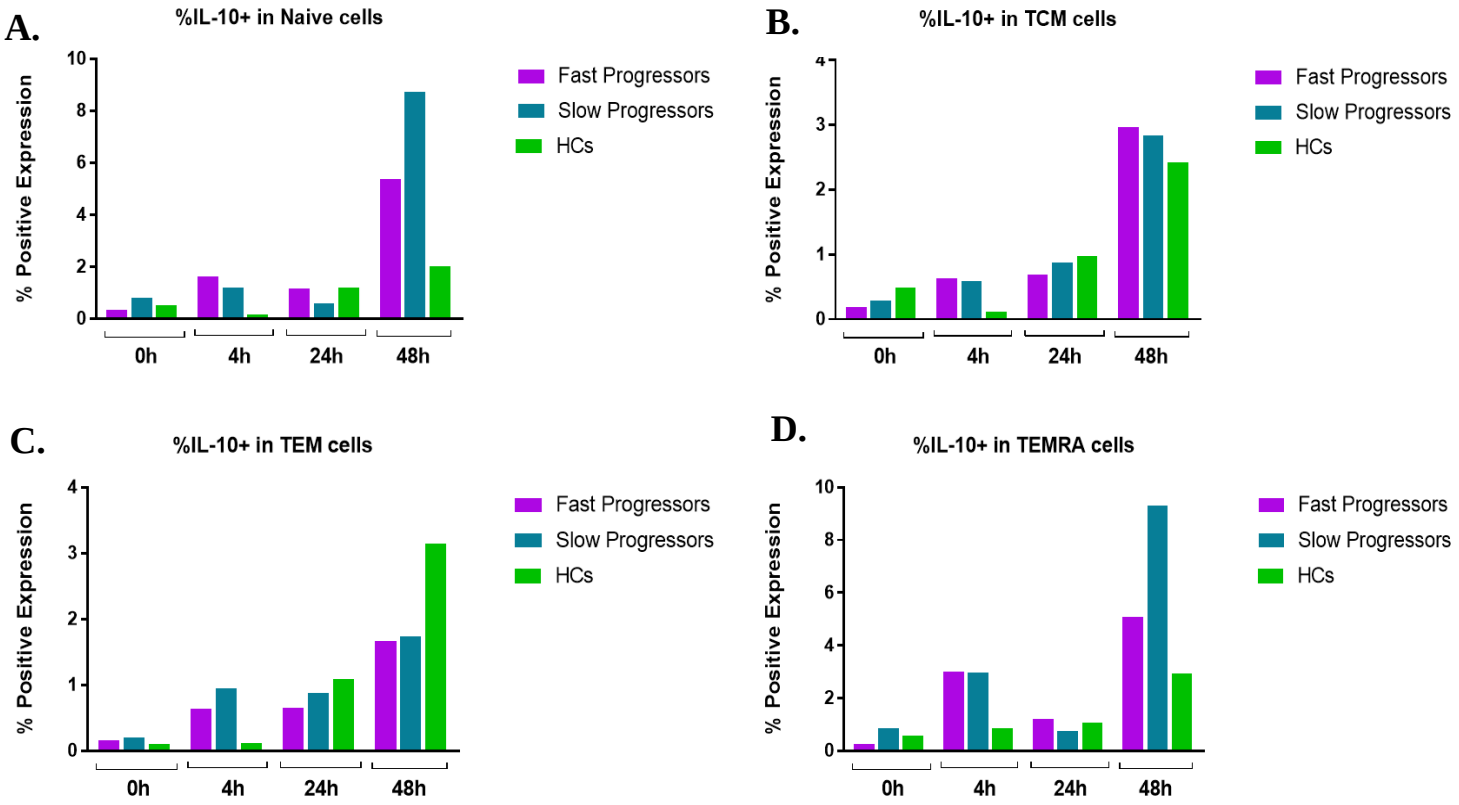

Figure 14: A) Naïve: At 48hrs, the proportion of IL-10+ cells was significantly higher in slow progressors than HCs, but not fast progressors (P=0.004). **B) TCM:** At 48h, the proportion of IL-10+ cells did not differ between any participant group (P>0.05). **C) TEM:** At 48h, the proportion of IL-10+ cells did not differ between any participant group (P>0.05). **D) TEMRA:** At 48hrs, the proportion of IL-10+ cells is significantly higher in slow progressors than in HCs, but not fast progressors (P=0.02). Medians shown.

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