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Disease Specific Dysregulation Of Stat1 And Stat5 Activation In Mature Cd4+ And Cd8+ T Cells Influences Enhanced Kinetic Responses And Inflammation In Patients With Treatment-Naïve Relapsing Remitting Multiple Sclerosis (Rrms)

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DISEASE SPECIFIC DYSREGULATION OF STAT1 AND STAT5 ACTIVATION IN MATURE $CD4^+$ AND $CD8^+$ T CELLS INFLUENCES ENHANCED KINETIC RESPONSES AND INFLAMMATION IN PATIENTS WITH TREATMENT-NAÏVE RELAPSING REMITTING MULTIPLE SCLEROSIS (RRMS)

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CELL, MOLECULAR, AND DEVELOPMENTAL BIOLOGY

ABSTRACT

Autoimmunity is propagated through the lack of effective mechanisms of central and peripheral tolerance. In relapsing remitting multiple sclerosis (RRMS), dysregulated adaptive immune cells have been shown to contribute to disease morbidity through the influence of immune-modulating cytokines in the peripheral blood. Interleukin-2 (IL-2) is a cytokine with several key functions in regulating homeostatic mechanisms within the human immune system. Interferon gamma (IFNγ) is a type II interferon that is needed for an effective response to intracellular bacteria infection through the actions of Th1 cells. IL-2 and IFNγ signaling occurs primarily through signal transducer and activator of transcription (STAT) activation. Genome-wide association studies (GWAS) have determined that single nucleotide polymorphisms in gene loci for IFNγ and IL-2 contribute to disease susceptibility and progression in MS. The role that IL-2/IFNγ play in the concurrent activation of $CD4^+$ and $CD8^+$ T lymphocytes in the context of early treatment naïve individuals with RRMS is equivocal. This study examined the outcome of IL-2 and/or IFNγ induced stimulation and found distinct signaling outcomes, over short stimulation durations, that led to phenotypic changes in subsets of regulatory and non-regulatory $CD4^+$ and $CD8^+$ T lymphocytes. Furthermore, we showed that over the course of short term IL-2 induced STAT5 signaling and IL-2 dependent IFNγ

induced STAT1 activation, the proportion of various effector $CD4^+$ and $CD8^+$ T lymphocytes subsets from patients with RRMS exhibited specific hyperactivation responses. Through unbiased approaches of phenotypic comparison in these cells, we uncovered heterogeneous populations of non regulatory and regulatory lymphocytes in healthy individuals and/or patients with RRMS. Moreover, in this treatment naïve cohort of RRMS patients, we saw that STAT1 or STAT5 activation did not correlate with common measures of disease status in IFNγ dependent STAT1 activation, IL-2 dependent STAT5 activation, or in IL-2 induced IFNγ dependent STAT1 activation. These approaches can better identify STAT dependent immune cell activation and functional outcomes, independent of clinical disease measures that assay disease accumulation. These findings apply to approaches that could potentially identify more effective therapeutic measures that constrain $CD4^+$ and $CD8^+$ T lymphocytes in the context of early treatment naïve individuals with RRMS.

Keywords: Relapsing Remitting Multiple Sclerosis, STAT5 activation, $CD4^+$ T lymphocytes, Multiple Sclerosis

DEDICATION

This work is dedicated to family members, friends, and colleagues that have believed in and wholeheartedly supported, both my goals and aspirations, more than I ever could for myself.

I want to specifically thank my late mother Sharron L.G. Pope and my father Jack Pope for their unrelenting faith in me and love for me throughout my graduate school career and life.

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Lastly, I would like to recognize the individuals who so graciously volunteered to participate in this research that are currently battling the ravages that multiple sclerosis can cause. You all have imparted in me a drive to take action to strive to find a cure for this terrible disease.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte antigen -4
DMT	Disease-modifying therapy
EAE	Experimental autoimmune encephalomyelitis
EDSS	Expanded Disability Status Scale
HC	Healthy controls
IFN	Interferon
IL	Interleukin
JAK	Janus Kinase
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
PPMS	Primary progressive multiple sclerosis
RRMS	Relapsing remitting multiple sclerosis
STAT	Signal transducer and activator of transcription protein
SPMS	Secondary progressive multiple sclerosis

Treg	$CD3^+CD4^+$ $CD25^+$ regulatory T lymphocytes
iTreg	in vitro induced $CD3^+CD4^+$ $CD25^+$ regulatory T lymphocytes
pTreg	peripheral $CD3^+CD4^+$ $CD25^+$ regulatory T lymphocytes
tTreg	thymic derived $CD3^+CD4^+$ $CD25^+$ regulatory T lymphocytes
t-SNE	t distributed stochastic neighbor embbing
US	Unstimulated

INTRODUCTION

Multiple Sclerosis: Presentation, Prevalence, Pathogenesis, Clinical Subtypes, and Therapeutic Strategies

Autoimmunity occurs when one's immune system mounts inappropriate inflammatory responses that lead to self-directed pathological damage and the breakdown of tolerance (Reviewed in (1) and (2)). Multiple sclerosis (MS) is the most common demyelinating disease in adults, where there are estimated as many as 2.5 million individuals worldwide and as many as 750,000 individuals in the US with MS (3-5). In the US, the overall incidence rate of MS was 7.3 per 100,000 as of the year 2000 (6), where the average age of clinical onset is between 20 - 40 years of age. MS is an autoimmune disease where individuals endure sub-acute periods of symptomatology that have been shown to devolve into a chronic progressive constellation of deficits over years to decades (7). The diagnosis of MS is made clinically based upon the presence of magnetic resonance imaging (MRI) that shows demyelinating lesions in the central nervous system (CNS) that are separated by space and time (8). Commonly experienced symptoms that occur in patients with MS are visual disturbances, cognitive impairment, bowel and bladder dysfunction, and gait instability (9). MS symptoms are caused because of inflammatory lesions in neuronal networks that perpetuate pathological damage to the CNS. These pathological insults can impact the proper function of many

different areas of the body due to the interconnected nature of the CNS in the homeostasis of many different organ systems (10, 11).

Many of the lesions in the CNS resolve as acute immune cell infiltration wanes at those locations but lasting deficits in these areas can potentially persist (12). Moreover, these pathological insults in MS, specifically insults in the gray matter, are associated with the accumulation of disability long term (13). The pathogenesis of MS is thought to be the result of the loss of the conductive covering of neurons that helps to transmit neurological impulses throughout axons from one area of the CNS to another connected area of the CNS (14). Myelin damage in the neurons of individuals with MS has also been shown to perpetuate metabolic derangements in affected neurons and surrounding neurological structures potentially leading to Wallerian degeneration (15). Axonal damage, as a result of demyelination that occurs in MS, is correlated with worse scores on measures of clinically defined acute disease status scales (i.e EDSS), axonal transection, and accumulation of intra-axonal cytoskeletal proteins found in the cerebrospinal fluid (CSF) (16-18). Over time, the loss of neurons and associated supportive connections can underlie long-term degenerative losses in CNS volume and can also potentially portend wide-spread neurodegeneration (19).

MS is clinically separated into three subtypes based upon an individual's course of disease-associated symptoms and disease progression profile. Most individuals present initially with relapsing remitting MS (RRMS), where these patients show subacute disease-associated symptoms that normally remit over time. MS symptoms can potentially involve any area of the CNS and it can be

common to see individuals present with deficits in areas of the CNS that are highly myelinated white matter areas (20). Most patients that develop RRMS will eventually go on to develop secondary progressive MS (SPMS). SPMS is marked by extensive unresolved disease burden and morbidity associated with the progressive loss of highly myelinated white matter areas, as well as the loss of function in grey matter areas (21). Lastly, primary progressive MS (PPMS) is a disease subtype where patients show a progressive pattern of disease-associated symptom exacerbation as early as the onset of disease that continues throughout the course of disease. Patients with SPMS and PPMS commonly have disease burden and morbidity that accumulate over time and do not resolve (22). Regardless of the initial disease subtype, many patients with MS can accumulate disease-associated deficits that can lead to ambulatory, visual, and cognitive decline many years after their diagnosis.

In recent years, the wealth of information discovered about MS and its various subtypes has enabled researchers and clinicians to develop novel therapeutic methods that address the symptoms that patients with MS endure. Disease modifying treatments (DMT) in MS have been a mainstay for the chronic treatment of these patients, as these therapies have been shown empirically to reduce the frequency of MS associated relapses in patients. As of 2020, there are 20 or more immunomodulatory drugs that are FDA approved to be used as DMTs in MS (23, 24). These drugs vary markedly in their methods of administration and their purported mechanisms of action but are generally aimed at immunosuppression or immunomodulation of inappropriately activated immune

cell populations (25). Some DMTs have mechanisms of action that primarily target cells in the innate immune system and some target cells primarily in the adaptive immune system. For example, Interferon beta (IFN-β) and analogous compounds are thought to induce antiviral, antiproliferative, and immunomodulatory genes that ultimately lead to a decrease in the activation and influence of antigen-presenting cells (APCs)(26). IFN-β is still used as a first line therapy for many individuals with RRMS. Also, glatiramer acetate is another immunomodulatory DMT that is used in RRMS. It was shown in a mouse model of MS to inhibit demyelination but the exact mechanisms of action are equivocal and broadly +affect many immune cell lineages (27).On the other hand, natalizumab, cladribine, ocrelizumab, and alemtuzumab are all FDA approved DMTs whose mechanisms of action lead to either a 1) decrease in the number of mature activated adaptive lymphocytes (28), 2) sequestration of these cells in secondary lymphoid structures (29), or 3) preventing the ability of these cells to reach the CNS from the periphery by blocking transmigration across the blood brain barrier (30). Natalizumab is an example of a newer DMT that has shown great promise as randomized controlled trials of this drug have shown a 92% reduction in overall gadolinium-enhancing MRI lesions, an 83% reduction new MRI lesions, and a 42% relative reduction in disability progression compared to placebo (31).

The therapeutic benefit of these DMTs to patients is best exhibited in their ability to decrease the frequency of relapses that ultimately result in disease morbidity. IFN-β was shown to decrease the relapse frequency by around 34% across the different formulations available. The relative risk reduction in the

number of relapses was assessed to be decreased by 31% to 68% by some of these newer agents (i.e. natalizumab, fingolimod, ocrelizumab, etc.) when compared to either placebo or older therapies. Although they are more effective at decreasing relapses in patients, many of the DMTs more recently developed have been shown to come at the cost of greater drug contraindications and adverse effects as a whole (32). Moreover, these DMTs have a list of adverse effects that include the possibility of severe infections with opportunistic organisms and an increased risk of malignancy (Reviewed in (25, 32)).

$CD4^+$ and $CD8^+$ T cell subsets and RRMS

Of the many cell types found within the human immune system, the roles that $CD4^+$ and $CD8^+$ T lymphocytes play in eliminating pathogens, based upon specific antigenic features of those pathogens, are paramount. $CD4^+$ and $CD8^+$ T lymphocytes acquire their specific antigen specificities as a result of an elaborate process of selection and V, D, and J segment recombination events that occur early in the thymus of humans (33). These cells respond to a specific antigen in the context of the proper processing of that antigen, major histocompatibility complex (MHC) molecule interaction, cytokine microenvironment present, and coreceptor type on the responding T lymphocyte (34). $CD4^+$ and $CD8^+$ T lymphocytes become inappropriately activated in the context of autoimmunity and contribute to disease pathogenesis (35). $CD4^+$ and $CD8^+$ T lymphocytes have been shown to have the capacity and propensity to perpetuate CNS damage in MS (36, 37). Dysregulation of $CD4^+$ and $CD8^+$ T lymphocytes in autoimmunity happens

because of a lapse in either central and/or peripheral tolerance. Moreover, T cells were discovered in all histopathological subtypes of MS examined from active demyelinating lesions (38). These data lead to the notion that MS is, at least partially, a T cell-mediated autoimmune disease driven by the inappropriate actions of effector and/or regulatory $CD4^+$ and $CD8^+$ T lymphocytes (39).

Non regulatory $CD4^+$ T lymphocytes in RRMS

$CD4^+$T lymphocytes, specifically helper $CD4^+$T lymphocytes, are important for coordinating the immune system's response to various pathogens. $CD4^+$T lymphocytes are essential for the maturation process of B lymphocytes, secrete various cytokines that elicit the function of other immune cells in their immediate environment, and can produce cytokines that promote their own differentiation. Activated $CD4^+$ T helper lymphocytes have also been found in brain tissue, CSF fluid preparation, and peripheral blood of patients that have MS (40). These cells have antigen specificities that are specific to various proteins that are normally found in oligodendrocytes in the CNS. Moreover, activated $CD4^+$T helper lymphocytes, capable of producing IFNγ, were found to be specific for autoantigenic proteins found in the CNS at a higher rate than matched healthy controls (HC) and bind more avidly to these autoantigenic targets (41).

Activated $CD4^+$T helper lymphocytes are cells that have a good degree of plasticity in their ability to differentiate based upon the cytokines and the molecular context of their target tissue. Cytokines activate specific signaling mechanisms found within these activated $CD4^+$T helper lymphocytes that enable master

transcriptional regulators to impose specific patterns of gene expression onto these cells that inform their future function (34). These cells have been shown to produce cytokines and chemokines that have the effect of perpetuating the activation and recruitment of various effector lineages in the CNS. Arguably, the most commonly studied activated CD4 $^{+}$T helper lymphocytes lineages in the context of MS pathogenesis have been the Th1 and Th17 cells that all have functions that are influenced by the chronic inflammatory microenvironment that the MS CNS represents.

Naïve CD4^{+} T lymphocytes, activated by cognate antigen, differentiate into Th1 cells in response primarily to the cytokine IL-12. Th1 cells activate downstream pathways that enable these cells to enhance aspects of cellular immunity through macrophage activation. Th1 cells produce IFNγ in greater amounts than any other immune cell. IFNγ is a critical factor involved in conferring activated macrophages with the ability to induce interleukin -12 (IL-12), nitric oxide, and superoxide compounds as a response to intracellular bacteria (42). IFNγ stimulation in immune cells acts primarily through the actions of the Janus kinase (JAK)/STAT pathway and is regulated by various accessory signal transduction proteins, such as the interferon-regulatory factor (IRF) family of proteins (43). IFNγ production can also be sustained by other soluble factors that play important roles in the context of health and disease. For example, IL-12 drives the expression of T-box expressed in T cells (T-bet) in Th1 cells, which increases IFNγ production. (44, 45). IFNγ can promote class switching in B cells which has been shown to contribute to autoantibody responses in patients with lupus (46). IFNγ can also lead potentially

to inflammation in the context of autoimmunity as a result of the actions of autoreactive Th1 cells in vivo (47). Conversely due to the positive impact of IFN-β as a DMT for MS in individuals with RRMS, there was the notion that IFNγ could be a valuable potential therapy for patients with MS. Moreover, in a mouse model of MS, it was shown that IFNγ acted to protect oligodendrocytes by inducing an ERK-dependent stress pathway (44, 45). In the context of MS, the administration of IFNγ to patients with MS was shown to precipitate more frequent clinical relapses (48). Also, IFNγ production was enhanced in the context of peripheral blood mononuclear cells (PBMCs) isolated from patients with MS and restimulated ex-vivo (49).

Naïve $CD4^+$ T lymphocytes can differentiate into Th17 cells in response to IL-6, where these cells activate downstream pathways and cytokines that best inform the immune system to fight off extracellular pathogens via activating innate cells (i.e. neutrophils). Cytokines produced by Th17 cells were found at increased levels in the CSF of patients with more severe forms of MS (50). Also, the levels of IL-17and IL-8, both of these being cytokines that Th17s can produce, were found to be correlated with spinal cord inflammation and leakage of albumin into the CSF of patients with MS (50). Th17 cells can also produce IFNγ under the proper context and cytokine microenvironment (51). Studies have shown that in vitro Th17 cells in response to IL-12 and IL-4 can be converted into Th1 or Th2 cells. Ex vivo Th17 cells were shown to maintain their ability to produce IL-17. Thus, the magnitude of various cytokines in the microenvironment can influence the function of this subset of helper cells (52). Another group has shown how in certain sites of

chronic inflammation in humans, IL-12 drives the induction of T-bet in Th17 and this process is permissive for IFNγ production in Th17 cells (53, 54). This notion is particularly important in the context of MS that can have a varied array of cytokines/chemokines that are produced from a myriad of cells involved in both the periphery and in the CNS.

Regulatory CD4$^+$ T lymphocytes in RRMS

Regulatory CD4$^+$ T cells (Tregs) are a critical lineage of adaptive immune cells that functionally contribute to effective peripheral tolerance in the context of health and disease. Forkhead box P3 (Foxp3) is the master transcription factor associated with the function, homeostasis, and persistence of mature Tregs (55). As a transcription factor, Foxp3 modulates the function of a vast number of proteins that are involved in the broader transcriptional landscape of regulatory T cells (56). The actions of Foxp3 are important for repressing the expression of effector cytokines that contribute to the plasticity of non-regulatory helper T cell programs in Tregs, amplifying the expression of IL2Rα and other genes necessary for Treg function, and activating factors that are critical for proper chromatin remodeling in these cells (57).

In vivo populations of regulatory T cells form as a result of 1) the process of T cell development in the thymus at the single positive thymocyte stage (tTregs) or 2) as a differentiated population of peripheral CD25$^-$ naïve cells that receive permissive cytokine and chemokine derived activation cues that activate the Foxp3 gene locus in this specific cell lineage (pTregs) (58, 59). There are also in vitro

methods that have been discovered that lead to the generation of “induced” regulatory cells (iTregs) (60). The specific mechanistic distinctions between these three separate populations of Tregs vary and ultimately is subject to further investigation because currently there are no markers that exclusively identify specific populations of Tregs.

The absence of Tregs was shown to lead to an early onset syndrome of clinical symptoms that are characterized by inflammation in multiple organ systems, antibody-mediated cytopenias, and genetic mutations in the Foxp3 locus (61). These cells exert their effects in secondary lymphoid structures and in peripheral tissues at various sites of inflammation, where cytokines specific to certain tissues niches impact their function. There is also the notion that as a result of being resident within specific tissue compartments that Tregs can differentiate to more specialized cells with functions that are uniquely suited to those tissues (62). Little is known about the phenotypic or functional specialization that occurs within Tregs that take up residence in the CNS and how this specifically impacts patients with MS. Recently, a group in the literature found a mechanism where Tregs can indirectly promote myelin regeneration in the CNS through their ability to drive oligodendrocyte development (63). Results similar to these are intriguing as they provide for a role for the protective capacity of Tregs outside of their ability to first directly influence the function of mature immune cells.

Tregs thrive on IL-2, as they are known to constitutively express the high affinity IL-2 receptor. IL-2 is involved in the differentiation of Tregs in the thymus during development, through its actions on promoter elements associated with

Foxp3 (64). Studies in the literature have shown that IL-2R dependent activation of STAT5 in mature Tregs is required for their ability to effectively suppress T cell proliferation (65). Genetic deletion of STAT5 in mature Tregs impairs their function and driving STAT5 function in mice boosts immunosuppression in Tregs (65). Moreover, IL-2 and TGF-b have been shown to potentiate the differentiation of pTregs in vitro (60).

Tregs constrain activated $CD4^+$ and $CD8^+$ T helper lymphocytes in humans through a variety of mechanisms (66-68). Tregs accomplish this ability through contact independent mechanisms such as 1) acting as a sink for extracellular IL-2 produced locally by recently activated cells, 2) producing cytokines that promote immunosuppression directly and inhibit non regulatory T cell effector function, and 3) producing metabolic intermediates that directly inhibit the proliferation of effector cells. IL-2 drives the proliferation of activated $CD4^+$ and $CD8^+$ T lymphocytes in the context of antigen specific cognate receptor activation. High levels of IL-2R levels that are found on mature Tregs helps to decrease this reservoir of IL-2, produced by activated naïve T lymphocytes, that can lead to autocrine effects in the secreting cells and paracrine actions on the surrounding cells (69). Tregs secrete IL-10, which downregulates the expression of costimulatory molecules and chemokines on APCs and inhibits TNFα production in T lymphocytes (70). Moreover, Tregs in IL10-deficient mice failed to prevent autoimmune inflammation in mice (71). Tregs also secrete IL-35 and transforming growth factor beta (TGF-β), where both of these cytokines have the added capacity to be able to further potentiate the suppressive functions of Tregs while suppressing other cell types.

IL-35, is exclusively secreted by Tregs, and contributes to Tregs ability to inhibit the proliferation of effector T cell lineages through potentially inducing cell cycle arrest in these cells, although the exact mechanisms of this biology are not known (72). IL-35 has also been purported to have the capacity to be useful as a diagnostic biomarker in MS since DMT in patients with MS induced beneficial effects that correlated with an increase in IL-35 production (73). As an inducer of Foxp3, TGF-β is a potent inducer of iTreg/pTreg development and differentiation (60, 74). Treg specific TGF-β was shown to constrain $CD8^+$ cytotoxic T lymphocytes and the differentiation of activated $CD4^+$ naïve T lymphocytes to Th1 cells (75, 76). Lastly, Tregs are able to utilize the actions of CD39 and CD73 on their cell surface in order to catalyze the removal of phosphate groups from extracellular AMP/ADP to form adenosine. Adenosine is then taken up by effector T cells through adenosine receptors and shown to inhibit T cell proliferation (77).

In addition to contact independent mechanisms of suppression, Tregs can use cell surface receptors to impair effector immune cell proliferation and response in contact dependent mechanisms. To accomplish these efforts Tregs can 1) induce inhibition directly through the activation of inhibitory receptors located on effector lineages and 2) secrete granzyme and perforin following cell to cell contact. Cytotoxic T-lymphocyte associated protein 4 (CTLA-4) mediated suppression is one contact dependent mechanism that Tregs use to send inhibitory signals to activate effector cells. CTLA-4 has been shown to inhibit $CD4^+$ T cell differentiation and proliferation by binding to cell surface molecules on effector cells and propagating inhibitory signals. These signals inhibit cell cycle progression

and IL-2 production within these cells (78). CTLA-4 is constitutively expressed on Tregs in humans. Polymorphisms in the gene for CTLA-4 can result in differential expression of CTLA-4 and has been associated with an increased risk of MS (79). The exact mechanisms of how indispensable CTLA-4 is for direct Treg suppression is still under investigation but Treg specific CTLA-4 function, in conjunction with IL-10 production by Tregs, was shown to be able to regulate inflammation in a model of colitis (80). Tregs can also utilize the granzyme/perforin pathways to directly suppress various cell types to control immune responses. Through the use of granzyme B upon activation, activated ex vivo Tregs could cause cell death in activated $CD4^+$, $CD8^+$, and $CD14^+$ target cells in a perforin dependent manner (81).

Many different classical proinflammatory cytokines can influence the specific function and phenotype of Treg cells. There is sufficient debate about the overall effect that these proinflammatory cytokines have on Tregs ability to participate effectively in peripheral tolerance as there are instances that show when Tregs are influenced by these cytokines they can either contribute to an effective immune response and disease resolution or perpetuate inflammation directly. Tregs can acquire the ability to produce IFNγ through Tbet dependent mechanisms, in the context of low amounts of IL-2 and *Toxoplasma gondii* infection. These Tregs ultimately contributed to intestinal inflammation and immunopathology in this model (82). Conversely Tregs in the context of skin graph transplantation can acquire the ability to produce IFNγ in response to alloantigen exposure, and this was shown to be important for graft acceptance (83). Ultimately,

the exact cytokines that are present in the context of chronic inflammation play major roles in stimulating or attenuating Treg function in vivo. This is an area that is largely still unresolved and primed to influence the way that Tregs are utilized in clinical applications for therapeutic strategies.

The plasticity of the phenotypic and functional program of Tregs in the context of patients with RRMS has been previously studied. Ex vivo Tregs isolated from patients with RRMS expressed higher amounts of IFNγ than ex vivo Tregs from healthy individuals. These cells were also greater in number in patients with RRMS, compared to controls. Also, IFNγ production from the ex vivo Tregs in RRMS patients was driven by IL-12. Moreover, these cells were found to be less suppressive when cocultured with responder T cells (84). Other studies from patients with early RRMS also show where Tregs contribute to the ongoing inflammation that occurs in MS by adopting an effector-like production of proinflammatory cytokines and subsequent decrease in their functional ability to suppress effector cells (85-90).

$CD8^+$ T lymphocytes in RRMS

Cytotoxic $CD8^+$ T lymphocytes can produce IFNγ, tumor necrosis alpha (TNFα), perforin, granzymes, and upregulate molecules necessary for migration in response to antigen-dependent activation and chemokine cues (91). $CD8^+$ T lymphocytes capable of causing inflammation have been found in brain tissue, CSF, and the peripheral blood of patients with MS (92). Moreover, $CD8^+$ T lymphocytes have been found in higher numbers in these target areas than the

number of $CD4^+$ T lymphocyte populations in some reports (93). Cytotoxic $CD8^+$ T lymphocytes can perform effector functions and MHC class I restricted killing of neurons through such processes as Fas/Fas-L mediated cytolysis and the perpetuation of inflammatory cytokines that can lead to disease-associated inflammation and CNS symptoms in MS patients (94). Brain-infiltrating inflammatory $CD8^+$ T lymphocytes clones have even been shown to have the capacity to persist for years in the blood or CSF of MS patients (92). Also, when compared across specific clinical subtypes of MS, individuals with RRMS and SPMS showed significantly higher numbers of inflammatory $CD8^+$ T lymphocytes than healthy controls and patients with PPMS. These data contribute to the notion that $CD8^+$ T lymphocytes, in particular cytotoxic T lymphocytes (CTLs), are poised to play a pivotal role in the way that CNS derived symptoms and disease-associated morbidity in MS are perpetuated.

JAK/STAT family of cellular receptors and transcription factors in $CD4^+$ and $CD8^+$ T cell subsets in RRMS

JAK/STAT proteins are pivotal signaling cascades that have broad and wide-ranging effects on many biological processes in the context of immunology. At the current time, there are four JAK proteins (JAK1, JAK2, JAK3, TYK2) and seven STAT proteins (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6) that play roles in processes as varied as the actions of colony-stimulating factors, interleukins, and cytokines (95). The JAK/STAT pathway has been shown to impact the cellular function and homeostasis of a wide array of cell types ranging from neutrophils, blood vessels, adaptive immune cell lineages, even including

epithelial cells. The JAK/STAT pathway is evolutionarily conserved throughout mammals and has important implications in the foundation and propagation of autoimmune disease when tolerance is broken (96). The classically defined method through which JAK/STAT proteins send signals from outside the cell to the nucleus occurs initially as a result of cognate ligand binding of specific receptors, phosphorylation, and conformational change of JAKs. This event causes the recruitment and activation of STATs that can form either homogenous or heterologous complexes with other STAT proteins. These activated STAT complexes are then able to translocate into the nucleus and inform gene expression by interacting with specific genetic sequences in promoter/enhancer regions of the DNA encouraging gene transcription. Moreover, activated STATs have even been shown to be involved in several aspects of broader transcriptional control, such as their role in overall chromatin structure and influence on microRNA production (97, 98). The outcome of STAT signaling is the product of the transcriptional events that are governed by STAT binding to the DNA, non-canonical STAT signaling, and the outcome of additional accessory transcription factor binding that happens after activation and not merely dependent on STAT signaling alone (99).

STAT5a/b and STAT1 activation are important in the function of $CD4^+$ and $CD8^+$ T cell subsets in health and disease. IL-2 signals primarily through the actions of STAT5a/b. STAT5 signaling is critical in the development and initial differentiation of many different $CD4^+$ and $CD8^+$ T cell subsets. All lymphocytes express STAT5a/b and many other types of non-lymphocyte populations express

STAT5a/b as well (100). The function of STAT5a/b are broadly similar and have mainly been shown to diverge when there are unequal amount of STAT5a or STAT5b. STAT5 signaling is critical during early T cell selection where these pathways enable the expansion of double-negative thymocytes (101). Later after selection, STAT5a/b are important in potentiating the Th1, Th2, and Treg lineages through the expression of critical signaling receptors (IL-12Rβ2 for Th1 and IL4Rα for Th2) or binding to the Foxp3 promoter sequence (100, 102). STAT5a/b signaling also opposes the proliferation of Th17 and Tfh cells (103, 104) and maintains $CD8^+$ effector T cell responses (105). STAT1 signaling is critical for type I/II interferon signaling, naïve $CD4^+$ T cell differentiation into Th1 cells, $CD8^+$ T cell clonal expansion and memory formation (106-108). STAT1 induced IFNγ induction in Th1 cells is one of the major methods that Th1 cells utilize to coordinate broad gene responses and promote intracellular pathogen defense (109).

Interleukin induced STAT dependent activation in $CD4^+$ and $CD8^+$ T cell subsets in RRMS

Interleukins (ILs), first seen to be expressed in immune cells, are a group of cytokines that have a wide array of functions in the normal homeostasis of many immune cell lineages in the human immune system (110). ILs are best organized by the structural homology of their receptor subunits and receptor function. In broad terms, ILs can be subdivided into cytokine families: the IL-1, common gamma chain, and IL-12 families of cytokines, cytokines of type 2 immune responses, and ILs with chemokine activity (111). The impact that ILs have on disease progression and pathogenesis in the context of MS is broad and cannot

be summarized here in its entirety but within the $CD4^+$ and $CD8^+$ T cell subsets that most influence RRMS, many ILs are dysregulated. For example, enhanced IL-6 signaling was shown to contribute to $CD4^+$ T effector subsets resistance to Treg mediated suppression in patients with active RRMS, as compared to controls in vitro (112). Both IL-17 and IL-22 were shown to disrupt the integrity of the blood-brain barrier in humans with MS, thus facilitating the migration of immune cells into the CNS (113). IL-1β was shown to be expressed in the lesions of MS patients at increased levels (114). Low levels of IL-10 production was associated with higher EDSS scores and a lower T2 lesion burden in patients with established MS (115). IL-12 levels were regulated by IFNβ treatment and were increased in patients with established MS (116).

Rationale of Dissertation Study

Effector lineages of $CD4^+$ and $CD8^+$ T lymphocytes play crucial roles in the development and maintenance of inflammation and damage in the CNS of individuals with established RRMS. The goal of this dissertation is to elucidate the contextual nature of IFNγ and IL-2 signaling in early treatment naïve RRMS patients, with a particular emphasis on how these molecular mediators can influence the subsequent activation and recruitment of downstream STAT pathways in various $CD4^+$ and $CD8^+$ T lymphocytes lineages, as compared to healthy controls. We have also endeavored to examine how IFNγ and IL-2 can influence the nature of the role that $CD4^+$ and $CD8^+$ T lymphocytes lineages play in disease pathogenesis in the context of early untreated RRMS patients. It is

therefore important to elucidate how stimulating various $CD4^+$ and $CD8^+$ T lymphocytes lineages with IFNγ and/or IL-2 and the subsequent response can be utilized to impact patients clinically.

ENHANCED IL-2 INDUCED ACTIVATION OF STAT5 IN $CD4^+$ AND $CD8^+$ T CELL POPULATIONS FROM NEWLY DIAGNOSED RELAPSING REMITTING MULTIPLE SCLEROSIS

by

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ABSTRACT

Polymorphisms within the *IL2RA* gene (CD25) are a risk factor for multiple sclerosis and anti-CD25 (Daclizumab) is currently a treatment for multiple sclerosis (MS). This indicates that IL-2 signals contribute to pathogenesis in MS. The major STAT activated in response to IL-2 is STAT5. We found that the activation of STAT5 in several non-regulatory $CD4^+$ and $CD8^+$ T cell populations was significantly greater in newly confirmed, treatment naïve relapsing remitting MS (RRMS) patients compared to healthy controls (HC). Comparing patients with RRMS to HC, the proportion of IL-2 responding $CD4^+$ and $CD8^+$ T cell populations were similar, however, the levels of IL-2 induced activation of STAT5 was much greater in RRMS patients. From t-SNE and FlowSOM analysis, we identified non-overlapping $CD4^+$ and $CD8^+$ T cell populations between RRMS and HC. Our analysis of $CD4^+$Treg cells using algorithm-based approaches revealed that RRMS patients and HC have distinct populations of Tregs both in phenotype and the ability to be activated by IL-2. However, the absolute levels of IL-2 induced activation of STAT5 found within $CD4^+$ Tregs were similar in RRMS and HC. Overall, this study has identified qualitative and quantitative differences between patients with RRMS and HC in the pattern of IL-2 induced activation of STAT5 found within $CD4^+$ and $CD8^+$ T cell populations that may have implications in the progression of disease.

INTRODUCTION

Multiple sclerosis (MS) is the most prevalent inflammatory disorder of the central nervous system in adults (4). The majority of individuals with MS exhibit periods of symptom exacerbation for days to weeks followed by complete or incomplete disease resolution called relapsing remitting multiple sclerosis (RRMS) (117). Adaptive immune cells play a major role in the immunopathogenesis of RRMS as mediators of inflammation directed towards self-antigens that activate effector lineages to mount inappropriate immune responses in the CNS(36). Moreover, many cytokines that are produced by adaptive immune cell lineages in the context of MS potentiate intracellular responses through the Janus Kinase/Signal Transducer and Activators of Transcription (JAK/STAT) signaling pathway (118). Previous reports in the field illustrate a role for $CD3^+$ T cell inflammatory infiltration in the CNS of newly diagnosed RRMS and clinically isolated patients (CIS) (93, 119-121). Many disease modifying therapies (DMTs) in RRMS are effective in attenuating activated immune cell migration or function (24, 122).

Interleukin-2 (IL-2) is a potent stimulus that potentiates the differentiation and polarization of recently activated $CD4^+$ and $CD8^+$ T cell subsets. It is also critical in the expansion/attenuation of terminally differentiated adaptive immune cells (123). IL-2 is secreted by several immune cells, where CD4, CD8, and NKT cells are the predominant producers in the context of immune cell activation in secondary lymphoid organs (123). IL-2 is also vital for immunoregulation through its action on $CD4^+CD25^+$regulatory T cells (124-127).

Early reports in the literature establish a role for the activation of IL-2 and the upregulation of IL-2 receptor complex in adaptive lymphocytes in the peripheral blood and CSF of patients with established RRMS (128-131). Furthermore, novel and established efforts to modulate the levels of IL-2 or activity of the IL2 receptor complex are being leveraged as potential therapies in RRMS (132, 133). Either the loss of proper regulatory cell function (86) or acquisition of pathogenic effector function in this lineage (134, 135) has also been purported to be a driver of the loss of effective peripheral tolerance in MS.

The IL-2 receptor is canonically found in two forms, the high (trimer of CD25/CD122/CD132) and low (CD122/CD132) affinity receptors, where the high affinity receptor is believed in experimental models to elicit many of the functions of IL-2 in $CD4^+$ and $CD8^+$ T cell lineages (136). IL2RA has been identified as a risk allele in genome wide association studies of patients with MS (137, 138). Genetic polymorphisms in IL2RA from MS patients have been previously shown to induce the expansion of pathogenic T helper cells that produce cytokines that mediate inflammation (139). IL-2 binds to the IL-2 receptor leading to the activation of JAK 1/3 by phosphorylation. The activation of JAKs are permissive for the tyrosine phosphorylation of STAT5 proteins (pSTAT5) that can then dimerize and translocate the nucleus to inform gene transcription (140). Overall, many studies reveal a biology for IL-2 in, both, the pathogenesis and regulation of autoimmunity in MS. However, the underlying mechanisms remain unclear. As a first step, the objective of this study was to determine if IL-2 signaling in specific circulating $CD4^+$ or $CD8^+$ T cell populations are altered in relapsing remitting MS (RRMS) as

compared to healthy controls (HC). The response to IL-2 was measured by assaying for the activation of STAT5 through phosphorylation, which is the first step of IL-2 response. To address this goal, we obtained peripheral blood from newly confirmed, treatment naïve RRMS and healthy controls. The activation of STAT5 (pSTAT-Y694) induced by IL-2 was determined in naïve, memory and effector $CD4^+$ and $CD8^+$ T cell populations and Treg cells by flow cytometry.

MATERIALS AND METHODS

Sample Collection and Patient Characteristics

Blood samples were volunteered by recruited patients with relapsing remitting multiple sclerosis that had been recently diagnosed at the UAB Multiple Sclerosis Center (MSC) to assess options for clinical symptom alleviation and treatment. Multiple sclerosis was verified by board certified, fellowship trained MS neurologists at the UAB MSC or associated specialty clinics. RRMS patients were verified to have fulfilled the most recent MAGNIMS criteria for multiple sclerosis diagnosis through the presence of clinical symptoms that satisfy the criteria of dissemination in space and time (8). All patients who volunteered for the study had not been treated with disease modifying treatments (DMTs) and were recently made aware of their diagnosis of multiple sclerosis. The characteristics of RRMS and healthy controls (HC) recruited for this study are shown in Table 1.

HC were identified from persons that matched the demographics of RRMS cohort and agreed to provide their informed consent. Healthy individuals were devoid of chronic diseases of autoimmunity and acute illness at the time of blood collection

as self-reported in a demographic document as an addendum to the informed consent documents. Routine venipuncture was performed on all samples to acquire peripheral blood mononuclear cells (PBMC) from whole venous blood after the patients and healthy individuals were properly made aware of the associated risks and provided their informed consent.

All samples were assigned double-blinded identification numbers that served to blind both patients and researchers to individualized patient clinical parameters and demographics. All protocols were approved by the Institutional Review Board at the University of Alabama at Birmingham.

Expanded Disability Status Scale (EDSS)

In order to assess each RRMS patients' level of morbidity, disease status, and symptom burden, patients' electronic medical record was mined in the period surrounding enrollment. The Kurtzke Expanded Disability Status Scale was used to compute the patients' deficits concerning each of the functional system scores (FSS). The composition of the functional system scores for each patient and ambulatory ability were used per (141).

T2 lesion count using Magnetic Resonance Imaging (MRI)

MRIs were performed as a part of the patient's normal workup specific to their chief complaint and newly diagnosed status. In order to assess the burden that brain lesions in RRMS patients might potentially have contributed to their disease symptoms, the number of MS lesions observed were documented from study

participants' most recent T2 weighted MRI surrounding their date of enrollment. Only T2 sequences were used to interrogate the number and location of hyperintense brain lesions that were suggestive of MS lesions. The total number of hyperintense brain lesions from all areas were summed as the total number of T2 brain lesions. Lesions found within the spinal cord were not included in this count as spinal cord sequences were not available for each of the patients. The accuracy of the number of T2 lesions observed was independently confirmed post hoc through a recount of a subset of the original T2 weighted images in a blinded fashion. In addition to the criteria enumerated here, consideration was taken to be congruent with the recommendations outlined in Filippi *et al (142).*

Quantitative Real-Time PCR

PBMC were isolated from whole venous blood of RRMS patients via density centrifugation and frozen at -80°C. PBMC were subsequently thawed, allowed to equilibrate to room temperature, stained for surface T lymphocyte phenotypic markers (CD4, CD8, CD45RA, CCR7) and/or markers used for the purpose of exclusion (CD19 and CD14). PBMCs were flow sorted into $CD4^+$ and $CD8^+$ T lymphocyte subsets (naïve, central memory, effector memory, and effector) using a BD FACS Aria directly into Trizol LS reagent. Central memory T cells were $CD45RA^-/CCR7^+$, effector T cells were $CD45RA^+/CCR7^-$, effector memory T cells were $CD45RA^-/CCR7^-$, and naïve T cells were $CD45RA^+/CCR7^+$. 2000 individual nonredundant cells was identified as the collection goal from each subset for each individual. From there, lysed cells were aliquoted directly into TRIZOL LS reagent

and frozen at -80°C. Lysed cells were subsequently thawed, treated with DNAse1 to clear DNA contamination. cDNA was synthesized using Thermofisher's Superscript VILO cDNA synthesis kit and the entire cDNA product was unbiasedly preamplified to enrich for housekeeping controls (18S and Beta Actin) and respective genes of interest (IL2RA, IL2RB, and IL2Ry). Preamplified cDNA transcripts were quantified on Applied Biosystems' Quantstudio 6 Flex Real-Time PCR System using Taqman assays empirically tested to be specific for extronic gene segments of each control and gene of interest. dCT values were collected for each gene of interest and normalized to 18S as a ratio of $dCT^{\text{gene of interest}}$ - $dCT^{18s.}$ Both housekeeping controls were internally examined to establish that they were consistent in the direction of mRNA expression.

Phosphoflow cytometry

PBMC were isolated from whole venous blood of patients and/or controls via density centrifugation and frozen at -80C. Technical bias associated with disease cohort status was controlled for by including samples from each cohort in every experimental run. PBMCs were rapidly thawed and allowed to equilibrate to room temperature over the course of 45 minutes. From there, PBMCs were stimulated with 25ng/ml of human IL-2 as a single pulse or over a time period of up to 24 hours to define an associated functional kinetic response. The concentration of human IL-2 was determined empirically through experiments on PBMC from a cohort of healthy individuals to establish a threshold for stimulation that would enable the quantification of activation of downstream STAT5 activation.

Lyophilized human IL2 (Cat# 2020IL010) was purchased from R+D Systems and was reconstituted appropriately per manufacturer's protocols. hIL-2 was used at $2.1* 10^4$ IU/ug.

Following stimulation, cells were stained for different functional and phenotypic T cell antibodies and assayed on a Thermofischer Scientific Attune NxT acoustic focusing flow cytometer with a 4 laser configuration. A live/dead stain and FSC/SSC discrimination were always included to ensure that only live single cells were analyzed in each experiment. A subset of patients was stained with antibodies specific to IL2 receptor subunit components (CD122 and CD25) and regulatory $CD4^+$ T lymphocytes (CD127 and CD25), respectively, pertinent to the experimental hypothesis in question.

All cells were fixed with methanol in order to expose nuclear epitopes associated with translocated nuclear proteins and stained for antibodies specific to phosphorylated sites on various STAT proteins (STAT1/3/5). STAT3 activation did not respond to hIL2 in patients/ controls and was not found to elicit a molecular pathway important in IL2 signaling and thus was not examined for further significance relevant to the experimental hypothesis. Analysis of the phosphorylation state of STAT1 (pY701), STAT3 (pY705) and STAT5 (pY694) on these cell populations was examined following stimulation. The activation of STATs was calculated as Log_2 fold of MFI obtained from isotype control that was included for each patient sample. This approach allowed for normalization between samples analyzed on different days under similar experimental conditions.

Flow Cytometry Analysis

After staining cells and collecting data, fcs files were analyzed using Flow Jo version 10.6.2. (Treestar). All mononuclear leukocytes were gated on forward scatter area and height. Next, cellular debris was excluded and live single cells were gated for staining markers (CD4, CD8, CCR7, CD45RA) to investigate the function and relevance of $CD4^+$ and $CD8^+$ T lymphocytes subsets across the patient cohorts. Live single cells were also analyzed unbiasedly using t-distributed Stochastic Neighbor Embedding (tSNE) and FLOWSOM plugin software.

tSNE/ FLOWSOM

$CD4^+$ and $CD8^+$T lymphocytes from each experiment analyzed were exported from each signaling experiment based upon the stimulation condition (basal, 15mins, 4hrs), independently. Data collected from exported cells was then concatenated based upon disease cohort and stimulation condition using all compensated parameters. Concatenated cells were exported as .fcs files based upon disease cohort and stimulation conditions. Samples used for these analyses were gated for live, single cells, downsampled to identical cell number across disease cohorts (to control for bias in node size) and used as input files for either FLOWSOM (143) or Barnes-Hut tSNE. CD4 BV421/CD8 BV510, pSTAT5 AF 647, CD45RA BV650, and CCR7 BV711 were used as input parameters to organize the self-organizing map (SOM) and t-SNE plots. The number of meta clusters selected corresponded to the number of input parameters to avoid bias in the number of proportions

selected. A random seed was set at the number 3 for each run of the plugin to provide for reproducibility across various runs.

Statistical Analysis

Statistical analysis was using either Graphpad Prism (GraphPad Software, LLC) or R (The R Foundation). Descriptive, inferential, or summary statistics were used, where appropriate, to assess the validity and context of each experimental design. An alpha level of > .05 was considered significant, except where an adjusted alpha level was determined using Bonferroni's correction. Data was consistently presented using mean +/- SEM.

RESULTS

Circulating $CD4^+$ and $CD8^+$ T cell populations in the peripheral blood of RRMS vs HC

We observed no difference in the proportion of naïve, central memory (CM), effector memory (EM) or effector $CD4^+$ T cells and Treg cells between RRMS and HC (Supplementary Figure 1A). Among CD8 T cell populations, only naïve $CD8^+$ T cells were significantly greater in RRMS than HC (Supplementary Figure 1B). Total CD8 T cells were similar between RRMS and HC (data not shown).

Enhanced IL-2 induced activation of STAT5 in $CD4^+$ and $CD8^+$ T cell populations from RRMS

PBMC from RRMS and HC were unstimulated or stimulated for various time periods and activation of STAT5 (pSTAT5-Y694) was determined in naïve,

memory (CM and EM) and effector $CD4^+$ and $CD8^+$ T cell populations. The activation of STAT5 at all time points in naïve and CM and at 15 and 60 min post IL-2 stimulation in EM and effector $CD4^+$ T cells was significantly greater in RRMS than HC (Figure 1). Within $CD8^+$ T cell populations, enhanced IL-2 induced activation of STAT5 was observed in naïve, CM and effector populations from RRMS compared to HC; in EM cells the activation of STAT5 was similar in RRMS and HC (Figure 1). IL-2 continued to activate STAT5 up to 24 h of stimulation, but by this time point the levels of pSTAT5 in all CD4 and CD8 populations were similar in RRMS and HC (Supplemental Figure 2). To further compare IL-2 induced activation between RRMS and HC CD4 and CD8 T cell populations, we focused on high IL-2 responders represented by the proportion of cells above pSTAT5 levels at basal (Supplementary Figure 3). The proportion of IL-2 responding naïve, memory and effector CD4 and CD8 T cell populations from RRMS and HC were similar (Figure 2A and 2B). However, the level of IL-2 induced activation of STAT5 was significantly greater within the responder T cells from RRMS compared to HC in all CD4 T cell populations; in CD8, significant difference between the cohorts was observed only in naïve and CM T cells (Figure 2C and 2D). The levels of expression of IL2RA (CD25) and IL2RB (CD122) on all CD4 and CD8 T cell populations were not significantly different between RRMS and HC (Supplemental Figure 4 and data not shown). This indicates to us that enhanced IL-2 induced activation observed in several $CD4^+$ and $CD8^+$ T cell populations from peripheral blood of RRMS compared to HC is independent of IL2R levels.

We next analyzed the data to determine to what extent are there differences in IL-2 induced activation of STAT5 between naïve, memory and effector populations of CD4 T cells or CD8 T cells. IL-2 induced activation of STAT5 in CM CD4 was significantly greater than naïve, EM or effector CD4 T cells from RRMS at 15 min and maintained until 240 min of stimulation (Supplemental Figure 4A and 4B). Similar results were observed within $CD8^+$ T cell populations from RRMS (Supplemental Figure 4C and 4D). With HC, the activation of STAT5 by IL-2 was similar in all populations, but this may reflect overall lower IL-2 induced activation of STAT5 (Supplemental Figure 4 A-D). Overall, these results indicate that in newly confirmed treatment naïve RRMS, enhanced response to IL-2 is frequently observed in several non-regulatory $CD4^+$ and $CD8^+$ T cell populations.

Overlapping and distinct populations of CD4 and CD8 T cells in RRMS and HC

Conventional flow cytometry analysis unavoidably requires biased sequential gating based recognized expression of phenotypic markers. The approach does not allow for simultaneous expression analysis of several proteins in multiple individuals. Because of these downfalls, the simultaneous visualization of heterogeneity that defines subpopulations is easily overlooked. To overcome this, we performed t-SNE (see Materials and Methods for details), to visualize simultaneously, the expression of CD45RA, CCR7, and pSTAT5 on $CD4^+$ and $CD8^+$ T cell populations, basal and stimulated with IL-2. At basal, overlapping and distinct clusters of RRMS (red) and HC (blue) $CD4^+$ and $CD8^+$ T cell populations

were seen (Figure 3A and 3B). Such differences were also observed in cells following activation with IL-2 for 15 min or 240 min.

Although the t-SNE analysis is a powerful data visualization tool to identify heterogeneity in populations from multi-dimensional data, the location of the clusters does not offer insight into the degree of relatedness between neighboring clusters. To determine if sufficient overlap between distinct expression patterns of phenotypic markers and the activation of STAT5 exists between RRMS and HC, we employed FlowSOM, which is a neural-network based algorithm. The data reveals distinct patterns between RRMS and HC both in the number of cells that comprise each population of cells and activation profile following stimulation by IL-2 (Figure 4). Overall, these data identify $CD4^+$ and $CD8^+$ T cell populations in RRMS that are distinct, with respect to activation threshold and population dynamics, from that in HC.

IL-2 response does not correlate with disease severity in newly confirmed RRMS

We interrogated if there was any association between IL-2 induced activation of STAT5 in $CD4^+$ and/or $CD8^+$ T cell populations and disease severity. We found no correlation between RRMS disease activity as measured by EDSS or the number of T2 lesions and levels of IL-2 induced activation of STAT5 (Supplementary Figure 5 and data not shown). We also found no correlation between expression of *IL2RA*, *IL2RB*, *IL2RG* (Supplementary Figure 6). Since all the patients are newly confirmed RRMS, these data are not surprising. It is

probable that IL-2 response at this early time point may reflect future disease progression, rather than directly correlate with current disease morbidity.

Distinct populations of IL-2 responding Treg cells between RRMS and HC

It is well established that IL-2 signals are essential for human Treg stability and function (144, 145). The enhanced IL-2 induced activation of STAT5 in non-regulatory $CD4^+$ T cell populations from RRMS led us to question if this also was a phenotype of RRMS Treg cells. Remarkably, we found no difference in IL-2 induced activation of STAT5 in Treg between RRMS and HC (Figure 5A). Similarly, the proportion of IL-2 responders or activation of STAT5 in responders were not different between RRMS and HC (Figure 5B and 5C). We further analyzed data using t-SNE.

Unexpectedly, at basal and following 15 min of stimulation with IL-2, we found areas of distinct $CD4^+$ Treg cells from RRMS and HC that clustered independently into distinct clusters indicating that they represented populations with different phenotypic characteristics (Figure 6). The clusters that showed maximal activation of STAT5 following 15 min IL-2 stimulation in RRMS and HC did not overlap. Following stimulation for 240 min with IL-2, we observed remarkable overlap between RRMS and HC (Figure 6). We further analyzed the data employing FlowSOM. Here again, we observed striking differences between Treg cell populations from RRMS and HC (Figure 7). There is a striking increase in the number of cells that had high representative expression of lineage defining markers CD127 and CD25 in populations of cells from HC as compared to a

contraction of these populations of cells in RRMS. Overall these data have identified phenotypic differences Tregs from RRMS and HC. The functional significance of this finding needs to be elucidated through future study.

DISCUSSION

In this study, we determined IL-2 induced activation of STAT5 is significantly greater in naïve, memory (CM and EM) and effector CD4 T cells in peripheral blood from RRMS as compared to HC. Among CD8 T cell populations, IL-2 induced greater activation of STAT5 in naïve, CM, and effector but not in EM cells. The enhanced activation was an early event, up to four hours in some populations and by 24 h the levels of pSTAT5 were similar in RRMS and HC. It is unclear if this enhanced IL-2 induced activation in non-regulatory CD4 and CD8 T cell populations from RRMS will contribute to disease severity. In this study, we only examined circulating $CD4^+$ and $CD8^+$ T cells from newly-confirmed, treatment naïve RRMS patients. Within this cohort, we found no association between RRMS disease severity and IL-2 induced activation of STAT5 within any CD4 or CD8 T cell population. However, the majority of the patients had only minimal disability (EDSS≤3.0) (141). Polymorphism in the *IL2RA* (CD25) is a risk allele for MS (137, 138). It is also associated with an increased proportion of GM-CSF expression in human Th cells (139). In fact, IL-2 treatment of human Th cell induces expression of GM-CSF through a STAT5 dependent mechanism. GM-CSF is being increasingly recognized as a major cytokine promoting the pathogenesis of MS (146-150). Humanized anti-CD5 (Daclizumab) until recently was an FDA approved

therapy for treatment of RRMS (151). It was recently removed from the market voluntarily due to cases of encephalitis after its use. Overall, these studies indicate that hyper-IL2 activation of CD4 T cells and perhaps CD8 T cells will promote pathogenesis in MS.

For a better understanding of cell populations activated by IL-2, we employed the use of t-SNE and FlowSOM. These data reductionist approaches enabled us to concatenate together all RRMS and controls and analyze them as a composite, un-biasedly. From these clustering approaches, we discovered the existence of distinct populations of cells between RRMS and HC. FlowSOM revealed nodes that show active IL-2 induced STAT5 in RRMS absent in HC. These neural-network based learning approaches have been used to identify unique signatures in cell populations that were previously overlooked in MS (148). A caveat of our study is the number of controls in our study is lower than RRMS and cohorts do not match precisely. However, we believe its impact is minimal because our patient cohort are all newly-confirmed RRMS, with mild disease and not taking any disease modifying therapies.

A major function of IL-2 is promoting stability and enhancing the function of Treg cells (144, 145). We observed that IL-2 activation of STAT5 was similar in RRMS and HC. Surprisingly, t-SNE analysis revealed major non-overlapping Treg clusters between HC and RRMS. A large proportion of cells that responded to IL-2 were also distinct between RRMS and HC. Neural network analysis using FlowSOM revealed near complete reduction of trees and clusters in RRMS compared to HC. Overall, these data indicate that Treg populations in early RRMS

and HC are phenotypically different and functionally to extent of IL-2 activation of STAT5.

This study has revealed that IL-2 may promote pathogenesis in MS by inducing hyperactivation of STAT5 in non-regulatory CD4 and CD8 T cells. Currently, we do not know the mechanism for the enhanced IL-2 induced STAT5 in RRMS T cell populations. The levels of IL-2 receptor CD25 and CD122 were not different between RRMS and HC T cells, thus excluding expression as a mechanism for enhanced activation of STAT5. We propose that it might reflect the functional absence of STAT5 regulation such as a STAT5 phosphatase in RRMS. These questions will be addressed in subsequent studies. Our data also reveal heterogeneity in the IL-2 response within RRMS ranging from normal to enhanced STAT5 activation. This may have significance as a biomarker for disease outcome. We will be able address this question as patients in this cohort are being longitudinally followed by physicians in the associated specialty clinics.

Table 1. **Demographic** Information for MS patients and healthy individuals

	HC (n = 15)	**RRMS (n = 37)**	**p value**
Gender (% female)	60%	75%	
Race/Ethnicity (%AA,%EA)	53%,46%	37%,63%	
Age at Enrollment (years, SD)	50.13 (12.65)	36.36 (8.1)	p < .0001
EDSS (SD)	NA	2.54 (1.04)	
Number of T2 MRI lesions (SD)	NA	8.86 (10.66)	
DMT Use	NA	NA	
Most Recent Corticosteroid Use (months)	NA	6.78	
Disease Duration (SD)	NA	2.67 (1.35)	
Age at First Reported Symptoms of RRMS (years)	NA	32.43 (6.43)	

HC- Healthy Controls; RRMS – Relapsing Remitting Multiple Sclerosis; AA – African American ancestry; EA – European American ancestry; EDSS – Expanded Disability Status Scale; DMT – Disease Modifying Therapy; SD – Standard Deviation; T2 MRI – T2 Weighted Magnetic Resonance Image; Unpaired student t-test with Welch's correction was used to determine statistical significance.

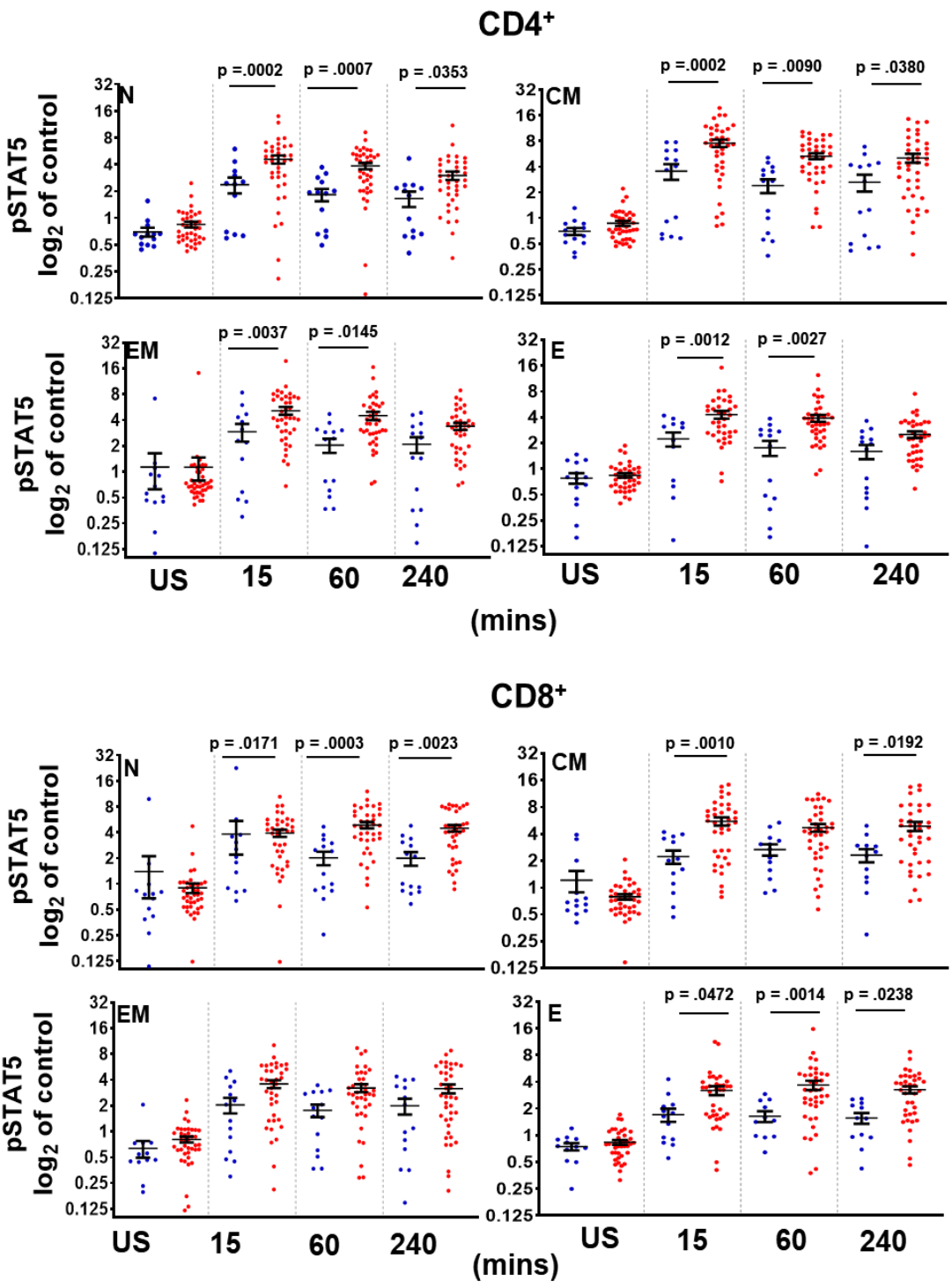

CD4+
N
CM
EM
E
p =.0002
p = .0007
p = .0353
p =.0002
p = .0090
p = .0380
p = .0037
p = .0145
p = .0012
p = .0027
pSTAT5
log2 of control
32
16
8
4
2
1
0.5
0.25
0.125
US
15
60
240
(mins)
CD8+
p = .0171
p = .0003
p = .0023
p = .0010
p = .0192
p = .0472
p = .0014
p = .0238

Figure 1**: Enhanced IL2-induced activation of STAT5 in RRMS CD4 and CD8 T cell populations.** Flow cytometry analysis of pSTAT5 levels in gated subpopulations of $CD4^+$ and $CD8^+$ T lymphocytes from HC (blue) or RRMS (red). PBMC from RRMS or HC were unstimulated (US) or stimulated with IL-2 for various time periods (15 – 240 min) and levels of pSTAT5 (pY694) was determined in naïve (N), central memory (CM), effector memory (EM) and effector (E) populations. The activation of STAT5 was normalized to isotype control for each individual and data represented as Log_2. Each dot represents and individual control or patient. Two-way repeated measures analysis of variance (ANOVA) was performed to assess the effect of disease, stimulation time, and the interaction of these two factors as they relate to pSTAT5 expression. Post hoc Student's t tests were performed using Bonferroni's correction to approximate the appropriate familywise error rate. p values were listed exactly as calculated from aforementioned statistical tests.

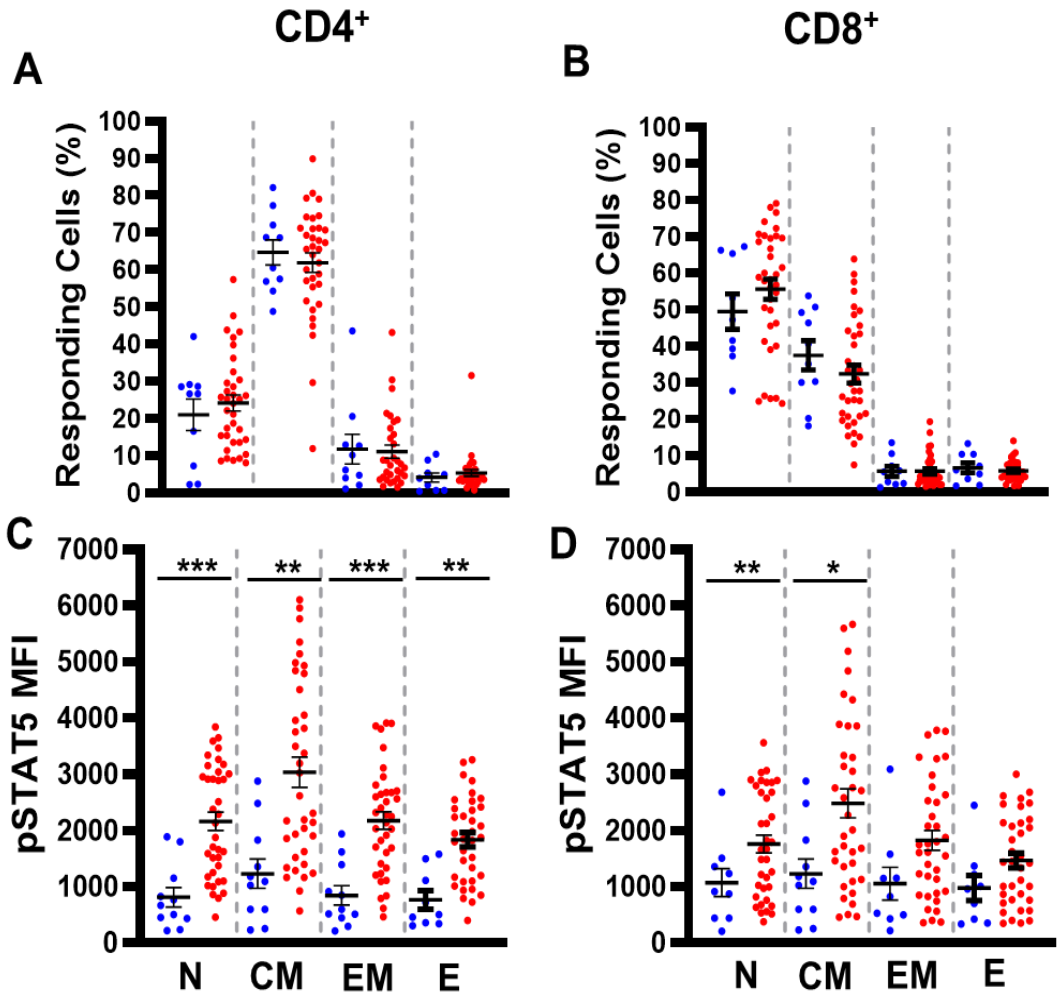


Figure 2**: IL-2 high responder and STAT5 activation in CD4 and CD8 T cell populations from RRMS and HC.** The frequency of high responding CD4$^+$ (*A*) and CD8$^+$ *(B)* T lymphocytes at 15 min post IL-2 stimulation in gated populations from RRMS (red) and HC (blue). The levels of pSTAT5 within high responders from CD4$^+$ (*C*) and CD8$^+$ (*D*) T cell populations from RRMS and HC. Two-way repeated measures analysis of variance (ANOVA) was performed to assess the effect of disease, stimulation time, and the interaction of these two factors as they relate to responder frequency. Post hoc Student's t tests were performed using Bonferroni's correction to approximate the appropriate familywise error rate. p values calculated for subset pSTAT5 MFI are presented as: *= p<.05, ** = p <.01, *** = p<.001.

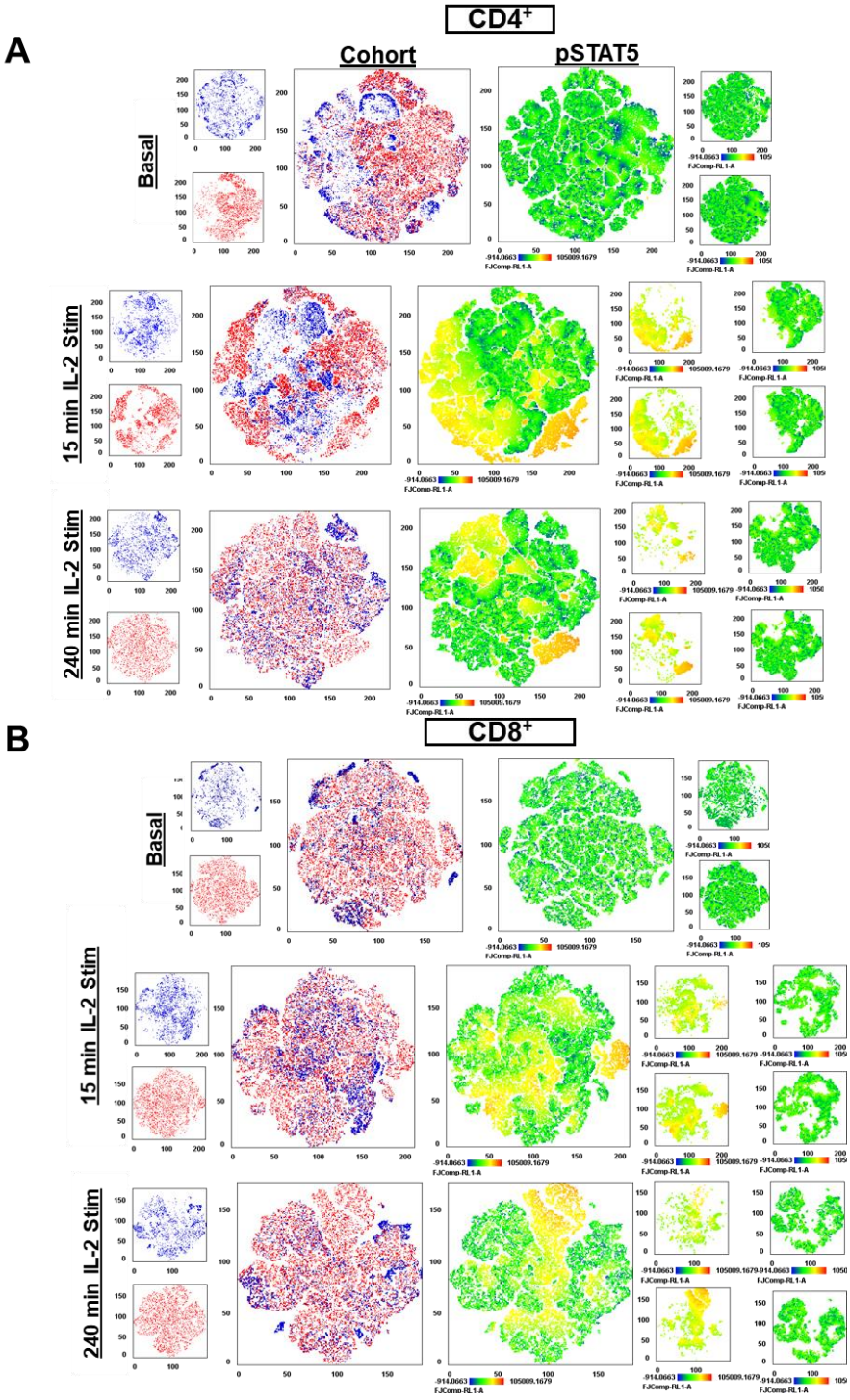

A
CD4+
Cohort
pSTAT5
Basal
15 min IL-2 Stim
240 min IL-2 Stim
B
CD8+
Basal
15 min IL-2 Stim
240 min IL-2 Stim

Figure 3**: Unbiased automated algorithm-based data analysis of $CD4^+$ and $CD8^+$ T cells shows non-overlapping populations RRMS and HC that are activated by IL-2.** Barnes-Hut t distributed stochastic neighborhood embedding (tSNE) was performed on concatenated files of either $CD4^+$ or $CD8^+$ T lymphocytes from individuals with RRMS (red) or healthy individuals (blue) that were either unstimulated or stimulated with IL-2 for 15 minutes or 240 minutes. pSTAT5 expression was superimposed as a mapping overlay over the tSNE plot specific to each stimulation condition. Insets represent pSTAT5 expression for high responding/low responding populations organized by disease cohort. The intensity of pSTAT5 expression is represented on a scale plotting the lowest mean pSTAT5 MFI (dark blue/green) to the highest pSTAT5 MFI (orange) in superimposed plots.

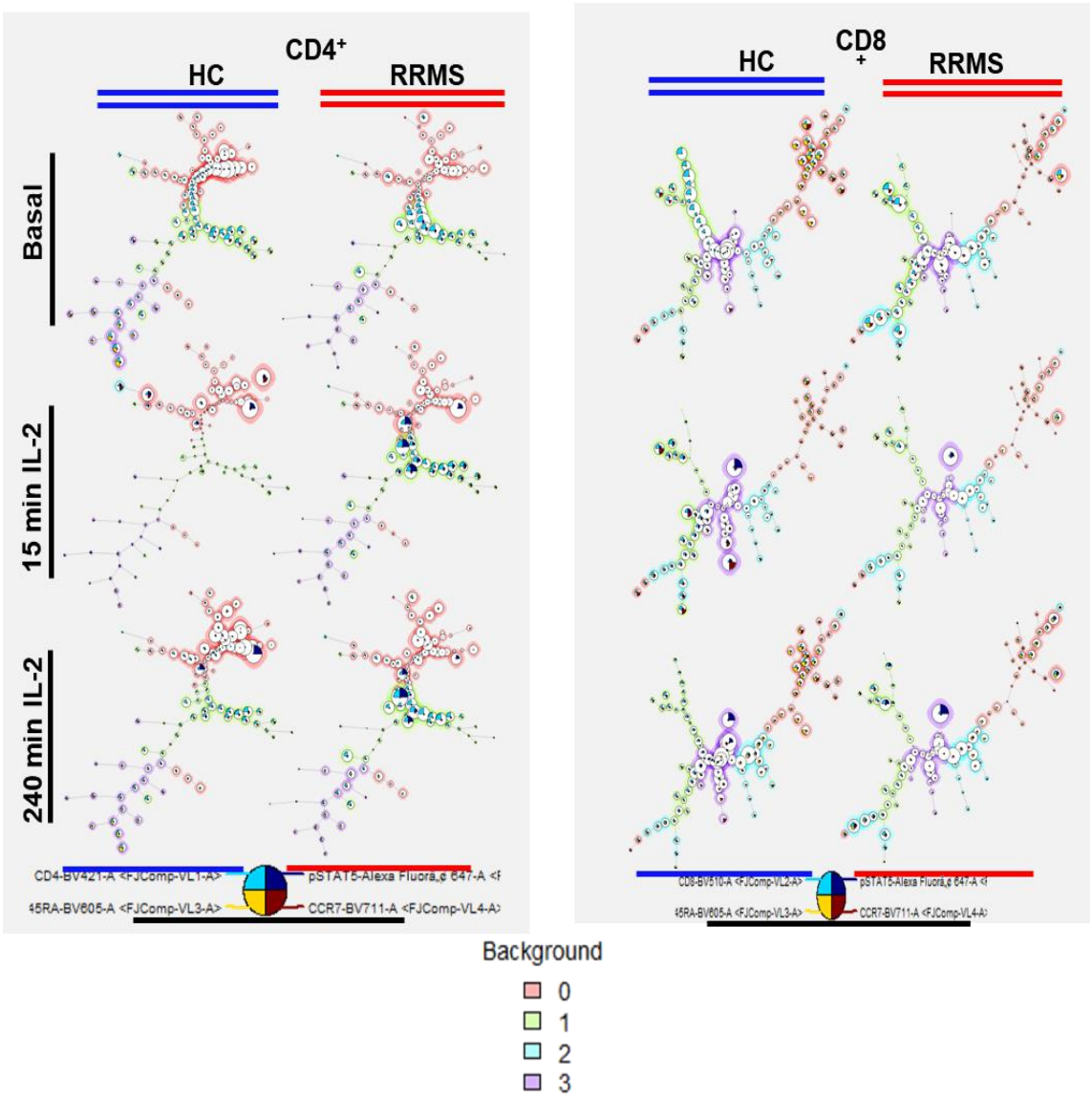


Figure 4**: Automated clustering nodes show difference in cell populations and IL-2 induced activation of STAT5 in RRMS and HC.** FLOWSOM analysis of the expression of CD4 (*left*) and CD8 (*right*) along with CD45RA, CCR7, and pSTAT5 in HC and RRMS at basal and following stimulation with IL-2 for 15 min or 240 min. Minimum spanning tree structure were compared qualitatively to assess the dynamics of fluctuations in the contraction and/or expansion of groups of nodes and marker profile of specific nodes clusters. Phenotypically related clusters of nodes are emphasized by the corresponding background color surrounding each node.

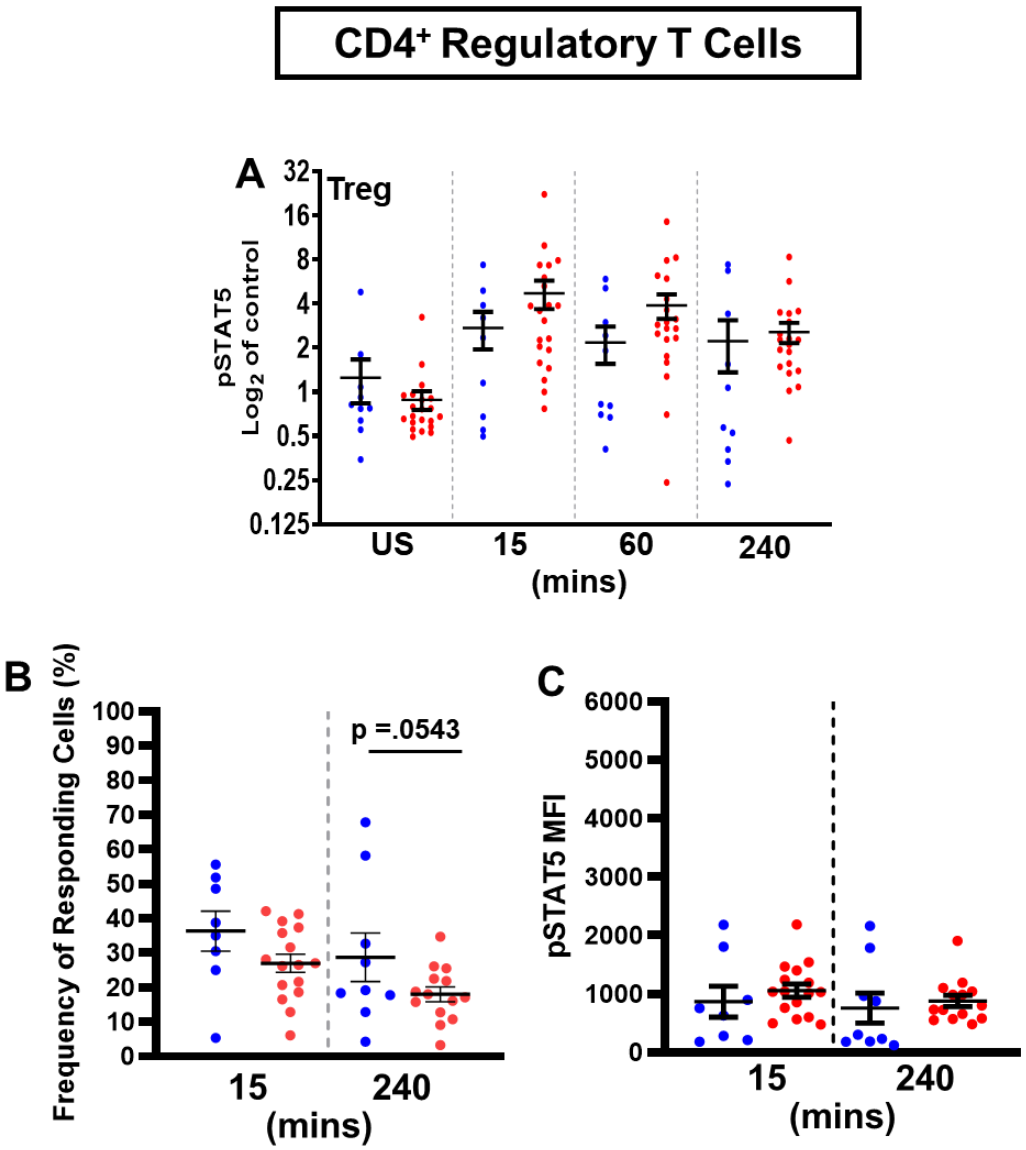


Figure 5**: IL-2 induced activation of STAT5 in Treg cells from RRMS and HC are similar.** A. pSTAT5 levels in Treg cells ($CD25^{hi}$, $CD127^{lo}$) from HC (blue) or RRMS (red) at basal (US) or following stimulation with IL-2 for various time points as indicated. B. Proportion of high IL-2 responders and C. Levels of pSTAT5 in high responders. Two-way repeated measures analysis of variance (ANOVA) was performed to assess the effect of disease, stimulation time, and the interaction of these two factors as they relate to pSTAT5 expression. Post hoc Student's t tests were performed using Bonferroni's correction to approximate the appropriate familywise error rate. p values were listed exactly as calculated from aforementioned statistical tests.

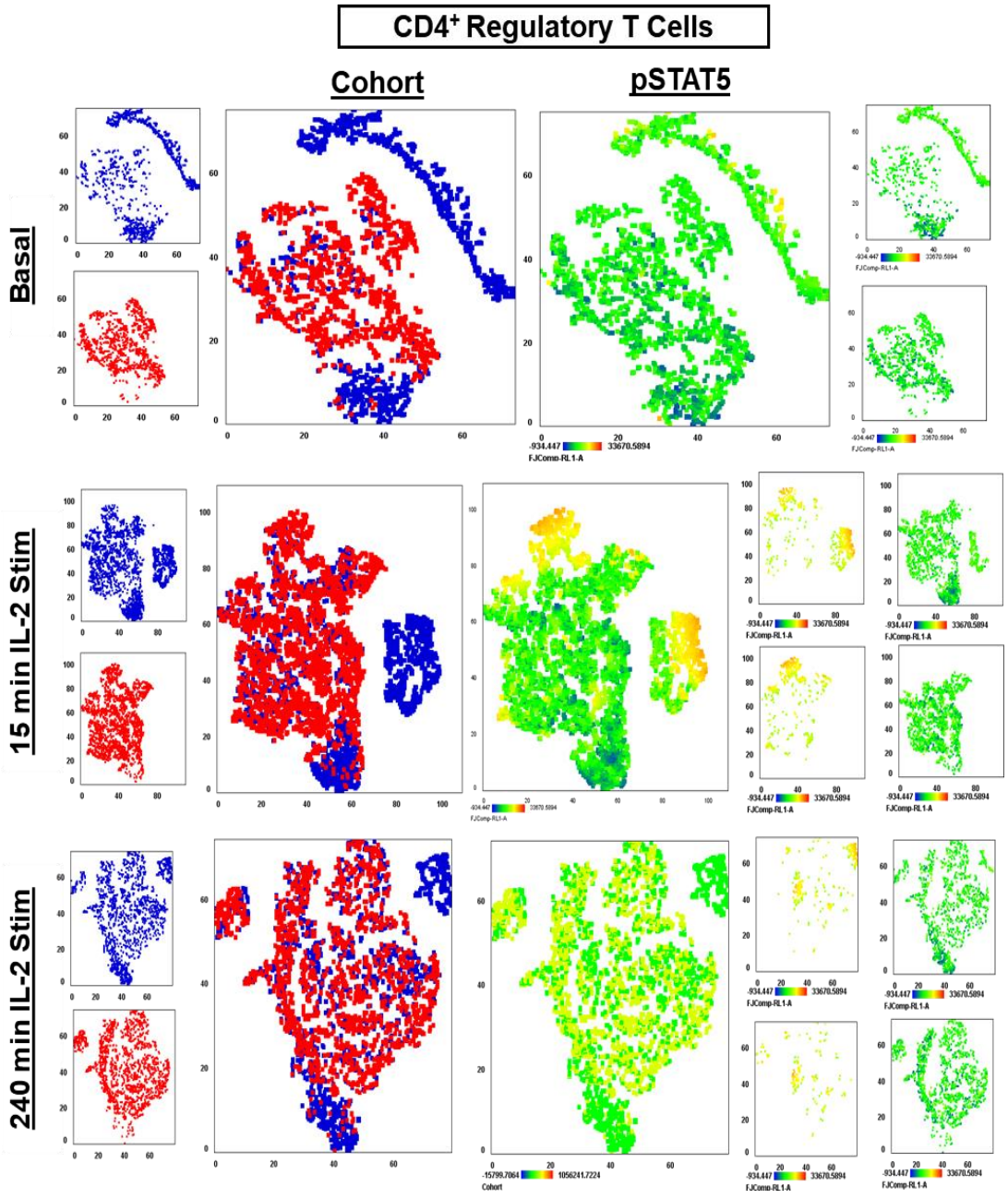

CD4+ Regulatory T Cells
Cohort
pSTAT5
Basal
15 min IL-2 Stim
240 min IL-2 Stim

Figure 6**: Distinct populations of Treg cells between RRMS and HC.** Barnes-Hut t distributed stochastic neighborhood embedding (tSNE) was performed on concatenated files of $CD4^+$ regulatory T lymphocytes from HC (blue) and RRMS (red) at basal or following stimulation with IL-2 for 15 minutes or 240 minutes. pSTAT5 expression was superimposed as a mapping overlay over the tSNE plot specific to each stimulation condition. Insets represent pSTAT5 expression for high responding/low responding populations organized by disease cohort. The intensity of pSTAT5 expression is represented on a scale plotting the lowest mean pSTAT5 MFI (dark blue/green) to the highest pSTAT5 MFI (orange) in superimposed plots.

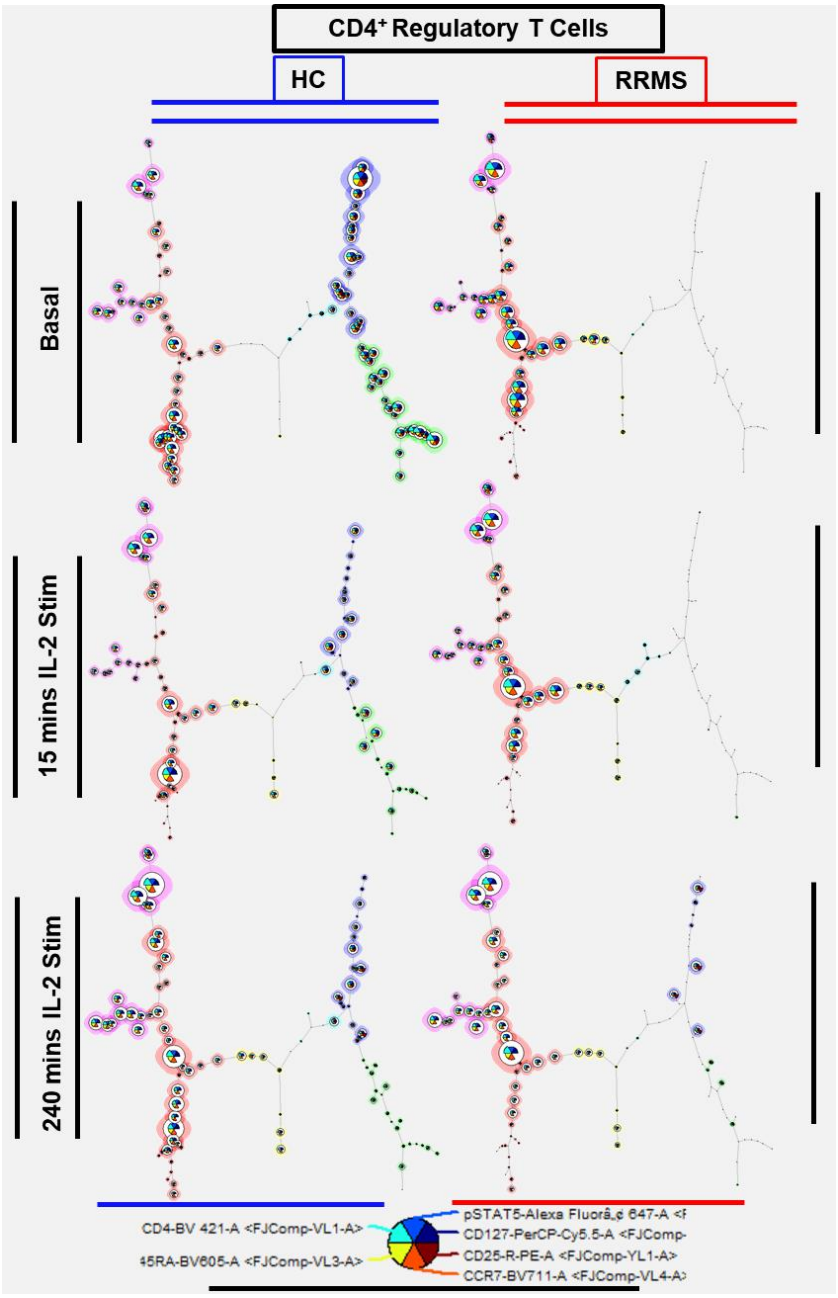
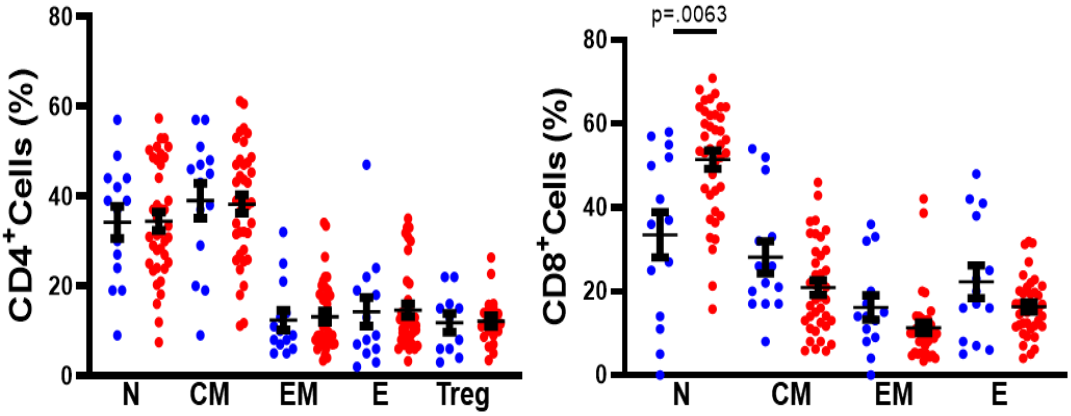
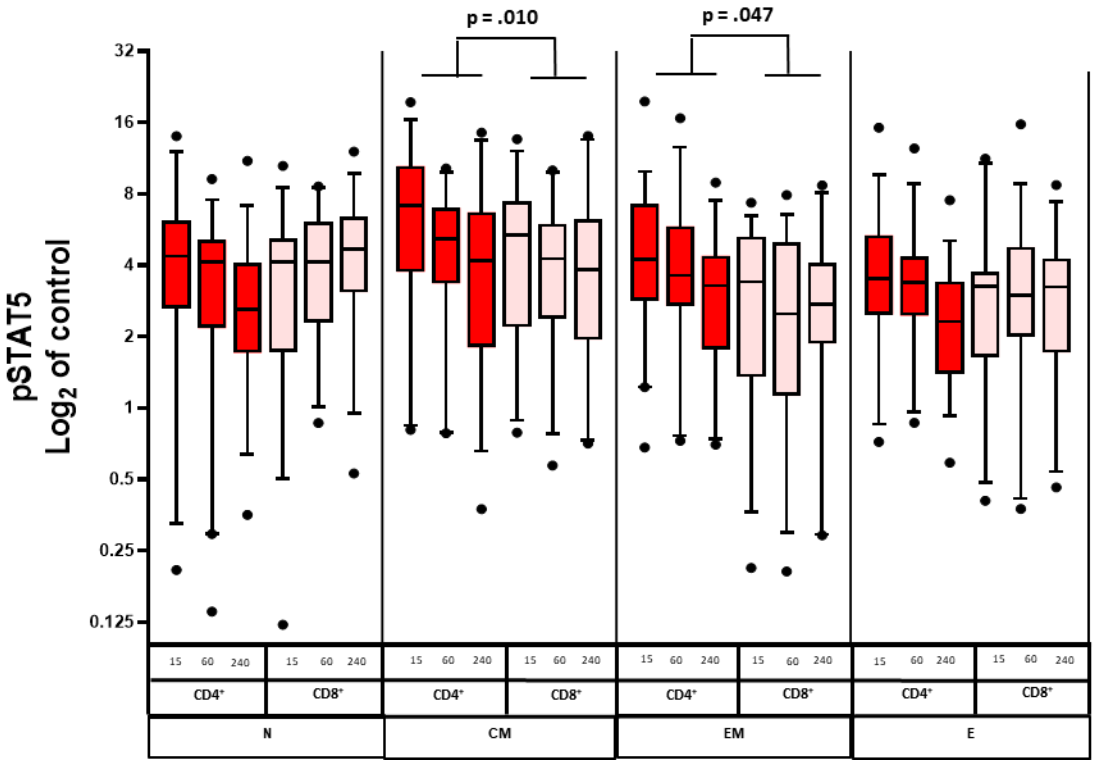

CD4+ Regulatory T Cells
HC
RRMS
Basal
15 mins IL-2 Stim
240 mins IL-2 Stim
CD4-BV 421-A <FJComp-VL1-A>
45RA-BV605-A <FJComp-VL3-A>
pSTAT5-Alexa Fluor® 647-A <F
CD127-PerCP-Cy5.5-A <FJComp-
CD25-R-PE-A <FJComp-YL1-A>
CCR7-BV711-A <FJComp-VL4-A>

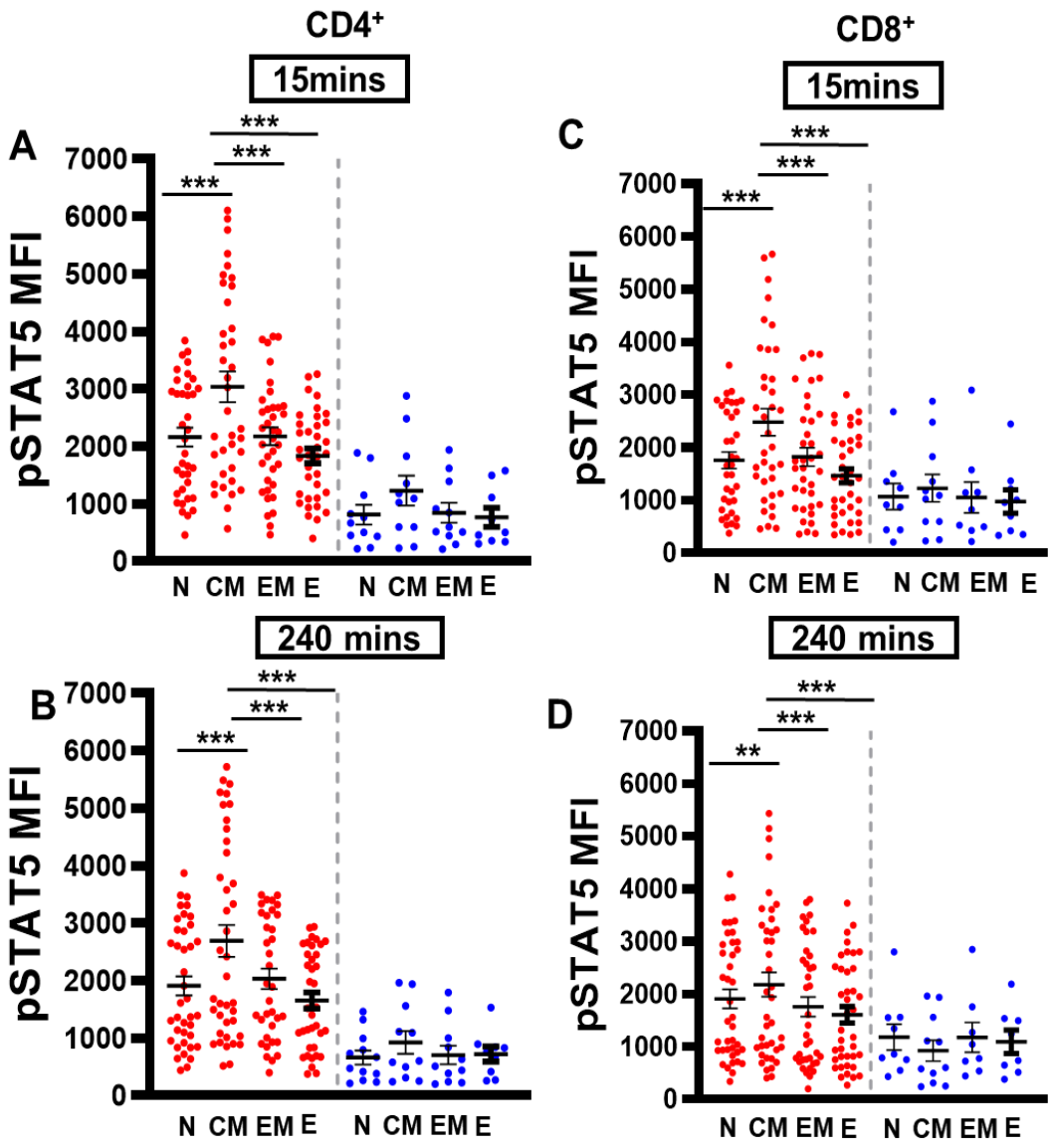
Figure 7**: Contraction of specific populations of clustered nodes of Tregs in RRMS.** FLOWSOM analysis of the expression of CD4, CD45RA, CCR7, CD25, CD127, and pSTAT5 in HC and RRMS at basal and following stimulation with IL-2. Minimum spanning tree structure were compared qualitatively to assess the dynamics of fluctuations in the contraction and/or expansion of groups of nodes and marker profile of specific nodes clusters. Phenotypically related clusters of nodes are emphasized by the corresponding background color surrounding each node.



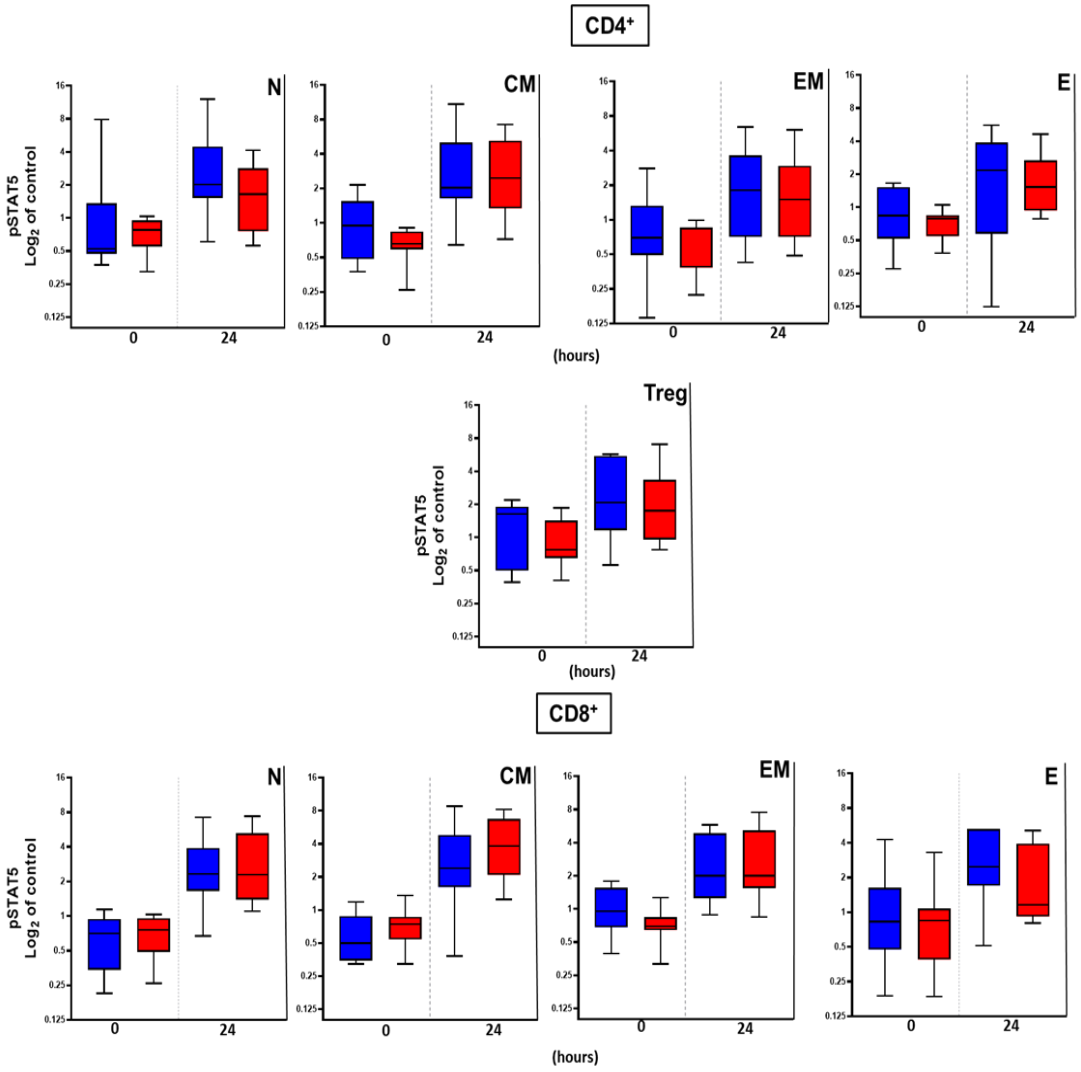
Supplemental Figure 1: **No difference in the frequency of CD4⁺ T lymphocytes and CD8⁺ central memory, effector memory, or effector T lymphocytes across early untreated RRMS patients and matched healthy individuals.** Quantitative comparison of the frequency of CD4⁺ and CD8⁺ T lymphocytes subsets across disease cohort were performed. Unpaired Students t tests using Welch's correction were performed to assess whether there was a significant difference between the frequency of cellular subsets. p values listed are those that were calculated from aforementioned statistical tests when significance was observed. Red circles depict data points obtained from RRMS patients, while blue circles depict data points obtained from healthy individuals.



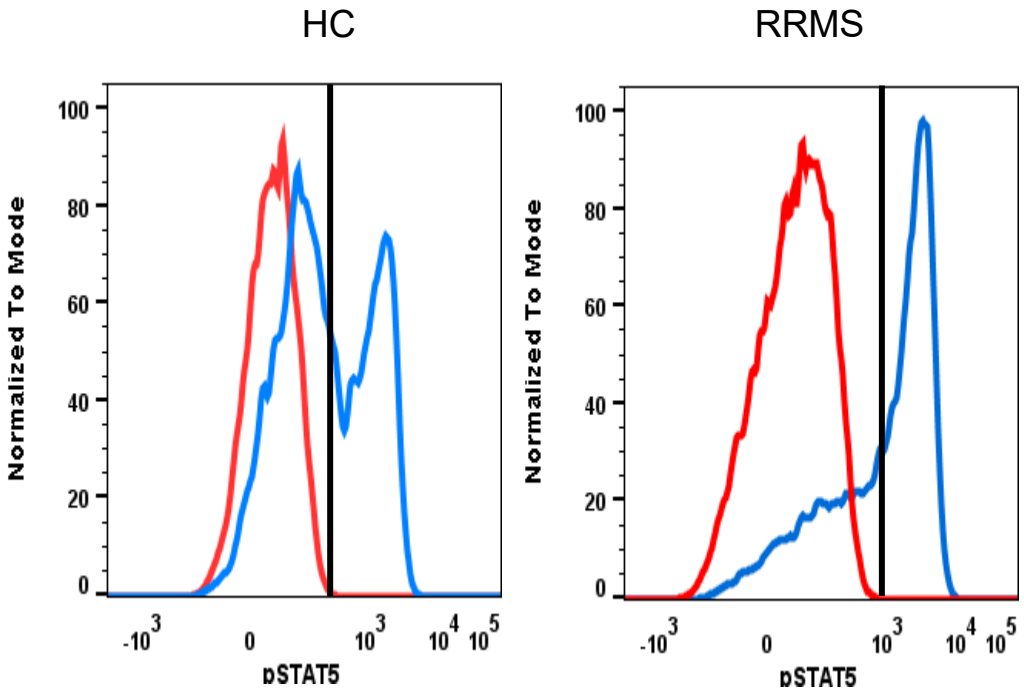
Supplemental Figure 2**: The kinetic response to IL-2 stimulation varies significantly between CD4⁺ and CD8⁺ T lymphocyte memory subsets in early untreated RRMS patients with respect to the induction and persistence of activated STAT5.** Flow cytometric analysis of the mean fluorescent intensity (MFI) of pSTAT5 AF647 in CD4⁺ and CD8⁺ T lymphocytes from individuals with RRMS were stimulated for either 15 minutes, 60 minutes, or 240 minutes with IL-2. Two-way repeated measures analysis of variance (ANOVA) was performed to assess the effect coreceptor type, stimulation time, and the interaction of these two factors as they relate to pSTAT5 MFI. p values were listed exactly as calculated from aforementioned statistical tests. Data is depicted in a box and whisker format. Outliers are defined by black circles and the remaining data represents 95% of values found within the whiskers.



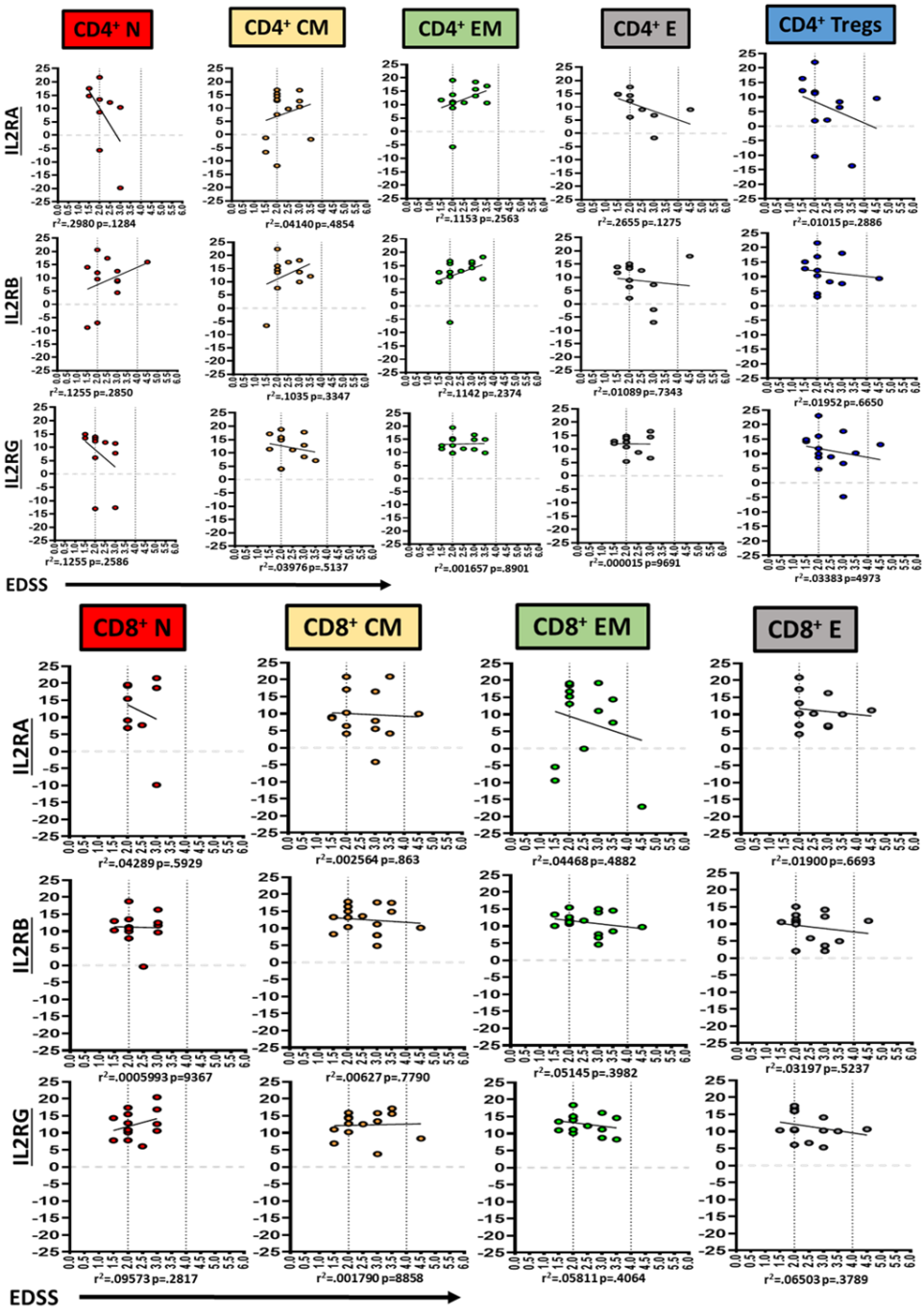
Supplemental Figure 3: **High responding CD4$^+$ and CD8$^+$ T lymphocytes from RRMS patients have higher pSTAT5 mean fluorescent intensity (MFI) over the course of four hours of IL-2 stimulation.** Flow cytometric analysis of the mean fluorescent intensity (MFI) of pSTAT5 AF647 across disease cohorts in high responding CD4+ and CD8+ T lymphocytes stimulated for either 15 minutes or 240 minutes with IL-2. Two-way repeated measures analysis of variance (ANOVA) was performed to assess the effect of disease, stimulation time, and the interaction of these two factors as they relate to responder MFI. Post hoc Student's t tests were performed using Bonferroni's correction to approximate the appropriate familywise error rate. p values from total CD4+ and CD8+ were calculated from aforementioned statistical tests and presented as: *= p<.05, ** = p <.01, *** = p<.001.



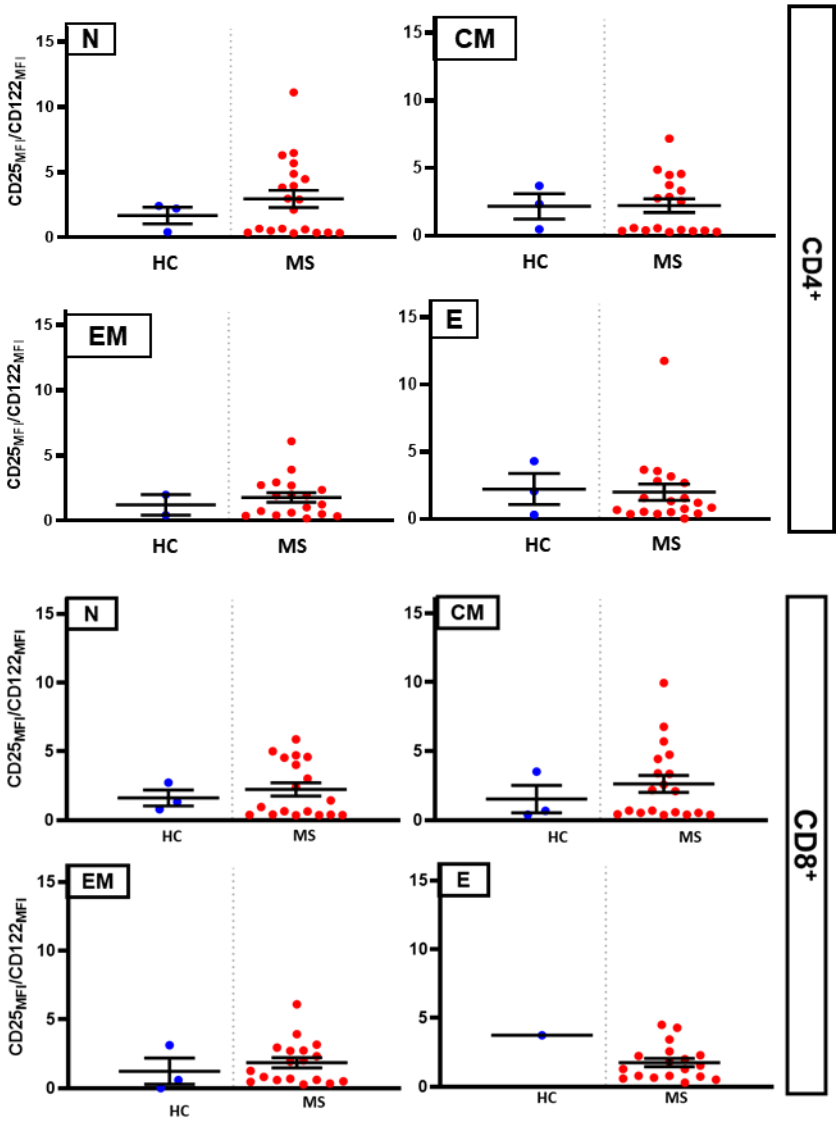
Supplemental Figure 4: **All CD4$^+$ and CD8$^+$ T lymphocytes subsets from early untreated RRMS and healthy individuals examined showed similar levels of activated STAT5 with extended exposure to IL-2 stimulation.** Flow cytometric analysis of the mean fluorescent intensity (MFI) of pSTAT5 AF647 in CD4$^+$ and CD8$^+$ T lymphocytes subsets from individuals with RRMS and healthy individuals were either unstimulated or stimulated for 24 hours with IL-2. Two-way repeated measures analysis of variance (ANOVA) was performed to assess the effect disease, stimulation time, and the interaction of these two factors as they relate to pSTAT5 MFI. Data is depicted in a box and whisker format. No outliers were observed and the remaining data recorded represents values found within the whiskers. Red boxes depict data points from RRMS patients, while blue boxes depict data points obtained from healthy individuals.



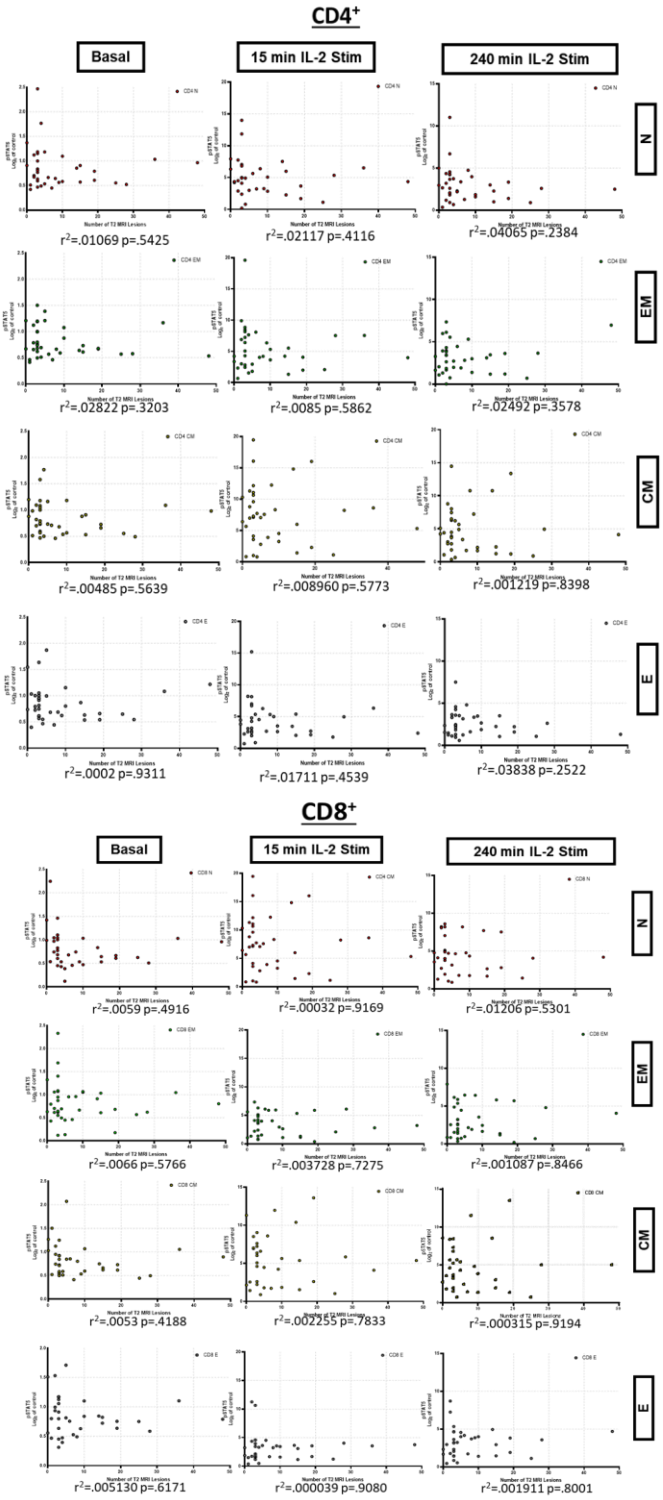
Supplemental Figure 5: **Gating strategy used to identify high responding cellular subsets with respect to pSTAT5 expression.** The mean fluorescent intensity (MFI) of unstimulated $CD4^+$ and $CD8^+$ T lymphocytes (red histograms) were used as a baseline to compare the MFI of IL-2 stimulated $CD4^+$ and $CD8^+$ T lymphocytes (blue histograms) between the different cohorts. The proportion of lymphocytes denoted to the right of the unstimulated $CD4^+$ and $CD8^+$ T lymphocytes were identified as responding cells. The total number of cells represented is depicted as a normalized ratio to the mode of each group.


CD4+ N
CD4+ CM
CD4+ EM
CD4+ E
CD4+ Tregs
IL2RA
IL2RB
IL2RG
r²=.2980 p=.1284
r²=.04140 p=.4854
r²=.1153 p=.2563
r²=.2655 p=.1275
r²=.01015 p=.2886
r²=.1255 p=.2850
r²=.1035 p=.3347
r²=.1142 p=.2374
r²=.01089 p=.7343
r²=.01952 p=.6650
r²=.1255 p=.2586
r²=.03976 p=.5137
r²=.001657 p=.8901
r²=.000015 p=9691
r²=.03383 p=4973
EDSS
CD8+ N
CD8+ CM
CD8+ EM
CD8+ E
IL2RA
IL2RB
IL2RG
r²=.04289 p=.5929
r²=.002564 p=.863
r²=.04468 p=.4882
r²=.01900 p=.6693
r²=.0005993 p=9367
r²=.00627 p=.7790
r²=.05145 p=.3982
r²=.03197 p=.5237
r²=.09573 p=.2817
r²=.001790 p=8858
r²=.05811 p=.4064
r²=.06503 p=.3789
EDSS

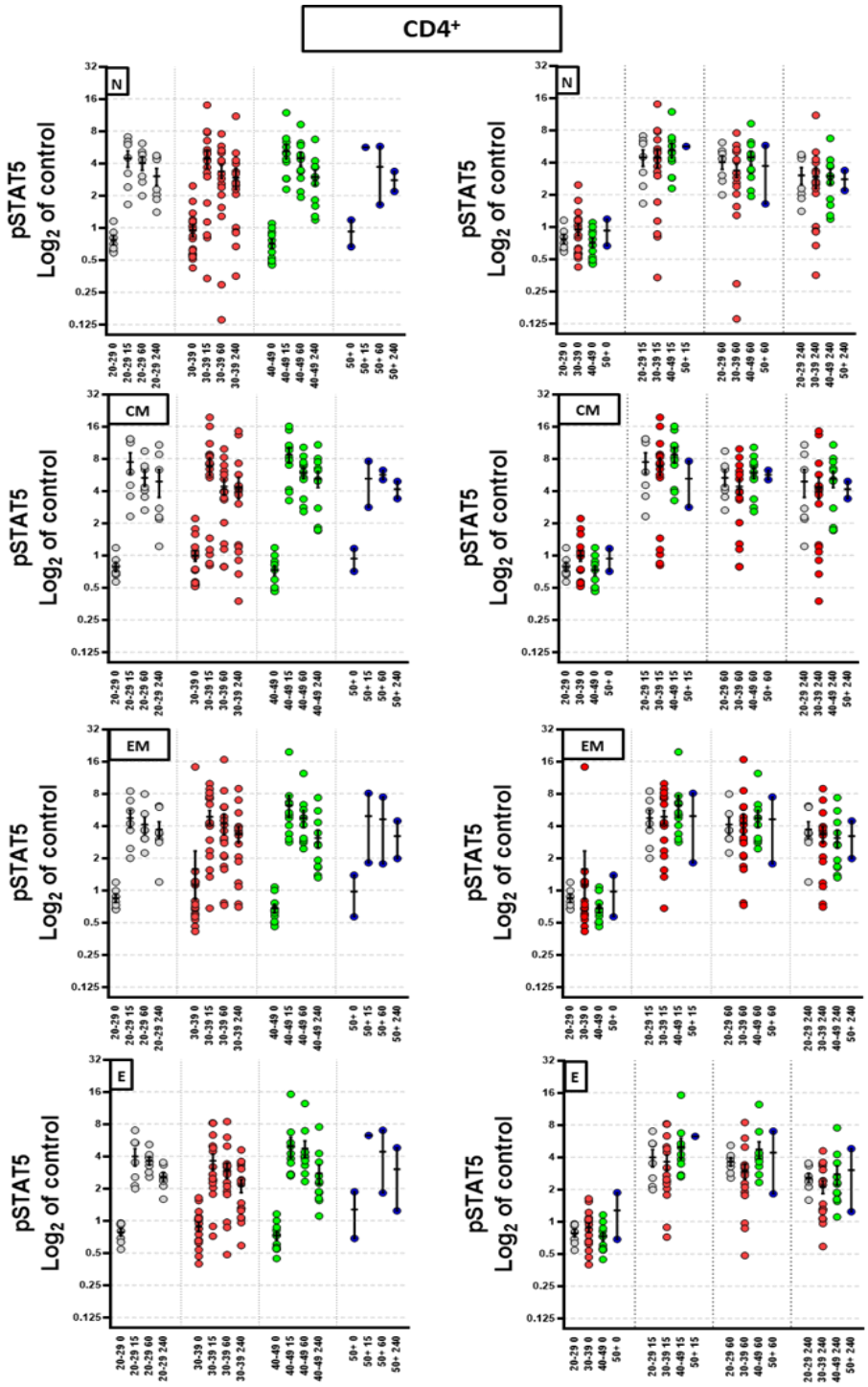
Supplemental Figure 6: **There is no correlation between IL-2 receptor subunit mRNA expression amongst $CD4^+$ and $CD8^+$ T lymphocyte subsets and disease status in early untreated RRMS patients as measured by EDSS.** IL2 receptor subunit mRNA expression was assessed in $CD4^+$ and $CD8^+$ T lymphocyte subsets that were previously sorted based upon their expression of CD45RA and CCR7. RRMS patient's expanded disability status scale (EDSS) at time of enrollment was collected to determine if there is an association present amongst IL2 receptor subunits and disability status. Correlation of determination (r^2) and significance value (p) for each test are presented with respect to each corresponding correlation plot.

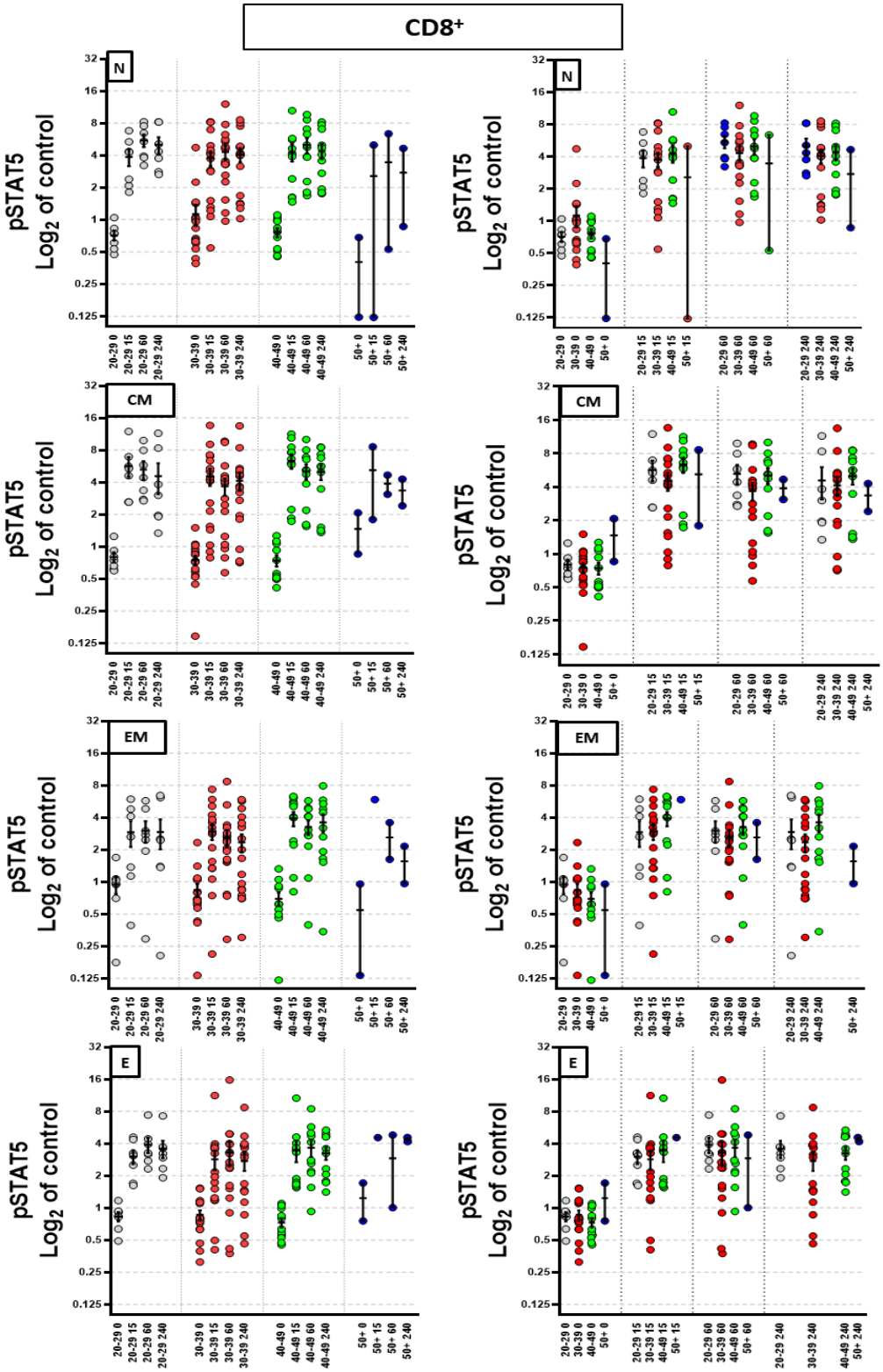


Supplemental Figure 7: **There was no difference observed in the abundance of IL-2 receptor subunit availability in CD4⁺ or CD8⁺T lymphocyte subsets across early untreated RRMS patients and healthy individuals.** The mean fluorescent intensity (MFI) of CD25 and the MFI of CD122 was collected from a subsample of healthy individuals and individuals with RRMS. The ratio of CD25 MFI /CD122 MFI was determined and presented for each patient in each CD4⁺ and CD8⁺ T lymphocytes subset as inferred by CD45RA and CCR7 expression. No differences were found between disease cohort in this sample using unpaired Student's t test with Welch's correction.

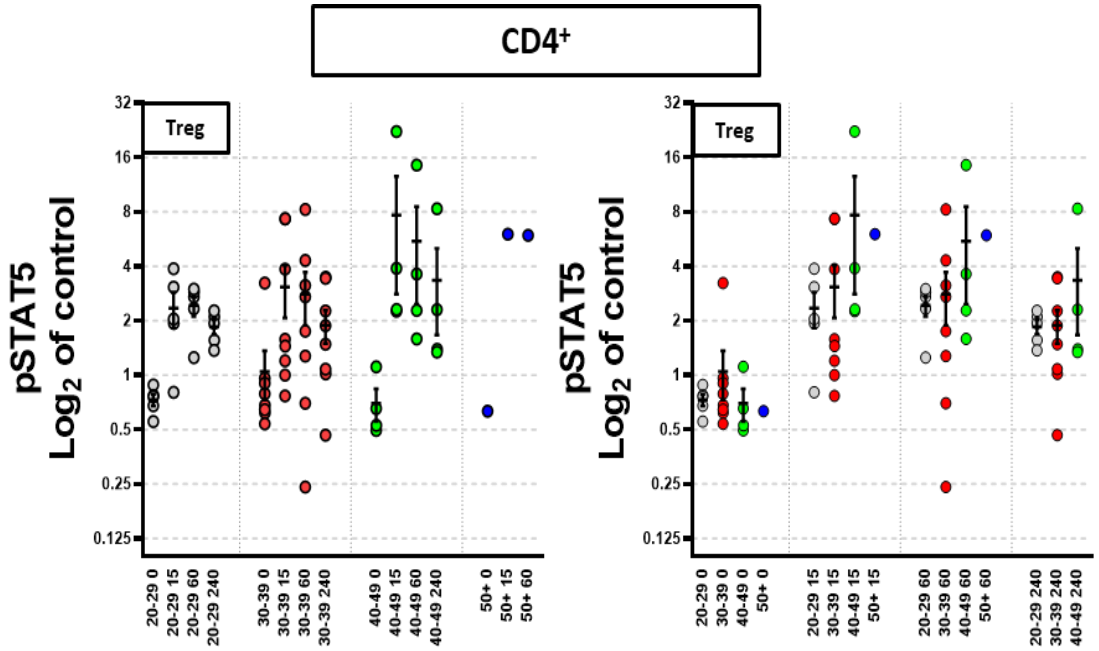

CD4+
Basal
15 min IL-2 Stim
240 min IL-2 Stim
N
EM
CM
E
r2=.01069 p=.5425
r2=.02117 p=.4116
r2=.04065 p=.2384
r2=.02822 p=.3203
r2=.0085 p=.5862
r2=.02492 p=.3578
r2=.00485 p=.5639
r2=.008960 p=.5773
r2=.001219 p=.8398
r2=.0002 p=.9311
r2=.01711 p=.4539
r2=.03838 p=.2522
CD8+
Basal
15 min IL-2 Stim
240 min IL-2 Stim
N
EM
CM
E
r2=.0059 p=.4916
r2=.00032 p=.9169
r2=.01206 p=.5301
r2=.0066 p=.5766
r2=.003728 p=.7275
r2=.001087 p=.8466
r2=.0053 p=.4188
r2=.002255 p=.7833
r2=.000315 p=.9194
r2=.005130 p=.6171
r2=.000039 p=.9080
r2=.001911 p=.8001

Supplemental Figure 8**: There is no correlation between the number of T2 MRI lesions and basal or IL-2 induced STAT5 activation in early untreated RRMS patients' $CD4^+$ or $CD8^+$ T lymphocytes.** Patients' total number of MRI lesions assessed by T2 weighted MRI sequences around the time of enrollment across different $CD4^+$ and $CD8^+$ T lymphocyte subsets was compared to patients' EDSS scores at time of enrollment. Correlation of determination (r^2) and significance value for each test are presented beneath each corresponding correlation plot.

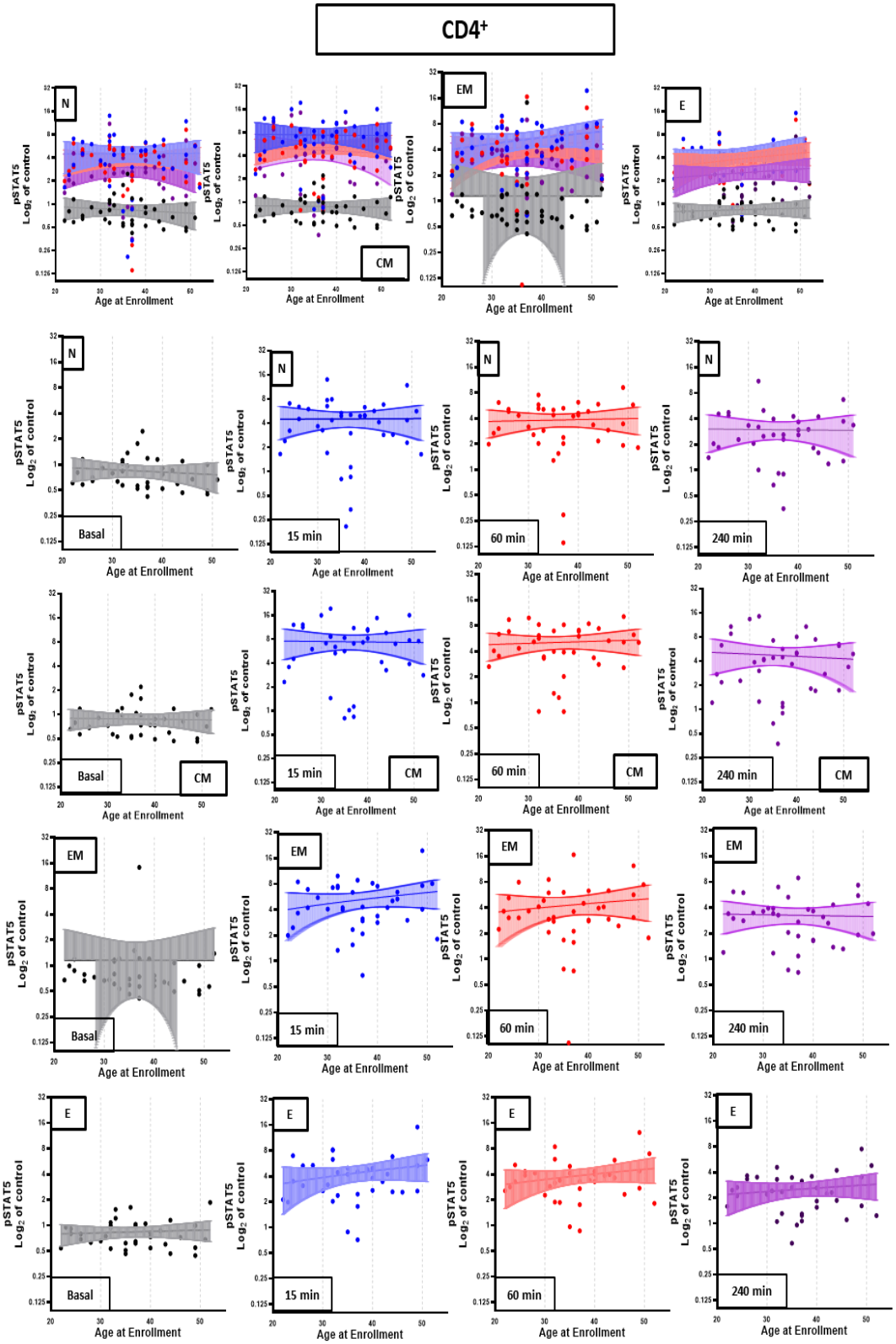

CD4+
N
CM
EM
E
pSTAT5
Log2 of control
32
16
8
4
2
1
0.5
0.25
0.125
20-29 0
20-29 15
20-29 60
20-29 240
30-39 0
30-39 15
30-39 60
30-39 240
40-49 0
40-49 15
40-49 60
40-49 240
50+ 0
50+ 15
50+ 60
50+ 240

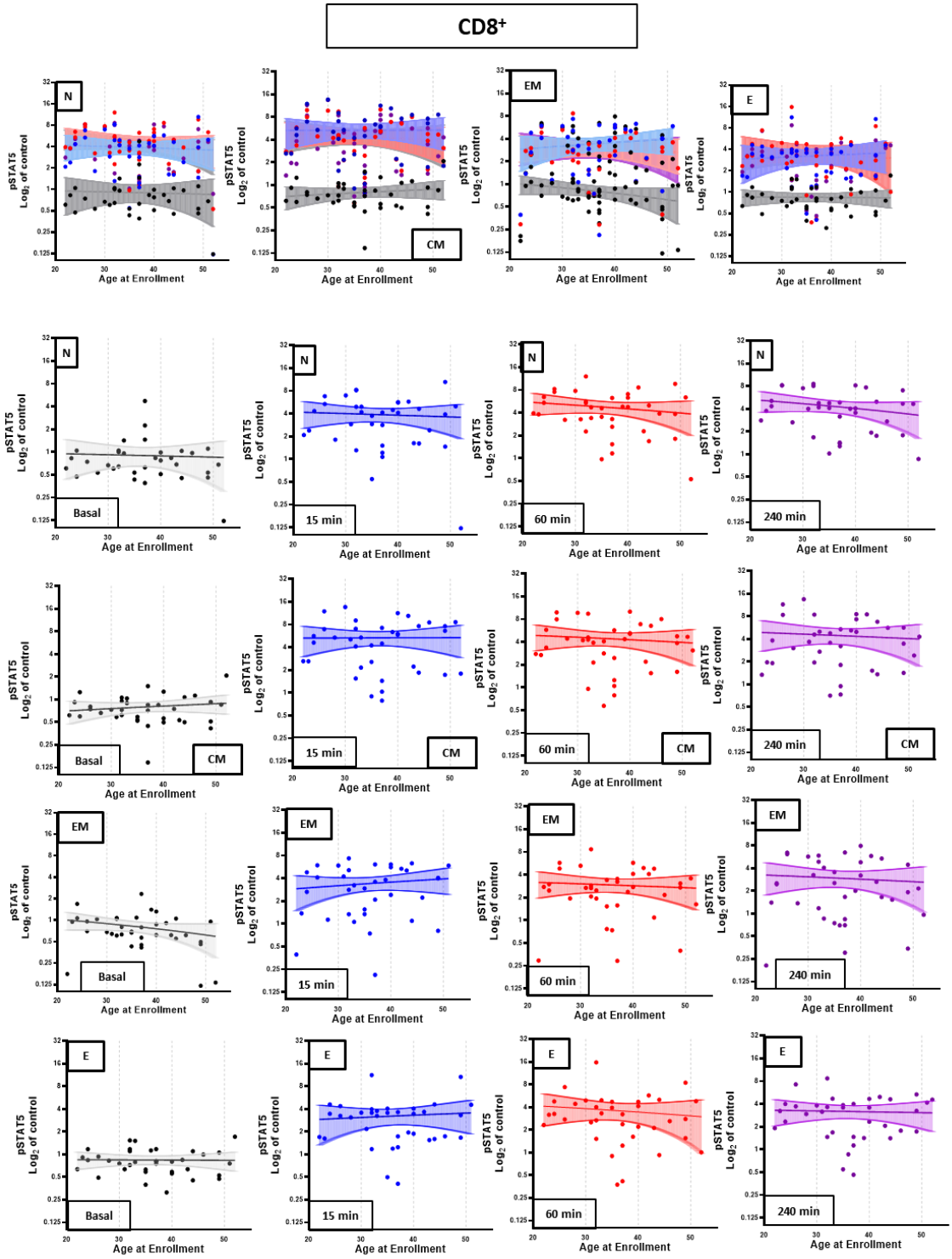

CD8+
N
CM
EM
E
pSTAT5
Log2 of control
32
16
8
4
2
1
0.5
0.25
0.125
20-29 0
20-29 15
20-29 60
20-29 240
30-39 0
30-39 15
30-39 60
30-39 240
40-49 0
40-49 15
40-49 60
40-49 240
50+ 0
50+ 15
50+ 60
50+ 240
20-29 0
30-39 0
40-49 0
50+ 0
20-29 15
30-39 15
40-49 15
50+ 15
20-29 60
30-39 60
40-49 60
50+ 60
20-29 240
30-39 240
40-49 240
50+ 240

Supplemental Figure 9**: There is no difference in $CD4^+$ or $CD8^+$ T lymphocyte subsets basal or IL-2 induced STAT5 activation across age of enrollment in early untreated RRMS.** Patient age at time of enrollment was collected and patients were organized into groups specific to each decade observed. From there, the expression of pSTAT5 was compared to patients' enrollment age to ascertain if there enhanced STAT5 activation with advanced age at onset in different $CD4^+$ or $CD8^+$ T lymphocytes subsets.

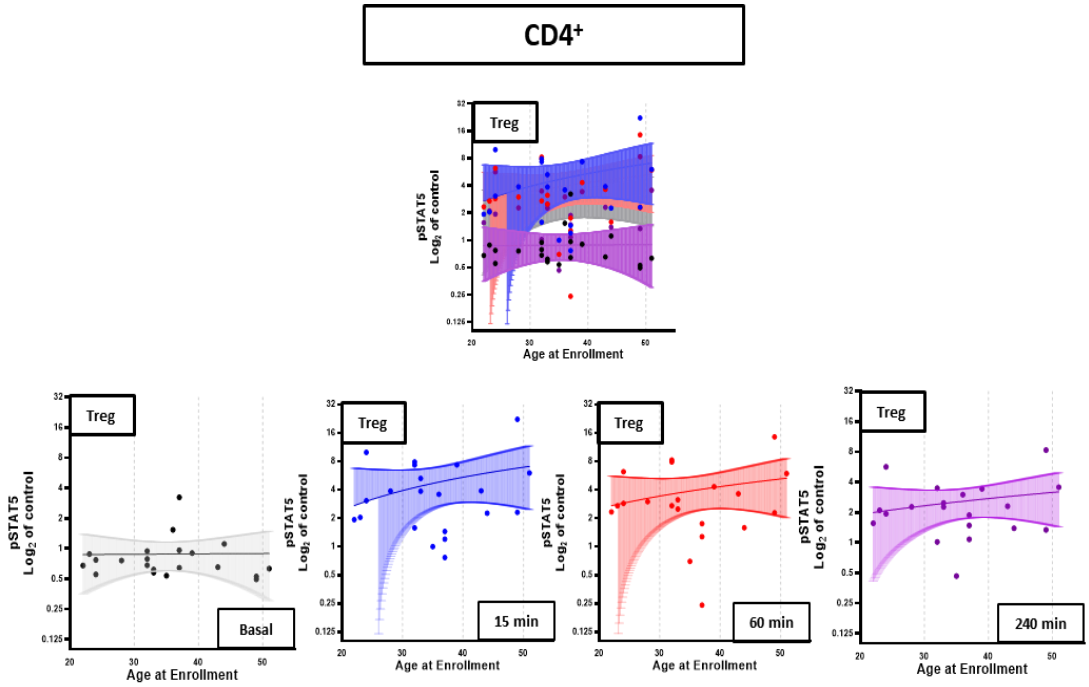


Supplemental Figure 10: **There is no difference in CD4$^+$ regulatory T lymphocyte basal or IL-2 induced STAT5 activation across age of enrollment in early untreated RRMS.** Patient age at time of enrollment was collected and patients were organized into groups specific to each decade observed. From there, the expression of pSTAT5 was compared to patients' enrollment age to ascertain if there enhanced STAT5 activation with advanced age at onset in different CD4$^+$ or CD8$^+$ T lymphocytes subsets.


CD4+
N
CM
EM
E
Basal
15 min
60 min
240 min
pSTAT5
Log2 of control
Age at Enrollment


CD8+
N
CM
EM
E
Basal
15 min
60 min
240 min
pSTAT5
Log2 of control
Age at Enrollment

Supplemental Figure 11: **There is no correlation patient's age at enrollment and basal or IL-2 induced STAT5 activation in early untreated RRMS patients' $CD4^+$ or $CD8^+$ T lymphocyte subsets.** Patients' overall age was collected at time of enrollment and compared to the expression of pSTAT5 across $CD4^+$ and $CD8^+$ T lymphocyte subsets from individuals with RRMS. Correlation of determination (r^2) and significance value for each test are presented beneath each corresponding correlation plot.



Supplemental Figure 12: **There is no correlation patient's age at enrollment and basal or IL-2 induced STAT5 activation in early untreated RRMS patients' CD4$^+$ regulatory T lymphocytes.** Patients' overall age was collected at time of enrollment and compared to the expression of pSTAT5 across CD4$^+$ regulatory T cells from individuals with RRMS. Correlation of determination (r^2) and significance value for each test are presented beneath each corresponding correlation plot.

INTERLEUKIN - 2 MODULATES INTERFERON GAMMA DEPENDENT STAT1 ACTIVATION IN SUBSETS OF $CD4^+$ AND $CD8^+$ T CELL SUBSETS IN PATIENTS WITH RELAPSING REMITTING MULTIPLE SCLEROSIS

by

BRANDON POPE, VISHAL SHARMA, RICHARD REYNOLDS, WILLIAM MEADOR, ALEXANDER SZALAI, KHURRAM BASHIR, S.LOUIS BRIDGES, JR., CHANDER RAMAN

In Preparation

Format adapted for dissertation

ABSTRACT

Disease modifying therapies (DMTs) in multiple sclerosis (MS) contribute to decreasing symptom burden that patients face, although novel agents have considerable adverse effects. Administration of IFNγ and the blockade of IL-2R have both been considered as potential therapeutic possibilities in relapsing remitting MS (RRMS), as they have the potential to induce immunomodulation and peripheral tolerance mechanisms. Moreover, polymorphisms in IFNγ and IL2 associated gene loci have been previously shown to confer risk in the susceptibility and pathogenesis of MS. Both IFNγ and IL-2 signal through JAK/STAT proteins, STAT1 and STAT5 being their primary signal transducers, respectively. We show within how IL-2 can modulate IFNγ induced STAT1 activation in $CD4^+$ and $CD8^+$ T lymphocyte subsets from peripheral blood mononuclear cells isolated ex vivo from patients with RRMS, as compared to healthy controls. Regulatory CD4+ T lymphocytes only showed this IL-2 dependent IFNγ induced elevation in STAT1 levels during short term concurrent stimulation and only $CD8^+$ naïve T lymphocytes exhibited a similar trend. Through the use of FlowSOM, we examined how STAT activation in $CD4^+$ and $CD8^+$T lymphocytes correlates with CD45RA and CCR7 expression across in MS. We found that $CD4^+$ T lymphocytes in RRMS patients showed distinct differences across populations of cells either unstimulated or stimulated with IFNγ alone or with concurrent IFNγ/IL-2 stimulation. This was not the case for $CD8^+$ T lymphocyte populations in RRMS patients as the dynamics of these populations largely mirrored that of healthy individuals under similar stimulation conditions. This study can shed light on qualitative and quantitative

differences that exist within T lymphocyte subsets across patients with RRMS and healthy individuals when stimulated with concurrent IFNγ/IL-2 stimulation. We have also begun to uncover a potential role for IL-2 induced IFNγ dependent STAT1 activation in patients with RRMS that could potentially be leveraged as an important clinically relevant therapeutic target.

INTRODUCTION

Multiple sclerosis (MS) is a chronic demyelinating disease that is most prevalent in individuals between the 2nd and 4th decades of life (5). Often in multiple sclerosis, patients experience periods of acute symptom worsening that frequently waxes and wanes in the form of relapses and remission, respectively. Activated antigen specific effector $CD4^+$ and $CD8^+$ T lymphocytes have been shown to be critical mediators of CNS targeted inflammation and pathological changes in MS (36). The activation and recruitment of Janus Kinase/Signal Transducer and Activators of Transcription (JAK/STAT) signaling pathway proteins in activated effector immune cells in MS is important in the pathogenesis of MS. Due to the contribution of these effector lineages to pathology in MS, several disease modifying therapies have been discovered in MS whose targets are either immunosuppressive or immunomodulatory in nature. STAT5a/b and STAT1 activation are important in the function of $CD4^+$ and $CD8^+$ T cell subsets in health and disease.

IL-2 and IFNγ are known to play critical roles in the immune system in the context of health and disease. IL-2 has long been established as a T cell growth

factor that induces the proliferation of naïve $CD4^+$ and $CD8^+$ T lymphocytes following antigen specific receptor activation (123). IL-2 also plays an important role in the normal homeostatic control and function of regulatory $CD4^+$ T cells(125). IFNγ is a type II interferon and is primarily produced by Th1 cells to direct an antiviral response to intracellular bacteria (152). Both IL-2Ra and IFNγ have been investigated as therapeutic options in MS, where Daclizumab received FDA approval as a DMT for RRMS until it was discovered that the risk of CNS infection in patients on this therapy significantly increases (45, 132, 153). Moreover, genome wide association studies have shown that polymorphisms in, both, Interleukin - 2 receptor subunit alpha (IL-2Ra) and Interferon gamma (IFNγ) are risk factors in MS (154, 155).

This study examines the influence that concurrent IFNγ/IL-2 stimulation has on induced STAT1/5 activation in non regulatory and regulatory populations of $CD4^+$ and $CD8^+$ T lymphocytes from patients with RRMS, as compared to healthy controls. Moreover, we used ex vivo PBMCs from patients to address whether IFNγ and/or IL-2 stimulation correlates with measures of disease progression and disability status in MS. Overall, we found that IL-2 stimulation can enhance $CD4^+$ T lymphocyte STAT1 activation above the activation level that was found in $CD4^+$ T lymphocyte subsets that had been stimulated with IFNγ alone. These results suggest a potential role for differential IFNγ induced STAT1 response in the context of high levels of IL-2 in vivo. We did not see this effect in $CD8^+$ T lymphocytes subsets, as the level of STAT1 activation in response to IFNγ alone was similar to that of healthy controls.

MATERIALS AND METHODS

Sample Collection and Patient Characteristics

Blood samples were volunteered by recruited patients with relapsing remitting multiple sclerosis that had been recently diagnosed at the UAB Multiple Sclerosis Center (MSC) to assess options for clinical symptom alleviation and treatment. Multiple sclerosis was verified by board certified, fellowship trained MS neurologists at the UAB MSC or associated specialty clinics. RRMS patients were verified to have fulfilled the most recent MAGNIMS criteria for multiple sclerosis diagnosis through the presence of clinical symptoms that satisfy the criteria of dissemination in space and time (8). All patients who volunteered for the study had not been treated with disease modifying treatments (DMTs) and were recently made aware of their diagnosis of multiple sclerosis. The characteristics of RRMS and healthy controls (HC) recruited for this study are shown in Table 1.

HC were identified from persons that matched the demographics of RRMS cohort and agreed to provide their informed consent. Healthy individuals were devoid of chronic diseases of autoimmunity and acute illness at the time of blood collection as self-reported in a demographic document as an addendum to the informed consent documents. Routine venipuncture was performed on all samples to acquire peripheral blood mononuclear cells (PBMC) from whole venous blood after the patients and healthy individuals were properly made aware of the associated risks and provided their informed consent.

All samples were assigned double-blinded identification numbers that served to blind both patients and researchers to individualized patient clinical

parameters and demographics. All protocols were approved by the Institutional Review Board at the University of Alabama at Birmingham.

Phosphoflowcytometry

PBMC were isolated from whole venous blood of patients and/or controls via density centrifugation and frozen at -80C. Technical bias associated with disease cohort status was controlled for by including samples from each cohort in every experimental run. PBMCs were rapidly thawed and allowed to equilibrate to room temperature over the course of 45 minutes. From there, PBMCs were stimulated with 25ng/ml of human IL-2 and/or 50ng/ml human IFNγ as a single pulse or over a time period of up to 4 hours to define an associated functional kinetic response. The concentrations of human IL-2 and human IFNγ were determined empirically through experiments on PBMC from a cohort of healthy individuals to establish a threshold for stimulation that would enable the quantification of activation of downstream STAT1/5 activation. Lyophilized human IL2 (Cat# 2020IL010) was purchased from R+D Systems and was reconstituted appropriately per manufacturer's protocols. hIL-2 was used at $2.1* 10^4$ IU/ug. Lyophilized human IFNγ(Cat#) was purchased form and was reconstituted appropriately per manufacturer's protocols.

Following stimulation, cells were stained for different functional and phenotypic T cell antibodies and assayed on a Thermofischer Scientific Attune NxT acoustic focusing flow cytometer with a 4 laser configuration. A live/dead stain and FSC/SSC discrimination were always included to ensure that only live single cells

were analyzed in each experiment. A subset of patients was stained with antibodies specific to regulatory $CD4^+$ T lymphocytes (CD127 and CD25), respectively, pertinent to the experimental hypothesis in question.

All cells were fixed with methanol in order to expose nuclear epitopes associated with translocated nuclear proteins and stained for antibodies specific to phosphorylated sites on various STAT proteins (STAT1/3/5). STAT3 activation did not respond to hIL2 or hIFNγ in patients/controls and was not found to elicit a molecular pathway important in IL2/IFNγ signaling and thus was not examined for further significance relevant to the experimental hypothesis.

Analysis of the phosphorylation state of STAT1 (pY701), STAT3 (pY705) and STAT5 (pY694) on these cell populations was examined following stimulation. The activation of STATs was calculated as Log_2 fold of MFI obtained from isotype control that was included for each patient sample. This approach allowed for normalization between samples analyzed on different days under similar experimental conditions.

Flow Cytometry Analysis

After staining cells and collecting data, fcs files were analyzed using Flow Jo version 10.6.2. (Treestar). All mononuclear leukocytes were gated on forward scatter area and height. Next, cellular debris was excluded and live single cells were gated for staining markers (CD4, CD8, CCR7, CD45RA) to investigate the function and relevance of $CD4^+$ and $CD8^+$ T lymphocytes subsets across the

patient cohorts. Live single cells were also analyzed unbiasedly using t-distributed Stochastic Neighbor Embedding (tSNE) and FLOWSOM plugin software.

tSNE/ FLOWSOM

$CD4^+$ and $CD8^+$T lymphocytes from each experiment analyzed were exported from each signaling experiment based upon the stimulation condition (basal, 15mins, 4hrs), independently. Data collected from exported cells was then concatenated based upon disease cohort and stimulation condition using all compensated parameters. Concatenated cells were exported as .fcs files based upon disease cohort and stimulation conditions. Samples used for these analyses were gated for live, single cells, downsampled to identical cell number across disease cohorts (to control for bias in node size) and used as input files for either FLOWSOM (143) or Barnes-Hut tSNE. CD4 BV421/CD8 BV510, pSTAT5 AF 647, CD45RA BV650, and CCR7 BV711 were used as input parameters to organize the self-organizing map (SOM) and t-SNE plots. The number of meta clusters selected corresponded to the number of input parameters to avoid bias in the number of proportions selected. A random seed was set at the number 3 for each run of the plugin to provide for reproducibility across various runs.

Statistical Analysis

Statistical analysis was using either Graphpad Prism (GraphPad Software, LLC) or R (The R Foundation). Descriptive, inferential, or summary statistics were used, where appropriate, to assess the validity and context of each experimental

design. An alpha level of > .05 was considered significant, except where an adjusted alpha level was determined using Bonferroni's correction. Data was consistently presented using mean +/- SEM.

RESULTS

There was no difference in the basal IL-2 response or combined IFNγ/IL-2 response between patients with RRMS and healthy controls in $CD4^+$ and $CD8^+$ T lymphocyte subsets. We observed no difference in the proportion of naïve (N), central memory (CM), effector memory (EM) or effector (E) $CD4^+$ T cells and Treg cells between RRMS and HC (Supplementary Figure 1A). Among $CD8^+$ T cell populations, only naïve CD8 T cells were significantly greater in RRMS than HC (Supplementary Figure 1B). Total $CD8^+$ T cells were similar between RRMS and HC (data not shown). Moreover, there was no difference in the basal level of STAT1 activation when you compare across the disease cohort or between specific $CD4^+$ and $CD8^+$ T lymphocyte subsets (Figure 1).

Non regulatory $CD4^+$ T lymphocyte subsets from RRMS patients exhibit a hyperactive IFNγ STAT1 response when stimulated with IL-2. PBMC isolated from patients with RRMS or healthy individuals were unstimulated or stimulated with for up to 240 minutes with IFNγ and/or IL-2. The activation of STAT5 (pSTAT5-Y694) and STAT1 (pSTAT1-Y701) across all time points was assessed in $CD4^+$ and $CD8^+$ naïve, effector, and memory subsets (central memory and effector memory). IFNγ did not activate STAT5 alone or during concurrent stimulation (data not shown). Also, IFNγ was shown to activate STAT1 robustly and there was no evidence of

disease specific enhancement of STAT1 levels with IFNγ stimulation alone (Figure 1). But, in all $CD4^+$ T lymphocytes, except Tregs, IL-2 was able to enhance the activation of STAT1 with concurrent IFNγ stimulation for up to 4 hours (Figure 1) in RRMS patients. The levels of expression of IL2 receptor subunits were not significantly different (data not shown). These results inform us that IL-2 stimulation in $CD4^+$ and $CD8^+$ T lymphocytes can contribute to IFNγ induced STAT1 activation in patients with RRMS.

Regulatory $CD4^+$ T cells from RRMS show a hyperactive IFNγ STAT1 response when pulsed with IL-2 followed by a sustained similar IFNγ STAT1 response. In regulatory $CD4^+$ T cells, IL-2 enhanced IFNγ induced STAT1 activation at 15 mins post concurrent IFNγ/IL-2 stimulation (Figure 1). During longer periods of concurrent IFNγ/IL-2 stimulation, STAT1 levels between patients with RRMS and healthy individuals were no different. Also, in stimulation duration longer than 15 mins post stimulation, regulatory $CD4^+$ T cell subsets from healthy controls showed enhanced levels of STAT1 activation in certain individuals assayed.

$CD4^+$ T lymphocyte subsets in RRMS show a distinct response from healthy individuals to IFNγ/IL-2 stimulation when analyzed unbiasedly**.** We sought to clarify whether subpopulations of $CD4^+$ T lymphocytes exist in RRMS patients and how more could be uncovered about how the phenotypic markers utilized in these cells correlate with STAT1/5 activation ex vivo. To accomplish this goal, we performed FlowSOM (see Materials and Methods for details), on $CD4^+$ T lymphocytes that were either unstimulated or stimulated IFNγ alone or with concurrent IFNγ/IL-2

stimulation for 15 mins and 4 hours. This enabled us to simultaneously visualize CD45RA, CCR7, STAT1, and STAT5 activation unbiasedly in these cellular subsets. At basal, there was a relative contraction in populations of nodes in patients with RRMS, specifically in the nodes of cells clustered in green (Figure 3). This contraction persisted in these nodes throughout IFNγ and concurrent stimulation. Healthy controls, at basal, showed a contraction of clustered cells at 15mins post IFNγ stimulation and in both stimulation durations with concurrent IFNγ/IL-2 stimulation. There is also a contraction of the nodes of cells clustered in red, across both cohorts, that have undergone stimulation with concurrent IFNγ/IL-2 stimulation, although this trend is more pronounced in healthy individuals. Overall, these results elucidate specific distinct differences in the magnitude and fluctuations in the stability of these subpopulations of $CD4^+$ T lymphocytes across patients with RRMS and healthy controls exposed to either IFNγ stimulation or concurrent IFNγ/IL-2 stimulation over time. We questioned whether differences in the magnitude or relative frequency of $CD8^+$ T lymphocyte subsets across patients with RRMS and healthy controls existed as well. There were no apparent relevant differences in these subpopulations of $CD8^+$ T lymphocyte subsets as examined through FlowSOM (Supplemental Figure 2). We did see instances where individual nodes differed in their overall magnitude in one cohort compared to the other but believe that these changes most likely do not represent major changes or inform our perspective of how CD45RA and CCR7 might be influenced by STAT activation in these cells.

$CD8^+$ naïve T lymphocyte subsets from RRMS patients exhibit a hyperactive IFNγ response with concurrent IFNγ/IL-2 stimulation. At 4 hours post stimulation with concurrent IFNγ/IL-2, $CD8^+$ naïve T lymphocytes exhibited significantly higher STAT1 activation, as compared to IFNγ stimulation alone at the same time point (Figure 2). $CD8^+$ T lymphocytes from healthy individuals showed drastic inductions of STAT1 activation as a result of concurrent IFNγ/IL-2 stimulation. This was intriguing as it mirrored what was seen in regulatory $CD4^+$ T lymphocytes in similar experimental conditions.

DISCUSSION

In this study, we examined the potential role that concurrent stimulation with IFNγ/IL-2 could have on influencing the level of STAT protein activation in the context of peripheral populations of $CD4^+$ and $CD8^+$ T lymphocyte subsets in patients with RRMS and controls. We found that there were no differences in the levels of STAT1 activation in these populations across each cohort with stimulation of IFNγ alone or in the absence of stimulation. Conversely, IL-2 was shown to modulate the levels of STAT1 activation in $CD4^+$ and $CD8^+$ naïve T lymphocyte subsets in patients with RRMS in an IFNγ dependent manner. Regulatory T lymphocytes from RRMS patients concurrently stimulated with IFNγ and IL-2 were not significantly different from those that were stimulated with IFNγ alone. In $CD8^+$ T lymphocyte effector and memory subsets from patients with RRMS, IL-2 did not seem to elevate STAT1 levels. All $CD4^+$ and $CD8^+$ T lymphocyte subsets were able to induce STAT1 activation when stimulated with IFNγ alone. We also found no

association with the IFNγ alone or in concurrent IFNγ/IL-2 stimulation with expanded disability status scale scores (data not shown). Overall, these results are novel in that they show how IL-2 can modulate IFNγ signaling over time intervals that presumably exclude the possibility of de novo protein translation or cell to cell effects of cytokine secretion. We posit that IL-2 could potentially be mediating these effects through the impact of either activating non-canonical signaling proteins that can associate with and activate IFNγ dependent STAT1 responses or through directly modulating the actions of regulators of STAT1 signaling, such as suppressor of cytokine signaling (SOCS) or protein tyrosine phosphate (PTP) proteins. For example, previous studies published by Rowse et. al. show a role for how GSK3 can regulate STAT1 levels independent of IFNγ, in a mouse model of EAE(156). IL-2 is also able to activate GSK3 signaling pathway in conjunction with the traditional activation of STAT5(140). It is therefore plausible that IL-2 dependent GSK3 activation can influence STAT1 activation and gene transcription during concurrent IFNγ/IL-2 stimulation. Our lab will address these separate mechanistic possibilities in future studies featuring patient samples from patients with RRMS.

In this study, we also sought to examine how we could use unbiased techniques to assess how CD45RA and CCR7 expression correlate with STAT1/5 levels in peripherally isolated $CD4^+$ and $CD8^+$ T lymphocyte subsets from patients with RRMS. We showed that FlowSOM was able to uncover distinct changes in the relative frequencies of populations of $CD4^+$ T lymphocytes across disease reliably and efficiently. This analysis enabled us to track the changes in population

dynamics across these disease cohorts and how this is altered by concurrent IFNγ/IL-2 stimulation or IFNγ stimulation alone. We saw a contraction in specific populations of cells that were disease specific and could potentially be used as a biological marker to assess how the frequency of populations of nodes correlates with STAT levels, disease status, or treatment response to specific DMT regimens. We did not see significant changes in $CD8^+$ T lymphocyte subsets composition in our unbiased FlowSOM analysis. These results are intriguing as these findings did not seem to explain the ability of IL-2 to induce STAT1 levels in an IFNγ dependent manner in naïve $CD8^+$ lymphocytes from patients with RRMS. The implications for these findings are equivocal but could be a representation of small discrete changes across many clustered nodes, as opposed to more significant changes in a small group of clustered nodes as was seem for $CD4^+$ T lymphocytes cells. We will refine these studies by enriching for naïve $CD8^+$ T lymphocytes specifically and assaying these cells separately across disease to attempt to uncover differences in this subset, as this was the only subset in RRMS patients in which IL-2 could modulate IFNγ induced STAT1 levels. Nonetheless, this study provides significant rationale to utilize and monitor the phenotypic marker expression markers and kinetic response in peripheral $CD4^+$ and $CD8^+$ T lymphocytes function in MS as one plausible biological tool that can help to guide treatment strategies clinically.

Table 1. **Demographic** Information for MS patients and healthy individuals

	HC (n = 15)	**RRMS (n = 37)**	**p value**
Gender (% female)	60%	75%	
Race/Ethnicity (%AA,%EA)	53%,46%	37%,63%	
Age at Enrollment (years, SD)	50.13 (12.65)	36.36 (8.1)	$p < .0001$
EDSS (SD)	NA	2.54 (1.04)	
Number of T2 MRI lesions (SD)	NA	8.86 (10.66)	
DMT Use	NA	NA	
Most Recent Corticosteroid Use (months)	NA	6.78	
Disease Duration (SD)	NA	2.67 (1.35)	
Age at First Reported Symptoms of RRMS (years)	NA	32.43 (6.43)	

HC- Healthy Controls; RRMS – Relapsing Remitting Multiple Sclerosis; AA – African American ancestry; EA – European American ancestry; EDSS – Expanded Disability Status Scale; DMT – Disease Modifying Therapy; SD – Standard Deviation; T2 MRI – T2 Weighted Magnetic Resonance Image; Unpaired student t-test with Welch's correction was used to determine statistical significance.

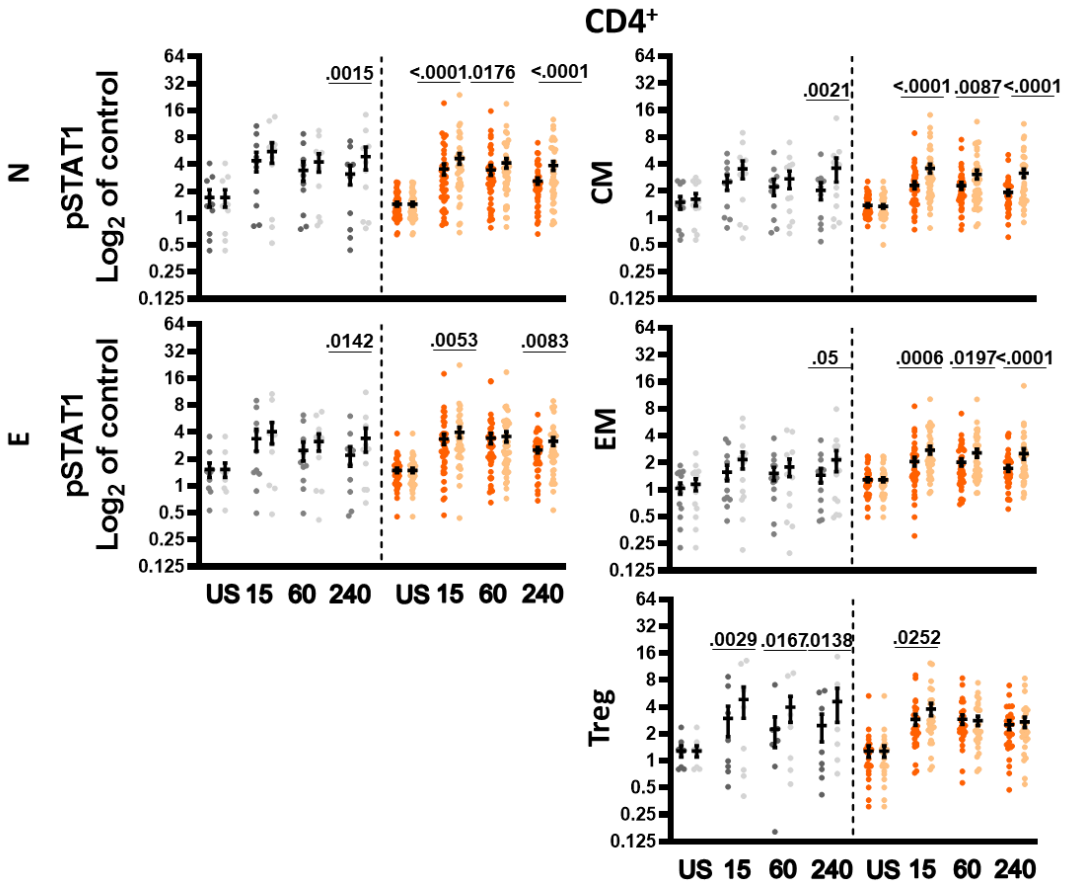


Figure 1**: CD4⁺ T lymphocyte subsets in RRMS patients show elevated STAT1 response to IFNγ/IL-2 stimulation as compared to healthy individuals.** Flow cytometry analysis of pSTAT1 levels in gated subpopulations of CD4⁺ T lymphocytes from HC (grey) or RRMS (orange). PBMC from RRMS or HC were unstimulated (US) or stimulated with IL-2 for various time periods (15 – 240 min) and levels of pSTAT1 (pY721) was determined in naïve (N), central memory (CM), effector memory (EM), effector (E), and regulatory (Treg) populations. Cellular subsets stimulated with IFNγ alone and IL-2 alone are represented by dark grey and dark orange dots, respectively. Cellular subsets stimulated with IFNγ and IL-2 are represented by light grey and light orange, respectively. The activation of STAT1 was normalized to isotype control for each individual and data represented as Log_2. Each dot represents and individual control or patient sample. Three-way repeated measures analysis of variance (ANOVA) was performed to assess the effect of disease, stimulation time, stimulation duration, and the interaction of these three factors as they relate to pSTAT1 expression. Post hoc Student's t tests were performed using Bonferroni's correction to approximate the appropriate familywise error rate. p values were listed exactly as calculated from aforementioned statistical tests.

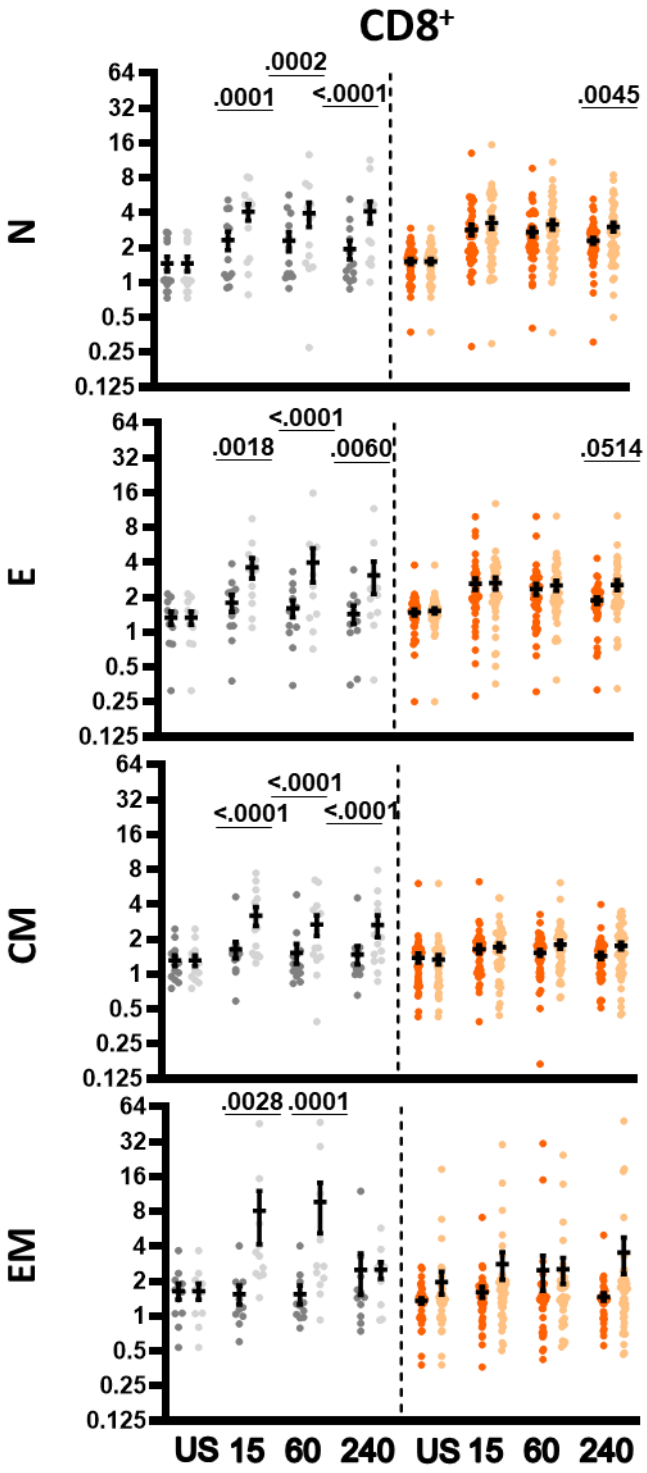

CD8+
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Figure 2**: IFNγ induced STAT1 response is unchanged in response to combined IFNγ/IL-2 stimulation, as compared to IFNγ stimulation alone in CD8$^+$ T lymphocyte subsets in early untreated RRMS patients.** Flow cytometry analysis of pSTAT1 levels in gated subpopulations of CD8$^+$ T lymphocytes from HC (grey) or RRMS (orange). PBMC from RRMS or HC were unstimulated (US) or stimulated with IFNγ and/or IL-2for various time periods (15 – 240 min) and levels of pSTAT1 (pY721) was determined in naïve (N), central memory (CM), effector memory (EM), effector (E), and regulatory (Treg) populations. Cellular subsets stimulated with IFNγ alone and IL-2 alone are represented by dark grey and dark orange dots, respectively. Cellular subsets stimulated with IFNγ and IL-2 are represented by light grey and light orange, respectively. The activation of STAT1 was normalized to isotype control for each individual and data represented as Log_2. Each dot represents and individual control or patient sample. Three-way repeated measures analysis of variance (ANOVA) was performed to assess the effect of disease, stimulation time, stimulation duration, and the interaction of these three factors as they relate to pSTAT1 expression. Post hoc Student's t tests were performed using Bonferroni's correction to approximate the appropriate familywise error rate. p values were listed exactly as calculated from aforementioned statistical tests.

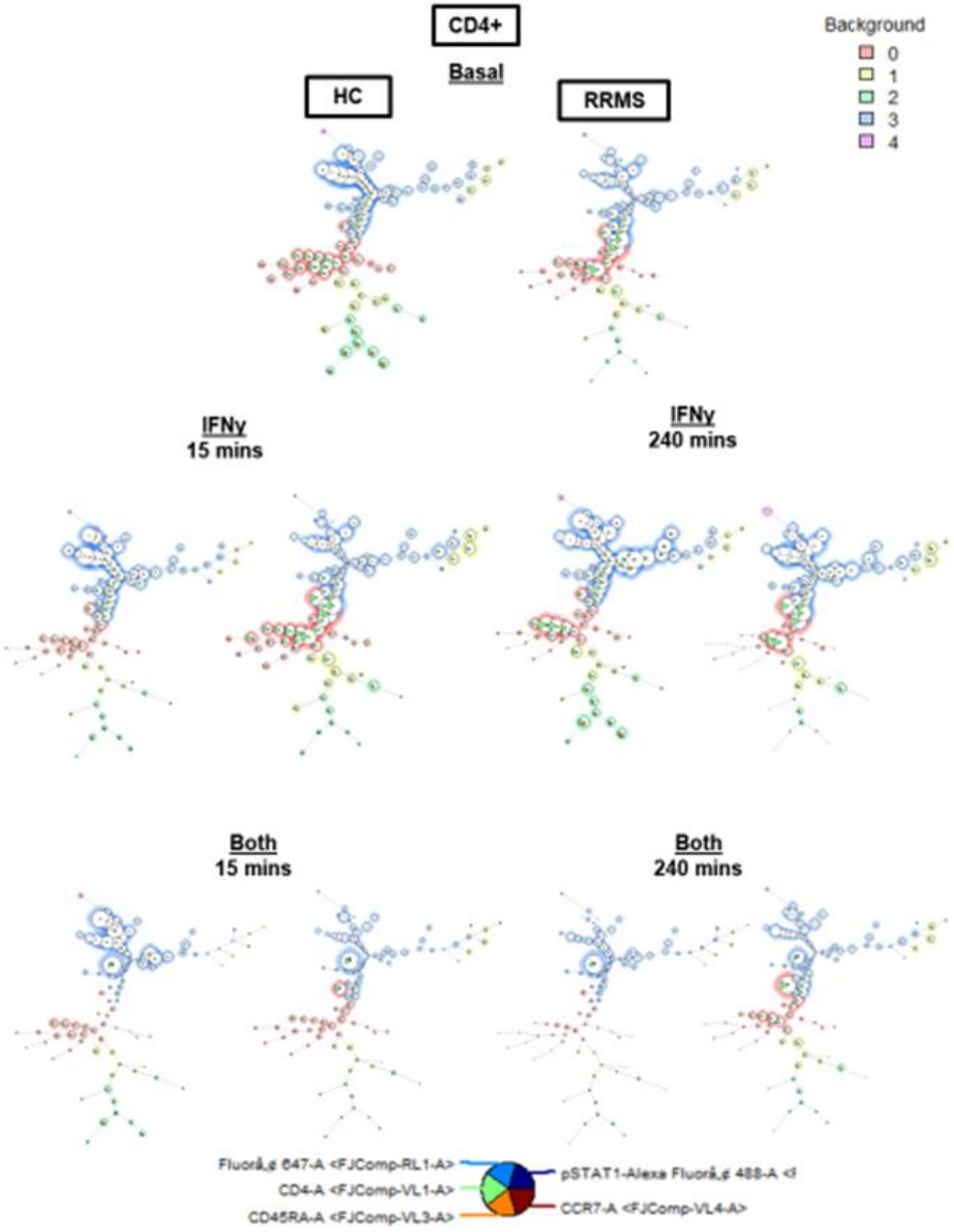
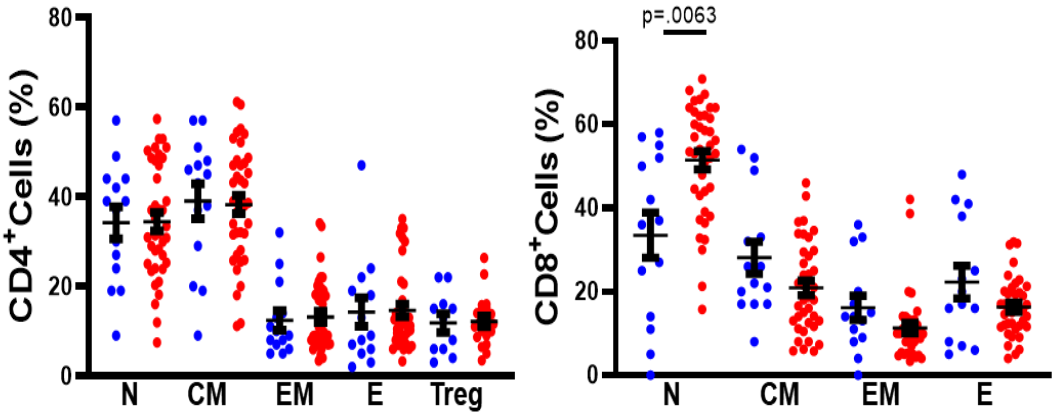
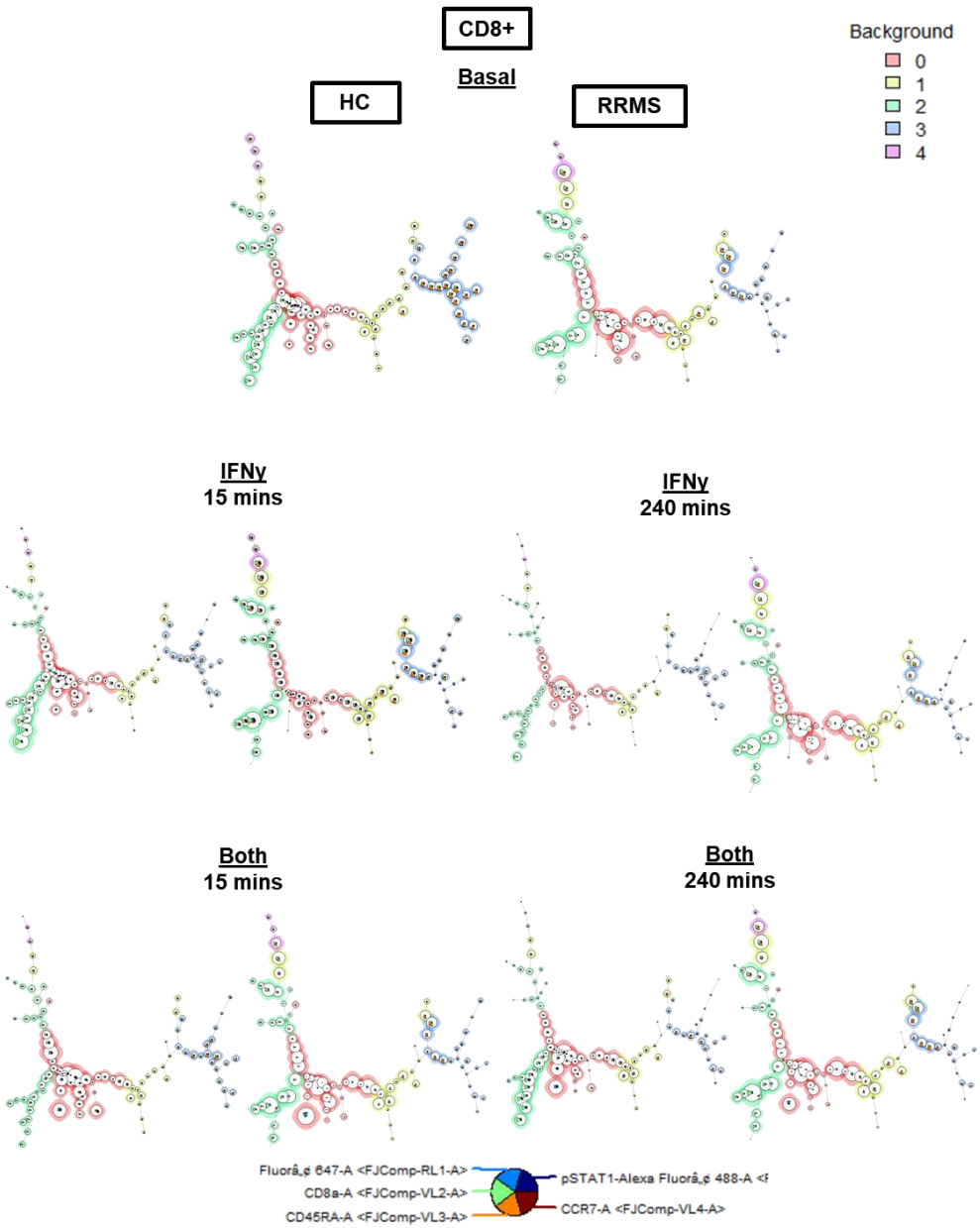


Figure 3**: RRMS patient's CD4⁺ T lymphocytes show contractions in relative node size compared to basal STAT1 response in combined IFNγ/IL-2 stimulation.** FLOWSOM analysis of the expression of CD4, CD45RA, CCR7, pSTAT1, and pSTAT5 in HC and RRMS at basal and following stimulation with IL-2. Minimum spanning tree structure were compared qualitatively to assess the dynamics of fluctuations in the contraction and/or expansion of groups of nodes and marker profile of specific nodes clusters. Phenotypically related clusters of nodes are emphasized by the corresponding background color surrounding each node.



Supplemental Figure 1: **No difference in the frequency of CD4$^+$ T lymphocytes and CD8$^+$ central memory, effector memory, or effector T lymphocytes across early untreated RRMS patients and matched healthy individuals.** Quantitative comparison of the frequency of CD4$^+$ and CD8$^+$ T lymphocytes subsets across disease cohort were performed. Unpaired Students t tests using Welch's correction were performed to assess whether there was a significant difference between the frequency of cellular subsets. p values listed are those that were calculated from aforementioned statistical tests when significance was observed. Red circles depict data points obtained from RRMS patients, while blue circles depict data points obtained from healthy individuals.



Supplemental Figure 2**: RRMS patients CD8⁺ T lymphocytes do not show major changes in relative node size or phenotypic marker expression despite differential response to combined IFNγ/IL-2 stimulation, as compared to healthy controls.** FLOWSOM analysis of the expression of CD4, CD45RA, CCR7, pSTAT1, and pSTAT5 in HC and RRMS at basal and following stimulation with IL-2. Minimum spanning tree structure were compared qualitatively to assess the dynamics of fluctuations in the contraction and/or expansion of groups of nodes and marker profile of specific nodes clusters. Phenotypically related clusters of nodes are emphasized by the corresponding background color surrounding each node.

DISCUSSION

This study demonstrates the profound short-term differences in the responses that result from the activation of IFNγ/IL-2 dependent JAK/STAT proteins in $CD4^+$ and $CD8^+$ T lymphocytes subsets in the context of RRMS, as compared to healthy individuals. Furthermore, we endeavored to examine the congruence, or lack thereof, of the disease-specific activation of these pathways in our patient cohorts and/or controls as compared to the functional phenotype in the $CD4^+$ and $CD8^+$ T lymphocytes themselves. We showed that several T lymphocytes subsets isolated ex-vivo from patients with early untreated RRMS expressed higher levels of either pSTAT1 or pSTAT5 when stimulated with either IFNγ or IL-2 respectively. This data was despite significant similarity observed in the proportion of cellular subsets recovered across patients with RRMS and healthy individuals. From this observation, the data suggest that the mechanisms that govern the outright proliferation of various $CD4^+$ and $CD8^+$ T lymphocyte subsets across this specific cohort of patients and controls are potentially alternatively regulated or not specifically associated with mechanisms that govern the cytokine induced activation of these cells.

We saw a broad induction of IFNγ and IL-2 STAT dependent signaling activation in populations of $CD4^+$ and $CD8^+$ T lymphocyte subsets. This could be expected in the case of IL-2 as IL-2 has been shown to be a growth factor that induces the proliferation of $CD4^+$ and $CD8^+$ T lymphocytes. However, what was not

initially appreciated in the field was the notion that IL-2 would differentially activate STAT5 in individuals with RRMS and that this IL-2 hyperactivation of STAT5 in nonregulatory cellular subsets would be found in concert with similar levels of STAT5 activation in regulatory populations from patients, as compared to controls. We believe that these findings are particularly important as it suggests a role for an increase in the propensity of nonregulatory T cell lineages to increase in number in the patients with RRMS due to the essential nature of IL-2 function in mediating T cell proliferation after receptor activation has occurred. This scenario could potentially perpetuate an increase in activated populations of non regulatory T cell lineages in patients with RRMS. Studies have found shared T cell receptor repertoires from activated $CD4^+$ and $CD8^+$ T cells, in the brain tissue of patients with MS, that are present in the periphery (157). These cells have acquired the ability to target self antigens, migrate to the CNS, clonally expand, and potentially propagate inflammation. We did not enrich for peripheral $CD4^+$ and $CD8^+$ T cell populations that can specifically target CNS peptides or examine immune cells in the CSF specifically in this study so we present this outcome as only one of a set of potential endpoints. These cells could potentially contribute to the relapse rate in patients and their disability status due to their functions as expanded clones in the CNS and thus drive disease progression on the aggregate. We suggest that it would be advantageous to constrain the amount of IL-2 in patients that showed the highest IL-2 dependent responses as this could potentially serve as a valuable course of therapeutic intervention that could decrease this aberrant ability of activated $CD4^+$ and $CD8^+$ T cells in the periphery to proliferate. There might also

be a role for the introduction of regulatory populations of Tregs that could be provided therapeutically that could contribute to peripheral tolerance in these individuals by acting as a sink for excess IL-2, that could be used by effector lineages to propagate and potentially cause inflammation.

From the perspective of IFNγ, we also saw a broad STAT1 response from the $CD4^+$ and $CD8^+$ T lymphocyte subsets that were stimulated with IL-2. The data suggest that IFNγ dependent STAT1 activation is modulated by IL-2. We also saw that IL-2 induced IFNγ dependent STAT1 activation was elevated in $CD4^+$ T lymphocytes, as compared to controls, while only naïve $CD8^+$ T lymphocytes showed enhancement in their IL-2 induced IFNγ dependent STAT1 activation. This is in contrast to the data for STAT5 activation that show a hyperactive IL-2 dependent STAT5 activation in some $CD4^+$ and $CD8^+$ T lymphocytes.

$CD4^+$ T lymphocyte cell subsets from patients with RRMS showed a hyperactive STAT1 response when stimulated with IFNγ/IL-2 as compared to healthy controls. Most $CD8^+$ T lymphocyte cell subsets showed a similar level of STAT1 activation when stimulated with IFNγ/IL-2 as compared to healthy controls. These data show that there is potentially a regulatory mechanism between the acute activation of IFNγ induced STAT1 and the ability of IL-2 to modulate STAT1 levels. IL-2 has not been shown to be able to activate STAT1 activation directly, as it signals primarily through STAT5. This data is both novel and intriguing as there are reports in the literature that suggests pathways by which IL-2 can induce STAT1 signaling indirectly through the actions of GSK3, which is a signaling pathway downstream of IL-2R activation (156). This hypothesis was not directly

tested here but would provide data that could be important in understanding the role that IL-2 can have on immune cells populations in the context of RRMS. Moreover, the implications of this data would be directly clinically relevant in MS as polymorphisms in gene locus for both of these cytokines have been shown to contribute to disease susceptibility (154, 155). We surmise that the hyperactive IFNγ/IL-2 response in $CD4^+$ T lymphocyte subsets in RRMS would potentially contribute to inflammation as a subset of these cells will have the capacity to produce IFNγ and hyperactivate IL-2. These cells have the potential to indirectly increase antigen presentation and cytokine production in APCs, through IFNγ's action on these cells. Also, IFNγ increases the transcription of iNOS that can cause inflammation and the propagation of damaging free radical species (158). Overall, this would contribute to relapses and disability in patients with RRMS in a $CD4^+$ T lymphocyte dependent manner. Many of the DMTs that are currently available might have the potential to be an effective solution for these aberrant inflammatory cells as they have been shown to be effective at sequestering or killing mature activated adaptive immune cells (25). We also see a greater therapeutic potential from the implication of this research. The notion that high levels of IFNγ and IL-2 in specific patient populations show this hyperactive IL-2 induced IFNγ dependent STAT1 response could contribute to disease pathogenesis and could serve as an effective biological marker that clinicians could use to steer patient's treatment to DMTs that are most useful in decreasing $CD4^+$ T cell lineages. This would be directly in opposition to those DMTs that are thought to exert their effects primarily on cells of the innate immune system.

This data extends over the course of 4 hours of stimulation, up to 24 hours of stimulations in the case of specific experiments, with either IFNγ or IL-2, in such a way that the different T cell subsets have appeared to have different stimulation dynamics that are affected by the presence of IL-2. This data furthers the notion that the cytokine microenvironment found, even peripherally, can influence the overall stimulation and activation of these cells in vivo in the context of RRMS. Moreover, our study shows that the specific response, guided by the milieu of cytokines and immune cells present in the microenvironment can have divergent effects on $CD4^+$ and $CD8^+$ T lymphocyte subsets from different patient populations. It is important to also consider the impact that other peripheral blood populations could potentially have on the different T cell subsets in our experiments. There is a role for macrophage/monocyte populations to act as APCs and also produce cytokines, although we believe that this is not the most prevalent explanation for the induction of different T cell subsets in our experiments due to the low frequency with which these cells are found in the peripheral blood (4%-10% reference range) and much lower proportion per experimental well (159). We acknowledge the potential for monocytes/macrophages to produce IFNγ and/or IL-2 that can influence the overall activation and stimulation of $CD4^+$ and $CD8^+$ T cell subsets within our experimental design. Also, naïve $CD4^+$ and $CD8^+$ T cells both proliferate and produce IL-2. These naïve $CD4^+$ and $CD8^+$ T cells could also potentially stimulate one another through cytokine secretion, irrespective of exogenous stimulation provided experimentally. We would endeavor, therefore, in the future to better ascertain the role that the microenvironment and associated immune cell

populations in the context of these ex-vivo experiments can provide reliable information about the role of the microenvironment in the context of patients.

Our studies also showed data that suggests that regulatory T lymphocytes in patients with RRMS exhibit profound phenotypic differences when stimulated with IL-2, as compared to regulatory T lymphocytes from healthy individuals. These regulatory populations were similar with respect to their initial level of IL-2 induced STAT5 activation, as well as the proportion of these cells isolated ex-vivo, but they did exhibit different patterns of phenotypic marker expression through unbiased approaches. These differences examined can be critical due to the important role that this cellular population plays in promoting immunoregulation in the context of RRMS and autoimmunity in general. There is also a role for the data observed in this cellular subset to better inform both the characterization and knowledge of the impact that IL-2 and IFNγ can have on Tregs in a variety of contexts. We will continue this line of investigation into identifying a functional signature of the subset of Tregs. It is still not completely appreciated if these cells play a role in constraining the hyperactivity of the non-regulatory cell populations shown in the data or if this population can effectively contribute to activation directly. There is also another area of interest that lies in examining the direct influence that the products of these Treg populations can have on one another. Logically, it would increase our understanding of the intrinsic plasticity of these cell populations through experiments that could examine the transfer or addition of media primed from healthy individuals supplied to Tregs from patients with RRMS, as well as the opposite experiment where RRMS primed media is provided to

healthy controls. We surmise that these types of mimic experiments would be informative about the nature in which the potential of pharmacological intervention could be used to influence either population towards a clinically targetable outcome.

Although the RRMS patient cohort examined in this work received their diagnosis relatively recently and do not overtly show extensive clinical deficits, these early untreated patients do exhibit a relative disruption in their effector T cell/Treg balance that is found in many autoimmune disorders. The dysfunction of this balance details how even in early autoimmune disease processes, specifically RRMS, we see that chronic derangements in peripheral tolerance can precede progressive forms of disease and disease pathogenesis. There were no specific constellations of clinical derangements that were found to have an association with a specific outcome of STAT activation, as many of the patients had mild but varied symptoms. This observation, in conjunction with the hyperactivity found in the context of specific RRMS patients' $CD4^+$ and $CD8^+$ T lymphocyte subsets could be an interesting method in which to test whether ameliorating persistently high levels of STAT5 or STAT1 might potentially impact patients symptoms in a broad nonselective way by decreasing the proliferation, inflammation, and thus the induction of these cellular lineages. Furthermore, these studies do not preclude a role for increased resistance to normal homeostatic methods of effector T cell regulation that enables these lineages to become inappropriately activated or the role for inadequate control of effector T cell function mediated by Tregs.

Recently, technological improvements have enabled researchers to apply principles of bioinformatics and parallel computing to the study of immune cells in the context of health and disease. Many of these techniques have the unique strength of being 1) highly effective at unbiasedly delineating related subsets of cells within a broader collection of cells assayed based upon marker expression and underlying cellular heterogeneity, 2) detecting rare populations of cells that otherwise might have been missed upon manual inspection, and 3) accommodating ever expanding datasets of biological data that needs to be processed efficiently (160). Our study utilized a select set of these technologies in order to better address the specific experimental hypotheses that we had and probe the data in ways that could uncover potential biologically relevant trends. Another benefit of the use of these technologies is that we were able to assess how disease, as an experimental variable, contributed to the results that we found throughout the data. There were significantly different responses across disease, as was predicted, due to the biological difference between the homeostasis and regulation of immune cells from healthy individuals and those that have MS. These methods broadly enhance our ability to critically analyze massive amounts of multidimensional data on a rigorous and efficient basis unbiasedly. The major technical limitations of these approaches are that they are computationally intensive, the interpretation of the data is dependent upon marker selection, and software acquisition and availability. Also due to the unsupervised nature of these analysis, the appropriate preprocessing of the input data and input parameters are necessary to ensure that there is sufficient ability for rigorous reproducibility. We

ensured that this was the case in our experiments by making sure to control all experiments for cell count across samples analyzed, exclude all doublets and dead cells using size and viability markers, and balance our experimental design across disease to avoid batch effects. We also set the same seed throughout our analysis in order to provide for reproducibility in the outcome of the mathematical computations performed on our datasets.

One such method that we utilized was Barnes-Hut-t-distributed stochastic neighbor embedding (Barnes-Hut-t-SNE). tSNE is a technique that attempts to approximate the intrinsic structure and heterogeneity of data that is represented across many dimensions into a single two dimensional visualization. tSNE was updated from a previous approach, SNE, that sought the same goal of approximating the similarity across different points on a multidimensional distribution into a two dimensional space. A major benefit between SNE and t-SNE is represented by the fact that t-SNE has the underlying data points, in our study this data was individual immune cells, centered on a t-distributed distribution, instead of a Gaussian distribution. The reason that this change in the visualization is beneficial is the fact that t-SNE has a lower tendency of crowding point erroneously in the center of the visualization, irrespective of the intrinsic relatedness of the input data (161). Moreover, the specific implementation of t-SNE used was Barnes-Hut-t-SNE due to refinements that were made that enable the more efficient use of computational resources by relying on the formation of vantage-point trees to compute the initial pairwise similarities (162). Barnes-Hut-tSNE enabled us to look specifically at the cellular heterogeneity that exists in the

STAT response across patient and control cohorts, either when stimulated with IFNγ/IL-2 or at basal levels. We saw substantial trends in cellular heterogeneity that could potentially be useful as biological markers of the response of non regulatory and regulatory populations of $CD4^+$ and $CD8^+$ T lymphocytes to IFNγ and/or IL-2 stimulation ex vivo. A majority of the T lymphocytes that were isolated from individuals with RRMS had undergone previous antigen challenge, as inferred by their loss of $CD45RA^+$ and apparent central memory phenotype. Using the strengths of this approach to better appreciate individual differences within cellular subsets could be important in the goal of contributing to personalized therapeutic strategies in individual patients with RRMS. This paired with the use of self-reactive antigen specific targets or techniques to enrich for self-reactive cells can be particularly insightful clinically.

Another method that we used was FlowSOM. FlowSOM is another unsupervised clustering algorithm that is used to approximate a visual representation of multidimensional data in a two dimensional space (143). FlowSOM also utilizes hierarchical clustering methods, similar to Barnes-Hut-t-SNE, although the exact methods used to reach the final clustered populations are different as the algorithms differ and the distributions that each are based on are different (t distribution vs. F distribution). Nonetheless, FlowSOM is much more effective at capturing the relative expression of multiple markers across various groups of cells and depicting this data for each group. This is because after data are clustered there is an unsupervised processing step that depicts the aggregate relative expression of the staining parameters across each group in a pie chart. It

is then much easier for interpretation of major differences in these staining parameters across the clustered groups. We utilized this advantage of FlowSOM to address how the phenotypic expression of CD45RA/CCR7 correlated with the IFNγ/IL-2 response across disease and stimulation duration in non regulatory and regulatory populations of $CD4^+$ and $CD8^+$ T lymphocytes. In this way, we were able to appreciate the role of how these populations of cells might be functionally influenced by acute and chronic STAT activation.

These hypotheses, as well as the mechanisms through which they might operate, are all areas of open investigation in early untreated patients with RRMS. Again, an important limitation of the study is that the results provided here do have to be viewed on aggregate, as we did not currently perform assays that allow for the segregation and effective enrichment of T lymphocyte populations that respond specifically to myelin proteins found in the human CNS. Future experiments that would seek to uncover the specific T cell clones that are most contributory to the hyperactivity profile seen in effector lineages from $CD4^+$ and $CD8^+$ T lymphocytes in patients with RRMS or the hypoactive profile seen from $CD4^+$ regulatory subsets in RRMS could also shed light on how these antigenic peptides can help to guide a dysregulated immune response due to the impact that they play on gene transcription in the context of disease.

In conclusion, this study examined differential IFNγ/IL-2 induced activation responses in $CD4^+$ and $CD8^+$ T lymphocyte subsets in the context of RRMS patients and healthy controls that helped to elucidate STAT dependent phenotypic and functional outcome, irrespective of clinical disease measures that assay

disease accumulation. We find that IL-2 can induce the enhancement of STAT5 dependent signaling and elevate IFNγ dependent STAT1 signaling in patients that have RRMS. The enhancement of IL-2 dependent STAT5 signaling in nonregulatory lineages and hypoactivation of IL-2 dependent STAT5 signaling in regulatory lineages can potentially contribute to an overall imbalance between effector function and peripheral tolerance mechanisms in patients with RRMS. Moreover, the ability of IL-2 to elevate IFNγ dependent STAT1 signaling can lead to exacerbated T cell specific autoimmune responses in patients with RRMS that can increase disease burden. In future studies, it will be important to delineate the specific responses of self reactive $CD4^+$ and $CD8^+$ T cell niches in patients with RRMS and develop strategies that constrain these populations specifically. Overall, these studies indicate that IL-2 can have a profound influence on JAK/STAT dependent signaling pathways in $CD4^+$ and $CD8^+$ T lymphocytes subsets that can potentially perpetuate inflammation, disease associated morbidity, and disease progression. We suggest that similar approaches should be utilized to provide for biological markers of STAT dependent signaling pathways in $CD4^+$ and $CD8^+$ T lymphocytes subsets, in order to better appreciate the role that these pathways contribute to phenotype and function longitudinally in patients with RRMS.

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APPENDIX

INSTITUTIONAL REVIEW BOARD APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Office of the Institutional Review Board for Human Use

470 Administration Building
701 20th Street South
Birmingham, AL 35294-0104
205.934.3789 | Fax 205.934.1301 |
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Approval Letter

To: Meador, William Ray

FROM: University of Alabama at Birmingham Institutional Review Board
Federalwide Assurance # FWA00005960
IORG Registration # IRB00000196 (IRB 01)
IORG Registration # IRB00000726 (IRB 02)
IORG Registration # IRB00012550 (IRB 03)

DATE: July 9, 2020

RE: IRB-140912002
UAB Transverse Myelitis Association Patient Registry

The IRB reviewed and approved the Personnel Amendment submitted on Jul 2 2020 for the above referenced project. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services.

Type of Review: Expedited
Expedited Catergories: 5,
Determination: Approved
Approval Date: Jul 8 2020
Expiration Date: Jun 24 2021

The following populations are approved for inclusion in this project:

Children – CRL 1

The following apply to this project related to informed consent and/or assent:

Waiver of Informed Consent,Waiver of HIPAA

To access approved documents and/or the stamped consent/assent forms:

1.Open your protocol in IRAP.

2.On the Submissions page, open the submission corresponding to this approval letter. NOTE: The Determination for the submission will be "Approved."

3.In the list of documents, select and download the desired approved documents. The stamped consent/assent form(s) will be listed with a category of Consent/Assent Document (CF, AF, Info Sheet, Phone Script, etc.).