$CD4^+$ AND $CD8^+$ NAÏVE T-CELL HOMEOSTASIS IN PRIMARY PROGRESSIVE MULTIPLE SCLEROSIS

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ABSTRACT

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Multiple Sclerosis (MS) is a chronic inflammatory and demyelinating disease of the central nervous system. The etiology of MS is unknown but many researchers believe that it is autoimmune mediated. This study investigated naïve $CD4^+$ and naïve $CD8^+$ T-cell homeostasis in patients with Primary Progressive Multiple Sclerosis and Relapsing Remitting Multiple Sclerosis. The naïve T-cell compartment involves a balance between thymic production of naïve T-cells, homeostatic proliferation and the delivery of death and survival signals. Naïve T-cell production was quantified by measuring signal joint T-cell receptor excision circles (sj-TRECs); episomal byproducts formed during V(D)J T-cell receptor rearrangement.

Homeostatic proliferation was quantified by flow cytometry analysis of % expression of CD31 and Ki-67. CD31 is a marker found on $CD4^+$ recent thymic emigrants (RTE) but not on naïve T-cells that have undergone homeostatic proliferation. CD31 can be used as a marker of the proliferation history of naïve $CD4^+$ T-cells. Ki-67 is a nuclear and nucleolar antigen found in actively cycling cells. It can be used as a marker of cell proliferation at the moment of isolation. Cell survival was measured by quantifying plasma IL-7 levels and by measuring Bcl-2 expressions. IL-7 plays an important role in maintaining and restoring peripheral naïve T-cell homeostasis. It stimulates naïve T-cell proliferation and prevents the reduction of Bcl-2, an antiapoptotic protein.

In this study, PPMS patients had significantly reduced naïve $CD4^+$ T-cell sj-TRECs compared to healthy controls ($p = 0.0007$) and compared to RRMS patients ($p = 0.0010$). RRMS patients had fewer sj-TRECs than healthy controls but this difference was not significant ($p = 0.4652$). Similarly, in PPMS, naïve $CD4^+$ T-cells had significantly lower CD31 expression than healthy controls ($p = 0.0017$) and RRMS patients ($p = 0.0032$). This finding indicates increased homeostatic proliferation in naïve $CD4^+$ T-cells in PPMS, most probably a response to decreased thymic export as marked by the decreased naïve $CD4^+$ T-cell sj-TRECs. % CD31 expression in naïve $CD4^+$ T-cells did not differ significantly in RRMS compared to healthy controls ($p = 0.7455$) which is consistent with their naïve $CD4^+$ sj-TREC levels.

Naïve $CD8^+$ T-cell sj-TRECs were significantly reduced in PPMS patients compared to healthy controls ($p = 0.0212$) but not compared to RRMS patients ($p = 0.2379$). RRMS patients had fewer naïve $CD8^+$ T-cell sj-TRECs compared to healthy controls but this difference was not significant ($p = 0.1517$). PPMS patients expressed increased Bcl-2 levels in their naïve $CD8^+$ T-cells. This finding indicates upregulation of survival signals, most probably a consequence of reduced thymic export of naïve $CD8^+$ T-cells.

The data from this study indicate that PPMS is different from RRMS in their naïve $CD4^+$ T-cell sj-TRECs and naïve $CD4^+$ T-cell % CD31 expression but is similar to RRMS in their naïve $CD8^+$ T-cell sj-TRECs. This study concludes, therefore, that both PPMS and RRMS patients have altered naïve T-cell homeostasis.

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EXTRAIT

Homéostasie des lymphocytes T- $CD4^+$ et T-$CD8^+$ naïfs

chez les patients atteints de sclérose en plaques progressive primaire

Par : Jessica Hackenbroch

La sclérose en plaques (SP) est une maladie inflammatoire et de démyélinisation chronique du système nerveux central. L'étiologie de la SP est encore inconnue mais de nombreux chercheurs croient qu'elle est auto-immune. La présente étude s'est penchée sur l'homéostasie des lymphocytes T-$CD4^+$ et T-$CD8^+$ naïfs chez les patients atteints de sclérose en plaques progressive primaire et de sclérose en plaques cyclique. Le compartiment du lymphocyte T naïf implique un équilibre entre la production thymique de lymphocytes T naïfs, la prolifération homéostatique, et l'émission de signaux de mort et de survie. On a quantifié la production de lymphocytes T naïfs en mesurant les cercles d'excision du récepteur des cellules T sj-TREC, des sous-produits épisomiques formés durant le réarrangement de récepteurs des lymphocytes T V(D)J.

On a quantifié la prolifération homéostatique par analyse de cytométrie de flux exprimée en pourcentage de CD31 et de Ki-67. CD31 est un marqueur qu'on retrouve sur les récents émigrants thymiques $CD4^+$ mais non pas sur les lymphocytes T naïfs qui ont fait l'objet d'une prolifération homéostatique. CD31 peut s'utiliser comme marqueur de l'historique de prolifération des lymphocytes T-$CD4^+$ naïfs. Ki-67 est un antigène nucléaire et nucléolaire qui se retrouve dans les cellules en cycle actif. On peut l'utiliser comme marqueur de prolifération cellulaire au moment de l'isolement. On a mesuré la survie des cellules en quantifiant les niveaux de plasma IL-7 et en mesurant les expressions de Bcl-2. IL-7 joue un rôle important dans le maintien et la restauration de l'homéostasie périphérique des lymphocytes T naïfs car il stimule la prolifération des lymphocytes T naïfs et prévient la réduction de Bcl-2, une protéine anti-apoptotique.

Dans le cadre de la présente étude, les patients atteints de sclérose en plaques progressive primaire affichaient des cellules sj-TREC des lymphocytes T-CD4$^+$ considérablement réduits comparativement aux groupes témoins en santé ($p = 0,0007$) et aux patients atteints de sclérose en plaques cyclique ($p = 0,0010$). Les patients atteints de sclérose en plaques cyclique affichaient moins de cellules sj-TREC que les groupes témoins en santé mais cet écart n'était pas important ($p = 0,4652$). De même, dans le cas de la sclérose en plaques progressive primaire, les lymphocytes T-CD4$^+$ naïfs démontraient une expression de CD31 plus faible que les groupes témoins en santé ($p = 0,0017$) et les patients atteints de sclérose en plaques cyclique ($p = 0,0032$). Cette conclusion indique une prolifération homéostatique accrue des lymphocytes T-CD4$^+$ naïfs chez les patients atteints de sclérose en plaques progressive primaire, fort probablement une réaction à l'exportation thymique réduite comme l'indique la diminution du nombre de cellules sj-TREC des lymphocytes T-CD4$^+$ naïfs. L'expression en pourcentage de CD31 chez les lymphocytes T-CD4$^+$ naïfs ne différait pas de beaucoup chez les patients atteints de sclérose en plaques cyclique comparativement aux groupes témoins en santé ($p = 0,7455$), ce qui est cohérent avec leurs niveaux de cellules sj-TREC des lymphocytes T-CD4$^+$ naïfs.

Les cellules sj-TREC des lymphocytes T-CD8$^+$ naïfs ont été considérablement réduits chez les patients atteints de sclérose en plaques progressive primaire comparativement aux groupes témoins en santé ($p = 0,0212$) mais non pas comparativement aux patients atteints de sclérose en plaques cyclique ($p = 0,2379$). Les patients atteints de sclérose en plaques cyclique affichaient un moins grand nombre de cellules sj-TREC des lymphocytes CD8$^+$ comparativement aux groupes témoins en santé mais cet écart n'était pas important ($p = 0,1517$). Les patients atteints de sclérose en plaques progressive primaire ont affiché des niveaux de Bcl-2 accrus dans leurs lymphocytes T-CD8$^+$ naïfs. Cette conclusion indique une régulation à la hausse des signaux de survie découlant fort probablement d'une exportation thymique réduite de lymphocytes T-CD8$^+$ naïfs.

Les données recueillies à la suite de cette étude indiquent que la sclérose en plaques progressive primaire diffère de la sclérose en plaques cyclique sur les plans des cellules

sj-TREC des lymphocytes T-CD4$^+$ naïfs et de l'expression en pourcentage de CD31 des lymphocytes T-CD4$^+$ naïfs mais qu'elle est semblable à la sclérose en plaques cyclique sur le plan des cellules sj-TREC des lymphocytes T-CD8$^+$ naïfs. Cette étude conclue donc que tant les patients atteints de sclérose en plaques progressive primaire que ceux atteints de sclérose en plaques cyclique affichent une homéostasie des lymphocytes T naïfs modifiée.

Chapter 1: Introduction and Literature Review

Chapter 1:
INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Multiple Sclerosis (MS) is a chronic inflammatory and demyelinating disease of the central nervous system (CNS), i.e. the brain and spinal cord.[1] An anti-myelin autoimmune response occurs in MS patients and its animal models.[2] The etiology of MS remains unclear but current data suggest that the disease develops in genetically susceptible individuals but requires additional environmental triggers.[3] Multiple Sclerosis can lead to substantial disability through deficits of sensation and of motor, autonomic, and neurocognitive function.[3]

The prevalence of MS in the world varies between 60-200/100,000 in Northern Europe and North America and 6-20/200,000 in low risk areas such as Japan.[3] First degree relatives of affected individuals have a 2-5% increased risk of developing MS and concordance rates between monozygotic twins are ~25%.[4] The prevalence of MS shows a latitudinal gradient in the Northern and Southern hemispheres, a finding that many think supports an environmental component in susceptibility to MS. Among proposed environmental factors, infectious, behavioral or lifestyle influences have been proposed to induce or contribute to disease expression.[5]

Primary Progressive Multiple Sclerosis (PPMS), one of four subtypes of MS, is typically perceived as having the most devastating natural history with no available therapies. Nevertheless, it has been studied the least historically.[6] Possible reasons include the difficulty in recruiting these patients, since the frequency of PPMS is much less than relapsing remitting multiple sclerosis (RRMS). PPMS patients also tend to be older than RRMS patients with increased disabilities making it more difficult for them to participate in studies. In spite of these obstacles, it is important to continue broadening the current knowledge of PPMS.

MS is highly heterogeneous in terms of its genetics, clinical course[7], pathology, immunology[8,9] and response to treatment[10,8]. This raises the question as to whether MS represents one or several distinct entities. In an attempt to partly address this question, this study investigated several aspects of $CD4^+$ and $CD8^+$ naïve T-cell homeostasis in patients with PPMS, compared to RRMS and healthy controls. More specifically, it investigated thymic production of naïve-T cells, naïve-T cell homeostatic proliferation and survival.

Chapter 1 begins by reviewing the four clinically defined subtypes of MS with an emphasis on PPMS. The chapter then delves into a discussion about the immune system, the diversity of the naïve-T cell receptor repertoire, naïve T-cell homeostasis and how to study naïve T-cell homeostasis.

Chapter 2 reviews the materials and methods used. This includes: the patient inclusion criteria, cell isolation and staining methodology, whole blood staining methodology, flow cytometry analyses, ELISA tests and the procedures involved in sj-TREC quantification by real-time PCR. This chapter will conclude with a discussion of the statistical methods employed.

Chapter 3 will provide the results from the phenotype studies by flow cytometry, the sj-TREC quantification results as well as the ELISA test results. Statistical comparisons between patient and control groups will be analyzed.

The final chapter will attempt to explain the results mentioned in chapter 3 as well as its implications. A discussion relating to other studies will be reviewed. Future research directions will also be stated.

1.2 Clinically Defined Subtypes of MS

MS follows four different clinical patterns as defined by the international survey of neurologists in 1996:[7]

Relapsing Remitting Multiple Sclerosis (RRMS) describes the initial course of 80-90% of MS cases. This form of MS is characterized by relapses/attacks followed by periods of remission. Attacks are defined as neurological disturbances which last a minimum of 24 hours and do not include pseudoattacks. Two separate attacks are defined by a time difference of 30 days between onset of event 1 and onset of event 2.[11] RRMS patients are usually diagnosed between the ages of 20 and 30 and twice as many women are affected as men.

Secondary Progressive Multiple Sclerosis (SPMS) describes patients who initially followed a relapsing-remitting MS course but have now developed a progressive neurological decline without definite periods of remission. This subtype describes 80% of RRMS patients,[7] and typically occurs 10-15 years after the onset of RRMS.

Progressive Relapsing Multiple Sclerosis (PRMS) describes patients who follow a progressive neurological decline from the onset of their disease but also have superimposed attacks. Although there may be some recovery immediately following an attack, there is a gradual worsening of symptoms and signs between these relapses.

Primary Progressive Multiple Sclerosis (PPMS), present in ~10-20% of MS cases, describes patients who follow a progressive form of the disease from its onset with no relapses. PPMS patients are diagnosed at a later age than RRMS patients, typically in the late thirties and early forties, and men are as likely to develop the disease as women.

1.3 Primary Progressive Multiple Sclerosis

As mentioned earlier, PPMS patients tend to have a later age of onset than the general MS population and the female to male ratio is equivalent. However, there are many other additional characteristics that distinguish this group of patients from RRMS. Some claim that the HLA profile is different in PPMS than in RRMS.[12] The most frequent immunological abnormality in PPMS is the increased synthesis of IgG in the cerebral spinal fluid (CSF) and the appearance of oligoclonal bands.[13] In terms of pathology, PPMS patients seem to have less inflammation in the brain, as seen by gadolinium-enhancing MRIs, than do RRMS patients. PPMS patients have been reported to have fewer and smaller focal brain lesions than RRMS patients, as well as the development of fewer new lesions over time. However, the focal lesion load in the spinal cord is similar in RRMS and PPMS. Thus, in general, the proportion of total MRI lesions in the spinal cord is higher in PPMS compared to RRMS.[14,15] Furthermore, the expression of adhesion molecules on blood leukocytes has also been reported to be different in PPMS compared to RRMS.[16] The most common presenting feature in PPMS is a slowly progressing spastic paraparesis rather than the non-visible sensory symptoms such as optic neuritis that are common in RRMS.[17] There are also no available treatments for PPMS patients.

PPMS is more difficult to diagnose than RRMS for several reasons: First, the clinical course that RRMS follows is uncommon in other neurological diseases; whereas the progressive pattern seen in PPMS is typical of many other neurological diseases. Second, there is a smaller variety of clinical symptoms and signs in PPMS which reduces the distinctiveness of this group.[18] Thirdly, the increased IgG synthesis typical of PPMS occurs less frequently in male patients with late disease onset and progressive myelopathy.[13] This makes diagnosis difficult particularly since the new diagnostic criteria for PPMS includes a positive CSF, as mentioned in the next section. Finally, the fact that there are fewer and smaller focal lesions in PPMS by MRI, compared to RRMS, as mentioned above, also increases the difficulty of diagnoses. The failure of MRI findings to correlate with the clinical phenotype of these patients further challenges clinicians to make a correct patient diagnosis.

1.3.1 The McDonald Criteria for PPMS Patients

In 2001, the International Panel on the Diagnosis of Multiple Sclerosis set out new diagnostic criteria which integrated MRIs into the diagnostic formulation and included for the first time, a scheme for diagnosing PPMS. These criteria are termed the McDonald Criteria.[6,10]

According to these criteria, a clinical presentation with an insidious neurological progression suggestive of MS must be present in PPMS patients. In addition, patients must show a positive CSF, signified either by increased oligoclonal IgG bands in the CSF but not the serum, or by an increased IgG index. Furthermore, patients must also show lesion dissemination in space and dissemination in time, which is also defined by specific criteria. If the criteria are not all met, the diagnosis is "possible MS." [10]

1.4 The Immune System

The immune system consist of the Innate Immune System and the Adaptive Immune System, both working together synergistically to mediate host responses to infection and tissue injury. The innate immune system includes a collection of host defenses that range from the non-specific barrier of the skin to the highly selective recognition of pathogen through the use of germ-line encoded receptors.[19] The cells of the innate immune system include dendritic cells, monocytes, macrophages, granulocytes and natural killer T-cells. The adaptive immune, in contrast, uses somatically rearranged antigen receptor genes to create receptors for nearly any antigen. This response is slower but more flexible and can fight infections that have escaped the innate immune response.[19] The adaptive immune system includes Humoral Immunity, in which B cells produce antibodies, as well Cellular Immunity which involves T-helper cells ($CD4^+$) and T-cytotoxic cells ($CD8^+$).

1.4.1 $CD4^+$ and $CD8^+$ T Lymphocytes

The main role of $CD4^+$ T-cells is to help in the generation of an immune response. Most antigens (Ags) that elicit an immune response are thymic dependent and require $CD4^+$ T-cells to initiate an immune response. For many years $CD4^+$ T-cells were divided into two fundamental populations termed Th1 and Th2, based on their role in the immune response and the cytokines they produced. More recently, a third major subtype of $CD4^+$ T-cells has been defined, termed Th17 $CD4^+$ T-cells. Some of the functions originally attributed to Th1 cells are now thought to be mediated, at least in part, by this third T-cell population. [20]

$CD8^+$ T-cells are cytotoxic and kill the cells to which they bind. Every nucleated cell in the body expresses major histocompatability complex (MHC) class I molecules. $CD8^+$ T-cells cells bind antigenic epitopes consisting of Ag lying in the groove of MHC class I molecules. These T-cells normally monitor cells of the body and destroy cells that present foreign peptides, such as viral antigens. When $CD8^+$ T-cells recognize Ag, they undergo clonal expansion followed by differentiation into effector cells. Effector cells exocytose cytoplasmic granules containing perforin and granzymes. Perforin molecules insert themselves into the plasma membrane of target cells which allows granzymes to enter these cells, resulting in apoptosis. Effector cytotoxic cells may otherwise kill target cells via their transmembrane protein FasL. This binds to receptor Fas on target cells and induces apoptosis.

1.4.2 $CD4^+$ and $CD8^+$ T Lymphocytes in MS

Many investigators believe that MS is an autoimmune mediated disease.[2,21] This view is based on the cellular composition of the brain and cerebrospinal fluid (CSF) as well as the data from the animal model of MS, experimental allergic encephalomyelitis (EAE).[22] In this model, the injection of myelin components into a susceptible animal, leads to an autoimmune response mediated by $CD4^+$ T-cells; similar to that of MS.[22] EAE can be transferred to naïve animals by in vitro transfer of autoreactive $CD4^+$ T-cells but EAE

could not be transferred by antibodies.[22] Extrapolation from animal models led to the hypothesis that MS is a T-cell mediated autoimmune disease. However, problems with this model reflect the use of inbred mouse strains that are genetically similar and show a marked restriction of T-cell receptor (TCR) gene usage. Furthermore, EAE studies in marmoset monkey outbred animals, failed to show restricted TCR gene usage. Studies in MS patients show that $CD4^+$ TCRs are oligoclonal and restricted in some MS patients but diverse between patients.[23] Identical twins discordant for MS have a shift in the overall diversity of their naïve $CD4^+$ TCR J beta repertoires.[24] $CD8^+$ T-cells have also been shown to contribute to demyelination and neurological disease in the viral model of MS, Theiler's murine encephalomyelitis virus (TMEV).[25] Furthermore, human oligodendrocytes were shown to be capable of expressing MHC class I molecules in vitro[26] and susceptible to lysis by $CD8^+$ T-cells specific for myelin basic protein.[27] Good evidence that MS is autoimmune-mediated comes from the work of Lucchinetti et al.[28] These investigators examined numerous autopsy brains and biopsies of MS patients. They identified four different patterns. The first two are by far the most common and show infiltrations by T-cells and macrophages or deposition of antibody and compliment. They argued that all patterns, and certainly the most common patterns, are mediated by T-cells. In view of the literature indicating the importance of helper T-cells and cytotoxic T-cells in MS pathogenesis, this study aimed to incorporate both groups of cells.

1.4.3 Regulatory T Cells (nTregs)

Immunological tolerance and protection from autoimmunity are conferred by central and peripheral mechanisms such as clonal deletion of self-reactive T-cells in the thymus and the induction of anergy upon encounter with autoantigens in the periphery.[29] More recently, the importance of specialized suppressor T-cells has been recognized in the maintenance of peripheral immune tolerance. One important subset is the naturally occurring regulatory T-cells (nTregs). They are $CD4^+$ T-cells identified by their expression of the interleukin-2 (IL-2) receptor alpha chain, CD25. These $CD4^+CD25^+$ T regulatory cells comprise 5-10% of peripheral $CD4^+$ T-cells in humans.[30] Unlike

conventional T cells, CD25 is constitutively expressed at a higher mean density, even on resting nTregs.[30] These cells are capable of suppressing immune cells from the innate and adaptive immune system but the mechanism of suppression still remains unclear. Nevertheless, there is ample evidence that cell-cell contact mediates suppression.[31]

nTregs are often associated with autoimmune diseases. In RRMS, nTregs have a diminished ability to suppress conventional T-cell proliferation and cytokine production.[32] However, the frequencies of nTregs in blood and CSF are similar in RRMS and healthy controls.[33] Other autoimmune diseases such as Kawasaki disease, or mucocutaneous lymph node syndrome, an immune vasculitis occurring in children under the age of five, show a significantly lower frequency of nTregs in peripheral blood mononuclear cells (PBMC), compared to healthy controls.[34] Acute phase patients also had lower absolute numbers of nTregs.[34]

1.5 T-cell Receptor Generation

The immune system is capable of recognizing an array of human pathogens. This is achieved by ensuring a vast diversity of T-cell receptors (TCR) expressed on naïve $CD4^+$ and $CD8^+$ T-cell lineages of the adaptive immune system. The majority of T-cells express alpha/beta ($\alpha\beta$) TCR heterodimers with only 0.5%-10% of peripheral T-cells expressing gamma/delta ($\gamma\delta$) TCR heterodimers.[35] The TCR is composed of a variable domain- responsible for antigen recognition, and a constant domain. The antigen-recognizing variable domain of the α and β chains are encoded by combinations of variable (V), diversity (D), and joining (J) gene segments in the TCRβ chain and V and J gene segments in the TCRα chain. (Figure 1)

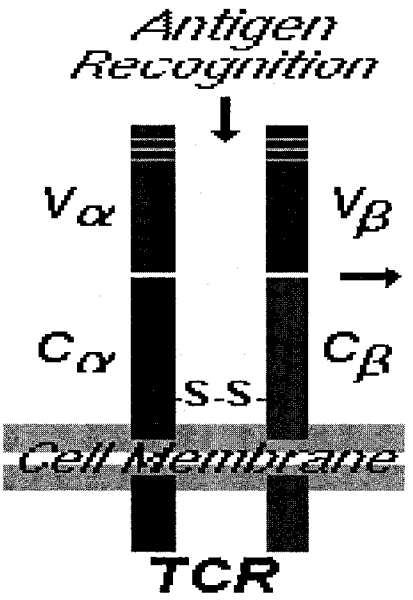


Figure 1: The T-Cell Receptor

The huge heterogeneity of TCR is not encoded in the germ line but is rather generated during T-cell differentiation in the thymus.[36] The developmental process begins when T-cell progenitors migrate from the primary sites of haematopoiesis (embryonic yolk sac, fetal liver, then bone marrow) to the thymic cortex where they proliferate extensively.[36] Thymocytes then make intimate contacts with thymic epithelial cells and nurse cells which promote IL-7 synthesis and activate **r**ecombination **a**ctivating **g**enes: RAG1 and RAG2.[37] These proteins are important in initiating the recombination events. They recognize **r**ecombination **s**ignal **s**equences (RSS) that flank the coding V(D)J gene segments and introduce site-specific cleavages.[36] Fusion between distinct V, (D) and J gene segments contributes to a diverse TCR repertoire.

During any rearrangement process, the DNA located between the RSS is excised and circularized. The resulting fragment is called a **T**-cell **r**eceptor **e**xcision **c**ircle (TREC). During TCRα rearrangement, the TCRδ locus located within the α locus, is excised. This is an important step in thymocyte development and indicates definite commitment of the thymocyte to the αβ T-cell lineage.[36] The by-product of this rearrangement is termed a signal-joint TREC (sj-TREC) or δRec-ΨJα TREC. (Figure 2) The importance of this product will be discussed in the next section.

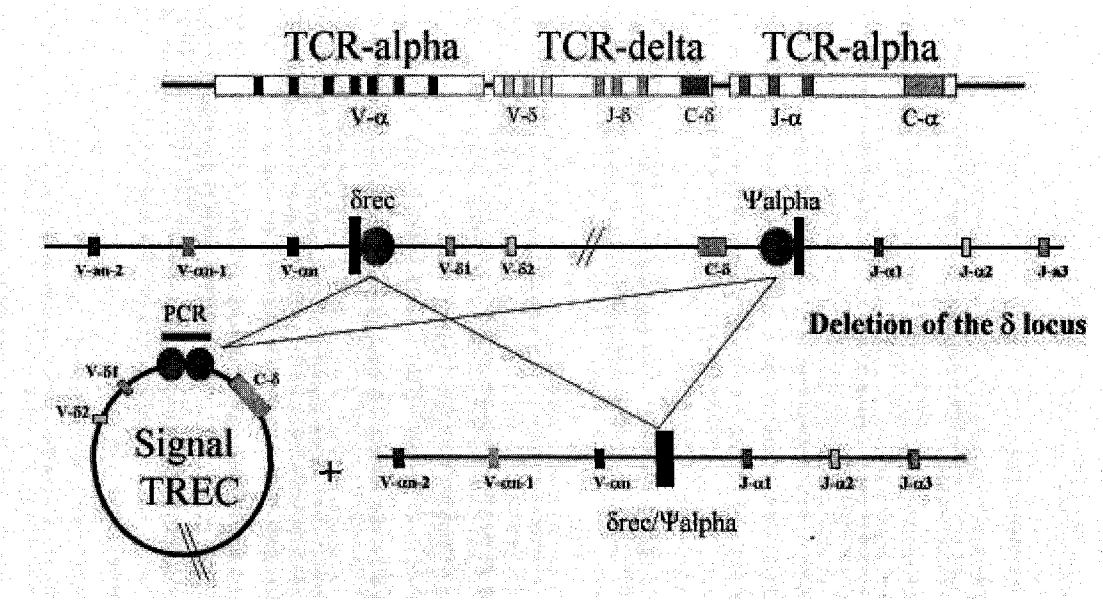


Figure 2: TCR- Rearrangement

1.6 Naïve T-Cell Homeostasis

The hypothesis that MS is a T-cell mediated autoimmune disease is complicated by the observation that potentially autoaggresive T-cells have also been found in healthy controls.[38,39] This raises the question as to whether a dysfunction in peripheral immune tolerance contributes to MS. In an attempt to address this question, this study investigated naïve T-cell homeostasis, which is one factor controlling activation of autoaggressive T-cells.[40] By definition, naïve T-cell homeostasis is a balance between thymic production, homeostatic proliferation of naïve T-cells, and the delivery of death and survival signals.[41]

1.6.1 Thymic Production

Naïve T-cells are T-cells that have not encountered antigen and typically express CD45RA. Memory T-cells represent T-cells that have been triggered by antigen and have enhanced capacity to respond to antigen upon re-exposure. These cells are typically $CD45RO^{+}$. Naïve and memory T-cells occupy separate homeostatic niches which are

vital to maintain both, a diverse naïve T-cell repertoire that can respond to invasion by unknown pathogens and a memory pool that can respond rapidly to previously encountered pathogens.[42] Memory cells occasionally revert from $CD45RO^+$ to $CD45RA^+$.[43] However, data from patients with viral infections, suggest that the majority of expanded $CD45RO^+$ T-cells die by apoptosis rather then revert to $CD45RA^+$.[44] In other words, reversion from $CD45RO^+$ memory cells to a $CD45RA^+$ naïve phenotype in humans is rare.

Naïve T-cells that have recently migrated from the thymus to the periphery are known as recent thymic emigrants (RTE). RTE leave the thymus with a specificity for antigens determined by the productive rearrangement and expression of the TCR genes.[45] Memory subsets have a restricted TCR repertoire as a consequence of antigenic stimulation. The accumulation of RTE provides a diverse T-cell pool capable of recognizing a vast array of pathogens and contraction of naïve T-cell diversity has been associated with autoimmune phenomena.[46] Therefore, constant thymic efflux is one way of ensuring naïve T-cell diversity and should be considered when assessing T-cell homeostasis.

Earlier, the importance of sj-TRECs during thymocyte development was stated. To reiterate, sj-TRECs are generated in double positive thymocytes ($CD4^+CD8^+$) by excising the TCR alpha locus during TCR gene rearrangements. sj-TRECs persist as circular, extrachromosomal, intracellular fragments in newly synthesized naïve $CD4^+$ and naïve $CD8^+$ T-cells.[47] sj-TRECs do not replicate during mitosis and, as a result, are diluted between daughter cells following proliferation.[48] As a consequence, sj-TRECs can be used as a marker of thymic function, i.e. to quantify the frequency of RTE within the periphery. sj-TRECs are present in all T-cell subsets at different levels which indicates that changes in sj-TREC concentrations could theoretically result from changes in proliferation and survival of all T-cell subsets, not only RTE.[48, 49] However, isolating naïve $CD4^+$ and naïve $CD8^+$ T-cells and then quantifying sj-TRECs would represent a more accurate estimate of RTE frequency. By further assessing homeostatic proliferation

of naïve T-cells as well as study of survival and death signals, non-thymic influences could be taken into account in evaluating sj-TREC levels.

1.6.2 Homeostatic Proliferation

Thymic involution occurs with increasing age[50] and should be taken into account when calculating sj-TREC frequencies. Douek et al. [51] found that the number of sj-TRECs in naïve $CD4^+$ and naïve $CD8^+$ T-cells decreases by 50-100-fold with increasing age, a finding which is compatible with the thymic involution that begins after puberty. However, absolute numbers of naïve $CD4^+$ and naïve $CD8^+$ T-cells only decrease by 2-3 fold.[52,53] This indicates that there is extensive peripheral expansion of naïve T-cells with increasing age. In order to measure homeostatic proliferation, this study used two makers: CD31 and Ki-67 expression.

CD31 is a marker expressed on $CD4^+$ RTE but not on naïve $CD4^+$ T-cells that have undergone homeostatic proliferation.[54] Accordingly, $CD4^+$ naïve T-cells located within the thymus, co-express CD45RA and CD31. These naïve cells have a high sj-TREC content. When they migrate from the thymus, RTE can be activated by foreign antigens to proliferate and differentiate into memory cells which are $CD31^-CD45RA^-CD45RO^+$. These cells have extremely low sj-TREC content. However, with increasing age, RTE may be activated by self-peptides and MHC. This allows RTE to proliferate homeostatically, resulting in a $CD31^-CD45RA^+$ phenotype and a very low sj-TREC content.[54] Consequently, percent CD31 expression can serve as a marker for the proliferation history of $CD4^+$ naive T-cells. As expected, there is a strong negative correlation between CD31 expression and age as well as a strong positive correlation between CD31 expression and sj-TREC content in healthy individuals.[47]

Ki-67 is a nuclear and nucleolar protein which is tightly associated with somatic cell proliferation.[55] The phosphorylation and dephosphorylation of Ki-67, coincides with the transit of cells through mitosis.[56] Ki-67 antibodies label actively cycling cells in all

phases of the cell cycle and provide a snapshot of cell proliferation at a given instant in time.[57] The function of this protein is unknown.

1.6.3 Death and Survival Signals

In order to measure peripheral events such as death and survival signals, this study quantified interleukin-7 (IL-7) levels and Bcl-2 expression.

IL-7 is a member of the type I cytokine family that signals through the common cytokine signalling γ-chain and IL-7Rα (CD127). It downregulates its own receptor and is produced by non-hematopoietic stromal cells of multiple organs including the thymus.[58] Basal levels of IL-7 are required for thymopoiesis, T-cell development in the thymus and post-thymic lymphocyte development.[58] IL-7 also plays a critical role in maintaining and restoring peripheral naïve T-cell homeostasis. It stimulates naïve T-cell homeostatic expansion and prevents Bcl-2 reduction through its receptor (CD127). Briefly, Bcl-2 is an anti-apoptotic protein found in the outer mitochondrial membrane and in the membrane of the endoplasmic reticulum. Over-expression of Bcl-2 blocks cytochrome C release, preventing the caspase cascade that leads to apoptosis.[44]

1.7 sj-TRECs in Various Diseases

sj-TRECs have been studied in other putative autoimmune diseases such as Rheumatoid Arthritis, Ankylosing Spondylitis, Psoriatic Arthritis, Sjogren Syndrome, Systemic Lupus Erythematosus, Myasthenia Gravis and Relapsing-Remitting Multiple Sclerosis.[59] The following discusses briefly some observations of two of them.

1.7.1 Rheumatoid Arthritis (RA)

Rheumatoid Arthritis is a chronic, inflammatory, autoimmune disease of the synovial membrane which eventually causes irreversible destruction of tendons, cartilage, and bone.[60] In RA, the majority of T-cells are clonally expanded, causing a decreased diversity of TCRs. Koetz et al.[60] investigated whether contraction of the naïve $CD4^+$ T-cell repertoire was caused by a defect in generating new T-cells. They measured thymic function by quantifying sj-TRECs and homeostatic proliferation by measuring telomeres. Telomeres are specialized ends of chromosomes that shorten with cellular proliferation. Therefore, telomere lengths can be used to estimate replicative history of cells. Naïve $CD4^+$ T-cells from RA patients had reduced sj-TRECs with increased telomeric loss indicating either a primary defect in peripheral T-cell homeostasis or an impaired thymic function with compensatory homeostatic proliferation of naïve T-cells.[60]

1.7.2 Relapsing-Remitting Multiple Sclerosis (RRMS)

Duszczyszyn et al.[47] quantified sj-TRECs in naïve $CD4^+$ and naïve $CD8^+$ T-cells of RRMS patients. Accordingly, RRMS patients and controls did not have significant differences in their naïve $CD4^+$ T-cell sj-TREC levels. However, it was found that RRMS patients had significantly lower CD31 expression in naïve $CD4^+$ T-cells compared to healthy controls (**$p = 0.03$**) . Correlation analysis suggested that peripheral, non-thymic factors could have strong influences on naïve $CD4^+$ sj-TREC levels of RRMS patients. RRMS patients from this study had significantly lower sj-TRECs in their naïve $CD8^+$ T-cells compared to healthy controls (**$p = 0.012$**) and significant increased expression of Ki-67 compared to healthy controls (**$p = 0.04$**). This led Duszczyszyn et al.[47] to hypothesize that some peripheral, non-thymic, process in RRMS induces either a sudden or sustained loss of naïve $CD8^+$ T-cells at an age when the thymus has limited ability to compensate for T-cell lymphopenia.

1.8 Hypothesis and Objectives

Hypothesis: PPMS patients have altered naïve $CD4^+$ and $CD8^+$ T-cell homeostasis, compared with healthy controls.

Objectives: The objectives of this project are to answer the following questions:

1) Is naïve T-cell homeostasis perturbed in patients with PPMS compared to healthy controls?

2) How does naïve T-cell homeostasis in PPMS patients compare to RRMS patients?

Chapter 2: Patients, Materials and Methods

Chapter 2
PATIENTS, MATERIALS AND METHODS

2.1 Patients and Controls

PPMS and RRMS patients were recruited from the Multiple Sclerosis Clinics at the Montreal Neurological Institute with the collaboration of Dr. J. Antel, Dr. Y. Lapierre and Dr. A. Bar-Or. All patients had definite MS according to the accepted McDonald Criteria for the diagnosis of Multiple Sclerosis.[6,10] None of the patients had received corticosteroids or immunomodulatory treatments in the two years preceding the study. Healthy controls with no history of autoimmune disease were recruited. All patients and controls signed consent forms, which were approved by the Institutional Review Board of the Faculty of Medicine at McGill University. (See appendix) 20 PPMS patients with a mean and median age of 47.9 and 49, respectively were recruited. 33 RRMS patients with a mean and median age of 47.44 and 47 respectively, and 34 controls with a mean and median age of 44.75 and 45 respectively were recruited to match the PPMS patients selected. Because the focus of the study was PPMS, only 20 age-matched patients, corresponding RRMS patients and controls were incorporated in the study.

2.2 Isolation of Naïve $CD4^+$ and Naïve $CD8^+$ T Lymphocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from 60 ml of ethylenediamine tetraacetic acid (EDTA) -treated blood by density gradient centrifugation using Ficoll-Paque™ PLUS (Amersham Biosciences, Uppsala, Sweden). PBMCs were separated into $CD4^+$ and $CD8^+$ T-cells using magnetic anti-CD4 and anti-CD8 microbeads (Miltenyi Biotech, Foster City, CA, USA) according to the manufacturer's instructions for magnetic associated cell sorting (MACS). $CD4^+$ and

$CD8^+$ lymphocytes were treated with a release reagent (Miltenyi Biotech) to remove the magnetic beads. Isolation of naïve T-cells was then achieved by negative selection using anti-CD45RO microbeads (Miltenyi Biotech) resulting in $CD4^+CD45RA^+$ and $CD8^+CD45RA^+$ cells. The purities of naïve $CD4^+$ and naive $CD8^+$ T cells were ≥ 95% and ≥ 90%, respectively.

2.3 Whole Blood Preparation

Whole blood samples were incubated with single and triple stains for 30 minutes in the dark at 4°C. Whole blood was subjected to red cell lysis using 1x Pharmlyse (BD Biosciences) followed by two washes and fixed with 2% formaldehyde (Sigma-Aldrich) in PBS before flow cytometry analysis.

2.4 Immunophenotypic Analysis by Flow Cytometry

For immunophenotypic analysis of naïve $CD4^+$ and naïve $CD8^+$ T-cells, single and double stains were performed with fluorescent-conjugated monoclonal antibodies (mAbs) specific for cell surface markers. Analysis by flow cytometry using a FACSCalibur™ (Becton Dickinson, San Diego, CA, USA) with Cellquest® Software followed. FITC-labeled anti-CD4, anti-CD8, anti-CD45RO, PE-Cy5-labeled anti-CD45RA, and PE-labeled anti-CD31, and anti-CD25 were purchased from BD PharMingen (Mississauga, ON, Canada) and PE-labeled anti-CD127 was purchased from Beckman Coulter (Mississauga, ON, Canada). Single stains were performed with each of these antibodies and double stains included FITC-labeled anti-CD4/PE-Cy5-labeled anti-CD45RA and PE-labeled anti-CD25/PE-Cy5-labeled anti-CD45RA.

Intracellular staining for Bcl-2, Ki-67 and isotype matched controls (BD Pharminogen) was performed following membrane permeabilization with 1x Cytofix/Cytosperm (BD Biosciences, Mississauga, ON, Canada).

Immunophenotypic analysis of whole blood was performed using the following single stains: FITC-labeled anti-CD4, anti-CD8, anti-CD45RO, PE-labeled anti-CD8, anti-CD45RA, anti-CD62L, anti-CD3, anti-CD31, anti-CD127, anti-CD25, and PE-Cy5 labeled anti-CD4, anti-CD8, and anti-CD45RA. The following triple stains were performed: PE-Cy5-labeled anti-CD4/FITC-labeled anti-CD45RO/PE-labeled anti-CD25, FITC-labeled anti-CD4/PE-Cy5-labeled anti-CD45RA/PE-labeled anti-CD25, FITC-labeled CD4/PE-Cy5-labeled anti-CD45RA/PE-labeled anti-CD31, FITC-labeled anti-CD8/PE-Cy5 labeled anti-CD45RA/PE-labeled anti-CD31, PE-Cy5-labeled anti-CD4/FITC-labeled anti-CD45RO/PE-labeled anti-CD45RA, PE-Cy5-labeled anti-CD8/FITC-labeled antiCD45RO/PE-labeled anti-CD45RA, PE-Cy5-labeled anti-CD4/FITC-labeled anti-CD45RO/PE-labeled anti-CD127, FITC-labeled anti-CD4/PE-Cy5-labeled anti-CD45RA/PE-labeled anti-CD127, PE-Cy5-labeled anti-CD8/FITC-labeled anti-CD45RO/PE-labeled anti-CD127, FITC-labeled anti-CD8/PE-Cy5-labeled anti-CD45RA/PE-labeled anti-CD127, PE-labeled anti-CD62L/FITC-labeled anti-CD4/PE-Cy5-labeled anti-CD45RA, PE-labeled anti-CD62L/FITC-labeled anti-CD8/PE-Cy5-labeled anti-CD45RA, FITC-labeled anti-CD4/PE-Cy5-labeled anti-CD8/PE-labeled anti-CD3.

Analysis by flow cytometry using a FACSCalibur™ (Becton Dickinson, San Diego, CA, USA) with Cellquest® Software followed.

2.5 IL-7 Quantification

Plasma was obtained from patients and controls after density gradient centrifugation and then immediately stored at -20°C. Plasma was thawed and IL-7 levels were measured by a quantitative sandwich enzyme immunoassay technique (Quantikine® HS Human IL-7 Immunoassay; R&D Systems, Minneapolis, Minn.) following the manufacturer's instructions. All samples were analyzed in duplicates and mean values were obtained.

2.6 Quantification of sj-TRECs

2.6.1 DNA Extraction

DNA was extracted from naïve $CD4^+$ and naïve $CD8^+$ T-cells using a DNA Isolation kit (Gentra, Minneapolis, Minn.), according to the manufacturer's instructions.

2.6.2 Real- Time PCR TaqMan® Chemistry

sj-TRECs were quantified by Real-Time PCR using TaqMan® technology. The ABI Prism® 7000 Sequence Detection System (Applied Biosystems) was used. Real-Time PCR permits observation and measurement of the PCR product as it forms, i.e. in "real time." Data are collected throughout the PCR process rather than at the end of the PCR process. sj-TREC forward and reverse primers (Sigma-Aldrich) and an sj-TREC probe (Sigma-Aldrich) were used. The probe is constructed with a reporter dye (FAM^{TM}) on the 5′ end and a quencher dye ($TAMRA^{TM}$) on the 3′ end. If the target sequence is present, i.e. the sj-TREC sequence, then the probe anneals between the primer binding sites. During each extension (replication) cycle, the 5′ nuclease activity of AmpliTaq Gold polymerase cleaves the reporter dye from the probe. When the probe is cleaved, the reporter dye becomes separated from the quencher dye allowing it to emit its characteristic fluorescence. Additional reporter dye molecules are cleaved from their respective quenchers with each cycle, resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced.

Real-time PCR was used to quantify sj-TRECs because this method requires only small amounts of DNA, uses a simple protocol and generates an easily quantitated signal.[61] A low DNA requirement allows for multiple sample replicates, making analysis reliable and accurate.

2.6.3 Generation of Standard Curves

Our laboratory previously cloned sj-TREC (376 base pairs (bp)) and albumin gene (72 bp) fragments into plasmids pCR®2·1-TOPO® (Invitrogen, Carsbad, CA, USA).[47] The plasmids were then transfected into TOP 10 *Escherichia coli* and the bacteria were then grown in Luria-Bertani broth. The plasmids were isolated by MidiPrep® (Qiagen, Mississauga,ON, Canada), according to the manufacturer's instructions. The absolute numbers of molecules of sj-TREC external standard and of albumin internal standard per 1μg of plasmid DNA were calculated. This was then used to prepare stock dilutions of sj-TREC plasmid and Albumin plasmid: 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 per 5 μl, which were then stored at -80°C. sj-TREC plasmids served as an external control while the albumin plasmids served as an internal control, normalizing for input DNA.

2.6.4 Reagent Preparation

Real-time PCR reagents were prepared in advance and stored at -20°C. sj-TREC primers and albumin primers (Sigma-Aldrich) were aliquoted at a concentration of 900 nM. Corresponding probes (Applied Biosystems) were also aliquoted at a concentration of 200nM. sj-TREC and Albumin cocktails containing 1.0 x Buffer A, 5.0 mM $MgCl_2$, 200 μM dATP, dGTP, dCTP, 400 μM dUTP, 0.5 U Amperase, 0.25 U AmpliTaq Gold (Applied Biosystems) and dH_2O were prepared.

2.6.5 Real-Time PCR Run

For each experiment, previously prepared reagents were thawed. 5μl of sj-TREC and albumin probes, 222 μl of sj-TREC primers (111 μl of forward and 111 μl of reverse) and 80μl of albumin primers (40 μl of forward and 40 μl of reverse) were added to their corresponding cocktails. 5 μl of plasmid for generation of external and internal standard curves with the addition of 45 μl of corresponding cocktails were run in triplicates at each dilution: 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2. The remaining wells contained 45μl of cocktail and 5 ul of DNA from an age matched PPMS patient, RRMS patient and a

healthy control- also run in triplicates. In addition, a no template control (NTC) containing 5µl of DNase free water instead of DNA or plasmid was used to verify the amplification quality. (See Table 1) Real-time PCR was performed as a single-plex reaction. The prerun thermal cycling conditions were 2 min at 50°C and 10 min at 95°C. Thermal cycling conditions were 50 cycles at 95°C for 15 sec. and 60°C for 1 minute.

10^2	10^2	10^2	PPMS CD4	PPMS CD4	PPMS CD4	PPMS CD4	PPMS CD4	PPMS CD4	10^2	10^2	10^2
10^3	10^3	10^3	PPMS CD8	PPMS CD8	PPMS CD8	PPMS CD8	PPMS CD8	PPMS CD8	10^3	10^3	10^3
10^4	10^4	10^4	RRMS CD4	RRMS CD4	RRMS CD4	RRMS CD4	RRMS CD4	RRMS CD4	10^4	10^4	10^4
10^5	10^5	10^5	RRMS CD8	RRMS CD8	RRMS CD8	RRMS CD8	RRMS CD8	RRMS CD8	10^5	10^5	10^5
10^6	10^6	10^6	Cont. CD4	Cont. CD4	Cont. CD4	Cont. CD4	Cont. CD4	Cont. CD4	10^6	10^6	10^6
10^7	10^7	10^7	Cont. CD8	Cont. CD8	Cont. CD8	Cont. CD8	Cont. CD8	Cont. CD8	10^7	10^7	10^7
10^8	10^8	10^8							10^8	10^8	10^8
10^9	10^9	10^9	NTC	NTC	NTC	NTC	NTC	NTC	10^9	10^9	10^9

sj-TREC Standard Curves

Patient and Control DNA

Albumin Standard Curves

Table 1: Plate Design

2.6.6 Real-Time PCR Analysis

The amplification plot is a graphical display of the fluorescence signal versus cycle number. The threshold for all PCR runs was set at 0.08310 and 0.20000 for sj-TREC and albumin amplifications, respectively. At this threshold, the sigmoidal curves were in the exponential phase. The threshold cycle (C_T) is the cycle number at which the fluorescence passes the threshold. By using these C_T values, we calculated sj-TREC numbers per million cells by absolute quantification as described by Litvak et al.[62] Absolute quantification determines the input copy number by relating the PCR signal to a standard curve. As an endogenous control, exon 12 of the albumin gene[63] which has two genomic copies per cell and no pseudogenes, was used to normalize for DNA input. Since there are 2 copies of the albumin gene per cell, the mean albumin quantity was divided by 2 and then used to calculate sj-TREC numbers. Reproducibility of sj-TREC levels is summarized in sections 3.4 and 3.5.

2.6.5 Statistical Analyses

In order to compare the various patient and control samples and determine whether they show significant differences, it is necessary first to determine whether the samples have a standard normal distribution. Parametric statistics assume that the samples are distributed normally, whereas non-parametric statistics make no such assumptions and are more useful when the number of samples is few. In order to determine the distribution of all the samples, Analyse-It® for Microsoft® Excel®, Continuous Descriptives Statistics was used.[64] This program provides a frequency histogram and a normal plot in which normality can be visually assessed. The frequency histogram shows the distribution of the sample as a bell-shaped curve, whereas the normal plot shows the observations plotted against a cumulative frequency distribution. The chart transforms the observations so that they can be compared against a straight line. Observations for a normally distributed sample should closely follow the blue-straight line. (Data shown in results, chapter 3) Normality can also be tested more formally using the Shapiro-Wilk W Test. Lower p-values indicate the sample is non-normally

distributed. Skewness is a measure of symmetry of a distribution around its mean with positive coefficient values indicating a right-tail skew and negative coefficient values indicating a left-tail skew. A value of "0" indicates that the sample is normally distributed. Kurtosis is a measure of peakiness of a distribution. Positive values indicate that the distribution is more peaked than normal, i.e. there are more observations clustered around the mean with fewer in the tails than normal. Negative values indicate the reverse and a coefficient of "0" indicates a normally distributed sample.[64] With this assessment it was then possible to determine the appropriate statistical methods.

Chapter 3: RESULTS

Chapter 3:
RESULTS

3.1 Immunophenotypic Results

3.1.1 Naïve $CD4^+$ T-Cells

The isolated $CD4^+$ naïve T-cells were stained singly and doubly with CD4 and CD45RA.

In healthy controls, the mean, median and range of percent naïve cells expressing CD4 was 98.79, 99.34 and 93.16 – 99.77; respectively. The mean, median and range of percent naïve cells expressing CD45RA was 95.85, 97.18 and 91.05– 99.70; respectively. Finally, the mean, median and range of percent of naïve $CD4^+$ T-cells co-expressing CD4 and CD45RA was 97.48, 98.25 and 90.51 – 99.85; respectively.

In PPMS patients, the mean, median and range of isolated cells expressing percent CD4 was 98.27, 99.13, and 94.90 - 99.89; respectively. The mean, median and range of percent naïve cells expressing CD45RA was 95.20, 97.34 and 83.50 – 99.95; respectively. Finally, the mean, median and range of percent naïve $CD4^+$ T-cells co-expressing CD4 and CD45RA was 95.28, 96.71 and 94.35– 99.32; respectively.

In RRMS patients, the mean, median and range of isolated cells expressing percent CD4 was 98.08, 99.03 and 93.80 – 99.84; respectively. The mean, median and range of percent naïve cells expressing CD45RA was 96.88, 98.14 and 87.72 – 99.70; respectively. Finally, the mean, median and range of percent naïve $CD4^+$ T-cells co-expressing CD4 and CD45RA was 97.36, 98.09 and 94.41 – 99.66; respectively.

These results confirm that the majority of cells isolated were indeed naïve $CD4^+$ T-cells.

3.1.2 Naïve $CD8^+$ T-Cells

The isolated $CD8^+$ naïve T-cells were stained singly with CD8 and CD45RA. These naïve $CD8^+$ T-cells were also doubly stained with CD8 and CD45RA.

In healthy controls, the mean, median and range of percent naïve cells expressing CD8 was 97.58, 98.16 and 87.10 – 99.26; respectively. The mean, median and range of percent naïve cells expressing CD45RA was 99.25, 99.37 and 95.34 – 99.89; respectively. Finally, the mean, median and range of percent naïve $CD8^+$ T-cells co-expressing CD8 and CD45RA was 97.92, 98.52 and 92.87 – 99.60; respectively.

In PPMS patients, the mean, median and range of percent naïve cells expressing CD8 was 96.33, 97.72 and 88.11 – 98.80; respectively. The mean, median and range of percent naïve cells expressing CD45RA was 98.44, 98.58 and 96.80 – 99.82; respectively. Finally, the mean, median and range of percent naïve $CD8^+$ T-cells co-expressing CD8 and CD45RA was 96.26, 97.25 and 92.55 – 99.27.

In RRMS patients, the mean, median and range of percent naive cells expressing CD8 was 97.04, 97.61 and 93.91 – 98.98; respectively. The mean, median and range of percent naïve cells expressing CD45RA was 98.55, 99.08 and 94.94 – 99.78; respectively. Finally, the mean, median and range of percent naïve $CD8^+$ T-cells co-expressing CD8 and CD45RA was 97.33, 97.59 and 94.70 – 99.35; respectively.

These results confirm that the majority of cells isolated were indeed naïve $CD8^+$ T-cells.

3.2 IL-7 Results

IL-7 plays a critical role in maintaining and restoring peripheral naïve T-cell homeostasis. It stimulates naïve T-cell homeostatic expansion and prevents though its receptor (CD127) Bcl-2 reduction. See section 1.6.3 for more details.

In healthy controls, the mean, median and range of plasma IL-7 concentration was 4.13 pg/mL, 3.50 pg/mL, and 0.99 - 14.21 pg/mL; respectively. In PPMS patients, the mean median and range of plasma IL-7 concentration was 3.55 pg/ml, 3.12 pg/ml and 1.22 – 6.02 pg/mL; respectively. In RRMS patients, the mean, median and range of plasma IL-7 concentration was 4.92 pg/mL, 4.98pg/mL and 1.66 – 10.56 pg/mL; respectively. See figure 3 for a sample of an IL-7 standard curve. Statistical comparisons are analyzed in section 3.6.7.

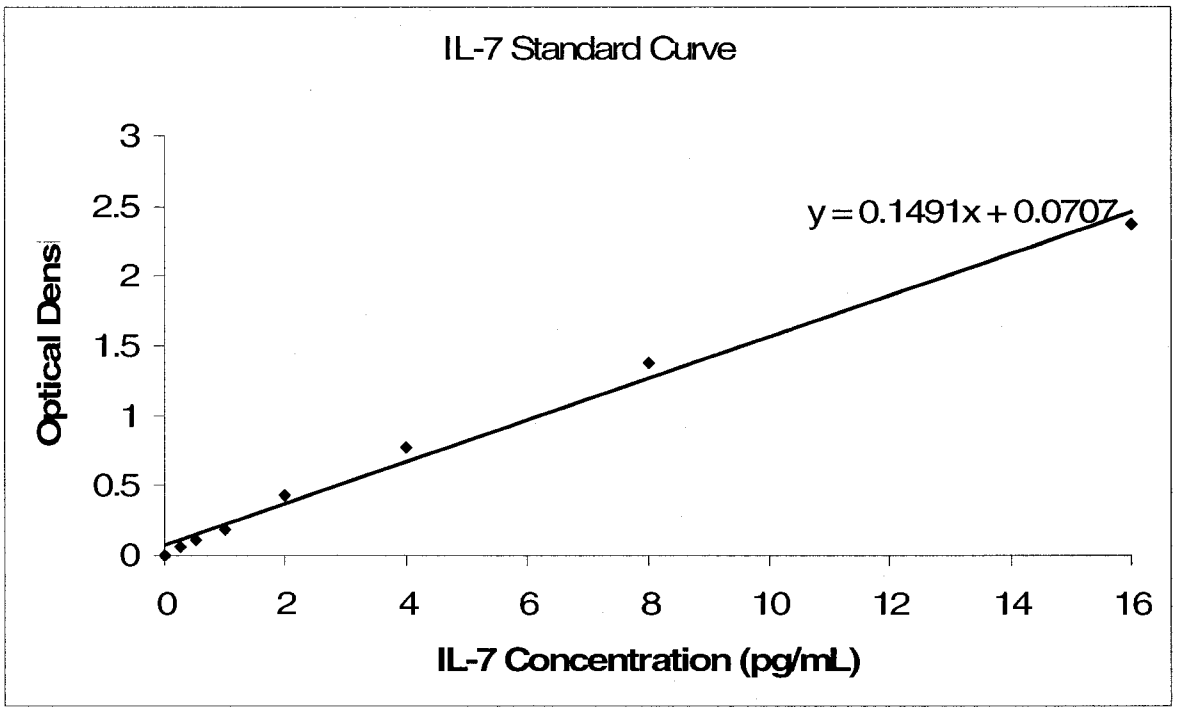


Figure 3: IL-7 Standard Curve

3.3 TREC and Albumin Standard Curves

The external and internal standard curves were run in triplicates with real-time PCR. All acceptable standard curves had $R^2 = 0.99$ which indicates that they were suitable for extrapolating data. (Data not shown.)

3.4 sj-TREC Quantification in Naïve $CD4^+$ T Cells

sj-TRECs are stable extrachromosomal DNA fragments formed during V(D)J receptor rearrangements. Quantifying sj-TRECs is valuable in assessing thymic function. For more details, see section 1.6.1.

In healthy controls, the mean and median number of sj-TRECs per million naïve $CD4^+$ T-cells was 64,442.56 and 18,062.26; respectively. In PPMS patients, the mean and median number of sj-TRECs per million naïve $CD4^+$ T-cells was 6,096.37 and 3,205.49; respectively. In RRMS patients, the mean and median number of sj-TRECs per million naïve $CD4^+$ T-cells was 24,630.04 and 14,614.03; respectively. The results from naïve $CD4^+$ sj-TREC quantification are summarized in table 2. Statistical comparisons are made in section 3.6. sj-TRECs were measured at different time points in two PPMS patients and one healthy control. The results obtained indicated that sj-TREC quantification is reproducible.

PPMS			RRMS			Controls		
N	Age	sj-TRECs	N	Age	sj-TRECs	N	Age	sj-TRECs
176	52	864.48	158/67	35	71,983.68	11	34	33,307.08
178	63	1,970.33	164	37	19,892.80	**35**	43	**2,287.21 2,317.87**
180	35	4,313.07	191	45	6,714.62	166	56	1,955.86
183	58	5,549.15	192	29	12,281.82	167	57	19,237.25
187	48	2,803.68	193	43	37,006.40	168	46	39,194.99
189	65	10,558.63	195	56	30,887.22	170	63	8,101.83
194	54	0.00	204	29	8,633.86	175	45	27,138.12
201	53	0.00	205	45	2,316.14	177	52	7,218.72
207	57	3,205.49	206	49	3,893.04	184	52	69,996.89
218	45	5,282.01	208	57	0.00	186	30	18,299.23
221	50	**1,370.67 1,707.20**	210	45	26,468.21	190	54	11,412.16
226	56	No DNA	216	44	79,125.99	196	51	1,136.10
228	45	**5,374.77 5,252.07 5,208..76**	263	51	21,904.53	199	36	18,589.01
259	30	19,221.28	264	63	124,868.71	232	29	63,668.13
260	45	6,706.77	265	63	11,849.12	235	44	31,661.12
261	29	40,150.42	266	65	1,872.29	238	35	38,993.79
269	46	2,591.57	271	49.5	22,340.43	240	48.5	6,199.04
273	42.5	0.00	272	47	8,513.37	242	55	17,825.28
275	50	5,173.65	276	57	26,241.34	245	47	9,972.50
279	35	695.08	277	52	6,396.78	247	63	3,608.96
			278	53	1,724.30	251	50	45,215.96
			280	59	16,946.24	254	44	1,066,217.02
						257	45	4,158.19
						262	56	1,227.02

Table 2: sj-TRECs in naive $CD4^+$ T-cells

3.5 sj-TREC Quantification in Naïve $CD8^+$ T Cells

In healthy controls, the mean and median number of sj-TRECs per million naïve $CD8^+$ T-cells was 84,334.63 and 19,224.01; respectively. In PPMS patients, the mean and median number of sj-TRECs per million naïve $CD8^+$ T-cells was 22,212.00 and 6, 928.18; respectively. In RRMS patients, the mean and median number of sj-TRECs per million naïve $CD8^+$ T-cells was 23,265.37 and 13,435.24; respectively. The results from sj-TREC quantification in naïve $CD8^+$ T-cells are summarized table 3. Statistical comparisons can be viewed in section 3.6. sj-TRECs were measured at different time point in 3 PPMS patients, 3 RRMS patients and 2 healthy controls. The data indicated that sj-TREC quantification is reproducible.

PPMS			RRMS			Controls		
N	Age	sj-TRECs	N	Age	sj-TRECs	N	Age	sj-TRECs
176	52	2,829.91	18	46	4,477.34	35	43	33,844.20
178	63	1,908.62	158/67	35	2,210.90	166	56	2,863.65
180	35	17,272.95	164	37	29,442.72	167	57	12,629.96
183	58	68,028.71	191	45	9,581.42	168	46	1,242.58
187	48	6,928.18	192	29	56,107.66	170	63	3,797.28
189	65	138.23	193	43	27,816.14	175	45	83,335.16
194	54	64.36	195	56	12,343.69	**177**	**52**	**4,813.67 6,060.30**
201	53	37,473.58	204	29	6,561.80	179	43	14,310.47
207	57	2,829.91	**205**	**45**	**11,894.09 13,137.16**	184	52	102,557.27
218	**45**	**4,105.93 4,507.41**	206	49	3,760.98	186	30	13,781.24
221	**50**	**3,042.42 4,414.70**	208	57	2,886.85	190	54	19,224.01
226	56	6,295.75	210	45	14,526.78	196	51	12,528.29
228	45	N/A	216	44	66,649.51	198	33	192,631.43
259	30	71,461.37	263	51	5,709.84	**199**	**36**	**14,709.49 13,533.06**
260	45	11,786.52	264	63	42,339.34	232	29	133,271.66
261	29	140,716.11	**265**	**63**	**23,150.34 23,134.52**	235	44	84,493.80
269	46	11,731.69	266	65	12,255.91	238	35	42,035.36
273	42.5	15,331.16	**271**	**49.5**	**115,164.99 118, 526.00**	240	48.5	239,459.73
275	**50**	**19,069.15 19,115.84**	272	47	28,463.64	242	55	14,310.47
279	35	1,013.44	276	57	15,093.31	245	47	7,344.93
			277	52	1,604.22	247	63	8,336.46
			278	53	19,796.65	251	50	56,594.10
			280	59	N/A	254	44	298,716.74
						257	45	676,652.63
						262	56	34,881.14

Table 3: sj-TRECs in Naive $CD8^+$ T-cells

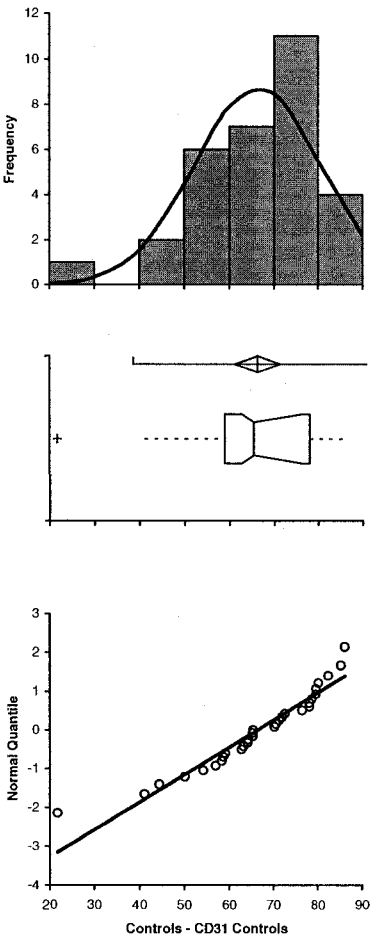
3.6 Statistical Analyses

3.6.1 Distribution Analyses

It was determined that all the samples were non-normally distributed. As a result, non-parametric statistics were used to compare the samples, make inferences and determine correlation. A p-value < 0.05 was determined to be significant. (See for example a non-normal distribution in figure 4)

analysed with: Analyse-it + General 1.73

Test	**Continuous summary descriptives**		
Variable	Controls - CD31 Controls		
Performed by	jessica hackenbroch	**Date**	23 April 2007



n	31 (cases excluded: 3 due to missing value
Mean	66.339
95% CI	61.149 to 71.529
Variance	200.2152
SD	14.1497
SE	2.5414
CV	21%
Median	65.460
97.1% CI	62.815 to 76.375
Range	64.29
IQR	19.09

Percentile	
2.5th	-
25th	58.980
50th	65.460
75th	78.068
97.5th	-

	Coefficient	**p**
Shapiro-Wilk	0.9239	0.0300
Skewness	-1.1421	0.0108
Kurtosis	1.9671	0.0560

Figure 4: Non Normal Distribution

3.6.2 CD31$^+$ Comparisons in Naïve CD4$^+$ T Cells

CD31 is a marker for CD4$^+$ RTE but not naïve CD4$^+$ T-cells that have undergone homeostatic proliferation. The expression of CD31 is non-normally distributed and requires a Mann-Whitney test to determine whether any observed differences are significant. This statistical method tests the null hypothesis that two populations have equal medians. The median percent expression of CD31 in naïve CD4$^+$ T-cells was 65.39 in healthy controls, 51.25 in PPMS patients and 64.98 in RRMS patients.

CD31 percent expression in naïve CD4$^+$ T-cells was significantly reduced in PPMS patients compared to healthy controls (p-value: 0.0017) and significantly reduced in PPMS compared to RRMS (p-value: 0.0032). The percent expression of CD31 in naïve CD4$^+$ T-cells did not differ significantly between RRMS patients and healthy controls (p-value: 0.7475). See Figures 5 and 6.

The mean fluorescence intensity (MFI) of CD31 was also compared between the 3 cohorts. The data indicate a trend with a lower MFI in PPMS compared to healthy controls (control median: 126.11. PPMS median: 107.00. p-value: 0.0815) and a lower MFI in PPMS compared to RRMS patients (RRMS median: 125.34, p-value: 0.0561).

These data are further supported by the percent expression of CD31 in whole blood of healthy controls, PPMS patients, and RRMS patients. (Median CD31 expression in whole blood: 66.11 in healthy controls, 60.70 in PPMS patients, and 62.38 in RRMS patients).

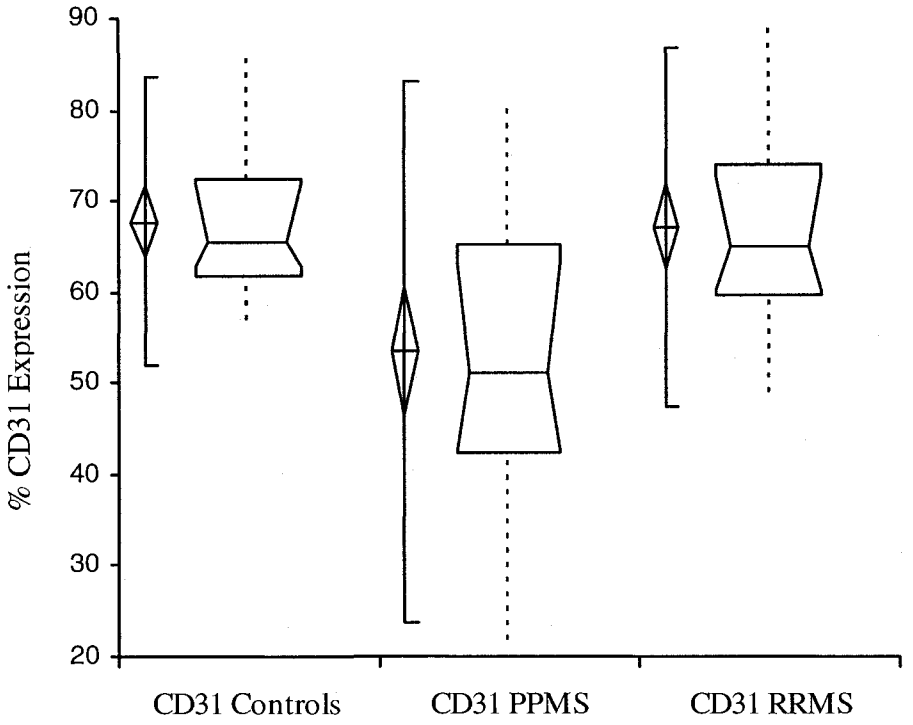


Figure 5: CD31 Box-Plot

Box Plot comparing % CD31 expression in naïve $CD4^+$ T-cells in controls and two patient groups

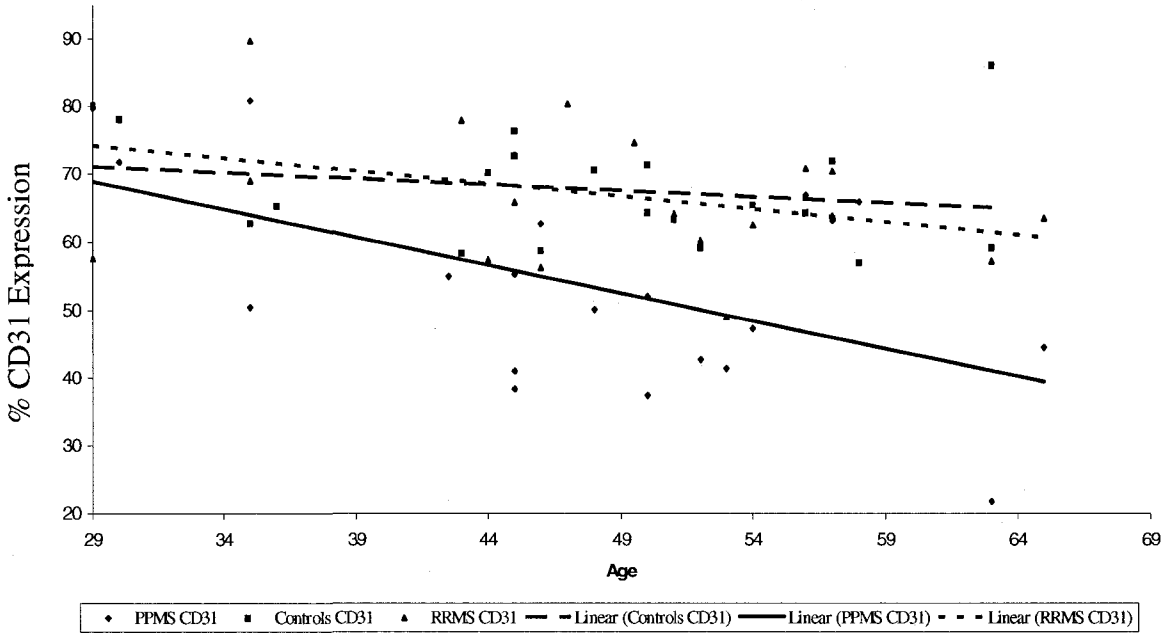


Figure 6: CD31 versus Age

Percent CD31 expression with increasing age
in controls and in patients with PPMS and RRMS

3.6.3 Bcl-2 Comparisons in Naïve $CD8^+$ T Cells

Bcl-2 is an anti-apoptotic protein that can be used to measure cell survival. The percent expression and MFI of Bcl-2 in naïve $CD4^+$ and naïve $CD8^+$ T-cells was investigated. The percent expression of Bcl-2 did not differ significantly in naïve $CD4^+$ T-cells but the data indicated some differences in the naïve $CD8^+$ T-cell population.

Healthy controls had a median % Bcl-2 expression of 93.21. In PPMS patients, the median % Bcl-2 expression was 97.94. These data indicate that % Bcl-2 is significantly higher in PPMS compared to healthy controls (p-value: 0.0029). RRMS patients had a median % Bcl-2 expression of 97.48. A trend is observed in comparing RRMS patients and healthy controls (p-value: 0.0742). There is no significant difference in the % Bcl-2 expression between PPMS with RRMS patients (p-value: 0.1441). See figure 7.

The MFI was also higher in PPMS and RRMS patients compared to healthy controls; however these differences were not significant. (Data not shown)

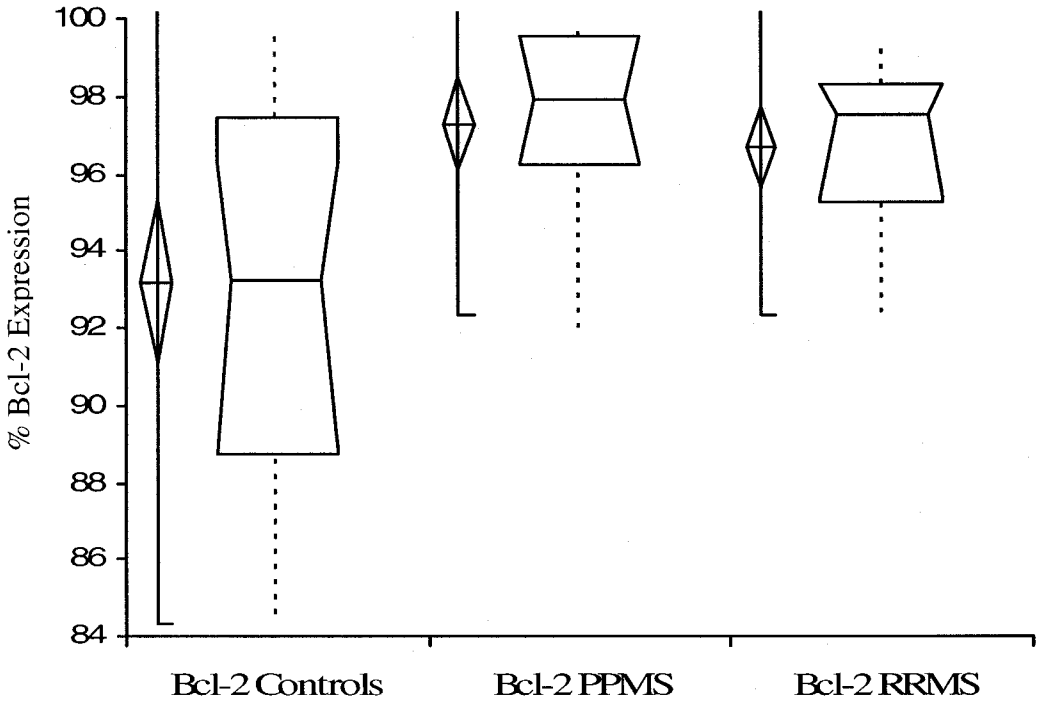


Figure 7: Bcl-2 Box-Plot

Box-plot showing expression of Bcl-2 in
Controls versus two patient groups

3.6.4 Other Stains: CD25, CD127, and ki-67

CD25 is a marker for regulatory T-cells, CD127 is the IL-7 receptor and Ki-67 is a nuclear and nucleolar proliferation antigen. The Mann-Whitney test was used to compare the percent expression of these markers in naïve T-cells and on whole blood. No significant differences were observed.

3.6.5 sj-TREC Comparison in Naïve $CD4^+$ T-Cells

The number of naïve $CD4^+$ T-cell sj-TRECs per million cells was compared between the three cohorts. (Figure 8) The Mann-Whitney test indicated that naïve $CD4^+$ sj-TRECs were significantly reduced in PPMS patients compared to healthy controls and RRMS patients ($p = 0.0007$, $p = 0.0010$). However, the number of naïve $CD4^+$ sj-TRECs did not differ significantly in RRMS patients compared to healthy ($p = 0.4652$). Control #254 is a clear outlier with unusually high sj-TRECs. Control #254 has been omitted. See figure 8.

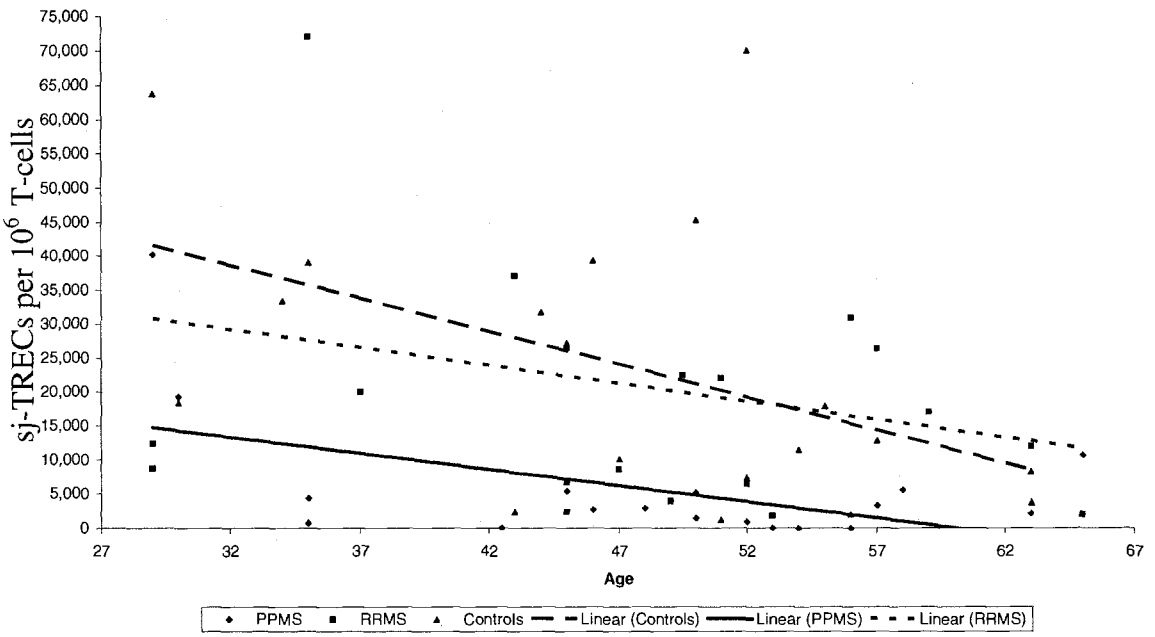


Figure 8: sj-TRECs in Naive $CD4^+$ T-cells versus Age

3.6.6 sj-TREC Comparison in Naïve $CD8^+$ T-Cells

The number of naïve $CD8^+$ T-cell sj-TRECs per million cells was calculated and compared between the three cohorts. (Figure 9). The Mann-Whitney test indicated that naïve $CD8^+$ T-cell sj-TRECs were significantly reduced in PPMS patients compared to healthy controls ($p = 0.0212$). The naïve $CD8^+$ T-cell sj-TRECs of RRMS patients did not differ significantly compared to PPMS patients ($p = 0.2379$) nor healthy controls ($p=0.1517$). Patient #261 is an outlier and has been omitted. See figure 9.

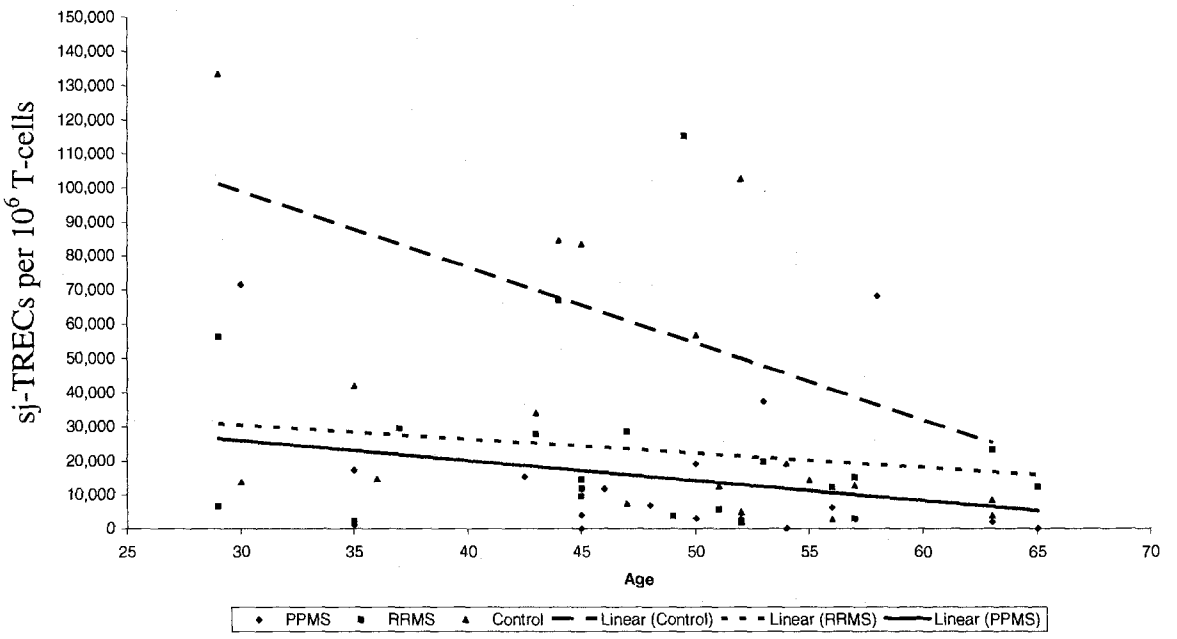


Figure 9: sj-TRECs in naïve $CD8^+$ T-cells versus age

3.6.7 IL-7 Comparison

IL-7 is part of the cytokine type I family that signals through the common cytokine signaling γ-chain and IL-7Rα (CD127). IL-7 stimulates naïve T-cell homeostatic expansion and prevents Bcl-2 reduction.[44] IL-7 plasma levels were measured in the 3 cohorts. The Mann Whitney test indicated no significant differences between plasma IL-7 levels in PPMS patients compared to healthy controls ($p = 0.4819$). RRMS patients had a trend towards higher IL-7 levels than PPMS patients ($p = 0.0810$), but significantly higher IL-7 levels than healthy controls ($p = 0.0453$).

3.6.8 Correlation Analysis: Age and CD31

Correlation analysis tests the degree of association between 2 related variables. The correlation coefficient (rs value) is a value between +1 and -1. A coefficient near 0 indicates that no correlation exists between the variables, i.e. the variables are independent and do not influence each other. A positive rs value indicates a positive correlation between the variables and a negative value indicates a negative correlation between the associated variables.[64]

Thymic involution occurs with increasing age and, in parallel with this age-associated thymic involution, CD31 expression on naïve $CD4^+$ T-cells decreases.[47,54] Using the Spearman rank correlation, we found a strong negative correlation (rs statistic = -0.37, p = 0.03) between age and CD31 expression in healthy controls. However, correlation analyses did not show a significant correlation between age and CD31 in PPMS patients and RRMS patients (PPMS: rs statistic = -0.36, p = 0.12 and RRMS: rs statistic = -0.34, p = 0.13). The correlation analysis in healthy controls was based on a larger sample (N value) with a mean age of ~45 compared to the patients who had a mean age of ~48. We then repeated the correlation analysis with 20 healthy controls that were matched to the PPMS and RRMS patient cohorts with a mean age ~48 and found no significant correlation (rs statistic = -0.26, p = 0.27). See figures 10-13.

n	33 (cases excluded: 1 due to missing values)
rs statistic	-0.37
95% CI	-0.63 to -0.03
2-tailed p	0.0333 (t approximation, corrected for ties)

Figure 10: Correlation Analysis: Controls- %CD31 versus Age

n	20
rs statistic	-0.36
95% CI	-0.69 to 0.10
2-tailed p	0.1212 (t approximation, corrected for ties)

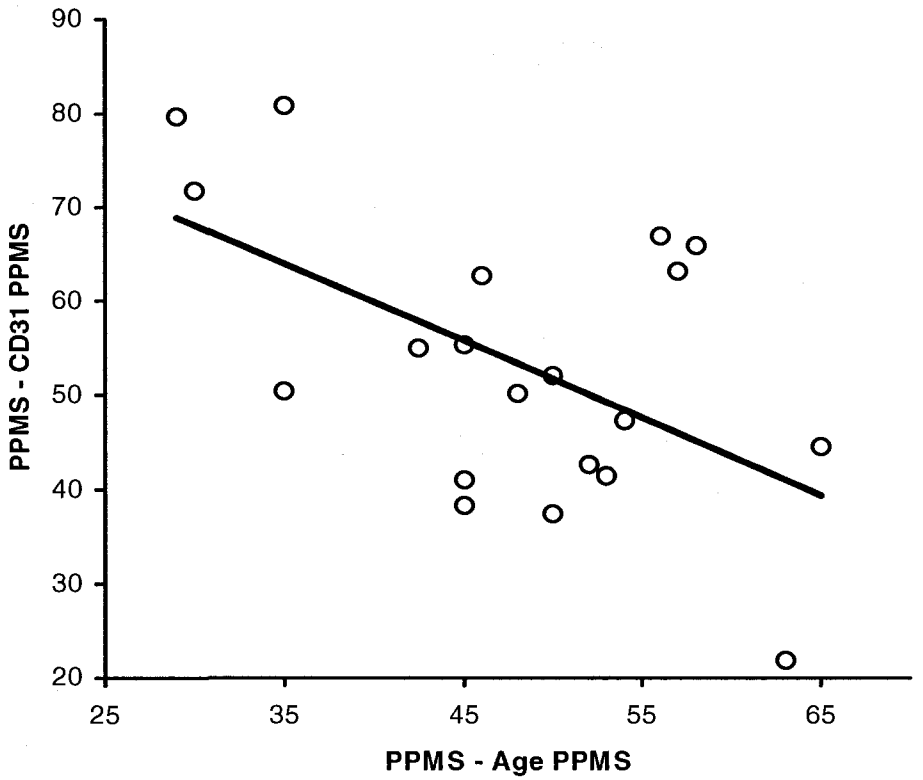


Figure 11: Correlation Analysis: PPMS- %CD31 versus Age

n	20
rs statistic	-0.34
95% CI	-0.68 to 0.12
2-tailed p	0.1392 (t approximation, corrected for ties)

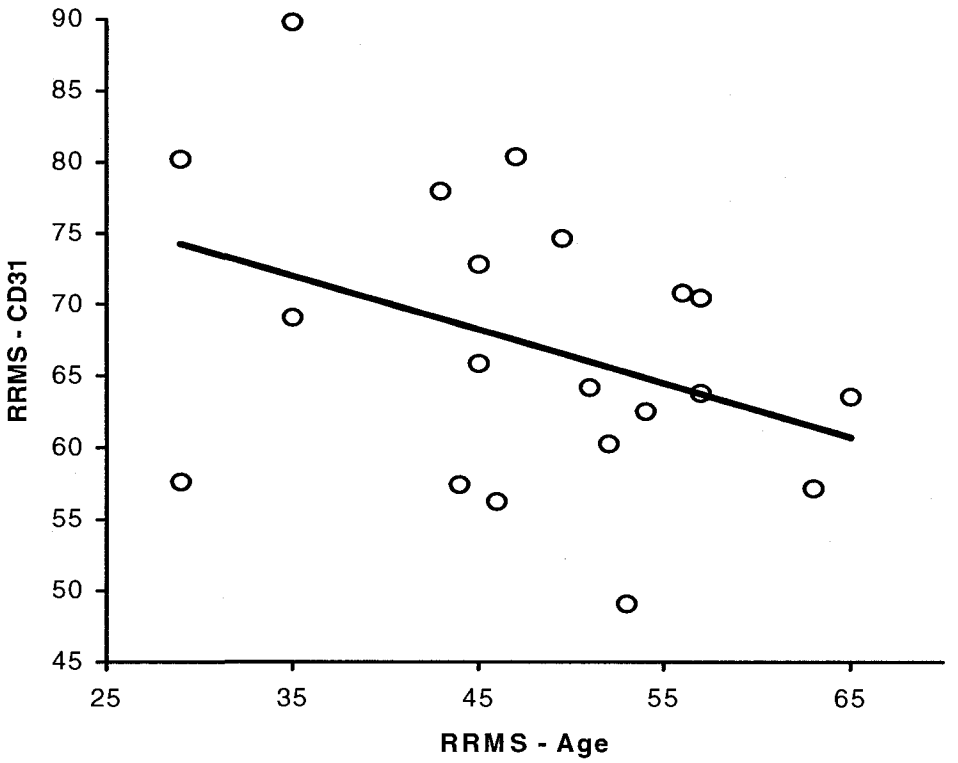


Figure 12: Correlation Analysis: RRMS- %CD31 versus Age

n	20
rs statistic	-0.26
95% CI	-0.63 to 0.21
2-tailed p	0.2689 (t approximation, corrected for ties)

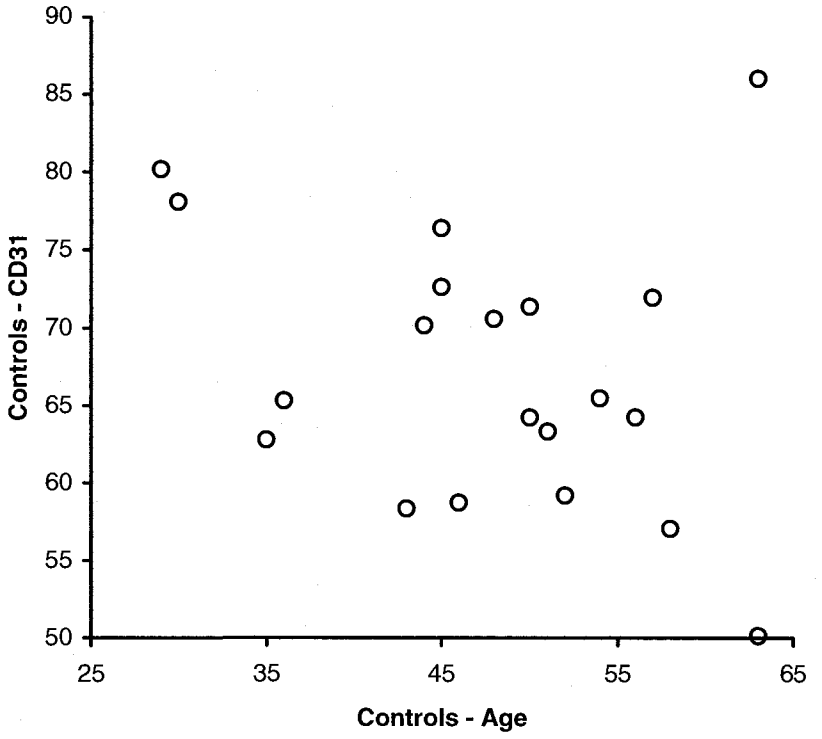


Figure 13: Correlation Analysis: Controls- %CD31 versus Age

3.6.9 Correlation Analysis: Age and sj-TRECs

Correlation between naïve $CD4^+$ T-cell sj-TRECs with age was done using Spearman rank correlation with 20 age-matched individuals from each cohort. The data indicated a significantly strong negative correlation between naïve $CD4^+$ T-cell sj-TRECs and age in healthy controls (rs = -0.50, p = 0.0261). These data are compatible with the current literature indicating that sj-TRECs decrease with increasing age.[50,51] However, correlation between naïve $CD4^+$ sj-TRECs and age was weak and insignificant in PPMS patients and RRMS patients (PPMS: rs = -0.22, p = 0.3629; RRMS: rs = -0.29, p = 0.2186) See figures 14, 15 and 16.

Correlation analysis was similarly done for naïve $CD8^+$ T-cell sj-TRECs with age. There exists a strong negative correlation between naïve $CD8^+$ T-cell sj-TRECs and age in healthy controls and PPMS patients (Controls: rs = -0.58, p = 0.0072; PPMS: rs = -0.48, p = 0.0388). However, in RRMS, correlation analysis indicates no association between naïve $CD8^+$ T-cell sj-TRECs and age (rs = -0.017, p = 0.4788). See figures 17, 18 and 19.

n	20
rs statistic	-0.50
95% CI	-0.77 to -0.07
2-tailed p	0.0261 (t approximation, corrected for ties)

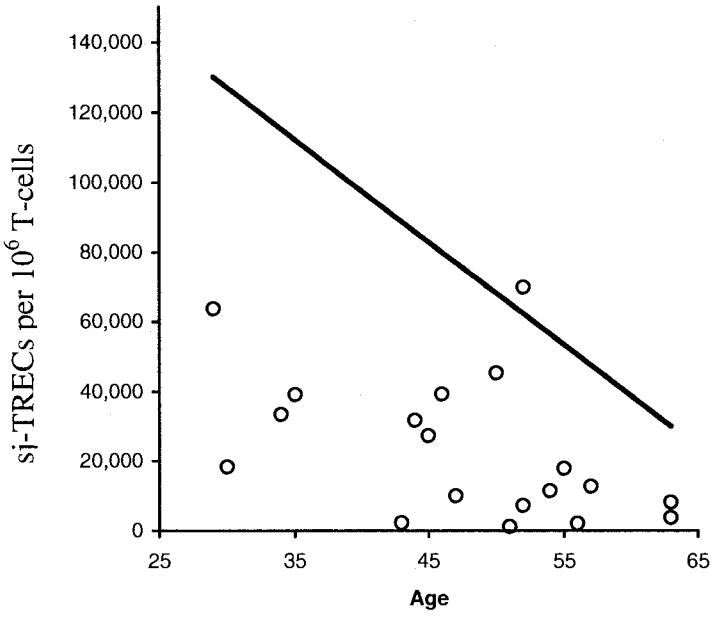


Figure 14: Controls- Correlation Analysis: Naive $CD4^+$ T-cell sj-TRECs with Age

n	19 (cases excluded: 1 due to missing values)
rs statistic	-0.22
95% CI	-0.61 to 0.26
2-tailed p	0.3629 (t approximation, corrected for ties)

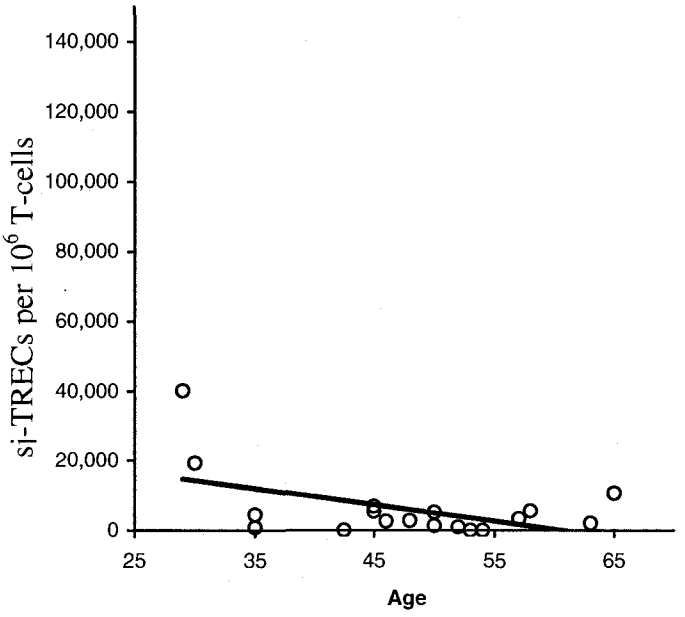


Figure 15: PPMS- Correlation Analysis: Naive $CD4^+$ T-cell sj-TRECs with Age

n	20
rs statistic	-0.29
95% CI	-0.65 to 0.18
2-tailed p	0.2186 (t approximation, corrected for ties)

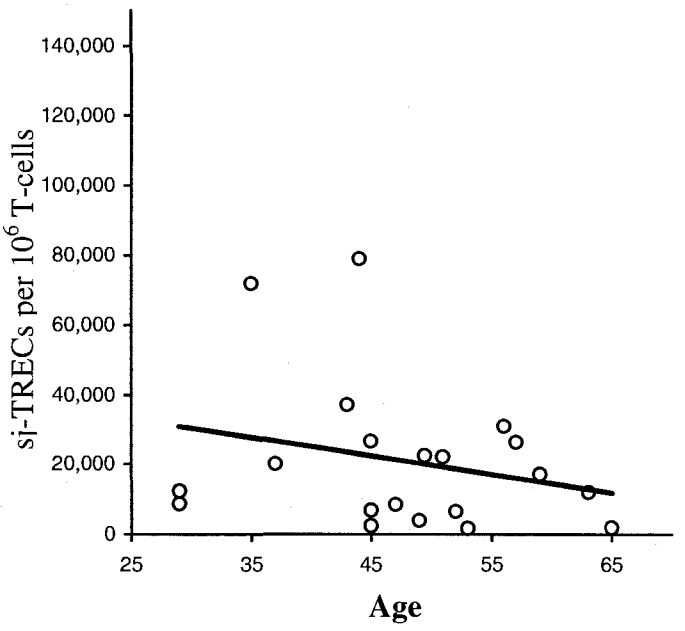


Figure 16: RRMS- Correlation Analysis: Naive $CD4^+$ T-cell sj-TRECs with Age

n	20
rs statistic	-0.58
95% CI	-0.81 to -0.19
2-tailed p	0.0072 (t approximation, corrected for ties)

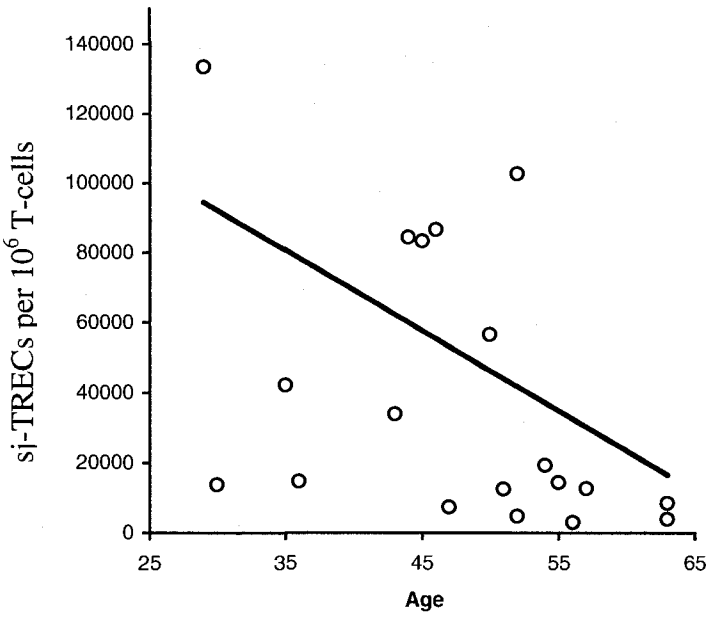


Figure 17: Controls- Correlation Analysis: Naive $CD8^+$ T-cell sj-TRECs with Age

n	19 (cases excluded: 1 due to missing values)
rs statistic	-0.48
95% CI	-0.77 to -0.03
2-tailed p	0.0388 (t approximation, corrected for ties)

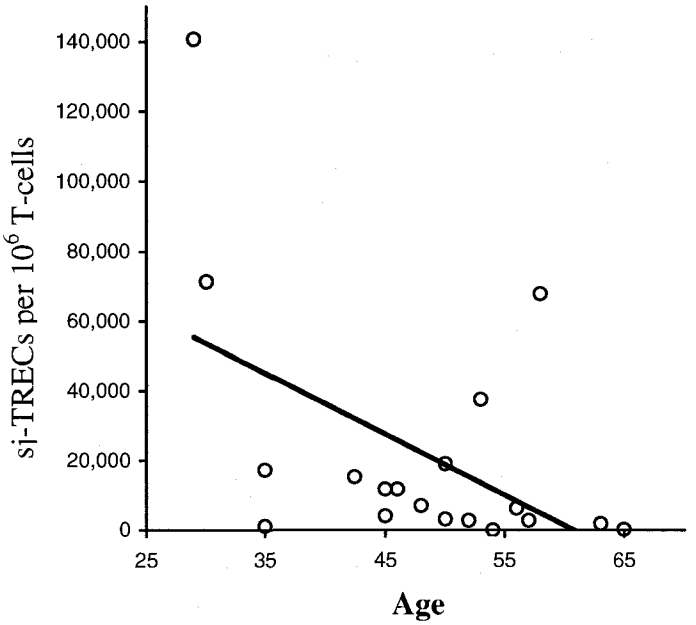


Figure 18: PPMS- Correlation Analysis: Naive $CD8^+$ T-cell sj-TRECs with Age

n	20
rs statistic	-0.17
95% CI	-0.57 to 0.30
2-tailed p	0.4788 (t approximation, corrected for ties)

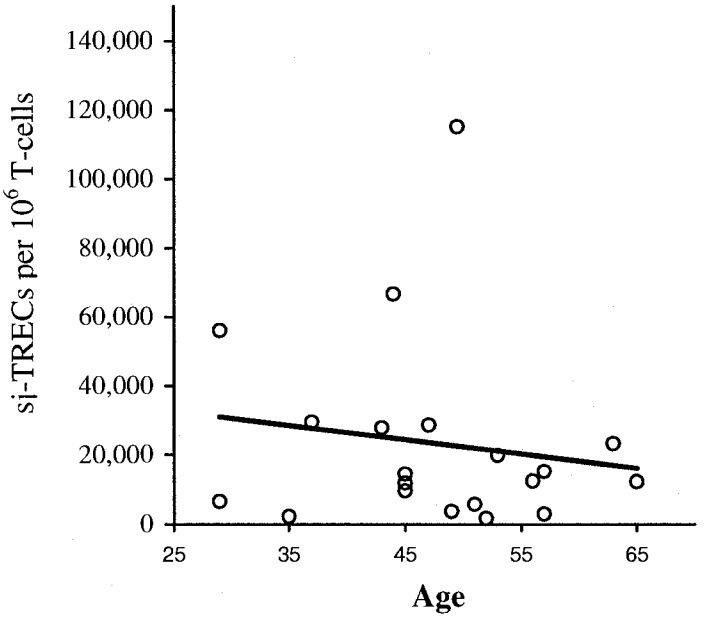


Figure 19: RRMS- Correlation Analysis: Naive $CD8^+$ T-cell sj-TRECs and age

3.6.10 Correlation Analysis: %CD31 and sj-TRECs

Correlation analysis of CD31 expression in naïve $CD4^+$ T-cells with sj-TRECs in naïve $CD4^+$ T-cells was done with Spearman rank correlation in all three cohorts. PPMS correlation analysis indicated a weak positive correlation ($rs = 0.25$, $p = 0.3099$). RRMS correlation analysis also indicated a weak positive correlation ($rs = 0.33$, $p = 0.1476$) and healthy controls indicated barely any correlation at all ($rs = 0.04$, $p = 0.8403$). Note that in all 3 cohorts the correlation is not significant. See figures 20, 21 and 22.

n	19 (cases excluded: 1 due to missing values)
rs statistic	0.25
95% CI	-0.23 to 0.63
2-tailed p	0.3099 (t approximation, corrected for ties)

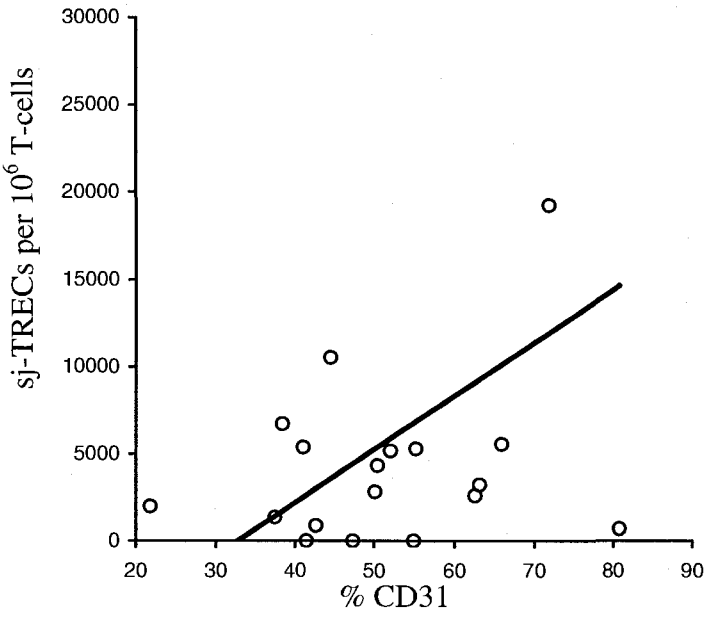


Figure 20: PPMS- Correlation Analysis: sj-TRECs and CD31

n	21 (cases excluded: 13 due to missing values)
rs statistic	0.33
95% CI	-0.12 to 0.67
2-tailed p	0.1476 (t approximation)

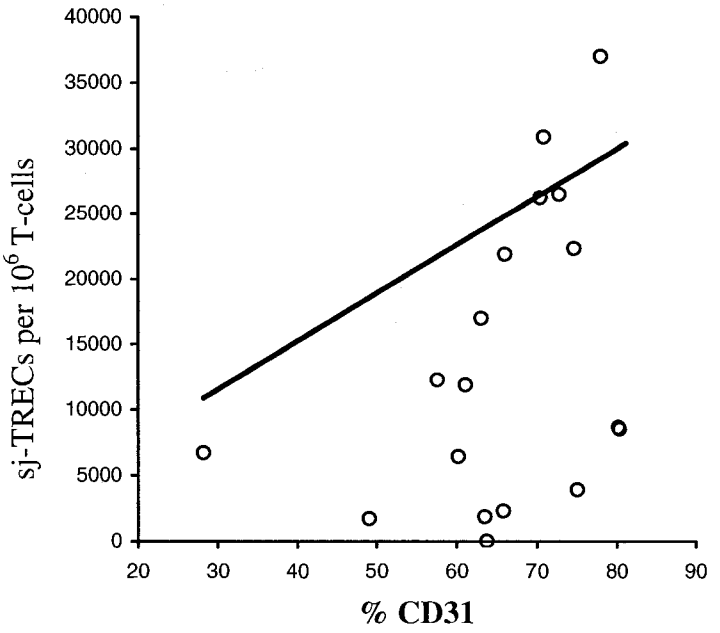


Figure 21: RRMS- Correlation Analysis: RRMS- sj-TRECs and CD31

n	23 (cases excluded: 11 due to missing values)
rs statistic	0.04
95% CI	-0.37 to 0.45
2-tailed p	0.8403 (t approximation)

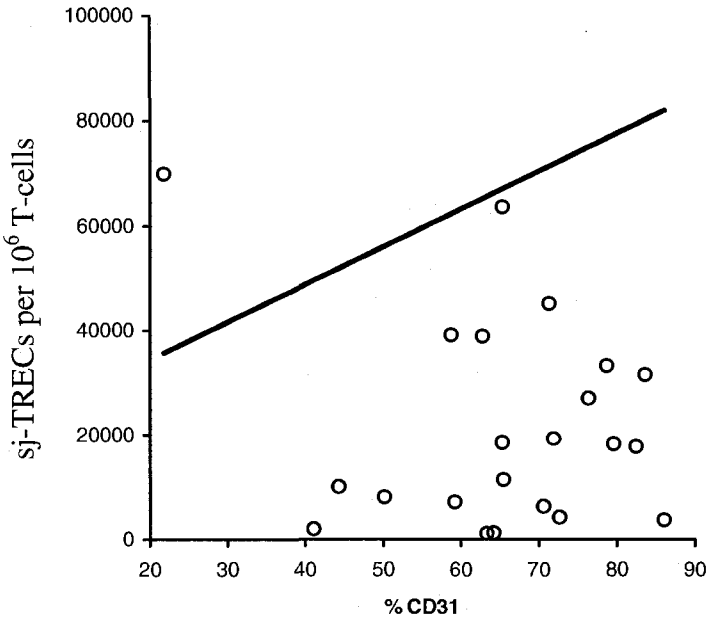


Figure 22: Controls- Correlation Analysis: sj-TRECs and CD31

CHAPTER 4: DISCUSSION

Chapter 4:
DISCUSSION

4.1 Introductory Remarks

Multiple Sclerosis is an inflammatory and demyelinating disease of the CNS of unknown etiology. MS is highly heterogeneous in terms of its genetics, clinical course[7], pathology, immunology[8,9] and response to treatment[8,10]. MS may represent one or several distinct entities. In an attempt to partly address this issue, this study investigated $CD4^+$ and $CD8^+$ naïve T-cell homeostasis in patients with PPMS, RRMS and healthy controls. Naïve T-cell homeostasis is defined as a balance between thymic production, homeostatic proliferation and the delivery of death and survival signals.[41] Thymic production was measured by quantifying sj-TRECs, an episomal by-product of TCR gene rearrangements.[36,37] Homeostatic proliferation was measure by flow cytometry analysis of CD31 and Ki-67 expression. CD31 is a marker present on RTE but not on naïve T-cells that have undergone homeostatic proliferation.[54] Ki-67 is a nuclear and nucleolar antigen found in actively cycling cells and staining of this antigen provides a 'snapshot' of cell proliferation at the moment of isolation.[55,57] Cell survival was measured by quantifying plasma IL-7 levels as well as flow cytometry analysis of Bcl-2 expression. IL-7 plays an important role in maintaining and restoring naïve T-cell homeostasis.[58] It stimulates naïve T-cell proliferation and prevents reduction of Bcl-2, an anti-apoptotic protein. [44]

4.2 Thymic Production: Naïve $CD4^+$ T-cell sj-TRECs

Naïve $CD4^+$ T-cell sj-TRECs were significantly reduced in PPMS patients compared to RRMS patients ($p = 0.0010$) and healthy controls ($p = 0.0007$). However, naïve $CD4^+$ T-cell sj-TRECs did not differ significantly in RRMS patients compared to healthy controls ($p = 0.4652$). sj-TRECs have been widely accepted as a surrogate marker of naïve T-cell production[51], thus the data indicate that there is a reduced thymic production of naïve $CD4^+$ T-cells in PPMS. This finding further indicates altered naïve $CD4^+$ T-cell homeostasis in PPMS patients. Duszczyszyn et al.[47] found that naïve $CD4^+$ T-cell sj-TRECs were reduced in RRMS patients compared to healthy controls but this difference was not significant. The data from our study correlate well with this earlier work[47] since no significant differences were found in naïve $CD4^+$ T-cell sj-TRECs of RRMS patients compared to healthy controls.

4.3 Thymic Production: Naïve $CD8^+$ T-cell sj-TRECs

Naïve $CD8^+$ T-cell sj-TRECs were significantly reduced in PPMS patients compared to healthy controls ($p = 0.0212$) but not reduced compared to RRMS patients ($p = 0.2379$). RRMS patients had fewer sj-TRECs than healthy controls but this difference was not significant ($p = 0.1517$). These data indicate that there is reduced thymic production of naïve $CD8^+$ T-cells in PPMS patients and thus, altered naïve $CD8^+$ T-cell homeostasis. Duszczyszyn et al.[47] found that naïve $CD8^+$ T-cell sj-TRECs were significantly reduced in RRMS patients compared to healthy controls. However, our study did not find a significant difference in the number of naïve $CD8^+$ T-cell sj-TRECs in RRMS patients compared to healthy controls. The difference in the results of the two studies of RRMS patients can be explained largely by the higher age of the patients and controls in our study. It should be emphasized that in our study PPMS patients were selected initially, and then RRMS patients and controls were selected to match the age of the PPMS patients. In PPMS, unlike RRMS, naïve $CD8^+$ sj-TRECs are reduced despite the increased age of the patient group.

4.4 Homeostatic Proliferation: % CD31 Expression

Flow cytometry analysis indicated that the % CD31 expression in naive $CD4^+$ T-cells was significantly reduced in PPMS patients compared to both healthy controls ($p = 0.0017$) and RRMS patients ($p = 0.0032$). In contrast, there was no significant difference in % CD31 expression in RRMS patients compared to healthy controls ($p = 0.7455$). Duszczyszyn et al.[47] found that RRMS patients had significantly lower % CD31 expression than healthy controls. Again, the difference in results of the two studies can be partially attributed to the different median ages of the RRMS patients in the two studies. The reduction in % CD31 expression in PPMS patients indicates increased homeostatic expansion of naïve $CD4^+$ T-cells. Naïve T-cell proliferation is a compensatory mechanism that occurs with decreased thymic output.[52,53] PPMS patients had reduced sj-TRECs in their naïve $CD4^+$ T-cells with increased homeostatic compensation as seen by the reduced % CD31 expression. In contrast, RRMS patients did not have reduced sj-TRECs in their naïve $CD4^+$ T-cells, a finding which is consistent with their CD31 counts.

4.5 Delivery of Death and Survival Signals

The % Bcl-2 expression in naïve $CD8^+$ T-cells was significantly increased in PPMS patients compared to healthy controls ($p = 0.0029$) but not compared to RRMS patients ($p = 0.1441$). RRMS patients showed a trend towards increased Bcl-2 expression compared to healthy controls ($p= 0.0742$). Since Bcl-2 is an anti-apoptotic protein,[44] these data indicate that naïve $CD8^+$ T-cells in PPMS have upregulation of at least one survival signal. This upregulation may be a homeostatic compensatory mechanism due, in part, to decreased thymic output of naïve $CD8^+$ T-cells, as indicated by the reduced sj-TRECs levels in these cells. IL-7 also plays an important role in maintaining and restoring naïve T-cell homeostasis.[58] It stimulates homeostatic expansion and prevents Bcl-2 reduction.[58,44] IL-7 levels did not differ significantly in PPMS patients compared to healthy controls ($p = 0.4819$). This finding indicates that increased % Bcl-2 expression is unlikely to be due to increased IL-7 levels in PPMS patients. However, IL-7 levels were significantly increased in RRMS patients compared to healthy controls (p

= 0.0453), which is consistent with the trend towards increased Bcl-2 expression in these patients. Thus, increased % Bcl-2 expression in RRMS patients may be due, in part, to increased IL-7 levels. The findings suggest a profound thymic disturbance in PPMS patients which is supported in part by the increased Bcl-2 expression in naïve $CD8^+$ T-cells. The RRMS data are less compelling but the increased IL-7 levels suggest some response to homeostatic alterations, as demonstrated in earlier work in a younger patient cohort.[47]

In summary, our data indicate that PPMS patients but not RRMS patients, have significantly lower sj-TRECs in their naïve $CD4^+$ and $CD8^+$ T-cells than do healthy controls. The % CD31 expression in naïve $CD4^+$ T-cells was also lower in PPMS patients than in healthy controls and RRMS patients.

4.6 *$CD4^+$ versus $CD8^+$ Naïve T-Cell Homeostasis*

The data from this study indicate that in PPMS compared to healthy controls, naïve $CD4^+$ T-cell homeostasis is slightly more affected than naïve $CD8^+$ T-cell homeostasis. However, Bcl-2 levels were significantly abnormal in naïve $CD8^+$ T-cells but not in $CD4^+$ T cells. We believe that these differences are a consequence of differential regulation between the naïve $CD4^+$ and naïve $CD8^+$ T-cell compartments. This data is in accordance with transgenic mice studies by Ferreira et al.[65] suggesting that naïve $CD4^+$ T-cells and naïve $CD8^+$ T-cell survival is relayed in differential manners. Similarly, data from neonatal thymectomy in young lambs by Cunningham et al.[66] suggest that the thymus exports mixed populations of T-cells with different homing properties, lifespans and post-thymic expansion potential. Therefore, different processes influence naïve $CD4^+$ and naïve $CD8^+$ T-cell homeostasis in PPMS.

4.7 Sj/β Trec Ratio

As discussed earlier in section 1.5, the TCRα chain and the TCRβ chain undergo rearrangements to form TRECs. The major byproduct of excising the TCRδ locus located within the α locus is termed an sj-TREC. Quantification of sj-TRECs has been studied extensively as a surrogate marker for thymic function.[48,49] However, recently, a new method of using TRECs to **assess** thymic function has been developed.[67] Based on the hypothesis that thymic output is determined by the level of intrathymic proliferation of precursor T cells, Dion et al.[67] developed an assay that extends the use of TRECs to include Dβ-Jβ TRECs. These TRECs are created during the first step of TCRβ rearrangement. There is extensive thymocyte proliferation during these two rearrangement events which leads to a dilution of the Dβ-Jβ TRECs.[37] As a consequence, there is a decreased proportion of Dβ-Jβ TRECs relative to that of sj-TRECs in mature thymocytes and RTE. Therefore, Dion et al.[67] concluded that intrathymic precursor T-cell ($TCR\beta^{+}\alpha^{-}$ thymocytes) proliferation can be assessed by measuring the sj/β TREC ratio in peripheral blood mononuclear cells (PBMCs). Analysis of sj/β TREC ratios might provide further insight into naïve T-cell homeostasis in PPMS. However, such analysis requires cloning of multiple Dβ-Jβ fragments into plasmids, isolation and purification of the plasmids, creation of standard curves for each fragment and subsequent testing of each DNA sample from patients and controls. We decided this was beyond the scope of this Master's degree thesis.

4.8 Correlation Analysis

The % CD31 expression in naïve $CD4^{+}$ T-cells has been found to decrease with increasing age.[40,47] These data correlate well with the thymic involution that is known to occur with increasing age.[50] However, the data from our study did not find a strong correlation between % CD31 expression and age in healthy controls, matched for age with the PPMS patients ($r_s = -0.26$, $p = 0.2689$). When correlation analysis was repeated with a more diverse age group of healthy controls, a strong negative correlation was found ($r_s = -0.38$, $p = 0.0353$). This indicates that % CD31 expression decreases with increasing age in healthy controls but when the age range is skewed, towards an older

age group, correlation is weak and insignificant. Since PPMS has a late age of disease onset, the patients recruited to this study had a skewed age range; making it more difficult to interpret correlation analyses. No correlation was found between % CD31 expression and age in PPMS patients (rs = -0.36, p = -0.1212) and RRMS patients (rs = -0.34, p = 0.1392). These results likely reflect the skewed age range of the patients in our study.

Naïve $CD4^+$ T-cell and naïve $CD8^+$ T-cell sj-TRECs have also been found to decrease with increasing age. This finding is consistent with the thymic involution that occurs with increasing age.[50] The results from our study further indicate that sj-TRECs decrease with increasing age. Healthy controls had a strong negative correlation between naïve $CD4^+$ T-cell sj-TRECs and age (rs = -0.50, p = 0.0261) and naïve $CD8^+$ T-cell sj-TRECs and age (rs = -0.58, p = 0.0072). PPMS patients did not show a negative correlation with age in their naïve $CD4^+$ T-cell sj-TRECs (rs = 0.22, p = 0.3629) but did show a strong negative correlation with age in their naïve $CD8^+$ sj-TRECs (rs = -0.48, p = 0.0388). RRMS patients did not show a negative correlation with age in their naive $CD4^+$ T-cell sj-TRECs (rs = -0.29, p = 0.2186) nor in their naïve $CD8^+$ T-cell sj-TRECs (rs = -0.17, p = 0.4788). Possibly, these findings, particularly in RRMS, reflect the skewed age of the patient population.

The literature indicates that there is a strong positive correlation between % CD31 expression and $CD4^+$ T-cell sj-TRECs.[40,47] However, we detected no such correlation in controls as in the two patient groups, probably because of the skewed age range of our study groups.

4.9 PPMS versus RRMS

Some researchers consider PPMS to be a different disease than RRMS while others believe that PPMS and RRMS are the same disease.[28] This study investigated naïve $CD4^+$ T-cell and naive $CD8^+$ T-cell homeostasis in both PPMS and RRMS. Some important differences and similarities have been found. First, naïve $CD4^+$ T-cell sj-

TRECs were significantly lower in PPMS compared to RRMS and healthy controls. This decrease was accompanied by increased homeostatic proliferation as marked by decreased % CD31 expression. RRMS patients had naïve $CD4^+$ T-cell sj-TRECs similar to healthy controls and consequently similar % CD31 expression. In this respect, PPMS is different from RRMS. Second, naïve $CD8^+$ T-cell sj-TRECs were similar in PPMS and RRMS, both being reduced compared to healthy controls. Therefore, it seems that PPMS and RRMS patients are different in their naïve $CD4^+$ T-cell sj-TRECs but similar in their naïve $CD8^+$ T-cell sj-TRECs. Third, PPMS and RRMS patients show some homeostatic differences. Strikingly, IL-7 levels were significantly higher in RRMS compared to healthy controls but not higher in PPMS. Thus, overall the findings show some similarities between PPMS and RRMS patients but also a number of important differences in naïve T-cell homeostasis. One possible interpretation is that both PPMS and RRMS patients have a thymic alteration leading to reduced naïve T-cell generation but that the alteration is more profound in PPMS.

4.10 Conclusions and Future Directions

The results from our study indicate that PPMS is similar to RRMS in some ways but different from RRMS in other ways. In both cases naïve T-cell homeostasis is perturbed yet discrete differences are observed (see section 4.6).

In the future it would be of interest to study naïve T-cell homeostasis in Secondary Progressive Multiple Sclerosis (SPMS). SPMS defines patients who initially followed a RRMS course but have developed a progressive phase.[7] It would be of interest to determine whether SPMS follows the same pattern as RRMS, PPMS or a completely different pattern from either of those. SPMS tends to have its onset at a similar age to patients with PPMS and some think PPMS corresponds to SPMS without a prior relapsing cause.[68] Study of naïve T-cell homeostasis could provide some insight as to whether SPMS and PPMS are similar or different disease entities.

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APPENDIX

Consent Form and Ethics Approval Documents

INFORMED CONSENT

Study title: Altered naïve T-cell homeostasis in multiple sclerosis

Site: Montreal Neurological Hospital
3801 University
Montreal, QC, H3A 2B4
Phone: 514-398-8550

Principal Investigator: Dr. David Haegert
Site Investigator: Dr. Jack Antel

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1. RESEARCH PROJECT DESCRIPTION

1.1 Justification:

- **Purpose of the research**:

The purpose of this project is to enhance our understanding of the role of the immune system in causing the damage to the brain that occurs in patients with MS. The study is aimed at identifying the process by which alterations occur in T-cells, and how alterations in these T-cells contribute to the immune response in MS. We will study tiny fragments of DNA that are produced during T-cell development and we will study RNA that the T-cell produces as part of its contribution to the immune response.

- **Explanation of relevant terms**

DNA: Molecule containing all the transmissible genetic information, which controls the activities of the body cells. DNA provides the instructions for determining the hereditary characterisitics of a person such as eye color or blood type.
RNA: A molecule, which is an exact copy of a portion of DNA, and transmits instructions from DNA molecules within cells.
T-cells: a population of immune cells in the blood.

1.2 Request for participation

We are asking you for your participation in this research project.

2. PROGRESSION OF THE RESEARCH PROJECT

2.1 Procedures. Blood will be drawn from a vein in your arm on 1-4 separate occasions over the course of the study. At each time point, between 55-90 ml (4 to 6 tablespoons) of blood will be drawn.

2.2 Duration of the research project. This study will require three years for its completion.
2.3 Scope of the research project. This research will take place at McGill University with collaboration by several investigators in Canada.
2.4 Access to your medical record. The research team will consult your medical record to obtain information, which is pertinent to the research project.

3.STORAGE AND SAFEKEEPING OF SAMPLES

3.1 Identification of the sample (Coded and anonymized samples). We will protect the confidentiality of the samples by assigning them a specific code. Your DNA and RNA samples will not be specifically identified but a code will link you to the sample. Decoding can only be performed by the principal researcher or an individual authorized by the latter. The researcher may decide to include specific information with the sample (such as your age, your gender, or certain clinical data); this information, however, would not allow you to be identified or retraced.

3.2 Length of storage of samples. Samples of your DNA and RNA will be stored at the Duff Medical Building, McGill University under the responsibility of Dr. David Haegert, for one year after the end of the research project. After this time, all samples will be destroyed. However, some samples of your DNA may be kept in the form of immortalized cell lines, hence for an indefinite period at the Duff Medical Building, McGill University.

3.3 Other Research. At the end of the research project, instead of destroying your sample, could we anonymize it so that it can be used for additional research on multiple sclerosis approved by the research ethics board? By anonymizing the sample, you would no longer be identifiable.

Yes
No

4. BENEFITS

You will receive no personal benefit from your participation in this research project. We hope, however, that the results obtained will permit us to further our knowledge in the area of multiple sclerosis by way of understanding changes in T-cells that contribute to the development of multiple sclerosis, and eventually, benefit society as a whole.

5.RISKS

5.1 Physical risks. Although the taking of the blood sample causes no serious problems for most people, it can cause some bleeding, bruising, malaise, dizziness, infection and/or discomfort at the site of the needle puncture. The most commonly encountered side effect is bruising at the site of the needle puncture.

5.2 Other risks. There are no other risks.

6.CONFIDENTIALITY

6.1 Safety/security of the data. All of the information obtained about you and the results of the research will be treated confidentially. This information will be coded and kept under lock and key. The study file will be kept at the Duff Medical Building, McGill University under the responsibility of Dr. David Haegert, and also in the electronic files of Dr. David Haegert.

Your participation and the results of the research will not appear in your medical record.

The results of the study may be published or communicated in other ways but it will be impossible to identify you.

6.2 Third-party access to results. Unless you have provided specific authorization or where the law permits or a court order has been obtained, your personal results will not be made available to third parties such as employers, governmental organizations, insurance companies or educational institutions. This also applies to your spouse, other members of your family and your physician.

However, for the purposes of ensuring the proper management of the research, it is possible that a member of an ethics committee, a Healthy Canada representative or McGill University or the Montreal Neurological Hospital may consult your research data as well as your medical record.

7. COMMUNICATION OF RESULTS

Since your data have been anonymized, it is impossible to communicate personal results. Nevertheless, you can communicate with the research team to obtain information on the status of the work or the general results of the research project. Project updates will be sent by mail at the end of the project.

8. CONFLICT OF INTEREST

There is no conflict of interest. This research is supported by a grant from the Multiple Sclerosis Society of Canada to the principal researcher.

9. FREEDOM OF PARTICIPATION AND PERIOD OF REFLECTION.

Your participation is completely free and voluntary. The quality of medical services available to you will not be affected by your decision. You may take the time necessary to reflect on your decision and discuss your participation in the project with persons close to you before giving us your answer.

Your are free to withdraw from the study at any time. If you withdraw, your samples will be retraced and destroyed.

10. COMPENSATION FOR EXPENSES INCURRED AND FOR INCONVENIENCE

If you incur expenses by reason of your participation (for example: parking, travel, child-care, meals), you will be reimbursed upon presentation of receipts.

11. CIVIL LIABILITY

If you suffer any injury as a result of your collaboration in this project, you retain all legal recourses against the research collaborators.

12. RESOURCE PERSONS

Member of the research team: If you would like additional information regarding the progression of the research project or would like to communicate any change of address to us, you can contact Dr. David Haegert, Professor, McGill University at the following phone number: 514-398-7192 Extension 00510#.

Authorized persons: If you would like to discuss your participation with an individual not directly involved in the project, we invite you to contact the Patients Committee (a group established to protect the rights of research subjects) at the following phone number:514-398-1923.

13. FINAL WORD: UNDERSTANDING, FREEDOM, QUESTIONS

Dr._____explained the nature and the progression of the research project. I have familiarized myself with the consent form and have received a copy. I have had the opportunity to ask questions that have been answered. Upon reflection, I agree to participate in this research project.

14. SIGNATURE, NAME, DATE

Name: **Surname:**
Address:

Telephone (home): **Work:**

I will inform the principal researcher of any change of address.

Signature of Participant:
Date:

Date of Birth: