USING B CELL CHARACTERISTICS AS PREDICTORS OF MULTIPLE SCLEROSIS IN CLINICALLY ISOLATED SYNDROME PATIENTS

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DEDICATION

I would like to thank my friend and mentor, Dr. Nancy Monson, who believed in me when few people did, including myself. I would also not be at this point without my Graduate Committee—Drs. Sally Ward, Chandra Mohan, Christopher Lu, Steven Vernino, and Olaf Stüve. I especially want to extend extreme gratitude to my mother, stepfather, my sister, and brothers for supporting me in life, whether it is in continuing my education, my health, or my happiness; I absolutely could not have survived sane without you, your love, and your support!

I owe all my self and my accomplishments to the Lord, Christ Jesus,
who truly enacted his own words: "Greater love hath no man than this, that a man lay down his life for his friends." John 15:13

USING B CELL CHARACTERISTICS AS PREDICTORS OF MULTIPLE SCLEROSIS IN CLINICALLY ISOLATED SYNDROME PATIENTS

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USING B CELL CHARACTERISTICS AS PREDICTORS OF MULTIPLE SCLEROSIS IN CLINICALLY ISOLATED SYNDROME PATIENTS

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Clinically isolated syndrome (CIS) is the diagnosis of patients who have experienced a single event due to nerve demyelination of the white matter of the central nervous system. This can be due to numerous causes, both autoimmune and infectious. We hypothesized that CIS patients with B cell characteristics like those seen in multiple sclerosis (MS) patients would develop clinically definite MS. We have determined that, like MS patients, several CIS patients have an increased frequency of VH4-expressing $CD19^+$ B cells in their cerebrospinal fluid (CSF) compared to peripheral B cells from healthy donors (HCPB) or CSF B cells

from patients with neurological diseases not related to MS. However, VH4 bias was a moderate predictor for conversion to MS.

Nevertheless, detailed analysis of antibody V-gene repertoires revealed eight codons that are significantly more mutated in the MS CSF than HCPB VH4-expressing B cells. This MS-specific antibody signature includes 25% of all mutations within the repertoires of CSF-derived B cells from MS patients. We then used the prevalence of this signature to predict if CIS patients converted to MS within two years of repertoire sampling. Indeed, we accurately predicted conversion to MS in 10 of 11 CIS patients. The B cell VH4 antibody signature can potentially be used as a diagnostic and prognostic tool for MS.

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Ab=antibody

Ag=antigen

AID=activation-induced (cytidine) deaminase

APC=antigen presenting cell

BAE or BAN=β-actin external or β-actin nested PCR reactions

BCR=B cell receptor

BDNF=brain-derived neurotrophic factor

CD=cluster of differentiation; family of glycoproteins involved in immunology

CDMS=clinically definite multiple sclerosis

CDR=complementary determining region

CIS=clinically isolated syndrome

CISCSF=clinically isolated syndrome cerebrospinal fluid

CNS=central nervous system

CS=class switched

CSF=cerebrospinal fluid

DW=DGYW/WRCH motif

EAE=experimental autoimmune encephalomyelitis

FAC=fluorescent-activated cell sorting

FBS=fetal bovine serum

FcRn=neonatal Fc (portion of antibody with isotype constant regions) receptor for IgG

FDA=United States of America Food and Drug Administration

FR=framework

HC=healthy control

HCPB=healthy control peripheral blood

$HCPB_{VH4}$=database containing VH4-expressing B cells from the HCPB

IEF=isoelectric focusing

IFN=interferon

Ig=immunoglobulin

IM=intramuscularly

IV=intravenously

IVIg=intravenously-infused immunoglobulin

LP=lumbar puncture

MBP=myelin basic protein

MF=mutational frequency

MHC=major histocompatibility complex

μL=microliter

mL=milliliter

MOG=myelin oligodendrocyte protein

MRI=magnetic resonance imaging

MS=multiple sclerosis

MSCSF=multiple sclerosis cerebrospinal fluid

$MSCSF_{VH4}$= database containing VH4-expressing B cells from the MSCSF

MSPB=multiple sclerosis peripheral blood

N:M=naïve B cell ($CD27^-$) to memory B cell ($CD27^+$) ratio

NGF=nerve growth factor

NMO=neuromyelitis optica, also known as Devic's disease

OCB=oligoclonal bands

OND=other neurological disease (besides MS)

ONDCSF=other neurological disease cerebrospinal fluid

PB=peripheral blood

PBS=phosphate buffered saline

PCR=polymerase chain reaction

PEP=primer extension preamplification

PLP=proteolipid protein

PPMS=primary progressive multiple sclerosis

PRMS=progressive relapsing multiple sclerosis

R=replacement

rpm=revolutions per minute

RRMS=relapsing remitting multiple sclerosis

S=silent

SC=subcutaneous

SD=standard deviation

SEM=standard error of the mean

SHM=somatic hypermutation

SPMS=secondary progressive multiple sclerosis

TdT=terminal deoxynucleotidyl transferase

TNF=tumor necrosis factor

VH4=variable heavy chain-4

CHAPTER ONE
Introduction

Clinically isolated syndrome (CIS) is the diagnosis of patients who have experienced a single demyelinating event within the white matter of the central nervous system (CNS). CIS patients can be categorized as clinically definite multiple sclerosis (CDMS), laboratory-supported definite multiple sclerosis (MS), clinically probable MS, laboratory-supported probable MS, or not MS (McDonald et al., 2001). Early immunomodulatory treatment can delay and dampen signs of MS, and so determining which CIS patients are more likely to convert to MS will allow for earlier treatment. We hypothesized that changes in humoral immunity (B cells and their antibody products) provide a more definite and earlier identification of CIS patients that will convert to MS than what current methods allow. The following introductory sections provide a detailed foundation for this hypothesis.

I. Multiple Sclerosis

MS is a chronic disease affecting the degradation of nervous tissue function via inflammatory destruction of myelin and nerve axons in the white and grey matter of the CNS (Bitsch et al., 2000; Geurts et al., 2005; Trapp et al., 1998). It has a high degree of variability, as the symptoms are dependent on which myelinated nerve(s) of the CNS is or are affected by the inflammation and destruction. During an MS "attack" (exacerbation or relapse), the symptoms

develop in hours to days and can persist for several days, weeks, or even never resolve (Brass et al., 2006).

CDMS comes in 4 sub-types, relapsing remitting multiple sclerosis (RRMS), primary progressive multiple sclerosis (PPMS), secondary progressive multiple sclerosis (SPMS), and progressive relapsing (PRMS) (Lublin and Reingold, 1996) (Figure 1-1). Approximately 85% of CDMS patients have the relapsing remitting form of the disease in which periods of disability (often up to 4 weeks in duration) occur interspersed with periods of normality (Brass et al., 2006) (Figure 1-2). Approximately 40-60% of these RRMS patients will go on to develop SPMS in which the disability becomes increasingly more considerable (Brass et al., 2006) (Figure 1-2). The patients may still have further relapses, but also experience an underlying progression of disability. These patients often will respond to immunotherapy during the relapsing remitting stage, but not as clearly in the progressive stage (1998; Cohen et al., 2002; Li et al., 2001). This could indicate that while inflammation is important during the early stages, it is less important to debilitation later in disease; whether immune modulators could prevent the occurrence or impact the timing of the progressive stage remains to be seen (Brass et al., 2006). The remaining 15% of patients have PPMS, and similar to SPMS, the functional ability the patients experience continues to worsen (Brass et al., 2006). Also similar to SPMS, PPMS is often impervious to immunotherapy (Leary et al., 2003). Progressive relapsing MS (PRMS) is the least common form

of MS and is similar in presentation to secondary progressive in which individuals have a steady neurological decline with superimposed attacks. All types of MS show the presence of lesions, and active MS lesions have several types of immune cells in them, one of which is B cells.

II. B lymphocytes: 1. B cell development

B lymphocytes, or B cells, are immune cells that express antibodies that are secreted into the circulation at later stages of B cell maturation. B cells arise from pluripotent stem cells in the bone marrow, and the stages of B cell development are demarcated by the stage of antibody rearrangement (section II-2 below; Figure 1-3) and directed by cytokines from the bone marrow stromal cells. B cells leave the bone marrow at the immature B cell stage when a functional unique antibody is expressed on the surface (reviewed in (Murphy et al., 2008b; Tangye and Mackay, 2006)). The immature B cell migrates to the spleen and becomes a transitional B cell; most transitional B cell will be capable of leaving as a mature naïve B cell (Figure 1-3). The mature B cell circulates between lymph nodes and the blood until it encounters the antigen it recognizes and interacts with a T cell (Figure 1-4). The lymph node microenvironment including the T cells, the follicular dendritic cells, and cytokines, will then cause the B cell to undergo somatic hypermutation (Chapter 1, section II-3), isotype switching, and affinity maturation/selection in a germinal center of the lymph node (Figure 1-4) (reviewed in (Murphy et al., 2008b; Tangye and Mackay, 2006)). The B cell

then leaves the lymph node as a plasma blast or memory cell. B cell activation can also occur by a T cell independent response created by a mitogen or polymeric antigen such as polysaccharides or lipids capable of binding multiple IgM complexes on a single B cell (which usually only creates IgM responses) (Figure 1-4) (reviewed in (Murphy et al., 2008b; Tangye and Mackay, 2006)).

II-2. VDJ recombination

B cell development is demarcated by the status of antibody rearrangement (Figure 1-3). Once the antibody heavy chain starts rearranging, the future B cell is known as a proB cell. The RAG enzymes and terminal deoxynucleotidyl transferase (TdT) enzyme are involved in causing the gene rearrangement of the D to J rearrangement followed by the V segment addition at the preBI stage (Figure 1-3 and Figure 1-5) (reviewed in (Murphy et al., 2008a)). If this combination is capable of producing a functional heavy chain, it is expressed on the cell surface in association with the surrogate light chain consisting of the VpreB protein (which resembles a light chain variable domain) and the λ5 protein (which forms a disulfide bond with the heavy chain), and demarcates the large PreBII stage. This preBCR also contains CD79 (a and b also known as Igα and Igβ), and interacts with CD19 and CD20 signaling molecules on the surface of the cell. The heavy chain is always of the IgM isotype at this stage in development. The B cell then rearranges the light chain genes at the small PreBII stage of development, and if capable of successfully rearranging one of these, becomes the

immature B cell (Figure 1-3) (reviewed in (Murphy et al., 2008a)). The number of possible combinations of heavy and light chains, along with unique junction nucleotides creates a large amount of diversity to encounter potential antigens (estimated to be at least 10^{11} (Murphy et al., 2008a)). Affinity maturation, in which somatic mutation accumulation results in higher affinity for the antigen further expands the diversity of the repertoire.

II-3. Somatic hypermutation and affinity maturation

During the process of activation, affinity maturation (somatic hypermutation (SHM) and clonal selection) occurs to create an antibody with increasingly greater affinity for the antigen. Particular regions of the antibody are more likely to be mutated, with the constant regions being the least likely, and the variable region (VDJ) the most likely (reviewed in (Casali et al., 2006; Diaz and Casali, 2002; Murphy et al., 2008a; Tangye and Mackay, 2006)). In the variable gene segment, somatic mutations are more prevalent in complementary determining regions (CDR) and less common in the framework regions (FR). These regions are targeted differently due the mutability of the component sequences (how likely a mutation at each site in the codon will result in a different amino acid) (Shapiro et al., 1999; Shapiro et al., 2002) as well as being enriched with nucleotide sequences or motifs within the variable gene segments that are targets for SHM by the mutational polymerases (Rogozin and Diaz, 2004).

SHM involves an enzyme, activation-induced (cytidine) deaminase (AID), which causes deamination of cytidine to uracil during DNA replication of cell division. This enables other repair enzymes (uracil-DNA glycosylase (UNG) followed by apurinic/apyrimidinic endonuclease (APE1)) to alter and cause a single (or double) strand break in the DNA creating a location requiring an error-prone polymerase to repair. The DNA polymerases η and ι are highly prone to error causing a mismatch with the opposite DNA strand, and the ζ polymerase is prone to extend from the mismatch, together causing multiple mutations in the variable region (reviewed in (Casali et al., 2006; Diaz and Casali, 2002; Murphy et al., 2008a; Tangye and Mackay, 2006)). The η DNA polymerase targets motifs on a single strand with the sequence of WA, and the AID DNA enzyme targets either DNA strand with a motif of DGYW or WRCH (reviewed in (Rogozin and Diaz, 2004)) (also Chapter 3, section V-3 and Table 2-12).

After recognizing the antigen and interacting with a T cell, the B cells clonally expand before undergoing hypermutation, which creates a collection of B cells that differ only in which mutations occurred in that particular cell. The B cell receptor is then "tested" against the antigen, and only B cells that retain or enhance affinity for the antigen survive to leave the germinal center. This process occurs each time the B cell undergoes a germinal center reaction in response to antigen, thus causing the B cell to accumulate mutations. B cells that receive the proper co-stimulatory signals (in either T-dependent or T-independent) become

antibody secretors, and some become memory B cells, cells that circulate in the body and survive without antigen for months to years capable of responding promptly if the antigen is reintroduced (reviewed in (Casali et al., 2006; Diaz and Casali, 2002; Murphy et al., 2008a; Tangye and Mackay, 2006)). Co-stimulatory signals in T-independent antigenic stimulation are from polymeric antigens capable of binding to multiple antibodies and co-localizing the signaling molecules with the antibody-antigen interaction. In T-dependent responses, the primary B cell co-stimulator is CD40, which interacts with CD40L on the T cell surface, though the B7 molecules interacting with CD28 on the T cell surface can also have an effect. These cells can rearrange the DNA to produce a particular isotype of antibody via input from cytokine environment, a process termed class switching. In mice, class switching occurs to IgG2a after IFN-γ, to IgG2b and IgA with TGF-β, and IgG1 and IgE with IL-4 (reviewed in (Amel Kashipaz et al., 2003; Fear et al., 2004; Harris et al., 2000)). In humans, the class switch stimuli is less understood, as class switching to all isotypes can be induced in human B cells by IL-4 and anti-CD40 (Fujieda et al., 1995; Jumper et al., 1994; Splawski et al., 1993), though the discovery of IL-21 and the ability of this cytokine to assist in causing switching to IgG and IgA clarified the field (Avery et al., 2008). The RNA during antibody production can also undergo transcription to be either secreted or membrane-bound on the B cell surface. Activated B cells can also become short-lived plasma blasts, temporary antibody machines, or long-lived

plasma cells, secreting basal levels of antibody. These cells lose most of the signaling molecules on the B cell surface, including the BCR, CD19, and CD20, but express a CD138 molecule, allowing us to recognize cells at this stage of maturation.

Autoimmune B cells are thought to bind self antigen with insufficient affinity to cause apoptosis in the bone marrow, and thus escape to the periphery. Multiple sclerosis is believed to be one of these autoimmune diseases, as discussed below.

III. Immune contributions to MS

The concept that MS is an autoimmune disease is supported by several pieces of evidence. First, inflammatory infiltrates including T lymphocytes, B lymphocytes, dendritic cells, and monocytes have been described in perivascular spaces and lesions of all sub-types of MS patients (Kerschensteiner et al., 1999; Prineas, 1979; Zhang et al., 2005b). Second, there is a genetic propensity for MS since monozygotic twins and first-degree relatives of MS patients have an increased risk of acquiring the disease (Sadovnick et al., 1988). Over 10% of discordant twins (one having CDMS and the other without CDMS) show silent lesions on MRI (Hogancamp et al., 1997; Thorpe et al., 1994; Uitdehaag et al., 1989), and other MS-like abnormalities such as evoked potentials (Nuwer et al., 1985), and cerebrospinal fluid (CSF) oligoclonal bands (OCB) (Xu and McFarlin, 1984) have been observed, even though no overt symptoms of MS are evident.

Interestingly, however, in cases where both parents are afflicted with MS, only 9% of the children develop the disease (Hogancamp et al., 1997), thus supporting influence of unknown environmental factors. Finally, the animal models of MS, experimental autoimmune encephalomyelitis (EAE), can be transferred from animal to animal with immune cells, and caused by an induction of inflammatory responses (via injection(s) of antigen and immune reactionary agent) in monkeys and rodents (Lalive et al., 2006; Lyons et al., 1999; Marta et al., 2005; McClain et al., 2007; Morris-Downes et al., 2002; Peterson et al., 2007; Smith et al., 2005; von Budingen et al., 2006; Zephir et al., 2006) (reviewed in (Cross et al., 2001; Ziemssen and Ziemssen, 2005)). EAE can be transferred from an animal with disease to a naïve animal through the transfer of lymph node cells (Paterson, 1960; Stone, 1961), and specifically activated, but not resting, $CD4^+$ T cells (Ben-Nun et al., 1981; Pettinelli and McFarlin, 1981; Zamvil et al., 1985). B cells cannot transfer disease (reviewed in (Cross et al., 2001)), but when B cells are appropriately activated using whole protein (rather than peptide), EAE onset, severity and duration are similar to classical EAE induction protocols (Lyons et al., 1999; Wolf et al., 1996) (reviewed in (Martin Mdel and Monson, 2007)).

This evidence does not exclude the possibility that initiation of MS is related to infection, although no microbial or viral agent has been demonstrated to orchestrate MS instigation or perpetuation (Brass et al., 2006). Many antigens have been proposed to initiate or exacerbate MS, including proteolipid protein

(PLP), myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and others, including viruses such as varicella (Mancuso et al., 2007). These antigens can cause MS-like disease in rodents and non-human primates (Lalive et al., 2006; Lyons et al., 1999; Marta et al., 2005; McClain et al., 2007; Morris-Downes et al., 2002; Peterson et al., 2007; Smith et al., 2005; von Budingen et al., 2006; Zephir et al., 2006) (reviewed in (Cross et al., 2001; Ziemssen and Ziemssen, 2005)), and antibodies against these antigens can be found (Andersson et al., 2002; Annunziata et al., 1997; Egg et al., 2001; Genain et al., 1999; Lambracht-Washington et al., 2007; Raine et al., 1999; Reindl et al., 1999; Sellebjerg et al., 2000; Warren and Catz, 1994; Warren et al., 1994) (reviewed in (Cross et al., 2001; Ziemssen and Ziemssen, 2005)). Also, human B lymphocytes and monocytes (and some T cells) express CD1c and CD1d, non-classical MHC molecules that present lipids to other cells (Ulanova et al., 2000). Since myelin is composed of approximately 80% lipid and 20% protein (Voet and Voet, 1995), these MHC-like molecules may very well be inducing an immune reaction to these lipids, and antibodies to lipids can be found in CSF from MS patients (Villar et al., 2005). MS antigens may also change over the disease course due to axonal damage revealing new antigens (McFarland, 1999), and each lesion within a single patient may be heterogeneous in composition of inflammatory presentation and lesion pathogenesis (Gerhard et al., 1985; Glynn et al., 1982; Mattson et al., 1980). Four different types of lesions have been described (Lucchinetti et al.,

2000), but once MS has been established, the active lesions may become more homogeneous containing complement and antibodies associated with macrophages in the lesion (Breij et al., 2008). However, it has been suggested that autoreactive features of the adaptive immune system, T and B lymphocytes, are more important in the relapsing-remitting stage while the innate immune system is more important in the progressive stage (Vaknin-Dembinsky and Weiner, 2007), or does not involve immune-mediated mechanisms at all.

The CNS was once believed to be an immune privileged locale, but recent evidence confirms that a relatively low number of immune cells trafficking from the periphery survey that compartment for pathogenic entities (Pedemonte et al., 2006). When T cells and B cells are prevented from entering the CNS as occurs after natalizumab treatment, progressive multifocal leukoencephalopathy may occur due to an opportunistic virus (JC polyomavirus) (Stuve et al., 2007) disrupting latency. The antigen presenting cells (APC) perivascular cells and monocytes regularly survey the CNS in animals (Hickey et al., 1992; Imai et al., 1997), and both hematopoietic (immunological) and resident (CNS) APC are required for disease initiation (Myers et al., 1993).

The presence of the physical barriers of the blood-brain barrier and blood-cerebrospinal fluid barrier, the absence of demarcated lymph nodes and lymphatic vessels, limited methods of entry and exit into the CNS, the pro-apoptotic milieu of the brain parenchyma, and the limited number of cells expressing MHC

molecules all may contribute to a limited but accessible location (Pedemonte et al., 2006). Leukocytes, including T cells, B cells, and monocytes, can enter the CNS by traveling from the blood to: 1. parenchymal perivascular space, 2. subarachnoid space (the interface between vascular tissue and CSF), and 3. CSF at the choroid plexus where the CSF is created (Pedemonte et al., 2006). In fact, activated T lymphocytes can enter rat brain parenchyma directly in an antigen independent manner (Hickey et al., 1991). Thus, this route is likely a normal passage for all or most recently activated (blasting stage) lymphocytes, and may be regulated by chemokines. It is likely that leukocyte trafficking may be dissimilar in the progressive and the relapsing forms of MS (Eikelenboom et al., 2005; Sorensen and Sellebjerg, 2001).

Five drugs have been approved by the FDA to diminish the occurrence and severity of MS relapses, all of which are immunoregulatory in nature—IFN-β-1a (Avonex® administered IM weekly or Rebif® administered SC 3 times weekly), IFN-β-1b (Betaseron® administered SC every other day), glatiramer acetate (a random copolymer of glutamic acid, lysine, alanine, and tyrosine, Copaxone® administered SC daily), mitoxantrone (a topoisomerase inhibitor, Novantrone® administered IV every 3 months) and natalizumab (an antibody against VLA-4, Tysabri® administered IV monthly) (reviewed in (Brass et al., 2006; Stuve et al., 2008)). The interferons, glatiramer acetate, and natalizumab are indicated for RRMS, mitoxantrone for SPMS and for worsening forms of

RRMS. Polyclonal intravenous immunoglobulin (IVIg) treatment derived from healthy donors is used in several autoimmune diseases, systemic inflammatory diseases and transplantations for its immunomodulatory effects (Kaveri et al., 1994). The exact mechanism is controversial, it is believed to be by both V region dependent and Fc dependent mechanisms, as $F(ab')_2$ fragments can neutralize the ability of autoantibodies from binding autoantigen (Kaveri et al., 1994; Wurster and Haas, 1994). One mechanism is utilizing available and saturable Fc receptors (FcRn, e.g.) so that pathogenic (endogenous) antibodies are in excess and cleared from the blood (reviewed in (Lobo et al., 2004)). The FcRn is responsible for monitoring IgG turnover and in addition to being highly expressed in vascular endothelium and myeloid-derived APC, is also highly expressed in epithelium, including CNS epithelium (reviewed in (Roopenian and Akilesh, 2007)). Antibodies endocytosed, but not capable of binding to FcRn, for example when IgG is in excess, get degraded in the lysosomes of these cells (reviewed in (Roopenian and Akilesh, 2007)). Enhanced degradation of endogenous IgG can therefore be achieved by delivering IVIg (Hansen and Balthasar, 2002). This methodology has been shown to be crucial in murine IVIg therapy for other autoimmune diseases (autoimmune skin blistering diseases, immune thrombocytopenia purpura) (Deng and Balthasar, 2007; Jin and Balthasar, 2005; Li et al., 2005). Additional possible mechanisms include binding of complement (Basta et al., 1989), blockade of Fcγ-receptors on phagocytic cells

(Jin and Balthasar, 2005), inhibition of cytokine production (including IL-1, IL-8, and TNF) (Kaveri et al., 1994; Ruiz de Souza et al., 1995), possibly even remyelination (van Engelen et al., 1992), or idiotypic anti-idiotypic interactions to the autoantibodies (Ronda et al., 1994; Rossi and Kazatchkine, 1989). It is unknown if it is necessary for the IVIg to access the CNS, though most MS patients do have disruption of the blood brain barrier as shown by gadolinium access to CNS lesions (Kaveri et al., 1994; Wurster and Haas, 1994). Azathioprine (unknown immunosuppressive mechanism of action inhibiting cell proliferation, Imuran®), corticosteroids (glucocorticoids hydrocortisone or corticosterone, potent anti-inflammatory agent), methotrexate (DNA synthesis inhibitor used as chemotherapy, Trexall® or Rheumatrex®), and mycophenolate (unknown immunomodulatory mechanism, usually given concordant with glucocorticoids, CellCept® or Myfortic®) have also been used in patients refractory to standard therapy, but it is unknown if these non-conventional agents slow disease progression (Frohman et al., 2005). Promising investigational therapeutic agents include rituximab (antibody which depletes CD20-bearing cells, Rituxan®), cyclophosphamide (alkylating chemotherapeutic agent, Cytoxan® or Neosar®), daclizumab (antibody against CD25, Zenapax®), cholesterol-lowering HMG-CoA reductase inhibitors (statins) (Frohman et al., 2005; Stuve et al., 2008; Stuve et al., 2002; Stuve et al., 2006b), and bone marrow transplantation (Gualandi et al., 2007) (reviewed in (Brass et al., 2006; Stuve et

al., 2008)). The interferons have been revealed in clinical trials to delay the occurrence of the second event, and therefore CDMS (CHAMPS/CHAMPIONS studies, ETOMS study, BENEFIT study, PRISMS studies (reviewed in (Stuve et al., 2008))). Since severe side effects can occur with these medications (e.g., liver injury with IFN-β (Tremlett et al., 2004; Wallack and Callon, 2004)), the benefit to the patient in frequency or severity of exacerbations must exceed the risk.

Other medications whose method of action involve altering the nervous system could be considered, because it is known that cross-talk between the nervous system and the immune system occurs (reviewed in (Kern and Ziemssen, 2008; Kerschensteiner et al., 2003; Steinman, 2004)), though many of the methods are as yet unclear. This approach of immune modulation may be complicated by many factors involving the local environment at the time of administration and whether immune or CNS targeting could be accomplished. The level of expression of β2 adrenoreceptors on peripheral blood mononuclear cells correlates well with disease activity in RRMS and PPMS but not in SPMS (Zoukos et al., 1994; Zoukos et al., 2003). Glucocorticoids are complex immune modulators that can either enhance or inhibit immune function depending on the situation; while glucocorticoid levels and immune cell receptor density were similar to controls in RRMS patients, an overall decreased sensitivity to glucocorticoids in immune cells from RRMS patients have been observed (reviewed in (Kern and Ziemssen, 2008)). This may explain the differences in

glucocorticoid responses between individual MS patients (van Winsen et al., 2005). In the peripheral blood of healthy controls (HCPB), oral terbutaline (β-receptor agonist) induces an anti-inflammatory cytokine (IL-10) and inhibits IFN-γ and TNF-α release, but not in peripheral blood cells from MS patients (MSPB) (Heesen et al., 2002). This may indicate that the nervous system creates an environment where the threshold for activation is increased over that in the periphery, but this threshold is not increased (in comparison to the periphery) in MS patients. Immune cells can also influence nerve function as T cells, B cells, and monocytes are all capable of producing nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). Interestingly, NGF can cause B cells to proliferate, produce immunoglobulin, and enhance cell survival (reviewed in (Kerschensteiner et al., 2003)), which may be the underlying support for heightened B cell activity in the CNS of MS patients.

Studying CNS lesions in MS is ideal for determining the immunological and physiological causes and distinctions of the lesions, but is impractical to do on a large number of living patients, and autopsy samples are not always readily available. CSF still requires a lumbar puncture, but not a biopsy of the individual's lesion(s). CSF is the fluid that cushions the brain, and is generally thought of as the draining fluid of the CNS. Thus cells circulating through the CNS tissues will be located in the CSF. Indeed, a relative high correlation between MS CSF B cell clones and OCBs found in individual RRMS patients

provides suggestion that the CSF is a clear representation of MS lesion cells and antibodies (Obermeier et al., 2008). However, it is unlikely that all of the individual cells and clones are adequately represented at similar frequencies in the CSF as in the lesion locale. CSF analysis is crucial in a correct differential diagnosis of CDMS, and the MS Therapy Consensus Group recommends positive CSF findings before immunomodulatory treatments are considered (Rieckmann and Toyka, 1999; Rieckmann et al., 2004). Positive CSF findings include an elevated white blood cell (WBC) count, OCB, or IgG index greater than an accepted value (actual value varies with laboratories, but generally considered to be above 0.8 (Mayringer et al., 2005)) (McDonald et al., 2001). Many other neurological diseases can mimic MS, and CSF findings may help indicate a definite diagnosis or eliminate other possibilities (reviewed in (Mehling et al., 2008)).

IV. B cells in MS

B cells have historically been implicated in the pathogenesis of MS since elevated CNS immunoglobulins and oligoclonal bands (OCB) were first described in MS patients in the 1940s (Kabat et al., 1950; Kabat et al., 1948; Kabat et al., 1942). In fact, up to 94% of MS patients have OCB (Link and Muller, 1971), which are small discrete bands located between pH 6 to 10 in an IEF separation of spinal fluid proteins. Additional evidence of B cell involvement in MS pathogenesis includes the presence of B cells in MS lesions (Raine et al., 1999),

and presence of B cells trafficking into the CNS during lesion development (Esiri, 1977) that intensifies with disease duration (Ozawa et al., 1994). Furthermore, the ratio of B cells to monocytes is stable over the disease course, but those patients with a prevalence of B cells have a more expeditious disease progression than those with monocyte predominance (Cepok et al., 2001). Antibodies in juxtaposition with complement have also been identified in MS lesions intermingled with disintegrating myelin, suggesting a potential causative role played by the immune elements in lesion development (Genain et al., 1999; Storch and Lassmann, 1997).

Much of what is known about MS was achieved through the use of an animal model, EAE. EAE in mice is induced in susceptible mouse strains by the transfer of myelin-reactive T cells or by active immunizations with myelin peptides, myelin proteins, or spinal cord homogenate. The presence or absence of B cells can affect the severity of disease, though in mice devoid of B cells disease can still be initiated with peptide (Wolf et al., 1996) (reviewed in (Cross et al., 2001; Martin Mdel and Monson, 2007)). Transfer of autoreactive B cells or antibody by itself is also capable of inducing disease in mice when EAE is induced with complex protein preparations (Lyons et al., 2002; Lyons et al., 1999) (reviewed in (Cross, 2000; Cross and Stark, 2005)). Interestingly, mice devoid of B cells develop EAE when peptides are used as antigens (Lyons et al., 1999). Complicating interpretation of EAE outcomes are differences in strains of mice

used, dosages, and immunizing antigen. For example, immunization of C57BL/6 mice with rat MOG protein or rat MOG peptide had a B cell-independent disease course (Hjelmstrom et al., 1998), but mice immunized with human MOG protein elicited a B cell-dependent disease (Lyons et al., 1999; Marta et al., 2005; Oliver et al., 2003). This is despite obtaining comparable antibody titers of similar immunoglobulin isotype and subclass when either rat or human MOG protein was used as the immunogen (Oliver et al., 2003), though only the antibodies capable of creating pathogenesis in cultured oligodendrocytes bound the human MOG glycoprotein while the non-pathogenic antibodies bound the unglycosylated MOG (Marta et al., 2005). Anti-myelin antibodies are also critical to successful EAE induction in marmosets (Genain and Hauser, 1996), and demyelination in Lewis rat EAE only occurs when antibodies are present (Linington et al., 1988). Double knock-in mice with both transgenic TCR and BCR that recognize a particular MOG peptide were necessary to develop spontaneous disease (the MS-like neuromyelitis optica (see Chapter 1, section V)) when immunized with rat peptide, while neither single transgenic had a high penetrance of disease (Bettelli et al., 2006). In conclusion, B cells can play an important role in EAE, the animal model of MS.

A more recent indication that B cells can play an important role in MS pathogenesis is the success of Rituxan® (rituximab), a humanized antibody targeting mature B cells, in treatment of RRMS patients. The neurological

improvements in the patients occurs too rapidly for the treatment's effect to be explained by the loss of antibodies to self antigen alone, thus an additional role of B cells other than antibody production (antigen presentation, T cell co-stimulation, inflammatory cell recruitment through the production of cytokines such as CXCL12 (SDF-1) a potent chemoattractant (Corcione et al., 2004)) in this disease is suggested. However, this does not suggest the increase in antibody production and the emergence of oligoclonal banding in the CSF of MS patients is not imperative to MS development or progression (Esiri, 1977; Kabat et al., 1950; Lucchinetti et al., 2000; Villar et al., 2002). Of note, however, OCB are not unique to MS, since patients with other neurological diseases can also be OCB positive (reviewed in (Reske et al., 2005) and (Brass et al., 2006)).

V. Other Neurological Diseases

In fact, any pathological process that causes transient or progressive injury to the central nervous system can mimic MS, and make a definite diagnosis of MS, which is variable in presentation, complicated by the necessity to consider many other possible disorders and lab evaluations to achieve a definite differential diagnosis. Inflammatory and autoimmune diagnoses resembling MS in presentation (albeit less common) include systemic lupus erythematosus (between 25 to 80% can have nervous system manifestations), antiphospholipid (Hughes) syndrome, Sjögren's syndrome (20% have CNS symptoms), neurosarcoidosis (5-10% of patients have CNS involvement, but often initially presents with CNS

signs), neuro-Behcet's disease, primary angiitis of the CNS, polyarteritis nodosa (up to 40% have CNS involvement), Wegener's granulomatosis, and Susac syndrome (Fadil et al., 2007). Infectious origins of MS-like symptoms include lyme disease, neurosyphilis, progressive multifocal leukoencephalopathy, tropical spastic paraparesis due to HTLV-1, and HIV (Fadil et al., 2007); other demyelinating disorders include metachromatic leukodystrophy, multiple sulfatase deficiency, Alexander disease, adrenoleukodystropy, Pelizaeus-Merzbacher disease, acute disseminated encephalomyelitis, concentric sclerosis (Balo's disease), and Schilder's disease (Fadil et al., 2007). In some of these disorders, involvement of other organ systems, specific antibodies in the CSF or serum, MRI and genetic tests, and instability of OCB banding that dissipates with corticosteroid treatment can suggest a non-MS diagnosis (Fadil et al., 2007; Reske et al., 2005). If an MS diagnosis is suspected, MRI and CSF results are relied upon to either provide evidence for or against this diagnosis, and can cause a misdiagnosis (Steven Vernino, M.D., Ph.D., personal communication).

Contention about whether neuromyelitis optica (NMO) represents a variety of MS or is a distinct disease entity is complicated by the fact that both NMO and MS can present in a range of phenotypes. NMO is rarely responsive to interferons and glatiramer acetate therapies, but plasma exchange and a small study with rituximab suggest these may be beneficial to this disorder (Frohman and Kerr, 2007). In addition, it has been demonstrated that serum antibodies from

NMO patients react to aquaporin 4 water channels, whereas serum antibodies from MS patients do not (Lennon et al., 2005; Lennon et al., 2004). In the research presented here, one of the CIS patients did obtain a diagnosis of NMO, and was considered to have CDMS, though an argument can be made that it is not MS.

VI. Clinically Isolated Syndromes

CIS presentation varies, but does typically emerge as isolated neurological disability in optic nerves, brainstem, or spinal cord, though many appear as multifocal abnormalities (Miller et al., 2005). Optic neuritis is the most common presentation and is caused by inflammation in the optic nerve, causing pain and impaired vision (Soderstrom, 2001). Transverse myelitis and brainstem syndromes are other common presentations of CIS (Pelidou et al., 2008).

Analysis of MRI lesion distribution in both time and space is necessary to prevent a misdiagnosis of another disorder (McDonald et al., 2001). Most MS treatment centers do not have the ability to perform frequent MRI and so diagnoses are made without the benefit of a second MRI (Polman, 2008). A current recommendation is to have the patient obtain an MRI every 3 months to determine a definite diagnosis of MS as early as possible (Pelidou et al., 2008). These patients have MS, but the medical and scientific communities lack the ability to distinguish the patients with MS from those with OND of similar features, as there is no single diagnostic test for MS. Another complication is

retrospective recall by patient of other possible demyelinating events that have occurred earlier, but that was not documented as to exact dates, clinical examination or MRI, and symptom duration. It has also been speculated that subclinical inflammation, demyelination and neurodegeneration may be present for a significant time (months to years) prior to detectable clinical symptoms (Stuve et al., 2008). Subclinical MRI lesions have been reported in up to 18% of healthy monozygotic twin of an MS patient (Stuve et al., 2008). Regardless, though CNS tissue damage may already have occurred, in those patients who will end up converting to MS, treatment should begin as soon as possible to deter further axonal and neuronal deterioration that advances with disease progression (Stuve et al., 2008). However, the immunomodulatory drugs do have side effects, so treating CIS patients who are not going to develop an autoimmune phenotype is not optimal. Finding new biomarkers for diagnosis of CDMS may be a means to increase likelihood of correct diagnosis and earlier treatment.

VII. Humoral Role in MS

Several unusual characteristics of B cells and their antibody products derived from the CSF of MS patients indicate that one such biomarker may be identified in this population. For example, several groups including ours have demonstrated that the distribution of genes used to generate antibodies in B cells from the cerebrospinal fluid (CSF) and brain lesions of MS patients are different from expected distributions. Indeed, the distributions are different in some cases,

particularly with regard to a family of variable heavy chains (VH4) which are significantly increased in frequency compared to expected distributions in the CSF (Colombo et al., 2000; Monson et al., 2005; Owens et al., 2003; Qin et al., 1998; Ritchie et al., 2004) and brain lesions (Baranzini et al., 1999; Owens et al., 1998) of MS patients. This finding was initially unremarkable since clonally expanding and autoreactive B cells from the CSF of MS patients could be found utilizing variable genes from any of the heavy (and light) chain families (Buluwela and Rabbitts, 1988; Humphries et al., 1988; Kodaira et al., 1986; Lee et al., 1987; Shen et al., 1987). However, emerging evidence that the VH4 expressing B cell population harbors autoreactive B cells (Koelsch et al., 2007) combined with the established observation that VH4 expressing B cells are overrepresented in CNS-derived B cell populations from MS patients (Baranzini et al., 1999; Colombo et al., 2000; Owens et al., 1998; Owens et al., 2003; Qin et al., 1998; Ritchie et al., 2004) prompted us to hypothesize that VH4 expressing B cells of CSF-derived B cells from MS patients would have evidence of dysregulation as assessed by mutation patterns associated with germinal center selection, as well as other features of autoreactive B cells such as high JH6 usage and long CDR3s. To address this, we utilized our MSCSF database that consists of B cell antibody repertoires from the CSF of eleven CDMS patients to compare repertoire characteristics to that of healthy controls or other CNS-related disorders. The VH4 over-expression seen in the CDMS patients is due to changes

in use of many of the genes in the VH4 family, and mutational analysis suggests antigen-driven selection in the context of classical germinal centers is intact. The hypothesis driving this dissertation was that unique patterns of mutations in VH4 expressing CSF-derived B cells of MS patients would provide a useful molecular diagnostic tool for MS. This hypothesis was tested by methods provided in the next chapter.

FIGURE LEGENDS

Figure 1-1. Sub-types of multiple sclerosis. Depiction of an average case of each of the four sub-types of MS. Figure created by Doug Arnold, M.D., Montreal Neurological Institute and Hospital, and adapted from (Lublin and Reingold, 1996).

Figure 1-2. Multiple sclerosis manifestations and progression. MS demographics based on study of 1,099 untreated patients evaluated at the MS Clinic at the University Hospital in London, Canada between 1972 and 1984. Figure created by Doug Arnold, M.D., Montreal Neurological Institute and Hospital, and adapted from (Weinshenker et al., 1989).

Figure 1-3. Stages of B cell maturation. B cell stage of development is demarcated by rearrangement of the BCR. Purple dotted lines show the preBCR, while purple solid lines are IgM, and red solid lines are IgD. Additional abbreviations: BM=bone marrow, GL=germline, SLN=secondary lymph node.

Figure 1-4. T cell independent (TI) and T cell dependent (TD) antigen driven activation. Purple solid lines are IgM, red solid lines are IgD, striped lines are Ig undergoing mutational and possibly isotype changes, peach lines are isotype switched—these can be IgG, IgE, IgA, IgM, or IgD, but each cell only produces one isotype. Figure adapted from (Tangye and Mackay, 2006).

Figure 1-5. VH recombination. Depiction of genetic changes and transcriptional adaptions involved in creating an antibody. Genetic changes occur during B cell development in the bone marrow, transcriptional changes occur each time the B cell creates the antibody. Further segments are added to determine if the resultant antibody will be secreted or membrane bound. Blue boxes depict the Variable (V) segments, the green boxes the Diversity (D) segments, the purple boxes are Joining (J) segments, and the peach boxes are the constant (C) regions. Drawing not to scale.

Figure 1-1

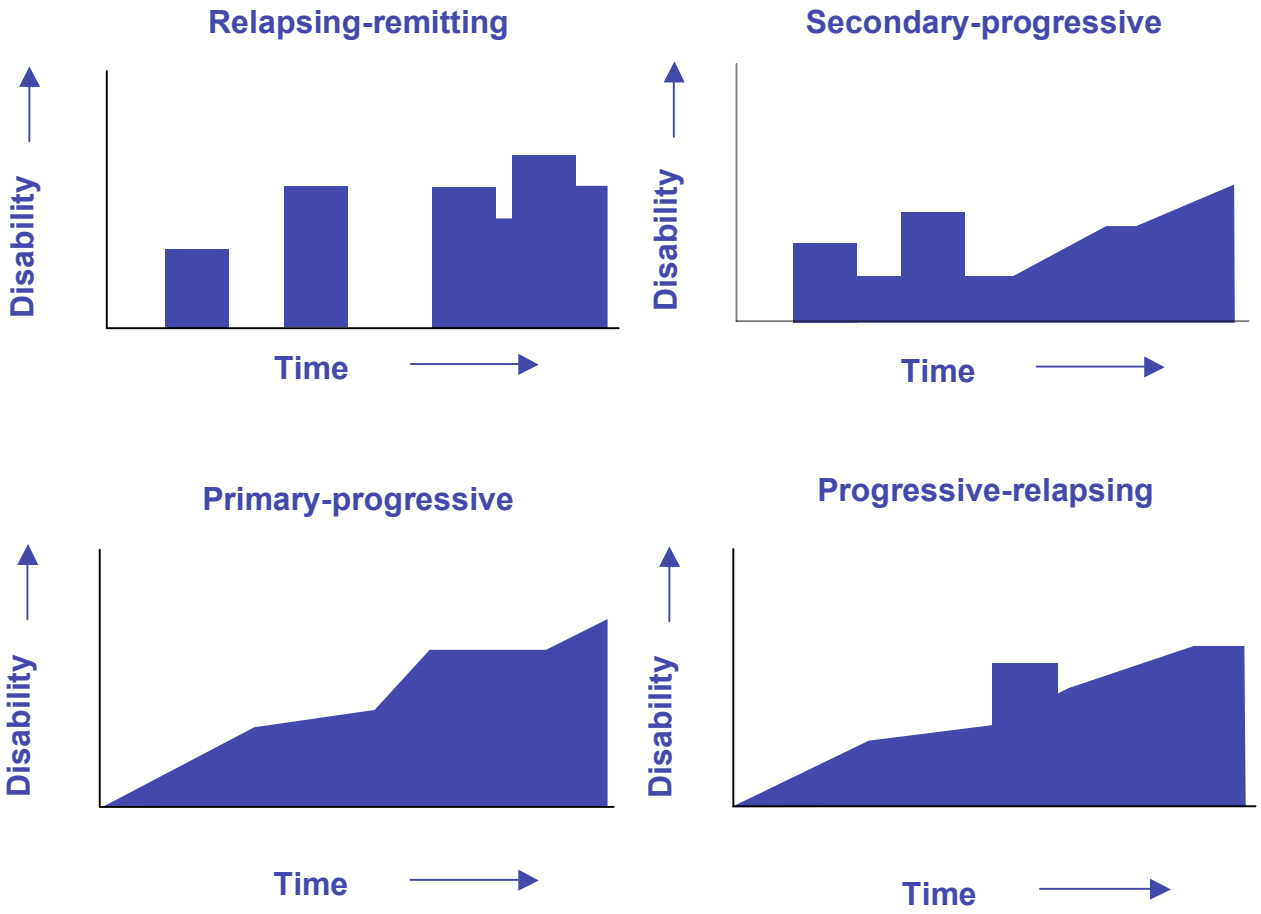
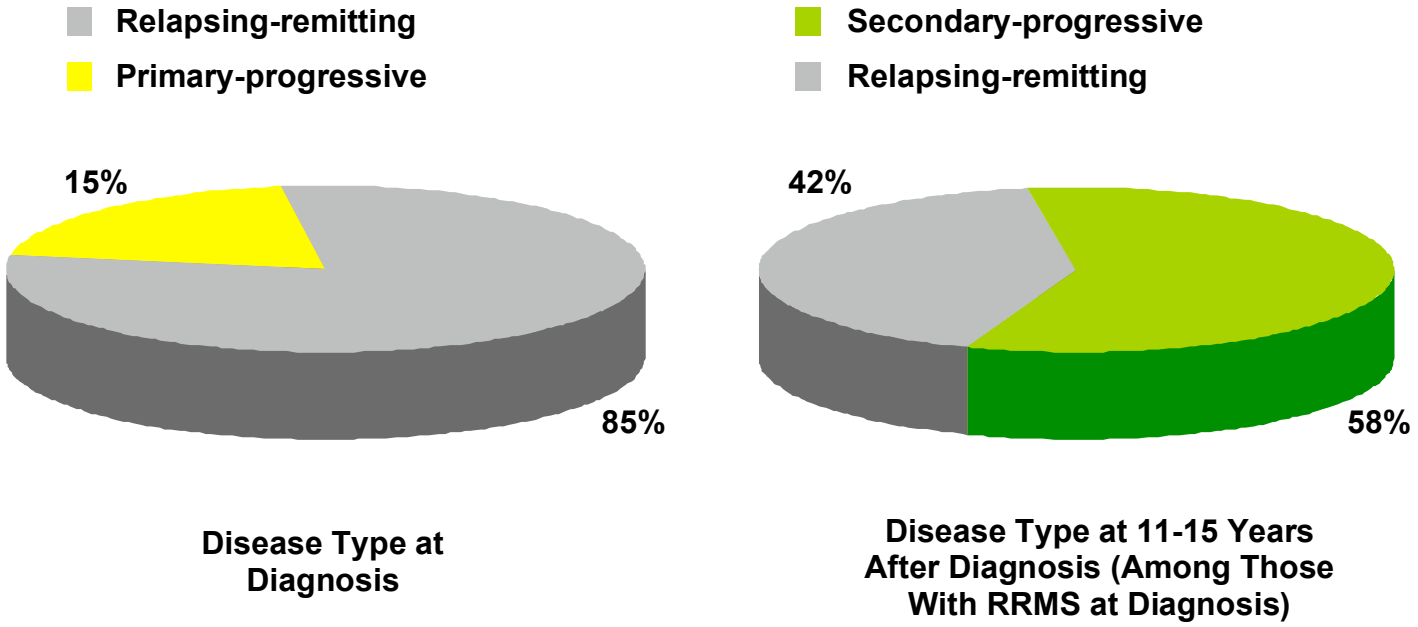

Relapsing-remitting
Disability
Time
Secondary-progressive
Disability
Time
Primary-progressive
Disability
Time
Progressive-relapsing
Disability
Time

Figure 1-2



Disease Type at Diagnosis

Disease Type at 11-15 Years After Diagnosis (Among Those With RRMS at Diagnosis)

Figure 1-3

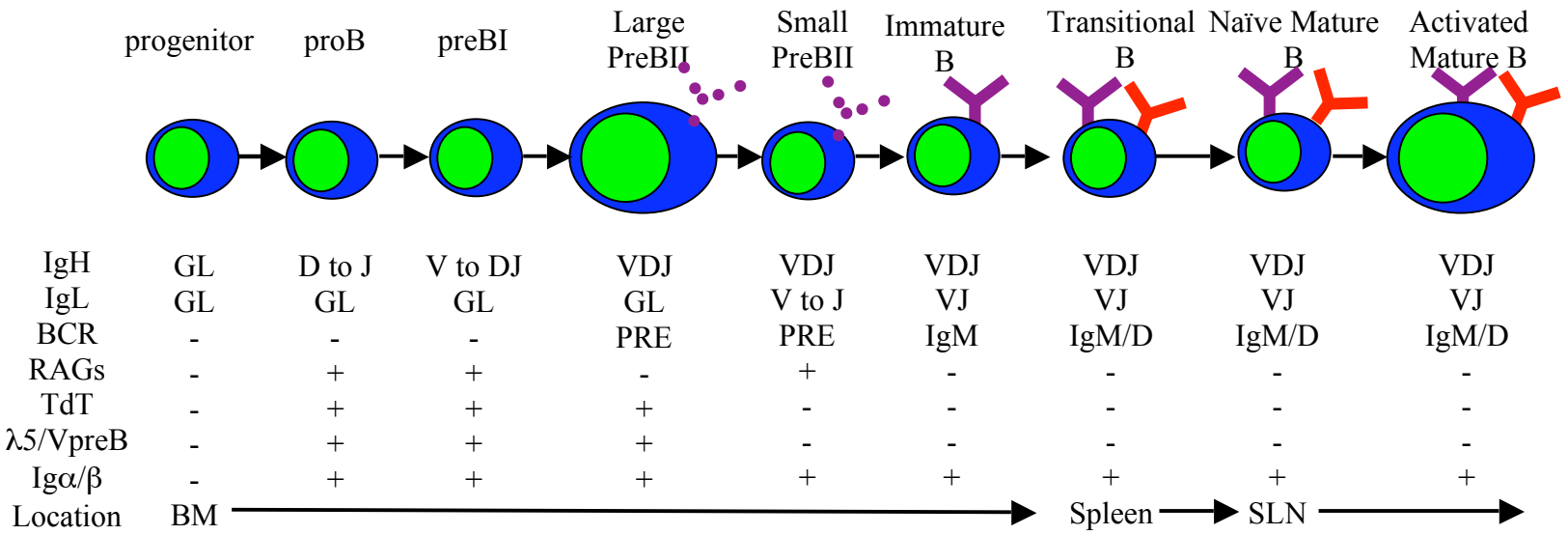

progenitor
proB
preBI
Large PreBII
Small PreBII
Immature B
Transitional B
Naïve Mature B
Activated Mature B
IgH GL D to J V to DJ VDJ VDJ VDJ VDJ VDJ VDJ
IgL GL GL GL GL V to J VJ VJ VJ VJ
BCR - - - PRE PRE IgM IgM/D IgM/D IgM/D
RAGs - + + - + - - - -
TdT - + + + - - - - -
λ5/VpreB - + + + - - - - -
Igα/β - + + + + + + + +
Location BM Spleen SLN

Figure 1-4

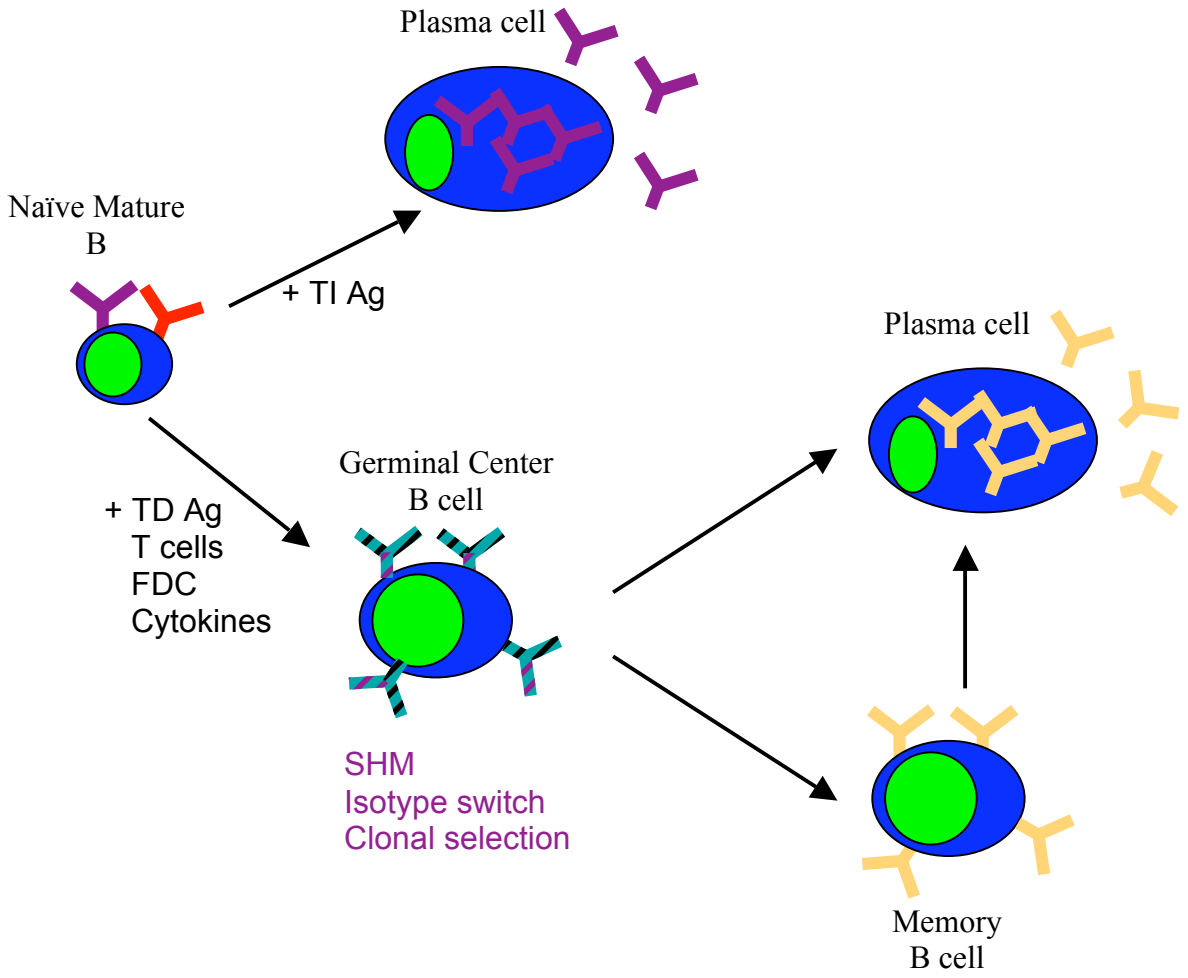
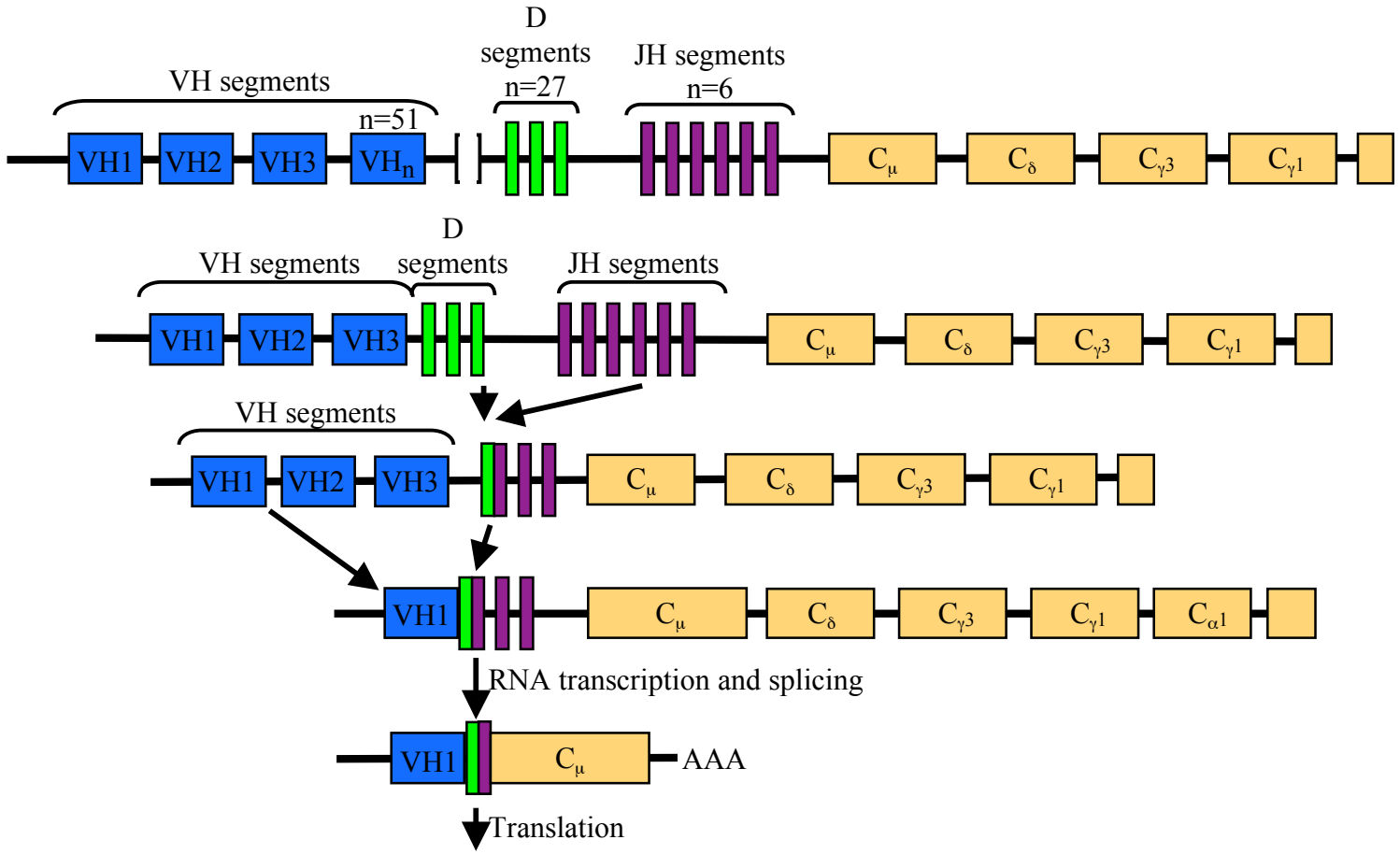

Plasma cell
Naïve Mature
B
+ TI Ag
Plasma cell
Germinal Center
B cell
+ TD Ag
T cells
FDC
Cytokines
SHM
Isotype switch
Clonal selection
Memory
B cell

Figure 1-5



Processing of protein and Pairing of Heavy Chain and Pseudo Light Chains to make Pre-BCR

CHAPTER TWO
Materials and Methods

I. Brief overview of antibody repertoire procurement (Figure 2-1)

Cerebrospinal fluid (CSF) was obtained by lumbar puncture and peripheral blood (PB) by venipuncture on the same day from patients recruited to be in the study at UT Southwestern Medical Center. The PB cells were centrifuged after being underlaid with a polysaccharide gradient that collects the mononuclear cells in a separate layer; the cells were then washed, counted, stained with labeling antibodies, and sorted. CSF cells were collected as a pellet after centrifugation, washed, counted, stained and sorted for single cells. During the sort, flow cytometry was collected to measure expression of B cell markers of interest. The plasma and CSF supernatants were aliquoted and archived. After the sort, a primer extension preamplification (PEP) was used to amplify the original DNA. Several rounds of polymerase chain reactions (PCR) were then performed to further amplify the antibody sequence rearrangement in each B cell. The PCR products were purified, sequenced, and analyzed for mutation characteristics and catalogued in databases. The CSF supernatant was analyzed for the presence of IgM, IgA, IgG, kappa, and lambda chains with the patient's plasma as a positive internal control.

II. CSF patient sample database composition: 1. Multiple Sclerosis

The MSCSF $CD19^+$ B cell antibody database was collected from ten RRMS patients and one PPMS patient at UT Southwestern Medical Center, in accordance with the UT Southwestern Institutional Review Board (refer to Table 2-1 for patient phenotypic details and Table 2-2 for individual stain and sort particulars).

II. 2. Clinically isolated syndromes

CSF from ten patients that had experienced a clinically isolated event (diagnosed as a patient with a clinically isolated syndrome (CIS) were recruited to be in the study at UT Southwestern Medical Center, in accordance with UT Southwestern Institutional Review Board at the time of the diagnostic lumbar puncture (LP). CSF antibody repertoires from two of these patients provided adequate sequence numbers for inclusion into the CIS antibody database. The remaining 8 patient samples were used for flow cytometry and isoelectric focusing (IEF). Antibody repertoires from the CSF of 9 CIS patients were obtained from our collaborators at University of Colorado Denver in accordance with the Institutional Review Board and published in Ritchie, et al. (Ritchie et al., 2004) or Bennett, et al. (Bennett et al., 2008). The patients included in the CISCSF database for repertoire analyses are listed in Table 2-3.

II. 3. Other neurological diseases

CSF from three OND patients was collected at UT Southwestern Medical Center, in accordance with UT Southwestern Institutional Review Board (Table 2-5). The patients had diagnoses of an inflammatory nature (OND341 and OND116) and non-inflammatory (OND758), and served as a control CSF population.

III. PB patient sample database composition: 1. Healthy Control

The healthy control peripheral blood (HCPB) antibody database was a combination of an in-house antibody database that has been used in multiple studies (Brezinschek et al., 1997; Brezinschek et al., 1998; Dorner et al., 1997; Dorner et al., 1998a, b; Dorner et al., 1998c; Farner et al., 1999; Hansen et al., 2000; Monson et al., 2000). Sequences with more than 4 mutations (less than 98% germline) from the in-house antibody database (Brezinschek et al., 1997; Brezinschek et al., 1998; Dorner et al., 1997; Dorner et al., 1998a, b; Dorner et al., 1998c; Farner et al., 1999; Hansen et al., 2000; Monson et al., 2000) were combined with the class-switched $IgD^{-}CD27^{+}$ database described in (Tian et al., 2007) (Genbank 535266-535274, 535324-535368, 535381-535408, and 535416-535418) to create a memory HCPB antibody database (mHCPB). The $IgD^{-}CD27^{+}$ database was collected under the Vanderbilt University Medical Center Institutional Review Board (Tian et al., 2007). The sequences from the $IgD^{-}CD27^{+}$ database were re-analyzed by our laboratory using IgBlast to obtain

specifics on gene usage, mutational number, and mutational codon location and type (replacement or silent) and to ensure any differences seen were not biased by inherent prejudice in evaluation tools. The sequences with less than 98% germline identity can be considered memory because this is similar to the mutation rate found in $CD27^+$ memory B cells (Klein et al., 1998) and resembles the mutation rate of IgG rather than IgM expressing B cells (Huang and Stollar, 1993); this mutational rate is significantly greater than can be acquired by Taq polymerase error (conservatively 1×10^{-4} mutations per basepair (Farner et al., 1999)). The patients included in this HCPB database are listed in Table 2-6, and the staining and sort details in Table 2-7.

III. 2. Multiple Sclerosis

The MS peripheral blood (MSPB) antibody database was collected from the peripheral blood of three relapsing remitting MS patients (M125, M354, and M484) (Tables 2-1 and 2-2) at the time of CSF sampling. This database was used to determine how specific the abnormalities seen in the CSF were in comparison to the overall B cell population from MS patients. Because most of the B cells found in the CSF are of the memory phenotype (Harp et al., 2007), peripheral blood antibody sequences from these 3 MS patients with more than 4 mutations were used as a memory population (mMSPB) for optimal comparison due to the predominance of memory cells in the CSF B cell population.

IV. Peripheral blood handling and processing

Approximately 5 to 10 milliliters of PB was acquired by venipuncture. The PB samples were diluted with an equal amount of phosphate buffered saline (PBS) then underlaid with the same amount (equal to the original amount of PB sample collected) of Ficoll® polysaccharide (Ficoll-Paque Plus, GE Healthcare Bio-Sciences, Corp., Piscataway, NJ) and centrifuged at room temperature at 726g (1900 rpm on Eppendorf 5810R with A-4-62 rotor (18 cm), Eppendorf, Westbury, NY) for 25 minutes without a brake to separate the mononuclear cells from the plasma and erythrocytes. The plasma was removed and aliquoted for freezing at -80°C to serve as a comparative sample and positive control to CSF supernatant in the antibody detection assays. The mononuclear cells were removed from the interface between the plasma layer and the high-density cells such as erythrocytes and granulocytes. The cells were diluted and washed to remove Ficoll, then counted to determine the concentration of cells in the initial sample volume and diluted to two million cells per milliliter (mL) for staining. Cells were counted in a hemacytometer by trypan blue (0.04% in PBS) or by Turk's solution (trace of gentian violet to 100 cm^3 of 1% acetic acid in water) exclusion. PB cells were sorted at 1 cell per well (see Chapter 2, section VII), and then subjected to single cell PCR as described in Chapter 2, sections IX and X. Flow cytometry details are provided in Chapter 2, section VI.

Peripheral blood mononuclear cells from healthy volunteers at Vanderbilt University were separated by a Ficoll-Hypaque density gradient, stained, and sorted by flow cytometry using a FACS Aria cytometer (Becton, Dickinson, and Company, San Jose, CA) (Tian et al., 2007). Cells were stained and sorted for $CD19^{+}IgD^{-}CD27^{+}$ that the authors called class switch memory cells (Tian et al., 2007). Single B cells were then expanded in a 96-well plate using feeder cells, supernatant from non-specifically activated T cells, IL-2 and IL-4 in a 3-week culture system. Human Ig capture ELISA were performed on each set of identical B cells to determine if the cells were producing IgM, IgG, or IgA. Reverse transcription PCR was then done on the mRNA isolated from each set of B cells that secreted antibody. Immunologic sequences from each set of identical B cells were then obtained by use of an automated DNA sequencer (Applied Biosystems, Foster City, CA) (Tian et al., 2007).

V. Cerebrospinal fluid handling and processing

Twelve to 15 milliliters of CSF (range of 4 to 25 mL) was collected by lumbar puncture. After procurement, CSF samples were centrifuged at 4°C at a speed of 201g (1000 rpm on Eppendorf 5810R with A-4-62 rotor (18 cm), Eppendorf, Westbury, NY) for 12 minutes, and the supernatant removed and aliquoted for freezing at -80°C for later antibody detection assays. The CSF cell pellet was washed and resuspended in one milliliter PBS/FBS (2 to 4% FBS), then stained with the same panel of antibodies used to stain the PB for the same length

of time. Collection materials were prepared during the staining incubation; following the incubation, the CSF cells were washed, resuspended in PBS/FBS (2 to 4%) and taken for single cell sorting and data collection (see Tables 2-2, 2-4, 2-5 and 2-7).

VI. Flow cytometry analysis

After the PB was resuspended to two million cells per mL, 250 microliters (μL) of PB cells ($5*10^5$ cells) were placed into each 5-mL tube to be used for compensation. Polypropylene tubes (12*75 mm round bottom; BD Falcon Catalog 352063; Becton, Dickinson, and Company, Bedford, MA) were used for MoFlo sorts; polystyrene tubes (12*75 mm round bottom; BD Falcon Catalog 352054; Becton, Dickinson, and Company, Bedford, MA) were used for Vantage sorts; Aria sorts were done with either type of tube. One mL of PB cells ($2*10^6$ cells) was used for the PB 6-color staining; the CSF cells were resuspended in 1 mL as long as there were less than $2 *10^6$ cells in the sample. All cells were centrifuged for 5 minutes at 4°C at 453g (1500 rpm on Eppendorf 5810R with A-4-62 rotor (18 cm), Eppendorf, Westbury, NY). After centrifugation, most of the supernatant was removed and the cells resuspended in the remaining supernatant. Either 20 or 40 μL of blocking buffer (made with 780 μL 2% FBS/PBS with 20 μL 1 mg/mL mouse sera added to it) was then added to the resuspended cells and incubated on ice for at least 10 minutes. Fluorochrome-labeled antibodies (Table 2-8) were then added to appropriate tubes according to manufacturer's

recommendations. Individual patient staining details can be found in Tables 2-2, 2-4, 2-5, and 2-7. All stains were used by themselves for the compensation except CD8 PE was used for PE compensation, as not enough cells in the PB express CD138 under normal circumstances; 10 μL of antibody was the typical amount added, though less was typically used with CD45 APC-Cy7 (2.5 μL), CD4 PE-Cy7 (3 μL), and CD8 APC (5 μL). Up to 40 μL of each antibody was used in the PBL and CSF tubes with antibodies of up to 6 colors total. Cells were incubated with the antibodies on ice for at least 30 minutes covered in aluminum foil to minimize light exposure. If necessary, plates with lysing solution were made up during this incubation (see Chapter 2, section VII). After incubation, 1 mL of 2% FBS in PBS was added to each tube to dilute excess non-bound antibody, then cells were centrifuged again for 5 minutes at 4°C at 453g (1500 rpm on Eppendorf 5810R with A-4-62 rotor (18 cm), Eppendorf, Westbury, NY). The supernatant was subsequently removed, and the cells resuspended with either 250 μL or 500 μL of 2% FBS in PBS and placed on ice.

Flow data was collected during cell sorting by the MoFlo (Cytomation, Beckman Coulter, Fort Collins, CO), FAC Star, Vantage, or Aria (Becton, Dickinson, and Company Biosystems, San Jose, CA). Staining was later analyzed in FlowJo (Tree Star, Inc., Ashland, OR) by selecting single cells by forward scatter width (FSC-W) if available, then live cells by forward scatter area (FSC-A) and side scatter (SSC). The single, live cells were then analyzed for

immunological characteristics. MS patient and CIS patient peripheral blood and CSF were examined for CD19, CD27, CD138, IgM, CD45, and CD16 expression (see Table 2-8 for antibodies and Tables 2-2 and 2-4 for patient particulars). HLA-DR was intermittently substituted in the protocol for CD45. OND patients (Table 2-5) were assessed for CD19, CD27, and CD138 expression as well as to CD4, CD8, and HLA-DR. The flow cytometry data from CSF samples were always compared to the PB of the same patient, to other patient CSF samples in the same patient group, and to control patient CSF samples.

VII. B cell single cell sorting and lysis

One $CD19^+$ B cell was sorted into each well of a 96-well plate containing 10 or 20 μL of lysis buffer (proteinase K (USB Corporation, Cleveland, OH) usually diluted or dissolved in Tris-HCl pH 8.0 mixed to a final volume of 400 μg/mL in 1x PCR buffer) using MoFlo (Cytomation, Beckman Coulter, Fort Collins, CO), FACStar, Vantage, or Aria (Becton, Dickinson, and Company Biosystems, San Jose, CA) (see Tables 2-2, 2-4, 2-5, and 2-7 for each patient's sort specifics). Samples were on ice except for the span of time the single cell sort expended. At least 2 wells of each plate were loaded with 10 cells to serve as an internal positive control. After covering the lysate with 10 μL of mineral oil to prevent evaporation and pulse spinning the plates (spinning at least 1000 rpm for 1 to 2 seconds), complete lysis was performed by heating the plates at 55°C for one hour followed by 95°C for 10 minutes in PTC-100 Programmable Thermal

Controller (MJ Research, Incorporated, Waltham, MA) or EP gradient S Mastercyclers (Eppendorf, Brinkmann Instruments, Inc., Westbury, NY). Current protocols using the REPLI-g Screening kit (QIAGEN, Germantown, MD, Catalog 150126) do not use lysis buffer to sort cells into, and instead sort each $CD19^+$ B cell into 3.5 μL of PBS (see end of Chapter 2, section VIII for further REPLI-g protocol).

VIII. B cell Primer Extension Preamplification

An initial non-specific primer extension preamplification (PEP) was performed using random 15mers (Operon BioTechnologies, Inc., Huntsville, AL) and wax pellets in a 62 cycle PCR with each cycle including a 3-minute gradient annealing temperature (starts at 37°C and increases 1°C every 10 seconds for 3 minutes each cycle for 60 seconds). Magnesium, dNTPs, and water (see Table 2-9 for concentrations) were mixed together on a 96-well PCR plate (TempPlate II, 1402-9600, USA Scientific, Inc., Ocala, FL) and a wax pellet was placed on top and melted at 80° for 5 minutes. Wax pellets were used in all single cell reactions to seal off the magnesium, primers, and dNTPs to prevent non-specific binding by keeping these reagents from mixing with the Taq polymerase enzyme and template until the reaction is underway (also called hot start) (see Figure 2-3 for an example of a hot start protocol). PCR buffer, random primer (Random 15mer, Operon Biotechnologies, Huntsville, AL, Catalog SP180), Taq enzyme and

additional water were then placed on top, and incubated as called for in the PEP program (see Table 2-10).

The wax pellets were created by melting wax on continual low heat and creating wax "dots" of 15 μL on aluminum foil, which were subsequently allowed to cool to solidify. Current protocol (REPLI-g Screening Kit, QIAGEN, Germantown, MD, Catalog 150126) does not use wax pellets. For the REPLI-g Screening kit, 17 μL of Buffer SB1 was added to each well, then after vortexing and spinning, plates were incubated at 65°C for 5 minutes (program '65' on Eppendorf Mastercyclers) and allowed to cool to room temperature (because polymerase is not thermo stable). One μL of the polymerase included with the kit was added in a mixture with 17 μL of Buffer SB2, so 18 μL of the mixture was added to each well. The plate was then incubated at 30°C for 16 hours (program '30' on Eppendorf Mastercyclers).

IX. Sort efficiency determination

Sort efficiencies were determined by β-actin PCR. A portion of the sort underwent an external and nested round of PCR (named BAE and BAN respectively) using the PCR product of the PEP as template. The result was displayed in a 1% agarose gel; sort efficiencies varied between experiments, generally being between 20 to 80%. β-actin external (BAE) PCR was performed using 5 μL of PEP template, and nested PCR was performed using 5 μL of the BAE PCR reaction as the template. The concentrations of PCR reagents are

shown in Table 2-9, the PCR program used for the β-actin external PCR is in Table 2-10, and the primers (Integrated DNA Technologies, Inc., Coralville, IA) for these reactions are listed in Table 2-11.

X. Heavy or light chain rearrangement amplification

For heavy chain PCR reactions, 5 μL of the random primer extension pre-amplification reaction (PEP) was used for an external reaction (named VHE), conducted at a gradient temperature (temperature changes from 50° to 58° for 30 seconds with each cycle; see Table 2-10) with a final volume of 50 μL; subsequently, 5 μL of the external reaction used for each nested (family specific) reaction (Table 2-9 and 2-10). Similar experiments were performed to obtain light chain sequences, with 5 μL of the PEP product being used for an external reaction, and 5 μL of the external reaction used for each nested (family specific) reaction. Magnesium chloride concentration and annealing temperatures were optimized for each external and nested PCR reaction, and are noted in Table 2-9. Primer sequences have been used in previous publications (Harp et al., 2007; Monson et al., 2005), and are shown in Table 2-11 (Integrated DNA Technologies, Inc., Coralville, IA). Taq polymerase error has been previously calculated to be 1×10^{-4} mutations per basepair as a conservative rate (Farner et al., 1999), although more recent variations of this enzyme can be as efficient as 4.4×10^{-7} (Phusion High-Fidelity DNA polymerase Finnzymes Oy, Espoo, Finland). This calculation would translate into 0.02 mutations in each VH

rearrangement (maximum of 215 basepairs) that may be attributable to Taq polymerase error. Bulk genomic DNA from the peripheral blood of a healthy control was used as an additional positive control for primer integrity. Example protocols for two different total volume reactions (both examples are VH4) are shown in Figures 2-2 (current protocol) and 2-3 (former hot-start protocol).

XI. PCR product visualization, processing, and sequencing

One fifth (10 μL) of the product of the nested reaction was visualized on a 1 to 2% agarose (various vendors) gel in 0.5x TBE (5.4 g Tris-Base, 2.75g Boric Acid (Fisher Scientific, Fair Lawn, NJ Catalog BP168-500), and 2 mL 0.5M EDTA per liter). Ten μL of 100bp DNA mass Ladder (New England BioLabs, Inc., Ipswich, MA, Catalog N3231S) at a final concentration of 0.1 μg/μL was included in each gel to use in size (number of basepairs) comparison (100bp DNA Ladder (Promega, Madison, WI Catalog #G2101) was used if New England BioLabs ladder was not available). The gel was run on Econo-Submarine Gel Unit (Model #SGE-030-02, CBS Scientific Co., Del Mar, CA) at 100 to 300 Volt-hours (typical gel of any size was run at a maximum of 115 milliamps). Resultant PCR products were purified by column or gel purification. Columns were used from various sources, but preferably QIAquick Columns (part of QIAquick PCR Purification Kit, Catalog #28106 QIAGEN, Germantown, MD). One hundred μL of Buffer PBI from the kit was added to each PCR well to be purified. The PCR reaction and PBI total volume was transferred to a column, and an additional 100

μL of Buffer PBI added. The columns were centrifuged at 15,996g (14,000 rpm on Eppendorf 5415C (7.3 cm), Eppendorf, Westbury, NY) for one minute, and then rinsed with 750 μL of Buffer PE (after ethanol had been added to it) for five minute at room temperature; these columns retain PCR products larger than 100 basepairs. The columns were centrifuged again (same speed and length of time), the liquid that had run through the column was disposed of, and then the columns were centrifuged a third time to ensure the Buffer PE had been completely removed. The membrane was wet with approximately 50 μL of water (molecular biology grade sterile) to elute the PCR products. The columns were placed in a 1.5-mL tube and centrifuged again at 15,996g (14,000 rpm on Eppendorf 5415C (7.3 cm), Eppendorf, Westbury, NY) for one minute at room temperature to elute PCR products larger than 100 basepairs from the column into the 1.5-mL tube. Gel purification was sometimes performed instead of column purification, using QIAGEN gel purification kit (QIAquick Nucleotide Removal Kit, QIAGEN, Germantown, MD). The band was cut out of the 1 to 2% agarose gel, placed into a 1.5-mL tube, and 3 times the weight volumes of buffer QG (300 μL buffer for each 100 mg of gel) was added. To check the efficiency of purification process, the purification products were then run on a 1 or 2% agarose gel to determine concentration of product obtained. Long Ranger Singels (Lonza, Fisher Scientific, Catalog BMA50694) system was used to sequence after at least 2 hours of polymerization. Sequencing was done with ABI Prism Dye Termination

Cycle Sequencing Kit in automated DNA sequencer (both of Perkin Elmer Corp.). Later, it was determined that it was as accurate and more cost effective to submit 6 μL of the purified PCR product (along with 6 μL of 1M appropriate V or J primer) for sequencing at UT Southwestern Medical Center Sequencing Core, which used Applied Biosystems Inc. (ABI) Big Dye Terminator 3.1 chemistry to collect data on ABI capillary instruments.

XII. Identification of V gene and mutational analyses: 1. V gene identification and length determination

B cell repertoire sequences from these patients were analyzed using Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, Michigan), and compared to the V-base human germline variable region sequence database (MRC Centre for Protein Engineering, Cambridge, UK). Differences from the IgBlast (NCBI www.ncbi.nlm.nih.gov/igblast) were evaluated, but few changes in gene usage were noted (e.g., variable heavy chain 4-30.1 sequences were changed to 4-31, and particular sequences were evaluated as particular allelic variations of other genes in the same family). B cell repertoire sequences from these patients were re-analyzed using the IgBlast (NCBI www.ncbi.nlm.nih.gov/igblast).

VH read length was defined as codons 31 through 95 (CDR1 and 2, FR2 and 3) (Table 2-12) for heavy chains, and codons 24 through 95 (CDR1, 2, and 3, FR2 and 3) for light chains. The 3' end of the V gene segment was defined as codon 95 on the condition that the germline variable sequence was present.

Sequences were considered productive provided that codon 103 (FR4) for heavy chain rearrangements or codon 98 (FR4) for lambda or kappa rearrangement remained in frame and no stop codons were inserted in the length of the V gene. Each nucleotide mutation in CDR1, FR2, CDR2, and FR3 (and the portion of CDR3 in light chain variable region) was counted once in mutational frequency and CDR targeting analyses, and each codon only counted once for replacement:silent ratios and replacement mutation frequencies.

XII. 2. Regional and sequence mutational frequency and codon replacement frequency

Mutational frequency was determined by dividing the number of mutations in a given germline VH, VK, or VL rearrangement by the read length, not including FR1. Mutational frequency in CDR or FR was calculated in the same manner, with the number of mutations in the regions divided by the total number of nucleotides in that region. For example, a sequence of 206 basepairs with 1 mutation would have a mutational frequency of 1/206 or 0.48%, which can be translated into 99.52% identical to the germline variable gene. Mutational position frequency was calculated as the number of replacement mutations at each codon location divided by the total number of replacement mutations in each particular database or subdatabase analyzed; this was evaluated using only replacement mutation frequency (RF) so that silent mutation bias was avoided. For heavy chains, codons 24 to 31 of FR1 (Table 2-12) were included in this

analysis because most of the sequences did include these locations, and thus might be biased, but conservatively. Codon 24 is the beginning of CDR1 in light chains. Codon domains were defined by (Kabat et al., 1983) and are shown in Table 2-12, and codon numbers were defined by Tomlinson in V-base (vbase.mrc-cpe.cam.ac.uk/).

XII. 3. Clonal expansion determination

Clonally expanded B cells were defined as those B cells whose VH or VL rearrangements were represented two or more times in the repertoire. Clonally related B cells were defined as having the same VH/DH/JH or VL/JL usage, CDR3 length and composition; and share a subset of mutations. In some analyses, repertoires were "normalized" so that the clones were only counted a single time.

XII. 4. Mutational machinery motifs

Targeting to DGYW/WRCH motifs (Table 2-12) was evaluated by mutational frequency in a motif; this number was generated using the number of mutations in a motif divided by the total number of nucleotides in DGYW/WRCH motifs (the number of nucleotides in a motif of each gene multiplied by the number of times this gene was used in the repertoire).

XII. 5. Replacement:silent ratios

Replacement to silent ratios (R:S ratios) were used as an indication of antigen selection (Jukes and King, 1979; Shlomchik et al., 1987), as mutations

leading to replacement of the amino acid would have greater potential to change the affinity of the antibody or B cell receptor for the antigen. Similarly by comparing the R:S ratios in the complementary determining regions (CDR) to that in the framework regions (FR), indicating antigen selection occurred in a typical germinal center reaction. The total number of codons with mutations leading to replacement of the germline amino acid with another was divided by the total number of codons with mutations that maintained the germline amino acid expressed in the resultant protein. This was done with each entire sequence in addition to the CDR and FR sections separately.

XIII. Signature Identification

Codons included in the signature were identified using three criteria. First, we identified codons that had statistically different RF values in the $MSCSF_{VH4}$ database compared to $HCPB_{VH4}$ by Goodness of Fit test where the expected frequency is the RF calculated in $HCPB_{VH4}$. Twenty-four codons passed this criterion. Next, codon positions that had an RF in both the $MSCSF_{VH4}$ and $HCPB_{VH4}$ databases that was less than the average + 2 S.D. of the memory $HCPB_{VH4}$ subdatabase were excluded. Thus, since the average ± S.D. RF of the memory $HCPB_{VH4}$ database was 0.68 ± 0.59, any individual codon RF less than 1.86 in both databases was excluded. Fourteen codons passed this additional criterion. Eight of these 14 codons (31B, 32, 40, 56, 57, 60, 81, and 89) were defined as "hot" since the RF at that codon position within the $MSCSF_{VH4}$

database was statistically higher compared to the $HCPB_{VH4}$ database. Six of these 14 codons (30, 43, 52, 77, 82 and 82a) were defined as "cold" since the RF at that codon position within the $MSCSF_{VH4}$ database was statistically less compared to the $HCPB_{VH4}$ database. Two of the 6 "cold" codons (52 and 82a) were excluded because the RF value in the $MSCSF_{VH4}$ database at that codon position was significantly higher than 1.86 (the average + 2 S.D. of the memory $HCPB_{VH4}$ subdatabase). The overall signature consequently consisted of codons 30, 31B, 32, 40, 43, 56, 57, 60, 77, 81, 82, and 89.

XIV. Signature score computation

Signature scores were generated by calculating Z-scores for the RF values at the 6 codons within the signature (31B, 40, 56, 57, 81 and 89) that had the most significant difference in RF compared to $HCPB_{VH4}$ at each codon position. The Z-score formula is: (RF at codon X minus the average RF in $HCPB_{VH4}$) divided by the standard deviation of the average RF in $HCPB_{VH4}$. For example, the average RF in $HCPB_{VH4}$ within the 6 signature codons was 1.6 ± 0.9 and so an RF of 4.4 at codon 31B would be assigned a score of 3.1 ((Z score=(4.4-1.6)/0.9)). Individual z-scores at each of the 6 codon positions were then added to generate the composite signature z-score. The average composite signature score in the $MSCSF_{VH4}$ database was 10.9 ±2.0 (range 7.6 to 11.9) (Figure 3-6) and so any signature score of an individual CIS patient above 6.8 (average – 2 S.D.) was predicted to convert to CDMS (Figure 3-6). Since the threshold of where to

"draw the line" between CIS patients is crucial to interpretation of the signature penetrance, we chose two standard deviations before examining the signature score distribution in the CIS patients, which created a distribution that would contain 95.4% of the CDMS patients by definition, and would be the lower threshold for 95% confidence interval. $CD19^+$ B cell and $CD138^+$ plasma cell mutation positions both contributed to each CIS patient's signature score. The $MSCSF_{VH4}$ signature scores were only composed of $CD19^+$ B cells.

XV. VH4 Structure

A human VH4-30.4 and VL2-8 antibody structure was obtained from the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (www.rcsb.org) under the identification moniker 1MCO, and adapted using the RasMol program (RasMac v2.6 available at mc2.cchem.berkeley.edu/Rasmol/ (Sayle and Milner-White, 1995)). The structure was described in (Guddat et al., 1993), and deposited in the data bank by the authors. The adaptations made were to show only the variable regions (including VDJ of the heavy and VJ of the light), and to highlight the codons included in the signature. Included in this structure is human VH4-30.4 (allele 01) in yellow and human lambda 2-8 (formerly known as 2c; allele 01) in gray. Note that Tomlinson numbering is different from that used by RasMol, e.g., 31B (Tomlinson) in the heavy and light chain is codon 33 (RasMol). VH4 structures are shown in Figures 4-1 and 4-2.

XVI. Isoelectric Focusing Gels

One to two milliliter aliquots of a patient's CSF supernatants was thawed then condensed using an iCON Concentrator (Catalog #89884, Pierce, Rockford, IL) approximately 10 to 40 fold by centrifugation at 3000 rpm for 40 minutes at 19°C. Three microliters of the condensed CSF supernatant was used in each well or lane (with the exception of the control well) of an agarose gel from the Titan Gel ImmunoFix IEF kit (Catalog #3046, Helena Laboratories, Beaumont, TX). The CSF supernatant was incubated at room temperature for 5 minutes to allow the gel to absorb the sample into the appropriate well; the excess was removed by blotting. Gel was run at 120V for 20 minutes (or 40V-hrs) in provided buffer (resuspended in 1.5 L deionized or distilled water) on the Titan IFE gel box (Model G4063000, Helena Laboratories, Beaumont, TX). Antisera was incubated with the gel for 10 minutes at room temperature to stain the samples for IgM, IgG, IgA, kappa and lambda in separate lanes of a single gel. After washing and drying, gel was stained in acid blue stain (provided in the Titan kit, dissolved in 1 L 5% acetic acid). Resultant stains were compared with a serum control gel in which after dilution, a manufacturer control of lyophilized pooled human serum with stabilizers added (SPE Normal Control, catalog #3424, Helena Laboratories, Beaumont, TX) was stained in a similar manner.

Each gel was run with a control serum sample (SPE Normal Control, catalog #3424, Helena Laboratories, Beaumont, TX) or the patient serum sample

diluted 1:2 in PBS. A fixative is added to the control well at the time the antisera were used; this was done to stain for overall protein to serve as a positive control for each gel. Usually, isoelectric focusing gels are compared against a patient plasma sample to determine if the oligoclonal bands are exclusive to the cerebrospinal fluid.

ELISA was used to determine the concentration of antibody in the CSF and to determine the final concentration used in the Titan IFE gels. ELISA plates (Thermo Fisher Scientific, Fair Lawn, NJ, Catalog 3655-1424579) were coated with anti-IgG (donkey-anti-human IgG (H+L), Jackson Immunoresearch, West Grove, PA, catalog 709-005-149) and anti-IgM (goat-anti-human IgM, Jackson Immunoresearch, West Grove, PA, catalog 109-005-129) were diluted to 2 μg/mL and 50 μL was added to each well then heated to 37°C for 30 minutes in an incubator (Quincy Lab, Inc., Chicago, IL, model 12-140). The wells in the plates were washed with PBS/Tween (0.5% Tween-20 (Sigma-Aldrich, Inc., St. Louis, MO, Catalog P7949) in PBS) in a plate washer (Wellwash 4 Mk 2, Labsystems, Thermo Fisher Scientific, Fair Lawn, NJ). Plates were blocked with 1% BSA in PBS at 4°C overnight. The next day, plates were washed, samples added and allowed to bind for 30 minutes at 37°C in an incubator. Plates were washed to remove unbound sample, then diluted (1:5000) alkaline phosphotase-conjugated antibodies to IgG (goat-anti-human IgG (H+L), Jackson Immunoresearch, West Grove, PA, catalog 109-055-008) and IgM (donkey-anti-human IgM, Jackson

Immunoresearch, West Grove, PA, catalog 709-055-073) were added and allowed to bind for 30 minutes at 37°C. Wells were washed, then 50 μL of substrate developing solution is added (2.5 μL 1M magnesium chloride, 125 μL 1M sodium bicarbonate, and 1 para-nitrophenyl tablet (5 mg, Sigma-Aldrich, Inc., St. Louis, MO, Catalog S529818) in 4.875 mL 1%BSA/PBS. The plates were incubated for 20 to 30 minutes until yellow color developed. Absorbance at 405 nm was measured on a microplate reader (Emax, Molecular Devices Corporation, Sunnyvale, CA).

XVII. Statistical analysis

Family usage, VH and VL gene usages, mutational frequencies (MF), naive: memory B cell ratios, D and J segment analyses, and CDR3 charge were compared using chi square analysis. CDR3 lengths were analyzed by ANOVA analysis, using the Kruskal-Wallis test. Mutational position was compared between MS and HCPB using the Goodness of Fit test. Signature statistics are detailed in Chapter 2, section XIII, and signature score statistics are detailed in Chapter 2, section XIV. Sensitivity, specificity, positive predictive value, and negative predictive value are explained following for the VH4 signature, but VH4 bias and clonal expansion numbers were computed in similar manners. Sensitivity was computed by those CIS patients that converted to CDMS that had signature and all of the CDMS patients with signature (true positives) divided by all of the patients that converted (including those that were without signature but

converted to CDMS (false negatives)). Specificity was computed by those CIS patients that had signature but did not convert to CDMS (false positives) divided by all of the patients that did not convert to CDMS (false positives and true negatives). Positive predictive value was computed by taking the true positives (those with signature and conversion to CDMS) divided by all the patients with signature, conversion or not. Negative predictive value was computed by those without signature that did not convert to CDMS (true negatives) by all of the patients without signature. p-values equal to or less than 0.05 were considered significant.

FIGURE LEGENDS

Figure 2-1. Summary of patient sample to antibody sequence procedure. Flow chart visualizing portions of this chapter pertaining to patient CSF and blood handling and subsequent PCR.

Figure 2-2. Example of low volume VH4 PCR protocol using heated primers. Following external VHE reaction, this protocol is the current method of amplifying VH4 rearrangements by PCR. A portion of the resultant sample is then loaded onto a 2% agarose gel to determine the number of wells that contained a $VH4^+$ B cell.

Figure 2-3. Example of hot-start VH4 protocol utilizing wax. Former method of VH4 amplification which divides portions of PCRs with a pre-made wax pellet to prevent primers from interacting with the Taq polymerase enzyme and DNA template until the initial heating step of the PCR program.

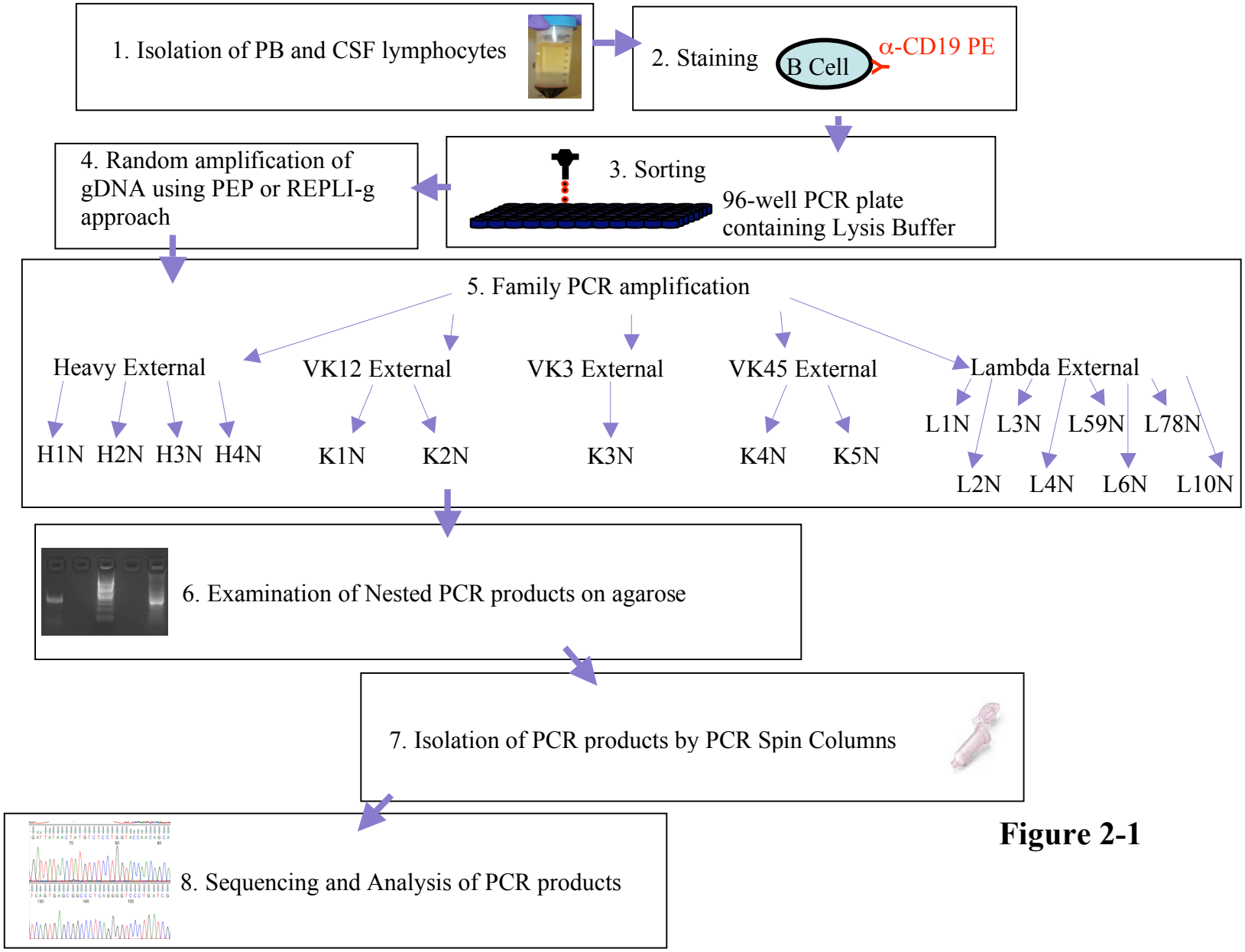

1. Isolation of PB and CSF lymphocytes
2. Staining
B Cell
α-CD19 PE
3. Sorting
96-well PCR plate containing Lysis Buffer
4. Random amplification of gDNA using PEP or REPLI-g approach
5. Family PCR amplification
Heavy External
VK12 External
VK3 External
VK45 External
Lambda External
H1N H2N H3N H4N
K1N K2N
K3N
K4N K5N
L1N L3N L59N L78N
L2N L4N L6N L10N
6. Examination of Nested PCR products on agarose
7. Isolation of PCR products by PCR Spin Columns
8. Sequencing and Analysis of PCR products

Figure 2-1

Figure 2-2

Purpose: VH4 amplification from Patient ID#XXX
Template: Patient ID# VHE
Primers: VH4N, JH1245N, JH36N

1 Dilute Primers 1:10 (originally at 100 uM)

2 Assemble the PCR Master Mix in a tube on ice.

Add the reagents in the yellow boxes *in the order listed*.

		enter total number of reactions here:		**# rxns**	
reagent	**reagent concentration**	**final concentration**	**ul per reaction**	**total volume to add**	
sterile mQH_2O			12.05	=12.05*# rxns*1.2	
VH4N	10.0 μM	0.5 μM	1.25	=1.25*# rxns*1.2	
JH1245N	10.0 μM	0.5 μM	1.25	=1.25*# rxns*1.2	
JH36N	10.0 μM	0.5 μM	1.25	=1.25*# rxns*1.2	
Heat primer mixture to 95 degrees C for five minutes before adding remaining ingredients.					
5X PCR Buffer (no Mg2+)	5 X	1 X	5.00	=5.0*# rxns*1.2	
$MgCl_2$	25 mM	1.5 mM	1.50	=1.50*# rxns*1.2	
dNTPs	10 mM	0.2 mM	0.50	=0.50*# rxns*1.2	
Taq (Promega)	5 units/μl	1 unit/rxn	0.200	=0.20*# rxns*1.2	
total volume		ul Master Mix	23.00	=23.0*# rxns*1.2	total volume
		ul DNA	2.00		
		total reaction volume	25.00		

3 Vortex the Master Mix and spin briefly in the Minifuge. Keep the mix on ice.

4 Pipette 23 ul Master Mix into each 0.2 ml tube or reaction well

Note: depending on how many reactions you have, you can either use individual 0.2 ml PCR tubes, 0.2 ml strip tubes, or a 96-well PCR reaction plate

5 Add template to each well.

6 Top with 15 uL mineral oil and cap the wells

7 Briefly spin the tubes (or plate).

8 Set up the PCR machine and start the reaction

9 Run 'VgN' PCR program

10 Dilute PCR product with 25 uL 1x PCR buffer

11 Load 2% agarose gel with 10 uL diluted PCR product or 10 uL loading dye

Figure 2-3

Purpose: VH4 amplification from Patient ID#XXX
Template: Patient ID #XXX VHE
Primers: VH4 Nested: VH4N + JH1245N + JH36N

LRM

	[Stock]	[Final]	Vol/rxn	* # rxns	Total V
ddH_2O	--	--	17.4		17.4 * # rxns
$Mg^{(2+)}$	25 mM	1.5 mM	4.8		4.8 * # rxns
Primers	100 mM	1 mM	0.4 * 3		0.4 * # rxns * 3
dNTPs	10 mM	200 mM	1.6		1.6 * # rxns
			25		25.0 * # rxns

Aliquot 25 mL/well
(Either use pre-waxed plates or add wax pellet with 18G needle)
Pulse spin
Run 'MELT' 80° 5', 4° until end

URM

	[Stock]	[Final]	Vol/rxn	* # rxns	Total V
ddH_2O	--	--	33.6		33.6 * # rxns
PCR buffer	5x	1x	16		16.0 * # rxns
TAQ	5U/ml	2U/rxn	0.4		0.4 * # rxns
			50		50.0 * # rxns

Aliquot 50 mL/well
Add 5 mL VHE external PCR product as template
Pulse spin
Run 'VgN' T_m=64° 40 cycles

Table 2-1: MS Patient Summary[1, 2]

	M125[3,4]	M199	M217	M354[3,4]	M368[3,4]	M376[4]	M484[3,4]	M522[4]	M584[4]	M875[3,4]	M887
Type of MS	RR	RR	RR	RR	RR	RR	PP	RR	RR	RR	RR
Time since MS diagnosis	<1 year	4 months	18 years	<1 year	15 years	20 years	3 months	3 years		1 month	13 years
Age/Sex	32/F	26/F	45/F	44/F	41/F	56/F	46/F	35/F	44/F	35/F	50/F
Clinical Presentation	ON	paresthesias	dystonia	TM	TM	ON	myelitis	TM	TM	ON	ON
MRI Brain Lesions	GD^+	GD^+	WML	WML	WML	WML	WML	GD^+ WML	GD^+	GD^+, WML	WML
Oligoclonal Bands	No	ND	ND	Yes	Yes	No	Yes	Yes	ND	ND	No
Ig Index	NL	ND	High	NL	High	ND	High	High	ND	ND	NL
Ig Synthesis	ND	ND	ND	NL	High	ND	High	ND	ND	ND	NL
No. CD19 VH Sequences	100 CSF 76 PB	19	1	6 CSF 19 PB	49	8	10 CSF 77 PB	71	85	21	3

Abbreviations: RR=Relapsing Remitting; SP=Secondary Progressive; ON=Optic Neuritis; TM=Transverse Myelitis; GD^+=gadolinium enhancing; WML=White Matter Lesions; NR=not reported; ND=not done; NL=normal

[1]All patients had CSF white blood cell (WBC) counts in the range of 1 $X10^3$ to 1 $X10^4$ per mL, typical of MS patients at UTSWMC (Stuve et al., 2006a)

[2]Three additional patients M918 (RRMS), M927 (RRMS), and M944 (SPMS) were not used in mutational signature analysis, but were used in flow cytometry and IEF analyses

[3]Patient clonal analysis previously published in (Monson et al., 2005)

[4]Patient repertoire or mutational analysis previously published in (Harp et al., 2007)

Descriptions of MS2-19, MS2-24, and MS3-7 are in figure legend of Figure 5-1

Table 2-2 MS Patient Sort Details

Patient Code	Date	Stain	Machine	Number of Plates Sorted	Cell Concentration[7] Volume PB received	Cell Concentration[7] Volume CSF received	Notes
M354	6-15-00	CD19 PE	FACStar[1]	2 PB 1 CSF	ND 10 cc	ND 25 cc	
M484	7-13-00	CD19 PE	FACStar[1]	1 PB 3 CSF	ND 10 cc	ND 20 cc	
M368	2-8-01	IgM FITC; IgD PE; CD19 PE-Cy5	FACStar[1]	2 PB 2 CSF	ND 10 cc	ND 30 cc	2 days with high FBS O/N
M125	7-27-01	IgM FITC; CD27 PE; CD19 PE-Cy5	FACStar[1]	2 PB 4 CSF	ND 10 cc	ND 15 cc	
M875	8-26-02	IgD FITC; CD27 PE; CD19 PE-Cy5	FACStar[3]	2 PB 1 CSF	ND 12 cc	$1.4 * 10^4$ 25 cc	
M887	1-22-03	IgD FITC; CD27 PE; CD19 PE-Cy5	MoFlo	2 PB 2 plates & 85 wells CSF	$1.8 * 10^6$ 7.5 cc	ND 22.5 cc	
M217	4-16-03	IgD FITC; CD27 PE; CD19 PE-Cy5; IgM APC	Vantage	12 wells PB 11 wells CSF	$2.3 * 10^6$ 10 cc	$7.7 * 10^4$ 15 cc	
M522-1	7-10-03	IgD FITC; CD27 PE; CD19 PE-Cy5; IgM APC	Vantage	2 PB 2 plates and 44 wells CSF	$7.0 * 10^5$ 10 cc	$1.3 * 10^3$ 23 cc	
M584-3	4-12-04	IgD FITC; CD27 PE; CD19 PE-Cy5; IgM APC	Vantage	2 PB 4 CSF	$2.7 * 10^6$ 20 cc	$3.1 * 10^4$ 20 cc	
M376	5-5-04	IgD FITC; CD27 PE; CD19 PE-Cy5; IgM APC; CD20 APC-Cy7	Vantage	1 PB 2 CSF	$2.4 * 10^6$ 25 cc	$9.3 * 10^2$ 27 cc	
M522-2	7-28-04	IgD FITC; CD27 PE; CD19 PE-Cy5; IgM APC; CD20 APC-Cy7	MoFlo[4]	2 PB 4 CSF	$1.1 * 10^6$ 12 cc	$7.5 * 10^3$ 22 cc	

Table 2-2 continued

Patient Code	Date	Stain	Machine	Number of Plates Sorted	Cell Concentration[7] Volume PB received	Cell Concentration[7] Volume CSF received	Notes
M199	2-25-05	IgD FITC; CD27 PE; CD19 PE-Cy5; IgM APC	Vantage[4]	2 PB 4 CSF	$4.0 * 10^6$ 10 cc	$1.7 * 10^3$ 22 cc	
M896/M4 12	7-25-07	CD27 FITC; CD138 PE; CD19 PE-Cy5; CD3 APC; CD16 PE-Cy7; HLA-DR APC-Cy7	Aria[2]	1 PB 1 CSF	$3.5 * 10^6$ 10 cc	$4.6 * 10^3$ 13 cc	2 days with cocktail
MS2-19	NR	Tube 1-CD3 FITC; CD138 PE Tube 2-CD3 FITC; CD19 PE	NR [5]	1 CSF CD19[6] 55 wells CSF CD138	NR NR	10 cells/mm^3 5 to 25 mL	Sort completed by Ritchie, et al. (Ritchie et al., 2004)
MS2-24	NR	Tube 1-CD3 FITC; CD138 PE Tube 2-CD3 FITC; CD19 PE	NR [5]	1 CSF CD19[6] 1 CSF CD138[6]	NR NR	17 cells/mm^3 5 to 25 mL	Sort completed by Ritchie, et al. (Ritchie et al., 2004)
MS3-7	NR	CD3 FITC; CD138 PE; CD19 APC	MoFlo[5]	1 CSF CD138	NR NR	10 cells/mm^3 5 to 25 mL	Sort completed by Owens, et al. (Owens et al., 2007)

Abbreviations: ND=not done; M=multiple sclerosis; PB=peripheral blood; CSF=cerebrospinal fluid; FITC=fluorescein isothiocyanate; PE=R-phycoerythrin; APC=allophycocyanin; NR=not reported

[1]Each well received 10 μL 1x PCR buffer before sort and 10 μL 1x PCR buffer with proteinase K (final concentration 400 μg/mL (dissolved in Tris-HCl pH 8.0) in 1x PCR buffer) after sort

[2]Sorted into 10 μL PCR buffer with proteinase K (final concentration 400 μg/mL (dissolved in Tris-HCl pH 8.0) in 1x PCR buffer)

[3]Each well received 20 μL 1x PCR buffer before sort and 20 μL 1x PCR buffer with proteinase K (final concentration 400 μg/mL (dissolved in Tris-HCl pH 8.0) in 1x PCR buffer) after sort

[4]Sorted into 20 μL PCR buffer with proteinase K (final concentration 400 μg/mL (dissolved in Tris-HCl pH 8.0) in 1x PCR buffer)

[5]Sorted into 20 μL 1x reverse transcriptase buffer

[6]Up to 3 plates sorted, but only 1 used for PCR

[7]in cells/cc for cell concentration and cc (equal to mL) for volume received

Table 2-3: CIS Repertoire Analysis Patient Summary

Subject No.[1]	CIS132	CIS429	CIS3-1	ON3-1	ON3-3	ON3-4	ON3-5	ON4-7	ON4-8	ON4-10	ON5-2
Age (years)/ Sex	24/F	62/M	25/F	44/F	32/M	25/F	23/F	27/F	49/F	39/M	35/F
Clinical Presentation	Diplopia	Optic Neuritis	Myelo-pathy	Optic Neuritis	Optic Neuritis	Optic Neuritis	Optic Neuritis	Optic Neuritis	Optic Neuritis	Optic Neuritis	Optic Neuritis
Time to LP[2]	1	1	2	10	4	1.5	1.75	3	1.5	1.25	1
Time to CDMS[2]	18	2	3	NA	3	NA	2	5	5	NA	3
MRI Brain Lesions	GD^+	GD^+	WML	WML	GD^+	None[3]	GD^+	None[3]	WML	WML	GD^+
Oligoclonal Bands	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	No	Yes
Ig Index	ND	0.7	1.4	2.0	0.5	0.5	2.1	1.2	0.8	0.5	1.8
Number of CD19 IgH sequences	19	56	24	23	39	28	35	17	NA	31	29
Number of CD138 IgH sequences	NA	NA	76	45	13	NA	44	20	18	NA	12

Abbreviations: CIS=Clinically Isolated Syndrome; ON=Optic Neuritis; LP=lumbar puncture; GD^+=gadolinium enhancing lesion positive; WML=white matter lesions by T2; OCB=oligoclonal bands; CDMS=clinically definite MS; NA=not applicable

[1]CIS132 and CIS429 were generated at UTSWMC; the remaining patient CSF B cell repertoires were generated at UCHSC.

[2]in months

[3]One spinal cord lesion was observed by T2 weighted MRI

Table 2-4 CIS Patient Sort Details

Patient Code	Date	Stain	Machine	Number of Plates Sorted	Cell Concentration[4] & Volume PB received[4]	Cell Concentration[4] & Volume CSF received[4]	Notes
CIS132	1-6-04	IgD FITC; CD27 PE; CD19 PE-Cy5; IgM APC	Vantage[1]	2 PB 4 CSF	$8.8 * 10^5$ 21 cc	$5.2 * 10^3$ 22 cc	
CIS429	9-26-05	CD27 FITC; CD138 PE; CD19 PE-Cy5; CD3 APC; CD16 PE-Cy7	Vantage[1]	2 ½ PB 1 ½ CSF	$1.8 * 10^6$ 15 cc	$2.0 * 10^3$ 22 cc	cDNA and gDNA single $CD19^+$ cells sorted
CIS3-1	NR	CD3 FITC; CD138 PE; CD19 APC	NR[2]	1 CSF $CD19^3$ 1 CSF $CD138^3$	ND	less than 1 cells/mm^3	Sort completed by Ritchie, et al. (Ritchie et al., 2004)
ON3-1	NR	CD3 FITC; CD138 PE; CD19 APC	MoFlo[2]	1 CSF $CD19^3$ 1 CSF $CD138^3$	ND	5 cells/mm^3	Sort completed by Bennett, et al. (Bennett et al., 2008)
ON3-3	NR	CD3 FITC; CD138 PE; CD19 APC	MoFlo[2]	1 CSF $CD19^3$ 1 CSF $CD138^3$	ND	1 cell/mm^3	Sort completed by Bennett, et al. (Bennett et al., 2008)
ON3-4	NR	CD3 FITC; CD138 PE; CD19 APC	MoFlo[2]	1 CSF $CD19^3$	ND	2 cells/mm^3	Sort completed by Bennett, et al. (Bennett et al., 2008)
ON3-5	NR	CD3 FITC; CD138 PE; CD19 APC	MoFlo[2]	1 CSF $CD19^3$ 1 CSF $CD138^3$	ND	32 cells/mm^3	Sort completed by Bennett, et al. (Bennett et al., 2008)
ON4-7	NR	CD3 FITC; CD138 PE; CD19 APC	MoFlo[2]	1 CSF $CD19^3$ 1 CSF $CD138^3$	ND	6 cells/mm^3	Sort completed by Bennett, et al. (Bennett et al., 2008)
ON4-8	NR	CD3 FITC; CD138 PE; CD19 APC	MoFlo[2]	1 CSF $CD138^3$	ND	10 cells/mm^3	Sort completed by Bennett, et al. (Bennett et al., 2008)
ON4-10	NR	CD3 FITC; CD138 PE; CD19 APC	MoFlo[2]	1 CSF $CD19^3$	ND	3 cells/mm^3	Sort completed by Bennett, et al. (Bennett et al., 2008)
ON5-2	NR	CD3 FITC; CD138 PE; CD19 APC	MoFlo[2]	1 CSF $CD19^3$ 1 CSF $CD138^3$	ND	12 cells/mm^3	Sort completed by Bennett, et al. (Bennett et al., 2008)

Abbreviations: ND=not done; CIS=clinically isolated syndrome; ON=optic neuritis; PB=peripheral blood; CSF=cerebrospinal fluid; FITC=fluorescein isothiocyanate; PE=R-phycoerythrin; APC=allophycocyanin; NR=not reported

[1]Sorted into 10 μL PCR buffer with proteinase K (final concentration 400 μg/mL (dissolved in Tris-HCl pH 8.0) in 1x PCR buffer)

[2]Sorted into 20 μL 1x reverse transcriptase buffer

[3]2 to 3 plates sorted, but only 1 used for PCR

[4]in cells/cc for cell concentration and cc (equal to mL) for volume received

Table 2-5 OND Patient Sort Details

Patient Code	Date	Stain	Machine	Number of Plates Sorted	Cell Concentration[2] and Volume PB received	Cell Concentration[2] and Volume received	Notes
OND116-1[3]	3-29-01	CD19 PE	FACStar[1]	2 PB 4 CSF	ND 10 cc	ND 20 cc	Before Rituximab; IVIg treatment previous
OND116-2	6-19-01	CD38 FITC; CD19 PE	FACStar[1]	2 PB 61 wells CSF	ND 10 cc	ND 22 cc	Post Rituximab
OND341[4]	11-15-01	CD38 FITC; CD27 PE; CD19 PE-Cy5	FACStar[1]	2 PB 1 CSF	ND 7 cc	ND 25 cc	
OND758[5]	11-3-03	IgD FITC; CD27 PE; CD19 PE-Cy5; IgM APC	Vantage	2 PB 1 plate & 13 wells CSF	$8.7 * 10^5$ 11 cc	$3.1 * 10^4$ 20 cc	

Abbreviations: ND=not done; OND=other neurological disease; PB=peripheral blood; CSF=cerebrospinal fluid; FITC=fluorescein isothiocyanate; PE=R-phycoerythrin; APC=allophycocyanin

[1]Each well received 10 μL 1x PCR buffer before sort and 10 μL 1x PCR buffer with proteinase K (final concentration 400 μg/mL (dissolved in Tris-HCl pH 8.0) in 1x PCR buffer) after sort

[2]in cells/cc for cell concentration and cc (equal to mL) for volume received

[3]Patient has inflammatory neurological disease other than MS, is a 58 yr. old female whom presented with chronic inflammatory demyelinating polyneuropathy. Patient sample was used for flow cytometry, and 14 $CD19^+$ VH sequences were used for repertoire analyses

[4]Patient has non-inflammatory neurological disease other than MS, is a 45 yr. old female whom presented with headache. Patient sample was used for flow cytometry, and 19 $CD19^+$ VH sequences were used for repertoire analyses. Patient's repertoire was previously published in (Dorner et al., 1999; Hansen et al., 2000)

[5]Patient has inflammatory neurological disease other than MS, is a 70 yr. old male whom presented with ataxia and was diagnosed with paraneoplastic syndrome. Patient sample was used for flow cytometry, and 32 $CD19^+$ VH sequences were used for repertoire analyses. Patient's repertoire was previously published in (Dorner et al., 1999; Hansen et al., 2000)

Table 2-6: HCPB Patient Summary

	HA[1]	HB[1]	HC[1]	HE[1]	BF1[2]	BF2[2]
Age/Sex	20-40 y.o.	20-40 y.o	20-40 y.o	20-40 y.o	26/M	45/M
No. Productive VH Sequences	9 CS memory	44 CS memory	26 CS memory	3 CS memory	67 $CD19^+$	281 $CD19^+$

Abbreviations: HCPB=healthy control peripheral blood; No.=number; VH=variable heavy chain; CS memory=$CD19^+CD27^+IgD^-$ cells

[1]These patients' repertoires published in (Tian et al., 2007) and the VH sequences comprise a portion of the mHCPB database

[2]Referred to as the "in-house" database; previously published in (Brezinschek et al., 1997; Brezinschek et al., 1998; Dorner et al., 1997; Dorner et al., 1998a, b; Dorner et al., 1998c; Farner et al., 1999; Hansen et al., 2000; Monson et al., 2000) and the VH sequences comprise the HCPB database and the sequences with more than 4 mutations comprise a portion of the mHCPB database

Table 2-7 HCPB Sort Details

Patient Code	Stain	Machine	Notes
BF1	IgM FITC; CD19 PE; CD5 biotin; RED613 Strepavidin	FACStar[1]	
BF2	IgM FITC; CD19 PE; CD5 biotin; RED613 Strepavidin	FACStar[1]	
HA	IgD PE; CD27 APC; CD3/CD14 APC-Cy7; CD19 PE-Cy7	Aria[2]	Sort completed by Tian, et al. (Tian et al., 2007)
HB	IgD PE; CD27 APC; CD3/CD14 APC-Cy7; CD19 PE-Cy7	Aria[2]	Sort completed by Tian, et al. (Tian et al., 2007)
HC	IgD PE; CD27 APC; CD3/CD14 APC-Cy7; CD19 PE-Cy7	Aria[2]	Sort completed by Tian, et al. (Tian et al., 2007)
HE	IgD PE; CD27 APC; CD3/CD14 APC-Cy7; CD19 PE-Cy7	Aria[2]	Sort completed by Tian, et al. (Tian et al., 2007)

Abbreviations: NR=not reported; HC=healthy control; PB=peripheral blood; FITC=fluorescein isothiocyanate; PE=R-phycoerythrin; APC=allophycocyanin

[1]Each well received 5 μL alkaline lysing solution (200 mM KOH with 50 mM DTT)

[2]Additional sorting details not reported

Table 2-8 Current protocol staining specifics

Target	Format	Clone	Catalog Number	Patient Type
CD19	PE-Cy5	HIB19	555414[1]	All
CD27	FITC	L128	340424[1]	All
CD138	PE	MI15	347192[1]	All
HLA-DR	APC-Cy7	L243	335796[1]	All
CD3	APC	UCHT1	555335[1]	MS and CIS
CD4	PE-Cy7	SK3	348799[1]	OND
CD8	APC	SK1	340584[1]	OND
CD16	PE-Cy7	B73.1	335806[1]	CIS
CD45	APC-Cy7	2D1	348805[1]	CIS
CD3	FITC	UCHT1 or S4.1	CD0301 or MHCD0301[2]	MS and CIS (Bennett et al., 2008; Ritchie et al., 2004)
CD138	PE	1D4	MHCD13804[2]	MS and CIS (Bennett et al., 2008; Ritchie et al., 2004)
CD19	APC	SJ35-C1	MHCD1904[2]	CIS (Bennett et al., 2008)

Abbreviations: MS=multiple sclerosis; CIS=clinically isolated syndrome; OND=other neurological disease; FITC=fluorescein isothiocyanate; PE=R-phycoerythrin; APC=allophycocyanin

[1]Purchased from BD Biosciences (San Jose, CA); all catalog numbers beginning with 5 (CD19 and IgM) are through the BD Pharmingen division

[2]Purchased from Caltag (Burlingame, CA)

Stains by Patient Type

	MS	CIS	OND
FITC	CD27	CD27	CD27
PE	CD138	CD138	CD138
PE-Cy5	CD19	CD19	CD19
APC	CD3	CD3	CD8
APC-Cy7	HLA-DR	CD45 or HLA-DR	HLA-DR
PE-Cy7	CD16	CD16	CD4

Table 2-9 PCR components and reaction concentrations

PCR Name	Primers[1]	Primer Concentration	Magnesium Concentration	dNTP[2] Concentration	Taq[3] Concentration
PEP[4]	Random	66.7 mM	2.5 mM	0.4 mM	5 units per reaction
BAE	BALE + BARE	0.5 mM	2.5 mM	0.2 mM	1 unit per reaction
BAN	BALN + BARN	0.5 mM	2.5 mM	0.2 mM	1 unit per reaction
VHE	VH1E, VH2E, VH3E, VH4E, JHE	0.5 mM	1.5 mM	0.2 mM	1 unit per reaction
VH1N	VH1N, JH1245N, JH36N	0.5 mM	1.5 mM	0.2 mM	1 unit per reaction
VH2N	VH2N, JH1245N, JH36N	0.5 mM	1.5 mM	0.2 mM	1 unit per reaction
VH3N	VH3N1, VH3N2, VH3N3, JH1245N, JH36N	0.5 mM	1.5 mM	0.2 mM	1 unit per reaction
VH4N	VH4N, JH1245N, JH36N	0.5 mM	1.5 mM	0.2 mM	1 unit per reaction
VK12E	VK12E, JK2E, JK5E	0.5 mM	3.5 mM	0.2 mM	1 unit per reaction
VK1N	VK1N, JK2N, JK5N	0.5 mM	3.5 mM	0.2 mM	1 unit per reaction
VK2N	VK2N, JK2N, JK5N	0.5 mM	3.5 mM	0.2 mM	1 unit per reaction
VK3E	VK3E, JK2E, JK5E	0.5 mM	2.5 mM	0.2 mM	1 unit per reaction
VK3N	VK3N, JK2N, JK5N	0.5 mM	2.5 mM	0.2 mM	1 unit per reaction
VK45E	VK45E, JK2E, JK5E	0.5 mM	2.0 mM	0.2 mM	1 unit per reaction
VK4N	VK4N, JK2N, JK5N	0.5 mM	2.0 mM	0.2 mM	1 unit per reaction
VK5N	VK5N, JK2N, JK5N	0.5 mM	2.0 mM	0.2 mM	1 unit per reaction
VLE	VL1E, VL2E, VL3E, VL4E, VL59E, VL6E, VL78E, VL10E, JLE	0.5 mM	2.0 mM	0.2 mM	1 unit per reaction
VL1N	VL1N, JL1N, JL237N	0.5 mM	2.0 mM	0.2 mM	1 unit per reaction
VL2N	VL2N, JL1N, JL237N	0.5 mM	2.0 mM	0.2 mM	1 unit per reaction
VL3N	VL3N1, VL3N2, JL1N, JL237N	0.5 mM	2.0 mM	0.2 mM	1 unit per reaction
VL4N	VL4N1, VL4N2, JL1N, JL237N	0.5 mM	2.0 mM	0.2 mM	1 unit per reaction
VL59N	VL59N, JL1N, JL237N	0.5 mM	2.0 mM	0.2 mM	1 unit per reaction
VL6N	VL6N, JL1N, JL237N	0.5 mM	2.0 mM	0.2 mM	1 unit per reaction
VL78N	VL78N, JL1N, JL237N	0.5 mM	2.0 mM	0.2 mM	1 unit per reaction
VL10N	VL10N, JL1N, JL237N	0.5 mM	2.0 mM	0.2 mM	1 unit per reaction

[1]All primers were purchased from (Integrated DNA Technologies, Inc., Coralville, IA) and resuspended at 100 mM
[2]dNTP Mix (Promega, Madison, WI) Catalog #U1515 at 10 mM
[3]GoTaq Flexi DNA Polymerase (Promega, Madison, WI) Catalog #M8295 at 5000 u/mL
[4]This is for original method of performing PEP, not REPLI-g method

Table 2-10 PCR Programs

Program Name	Program
PEP gDNA	STEP1-95° 10 min; STEP2-95° 2 min; STEP3-37° 2 min; STEP4-37° *3min→temp 37°, increase +1°C, time 3 min, increase +10 sec, 100% ramp; STEP5-55° 4 min; STEP6-94° 1 min; STEP7-37° 2 min; STEP8-37° *3min→temp 37°, increase +1°C, time 3 min, increase +10 sec, 100% ramp; STEP9-repeat STEPS5 to 8 for 59 cycles; STEP10-55° 10 min; STEP11-4° ∞
BAE	STEP1-95° 7 min; STEP2-50° 1 min; STEP3-72° 1 min; STEP4-94° 1 min; STEP5-50° 30 sec; STEP6-72° 1 ½ min; STEP7-repeat STEPS4 to 6 for 35 cycles; STEP8-72° 10 min; STEP9-4° ∞
BAN	STEP1-95° 7 min; STEP2-60° 1 min; STEP3-72° 1 min; STEP4-94° 1 min; STEP5-60° 30 sec; STEP6-72° 1 ½ min; STEP7-repeat STEPS4 to 6 for 35 cycles; STEP8-72° 10 min; STEP9-4° ∞
VHE	STEP1-95° 5 min; STEP2-48° 1 min; STEP3-72° 1 min; STEP4-94° 1 min; STEP5-48° 30 sec; STEP6-72° 1 min; STEP7-repeat STEPS4 to 6 for 34 cycles; STEP8-72° 5 min; STEP9-4° ∞
VHN	STEP1-95° 7 min; STEP2-65° 1 min; STEP3-72° 1 min; STEP4-94° 1 min; STEP5-65° 30 sec; STEP6-72° 1 ½ min; STEP7-repeat STEPS4 to 6 for 30 cycles; STEP8-72° 5 min; STEP9-4° ∞
VKE	STEP1-95° 7 min; STEP2-56° 1 min; STEP3-72° 1 ½ min; STEP4-94° 1 min; STEP5-56° 30 sec; STEP6-72° 1 ½ min; STEP7-repeat STEPS4 to 6 for 40 cycles; STEP8-72° 5 min; STEP9-4° ∞
VKN	STEP1-95° 7 min; STEP2-65° 1 min; STEP3-72° 1 ½ min; STEP4-94° 1 min; STEP5-65° 30 sec; STEP6-72° 1 ½ min; STEP7-repeat STEPS4 to 6 for 40 cycles; STEP8-72° 5 min; STEP9-4° ∞
VLE	STEP1-95° 7 min; STEP2-60° 1 min; STEP3-72° 1 ½ min; STEP4-94° 1 min; STEP5-50° 30 sec; STEP6-72° 1 ½ min; STEP7-repeat STEPS4 to 6 for 40 cycles; STEP8-72° 10 min; STEP9-4° ∞
VLN	STEP1-95° 7 min; STEP2-62° 1 min; STEP3-72° 1 ½ min; STEP4-94° 1 min; STEP5-62° 30 sec; STEP6-72° 1 ½ min; STEP7-repeat STEPS4 to 6 for 40 cycles; STEP8-72° 10 min; STEP9-4° ∞
VgE	STEP1-95° 7 min; STEP2-94° 1 min; STEP3-50° 30 sec; STEP4-72° 1 ½ min; STEP5-repeat STEPS2 to 4 for 45 cycles; STEP8-72° 10 min; STEP9-4° ∞
VgN	STEP1-95° 7 min; STEP2-94° 1 min; STEP3-64° 30 sec; STEP4-72° 1 ½ min; STEP5-repeat STEPS2 to 4 for 40 cycles; STEP8-72° 10 min; STEP9-4° ∞
MELT	STEP1-80° 5 min; STEP2-4° ∞

Table 2-11 Primer Sequences

Primer Name	Sequence
VH1E	CAG CTK GTR CAG TCT GG
VH2E	CAG RTC ACC TTG AAG GA
VH3E	GTG CAG CTG KTG GAG
VH4E	CAG SAG TSG GGC BCA G
JHE	CTG ARG AGA CRG TGA C
VH1N	GTC TGG RSC TGA GGT GAA GAA G
VH2N	GAG TCT GGT CCT RYG CTG GTG
VH3N1	GGA GTC TGG GGG AGG YBT GGT
VH3N2	GAG KCK TGG TMC AGC CTG GG
VH3N3	GGA GGC TTR RTY CAG CCT GGG
VH4N	GGC BCA GGA CTG KTG AAG CCT T
JH1245N	GTG ACC RTK GTC CCT TGG CCC
JH36N	TGA CCA GGG TKC CMY GGC CC
VK12E	GCT CAG CTC CTG GGG CT
VK3E	GGA ARC CCC AGC DCA GC
VK45E	CTS TTS CTY TGG ATC TCT G
VK67E	CTS CTG CTC TGG GYT CC
JK2E	ACG TTT GAT CTC CAG CTT G
JK5E	CTT ACG TTT AAT CTC CAG TC
VK1N	CAT CCA GWT GAC CCA GTC TCC
VK2N	TCC AGT GGG GAT ATT GTG ATG AC
VK3N	GTC TKT GTC TCC AGG GGA AAG AG
VK4N	GAC ATC GTG ATG ACC CAG TCT C
VK5N	GGG CAG AAA CGA CAC TCA CGC A
JK2N	CAG CTT GGT CCC CTG GCC AAA
JK5N	CCA GTC GTG TCC CTT GGC CG

Table 2-11 continued

Primer Name	Sequence
VL1E	CCT GGG CCC AGT CTG TG
VL2E	CTC CTC ASY CTC CTC ACT
VL3E	TCY TAT GWG CTG ACW CAG
VL4E	CWG CYT GTG CTG ACT CA
VL59E	CCC TCT CSC AGS CTG TG
VL6E	TCT TGG GCC AAT TTT ATG C
VL78E	ATT CYC AGR CTG TGG TGA C
VL10E	CAG TGG TCC AGG CAG GG
JLE	AGG ACG GTS ASC TKG GT
VL1N	CCA GTC TGT GYT GAC KCA GCC
VL2N	CAG TCT GCC CTG ACT CAG CC
VL3N1	TGA CTC AGG ACC CTG CTG TGT C
VL3N2	TGA CWC AGC CAC YCT CRG TGT C
VL4N1	TCT GCC TCT GCY TCC CTG GG
VL4N2	TCT GCA TCT GCC TTG CTG GG
VL59N	CAG SCT GTG CTG ACT CAG CC
VL6N	CCA ATT TTA TGC TGA CTC AGC CC
VL78N	CTG TGG TGA CYC AGG AGC
VL10N	CAG GCA GGG CWG ACT CAG C
JL1N	GGT SAC CTT GGT SCC AST KCC
JL237N	GGT CAG CTK GGT SCC TCC KCC
BARE	TCT CCA CTC ACC CAG G
BALE	CTT ACA GAT CAT GTT TGA G
BALN	GTT TGA GAC CTT CAA CAC CCC
BARN	CCA GGA AGG AAG GCT GGA AG

Table 2-12 Variable Region Sections and DGYW/WRCH Motifs

	Variable Region Sections											
	FR1		CDR1		FR2		CDR2		FR3		CDR3	
Family	Codons Names	# of Codons[4]	Codons Names	# of Codons[4]	Codons Names	# of Codons[4]	Codons Names	# of Codons[4]	Codons Names	# of Codons[4]	Codons Names	# of Codons[4,5]
H1[1]	1 – 30	30	31 – 35	5	36 – 49	14	50 – 65	17	66 – 94	32	NA	NA
H2[1]	1 – 30	30	31 – 35	7	36 – 49	14	50 – 65	16	66 – 95	33	NA	NA
H3[1]	1 – 30	30	31 – 35	5	36 – 49	14	50 – 65	15 to 19	66 – 94 or 95	32 to 33	NA	NA
H4[1]	1 – 30	30	31 – 35	5 to 7	36 – 49	14	50 – 65	16	66 – 94	32	NA	NA
H5[1]	1 – 30	30	31 – 35	5	36 – 49	14	50 – 65	17	66 – 94	32	NA	NA
H6[1]	1 – 30	30	31 – 35	7	36 – 49	14	50 – 65	18	66 – 94	32	NA	NA
H7[1]	1 – 30	30	31 – 35	5	36 – 49	14	50 – 65	17	66 – 94	32	NA	NA
K1[2]	1 – 23	23	24 – 34	11	35 – 49	15	50 – 56	7	57 – 88	32	89 – 95	7
K2[2]	1 – 23	23	24 – 34	16 to 17	35 – 49	15	50 – 56	7	57 – 88	32	89 – 95	7
K3[2]	1 – 23	23	24 – 34	11 to 12	35 – 49	15	50 – 56	7	57 – 88	32	89 – 95	7
K4[2]	1 – 23	23	24 – 34	17	35 – 49	15	50 – 56	7	57 – 88	32	89 – 95	7
K5[2]	1 – 23	23	24 – 34	11	35 – 49	15	50 – 56	7	57 – 88	32	89 – 95	7
K6[2]	1 – 23	23	24 – 34	11	35 – 49	15	50 – 56	7	57 – 88	32	89 – 95	7
L1[3]	1 – 23	23	24 – 34	13 to 14	35 – 49	15	50 – 56	7	57 – 88	32	89 – 95b	9
L2[3]	1 – 23	23	24 – 34	14	35 – 49	15	50 – 56	7	57 – 88	32	89 – 95b	9
L3[3]	1 – 23	23	24 – 34	11	35 – 49	15	50 – 56	7	57 – 88	32	89 – 95, 95a or 95b	7 to 9
L4[3]	1 – 23	23	24 – 34	12	35 – 49	15	50 – 56	11	57 – 88	32	89 – 95 or 95e	7 to 12
L5[3]	1 – 23	23	24 – 34	14	35 – 49	15	50 – 56	11	57 – 88	34	89 – 95a or 95b	8 to 9
L6[3]	1 – 23	23	24 – 34	13	35 – 49	15	50 – 56	7	57 – 88	32	89 – 95	7
L7[3]	1 – 23	23	24 – 34	14	35 – 49	15	50 – 56	7	57 – 88	32	89 – 95a	8

Table 2-12 continued

Variable Region Sections												
	FR1		CDR1		FR2		CDR2		FR3		CDR3	
Family	Codons Names	# of Codons[4]	Codons Names	# of Codons[4]	Codons Names	# of Codons[4]	Codons Names	# of Codons[4]	Codons Names	# of Codons[4]	Codons Names	# of Codons[4,5]
L8[3]	1 – 23	23	24 – 34	14	35 – 49	15	50 – 56	7	57 – 88	32	89 – 95a	8
L9[3]	1 – 23	23	24 – 34	12	35 – 49	15	50 – 56	12	57 – 88	32	89 – 95e	11
L10[3]	1 – 23	23	24 – 34	13	35 – 49	15	50 – 56	7	57 – 88	32	89 – 95b	9

[1]Heavy Family Number shortened to HX
[2]Kappa Family Number shortened to KX
[3]Lambda Family Number shortened to LX
[4]Number of full codons
[5]CDR3 portion encoded by the variable gene; heavy chains do not encode for any portion of CDR3 as the D and J segments comprise the CDR3 in it's entirety

DGYW/WRCH Motifs	
DGYW	AGCT, AGCA, AGTT, AGTA, GGCT, GGCA, GGTT, GGTA, TGCA, TGTA, TGTT
WRCH	TACC, TACT, TGCC, TGCT, AACC, AACT, AGCC, AGCT, AACA, TACA

CHAPTER THREE
Results

Current methods of determining which CIS patients will convert to CDMS include detection of OCB in CSF and identification of multiple white matter lesions by MRI (Andersson et al., 1994; Frohman et al., 2003). In fact, the most recent diagnostic criteria include these two findings as part of laboratory-confirmed MS (McDonald et al., 2001). However, neither of these two findings is specific for MS. We wanted to evaluate a series of CIS patients by these standard measures and then determine if an analysis of other B cell characteristics would increase our ability to accurately identify patients likely to convert to CDMS. Several of the methods described here are equally effective as OCB and MRI lesions at conversion prediction (VH4 bias and clonal expansion). Others, such as absolute number of CSF B cells and B cell activation status (i.e. whether a B cell has ever encountered antigen (memory) or not (naïve)) were ineffective at predicting which patients would convert to MS. When we examined mutational patterns in CSF B cells from CDMS patients, a "signature" mutation pattern emerged. We found twelve codon positions in VH4 expressing B cells where replacement mutational frequency was significantly different in all CDMS patients examined. Prevalence of this signature was converted into a scoring system that was used to accurately predict the likelihood of MS conversion in a cohort of CIS patients. These results will be discussed in more detail below.

I. OCB occurrence in CISCSF and number of lesions observed by MRI in CIS CNS moderately predicts conversion to CDMS. Traditionally, the presence of OCB has been used in combination with other clinical features to predict whether or not a particular patient will convert to CDMS. However, depending on the inclusion criteria, the success of this measure for prediction purposes varies (Compston et al., 1978; Miller et al., 2005). Physicians commonly use this observation with the presence or absence of multiple lesions on an MRI for a predictive feature and more than one lesion is part of the diagnostic criteria for CDMS (McDonald et al., 2001; Poser et al., 1979). The hypothesis was that this criterion would be of value in predicting conversion, either alone or in combination with another feature.

All (10/10) of the CIS patients evaluated at UTSW Medical Center for this assay were positive for the presence of these immunoglobulins, whereas not all OND patients expressed immunoglobulin bands (Table 3-1 and data not shown). All of the patients showed the presence of IgG (10/10) and most for kappa light chain (9/10), some also for IgA (5/10) and/or lambda (6/10). None of the patients were positive for IgM. Most of the patients with IgG and kappa bands did convert, but CIS681 and CIS951 also had these features and did not convert (Table 3-1). CIS636, interestingly, had an increase of IgG, but also of lambda, not kappa. So the presence of immunoglobulin does predict conversion, but only

moderately since only 70% of the patients who were positive for immunoglobulin in the CSF went on to convert to CDMS (Table 3-1).

II. CISCSF cell concentration and $CD19^+$ B cell concentration of no predictive value to conversion. Although it has historically been considered immune privileged, lymphocytes do traffic through the CNS ((Anthony et al., 2003) reviewed in (Pedemonte et al., 2006)). It was hypothesized that the patients with unusually elevated numbers of cells in the CNS must have a breach in the blood brain barrier or local expansion of trafficking lymphocytes, and thus may be an indicator of patients which would subsequently convert to CDMS. However, the CIS patients had a large variability in the concentration of B cells. Seven of the 9 of the patients converted to MS, however the patients that did not develop CDMS had no difference in the overall or $CD19^+$ cell number from those that converted (Table 3-2). The difficulty in using this as a predictive tool is the variability of number and that this feature is not unique to MS, as patients with other inflammatory neurological diseases can also have large numbers of B cells, in some cases over 2800 B cells per mL CSF (OND758; OND average 307 $CD19^+$ B cells per mL CSF, ±651 cells (SEM)) and have no symptoms indicative of MS. MS patients also have a wide range of $CD19^+$ B cells in the CSF, ranging from 21 B cells per mL to over 1800 B cells per mL (average 340 $CD19^+$ B cells per mL ±611 cells (SEM)). Non-inflammatory neurological diseases still had B cells in the CSF, ranging from less than 4 B cells per mL (OND823) to 63 B cells

per mL (OND315) with an average of 23 B cells per mL ±21 cells (SEM) (Table 3-2), confirming other reports of detectable trafficking to this compartment in the absence of acute inflammation (reviewed in (Pedemonte et al., 2006)). So while this feature is indicative of inflammation, it is not constructive as a predictive tool for CDMS.

III. CISCSF naïve: memory ratios are similar to OND and MS patients.

$CD19^+$ B cells found in the CSF of MS patients have a memory phenotype, documented by both flow cytometry (expression of the human memory B cell marker CD27 (Klein et al., 1998)) and high antibody mutation accumulation (4.9% in our MSCSF database) (Brezinschek et al., 1997; Brezinschek et al., 1998; Dorner et al., 1997; Dorner et al., 1998a, b; Dorner et al., 1998c; Farner et al., 1999; Hansen et al., 2000; Monson et al., 2000). The naïve to memory (N:M) ratio is a way to numerically compare the patients, with a number >1.0 showing a predominance of naïve B cells and a number <1.0 having an enrichment of memory B cells. MS patients' CSF naïve:memory B cell ratios range from 0.20 to 1.37, all showing the enhancement of memory B cells over what is seen in the periphery, with an overall N:M B cell ratio of 0.73 (±1.07 SD) (Table 3-2). It was therefore hypothesized that this phenomenon would also be shown in the CIS patient CSF B cells, though maybe to a lesser extent. The CIS patients had N:M B cell ratios of 0.15 to 1.32, with an overall N:M ratio of 0.55 (±1.15 SD) (Table 3-2). Six of the 9 patients converted to CDMS, with one additional patient being

diagnosed with NMO (Table 3-2). However, OND patients, either inflammatory or non-inflammatory, also express this enrichment of memory cells in the CSF $CD19^+$ B cells in most cases (0.59 overall N:M B cell ratio; 1.8 SD, range 0.14 to 1.46) (Table 3-2) (Harp et al., 2007). So while the N:M B cell ratio is different from the PB, it is present in both patients with MS and without MS, and thus not beneficial to use as a predictive tool.

IV. Repertoire abnormalities in MSCSF include VH4 enrichment and clonal expansion. MSCSF B cells, as a whole, reflect characteristics of memory B cells in the periphery including germinal center-introduced mutational characteristics (Harp et al., 2007). However, the B cells in MSCSF differ in 2 characteristics, clonal expansion (Buluwela and Rabbitts, 1988; Humphries et al., 1988; Kodaira et al., 1986; Lee et al., 1987; Shen et al., 1987) and VH4 family overrepresentation (Baranzini et al., 1999; Colombo et al., 2000; Monson et al., 2005; Owens et al., 1998; Owens et al., 2003; Qin et al., 1998; Ritchie et al., 2004). The hypothesis is that if one or especially both of these characteristics are present in the CSF B cell pool of a CIS patient, this patient is at an early stage of CDMS.

IV.1a. VH4 enrichment is observed in CSF B cells from MS patients, but only moderately accurate in determining CDMS conversion in CISCSF. Healthy control peripheral blood (HCPB) displays a particular distribution in the frequency of the variable heavy chain gene families that are expressed in the B

cell repertoire. VH3 was utilized the most frequently by B cells in HCPB (55.2%), followed by VH4 (21.8%), then VH1 (15.5%), while VH2 (2.0%), VH5 (2.3%), VH6 (2.0%), and VH7 (1.2%) are used less frequently (Figure 3-2 and Table 3-3). In contrast to HCPB, $CD19^+$ B cells from MSCSF utilize a significantly higher frequency of VH4 family genes in comparison to HCPB (34.3% vs. 21.8%, p=0.0002 by χ^2 test) and in comparison to the memory HCPB database (34.3% vs. 16.6%, p=0.001 by χ^2 test) (Figure 3-2 and Table 3-3), with a range of 16.3 to 63.4 percent of CSF B cells utilizing VH4 family genes in individual MS patient repertoires. Although the CDMS patient group overall demonstrated VH4 bias, when each CDMS patient CSF B cell antibody repertoire was considered separately, only 2 ((M522 (n=71) and M584 (n=85)) exhibited the VH4 bias. M125 (n=101), M199 (n=19), M368 (n=49), and M875 (n=21) did not exhibit VH4 bias individually).

The CISCSF antibody database consisting of $CD19^+$ B cells only had a similar frequency of B cells that utilize VH4 family genes in comparison to HCPB (26.2% vs. 21.8%, p=0.20 by χ^2 test) (Figure 3-2 and Table 3-3), and a higher frequency of B cells that utilize VH4 family genes in comparison to the mHCPB database (26.2% vs. 16.6%, p=0.01 by χ^2 test) (Figure 3-2 and Table 3-3). The CISCSF antibody database consisting of both $CD19^+$ B cells and $CD138^+$ plasma cells also had a higher frequency of B cells that utilize VH4 family genes in comparison to HCPB (35.0% vs. 21.8%, p=0.00001 by χ^2 test) (Figure 3-2 and

Table 3-3), and a higher frequency of B cells that utilize VH4 family genes in comparison to the mHCPB database (35.0% vs. 16.6%, p=0.00001 by χ^2 test) (Figure 3-2 and Table 3-3). Four of the eleven individual CIS patients examined had evidence of VH4 bias in $CD19^+$ B cells in the CSF (CIS3-1, ON3-5, ON4-10, and ON5-2) (Table 3-4). Three of these patients also had bias in the $CD138^+$ CSF cells (Table 3-4). One additional patient had VH4 bias in the CSF $CD138^+$ repertoire, but did not have a recoverable $CD19^+$ repertoire (ON4-8). In total, five of the eleven patients showed evidence of VH4 bias, and four of the five with VH4 bias converted to CDMS (ON4-10 did not convert). CSF-derived B cell antibody repertoires from patients with Other Neurological Diseases (OND) were not enriched for VH4-expressing CSF B cells in comparison to HCPB (23.1% vs. 21.8%, p=0.61 by χ^2 test) (Figure 3-2 and Table 3-3), indicating that VH4 over-expression in the CSF of MS patients was specific to MS and not due to bias in the ability of VH4 expressing B cells to enter the CNS.

MS patients also have an increase in the number of CSF B cells expressing VH1 (27.1% compared to HCPB 15.5%, p=0.0002) (Figure 3-2 and Table 3-3). $CD138^+$ cells from MSCSF, while having the VH4 bias seen in the $CD19^+$ population, do not show the VH1 bias seen in the $CD19^+$ cells ($MSCSF_{VH1}$ $CD19^+$ 27.1% compared to $MSCSF_{VH1}$ $CD138^+$ 0.7%, p<0.0001). While MSCSF $CD19^+$ B cells show VH1 bias, no VH1 bias in either the $CD19^+$ or $CD138^+$ repertoires is observed in any of the CIS patients.

While not seen in MSCSF, VH2 bias has also been reported to predict conversion to CDMS (Bennett et al., 2008). Two CIS patients (ON3-5 and ON4-7) demonstrate this bias in both the $CD19^+$ CSF B and $CD138^+$ CSF plasma cells (Table 3-4). One of these patients also had VH4 bias (ON3-5), and one patient (ON4-7) had a bias in VH2, but not VH4. ON3-1 $CD138^+$ cells have significant increase in VH2 expression compared to HCPB $CD19^+$ cells, but no increase in VH2 expression by $CD19^+$ cells.

In conclusion, VH2 and VH4 but not VH1 bias is present in some CIS patients, and 73% accurate in predicting conversion to CDMS with specificity of 66.7% (2/3), sensitivity of 42.9% (6/14), positive predictive value of 85.7% (6/7), and negative predictive value of 20.0% (2/10). Therefore, an absence of VH2 or VH4 does not accurately predict non-conversion.

IV.1b. MSCSF and CISCSF B cell databases reveal no restriction in individual VH4 gene usage. The increase of VH4 family usage in the MSCSF database in comparison to HCPB could be attributed to an increased usage frequency of all nine individual heavy chain genes that comprise the VH4 family, or preferential use of one or more of the VH4 family genes. To differentiate between these two possibilities, the frequency usage of the 9 individual genes that comprise the VH4 family in the MSCSF database was compared to the HCPB and mHCPB databases (Figure 3-3 and Table 3-5). Individual VH4 gene frequencies in MSCSF were similar to HCPB and mHCPB by χ^2 test using a Bonferroni

corrected p-value of 0.004 (Figure 3-3 and Table 3-5). CISCSF, either just $CD19^+$ B cells or the combination of $CD19^+$ and $CD138^+$ cells, both had similar VH4 gene frequencies as MSCSF, HCPB, and mHCPB. Therefore the increase in VH4 family usage is not due to bias in frequency of any particular VH4 gene, but an increase in the frequency of all of the genes comparatively equally.

IV.2. Clonal expansion in CSF B cells from CIS patients predicts conversion to CDMS. B cells found in MSCSF have frequently undergone clonal expansion (seen in 10 of 11 CDMS patients). The presence or absence of clonal expansion in a CISCSF was predicted to be useful in determining whether the patient would convert to MSCSF in the future. Clonal expansion was detected in seven of the eleven CIS patients (Table 3-4), but only corresponded with conversion in nine of the eleven CIS patients (Table 3-4). Thus clonal expansion is useful in predicting conversion, but by itself does not reach satisfactory accuracy in conversion prediction (73% accurately predicted; specificity of 66.7%, sensitivity of 78.6%, positive predictive value of 91.7%, negative predictive value 40.0%).

V. MSCSF and CISCSF repertoire characteristics normal in other analyses. Mutational characteristics of the antibody variable region can confirm whether appropriate targeting of mutations that are associated with antigenic selection occur within the context of a classic germinal center (Harp et al., 2007). The MSCSF database maintains typical germinal center features including targeting to CDRs and particular motifs within the CDRs (Harp et al., 2007). CSF derived B

cell clones from MS patients have more atypical features (Monson et al., 2005), suggesting that some clonally expanded B cells in the CSF are not selected in the context of a classical germinal center. Mutational characteristics attributed to germinal center education of B cells were evaluated to see if any abnormalities were seen in $MSCSF_{VH4}$, and if so, could these be used for prediction tools for CIS patients. Sequences were evaluated and each mutation characterized as described in Materials and Methods (Chapter 2, Section XII).

V. 1. $MSCSF_{VH4}$ and $CISCSF_{VH4}$ have an increased mutational frequency in comparison to $HCPB_{VH4}$. Previously, we established that the MSCSF B cell database had an enhanced mutational frequency (MF) in comparison to HCPB (Harp et al., 2007; Monson et al., 2005). We hypothesized that VH4 expressing CSF-derived B cells would also have a higher MF than what is observed in the population as a whole, especially considering the increased frequency of VH4 expressing B cells, suggesting local expansion of this population. In order to test this hypothesis, we compared the MFs of the B cell databases as a whole to the MFs of the sub-database of only those B cells expressing VH4 genes (Table 3-6). As expected, the inclusive HCPB repertoire has an MF of 2.3% because the majority of the B cells in this compartment are naïve and, as expected, the memory B cell subpopulation of this database (mHCPB) had a much higher MF (compare 2.3% in HCPB to 6.6% in mHCPB, $p<0.001$). Interestingly, the VH4 expressing B cell subpopulation of this database ($HCPB_{VH4}$) had a MF of 2.0%,

which was significantly less than the MF of the overall repertoire without VH4 (compare 2.0% in $HCPB_{VH4}$ to 2.3% in $HCPB_{All\text{-}VH4}$, p=0.02) (Table 3-6). In contrast, the $MSPB_{VH4}$ and the $MSCSF_{VH4}$ sub-databases had MFs that were statistically greater than the MF of the overall MSPB and MSCSF databases (compare 2.9% in $MSPB_{VH4}$ to 1.7% in MSPB ($p<0.001$) and 5.9% in $MSCSF_{VH4}$ to 4.9% in MSCSF ($p<0.001$)) (Table 3-6).

CISCSF $CD19^+$ B cells are similar to MSCSF $CD19^+$ B cells in that they reflect memory characteristics, both in CD27 expression (see Chapter 3, section II.), and MF (Table 3-6). The $CISCSF_{VH4}$ $CD19^+$ B cells have a MF (5.8%) similar to that of the overall CISCSF $CD19^+$ database (5.6%, $p>0.05$ (comparing $CISCSF_{VH4}$ $CD19^+$ to $CISCSF_{non\text{-}VH4}$ $CD19^+$)). The CISCSF $CD19^+$ and $CD138^+$ combination overall database (abbreviated CD19+CD138) had a MF statistically greater than the $CD19^+$ database alone (compare 5.8% to 5.4%, p=0.002), but both had VH4 sub-databases that were similar to each other (compare $CD19_{VH4}$ 5.8% to $CD19{+}CD138_{VH4}$ 6.1%, $p>0.05$). The CISCSF $CD19{+}CD138_{All}$ database did have a statistically smaller MF to the $CD19{+}CD138_{VH4}$ (compare 5.8% to 6.1%, p=0.004) (Table 3-6).

The VH4 sub-database was less frequently mutated than the overall HCPB database. In MSPB, MSCSF, and CISCSF (when $CD138^+$ cells are included) the VH4 sub-database was more frequently mutated than the parent database. While this is not useful for conversion purposes, it is interesting to note that both

CISCSF $CD19^{+}_{VH4}$ B cells and CISCSF CD19+$CD138_{VH4}$ cells are already as mutated as they are in $MSCSF_{VH4}$ B cells.

V.2. J segment usage not biased in $MSCSF_{VH4}$ or $CISCSF_{VH4}$ populations, but is in $MSPB_{VH4}$. The extensive VH4 family usage by the MSCSF database prompted analysis of the J segment usage within the VH4 expressing B cell databases. Autoreactive B cells in peripheral germinal centers are known to utilize JH6 segments more frequently (Zheng et al., 2004), so we reasoned that VH4 expressing B cells from the CSF of MS patients may also be enriched for JH6 usage. JH4 is the most common J segment used in the HCPB B cell repertoire database as described by us (Figure 3-4, Table 3-7, and (Harp et al., 2007; Monson et al., 2005)) and others (Brezinschek et al., 1995), even when only those B cells expressing VH4 family genes are considered (Figure 3-4 and Table 3-7, $HCPB_{VH4}$ =56.6%). The $mHCPB_{VH4}$ family genes also utilized the JH4 segment most frequently (54.1%). In contrast, the $MSPB_{VH4}$ database utilized JH6 segments more frequently than $mHCPB_{VH4}$ (compare 40% $MSPB_{VH4}$ to 14% $mHCPB_{VH4}$, $p=0.009$). Curiously, this enrichment of JH6 utilization by $MSPB_{VH4}$ was not observed in $MSCSF_{VH4}$ (compare 40% $MSPB_{VH4}$ to 14% $MSCSF_{VH4}$, $p<0.001$) (Figure 3-4 and Table 3-7). $CISCSF_{VH4}$, both $CD19^{+}_{VH4}$ B cells and CISCSF CD19+$CD138_{VH4}$ cells, have similar J segment expression frequency as seen in $MSCSF_{VH4}$ and $mHCPB_{VH4}$ (Figure 3-4 and Table 3-7). Since there are no differences between MS patients' CSF B cell J segment usage and mHCPB,

and no differences between either of these two groups and CIS patients' J segment expression this factor is not functional in evaluating the likelihood of converting to CDMS.

V. 3. $MSCSF_{VH4}$ and $CISCSF_{VH4}$ mutational characteristics retain targeting to CDR and DGYW/WRCH motifs. Targeting, both to regions (CDR) and particular motifs (DGYW/WRCH) might be abnormal in ectopic B cell expansions. Therefore, we hypothesized that the VH4 cells in MSCSF might be different from peripheral B cells in targeting mutations to CDRs and DGYW/WRCH (abbreviated "DW"), and that this has a possibility of being used to predict conversion to CDMS.

V. 3a. Mutational targeting measured by R:S ratios in $MSCSF_{VH4}$ and $CISCSF_{VH4}$ CDR is preserved. As one would expect in a typical germinal center reaction, the $MSCSF_{VH4}$ CDRs have a much higher R:S ratio than FRs (4.2 to 1.2, $p<0.001$) (Figure 3-5 and Table 3-8). Both CDR and FR R:S ratios are similar in the $MSCSF_{VH4}$ to $HCPB_{VH4}$ and $mHCPB_{VH4}$. This data confirms that targeting mutations to CDR is preserved in the VH4 expressing B cells from MSCSF. $CISCSF_{VH4}$, both $CD19^{+}{}_{VH4}$ B cells and CISCSF CD19+$CD138_{VH4}$ cells, had similar R:S ratios to what was seen in $MSCSF_{VH4}$, $HCPB_{VH4}$, and $mHCPB_{VH4}$, and thus not useful for predicting conversion to CDMS.

V. 3b. Targeting to DGYW/WRCH motifs is preserved in $MSCSF_{VH4}$

Somatic hypermutation occurring in the context of a classical germinal center is

predominantly targeted to DW motifs within variable immunoglobulin genes (Rogozin and Diaz, 2004). If VH4 expressing B cells undergo antigen driven selection in the context of a classical germinal center, then targeting to these motifs should be preserved. In order to determine whether appropriate targeting to DW motifs occurred in $MSCSF_{VH4}$ in comparison to the VH4 sub-database of the control groups, MFs in the motifs were determined. $HCPB_{VH4}$ B cells had a MF within DW motifs of 2.7%, while the $mHCPB_{VH4}$ B cells had a MF of 8.9% ($p<0.001$) (Figure 3-5 and Table 3-8). $MSCSF_{VH4}$ B cells had a MF of 8.0% within DW motifs, which was statistically greater than what was observed in $HCPB_{VH4}$ B cells (2.7%, $p<0.001$) yet similar to what was observed in mHCPB (8.9%, $p>0.05$). Similar to $MSCSF_{VH4}$ B cells, $CISCSF_{VH4}$, both $CD19^{+}{}_{VH4}$ B cells and CISCSF $CD19+CD138_{VH4}$ cells, had DW MF greater than $HCPB_{VH4}$ (8.9% and 9.3% compared to 2.7%, both $p<0.001$) and similar to $mHCPB_{VH4}$ (compared to 8.9%, both $p>0.05$) (Figure 3-5 and Table 3-8). Thus mutational characteristics involving targeting are not effective as predictive tools.

VI. Multiple Sclerosis VH4 Signature 1. Identification of codons within MSCSF that are enriched for replacement mutations. Since VH4 expressing B cells are enriched in the CSF of MS patients, we predicted that mutational analysis would reveal a pattern of replacement mutations (i.e. "signature") that is unique to VH4 expressing B cells from the CNS of MS patients in comparison to HCPB. In order to test this hypothesis, the percentage of replacement mutations

(RF) at each codon within the $MSCSF_{VH4}$ subdatabase was determined and compared to the RF at each codon position within the $HCPB_{VH4}$ subdatabase. Hot spots were defined as those codon positions within $MSCSF_{VH4}$ with a statistically higher RF at a particular codon position in comparison to $HCPB_{VH4}$ (Table 3-9). Using this approach, 8 codon positions (31B, 32, 40, 56, 57, 60, 81, and 89) were identified that have a total RF value in $MSCSF_{VH4}$ (25.0%) that was statistically higher than in $HCPB_{VH4}$ (12.6%) ($p=0.001$ by χ^2 test) (Table 3-9). Previous analysis had identified codon 56 as a replacement mutation hotspot in HCPB (Dorner et al., 1998a), which intensified as a hot spot in $MSCSF_{VH4}$ since a significantly greater percentage of replacement mutations were found in $MSCSF_{VH4}$ at codon 56 compared to $HCPB_{VH4}$. Of note, there was a 7.0 fold increase in replacement accumulation at codon 31B in the $MSCSF_{VH4}$ database in comparison to $HCPB_{VH4}$ that is likely due to the use of this codon by only a subset of VH4 genes (4-30, 4-31, 4-39 and 4-61). Four codons (30, 43, 77 and 82) were also identified as cold spots, since the total RF value in $MSCSF_{VH4}$ (5.1%) were statistically less than $HCPB_{VH4}$ (8.5%)($p=0.001$ by χ^2 test). Individual MS patient RFs within the 8 hot spot codons of the signature ranged from 22.5 to 34.1 percent (Figure 3-6), indicating that some individual patient MSCSF repertoires had a greater enrichment of replacements at these 8 codon positions than others. There was also variability of RF values within the 8 hot spot codons of the signature in individual VH4 genes in $MSCSF_{VH4}$ ranging from 14.5 to 36 percent

(Table 3-10), indicating that some individual VH4 genes had a greater enrichment of replacements at these 8 hot spot codon positions than others. An example of a signature-enriched VH4 antibody gene rearrangement from a CSF-derived B cell of an MS patient is provided in Figure 3-1. Of note, 5 of the 8 hot spot codons of the signature retained higher RF values in MSCSF compared to the memory HCPB database (31B, 40, 56, 57, and 60), emphasizing that the signature does not simply reflect enrichment of memory B cells in the CSF.

VI. 2. Potency of signature score to predict development of clinically definite MS. Finally, we reasoned that prevalence of the signature might allow us to identify patients at risk to develop MS who subsequently convert to CDMS. CISCSF $CD19^+$ B cell and $CD138^+$ plasma cell repertoires from the CSF of two CIS patients at UTSWMC and nine patients at UCHSC were generated and analyzed for RF values within the 6 codons of the signature defined from the MSCSF master database that had the most significant difference in RF compared to $HCPB_{VH4}$ at each codon position (codons 31B, 40, 56, 57, 81 and 89). RF values were combined using a signature score that accounts for RF variance, as signature scores were generated by calculating Z-scores for the RF values at the 6 codons within the signature (31B, 40, 56, 57, 81 and 89) that had the most significant difference in RF compared to $HCPB_{VH4}$ at each codon position. The average signature score in the MSCSF master database was 10.9 ±2.0, and so any individual CIS patient score that was 6.8 (average signature score – 2 S.D.) or

higher was predicted to develop MS (Figure 3-6). As indicated in Table 3-11 and shown in Figure 3-6, prediction of conversion to CDMS using this system was accurate in 8 of 8 CIS patients that converted to CDMS. Lack of signature prevalence also accurately predicted that 2 of 2 patients who had recently experienced a first demyelinating event (ON3-1 and ON4-10) would not develop CDMS, and indeed, have not developed CDMS up to 2 years after initial sampling. One additional patient who had recently experienced a first demyelinating event (ON3-4) had a high signature score (11.3), but had not converted to CDMS at the two-year follow-up. This yielded a specificity of 66.7%, 100% sensitivity, 93.3% positive predictive value, and 100% negative predictive value. Most patients converted to CDMS within 3-6 months of repertoire sampling, although predictions could be made based on signature score even 18 months prior to conversion to CDMS (CIS132) (Table 3-11). MRI, OCB and VH4/VH2 bias are also useful in assessing probability of MS conversion (Bennett et al., 2008; Freedman et al., 2005; Frohman et al., 2003; Korteweg et al., 2006; Paolino et al., 1996; Soderstrom et al., 1998), but were not considered in calculating the signature score.

Figure Legend

Figure 3-1. Example of VH4 comparison. A VH4-30.4 sequence is listed as the germline configuration (allele 01) and compared to a patient $CD19^+$ B cell sequence. The germline protein conversion and the changes made by replacement mutations in the patient sequence are noted. Signature codons are boxed, with the dashed boxes demarcating cold spots, and the solid boxes demarcating hot spots. CDRs as defined by (Kabat et al., 1983) are shaded.

Figure 3-2. Frequency of VH family usage. Values provided are percent of productive VH rearrangements of 348 HCPB B cell sequences from 2 donors (BF1 n=67 and BF2 n=281), 205 mHCPB ($CD19^+$ memory B cells with mutation accumulation (less than 98% homologous to germline) and IgD^-CD27^+ class switched B cells) from 6 donors (BF1 n=18, BF2 n=105, HA n=9, HB n=44, HC n=26, and HE n=3) (Tian et al., 2007). MSPB included 168 $CD19^+$ sequences from 3 donors (M125 n=74, M354 n=18, and M484 n=76). MSCSF included 373 $CD19^+$ sequences from 11 donors (M125 n=101, M199 n=19, M217 n=1, M354 n=6, M368 n=49, M376 n=8, M484 n=9, M522 n=71, M584 n=85, M875 n=21, and M887 n=3). CISCSF included 302 $CD19^+$ sequences from 10 donors (CIS132 n=19, CIS429 n=57, CIS3-1 n=24, ON3-1 n=23, ON3-3 n=39, ON3-4 n=28, ON3-5 n=35, ON4-7 n=17, ON4-10 n=31, and ON5-2 n=29) and the CD19+CD138 group also included $CD138^+$ sequences from CIS3-1 n=76, ON3-1 n=45, ON3-3 n=12, ON3-5 n=44, ON4-7 n=17, ON4-10 n=17, and ON5-2 n=12,

which brings the patient total to 11 CIS patients, as ON4-8 did not have $CD19^+$ cell sequences, only $CD138^+$, and 528 sequences. The ONDCSF group included 65 $CD19^+$ sequences from 3 donors (OND341 n=32, OND758 n=19, and OND116 n=14). Frequencies significantly different from HCPB frequency ($p \leq 0.05$) are marked as *, and those significantly different from mHCPB frequency ($p \leq 0.05$) are marked as ◆. Precise frequencies can be found in Table 3-3.

Figure 3-3. VH4 individual gene usage frequency in peripheral blood or spinal fluid. VH4-30 includes both 4-30.2 and 4-30.4 sub-genes, and none of the groups used VH4-28, and so it was eliminated from this figure. Values provided are percent of productive VH4 rearrangements of 76 HCPB B cell sequences (348 total productive VH sequences), 38 mHCPB (205 total productive VH sequences), 40 MSPB VH4-expressing B cells (168 total productive VH sequences) 128 MSCSF (373 total productive VH sequences), 80 CISCSF $CD19^+$ sequences (302 total productive VH sequences) 180 from the CD19+CD138 group (528 total productive VH sequences, and 15 ONDCSF VH4-expressing B cell sequences (65 total productive VH sequences). None of the frequencies were significantly different from HCPB frequency or MSCSF frequency. Precise frequencies can be found in Table 3-5.

Figure 3-4. Frequency of JH family usage. The HCPB group includes $CD19^+$ B cell antibody sequences from healthy controls BF1 (n=67) and BF2 (n=281), with 76 VH4 sequences from these 2 donors. The memory HCPB (mHCPB) group includes $CD19^+$ B cell antibody sequences from healthy controls BF1 (n=18) and BF2 (n=105) with 4 or more mutations (less than 98% homology to germline) and IgD^-CD27^+ B cell antibody sequences from healthy controls HA (n=9), HB (n=44), HC (n=26), and HE (n=3) (Tian et al., 2007), with 37 VH4 sequences from the 6 donors. The MSPB group includes $CD19^+$ sequences from MS patients M125 (n=74), M354 (n=18), and M484 (n=76), with 40 VH4 sequences from the 3 donors. The MSCSF group includes $CD19^+$ sequences from MS patients M125 (n=101), M199 (n=19), M354 (n=6), M368 (n=49), M376 (n=8), M484 (n=9), M522 (n=71), M584 (n=85), M875 (n=21), M217 (n=1), and M887 (n=3), with 127 VH4 sequences from 10 donors. The CISCSF group includes $CD19^+$ sequences from CIS patients CIS132 (n=19), CIS429 (n=57), ON3-1 (n=23), ON3-3 (n=39), ON3-4 (n=28), ON3-5 (n=35), ON4-7 (n=17), ON4-10 (n=31), and ON5-2 (n=29), with 80 VH4 sequences from 10 donors. The CISCSF group with $CD138^+$ sequences in addition to the $CD19^+$ sequences listed above included $CD138^+$ sequences from CIS3-1 (n=76), ON3-1 (n=45), ON3-3 (n=12), ON3-5 (n=44), ON4-7 (n=20), ON4-8 (n=17), and ON5-2 (n=12), with 180 VH4 sequences between the $CD19^+$ and $CD138^+$ sequences from 11 total donors. Frequencies significantly different from HCPB frequency ($p \leq 0.05$) are

marked as *, and those significantly different from mHCPB frequency ($p \leq 0.05$) are marked as ◆. Precise frequencies can be found in Table 3-7.

Figure 3-5. VH4 mutational characteristics. Methods of how these calculations were determined can be found in Chapter 2, sections XII.4 and XII.5. VH4 sequences included in this analysis were 76 from HCPB donors, 38 from mHCPB donors, 40 from MSPB donors, 128 from MSCSF donors, 74 from CISCSF $CD19^+$ cells, and 180 from CISCSF $CD19^+$ and $CD138^+$ cells. CDR and FR determinations were taken from (Kabat et al., 1983). Characteristics significantly different from HCPB ($p \leq 0.05$) are marked as *, and those significantly different from mHCPB ($p \leq 0.05$) are marked as ◆. Precise frequencies can be found in Table 3-8.

Figure 3-6. Signature score in individual MS and CIS patients. Signature scores were generated by calculating Z-scores for the RF values at the 6 codons within the signature (31B, 40, 56, 57, 81 and 89). Individual z-scores at each of the codon positions were then added to generate the composite signature z-score. MS patient signature scores are shown as black circles (●), CIS patient signature scores that resulted in prediction of CDMS are black squares (■), and CIS patient signature scores that resulted in prediction of another neurological disorder other than MS are in open squares (□). The average composite signature score in the

$MSCSF_{VH4}$ database was 10.9 ±2.0 (black line) and so any signature score of an individual CIS patient above 6.8 (average – 2 S.D.; threshold shown as red line) was predicted to convert to CDMS. $CD19^+$ B cell and $CD138^+$ plasma cell mutation positions both contributed to each CIS patient's signature score. The $MSCSF_{VH4}$ signature scores were only composed of $CD19^+$ B cells.

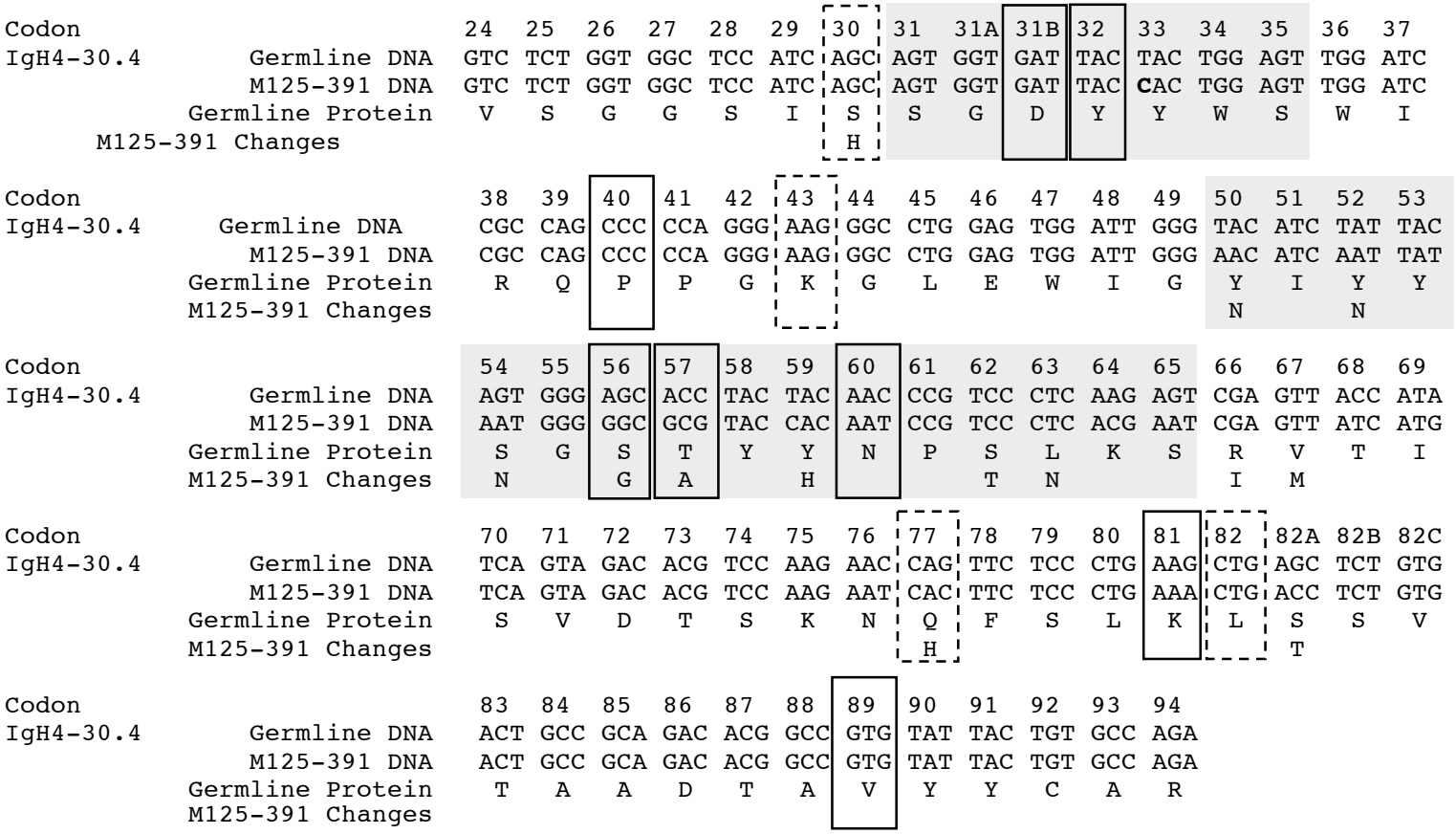

Codon
IgH4-30.4 Germline DNA
M125-391 DNA
Germline Protein
M125-391 Changes
24 25 26 27 28 29 30 31 31A 31B 32 33 34 35 36 37
GTC TCT GGT GGC TCC ATC AGC AGT GGT GAT TAC TAC TGG AGT TGG ATC
GTC TCT GGT GGC TCC ATC AGC AGT GGT GAT TAC CAC TGG AGT TGG ATC
V S G G S I S S G D Y Y W S W I
H
38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53
CGC CAG CCC CCA GGG AAG GGC CTG GAG TGG ATT GGG TAC ATC TAT TAC
CGC CAG CCC CCA GGG AAG GGC CTG GAG TGG ATT GGG AAC ATC AAT TAT
R Q P P G K G L E W I G Y I Y Y
N N
54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69
AGT GGG AGC ACC TAC TAC AAC CCG TCC CTC AAG AGT CGA GTT ACC ATA
AAT GGG GGC GCG TAC CAC AAT CCG TCC CTC ACG AAT CGA GTT ATC ATG
S G S T Y Y N P S L K S R V T I
N G A H T N I M
70 71 72 73 74 75 76 77 78 79 80 81 82 82A 82B 82C
TCA GTA GAC ACG TCC AAG AAC CAG TTC TCC CTG AAG CTG AGC TCT GTG
TCA GTA GAC ACG TCC AAG AAT CAC TTC TCC CTG AAA CTG ACC TCT GTG
S V D T S K N Q F S L K L S S V
H T
83 84 85 86 87 88 89 90 91 92 93 94
ACT GCC GCA GAC ACG GCC GTG TAT TAC TGT GCC AGA
ACT GCC GCA GAC ACG GCC GTG TAT TAC TGT GCC AGA
T A A D T A V Y Y C A R

Figure 3-1

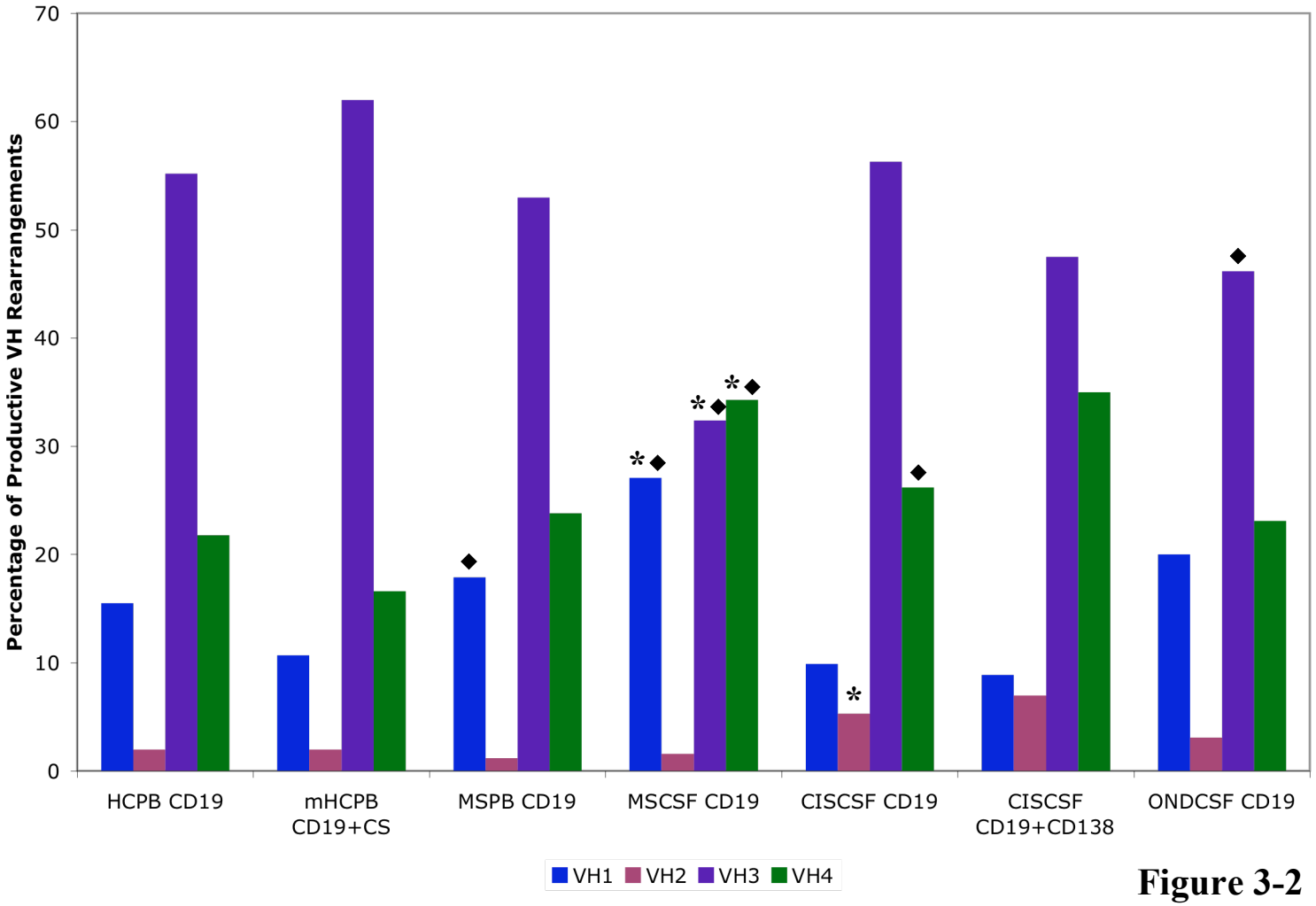

Percentage of Productive VH Rearrangements
70
60
50
40
30
20
10
0
HCPB CD19
mHCPB CD19+CS
MSPB CD19
MSCSF CD19
CISCSF CD19
CISCSF CD19+CD138
ONDCSF CD19
VH1
VH2
VH3
VH4

Figure 3-2

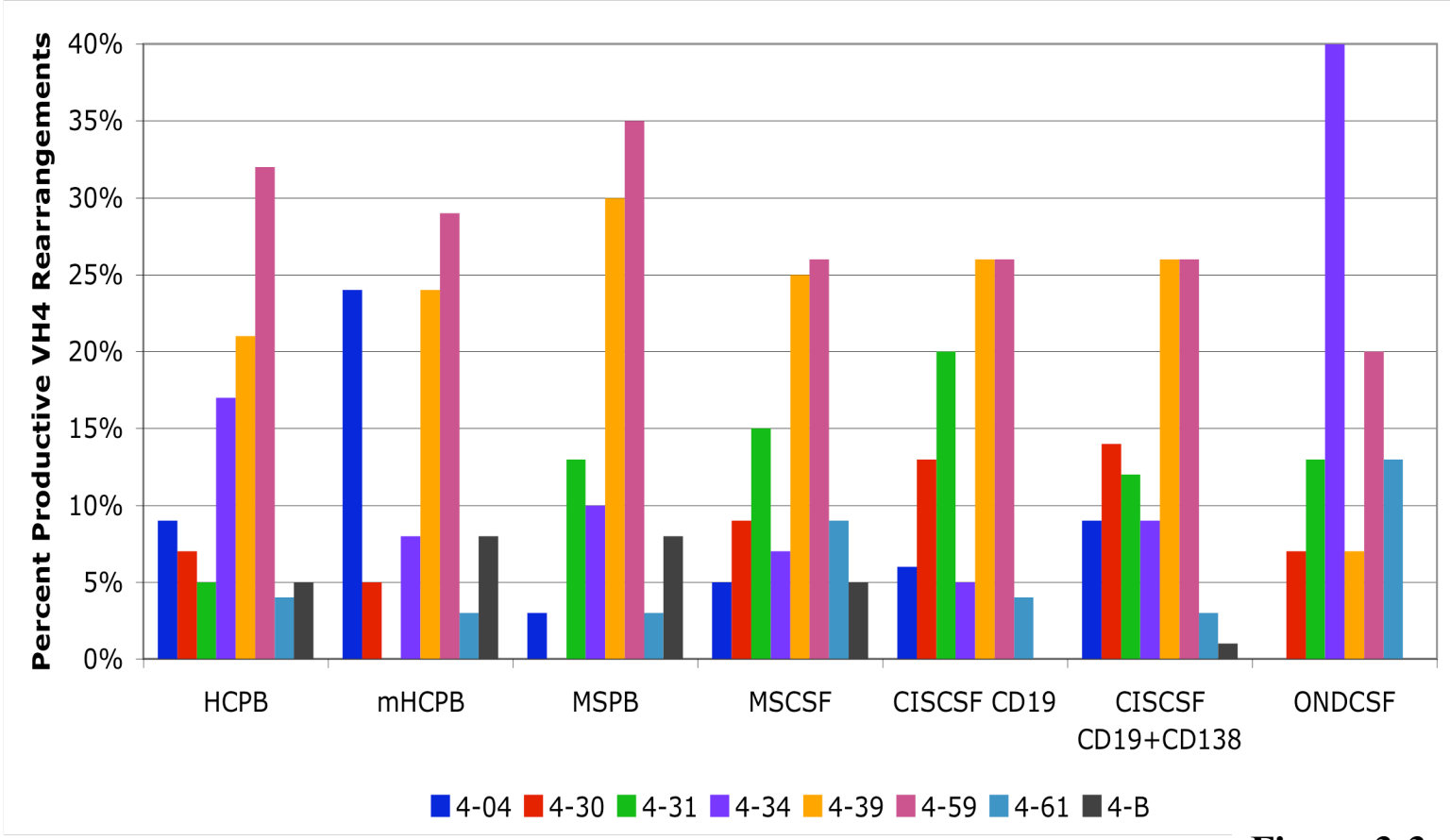
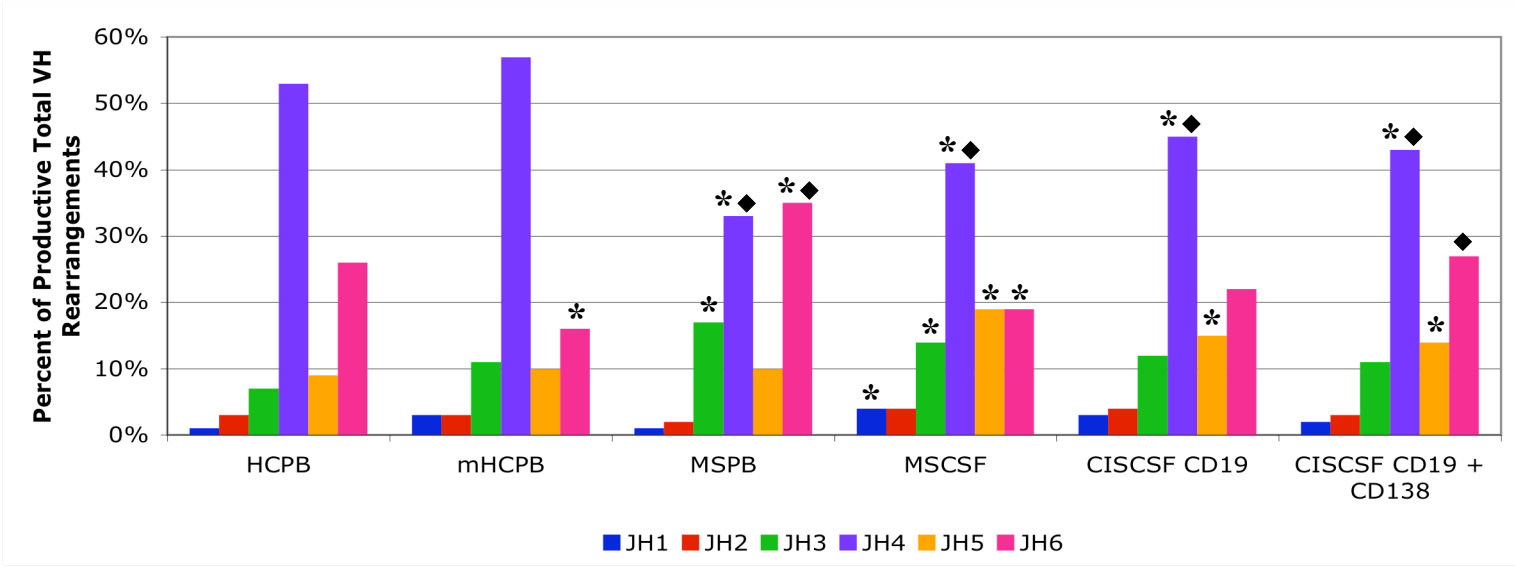

Percent Productive VH4 Rearrangements
40%
35%
30%
25%
20%
15%
10%
5%
0%
HCPB
mHCPB
MSPB
MSCSF
CISCSF CD19
CISCSF CD19+CD138
ONDCSF
4-04
4-30
4-31
4-34
4-39
4-59
4-61
4-B

Figure 3-3


Percent of Productive Total VH Rearrangements
60%
50%
40%
30%
20%
10%
0%
HCPB
mHCPB
MSPB
MSCSF
CISCSF CD19
CISCSF CD19 + CD138
JH1 JH2 JH3 JH4 JH5 JH6

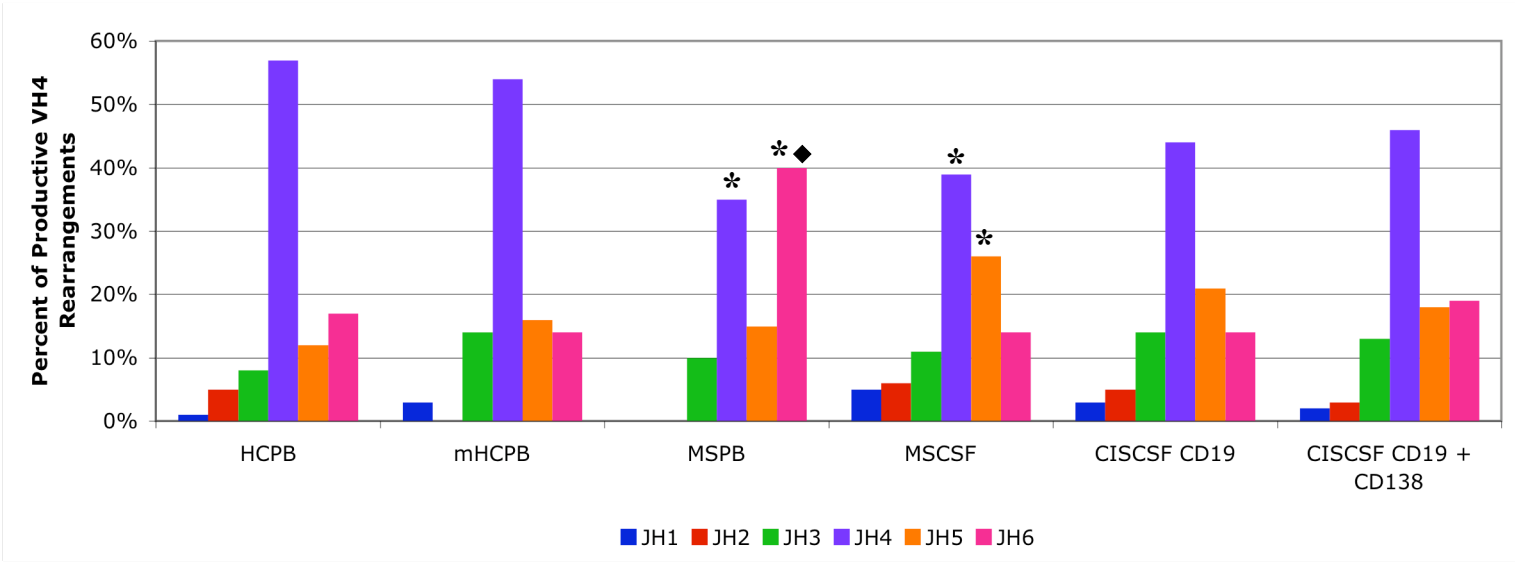
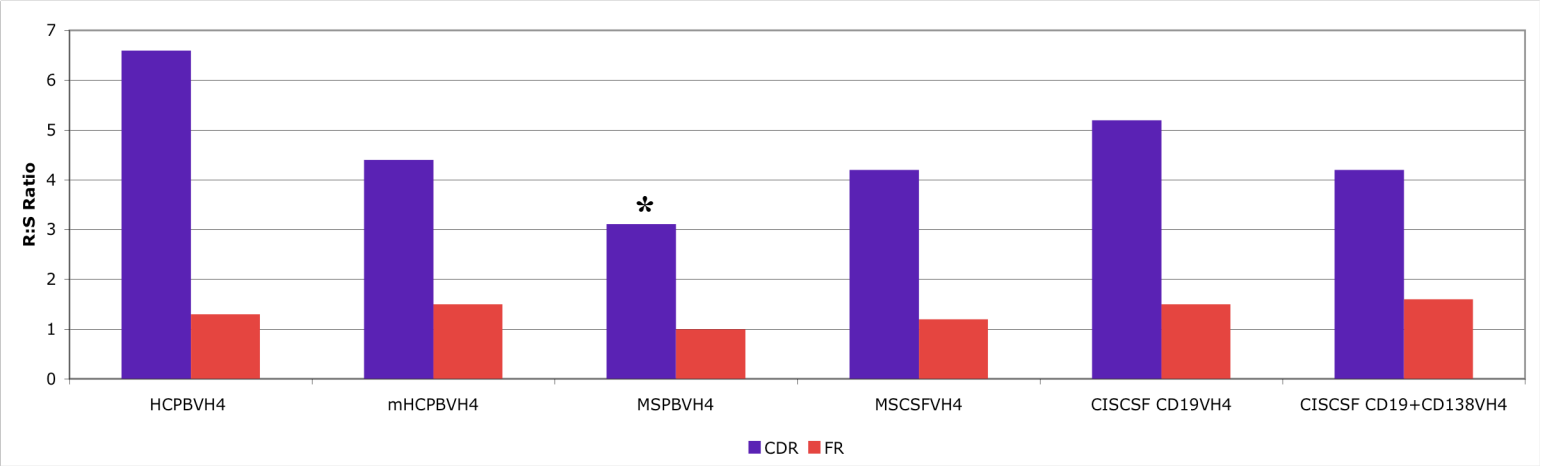

Percent of Productive VH4 Rearrangements
60%
50%
40%
30%
20%
10%
0%
HCPB
mHCPB
MSPB
MSCSF
CISCSF CD19
CISCSF CD19 + CD138
JH1 JH2 JH3 JH4 JH5 JH6

Figure 3-4


R:S Ratio
7
6
5
4
3
2
1
0
*
HCPBVH4
mHCPBVH4
MSPBVH4
MSCSFVH4
CISCSF CD19VH4
CISCSF CD19+CD138VH4
CDR
FR

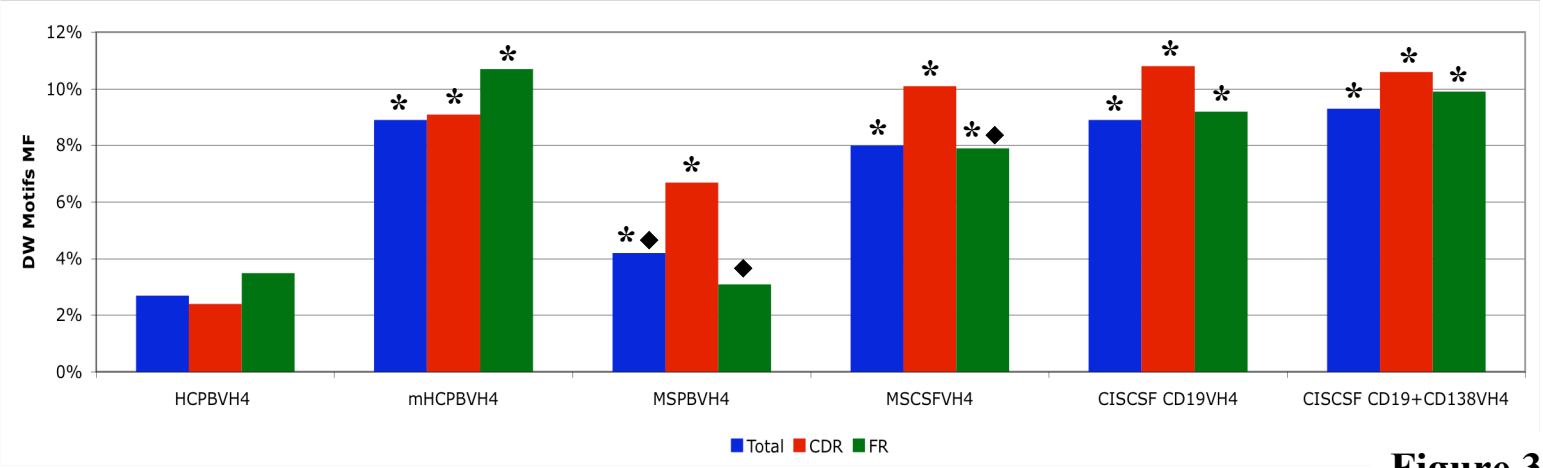

DW Motifs MF
12%
10%
8%
6%
4%
2%
0%
*
◆
HCPBVH4
mHCPBVH4
MSPBVH4
MSCSFVH4
CISCSF CD19VH4
CISCSF CD19+CD138VH4
Total
CDR
FR

Figure 3-5

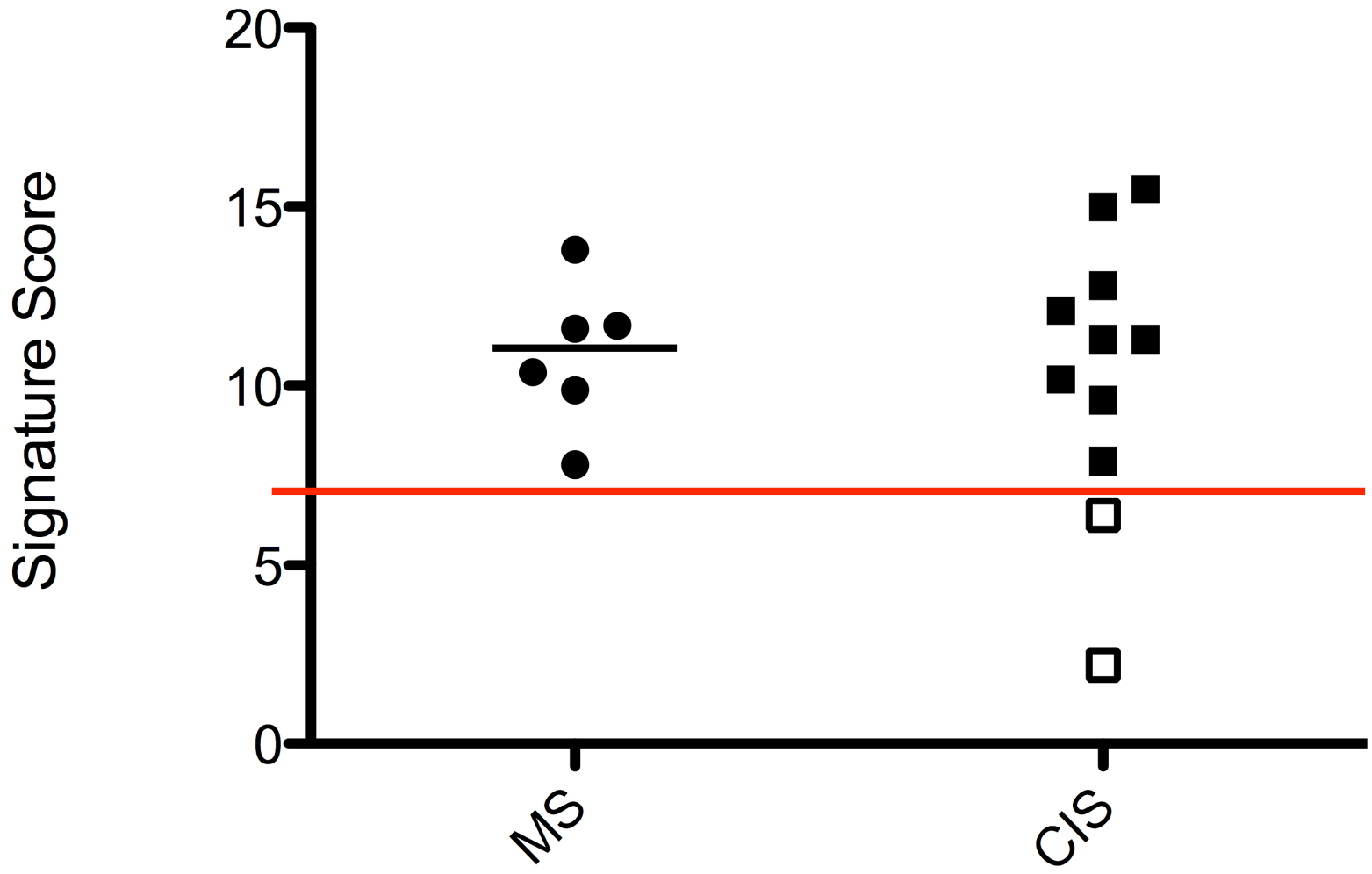


Figure 3-6

Table 3-1 Oligoclonal Banding in CISCSF

	IgG	IgA	IgM	Kappa	Lambda	CDMS
CIS132	Yes	No	No	Yes	No	Yes
CIS232	Yes	No	No	Yes	No	Yes
CIS429	Yes	Yes	No	Yes	Yes	Yes
CIS558	Yes	No	No	Yes	Yes	Yes
CIS599	Yes	Yes	No	Yes	Yes	Yes
CIS636	Yes	Yes	No	No	Yes	No
CIS681	Yes	No	No	Yes	No	No
CIS736	Yes	No	No	Yes	Yes	Yes
CIS896	Yes	Yes	No	Yes	Yes	Yes[1]
CIS951	Yes	Yes	No	Yes	No	No
Total CIS	10/10	5/10	0/10	9/10	6/10	7/10

Abbreviations: CIS=clinically isolated syndrome; CSF=cerebrospinal fluid; CDMS=clinically definite MS; MS=multiple sclerosis; NMO=neuromyelitis optica

[1]Converted to NMO

Table 3-2 MN Cell Number and Predictive Value

Patient Code	Percent of CSF CD19^{+}[1]	Absolute Number CSF CD19^{+}[2]	CSF CD19^{+} N:M Ratio	CDMS
ONDCSF	4.9%[3]	307[3]	0.59[4]	No
MSCSF	3.0%[5]	340[5]	0.73[6]	Yes
CIS132	6.5%	335	1.26	Yes
CIS232	2.8%	830	0.42	Yes
CIS429	0.6%	11	0.40	Yes
CIS558	2.2%	157	1.32	Yes
CIS599	0.1%	41	0.92	Yes
CIS681	6.2%	518	4.00	No
CIS736	14.1%	704	0.15	Yes
CIS767	0.2%	14	0.98	No
CIS896	0.5%	14	0.68	Yes[7]
Total CIS	2.3%	146	0.55	7/9 (78%)

Abbreviations: CIS=clinically isolated syndrome; CSF=cerebrospinal fluid; CDMS=clinically definite MS; MS=multiple sclerosis; NMO=neuromyelitis optica; OND=other neurological diseases; N:M=naïve:memory

[1]Percent of live cells

[2]Cells per milliliter CSF

[3]Includes OND117, OND142, OND315, OND336, OND382, OND456, OND475, OND492, OND648, OND658, OND758, OND766, OND788-2, OND823, OND859-1, OND859-2, OND935, OND936

[4]Includes OND patients OND142, OND315, OND336, OND341, OND382, OND456, OND475, OND492, OND648, OND658, OND758, OND766, OND788-2, OND823, OND859-2, OND935, OND936

[5]Includes MS patients M199, M217, M376, M522-1, M522-2, M584-3, M875, M927

[6]Includes MS patients M125, M217, M376, M522-1, M522-2, M584-3, M875, M887

[7]This patient was diagnosed with NMO

Table 3-3: Frequency of VH family usage[1]

	HCPB[2]	mHCPB[3]	MSPB	MSCSF[4]	CISCSF[5]	CISCSF[6]	ONDCSF[7]
B cell source	$CD19^+$	$CD19^+$ IgD^-CD27^+	$CD19^+$	$CD19^+$	$CD19^+$	$CD19^+$ $CD138^+$	$CD19^+$
VH1	15.5	10.7	17.9[9]	27.1[7,9]	9.9	8.9	20.0
VH2	2.0	2.0	1.2	1.6	5.3[8]	7.0	3.1
VH3	55.2	62.0	53.0	32.4[8,9]	56.3	47.5	46.2[9]
VH4	21.8	16.6	23.8	34.3[8,9]	26.2[9]	35.0	23.1
Total Sequences	348	205	168	373	302	528	65
Number of Donors	2	6	3	11	10	11	3

Abbreviations: VH=variable heavy chain; HCPB=healthy control peripheral blood; mHCPB=memory HCPB; MSPB=MS peripheral blood; MS=multiple sclerosis; CSF=cerebrospinal fluid; CIS=clinically isolated syndrome; OND=other neurological diseases

[1]Values provided in percent

[2]The HCPB group includes $CD19^+$ B cell antibody sequences from healthy controls BF1 (n=67) and BF2 (n=281).

[3]The mHCPB group includes $CD19^+$ B cell antibody sequences from healthy controls BF1 (n=18) and BF2 (n=105) with 4 or more mutations (less than 98% homology to germline) and IgD^-CD27^+ B cell antibody sequences from healthy controls HA (n=9), HB (n=44), HC (n=26), and HE (n=3) (Tian et al., 2007).

[4]The MSCSF group includes $CD19^+$ sequences from MS patients M125 (n=101), M199 (n=19), M354 (n=6), M368 (n=49), M376 (n=8), M484 (n=9), M522 (n=71), M584 (n=85), M875 (n=21), M217 (n=1), and M887 (n=3).

[5]The CISCSF group includes $CD19^+$ sequences from CIS patients CIS132 (n=19), CIS429 (n=57), CIS3-1 (n=24), ON3-1 (n=23), ON3-3 (n=39), ON3-4 (n=28), ON3-5 (n=35), ON4-7 (n=17), ON4-10 (n=31), and ON5-2 (n=29).

[6]In addition to those $CD19^+$ sequences listed in [5], this group includes $CD138^+$ sequences from CIS patients CIS3-1 (n=76), ON3-1 (n=45), ON3-3 (n=12), ON3-5 (n=44), ON4-7 (n=20), ON4-8 (n=17), ON5-2 (n=12).

[7]The ONDCSF group includes $CD19^+$ B cell antibody sequences from OND patients OND341 (n=32), OND758 (n=19), and OND116 (n=14).

[8]Significantly different from HCPB frequency

[9]Significantly different from mHCPB frequency

Table 3-4 CIS Characteristics Conventionally used for Predictive Purposes

Patient	MRI	Clonal Expansion	CD19/CD138 VH4 Bias[1]	CD19/CD138 VH2 Bias[1]	CD19/CD138 VH1 Bias[1]	Convert to CDMS
HCPB	NA	Not detectable	NA	NA	NA	No
MSCSF[2]	Yes	Yes	Yes / Yes	No / Yes	Yes / No	NA
CIS132	GD^+	No	No / NA[3]	No / NA[3]	No / NA[3]	Yes
CIS429	GD^+	Yes	No / NA[3]	No / NA[3]	No / NA[3]	Yes
CIS3-1	WML	Yes	Yes / Yes	No / No	No / No	Yes
ON3-1	WML	Yes	No / No	No / Yes	No / No	No
ON3-3	GD^+	Yes	No / No	No / No	No / No	Yes
ON3-4	None	No	No / NA[3]	No / NA[3]	No / NA[3]	No
ON3-5	GD^+	Yes	Yes / Yes	Yes / Yes	No / No	Yes
ON4-7	None	Yes	No / No	Yes / Yes	No / No	Yes
ON4-8	WML	No	NA[3] / Yes	NA[3] / No	NA[3] / No	Yes
ON4-10	WML	No	Yes / NA[3]	No / NA[3]	No / NA[3]	No
ON5-2	GD^+	Yes	Yes / Yes	No / No	No / No	Yes

Abbreviations: CIS=clinically isolated syndrome; CSF=cerebrospinal fluid; CDMS=clinically definite MS; MS=multiple sclerosis; NMO=neuromyelitis optica; GD=gadolinium; WML=white matter lesion; NA=not applicable; HCPB=healthy control peripheral blood; ON=optic neuritis

[1]Bias considered significantly higher than HCPB

[2]MS Patients include M125, M199, M354, M368, M376, M484, M522, M584, M875, M217, and M887

[3]no cell sequences recovered from this group

Table 3-5: VH4 individual gene usage frequency in peripheral blood or spinal fluid

Genes	HCPB	mHCPB	MSPB	MSCSF	CISCSF CD19	CISCSF CD19 + CD138	ONDCSF
4-04	9%	24%	3%	5%	6%	9%	0%
4-28	0%	0%	0%	0%	0%	0%	0%
4-30[1]	7%	5%	0%	9%	13%	14%	7%
4-31	5%	0%	13%	15%	20%	12%	13%
4-34	17%	8%	10%	7%	5%	9%	40%
4-39	21%	24%	30%	25%	26%	26%	7%
4-59	32%	29%	35%	26%	26%	26%	20%
4-61	4%	3%	3%	9%	4%	3%	13%
4-B	5%	8%	8%	5%	0%	1%	0%
VH4 N[2]	76	38	40	128	80	180	15
Total N[3]	348	205	168	373	302	528	65

Abbreviations: VH=variable heavy chain; HCPB=healthy control peripheral blood; mHCPB=memory HCPB; MSPB=MS peripheral blood; MS=multiple sclerosis; CSF=cerebrospinal fluid; CIS=clinically isolated syndrome; OND=other neurological diseases
[1]Includes all sub-genes (4-30.2 and 4-30.4)
[2]Number of productive VH4 sequences analyzed in each group
[3]Number of productive VH sequences overall
[4]Absolute number (% of total VH4 sequences)
[5]Different from HCPB and/or MSCSF

Table 3-6: Mutational Frequency in B cell Repertoires

	All				VH4				Total MF[3] All vs. VH4 p-value
	Total	CDR	FR	CDR vs. FR p-value	Total	CDR	FR	CDR vs. FR p-value	
HCPB	2.3%[1,2]	3.5%[2]	1.7%[2]	p<0.00001	2.0%[1,2]	2.4%[2]	1.9%[2]	p<0.00001	p=0.018
mHCPB	6.6%[2]	10.6%[1,2]	4.7%[1,2]	p<0.00001	6.7%[2]	8.4%[1]	5.9%[1,2]	p<0.00001	p=0.746
MSPB	1.7%[1,2]	3.3%[2]	1.0%[1,2]	p<0.00001	2.9%[1,2]	5.2%[1,2]	1.9%[2]	p=0.00011	p<0.001
MSCSF	4.9%[2]	7.9%[1]	3.5%[1]	p<0.00001	5.9%[1]	8.7%[1]	4.6%[1]	p<0.00001	p<0.001
CISCSF CD19	5.4%[1,2]	8.8%[1,2]	3.9%[1,2]	p<0.00001	5.8%[1]	8.0%[1]	4.8%[1]	p<0.00001	p=0.366
CISCSF CD19 + CD138	5.8%[1,2]	9.0%[1,2]	4.4%[1,2]	p<0.00001	6.1%[1]	7.9%[1]	5.2%[1,2]	p<0.00001	p=0.004

Abbreviations: VH=variable heavy chain; HCPB=healthy control peripheral blood; mHCPB=memory HCPB; MSPB=MS peripheral blood; MS=multiple sclerosis; CSF=cerebrospinal fluid; CIS=clinically isolated syndrome; CDR=complementary determining region; FR=framework

[1]Different from HCPB memory ($p \leq 0.05$)

[2]Different from MSCSF ($p \leq 0.05$)

[3]Comparing All VH sequences except VH4 to VH4 only

Table 3-7: Frequency of JH family usage

	HCPB[1]		mHCPB[2]		MSPB		MSCSF[3]		CISCSF CD19[4]		CISCSF CD19 + CD138	
	All	VH4	All	VH4	All	VH4	All	VH4	All	VH4	All	VH4
JH1	1%	1%	3%	3%	1%	0%	4%	5%	3%	3%	2%	2%
JH2	3%	5%	3%	0%	2%	0%	4%	6%	4%	5%	3%	3%
JH3	7%	8%	11%	14%	17%	10%	14%	11%	12%	14%	11%	13%
JH4	53%	57%	57%	54%	33%	35%	41%	39%	45%	44%	43%	46%
JH5	9%	12%	10%	16%	10%	15%	19%	26%	15%	21%	14%	18%
JH6	26%	17%	16%	14%	35%	40%	19%	14%	22%	14%	27%	19%
Total Number of Sequences	348	76	212	37	162	40	370	127	303	80	528	180
Number of Donors	2		6		3		10		10		11	

Abbreviations: VH=variable heavy; HCPB=healthy control peripheral blood; mHCPB=memory healthy control peripheral blood; MSPB=multiple sclerosis peripheral blood; MSCSF=multiple sclerosis cerebrospinal fluid; CISCSF=clinically isolated syndrome cerebrospinal fluid

[1]The HCPB group includes $CD19^+$ B cell antibody sequences from healthy controls BF1 (n=67) and BF2 (n=281).

[2]The memory HCPB (mHCPB) group includes $CD19^+$ B cell antibody sequences from healthy controls BF1 (n=18) and BF2 (n=105) with 4 or more mutations (less than 98% homology to germline) and IgD^-CD27^+ B cell antibody sequences from healthy controls HA (n=9), HB (n=44), HC (n=26), and HE (n=3) (Tian et al., 2007).

[3]The MSCSF group includes $CD19^+$ sequences from MS patients M125 (n=101), M199 (n=19), M354 (n=6), M368 (n=49), M376 (n=8), M484 (n=9), M522 (n=71), M584 (n=85), M875 (n=21), M217 (n=1), and M887 (n=3).

[4]The CISCSF group includes $CD19^+$ sequences from CIS patients CIS132 (n=19), CIS429 (n=57), ON3-1 (n=23), ON3-3 (n=39), ON3-44 (n=28), ON3-5 (n=35), ON4-7 (n=17), ON4-10 (n=31), and ON5-2 (n=29).

Table 3-8: VH4 Mutational Characteristics

	$HCPB_{VH4}$	$mHCPB_{VH4}$	$MSPB_{VH4}$	$MSCSF_{VH4}$	CISCSF $CD19_{VH4}$	CISCSF $CD19 + CD138_{VH4}$
R:S Ratio CDR	6.6	4.4	3.1[1]	4.2	5.2	4.2
R:S Ratio FR	1.3	1.5	1.0	1.2	1.5	1.6
DW Motifs MF Total	2.7%	8.9%[1]	4.2%[1,2]	8.0%[1]	8.9%[1]	9.3%[1]
DW Motifs MF CDR	2.4%	9.1%[1]	6.7%[1]	10.1%[1]	10.8%[1]	10.6%[1]
DW Motifs MF FR	3.5%	10.7%[1]	3.1%[2]	7.9%[1,2]	9.2%[1]	9.9%[1]

Abbreviations: VH=variable heavy chain; HCPB=healthy control peripheral blood; mHCPB=memory HCPB; MSPB=MS peripheral blood; MS=multiple sclerosis; CSF=cerebrospinal fluid; CIS=clinically isolated syndrome; CDR=complementary determining region; FR=framework; MF=mutational frequency; DW=DGYW/WRCH

[1]Different from $HCPB_{VH4}$ ($p \leq 0.05$)

[2]Different from $mHCPB_{VH4}$ ($p \leq 0.05$)

Table 3-9: Percentage of Replacement Mutations in Each Signature Codon

		$MSCSF_{VH4}$	$HCPB_{VH4}$			$mHCPB_{VH4}$		
Codon	**Location[2]**	**RF**	**RF**	**Fold Increase**	**p-value[4]**	**RF**	**Fold Increase**	**p-value[4]**
31B	CDR1	3.5[5]	0.5[5]	7.0	0.001	0.8	4.4	0.001
32	CDR1	2.3	1.5	1.5	0.05	2.1	1.1	NS
40	FR2	2.7	1.0	2.7	0.001	1.1	2.5	0.001
56[1]	CDR2	5.5	3.0	1.8	0.001	3.2	1.7	0.001
57	CDR2	2.0	1.0	2.0	0.005	0.5	3.7	0.001
60	CDR2	2.4	1.5	1.6	0.05	1.1	2.2	0.001
81	FR3	4.7	3.0	1.5	0.005	3.7	1.3	NS
89	FR3	2.0	1.0	2.0	0.005	1.3	1.5	NS
Hotspot Total[3]		**25.0**	**12.6**	**2.0**	**0.001**	**13.8**	**1.8**	**0.001**
30	FR1	2.0	4.0	0.5	0.005	2.9	0.7	NS
43	FR2	0.9	2.0	0.5	0.025	1.3	0.7	NS
77	FR3	1.5	2.5	0.6	0.05	1.6	0.9	NS
82	FR3	0.7	2.5	0.3	0.001	1.6	0.5	0.05
Coldspot Total[3]		**5.1**	**8.5**	**0.5**	**0.001**	**7.4**	**0.7**	**0.01**

Abbreviations: VH=variable heavy chain; HCPB=healthy control peripheral blood; mHCPB=memory HCPB; MS=multiple sclerosis; CSF=cerebrospinal fluid; CDR=complementary determining region; FR=framework; RF=replacement frequency

[1]Previously published replacement hotspot (Dorner et al., 1997; Dorner et al., 1998a)

[2]As defined by (Kabat et al., 1983)

[3]"Hotspot Total" is the total RF within codons 31B, 32, 40, 56, 60, and 81. "Coldspot Total" is the total RF within codons 30, 77 and 82. 965, 199, and 337 replacements respectively were included in this analysis for $MSCSF_{VH4}$ $HCPB_{VH4}$, and $mHCPB_{VH4}$.

[4]Comparing $HCPB_{VH4}$ or $mHCPB_{VH4}$ to $MSCSF_{VH4}$ RFs at each codon position using χ^2 goodness-of-fit where expected frequency is the RF calculated in $HCPB_{VH4}$ and $mHCPB_{VH4}$ respectively.

[5]We also calculated the frequency of possible mutations (the number of replacements at each codon divided by the total number of replacement mutations in genes which contain that codon) to determine if the frequency of genes utilizing 31B impacted the MF, and $MSCSF_{VH4}$ still had a significant increase in the number of replacement mutations located at that codon (compare 4.9% $MSCSF_{VH4}$ to 1.6% $HCPB_{VH4}$, fold increase = 3.1, $p<0.001$).

Table 3-10: MSCSF VH4 Gene RF at Signature Codons

	4-04	**4-30**[1]	**4-31**	**4-34**	**4-39**	**4-59**	**4-61**	**4-B**	**Total**[4]
% total VH4 mutations[2]	3%	10%	16%	7%	28%	23%	12%	3%	977
% total VH4 use	5%	9%	15%	7%	25%	26%	9%	5%	128
31B	NA[3]	0%	4.6%	NA[3]	6.9%	NA[3]	7.0%	NA[3]	3.5%[4]
32	7.7%	1.1%	5.3%	0%	2.2%	0.4%	2.6%	3.8%	2.3%[4]
40	0%	2.2%	4.6%	6.2%	2.2%	3.1%	0%	0%	2.7%[4]
56	3.8%	6.5%	5.3%	3.1%	5.8%	4.4%	7.0%	7.7%	5.5%[4]
57	3.8%	3.2%	0%	6.2%	1.8%	0.9%	2.6%	3.8%	2.0%[4]
60	3.8%	1.1%	3.3%	0%	2.6%	2.2%	3.5%	0%	2.4%[4]
81	11.5%	3.2%	6.6%	4.6%	5.5%	1.8%	4.4%	7.7%	4.7%[4]
89	3.8%	0%	1.3%	4.6%	1.5%	1.8%	2.6%	7.7%	2.0%[4]
Hotspot Total	34.6%	17.2%	31.1%	24.6%	28.5%	14.5%	29.8%	30.8%	25.0%[4]
30	3.8%	1.1%	0.7%	4.6%	2.2%	2.6%	0.9%	0%	2.0%[4]
43	0%	2.2%	0.7%	1.5%	1.1%	0.4%	0.9%	0%	0.9%[4]
77	0%	3.2%	2.6%	0%	1.5%	1.3%	0%	0%	1.5%[4]
82	0%	1.1%	0%	3.1%	0.4%	1.3%	0%	0%	0.7%[4]
Coldspot Total	3.8%	7.5%	4.0%	9.2%	5.1%	5.7%	1.8%	0%	5.1%[4]

Abbreviations: VH=variable heavy chain; MSCSF=multiple sclerosis cerebrospinal fluid; RF=replacement frequency

[1] 4-30 includes all sub-genes

[2] Percent of total R mutations in each gene

[3] Gene does not contain codon 31B

[4] Overall VH4 mutational frequency (see Table 3-4)

Table 3-11: CIS Patient Summary and Signature Score Predictions

Subject No.[1]	MRI Brain Lesions	OCB	CD19 / CD138[3]	CD19 VH4 bias[4]	CD138 VH4 bias[4]	Signature Score	Prediction based on signature score	Definite MS	Time to MS Diagnosis[2]
HCPB	NA	NA	348 / NA	No	NA	NA	NA	No	NA
MSCSF	Yes	Yes	370 / NA	Yes	NA	10.9	NA	Yes	NA
CIS132	Yes	Yes	19 / NA	No	NA	12.1	CDMS	Clinical	18
CIS429	Yes	Yes	56 / NA	No	NA	15.0	CDMS	Clinical	3
CIS3-1	Yes	Yes	24 / 76	Yes	Yes	15.5	CDMS	MRI	3
ON3-1	Yes	Yes	23 / 45	No	No	6.4	No	No	NA
ON3-3	Yes	Yes	39 / 13	No	No	11.3	CDMS	Clinical	3
ON3-4	No[5]	No	28 / NA	No	NA	11.3	CDMS	No	NA
ON3-5	Yes	Yes	35 / 44	Yes	Yes	12.8	CDMS	Clinical	2
ON4-7	No[5]	Yes	17 / 20	No	Yes	10.2	CDMS	Clinical	5
ON4-8	Yes	Yes	NA / 18	NA	Yes	9.6	CDMS	Clinical	5
ON4-10	Yes	No	31 / NA	No	NA	2.2	No	No	NA
ON5-2	Yes	Yes	29 / 12	Yes	Yes	7.9	CDMS	Clinical	3

Abbreviations: CIS, Clinically Isolated Syndrome; ON, Optic Neuritis; LP, lumbar puncture; GD, gadolinium enhancing lesion positive; WML, white matter lesions by T2; OCB, oligoclonal bands; CDMS, clinically definite MS; NA, not applicable

[1]CIS132 and CIS429 were generated at UTSWMC; the remaining patient CSF B cell repertoires were generated at UCHSC. Patient information can be found in (Bennett et al., 2008; Harp et al., 2007)

[2]in months

[3]Values given are number of sequences in CD19 repertoire/CD138 repertoire

[4]Bias was considered significantly different from expected frequency in HCPB (Brezinschek et al., 1997; Brezinschek et al., 1998; Dorner et al., 1997; Dorner et al., 1998a, b; Dorner et al., 1998c; Farner et al., 1999; Hansen et al., 2000; Monson et al., 2000).

[5]One spinal cord lesion was observed by T2 weighted MRI

CHAPTER FOUR
Discussion

Current diagnostic criteria require multiple episodes to distinguish MS from other possible neurological diseases or infectious causes; this lack of exact diagnosis causes unnecessary delays in optimal treatment. The current accepted diagnostic criteria for MS, the McDonald criteria, were introduced in 2001 (McDonald et al., 2001), updated in 2005 (Polman et al., 2005), and have a general requirement for separation of time and space between clinical episodes (reviewed in (Frohman et al., 2003)). Neurologists generally consider a patient exhibiting the diagnostic criteria (the presence of OCB or MRI lesions) as an indication of a much greater likelihood of clinically definite MS diagnosis with conversion within months to a few years (Frohman et al., 2003; Tintore et al., 2008), even without fulfilling the full diagnostic criteria. However, CSF abnormalities are not required for diagnosis, except for primary progressive MS (approximately 10-15% of MS cases) (Link and Huang, 2006; McDonald et al., 2001). Several diseases, present in a smaller percentage of the population, can have features (OCB and high immunoglobulin synthesis) that make them difficult to distinguish from classical MS (Reske et al., 2005), such as neuropsychiatric lupus erythematodes, neurosarcoidosis, Behçet, and Sjögren's syndrome (Reske et al., 2005). So we investigated humoral immune characteristics in MSCSF to

determine if any single B cell related criteria would be more specific and accurate than current methods to identify patients that would convert to CDMS.

I. OCB occurrence in CISCSF and more than two lesions observed by MRI in CIS CNS predicts conversion.

Though the presence of OCB was universally seen in our patient cohort, the patients did not all fulfill the diagnosis of CDMS. Similarly, the presence of multiple MRI lesions, though satisfying part of the requirement of separation in time and space, increases the likelihood of a CDMS diagnosis (73% accurate in our cohort of 11 CIS patients). Together, however, these 2 methods are the most accurate indicator of the possibility of CDMS in the future of the CIS patients' disease progression.

II. CISCSF cell concentration and $CD19^+$ B cell concentration of no predictive value to CDMS conversion. Increased numbers of immune cells and B cells in the CSF of CIS patients is indicative of inflammation, and in itself, has no predictive value, as inflammatory OND patients with no significant symptoms of CDMS had similar or increased numbers of total cells (or total lymphocytes) and particularly $CD19^+$ B cells in their CSF. This is not a novel finding, but we had originally rationalized that increasing the number of patients and grouping both the MS and OND patients may have changed the impact of this observation. This suggests that the chronic "attacks" of inflammation itself does not create MS, but that some difference in the immune response in these cases of inflammation,

either inherent in the patient or environmentally caused, is causing the difference between MS and other inflammatory OND.

III. CISCSF naïve:memory ratios no different from OND or MS patients. It has been shown that B cells can enter the CNS, though usually in low numbers (Anthony et al., 2003), presumably for maintenance surveillance. B cells that enter the brain parenchyma show a more activated phenotype by nature (expressing CD23 in this study), and intravascular B cells have a lower expression of activation markers (Anthony et al., 2003). In rats, it has been shown that T cells must be activated to enter the normal CNS and must encounter antigen rapidly (within 1 to 2 days of entry) to remain there (Hickey et al., 1991). Perhaps a similar requirement enriches this compartment with memory B cells in CIS, OND, and MS CSF, presumably with retention of B cells responding to resident, disease-specific antigen (as seen in (Hickey et al., 1991)).

IV.1a. VH4 enrichment is seen in CSF B cells from MS patients and helpful, but not precisely accurate, in determining CDMS conversion in CISCSF. If B cells with disease relevant antigen specificity are more likely retained in the CNS than other trafficking B cells, then there should be some B cell antibody characteristics that are unique to antibodies found against MS antigens in comparison to OND. Indeed, while VH4 expressing B cells from healthy donor B cell repertoires are negatively selected (Brezinschek et al., 1995; Pugh-Bernard et al., 2001; Zheng et al., 2004), several groups investigating the role of B cells in

MS have reported that there is an increased frequency of VH4 expressing B cells in the CSF and brain lesions of patients with MS (Baranzini et al., 1999; Colombo et al., 2000; Harp et al., 2007; Monson et al., 2005; Owens et al., 1998; Owens et al., 2003; Owens et al., 2007; Qin et al., 1998). We predicted that B cells from the CSF of MS patients specifically expressing VH4 genes may be enriched for features associated with autoreactivity since 1) VH4 expressing B cells from patients with autoimmune diseases (including SLE and RA) are enriched for autoreactivity (Hayashi et al., 2007; Huang et al., 1998; Mockridge et al., 2004; Pugh-Bernard et al., 2001; Voswinkel et al., 1997; Zheng et al., 2004), and 2) some autoreactive clonally expanded CSF-derived B cells from MS patients use VH4 in their antibody rearrangements (Lambracht-Washington et al., 2007). Features that we were particularly interested in were those known to be associated with autoreactivity including bias towards VH4-34 usage (Zheng et al., 2004) and features associated with receptor editing including bias in JH6 usage (Meffre et al., 2000; Zheng et al., 2004). Diminished mutational frequency has been associated with receptor editing (Meffre et al., 2000), and diminished mutation targeting has been associated with clonally expanded CSF derived B cell populations in MS patients (Monson et al., 2005) and thus were also included in the analyses.

In order to perform this VH4-specific analysis, we constructed an extensive CSF B cell database containing CSF-derived B cells from MS patients

and CIS patients' antibody characteristics to determine if any of these attributes could accurately determine the likelihood of these patients converting to CDMS. Overrepresentation of VH4 expressing $CD19^+$ B cells in the CSF of MS patients was present in the CSF but not the PB of MS patients, and was different from the frequencies found in HCPB and CSF B cells from patients with OND, either non-inflammatory or inflammatory in nature. In fact, further analysis of those VH4 expressing B cells from the peripheral blood of MS patients within our cohort suggest that this group of B cells were likely recognized for their autoreactive potential (as evidenced by high JH6 usage and long CDR3 length), and were denied further selection (as evidenced by low mutational frequencies). The lack of VH4 overrepresentation in the OND patients indicates that over-representation of VH4 expressing B cells in the CSF of MS patients is not due to bias in the ability of VH4 expressing B cells to enter the CNS. Taken together, these data suggest that VH4 expressing B cells are selected into the CSF B cell repertoire of MS patients in particular, and is further validated by the high mutational frequencies and punctuated mutational targeting observed in this population.

Since VH4 is increased in CDMS patients and VH4-expressing B cells can be found at MS lesion sites (Baranzini et al., 1999; Owens et al., 1998), we hypothesized that the CIS patients that had an overrepresentation of CSF B cells expressing VH4 would convert to CDMS. Already this early in disease the VH4 compartment has increased in some patients, and the patients with VH4 bias have

a propensity to convert to CDMS (Table 3-3); these facets suggest the role these VH4-expressing CSF B cells are playing is important even early in disease development.

Since VH4-expressing B cells are not reaching the CNS more readily than B cells expressing other VH families (as evidenced by ONDCSF), the increase in VH4 frequency is most likely due to antigen response, but other possibilities have not been eliminated. It is possible that B cells expressing VH4 survive in the CSF more readily than B cells expressing other heavy chain family members, perhaps by expressing higher levels of BAFF receptors, for example, which heighten B cell survival ((Avery et al., 2003) and reviewed in (Crowley et al., 2005; Mackay et al., 2003; Zhang et al., 2005a)). Interestingly, high levels of BAFF have been observed in MS lesions (Krumbholz et al., 2005; Thangarajh et al., 2004). However, such pathways would maintain a requirement for antigen stimulation, and thus it is reasonable that in the CSF of MS and CIS patients preferential survival of VH4 expressing $CD19^+$ B cells is due to increased proliferation in response to antigen found in this compartment. Also contributing to this hypothesis is the presence of matching clones in both the $CD19^+$ and $CD138^+$ populations (Martin Mdel and Monson, 2007), suggesting that the plasma cells and plasma blasts are arising out of the $CD19^+$ population, but do not linger in the memory pool long, and are signaled to differentiate rapidly into plasma blasts and plasma cells. This hypothesis is also further substantiated by the lack of receptor

editing in the VH4 expressing CSF-derived B cells from these patients (as assessed by normal JH6 usage and CDR3 length), as well as documentation that plasma blasts and plasma cells are highly enriched in the CSF of these patients (Cepok et al., 2005; Winges et al., 2007). Dysregulation of these VH4 cells at the initiation of disease processes may be a central component of ongoing pathogenesis.

IV.1b. MSCSF and CISCSF B cell databases reveals no restriction in individual VH4 gene usage.

We expected the increase in VH4 family usage would correspond to an increase in particular VH4 genes used most frequently in MS lesions and in the clones found in MSCSF such as 4-34, 4-39 and 4-59 (Monson et al., 2005; Owens et al., 1998). However, usage frequency of individual VH4 genes within the VH4 expressing CSF B cell subdatabase was no different than in HCPB or the periphery of the MS patients. It is possible that B cells from the MS patients examined were responding to a variety of VH4-binding antigens, so that the combination of these made an increase in a single gene indeterminable. Another possibility is an antigen may bind to the VH4 genes and cause a superantigen response in only the B cells expressing VH4, similar to what is seen with staphylococcal enterotoxin A with VH3-expressing B cells (Domiati-Saad and Lipsky, 1998). However, superantigen binding capacity is diminished with high mutation accumulation (Oppezzo et al., 2004), and so a classical superantigen

response is unlikely. In contrast, EBV infected memory B cells tend to have high mutational frequencies and prevalent mutational targeting (Souza et al., 2007) similar to what we described in the MSCSF database presented here. However, no mechanism of EBV infection susceptibility or immune response to the virus has been reported that favors VH4 expressing B cells over other heavy chain family expression. Nevertheless, the elevated mutational frequency observed in VH4 expressing B cells from the CSF of MS patients extends our previous hypothesis that CSF-derived B cells responding to antigen in the CNS are heavily driven within the CNS itself to suggest that much of this heightened activity is occurring within the VH4 expressing CSF-derived B cell populations.

Interestingly, some MSCSF antibody repertoires are enriched in the frequency of VH1 expressing $CD19^+$ cells, but not in $CD138^+$ plasma cells. This bias is also seen in MSPB, so this increase in the CSF could be a direct result of PB bias, so more VH1 expressing B cells are available to survey the CNS. Another potential explanation of the VH1 bias in some patient repertoires is that these cells either do not expand as well in the CSF as the VH4-expressing $CD19^+$ B cells or an unrecognized regulation is occurring in the VH1 expressing B cells that is not occurring in the VH4. This regulation could be the VH1 expressing B cells acting as regulatory B cells, being selectively destroyed by a cytotoxic cell, or reacting more powerfully to a CSF agent like NGF than the VH4-expressors.

IV.2. Clonal expansion in CSF B cells from CIS patients predicts conversion.

Similar to VH4 and VH2 bias, clonal expansion when present accurately predicts conversion to CDMS; however, the lack of clonal expansion did not accurately predict that a patient had an OND or idiopathic CIS. Surprisingly, when the MSCSF B cells are normalized, such that each clone was only represented a single time, an overrepresentation of VH4 is still observed. It may be that these cells are indeed clones, but were at a frequency below our level of detection; possibly the other family members are located in lesions, with only a non-detectable portion in the CSF. It is thought that different lesions can contain different clones because OCB patterns from a single patient's lesions are not identical (Lolli et al., 1989; Mattson et al., 1980). It could also be that some of these cells are just in the beginning of the clonal expansion, and the sister(s) (fellow clones) did not exist at the time of sampling.

V. MSCSF and CISCSF repertoire normal in other analyses. We were anticipating that the increase in VH4, suggesting that these cells are reacting in the CNS, would also be compatible with the idea that they might be activated in an ectopic germinal center similar to those that have been described in secondary progressive MS patients (Corcione et al., 2004). Interestingly, in support of this idea, at least one of the CIS patients (CIS429) had clones that were present in both IgM and IgG isotypes (data not shown). However, we did not observe that these B cells were maturing in an atypical germinal center (Figure 3-5).

Whether these B cells are responding to self-antigens or valid foreign targets remains controversial. However, mutational analysis indicates that the VH4 expressing CSF-derived B cells from MS patients had gone through a typical germinal center, since mutational targeting to CDR and to DGYW/WRCH motifs is intact, unlike what has been observed in the individual clonal populations from MS patients in the cohort (Monson et al., 2005). In addition, targeting was actually increased in the $MSCSF_{VH4}$ subdatabase, most likely because the number of rounds of somatic hypermutation the B cells had undergone in response to antigen was extensive (evidenced by the high mutation frequency). Defining the antigen specificity of highly mutated, VH4 expressing CSF-derived B cells from MS patients will be paramount to resolving the mechanism of this unique selection of VH4 expressing B cells in the CSF of MS patients.

MS patients did have an increase in JH6, but in the PB, not the CSF. It is interesting that the mHCPB sub-database had a decreased frequency of JH6 in comparison to the inclusive HCPB, but if we just took the memory from the MSPB database, the frequency of JH6 was actually increased in comparison to the inclusive MSPB database. This could be a symptom of dysregulation, or that autoreactive VH4 cells are being edited in the PB, but not in the CSF.

VI. 1. Identification of codons within MSCSF that are enriched for replacement mutations. Hyperintense mutation accumulation in $MSCSF_{VH4}$ enabled us to identify a unique antibody gene signature enriched for replacement

accumulation at codons 31B, 32, 40, 56, 57, 60, 81 and 89 that was not observed in $HCPB_{VH4}$.

In order to define this VH4 antibody signature, the first criterion was a statistical difference at each codon between $MSCSF_{VH4}$ and $HCPB_{VH4}$ databases. This criterion was not stringent, as it is possible that this signature simply reflects enrichment of mutation accumulation in CNS derived B cells compared to peripheral B cells since the former consists of mostly memory B cells and peripheral B cells are mostly naïve. However, tabulating only those sequences with mutations resulting in amino acid replacements minimized the effect of naïve B cells on the RF calculation. Second, we discarded codons from signature consideration when the RF value did not exceed 1.86, which was the average + 2 S.D. of the memory $HCPB_{VH4}$ subdatabase. This criterion also minimized the effect of naïve B cells in the signature identification process. Third, 5 of the 8 hot codons of the signature retain higher RF values in the MSCSF database compared to the memory HCPB database. And finally, if signature score simply reflects enrichment of mutation accumulation due to the repertoire's high memory representation, then all signature scores from the CISCSF antibody repertoires should have been high since they were all heavily enriched for memory B cells. This was not the case, since CISCSF repertoires ON3-1 and ON4-10, despite being heavily enriched for memory B cells (with mutation frequencies of 5.2%

and 6.7%, respectively), had very low signature scores (ON3-1 score=6.4, ON4-10 score=2.2; Table 3-7).

Codon composition can also influence the protein structure of antibody variable regions (Chothia et al., 1992). VH4-34 and 4-59 have a similar structure, as they have neither codons 31A or 31B; VH4-04, 4-B, and 4-28 have only codon 31A; and the 4-30 sub-genes, 4-39, 4-61, and 4-31 have both codons 31A and 31B. In addition, several crucial codons are needed to maintain structure; none of the VH4 signature codons are key residues that would change the structure of the antibody (Chothia and Lesk, 1987; Chothia et al., 1992). This infers that genes of similar structure have similar antigen-binding sites, though the exact placement may differ due to the size, hydrophilicity, and polarity of surrounding residues. The site of the antigen binding (often a cavity or groove) is determined largely by the conformation of the heavy chain CDR3, but the other CDRs contribute as well (Ramsland and Farrugia, 2002). By the method designated by Chothia, et al. (Chothia et al., 1992), CDR1 is comprised of residues 26 through 32 because these are outside the framework β-sheets and form a loop involved in the antigen binding pocket, and CDR2 is only residues 50 through 58; this translates into codon 30, 31B, 32, 56, and 57 are all predicted by the proposed structure to be in direct contact with the antigen (Figure 4-1), while 60 is between the antigen binding pocket and another surface loop not directly involved with antigen binding (Chothia et al., 1992). Therefore, codon 30 is likely “cold” to maintain

efficient antibody interaction with the antigen, while variation in codons 31B (in the few genes it is in), 32, 56, and 57 provide more effective binding to their antigen with different size, hydrophilicity, or polarity properties (Table 4-1). Codon 31B was particularly interesting because it accumulated replacement mutations up to 7-fold higher than in HCPB, suggesting that this codon plays a pivotal role in antigen-antibody interactions. Mutations at this codon position are well regulated in the peripheral blood of healthy donors since the MF at codon 31B in the $HCPB_{VH4}$ database is 0.5%, and only 0.9% in $mHCPB_{VH4}$. It is less clear why residues 40, 81, and 89 are "hot" or residues 43 and 77 are "cold", and how replacement mutations at these positions (Table 4-1) affect VH4 antigen binding (Figure 4-1). Others have demonstrated that mutations in FR, which are not thought to have direct contact with the antigen, nevertheless increase affinity for the antigen (Clark et al., 2006; Daugherty et al., 2000; Zahnd et al., 2004) via electrostatic optimization and side chain repacking (Clark et al., 2006).

Replacement mutations at codons far from the CDRs might have spatial constraints as well. We examined the codons in the signature for changes that have a predictable modification in the resultant amino acid (Table 4-1). At codon 40 in gene 4-31, for example, a histidine is most often mutated to proline (Table 4-1). Since a proline causes the resultant protein to have a more rigid conformation and reduces flexibility (Nelson and Cox, 2000), mutating into this amino acid could have drastic structural implications. However, at codon 81,

where all VH4 genes have a germline lysine, in $MSCSF_{VH4}$ B cells this is equally often mutated to an arginine or an asparagine. As both lysine and arginine are basic amino acids, and asparagine is hydrophilic, the change at this position is more ambiguous. Also interesting is the change at position 89 of 16% of the VH4 genes being mutated to methionine, as methionine is relatively rarely seen (e.g., 0.6 percent of human collagen, 0.2 percent of mammalian elastin, and 1 percent of human hemoglobin (Schultz and Liebman, 1997)). Finally, some of the signature codons are located within DW motifs, making these codons naturally more mutable, though the mutations in the $HCPB_{VH4}$ B cells would be just as likely at these positions. Also, individually, these codons may influence binding, but when working in concert have a more dramatic effect on antigen binding (Clark et al., 2006). Investigating the impact of replacement mutations at the signature positions will provide important clues regarding the interaction of these VH4 utilizing antibodies with self-antigens in the CNS.

Such studies will likely indicate that different combinations of residue replacements affect binding to discreet antigens. For example, perhaps the combination of replacements at codons A, B and C mediate high affinity binding to antigen X, while replacements at codons BDE mediate high affinity binding to antigen Y. This would explain the differences in replacement mutation positions in different VH4 genes; codon positions ABC are needed for 4-31 to bind antigen X, while codon positions BDE are needed for 4-39 to bind antigen Y. In support

of this, we found that different VH4 genes do selectively use the MS signature mutations at varying levels; for example, VH4-34 has more mutations in codons 40 and 57, while VH4-39 tends to accumulate mutations more rapidly in codons 31B, 56, and 81 (Table 3-6). MS patient antigen specificity may vary within the individual, over time, and between patients. However, the $MSCSF_{VH4}$ signature was found in all 11 CDMS patients examined, even one with PPMS sub-type of MS; the $MSCSF_{VH4}$ signature of mutational frequency changes is most likely more straightforward to track than response to any particular antigen.

It is possible that other combinations of the codon hot spots may be required for efficient binding to other CNS-derived antigens, such as MOG (Genain et al., 1999; Reindl et al., 1999; Tomassini et al., 2007) or alpha-beta crystallin (Dunn et al., 2007; van Noort et al., 2006). For example, codon 31B is only present in the 4-30, 4-31, 4-39, and 4-61 genes; in the majority of 4-30 genes (all except 4-30.4) and 4-31, this is a glycine, while in 4-39 and 4-61 this codon reads for a serine. Most (67%) of the MSCSF B cells employing 4-61 have a mutation at this codon, and many (59%) of the 4-39 and 4-31 (37%) sequences do as well; in comparison, none of the 4-30 genes had mutations at this codon (comparison of 4-30 to 4-61, $p<0.001$, 4-30 to 4-39, $p<0.001$, 4-30 to 4-31, $p<0.03$, all other three compared to one another, $p>0.05$). The HCPB database had only 1 mutation at this position, in a 4-30.4-using B cell; the CS memory database had 2 mutations at 31B, both in 4-39 genes. It is therefore tempting to

speculate that an antigen favors mutations at this site for binding, especially to 4-39 and 4-61 encoding B cells, and possibly that mutations at 31B help VH4 encoding B cells to be autoreactive. The mutational machinery is targeting properly, as both of these codons are inside DW motifs, but no obvious changes in hydrophobicity, size, or polarity could be found.

VI. 2. Potency of signature score to predict development of clinically definite MS. It was compelling to investigate whether a mutational signature may be of value to predict disease risk, since early and accurate diagnosis of MS is of tantamount importance in clinical care (Stuve et al., 2008), and the clinical presentation of a first demyelination event does not readily lend itself to correct diagnosis (Miller et al., 2005). Indeed, signature prevalence could be used as an independent tool to identify patients who would be diagnosed with CDMS within 3-18 months of experiencing their first demyelinating event. Of note, patient ON3-4 had a signature score that indicated this patient would convert to CDMS (score=11.3, Table 3-7), but did not demonstrate a lesion load by MRI, banding by OCB, or VH4/VH2 bias, and had not developed CDMS up to 2 years after sampling was performed. It will be interesting to determine whether this patient is diagnosed with CDMS over time. It is also important to note that the majority of patients in this cohort already had evidence of MS risk as indicated by positive MRI and OCB. Patient ON4-7, however, did not present with brain lesions by MRI, but had a signature score that indicated this patient would be diagnosed with

CDMS (score=10.2). Indeed, this patient did convert to CDMS within 5 months of CSF B cell antibody repertoire sampling, and provides a reasonable example of how signature prevalence may predict CDMS diagnosis in patients that either do not present with brain lesions by MRI, or who are not evaluated by MRI at this stage of their disease. In addition, signature prevalence may provide an evaluation mechanism to identify the most appropriate patient candidates to receive B cell depletion therapies. Certainly this is a priority since efficacy of Rituximab in RRMS patients was quite profound (Hauser et al., 2008), but did not meet endpoint criteria in PPMS patients (www.gene.com/gene/news/press-releases/display.do?method=detail&id=11147). Although such results may suggest that B cells do not play a significant role in the pathogenesis of PPMS (but do in RRMS), it is likely that some PPMS patients within the cohort did respond positively to B cell depletion therapy. Signature prevalence may be a useful tool to negotiate continued Rituximab therapy in such patients. Further experimentation is underway to evaluate the utility of the MS specific antibody signature for these purposes.

Given the urgency for early identification of MS and the rapid initiation of disease modifying therapy, presentation of a molecular signature in the CSF B cells of CIS patients who develop MS may provide an unique tool for identifying at-risk individuals. In addition, many early MS patients have atypical clinical presentations or unremarkable MRI scans, and patients with alternative

inflammatory conditions may mimic idiopathic demyelinating disease. In these circumstances, the advent of a molecular diagnostic signature would increase diagnostic sensitivity and specificity.

The presence of a mutational signature among clonally expanded VH4 germline antibodies in MSCSF may be helpful in understanding disease pathogenesis. The VH4 germline mutational signature may be the direct result of antigen targeting in the humoral immune response. Therefore, determining the antigen specificity of signature-enriched antibodies from CSF B cells of patients with definite MS and CIS is one of the first steps towards dissecting whether signature-enriched B cells have the potential to participate in MS pathogenesis. Of note, 5 of the 8 signature codons (31B, 32, 56, 57 and 60) we identified as having a unique accumulation of amino acid replacements in $MSCSF_{VH4}$ are predicted to have direct antigen contact since they reside in CDRs (Figure 4-1). Dissecting the relative contribution of replacement mutations at each of these signature codons as well as those outside of the antigen-binding region will address the impact of both codon classifications (direct and indirect antigen binding capacity) on antigen binding affinity.

Interestingly, the signature of VH4 replacement mutations can be found to a certain extent in the $MSPB_{VH4}$ B cells (data not shown) and even the MSPB as a whole (data not shown), though the differences in the MSPB (inclusive and VH4) at codon 31B was the only signature codon strikingly different than the HCPB

inclusive database (RF of 1.3 in inclusive MSPB and 2.9 in VH4 compared to HCPB of 0.4, $p<0.005$ and $p<0.001$ respectively). Obtaining a blood sample is significantly less traumatic than a lumbar puncture, so determining the extent of penetration of the signature and the threshold for detection of that signature in the PB will be vital. Particular mutation positions are significantly different in MSPB than HCPB; whether changes in the footprint occur in correlation with particular treatment regimens, and how these changes relate to disease severity would be of considerable interest, and may provide a unique tool to categorize MS patients with regard to probability to develop MS or predict exacerbations related to activation and clonal expansion of VH4-expressing B cells resulting in higher frequency detection of the footprint, possibly before any overt symptoms appear. Such an approach has been used to determine the presence of sub-clinical disease in melanoma after treatment to track the cancer before it is detectable by other methods (Hoon et al., 2000; Hoon et al., 2001; Koyanagi et al., 2005a; Koyanagi et al., 2006; Koyanagi et al., 2005b). In addition, if PB B cells from MS patients also carry the signature, then perhaps methods can be developed to detect the signal in PB rather than CSF, which would be more cost efficient and less traumatic for MS and CIS patients.

In summary, multiple sclerosis is a chronic debilitating disease. VH4 family usage is substantially increased in both $CD19^+$ B cells and $CD138^+$ plasma cells isolated from the central nervous system of MS patients, (Figure 3-2 and

(Owens et al., 2007)), but not in healthy controls, or patients with other CNS-related diseases. More importantly, a unique footprint of mutational characteristics can be found in the $MSCSF_{VH4}$ database that is not observed in healthy control peripheral blood or CSF-derived B cells from patients with other neurological diseases. This signature is most likely a combination of sub-signatures that mediate effective binding to antigens present in the CNS. This signature can be used to determine whether a particular patient will convert to CDMS, but needs to be tested on a larger cohort of patients before being used diagnostically. The signature has the potential to be used for a variety of other applications, especially if it can be adapted to sampling by venipuncture rather than lumbar puncture. Though we do not expect the VH4 signature to supplant the data provided by an MRI, it is foreseeable to use it to predict conversion of MS, to predict which patients might have a most advantageous result to rituximab, and to be used for MS prevention studies. It might also lead investigation into which potential self antigens are leading the disease, whether these differ at different time points in disease severity or sub-types of MS, or if the self antigen is variable. In conclusion, the MS-specific VH4 antibody signature provides a new focus to a more complete understanding of the disease and what occurs in the immune system in response to treatment or environmental exposures, and therefore, possibly even the causes of MS.

FIGURE LEGENDS

Figure 4-1. Model of VH4 structure. A human VH4-30.4 and VL2-8 antibody structure (Guddat et al., 1993) was obtained from the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (www.rcsb.org) under the identification moniker 1MCO, and adapted using the RasMol program (RasMac v2.6 available at mc2.cchem.berkeley.edu/Rasmol/ (Sayle and Milner-White, 1995)). Two orientations of the structure are provided in Panels A and B. The light chain variable domain is included for reference and is encoded in gray, while the heavy chain backbone is in yellow. The VH4 signature has been demarcated, with "hot" spots in blue (residues 31B, 32, 40, 56, 57, 60, 81, and 89), and "cold" spots in green (residues 30, 43, 77, and 82). Residues contained within CDR1 and 2 are boxed. The CDR3 is that of the original structure, and not from any VH4 rearrangement discussed here.

Figure 4-1A

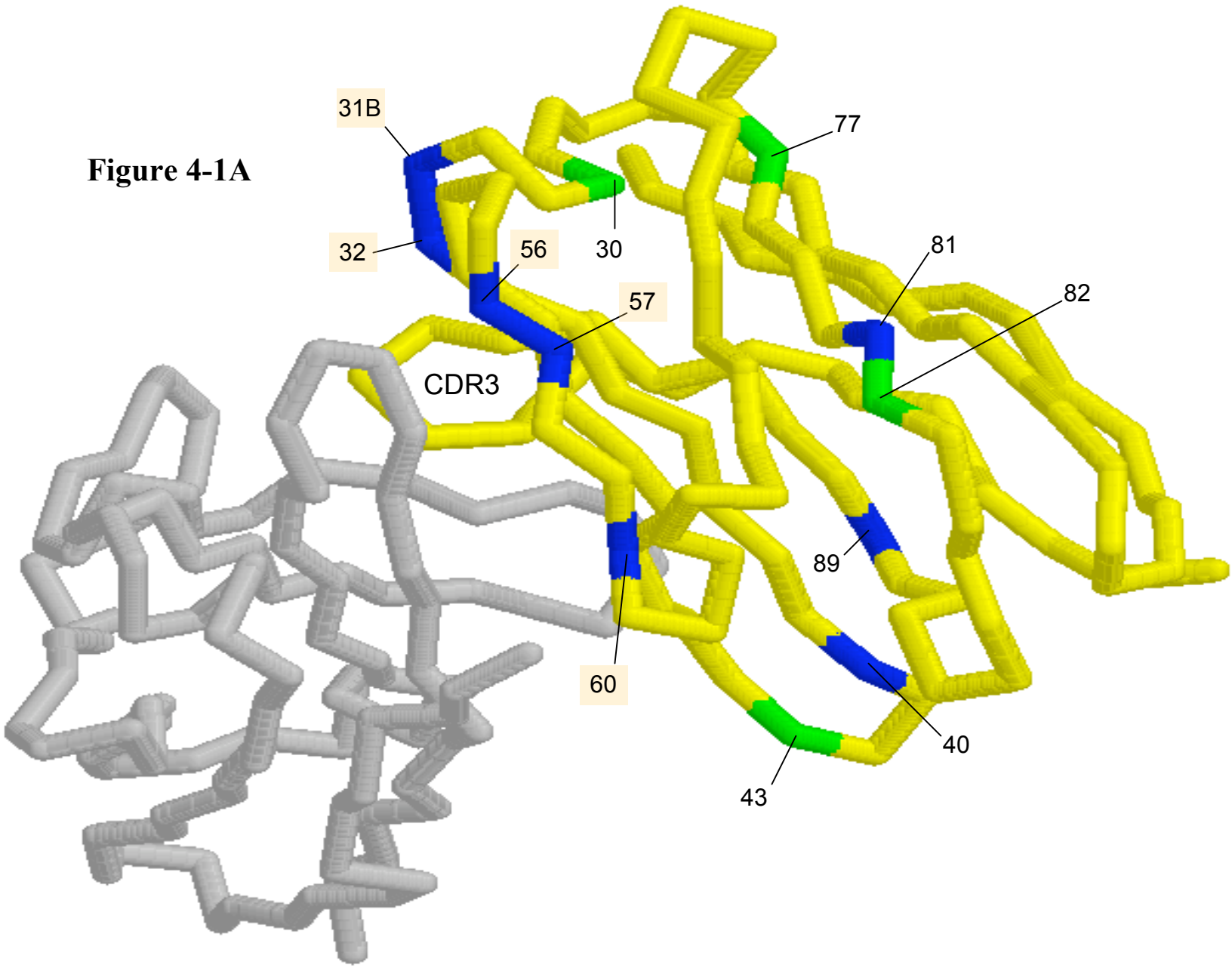


Figure 4-1B

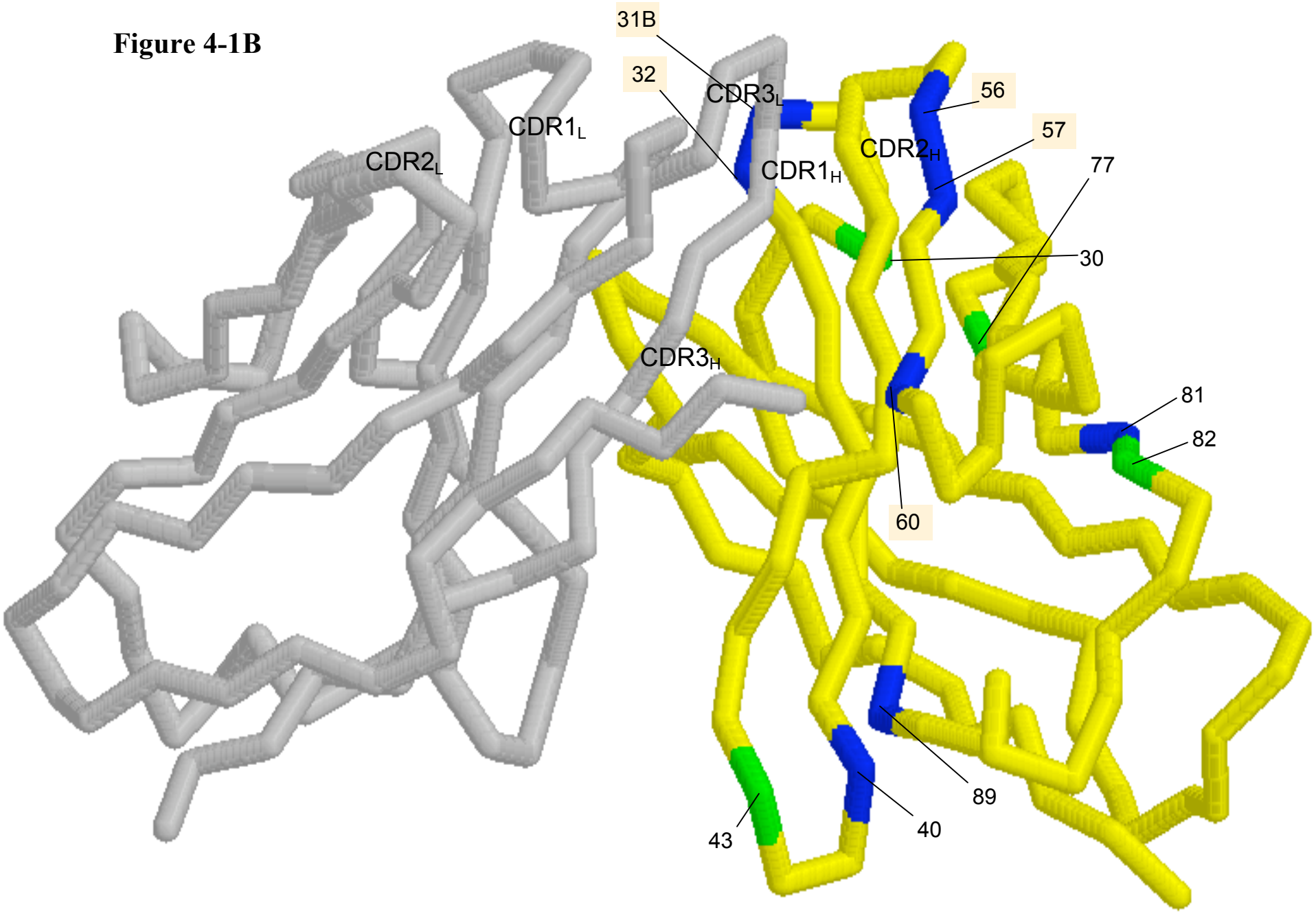

31B
32
CDR3L
56
CDR1L
CDR2H
57
CDR2L
CDR1H
77
30
CDR3H
81
82
60
89
40
43

Table 4-1: Signature hotspot codons and resultant amino acid changes					
Codon	Gene	Germline	Replacement AA	Percent of mutations with this replacement[1]	Total sample size
31B	4-30 & 4-31[2]	G	A	10	30
			D	63	
			R	17	
	4-39 & 4-61	S	G	19	54
			N	30	
			T	24	
32	4-04	N	D	40	5
			H	40	
			Y	20	
	Other VH4	Y	D	13	47
			H	40	
			S	21	
40	4-31	H	A	10	29
			L	17	
			P	38	
			S	10	
			Y	17	
	Other VH4	P	A	30	44
			S	53	
			T	11	
56	All VH4	S	G	10	132
			I	11	
			N	27	
			R	11	
			T	27	
57	All VH4	T	A	40	43
			P	19	
			S	33	
60	All VH4	N	K	25	53
			S	42	
			T	19	
81	All VH4	K	N	39	138
			R	37	
89	All VH4	V	I	49	57
			L	23	
			M	16	

Abbreviations: A=alanine, AA=amino acid, D=aspartic acid, E=glutamic acid, G=glycine, H=histidine, I=isoleucine, K=lysine, L=leucine, M=methionine, N=asparagine, P=proline, R=arginine, S=serine, T=threonine, V=valine, Y=tyrosine

[1]Not showing other alleles with only 1 mutation each

[2]Only showing replacements with more than 10% of the mutations causing this replacement

CHAPTER FIVE
Future Directions

The VH4 signature found in CDMS patients can be used to determine the CIS patients that would convert to CDMS in our patient cohort. In order to validate the use of the signature as a predictive tool, patients in the comparison group (CIS patients which obtain a diagnosis other than CDMS) need to have CSF single cell analysis performed at the mutational level. We performed a power analysis in which a conversion rate of 85% in the CIS group converting to CDMS was assumed, which approximated the actual conversion rate of 88% (15 of 17 patients including those that had been previously diagnosed with MS) and a conversion rate of 5% of the CIS group not converting to CDMS (though the sensitivity of the VH4 signature predicting CDMS conversion was 100%). For a statistical test at the 5% significance level, 9 patients are needed from each group for a 95% power. Thus seven additional patients that did not convert to CDMS and did not have the VH4 signature would be necessary to validate this tool. Alternatively, OND patients could be substituted or used in addition to non-converting CIS patients.

If we look at a worse case with only 60% of CIS patients with the signature converting and 5% CIS patients without the signature converting, then 19 patients in each group would be required. Since there are 14 in the signature group, this means that 5 additional CIS patients with signature will be needed that convert to CDMS, and 17 additional CIS patients that do not have signature (or alternatively OND patients) would need to have their signature assessed to achieve the same power at the 0.05 significance level.

Once confirmed in larger cohorts of RRMS, CIS, and OND patients, there are several potential directions for the VH4 signature project. First is to determine how early in the disease the VH4 signature can be detected, so at-risk populations (first degree relatives of CDMS patients) could be periodically monitored by bulk B cells using real-time PCR to examine VH4 signature prevalence in PB. Next is to determine the specifics of antigen binding, the antigen specificity of antibodies containing the VH4 signature mutations. This will be achieved by determining which codon positions and changes are necessary or sufficient to change the antibody affinity for important antigen(s) and to determine if a correlation exists between the codons in order to bind to the particular antigen. Third, there is a need to examine light chain usage, to determine if the MSCSF B cell repertoire has abnormalities in family frequencies or mutational patterns, and to determine if a signature exists in the light chains, especially those that pair with a VH4 heavy chain. Also, we could determine if the signature penetrance is similar or different in PPMS compared to RRMS, to determine if the signature could be used to predict the disease occurrence in these patients. Interestingly, one patient in this study (M484) is of the PPMS sub-type, and has a signature score within the threshold of MS patient signature scores (though the lowest score of the group of CDMS patients). Fifth, it would be of considerable interest to determine if the signature fluctuates over time, correlates with relapses, disappears upon conversion to SPMS if RRMS, or changes after treatment with disease modifying agent such as rituximab (Rituxan®). If so, the signature may provide a new measurement to identify patients that will have a higher probability of successful outcomes with a drug like rituximab (Rituxan®), or allow us to postulate that the success of rituximab could be caused by the loss of signature bearing B cells. If the

signature fluctuates over time, it might allow evidence into disease mechanisms, and allow us to identify which types of patients the signature would be most relevant. If the signature presence changes over the course of disease in regards to being proportional with relapses and remissions it might allow predictions of the relapses, or even predictions on relapse severity. This also relates to why determining if the signature changes when the patient converts to SPMS from RRMS could give us suspicion to changes in disease mechanisms and possibly to predict this progression of patient disease.

Lastly, the $CD19^+$ B cells need to be examined for a VH1-specific signature, as this family is also over-represented in the MSCSF $CD19^+$ B cells when compared to HCPB B cells (Figure 3-2 and Table 3-3). Interestingly, however, this family is not over-represented in the MSCSF $CD138^+$ plasma cells. Investigating why this dichotomy exists between $CD19^+$ CSF B cells and plasma cells, whether VH1 expressing plasma cells do not expand as efficiently in the CSF as the VH4 expressing plasma, or an unrecognized regulation is occurring in the VH1 expressing CSF B cells that is not occurring in the VH4 expressing CSF B cells. This regulation could be the VH1 expressing B cells acting as regulatory B cells, being selectively destroyed, or reacting more powerfully to a CSF agent like BDNF than the VH4-expressing B cells, and may justify investigation of reagents that are designed to suppress B cells using particular variable heavy chains, while leaving the rest of the B cell pool intact. Of course, since not all MS patients or CIS patients who converted to CDMS had evidence of VH4 bias, but did all have high antibody signature prevalence, it is also reasonable to suggest that signature enriched B cells may be more specific and effective targets for new immunotherapies in the future as well.

FIGURE LEGENDS

Figure 5-1. Frequency of MSCSF VH family usage. Values provided are percent of productive VH rearrangements of MSCSF. This included 373 $CD19^+$ sequences from 11 donors (M125 n=101, M199 n=19, M217 n=1, M354 n=6, M368 n=49, M376 n=8, M484 n=9, M522 n=71, M584 n=85, M875 n=21, and M887 n=3) and 150 $CD138^+$ sequences from 3 donors (MS2-19 n=21, MS2-24 n=66, MS3-7 n=63). Results were similar if MS2-19 and MS2-24 $CD19^+$ cells were added to the other MS patients (no $CD19^+$ cells recovered from MS3-7). Patient descriptions of MS2-19 and MS2-24 are in (Ritchie et al., 2004), of MS3-7 are in (Owens et al., 2007) and described briefly below. At time of lumbar puncture, patient MS2-19 was a 46-year-old female diagnosed with PPMS 3 years previous to lumbar puncture. Patient MS2-24 was a 39-year-old female diagnosed with SPMS and she had CDMS for 20 years. Patient MS3-7 was a 42-year-old female diagnosed with RRMS 4 years previous to lumbar puncture.

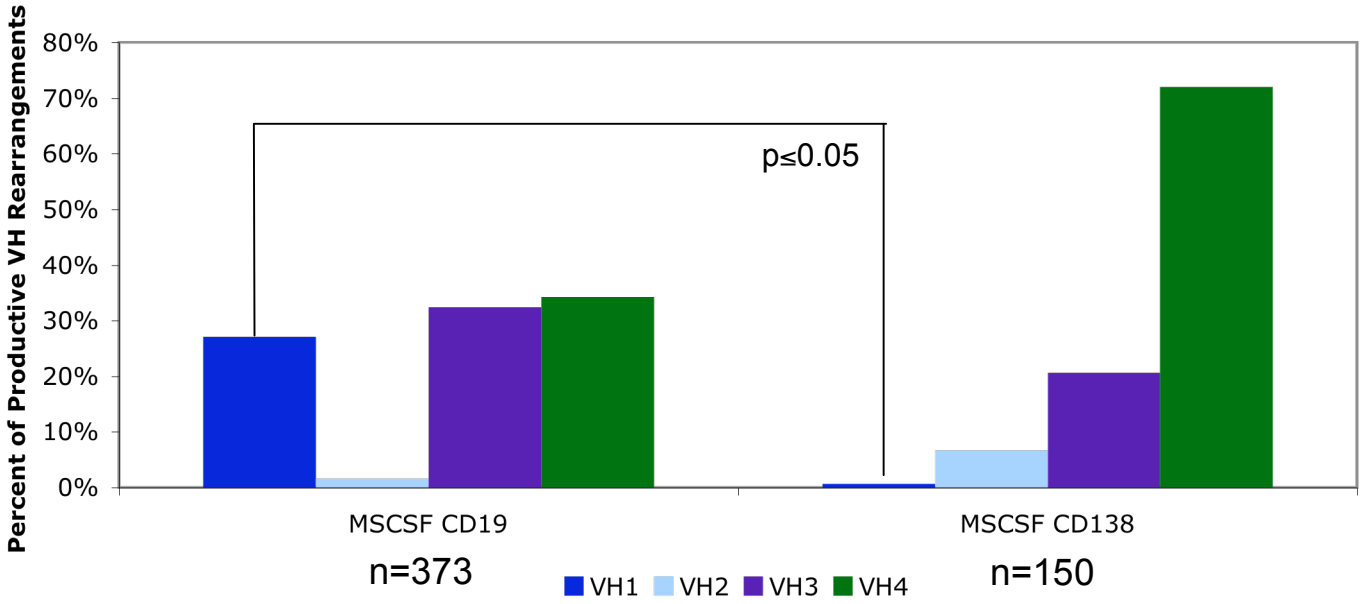

Percent of Productive VH Rearrangements
80%
70%
60%
50%
40%
30%
20%
10%
0%
p≤0.05
MSCSF CD19
n=373
MSCSF CD138
n=150
VH1
VH2
VH3
VH4

Figure 5-1

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