T Cell Repertoire and Autoimmunity in Multiple Sclerosis Patients in Sardinia

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Abstract

Multiple sclerosis (MS) is an autoimmune disease characterized by destruction of the myelin sheath and axon loss within the central nervous system. Sardinia is a high-risk area of MS(multiple sclerosis). A genetically homogeneous population resulting from geographical isolation may be a critical factor that contributes to the high prevalence of the MS in Sardinia. It has been found that specific HLA genotypes in the Sardinian population are highly correlated with MS. The mechanism of how HLA impacts the adaptive immune response and triggers autoimmunity in the MS patient remains unknown. The total clonotypes of TCRs in one individual at a given time point is known as the T-Cell Receptor repertoire. By using next-generation sequencing, we have identified the TCR repertoire of MS patients in Sardinia, Italy. We found that MS patients have different TCR repertoires compared to those of healthy controls. MS-associated CDR3 sequences have been screened out in this study. BMLF-1 associated CDR3 are screened out in the MS-associated CDR3, indicating a higher activity of EBV in MS patients. TRBV20.1$TRBJ2.1 and TRBV20.1$TRBJ1.5 and other V-J recombinations show significant increases in MS individuals. These results suggest that the TCR repertoire may be a promising diagnostic method for disease status in MS patients. V-J recombination may be a biomarker for MS, especially in Sardinian people.

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Abbreviations

APC	Antigen-presenting cell
BBB	Blood-brain barrier
CDR3	Complementarity determining region 3
CDR3-LD	Complementarity determining region 3 length distribution
CNS	Central Nervous System
CSF	Cerebrospinal fluid
DCs	Dendritic cells
EAE	Experimental autoimmune encephalomyelitis
EBNA	Epstein-Barr Virus nuclear antigen-1
EBV	Epstein-Barr Virus
FACS	Fluorescence-activated cell sorting
HA	Haemagglutinin
HSV	Human herpesvirus
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HSCs	Haematopoietic stem cells
IMGT	International ImMunoGeneTics
MAG	Myelin-associated glycoprotein
MAIT	Mucosal-associated invariant T cells
MBP	Myelin basic protein
MOG	Myelin oligodendrocyte glycoprotein
MR1	Major histocompatibility complex class I related protein I
MS	Multiple sclerosis
OND	Other neurological disorders
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PLP	Proteolipid protein
pMHC	Peptide/MHC
PPMS	Primary progressive Multiple sclerosis
RRMS	Relapsing-remitting Multiple sclerosis
RT-PCR	Reverse transcription polymerase chain reaction
TCR	T cell receptor
TCRVa	Alpha chain of T cell receptor
TCRVb	Beta chain of T cell receptor
Th1	T helper 1 cells
Th7	T helper 7 cells
Tregs	Regulatory T cells

Chapter I

Introduction

1.1 The Immune System

The immune system can be divided into two subsystems based on their immunological roles and functions, the innate immune system and the adaptive immune system. Innate immunity or non-specific immunity is the nonspecific recognition of microbiomes and foreign molecules in the host. Innate immunity rapidly recognizes and reacts with the infectious agent, and therefore serves as the frontier of the immune system. Another essential function of innate immunity is to present antigens to the adaptive immune system[1]. Adaptive immunity the capacity to recognize the small regions of an antigen with specificity. These small regions are known as epitopes.

The adaptive arm of the immune system adapts to the different antigen epitopes with the proliferation of clones of lymphocytes that distinctly recognize individual epitopes of the antigen. The antigen-specific clones of lymphocytes are known as“clonotypes” and produce antibodies and T effector cells that have different functions in combating infectious disease. Humoral immunity is the arm of the immune system that produces antibodies and involves T cells and B cells. “Cellular Immunity” is the arm of the immune system where antigen-specific T cells attack infectious agents and infected cells. The two arms of the adaptive immune system are composed of the lymphocytes subsets, T cells and B cells.

1.1.2 T cell immunity

1.1.2.2 T cell development in the thymus

Hematopoietic stem cells (HSC) in bone marrow (BM) are the precursors of myeloid and lymphoid lineages[2-5]. T lineage progenitors (ETPs)emigrate from the bone marrow and migrate to the thymus for further differentiation[6-9].

The T cell progenitors in the thymus cortex region lack expression of cell surface CD4 and CD8, as well as the T cell receptor(TCR), and are defined as the double-negative(DN) thymocytes. DN T cells undergo four stages of maturation, DN1-DN4 maturational stages. During the DN2-DN4 phase, thymocytes express pre-TCR without rearrangement[10]. The initial expression of pre-TCR promotes ND4 thymocyte proliferation and expression of CD4 as well as CD8.Double positive(CD4+CD8+cellspre-TCR,)thymocytes further rearrange TCR differentiate into TCR double positive cells[11, 12]. DP thymocytes migrate into the medulla region and interact with epithelial cells. Cortical epithelial cells highly express MHC class, I or class II molecules loaded with self-antigen. The committed CD8 +DP thymocytes show a proper affinity with MHC class I[13], TCR signaling further enhance the CD8 expression of the CD8 +DP thymocytes. Committed CD4 +DP thymocytes with in optimal affinity for MHC class II, interact with class II expressing epithelial cells and TCR signaling enhancesCD4 expression. Neither high affinity nor low-affinity of TCR-MHC Class interactions CD4+ or high affinity or low-affinity TCR-MHC class I interactions onCD8+wouldbe selected for in T cell-epithelial interactions. This process of CD4/CD8 and TCR selection is termed as the positive selection.

Before T cells can mature and emigrate to the peripheral lymphoid sites, committed thymocytes must interact with dendritic cells(DC) expressing high levels of MHC class I or class II molecules loaded with self-antigen. If the TCR on CD4+ or CD8+ T cells has a

high affinity for MHC-self antigen complexes, stimulated signaling will lead to cell apoptosis. Naive T cells with functional TCR and single CD8 or CD4 expression will not react with self-antigen and will migrate to the peripheral lymphoid sites.

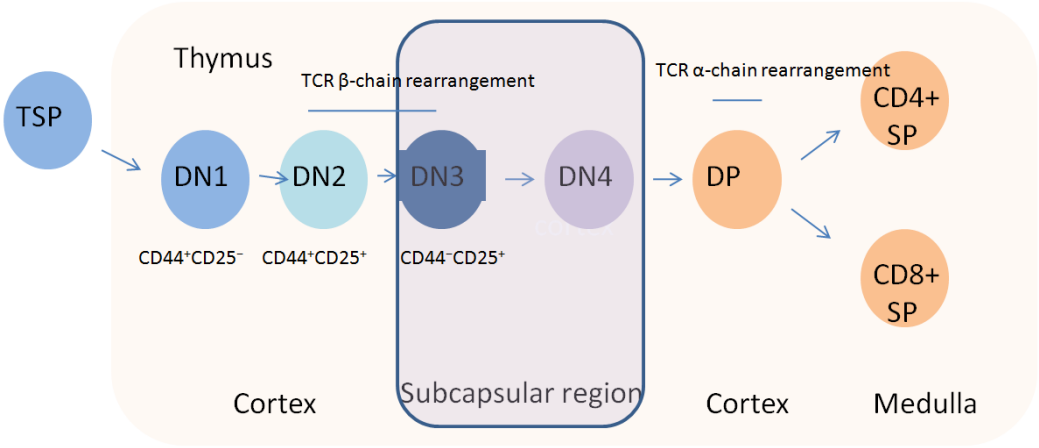

Figure 1.1. T cell development in the thymus[14]T cell development in the thymus

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The thymus-settling progenitors (TSP) migrate into the thymus from bone marrow (BM). As these progenitors differentiate through the CD4–CD8– double-negative (DN)stages they lack expression of TCR and they migrate from the cortico-medullary junction to the cortex. The process of thymocyte differentiation can be further subdivided into DN1-DN4 four stages. TCR β-chain rearrangement in the DN2-DN3 stage combines with the non-rearranging pre-Tα chain. TCR α chain is not rearranged until the DP stage and generate complete αβ TCR. Interaction with cortical epithelial cells expressing MHC class I and class II, theαβ-TCR+CD4+CD8+ DP thymocytes further differentiate into CD8+ T cells,orCD4+ T cells based on the signaling that is mediated by the interaction of the TCR with this self-peptide–MHC ligands. After differentiation they eventually leave the thymus.

1.1.2.3Tcellreceptor

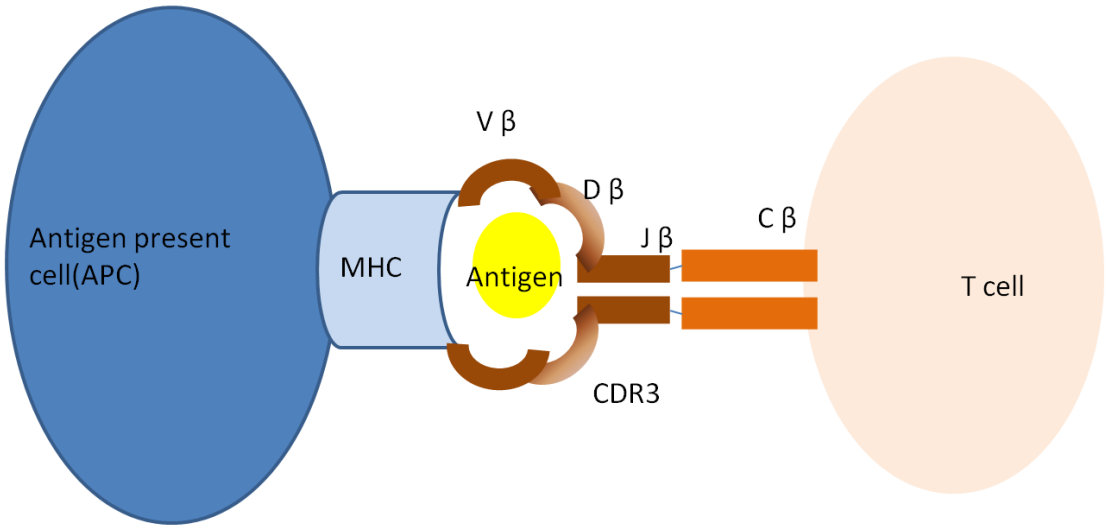

Figure 1.2. The T-cell receptor interacts with a peptide–MHC complex

Figure 1.2. The T-cell receptor interacts with a peptide–MHC complex

A functional αβT-cell receptor consisting of α- and β-subunit chains. Each subunit chain, the TCRβchain for example, consists of the variable (V), joining (J), diversity (D),and constant (C) region segments. The CDR3 region is the joint region of V,D, and J segments, which is crucial for antigen recognition. The TCR is present on the T-cell surface, binding with the antigen present with MHC molecule on the surface of an APC.

The antigen-specific nature of T lymphocytes is based on the surface expression of the T Cell Receptor (TCR). Expressed as a heterodimer, the TCR plays a crucial role in antigen-specific recognition. TCR is a cell surface heterodimer consisting of αβ chains or δγ chains [15, 16].The αβ T cell is the most common, consisting of about 90%-95%of the T cells in the total T cell population. Highly homologous with the Immunoglobulin (Ig)-

superfamily, TCR molecules have a variable region and conserved regions. The X-ray crystal structure of the αβ TCR-antigen-pMHC complex was first described in1996[17]. Hypervariable regions located in the variable region of the TCR have been found to bind to and recognize the antigen presented in the context of the major histocompatibility complex (MHC). This region is known as the complementarity-determining region(CDR)[18-21].Complementarity determining regions(CDR) include regions CDR1, CDR2, and CDR3. CDR1 and CDR2 are encoded by the V segment gene from the genome, whereas the CDR3 is combination of the V segment gene, D gene segment, and J gene segment. The CDR3 loop with high diversity undertakes the role for the antigen-specific recognition[22].

The investigation into the physical nature of CD3-antigen-MHC interactions demonstrated a two-step process of TCR MHC interactions. Initially, measurement of the affinity shows a low-affinity interaction of the complex, which may act as low sensitivity of the detector[23]. With dynamic observations of TCR-polymorphic MHC-antigen interactions using a BIAcore TM (Pharmacia Biosensor), Wu et al. [24] found two phases of binding and recognition. Initially, the TCR engages the MHC independent of a different antigen peptide. Later on, the TCR contacts the antigen peptide, and the MHC-antigen-TCR complex then facilitates intracellular signaling resulting in antigen-specific T cell activation.

1.1.2.3T cell receptor recombination

1.1.2.3.1 T cell receptor

As a member of the Ig-superfamily, the germline gene of the TCR gene is formed as a gene cluster into groups with different gene segments[25]. The human TRA(T cell

Receptor Alpha)gene and the TRD(T cell Receptor delta) gene are located on chromosome 14and comprise theTCRα/δlocus[26, 27]. TRA gene is organized in tandem-aligned pattern as Vα-Jα-Cα. The TRD gene is rearranged the same rule of TRA gene. The TRB(T cell Receptor Beta)locus located on chromosome 7 inhuman(7q34)encodes TCR βand is distinguished from the TRG(T cell Receptor Gamma) which is also located on chromosome 7[28].

Table 1.1. TCR gene location and classification

	Positions on chromosomes	V segments	D segments	J segments	C segments
TRA	*14q11.2*	*54*	*-61*	*61*	*1*
TRB	*7q34*	*64*	*2*	*14*	*2*
TRG	*7p14*	*8*	*0*	*2*	*2*
TRD	*14q11.2*	*3*	*3*	*3*	*1*

The TRA tandem-alignment pattern is arranged as Vα-Jα-Cα.TRB insert the D gene in the tandem-aligned pattern arranged asVβ-Dβ-Jβ-Cβ. Generally, TCR gene segments are flanked by short complementary palindromic sequences called recombination signal sequences (RSS). These signals are crucial elements for the somatic recombination of the TCR segments [29-31].Recombination signal sequences (RSS)are highly conserved 7mer(heptamer),typically CACTGTG, and 9mer(nonamer), typically GGTTTTTGT, joined by a 12/23 constant sequence length. TCRB, for instance, each has a V gene 3' flanking RSS(7mer-23- 9 mer), with each J gene 5' region starting with RSS(9mer-12-7mer), and the D gene arrangement has the RSS on both sides[29-31].

1.1.2.3.2.V(D)J recombination

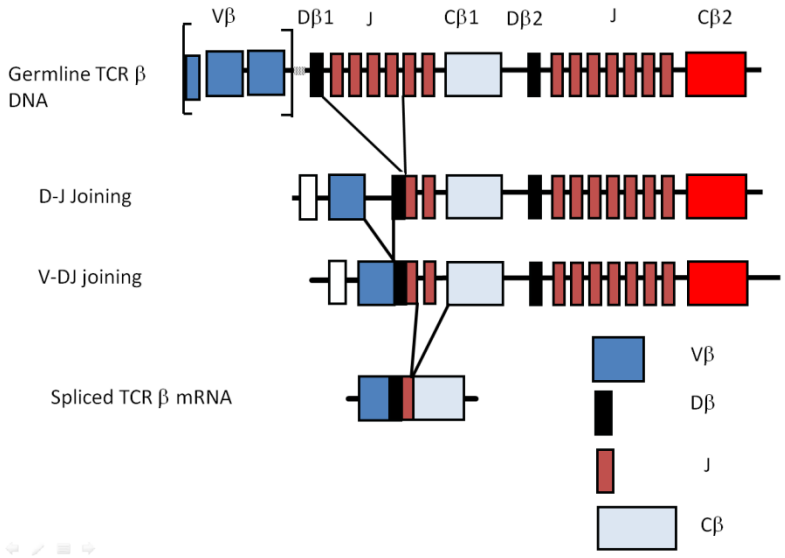

Figure 1.3. T cell receptor recombination

αβ functional TCRs are heterodimers consisting ofα- and β-subunit chains that are generated by germline DNA recombination of variable (V), diversity (D) and junctional (J) gene segments for the β-chain. In the human TCR loci, there are 64 Vβ, 2 Dβ, 14 Jβ, 54 Vα,and61 Jα functional gene segments. During T-cell differentiation, gene segments go through rearrangement and are further spliced with the constant region (C) to construct theαβ TCR,with each cell expressing a unique clonotype type of the recombined receptor.

In T cell development, as previously described, T cells express pre-TCR without rearrangement until the double positive(CD4+&CD8+) stage. Thymocytes at the double positive stage first begin recombination by rearrangement between the Dβ gene and Jβ gene[32-35]. The recombination is dependent on the 12-23 rule[36-38]: First,the RAG1(recombination activating gene-1) and RAG2 (recombination activating gene -2) protein complexes recognise the two RSSs (generally one RSS(7mer-23- 9 mer) and one RSS(9mer-12-7mer) of the Dβ gene and the Jβgene and bring these gene segments into close proximity to form a stable synaptic complex. The RAG complex mediates the pairing of double-strand DNA cleavage in the synaptic complex. Next the dissociated

blunt-end DNA are repaired by the PolX family proteins (TdT, pol μ, pol λ),and DNA-PKcs(DNA-dependent protein kinase catalytic subunit)and other proteins[39].There combined DJ gene continues to rearrange with the V segment gene with similar rules[40].

1.1.2.1. T cell subsets and functions and their association with the autoimmunity

T cell has been characterized into several subsets according to their different functions, surface phenotypes, transcription factors, and cytokine-secreting.The classification of the T cell subsets is still unfixed and plastic and can interconvert from one subset phenotype to another subset. Figure(1.4.)shows the most typical T cell subsets, including CD4+ original T helper cells and CD8+ T cells.

T helper type 1 (Th1) cells play an important role in adaptive immunity against intracellular viral and bacterial pathogens[41]. Th1 mainly produce IFN-γ; however, whether it plays a pathogenic or protective role in autoimmune disease remains controversial. It was first reported thatIFN-γ therapy for MS patients results in disease exacerbation[42].Subsequent studies, including human as well as animal models, further confirmed that the Th1 cells producing IFN-γare pathogenic[43-45]. This conclusion has also been challenged by some studies[46].T helper type 2 (Th2) cells are crucial for host defense against parasites and large extracellular pathogens and would induce allergic responses. For autoimmune disease,it has been found that Th2 plays a pathogenetic role which may be associated with B cell activation[47].

T helper type 17 (Th17) cells have been reported to be associated with autoimmune disease[48] and mucosal immunity[49, 50]. Th17 lineage is plastic would also be

interconverted into regulatory T (Treg) cells under TGF-βregulation[51].Th17 cells have been found to produce IL-17 in both mice and humans[49].Regulatory T (Treg) cells have been found to play an important role as immunoregulators in many autoimmune diseases such as MS[52], asthma[53],and type 1 diabetes [54].

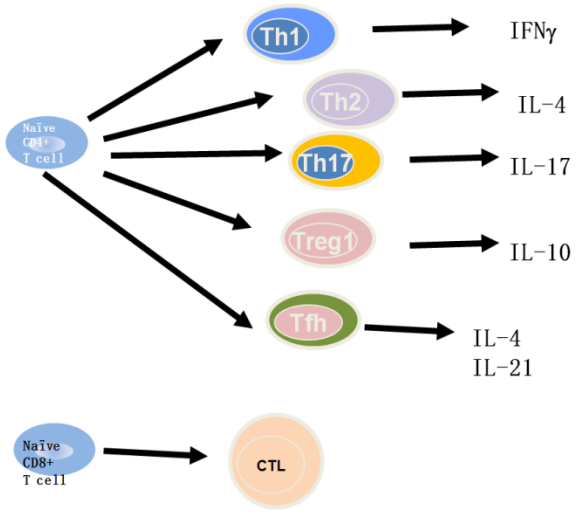

Figure 1.4. T cell subsets and their signature cytokine production

Different CD4+ subsets were generated from naive CD4+ T cells include Th1, Th2, Th17, Treg1,Tfh.

Apart from MHC-restricted T cells, some unconventionalαβT cells,which are not MHC-restricted T cells (figure 1.5), have also been identified [55]. The type most related to this study is the mucosal-associated invariant T lymphocyte (MAIT lymphocytes),which is an innate-like T cell with an invariant TCR α chain, highly conserved recombination with TCRA1-2 and TCRJ33 in humans [56]. MAIT has an antibiotic function would be activated by some bacterial metabolites

presented by the major classes of MHC Class I (MHC I)-Related Protein 1 (MR1). Pro-inflammation cytokines have also been reported as activators of the MAIT lymphocytes.

MAIT has been proved to be associated with autoimmune disease[57-60]; however, its association with multiple sclerosis remains controversial. MAIT-like T cells with the clonotype of Va7.2-Ja33 T cells were first be found accumulated in a lesion of MS patients [61]. It is essential to determine the role MAIT plays in the MS patient, whether it is just stand-by a role in the MS lesion or whether it protects the CNS from autoimmune injury, or even whether it is a critical cell type that trigger autoimmunity.

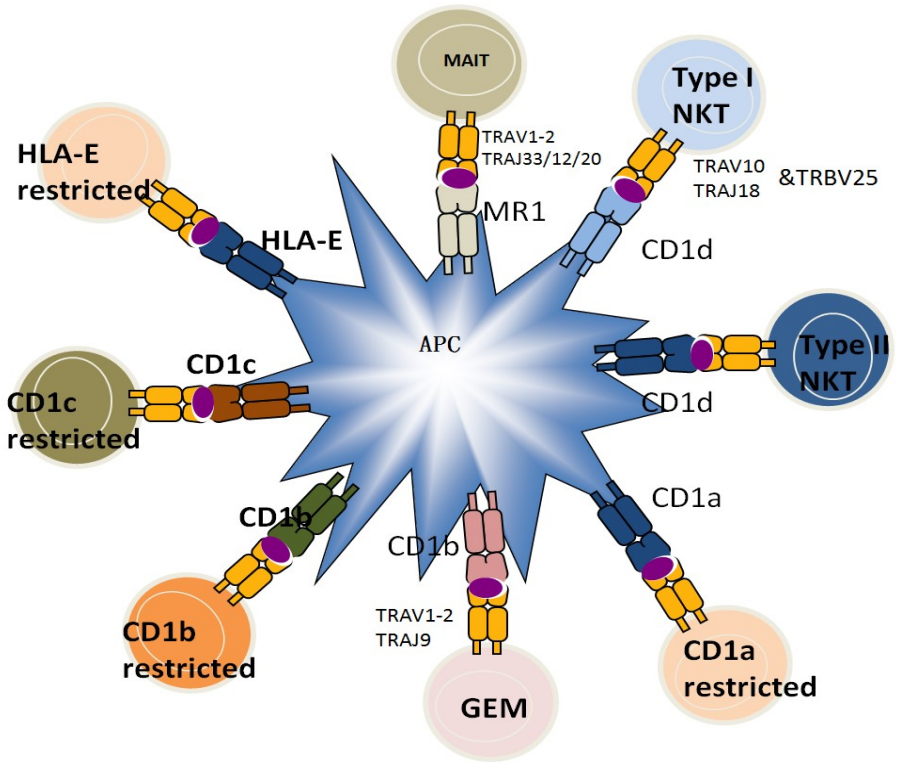

Figure 1.5. Overview of the unconventional αβ T cell family

Interactions of unconventional αβ T cells with their antigen targets via their TCRs in human cells

1.1.2.2.Cross-reactivity and autoimmunity

Natural selection theory postulated by Niels K. Jerne in1955put forward a hypothesis that various antibodies to antigens naturally exist [62]. Later on, Burnet further adapted the natural selection theory and proposed the clonal selection theory which is widely accepted as" One cell -one-clonotype–one-specificity"[63]. After the work of several generations of immunologists, the basic form of the adaptive immune the TCR-MHC complex is the key to specificity of recognition-self antigens as well as recognition of neoantigens and thereby protect the body from disease and autoimmunity. However,

pathogenic immune responses derived from the improper interaction of TCR-MHC, which mistakes self-antigens as pathogenic antigens, causes autoimmune disease.Theoretically, autoreactivity T cells should have been eliminated by clonal deletion or inactivation during the negative selection and positive selection during T cell development.

However, the above concept has been challenged by Don Masonwhoproposed that a high level of cross-reactivity and recognition of T cells and antigen is necessary for adaptive immune development and function [64]. Don Masonpredictsthe adaptive immune system needs 10^15 T cells to cover the different antigens, and this total amount of T cells is about 500kg in weight, which far exceeds normal human weight. It is impossible. So the natural selection theory needs further investigation to determine which cross-reactive combinations of TCR are actually in the adaptive immune system.T-cell cross-reactivity is defined as the ability of a given T-cell to be activated by two or more heterologous pMHCs. It has been found that memory HIV specific T cells were detected in the unexposed healthy population by Davis MM et al.[65]. Moreover, those HIV specific T cells exhibit cross-reactivity to other antigens.

1.1.3 Immune Repertoire Research the Past and Present and the Future

Originally, Southern blot and northern blot analysis were applied to immune repertoire research for the detection of TCR and PCR gene rearrangement patterns[66].CDR3 spectratyping, originally called Immunoscope, was carried out to renew the analysis of the CDR3 length distribution[67, 68]. It was found that normal CDR3 length followed

Gaussian-like distribution[69-71]. This distribution was found to be perturbed in unhealthy conditions including infectious disease, cancer, and autoimmunity[67, 69, 71-74].Multiplex genomic PCR combined with Sanger sequencing enriched the spectratyping analysis but was still unable to fully resolve the diversity of immune repertoire[75, 76]. Most studies on antigen-specific recognition of the immune cell receptor have been conducted at the protein level, such as immunoassays including ELISA and western blot to detect the specific antibody. Antigen-specific tetramers and the Vβ/Vα antibody combined with flow cytometry single-cell analysis has detected some antigen-specific CDR3s[77, 78]. Additionally, FACS and PANAMA-blot technology or antigen micro-array chips have also been applied in the identification of specific antigens for repertoire investigation[79].

The technical bottleneck of the immune repertoire studies is the vast diversity of the V gene and their massive recombination possibilities. With the development of next-generation sequencing(NGS) techniques, it is now possible to amplify nearly all TCR or BCRof an immune repertoire and obtain sequence data and analysis from millions of individual TCRs[79, 80]. Consequently, the tremendous amount of immune repertoire data requires sophisticated and automated bioinformatics analysis. Apart from the basic analysis of the immune repertoire, CDR3'antigen-specific mapping still requires additional analysis. VDJdb, are recently developed a database of TCR sequences with known antigen specificity[81], makes it possible to analyze TCR antigen specificity among the TCR repertoire. However, the annotated TCR is only the tip of the iceberg, as most of the TCR remains unknown. Determining the network of the TCR repertoire would be a solution to predict the function the unknown TCR. The immune repertoire

applied to the different disease models, including cancer and autoimmune diseases as well as vaccination studies, may provide new insight into the adaptive immune response in different conditions. For instance, a functional association of the different clonotypes of T cells associated with antigen-specific T cell clonotypes may help define the pathogenic mechanisms of autoimmune diseases.

1.2 Multiple sclerosis

1.2.1 Autoimmune Disease overview

Generally, autoimmune disease can be classified into organ-specific autoimmune disease and systemic autoimmune disease, based on the disease symptoms and pathology. Only one or a limited number of tissues and organs are affected in organ-specific autoimmune diseases, which may be caused by autoreactive T cells or antibodies to the tissue-specific antigens of the affected corresponding organ. The etiology of Hashimoto's Thyroiditis, for instance, appears to be related to auto-antibodies and auto-reactive cytotoxic T lymphocytes(CTLs) against thyroid-specific proteins [82]. Systemic autoimmune disease, on the other hand, involves multiple organs or tissues injured through autoimmune mechanisms. The clinical presentation is more diversified and complicated than tissue-specific autoimmunity. Systemic Lupus Erythrocytosis (SLE) involves autoimmune antibodies targeting same molecules, including single-stranded DNA, and results in autoimmunity involving multiple organs including kidney, brain, and connective tissue[83, 84].

Even though vast amounts of research has been conducted on autoimmune diseases and advances have been achieved, how the immunological mechanisms of autoimmune diseases contribute to disease progression is just beginning to be understood.

1.2.1.1. Molecular Mechanisms of Autoimmune Disease

Molecular mimicry is referred to as the pathogenic antigen sharing the similar epitope with the auto-antigen, which then causes the immune system to mistakes the self as non-self. Amino acid sequence homology comparison is the most common method used to analyze the pathogen antigen with the self-antigen.

Infectious pathogen epitope-mimicking autoimmune diseases have been summarized in table1.2.Theoretically, the high-affinity TCR-pMHC of the self-antigens depleted during cell development, so when healthy, the T cell is tolerant of the autoimmune disease target antigen. Somewhat paradoxically, the first encounter with the mimicking antigen of the pathogen should also be tolerant to the mimicking antigen, unless another mechanism is cooperating with the antigen mimicry to trigger the adaptive response.

1.2.1.1.1. Exposure to the Self-antigens in Immunologically Privilege Tissues

Some organs and tissues such as the central nervous system(CNS), the eye, the testis, and the pregnant uterus have natural anatomical barriers which lack immuno surveillance. So self-antigen from these sites are not recognized. These protected areas are often referred to as immune privileged sites [85, 86]. At times trauma or inflammation or surgery of these barriers allow the release of isolated self-antigen from these sites to the immune system and trigger adaptive immune responses against these self-antigens[87]. The immune privileged concept has been challenged in recent years. For

instance, it was reported that meningeal lymphatic vessels present in mouse brain demonstrate, for the first time, a functional lymphatic system in the central nervous system[88], which indicates that the CNS is not absolutely isolated from the immune system.

1.2.1.1.2. Auto-antigen Structure Alteration

Self-antigens may undergo modification by biological or chemical factors there by modifying self-antigens to undergo modifications to eventually become auto-antigen and cause an autoimmune response and disease[89, 90]. It has been reported that chlorination could modify self-antigens to generate auto-antigens and cause autoimmune disease[91]. It has been found that inSjögren' syndrome autoantigen alteration may be implicated in autoimmune injury[92].

1.2.1.1.3. Molecular Mimicry

Molecular mimicry is often referred to as antigens originating from an infectious pathogen sharing antigen epitopes with similar epitopes of a self-antigen. The immune system in reacting against the foreign infectious disease attacks the pathogenic antigens and mistakenly also reacts with the similar self-antigens. The self-antigens now become auto-antigens, and immune reactivity with the host organs and tissues results in autoimmune disease. Amino acid sequence homology comparison analysis is the most common method to determine if the infectious pathogenic antigen can elicit self-antigenic responses by the immune system [93, 94].

Some infectious pathogenic epitopes that mimic self-antigens and cause autoimmune disease are summarized in table1.2.Theoretically, the high-affinity TCR-pMHC presentation of the self-antigens depleted duringT cell development. Under normal health

conditions, T cells are tolerant to autoimmune target antigens that can cause disease. Molecular mimicry appears paradoxical; however, epitope spreading and low-affinity antigens may play a role in molecular mimicry and autoimmune disease.

1.2.1.1.4.Major Histocompatibility complex (MHC) associated with Autoimmune disease

Epidemiology studies have established that a strong link exists with specific major histocompatibility complexes (HLA or MHC)antigens and the likelihood to develop autoimmune disease. One HLA type DRB1*0402is found to have a pocket structure that carries a higher risk for autoimmune diseases such rheumatoid arthritis(RA)[95].Large cohort genome-wide association studies(GWAS)from differentgeographicregionsalsofoundthatdifferentautoimmunediseasesshowanHLA bias for specific autoimmune diseases [96].

1.2.1.1.5. Dysfunction of the Treg Cell.

Regulatory T (Treg) cells mediate homeostatic peripheral tolerance by suppressing auto-reactive T cells[97, 98].

Table 1.2. Autoimmune Disease and possible associated infectious diseases

	Disease	Self-antigen	Infectious agent implicated	Reference	Molecular mimicry	Reference
Organ-Specific Autoimmune Disease	Type 1 diabetes	Beta cell antigens				
	Rheumatic heart disease	Cardiac myosin			M protein	[99, 100]
	Rheumatoid arthritis	Connective tissue, IgG	Mycoplasma, EBV, MCV	[101-103]		[104]
	Rasmussen's encephalitis	CNS-Antiglutamate receptor (GLUR3)	Herpes Simplex Virus (HSV)	[105-107]	-	-
	Guillain-Barre syndrome	Gangliosides and peripheral nerve	Zika Virus	[108]	Campylobacter jeuni	[109, 110]
	Multiple sclerosis	CNS-MBP	Herpes Simplex Virus (HSV)			[111]
	Addison's disease	Cytochrome P-450 antigens				
	Hashimoto's thyroiditis	Thyroid cell antigens (e.g., thyroglobulin)	Hepatitis C Virus(HCV), HTLV-I	[112-114]	-	-
	Graves' disease	Thyroid-stimulating hormone receptor				
	Autoimmune hepatitis	Hepatocyte antigens (cytochrome P450 2D6)	Hepatitis C Virus	[115-117]	HCV	[118]
	Autoimmune hemolytic anemia	Red blood cell membrane proteins	Hepatitis C Virus,EBV	[119, 120]	-	-
	Idiopathic thrombocytopenic purpura	Platelet antigens (GP IIb/IIIa)	Hepatitis C Virus,EBV	[121, 122]	HIV Helicobacter pylori	[123, 124]
Systemic Autoimmune Disease	systemic lupus erythematosus	DNA, nuclear protein, RBC and platelet membranes	EBV	[125, 126]	-	-
	Sjogren's syndrome	Salivary gland, liver, kidney, thyroid	EBV,HCV	[127, 128]	-	-

1.2.2. Multiple sclerosis (MS)overviews

Multiple sclerosis (MS) is an autoimmune disease characterized by destruction of the myelin sheath and axon loss within the central nervous system[129-133].

1.2.2.1 Epidemiology of multiple sclerosis

More than 2.3 million people in the world population are suffering from MS according to the Atlas of Multiple Sclerosis 2013 report revealed by the Multiple Sclerosis International Federation (MSIF) and the World Health Organization (WHO) joint project[134].The global prevalence in 2013 was 33 per 100,000. The prevalence varies with the geographical region. Europe and North America have a high prevalence according to the Kurtzke classification[135] and show a correlation with latitude[136]. However, the high prevalence of MS in Sardinian does not fit the latitude gradient distribution[137-139]. In Asia, especially, the prevalence of MS is low, but the incidence is reported to be increasing in recent years (ref)[140, 141]. For gender distribution, females are more at risk to develop multiple sclerosis than males [134]. The average age of MS onset is 30 years[134].

1.2.2.2. Clinical Manifestations and Course of Multiple sclerosis.

The clinical manifestations of MS are variable according to the location of the lesson suffering in the brain[142-144]. Generally, the clinical course is classified into four types including primary progressive (PP), secondary progressive (SP),relapsing-remitting (RR), and progressive relapsing (PR)[142].Relapsing-remittingMS(RRMS), which is the most common form of MS, relapse after periods of months to years of relative remission with no new signs of disease activity. Secondary Progressive Multiple

Sclerosis(SPMS) usually initiates with the RRMS.SPMS may have no remission after a secondary attack and cause a high percentage of disability. Primary Progressive Multiple Sclerosis(PPMS) is characterized by accumulative nerve injury and by a lack of distinct MS attacks. Different from PPMS,Progressive relapsing Multiple Sclerosis(PPMS)is characterized by accumulative nerve injury with distinct MS attacks.

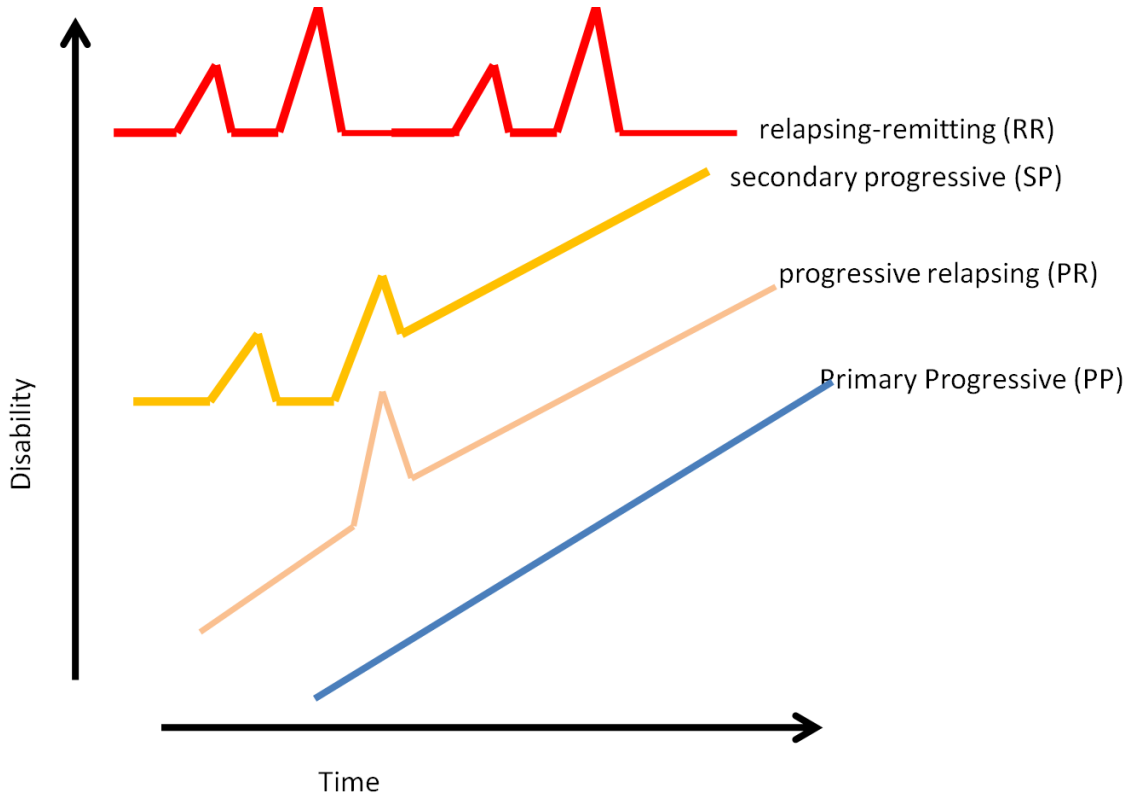

Figure 1.6. Clinical Course of Multiple Sclerosis [143]

MS can be classified into four clinical types, including primary progressive (PP), secondary progressive (SP), relapsing-remitting (RR), and progressive relapsing (PR)

1.2.2.3.Etiology of Multiple sclerosis.

The etiology of the MS is unknown, but genetic, infectious[145], environmental[146], and immunological factors may play roles in the possible causes and development of the disease[147-149].

1.2.2.3.1 The genetic variant associated with MS

HLA class II, as a key role in effector CD4+ T cell activation and function, has been extensively studied in different geographical regions using genome-wide association studies(GWAS). In Europe, HLA alleles includingDRB1*15: 01, DRB1*03:01 and DRB1*13:03are associated with MS[96](HLA alleles includingDRB1*15: 01, DRB1*03:01 and DRB1*13:03as the risk alleles with an average odds ratio (OR) more than 1). Interestingly, HLA-A*02:01shows a protective effect against the development ofMS.

1.2.2.3.2 Infectious Factors associated with Multiple sclerosis

The possible association of the development of MS after the infectious disease has been reported. Associated infectious microorganisms, include *Chlamydia pneumoniae*[150-154],HHV-6[155-158],EBV[159-165],Retroviruses[166-168], Coronaviruses[169, 170], and JC virus[171-173]. Direct virus isolation from the CSF or brain of the patients affected by the above-named infectious agents rarely reported, and most of the evidence for an association has been provided by serological research[157, 174, 175].

Molecular mimicry between the antigens expressed by microorganisms and self-antigens is a hypothesis that has been discussed for years. Sequence homology conserved

TCR recognizing motifs shared between viral peptides and self-antigens, and structural mimicry is known as the“molecular mimicry theory” [176]. MBP (85–99)-specific TCR and EBV peptide mimics have been reported in many studies[5, 7, 177].

1.2.2.4.Pathology of Multiple Sclerosis

1.2.2.4.1.CNS structure in homeostasis

The CNS is protected by the skull and the meninges anatomically. The meninges from inside out is comprised of pia mater, arachnoid mater, and vascularized dura mater[178].Cerebrospinal fluid(CSF) flows in the space between arachnid and pia mater and functions as a homeostatic factor for CNS function[178]. The blood-brain barrier(BBB) is another essential component that protects the CNS parenchyma from harmful substances such chemical compounds, peptides, and proteins, as well as microorganisms and some immune cells. It is widely accepted that the CNS is an immune-privileged organ, in part because of the BBB. During the homeostatic state, blood-borne immune cells circulate through the meningeal spaces and do not cross the blood-brain barrier (BBB). The microglia, considered to be CNS resident macrophages, take the responsibility of innate immune surveillance in the brain parenchyma[179].

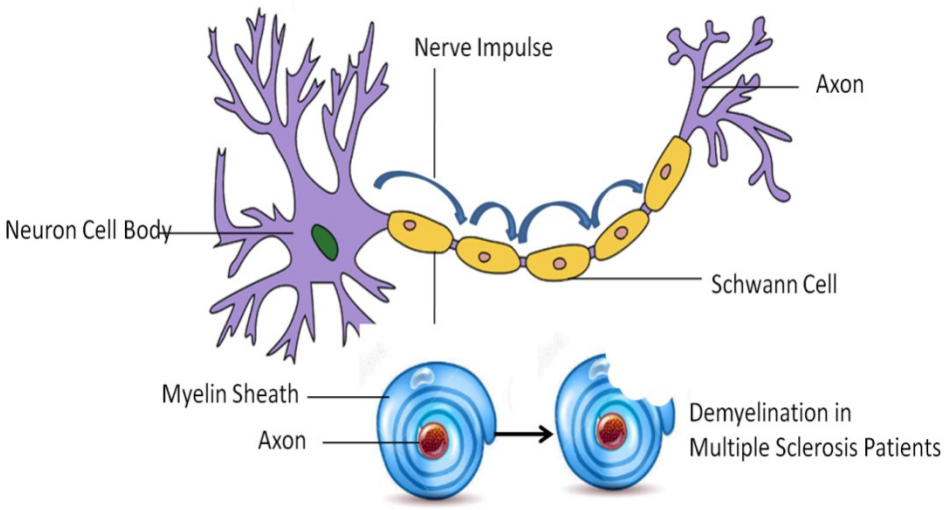

Figure 1.7. Myelin sheath on the healthy nerve of axon and Demyelination in MS.

The normal nerve conduction by saltatory conduction the propagation of action potentials through myelinated axons from one node of Ranvier to the next.Saltatory conduction is a very efficient way for the nerve conduction which is highly base on the healthy anatomical structure of the axon which is covered by a myelin sheath. In the pathological feature of MS are inflammation, demyelination which cause the block of the nerve conduction.

1.2.2.4.2.Pathology of Multiple Sclerosis

The pathological features of MS are inflammation, demyelination, and neuro-degeneration focally or diffusely throughout the white and grey matter in the brain and spinal cord, leading to remyelination and glial scar formation in the chronic disease process.

In the acute stage of MS lesions, the infiltration of lymphocytes along with the activation of microglial is found in perivascular and parenchymal areas[180].

In the classic MS lesion, demyelination plaques are the hallmark of all stages of MS patients. MOG-specific MBP-specific antibodies bound with myelin have been

detected in situ in the MS lesions, indicating the autoimmune response in MS pathology [181].

1.2.2.5. The immunological mechanism of Multiple Sclerosis (MS)

1.2.2.5.1.CD4+ T cells

The adaptive immune system plays central role in the immunopathogenesis of multiple sclerosis[182].Oligo clonal IgG bands (OCBs) detected in cerebrospinal fluid is a hallmark of multiple sclerosis(MS)[183-186] and indicate that plasma cells are responsible in part for the pathogenesis of MS. It is believed that the OCBs are products of the antigen-specific B cell clonal expansion[186]. However, recent studies using NGS of the BCR repertoire and using mass spectrometry revealed that the OCBs are partially heterogeneous auto-reactive antibodies and have an overlapping repertoire with that of peripheral blood cells [187]. The status of the TCR repertoire of T helper cells (Th), an indispensable part of T cell-dependent immunoglobulin production, is critical to the understanding of the role of Th cells in MS pathology. Naive CD4+ T cells recognize antigen presented by MHC class II on the antigen present cell(APC) and differentiate into several subsets of Th cells with different functions and phenotypes [188-190]. The mature Th cell further differentiates into Th1, Th2, and Th17 T helper cell subsets. These subsets are the basis of cytokine production and the phenotype of each cell type[188].Th2 cells are the major producers of IL-10 and may have a protective role in the development of autoimmune disease development[191, 192]. Th17 cells, the major producers of IL-17, recently have been demonstrated to be correlated with MS activation and relapse and are also highly correlated with other autoimmune diseases[193-195].

1.2.2.5.1.CD8+ T cells

CD4+ T cells were originally considered the main T cell subset for the induction of immunopathogenesis of MS[196]. However, recently, GWAS and histopathological studies have implicated that CD8 +T cells are critical in the pathogenesis of multiple sclerosis[143, 197]. Histopathological studies show cells (CTL) infiltrate multiple sclerosis plaques in the relapsing, persisting and the chronic stages of disease indicating thatCD8+ T cells may be directly involved in the immunopathogenesis of myelin sheath destruction[198, 199].In the mouse animal model, experimental autoimmune encephalomyelitis (EAE)studies demonstrated that-MOG specific CD8 T cells [200] and MBP specific CD8-T cells[201] induce autoimmune responses in CNS. Neuroantigen-specific T-cells have also been identified in the analysis of T cells in MS patients [72, 202-208]. These studies suggest T cells play a role in the autoimmune pathogenesis in the disease process. However, CFLs are not recruited, and clonal expansion does not take place in the CNS until inflammation occurs as the BBB(blood–brain barrier) restricts immune cell entry[209]. Due to the limited number of immune repertoire libraries and sequence data, further investigations are needed to identify the possible pathogenic T cell clonotypes involved in the destruction of the myelin sheaths and axons. Myelin basic protein (MBP)[210-213] and myelin oligodendrocyte glycoprotein (MOG)[214-217]and proteolipid protein (PLP)[213, 218, 219]from neurons have been widely studied as the relevant self-antigens in MS. However, the relationship between these proteins and T cell receptors and the etiology of multiple sclerosis still unknown.

It was long thought that the CNS lacked lymphatic vessels. However, the recent finding showing that meningeal lymphatic vessels are present in mouse brain

demonstrates for the first time a functional lymphatic system in the central nervous system[88]. Does it pose new questions for MS immunopathogenesis: Is the central nervous system under constant lymphocytic surveillance? If so, what are the regulatory mechanisms and regulatory cells? What happens during autoimmune disease to the regulatory mechanisms?

First of all, where is the site of pathogenic expansion for T cells and B cells during the development of MS? Does the peripheral blood and CSF share the clonotypes with the lesions of MS patients?

Secondly, what are the baselines of the autoreactive T cells and antibodies in the healthy population and the healthy state of the MS patient before disease onset?

Thirdly what is the initiating stimulant to the immune response causing the auto-reactive process in the CNS? What is the pathogen's epitope sharing similarity with the self-antigen, if molecular mimicry is the universal mechanism of autoimmune disease?

The last but the most interesting mystery is how the immune cells coordinate with each other and the different clonotypes and cause autoimmunity.

Sardinia is a high-risk area of MS(multiple sclerosis)[220-222]. The genetically homogeneous population resulting from geographical isolation may be a critical factor that contributes to the high prevalence of the MS in Sardinia.

Using next-generation sequencing, the TCR repertoire can be sequenced providing a snapshot and a general view of autoreactive T cells as well as possible virus-specific T cells and their role in the disease process. Here we sequenced the T cell repertoire of 13 cases of multiple sclerosis patients as well as 21 healthy volunteers. The TCR repertoires show differences between multiple sclerosis patients and healthy

controls. Multiple sclerosis patients have a higher repertoire of diversity than the healthy control patients.

1.3Specific aims of this thesis

I hypothesise that:

- Multiple Sclerosis patients have a unique TCR repertoire distinguished from the healthy population.
- Molecular mimicry and cross-reactivity of self-antigens and viral epitopes may be one the immunological mechanism of Multiple Sclerosis.

To address this hypothesis, we set the following aims for this project:

Analyze the TCRβ repertoire of multiple sclerosis patients and healthy controls.

Analyze the TCR α repertoire of multiple sclerosis patients and healthy controls.

Annotate the TCR repertoire and determine the possible association between the infectious agent-specific TCR with the auto-reactive TCR.

Chapter II

Materials& Methods

Table 2.1 Reagents

Reagents	Source	Catalog
DMSO	Sigma	D2650
RPMI-1640, sterile, L-glutamine and HEPES	Thermo Fisher	R7388
GE Healthcare Ficoll-Paque™ PLUS Media	Fisher Scientific	45-001-749
TRIzol™ Reagent	Thermo Fisher	15596018
Chloroform Solution	Sigma	613312-1EA
Isopropanol	Sangon Biotech	A507048
RNase-free water	Takara	9012
Fetal bovine serum (FBS)	Thermo Fisher	10099141
Trypan blue, 0.4% solution	Thermo Fisher	T10282
RNA zap	Thermo Fisher	AM9782
AMPure XP beads	Beckman	NC9933872
Qubit™ dsDNA HS Assay Kits	Thermo Fisher	MAN0002326
SMARTer® Human TCR a/b Profiling Kit	Clontech	635016

Table 2.2. Accessory Products and Instrumentations

Product	Source
BD Vacutainer™ CPT™ Tube with sodium heparin	BD Biosciences,
Disposable polystyrene serological pipette	Discovery Labware
Sterile polypropylene conical tube, 50 mL	Crystalgen
1.8 and/or 3.6 ml cryovial, Nunc or equivalenta	Nunc
Pipettes (0.5 – 10 µL,2 – 20 µL,10 – 100 µL,100 – 1,000 µL)	eppendorf
Biological safety cabinet	Eppendorf
Refrigerated centrifuge	Eppendorf
PCR Cyclers-Mastercycler® nexus	eppendorf
Agilent 2100 Bioanalyzer	Agilent
The Qubit Fluorometer	Thermo Fisher
PCR clean and sterile tips	Eppendorf

2.2.1. Peripheral blood mononuclear cells Isolation

4 ml of peripheral blood was collected from each participant with an EDTA tube. Centrifuge1000rmp for 15min at 4°C to separate the plasma from blood. Dilute remaining blood at the ratio1:2 with PBS/physiological saline solution. For instance,15ml blood + 35ml physiological saline solution. Layer up to 10 ml of the diluted sample over 5 ml ficolin a 15 ml tube(ratio 2:1).Centrifuge for 2200rmp for 30 minutes at 23°C. If blood is stored for more than 2 hours, increase centrifugation time to 30 min (as per gradient medium instructions).With the centrifuge brake off. Collect PMNC from the interface and transfer cells to a new 15 ml centrifuge tube. Wash PBMC with PBS by centrifugation at 1600rmp for 5 min at 23°C.Remove the supernatant into a new 15 ml tube for a further centrifuge. Resuspend the remaining cells with 15 ml PBS. Centrifugation at 1800rmp for 8 min at **23°C**. Remove the supernatant and pull out all the cell into one tube and add PBS/physiological saline solution at final volume 4ml. Dilute the cell solution with PBS in a 5 ml round-bottom test tubes at the ratio 1:10(20ul PBMC+ 180ul PBS/physiological saline solution)Counting the cell.Centrifuge the cell solution at 1800rmp for 8 min at **23°C**. Resuspend the remaining cells to 2 x 10^6 per ml in PBS

2.2.2. RNA extraction

- Add 750 ul of TRIzol® Reagent for each sample.Incubate in room temperature for 5 minutes. Add 200ul chloroform and shake the tube vigorously for **about 15**

sec. (at a ratio of 1 part chloroform to 5 parts TRIzol). Centrifuge the sample at 12,000 rpm for 15 minutes at 4°C. Place the aqueous phase into a new tube, Add 500ul of 100% isopropanol to the new tube Incubate at room temperature for 10 minutes. Centrifuge the sample at 12,000 rpm for 10 minutes at 4°C. Remove the supernatant from the tube.Wash the pellet, with one mL of 75% ethanol per 1 mL of TRIzol® Reagent used in the initial homogenization. Vortex the sample, then centrifuge the sample at 12000 rpm for 5 minutes at 4°C. Discard the wash. Dry the RNA pellet for 10 minutes. Resuspend the RNA pellet in RNase-free water. Incubate at 55–60°C for 10–15 minutes. Proceed to downstream applications, or store at –70°C.RNA concentration and purity were measured with Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA).

2.2.3.TCR library construction

SMARTer Human TCR a/b Profiling Kit was used for the TCR library construction. This kit is base on a 5' RACE-like approach to amplify complete V(D)J variable regions of TCR transcripts.

- First-strand cDNA synthesis

First-strand cDNA synthesis is performed by Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV)-derived SMARTScribe™ Reverse Transcriptase (RT) and dT-primed), which adds nucleotides upon reaching the 5' end of each mRNA template[223]. Non templated nucleotides anneals the 5' end of each mRNA template by the SMART-Seq® v4 Oligonucleotide and serves as a template for the reverse transcription into the

first-strand cDNA. This additional sequence, non templated nucleotides, —referred to as the "SMART sequence."The artificial knowing SMART sequence would serve as a primer-annealing site for subsequent rounds of PCR, ensuring TCR region from full-length cDNAs undergo amplification. The anneals SMART sequence overcome the bottleneck of the high diversity of TCR V segment gene.

- The First PCR Amplification

After reverse transcription-PCR, the first-strand cDNA as a template further amplify by SMART Primer 1 and reverse primer that is

complementary to the constant region. Both reverse primers may be included in a single reaction if analysis of both TCR subunit chains is desired. By priming from the SMART sequence and the constant(C)region, the first PCR specifically amplifies the entire V region and a considerable portion of the constant region of TCRα and TCR-β cDNA.

- The second PCR amplification

With the product of the first round PCR as a template, further, perform semi-nested PCR. The reverse primer is a portion of the constant region of TCR-α and TCR-β cDNA. Both forward primer and reverse primer contain the nucleotides of p5 and p7 which is the primer for Illumina sequencing. Following post-PCR purification, size selection, and quality assay, the library is ready for sequencing.

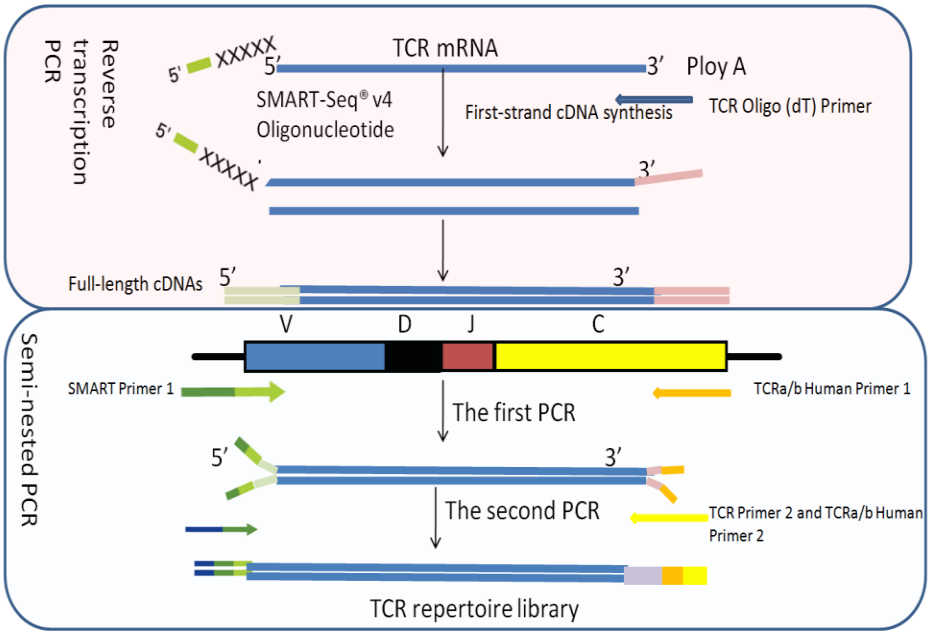

Figure 2.1. Workflow for SMARTer Human TCRa/b Profiling

2.2.4.The Quality Control of The NGS Library of TCR Immune Repertoire

Before the library sequencing on Illumina platforms. The library validation is necessary to have an idea of the library quality. The thefollow item should be taken into consideration.

①Mass Concentration Quantification：■Agilent 2100; ■Qubit quantitation assay; ■ Nanodrop

②Library FragmentValidation：■Agilent 2100

③Molar Concentration Quantification：■Q-PCR

Library Validation:

①Concentration Quantificationmeet the request concentration >1nM at least 50 ul on Miseq platform for BGI.

②TCR-α or TCR-β sequence fragments yield electropherogram maxima that tend toward the upper and lower ends of the 700–800 bp size spectrum.

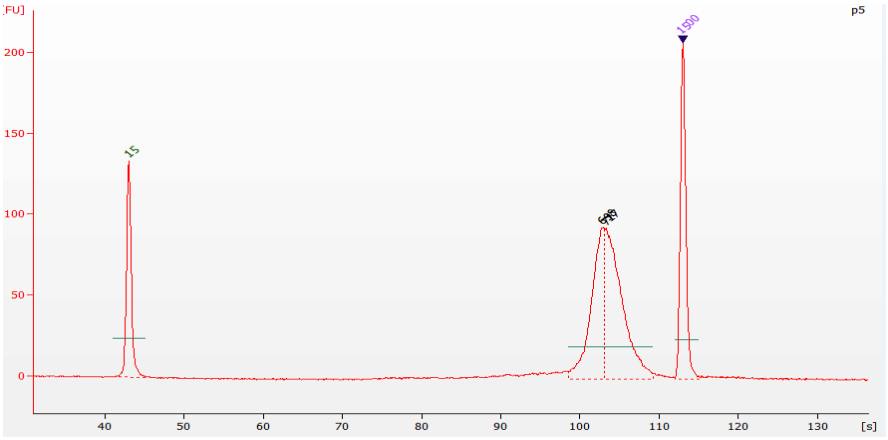

Figure 2.2. Example electropherogram results of the TCR repertoire library.

TCR-α or TCR-β sequence fragments yield electropherogram maxima that tend toward the upper and lower ends of the 700–800 bp size spectrum.

The final TCR library was shipped to NGS company BGI China (BGI diagnostic Laboratories, Shenzhen, China) perform. The library was sequencing on the IIlluminaMiSeq® sequencer using the 600-cycle MiSeq Reagent Kit v3 (Illumina, Cat. No. MS-102-3003) with paired-end, 2 x 300 base pair reads.

2.2.5.Next-generation sequencing data analysis

A first initial quality control assay was used FastQC. Adapter and quality trimming as well as pairing Fastq-join were performed by ea-utils. The joined fastq rerun the fastQC for the quality control. TCR alignment and αTCR/ βTCR clonotypes exported

by MIXCR for downstream analysis. VDJtoolswas applied for the analysis the data post-MIXCR for comparative post-analysis of TCR repertoires include basic statistical analysis, diversity estimation and repertoire overlap analysis.

Chapter III.

T Cell Receptor β Repertoire of Sardinian Multiple Sclerosis Patients

Results

To investigate the T Cell Receptor β Repertoire of individuals diagnosed with Multiple Sclerosis (MS), we sequenced the T Cell Receptor β(TCRβ) repertoire from peripheral blood obtained from MS patients and control individuals of Sardinian origin. The objective of this portion of the study was to identify the possible clonotypes associated with Multiple Sclerosis disease. Furthermore, if functional clonotypes were revealed we wanted to determine if any clonotypes were associated with recognition of antigens of infectious disease origin.

2.1. Basic analysis of TCR-β repertoires

TCR-β repertoires were sequenced and analysis performed from individuals with multiple sclerosis and healthy volunteers. We were able to obtain functional TCR-β CDR3 with an average of 95% (CI11507± 3612 N=13) for the MS group and an average of 95% (CI 19604 ± 3534 N=21) for the control group. To exclude cross-sample contamination, the data was processed with a decontamination filter. Differences in TCRBV CDR3 length (Fig. 3.1A) (P= 0.3837) was not observed between the MS patients (43.62 ± 0.06532 N=13) and healthy controls(43.38 ± 0.2095 N=21).Mean some nucleotides that lie between V and J segment sequences in CDR3 (Fig. 3.1B) was not significantly different between multiple sclerosis (12.65 ± 0.07071 N=13) and healthy volunteers (12.52 ± 0.2158 N=21). Mean some inserted random nucleotides in CDR3 sequence(Fig. 3.1C) was not significantly different between multiple sclerosis (4.731 ± 0.03622 N=13) and healthy volunteers (4.713 ± 0.1411 N=21). Convergence of mean number of unique CDR3 nucleotide sequences(Fig.3. 1D) that code for the same CDR3

amino acid is also similar(P=0.2645) between multiple sclerosis (1.007 ± 0.001305 N=13) and healthy controls (1.008 ± 0.001069 N=21).

Table 3.1. Basic information of Multiple Sclerosis patients.

.

ID	HLA	Gender	Age(years)	Period Between initial Onset (years)	Relapses
C1	HLA-DRB1*0301-	F	54	25	2015/12/1
C2	HLA-DRB1*0301-	F	47	13	NO
C3	HLA-DRB1*0301-	F	48	16	NO
C6	HLA-DRB1*0301-	F	43	11	NO
C7	HLA-DRB1*0301-	F	34	10	NO
C12*	HLA-DRB1*0301-	F	26	7	NO
C5*	HLA-DRB1*0301-	M	44	10	NO
C4*	HLA-DRB1*0405-	F	43	9	NO
C8	HLA-DRB1*0405-	F	47	22	NO
C9	HLA-DRB1*0405-	F	62	12	NO
C10	HLA-DRB1*0405-	M	66	50	2016/4/1
C11	HLA-DRB1*0405-	F	42	10	2015/11/1
C13*	HLA-DRB1*0405-	F	47	21	NO

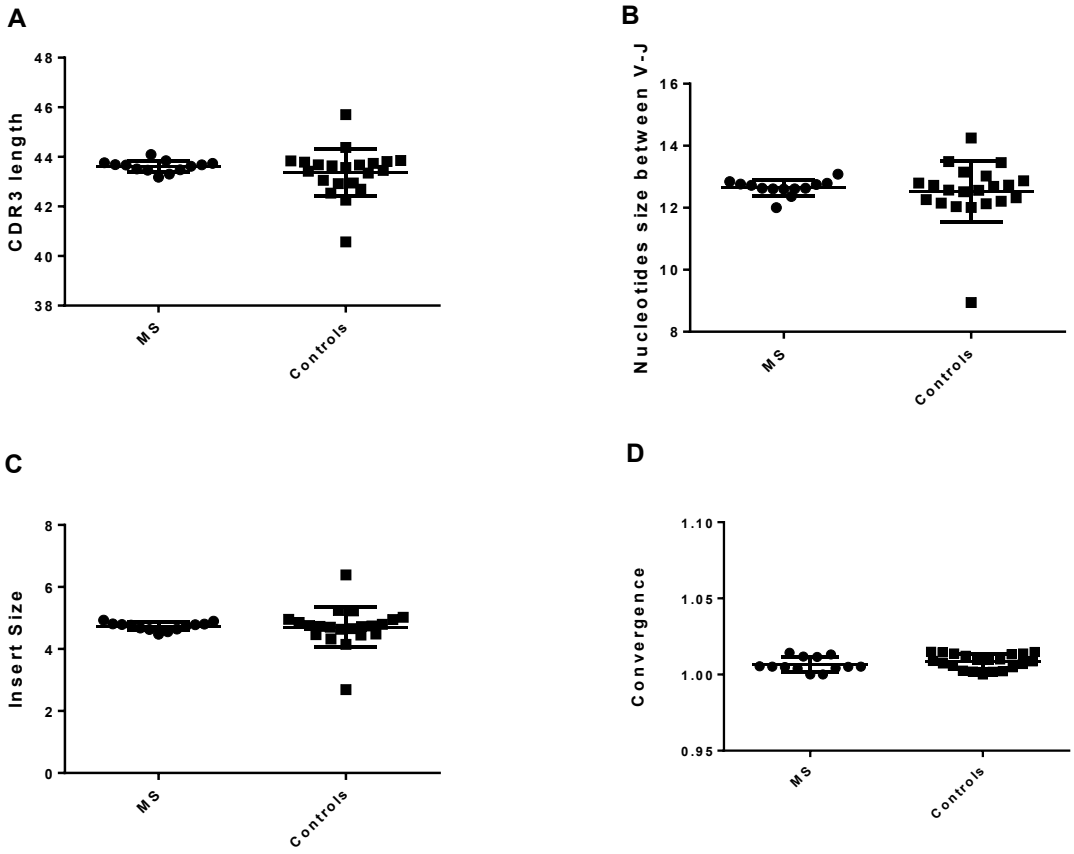

Figure3. 1. **A.** Mean length of CDR3 nucleotide sequence weighted by clonotype frequency. **C.** Mean number of inserted random nucleotides in the CDR3 sequence. **B.** Characterizes V-J insert for receptor chains without D segment, or a sum of V-D and D-J insert. **D** convergence means a number of unique CDR3 nucleotide sequences that code for the same CDR3 amino acid sequence.

2.2. Diversity estimation of TCR-β repertoires

We next compared the diversity by different indexes. Lower bound total diversity estimates (LBTE) include chao1(Fig 3. 2A) (p=0.5952) efron Thisted (Fig 3.2B) (p=0.5044) analysis which showed no difference between the MS patients with the healthy controls. The Shannon Weiner Index(Fig. 3.2C) (p=0.3700) and chaoE(Fig.3.2F) (p=0.3124) also agree with the LBTE results. However, whether diversity indices were used we obtained a different result: the normalized Shannon Weiner Index(Fig.3.2D)(p=0.0074)and Inverse Simpson index (Fig. 3.2E) showed multiple sclerosis patients have higher diversity than healthy controls. All sample were re-sampled and normalized to exclude a sampling depth effect. As chaoE is recommended to compared between samples, so we could generally consider MS, and the healthy controls are similar in total diversity estimation, but MS patient repertoires have higher entropy when compared with healthy controls. The amount of a difference in the clonotypes, which may contribute to the entropy of the repertoire diversity, between the two group of the TCRβ repertoire was the next question we analyzed.

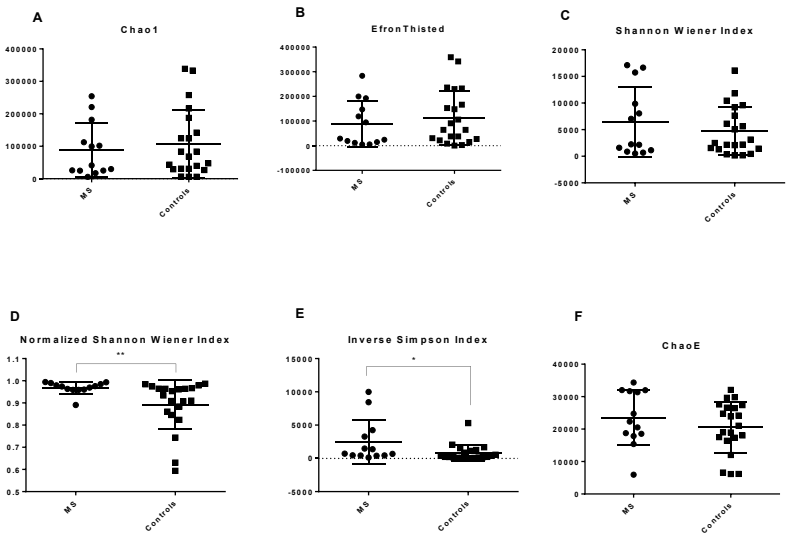

Figure 3.2. Comparison of TCRβ repertoire diversity index between MS and controls. (**A**) Chao estimate (denoted chao1) (**B**) Efron-Thisted estimate (**C**) Shannon-Wiener index (**D**)Normalized Shannon Weiner Index (**E**)Inverse Simpson index(**F**) chaos.

2.3.Clustering and overlap of TCR-β repertoires

We next used cluster analysis to investigate the overlap of the CDR3 amino acid sequences in each sample. After Hierarchical clustering analysis, the dendrogram (Fig.3.3A) shows that the MS group forms a distinct cluster. MDS (Multi-dimensional scaling) (Fig.3B) also verified that MS patients form a cluster distinguished from the healthy controls. Permutation testing (Fig.3.3C) based on MDS of the CDR3 shows that the TCR-β repertoires of the MS group show a significant difference from the healthy controls. We did not find an obvious diffusion in the MS group of functional CDR3 as has been recently reported[224]. However, in our study, we found a difference in the MS group of all clonotypes including the out-frame and in-frame clonotypes.

From this, we conclude that MS patients have a different TCR-β repertoire when compared with healthy controls. Our next step was to determine what are the unique clonotypes of the MS patients. Furthermore, are these clonotypes responsible for the pathological autoreactive responses of Multiple Sclerosis?

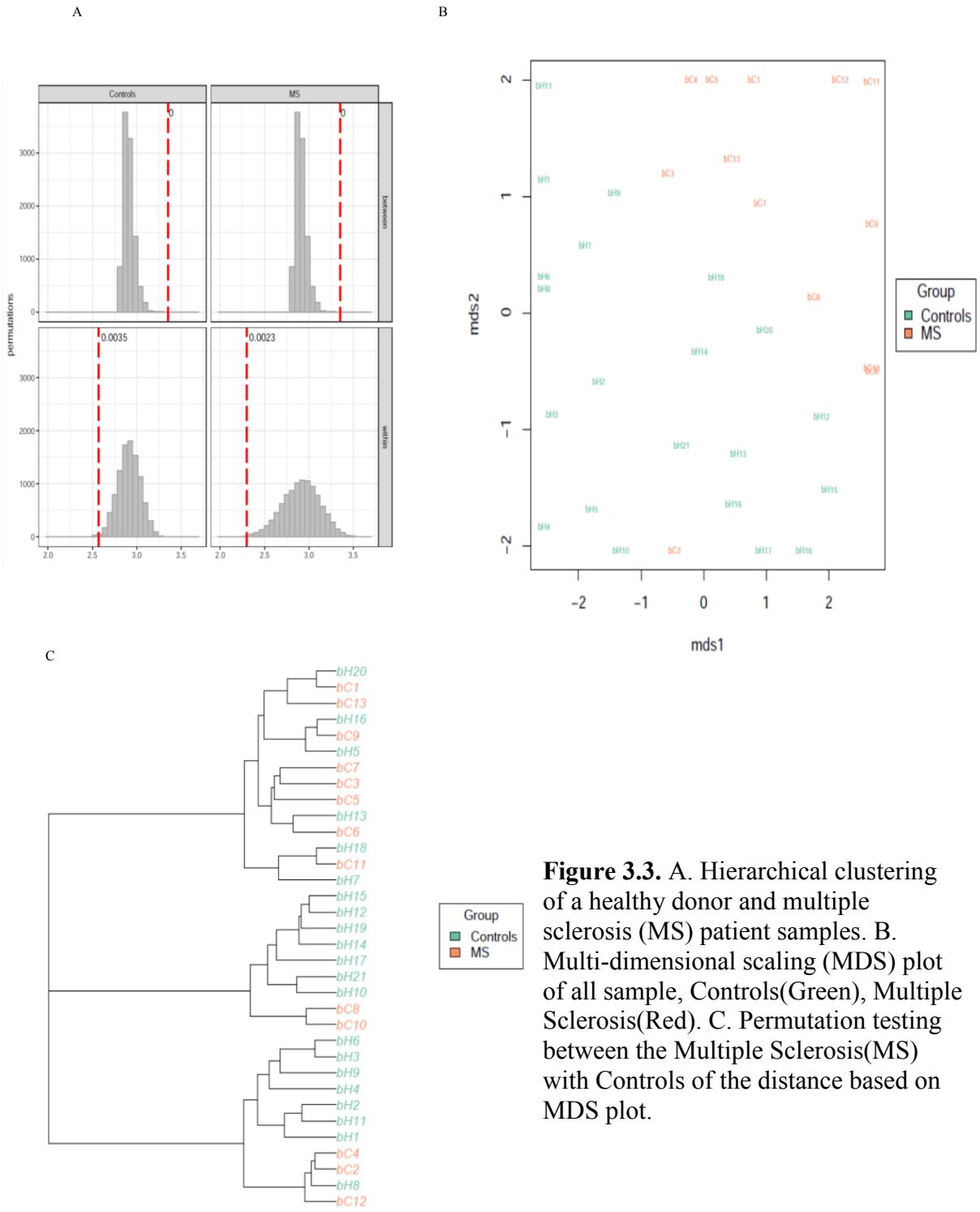

Figure 3.3. A. Hierarchical clustering of a healthy donor and multiple sclerosis (MS) patient samples. B. Multi-dimensional scaling (MDS) plot of all sample, Controls(Green), Multiple Sclerosis(Red). C. Permutation testing between the Multiple Sclerosis(MS) with Controls of the distance based on MDS plot.

4.4. The Variable (V) and Joining (J) segment usage

Previous work has been performed on the TRBV gene usage bias in MS patients [224, 225].V/J gene usage profile analysis by hierarchical clustering based on Euclidean distance was also performed on our data to determine TCRβ segment usage in our MS patients and healthy controls. The heatmap of the V/J gene segment usage (Fig.3.4) did not show any obvious clustering differences between MS patients and the healthy controls. For the V gene segment, a cluster of the (C3,C6,C2,C1,C9,C4), and J gene (C4,C1,C2,C13,C10) was observed (Fig.3.4). We next examined each gene usage and determined and performed statistical analysis. TRBV23.1 and TRBV3.1 have a higher statistically significant frequency in healthy controls. Table 3.2 shows the list of the important V gene usage in the MS patients and healthy controls. The previously reported MS associated variable genes including TRBV5-6 (P =0.98) and TRBV5-1 (P=0.4811) [224]has the same level frequency in MS group and healthy controls in our study. The TRBV20-1(p-value=0.0792) previously reported has a higher frequency in multiple sclerosis groups. This gene has also been reported in the Sardinian population, but the null allele did not influence the risk of multiple sclerosis, however, more data sets are needed to determine if statistical differences can be achieved. For the TRBJ gene usage,TRBJ1.5(p-value=0.004061) and TRBJ1.4 (p-value=0.03988) have a higher frequency of usage in the MS group.

Although the TRBV20-1 gene usage was not significantly different between the MS and the healthy controls, we did, however, find that the Variable-Joining segment rearrangement TRBV20.1$TRBJ2.1(p=0.01223) and TRBV20.1$TRBJ1.5(p=0.04875) (Table.3.4) show a significant increase the MS group. Interestingly, this rearrangement pattern frequency is more than 1%.

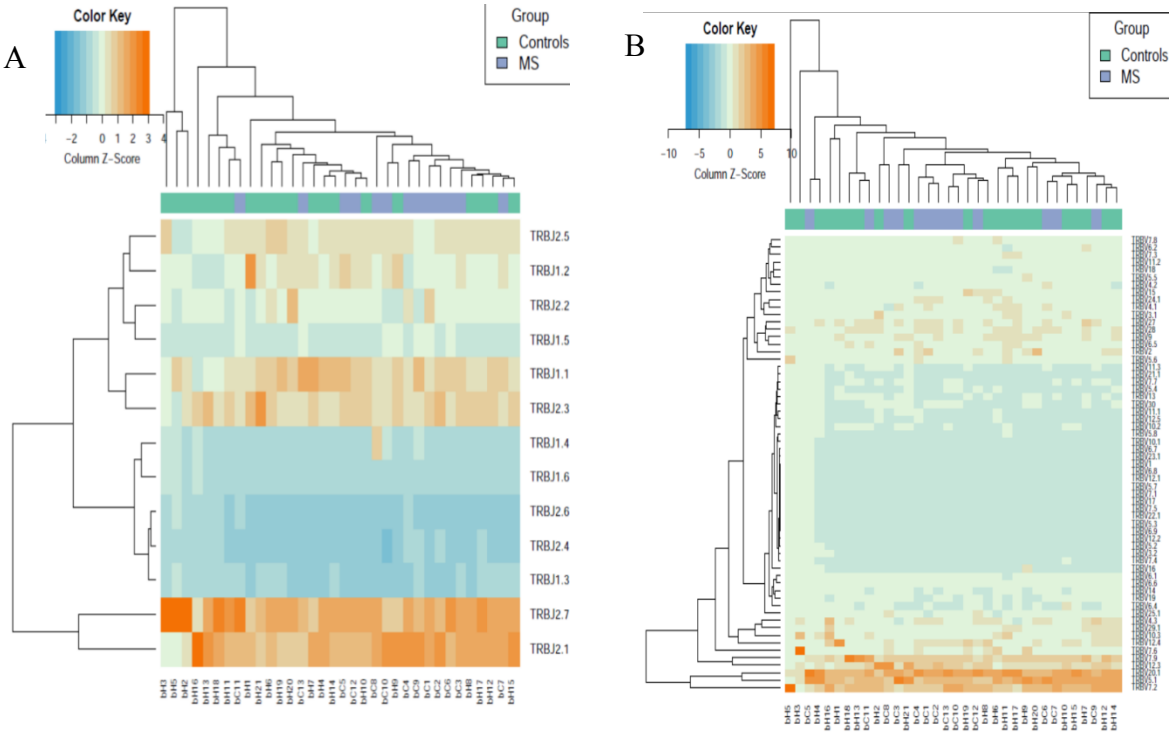

Figure3. 4. Hierarchical clustering of samples based on the Euclidean distance

A. J gene segment usage of TCR β chain. B. V segment gene usage of TCR β chain.

Table 3.2. V gene usage in MS patients and Healthy controls

V gene	Controls	MS	p-value
TRBV23.1	0.000333	0.000101	0.03571
TRBV21.1	0.002154	0.003022	0.05548
TRBV6.4	0.006353	0.003473	0.05544
TRBV5.6	0.019996	0.019841	0.98
TRBV3.1	0.026739	0.016178	0.002162
TRBV5.1	0.098168	0.10764	0.4811
TRBV20.1	0.104435	0.123383	0.0792

Table2. 3. J gene usage in MS patients and Healthy controls

J gene	Controls	MS	p-value
TRBJ1.4	0.027317	0.043298	0.03988
TRBJ1.5	0.055716	0.06732	0.004061

Table 2.4. V-J junction of MS patients and Healthy controls

V-J junction	Controls	MS	p-value
TRBV19$TRBJ1.3	5.3151E-05	0.00026	0.03939
TRBV21.1$TRBJ2.5	0.00011923	0.00048	0.03879
TRBV5.6$TRBJ1.5	0.00087559	0.00295	0.04661
TRBV5.5$TRBJ1.4	0.0003248913 0.0008485224	0.00085	0.01746
TRBV12.4$TRBJ2.4	0.00024834	0.00061	0.0412
TRBV4.3$TRBJ1.3	0.00041331	0.00096	0.00727
TRBV5.4$TRBJ2.1	0.00034233	0.00072	0.02828
TRBV5.6$TRBJ2.3	0.00102444	0.00211	0.00232
TRBV12.4$TRBJ2.3	0.00154772	0.0029	0.00326
TRBV7.6$TRBJ2.3	0.00139905	0.00253	0.01444
TRBV15$TRBJ1.2	0.00086822	0.00152	0.03137
TRBV4.1$TRBJ1.1	0.00169047	0.00287	0.03532
TRBV12.4$TRBJ1.1	0.00233696	0.00394	0.03131
TRBV4.3$TRBJ1.1	0.00166354	0.00274	0.0108
TRBV19$TRBJ2.7	0.00061349	0.00099	0.0323
TRBV12.4$TRBJ2.2	0.00158877	0.00257	0.02417
TRBV5.6$TRBJ2.1	0.00199412	0.00322	0.00034
TRBV6.6$TRBJ2.5	0.0004826	0.00077	0.01659
TRBV2$TRBJ2.7	0.0025739	0.00377	0.01242
TRBV20.1$TRBJ2.1	0.01718289	0.02225	0.01223
TRBV20.1$TRBJ1.5	0.00808951	0.01031	0.04875

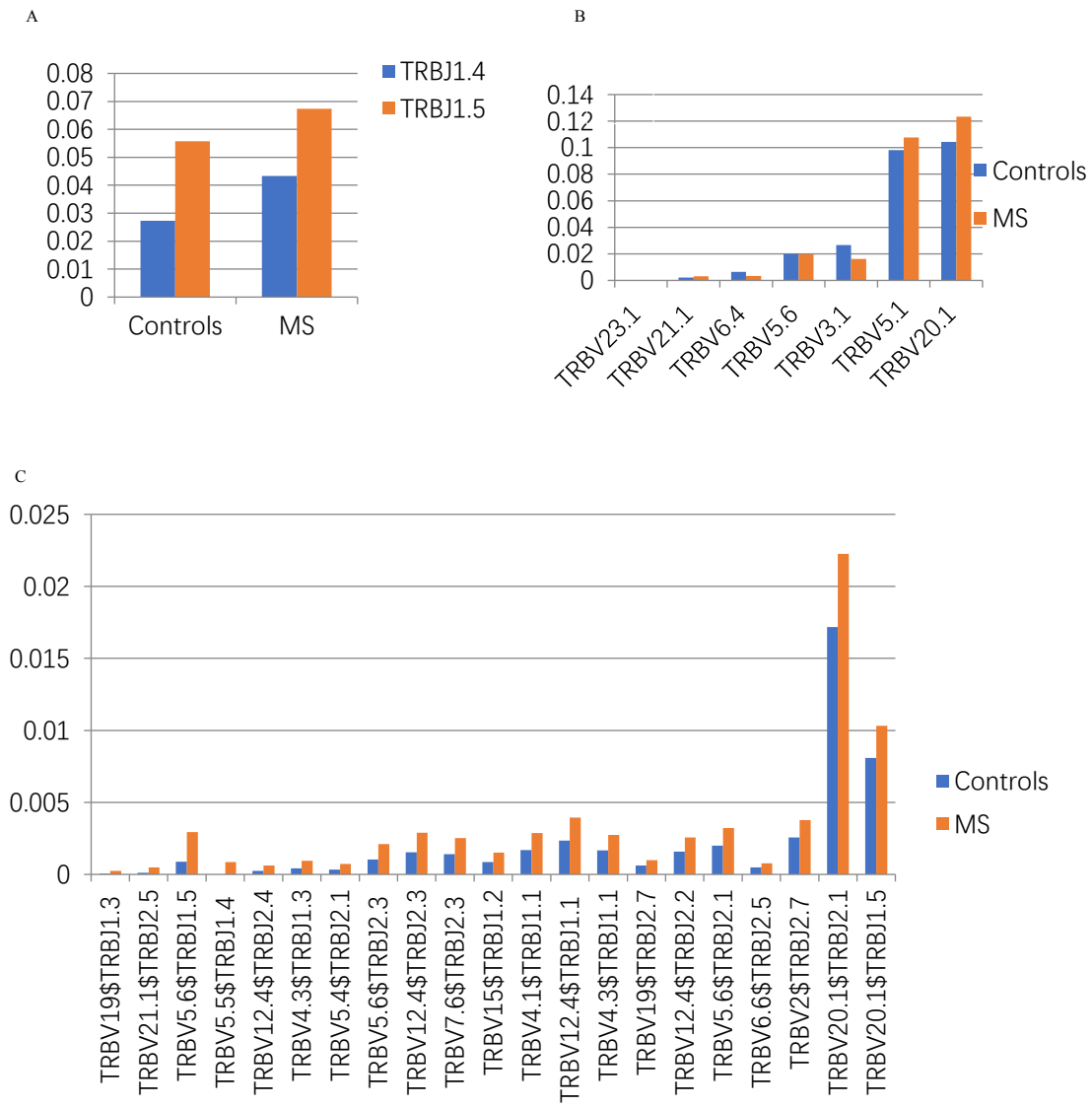

Figure 3.4. Significant usage of V&J gene between MS patients and the healthy controls. A. J gene segment usage of TCR β chain. B. V segment gene usage of TCR β chain. C.J gene&V gene rearrangment frenquency between the MS patient and the healthy controls.

3.5 MS associated CDR3 of TCR-β repertoires

We merged all the clonotypes of the MS patients and the healthy controls and determined the frequency of each unique clonotype using the Wilcoxon test. 119 unique TCRB CDR3($P<0.05$) were obtained that have a higher frequency in MS patients. To determine the function of that MS-associated CDR3, the high-frequency CDR3 clonotypes were annotated by using the database VDJdb[81]. One of the clonotypes with the predicted amino acid sequence, CASSFGQGNTEAFF, and having a p-value of ($p=0.0255$), is a BMLF1 specific CDR3 clonotype [226]. BMLF1 is an antigenic peptide found in the Epstein Bar Virus.

The MS associated CDR3 repertoire V-J recombination results also validate the previous V-J usage results above. TRBV20.1$TRBJ2.1 is the highest percentage V-J recombination in the MS associated CDR3 repertoire at 5% of all V-J recombinations. TRBV20.1$TRBJ2.1 at 2% is the dominant recombination in the MS group individually. However, in the control group, the V-J recombination with the highest percentage is TRBV7-2$ TRBJ2.7 at 2.6%. Spectratype profiling of the MS-associated CDR3 did not show obvious skewing comparing the total MS patients TCRB repertoire as well as the controls. However, the single V gene shows a bias feature; it is likely to be caused by the limited MS associated CDR3 clonotypes.

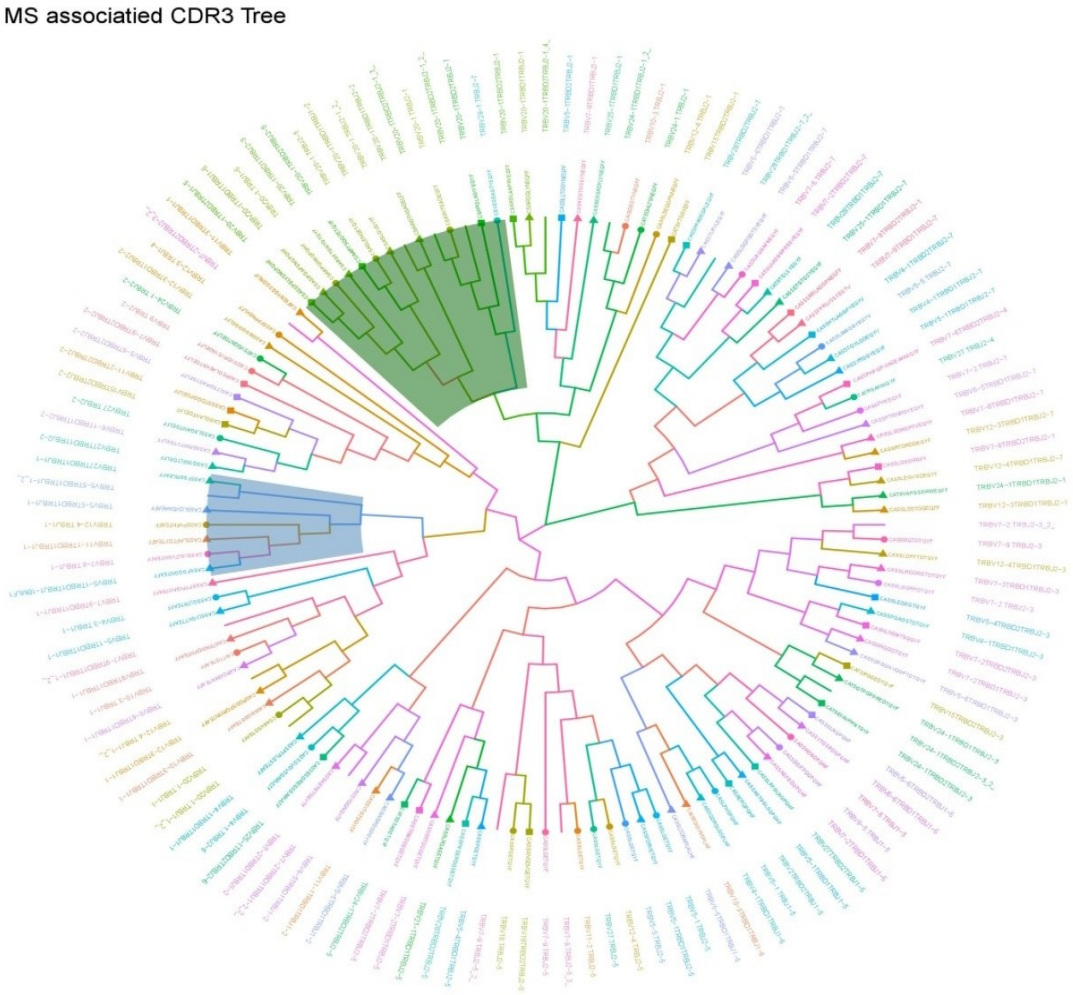

Figure 3.5. Significant usage of V&J gene between MS patients and the healthy controls.

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Chapter III Summary

In this chapter, I have highlighted that MS patients present a different TCR-β repertoire compared to the healthy populations in our Sardinia cohort. Dendrogram and Multi-dimensional scaling (MDS) plot base on the TCR-β repertoire shows that the MS patients split into a cluster distinguish with the healthy populations. Permutation testing based on MDS of the CDR3 further confirmed that the TCR-β repertoires of MS group show a difference with the healthy controls. Alterations in length of CDR3 of TCR-β repertoire was not observed in our MS patient cohort. According to our most recent analyses, diversity skew in MS patients TCR-β repertoire is not supported by our data.

To further identify what unique clonotypes MS patients have, we firstly analysis the frequent high variable (V) and joining (J) segment usage in MS patients compared with the healthy controls. We found TRBV23.1 and TRBV3.1 have a higher statistically significant frequency in healthy controls. For the TRBJ gene, usageTRBJ1.5 and TRBJ1.4 are more higher frequency usage in the MS group. We found that the Variable-Joining segment rearrangement TRBV20.1$TRBJ2.1and TRBV20.1$TRBJ1.5show a significant increase MS group.TRBV20-1 gene is high frequent used in Sardinia population. Meantime we found TRBV20-1 has certain rearrange pattern with J gene has a higher level in the MS patients in Sardinia. Our result highlighted thatTRBV20-1 gene is a critical variable (V) gene segment need further investigation.

Then we have identified 119 unique MS associated CDR3 of TCR-β clonotypes.BMLF1(EBV) specific CDR3(CASSFGQGNTEAFF) has been identified

among those MS associated CDR3 of TCR-β repertoire indicated that MS maybe have an association with the EBV infection.

Chapter IV.

TCell Receptor αRepertoire of Sardinian Multiple Sclerosis Patients

Results

To investigate the T Cell Receptor **α**Repertoire of individuals diagnosed with Multiple Sclerosis (MS), we sequenced the T Cell Receptor **α**(TCRA) repertoire from peripheral blood obtained from MS and control individuals of Sardinian origin. The objective of this portion of the study was to identify the possible clonotypes associated with Multiple Sclerosis disease. Furthermore, if functional clonotypes were revealed we wanted to determine if any clonotypes were associated with recognition of antigens of infectious disease origin.

4.1 Basic profiling of the TCRαrepertoires in MS Patients and Controls

The top 800 clonotypes were filtered from the decontamination TCR-α repertoires and analyzed to remove the PCR amplification bias. CDR3 length alterations of TCRA in MS patients compared to healthy controls (HC) is shown in Fig. 4 1C(MS, 42.73172±0.0315011 N=13 vs HC, 41.40594±2.6200671 N=21). The mean number of inserted random nucleotides in CDR3 sequences is not significantly different (p=0.4222) between multiple sclerosis (4.830006±0.4820947 N=13)and healthy controls (4.51952±1.1030651 N=21). MS spectratyping plot show (Fig.4. 1C) CALSDQKYSGGGADGLTF rearranged by TRAV9-2&TRAJ45 is the top 3 clonotype among the repertoire of MS patients.

The inserted nucleotides in the CDR3 region of TCR α chain does not appear to be the reason for the alteration of CDR3 length. If it were the biased usage of V/J gene would contribute to this difference. Convergence indicates the number of unique CDR3 nucleotide sequences that code for the same CDR3 amino acid is similar (P=0.883) in multiple sclerosis (1.012525±0.005950081 N=13) and healthy controls (1.008±0.006877180N=21).

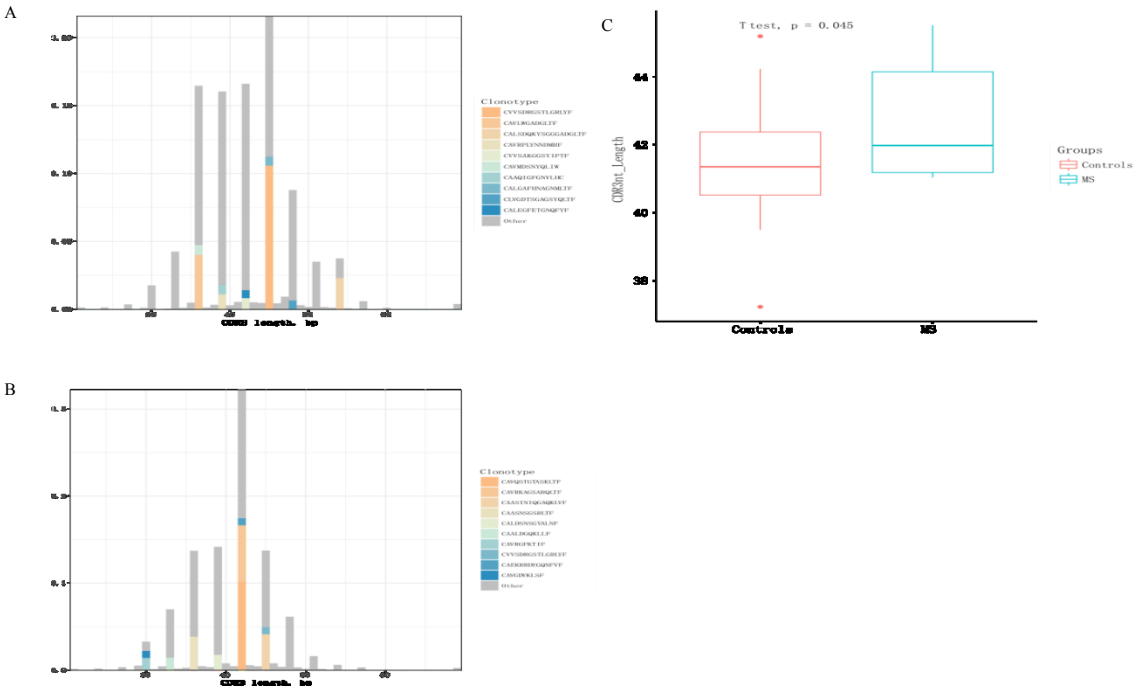

Figure 4.1. CDR3 Length Distribution of TCRα Chain. The spectral type plot displays of CDR3 lengths and top 10 clonotypes in MS patients (A), Controls (B), (C) CDR3 nt length distribution of TCRα Chain of controls and MS patients.

4.2.Clustering and overlap of TCR-α repertoires

Overlap analysis of CDR3 amino acid sequence in the repertoire from individual samples was processed and used to construct a hierarchical clustering tree. After Hierarchical clustering analysis, the dendrogram clustering tree(Fig.4.3.A) shows that the MS group has one major cluster. MDS(Fig.4.3.B) also verified that MS patients have a cluster distinguished from the healthy controls. Permutation testing (Fig.4.3.C) based on MDS of the CDR3 shows that the TCR-α repertoires of MS group show a difference with the healthy controls.

These results suggest that the MS patients have a different TCR-α repertoire when compared with healthy controls, and the results are consistent with our TCR βRepertoire results from Chapter III. Again, we ask if there are unique clonotypes of the MS patients that are specific for their pathology and disease. Mechanistically our question is where these clonotypes are responsible for the pathological autoreactive responses in MS patients.

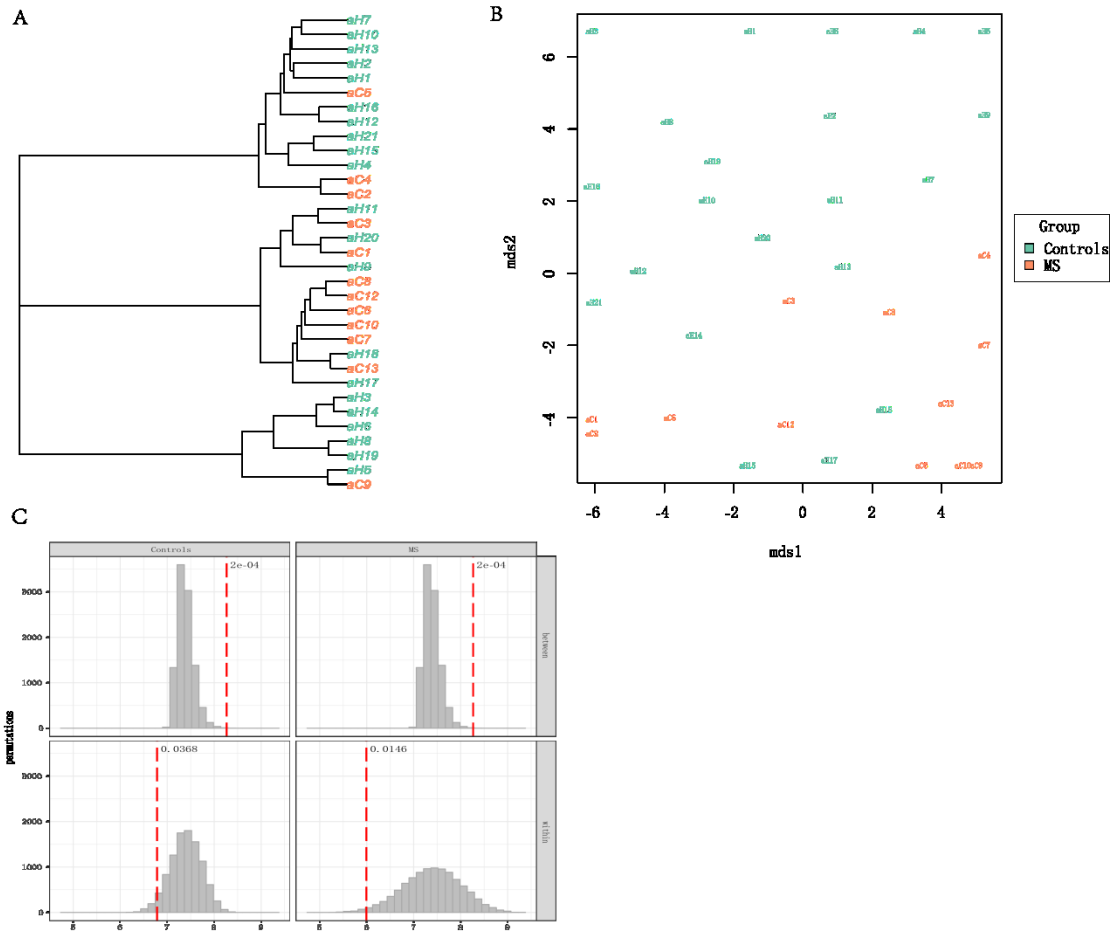

Figure 4.2. A. Hierarchical clustering of healthy donors and multiple sclerosis (MS) patients.B. Multi-dimensional scaling (MDS) plot of all ample, Controls(Green), Multiple Sclerosis(Red). C. Permutation testing between the Multiple Sclerosis(MS) and Controls (the distance is based on MDS plotting).

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4.3. Variable (V) and Joining (J) segment usage in MS patients

Hierarchical clustering of V/J gene usage profiles based on Euclidean distance was performed on our MS and control cohorts (Fig.4.3.). The heatmap of the V/J gene segment usage did not show any obvious clusters for either the MS group or the healthy control group (Figure 4.3.). For V segment gene usage however,there is a cluster of theC7,C2,C1,C9,C4, and for J gene a cluster C4,C1,C2,C9. We further examined each gene usage and performed statistical analysis. TRAV 12.3, TRAV27, TRAV38-2DV8, TRAV5as well as TRAJ11, TRAJ15, TRAJ17, TRAJ52, and TRAJ6. Figure 4.4.Shows significantly higher frequency of these genes in MS patients. Table4.2 displays the entire list of important V gene usage in the MS patients and healthy controls.

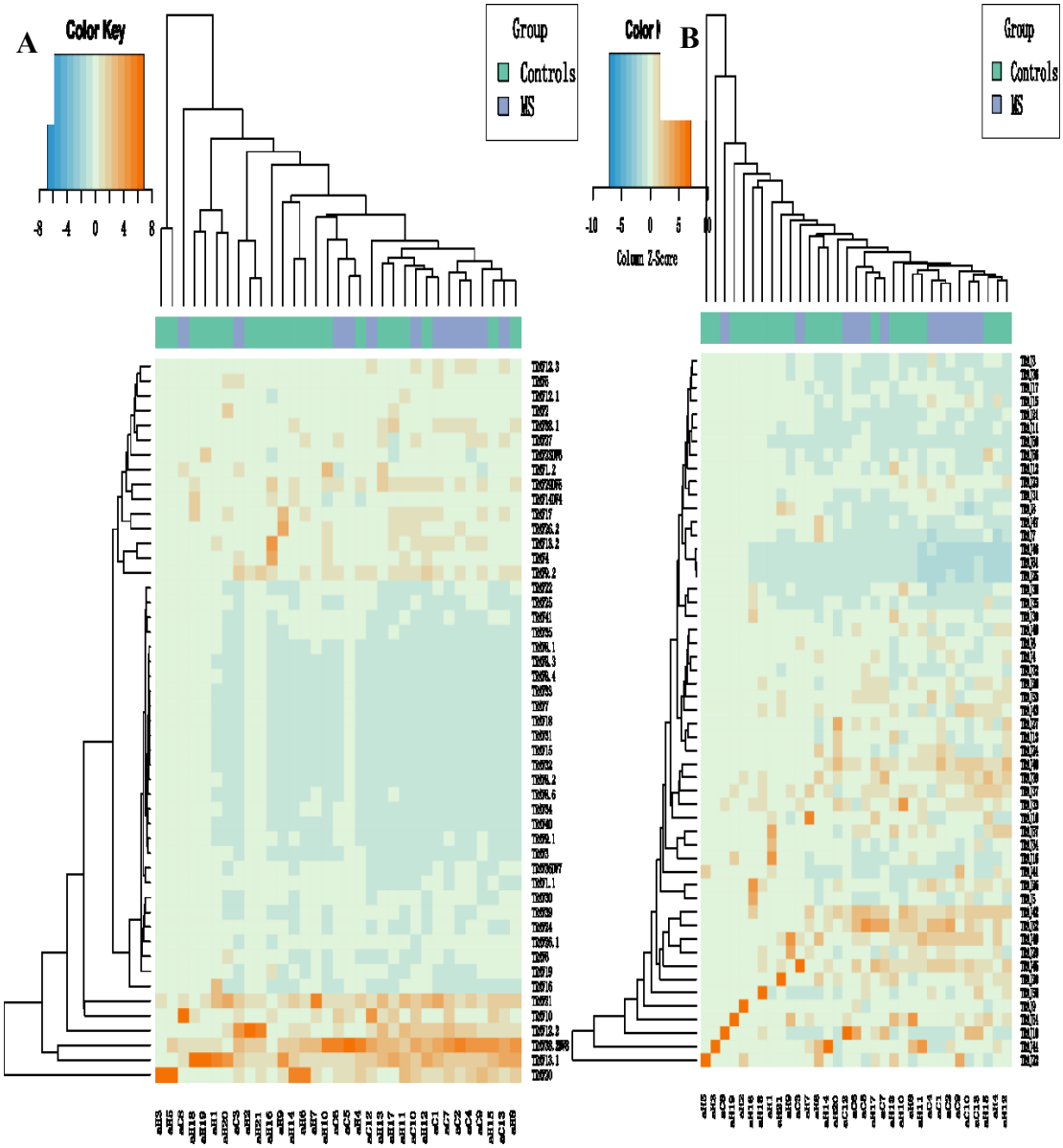

Figure 4.3. Hierarchical clustering of samples based on the Euclidean distance

A. V segment gene usage of TCRαchain. B. J gene segment usage of TCRαchain.

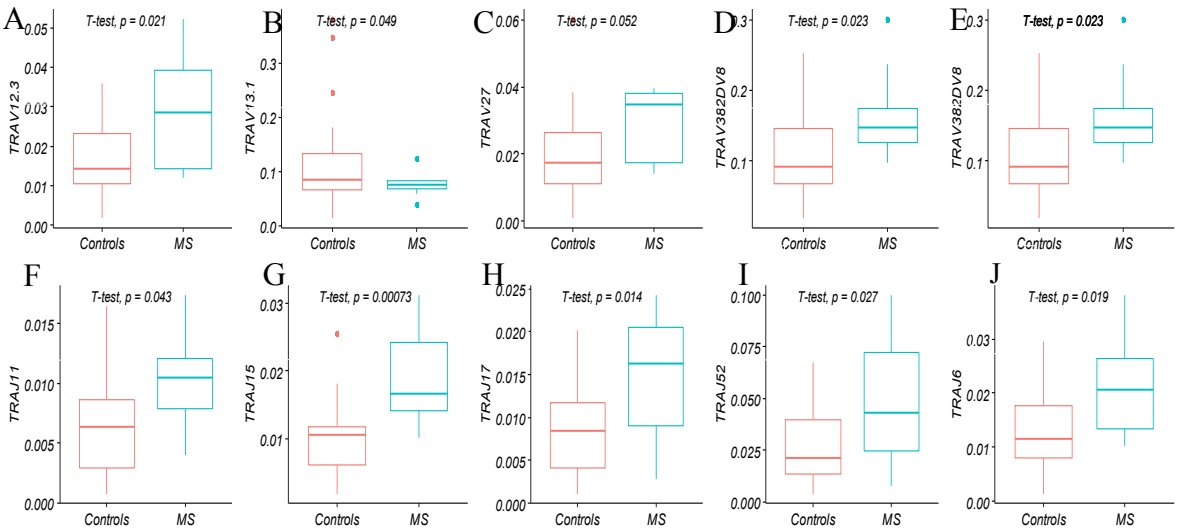

Figure 4.4.V&J gene usage in MS patients and Healthy controls

Table 4.1. V&J gene usage in MS patients and Healthy controls

TCRA	MS	Controls	p.value
TRAV12.3	0.029311	0.0169	0.021307
TRAV13.1	0.075794	0.121643	0.049075
TRAV27	0.029004	0.020036	0.052023
TRAV38-2DV8	0.159797	0.108271	0.02268
TRAV5	0.012193	0.021722	0.014338
TRAJ11	0.009943	0.006756	0.042563
TRAJ15	0.019014	0.009979	0.000728
TRAJ17	0.014712	0.008432	0.014006
TRAJ52	0.048084	0.024994	0.027462
TRAJ6	0.021035	0.013237	0.018615

4.4. Top20 sharing clonotypes between the MS patients and the healthy controls

We selected the overlapping clonotypes of the TCRα CDR3 among all MS and healthy control samples. The top 20 clonotypes of the shared repertoire were processed with hierarchical analysis(Fig4.5.A). The semi-invariant T cell includes Mucosal-associated invariant T cells(MAIT lymphocytes) and invariant natural killer T cell (iNK T cell). Dominant clonotypes among the top 20 include several that are associated with MAIT lymphocytes and iNK T cells. CAVRDSNYQLIW is shared between the most samples in our study; the controls had a significantly higher expression of this clonotype than MS patients (p= 0.05). CAVRDSNYQLIW has have also been reported in rhesus

macaques, mice, as well as cattle[227, 228]. More importantly, CAVRDSNYQLIW is also found in multiple sclerosis pathologic brain lesions [229].CAVLDSNYQLIW expressed higher in the MS group (p=0.02) than healthy controls.

CAVLDSNYQLIW（Fig4.5.B）has a higher level in the MS patient and shows BST2 specificity. CAVRDSNYQLIW is prevalent in most individuals and crosses the species barrier, and has specificity for the p65 of CMV antigen [230]. This corroborates by the high prevalence of CMV infection globally and throughout mammals.

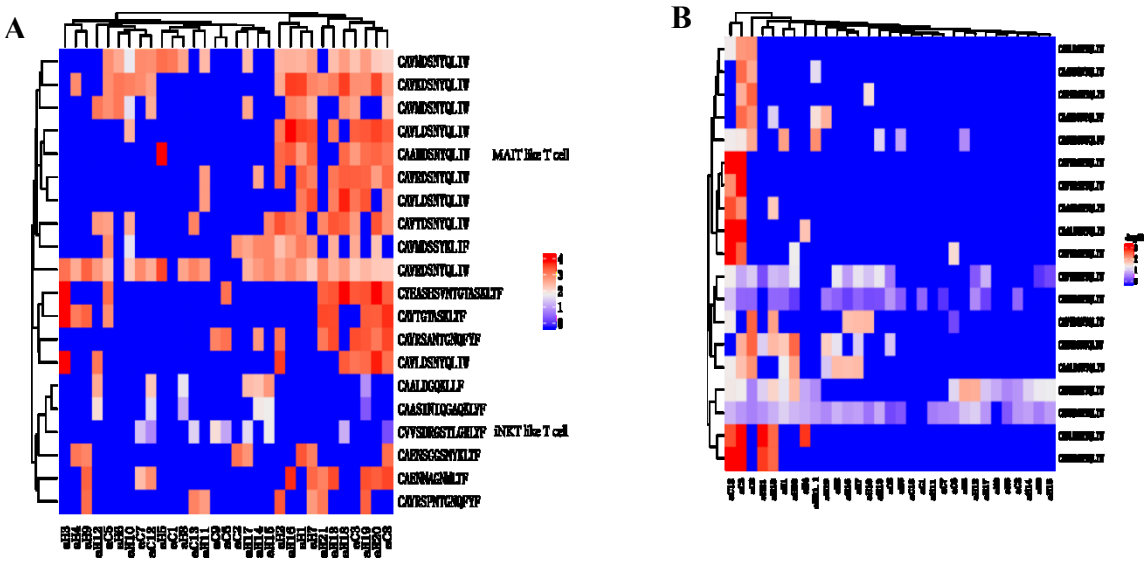

Figure 4.5. A. Top20 overlap clonotype of CDR3 of TCR a chain between the MS and healthy controls.

A. Top20 overlapping CDR3 of TCR a chain clonotypes between MS patients and healthy controls. B. Top20 overlapping CDR3clonotypes in MAIT cells in MS patients and healthy controls.

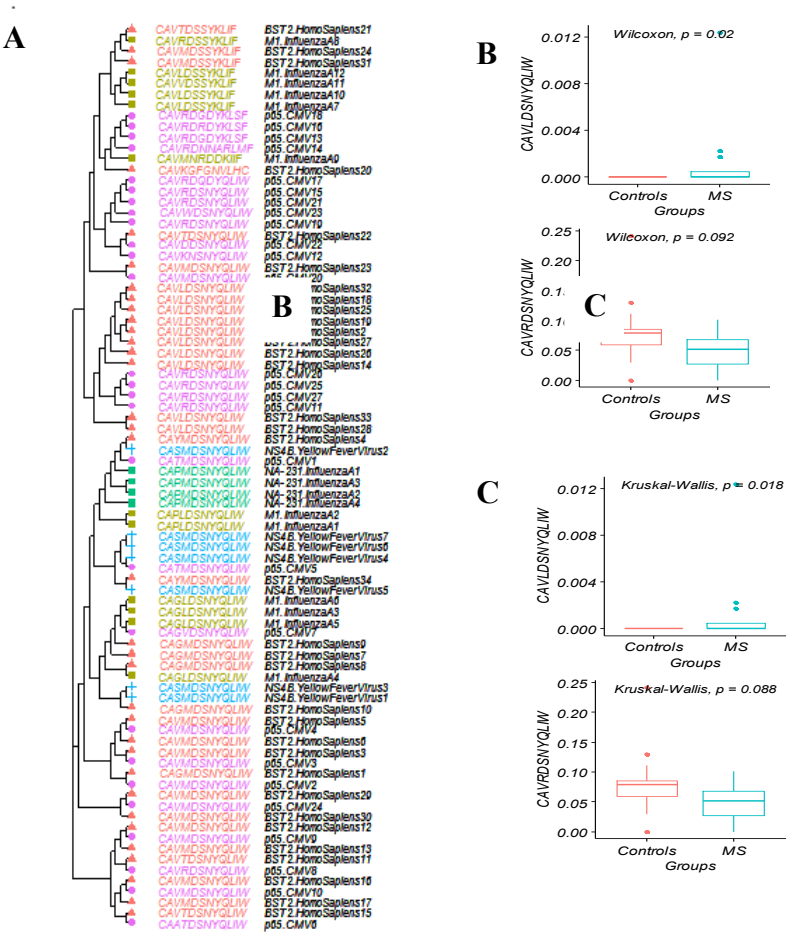

Figure 4.6. *A. Phylogenetic tree of unique MAIT clonotypes of the CDR3 TCR a chain with antigen-specific annotation. B. CAVLDSNYQLIW has significantly higher expression in the MS group(t-test) .C.Wilcoxon test reconfirmed that CAVLDSNYQLIW has significantly higher expression in the MS group.*

4.5. MAIT annotation

MAIT lymphocyte function has been reported to be related to microbial infections [231-237]. Moreover, it also appears to play a role in autoimmune disease[57-60]. According to the molecular mimicry theory, there would be cross-reactivity between the self-antigen and the non-self antigen[238-240]. The recently published database VDJDB can be used in mapping antigen specificity by matching unknown CDR3s with previously published antigen-specific CDR3s [81]. Therefore, we annotated all the unique MAIT like clonotypes with all samples that matched with the database. The phylogenetic tree was constructed with the annotated CDR3. Annotated MAIT CDR3s show specificity to virus antigens from CMV[230], influenza[241], Yellow fever virus[242], as well as the self-antigen BST2[243]after matched with the VDJDB.

4.6 MS associated CDR3 TCR-α repertoires

Furthermore, we merged all the clonotypes of the MS patients with clonotypes of healthy controls. The frequency of each unique clonotype was tested with the Wilcoxon test. Unique TCRA CDR3($P<0.05$) having a higher level in MS patients were identified. To determine the function of MS associated CDR3s, the CDR3s were annotated by the VDJdb[81]. Clonotypes with HCV specificity were higher in the multiple sclerosis patient groups than the Healthy Control group.

We annotated the TCRA CDR3 associated with multiple sclerosis with the VDJDB and found three clonotypes have previously identified antigen specific

information. As shown in table 2, CAVLDSNYQLIW which is a MAIT CDR3 shows BST2 specificity. CAVNTGNQFYF has cross-reactivity with CMV p65, influenza M1, and BST2. Additionally, the clonotype CAVSNTGNQFYF also shows BST2 specificity.

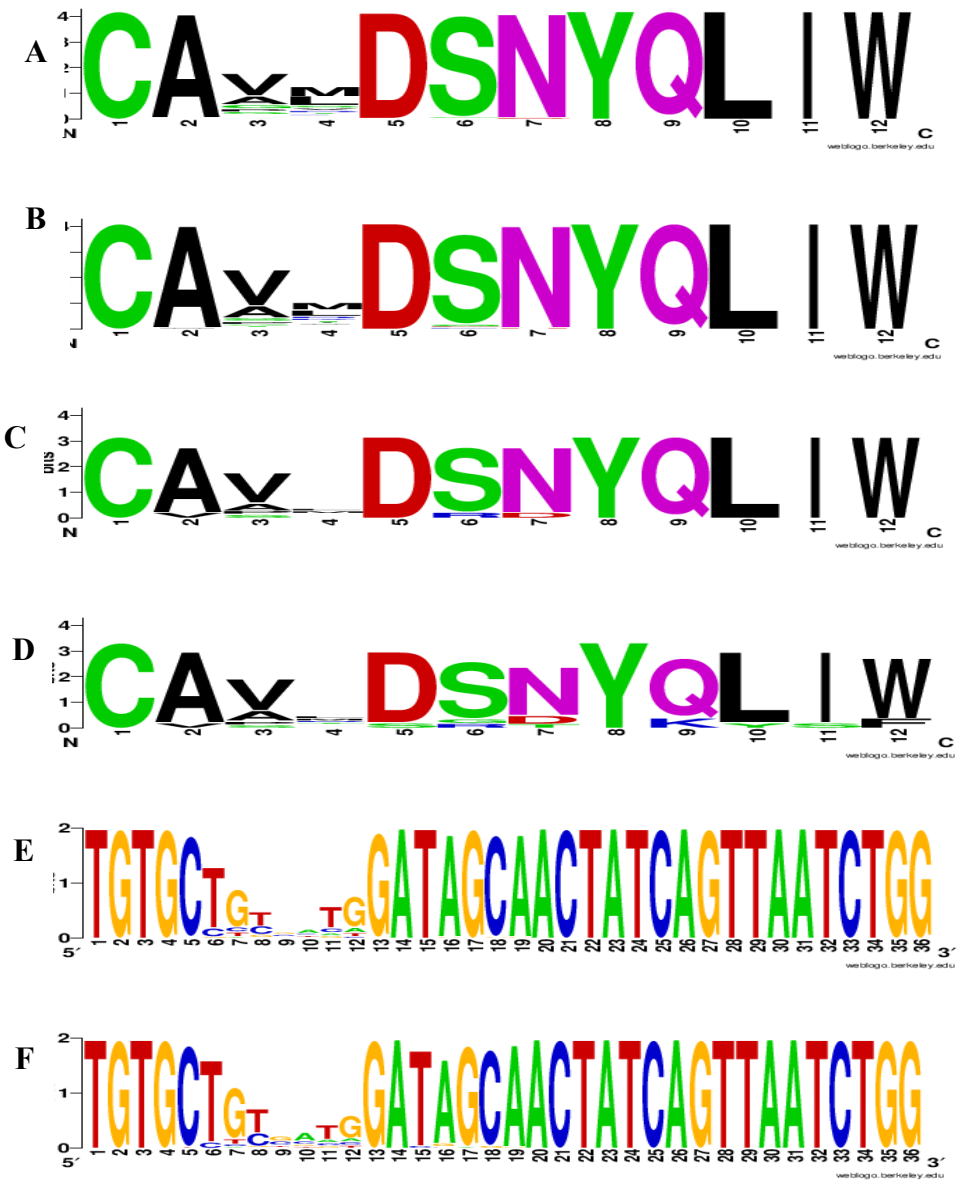

Figure 4.7. MAIT cell CDR3 aa Sequence logo.

A. Controls B. MS. C.Over expressed CDR3s in clonotypes. D.Over expressed CDR3s in MS clonotypes. E. CDR3 nucleotide logos of clonotypes. F. CDR3 nucleotide logos of MS clonotypes.

4.7.MS Associated CDR3 of TCR a chain

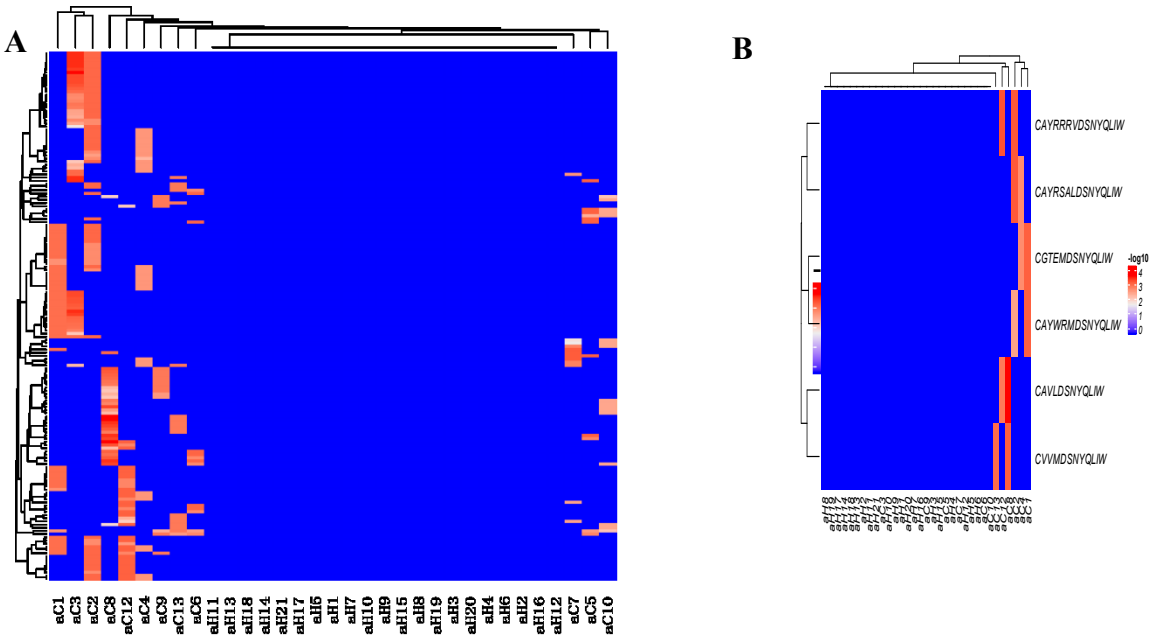

Figure 4.8. MS Associated CDR3 of TCR a chain

Figure 4.8. ***A. Heatmap of MS associated clonotype in of CDR3 in TCR a chain.B. MAIT like cell CDR3 in the MS associated clonotype.***

Table 4.2. MS Associated CDR3 Annotation

CDR3aa (Sample)	V (Sample)	J (Sample)	Epitope gene (DB)	Epitope species (DB)	Reference (DB)
CAVLDSNYQLIW	TRAV1-2	TRAJ33	BST2	HomoSapiens	https://github.com/antigenomics/vdjdb-db/issues/193
CAVNTGNQFYF	TRAV12-2	TRAJ49	p65	CMV	PMID:28423320
CAVNTGNQFYF	TRAV12-2	TRAJ49	M1	InfluenzaA	PMID:28636589
CAVNTGNQFYF	TRAV12-2	TRAJ49	BST2	HomoSapiens	https://github.com/antigenomics/vdjdb-db/issues/193
CAVSNTGNQFYF	TRAV12-2	TRAJ49	BST2	HomoSapiens	https://github.com/antigenomics/vdjdb-db/issues/193

4.8. CDR3 TCR-α repertoire Annotation.

To further explore the association of between the possible infectious disease clonotypes and auto-immune clonotypes, we annotated the CDR3 clonotypes in each. For most antigens including VP22 p65, NS4B, MLANA, M1, HA, and BST2 did not show a difference between multiple sclerosis patients and the healthy controls. However, we did find that the NS3-HCV specificity has a higher level in the multiple sclerosis patients than healthy controls.

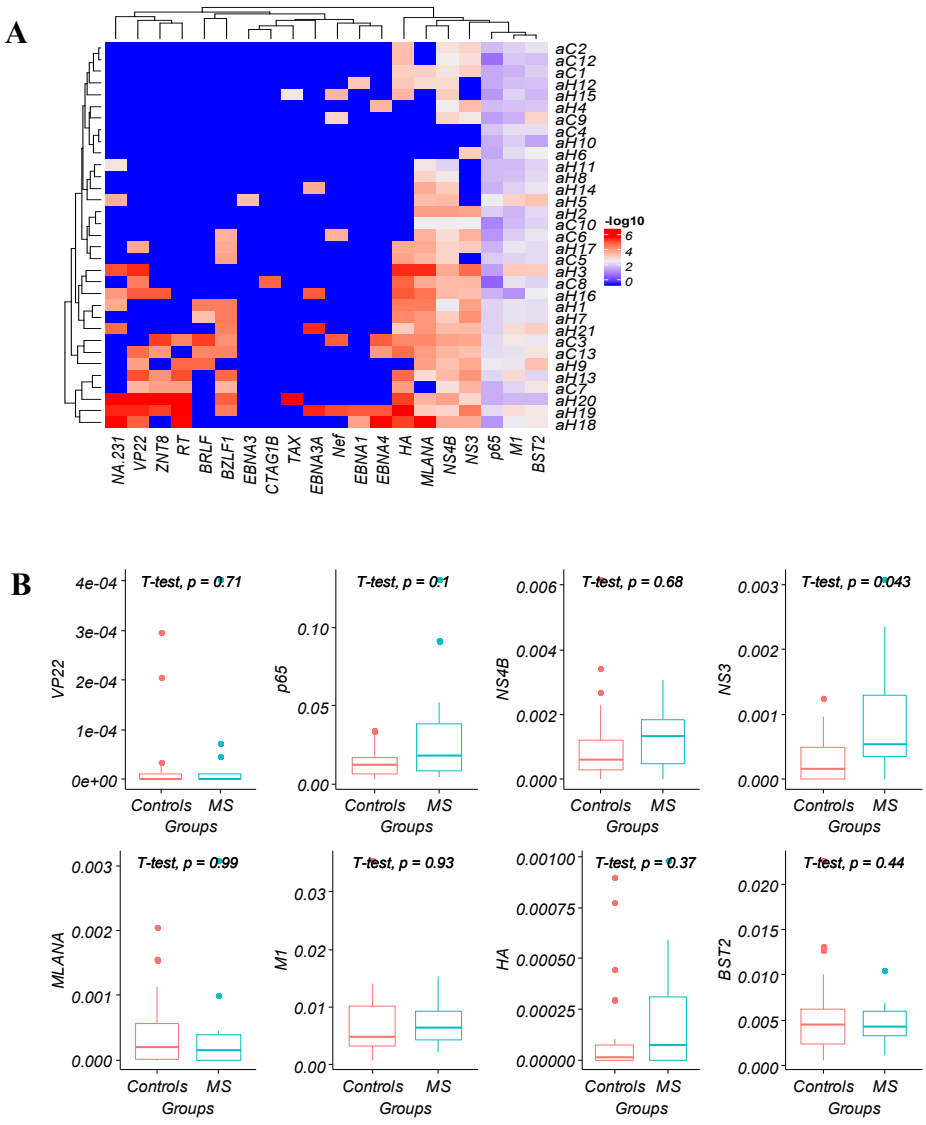

Figure4.9. A. Heatmap of clonotype annotation of CDR3 TCR chains. B. statistical analysis of the specific CDR3s in MS and controls.

Chapter III Summary

In this chapter, the TCR α repertoire of MS patients and healthy controls were analyzed by NGS deep sequencing, filtering, and annotation,Consistent with the TCR βrepertoire, MS patients also showed a difference from healthy control subjects in the

clonotypes of the TCR αrepertoire. CDR3 length alterations of TCRA repertoire was observed in MS patients compared with the healthy controls. However, the coverage and the inserted nucleotides show no difference between the MS patients and the healthy controls. In the V&J gene usage, MS patients have a cluster that is distinguished from that of healthy controls.TRAV 12.3, TRAV27, TRAV38-2DV8, TRAV5as well as TRAJ11 TRAJ15 TRAJ17 TRAJ52 TRAJ6 shows significantly higher usage in MS patients.

Mucosal-associated invariant T cell (MAIT lymphocytes) and invariant natural killer T cell (iNK T cell) are the dominant clonotypes among the top 20 sharing clonotypes between the MS patients and the healthy controls. Additional analysis of the clonotypes of MAIT among the TCR α repertoire showed that CAVRDSNYQLIW is commonly shared between most of the samples in our study. CAVRDSNYQLIW shows specificity with p65 of CMV. CAVLDSNYQLIW is highly expressed in the MS cohort. CAVLDSNYQLIW shows a BST2 specificity. We further examined unique MAIT-like clonotypes among all TCRα repertoires for possible annotation. We found MAIT-like shows specificity to the virologic antigens including CMV, influenza, Yellow fever virus as well as the self-antigen BST2.

MS associated CDR3 of TCR-α repertoires were also screened. After annotation CAVNTGNQFYF was found to be associated with withrecognizingCMVp65, influenza M1, and BST2. The clonotype CAVSNTGNQFYF also shows BST2 specificity. These results indicate cross specificity patterns may exist in T cell recognition and immunity. Also, we found that the NS3-HCV associated CDR3has a higher level in the multiple sclerosis patients than healthy controls.

Chapter V

Discussion

This is the first study to apply deep sequencing technology of the TCR repertoire in the Sardinians with multiple sclerosis. We found MS patients Sardinian origin present with a different TCR-β repertoire compared to the Sardinian healthy volunteers. Alterations in the length of TCRBV CDR3 was not observed in our MS patient cohort. According to our most recent analyses, diversity skew was not observed in our data. We found thatTRBV20-1 gene usage is high in the Sardinian population. We also found that TRBV20-1 J gene specific rearrangements were higher in the MS patients than healthy volunteers in Sardinia.

Other studies have reported that T cell repertoire CDR3 length distribution skewing was observed in MS patients compared with healthy controls[244]. Lapland's work also indicated that the alterations of TCRBV CDR3 length correlated with the disease progression; however, our study did not find any obvious alteration in TCRBV CDR3 length in the TCRB gene of MS patients. The fact that all the MS patients in our study were in the chronic stage and the analysis was based on all TCRB genes may explain the differences between our observations and Lapland's work. Further analysis of specific TCRBV genes individually may give new insight into CDR3 length distribution.

D gene insertion and the random nucleotide insertions and deletions in the VDJ region is another factor that could cause diversity and alter the CDR3 length of the TCR repertoire. Based on the analysis of the number of inserted random nucleotides between the MS patients and controls we have not found any significant variation of nucleotides in the CDR3 sequence. Since the analysis of the D gene and nucleotide insertions and deletions is highly dependent on the algorithms of the computational software used in our analysis investigation utilizing additional methods are needed to support our initial findings.

It is believed that in MS patients, there is more clonal expansion and skewing in the TCR repertoire compared with the healthy population. Recent studies on NGS of the TCR repertoire in MS patients give new insights regarding this question. Muraro et al.'s studies suggest higher TCR diversity after HSCT of the MS patient[245], while Shugay et al.'s study found lower diversity in MS samples compared with those of healthy controls and decreased diversity after HSCT in one MS patient[246]. Another NGS study has shown that the diversity of the TCRb repertoire is higher in the peripheral blood and cerebral spinal fluid (CSF) of MS patients compared with those of IIH(Idiopathic Intracranial Hypertension) controls[247]. Lossius et al. indicated that the TCR repertoire is high diverse in both the MS patients and the healthy controls, but EBV-specific CD8+ T cells were expanded in the CSF of MS patients.

So far, our result does not support the view that MS patients have a lower diversity of TCR repertoire in PBMCs compared with that of healthy controls. Generally, MS patients have the same level of diversity compared with that of controls, but the entropy of the repertoire diversity is higher in the MS patients. Further studies could

determine whether these results are artifactual. Another factor that should be taken into consideration is that all the MS patients in our study were in the chronic stage of the disease, which is different from the acute relapsing stage used in other studies. Furthermore, 10 of our 13 MS patients were undergoing therapy, which may also have affected the diversity of MS patients. We are designing further studies concerning the effects of therapy on MS patient and TCR repertoire diversity.

Our analysis showed a difference in the TCR-β repertoires of MS patients compared with those of healthy controls. The limited literature reported the TCR repertoire similarity or genetic distance between the MS and healthy control as the technical bottleneck as well as the suitable algorithm is not available. Marion 's work [248] comparing individual CSF and PBMCs CD4+ T cells and CD8+ T cells, found that TCR Vb repertoire is shared between CNS lesions, CSF, and blood CD8+ T cells. This finding is different from previous ideas that the CSF and MS lesions are based on oligoclonal expansion. Shugay's work[246] did not find a significant distance between the MS group and the healthy controls, but within the MS group, the TCR repertoire showed a diffusion between the samples. The diffusion was also observed in our CDR3 analysis, including that of the non-functional CDR3. Non-functional CDR3 is another important component thatshould be analyzed in the future.

In MS repertoires previously studied,Vβ 5.2 usage was high in MBP-specific T cells found by Kotzen al. in HLA-DR2 MS patients[203]. Vβ 5.2 was also r reported as significantly expanded in the blood of MS patients; moreover, Vβ 5.2 was most dominant in CSF and may be a target for immune therapy for MS[207, 249-252]. Our study, however, showed no-correlation of Vβ 5.2andMS patients in Sardinia. This result was

also found in other Vβ 5 family who reported has bias V gene usage in MS patient. In Shugay'sstudy TRBV genes, including TRBV5-6TRBV5-1, TRBV 5–8, TRBV 7–6, and TRBV20-1, are at a higher usage frequency in MS patients [246].

A study of the TCR repertoire of Sardinians revealed that the TCRBV20S1 null allele was significantly higher than in-Sardinian Caucasians[253, 254],but it did not indicate an increase in susceptibility to MS or type 1 diabetes (T1D) in the Sardinian population[255].

Analysis of the data in our study showed that e TRBV20-1(p-value=0.0792) has a higher frequency in the Sardinian population, but more data is needed to determine the significance of the null allele influence on the risk of multiple sclerosis. We found that the Variable-Joining segmentrearrangementTRBV20.1$TRBJ2.1(p=0.01223) and TRBV20.1$TRBJ1.5(p=0.00808951) showed a highly significant increase in the MS group. Furthermore, TRBV20.1$TRBJ2.1 is also dominant in screened MS associated with CDR3.TRBV20-1geneand was highly increased in MS patients in the Russian population[246]. The bias of the V gene usage could result in TCR repertoire skewing. Viral infection and autoimmune diseases, for instance, have been reported to have a TCR repertoire bias [256]; however, up until a point, we do not know what the was is the function of TRBV20.1 clonotypes.

By comparing the EBV-specific TCR repertoire with the CSF and blood TCR repertoires, Andreas's group found that EBV-reactive CD8+ T cells expand in multiple sclerosis patients, indicating the important role of EBV infection in those patients [257].

BMLF-1 is an early lytic protein expressed by EBV in the early lytic cycle. Increased BMLF-1 specific CD+8 T cells indicate either previous infection or viral

reactivation. The increased BMLF-1 associated CDR3 levels in MS patients give evidence in the TCR repertoire that EBV replication and activity maybe associated with the immunopathogenesis of MS.

Although we found that BMLF-1 associated CDR3 is increased in the blood of MS patients, the correlation of the blood TCR repertoire with the CSF TCR repertoire as well as the infiltrated T cells in MS lesions was not determined in this study. Also, since this study recruited MS patients in the chronic stage, it may not explain how the autoimmune response is initiated or activated. A follow-up cohort study covering a longer period should be done to provide an immune repertoire of the patients at all stages of the disease, including before disease onset, first attack, relapse, and chronic stage. Our study would be the first step in defining this type of cohort. We have screened MS associated CDR3 sequences, but the function and phenotype should be determined in further studies. Single cell sequencing of the immune cell would provide a promising method to solve this problem.

Generally, our TCR α repertoire results are in agreement with the TCR β repertoire analysis which indicates that MS patients show a difference from healthy control subjects in the clonotype of the TCR repertoire. In the V&J gene usage, MS patients have a cluster that is distinguished from that of healthy controls. The coverage and the number of inserted nucleotides show no difference between the MS patients and the healthy controls.

Mucosal-associated invariant T lymphocytes (MAIT lymphocytes) are an innate-like T cell with an invariant TCR α chain, and with highly conserved recombination with

TCRA1-2 and TCRJ33 in humans[56] MAIT cells have an antibiotic function that would be activated by some bacterial metabolites presented by the Major Classes of MHC Class I (MHC I)-Related Protein 1(MR1). Pro-inflammatory cytokines have also been reported as activators of the MAIT lymphocytes.

MAIT lymphocytes have been demonstrated to be associated with autoimmune diseases[57-60], but their association with multiple sclerosis is still controversial. MAIT-like T cells with the clonotype of Va7.2-Ja33 T cells were first found accumulated in a lesion of an MS patient[61]. It is important to determine the role MAIT plays in the MS pathogenesis:whether it has a standby role in the MS lesion or whether it protects the CNS from autoimmune injury, or even if it is a critical cell type that triggers autoimmunity.

An EAE model was built to investigate unconventional T cell include MAIT function in MS pathological progression[258]. This study shows MAIT lymphocytes in mice could play a regulatory role in the of the immune system network.

The role MAIT in human MS pathogenesis is still controversial as the frequency MAIT in the MS patients was found to be reduced compared with the healthy controls [204]. The author suggested that an immune-regulatory role of MAIT cells in MS through suppression of pathogenic T(h)1 cells was possible [259].

Another study that was designed around a cohort of six pairs of twins did not show that MAIT CD8 T cells were increased in MS patients, indicating that MAIT has a pathogenetic feature in MS[260].

Anautologoushematopoieticstem cell transplantation study with two years of follow-up post autologous hematopoietic stem cell transplantation (AHSCT) showed depleted

MAIT cells post transplantation[261], while a further study revealed antigen-driven MAIT expansion in the MS lesions by immunohistochemistry and PCR[262].

Recently it was further confirmed that the IL17+ MAIT cell was increased in MS patient peripheral blood[263].

In our study, we observed that there are several typical MAIT like cells,CAVRDSNYQLIW for example, which is widely found in most samples; however, some clonotypes show the higher levels in multiple sclerosis patients.

While we may infer that MAIT is highly conserved among the population , there are some low-frequency MAIT cells that would show a difference between the healthy population and multiple sclerosis patients. Further large-cohort studies that can be validated by other molecular methods should confirm this.

These results pose the question regarding how those differences developed and what role MAIT cells play in multiple sclerosis disease progression.

First, we may think about what role MR1 plays in MAIT development and immigration from the mucosa to other tissues. As we know MRI is highly conserved and expressed in most cell types.Whether there is a higher MR1 expression level in the CNS in multiple sclerosis remains to be seen.

Secondly, the normal frequency of MAIT in different tissues as well as in peripheral blood should be determined. The increased frequency of certain clonotypes of MAIT in MS patients is maybe caused by the circulating pathogenetic T cell expansion of that clonotype.

Third, as an important component of mucosal immunity, MAIT cells inevitably affect gut microbes. Correspondingly, the microbes react to the clonotype diversity of MAIT and come to a symbiotic balance.

Last, the fact that CMV and EBV belong to the HSV family and have a very high prevalence and long latent infection period the humans could also be a factor in the changes in the clonotypes of T cells and B cells.

With TCR annotation, some MAIT CDR3 shows specificity to virologic antigens including CMV, influenza, yellow fever virus, and HCV as well as self-antigens such as BST2.

Although the available CDR3 is limited in VDJDB and affect the annotation data output, the annotation data of the MAIT CDR3 in our study is provocative leading to the obvious question of the role of MAIT cells in regulating viral infections and autoimmunity.

To determine the antigen specificity of MAIT cells with different clonotypes, it is important first to understand the function of the MAIT cells. We matched MAIT cell CDR3 with previously published CDR3 and found several hits. Most antigens, including VP22 p65 NS4B, MLANA,M1 HA, and BST2, did not show any differences between multiple sclerosis patients and the healthy control subjects; however, we did find that NS3-HCVshowedhigher levels in multiple sclerosis patients.

In conclusion, we found that MS patients present a TCRβ repertoire that can be distinguished from the healthy controls in the Sardinian population. BMLF-1 associated CDR3 are screened out in the MS associated CDR3, indicating a higher activity of EBV in MS patients. TRBV20.1, especially when rearranged with TRBJ2.1, TRBJ1.5 are in

high usage in the Sardinian population. These are ample data from our study suggesting that MAIT cells may play a regulatory role in Multiple Sclerosis.

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