THE TCRBJ AND TCRBV REPERTOIRE IN NAIVE AND
MEMORY HUMAN T-CELLS

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The TCRBJ and TCRBV Repertoire in Naive and Memory Human T-Cells

by

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1. ABSTRACT

Previous studies suggested that human memory and naive T-cells can be distinguished based on the differential expression of two CD45 isoforms. It was believed that CD45RA is expressed only on naive cells and that CD45RO is expressed only on memory cells. The separation of cell subsets based on the differential expression of CD45 isoforms was fundamental to the experimental design used to test the hypotheses presented in this thesis.

The first hypothesis was that the memory T-cell TCRBJ and TCRBV repertoires are significantly different from those of naive T-cells within the same individual. Using a quantitative RT-PCR technique, it was found that the TCRBJ, and to a lesser extent, TCRBV repertoires of CD45RA$^+$ (naive) and CD45RO$^-$ (memory) T-cells differ. This result has relevance for future studies of the TCR repertoire and suggests that researchers must distinguish between these two T-cell subsets in their experimental designs.

Based on the commonly accepted belief that T-cell maturation in the thymus is HLA dependent, the second hypothesis of this thesis was that genetics and, more precisely, HLA affect the TCRBJ repertoire of CD4$^+$CD45RO$^-$ (naive) T-cells. Previous studies have attempted to test this hypothesis, but the conclusions drawn are weak in view of the lack of adequate statistical approaches to analyze TCR repertoires. By comparing the naive T-cell TCRBJ repertoires of

identical twins and unrelated individuals and by employing a novel method of statistical analysis, statistical evidence was found for a genetic effect on the TCRBJ repertoire of $CD4^+CD45RO^-$ (naive) T-cells. Similarly, family studies provided statistical evidence for an HLA effect on the $CD4^+CD45RO^-$ (naive) T-cell TCRBJ repertoire in comparisons of HLA identical and HLA non-identical siblings.

Statistical evidence was obtained in support of the third hypothesis which was that twin pairs discordant for multiple sclerosis (MS) have less similar $CD4^+CD45RO^-$ (naive) T-cell TCRBJ repertoires than do healthy twin pairs. In other words, this research provides evidence that the TCRBJ repertoires are altered in MS patients. The cause and timing of this alteration remains unknown.

It appears some $CD45RO^+$ (memory) T-cells may revert to the expression of the CD45RA (naive) isoform and retain their immunological memory. This recent evidence may refute the use of these markers for distinguishing naive and memory cells. These results, however, do not affect the interpretation of results for the first hypothesis, and results obtained for HLA identical vs. HLA non-identical siblings suggest that the effect of "revertant" cells was minimal.

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1. INTRODUCTION

"The immune system is a remarkably adaptive defense system that has evolved in vertebrates to protect them from invading pathogenic microorganisms and cancer. It is able to generate an enormous variety of cells and molecules capable of specifically recognizing and eliminating an apparently limitless variety of foreign invaders" (Kuby, 1994).

"Vertebrates use a complex and intricately interconnected array of weapons to mount an immunological defense against infectious foreign invaders" (Strange, 1995).

1.1 Brief History of Immunology

The earliest known record of immunity was made by the historian, Thucydides, in 430 BC, though there is evidence suggesting that the concept of immunity predates this. One definition of immunity is a "state of protection from infectious disease" (Kuby, 1994). Attempts to induce immunity through a technique known as variolation were made in the 15th century by the Chinese and the Turks. In 1798, Edward Jenner observed that milkmaids who contracted cowpox were not susceptible to the more severe disease, smallpox. As a result of this observation, Jenner improved the technique of variolation and produced the first vaccine.

For the next 100 years, there were very few developments in the field of immunology. Then in 1885, Pasteur developed the first attenuated human vaccine against rabies. Remarkably, these advancements were made while the mechanisms involved were very poorly understood. The accomplishments of Jenner and Pasteur made it possible to induce protective immunity against important pathogens. Together with a large number of twentieth century immunologists, beginning with most notably perhaps Paul Erlich, Edward Jenner and Louis Pasteur helped set the foundations from which the field of immunology has flourished (for review see Kuby, 1994 and Abbas *et al.*, 1994).

1.2 Innate and Acquired Immunity

Vertebrates possess three lines of defense against foreign antigen. First are physical barriers such as the skin and mucous membranes; second and third are the innate and acquired immune response (Strange, 1995).

1.2.1 Innate Immunity

The stomach's low pH is one example of how the human body protects itself non-specifically from foreign pathogens; (many pathogens die in an acidic environment.) This type of immunity is also known as innate immunity; it provides an antagonistic environment to many pathogens, but is not directed to any particular pathogen. Innate immunity can be divided into the following categories: 1) Physiologic barriers: temperature, pH, oxygen tension and

soluble factors; 2) Endocytic and phagocytic barriers; 3) Inflammatory response (Kuby, 1994).

1.2.2 Acquired Immunity

An important function of the immune system is to rid the body of pathogenic microorganisms. While innate immunity is often effective in achieving this goal, it is at times insufficient and lacks the ability to generate what are known as the hallmarks of immunity: specificity, diversity, self/non-self recognition and memory (Kuby, 1994), the characteristics of the acquired or specific immune response.

Lymphocytes and antigen presenting cells (APCs) are two types of cells necessary for the acquired immune response. T-cells, B-cells and natural killer cells constitute lymphocytes, while APCs include mainly macrophages, dendritic cells and B-cells. Together lymphocytes and APCs are capable of recognizing specific antigen and eliminating it from the system, thus achieving one of the most important functions of the immune system.

In this thesis, the cell of interest is the T-cell, therefore, the subsequent portion of this thesis will deal with T-cells and related topics.

1.3 Function and Role of T-cells

1.3.1 T-cells and the Hallmarks of the Immune System

T-cells are lymphocytes, and like other white blood cells. lymphocyte precursors originate in the bone marrow in a process known as hematopoiesis. Lymphocytes are responsible for the hallmarks of immunity mentioned above. The following paragraphs will expand upon how T-cells contribute to these hallmarks.

T-cells express a surface protein known as the T-cell receptor (TCR). The TCR does not bind antigen alone; it binds antigen in combination with a major histocompatibility complex (MHC) molecule. The tri-molecular complex is formed as a consequence of this binding. As its name suggests. the tri-molecular complex is composed of three molecules: TCR, antigen and MHC. The portion of the TCR which is implicated in the actual binding to the MHC and antigen is referred to as the combining site (Garboczi *et al.*,1996). The TCR combining site is the most diverse part of the TCR. That is, the amino acid sequence varies remarkably within the TCR combining site creating many different TCRs which are capable of binding to many different antigens, thus contributing to the **diversity** of the immune system. The mechanisms contributing to this variable amino acid sequence will be discussed in Sections 1.6 and 1.7.

Precursors to T-cells are released from the bone marrow and migrate to the thymus where they complete their maturation. In the thymus, there are two basic selection processes known as positive and negative selection. These selection processes will be discussed further in Section 1.6. Negative selection eliminates T-cells the receptors of which bind to self-MHC in combination with self-peptide with very high affinity. This process is an attempt to ensure that T-cells will not attack self tissues and helps create the immune system's hallmark of **self/non-self recognition**.

Each T-cell expresses on its surface many copies of one specific TCR. The TCR expressed varies from T-cell to T-cell, thus (if the phenomenon of cross reactivity is overlooked,) each T-cell is specific for a different antigen (Ag). When a T-cell binds Ag-MHC and receives all the necessary stimuli, it becomes activated and proliferates, producing progeny identical to itself. These are known as clonal progeny and are specific for the original antigen. The process is referred to as clonal expansion and enables the immune system to generate a response against a specific antigen. That is, it provides the immune system with the hallmark of **specificity**.

After proliferation, T-cells differentiate into effector or memory cells. Effector cells assist in clearing antigen, and have a short lifespan, dying very shortly after antigen clearance. Memory cells are long lived, and if the same antigen is encountered a second time, memory cells initiate a faster, more

efficient response. This provides the immune system with the hallmark of
memory (see Kuby, 1994 for more information on the hallmarks of immunity).

1.3.2 T-helper Cells

T-cells can be divided into two distinct and mutually exclusive groups.
Upon leaving the thymus, T-cells express either CD4 or CD8 molecules (see
Section 1.6). Upon differentiation, the T-cells expressing CD4 are T-helper cells
(Th) and those expressing CD8 are T-cytotoxic cells (Tc). There are exceptions
to this general rule, but they are inconsequential to this report.

Tc cells recognize MHC class I and exhibit cytotoxic function. Most cells in
the body express MHC class I. Provided with all the necessary co-stimulators, Tc
cells will lyse cells expressing self-MHC class I in combination with foreign
antigen.

The research presented in this thesis is focused exclusively on CD4$^+$
T-cells. CD4$^+$ T-cells were selected for two fundamental reasons. First, these
cells are more abundant in the peripheral blood of humans than CD8$^+$ T-cells.
Second, CD4$^+$ T-cells have been implicated in the pathogenesis of multiple
sclerosis (MS), a disease relevant to this thesis.

1.3.2.1 General Function

As their name implies, Th cells provide "help" to other cells in the immune system; they do this through three main functions. First, B-cells require a co-stimulatory signal from Th cells in order to differentiate into plasma cells. Second, Tc cells require IL-2, a cytokine secreted from Th cells in order to become activated. Finally, Th cells are involved in delayed type hypersensitivity (DTH) (Kuby, 1994).

1.3.2.2 Th1 and Th2 cells

Cytokines are soluble molecules that are capable of mediating numerous biological activities. They are secreted by immune cells including Th cells. Th cells can be separated into two categories based on their cytokine profile. Th1 cells secrete IL-2, IFN-γ, TNF-α and TNF-β, promoting Th and Tc cell proliferation, aiding IgG production and increasing the activity of macrophages. Th2 cells secrete IL4, IL-5, IL-6, IL-10 and IL-13, facilitating the allergic reaction and contributing to B-cell activation. Interestingly, the cytokines secreted by each type of Th cell have an inhibitory effect on the other type of Th cell (Amital *et al.*, 1995 and Kuby, 1994). Furthermore, Th1 and Th2 cells secrete at least some cytokines which have an autocrine function, promoting the growth of the very cells that secrete them (Amital *et al.*, 1995 and Kuby, 1994).

1.4 Structure of the T-cell Receptor

1.4.1 Nomenclature

The current World Health Organization standards for TCR nomenclature are used in this report (WHO-IUIS Nomenclature Subcommittee on TCR Designation, 1993).

1.4.2 Structure of the T-cell Receptor

The TCR is a glycosylated heterodimer (Bentley & Mariuzza, 1996). It is composed of two polypeptide chains linked by a cysteine bridge. Over 95% of TCRs are composed of one α-chain and one β-chain, known as $\alpha\beta$TCRs (Kay & Ollier, 1994). In this thesis, all mention of T-cells is in reference to those expressing the $\alpha\beta$TCR. The remaining 5% of TCRs are $\gamma\delta$TCRs; not only are these less abundant, but the method by which they recognize Ag is not as well understood (Bentley & Mariuzza, 1996).

Until recently, information on the three dimensional structure of the TCR has been extrapolated from the structure of immunoglobulin based on sequence homology (Bentley & Mariuzza, 1996). Normally, polypeptide structure is determined by X-ray crystallography. There are many obstacles to be overcome, however, for this process to be applicable to the TCR. The main obstacle occurs at the crystallization step; it is difficult to crystallize the TCR, because it is not

commonly found in soluble form and is normally associated with carbohydrates (Bentley & Mariuzza, 1996).

Advances in the X-ray crystallographic analysis of the TCR have provided the three dimensional structure of a TCR β-chain and of the V region of a TCR α-chain (Bentley *et al.*, 1995 and Fields *et al.*, 1995) and, most recently, have led to the determination of the three dimensional structure of a complete TCR in complex with antigenic peptide and MHC (Garboczi *et al.*, 1996). Research supports the hypothesis that the TCR is structurally similar to immunoglobulin (Fields & Mariuzza, 1996; Bentley & Mariuzza, 1996 and Garboczi *et al.*, 1996).

As stated earlier, the TCR is a heterodimer. Each chain of this heterodimer can be divided into two domains: a variable (V) domain and a constant (C) domain. The terms V and C reflect the extent of variation in the amino acid sequence of the domains: the V domain exhibits marked variability and the C domain is relatively conserved. The two domains do, however, show some similarity. Most notably, there is an intrachain disulfide loop of approximately 70 amino acids (Kuby, 1994). Furthermore, each chain is arranged in the T-cell membrane as follows: carboxy-terminus, short cytoplasmic tail, transmembrane region, hinge region (where the two chains are connected by a disulfide bridge), C-domain, V-domain and the amino-terminus (Kuby, 1994).

The variable domain participates in antigen binding. As with immunoglobulin, the most variable parts of the V-domain are referred to as the

complementarity determining (CDR) regions. The TCR includes four CDR regions. Recent evidence suggests that CDR1, CDR2 and CDR3 regions are involved in binding the MHC molecule (Garboczi *et al.*, 1996). In contrast, CDR3 alone is believed to play a major role in binding the antigen (Cothia *et al.*, 1988; Parham, 1996 and Garboczi *et al.*, 1996), although it should be noted that Garboczi *et al.* (1996) found that CDR1 also bound peptide. A fourth CDR region is apparent and may be involved in recognition of superantigen (Irwin & Gascoigne, 1993).

The germline DNA encoding the TCR is composed of many gene segments. These can be divided into categories. For the α-chain, there are variable (V) gene segments, joining (J) gene segments and constant (C) gene segments. The β-chain has all of these, and in addition, has diversity (D) gene segments. Together, the V, D and J segments make the variable (V) domain discussed above, and the C segment comprises the constant (C) domain. How these gene segments rearrange to produce a contiguous coding region will be discussed in Section 1.7.3 (Moss *et al.*, 1992).

The TCR is associated with another membrane-associated molecular complex termed CD3. After antigen recognition by the TCR, CD3 is involved in signal transduction. There are five invariant chains that combine to make CD3; these are γ, ε, δ, ξ and η. These chains form four possible dimers: $\gamma\varepsilon$, $\delta\varepsilon$, $\xi\xi$ and $\xi\eta$. 90% of all CD3 molecules have one $\gamma\varepsilon$ heterodimer, one $\delta\varepsilon$ heterodimer and

one $\xi\xi$ homodimer. The remaining 10% have a $\xi\eta$ heterodimer in the place of the $\xi\xi$ homodimer.

The interaction between a T-cell and an APC is strengthened by other accessory molecules. The most noteworthy of these are CD4 and CD8. CD4 and CD8 not only function as adhesion molecules, but as co-signaling coreceptors. As cell adhesion molecules, CD4 binds to class II MHC molecules, and CD8 binds to class I MHC molecules (see Kuby, 1994 and Abbas *et al.*, 1994 for further details on overall TCR structure, CD3, CD4 and CD8).

1.5 Structure and Role of the MHC

1.5.1 Structure of the MHC

The major histocompatibility complex (MHC) is a group of genes that encode, perhaps most notably, the MHC molecules recognized by T-cells. Class I, class II and class III are the three regions into which it is divided. In humans, the MHC is known historically as the Human Leukocyte Antigen (HLA) gene region and is present on chromosome 6. Each HLA class I and II region has three main subregions. The main class I subregions are termed A, B and C, and the main class II subregions are termed DP, DQ and DR. The MHC is highly polymorphic; each gene has many different forms or alleles. As a result, most individuals are heterozygous at each of the six regions mentioned above. MHC

molecules are codominantly expressed. A heterozygous individual would, thus, express twelve different MHC molecules. The MHC loci are closely linked, and are usually inherited as a linked set of alleles referred to as a haploid genotype or haplotype.

Class I MHC molecules consist of two polypeptides: an α-chain and β_2-microglobulin. The β_2-microglobulin shows little polymorphism. It appears to be necessary for the proper conformation and expression of class I MHC. The α-chain is encoded within the A, B and C regions of the HLA complex. It has three external domains of approximately 90 amino acids each, referred to as α_1, α_2 and α_3. There are two pairs of interacting domains. The β_2-microglobulin and the α_3 make up the membrane proximal domains. Interestingly, α_3 is very similar to β_2 microglobulin in size, orientation and structure. α_1 and α_2 make up the membrane distal domains. The α_1 and α_2 domains together form an antigen binding cleft: the bottom of the cleft consists of β-pleated sheets, and the sides consist of α-helices. The α-chain also has a transmembrane and a cytoplasmic domain.

Class II MHC molecules also consist of two polypeptides: an α-chain and a β-chain. These are encoded by the DP, DQ and DR regions of the HLA. Each chain has two domains: α_1, α_2, β_1 and β_2. Similar to class I MHC molecules, these domains interact with one another: α_2 and β_2 form the membrane proximal

domains, and α_1 and β_1 form the membrane distal domains that contribute to the antigen binding cleft. Transmembrane and cytoplasmic regions are also present on each chain.

Though both class I and class II MHC molecules form an antigen-binding cleft, differences in the quaternary structure of the two classes of molecules result in differences in the size and shape of the cleft. The size and the sequence of the antigenic peptides that bind to each class of MHC molecules vary (for further details on this subject see Kuby (1994) and/or Abbas *et al.* (1994)).

1.5.2 Role of the MHC in the Immune System

Class I and class II MHC molecules have many functional differences, including differences in cellular expression, involvement in antigen processing and T-cell recognition.

As a general rule class II MHC molecules present antigen processed via the endocytic pathway. In this process the internalization of antigen involves endocytosis and sometimes phagocytosis; (only specialized cells are capable of phagocytosis). Once internalized, the antigens are degraded into peptides in endosomes and lysosomes.

Class I MHC molecules usually present antigen processed via the cytosolic pathway. This process involves the degradation of proteins already present inside the cell. These intracellular proteins are continuously being formed and

degraded in cells. To be degraded, proteins are first covalently bound to ubiquitin, and the subsequent degradation of this complex occurs within proteosomes.

As a result of these two pathways, each class of MHC molecules, that is class I or II, present different types of antigen. The endocytic pathway processes exogenous antigen, and the cytosolic pathway processes endogenous antigen. Exogenous antigens are present outside the cell. Most bacteria are exogenous antigens; to be internalized, they must undergo endocytosis or phagocytosis. Endogenous antigen is an antigen that is present within the cell. For example, viruses are endogenous antigen; they enter the cell and replicate within. These antigens are subject to the same degradation processes to which naturally intracellular proteins are subject.

Provided with all of the necessary co-stimulators, binding of a T-cell to an MHC molecule and foreign peptide results in T-cell activation. As mentioned earlier, these T-cells then proliferate and differentiate into effector and memory cells. The two classes of MHC molecules are recognized by different T-cell subsets; thus, they lead to the expansion of different effector cells.

Class I molecules are expressed on most nucleated cells. Class I molecules present endogenous antigen and are recognized by CD8$^+$ T-cells. When the class I molecule is in combination with foreign antigen, activated CD8$^+$

T-cells become Tc cells which lyse infected cells. As a result, the immune response is able to eliminate infected cells and limit the spread of a pathogen.

Class II molecules are expressed on specialized antigen-presenting cells (APCs). Class II molecules present exogenous antigen and are recognized by $CD4^+$ T-cells. When the class II molecule is in combination with foreign antigen, the $CD4^+$ T-cell proliferates and its progeny become Th cells. As a result, the Th cells are able to provide help to Tc and B cells, allowing them to eliminate antigen. These Th cells may also attract macrophages to the site of infection (see Kuby, 1994 and Abbas et al., 1994 for further information on this subject).

1.6 T-cell Maturation

1.6.1 Hematopoiesis

By definition, hematopoiesis is the formation and development of red blood cells, white blood cells and platelets. In adult humans, hematopoiesis occurs mainly in the bone marrow. Pluripotent stem cells are present in the bone marrow. The term pluripotent indicates that the destiny of these stem cells has not yet been decided; depending on their immediate microenvironment, these cells have the potential to develop into numerous different types of cells. The actual number of pluripotent stem cells in the marrow is maintained by homeostatic mechanisms, and these cells are self-renewing. All hematopoietic

cells grow and mature on a network of stromal cells which support their growth and differentiation.

The first step of differentiation of pluripotent stem cells involves the separation between lymphoid and myeloid lineages. This, like all differentiation steps within the bone marrow, requires certain cytokines. The various stem cells must, therefore, express receptors for various cytokines at each step in their differentiation. Myeloid stem cells require GM-CSF and IL-3 for differentiation whereas lymphoid stem cells require IL-3. Consequently, these cells express the corresponding cytokine receptors. Myeloid cells continue differentiation to become macrophages, monocytes, neutrophils, eosinophils, basophils, mast cells, platelets, and red blood cells. It is the lymphoid lineage which is of interest in this thesis, however, as it is from these cells that the Th cells are derived.

The lymphoid stem cell has the potential to become either a T progenitor or a B progenitor. T progenitors are attracted to the thymus via a chemotactic factor secreted by thymic epithelial cells. On entry into the thymus, these cells are $CD4^-$ and $CD8^-$ and are referred to as double negatives. They also do not, as yet, express the T-cell receptor or CD3 complex, and are, therefore, $CD4^-CD8^-TCR^-CD3^-$. Cells of the lymphoid lineage in the thymus are termed thymocytes (see Kuby, 1994 for further details on hematopoiesis).

1.6.2 Gene Rearrangement

As mentioned earlier, the TCR DNA contains many different gene segments which rearrange during ontogeny in the thymus. The number of genes present in the germline DNA for each type of segment is shown in Table 1-1. In this table, TCRA and TCRB refers to the TCR α-chain genes and the TCR β-chain genes, respectively. The V, D, and J terms refer to the variable, diversity, joining and constant gene segments discussed in Section 1.4.2. The TCR β-chain genes are present on chromosome 7, and the TCR α-chain genes are present on chromosome 14. β-chain orphan (OR) genes are found on chromosome 9 and outside the β-chain complex on chromosome 7 (Kay & Ollier, 1994). These genes are not expressed, though "they have no obvious defects in their nucleotide sequence that would preclude translation and their heptamer/nonamer recombination site sequences [RSS] are conserved" (Kay & Ollier, 1994). (RSSs are discussed later in this section.) The focus in this thesis is on the TCRBV and TCRBJ gene segments. Table 1-1 shows that there are 64 TCRBV gene segment and these are categorized into 25 families on the basis of a minimum of 75% sequence homology (Kay & Ollier, 1994). Families may contain only one member or multiple members (Laroche & Robinson, 1996). Families may also contain pseudogenes (Laroque & Robinson, 1996). There are 13 TCRBJ gene segments, divided into two clusters or families based on proximity within the genome; in this case, the term families is confusing as these

particular clusters are based on their proximity in the genome and not based on sequence homology. The two TCRBC gene segments are very similar; they differ only by 6 amino acids in the translated region (Kay & Ollier, 1994).

Table 1.1: Number of TCR Gene Segments

	Number of Families	Number of Gene Segments	Reference
TCRBV	25	64	LaRoque & Robinson, 1996
TCRBJ	-	13	Kuby, 1994
TCRBD	-	2	Kuby, 1994
TCRBC	-	2	Kuby, 1994
TCRAV	32	70	Wei & Concannon, 1996 and Kay & Ollier, 1994
TCRAJ	-	75	Kay & Ollier, 1994
TCRAC	-	1	Kuby, 1994

The TCR gene segments are positioned on the chromosomes as follows. On chromosome 14 proceeding from 5' to 3', there are all the TCRAV gene segments followed by the TCRAJ gene segments and the constant gene region (C). Interestingly, the δ-chain genes are found between the TCRAV segments and the TCRAJ segments. On chromosome 7 from 5' to 3' are all the TCRBV segments, followed by TCRBD1 and six TCRBJ gene segments (i.e., the TCRBJ1 cluster) and the first constant region gene; this constitutes the first BD-BJ-BC cluster. Finally, there is a second BD-BJ-BC cluster; that is, TCRBD2 followed by seven TCRBJ segments (i.e., the TCRBJ2 cluster) and a second constant region gene (Kay and Ollier, 1994).

Conserved recognition signal sequences (RSSs) have been found flanking each gene segment. A recombinase enzyme recognizes these RSSs and catalyzes the joining of the segments. All rearrangements follow the one-turn/two-turn joining rule, ensuring that gene segments are joined in a proper order. The usual order of rearrangements is as follows: 1) TCRBD to TCRBJ; 2) TCRBV to the newly formed DJ segment; 3) TCRAV to TCRAJ (Godfrey *et al.*, 1993). Occasionally however, different rearrangements can occur; these are to be discussed further on in this section (Quiros-Roldan, 1995 and Kuby, 1994). These rearrangements contribute to TCR repertoire diversity (Kuby, 1994). For the TCR, the diversity generated through this mechanism is known as combinatorial joining and is represented by equations 1.1 and equation 1.2.

For the TCR β-chain - equation 1.1:

TCRBV gene segments x TCRBD gene segments x TCRBJ gene segments
$$64 \times 2 \times 13 = 1664$$

For the TCR α-chain - equation 1.2:

TCRAV gene segments x TCRAJ gene segments
$$70 \times 42 = 2940$$

Since the α and β-chain rearrangements are independent and the chains form heterodimers, we get equation 1.3.

Equation 1.3:
β-chain combinatorial joining x α-chain combinatorial joining
$$2940 \times 1664 = 4.9 \times 10^{6}$$

This gives almost 5 million possibilities for the αβTCR based on combinatorial joining. Other mechanisms increase this potential diversity: alternate joining of the D gene segments, exonucleolytic nibbling and N-nucleotide addition. The total potential T-cell repertoire is approximately 10^{12}, and is termed the potentially available repertoire. However, this potentially available repertoire cannot be expressed in a single individual, as the total number of lymphocytes in an adult is in the same range (approx. 10^{12}) (Shuurman, 1993). The actual repertoire is believed to be much less than this due to mechanisms to be discussed in Section 1.7.

Alternate joining of the D segment is observed as a result of the joining rule. If this rule is observed, it allows for a TCRBV segment to join either a DJ segment, which is the usual occurrence, or for a TCRBV segment to join a TCRBJ segment directly. This creates either a VDJ unit or a VJ unit (Kuby, 1994). There is also evidence of illegitimate rearrangements which produce a VDDJ unit (Quiros-Roldan et al., 1995).

Both exonucleolytic nibbling and nucleotide addition contribute to junctional diversity and both these processes are believed to be non-random (Candieas et al., 1991). Furthermore, these processes lead to many non-productive rearrangements, but they also produce productive rearrangements with alternative amino acid sequences at the coding joint, increasing the overall diversity (Kuby, 1994). Exonucleolytic nibbling is the deletion of nucleotides at the site where the two gene regions are joined (Quiros-Roldan, 1995). Nucleotide addition is carried out by an enzyme, terminal deoxynucleotidyl transferase (TDT), which adds up to six nucleotides at the coding joint (Kuby, 1994 and Komoriel et al., 1993).

If a non-productive rearrangement occurs at one allele, rearrangement of the second allele is attempted. If this also fails, the cell dies by apoptosis (Kuby, 1994).

A successful rearrangement at the TCRB locus is followed by the expression of a pre-TCR. This pre-TCR consists of a β-chain and an

alternate form of the CD3 complex. The expression of this complex terminates any further rearrangements at the TCRB locus and promotes expression of CD4 and CD8. These cells are now CD4$^+$CD8$^+$TCR$^-$CD3$^+$, and are known as double-positive cells. This process ensures that only cells with a productive β-chain rearrangement go on to rearrange the α-chain (Robey & Folkes, 1994).

α-chain rearrangement, in turn, is thought to be terminated by positive selection (Robey & Folkes, 1994). Therefore, just prior to positive selection T-cells are CD4$^+$CD8$^+$TCR$^+$CD3$^+$.

1.6.3 Thymic Selection

1.6.3.1 Positive and Negative Selection

Whether the peptides expressed during positive and negative selection are dominant or cryptic is the subject of much debate. In fact, many of the aspects of thymic selection are controversial. What follows is a general outline of the processes of positive and negative selection based on recent review articles: Anderson *et al.* (1996), Janeway (1994), Kiesielow & Miasek (1996), Kruisbeek (1993) and Ritter & Boyd (1993).

Human T-cells mature within the thymus from immature CD34$^+$ precursor cells that migrate via chemotaxis from the bone marrow. In the thymus,

maturation proceeds via an ordered and specific sequence of phenotypic changes (Vanhecke *et al.*, 1995). In the previous sections, T-cells were followed from their entrance into the thymus as $CD4^-CD8^-TCR^-CD3^-$ cells, to the rearrangement of the β-chain and the associated phenotype change to $CD4^+CD8^+TCR^-CD3^+$ and finally, to the α-chain rearrangement and the expression of a viable TCR, creating $CD4^+CD8^+TCR^+CD3^+$ cells.

In the thymus, the $CD4^-CD8^-$ or double-negative cells are situated in the outer cortical region. $CD4^+CD8^+$ or double-positive cells are found in the remainder of the cortex. $CD4^+$ and $CD8^+$ T-cells or single-positives are concentrated in the medulla where they eventually migrate to the periphery.

Double-positive cells undergo a selection process that ensures two things: that mature T-cells will recognize antigen only in the context of self-MHC and that T-cells expressing high affinity or avidity receptors are eliminated. This selection process includes the processes of positive and negative selection. It is estimated that 95 to 99% of thymocytes never leave the thymus (Janeway, 1994). At present, it is believed that the majority of these cells die by neglect; double positive cells have a finite lifespan, and if they are unable to undergo positive or negative selection within this lifespan, they die (Anderson *et al.*, 1996). This is considered death by neglect; the cells die via a process known as apoptosis. Positive and negative selection occur through biochemically distinct pathways

(Anderson *et al.*, 1996), and can occur simultaneously on double positive thymocytes (Kisielow & Miasek, 1996).

It appears that contact with the thymic epithelial cells is necessary for all stages of T-cell maturation (Anderson *et al.*, 1996). The MHC molecules on the thymic stroma and their interaction with the TCR are known to play a critical role in thymic selection (Kruisbeek, 1993). Besides the thymic epithelial and stromal cells, other cells playing a role in the selection process include dendritic cells and macrophages. Together, all these cells provide a combination of cell-to-cell contact and soluble factors which aid in the maturation of thymocytes (Anderson *et al.*, 1996). It has also been stated that there is "intrathymic symbiosis", an interdependence between thymocytes and their microenvironment (Ritter & Boyd, 1993); in other words, the thymic environment is dependent on the thymocytes and vice versa.

Most TCR$^+$CD8$^+$CD4$^+$ cells are small non-dividing cells that have a finite life span of a few days (Anderson *et al.*, 1996). Cells with a low level of expression of TCR molecules are subjected to positive and negative selection. The result is that "only cells bearing receptors with the potential to recognize foreign peptides in the context of self-MHC molecules are allowed to mature into single-positive CD4$^+$ or CD8$^+$ T-cells . . . Thus, low avidity interactions lead to positive selection and further differentiation, while high avidity interactions lead to negative selection and death by apoptosis" (Anderson *et al.*, 1996). Avidity,

however, is necessary for but not sufficient to promote positive or negative selection (Anderson *et al.*, 1996).

During positive selection, thymic epithelial cells provide a sustained interaction rather than a transient interaction with CD4$^+$CD8$^+$ thymocytes (Kiselow & Miasek, 1995 and Wilkinson *et al.*, 1995). Though other cells may contribute to positive selection, thymic epithelial cells appear to be the most efficient.

Far fewer APCs are required to induce negative selection, suggesting that these cells form multiple contacts with developing thymocytes (Anderson *et al.*, 1996). Thymocytes undergoing positive selection are capable of undergoing negative selection until TCR levels are upregulated (Dyall & Nikoloc-Zugic, 1995). This retention of susceptibility to negative selection provides a mechanism for elimination of self-reactive cells (Wilkinson *et al.*, 1995).

1.6.4 Activation and Proliferation

After leaving the thymus and entering the periphery, T-cells are naive; that is, they have not yet encountered antigen. Naive cells are also referred to as unprimed, antigen-inexperienced or virgin cells. Activation occurs upon antigenic stimulation and induces differentiation and proliferation. Proliferation proceeds by a process known as clonal expansion. Differentiation, as mentioned earlier, results in the production of effector and memory cells. Effector cells help rid the

body of antigen and are very short lived. Memory cells on the other hand are long-lived cells and upon a second exposure to antigen, mediate a faster and greater secondary response (see Section 1.3.1) (Kuby, 1994).

1.6.5 CD45 Isoforms

T-cells express CD45 as do all leukocytes. CD45 occurs in several isoforms, which are formed via differential RNA splicing (Stulnig et al., 1995). The alternative splicing of three consecutive NH_2-terminal exons of the CD45 transcripts, creates these isoforms (Shanafelt et al., 1996). Those of interest for this study are CD45RA and CD45RO; these two isoforms differ in molecular weight and surface epitopes. CD45RA is expressed primarily on naive cells, and CD45RO is expressed mainly on memory cells. The two subsets, thus, represent different maturational stages of the same lineage (Kristensson et al., 1990). Furthermore, the switch from CD45RA to CD45RO cannot take place without proliferation (Johanisson & Festin, 1996).

There is evidence in mice and in vitro in humans that cells bearing the CD45RO isoform can revert to the expression of the CD45RA isoform (Michie et al., 1992; Hargeaves & Bell, 1997 and Pilling et al., 1996). Though this reversion of CD45RA^{-} cells to CD45RA^{+} cells has not been demonstrated in vivo in humans, the possibility remains that CD45RO^{+} (memory) T-cells may revert to the CD45RA^{+} (naive) phenotype (Rothstein et al. in Richards et al., 1997).

Furthermore, these "revertant" cells appear to retain immunological memory (Richards *et al.*, 1997); it has been demonstrated in humans that CD45RA$^+$ cells demonstrate aspects of immunological memory usually associated with CD45RO$^+$ cells (Richards *et al.*, 1997). This finding may have profound implications on the results of the research presented in this thesis; these implications will be discussed in detail in Chapter 5. In this thesis, CD45RO$^-$ and CD45RA$^+$ T-cells are refered to as naive cells even though populations of cells bearing these phenotypes may include "revertant" memory cells.

It is interesting to note that the proportion of cells expressing the CD45RO isoform increases with age. These changes in CD45 isoform expression on T-cells are likely the result of a lifetime exposure to different antigens (McElhaney *et al.*, 1995).

There is also evidence for CD45RO$^+$ CD45RA$^+$ cells (Wallace & Beverley, 1990). These cells have been referred to as early activated cells. It is believed that this surface phenotype reflects cells in transition to the CD45RO isoform with a relative delay in the loss of CD45RA from the cell surface after stimulation (McElhaney *et al.*, 1995).

Very little is known about CD45, though knowledge surrounding its function and role in the immune system is expanding every day. CD45 is physically associated with CD4 on resting T-cells. (Bonnard *et al.*, 1997). CD45 may be involved in thymocyte differentiation (Poggi *et al.*, 1996) and apoptosis

regulation (Macino *et al.*, 1996). CD45's tyrosine phosphatase activity enable it to play a major role in regulating signal transduction (Shanafelt *et al.*, 1996).

1.7 The T-cell Receptor Repertoire

1.7.1 Overview

The T-cell receptor repertoire is defined as the total diversity of TCR molecules in a given T-cell population (Musette, 1996b). Many variables contribute to the diversity of the repertoire, including gene rearrangements, alternate joining of the D gene segments, exonucleolytic nibbling and N-nucleotide addition which have already been discussed.

In this thesis, the focus is on the expression of TCR gene segments on peripheral blood lymphocytes (PBLs). TCR gene segments are not expressed at equal levels in the periphery. Furthermore, this differential expression of each segment is not proportional to gene number (Robinson, 1992). Thus for this thesis, the TCRBJ repertoire is the percent expression of each TCRBJ gene segment and the TCRBV repertoire is the percent expression of each TCRBV family.

The naive T-cell repertoire is the product of combinatorial diversity, α and β-chain joining, thymic selection, allelic differences and perhaps also the effects of endogenous superantigen (Kuby, 1994). The memory T-cell repertoire reflects

the influence of antigen and of exogenous superantigen on the naive T-cell receptor repertoire. Some of these processes have already been discussed, the remainder will be discussed in the following sections.

1.7.2 Generation of Diversity

It has already been demonstrated that some diversity is created at the level of gene rearrangements. The rearrangement of TCR gene segments is not random, and certain recombinations occur with a higher than random frequency (Quiros-Roldan *et al.*, 1995). Thus, prior to thymic selection, there is skewing of the TCR repertoire.

The naive TCR repertoire may also be influenced by thymic selection. Both positive and negative selection involve binding to self-MHC in combination with self-peptide. It has been shown that binding to a particular MHC-antigen complex is dependent on the TCR gene segments expressed (Nanda *et al.*, 1992; Smith *et al.*, 1994 and Stryuk *et al.*, 1995). Since different TCRBV and TCRBJ gene segments would bind these MHC complexes with differential avidity, certain TCR gene segments would be more likely to survive thymic selection (Walser-Kuntz *et al.*, 1995b).

Allelic differences have also been demonstrated for TCRBV (Posnett, 1990 and Kay & Ollier, 1994) and for TCRAV (Kay & Ollier, 1994). Allelic differences include: 1) the presence of a stop codon that causes failure to

express the allele; 2) differences in the non-coding region of two gene segments that produce an identical protein but possibly lead to differences in the level of expression of that segment (Kay et al., 1994); and 3) differences in the sequence of TCR molecules that may cause differences in circulating levels, especially if the changes in sequence affect the avidity of binding during positive and negative selection. To summarize, different alleles may code for entirely different proteins, for premature stop codons, or for changes in sequence that lead to failure of expression of a particular gene (Kay & Ollier, 1994). What is vital here is that some polymorphisms influence the circulating repertoire (Kay & Ollier, 1994). Interestingly, alleles may vary in their expression levels among human populations (Barron et al., 1995 and De Inocencio et al., 1995).

Superantigens will bind to certain TCRBV families (Moss et al., 1992). Expression of superantigens in the thymus, can result in the deletion of an entire TCRBV family. Endogenous superantigens have been identified in the mouse but not yet in the human. It has recently been demonstrated that superantigens affect not only TCRBV, but TCRBJ (Irwin & Gascoigne, 1993; Pullen & Bogatzki, 1996 and Musette et al., 1996b).

The above factors contribute to the diversity of the naive T-cell repertoire. Once T-cells are in the periphery, their repertoire is modulated by exposure to various antigens and superantigens. Again since TCR gene segments may

preferentially bind different peptides, further skewing of the repertoire will occur
and is dependent on the antigens and superantigens encountered.

The potential TCR repertoire was stated in Section 1.6.2 as being 10^{12}. It
is believed, however, that as a result of many of the abovementioned processes
influencing T-cells (i.e., non-random rearrangements, non-random production of
junctional diversity and HLA-dependent thymic selection), the overall available
TCR repertoire is much less.

1.7.3 The Influence of Genetics on the T-Cell Receptor Repertoire

It is claimed that the genetic makeup of an individual affects the TCR
repertoire expressed by the individual (Malhotra et al., 1992). Researchers have
provided evidence for this by demonstrating that monozygotic twins are more
similar with respect to their TCRAV, TCRBV and TCRBJ repertoires than
unrelated individuals (Lovebridge et al., 1991; Gulwani-Akolkar et al., 1995 and
Nanki et al., 1996).

The known genetic region apparently having an influence on the TCR
repertoire is the HLA gene region. Various studies report that HLA identical
siblings exhibit more similar TCRBV and TCRAV repertoires than do HLA
non-identical siblings (Gulwani-Akolkar et al., 1995; Gulwani-Akolkar et al., 1991;
Akolkar et al., 1992 and Uhrberg et al., 1996). Other studies have demonstrated

a similar effect of HLA on the TCR repertoire in HLA-DR matched but unrelated individuals (Walser-Kuntz et al., 1995b).

As will be discussed (see Section 5.3), the evidence in the literature cited above, that genetics including HLA influences the TCR repertoires is actually weak in view of the fundamental lack of adequate statistical approaches to analyze TCR repertoires.

1.7.4 The T-Cell Receptor Repertoire and Disease

Analysis of the TCR repertoire has provided interesting results for three main types of disease: autoimmune disease, immunogenic tumors and chronic inflammatory disease (Musette, 1996a). The diseases studied include such diverse disorders as rheumatoid arthritis (Weyand et al., 1992; Walser-Kuntz et al., 1995b and Mu et al., 1996), malignant melanomas (Puisieux et al., 1995), carcinomas (Halapi et al., 1993), alopecia areata totalis (Hoffmann et al., 1996), pulmonary sarcoidosis (Silver et al., 1996) primary biliary cirrhosis (Mayo et al., 1996) and multiple sclerosis.

1.8 Multiple Sclerosis

1.8.1 Overview

1.8.1.1 Multiple Sclerosis

Multiple sclerosis is an inflammatory demyelinating disease of the central nervous system associated with destruction of the myelin sheath and gliosis. A hallmark of multiple sclerosis is the formation of white matter plaques of demyelination. These plaques occur most often in the optic nerve, brainstem, spinal cord and periventricular white matter (Luchinetti *et al.*, 1996).

Recently, multiple sclerosis has been classified into five categories based on the symptoms and progression of the disease. These classifications are: relapsing-remitting, secondary progressive, primary progressive, progressive-relapsing and benign multiple sclerosis (Thompson & Noseworthy, 1996).

There is presently no cure for multiple sclerosis, and for reasons not understood, multiple sclerosis is not a self-limiting disease (Vanderbark *et al.*, 1996).

It is generally accepted that the immune system contributes to the pathology of multiple sclerosis; it is considered an autoimmune disease. The initiating autoantigens involved in the disease are not known. Some possibilities

are myelin basic protein, proteolipid apoprotein and myelin oligodendrocyte glycoprotein (Carnaud & Bach, 1993).

Multiple sclerosis affects approximately one in a thousand individuals in Canada, and disease onset is more frequent in young adults. The disease is more common in women than in men. There is considerable evidence of genetic predisposition to multiple sclerosis (Ebers *et al.*, 1995). The concordance rate, however, for identical twins is between 25-50% (Ebers *et al.*, 1995), suggesting that other factors, presumably environmental are also involved (Coyle, 1996). One view is that in order to acquire multiple sclerosis, genetically susceptible individuals must be exposed to appropriate environmental factors (Coyle, 1996). An alternate explanation is presented in Section 2.3.

1.8.2 T-Cell Involvement in Multiple Sclerosis (MS)

In animal models of autoimmune diseases in general, T-cell involvement can be demonstrated by identification of T-cells at the site of the lesion, transfer of the disease from affected animals to healthy animals by T-cells, prevention of disease by T-cell directed immune intervention and by *in vitro* analysis of functional cellular reactivity to various autoantigens (Carnaud & Bach, 1993). T-cells have been implicated in the pathogenesis of MS (Luchinetti *et al.*, 1996). The experimental allergic encephalomyelitis (EAE) animal model of MS strongly implicates T-cells in disease pathogenesis. In humans, there is an increased

frequency of myelin basic protein reactive T-cells in the blood (Vanderbark *et al.*, 1996). CD4^{+} T-cells can be found throughout the lesional areas of the central nervous system in both acute MS and in chronic-active MS lesions (Brosnan & Raine, 1996). Furthermore, MS has been associated with certain HLA-DR molecules (Epplen *et al.*, 1995); since CD4^{+} T-cells are MHC class II restricted, it seems logical to implicate these cells in the pathogenesis of MS (Brosnan & Raine, 1996).

1.8.3 TCR Contribution to Multiple Sclerosis

Animal models of MS suggest that only a few TCRs contribute to the pathogenesis of EAE and that blockade of specific TCRs can inhibit induction and/or progression of the disease. It is clear in humans, in contrast, that the contribution of TCRs to MS is extremely complex and that it is unlikely that one or a few TCRs are involved in MS pathogenesis. This partly relates to the phenomenon of intermolecular and intramolecular determinant spreading with recruitment of TCRs directed against multiple CNS antigenic epitopes (Lehmann *et al.*, 1992). Nevertheless, there is evidence that particular TCRs may be preferentially skewed in MS, probably/possibly as a consequence of disease (Utz *et al.*, 1993).

Considerable data have accumulated on the TCR usage in MS lesions (see for example, Oksenberg *et al.*, 1993 and Hafler *et al.*, 1996) but there has

been no formal study of naive T-cells in MS. A precedent for such a study comes from the work of Walser-Kuntz *et al.* (1995b) who showed in rheumatoid arthritis that the TCR repertoire is skewed in naive T-cells.

1.9 Studying the T-Cell Receptor Repertoire

1.9.1 Reasons

Recall from section 1.7.4 that the analysis of the TCR repertoire is centralized around three main types of diseases. For the purpose of this thesis, a single putative autoimmune disease, MS, is a focus of interest. Current therapeutic approaches in the treatment of MS frequently involve non-specific suppression of the immune system. These have the potential to alleviate the patient's symptoms, but they also leave the patient immunocompromised; that is, these therapies may render the patient increasingly susceptible to everyday pathogens (Musette *et al.*, 1996b).

Studies of the TCR may, in contrast, lead to a more precise understanding of the T-cells involved in the pathogenesis of multiple sclerosis and have the potential to lead to highly specific immunotherapy directed against particular TCRs (Musette *et al.*, 1996b).

1.9.2 Methods

Various methods have been used in the analysis of the TCR repertoire. For example, Southern blots (Hall *et al.*, 1993) and heteroduplex analysis (Sotinni *et al.*, 1996) have been used to assess clonality, but these are relatively insensitive methods. Sequence analysis is useful for the study of CDR diversity, but is laborious for the analysis of gene segment usage.

The methods most often used, therefore, for the analysis of gene segment usage involve applications of monoclonal antibodies and the polymerase chain reaction (PCR) to amplify TCR cDNA. (PCR is discussed in Section 1.10.) Unfortunately, monoclonal antibodies specific for all the TCR gene segments are not available (Hall *et al.*, 1993). As a result, the most commonly used method is PCR-based.

The PCR method relevant to TCR repertoire analysis involves isolation of TCR mRNA and first strand cDNA synthesis and can be used basically in two ways. A single sided PCR or anchored PCR can be employed when the 5' sequence is unknown (Rosenberg *et al.*, 1992). Otherwise, family-specific or gene segment-specific PCR can be used. In this case, primers specific for a particular family of TCR gene segments or for particular TCR gene segments themselves are used in the PCR (Hall *et al.*, 1993). This method is termed reverse transcribed PCR (RT-PCR).

1.9.3 Difficulties

For this thesis, family and gene segment-specific quantitative PCR is used. The main difficulties in this PCR method arise in quantitation of individual TCR gene segments and families. Once the TCR gene segments have been amplified, the question arises as to whether it is possible to determine the original amount of mRNA for each TCR gene segment. This will be addressed in Chapter 3.

1.10 Quantitative PCR

1.10.1 Overview

The polymerase chain reaction (PCR) may be defined as primer-mediated enzymatic amplification of specific genomic DNA or cDNA sequences (Innis *et al.*, 1990). Thermostable DNA polymerases catalyze this cyclical reaction *in vitro*, and the product of one cycle is used as the substrate of subsequent cycles. This creates an exponential increase in the desired DNA.

This process is not 100% efficient even when the reaction conditions are optimized. Optimizing the individual reaction components and reaction temperatures does, however, ensure the most efficient reaction. A phenomenon known as the plateau effect limits the yield of the reaction.

A typical PCR reaction will proceed through an exponential phase, a quasi-linear phase and a plateau phase (see Figure 1-1).
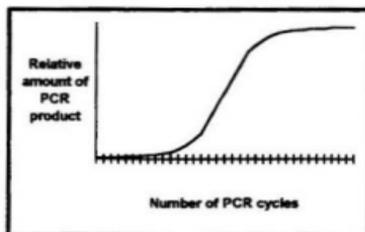
Figure 1-1: The three phases of a polymerase chain reaction.

1.10.2 Plateau Phase

The plateau phase is defined as the attenuation rate of the exponential product accumulation (Sardelli, 1993). According to Sardelli (1993), a number of components contribute to this plateau. First, there is the thermal inactivation of the Taq polymerase enzyme. The enzyme has a half-life of approximately 30 min. at 95° C. Therefore, if each cycle contains a one minute denaturation step, after 30 cycles one half-life will have elapsed. Since the enzyme is usually present in great excess to the DNA, the amount of enzyme is usually not a concern in the PCR unless a large number of cycles are run (Sardelli, 1993). Second, there is the limiting concentration of the enzyme. As the amount of DNA increases, the ratio of enzyme to DNA decreases. The efficiency of extension eventually decreases yielding less than full length products (Sardelli, 1993). Third, there is a reduction in denaturation efficiency. The melting temperature of the double stranded DNA increases as the concentration increases. This third obstacle can be overcome, however, by maintaining the denaturation temperature for each cycle at 94-95° C (Sardelli, 1993). Fourth, there is a decrease in the efficiency of primer annealing. As the concentration of DNA increases, there is an increasing probability that after the denaturation step, the DNA strands will reanneal to each other forming a very stable complex. This would make it impossible for primer annealing to take place. Finally, the enzyme, Taq polymerase, demonstrates exonuclease activity. If both a complementary strand

and a primer attempt to bind the same strand of DNA, it is possible that the complementary strand will be left single stranded. This is because the complementary strand cannot bind to the same site as the primer once the primer has bound. The enzyme would, at this point, cleave this single strand, destroying the product.

From this, it is obvious that the height of the plateau is dependent on various factors including the size of the substrate used and the concentration of reagents (Sardelli, 1993).

1.10.3 Why Quantitative PCR Must Not Be Permitted Beyond the Quasi-Linear Phase

For a quantitative PCR, the reaction must never be permitted to proceed beyond the quasi-linear phase. Take for example Figure 1-2. This demonstrates two hypothetical, identical PCRs; the only difference between the two is the amount of substrate available at the beginning of the reaction. The PCR represented by the dashed line has less substrate than the PCR represented by the solid line. Since the same substrate, reagent concentrations and enzyme are used for both, the PCRs plateau at the same level or the same relative amount of PCR product. During the quasi-linear phase, however, the amount of product is dependent on the amount of substrate. Figure 1-2 demonstrates that at X cycles the amount of product is directly proportional to the amount of the original substrate. In a quantitative PCR, the goal is to determine the amount of

substrate. Since the amount of PCR product is proportional to the amount of original substrate during the quasi-linear phase, a determination of the amount of PCR product during the quasi-linear phase provides an accurate reflection of the amount of starting or original substrate. In summary beyond the quasi-linear phase the amount of PCR product is not proportional to the amount of original substrate, therefore, quantitative PCR must not be permitted beyond the quasi-linear phase.
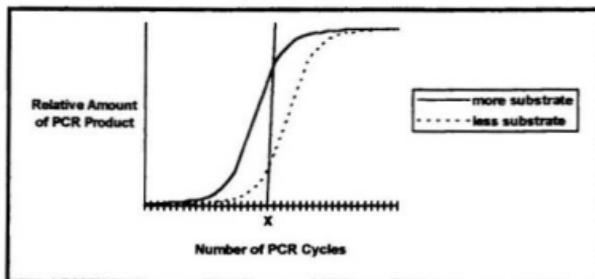
Figure 1-2: Dependence of PCR on the amount of substrate originally present.

1.10.4 Priming Efficiency

In the TCR-family-specific or gene segment-specific PCR used with radiolabelled primers (see Chapter 3), the efficiency of the individual primers must be determined. It is possible that the different TCR primers amplify with varying efficiencies. Failure to correct for these amplification biases could lead to spurious conclusions as to the relative expression levels of various TCR segments (Daniel & Haegert, 1996).

1.11 Summary

1.1 - Immunology is the study of the immune system, a system by which an organism defends itself from foreign pathogens.
  - Though the concept of immunity dates back hundreds of years, fundamental understanding of immune mechanisms have only come about in the last century.

1.2 - The immune system can be divided into two categories: innate and acquired immunity. It is acquired immunity which is responsible for the hallmarks of immunity: memory, specificity, self/non-self recognition and diversity. T-cells form one component that contributes to acquired immunity.

1.3 - T-cells can be divided into two classes based on their differential expression of surface molecules: $CD4^+$ and $CD8^+$ cells. These cells have different effector functions. Upon activation, they differentiate for the most part into Th and Tc cells, respectively.
  - $CD4^+$ cells are the cells of interest in this thesis.
  - As a broad generalization, Th cells, when activated, provide "help" to other immune cells, thus enhancing the immune response.

1.4 - The TCR is a protein expressed on the surface of T-cells. It is the receptor specific for antigen presented by MHC class I and class II molecules.
  - The most variable part of the TCR is the TCR combining site.

1.5 - CD4$^+$ cells recognize Ag when it is presented in association with class II MHC molecules.
   - Class II MHC molecules present exogeneous antigen processed via the endocytic pathway.

1.6 - T-cell precursors originate in the bone marrow from hematopoietic stem cells.
   - The germline TCR DNA contains numerous gene segments which rearrange. This process of rearrangement contributes to the TCR diversity.
   - The TCR β-chain has V, D and J gene segments.

1.7 - The peripheral T-cell receptor repertoire represents the total number of different TCRs available among peripheral T-cells for potential recognition of antigen.
   - Many different mechanisms contribute to this diversity, including gene rearrangements, thymic selection, superantigen, allelic polymorphisms, N-nucleotide addition, junctional diversity and exposure to foreign antigens.

1.8 - Multiple sclerosis is a putative autoimmune disease in which autoreactive T-cells are believed to attack myelin components causing demyelination in the central nervous system.

1.9 - In the case of multiple sclerosis and other putative autoimmune diseases, study of the TCR repertoire may lead to new highly specific immunosuppressive therapies.

1.10- A PCR has three phases: exponential, quasi-linear and plateau
   - Certain unavoidable factors associated with the PCR cause the reaction
     conditions to become limiting and the plateau phase to occur.
   - The main difficulty associated with quantitative PCR, is the actual
     quantitation itself.
   - For quantitative PCR, the reaction must be stopped before the plateau
     phase is reached.
   - For quantitative family-specific or gene segment-specific PCR, testing the
     efficiency of the individual primers is essential.

2. HYPOTHESES

2.1 First Hypothesis

2.1.1 Rationale

It is generally believed that mature, naive T-cells entering the peripheral blood from the thymus express a distinct TCRBV and TCRBJ repertoire, partly accounted for by gene rearrangements, positive selection, negative selection, allelic differences and possibly endogenous superantigen (see Chapter 1).

Upon recognition of antigen or superantigen in the periphery, these T-cells proliferate. Some become effector cells which hopefully aid in the elimination of pathogens. Others become memory cells.

Different TCRBV and TCRBJ segments will preferentially bind nominal antigen in association with self-MHC (Nanda *et al.*, 1992; Smith *et al.*, 1994 and Stryuk *et al.*, 1995). Thus, which cells become activated and differentiate into memory cells is at least partly dependent on the TCRBV and TCRBJ genes expressed. As a result, memory cells may express a TCR repertoire that is skewed in relation to the expressed naive cell repertoire. It is reasonable, therefore, to assume that environmental factors, including past and present

illnesses, vaccinations and asymptomatic infections may skew the TCR repertoire.

Memory cells and naive cells can be distinguished based on their cell surface expression of two CD45 isoforms. CD45RA is expressed on naive cells, and CD45RO is expressed on memory cells (see Section 1.6.5). Recent evidence for "revertant" cells (see Section 1.6.5) may refute the use of these markers for distinguishing naive and memory cells. The implications of these findings are discussed in Chapter 5.

This leads to the first hypothesis: **The TCRBJ and the TCRBV repertoires are significantly different for $CD4^+CD45RA^+$ (naive) and $CD4^+CD45RO^+$ (memory) T-cells isolated from the same individual.**

2.1.2 Experimental Design

2.1.2.1 For the TCRBJ Repertoire

1) Isolate $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$ T-cells from unrelated individuals.

2) Analyze the TCRBJ repertoire for each T-cell subset.

3) Correct data for priming efficiency.

4) Use Pearson correlation analysis to analyze the data statistically.

2.1.2.2 For the TCRBV Repertoire

1) Isolate $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$ T-cells from unrelated
individuals.

2) Analyze the TCRBV repertoire for each T-cell subset.

3) Correct data for priming efficiency.

4) Use Pearson correlation analysis to analyze the data statistically.

2.1.3 Possible Interpretation of Results

2.1.3.1 For TCRBJ and TCRBV

1) If no differences are demonstrated between the TCRBJ or TCRBV
repertoires of naive and memory T-cells, then it can be concluded that
antigenic exposure does not affect the overall repertoires. However, an
important potential caveat is that the methodology may be insufficiently
sensitive to detect subtle differences in TCR repertoires between T-cell
subsets. An alternate possibility is that the phenotypic markers do not
adequately divide T-cells into distinct subsets.

2) If differences are demonstrated between the TCRBJ or TCRBV
repertoires of the two subsets, then it can be concluded $CD4^+CD45RO^+$

and CD4⁺CD45RA⁺ T-cells have different TCR repertoires.

2.2 Second Hypothesis

2.2.1 Rationale

In the thymus, both positive and negative selection require T-cell interaction with self-MHC molecules associated with self-peptide as discussed in Section 1.6.

Since the TCRBV molecules are believed to be involved directly in binding to MHC molecules, the naive T-cell TCRBV repertoire may be dependent on MHC haplotype as a result of thymic selection. Evidence in support of this statement for total T-cells and specific T-cell subsets, but not for naive T-cells has been obtained by various researchers (Gulwani-Akolkar et al., 1995; Gulwani-Akolkar et al., 1991; Akolkar et al., 1992 and Uhrberg & Wernet, 1996).

Recent evidence suggests that TCRBJ segments are also involved in binding to the MHC (Garboczi et al., 1996). Thus, it would be legitimate to extend the above reasoning to the TCRBJ segment and conclude that the naive T-cell TCRBJ repertoire may be dependent on MHC haplotypes. In contrast to TCRBV, the TCRBJ segments are believed to be involved directly in binding to peptides presented by the MHC molecules. It is believed that in the thymus, not all self-peptides are equally represented and that dominant self-peptides play a

particular role in thymic selection (Walser-Kuntz et al., 1995b). Also, it is known that different MHC molecules present different self-peptides. From these two observations, it can be hypothesized that since various TCRBJ segments bind dominant self-peptides with differential avidity and the self-peptides presented are MHC-dependent, the naive T-cell TCRBJ repertoire would also be dependent on the MHC of the individual. It was decided to focus on naive rather than total memory CD4$^+$ T-cells as it was felt a priori that a genetic/HLA effect would more likely be demonstrable on T-cells that are presumed to be unaffected by post-thymic events.

To summarize, the second hypothesis is as follows: **genetic factors, particularly HLA molecules, have an effect on the CD4$^+$CD45RO$^-$ (naive) T-cell TCRBJ repertoire**.

2.2.2 Experimental Design

2.2.2.1 Demonstration of Genetic Effects on the Naive T-Cell TCRBJ Repertoire

1) Isolate CD4$^+$CD45RO$^-$ cells from sets of healthy monozygotic twins.

2) Analyze the TCRBJ repertoires of naive T-cells (CD4$^+$CD45RO$^-$ cells).

3) Correct data for priming efficiencies.

4) Statistically analyze the data (the method of analysis will be further

discussed in Chapters 4 and 5.)

2.2.2.2 Demonstration of HLA Effects on the Naive T-Cell TCRBJ Repertoire

1) Isolate CD4⁺CD45RO⁻ cells from three families (9, 7 and 6 members).

2) Analyze the TCRBJ repertoires of naive T-cells (CD4⁺CD45RO⁻ cells).

3) Correct data for priming efficiencies.

4) Statistically analyze the data (the method of analysis will be further

discussed in Chapters 4 and 5.)

2.2.3 Possible Interpretation of Results

2.2.3.1 Demonstration of Genetic Effects on the Naive T-Cell TCRBJ Repertoire

1) If the twin pairs demonstrate more similar TCRBJ repertoires than do

unrelated individuals, it can be concluded that genetic factors influence

the TCRBJ repertoire of naive T-cells.

2) If the twin pairs do not demonstrate more similar TCRBJ repertoires

than the unrelated individuals, it can be concluded no genetic effect is

detected by the quantitative PCR methodology.

2.2.3.2 Demonstration of HLA Effects on the Naive T-Cell TCRBJ Repertoire

1) If the HLA identical siblings do not demonstrate more similar naive T-cell TCRBJ repertoires than their HLA non-identical counterparts, one possible conclusion is that HLA has little effect on the naive T-cell repertoire. Alternatively, due to sharing of both environments and various non-HLA genes by siblings, HLA effects on the repertoire may be masked, or an HLA effect may not be detectable by the method used.

2) If the HLA identical siblings demonstrate more similar TCRBJ repertoires than their HLA non-identical counterparts, it can be concluded that HLA influences the TCRBJ repertoire of naive T-cells.

2.3 Third Hypothesis

2.3.1 Rationale

Autoimmunity reflects an immune response against self-antigens. Multiple sclerosis is believed to be a T-cell mediated autoimmune disease, in particular Th1 cells have been implicated in the pathogenesis of MS with secretion of cytokines and activation of macrophages (Abbas et al., 1996). It is likely that macrophages damage the myelin and lead to the focal demyelination in the central nervous system, typical of MS.

Ascertainment of patients from various MS clinics indicate that the concordance rate for MS among monozygotic twin pairs is 30%, i.e. 70% of identical pairs are discordant for MS; discordance means that one member of a twin pair has MS and one is healthy. The usual explanation for discordance is that the affected twin members but not the healthy twin members were exposed to environmental factor(s) that triggered the onset of MS. An alternate explanation is that random or stochastic events during thymic selection led to different TCR repertoires between the discordant twin members and the different repertoires are responsible for development/non-development of MS (Moller et al., 1990).

If evidence were obtained in support of the second hypothesis, i.e. that genetics influence the naive T-cell TCRBJ repertoire, one would expect healthy twin pairs and discordant twin pairs to have similar naive T-cell repertoires unless intrathymic selection or other factors in multiple sclerosis alter(s) the repertoire of naive T-cells. The third hypothesis is that **twin pairs discordant for MS have less similar CD4$^+$CD45RO$^-$ (naive) T-cell TCRBJ repertoires than do healthy twin pairs**.

Interestingly, an association has recently been demonstrated between rheumatoid arthritis, another autoimmune disease, and the TCRBJ repertoire of naive T-cells (Walser-Kuntz et al., 1995b).

2.3.2 Experimental Design

2.3.2.1 Multiple Sclerosis

1) Isolate CD4⁺CD45RO⁻ cells from sets of monozygotic twins

discordant for multiple sclerosis.

2) Analyze the TCRBJ repertoire of the naive T-cells.

3) Correct data for priming efficiency

4) Statistically analyze the data from discordant twin pairs and healthy

twin pairs (the method of analysis will be further discussed in Chapters 4

and 5.)

2.3.3 Possible Interpretation of Results

2.3.3.1 Multiple Sclerosis

1) If twins discordant for multiple sclerosis demonstrate less similar naive

T-cell TCRBJ repertoires than do healthy twins, then the naive T-cell

TCRBJ repertoire may be altered in multiple sclerosis.

2) If twins discordant for multiple sclerosis do not demonstrate less

similar TCRBJ repertoires than do healthy twins, then either the TCRBJ

repertoires are not altered in multiple sclerosis, or the methodology is

insufficiently sensitive to detect minor differences in the TCRBJ repertoire

between individuals. Alternatively, since twins have identical

genetic makeups and usually share similar environments at an early age,

disease related alterations in the TCRBJ repertoires may be masked.

2.4 Summary

2.1 - First hypothesis: The TCRBJ and TCRBV repertoires are significantly different between naive and memory T-cells isolated from the same individual.

2.2 - Second hypothesis: There exists a genetic and in particular, an HLA effect on the naive T-cell TCRBJ repertoire.

2.3 - Third hypothesis: The naive T-cell TCRBJ repertoire is altered in multiple sclerosis.

3. MATERIALS AND METHODS

The experiments presented in the following sections were designed with the following objective: to determine the level of expression of each of the 13 TCRBJ gene segments and/or the level of expression of each of 24 TCRBV gene families in naive and/or memory CD4$^+$ T-cells.

3.1 Blood Collection

The first step in any study involving human subjects is to gain the approval of the institution's ethics committee, in this case the Human Investigations Committee (HIC) of the Faculty of Medicine, Memorial University of Newfoundland (St. John's, NF, Canada). Formal approval was, therefore, obtained. The study was then explained to the volunteers, and time was taken to answer their questions and address their concerns. After the consent forms were signed, 10mL EDTA Vacutainer tubes were used to collect either 40mL or 60mL of peripheral blood, according to Table 3.1. A variety of anticoagulants are available; EDTA, however, is optimal for the purpose of this study (Panaccio *et al.*, 1993).

Table 3.1: Amount of Blood Required for Each Experiment

Experiment	Volume of Blood
1) CD45RO vs. CD45RA	60mL
2) Twins vs. unrelated individuals	40mL
3) HLA identical vs. HLA non-identical siblings	40mL

The 10mL of blood from each vacutainer tube was transferred to a 50mL polypropylene tube. Each Vacutainer was then rinsed with PBS (phosphate buffered saline). This rinse solution (PBS) was then added to the polypropylene tube containing the blood, creating a 1:1 dilution. This rinse was performed to ensure the transfer of a maximum number of lymphocytes to the polypropylene tube.

3.2 Isolation of Mononuclear Cells (Including Lymphocytes)

3.2.1 Theory

Ficoll-Paque contains Ficoll 400, sodium diatrizoate and calcium EDTA. Its osmolarity and density are optimal for the isolation of mononuclear cells. Mononuclear cells consist of lymphocytes and monocytes. After a tube of blood is centrifuged over Ficoll-Paque, the most dense particles can be found at the bottom of the tube and the least dense at the top. Four layers are created as a result of centrifugation. From bottom to top or in order of decreasing density these layers are red blood cells and platelets, Ficoll-Paque, white blood cells and plasma. The thin layer of white blood cells or mononuclear cells is often referred to as the buffy coat.

3.2.2 Materials

1) Ficoll-Paque (Pharmacia, Baie d'Urfe, Quebec, Canada)

2) PBS

3.2.3 Method

Using a graduated pipette, Ficoll-Paque was added beneath the blood, creating two distinct layers: whole blood and Ficoll-Paque. This was centrifuged at 1500 rpm for 35 min. The buffy coat was removed (along with a small amount of plasma) and Ficoll-Paque and placed in a new 50mL polypropylene tube. Mononuclear cells from a given individual were pooled into this 50mL tube, and the tube was centrifuged at 2000 rpm for 30 min. The majority of the liquid was decanted; the white blood cell pellet remained at the bottom of the tube. The pellet and the remaining liquid were transferred to a 12x75mm polypropylene tube. In order to ensure the transfer of all the cells, the 50mL tube was rinsed three times with 0.5mL of PBS, and this PBS was transferred to the polystyrene tube containing the mononuclear cells. The final volume was approximately 2mL.

3.3 Isolation of Cells Expressing CD4

3.3.1 Theory

According to Dynal (the manufacturer of Dynabeads), Dynabeads are "uniform supermagnetic polystyrene spheres" (Dynal, 1995). These spheres are coated with antibodies against a specific cell surface protein, for example CD4 and CD8. When beads are mixed with a heterogeneous cell population, the antibody on the beads is capable of binding to the cell surface protein for which it is specific. A magnet is then placed alongside the mixture. The Dynabeads are attracted to the magnet, (i.e., the Dynabeads and the cells to which they are attached via antibody are pulled towards the magnet). The magnet holds the beads and attached cells to the side of the tube facilitating the removal of the PBS and unbound cells. The magnet is then removed and the beads rinsed with PBS and resuspended in PBS; the resulting mixture contains only cells expressing the cell surface protein for which the Dynabeads are specific.

Dynabeads are satisfactory for the preparation of lymphocyte subsets based on the expression of various cell surface proteins, obtaining >90% purity by positive selection, see Section 3.1.4 (Manyonda *et al.*, 1992). There is no evidence for non-specific binding (Patell & Rickwood, 1995).

The optimal conditions for the isolation of naive and memory CD4$^+$ T-cell subsets using Dynabeads were determined through experimentation, and these

conditions are shown in Table 3.2. Published parameters are very similar (Patell & Rickwood, 1995); these results are also presented in Table 3.2.

Table 3.2: Conditions for Isolation of CD4⁺ T-cells with Dynabeads

	Test conditions used in this thesis	Published test conditions
Time	1 hour	1 hour
Temperature	4°C	25°C
Mixing	End-over-end rotation	End-over-end rotation
Bead to Cell Ratio	Not measured	10:1 (for positive selection)
Total Volume (PBS+cells+dynabeads)	2mL for CD4 0.5mL for CD45 isoforms	1mL

The major difference between the two sets of test conditions is the bead to cell ratio. Due to the large number of samples studied and the cost of Dynabeads, a lower bead to cell ratio was used for this study, and a larger number of cells was used for CD4$^+$ T-cell isolation to ensure adequate number of CD4 cells for the study. (In Section 3.4, positive and negative isolation are discussed; please note that for negative isolation, a much higher bead:cell ratio was used.)

In order to separate these CD4$^+$ cells into subsets based on their CD45 isoform, it was necessary to remove the CD4 Dynabeads. Detachabead removes CD4 or CD8 bound Dynabeads from cells very efficiently (Manyonda *et al*., 1992).

3.3.2 Materials

1) CD4 Dynabeads (Dynal, Lake Success, New York, USA)

2) Detachabead (Dynal, Lake Success, New York, USA)

3) PBS

3.3.3 Method

To the 2mL of mononuclear cells obtained from each volunteer, 30 million beads coated with anti-CD4 were added. This represents an approximate bead to cell ratio of 1:1. The cell/bead mixture was incubated for 1 hour at 4°C and rotated end-over-end. The CD4$^+$ cells were then separated from the mixture

using a magnet. The cells and the beads bound to them were rinsed and resuspended in 1mL of PBS. Fifty microliters of Detachabead was then added. After incubation for 1 hour at room temperature, a magnet was used to separate the Dynabeads from the mixture. The remaining mixture contained $CD4^+$ cells which were then ready to be separated based on CD45 isoform.

3.4 Isolation of Cells Expressing Various CD45 Isoforms

3.4.1 Theory

In order to separate the $CD4^+$ cells based on their expression of the CD45 isoforms, a primary and secondary antibody system was required, because Dynal does not manufacture Dynabeads specific for the CD45 isoforms. In this case, a primary antibody specific for one of the cell surface CD45 isoforms of interest was allowed to bind to the cells. Dynabeads coated with secondary antibody specific for the primary antibody were then added.

Using Dynabeads, cells can be either positively or negatively selected. (The reasons for choosing either positive or negative selection will be discussed in section 3.7.) After a sufficient incubation of cells with Dynabeads, a magnet divides the cells into two populations: the cells that bound to the Dynabeads, and those that did not. The population of cells that bound the antibody expresses the cell surface protein for which the antibody is specific. Isolation of this population is what is referred to as positive selection; the resulting population is pure, and it

only contains cells expressing a particular surface protein. Isolation of cells that did not bind the antibody is referred to as negative selection; the resulting population is depleted of cells expressing a particular surface protein.

Positive selection yielded either CD4$^+$CD45RO$^+$ or CD4$^+$CD45RA$^+$ populations depending on the antibodies used. Negative selection yielded either CD4$^+$CD45RO$^-$ or CD4$^+$CD45RA$^-$ populations depending on the antibodies used. The purity of T-cell subsets isolated using Dynabeads was determined by flow cytometry, and the results are presented in Section 4.1.

3.4.2 Materials

1) Primary Ab: 25µg/mL mouse IgG2a anti-CD45RO

25µg/mL mouse IgG1 anti-CD45RA (Becton Dickinson)

2) Dynabeads: rat anti-mouse IgG2a coated Dynabeads (Dynal, Lake Success, New York, USA)

rat anti-mouse IgG1 coated Dynabeads (Dynal, Lake Success, New York, USA)

3) PBS

3.4.3 Method

Fifty microliters of primary antibody was added to the CD4$^+$ cells, the cells were incubated on ice for 30 min., and then centrifuged at 4°C at 2000 rpm for 15 min. The cells were pelleted; the supernatant containing the excess antibody was discarded, and the cells were resuspended in 0.5mL PBS. Dynabeads coated with secondary antibody (rat anti-mouse IgG2a or rat anti-mouse IgG1) were added, and this mixture was incubated for an hour at 4°C with end-over-end rotation. T-cells bearing the various CD45 isoforms were then separated using a magnet.

3.5 RNA Extraction

3.5.1 Theory

Ribonucleases are a major obstacle in RNA extraction. These enzymes are very stable and very active (Ausubel *et al.* editors, 1996); their removal or inactivation is a necessary step in any protocol involving RNA.

MacDonald and colleagues (1987) separate RNA extraction into three distinct steps: 1) inhibition of endonucleases, 2) deproteination of RNA and 3) physical separation of RNA from the other cell components (1987).

Guanidinium thiocyanate and guanidinium chloride are among the best known and powerful protein denaturants (Ausubel *et al.* editors, 1996). DTT

(dithiothreitol) or 2-mercaptoethanol act to break disulfide bonds, bind irreversibly to ribonucleases and are often used to enhance denaturation (MacDonald *et al.*, 1987). Protein denaturation achieves steps one and two above. First, endonucleases are proteins, therefore, they are susceptible to and inhibited by denaturation. Second, RNA-binding proteins are denatured, leading to the release of the nucleic acid.

A high concentration of guanidinium salts has a second function. The high osmolarity lyses the cells and frees the intracellular contents.

Previously, completion of the third step was not only separated from the first two but lengthy and complicated. An example of one such method is the separation of RNA using a cesium chloride gradient, which requires ultracentrifugation (Chirgwin *et al.*, 1979). Now, there is a single-step method available which completes all three goals at once. It was first described by Chomczynski and Sacchi (1987).

This single-step method has been modified, and an appropriate RNA extraction reagent is distributed by Biotecx as Ultraspec. Ultraspec contains 14M guanidinium salts and urea (which also acts as a denaturant and provides a concentration suitable for cell lysis), detergents, buffers, phenol and stabilizers.

The theory behind the single-step method is that under conditions of high salt concentration and a pH of 4 (maintained by the buffers) and upon the addition of chloroform, RNA physically separates from the other cell components. Small

fragments of DNA (150 bases to 10 kbases) and protein remain in the organic layer. Some protein and some large DNA molecules remain at the interface, but the RNA remains soluble in the aqueous layer (Ausubel *et al.* editors, 1996).

The aqueous layer is easily removed with a pipette and precipitated in isopropanol. The RNA is stored in DEPC-treated water. DEPC (diethyl pyrocarbonate) inactivates any endonucleases present in the water, protecting the RNA from degradation (Ausubel *et al.* editors, 1996).

3.5.2 Materials

1) Ultraspec (Biotecx, Houston, Texas, USA)

2) chloroform

3) isopropanol

4) 75% ethanol in DEPC-treated water

3.5.3 Method

To the isolated cells, 1mL of Ultraspec and 0.1mL of chloroform were added in a 1.5mL tube. This was shaken vigorously for 15 seconds and placed at 4°C for 15 min. The sample was then centrifuged briefly to accelerate the separation of the organic and aqueous layers. The majority (80-90%) of the aqueous layer was removed and diluted 1:1 with isopropanol and frozen at -70°C

overnight. The next day, the sample was thawed and centrifuged at 4°C at

14000 rpm for 15 min. The supernatant was discarded and the pellet was rinsed

three times with 0.5mL of 75% ethanol in DEPC-treated water. The pellet was

then dried and resuspended in 20μL of DEPC water. This sample contained total

cellular RNA and was frozen at -70°C until cDNA synthesis was carried out.

Absorbance at 260nm and 280nm was determined on a spectrophotometer to

assess the quantity as well as the purity of the RNA.

3.6 cDNA Synthesis

3.6.1 Theory

The First-Strand cDNA Synthesis Kit contains DTT, primer and

Bulk-First-Strand Reaction Mix. DTT (dithiothreitol or Cleland's reagent) is a

reductant; it reduces any proteins present, including endonucleases. The primer

used was Not I-d(T)$_{18}$ bifunctional primer. The series of eighteen T residues at

the 3' end of this primer bind to the poly-A tail of the RNA and allow for cDNA

synthesis. The sequence of the Not I-d(T)$_{18}$ bifunctional primer is as follows:

5'-d[AAC TTG AAG AAT TCG CGG CCG CAG GAA T$_{18}$]-3'

The Bulk-First-Strand Reaction Mix contains cloned FPLC pure Murine Moloney Virus Reverse Transcriptase, RNA guard, RNase/DNase-Free BSA (bovine serum albumin), dATP, dCTP, dGTP and dTTP in aqueous buffer. The reverse transcriptase is optimally active at 37°C and is most efficient on denatured RNA. (RNA can be denatured by heating it to 65°C.)

3.6.2 Materials

1) First-Strand cDNA Synthesis Kit (Pharmacia, Baie d'Urfe, Quebec, Canada)

3.6.3 Method

Twenty microliters of total RNA was transferred to a 0.5mL tube and heated to 65°C for 10 min. To recapture any condensation, the sample was then cooled for 5 min. and briefly centrifuged. To this tube, 1μL of DTT, 1μL of Not I-d(T)$_{18}$ bifunctional primer and 11μL of Bulk-First-Strand Reaction Mix were added. This solution was incubated at 37°C for 60 minutes. A cDNA:RNA heteroduplex was formed and stored at -70°C until PCR amplification.

3.7 Primer Labeling

3.7.1 Theory

As discussed earlier (see Chapter 1), the ultimate goal of these experiments was to determine the relative amount of each TCRBJ segment present in RNA samples from different individuals and/or from different cell subsets. To do this, a method of quantification was necessary. Radiolabeling the reverse primers used in PCR is one of the methods of obtaining this goal.

The reaction is fairly straightforward and very efficient. T4 PNK (polynucleotide kinase) catalyzes the transfer of the terminal (γ) phosphate of ^{32}P-labeled ATP to the 5' hydroxy termini of the DNA primer. The enzyme T4 PNK is a product of T4 pseT gene; it is active at 37°C and is inactivated at 65°C.

After radiolabeling, it was necessary to separate the radiolabelled primers from the unincorporated nucleotides. This not only decreased the background counts, but prevented any unnecessary radiation exposure to the researchers.

The separation was accomplished through the use of Bio-Spin Chromatography Columns. The columns are packaged containing 0.8mL SSC (sodium saline citrate) and 2% sodium azide, which must be removed before the separation process can be initiated. BioGel P polyacrylamide gel matrix contained within the column permits separation based on size. The radiolabelled

primers are large and readily pass through the column, whereas the unincorporated nucleotides are held within the matrix.

3.7.2 Materials

1) Bio-Spin Chromatography Columns (Bio-Rad, Hercules, California, USA)

2) Reverse primers for PCR amplification (discussed further in section 3.5)

3) T4 PNK (Polynucleotide kinase) and 5X Forward Reaction Buffer (GibcoBRL, Burlington, Ontario, Canada)

4) ^{32}P γ labeled ATP (Mandell, Guelph, Ontario, Canada)

5) DEPC treated water

3.7.3 Method

Six and four-tenths of a microliter of 8.25µM of primer were added to a 0.5mL tube. To this was added 1.6µL of T4 PNK, 5µL of PNK buffer, 2-6µL radiolabelled ATP and enough DEPC treated water to give a total volume of 25µL. This was heated to 37°C for 60 min. and then to 65°C for 10 min. The Bio-Spin columns were centrifuged at 2300 rpm for 5 min. The columns were then washed three times with SSC, discarding the buffer each time. The mixture

containing the radiolabelled primer was carefully added to the center of the column, which was then centrifuged at 2300 rpm for 5 min. The radiolabelled primer was collected in a 1.5mL tube. The column containing the excess nucleotides was discarded. The purified radiolabelled primer was diluted to $0.075\mu M$ and stored at -20°C.

3.8 PCR Amplification

3.8.1 Theory

Each TCRBJ gene segment comprises only a small amount of the total RNA isolated from the cells. Once a cDNA strand had been synthesized using the total RNA as a template, PCR (polymerase chain reaction) techniques are used to amplify a specific segment of this DNA (Darnell, 1990).

PCR amplifications were performed as described by Choi *et al.* (1989) with a few modifications, (see Section 3.8.3). Most of the experiments used in this thesis involved the TCRBJ repertoire. Though some work was done on the TCRBV repertoire, the description of the method will focus on the TCRBJ gene segments for the sake of simplicity. It should be noted, however, that all the methods can be easily extrapolated to the TCRBV gene families by taking into account the number of primers used in TCRBV family analysis.

For each TCRBJ repertoire analysis, 14 PCRs were performed, one for each of the TCRBJ gene segments and one as a negative control. The negative control contained all the required reagents, but lacked cDNA to ensure that there was no contamination of samples.

The TCRBJ-specific primers were added to the 14 individual reaction tubes; a different primer to each tube. The TCRBJ primer specific for the 2S1 gene segment was added to the negative control as this appeared to be the most efficient primer. All the other reagents (everything except the primers) were added to a 2 mL tube and mixed thoroughly to form a master mix or a cocktail. The appropriate amount of this cocktail was then aliquoted to each of the 13 reaction tubes. The cocktail was aliquoted to the negative control before the addition of the cDNA. A master mix was used instead of adding each reagents individually to the reaction tubes, so as to reduce tube to tube variation in the concentration of the reagents.

3.8.2 *Materials*

1) Taq DNA polymerase kit (Promega, Madison, Wisconsin, USA)

2) dNTP's

3) distilled and autoclaved water

4) radiolabelled primer: Cα2 and Cβ (see Table 3.3)

6) primer:　specific for each TCRBJ gene segment (see Table 3.4)

　　　　　　　specific for each TCRBV gene segment (see Table 3.5)

　　　　　　　specific for each TCRBC and TCRAC region (see Table 3.3)

Table 3.3: *TCR Constant Region Primers*

Primer	Sequence (5' to 3')	Reference
TCRAC sense (Cα1)	GGA CCC TGA CCC TGC CGT GTA CC	Choi et al, 1989
TCRAC antisense (Cα2)	ATC ATA AAT TCG GGT AGG ATC C	Choi et al, 1989
TCRBC antisense (Cβ)	TTC TGA TGG CTC AAA CAC	Choi et al, 1989

Table 3.4 : TCRBJ Primers

Primer (TCRBJ)	Sequence	Reference
1S1	ACA AGG CAC CAG ACT CAC AGT	Pannetier *et al.*, 1993
1S2	TTC GGG GAC CAA GTT AAC CGT TGT	Pannetier *et al.*, 1993
1S3	GTT GCT CAC TGT TGT AGA GGA CC	Pannetier *et al.*, 1993
1S4	CAG TGG AAC CCA GCT CTC TCT CTT	Pannetier *et al.*, 1993
1S5	TGA TGG AAC TCG ACT CTC C	Pannetier *et al.*, 1993
1S6	AAT GGG ACC AGG CTC ACT GTG ACA	Pannetier *et al.*, 1993
2S1	TGA GCA GTT CTT CGG GCC AGG	Pannetier *et al.*, 1993
2S2	TCT AGG CTG ACC GTA CTG GAG GAC	Pannetier *et al.*, 1993
2S3	CGC AGT ATT TTG GCC CAG GCA	Pannetier *et al.*, 1993
2S4	TTC AGT ACT TCG GCG CGG GGA	Pannetier *et al.*, 1993
2S5	GCG GCT CCT GGT GCT CGA	Pannetier *et al.*, 1993
2S6	GAC TTT CGG GGC CGG CAG CAG	Pannetier *et al.*, 1993
2S7	CGG GCA CCA GGC TCA CGG TCA	Pannetier *et al.*, 1993

Table 3.5: TCRBV Primers

Primer (TCRBV)	Sequence	Reference
1	GCA CAA CAG TTC CCT GAC TTG CAC	Choi et al, 1989
2	TCA TCA ACC ATG AAG GCC TGA CCT	Choi et al, 1989
3	GTC TCT AGA GAG AAG AAG GAG CGC	Choi et al, 1989
4	ACA TAT GAG AGT GGA TTT GTC ATT	Choi et al, 1989
5	CAG AGA AAC GGA AAC TTC CCT GGT CGA	Wucherpfenning et al, 1990
6	GGG TGC GGC AGA TGA CTC AGG GCT GCC CAA	Wucherpfenning et al, 1990
7	CCT GAA TGC CCC AAC AGC TCT C	Choi et al, 1989
8	ATT TAC TTT AAC AAC AAC GTT CCG	Choi et al, 1989
9	CCT AAA TCT CCA GAC AAA GCT CAC	Choi et al, 1989
10	CTC CAA AAA CTC ATC CTG TAC CTT	Choi et al, 1989
11	TCA ACA GTC TCC AGA ATA AGG ACG	Choi et al, 1989
12	AAA GGA GAA GTC TCA GAT	Choi et al, 1989
13	GCA TGA CAC TGC AGT GTG CCC	Genevee et al., 1992
14	GTC TCT CGA AAA GAG AAG AGG AAT	Choi et al, 1989
15	AGT GTC TCT CGA CAG GCA CAG GCT	Choi et al, 1989
16	AAA GAG TCT AAA CAG GAT GAG TCC	Choi et al, 1989
17	CAG ATA GTA AAT GAC TTT CAG	Choi et al, 1989
18	GAT GAG TCA GGA ATG CCA AAG GAA	Choi et al, 1989
19	CAA GAA ACG GAG ATG CAC AAG AAG CGA TTC	Choi et al, 1989
20	AGC TCT GAG GTG CCC CAG AAT CTC	Choi et al, 1989
21	TCC AAC CTG CAA GGC TTG ACG ACT	Genevee et al., 1992
22	AAG TGA TCT TGC GCT GTG TCC CCA	Genevee et al., 1992
23	GCA GGG TCC AGG TCA GGA CCC CCA	Genevee et al., 1992
24	CCC AGT TTG GAA AGC CAG TGA CCC	Genevee et al., 1992

3.8.3 Method

The sequences for the 13 TCRBJ primers were derived from the work of Pannetier *et al.* (1993). These investigators used the TCRBJ primers as antisense primers. For the purpose of this thesis, the complementary sequences were derived and synthesized.

All the primer sequences for both TCRBJ and TCRBV were sent to GenBank to verify that the sequences were indeed specific for the TCRBV and TCRBJ gene segments.

Five microliters of the TCRBJ primers were added to each of 14 different 0.5mL tubes. Enough cocktail was prepared for 14 PCR reactions (see Table 3.6). As will be discussed shortly, TCRAC was used as an internal control; it is amplified simultaneously with the TCRBJ (or BV) segments. Before the cDNA was added to the cocktail, 14μL of the cocktail was added to the negative control. The cDNA was then added to the cocktail, and 15μL of the cocktail was added to each of the remaining tubes. The tubes were placed in a Perkin-Elmer Cetus Model 480 thermocycler under the conditions described in Table 3.7. The number of cycles was dependent on the plateau effect; the results are presented in Table 5-1.

Table 3.6: Reagents for PCR Amplification

Reagent	Volume Required per Reaction	Final Concentration
10X Buffer	2µL	1X
dNTP's	1.2µL	240µM
MgCl$_2$	1.2µL	1.5mM
TCRAC1	0.4µL	0.165µM
TCRAC2	0.4µL	0.15µM
TCRBC	0.4µL	0.15µM
TCRAC2 radiolabelled	0.4µL	0.015µM
TCRBC radiolabelled	0.4µL	0.015µM
Taq (DNA polymerase)	0.16µL	0.75 units
cDNA	1µL	0.2 ng/µL
Water	7.44µL	

Table 3.7: PCR Amplification Conditions

Step	Time	Temperature
Initial Denaturation	5 minutes	94°C
Denaturation	1 minute	94°C
Annealing	1 minute	55°C
Extension	1 minute	72°C
Final Extension	7 minutes	72°C

3.9 Quantitation of PCR Products

3.9.1 Theory

One of the obstacles in quantitative PCR is the development of an adequate and precise method of quantitation of PCR products (Choi *et al.*, 1989).

The primers used in this method were radiolabelled as discussed earlier. After the PCR, the unincorporated primers were separated from the PCR product by agarose gel electrophoresis. Electrophoresis separates DNA molecules based on size. Thus, it not only separates the amplified TCRAC product from the amplified TCRBJ product, but separates these from any other cDNA or DNA present in the solution (including unincorporated primers.)

The electrophoresis was performed on a Submarine Gel Apparatus, and to avoid contamination between the lanes, alternate wells were used. Bromophenol blue was found to interfere with the visibility of the TCRBJ bands. In order to avoid this, the dye was diluted with glycerol and TBE (tris-borate EDTA (ethylene diamine tetraacetic acid)).

Ethidium bromide was added to the buffer in the Submarine Gel Apparatus. Thus, UV light allowed the visualization of the TCRAC band and the TCRBJ band in each lane. The bands were cut out with a scalpel and placed in scintillation vials. Care was taken to clean the scalpel between excisions to

reduce the possibility of contamination. Scintillation fluid was then added to the vials and counts per minute (cpm) were measured in a scintillation counter.

As explained later (see section 3.10.3), the value, used as an indicator of how much of each TCRBJ gene segment was originally present, was percent relative counts (%RC), see Equations 3.1 and 3.2 (Daniel, 1994).

3.9.2 Materials

1) bromophenol blue

2) high gelling temperature agarose (Sigma, Oakville, Ontario, Canada)

3) TBE

4) Submarine Gel Apparatus (Bio-Rad, Hercules, California, USA)

5) ethidium bromide

3.9.3 Method

Nine microliters of PCR product and 1μL of bromophenol blue were added to the alternate wells of a 2% agarose gel, in 0.5X TBE buffer. A voltage of 80-90V was applied for 45 minutes. The gels were then stained with ethidium bromide. The DNA bands were excised using a scalpel and placed in scintillation vials. Five milliliters of Beckman Ready Value Scintillation Fluid was added to the

samples, and the samples were left overnight. The vials were then placed in the scintillation counter, and a reading of counts per minute (cpm) was obtained.

3.10 Testing PCR Variables

3.10.1 Plateau Phase

3.10.1.1 Theory

In Chapter 1, it was shown that for a quantitative PCR, the reaction must never be permitted to proceed beyond the quasi-linear phase. Thus, the number of cycles which progress before the onset of the plateau must be calculated. In this thesis, the TCR repertoire was determined using cDNA as a starting product or substrate. In addition, to test primer efficiencies the substrate required for the PCR is DNA that has been previously PCR-amplified (see Section 3.10.2). Since two different substrates were used (i.e., cDNA and PCR-amplified TCR DNA), the various phases of the PCR were determined using the two different substrates.

3.10.1.2 Method

3.10.1.2.1 Preparation of cDNA Substrate

cDNA was prepared from total cellular RNA (see section 3.6) and was diluted to $0.1\mu g/mL$.

3.10.1.2.2 Preparation of PCR-Amplified TCR DNA

For both the TCRBJ and the TCRBV primers, a representative primer was chosen to test the plateau phase; the criteria used in the selection of this primer will be discussed in Chapter 5. A non-radioactive PCR was used to amplify cDNA using the chosen TCR primer and a suitable antisense primer. This PCR product was diluted to $0.01 \mu M$.

3.10.1.2.3 Testing the Plateau Phase of the PCR

Approximately 30 identical PCR reactions were set up, using the representative TCRBJ and TCRBV primers discussed above and substrate (cDNA or PCR-amplified TCR DNA). The reverse primers were radiolabelled. The reaction mixtures were placed in the thermocycler and the PCR was allowed to proceed for 4 to 42 cycles. After a predetermined number of cycles, two tubes were removed and allowed to complete the final seven minute extension in a separate thermocycler. After electrophoresis and excision of the bands, a cpm reading was obtained from these. A graph was then compiled from which the number of cycles completed before the plateau occurs could be extrapolated.

3.10.2 Primer Efficiency

3.10.2.1 Theory

Chapter 1 and Chapter 5 discuss the importance of evaluating the efficiency of the primers. Without correction for amplification biases, erroneous conclusions may be made concerning the level of expression of various TCRBV families or TCRBJ gene segments in an individual or in a tissue (Daniel & Haegert, 1996)

3.10.2.2 Method

T-cell cDNA was obtained (see section 3.6). A first round PCR using non-radiolabelled primers specific for each of the TCRBJ gene segments was carried out for 40 cycles with no internal control. TCRAC was amplified in a separate reaction tube. The product of each of these first round PCRs was then diluted to 0.01µM. These products were then used as the substrates in a second round PCR. In this second PCR both amplified TCRAC and a TCRBJ were placed in the tube and radiolabelled primers were used. The relative counts were used as an indicator of primer efficiency.

3.11 Methodological Concerns

3.11.1 Cell Stimulation

If the amount of RNA isolated from the cells had been greater, less blood would have been required from the volunteers or more PCR reactions could have been performed with the same amount of blood. One method of increasing the amount of RNA obtained would have been to increase the CD4-specific Dynabead to cell ratio. As mentioned before however, the number of Dynabeads used was limited due to finances available. Another way to increase the amount of RNA obtained would have been to stimulate the cells with mitogen, IL-2, anti-CD3 antibody or a similar reagent. However, published data have shown that at least some of the substances listed may skew the repertoire (Jason, 1996). Therefore, the cells were not stimulated in any way.

3.11.2 Volume of Blood Required

The volume of blood listed in Table 3.1 was the minimum amount of blood necessary to ensure an adequate amount of RNA to complete amplification of the TCRBJ or TCRBV gene segments. These figures were determined through trial and error.

3.11.3 Internal Control

Once a cpm reading was available for each gene segment amplified, a method was necessary to compare the expression levels of the 13 TCRBJ gene segments. The amount of product at the end of a PCR is dependent on a number of factors. These include the quality and the concentration of the cDNA, the quality and the concentration of the reverse transcriptase, Mg ion concentration, temperature variations between the wells of the thermocycler, the efficiency of the thermocycler, etc (Innis *et al.*, 1990). As a result of these differing factors, it was impossible to compare cpm obtained as the products of separate PCRs. Thus, in order to account for the variations between reactions, an appropriate control was needed. Since the TCR is a heterodimer, the amount of β-chain and α-chain mRNA in a cell should be identical. The TCR-α chain was, therefore, used as an internal control. The internal control not only provided an indication of the individual reaction conditions within tubes, but it allowed for a comparison between reaction tubes.

So, in every PCR reaction tube, there were two amplifications underway: TCRBJ (or TCRBV) and TCRAC. There is no *a priori* reason to believe that these two amplifications interfere with one another.

Mathematically, to account for the variations between reactions. Equation 3.1 was used (Daniel, 1995).

$$\text{Relative Counts (RC}_n) = \frac{J\beta_x \text{ cpm}}{C\alpha_x \text{ cpm}} \qquad \text{Equation 3.1}$$

Using this equation, an RC value was obtained for every TCRBJ gene segment, designated RC$_n$ where n = 1-13. To calculate the frequency of each TCRBJ gene segment, equation 3.2 was used (Daniel, 1995).

$$\%RC_n = \frac{RC_n}{\sum_1^{13} RC_n} \times 100 \qquad \text{Equation 3.2}$$

Once again, these conditions and equations can easily be extrapolated to analyze the TCRBV families.

3.11.4 Amount of cDNA Required for PCR

Preliminary experiments established that $1\mu L$ of a $0.2\mu g/mL$ preparation of cDNA was suitable for PCR. Since the initial concentration of cDNA might affect the number of cycles at which the plateau occurs, every cDNA sample was diluted to $0.2\mu g/mL$ before a PCR was performed.

3.11.5 Reproducibility of Experiments

Experiments when repeated from day to day, must give the same results, or the validity of the test procedure must be questioned. Reproducibility tests

were performed, therefore, and statistically analyzed. It was found that the method employed is valid; results are presented in Chapter 4.

3.11.6 Statistics

The major statistic used in these experiments was Pearson correlation analysis. This statistic allows for the comparison of two sets of data to determine similarity. A correlation test gives an r-value, which ranges from -1 to 1. A value of close to $|1|$ indicates the two data sets are linearly related. An r-value close to zero indicates the two data sets are not linearly related (Daniel, 1994). All calculations were performed using the Minitab statistics program (Minitab 10 for Windows).

The statistical analysis described in this thesis was recommended by Dr. V. Gadag (Associate Professor of Biostatistics, Faculty of Medicine, Memorial University of Newfoundland.)

3.11.7 Positive vs. Negative Selection for CD45 Isoform

As discussed in Section 1, naive human T-cells express CD45RA, and memory cells express CD45RO although it is recognized that CD45RA$^+$ T-cells could contain some "revertant" memory T-cells (Richards et al., 1997 and Hargreaves & Bell, 1997). There exists a third set of cells that are CD45RO$^+$ and CD45RA$^+$; that is, they express both isoforms (McElhaney et al., 1995). These

cells are known as early activated T-cells. They do not appear to be as numerous as naive or memory cells. To eliminate these cells negative selection was performed. As discussed earlier, negative selection depletes the mixture of any cells that express a certain cell surface protein. For example, if naive cells were the desired population, CD45RO⁻ cells (rather than CD45RA⁺ cells) would be isolated. In this way, both memory and early activated cells would be depleted from the medium, for both express CD45RO.

At the commencement of the naive and memory T-cell experiments, I had no knowledge of early activated cells and so the first hypothesis was tested using positively selected CD45RA⁺ T-cells.

Table 3.8: Cell Type Isolated for Each Experiment

Experiment	Cell Type Isolated
1) CD45RO vs. CD45RA	CD4$^+$CD45RO$^+$ and CD4$^+$CD45RA$^+$
2) Twins vs. unrelated individuals	CD4$^+$CD45RO$^-$
3) HLA identical vs. HLA non-identical siblings	CD4$^+$CD45RO$^-$
4) Twins pairs discordant for multiple sclerosis vs. healthy twin pairs	CD4$^+$CD45RO$^-$

3.11.8 Correcting for Primer Efficiency

As discussed in Section 3.10.2, the efficiency of each TCRBJ and each TCRBV-specific primer was tested. Once again, the method used to correct for primer efficiency will be discussed for TCRBJ only; the method is, however, easily extrapolated to TCRBV. Every TCRBJ repertoire to be corrected is composed of 13 %RC values, one for each of the 13 TCRBJ segments(see equations 3.1 and 3.2). Relative counts were also used as an indicator of primer efficiency, the relative count value obtained for each primer will be designated RCP in order to avoid confusion. The 13 %RC values were corrected for primer efficiency according to equations 3.3 and 3.4; the 13 resulting corrected values were desigated %RCC.

$$CF_n = \frac{1}{RCP_n} \qquad \qquad \text{equation 3.3}$$

$$\%RCC_n = \frac{\%RC_n x CF_n}{\sum_{1}^{13} \%RC_n x CF_n} x100 \qquad \qquad \text{equation 3.4}$$

3.12 Summary

3.1 - Peripheral blood was collected from volunteers.

3.2 - Lymphocytes were isolated from whole blood.

3.3 - Cells expressing CD4 were isolated from the heterogeneous lymphocyte population using Dynabeads.

3.4 - $CD4^+$ cells expressing specific CD45 isoforms were isolated.

3.5 - The cells were lysed and RNA was extracted from the cells.

3.6 - A cDNA:RNA hybrid was synthesized from the RNA.

3.7 - Primers for use in subsequent PCR were labeled with ^{32}P.

3.8 - Quantitative PCR was performed. This includes a separate reaction for each TCRBV family and/or each TCRBJ gene segment.

3.9 - A cpm reading was obtained for each TCRBV family and/or each TCRBJ gene segment. Equation 3.1 and 3.2 were used to analyze these data.

3.10- The plateau phase and the primer efficiency were tested.

3.11- The following is a list of methodological concerns associated with this procedure: internal controls, amount of cDNA required, statistics, reproducibility, different CD45 isoforms and FACS analysis of the cell subsets.

4. RESULTS

4.1 FACS Analysis of Cell Subsets

Dynabeads have proven to be an efficient and highly specific method to isolate cell subsets based on surface epitopes (Manyonda et al., 1992). Nonetheless, a FACS analysis was performed on a T-cell sample to verify that the correct cell subsets were being isolated. The results proved that Dynabeads effectively isolated $CD4^+$ T-cells from a heterogeneous population of lymphocytes (98.5% were $CD4^+$), and that Dynabeads effectively depleted this population of $CD4^+$ T-cells of $CD45RO^-$ cells, creating a final population of $CD4^+CD45RO^-$ cells (97% were $CD45RO^-$).

4.2 Reproducibility

It was deemed important to establish the extent to which determination of the TCRBJ repertoire is reproducible. Blood was taken from a single subject (1.X.01) and $CD8^+$ T-cells were isolated on a single occasion. Initially, the $CD8^+$ T-cell repertoire of this individual was analyzed on two separate days using separate PCR cocktails but the same DNA. A correlation coefficient was obtained as a measure of the similarity between graphs. A t-test was used to determine whether or not this correlation coefficient indicated similarity or difference. The t-test gave $p < 0.07$; a $p < 0.05$ indicates similarity. Then, the test

was repeated on two separate days but this time, using the same PCR cocktail for the repeat testing. This produced more convincing evidence of test reproducibility ($p<0.001$); the results of this second test are plotted in Figure 4-1; even at first glance, the two lines are strikingly similar.

The $p<0.001$ confirms that the lines are indeed similar, i.e. that the TCRBJ repertoires are remarkably similar when performed on separate days but using the same PCR cocktail. That is, the results are reproducible. This finding was important and meant that all subsequent comparisons of repertoires had to be performed using the same PCR cocktail (or master mix).
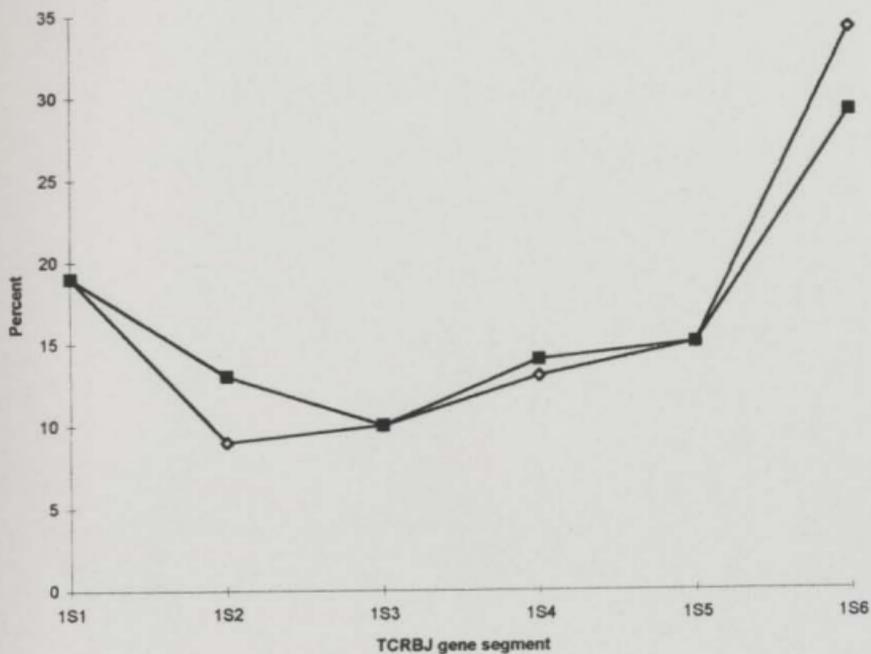
Figure 4-1: Partial TCRBJ Repertoire (including segments 1S1 to 1S6 inclusive) performed on two separate days but analyzed using the same PCR cocktail to demonstrate the reproducibility of the results.

4.3 Plateau Phase

As discussed in Chapters 1 and 3 in applying an RT-PCR method for TCR repertoire determination, it is critical to know when the PCR amplification conditions become limiting, i.e. when the plateau phase has been reached.

The plateau phase for the PCRs performed in this study was determined using the methodology described in Chapter 3. In order to accomplish this, a representative primer was chosen; the criteria used in this selection will be discussed in Chapter 5.

Two different substrates were used in the various PCRs of this research. The first substrate was PCR-amplified DNA of the gene segment for which the primer was specific. The second substrate was cDNA isolated from T-cells. The implications of the choice of substrate will also be discussed in Chapter 5.

The plateau was determined for TCRBJ using both PCR-amplified DNA (Figure 4-2) and cDNA (Figure 4-3) as substrates. For TCRBV, the plateau using cDNA as a substrate was determined (Figure 4-4). The TCRBV plateau using amplified DNA as a substrate was previously determined by E. Daniel (Daniel, 1995). Table 4-1 presents the results of these studies.

Table 4.1: Number of Cycles before the Plateau Effect Occurs

Primer set (and representative primer)	Substrate	Number of Cycles before the Plateau Effect Occurs
TCRBJ (1S5)	amplified DNA	18
TCRBJ (2S2)	cDNA	32
TCRBV	amplified DNA	30
TCRBV6	cDNA	35
TCRAC	amplified DNA	still linear at 30 cycles
TCRAC	cDNA	still linear at 35 cycles

To ensure that the internal control would also remain in the quasi-linear phase at the end of each PCR, the progression of a PCR was also determined for TCRAC using the specific primers. This had previously been determined using an amplified DNA substrate (Daniel, 1995). The results determined for a cDNA substrate are presented in Figure 4-5. These results are also presented in Table 4-1.
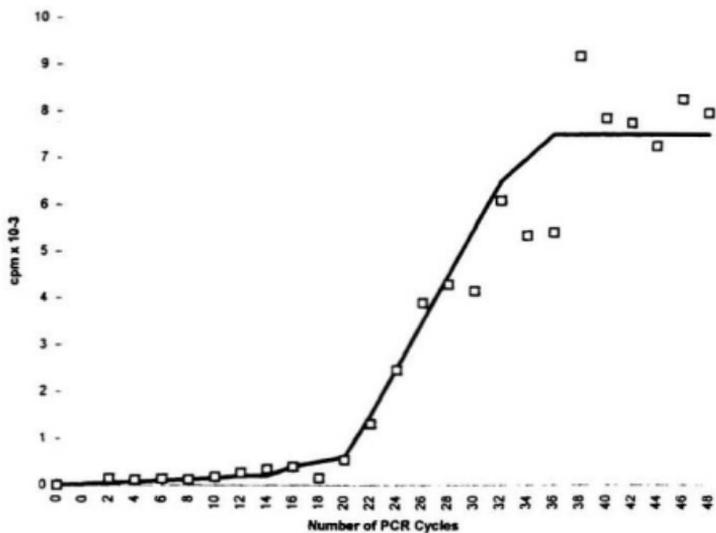
Figure 4-4: The progression of a PCR for TCRBV using TCRBV6 as a representative primer and cDNA as substrate.
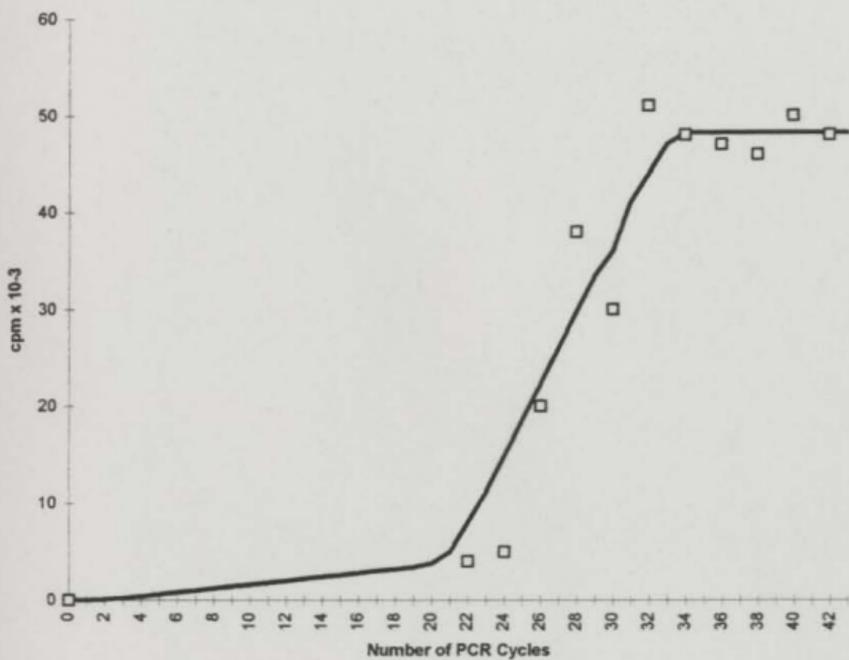
Figure 4-3: The progression of a PCR for TCRBJ primers using TCRBJ2S2 as a representative primer and cDNA as a substrate.
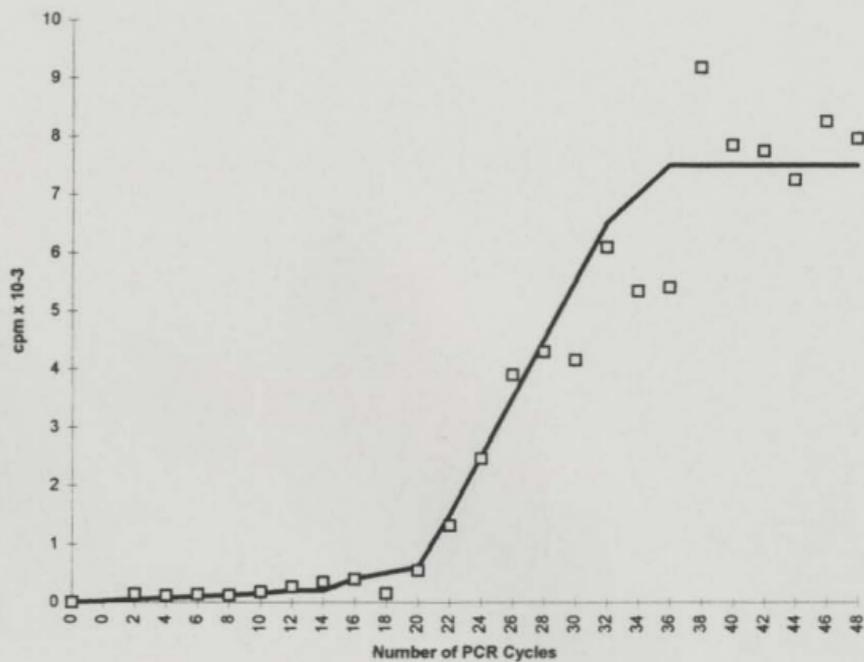
Figure 4-4: The progression of a PCR for TCRBV using TCRBV6 as a representative primer and cDNA as substrate.
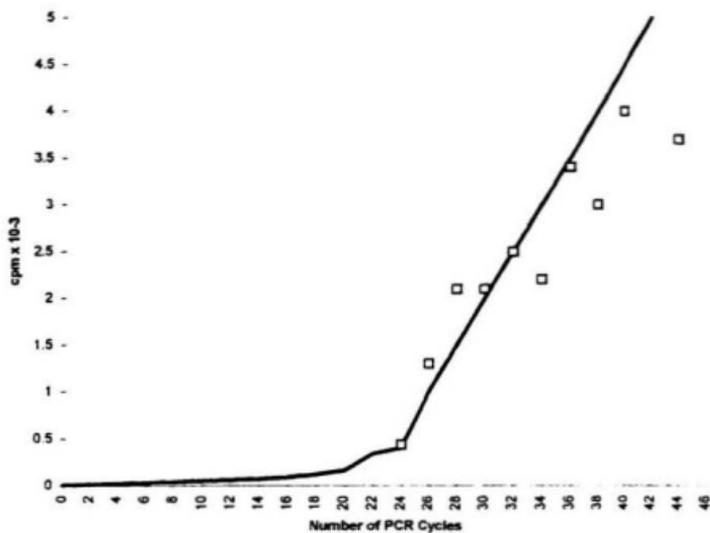
Figure 4-5: The progression of a PCR for TCRAC, using cDNA as a substrate.

4.4 Priming Efficiency

The importance of determining primer efficiency was discussed in Chapter 1. The primer efficiency was determined for the thirteen TCRBJ gene segment specific-primers and for the twenty-four TCRBV family specific primers following the method described in Chapter 3.

For the TCRBJ primers, five trials were performed; the results are presented in Figure 4-6. On inspection of the figure, there appears to be marked variability in priming efficiency; this is confirmed by a one-way ANOVA ($p<0.005$). Primers for TCRBJ2S1, 2S4 and 2S7 are most efficient, whereas primers for TCRBJ1S4 and 1S5 are least efficient.

For the TCRBV primers, two trials were performed; the results are presented in Figure 4-7. Again, there appears to be marked variability in priming efficiency, confirmed by a one-way ANOVA ($p<0.010$). Primers for TCRBV12, 13 and 14 are most efficient, whereas primers for TCRBV19 and 24 are least efficient.
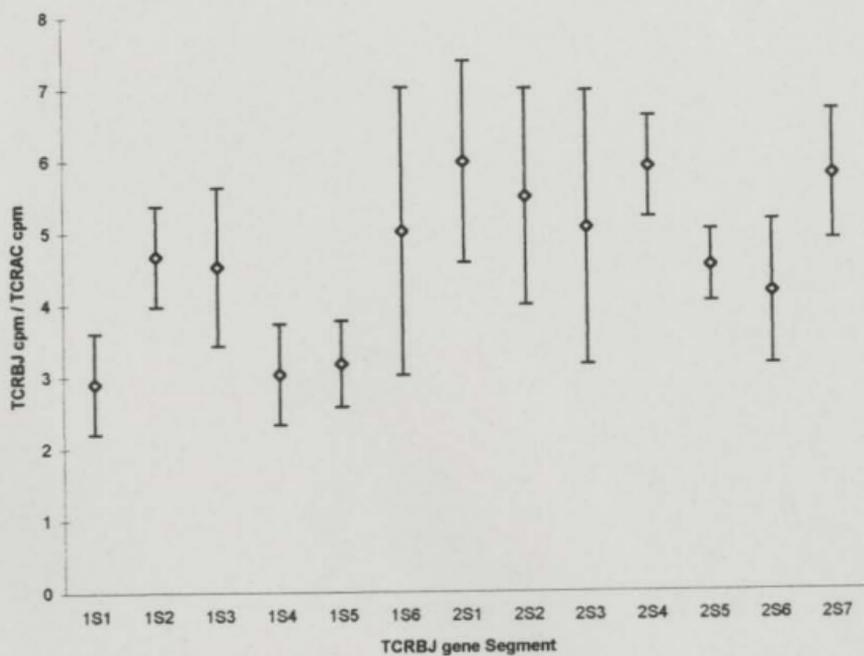
Figure 4-6: Mean priming efficiency of the thirteen TCRBJ specific primers. Error bars represent one standard deviation.
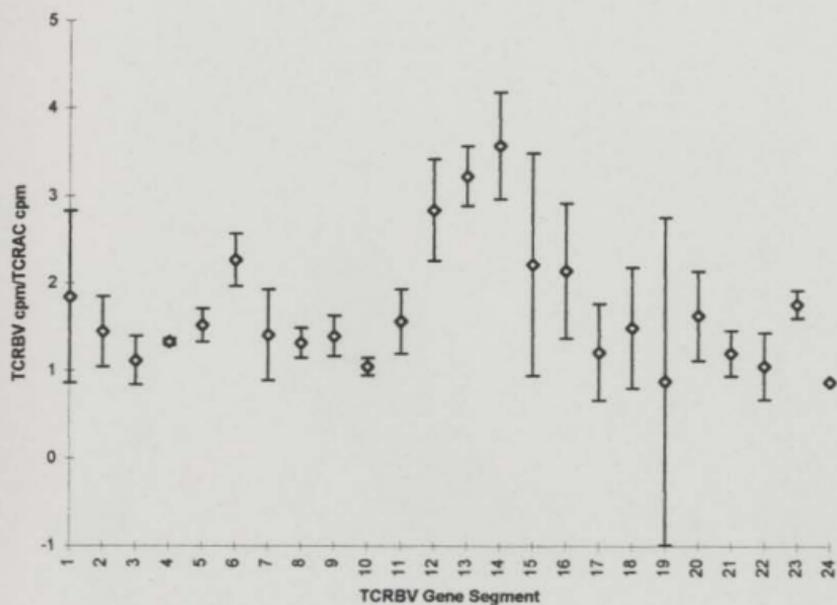
Figure 4-7: Mean priming efficiency of the twenty-four TCRBV specific primers. Error bars represent one standard deviation.

4.5 First Hypothesis

4.5.1 The TCR Repertoire in Naive (CD45RA⁺) vs. Memory (CD45RO⁺) T-cells

4.5.1.1 TCRBJ Analysis

The TCRBJ repertoires of naive and memory cells were determined for twenty unrelated volunteers, and the data were corrected for priming efficiencies. Figure 4-8 is an example of the TCRBJ repertoire for naive and memory cells of a single individual and is presented on the following page. The repertoires of the twenty subjects are presented as Figures A-1 to A-20 in Appendix A. The goal of this part of the study was to test whether there is a relationship between the memory and naive TCRBJ repertoires; to do this, Pearson correlation coefficients were used as discussed in Chapter 1. Each coefficient is designated 'r'; the r-values were calculated for each volunteer and the results are included in Figures 4-8 and A-1 to A-20.
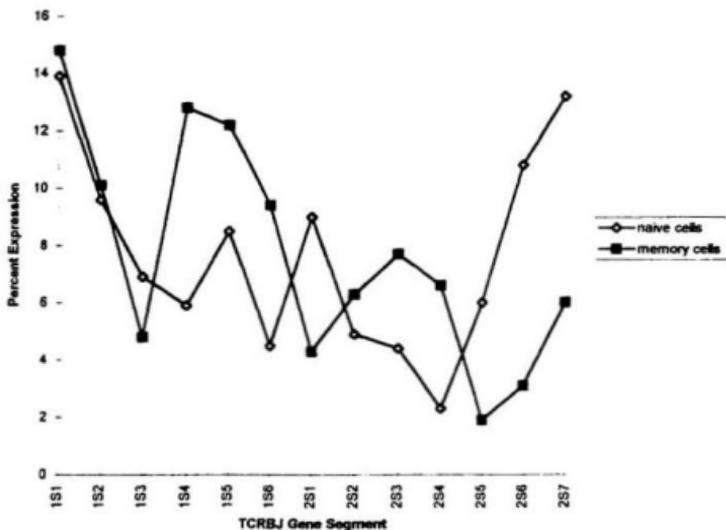
Figure 4-8: The TCRBJ repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) T-cells of subject 1.X.01. r = 0.189.

The question then arose as to whether the naive T-cell repertoire and memory T-cell repertoire within an individual are similar or different, see Section 2.1. In other words, how close does the correlation coefficient have to be to 1 to demonstrate a statistically significant correlation between the two cell subsets? A t-test reveals that all values above 0.55 for the correlation coefficient are statistically significant (see equation 4.1). Only 5 of the 20 individuals who participated in this study showed any statistically significant correlation (i.e., correlation coefficient greater than 0.55) between their naive CD4$^+$ T-cell and their memory CD4$^+$ T-cell TCRBJ repertoire, see Table 4-2. Correlation coefficients above 0.55 are indicated with an asterix.

$$t = r^2 \sqrt{\frac{n-2}{1-r^2}}$$
equation 4.1

In Table 4-2, the second digit of the code indicates memory or naive cell: one refers to memory and two refers to naive. The last digit of the code indicates the individual. The conclusion is, therefore that in 75% of the individuals tested the naive T-cell repertoire of a particular individual differed statistically from the memory T-cell repertoire of the same individual.

Table 4.2: Correlation Coefficients (r-values) between TCRBJ Repertoires of Naive and Memory T-Cells

Comparison between subsets	r-value
1.1.01 vs. 1.2.01	0.189
1.1.02 vs. 1.2.02	0.794*
1.1.04 vs. 1.2.04	0.591*
1.1.05 vs. 1.2.05	0.590*
1.1.06 vs. 1.2.06	0.495
1.1.07 vs. 1.2.07	0.342
1.1.08 vs. 1.2.08	0.674*
1.1.09 vs. 1.2.09	0.306
1.1.10 vs. 1.2.10	-0.345
1.1.11 vs. 1.2.11	0.578*
1.1.12 vs. 1.2.12	0.315
1.1.13 vs. 1.2.13	0.335
1.1.14 vs. 1.2.14	0.365
1.1.15 vs. 1.2.15	0.401
1.1.17 vs. 1.2.17	-0.478
1.1.18 vs. 1.2.18	0.269
1.1.20 vs. 1.2.20	-0.433
1.1.21 vs. 1.2.21	0.475
1.1.22 vs. 1.2.22	0.182
1.1.24 vs. 1.2.24	0.083
mean r-value	0.286

* indicates values above 0.55 (see text)

4.5.1.1.1 Age and the TCRBJ Repertoire of Naive and Memory T-Cells

There is reason to hypothesize that the correlation between the memory T-cell repertoire and the naive T-cell repertoire (represented by 'r') may decrease with age as a consequence of antigenic exposure throughout life. Table 4-3 shows the correlation coefficient (r-value) for each subject. The subjects are categorized by age and the mean correlation coefficient is reported at the bottom of the table. Individuals were arbitrarily divided into an under and an over thirty years of age category. The mean r-value for individuals under thirty years of age (mean age = 24) was 0.461. The mean r-value for individuals over thirty years of age (mean age = 45) was much lower, 0.073. These two means are indeed different; a t-test gave a p<0.02. From this it can be concluded that the differences between the memory T-cell repertoire and the naive T-cell TCRBJ repertoire are more pronounced above age 30 than under age 30. It is interesting to note that the extremely low correlations were all in individuals over 50 years of age.

Table 4.3: Correlation Coefficients (r-values) for Subjects Based on Age for naive vs. memory
T-Cells TCRBJ repertoires

Under 30 years of age	r-values	Over 30 years of age	r-values
1.X.01	0.189	1.X.07	0.342
1.X.02	0.794	1.X.09	0.306
1.X.04	0.591	1.X.10	-0.345
1.X.05	0.590	1.X.11	0.578
1.X.06	0.495	1.X.13	0.335
1.X.08	0.674	1.X.17	-0.478
1.X.12	0.315	1.X.18	0.269
1.X.14	0.365	1.X.20	-0.433
1.X.15	0.401	1.X.24	0.083
1.X.21	0.475		
1.X.22	0.182		
mean r-value	0.461	mean r-value	0.073

4.5.1.1.2 Gender and the TCRBJ Repertoire in Naive and Memory Cells

It is conceivable that the correlation coefficients between naive and memory TCRBJ repertoires vary with gender in view of the reported differences in immune reactivity between men and women (Grossman, 1989). Table 4-4 shows the correlation coefficient for each subject. The subjects are categorized by gender and the mean correlation coefficient is reported at the bottom of the table. The two categories are not statistically different; a t-test gave a $p < 0.51$.

Table 4.4: Correlation Coefficients (r-values) for Subjects Based on Gender for Naive vs Memory T-Cell TCRBJ Repertoire

male	r-value	female	r-value
1.X.04	0.591	1.X.01	0.189
1.X.05	0.590	1.X.02	0.794
1.X.06	0.495	1.X.08	0.674
1.X.07	0.342	1.X.11	0.578
1.X.09	0.306	1.X.13	0.335
1.X.10	-0.345	1 X.14	0.365
1.X.12	0.315	1.X.17	-0.478
1.X.15	0.401	1.X.21	0.475
1.X.18	0.269	1.X.22	0.182
1.X.20	-0.433		
1.X.24	0.083		
mean r-value	0.238	mean r-value	0.346

4.5.1.2 TCRBV Analysis

The TCRBV repertoires of naive and memory T-cells were determined for nine unrelated individuals, and the data were corrected for priming efficiencies. All these volunteers also participated in the TCRBJ repertoire study (Section 4.3.1.1). An example of the TCRBV repertoires for naive and memory T-cells of a single individual is presented on the following page as Figure 4-9. The repertoires of all the individuals can be found in Appendix B as Figures B-1 to B-9. The goal of this part of the study was to test the relation between the repertoires of memory and naive T-cells within an individual; once again, the Pearson correlation coefficients are presented on the graphs. These correlation coefficients are also presented in Table 4-5. Once again, a t-test can be used on the correlation coefficients to determine similarity between TCR repertoires of the two cell populations. The critical value in this instance is 0.40. In other words, for any individual, a correlation coefficient above 0.40 indicates a statistically significant correlation between the naive T-cell and memory T-cell TCRBV repertoires. Five individuals were found to have correlation coefficients above 0.40 for the comparison of the TCRBV repertoires of their naive and memory T-cells; these individuals are indicated by an asterix in Table 4-5. This means that five out of the nine individuals tested demonstrated a statistically significant correlation between their naive and memory TCRBV repertoires.

Due to the insufficient and disproportionate sample, it was impossible to determine whether or not the correlation between the memory T-cell TCRBV repertoire and the naive T-cell TCRBV repertoire decreases with age. Also, it was impossible to determine whether or not a statistically significant correlation between the naive T-cell repertoire and memory T-cell TCRBJ repertoire is a indicator for a similar correlation for the TCRBV repertoire. By disproportionate, it is meant that of the nine individuals, 66% were over the age of 30, and only one had demonstrated a statistically significant correlation between his/her memory T-cell repertoire and naive T-cell TCRBJ repertoire.

Table 4.5: Correlation Coefficients (r-values) between TCRBV Repertoires of Naive and Memory T-Cells

| Comparison between subsets	r-value
1.1.02 vs. 1.2.02	0.247
1.1.07 vs. 1.2.07	0.951*
1.1.09 vs. 1.2.09	0.099
1.1.10 vs. 1.2.10	0.681*
1.1.12 vs. 1.2.12	0.253
1.1.13 vs. 1.2.13	0.418*
1.1.15 vs. 1.2.15	0.901*
1.1.17 vs. 1.2.17	-0.029
1.1.18 vs. 1.2.18	0.429*
mean r	0.439

* indicates values above 0.40 (see text)
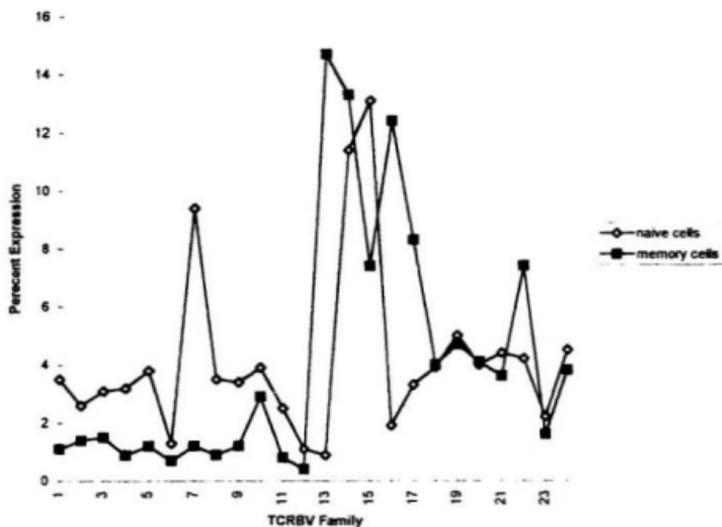
Figure 4-9: The TCRBV repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X.02. r = 0.247.

4.5.2 Overall TCRBJ Repertoire

4.5.2.1 Naive T-Cell TCRBJ Repertoire Analysis

Twenty individuals participated in this study (see section 4.3.1.1), and the mean expression level of each TCRBJ segment was calculated for these twenty individuals. For naive T-cells, this was done with both the corrected and uncorrected data; see Figures 4-10 and 4-11, respectively. Each column in these graphs, therefore, represents the mean expression of the TCRBJ gene segment signified. An ANOVA demonstrates that the mean levels of expression of the thirteen TCRBJ segments are significantly different in both instances, ($p<0.001$ for both).

The error bars on these graphs represent one standard deviation. Thus, the length of the error bar is proportional to the variability of expression of each TCRBJ segment within the given population. The error bars show that TCRBJ1S1, 1S3, 1S4, 2S1 and 2S7 are the most variable of the TCRBJ gene segments for the uncorrected data, and TCRBJ1S1, 1S4 and 2S7 are the most variable for the corrected data.

Figure 4-12 shows both the corrected and the uncorrected data for the TCRBJ repertoire in CD4$^+$CD45RA$^+$ cells. There is a striking difference between the two repertoires. The uncorrected data shows TCRBJ2S1 and 2S7 as the predominantly expressed TCRBJ gene segments and TCRBJ1S4, 2S4 and 2S6

as the least expressed. In contrast, the corrected data reveal that TCRBJ1S1, 1S5 and 2S7 are the predominantly expressed TCRBJ segments and TCRBJ 2S4 and 2S6 are the least expressed.

In conclusion taking into account the primer efficiencies, the naive T-cells of the study population express TCRBJ1S1, 1S5, 2S1 and 2S7 at higher levels than the other TCRBJ gene segments, and TCRBJ1S1, 1S4 and 2S7 exhibit the most variability.
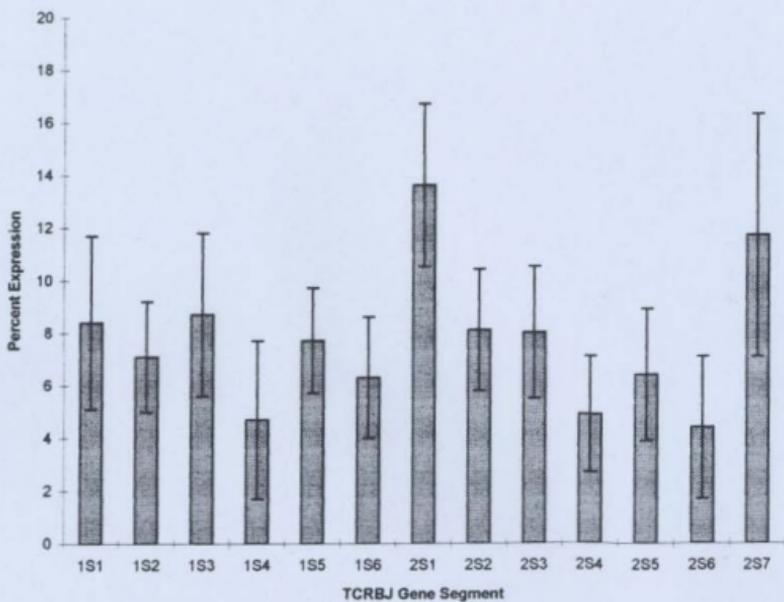
Figure 4-10: Mean percent expression of TCRBJ segments in naive T-cells from 20 unrelated individuals using data uncorrected for primer efficiency. Error bars represent one standard deviation. $p < 0.001$, ANOVA.
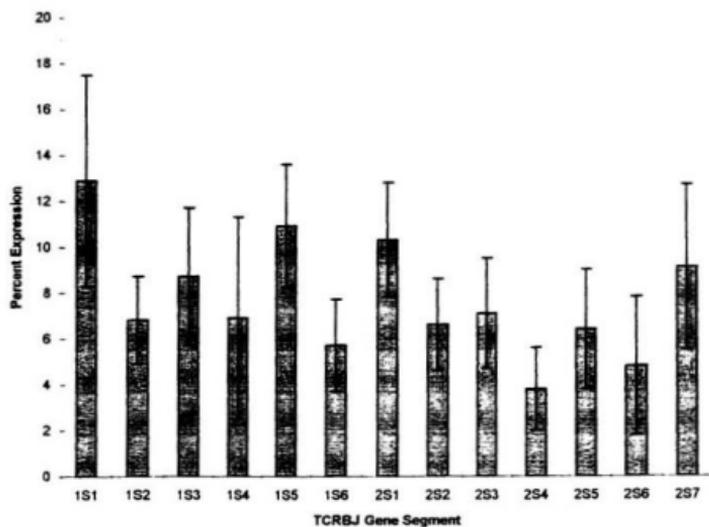
Figure 4-11: Mean percent expression of TCRBJ segments in naive T-cells from 20 unrelated individuals using data corrected for primer efficiency. Error bars represent one standard deviation. p<0.001, ANOVA.
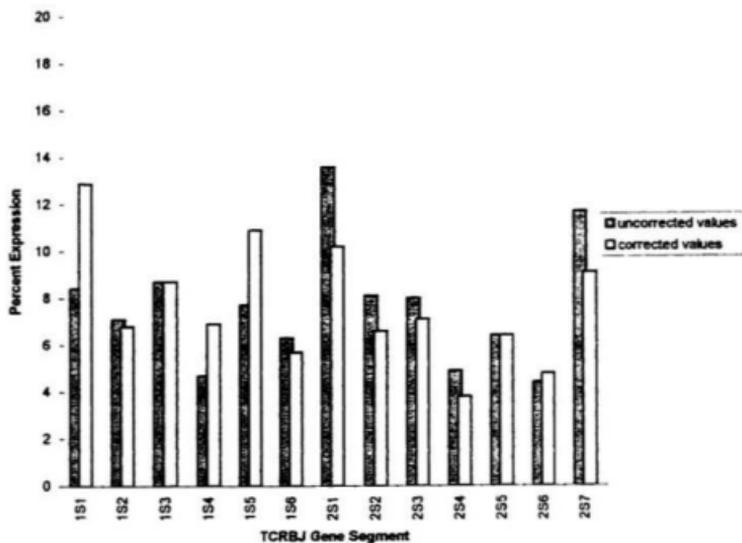
Figure 4-12: Comparison of corrected and uncorrected mean percent expression levels of TCRBJ segments from the naïve T-cells of 20 unrelated individuals.

4.5.2.2 Memory T-Cell TCRBJ Repertoire Analysis

For memory T-cells, data were compiled for both the corrected and uncorrected data; see Figures 4-13 and 4-14, respectively. An ANOVA demonstrates that for both corrected and uncorrected data there are significant differences in the levels of expression of the various TCRBJ segments (p<0.001 for both).

Again, the error bars on these graphs represent one standard deviation. This demonstrates that, for the uncorrected data, TCRBJ1S3, 2S1, 2S6 and 2S7 show the most variability, and for the corrected data 1S3, 1S5, 2S1 and 2S6 show the most variability.

Figure 4-15 shows both the corrected and the uncorrected data for the TCRBJ repertoire in CD4$^+$CD45RO$^+$ cells, i.e. in memory cells. The uncorrected data show that TCRBJ2S1 and 2S7 are the predominantly expressed TCRBJ segments. The corrected data, however, demonstrate no extreme values except it may be noted that TCRBJ2S4 is the least expressed TCRBJ segment.

Since primer efficiency must be accounted for, it can conclude from these graphs that memory cells in the study population express no predominant TCRBJ segment and TCRBJ1S3, 1S5, 2S1, and 2S6 exhibit the most variability in levels of expression.
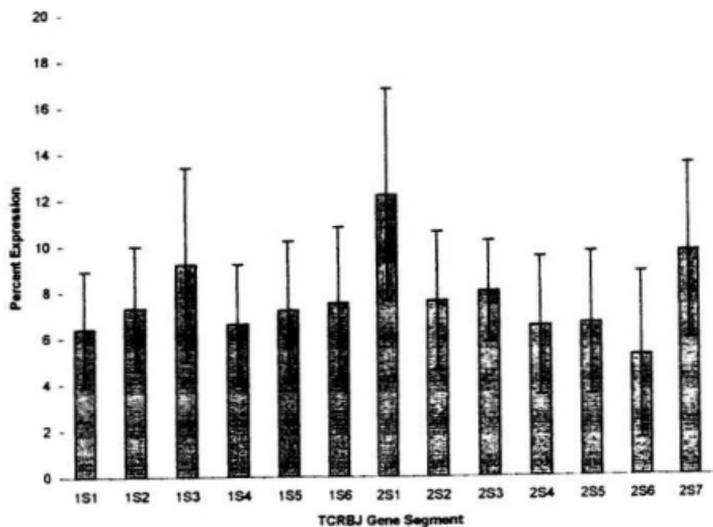
Figure 4-13: Mean percent expression of TCRBJ segments in memory T-cells from 20 unrelated individuals using data uncorrected for primer efficiency. Error bars represent one standard deviation. p<0.001, ANOVA.
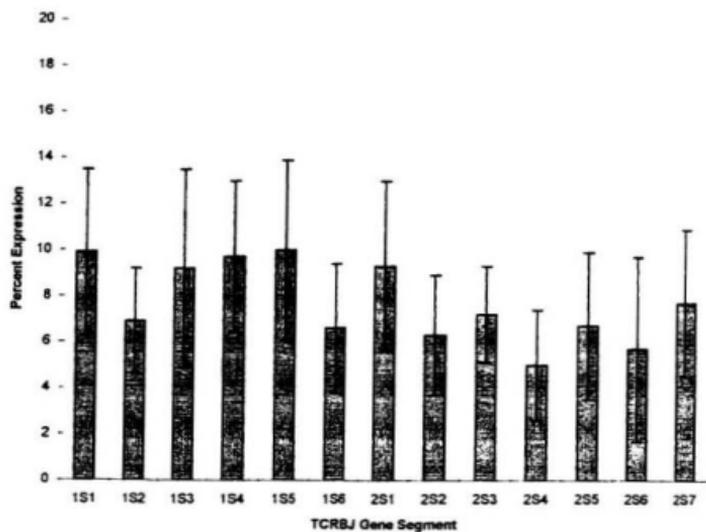
Figure 4-14: Mean percent expression of TCRBJ segments in memory T-cells from 20 unrelated individuals using data corrected for primer efficiency. Error bars represent one standard deviation. p<0.001, ANOVA.
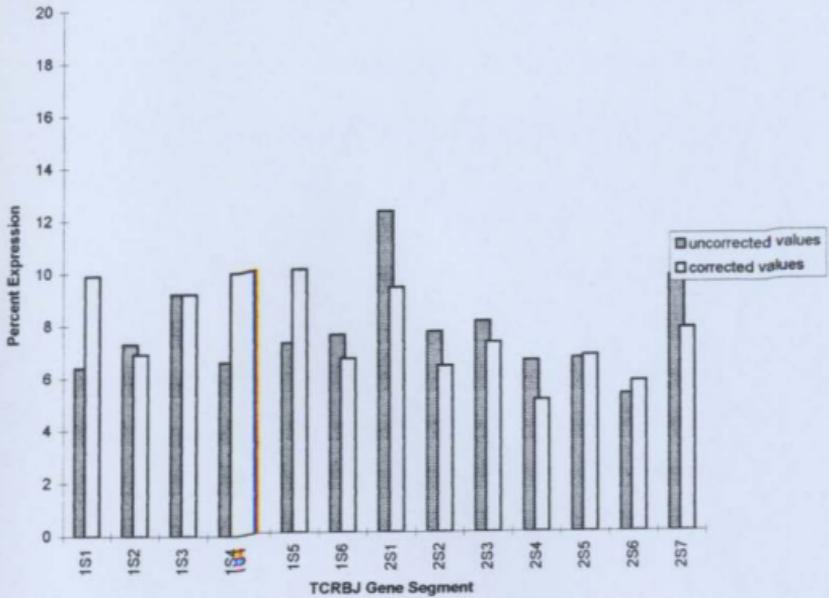
Figure 4-15: Comparison of corrected and uncorrected mean percent expression levels of TCRBJ segments from the memory T-cells of 20 unrelated individuals.

4.5.2.3 Mean Memory Cell Repertoire vs. Mean Naive Cell Repertoire

For the twenty unrelated participants in this study, an ANOVA shows a significant difference in the mean levels of expression of the various TCRBJ gene segments in both naive and memory cells (see Section 4.3.2.1 and 4.3.2.2). Figure 4-16 is a comparison of the mean levels of expression of TCRBJ segments between naive and memory T-cells of the population. This figure shows the greatest difference in the levels of expression of TCRBJ1S1 and 1S4.
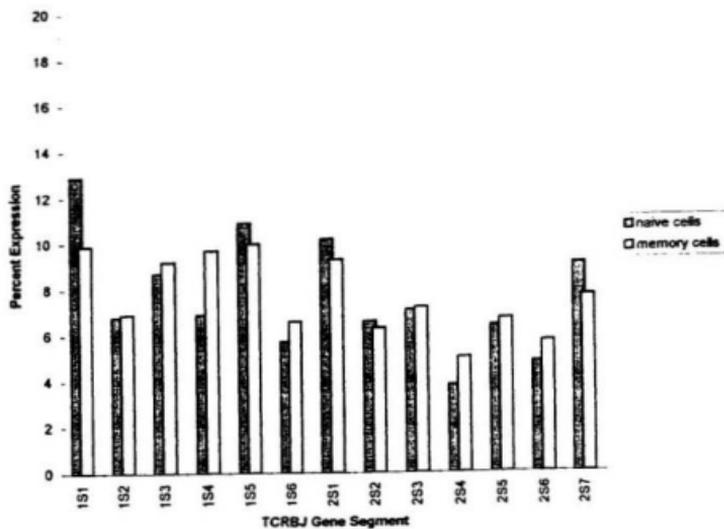
Figure 4-16: Comparison of the mean levels of expression of TCRBJ segments in naive and memory T-cells from 20 unrelated individuals.

4.5.3 Umbilical Cord Blood TCRBJ Analysis

Knowledge that the ratio of memory to naive cells increases with age (McElhaney *et al.*, 1995) and that the correlation between naive and memory TCRBJ repertoires decreases with age (see section 4.4.1), led to an interest in the examination of memory and naive T-cells in umbilical cord blood. The memory T-cell repertoire obviously changes with age, so it was of interest to study memory T-cells at a very young age i.e., in newborns. It was hypothesized that since newborns have been in contact with relatively few external antigens, their memory T-cell repertoires would be oligoclonal.

One sample of cord blood was analyzed by flow cytometry. Greater than 90% of the cells expressed both isoforms. Thus the findings that blood taken from the umbilical cord is mostly composed of $CD45RA^+CD45RO^+$ cells was confirmed (Yamada *et al.*, 1992; Byrne *et al.*, 1994 and Amlot *et al.*, 1996). In other words, there were no identifiable and distinct memory and naive T-cell populations. Thus, it was not possible to distinguish, let alone isolate memory T-cells.

A $CD4^+$ T-cell isolation was performed on three samples of umbilical cord blood from different donors. cDNA was prepared from the $CD4^+$ cells and PCR-amplified using the 13 TCRBJ primers. The PCR was run for 60 cycles to

ensure adequate amplification. Radioactive primers were not used in this case, for only the presence and not the quantity of the gene segments was of interest.

In two of the three cases, the presence of all thirteen TCRBJ segments was confirmed. In the third case, only 11 of the 13 gene segments were successfully amplified; in the wells where no amplification was visible for the TCRBJ segment, the positive control (TCRAC) was also absent, i.e. the experiment had failed technically.

4.6 Second Hypothesis

4.6.1 Monozygotic Twins vs. Unrelated Individuals: TCRBJ Analysis of Naive T-Cells

The TCRBJ repertoire of naive T-cells was determined for six sets of monozygotic twins (see Appendix C). The age range for these twin pairs was from 15 to 35 years old. A Pearson correlation coefficient was calculated from the data for each twin pair.

Six unrelated twin pairs were divided into groups, each group containing two twin pairs, i.e. four members. For each group, the same PCR cocktail was used, minimizing the variation between reactions (see Section 4.1 and 5.1). Test data for each group consisted of a Pearson correlation coefficient (r-value) for each twin pair; therefore, six test correlation coefficients were obtained. For example, suppose A1 and A2 are twins and B1 and B2 are twins. The test

statistics would include a comparison of A1 and A2 and a comparison of B1 and B2. The twin data were compared with data obtained from analysis of the TCRBJ repertoire in control individuals. Controls consisted of unrelated individuals. For example, A1 was compared to B1 and A2 was compared to B2. All possible Pearson correlation coefficients for twin pairs and unrelated pairs (controls) are presented in Figure 4-17.
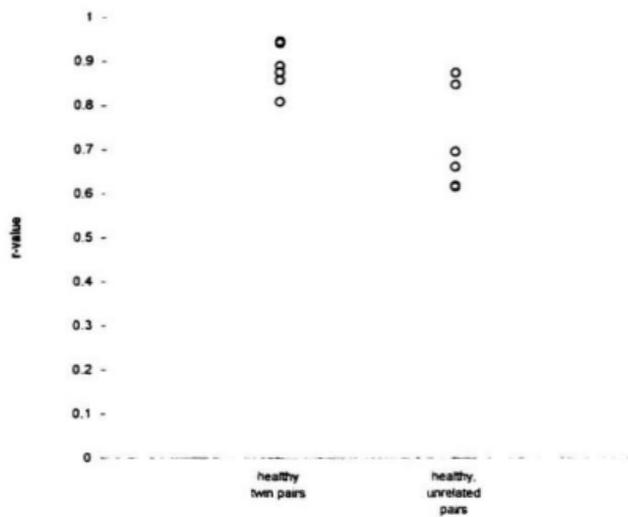
Figure 4-17: Correlation analysis of the naive T-cell TCRBJ repertoires in monozygotic twin pairs and unrelated individuals.

It appears in Figure 4-17 that the Pearson correlation coefficients for the twin pairs are greater than those for the unrelated pairs of individuals. Whether this difference is statistically significant is difficult to test due to the low number of data points in each repertoire, i.e. thirteen (one for each TCRBJ gene segment). To overcome this problem, the repertoires were grouped, enabling the potential comparison of twenty-six data points (or any multiple of thirteen) instead of thirteen. In actuality, only twelve data points were used for each individual permitting comparison of twenty-four data points; the expression level of TCRBJ2S7, the last TCRBJ segment measured, was omitted to achieve independence of the data. After increasing the number of data points in this manner, a comparison could be made using a z-test (see equations 4.2 and 4.3). There is one rule to be followed in these groupings, however; no individual can be included in the calculation of correlation coefficients for both the twins and the unrelated pairs. This leads to various possibilities for comparisons. For example, suppose there were four twin pairs: A1 and A2, B1 and B2, C1 and C2; and D1 and D2. Table 4-6 summarizes the six possible groups for this example. Each possibility will yield a different z-test result. To obtain the best overall comparison, therefore, all possibilities must be calculated.

The following are the equations used for the z-test. In equation 4.3, r_n represents the correlation coefficient for the nth comparison, and equation 4.2

represents a z-test for two comparisons, represented by the arbitrary digits 1 and 2.

$$z = \frac{z_1 - z_2}{\sqrt{\frac{1}{n_1 - 3} + \frac{1}{n_2 - 3}}}$$

equation 4.2

where

$$z_n = \frac{1}{2} \ln\left(\frac{1 + r_n}{1 - r_n}\right)$$

equation 4.3

Table 4.6: Hypothetical Example of Data Analysis

Possibility	Twins pairs	Unrelated pairs
1	A1B1 vs. A2B2	C1C2 vs. D1D2
2	A1C1 vs. A2C2	B1B2 vs. D1D2
3	A1D1 vs. A2D2	C1C2 vs. B1B2
4	B1C1 vs. B2C2	A1A2 vs. D1D2
5	B1D1 vs. B2D2	A1A2 vs. C1C2
6	C1D1 vs. C2D2	A1A2 vs. B1B2

For the six twin pairs in this study, there were 40 possibilities. For each possibility, one z-test statistic was calculated; these are presented in Table 4-7 and in Figure 4-18. The z-value must be greater then 1.96 for the results to be statistically significant with 95% confidence. Notice only five values are below this critical number. Therefore, 85% of the possibilities demonstrate a statistically significant difference between the correlation coefficients of the twins and those of the unrelated pairs of individuals.

Table 4.7: z-test Statistic for TCRBJ Repertoires of Monozygotic Twins vs. Unrelated Pairs

| z-values				
4.5	4.4	4.2	4.2	4.1
4.0	3.9	3.8	3.7	3.5
3.5	3.4	3.4	3.4	3.4
3.4	3.2	3.1	3.0	3.0
3.0	3.0	3.0	2.9	2.7
2.7	2.6	2.5	2.4	2.4
2.3	2.3	2.1	2.0	2.0
1.7	0.8	0.6	0.5	-0.5
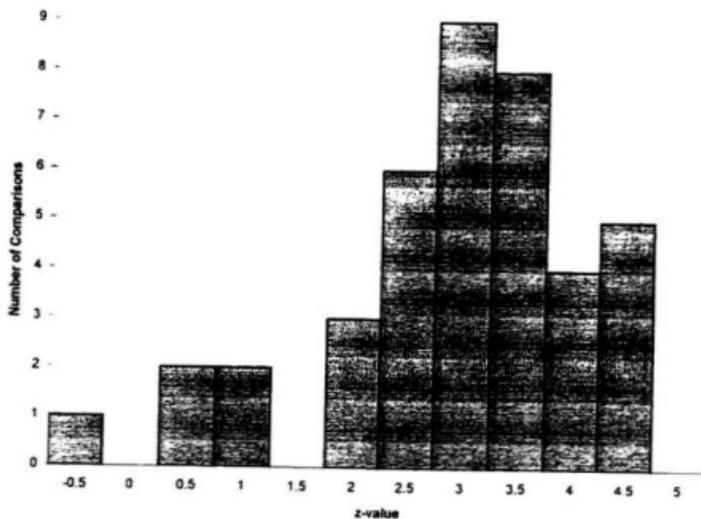
Figure 4-18: Distribution of z-values obtained from the 40 comparisons of correlation coefficients of twins vs. unrelated pairs of individuals.

4.6.2 HLA Identical Siblings vs. HLA Non-Identical Siblings: TCRBJ Analysis of Naïve T-Cells

The TCRBJ repertoires were analyzed in three families. The number of members in each of these families is presented in Table 4-8. Each individual was given a code to preserve anonymity and to distinguish parents from children within the families. The last digit represents the individual: the number one represents the father, the number two represents the mother and the remainder represent the children.

Table 4-9 shows the HLA haplotypes for all the family members. The haplotype designations were arbitrary. The actual haplotypes were determined by E. Daniel (1995). For each family a variety of comparisons could be made: between parents, between parents and children, between HLA identical siblings, between HLA haploidentical siblings and between HLA non-identical siblings. The Pearson correlation coefficients (r-values) for every possible comparison are presented in Figures 4-19 to 4-22, but for this thesis it was decided to analyze statistically only the differences between r-values for HLA identical and HLA non-identical sibling pairs (see below).

Table 4.8: Number of Members in Each Family

Family	Number of Members (including parents)
2.2.1.X	6
2.2.2.X	7
2.2.3.X	9
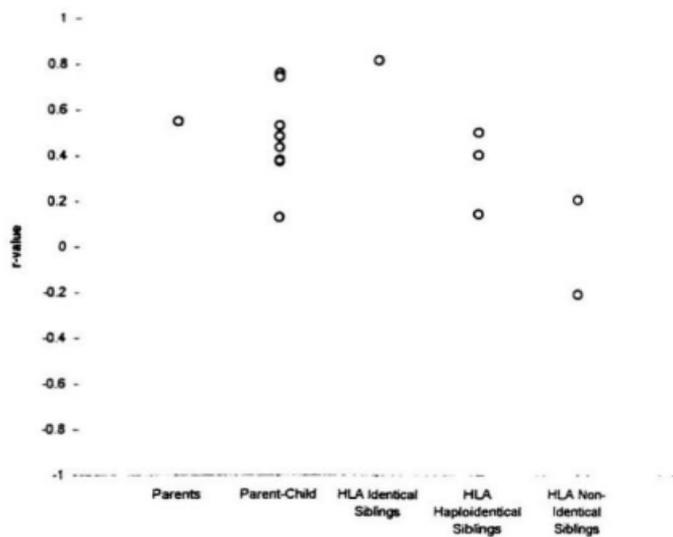
Figure 4-19: *Correlation analysis of the naive T-cell TCRBJ repertoires in family 2.2.1.X.*
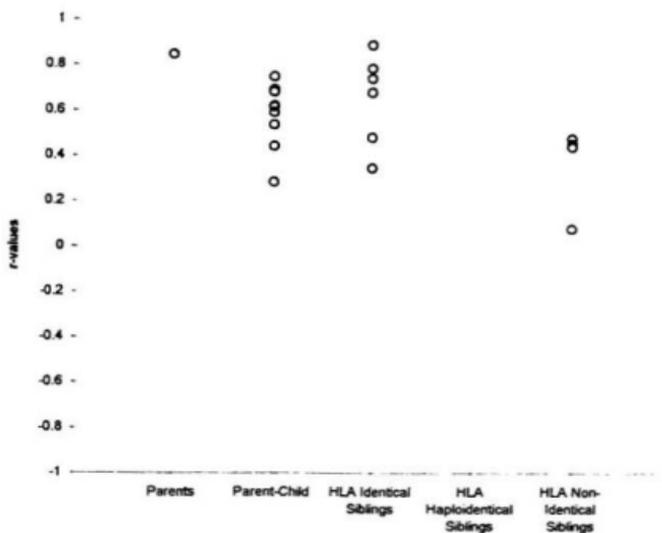
Figure 4-20: Correlation analysis of the naive T-cell TCRBJ repertoires in family 2.2.2.X; no HLA haploidentical siblings are present in this family.
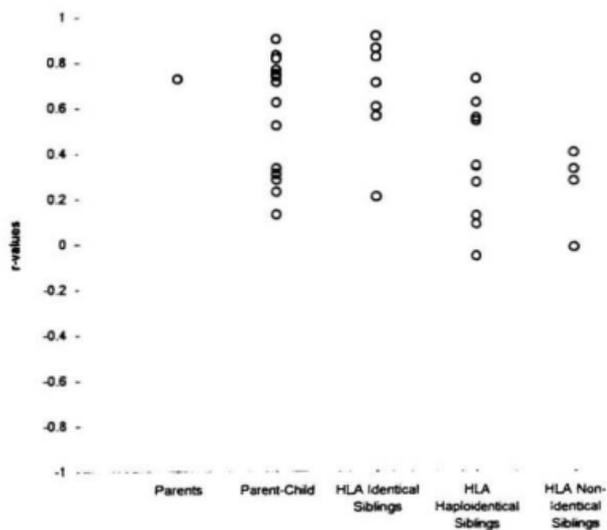
Figure 4-21: Correlation analysis of the naïve T-cell TCRBJ repertoires in family 2 2.3 X
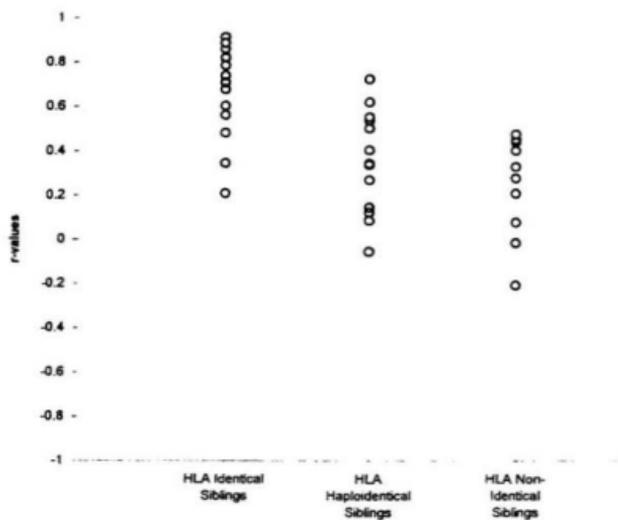
Figure 4-22: Correlation analysis of the naive T-cell TCRBJ repertoires in families 2.2.1.X, 2.2.2.X and 2.2.3.X.

Table 4.9: HLA Types for All Family Members

Family 2.2.1	HLA haplotype	Family 2.2.2	HLA haplotype	Family 2.2.3	HLA haplotype
2.2.1.1	A/B	2.2.2.1	E/F	2.2.3.1	I/J
2.2.1.2	C/D	2.2.2.2	G/H	2.2.3.2	K/L
2.2.1.3	A/C	2.2.2.3	E/G	2.2.3.3	I/K
2.2.1.4	A/C	2.2.2.4	E/G	2.2.3.4	I/K
2.2.1.5	B/D	2.2.2.5	E/G	2.2.3.5	I/K
2.2.1.6	B/C	2.2.2.6	E/G	2.2.3.6	I/K
		2.2.2.7	F/H	2.2.3.7	J/K
				2.2.3.8	J/K
				2.2.3.9	J/L

*actual HLA types can be found in: Daniel, E. S. "Multiple Factors Shape the TCR Vβ Repertoire in Humans." M.Sc.

Thesis, Memorial University of Newfoundland, 1995

Using the same method of data analysis as described for monozygotic twins (see section 4.5.1), a z-test was used to compare HLA identical sibling pairs to HLA non-identical sibling pairs. There are 288 possibilities for grouping these data points; the z-values for each of these possibilities are presented in Table 4-8 and Figure 4-23. Two hundred and sixty-one of these possibilities or 93.75% are above the critical z-value of 1.96, leading to the conclusion that HLA identical siblings are more similar with respect to their TCRBJ repertoires than HLA non-identical siblings.

Figure 4-22 also shows that the Pearson correlation coefficient increases with increased sharing of HLA haplotypes. In other words, the correlation coefficients increase as follows: HLA non-identical pairs < HLA haploidentical pairs < HLA identical pairs. This, however, is not statistical evidence. The preceding paragraphs have discussed the statistical evidence which indicate that the correlations are higher between HLA identical siblings than between HLA non-identical siblings. Since not all the possible z-test statistics demonstrated a significant difference for these comparisons, it was deemed unlikely that any definitive conclusions could be drawn concerning the haploidentical pairs vs. HLA identical or HLA non-identical pairs. Therefore, no z-tests were performed for these particular comparisons.

Table 4.10: z-test Statistic for the TCRBJ Repertoires of HLA Identical vs. HLA Non-identical Pairs.

| z-values									
5.3	5.1	5.1	5.0	5.0	4.9	4.9	4.9	4.9	4.8
4.8	4.8	4.7	4.7	4.7	4.7	4.7	4.7	4.7	4.6
4.6	4.6	4.6	4.5	4.5	4.5	4.5	4.5	4.5	4.5
4.5	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4
4.4	4.4	4.3	4.3	4.3	4.3	4.3	4.3	4.3	4.2
4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.1
4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.0	4.0
4.0	4.0	4.0	4.0	4.0	4.0	4.0	3.9	3.9	3.9
3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.8
3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8
3.8	3.8	3.7	3.7	3.7	3.7	3.7	3.7	3.7	3.6
3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6
3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
3.5	3.5	3.5	3.5	3.4	3.4	3.4	3.4	3.4	3.4
3.4	3.4	3.4	3.4	3.4	3.4	3.3	3.3	3.3	3.3
3.3	3.3	3.3	3.3	3.2	3.2	3.2	3.2	3.2	3.2
3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2
3.2	3.2	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1
3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
3.0	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9
2.9	2.9	2.8	2.8	2.8	2.8	2.8	2.7	2.7	2.7
2.7	2.7	2.7	2.7	2.7	2.6	2.6	2.6	2.6	2.5
2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
2.5	2.5	2.4	2.4	2.4	2.4	2.4	2.4	2.3	2.3
2.3	2.3	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2
2.2	2.2	2.2	2.2	2.2	2.1	2.1	2.1	2.1	2.0
2.0	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9
1.9	1.8	1.8	1.6	1.6	1.6	1.6	1.5	1.5	1.5
1.5	1.5	1.3	1.2	1.2	1.2	1.2	0.9		
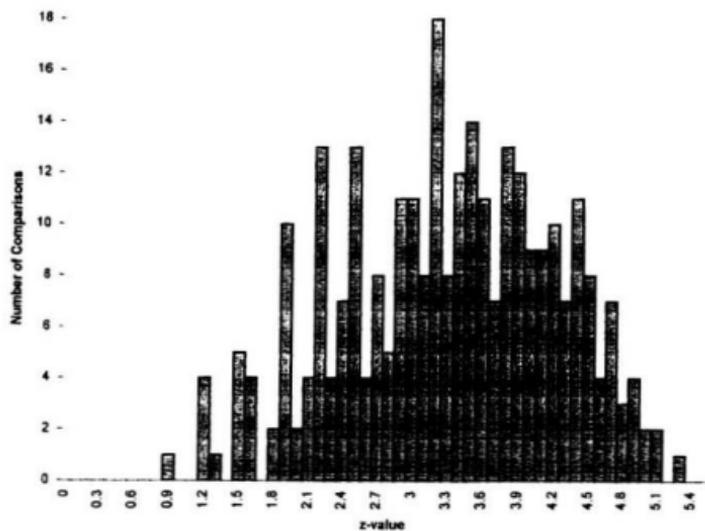
Figure 4-23: Distribution of z-values obtained from the 288 comparisons of correlation coefficients of HLA identical vs. HLA non-identical pairs.

4.7 Third Hypothesis

4.7.1 Monozygotic Twins Discordant for Multiple Sclerosis: TCRBJ Analysis

From six pairs of monozygotic twins discordant for multiple sclerosis, CD4+CD45RO− (naive Th) cells were isolated, and the TCRBJ repertoires were determined for each set of cells (see Appendix D). The average age of the discordant twin pairs was 40 years old. Please note that subject identification numbers ending in 1 represent individuals with MS and those ending with 2 represent healthy individuals. The correlation coefficients derived from the comparison of these repertoires are presented in Figure 4-24. Figure 4-24 depicts four possible pairs; these include comparisons 1) between healthy twin pairs (see Figure 4-17), 2) twin pairs discordant for MS, 3) between healthy, unrelated pairs (i.e., between the six healthy twin members from the discordant pairs), and 4) between unrelated MS patient pairs (i.e. between the six MS patients from the discordant pairs.)
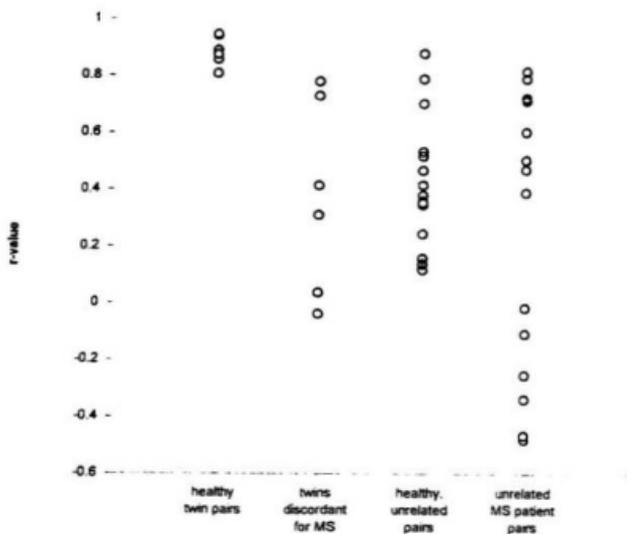
Figure 4-24: Correlation analysis of the naive T-cell TCRBJ repertoires in six healthy twin pairs, in six identical twin pairs discordant for MS, in healthy members of discordant twin pairs and in affected members of discordant twin pairs.

From Figure 4-24, three interesting observations can be made. First, the twin pairs discordant for MS appear to be less similar with respect to their TCRBJ naive T-cell repertoires than do healthy twin pairs. Confirming this, a z-test gives $p<0.001$. This z-test compared two r-values: one r-value for the healthy twin pairs and one r-value for the twin pairs discordant for MS. Second, no difference is observed between twin pairs discordant for MS and healthy, unrelated pairs. Using the method of analysis discussed in Section 4.5.1, healthy, unrelated pairs were compared to twin pairs discordant for MS; the results are presented in Figure 4-25. Figure 4-25 shows that of the 45 possible comparisons, not one was above the critical z-value of 1.96, indicating that the naive T-cell TCRBJ repertoires show no statistical difference between twin pairs discordant for MS and healthy, unrelated pairs. Third, Figure 4-24 shows that unrelated MS patient pairs do not appear to be more similar with respect to their naive T-cell TCRBJ repertoires than healthy, unrelated pairs; this was tested using the method of comparison described in Section 4.5.1. Of 675 possible comparisons between unrelated MS patient pairs and healthy, unrelated pairs, not one was statistically significant, see Figure 4-26.
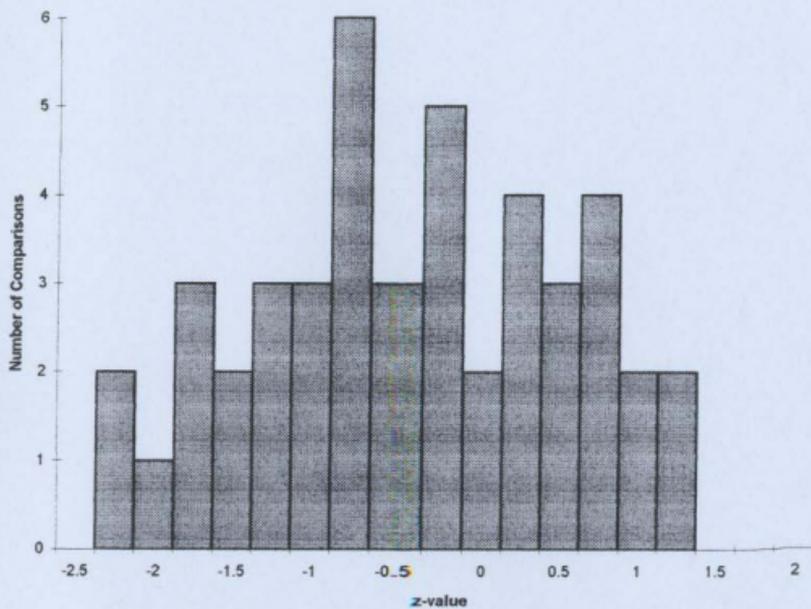
Figure 4-25: Distribution of z-values obtained from 45 comparisons of correlation coefficients of twin pairs discordant for MS and unrelated pairs.
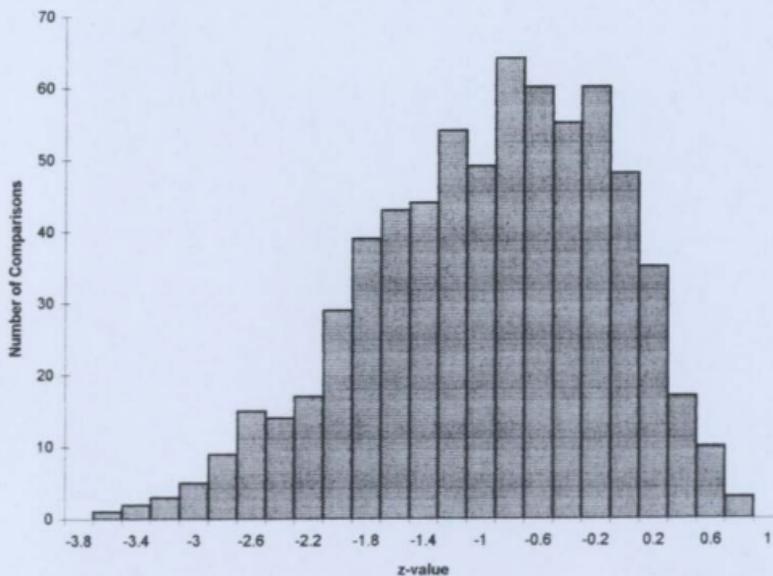
Figure 4-26: Distribution of z-values obtained from 675 comparisons of correlation coefficients of unrelated MS patient pairs vs. healthy unrelated pairs.

4.8 Summary

4.1 - The TCR repertoires obtained by the method described in Chapter 3 are reproducible.

4.2 - The number of PCR cycles after which the accumulation of product is no longer linear was determined for TCRBV primers and TCRBJ primers.

4.3 - The efficiency of the thirteen TCRBJ primers and the 24 TCRBV primers in the PCR was determined. These primers show statistically significant differences in their priming efficiencies.

4.4 - Statistical evidence was obtained that most individuals (15 of 20) have different TCRBJ repertoires in naive vs. memory T-cells but that a minority of individuals (4 of 9) have different TCRBV repertoires in naive vs. memory T-cells.
  - The difference between the TCRBJ repertoires of naive and memory cells increases with age.
  - TCRBJ repertoires were compiled for the twenty unrelated volunteers. The repertoires show statistically significant differences between quantitative use of each gene segment.

4.5 - Monozygotic twins have more similar TCRBJ repertoires than unrelated individuals.
  - HLA identical siblings have more similar TCRBJ repertoires than HLA non-identical siblings.

4.6 - Twins discordant for MS have less similar TCRBJ naive T-cell repertoire than healthy twins.

  - Twins discordant for MS are no more similar with respect to their naive T-cell TCRBJ repertoire than healthy unrelated individuals.

  - Unrelated MS patients are no more similar with respect to their naive T-cell TCRBJ repertoire than healthy unrelated individuals.

5. DISCUSSION

5.1 Materials and Methods

5.1.1 Reproducibility

If an experiment is not reproducible, its results are meaningless. It was crucial, therefore, to determine the reproducibility of the RT-PCR experimental approach designed to determine the TCR repertoire of different T-cell subsets. Correlation analysis was used to test reproducibility. Initially, when identical cDNA samples were prepared but analyzed using separate PCR cocktails, the results approached statistical significance ($p < 0.07$). The experimental determination of the TCR repertoire was only truly reproducible, however, when identical cDNA samples were analyzed using the same PCR cocktail ($p < 0.001$) (see Section 4.2). This was an important finding as it meant that the same PCR cocktail or master mix must be used when the experimental objective is to compare the TCR repertoires of different cDNA samples.

For example in order to be able to compare the TCR repertoires of unrelated individuals in the study on monozygotic twins, a PCR cocktail had to be prepared for each set of four individuals, i.e. two sets of twins. In this way, not only could the twins be compared but an individual from one twin pair could be compared to an individual from a second twin pair, thus providing a comparison of

unrelated individuals which is required as a control. Similarly, one PCR cocktail was prepared for analyses of the TCR repertoires of each individual participating in the study of naive vs. memory T-cell TCR repertoires. From this cocktail, both the naive cells and the memory T-cells were analyzed. Similarly, for the study on HLA identical vs. HLA non-identical siblings, a single PCR cocktail was prepared for TCR repertoire analyses of each entire family.

5.1.2 Plateau Phase

As discussed in section 1.10, every PCR has three phases: exponential, quasi-linear and plateau (Sardelli, 1993). Throughout the first two phases, the amount of product is proportional to the amount of substrate initially present in the reaction tube. The plateau phase, however, results when the PCR conditions become limiting and during this phase the amount of product is no longer an accurate reflection of the amount of substrate initially present. It is crucial, therefore, to determine the number of cycles which are still in the quasi-linear phase, i.e. the number of cycles before the onset of the plateau phase (see sections 1.10 and 3.8 to 3.10).

As discussed in section 3.10, there were two types of substrates used in PCR. The first was PCR-amplified DNA; the second was cDNA. cDNA was used in the analysis of the TCR repertoires, in the testing of the experimental reproducibility and in the first round PCR used to determine the priming

efficiencies. Amplified DNA was used in the second round PCR used to determine the priming efficiencies. The plateau was determined for both types of substrate.

It was reasoned that the most efficient primer should be used to determine the plateau, because the PCR product using this primer should reach a plateau in the least number of cycles. In other words, when the PCR product using the most efficient primer has reached its plateau, it can be assumed that the remainder of the PCR-products are still within the quasi-linear phase. Unfortunately, in order to determine the most efficient primer, the results of the plateau phase determination were needed and vice-versa, creating a dilemma.

To overcome this dilemma, the representative primers used to determine the plateau phase were chosen based on an estimate of the relative priming efficiencies of the primer sets. The brightness of the bands on an ethidium bromide stained agarose gel was used as a crude indicator of priming efficiency. It was assumed that the brighter the bands, the more efficient the primer.

The number of cycles chosen for the various PCRs was a little lower than the estimated beginning of the plateau, i.e. still in the quasi-linear phase. For example, the extrapolated plateau of the cDNA substrate for TCRBJ was 32 cycles, but subsequent PCRs carried out under the same conditions (i.e. using the same substrate and a TCRBJ primer from the same set as the representative primer) were run for 24 cycles. Similarly, the extrapolated plateaus for the

TCRBJ PCR-amplified DNA substrate, for the TCRBV cDNA substrate and for the TCRBV PCR-amplified DNA substrate were 18, 35 and 30 cycles, respectively; subsequent PCRs carried out under the same conditions were run for 14, 30 and 25 cycles, respectively.

Another interesting observation is that the plateau occurred much faster for an amplified DNA substrate than for a cDNA substrate, see Table 4-1. A cDNA substrate contains not only the cDNA for which the primer is specific but all the other T-cell cDNAs. This means that the actual cDNA substrate which is PCR amplified by a particular primer pair must represent only a very small fraction of the total cDNA and is likely to be several orders of magnitude less than the PCR-amplified DNA substrate. The obvious implication is that the application of the method described here to assess other primer sets require all due care that the PCR amplification in the second round is in the quasi-linear phase.

5.1.3 Primer Efficiency

The possibility that different TCR primers have different priming efficiency has been considered by some investigators but not others (reviewed by Daniel and Haegert, 1996). Daniel and Haegert (1996) first described a method to test and correct for differences in priming efficiency. These researchers showed how failure to account for primer efficiency in analyses of 23 TCRBV segments could

lead to incorrect conclusions as to levels of expression of TCRBV segments in a tissue or in an individual.

This thesis extended the earlier work of Daniel and Haegert (1996) to 24 TCRBV families and analyzed for the first time a set of 13 TCRBJ primers. Several observations were made. First, the various TCRBJ and TCRBV primers demonstrated statistically significant differences in their priming efficiencies (see section 4.3). Second, the most efficient TCRBV primers in this thesis were TCRBV12, 13 and 14 whereas Daniel and Haegert (1996) reported that TCRBV6 and 15 are most efficient. Since the set of TCRBV primers used in this thesis and the set used by Daniel and Haegert (1996) were not identical, this second finding has an important implication. That is, it is essential to analyze each primer set for primer efficiencies if the experimental objective is to determine levels of expression of particular TCRs in a tissue or in an individual.

It should be noted that only particular types of studies are affected by differences in priming efficiency. As discussed, studies in which multiple primers are used to amplify different genes could produce skewed results as a result of variations in priming efficiency. In contrast, if the same gene product is being PCR-amplified and compared in different people, different tissues or different cell types, biases in priming efficiency are irrelevant to the results. It is when the product of two sets of primers is compared, however, that it becomes critical to account for these differences.

The present and earlier results (Daniel & Haegert, 1996) call into question various findings on the frequency of usage of various TCR segments in a group of individuals (Cowan & Haegert, 1997). Examples of some studies, the results of which may have been affected by failure to account for priming efficiencies, are those of Gulwani-Akolkar et al., 1995 and Usuku et al., 1993, in which the relative expression levels of various TCR segments are reported.

It is important to note that the priming efficiencies must be tested in other laboratories even if the same primers are used, because differences in equipment and reagents may affect the primer efficiencies.

5.2 First Hypothesis

5.2.1 Interpretation of Results

5.2.1.1 Naive vs. Memory T-Cell TCR Repertoires

Earlier (Section 1.6.5) the issue of "revertant" memory T-cells among CD45RA$^+$ T-cells was discussed and the entire issue will be discussed in detail later (Section 5.5). The data obtained from testing the first hypothesis are of interest with regard to this issue. Section 4.5.1 shows that 75% of the individuals tested showed no correlation between the TCRBJ repertoires of CD4$^+$CD45RA$^+$ and CD4$^+$CD45RO$^+$ T-cells. Further 44% demonstrated no correlation between the TCRBV repertoires of the phenotypically distinct subsets. In other words, at

least for the TCRBJ repertoires, the results indicate that the two populations are distinct. Our data does not exclude the possibility that memory T-cells are peresent among CD45RA$^+$ T-cells. If these 'revertant' cells are present, their numbers are few; otherwise, no distinction could have been made between the memory T-cell and naive T-cell repertoires. For the remainder of the discussion the terms naive and memory will be synonymously with CD4$^+$CD45RA$^+$(RO$^-$) and CD4$^+$CD45RA$^-$, respectively.

Even though some individuals showed no statistical evidence of differences in repertoires between the two cell subsets, there is no method of accurately determining who will and who will not demonstrate a correlation between his/her memory T-cell and naive T-cell TCR repertoires. As a result, I suggest that differences between the T-cell repertoires of the two subsets must be accounted for in any experimental design focused on analyzing the T-cell TCR repertoire. The memory T-cell repertoire is influenced by post-thymic antigenic exposure and superantigen, whereas the naive T-cell repertoire likely is closely similar to that of T-cells exiting the thymus. Failing to account for this may explain why Daniel (1995) failed to demonstrate a genetic effect on the TCRBV repertoire in twins; T-cell subsets were compared in twin pairs and in unrelated pairs, but the T-cell subsets were not further subdivided on the basis of CD45 isoform expression. A focus on naive T-cells might have led to evidence of a genetic effect. Many other studies of the TCR repertoire examine T-cell subsets

but not memory or naive T-cells separately (see for example Lovebridge *et al.*, 1991 and Akolkar *et al.*, 1993).

A difference was detected between the naive T-cell TCR repertoires and the memory T-cell TCR repertoires with respect to the levels of expression of the 13 TCRBJ gene segments and the 24 TCRBV families. Recently, Walser-Kuntz *et al.* (1995a) obtained similar results; their study, however, only analyzed two TCRBV gene segments and not the entire repertoire. Almost certainly, these results reflect post-thymic events that affect particular TCR segments in memory T-cells. It is surprising to note that no other comprehensive study has evidently compared memory to naive T-cells for relative levels of expression of different TCRs.

The correlation of the TCRBJ decreases with age. In other words as a person ages, his/her memory T-cell TCRBJ repertoire progressively deviates from his/her naive T-cell repertoire. This stands to reason if the subsets are indeed distinct, because as an individual ages, there is increasing opportunity for contact with external antigen. With every contact with antigen, there is an accumulation of memory cells with an expanded range of TCR specificities. This is also supported by the finding that with age comes an increased proportion of CD45RO$^+$ cells (McElhaney *et al.*, 1995).

No evidence of a gender effect was apparent in the correlation between memory and naive T-cell TCRBJ repertoires. This may be for several reasons.

Most obviously, there may be no gender related difference in the TCR repertoire. This is not the only possible explanation, however. Perhaps, there were not enough people in the study; a larger sample population may have provided evidence for a gender difference. Furthermore, differences in age groups may have masked any gender difference. 55% of the males were over thirty years old, whereas, only 22% of the females fell into that age category. This may have skewed the results, causing the male group to have a much lower r-value than expected and vice versa. A study to specifically investigate possible gender related differences would analyze the TCRBJ repertoires in individuals from the same age categories.

5.2.1.2 Overall TCRBJ Repertoire

There is an obvious pattern in expression of the TCRBJ repertoire for $CD4^+CD45RA^+$ cells and $CD4^+CD45RO^+$ cells of the twenty individuals studied (see Figures 4-12 and 4-16). An ANOVA confirms that the mean levels of expression of the various TCRBJ segments are significantly different for both T-cell subsets. For the twenty unrelated individuals, analyses of naive T-cells showed that TCRBJ1S1, 1S5, 2S1 and 2S7 are expressed at the highest mean levels and TCRBJ2S4 and 2S6 at the lowest mean levels. By contrast, memory T-cells showed no extreme levels of TCRBJ segment expression although similar to naive T-cells, TCRBJ2S4 was expressed at the lowest level.

Comparison of the present results with published data reveals some interesting findings. Nanki *et al.* (1996) analyzed cDNA from CD4$^+$ T-cells from six healthy twin pairs, six twin pairs discordant for rheumatoid arthritis and five healthy individuals. The cDNA was PCR-amplified using TCRBV5, 13 and 17-specific primers. Then, the amplified products were hybridized with TCRBJ-specific probes to assess TCRBJ usage. Nanki *et al.* report that TCRBJ2S1 is the most frequently used TCRBJ segment, and 1S4, 1S6, 2S4 and 2S6 are used least frequently. Jeddhi-Tehrani *et al.* (1993) similarly analyzed cDNA from CD4$^+$ T-cells from seven individuals using TCRBV-specific primers and hybridization with TCRBJ-specific probes. These investigators reported that TCRBJ1S1, 2S1 and 2S7 are used most frequently and 1S3, 1S4, 1S6, 2S4 and 2S6 least frequently. Rosenberg *et al.* (1995) analyzed RNA from one twin pair and three unrelated individuals and sequenced cDNA from 50 clones. The most frequently used segments were 1S3, 1S4 and 2S6. Hall and Lanchbury (1995), in contrast, prepared RNA from PBLs of a single healthy individual and sequenced 129 cDNA clones to study TCRBJ usage. Finally, Walser-Kuntz *et al.* (1995b) analyzed cDNA from CD4$^+$CD45RO$^-$ PBLs using PCR-amplification of TCRBV5S1 and TCRBV8 followed by hybridization with TCRBJ-specific probes. The latter two groups of investigators reported that TCRBJ2S1 alone (Walser-Kuntz *et al.*, 1995b) or TCRBJ1S1, 1S2, 2S1, 2S5 and 2S7 (Hall & Lanchbury, 1995) were used most frequently. Interestingly, all groups reported that the TCRBJ2 gene cluster is used more frequently that the TCRBJ1 gene

cluster. There are several possible explanations for the discrepancies between the present and earlier findings. First, and most important, most of the cited studies did not address the possibility that naive and memory T-cells could have different TCRBJ repertoires; two of the studies did not even distinguish between CD4$^+$ and CD8$^+$ T-cells. Second, the repertoires determined in three of the studies were only based on analyses of TCRBJ segments in a limited number of TCRBVs; this is likely an important caveat since Walser-Kuntz et al. (1995b) demonstrated that the TCRBJ repertoire associated with each TCRBV family may differ. Third, two of the studies based conclusions on analyses of only a small number of TCR clones (50 and 129). Fourth, the various reports are from different parts of the world and we are, therefore, comparing populations with potentially different genetic backgrounds. This could affect the TCRBJ repertoire and may partially account for the discrepancies mentioned above. Finally, it is important to note that the present thesis is the first to my knowledge that provides a detailed RT-PCR analyses of the TCRBJ repertoire in T-cell subsets based on surface expression of CD45 isoforms using the RT-PCR technique.

Analyses of the mean levels of expression of the TCRBJ segments in naive T-cells (CD4$^+$CD45RA$^+$ T-cells) indicate that both the TCRBJ gene clusters are expressed at relatively equal levels. That is, TCRBJ1S1 to 1S6 segments make up 52% of the repertoire and TCR2S1 to 2S7 segments make up 48% of the repertoire. Interestingly, this is in contrast to what has been reported

previously (see above); most researchers find that TCRBJ2 gene segments are expressed at higher levels than TCRBJ1 gene segments. The reasons for this discrepancy may be found in the preceding paragraph, particularly the failure to distinguish between T-cell subsets.

5.2.1.3 Umbilical Cord Blood Analysis

Analysis of CD4$^+$ cells isolated from umbilical cord blood revealed that all thirteen TCRBJ segments are expressed but that most cells have the so-called early activated phenotype, i.e. most CD4$^+$ cells are CD45RO$^+$CD45RA$^+$. The present study, therefore, provides no insight into the presence/absence of memory T-cells at birth and no insight into TCRBJ segment usage in naive T-cells at birth. The present study also suggests that no comparison of the naive and memory T-cell repertoires are possible in the newborn using CD45 isoforms to select for different maturational stages.

5.3 Second Hypothesis

5.3.1 Interpretation of Results

5.3.1.1 Statistical Analysis of the Effect of Genetics/HLA on the TCRBJ Repertoire

To date, most studies of the TCRBJ, BV or AV repertoires involve the comparison of different groups of individuals, for example twins vs. unrelated individuals (Gulwani-Akolkar et al., 1991; Hawes et al., 1993 and Lovebridge et al., 1991), HLA identical siblings vs. HLA non-identical siblings (Gulwani-Akolkar et al.; 1995, Akolkar et al., 1993 and Uhrberg & Wernet, 1996) and individuals affected by a certain disease vs. healthy individuals (Walser-Kuntz et al., 1995b and Nanki et al., 1996).

The experimental design of any study comparing the TCR repertoire between two groups of individuals must contain not only an acceptable method of determining the levels of expression of the TCR gene segments, but also an appropriate statistical method for analyzing the data. Until recently, no satisfactory method of statistical analysis has been published (Daniel et al., 1997 in press).

Usually, individuals are compared in groups of two, i.e. using pairwise comparisons. There are, however, some studies which attempt to analyze larger

populations using cluster analysis (Walser-Kuntz *et al.* 1995b and Nanki *et al.*, 1996); this statistical approach will be briefly discussed later in this section.

Assuming use of pairwise comparisons, there are at least four necessary steps in the statistical analysis: 1) division of the individuals into pairs in an appropriate manner, 2) deletion of one of the TCR gene segments from the analysis in order to achieve independence of the data, 3) statistical analysis of the similarity between the TCR repertoire of each pair of individuals resulting in a test statistic for each pair and finally, 4) comparison of the test statistics obtained in step three.

The majority of the statistical analyses used in the current literature fail to achieve either one or both of steps 2 and 4.

Step 1: When dividing individuals into groups for statistical analysis, every individual must only take part in one pairwise comparison. For example, take two sets of twins, A1 & A2 and B1 & B2. If A1 is paired with A2 to form a twin pair, A1 cannot simultaneously pair with B1 to form a pair of unrelated individuals. Allowing the same individual to participate in more than one comparison would bias results (Daniel *et al.*, 1997 in press).

Step 2: Whether it is TCRAV, BV or BJ, the sum of all the levels of expression in any particular repertoire is 100%. The levels of expression, therefore, are not independent of one another. Take the thirteen TCRBJ gene segments for example. If the levels of expression of twelve of the TCRBJ gene

segments are known, and these levels of expression are known to add up to 90%, then the level of expression of the thirteenth TCRBJ segment is known, 10%. In order to achieve independence of the data, the level of expression of one gene segment must be removed from all the repertoires, and subsequent calculations. In order to ensure no bias of the results, the same TCR gene segment must be removed from the repertoire of every individual.

Step 3: There are two main methods of statistical analysis which have been employed to test the similarity of the TCR repertoires between various pairs of individuals: Δ-scores (Uhrberg & Wernet, 1996; Gulwani-Akolkar *et al.*, 1991 & 1995 Akolkar *et al.*, 1993 and Lovebridge *et al.*, 1991) and regression analysis (Hawes *et al.*, 1993). Regression analysis is not suitable for the statistical testing of the TCR repertoire. The test statistic obtained from a regression analysis is an r^2-value. An r^2-value close to one indicates a high correlation, and an r^2-value close to zero indicates a low correlation. A problem arises because the square root of the r^2-value, that is the r-value or the correlation coefficient, can be either a positive or a negative number. A negative number indicates a high negative correlation. If two individuals expressed highly different repertoires (i.e., the TCR gene segments expressed at high levels in one individual are expressed at low levels in a second individual), this would produce an r-value close to -1 and an r^2 of close to 1. In other words, regression analysis would lead researchers to make wrongful conclusions about two individuals expressing highly different repertoires.

Δ-scores are the sum of the absolute differences obtained between two individuals for each of the TCR gene segments. Problems arise in the statistical analysis using Δ-scores (see step 4).

Step 4: As mentioned earlier, the objective of many of the studies presently being discussed is to test the similarity of the TCR repertoires between two populations of individuals. Each population is divided into pairs of individuals, and a test statistic is obtained for each pair. Step 4 requires a statistical test to compare the test statistics obtained for each of the two populations of individuals. Though some studies have attempted to compare the Δ-scores obtained either by Kruskal-Wallis test (Gulwani-Akolkar *et al.*, 1995) or by ANOVA (Lovebridge *et al.*, 1991 and Gulwani-Akolkar *et al.*, 1991), the Δ-score represents a measure that cannot be analyzed statistically (Daniel *et al.*, 1997 in press).

Similar to the pairwise comparisons, analysis of two test populations simultaneously by cluster analysis does not provide statistical evidence for similarity or differences between the two populations being studied. Though it may appear initially that the two populations are different, when a cluster analysis shows that they form distinct groups based on the similarity of their TCR repertoire, the actual statistical significance of the formation of these clusters is not tested.

Daniel *et al.* (1997 in press) have recently published a method to analyze the TCRBV repertoire between pairs. In their novel approach, the investigators

use the r-value or correlation coefficient to compare the TCRBV repertoires of individuals. In order to test the closeness of the r-values obtained, Daniel *et al.* (1997 in press) have developed a method based on Fischer's Z-transformation which requires approximately 25 data points or more. In other words, it is necessary to determine the level of expression of approximately 25 gene segments in each individual. Fischer's Z-transformation usually permits comparison of only two r-values, but these investigators expanded the application of this test to include numerous r-values.

Unfortunately, this method cannot be applied to the TCRBJ repertoire due to the number of TCRBJ gene segments. As mentioned, approximately 25 or more gene segments are required for the data analysis and there exist only 13 TCRBJ gene segments. The method used in this thesis to analyze the TCRBJ repertoire makes use of both correlation coefficients and Fischer's Z-transformation and combines data from more than one individual (see Chapter 4). The drawbacks to this method are that it is extremely labour intensive requiring application of Fischer's Z-transformation to every possible combination of r-values and yet, may provide insufficient evidence to make a definite conclusion (see Chapter 4 for a more detailed explanation). Thus, the analysis of twin pairs vs. unrelated pairs required 40 comparisons, and the analysis of the HLA identical sibling pairs vs. the HLA non-identical sibling pairs required 288 comparisons. Over 85% and 94% of the comparisons showed

statistical significance, respectively. Since the vast majority of the comparisons demonstrated statistically significant differences between the TCRBJ naive T-cell repertoires of the groups being analyzed, it is reasonable to conclude the TCRBJ repertoires differ between identical twins and unrelated pairs and between HLA identical and non-identical siblings. These findings are noteworthy as the twin-unrelated pair studies provide the first statistical evidence that there is a genetic effect on the TCRBJ repertoire. Moreover, the family studies provide the first statistical evidence for an an HLA effect on the TCRBJ repertoire. In fact, these findings are the first that clearly indicate a genetic/HLA effect on any component of the TCR repertoire that is **supported by statistical evidence**.

5.4 Third hypothesis

5.4.1 Interpretation of Results

5.4.1.1 Monozygotic Twins Discordant for Multiple Sclerosis

The naive T-cell TCRBJ repertoire was determined for six sets of twins discordant for multiple sclerosis, and six sets of healthy twins (see Section 5.3). Initial analyses of naive T-cell TCRBJ repertoires provided statistical evidence that the naive T-cell TCRBJ repertoire is more similar among healthy monozygotic twins than among unrelated pairs (see Section 5.3). However, this increased similarity of the TCRBJ repertoire for naive T-cells in identical twins is

no longer found when identical twins are discordant for multiple sclerosis. Not only was there no statistical evidence that twin pairs discordant for MS are more similar with respect to their naive T-cell TCRBJ repertoire than unrelated individuals, but also these discordant twin pairs were statistically different from the healthy twin pairs, with the healthy twin pairs having significantly more similar repertoires ($p<0.01$).

The implications of the discordant MS twin findings are of interest. Clearly, the genetic effect demonstrable in healthy twin pairs is no longer evident in the discordant twin pairs and, in fact, the repertoires of the latter are similar to those of unrelated pairs. The interesting issue is how to explain the masking of the genetic effect in the discordant MS pairs. Several possibilities were considered but only one possibility is deemed likely. First, the possibility that different T-cell subsets had been analyzed in the different types of twin pairs can reasonably be excluded since the repertoires were determined on cells with identical phenotypes. The only caveat is that there could conceivably be more "revertant" memory cells among $CD4^+CD45RO^-$ T-cells in MS patients than in healthy individuals (see also Section 5.5). Second, alteration of the TCR repertoires in healthy members of discordant pairs could have led to protection against MS in individuals sharing susceptibility genes with their affected twin members. This seems an unlikely possibility as the question arises as to what factor could consistently alter the repertoires of **all** the healthy individuals in the discordant

pairs that would not have similarly altered the repertoires in at least some healthy twin pairs, thus leading to low r-values. The third possibility, and the simplest (see Occam's Razor), is that the TCR repertoires were actually altered in the patients having a disease known to affect TCR usage against myelin components, namely MS. Whether this alteration occurred within the thymus during thymic selection as a consequence of random or stochastic events, as postulated by Möller et al. (1990), or later in life, either preceding or as a consequence of MS, remains unknown. However, it seems unlikely that thymic selection events would have had such profound effects on the TCRBJ repertoires that are shown in Appendix D. Thus, among the healthy twin pairs the expression of individual TCRBJ segments usually varied only slightly between twin members and only three of the six pairs showed variation in a single TCRBJ segment of 4%. In contrast, among five of the six discordant MS pairs at least two and as many as eight TCRBJ segments varied by 4% or more between twin members. One discordant pair showed variation in a single TCRBJ segment but this variation was 8% between twin members.

The present work on discordant MS pairs has broader implications for the study of the TCR repertoire in disease states. An important question is how to show whether the overall TCR repertoires are altered in a particular autoimmune disease. Usually studies have focused on TCR usage in particular tissues (Hafler et al., 1996) or TCR usage against particular molecules (Hafler et al., 1996) but

have not focused on the overall TCR repertoire. This is because it is difficult to identify a reference point against which a patients' TCR repertoires can be compared in order to determine whether the overall TCR repertoire is altered in a disease state. The current work used healthy twins as the initial reference point and showed that the TCRBJ repertoires of the twin members are very similar with high r-values. The second reference point was then the healthy member of each discordant MS twin pair. The alteration of TCRBJ repertoires among the discordant MS twin pairs then becomes evidence that the overall TCRBJ repertoires in a phenotypically defined T-cell subset are actually altered in MS patients. To my knowledge this is the first evidence for alteration of a major component of the TCR repertoires in MS rather than evidence of alteration of TCR usage. The approach used here can be easily extended to other autoimmune disorders using the reference points described here.

5.5 Implications of immune memory in CD45RO⁻ cells

The research presented in this thesis initially assumed that the two CD45 isoforms, CD45RO and CD45RA, are accurate indicators of the different maturational stages of T-cells. In other words, it was assumed that CD45RO was expressed on memory cells and that CD45RA was expressed on naive cells. These isoforms are not expressed on mutually exclusive populations, therefore, cells were isolated by negative selection in order to exclude the small number of double positive cells (see Section 3.11.7).

Recent evidence suggests that the aforementioned assumption may be incorrect (see Section 1.6.5). More precisely, it appears that some CD45RO$^+$RA$^-$ (memory) cells may revert back to the so-called CD45RA$^+$RO$^-$ (naive) phenotype and that these "revertant" cells may retain their immunological memory; that is they may continue to behave as memory cells (see Section 1.6.5).

This finding does not affect the interpretation of the results obtained in support of the first hypothesis. That is, for many individuals the TCRBJ, and to a lesser extent, the TCRBV repertoires are significantly different for CD4$^+$CD45RA$^+$ (naive) and CD4$^+$CD45RO$^+$ (memory) T-cells isolated from the same individual. If the CD4$^+$CD45RA$^+$ cells were not purely naive cells, but were contaminated by "revertant" cells, it would have caused the TCRBV and TCRBJ repertoires of these two populations to appear more alike. Thus, in many individuals the TCR repertoires (either TCRBV or TCRBJ) of CD4$^+$CD45RA$^+$ cells and CD4$^+$CD45RO$^+$ cells were shown to be statistically different in spite of possible contaminating "revertant" cells which would have resulted in the cell populations being more alike. Thus, these data are strong evidence that CD45RA vs. CD45RO defines distinct T-cell populations. Also, as discussed in Section 5.2.1.1 these data suggest that if CD45RA$^+$ cells contain "revertant" memory cells, their numbers are few.

The same principle should apply to interpretation of most data resulting from tests of the second and third hypotheses. Thus, the finding that the TCRBJ

repertoires of CD4$^+$CD45RO$^-$ cells in healthy HLA-identical siblings differed from those of HLA non-identical siblings further suggests that the effect of "revertant" cells was minimal. Since the memory T-cell repertoire is influenced by antigen exposure, and siblings would be expected to have encountered similar antigens during childhood and if "revertant" T-cells influence the CD45RO$^-$ cell population, then the populations would have had more comparable TCRBJ repertoires than were found. The potential influence of "revertant " memory cells on the discordant MS twin data needs further brief consideration since all other data were obtained from studies of healthy individuals. If the number of "revertant" cells in MS patients were greater than in healthy individuals, then one would expect the present result, namely that the twin pairs discordant for MS have less similar repertoires in CD4$^+$CD45RO$^-$ T-cells than do healthy twin pairs. However, since all individuals have memory cells such an explanation for the discordant MS pair data would demand some, as yet, unknown mechanism to enhance the percentage of "revertant" cells among MS patients. To my knowledge there are no published data on increased numbers of "revertant" cells in MS or in other diseases. Thus the conclusion outlined earlier still stands, that is, that in MS the TCRBJ repertoire is altered in CD4$^+$CD45RO$^-$ T-cells.

5.6 Variations of the TCRBJ Repertoire in Relation to Variations of the TCRBV Repertoire

Quiros-Roldan *et al.* (1995) demonstrate that TCRBV gene segments preferentially rearrange to certain TCRBJ gene segments. Results presented by Walser-Kuntz *et al.* (1995b) demonstrate that a distinct TCRBJ repertoire is associated with two different TCRBV gene segments (TCRBV5S1 and TCRBV8), though no statistical evidence is presented. The implications of these two studies are that the TCRBJ repertoire may be secondary to the TCRBV repertoire. In other words, it is conceivable that the TCRBJ repertoire is dependent on or a reflection of the TCRBV repertoire.

If the TCRBJ repertoire was dependent on the TCRBV repertoire, it is presumable that those individuals who demonstrated a significant correlation between the memory T-cell and naive T-cell TCRBJ repertoire would demonstrate a similar correlation with respect to the TCRBV repertoire. It was discussed earlier, however, that due to the insufficient and disproportionate sample (see Section 4.5.1.2), no conclusion concerning the above statement could be drawn.

The last two hypotheses of this thesis focus on the TCRBJ repertoire and led to the following two conclusions: that genetics, in particular HLA, influences the TCRBJ repertoire and that the TCRBJ repertoire is altered in multiple sclerosis. If the TCRBJ repertoire is, in fact, secondary to the TCRBV repertoire,

then the results of these two hypotheses would implicate a genetic/HLA effect on the TCRBV repertoire and an alteration of the TCRBV repertoire in MS.

Previous studies by E. Daniel (1995) failed to show any genetic effect on the TCRBV repertoire in CD4$^+$ and in CD8$^+$ T-cell subsets. Daniel used the same methodology described in this thesis (though cells were not separated based on CD45 isoform) and used appropriate statistical tests. Since Daniel (1995) showed no evidence of a genetic/HLA effect on the TCRBV repertoire, it is reasonable to conclude that the present findings of a genetic/HLA effect on the TCRBJ repertoire are not simply the consequence of a genetic /HLA effect on the TCRBV repertoire.

6. CONCLUSION

Many cells participate in the immune response. Differentiation of CD4$^+$ T-cells leads to production of Th cells that "help" other immune cells, thus enhancing the immune response. These cells are short-lived. CD4$^+$ T-cells also differentiate into memory cells that have a longer lifespan and ensure a faster more efficient immune response upon a second contact with the antigen.

Prior to contact with foreign antigen, T-cells are considered to be virgin, unprimed or naive. Memory cells are also referred to as primed cells. These two cell subsets can be partly distinguished based on the expression of a surface protein known as CD45. CD45 has a variety of different isoforms including CD45RA and CD45RO. CD45RA is expressed by naive T-cells, and CD45RO is expressed by memory T-cells, but in addition early activated T-cells express both CD45 isoforms. Also, there is evidence that some CD45RO$^+$ memory cells revert in phenotype and express the CD45RA isoform.

T-cells also express a cell surface protein known as the T-cell receptor (TCR). In this thesis, it is the β-chain of the TCR which is of interest. The genomic DNA of this chain is composed of V, D, J and C segments. These segment rearrange before the T-cell is mature/functional, so that every mature T-cell normally expresses TCRs with only one V, one D, one J and one C segment.

The peripheral TCR repertoire is the number of different TCRs expressed in the peripheral T-cell population. Many different mechanisms contribute to the diversity of the TCR: gene rearrangement, α-chain-β-chain pairing, thymic selection, endogenous superantigen, allelic polymorphisms, N-nucleotide addition and exonucleolytic nibbling.

Multiple sclerosis is an autoimmune disease for which there is currently no cure. In patients with multiple sclerosis, it is hypothesized that autoreactive T-cells lead to demyelination of the central nervous system. Treatments for the disease include mainly non-specific suppression of the immune system. Studying the T-cell repertoire in relation to multiple sclerosis could theoretically lead to new highly specific immunosuppressive therapies.

The research carried out for this thesis focused on determining the percent expression of the 13 TCRBJ gene segments and the 24 TCRBV gene families. This was done using a radioactive RT-PCR technique.

First, PBLs from volunteers were collected. Then, specific cell subsets were isolated based on the surface expression of CD45RO and CD45RA isoforms. RNA was extracted from these cells and cDNA:RNA hybrids were synthesized. Using ^{32}P labeled reverse primers, PCRs were performed. Primers in this reaction were specific for each TCRBJ gene segment or TCRBV gene family. TCRAC was used as an internal control. The amplification of each gene segment or family was carried out in a separate tube. The amplified product was

isolated on an ethidium bromide stained agarose gel, and a cpm reading was obtained for each TCRBJ gene segment or TCRBV gene family. This reading is proportional to the level of expression of each segment or family.

PCR has three phases: exponential, quasi-linear and plateau. Certain unavoidable factors create limiting conditions and result in a plateau. For a quantitative PCR, the plateau phase must never be reached as in this phase the amount of product is no longer proportional to the amount of substrate initially present. The number of cycles which progressed before the plateau phase began was determined for the primers used in this research. These results were incorporated into the experimental design.

As multiple primers were used, it was also important to determine the primer efficiency. The TCRBJ and TCRBV primers were found to have varying efficiencies. For the TCRBJ primer set, TCRBJ2S1, 2S4 and 2S7 were found to be most efficient, and TCRBJ1S4 and 1S5 were found to be least efficient. For the TCRBV primer set, TCRBV12, 13 and 14 were found to be most efficient, and TCRBV19 and 24 were found to be least efficient. All data were corrected for biases in primer efficiency.

The hypotheses of this study were as follows: 1) the TCRBJ and TCRBV repertoire is significantly different between naive and memory T-cells isolated from a single individual, 2) there exists a genetic and an HLA effect on the

TCRBJ repertoire in naive T-cells, and 3) the naive T-cell TCRBJ repertoire is altered in multiple sclerosis.

With regards to the first hypothesis, it was found that the TCRBJ, and to a lesser extent, the TCRBV repertoires are significantly different between CD4$^+$CD45RA$^+$ and CD4$^+$CD45RO$^+$ T-cells in many individuals. This is relevant to any further studies of the TCR repertoire; researchers must in their experimental design account for these T-cell subsets differences. Furthermore, it was found that the correlation between the TCRBJ repertoires in CD4$^+$CD45RO$^+$ T-cells and CD4$^+$CD45RA$^+$ T-cells decreases with age, most likely the result of increased opportunity for exposure to external antigen. No relationship between gender and the correlation between the T-cell TCRBJ repertoires of the two T-cell subsets could be found. A larger study controlling for subjects' age would be necessary to establish any relationship with gender.

With regard to the second hypothesis, this research provides the first statistical evidence that the TCRBJ repertoire of CD4$^+$CD45RO$^+$ T-cells is influence by the genetic makeup including the HLA makeup of an individual. The research presented shows the first statistical evidence that identical twins have more similar TCRBJ repertoires in CD4$^+$CD45RO$^+$ T-cells than unrelated individuals (genetic effect) and that HLA identical siblings have more similar TCRBJ repertoires in CD4$^+$CD45RO$^+$ T-cells than HLA non-identical siblings (HLA effect).

Testing of the third hypothesis provided evidence that the naive T-cell TCRBJ repertoire of CD4$^+$CD45RO$^-$ T-cells in identical twins discordant for multiple sclerosis is altered when compared with healthy twins. Furthermore, no distinct TCRBJ repertoire pattern was found for MS patients.

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Appendix A : FIGURES A1 TO A20
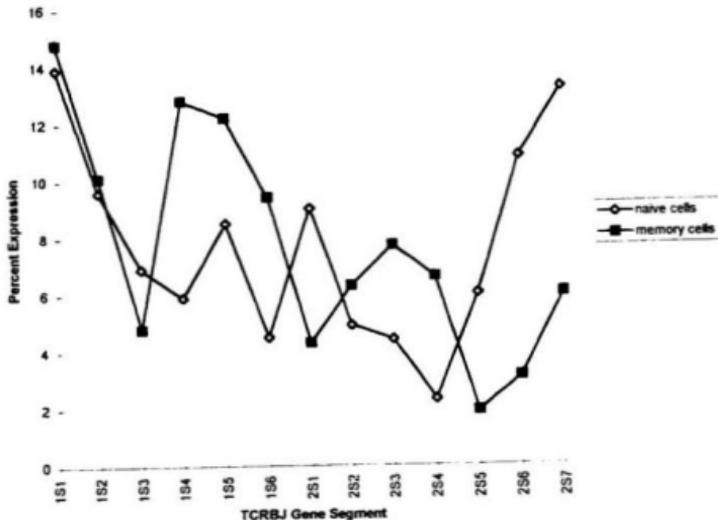
Figure A-1: The TCRBJ repertoire (corrected for primer efficiency) for the
CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1 X.01.
r-value = 0.189
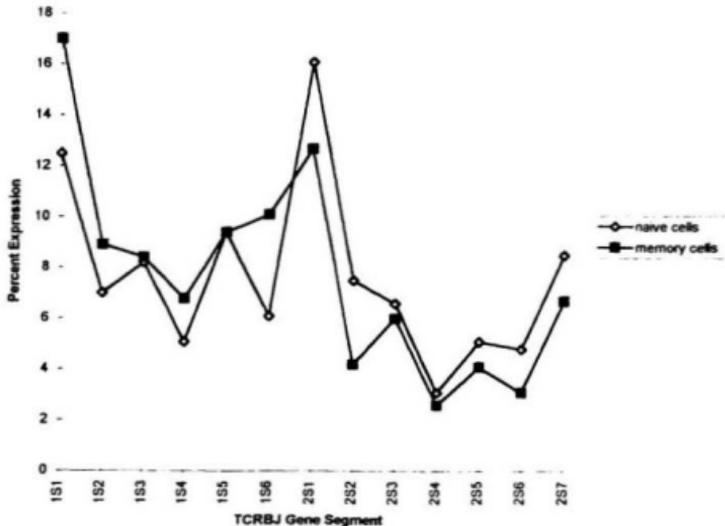
Figure A-2. The TCRBJ repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1 X 02. r-value = 0.794
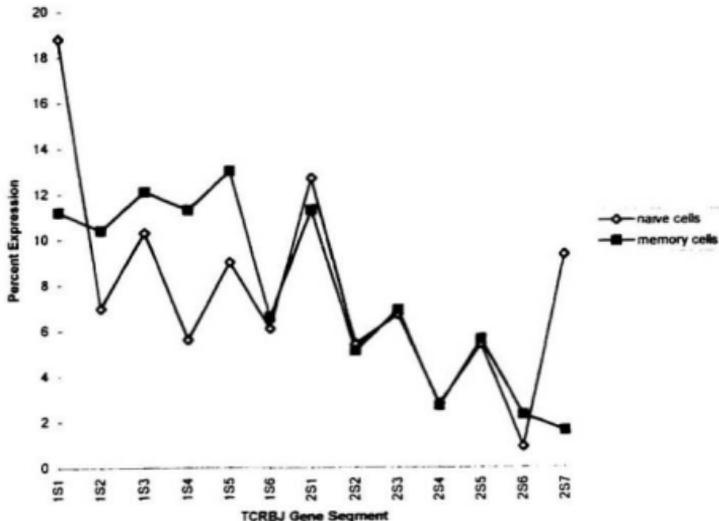
Figure A-3: The TCRBJ repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1 X.04
r-value = 0.591
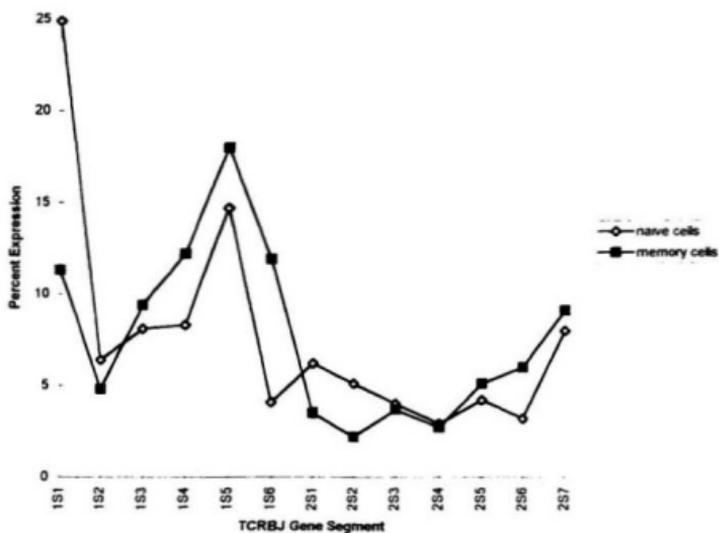
Figure A-4: The TCRBJ repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1 X 05 r-value = 0.590
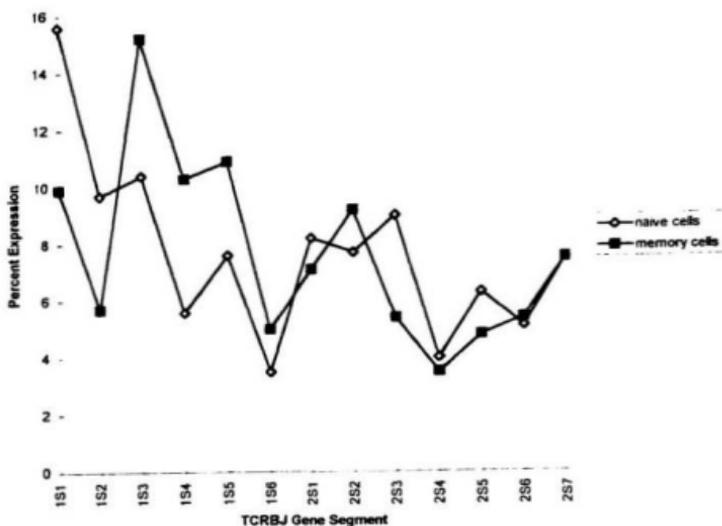
Figure A-5: The TCRBJ repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X.06. r-value = 0.495
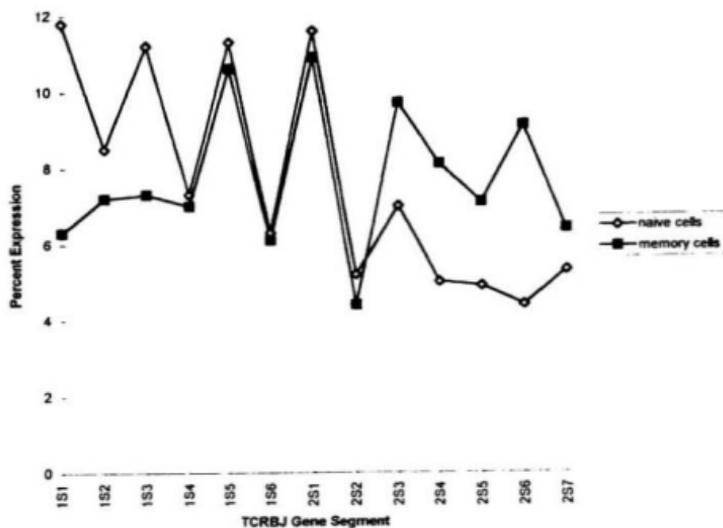
Figure A-6: The TCRBJ repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X.07 r-value = 0.342
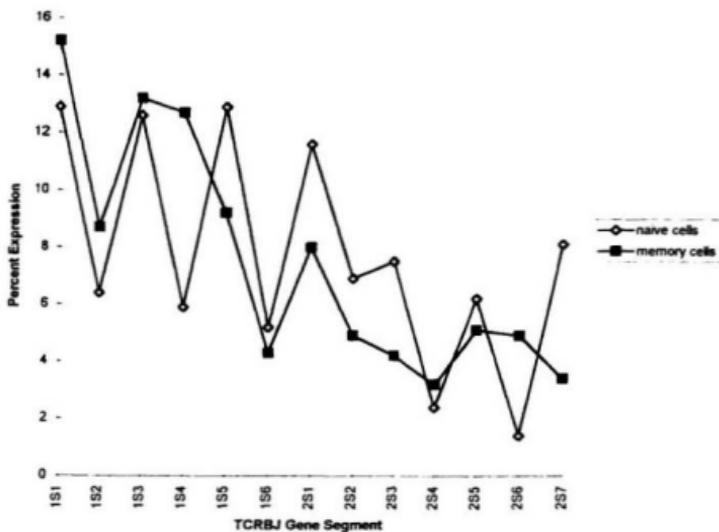
Figure A-7: The TCRBJ repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X.08. r-value = 0.674
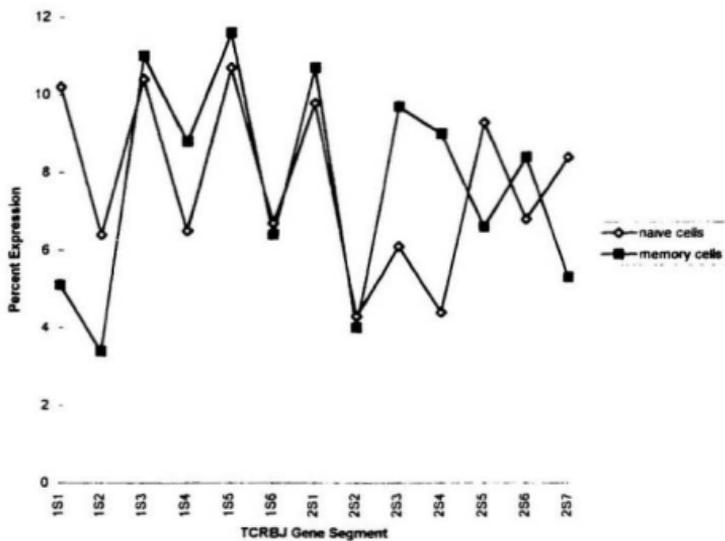
Figure A-8: The TCRBJ repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1 X.09 r-value = 0.306
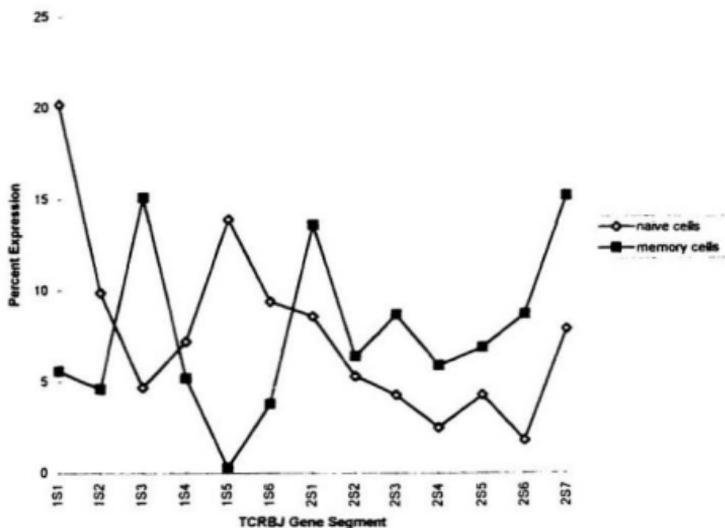
Figure A-9: The TCRBJ repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X.10. r-value = 0.345
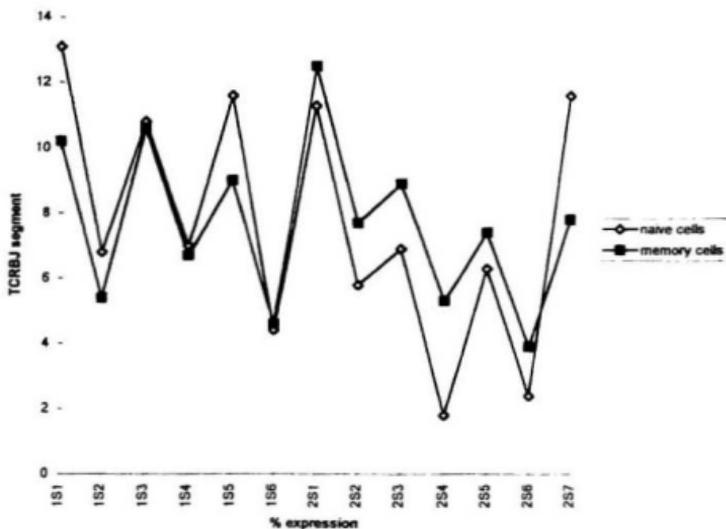
Figure A-10: The TCRBJ repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X.11
r-value = 0.578
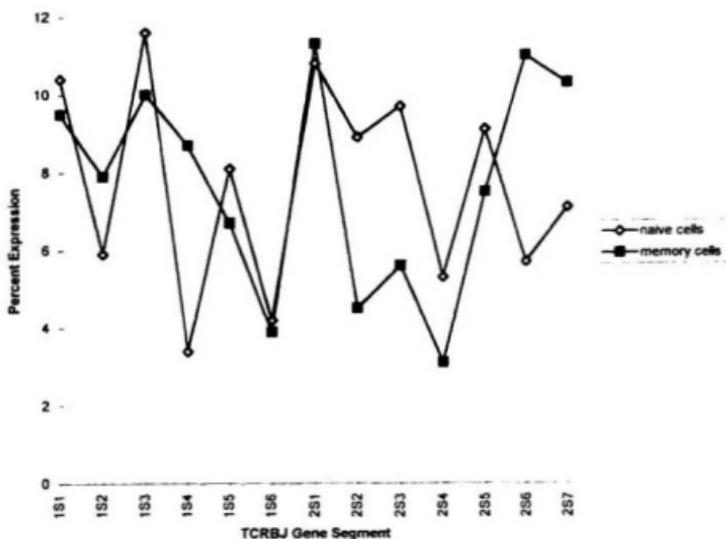
Figure A-11: The TCRBJ repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1 X 12. r-value = 0.315
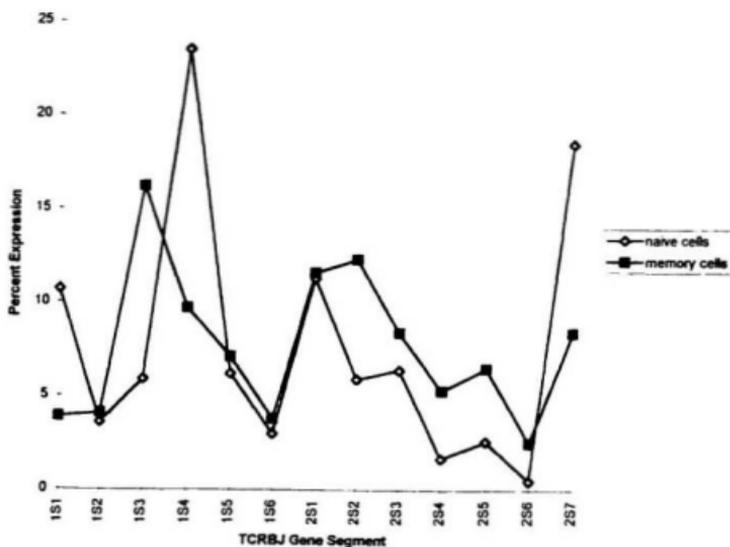
Figure A-12: The TCRBJ repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X.13. r-value = 0.335
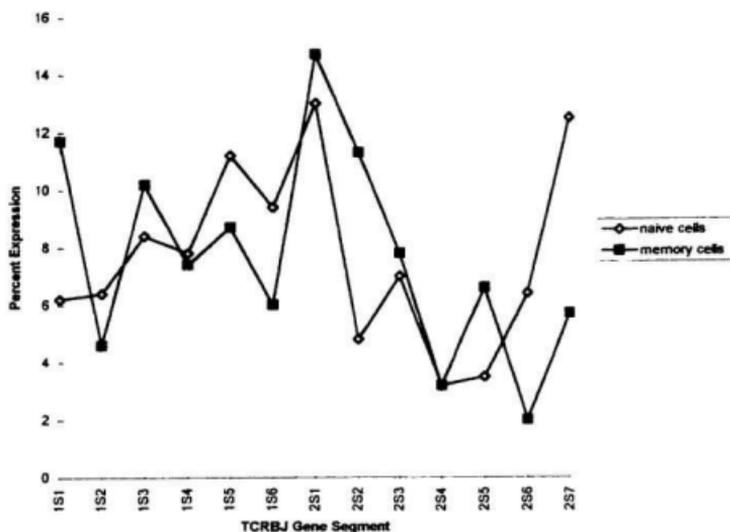
Figure A-13: The TCRBJ repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X.14. r-value = 0.365
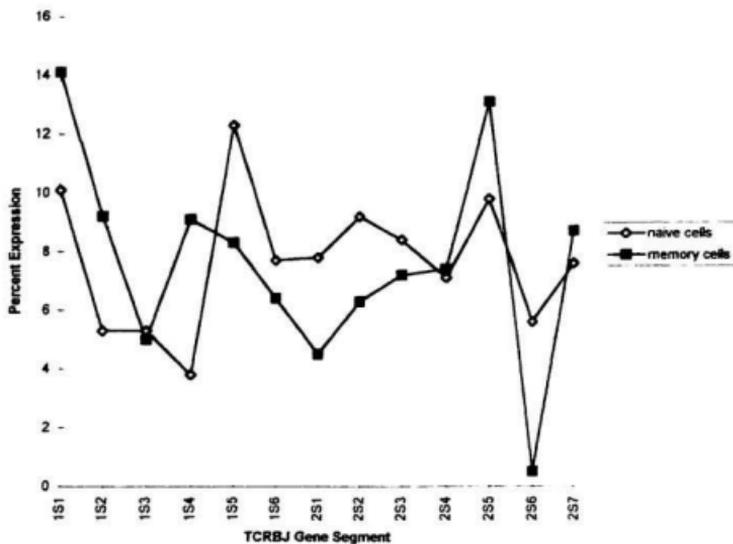
Figure A-14: The TCRBJ repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X.15. r-value = 0.401
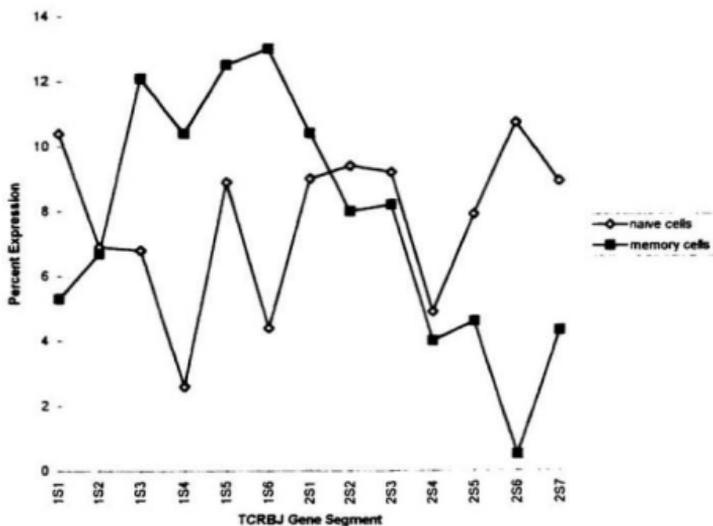
Figure A-15: The TCRBJ repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X.17. r-value = -0.478
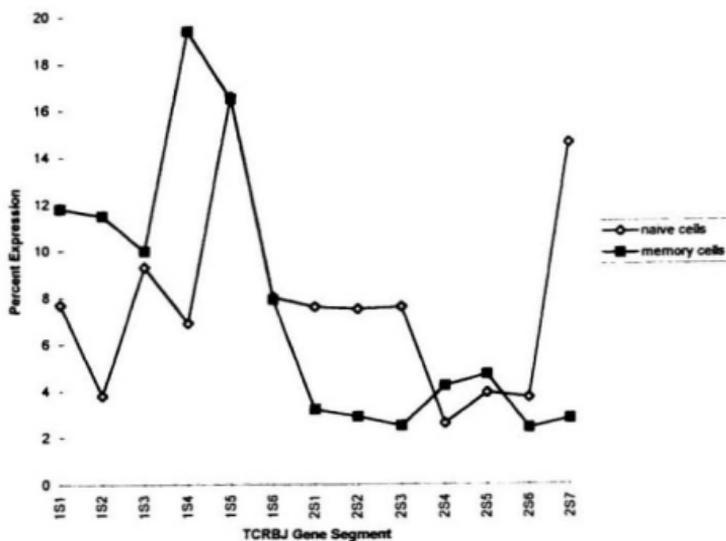
Figure A-16: The TCRBJ repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X.18. r-value = 0.269
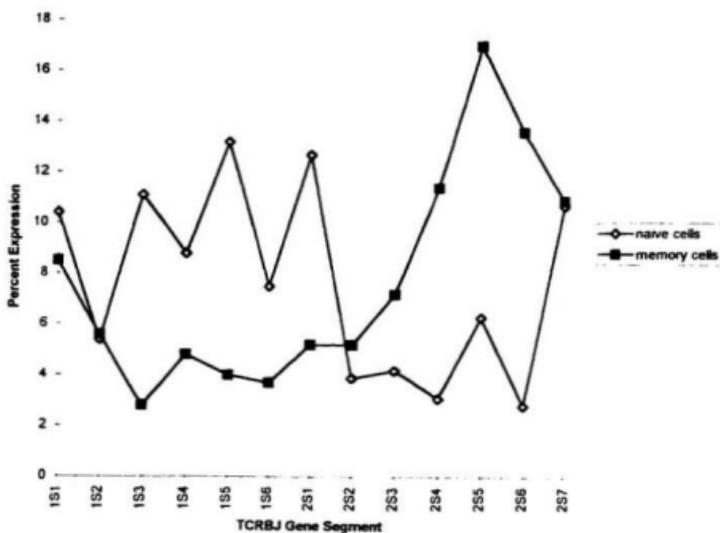
Figure A-17: The TCRBJ repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1 X 20. r-value = -0.433
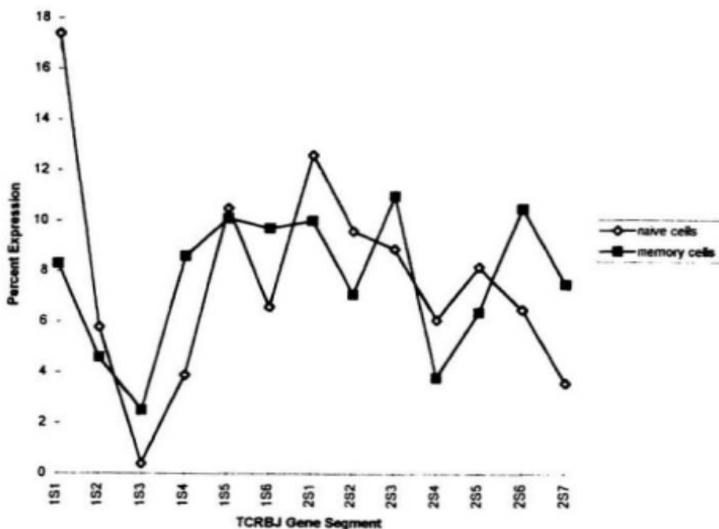
Figure A-18: The TCRBJ repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X.21
r-value = 0.475
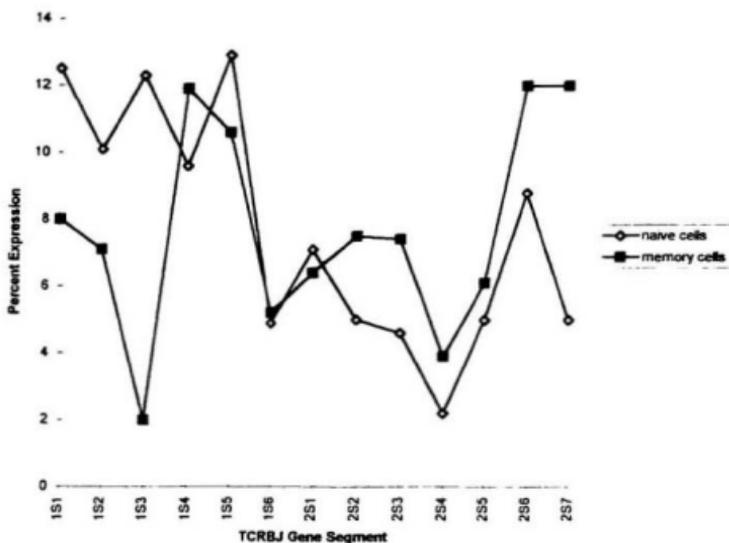
Figure A-19: The TCRBJ repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X.22. r-value = 0.182
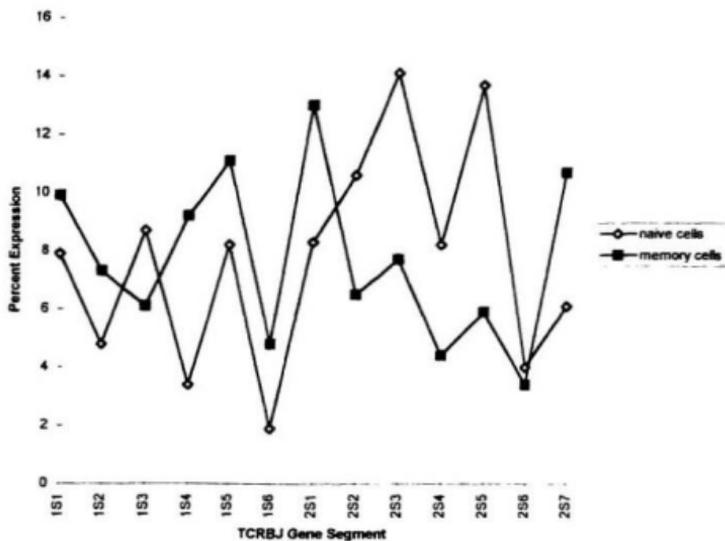
Figure A-20: The TCRBJ repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1 X 24 r-value = 0.083

Appendix B : FIGURES B-1 TO B-9
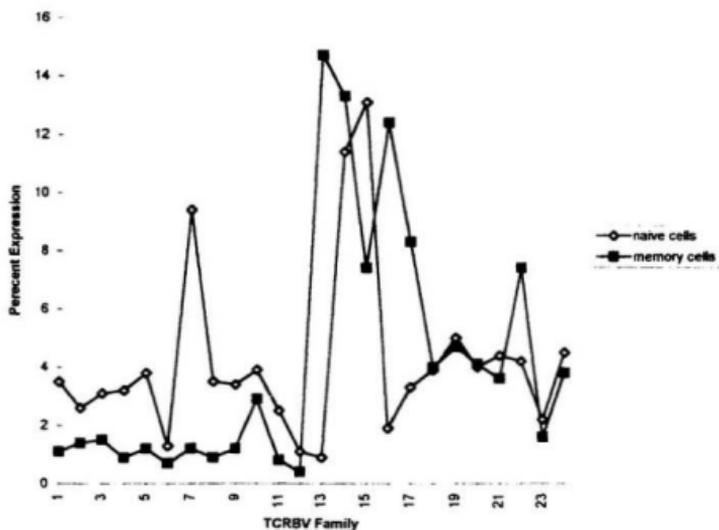
Figure B-1: The TCRBV repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X.02. r-value = 0.247
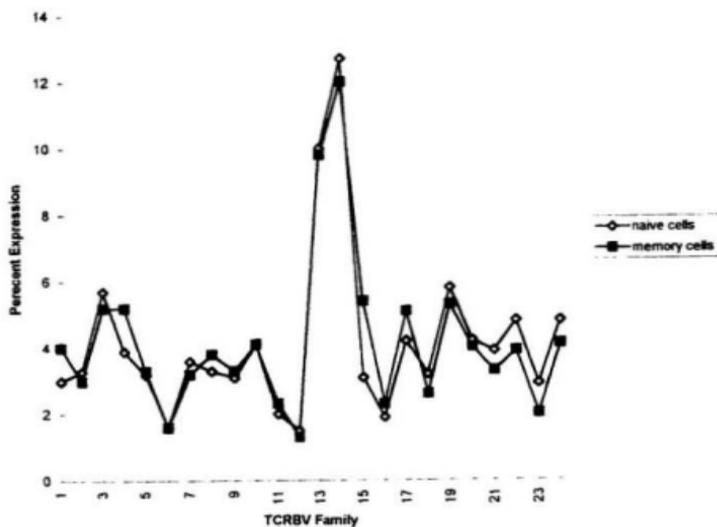
Figure B-2: The TCRBV repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X.07 r-value = 0.951
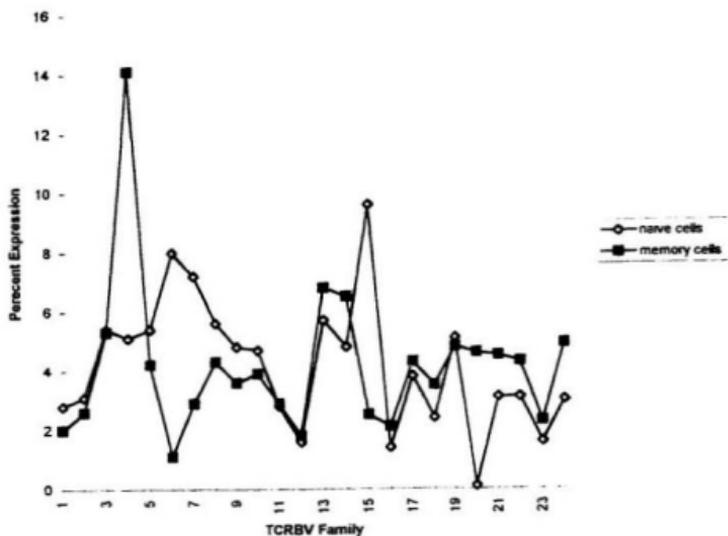
Figure B-3: The TCRBV repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X.09. r-value = 0.099
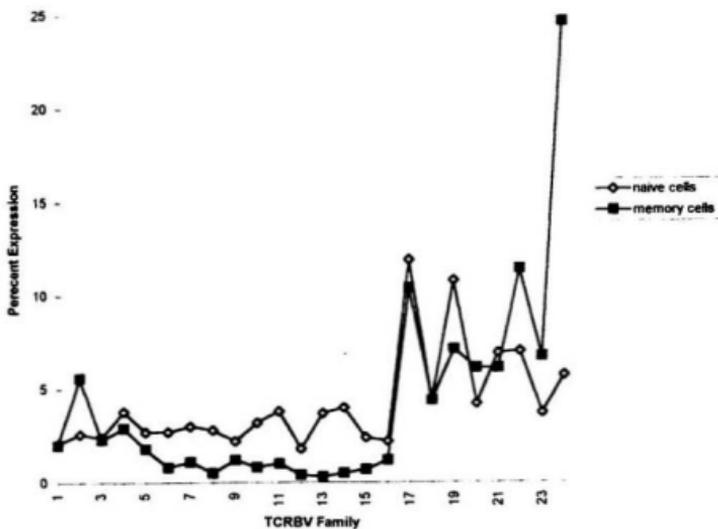
Figure B-4: The TCRBV repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X.10. r-value = 0.681
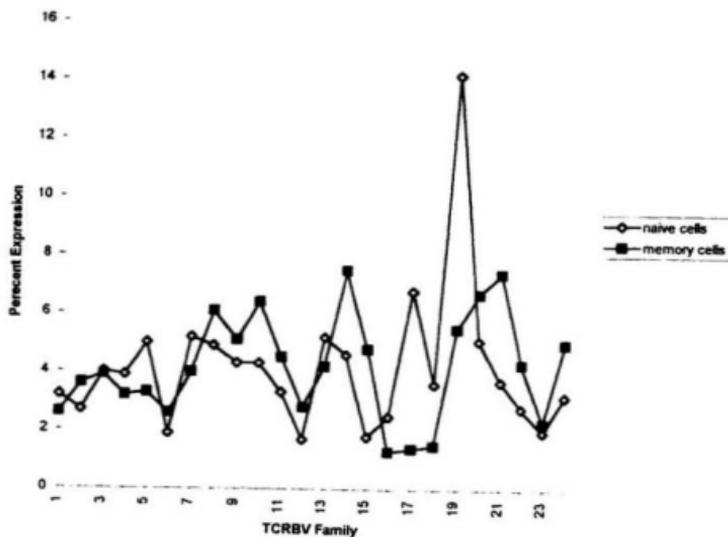
Figure B-5: The TCRBV repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X 12. r-value = 0.253
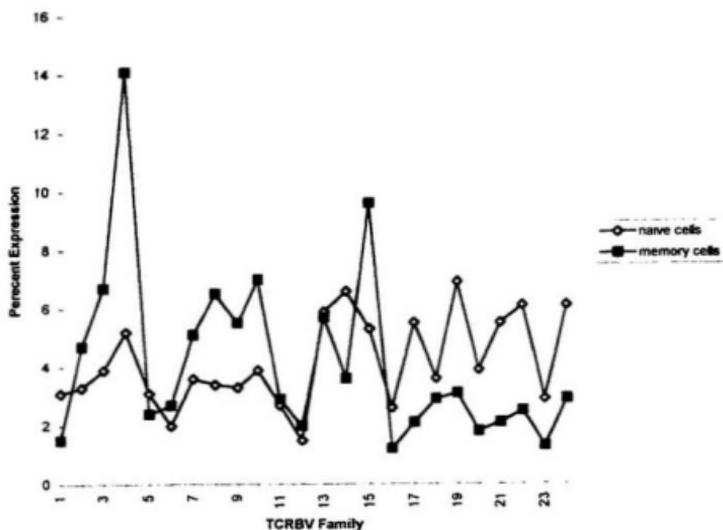
Figure B-6: The TCRBV repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X.13. r-value = 0.418
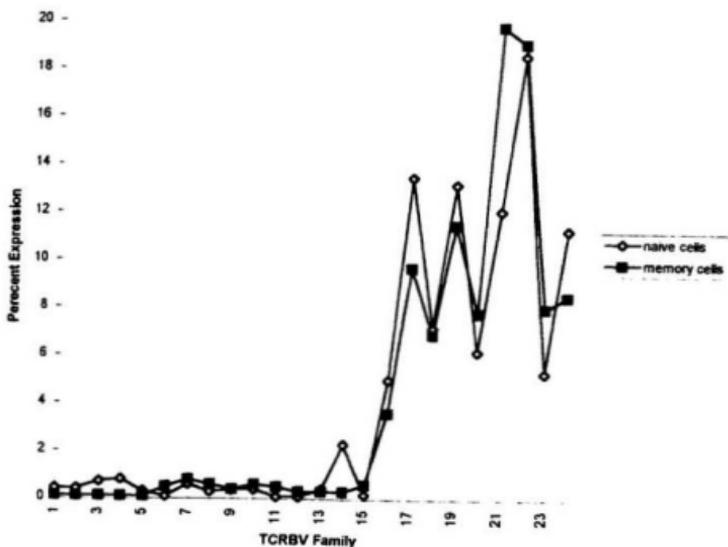
Figure B-7: The TCRBV repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X.15. r-value = 0.901
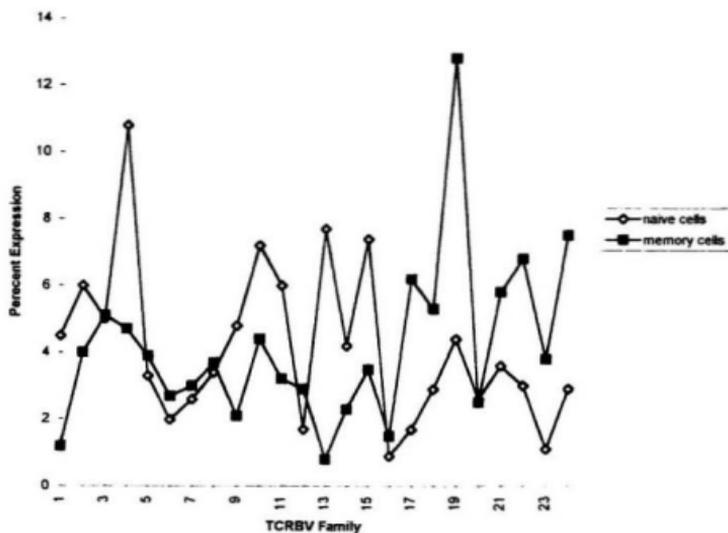
Figure B-8: The TCRBV repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X.17 r-value = -0.029
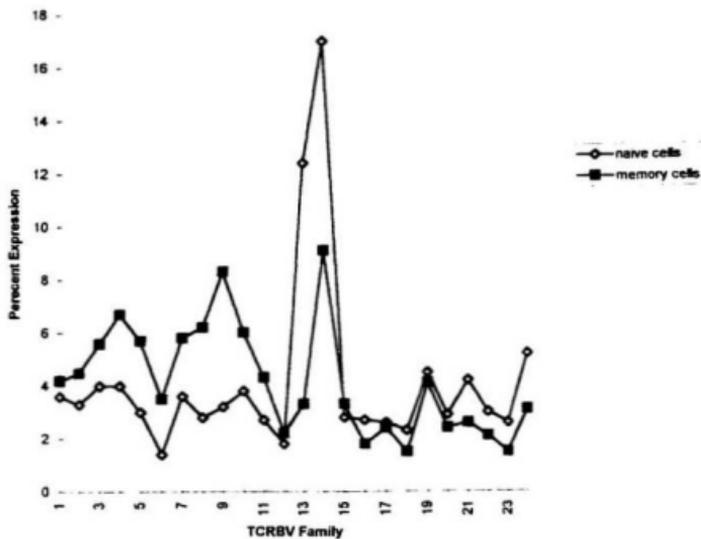
Figure B-9: The TCRBJ repertoire (corrected for primer efficiency) for the CD4+CD45RO+ (memory) and CD4+CD45RA+ (naive) cells of subject 1.X.18. r-value = 0.429

Appendix C : FIGURES C1-C6
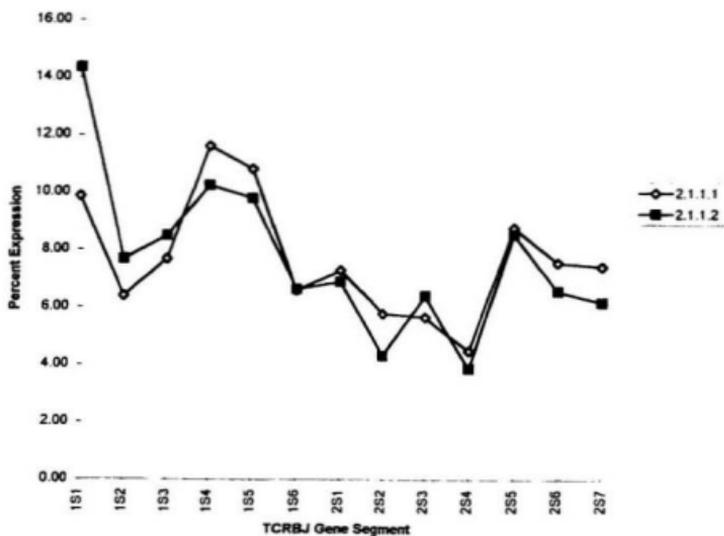
Figure C-1: The naive T-cell TCRBJ repertoires (corrected for priming efficiencies) for identical twins, 2.1.1.1 and 2.1.1.2. r-value = 0.809.
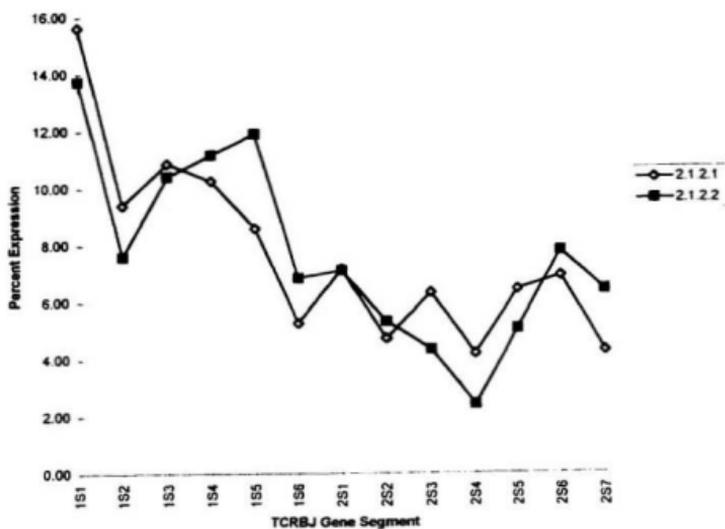
Figure C-2: The naive T-cell TCRBJ repertoires (corrected for priming efficiencies) for identical twins, 2.1.2.1 and 2.1.2.2. r-value = 0.858.
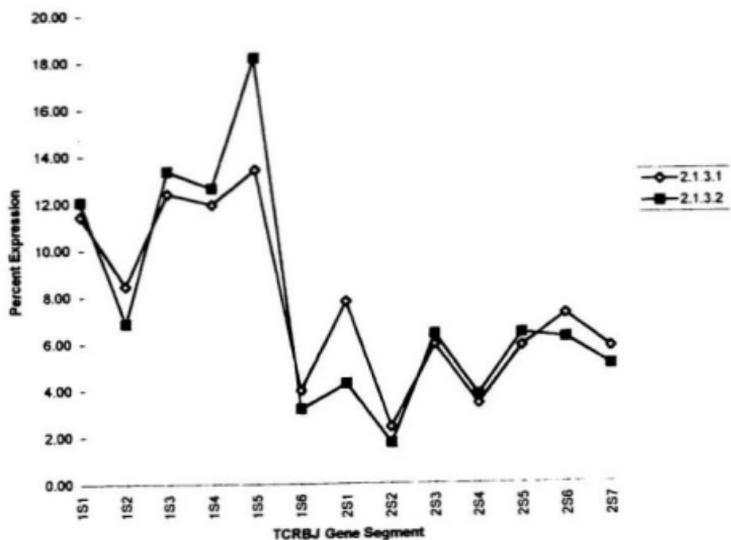
Figure C-3: The naive T-cell TCRBJ repertoires (corrected for priming efficiencies) for identical twins, 2.1.3.1 and 2.1.3.2. r-value = 0.942.
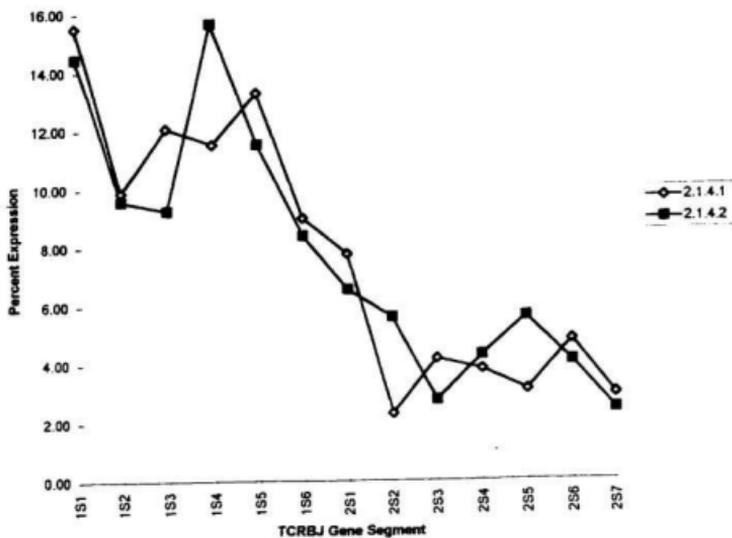
Figure C-4: The naive T-cell TCRBJ repertoires (corrected for priming efficiencies) for identical twins, 2.1.4.1 and 2.1.4.2. r-value = 0.890.
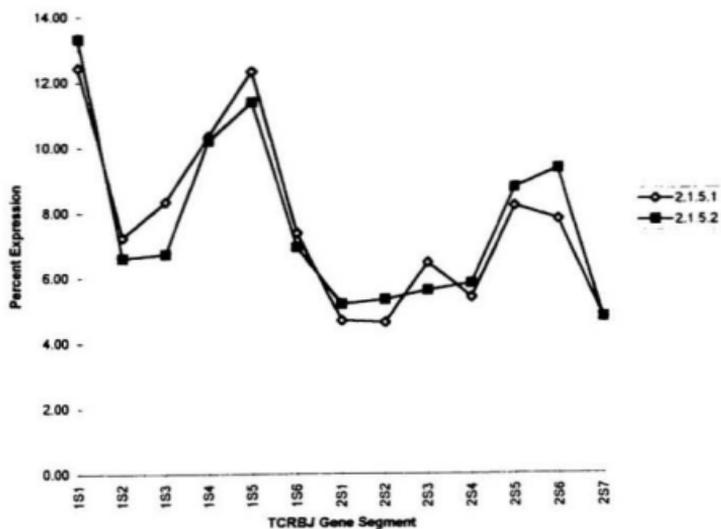
Figure C-5: The naive T-cell TCRBJ repertoires (corrected for priming efficiencies) for identical twins, 2.1.5.1 and 2.1.5.2. r-value = 0.946.
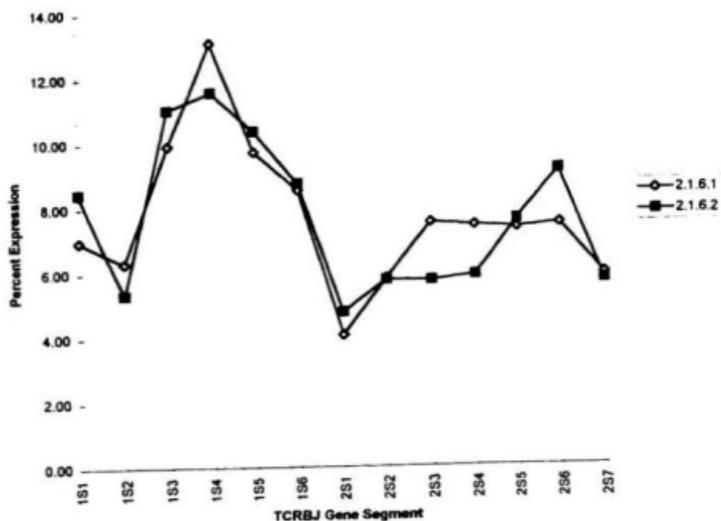
Figure C-6: The naive T-cell TCRBJ repertoires (corrected for priming efficiencies) for identical twins, 2.1.6.1 and 2.1.6.2. r-value = 0.876.

Appendix D : FIGURES D1-D6
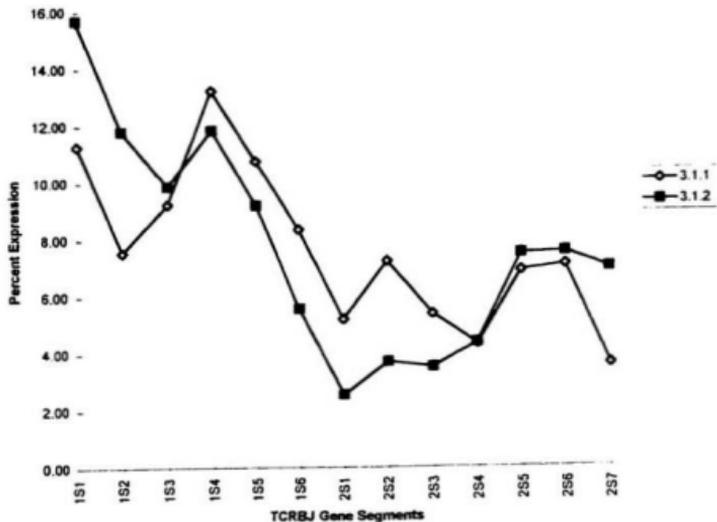
Figure D-1. The naive T-cell TCRBJ repertoires (corrected for priming efficiencies) for identical twins discordant for MS, 3.1.1 and 3.1.2. r-value = 0.783.
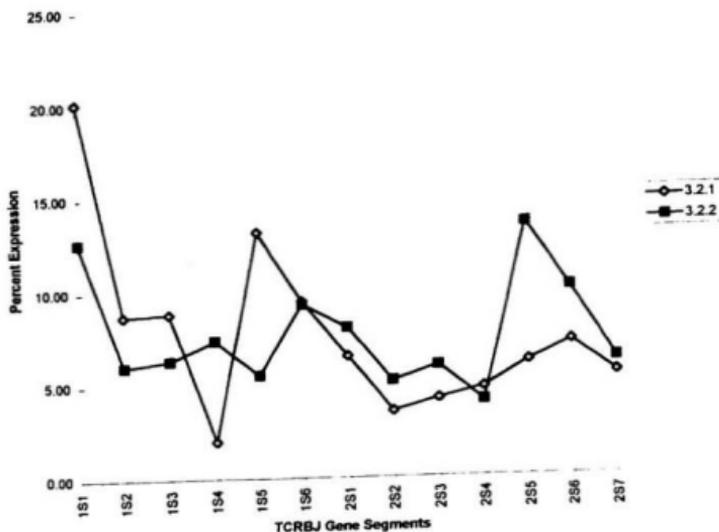
Figure D-2: The naive T-cell TCRBJ repertoires (corrected for priming efficiencies) for identical twins, 3.21 and 3.2.2. r-value = 0.417.
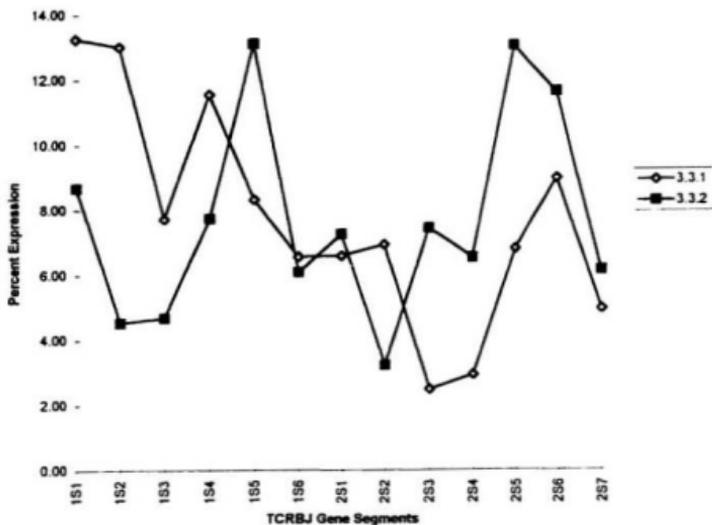
Figure D-3: The naive T-cell TCRBJ repertoires (corrected for priming efficiencies) for identical twins, 3.3.1 and 3.3.2. r-value = 0.039.
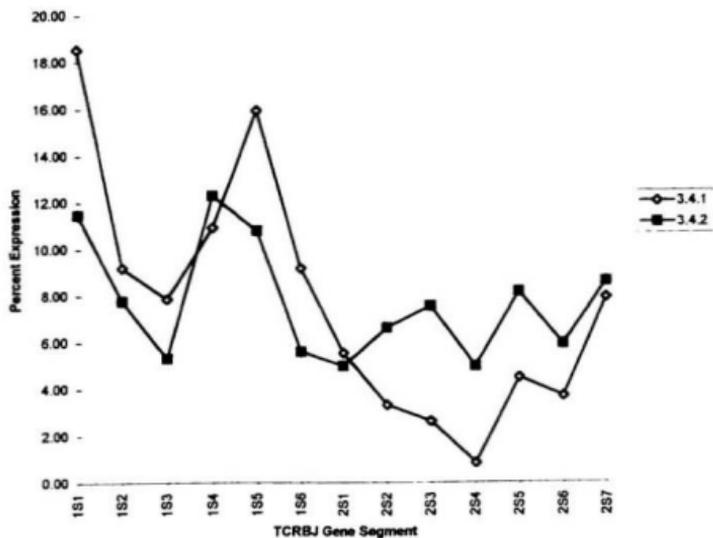
Figure D-4: The naïve T-cell TCRBJ repertoires (corrected for priming efficiencies) for identical twins, 3.4.1 and 3.4.2. r-value = 0.732.
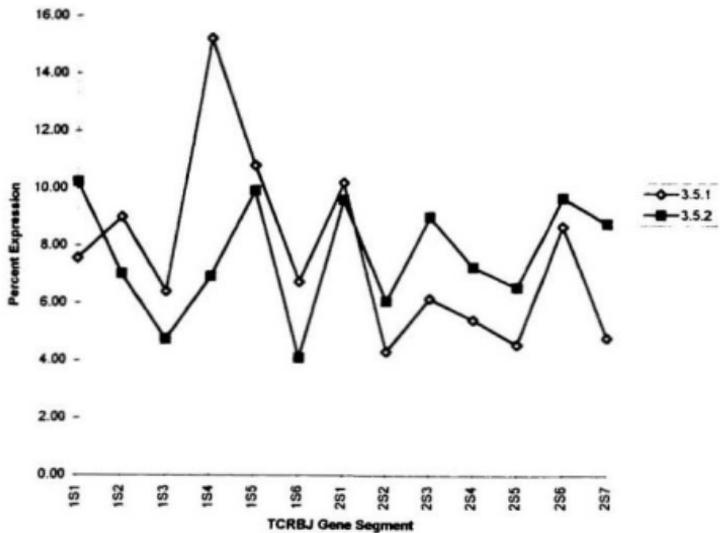
Figure D-5: The naive T-cell TCRBJ repertoires (corrected for priming efficiencies) for identical twins, 3.5.1 and 3.5.2. r-value = 0.312.
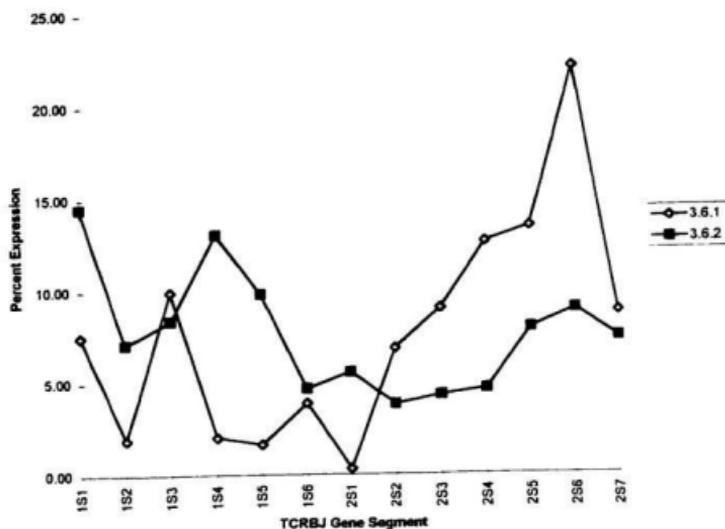
Figure D-6: The naive T-cell TCRBJ repertoires (corrected for priming efficiencies) for identical twins, 3.6.1 and 3.6.2. r-value = -0.037.