CHANGE OF TCR V BETA CDR3 LENGTH DISTRIBUTION IN CD4 T CELLS OF INFLUENZA VACCINATED SUBJECTS

By

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To my parents, Thomas and Magdalene, for all their love and support.

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TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AUC	Area under the curve
CD45RA	Cell surface molecule for naïve T cells
CD45RO	Cell surface molecule for memory T cells
cDNA	Complementary deoxyribonucleic acid
CDR 3	Complementary determining region 3
CTL	Cytotoxic lymphocyte
DF	Differentiation factor
DNA	Deoxyribonucleic acid
FBS	Fetal bovine serum
HA	Haemagglutinin
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
MHC	Major histocompatibility complex
NA	Neuraminidase
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNP	Ribonuclear proteins
RT	Reverse transcription
RT-PCR	Real time polymerase chain reaction
TCR	T cell receptor
Vβ or Vb	Variable beta chain of T cell receptor

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Observing TCR V beta (Vb) CDR3 length distribution tells us which specific TCR Vb families are used by a particular antigen. By observing the TCR Vb CDR3 length distribution in influenza vaccinated patients, we are able to determine which specific Vb families are used which may result in the development of a more effective influenza vaccine that targets those specific receptors.

The TCR Vb CDR3 length distribution was assessed by spectratyping. The condition to amplify 21 Vb families using the minimal amount of RNA from CD4 positive T cells of influenza vaccinated subjects was optimized using RNA from cord blood and purified CD8 positive T cells of healthy donors. The change of TCR Vb CDR3 length distribution in CD4 positive T cells of flu vaccinated adults was evaluated by comparison to the Gaussian distribution of TCR Vb CDR3 length in naive CD4 positive T cells from healthy children using statistical analytical tools.

The conditions needed for T cell receptor spectratyping have been optimized to perform spectratyping on 21 Vb families. Through the use of purified CD45RA and CD45RO CD8 positive T cells, the optimum condition of using the minimum amount of cDNA derived from the

minimum amount of RNA was discovered. It was found that between 5 to 10 x 10^6 peripheral blood mononuclear cells (PBMC) are need to obtain between 3 to 5 x 10^6 purified (CD45RA or CD45RO) T cells from which 0.25 micrograms of RNA is derived to yield 1 micro liter (2.5 percent of the total amount) of cDNA. This amount (1 micro liter or 2.5 percent of the total amount of cDNA derived from 0.25 micrograms of RNA) was sufficient to perform one RT reaction and amplify 21 Vb families.

Using the optimum condition for spectratyping obtained earlier, we sought to perform spectratyping on 21 Vb families for two influenza vaccinated subjects to study the T cell receptor Vb CDR3 repertoires within PBMC, CD4 positive, and CD4 negative cells. We only obtained results for the CD4 positive cells since the CD4 positive cells consist of a pure cell population whereas the PBMC and CD4 negative cells consist of a mixed cell population. In performing spectratyping on the CD4 positive cells, it was found that nine Vb families were perturbed in both subjects, two Vb families did not amplify in either subject (Vb 15 and 18) and one family (Vb 9 in Subject 1 and Vb 7 in Subject 2) was found to show monoclonal expansion. Only one family, Vb 3, was normal in both subjects. In comparing our results to the cited literature, of the nine families perturbed in our study, six were found to correspond with perturbations in the CD4 positive subset in at least one study in the literature including Vb 5, 8, 9, 12, 13, and 17. These perturbations may be carryover from previous antigen recognition. The two families that did not amplify may represent a minor population in the TCR repertoire. Monoclonal expansions were observed in Vb 12, 14, and 23 in the literature, however such expansions were not observed in Vb 9 and Vb 7 as was seen in our subjects. These monoclonal expansions may be due to the influenza vaccine; however this cannot be stated with certainty without further studies being conducted with pre and post vaccination sample time points.

CHAPTER 1
INTRODUCTION AND SPECIFIC AIMS

Introduction

Influenza causes severe respiratory illness in a significant portion of the population each year, even resulting in death for some individuals. There are two types of vaccines that are important to prevent the pathogenesis of the disease, however efficacy of the vaccine is difficult to assess prior to the display of symptoms from infection. The goal of this study is to evaluate a surrogate marker for effective vaccination using changes in TCR repertoire as the surrogate marker as assessed through spectratyping.

Influenza Virus

The influenza virus attacks the respiratory system through infection of the columnar epithelial cells. Symptoms of influenza infection include: coughing, sneezing, runny nose, headache, sore throat, chills, and body aches [1]. The virus is highly contagious with between 5 and 20% of the population contracting influenza each year. The influenza virus is an especially virulent virus that, according to the Centers for Disease Control (CDC), is thought to cause between 3 and 5 million cases of severe illness and approximately 36,000 deaths each year. Those most at risk for serious illness and death are the elderly aged 65 and above, children under the age of 2 years, and those who are immunocompromised [2].

The virus is primarily transmitted from person to person via droplets extruded from the nose or throat of an infected person by coughing or sneezing with close contact (no more than 3 to 6 feet) required for transmission. The virus is also spread through direct skin to skin contact as well as indirect contact by touching contaminated surfaces and then touching the nose, eyes, or mouth. Persons are considered infectious from 2 days before to 5 days after the onset of symptoms [3, 4]. The human infectious dose is between 100 and 1000 virus particles. A 0.1μL

aerosol particle contains more than 100 virus particles and within nasal secretions, millions of virus particles are shed [5]. If the virus is able to replicate early in the course of infection within the lower respiratory tract then smaller droplets with higher viral load and higher infectivity are seen [3].

The virus has three strains labeled A, B, and C although only strains A and B cause significant illness in humans [6]. Of these strains, strain A is the most virulent giving rise, through point mutations in the virus surface glycoproteins, to new variants every 1 to 2 years [4]. Some of the mutations cause changes to these viral proteins that affect the binding of host antibodies thus variants with these mutations are not effectively inhibited by host antibodies [6]. These variants are able to elude the body's host defenses and therefore do not permit lasting immunity against the virus whether by natural infection or by vaccination. These changes in the antigenicity of influenza A viruses are termed "antigenic drift" and are the basic cause for influenza epidemics [4].

In contrast to epidemics, pandemics occur approximately every 10 to 50 years [7]. There have been three major influenza pandemics within the twentieth century: 1918, 1957, and 1968. The 1918 pandemic was the most devastating with between 50 and 100 million deaths. One characteristic of a pandemic is a mortality shift towards younger age groups (those younger than age 65). Pandemics occur due to "antigenic shift" whereby there is a major change in the influenza A virus caused by either the reassortment of the genome of two viruses or by the gradual mutation of an animal virus. This major change results in new glycoproteins on the surface of the virus [4].

Influenza Structure

The influenza virus is an enveloped negative stranded RNA virus that has a segmented genome composed of eight single stranded RNA segments covered by the nucleocapsid protein.

Together these form the ribonucleoprotein (RNP) with each segment coding for a protein [5, 8]. The most important proteins for virulence of the virus are the surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). There are sixteen (H1 to H16) subtypes of HA and nine (N1 to N9) subtypes of NA. Different combinations of the HA and NA subtypes give rise to different types of the influenza A strain [1, 5]. The virulence of the virus depends on the compatibility of NA with HA meaning that mutations in HA need to have compensatory mutations in NA [3]. Currently, only three types of HA (H1, H2, H3) and two types of NA (N1, N2) have been seen in influenza virus infection in humans [1].

HA is a glycoprotein projecting outward from the surface of the virus. The HA protein is encoded by the fourth RNA segment of the genome. HA is involved in attaching the virus to the host cell through binding sialic acid residues, and induces penetration of the interior of the virus particle through membrane fusion. HA has five antigenic sites and is the main virus antigen against which neutralizing antibodies are produced. In antigenic drift, mutations in the antigenic sites reduce or inhibit the binding of neutralizing antibodies thus permitting a new subtype of the virus to enter the population and cause an epidemic. Genome reassortment or antigenic shift arises when the HA subtype is exchanged in a virus. Exchange of HA can lead to pandemics as discussed above [5, 8].

NA is also a surface glycoprotein. NA cleaves sialic acid thus facilitating virus infection by permitting the virus to be endocytosed into the cell. The cleavage of sialic acid by NA is the major component necessary for the release of progeny virus from the infected cell. NA is also involved in allowing penetration of the virus through the mucin layer of the respiratory epithelium. Like HA, NA also has antigenic sites that are capable of mutation and antigenic drift. Mutations in the amino acid residues can also cause resistance to antiviral drugs [5, 8].

In addition to the surface glycoproteins identified above, several other proteins are encoded by the genome. The M2 ion channel induces a low pH in the virus particle allowing the nucleocapsid to uncoat so that it can enter the cytoplasm of the host cell. The M2 protein then destabilizes protein binding thus allowing the nucleocapsid to be transported to the nucleus [5, 8].

The functions of the NS1 and NS2 proteins are not known for sure, however the NS1 protein is thought to inhibit the export of poly-A containing mRNA molecules from the nucleus which allows preference to be given to the viral RNA to be transported to the ribosome and translated. The NS2 protein is thought to facilitate the transport of the newly synthesized ribonuclear proteins (RNPs) from the nucleus to the cytoplasm in order to accelerate virus production [5, 8].

Influenza Life Cycle

The influenza virus particle is first introduced to the host cell through the binding of HA to the sialic acid on the host cell glycoproteins. After attachment, the virus particle is taken up by endocytosis into the host cell. The M2 ion channel induces a low pH which encourages the HA to fuse with the membrane of the endosome thus uncoating the virus and allowing the RNPs to enter into the cytosol of the host cell. Uncoating of the virus normally is completed within 20 to 30 minutes of virus attachment [5, 8]. The M1 protein is dissociated from the RNPs and the RNPs enter the nucleus of the cell through the nuclear pore. Once in the nucleus, the virus needs active host cell DNA to scavenge cap sequences from the nascent mRNA that is generated in the nucleus by transcription of the host cell DNA and attaches them to its own mRNA. This process is called “cap snatching” [8].

The viral mRNA is transported to the cytoplasm where it is translated by host ribosomes which then generate the viral proteins. Some of the newly synthesized viral proteins are

transported back to the nucleus where they bind to viral RNA to form RNPs. Other newly synthesized viral proteins are processed in the endoplasmic reticulum and the Golgi apparatus where glycosylation occurs. These modified proteins include the HA and NA glycoproteins. The HA and NA glycoproteins are transported to the cell membrane and insert into the lipid bilayer. When these glycoproteins reach a high enough concentration at the plasma membrane, RNPs and M1 proteins aggregate and condense to produce the viral particle. Finally, the newly synthesized viral particle is extruded from the membrane and cleaved from the host cell by NA activity. The time from viral entry into the host cell to the production of a new virus averages 6 hours [5].

Influenza Vaccine

The influenza vaccine is an important component to preventing the pathogenesis of the virus. The vaccine is normally administered in the fall season. The vaccine composition changes every year according to data collected by the World Health Organization throughout each year which determines what strains to put into the vaccine. The new vaccine takes approximately six months to produce. The vaccine is a trivalent vaccine consisting of two types A strains and one type B strain. Vaccine composition is named in the following particular nomenclature: type of influenza strain/where the strain was isolated/the isolate number/the date the strain was isolated. Following this designation in parentheses is the particular HA and NA subtypes represented. For example, in 2005, when the samples used in this study were collected, the vaccine contained the following strains: A/Solomon Islands/3/2006 (H1N1 like); A/Wisconsin/67/2005 (H3N2 like); and B/Malaysia/2506/2004 like [9]. For the 2007 influenza season, the vaccine contains the following strains: A/New Caledonia/20/1999 (H1N1 like); A/California/7/2004 (H3N2 like); and B/Shanghai/361/2002 like [2].

Vaccine Types

There are two main types of influenza vaccine: trivalent inactivated vaccine (TIV) and live, attenuated influenza virus vaccine (LAIV). TIV contains purified HA and NA while LAIV contains a weakened form of the virus [10].

Inactivated vaccines are further divided into whole, split, and subunit. The whole vaccine was the first developed. In this type of inactivated vaccine, the influenza virus is grown in eggs and subsequently purified, concentrated, and inactivated using formaldehyde or β-propiolactone. More recently, this method has been modified using centrifuge purification, density gradient purification, or filter-membrane purification [9].

Split vaccines are produced similarly to whole vaccine but the virus particles are disrupted through the use of detergents. In contrast, subunit vaccines are composed of only purified HA and NA proteins. The other viral proteins are removed [9]. The inactivated vaccines consist of killed viruses and cannot cause influenza. These vaccines are generally administered intramuscularly and cause few side effects [2].

In contrast to the inactivated vaccines, a live attenuated vaccine is available that is administered intranasally. The vaccine is comprised of a master attenuated virus inserted with HA and NA genes. This master vaccine is cold-adapted to grow ideally at 25 degrees Celsius. When the vaccine is administered to a person, the high human body temperature attenuates the virus by inhibiting its growth. The advantage of the live, attenuated vaccine administered intranasally is the development of local neutralizing immunity and the development of cell-mediated immune response which may lead to a cross-reactive and longer lasting immune response. The major disadvantages of live, attenuated vaccines are the production of mild flu-like symptoms, the inability of use on immunocompromised subjects, and the possibility of

genetic reversion and reassortment with wild-type virus causing new strains to be developed [2, 9].

Vaccine Effectiveness

Vaccine effectiveness is defined as the prevention of illness in vaccinated populations. Influenza vaccine effectiveness is between 70 and 90% in children and healthy adults less than 65 years of age and between 30 and 40% in those over age 65. However, the effectiveness of the vaccine in those over age 65 can be as high as 80% effective in preventing death among this age group [2, 9].

Vaccine efficacy is defined as the prevention of illness among vaccinated persons in controlled trials as measured by haemaglutination titers used as a serological marker of the immunological response to the vaccine. The efficacy in healthy primed adults and children is between 80 and 100% when vaccinated once. In unprimed adults, those who have never been vaccinated or encountered antigen, two vaccinations are needed to reach this efficacy. The vaccination efficacy for adults over age 60 and not living in nursing facilities was found to be between 30 and 70%. Vaccine efficacy for children was similar to adults, about 68%, although if two doses of vaccine were given, the efficacy of this group increased to 89% [2].

Immune Response

Humoral Immune Response to Vaccine

The immune response takes a few days to become effective against the virus but then helps to eradicate the virus as well as establish a memory response that results in long-lived resistance to that particular strain of virus. In the humoral immune response, B lymphocytes recognize antigen and differentiate into antibody secreting cells that produce antibodies to both the HA and NA glycoproteins. Peak antibodies are seen four to seven weeks after infection followed by a steady decline; however antibodies remain detectable for years after infection even without re-

exposure. The anti-HA antibody neutralizes virus infectivity and protects against disease and infection with a homologous virus [3].

In contrast to anti-HA, the anti-NA antibody reduces the release of progeny virus from the infected host cells. Since the NA glycoprotein of the virus is used to cleave the sialic acid residues and thus release progeny virus from the host cell, the anti-NA antibody effectively blocks this cleavage. Anti-NA antibody results in decreased virus shedding and severity of flu symptoms [3, 11].

Cellular Immune Response to Vaccine

In contrast to humoral immunity, T cells cannot recognize antigens directly but rather recognize antigen through surface peptide fragments that are displayed at the surface of the infected host cell. These peptide fragments are derived from the virus' proteins and are displayed on the cell surface by specialized peptide-binding host-glycoproteins named the major histocompatibility complex molecules (MHC). There are two types of MHC molecules: MHC I and MHC II. MHC I molecules present antigen to $CD8^+$ T cells while MHC II molecules present to $CD4^+$ T cells [12]. Peptides associated with MHC I molecules are endogenously produced while peptides associated with MHC II molecules are exogenously produced [13].

In cellular immunity, dendritic cells within the lung acquire antigen and become activated [3, 14]. These dendritic cells then travel to the lymph nodes where the antigen is processed and fixed on the dendritic cell surface as peptides which are presented to the T cells through the MHC molecules [3, 15]. An immune response is triggered by the dendritic cells for any T cell with a receptor that is specific for the foreign peptide-MHC complex on the dendritic cell surface [3, 16]. The activated T cells acquire effector cell functions and travel to the site of infection in the lungs of the respiratory system. These effector functions allow the T cells to help directly, release cytokines, or mediate cytotoxicity after recognition of antigen [3].

T lymphocyte responses peak around day fourteen post infection. In primary infection, viral clearance depends on $CD8^+$ T lymphocytes whereas upon re-infection both $CD4^+$ and $CD8^+$ T cells respond to the site of infection to mediate control of the influenza infection [3, 17]. Previous studies seem to indicate that virus-specific CTLs respond to influenza infection and are able to clear the virus from the lungs [18-21]. However, these virus-specific CTLs alone are not able to efficiently clear the virus from the lungs. In fact, both $CD4^+$ and $CD8^+$ T cells are required for effective virus clearance [18], and both also act as effectors in protective immunity against influenza viral infection [22]. Immune response to influenza requires cytotoxic $CD8^+$ cells, cytokine-secreting $CD4^+$ cells, and antibody secreting B cells [23].

The $CD4^+$ response

$CD4^+$ cells are important to the clearance of virus mainly through the augmentation of $CD8^+$ cells and B cell responses [1]. $CD4^+$ T lymphocytes assist B lymphocytes to produce anti-HA and anti-NA antibodies, however the HA epitopes recognized by the $CD4^+$ T helper cells are different from those recognized by the antibodies. $CD4^+$ cells secrete numerous cytokines to contribute to the immune response at the site of infection [1, 24, 25]. T helper cells are divided in to Th1 and Th2 cells depending on the type of cytokines they produce. Naïve $CD4^+$ T cells, when in the presence of IL-12 and IFN-γ, differentiate into Th1 cells but if in the presence of IL-4, differentiate into Th2 cells. Th1 cells produce IFN-γ, IL-2, and TNF-α while Th2 cells produce IL-4, IL-5, and IL-13 [23].

Viral infections predominantly induce Th1 responses that activate $CD8^+$ T cells. Correspondingly, protective immunity from influenza infection induces more of a Th1 response than a Th2 response [23]. There are conserved regions subunits of the HA glycoprotein that are homologous among different subtypes of the virus. $CD4^+$ T cells have been found to recognize

these conserved areas of the HA molecule which may explain the partial protection from vaccination. One study reported that some of the conserved regions were recognized by a large proportion of subjects in the study thus implying a broadly antigenic $CD4^+$ T cell response [26].

During influenza infection, the naïve $CD4^+$ T cell immune response is initiated in the lymph nodes and spleen. Upon stimulation these naïve $CD4^+$ T cells become a large effector cell population that contains many subsets. After these effector cells are created, only the most divided and differentiated cells travel to the lung, thus antigen-specific cells travel to the site of infection only after effector cells are created. The highly differentiated effector cells that travel to the lung have different functional abilities [27]. $CD4^+$ effector T cells in the lung are directly involved in viral clearance by direct cytolytic effects on infected cells and/or by recruiting other cells to the site of infection by rapid expression of cytokines and chemokines [23]. After viral clearance, a spectrum of resting cell subsets remains which indicates that heterogenous effector cells gives rise to corresponding memory cells [27].

$CD4^+$ cells are needed for a long lasting effective $CD8^+$ memory response. Memory $CD4^+$ T cells that have an activated phenotype and are capable of immediate effector function are present in high frequencies in the lungs after clearance of a respiratory viral infection and help confer effective cellular immunity [28]. There are multiple diverse subsets of $CD4^+$ memory T cells of which one subset of these primed $CD4^+$ cells is positioned in the lungs for immediate response to reinfection while another subset is in the lymph nodes and can be recruited later [23]. Antigen recognition is responsible for the later accumulation of both $CD8^+$ and $CD4^+$ T cells at the site of infection [29].

There are both similarities and differences in the responses of $CD4^+$ and $CD8^+$ T cells. For both types of T cells, transient exposure to antigen is enough to induce proliferation and

differentiation; however the strength and duration of the antigen stimulus can affect the differentiation process and regulate the function of the effector and memory cells that develop [30, 31]. Antigen exposure for naïve $CD8^+$ cells requires less time than that for naïve $CD4^+$ cells [32-35] and $CD8^+$ T cell responses are higher in frequency when compared to $CD4^+$ T cell responses [29, 36].

Initial activation of both $CD4^+$ and $CD8^+$ T cells results in a death phase where most effector cells (about 90%) are eliminated. Naïve $CD8^+$ T cells develop into effector cells more readily than $CD4^+$ cells after short-term primary stimulation. $CD8^+$ effector cells are more likely to develop into long lasting memory cells as compared to $CD4^+$ T cells since $CD4^+$ T cells have a unique characteristic that renders them more susceptible to death when they become IFN-γ producing cells [36].

$CD4^+$ and $CD8^+$ T cells are divided into two subsets based on the expression of molecular weight isoforms of the leukocyte common antigen, CD45RA and CD45RO. CD45 is cell surface protein that changes after exposure to an antigen. Repeated exposure to antigen on antigen-presenting cell allows cell to become effector cell [37]. CD45RA cells have a high molecular weight and contain naïve cells while CD45RO cells are memory cells that have encountered antigen and have a low molecular weight [38]. CD45RA cells have three variable exons (A, B, C) and do not associate with the TCR or coreceptor. In contrast, CD45RO cells have variable exons removed by alternative splicing and associates with both TCR and coreceptor so it transduces signals more effectively [37]. CD45RA cells, being naïve cells that have not encountered antigen, usually have a Gaussian distribution while CD45RO cells tend to show more differences in CDR3 length distributions [38]. Dominant T cell clones in peripheral blood may indicate previous exposure to antigen or previous or ongoing immune response.

Oligoclonal populations have also been detected in a number of chronic diseases indicating that these populations may be the consequence of continuous antigen stimulation [13].

The $CD8^+$ response

$CD8^+$ T cells can operate through direct lysis of infected cells or through the production of pro-inflammatory cytokines such as IFN-γ and TNF-α and are needed for effective clearance of the virus [1]. $CD8^+$ T cells require specific antigen for activation but memory cells can be recruited to the lungs in an antigen-nonspecific manner during influenza infection [29, 39]. Memory $CD8^+$ T cells are recruited to the lung during influenza infection in three distinct phases, the first two of which are non-proliferating populations. One population is present in the tissue and thus is in the lung at the outset of infection. A second population is antigen-dependent and recruited from the blood. The third population is produced in the lymph nodes and has proliferated in response to antigen [29, 40].

The T cells that respond to influenza infection undergo similar changes in the expression of cell surface markers. Both $CD4^+$ and $CD8^+$ cells divide at a similar rate but $CD8^+$ cells continue to proliferate after $CD4^+$ cells peak and then decline more slowly than $CD4^+$ cells. This continued proliferation and slow decline by $CD8^+$ T cells leads to a tenfold more increase in the number of cells. $CD4^+$ and $CD8^+$ cells are regulated differently to perform distinct functions [41].

Virus-specific activated $CD8^+$ T cells can remain in the lungs for several months post-influenza infection. Some level of T cell activation or inflammation in the lungs is needed for antigen-specific $CD8^+$ T cells to remain in the lungs. The T cells in the lungs after viral infection express surface markers that are normally found on recently activated effector cells. A prolonged effector T cell response may produce the chronic activation of antigen-specific $CD8^+$

T cells in the lungs. Persistent antigen stimulation may be required for maintaining activated $CD8^+$ T cells near the site of virus amplification in the lungs. Influenza virus antigens are retained in the draining lymph nodes and are presented to $CD8^+$ T cells for at least 2 months post-infection [42].

In memory responses with a large number of T cells before infection there is a longer delay in T cell expansion, and a limit on the number of effector T cells that can be produced which results in the same peak CTL number being reached over a wide range of pre-infection T cell levels. This seems to indicate that repeated vaccination could lead to a stronger early response after infection; however repeated vaccination could also lead to decreased proliferation in responding cells thus resulting in decreased responsiveness with chronic antigenic stimulation [43].

$CD8^+$ cytotoxic lymphocytes (CTL) may be either subtype specific, recognizing HA, or broadly cross-reactive with influenza strain A recognizing internal proteins such as the M protein [3]. Most CTL recognize one subtype of influenza strain A viruses that are currently circulating, but some CTL seem to be able to recognize both homosubtypic and heterosubtypic variants. Consecutive infection with viruses containing different variants of the same epitope will select for cross-reactive T cells that are reactive against both variants. The flexibility of the TCR of this subset of CTL recognizes naturally occurring variants of the epitope that may be escape mutants. This subset of cross-reactive CTL may contribute to protective immunity be increasing in number after repeated exposure to heterologous viruses [44].

Both $CD8^+$ and $CD4^+$ T cells as well as B cells contribute to heterosubtypic immunity, which is immunity against a different virus subtype than the original infection. There must be a properly diversified TCR repertoire in order to confer heterosubtypic immunity [45].

The T Cell Receptor (TCR)

The virus interacts with T cells through the T cell receptor (TCR). Each T cell has approximately 30,000 antigen-receptor molecules on its surface. Each T cell receptor is comprised of a constant region and a variable region. The rearrangement of V and J gene segments in the variable region produces the Vα chain while the recombination of V, D, and J gene segments produces the Vβ chain. Each of these variable region exons are transcribed and spliced to join the constant region of the TCR. The variable (V) regions of the TCR, the α chain (TCRα) and the β chain (TCRβ), are linked together by a disulfide bond [37, 46].

There are hypervariable regions within the Vα and Vβ chains that fold in close proximity to each other to form the TCR antigen binding site. These regions are called the complementary determining region (CDR) and are divided into CDR1, CDR2, and CDR3. The Vα chain contains only CDR1 and CDR2 regions while Vβ consists of CDR1, CDR2, and CDR3. The CDR1 and CDR2 regions are encoded by the V and J genes while the CDR3 region is encoded by the V, D, and J genes, thus making the CDR3 region the most diverse region [37].

The ability of the T cell immune system to recognize a vast array of pathogens rests on the diversity of the TCR Vβ CDR3 region. This diversity occurs mainly through two methods: (1) recombination of the variable (V), diversity (D), and joining (J) gene segments; or (2) removal or insertion of nucleotides at V-D and D-J junctions by terminal deoxynucleotidyltransferase (TdT) during the recombination process. Thus the CDR3 region of the TCR Vβ chain is variable in both length and amino acid sequence [38]. Each V gene segment can be grouped into families where each segment shares at least 80% DNA sequence identity with each other family member [12]. The CDR3 region may vary in length by as many as six to eight amino acids within each Vβ family [12, 47, 48]. TCR Vβ CDR3 length diversity can serve as an indicator of diversity of

antigenic specificity, and changes in the amino acid sequence and length of the CDR3 region can be used as a clonal marker to show the T cell response to an antigen in the course of an immune response [38, 47]. In regards to determining whether or not TCR Vβ spectratyping can be used as a surrogate marker of T cell activation due to vaccination, according to the literature, examining changes in the TCR Vβ repertoire can be used as a surrogate marker of T cell activation due to vaccination [49-53]. In addition, several studies have shown that examining which Vβ genes are involved in immune response is important since these molecules are expressed on the T cell surface and can be used as targets for immune therapy [49-51, 54, 55].

The majority of literature seems to indicate that vaccination does indeed cause TCR activation that can be measured, and different Vβ families show perturbation according to what vaccine was used. Studies on TCR Vβ repertoire activation have been done on tetanus, measles, and hepatitis B vaccinations with pre-vaccination and post-vaccination samples taken to determine if a particular TCR Vβ family perturbation was due to the effect of the vaccine as opposed to carry over from previous exposure to an antigen [49, 51, 52, 54]. Differences in TCR Vβ family activation within each specific antigen type may be due to differences in time points at which the samples were taken (ranging from 48 hours post vaccination to 14 days post vaccination). Additionally the differences in TCR Vβ gene usage may also be due to different HLA haplotypes between individuals [56]. Haplotype refers to each individual person having different MHC molecules that present different antigens. If a study examines subjects with a specific haplotype then more families may be shared [37]. Studies have also shown that there seems to be a difference in the TCR Vβ activation between measles vaccination and natural measles infection which may be due to the effects of the virus strain or the ages of the subjects [50, 53].

The diversity, clonality, and specificity of a TCR repertoire that is antigen-specific depend on the molecular, biophysical, and structural components of both the Vβ and Vα regions [57]. Theoretically the TCR repertoire could have a size of 10^{15} different TCRαβ dimers [48]. The actual diversity of the TCR repertoire is much less than the theoretical structural diversity of 10^{15} due to selection of T cells in the thymus [58, 59]. The actual number of αβTCR expressed in adults is closer to 10^7 with the complexity of the expressed repertoire varying between functionally distinct subpopulations of T cells [60].

There are differences in cord blood versus adult repertoires that represent those T cell subsets that have survived as a result of antigen selection [61]. Cord blood TCR expression is highly diverse while during aging the TCR repertoire changes by becoming less diverse and more oligoclonal [62]. This result is expected since as a person grows older, he or she is exposed more frequently to a diverse group of antigens which would invoke changes in the TCR repertoire.

A diverse TCR Vβ CDR3 repertoire allows greater protection from infection since many diverse subtypes of virus can be recognized as well as immune escape viral variants can be controlled. In influenza virus infections, it has been reported that a diverse TCR repertoire could be important in controlling viral CTL escape variants. Recent research has found a novel molecular mechanism to maintain overall diversity of the TCR repertoire in order to control the escape of heterogenous influenza A virus from the recognition by virus-specific CTLs. This mechanism is shown by pairing a diverse Vα chain with a biased Vβ chain. Diversity of Vα chains has not been extensively explored; however Vα chain diversity can potentially have a profound impact on overall TCR diversity [57].

All Vβ segments can be amplified ex vivo by PCR by using Vβ specific primers. CDR3 length distribution for each Vβ subfamily can be measured by having an average of eight peaks spaced by three nucleotides apart. Oligoclonality comprises a discrete number of specific T cells being used. In immune responses, there can be a public response where the response is shared among individuals or a private response which has T cells clones that are different between individuals. The rationale for determining CDR3 size is that it provides a picture of the repertoire without needing in vitro growth. The relative intensity of any given peak is proportional to the number of non-amplified cDNA molecules that share this CDR3 size. An increase in the height or area of a peak usually indicates a monoclonal expansion. Certain TCR are not selected at the nucleotide level but rather are selected at the amino acid level [47].

Spectratyping

Spectratyping is a PCR based assay designed to measure the length variation within a gene family. To measure length distribution in TCR Vβ CDR3 region, two rounds of PCR are performed. In the first round PCR, the forward primer is specific to a particular Vβ family and the reverse primer is located in the constant region of the TCR Vβ chain. In second round PCR, the first round products are used as a template and a pair of nested primers of which the forward primer is Vβ family specific and is nested 3' to the first round PCR primer while the reverse primer is constant and located 88 base pairs away from the CDR3 region. One of the second round primers is labeled with a flourochrome to allow the bands to be seen on a gel and signals to be detected by a computer. The PCR products are then run on a 6% acrylimide sequencing gel and analyzed using ABIPRISM GeneScan analysis software (Applied Biosystems) which calculates the relative intensity of each product to generate a histogram of CDR3 lengths for each Vβ family [63] (Figure 1-1).

The relative representation of the particular CDR3 sizes within an individual Vβ family is reflected in the intensity of an individual peak. Expressing this data as a percentage of the total area under the curves for each Vβ family can be used to accurately determine the degree of CDR3 length diversity within a particular Vβ family. These results allow comparisons of TCR diversity among different Vβ families or among different subjects [38].

In the absence of antigen stimulation, the distribution of CDR3 lengths within a Vβ family should be Gaussian or bell-shaped. Use of a Vβ family after antigen stimulation is demonstrated by the loss of the Gaussian distribution of CDR3 lengths (polyclonal expansion) or the predomination of particular CDR3 peaks within the family (monoclonal expansion), both of which constitute perturbation [64]. Spectratyping allows examination of the antigen specific CDR3 region of the TCR by examining CDR3 length distributions for 24 Vβ families. However, technically there are only 21 functional Vβ families since family #10 and #19 are pseudo-families [65].

Specific Aim 1: To Optimize the TCR Spectratyping Conditions Needed to Perform Spectratyping on 21 Vβ families Using Cord Blood

The hypothesis for specific aim 1 was that using the minimum amount of cDNA derived from the minimum amount of RNA would produce similar results as when using higher starting amounts of RNA and cDNA. We want to use the minimum concentration that still allows for sufficient representation of all Vβ families while eliminating false negatives and oligoclonality due to insufficient sample. Using the minimum amount of RNA and cDNA needed to obtain an optimal PCR result allows the patient's sample to be conserved. The objective was to find the minimum amount of cDNA derived from the minimum amount of RNA needed to amplify 21

families. The targeted optimal PCR condition was 1 μL or 2.5% of the total amount of cDNA derived from 0.25 μg of RNA.

Specific Aim 2: To Use the Optimal Conditions Found in Specific Aim 1 to Perform Spectratyping on 21 Vβ Families from Influenza Vaccinated Subjects to Measure the TCR Vβ Family Usage Caused by Vaccination

This aim was a pilot study done to determine the feasibility of time points in a larger study done in collaboration with the University of South Florida. The larger study was examining the breadth of TCR immune response following immunization with influenza as recall antigen among healthy and bone marrow transplant patients undergoing hematopoietic stem cell transplantation (HSCT) as well as examining the relationship between influenza-specific clonal expansions within the T cell repertoire and increases in influenza-specific T cell responses in healthy and post-HSCT subjects with a particular genotype. Samples from each subject would be drawn pre-vaccination, and 1 month and 3 months post vaccination.

Our pilot study was done with samples that were drawn at the time of vaccination and one month post vaccination to determine if there were indeed perturbations. The rationale for this pilot study was to examine post-vaccination samples first because if no perturbations were observed in the post-vaccination samples, then there would be no need to examine a pre-vaccination sample time point.

The hypothesis for the pilot study is that vaccination would cause perturbations in the TCR repertoire and that changes in T cell diversity could be used as a surrogate marker to determine the efficacy of the influenza vaccine. Spectratyping was used to examine the changes in PBMC, $CD4^+$ (enriched), and $CD4^-$ (depleted) T cell subsets from two influenza vaccinated subjects for 21 Vβ families.

Significance

The significance of this study was twofold. First, we wanted to determine the minimum amount of RNA that was needed to evaluate 21 Vβ families. This aim was significant because samples from subjects may be in limited supply. The optimization of conditions to use the minimum amount of RNA is applicable not only to this study but also to other studies going on in the lab.

The second aim is significant because as a pilot study, we can determine the appropriate time points for samples to be taken from subjects in order to observe TCR repertoire diversity. If perturbations are not observed post-vaccination, then pre-vaccination samples do not need to be taken. As part of a larger study, we can determine whether or not TCR diversity can be used as a surrogate marker to determine the efficacy of the influenza vaccine. By examining which specific families are perturbed, we can have a clearer idea as to what specific TCR are used during influenza viral infection. Knowing which families are perturbed can lead to development of a more effective influenza vaccine.

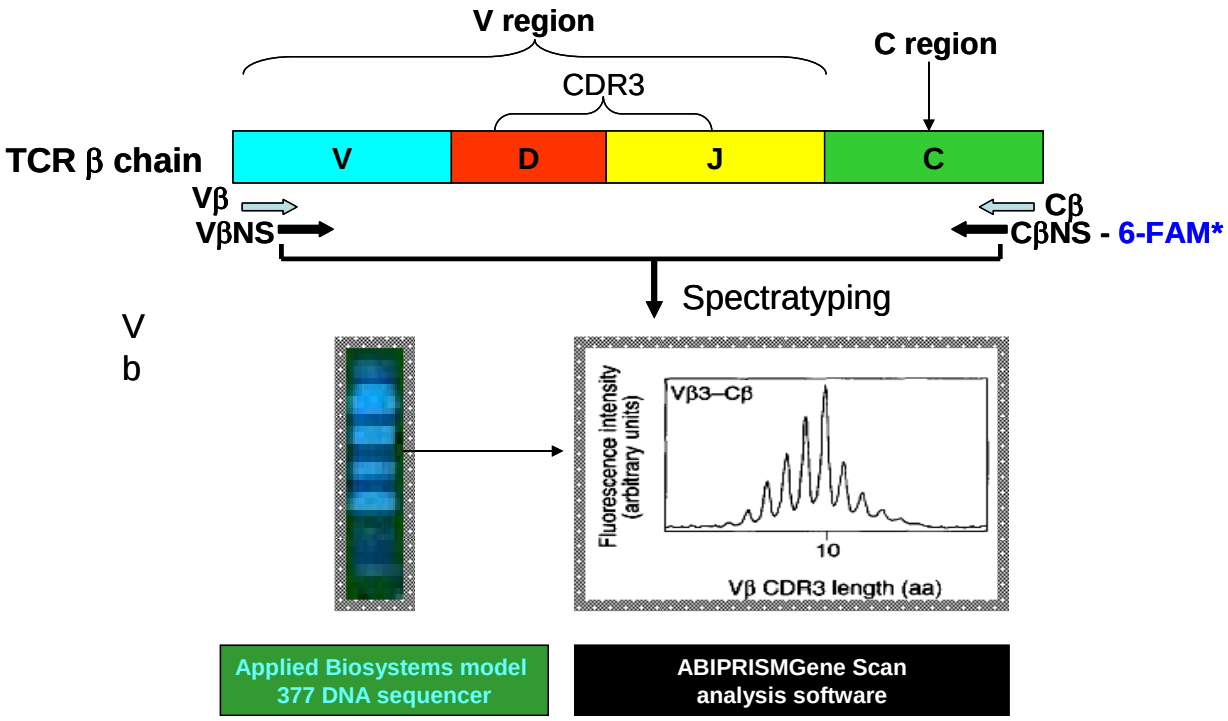


Figure 1-1. Spectratyping process can examine the antigen specific CDR3 region of the TCR by examining CDR3 length distributions for twenty four Vβ families. In first round PCR, we use a forward primer that is specific to the particular Vβ family and a reverse primer that is in the constant region. In second round PCR, we use the first round products as a template and a pair of nested primers of which the forward primer is Vβ family specific and is nested 3' to the first round PCR primer while the reverse primer was constant and located 88 bp away from the CDR3 region. This allows the CDR3 region to be amplified. The second round primers are labeled with fluorescence (6-FAM) to allow the bands to be seen on the gel. The PCR products were then run on a 6% acrylimide sequencing gel and analyzed with ABIPRISM GeneScan analysis software which calculates the relative intensity of each product to generate a histogram for each Vβ family. The area under each peak is calculated and a peak with an area that is >40% of the total area is considered perturbed which can indicate oligoclonal expansion. Each peak is one length and we can observe the length distribution of the CDR3 region (proportion, what length is, distribution) to determine TCR recognition of Ag

CHAPTER 2
MATERIALS AND METHODS

Cord Blood PBMC and $CD8^+$ T Cell Subsets

Cord blood PBMC (STEMCELL technologies) and CD45RA and CD45RO $CD8^+$ T cell subsets isolated from the PBMC of healthy donors were used to find the minimum number of cells which could provide enough RNA and subsequently cDNA to amplify 21 TCR Vβ families.

Separation of CD45RA and CD45RO CD8 T Cells from PBMC

Peripheral blood mononuclear cells (PBMC) were collected from leukopacks of healthy subjects (Civitan, Gainesville, FL) and frozen down in liquid nitrogen, were quickly thawed in a 37^o water bath. Normally one cryovial contains 20 x 10^6 PBMC in 1 mL of freezing medium. One half microliter of fetal bovine serum (FBS) was added drop-wisely to each vial and then incubated at room temperature for 15 minutes. The cells were then transferred drop-wisely into two 15 mL conical tubes, each containing 6 mL RT RPMI-1640 and mixed by pipetting. Each tube was centrifuged at 800 rpm in the Beckman TJ-6 centrifuge for 5 minutes at room temperature. The cell pellet was then resuspended in an appropriate amount of phosphate buffered saline (PBS) and mixed well by pipetting. After appropriate dilution, 10 μL of cells were placed on a hematocytometer and counted under the microscope. The concentration of cells was calculated by taking the mean of the total number of cells from the four quadrants and multiplying this number by the differentiation factor (DF) and multiplying this number by 10^4 (concentration of cells = mean of total x (DF) x 10^4 = number x 10^6 cells / mL). The concentration of cells was then multiplied by the volume of PBS used to resuspend the cell pellet to find the total number of cells (total number of cells = concentration cells / mL x volume of cell suspension in mL).

Prepare Cells for Cell Sorting

The tubes with the required number of PBMC were then centrifuged at 800 rpm in the Beckman TJ-6 centrifuge for 5 minutes at room temperature. When finished, the supernatant was pipetted off each tube as much as possible without disturbing the pellet.

Magnetic Labeling of CD8 Negative Cells

A $CD8^+$ T Cell Isolation Kit II by Miltenyi Biotech (Auburn, CA) was used to separate the $CD8^+$ T cells. The cell pellet was resuspended in wash buffer of 40 μL /10 x 10^6 cells. The $CD8^-$ cells were labeled by adding Biotin-Antibody Cocktail in the amounts of 20 μL /10 x 10^6 cells. The content of each tube was mixed by vortexing, incubated for 10 minutes at 4-8^o C, and vortexed every 3 minutes. Wash buffer was added at 30 μL /10 x 10^6 cells. Anti-Biotin Microbeads were added to the cells at 20 μL /10 x 10^6 cells. The cells were then mixed well by vortexing, incubated for 15 minutes at 4 to 8^o C, and vortexed every 3 minutes. The cells were washed with wash buffer of 20 x labeling volume and centrifuged at 800 rpm for 5 minutes at room temperature. After centrifugation, the supernatant was pipetted off completely and the cell pellet was resuspended in each tube with 500 μL of wash buffer (up to 100 x 10^6 cells in 500μL wash buffer).

Magnetic Separation of $CD8^+$ Cells from $CD8^-$ Cells

A column labeled $CD8^-$ was placed in the magnetic field of a MiniMACS Separator and the column was prepared by rinsing with 500 μL of wash buffer with the flow through discarded. One collection tube was placed under each of the three columns (representing each of the three tubes) and labeled $CD8^+$. The cell suspension (500 μL) was applied onto each column with the cells allowed to pass through and the effluent collected as a fraction with unlabeled cells which represents the enriched $CD8^+$ T cell fraction. Each column was then washed with 500 μL of

wash buffer three times, each time once the reservoir was empty. The $CD8^+$ cells were then counted (total number of cells = mean of total x DF x 10^4 x 2 mL) and the separation efficiency calculated (separation efficiency = cell number / total # of PBMC starting with).

Magnetic Labeling of $CD45RO^+$ CD8 T Cells

The $CD8^+$ T cells were centrifuged at 800 rpm for 5 minutes at room temperature. After centrifugation, the pellet was resuspended in wash buffer of 80 μL /10 x 10^6 cells. We added 80 μL of mouse anti-CD45RO-APC (BD PharMingen 340438) into each tube and mixed well. The tubes were incubated on ice for 20 minutes and vortexed every 5 minutes. The cells were washed carefully by adding 2 mL of buffer per 10 x 10^6 total cells and centrifuged at 800 rpm for 5 minutes. The wash step was then repeated once and the supernatant was pipetted off completely. The cell pellet was resuspended in 80 μL of buffer per 10 x 10^6 total cells. Twenty microliters per 10 x 10^6 total cells of MACS Anti-APC Microbeads was added to the cells and mixed well. The cells were then incubated for 15 minutes at 4 to 8^o C and vortexed every 3 minutes. The cells were washed by adding 2 mL of wash buffer per 10 x 10^6 cells and centrifuged at 800 rpm for 5 minutes at 4^o C. The supernatant was pipetted off completely and the cell pellet was resuspended in 500 μL of wash buffer (500 μL of wash buffer per 100 x 10^6 total cells).

Magnetic Separation of CD45RA and CD45RO CD8 T Cells

A column was placed in the magnetic field of the MiniMACS Separator and prepared by rinsing it with 500 μL of wash buffer. The cell suspension was applied onto the column and the unlabeled cells were allowed to pass through and the effluents were collected into a 4 mL clear collection tubes that were labeled CD45RA. The column was then washed with 500 μL of wash buffer three times, each time once the column reservoir was empty. The column was removed

from the separator and placed on a suitable collection tube labeled CD45RO. One milliliter of wash buffer was pipetted onto the column and the positive labeled cells were flushed out using the plunger supplied with the column. The column was washed once more with 1 mL of wash buffer. The cells were then counted and the separation efficiency calculated as described earlier for both the CD45RA and CD45RO cells. The cells were centrifuged at 800 rpm for 5 minutes at room temperature. Finally, the cells were lysed by adding 0.5 mL of lysis/binding solution (RNAqueous-4PCR kit, Ambion Cat. No. 1914), vortexed very well to break up any clumps, and left in -20^{o}C until the RNA was extracted.

Extraction of RNA

An RNAqueous-4PCR kit (Ambion, Austin, Texas) was used to extract the RNA from the lysed purified cells. The manufacturer's protocol was modified to include extra elutions. In extracting the RNA, first a 64% ethanol solution was prepared by adding 22.4 mL of ACS grade 100% ethanol to a bottle containing 12.6 mL of RNase-free water and mixing well. A wash solution #2/3 was prepared by adding 28 mL ACS grade 100% ethanol to bottle labeled "wash solution #2/3 concentrate" and mixing well. We heated 200 μL of elution solution in an RNase-free microfuge tube in a heat block set to 70 to 80^{o}C.

The cells in the lysis/binding solution obtained from the cell separation step or cord blood PBMC were thawed and an equal volume (500 μL) of 64% ethanol was added to the lysate and mixed by vortexing. A filter cartridge was inserted into a collection tube and the lysate/ethanol mix was pipetted onto the cartridge (cartridge can hold 700 μL at a time – can pass up to 1.8 mL of lysate/ethanol mixture without exceeding the RNA binding capacity). The collection tube containing the filter cartridge was centrifuged for 1 minute at 14,000 rpm. After centrifugation,

the flow through was discarded and the collection tube was reused for the subsequent washing steps.

We applied 700 μL of Wash Solution #1 to the filter and the tube was centrifuged for 1 minute at 14,000 rpm until all wash solution was through the filter. The flow through was discarded and the tube reused for subsequent steps. Five hundred micro liters of Wash Solution #2/3 was added to the filter and the tube was centrifuged for 1 minute at 14,000 rpm. Another 500μL of Wash Solution #2/3 was added to the filter and the tube was centrifuged for another 1 minute at 14,000 rpm. The wash solution was discarded and centrifugation was continued for another 10 to 30 additional seconds to remove the last traces of wash solution.

The filter was then placed into a fresh collection tube and 50 μL Elution Solution that was preheated to 70 to 80^{o}C was applied to the center of the filter and the cap of the tube was closed and the tube was centrifuged for 30 seconds at 14,000 rpm. The elution and centrifugation steps were repeated three times for a total of four elutions.

Twenty micro liters (0.1 volumes) of 10x DNase I Buffer and 2 μL of DNase I were added to the tube and mixed gently by flicking the tube. The tube was then incubated for 30 minutes at 37^{o}C. After incubation, 20 μL (0.1 volumes) of DNase Inactivation Reagent was added to the tube. The slurry was vortexed immediately prior to use. The tube contents were mixed gently by flicking the tube and incubated for 10 minutes at room temperature. The tube was then centrifuged at 14,000 rpm for 1 minute to pellet the DNase Inactivation Reagent. The supernatant was transferred to a new tube and the pellet discarded.

Twenty micro liters (0.1 volumes) of 5M Ammonium Acetate and 4 μL (0.02 volumes) of linear acrylamide were added to the tube containing the supernatant and the tube was vortexed to

mix the contents. After mixing, 500 μL (2 to 2.5 volumes) of 100% ethanol were added to the tube and mixed well. The tube was placed in -20^{o}C overnight.

The tube was centrifuged at 14,000 rpm for 15 minutes the next day. After centrifugation, the supernatant was removed and the tube centrifuged again for several seconds to remove the residual fluid. The RNA was then resuspended in 15 μL of elution solution and was stored in -80^{o}C for further use.

Quantification of Concentration of RNA

A spectrophotometer was used to calculate the concentration of RNA in our samples. Spectrophotometers compare the light transmitted through a sample to the light transmitted through a blank which in this case is a cuvette containing 100 μL of DEPC water. A spectrophotometer can be shown to be calibrated by using a standard curve of known RNA concentrations. Calibration is important because even a 1 nm displacement will significantly affect the absorption reading.

In spectrophotometry, a measurement of the absorbance of standards containing known concentrations of RNA is first taken. A standard curve is plotted with absorbance on the X-axis and RNA concentration on the y-axis. The absorbance of the unknown samples is then measured. The concentration of the unknown RNA is calculated based on the standard curve. In using the spectrophotometer to calculate the optic density of the subject samples, the absorbance at 260 nm is related to the concentration of RNA in the sample. If a sample containing pure RNA has an absorbance of 1 at 260 nm then it contains approximately 40 μg/μL of RNA. To find the concentrations of RNA for the samples, the 260 nm reading was multiplied by 4 μg /μL.

The concentration of RNA was measured using a Beckman DU-600 spectrophotometer at a wave length of 260 nm after blanking with DEPC water used to dilute the RNA sample. A 1:100

dilution was used in which 1 mL of RNA sample was added into a cuvette containing 99 mL of DEPC water and pipetted to mix. The cuvette was then placed into the Beckman DU-600 spectrophotometer and read at 260 nm. The concentration of the sample RNA was calculated as absorbance at 260 nm x 40 μg /μL because if a sample containing pure RNA has an absorbance of 1 at 260 nm then it contains approximately 40 μg /μL of RNA. An A260/A280 ratio of 2.0 is characteristic of pure RNA and a ratio of 1.8 to 2.0 is desirable. Any ratio below 1.7 indicates that there is probably a contaminant in the solution, usually a protein or phenol.

Reverse Transcription

Reverse transcription (RT) was used to synthesize cDNA from the RNA obtained earlier. One RT reaction produces 40 μL of cDNA. Two mixes were prepared for each RT reaction. The first mix included 1 μL of Oligo $(dT)_{12-18}$ Primer (Invitrogen, Cat. No. 18418-012) and 20 μL of RNA sample in DEPC water in a 250 μL PCR tube. The tube was then placed on ice and the second mix was prepared and added to each tube including 8 μL of 5x First Strand Buffer, 2.7 μL of 0.1M DTT, 1.3 μL of Rnasin Ribonuclease Inhibitor (Promega, Cat. No. N2111), 2.0 μL of 10mM dNTP (Amersham Biosciences, Cat. No. 27-2035-07), 4.0 μL of Bovine Serum Albumin, Acetylated (Promega, Cat. No. R3961), and 1.0 μL of SuperScript™ II Reverse Transcriptase. The 5x buffer, DTT, and SuperScript™ II Reverse Transcriptase were from Invitrogen (Cat. No. 18064-022). The tube was then centrifuged and run on the PCR machine for 60 minutes at 42°C and then at 95°C for 10 minutes, and held at 4°C.

Polymerase Chain Reaction (PCR)

After cDNA was synthesized by reverse transcription, specific regions of the cDNA were amplified through the use of primers that are specific to each Vβ family. Inner and outer primers specific for each of the 22 Vβ families were previously constructed in the lab to flank specific

regions of the cDNA strand (Table 2-1). The master mix for the first round of PCR consisted of: dH_2O, 1x buffer; $MgCl_2$ (2.8 mM); the 3' primer (Cβ) for each Vβ family (0.4 μM); the 5' (Vβ# of family) primer for each Vβ family (0.4 μM); and dNTPs (0.2 mM of each dNTP); Taq DNA Polymerase (0.05 U/μL). This master mix 1 was added to each well in the block plate along with water and the cDNA synthesized from the RT-PCR reaction. Water was used a negative control. The first round of PCR was performed on a thermocycler at 95°C for 2 minutes then 35 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute with the final extension at 72°C for 10 minutes. The plate was then held at 4°C until the gel was run.

The master mix for the second round of PCR consisted of: dH_2O; 1x buffer; $MgCl_2$ (2.8 mM); reverse primer CβNS-FAM (0.1 μM); forward primer VβNS-family # (0.1 μM); dNTPs (0.2 mM for each dNTP); and Taq DNA Polymerase (0.05 U/μL). The forward primer VβNS-family #) is nested 3' to the first-round Vβ-specific primer, and the reverse primer (CβNS-FAM) is located 88 base pairs away from the CDR3 region. The reverse primer (CβNS-FAM) contains a nontemplate sequence GTTTCTT which is placed on the 5' end of the CβNS primer and labeled at the 5' end with a blue fluorescent dye (6-FAM; Applied Biosystems, Foster City, CA). The primer concentrations were reduced in the second round of PCR to increase amplifying specificity. The master mix 2 was added to each well of a new block plate and 2 μL of first round PCR product was added to each well. The plate was run for second round PCR on a thermocycler at 95°C for 2 minutes, then 20 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute with a final extension cycle at 72°C for 10 minutes. The plate was then held at 4°C until the gel was run. The second round PCR product was run on an agarose gel after which bands were viewed and a picture was taken through the Stratagene Eagle Eye II.

Spectratyping

Spectratyping is a PCR based assay that is specially designed to measure the length variation within the same gene, for example, here we used spectratyping to measure the TCR Vβ CDR3 length distribution. Spectratyping allows oligoclonal expansion within T cell populations to be detected as dominant peaks within individual Vβ families. Spectratyping uses two rounds of PCR in order to amplify the CDR3 region of the TCR. In first round PCR, a forward primer is used that is specific to the particular Vβ family and a reverse primer is used that is in the constant region of the TCR. In second round PCR, the first round products are used as a template and a pair of nested primers of which the forward primer is Vβ family specific and is nested 3' to the first round PCR primer while the reverse primer is constant and located 88 base pairs away from the CDR3 region (Table 2-1). The second round primers are labeled with a blue fluorescent dye (6-FAM, Applied Biosystems) to allow the bands to be seen on a gel. The PCR products are then run on a 6% acrylimide sequencing gel and analyzed using ABIPRISM GeneScan analysis software (Applied Biosystems) which not only gives the CDR3 length distribution in base pairs but also calculates the relative intensity (area under the curve, AUC) of each length product to generate a histogram for each Vβ family [63].

In interpreting the histogram, the area under each peak is calculated and a peak with an area that is greater than 40% of the total area is considered perturbed which may indicate oligoclonal expansion. Each peak is one length and by observing the proportion, measurement, and distribution of the peaks, one can determine TCR recognition of antigen [66]. The intensity of each individual peak shows the relative representation of the particular CDR3 sizes within each individual Vβ family. The relative fluorescent intensity of individual CDR3 sizes can be quantified as the area under each peak [38, 48]. When this data is expressed as a percentage of

the total area under the curves for each Vβ family, it can be used to accurately determine the degree of CDR3 length diversity within any particular Vβ family. The relative contribution of each CDR3 size is determined by the relationship of each particular peak to the total area under the curve, the results of which are shown as a percentage [38].

In-frame transcripts of the CDR3 region of the TCR in adults shows six to eight peaks spaced three nucleotides apart to form a bell-shaped curve. The area under each peak is proportional to the amount of transcripts of the corresponding CDR3 size in the sample and each peak corresponds to a given CDR3 length that contains multiple distinct sequences. An increase in the height and area of a given peak that results in modification of the bell-shaped curve represents oligoclonal or monoclonal expansions due to immune stimulation [55].

A peak is considered a predominant peak if the area under the peak is greater than 40% of the total area under the curve. A predominant peak in any given CDR3 pattern could reflect either oligoclonal expansion of T cells expressing a particular TCR or a polyclonal collection of T cells that contain multiple TCRs of the same CDR3 size. Oligoclonal or polyclonal expansion can be determined by sequencing the amplified CDR3 regions [38]. Examining intensity can signify multiple species of the same size class with each having a low concentration or it could signify the expansion of one or a few species within the size class [67]. A Gaussian, or bell-shaped, distribution of peaks within each family is normally seen in healthy individuals consisting of multiple peaks (usually six to eight) that are three nucleotides apart [48, 65].

Some antigens use only a specific receptor which correlates to a specific peak while other antigens may use multiple receptors within one family. T cells for a specific antigen will activate and replicate thus allowing monoclonal or polyclonal expansion. By examining the distribution of the T cell repertoire, one can determine whether or not the virus has interacted

with one or more families thus allowing the use of the TCR as a surrogate marker to observe the effectiveness of a vaccine. The more diversified the response, the better protection from virus infection.

Determination of TCR Vβ Perturbation

Perturbation in TCR Vβ CDR3 repertoire was assessed by two methods. In the first method, any Vβ family exhibiting a predominant peak is considered perturbed. A predominant peak was defined as the AUC of one peak within a given CDR3 spectratype being greater than 40% of the sum of the total AUC. In the second method, if the distribution of TCR Vβ CDR3 lengths were significantly away from the Gaussian distribution they were considered perturbed. The method of analysis is described in detail below.

The AUC of each CDR3 length (*i)* in a V*β* family profile (*κ)* was translated into a *probability distribution,* $P^{\kappa}_{j}(i) = A^{\kappa}_{j\,i} / (\Sigma_i A^{\kappa}_{j\,i})$, using the fraction of the area (A_i*)* under the V*β* family profile for each CDR3 length, from minimal to maximal length in steps of three nucleotides. The letter *j* represents each sample. Generally there were ten possible amino acid lengths ($i_{(1 \text{ to } 10)}$) in each V*β* family. A control profile was established for each CDR3 length by calculating the *average probability distribution,* $P^{\kappa}_{c}(i) = (\Sigma_j [P^{\kappa}_{j}(i)]) / n_j$ of corresponding Vβ profiles, where *n* represents the number of healthy subjects. The resulting control profiles, $P^{\kappa}_{c}(i)$, conform to a Gaussian distribution.

To define the extent of perturbations in CDR3 length the distance (D) between the probability distributions of sample and average probability distributions of healthy controls (c) was calculated as $D^{\kappa}_{j}(i) = P^{\kappa}_{j}(i) - P^{\kappa}_{c}(i)$. The sum of the absolute distance, $D^{\kappa}_{j} = 100\ [\Sigma_i |D^{\kappa}_{j}(i)|\ ] / 2$, was calculated in each Vβ family. Overall, TCR length yield perturbations of the TCR profile in percentages. The average perturbation among all Vβ families studied for each individual j is

calculated as an average distance (AD), $AD_j = (\Sigma_{\kappa} D^{\kappa}{}_j)/ m_{\kappa}$, of all Vβ families examined. In this study, AD for a control sample is presented as $AD_c(j) = (\Sigma_{\kappa} D^{\kappa}{}_j) / m_{\kappa}$. Based on approximate Z test, a perturbation within each Vβ family was defined as $AD^{\kappa}_j > [\Sigma_j AD_c(j)] / n_j + 3$ Standard Deviations (SD) [68] (Figure 2-1).

TCR Vβ perturbation was evaluated using the first method (assessing predominant peaks) in Specific Aim 1 where all we wanted to determine is whether each Vβ family was successfully amplified. Unsuccessful amplification is mainly due to insufficiency of template that leads to either no curve, loss of CDR3 lengths exhibiting wobble at the baseline, or low fluorescent intensity (FI < 400). Unsuccessful amplification is distinguished from a successfully amplified but perturbed Vβ family by the latter exhibiting a predominant peak having a straight baseline and a high fluorescent intensity (FI > 400). TCR Vβ perturbation in Specific Aim 2 was evaluated using statistical analysis as described in the second method described above since we needed to know a detailed change in the CDR3 length distribution within each Vβ family in order to map out the Vβ family usage after influenza vaccination.

Samples from Influenza Vaccinated Healthy Adults

Changes in TCR Vβ CDR3 length repertoire after influenza vaccination were evaluated within $CD4^+$ T cells from two anonymous donors who each received a trivalent split influenza vaccine administered intramuscularly. The anonymous donors were part of a pilot study carried on at the University of South Florida (USF) clinic and All Children's Hospital to determine appropriate time points of sampling to be able to detect the response of T cells to influenza vaccine. Each subject received a split inactivated trivalent influenza vaccine intramuscularly and blood samples were taken at two separate time points: at vaccination and one month post vaccination.

This pilot study was done in collaboration with a larger study carried out at the University of South Florida that sought to measure the breadth of the T cell receptor immune response within naïve and activated memory/effector $CD8^+$ T cells following immunization with influenza as recall antigen for 15 healthy subjects as well as 15 post bone marrow transplant subjects. IRB approval was obtained for the larger study at the University of South Florida. The larger study design was designed to use MHC I: influenza tetramers, flow cytometry, and spectratyping to determine the magnitude and breadth of the memory immune response to influenza recall antigen. Tetramers are complexes of four MHC molecules which are bound to four antigenic peptides to form a complex that is recognized by the TCR. These tetramers are conjugated to a flourochrome and to fluorescently conjugated monoclonal antibodies thus facilitating the detection of the antigen specific T cell response through flow cytometry. The MHC I: peptide complexes are mutated to bind primarily to T cells of a specific HLA allele. Subjects in the larger study were HLA typed and only subjects with the HLA-A0201 genotype were included in the study.

The larger study sought to first use spectratyping to determine the memory effector response to influenza recall antigen at pre-vaccination, and 1 month and 3 months post-vaccination for 15 healthy adult subjects and 15 one-year post-transplant bone marrow patients with immune reconstitution. Each subject was to have the HLA-A0201 genotype in order to detect the influenza recall antigen with the use of specific tetramers sensitive to this genotype. The two anonymous subjects in this paper were excluded from the original larger study at the University of South Florida due to not having the specific HLA genotype. However, cell lysates from these two anonymous subject samples were sent to the Goodenow lab to perform a pilot study to determine if perturbations were seen within either PBMC or $CD4^+$ T cells in the 1

month post-vaccination sample when compared to the sample taken at vaccination. Unfortunately, the larger project was stopped before the 3 month post vaccination sample could be drawn.

Cell separation was performed at the University of South Florida. PBMC were isolated from each subject's sample and then $CD4^+$ and $CD4^-$ were separated and isolated using a T Cell Isolation Kit II (Miltenyi Biotech, Auburn, CA). Cell lysates were then sent to the Goodenow lab for RNA extraction and spectratyping. RNA was extracted from PBMC, $CD4^+$, and $CD4^-$ cells of each patient using the Ambion RNAqueous-4PCR kit according to the protocol stated earlier. Reverse transcription and PCR (described earlier in this section) were performed on each sample for all 21 Vβ families. Spectratyping (described earlier in this section) was finally used to measure the T cell receptor diversity.

Table 2-1. Variable beta chain primers for spectratyping

First-round PCR primers		Nested PCR primers	
Primer	Sequence	Primer	Sequence
Vβ1	5′-GAT TCT GGA GTC ACA CAA ACC CCA AAG-3′	Vβ1NS	5′-GAA CTA AAC CTG AGC TCT C-3′
Vβ2	5′-GAG CTG GGT TAT CTG TAA GAG-3′	Vβ2NS	5′-GCT TCT ACA TCT GCA GTG C-3′
Vβ3	5′-GAT GTG AAA GTA ACC CAG AGC TCG AG-3′	Vβ3NS	5′-CTG GAG TCC GCC AGC ACC-3′
Vβ4	5′-CAA GTC GAT AGC CAA GTC ACC ATG-3′	Vβ4NS	5′-GAA GAC AGC AGC ATA TAT C-3′
Vβ5.1	5′-CCC TGG TCG ATT CTC AGG GCG CCA G-3′	Vβ5.1NS	5′-CTC GGC CCT TTA TCT TTG CG-3′
Vβ6	5′-CTG GAG TCT CCC AGA ACC CCA GAC AC-3′	Vβ6NS	5′-GAT CCA GCG CAC AGA GCA G-3′
Vβ7	5′-CAT GGG AAT GAC AAA TAA GAA GTC-3′	Vβ7NS	5′-GAA TGC CCC AAC AGC TCT C-3′
Vβ8	5′-CGT TCC GAT AGA TGA TTC AGG GAT GC-3′	Vβ8NS	5′-GCC CTC AGA ACC CAG GGA C-3′
Vβ9	5′-GAA ACG ACA AGT CCA TTA AAT G-3′	Vβ9NS	5′-CCC TGG AGC TTG GTG ACT CTG-3′
Vβ10	5′-GAC ACC AAG GTC ACC CAG AGA CC-3′	Vβ10NS	5′-GGA GAT CCA GTC CAC GGA G-3′
Vβ11	5′-GAA GCT GAC ATC TAC CAG ACC CCA AG-3′	Vβ11NS	5′-GGA GTC TGC CAG GCC CTC-3′
Vβ12	5′-CTG ACA AAG GAG AAG TCT CAG-3′	Vβ12NS	5′-CTC TGG AGT CGC TAC CAG-3′
Vβ13	5′-GAC CCA GGC ATG GGG CTG AAG CTG-3′	Vβ13NS	5′-GAT TTC CCG CTC AGG CTG G-3′
Vβ14	5′-GAT GTT CCT GAA GGG TAC AAA G-3′	Vβ14NS	5′-CGA AAA GAG AAG AGG AAT TTC-3′
Vβ15	5′-GTG TCT CTC GAC AGG CAC AGG C-3′	Vβ15NS	5′-CCC TAG AGT CTG CCA TCC-3′
Vβ16	5′-CTT AGC TGA AAG GAC TGG AGG GAC G-3′	Vβ16NS	5′-CTG CAG AAC TGG AGG ATT C-3′
Vβ17	5′-CTG AAG GGT ACA GCG TCT CTC GGG AG-3′	Vβ17NS	5′-CCA AAA GAA CCC GAC AGC TTT C-3′
Vβ18	5′-CAA ATG CCG GCG TCA TGC AGA ACC-3′	Vβ18NS	5′-GCG AGG AGA TTC GGC AGC-3′
Vβ19	5′-GCC AAA GTC ACA CAG ACT CCA GG-3′	Vβ19NS	5′-GCC CCA AGA ACG CAC CCT GC-3′
Vβ20	5′-GAA TCT CTC AGC CTC CAG AC-3′	Vβ20NS	5′-CCA GGA CCG GCA GTT CAT C-3′
Vβ21	5′-GCA GCC TGT GGC TTT TTG GTG C-3′	Vβ21NS	5′-CCA GCC TGC AGA GCT TGG-3′
Vβ22	5′-CAG ATG GGA CAG GAA GTG-3′	Vβ22NS	5′-CTC TGA AGA TCC GGT CCA C-3′
Vβ23	5′-GAT CGA TTC TCA GCT CAA CAG-3′	Vβ23NS	5′-CTC CTT GGA GCT GGG GGA C-3′
Vβ24	5′-GGC CGA ACA CTT CTT TCT GC-3′	Vβ24NS	5′-GAC ATC CGC TCA CCA GGC CTG-3′
Cβ	5′-CCC CAG GCC TCG GCG CTG ACG ATC TGG G-3′	CβNS[a]	5′-GTT TCT TCT GCT TCT GAT GGC TCA AAC ACA G-3′

[a] 6-FAM labeling.

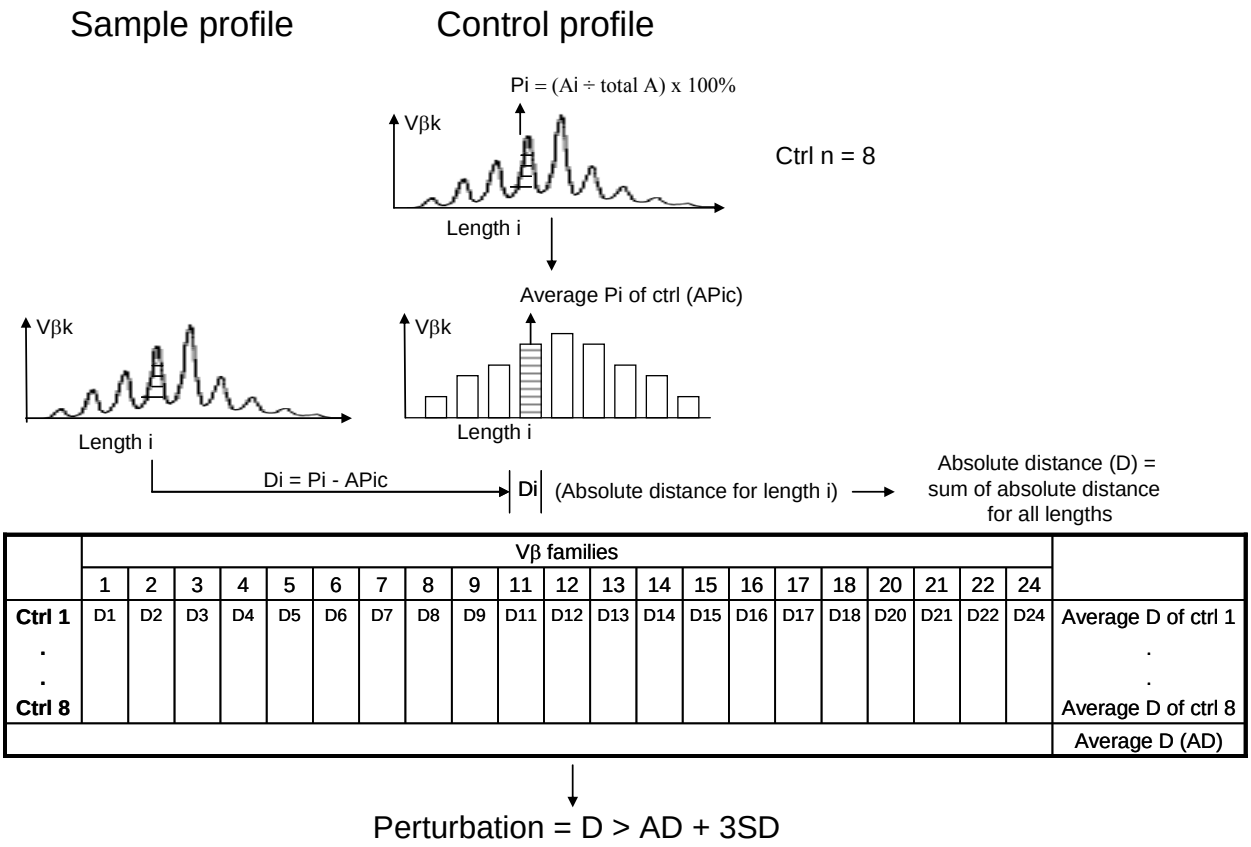


Figure 2-1. Statistical method for determination of TCR Vβ perturbations. First find area under each peak for each family for each control to get probability distribution (Pi) = (area under peak/total area) x 100. Second, average each peak for each family for all controls to get average probability distribution (APi) – ex=Vb1 for all 8 controls. Third, get absolute distance, Di, for each peak for each sample Di=Pi-APi. Fourth, add Di of all families for each control and divide by # of families (ex-Vb1+Vb2+…./21 = Average distance of each control=AD). Fifth, add average distance of each control together and divide by # controls = average D of AD or AAD. Sixth, perturbation of family if D of each family of each sample >AAD+3sd.

CHAPTER 3
RESULTS

Results Obtained for Aim 1

The objective of Specific Aim 1 was to define the minimum amount of cDNA derived from the minimum amount of RNA extracted from purified CD45RA and CD45RO $CD8^+$ T cells needed to be able to amplify 21 Vβ families. The strategies to achieve the goal were: (1) to use PBMC from cord blood to scale down the amount of RNA and the volume of cDNA used to amplify 21 Vβ families; and (2) to test the minimum amount of RNA and volume of cDNA determined in cord blood PBMC on purified CD45RA and CD45RO $CD8^+$ T cells from healthy donors to ensure the conditions also work for purified T cells. We chose cord blood PBMC because the T cells in cord blood PBMC always demonstrate a Gaussian distribution of CDR3 length for each Vβ family due to lack of antigen stimulation. We hypothesized that the minimum amount of RNA and cDNA determined in cord blood, a mixed population of mononuclear cells, will also apply to purified T cell subsets. The purpose of using cord blood and T cell subsets from healthy donors was to minimize the chance of Vβ perturbation during the optimization process.

Estimation of Targeted Minimum Amount of RNA and cDNA Required to Amplify 21 Vβ Families

Based on previous experience, normally 2 μg of RNA is used in each RT reaction to obtain 40 μL of cDNA. Two micrograms of RNA is extracted from approximately 3 x 10^6 cells. A subject's PBMC sample usually contains 5 x 10^6 to 10 x 10^6 PBMC per vial. Around 0.3 x 10^6 and 0.5 x 10^6 CD45RA and CD45RO $CD8^+$ T cells can be purified from 5 x 10^6 to 10 x 10^6 PBMC respectively from which 0.2 μg and 0.3 μg of RNA can be extracted. In our study, we wanted to determine if we could amplify 21 Vβ families from 40 μL of cDNA that was reverse

transcribed from 0.25 μg of RNA which was extracted from 0.2 – 0.3 x 10^6 purified CD45RA or CD45RO $CD8^+$ T cells (Figure 3-1).

Determination of the Minimum Amount of RNA and cDNA Using Cord Blood

PBMC from cord blood was used to scale down the amount of RNA and cDNA used to amplify 21 Vβ families. First, cord blood PBMC were used to derive differing amounts of RNA, ranging downward from 2 μg to 0.25 μg. These differing amounts of RNA were then used to make cDNA. Differing volumes of cDNA, ranging downward from 10 μL to 0.5 μL (40 μL total volume), were used to amplify the Vβ2 family. The Vβ2 family was amplified showing an acceptable CDR3 length distribution when lower limit conditions of 1 μL of cDNA reversely transcribed from 0.25 μg of RNA were used (Figure 3-2). We found the upper limit to be 6 μL of cDNA derived from 2 μg of RNA, given that amounts over this limit did not exhibit amplification of the Vβ2 family.

Next, 1 μL of cDNA derived from 0.25 μg RNA of cord blood PBMC was tested on four other Vβ families – two easy families (Vβ14 and Vβ21) and two hard families (Vβ5 and Vβ15). Families are classified as easy or hard according to the ease of which they have been successfully amplified in previous studies. The "easy" families have been amplified in almost all of the previous studies while the "hard" families have been amplified in fewer studies. The easy families may be more representative, i.e. they may have more cells expressing these families, or their primers may be more efficient, whereas the hard families may be less representative or have less efficient primers. The results showed that all four families amplified well with this amount of cDNA (Figure 3-3).

Since the minimum amount of RNA and cDNA was able to successfully amplify five representative Vβ families, all 21 Vβ families were amplified using 1 μL of cDNA derived from

0.25 μg RNA. However, using this condition, some families did not amplify well including Vβ4, 7, 8, 13, 16, 17, 18, and 22. These families were amplified after increasing the volume of cDNA to 3 μL thus indicating that the failure of amplification of these families was due to having insufficient template. Figure 3-4 demonstrates the histographs of Vβ4, 7, 13, and 17 with insufficient (1 μL) and sufficient (3 μL) template respectively. When the template is insufficient, there are distortions at the baseline, the fluorescent intensity is very low (FI < 400), or there are no peaks shown. In order to amplify all the families, the amount of RNA was increased to 0.5 μg and the amount of cDNA remained at 1 μL. This amount is equivalent to using 0.25 μg of RNA and 2 μL of cDNA. With this adjustment, all families were amplified successfully (Figure 3-5).

Application of the Minimum Amount of RNA and cDNA of Cord Blood to Purified CD45RA and CD45RO CD8 T Cell Subsets

The 1 μL cDNA derived from 0.25 μg RNA was used to amplify Vβ families of T cell subsets since these were pure T cells. Since cord blood is a mixed population of cells and only contains 17% T cells, if 1 μL cDNA derived from 0.5 μg RNA was able to amplify each Vβ family for cord blood PBMC, we hypothesized that 1 μL cDNA derived from 0.25 μg RNA should be able to amplify each Vβ family from purified T cells. As in the cord blood experiments, five Vβ families, three easy families (Vβ2, Vβ14, and Vβ21) and two hard families (Vβ5, Vβ15) were first amplified. These five families were amplified for $CD8^+$ CD45RA cells and $CD8^+$ CD45RO cells separately. The five Vβ families within $CD8^+$ CD45RA T cells showed a Gaussian distribution of CDR3 lengths, which is expected since these are naïve cells (Figure 3-6). However, one of the five Vβ families (Vβ14) in the $CD8^+$ CD45RO cells exhibited perturbation with a prominent CDR3 size of 201bp whose AUC is greater than 40%, indicating

monoclonal expansion. Sufficient template was used since the fluorescent intensity was greater than 400 for all five Vβ families (Figure 3-7). In healthy adults, perturbed Vβ families can often be seen in effector memory $CD8^+$ $CD45RO^+$ T cells. The $CD45RO^+$ T cells express a number of activation markers and respond to recall antigen readily thus these cells represent memory cells. A strongly positive expression of $CD45RO^+$ $CD8^+$ T cells is generally consistent with T cell activation [54, 69]. Patterns of perturbations in $CD45RO^+$ $CD8^+$ T cells have been observed to be persistent over many months in different healthy adults [54].

Given the encouraging results from amplifying five Vβ families, all 21 Vβ families were amplified using 1 μL cDNA derived from 0.25 μg RNA for CD45RA and CD45RO $CD8^+$ T cells separately. Eighteen families amplified well from $CD8^+$ CD45RA T cells and exhibited normal distributions (Figure 3-8). All 21 Vβ families were amplified from $CD8^+$ CD45RO T cells with four Vβ families exhibiting predominant peaks, including Vβ11, 14, 17, and 24 (Figure 3-9). Again, these predominant peaks were not due to having an insufficient template since the fluorescent intensity was greater than 400.

Calculation of the Number of PBMCs Needed to Amplify 21 Vβ Families from CD8 CD45RA and CD45RO T Cell Subsets

Based on the above results, 40 μL of cDNA derived from 0.25 μg RNA from purified CD45RA and CD45RO $CD8^+$ T cells was able to amplify 21 Vβ families using only 1 μL of cDNA per Vβ family. We obtained 0.25 μg of RNA from 0.3 to 0.5 x 10^6 purified CD45RA and CD45RO $CD8^+$ T cells which were purified from 5 to 10 x 10^6 PBMC. Therefore 5 to 10 x 10^6 PBMC are needed to successfully amplify 21 Vβ families of CD45RA and CD45RO $CD8^+$ T cell subsets (Table 3-2).

A greater quantity of RNA should be extracted from CD45RA and CD45RO $CD4^+$ T cells as compared to CD45RA and CD45RO $CD8^+$ T cells due to the PBMC of healthy adults having a higher percentage of $CD4^+$ T cells than $CD8^+$ T cells. The ratio of $CD4^+$ T cells to $CD8^+$ T cells is 1.5 to 1, meaning that for every one $CD8^+$ T cell in the PBMC of healthy adults, there are one to two $CD4^+$ T cells. In other words, for every 10 $CD8^+$ T cells, there are 15 to 20 $CD4^+$ T cells. This ratio seems to be constant across different studies. An average of 800 cells /μL has been observed for $CD4^+$ T cells in healthy adults while an average of 550 cells /μL has been observed for $CD8^+$ T cells in healthy adults [70, 71]. Given our results for specific aim 1, we estimate that 5 to 10 x 10^6 PBMC should contain 0.3 to 0.5 x 10^6 CD45RA or CD45RO $CD8^+$ T cells which should provide enough RNA and cDNA be able to successfully amplify 21 Vβ families from CD45RA and CD45RO $CD4^+$ T cell subsets.

Results Obtained for Aim 2

The results obtained for the second aim of this study are described in this part of the chapter. This aim uses the optimal conditions found in specific aim 1 to perform spectratyping on 21 Vβ families in two anonymous influenza vaccinated subjects. Spectratyping was used to examine the changes in PBMC, $CD4^+$, and $CD4^-$ T cells in two influenza vaccinated subjects for 21 Vβ families. The hypothesis for the pilot study is that vaccination would cause perturbations in the TCR Vβ repertoire and that changes in TCR Vβ CDR3 length distribution could be used as a surrogate marker to determine the efficacy of the influenza vaccine as measured by spectratyping.

In examining the RNA concentration for the samples examined in this study, we observed that all of the RNA concentrations for each of the cell types were below the concentrations that were expected based on the starting cell number. If there is too much or too little of a sample,

the spectrophotometer cannot read the absorbance accurately. Since the concentrations of RNA for the samples were low, perhaps our samples were too dilute and thus were outside of the linear range of the reading or that the starting cell number was incorrect. The linear range of absorbance values should be between 0.1 and 1.0. Given that the linear range of the samples here were below 0.1, it would seem as if our samples were too dilute and thus gave an inaccurate reading of absorbance (Table 3-3).

Cell lysates containing PBMC, $CD4^+$, and $CD4^-$ cells from two anonymous influenza vaccinated healthy adult subjects were sent to us by a collaborator to examine the change of TCR Vβ repertoire in each of the above cell types from vaccination to 1 month post vaccination. After RNA extraction, it was found that only $CD4^+$ T cells from both subjects had sufficient number to obtain the 0.25 μg RNA needed to amplify 21 Vβ families. Unfortunately, the PBMC and $CD4^-$ cells did not contain the minimum amount of RNA needed for TCR Vβ amplification, therefore TCR Vβ spectratyping was performed only on the two $CD4^+$ T cell samples from both subjects.

Eighteen Vβ families were amplified from the $CD4^+$ T cells from Subject 1. After statistical analysis, thirteen Vβ families exhibited a perturbation, including Vβ 1, 5, 7, 8, 9, 11, 12, 13, 14, 17, 20, 21, and 24. Of those Vβ families exhibiting a perturbation, only the Vβ9 family displayed a monoclonal expansion. Vβ15, 16, and 18 could not be amplified (Figure 3-10).

Fifteen Vβ families were amplified from Subject 2, fourteen of which showed perturbation including Vβ 1, 2, 4, 5, 6, 7, 8, 9, 11, 12, 13, 16, 17, and 22. Of these fourteen families exhibiting a perturbation, only Vβ7 displayed a monoclonal expansion. Vβ14, 15, 18, 20, 21, and 24 could not be amplified (Figure 3-11). The sample from Subject 2 had

significantly less RNA than the other subject which may explain why more families could not be amplified.

In comparing the two subjects, nine Vβ families were perturbed in both subjects including Vβ1, 5, 7, 8, 9, 11, 12, 13, and 17. These perturbations are not due to insufficient template since the fluorescent intensity is higher than 400 and there is no evidence of distortions at the baseline. Only Vβ3 was normal in both subjects. Two Vβ families, Vβ15 and 18, failed to amplify in either subject (Figure 3-12).

Table 3-1. Separation of CD45RA and CD45RO CD8 T cell subsets

Starting PBMC #	Cell type	Cell # after separation (x 10^6)	Separation efficiency (%)	RNA (μg)	RNA conc. (μg/μl)
$5x10^6$	$CD8^+$	0.7	14%	---------	----------
	$CD8^+$ $CD45RA^+$	0.24	34%	20.1	1.34
	$CD8^+$ $CD45RO^+$	0.3	42%	16.4	1.09
$10x10^6$	$CD8^+$	1.12	11%	---------	----------
	$CD8^+$ $CD45RA^+$	0.48	43%	16.7	1.13
	$CD8^+$ $CD45RO^+$	0.5	45%	17	1.11
$20x10^6$	$CD8^+$	2.38	12%	---------	----------
	$CD8^+$ $CD45RA^+$	0.56	24%	16.7	1.22
	$CD8^+$ $CD45RO^+$	0.58	25%	18.2	1.12

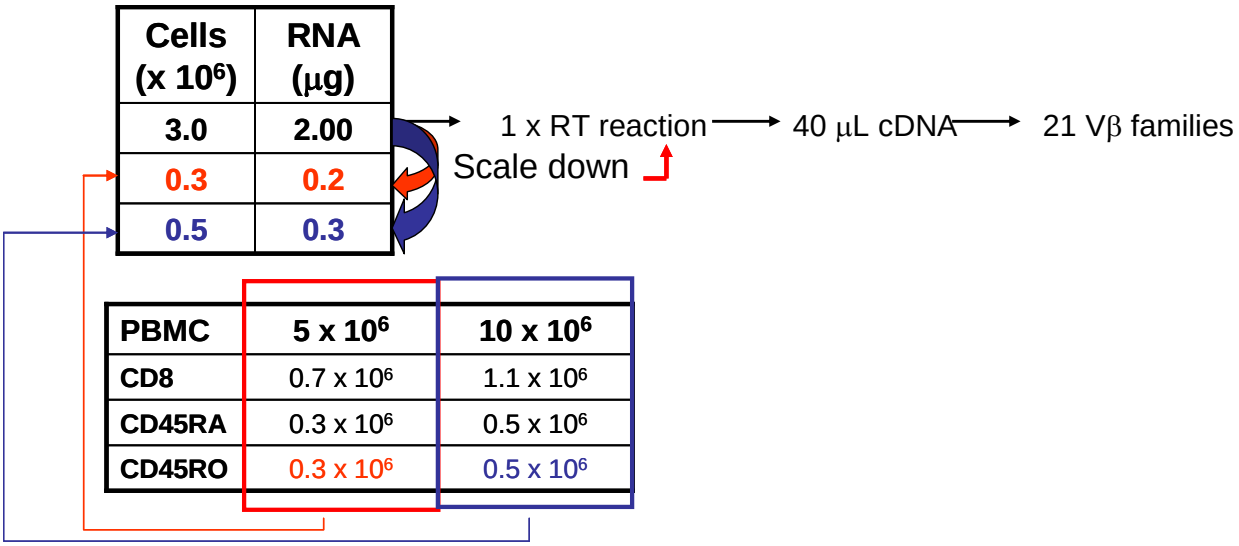


Figure 3-1. Objective is to find the minimum amount of cDNA that is derived from the minimum amount of RNA that can be used to amplify 21 Vβ families. The targeted optimal condition is 1μL cDNA (2.5% of total amount) derived from 0.25μg of RNA.

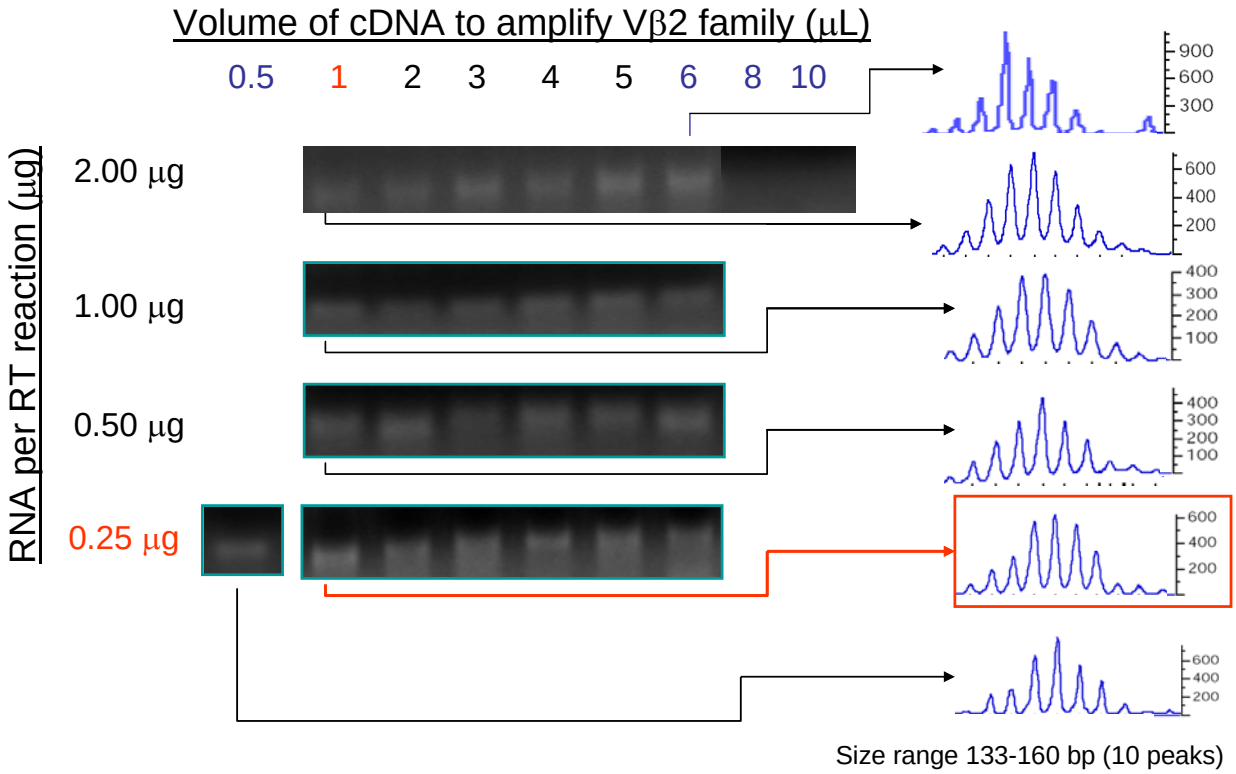


Figure 3-2. Scale down amount of RNA per RT using cord blood cells on Vβ2 family

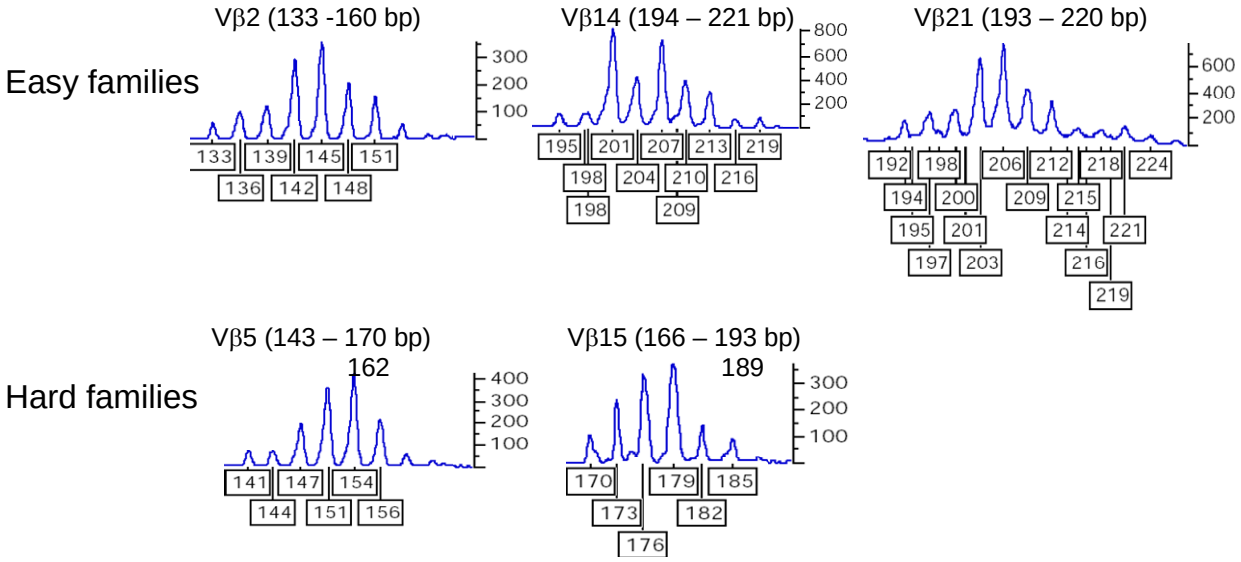


Figure 3-3. Cord blood amplification of five Vβ families using 1 μL or 2.5% of cDNA that was derived from 0.25 μg of cord blood RNA. The hard families length distribution is shorter possibly due to cord blood having shorter range, needing more template, or a smaller proportion of cells express this particular family.

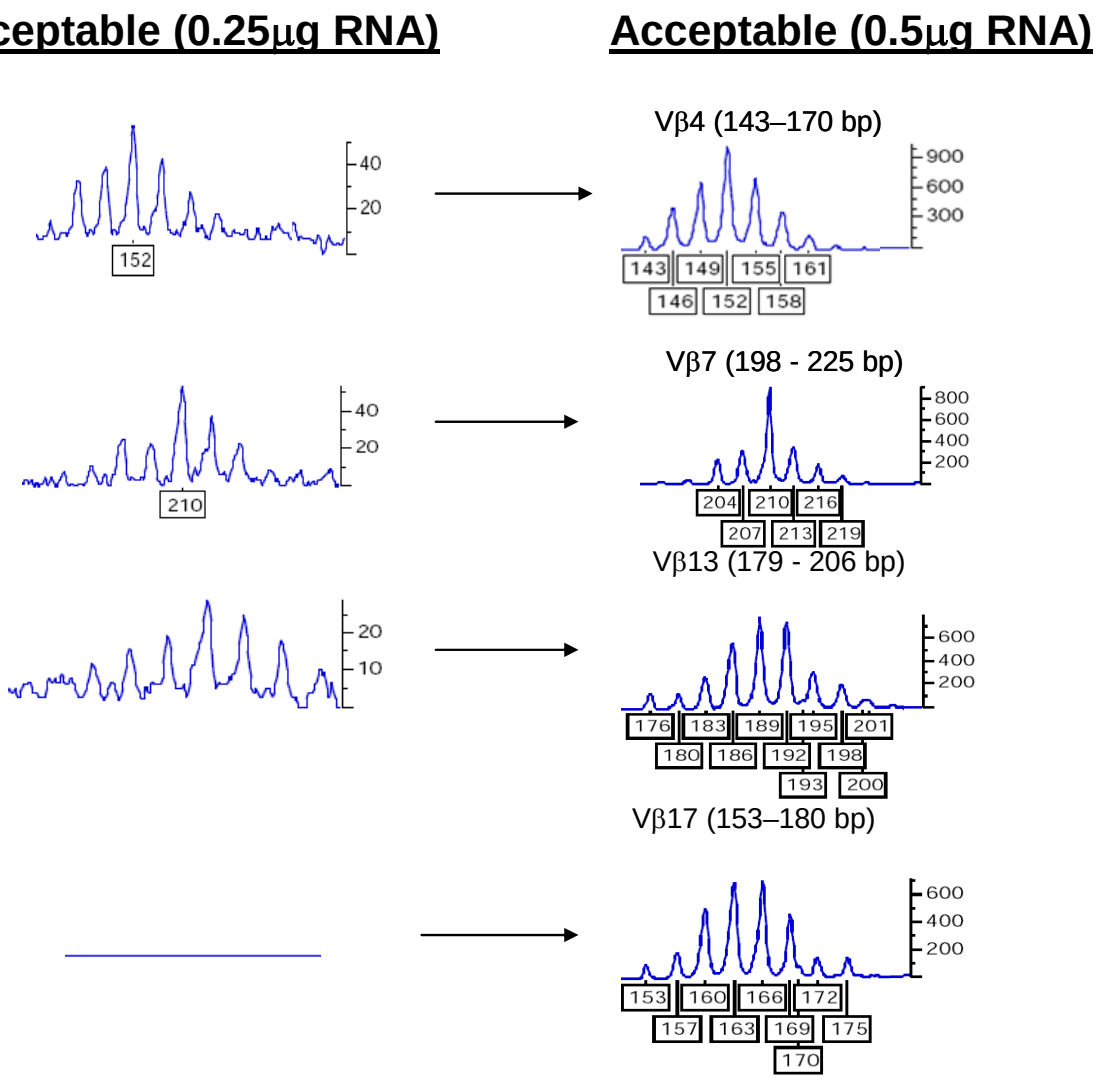


Figure 3-4. Comparison of unacceptable versus acceptable spectratyping results. Unacceptable results have distortions at the baseline, low fluorescent intensity, or lack of length distribution including no peaks. Acceptable results have an even length distribution, no wobble at the baseline, and good fluorescent intensity.

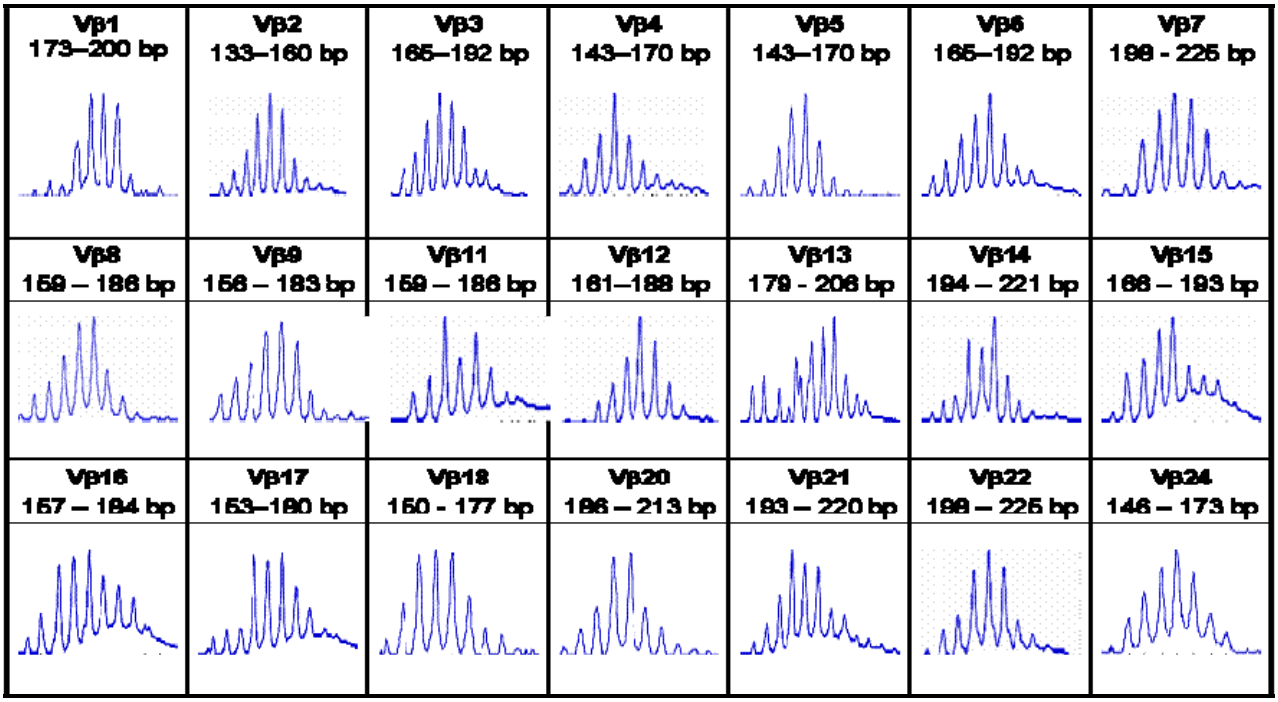


Figure 3-5. Cord blood amplification of 21 Vβ families using 1 µL of cDNA derived from 0.5 µg of RNA. This amount is equivalent to 3 µL cDNA derived from 0.25 µg RNA. The amount of RNA had to be increased due to the lack of amplification of some families when 0.25 µg RNA was used.

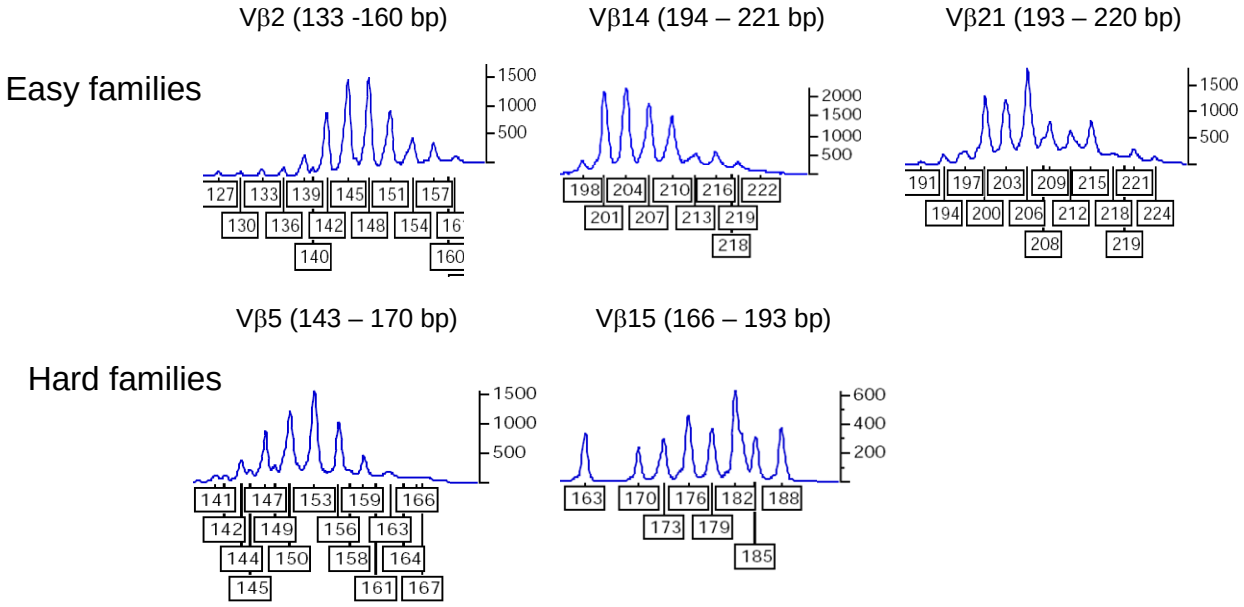


Figure 3-6. CD8^{+} CD45RA cells amplification of five Vβ families using 1 μL cDNA derived from 0.25 μg RNA.

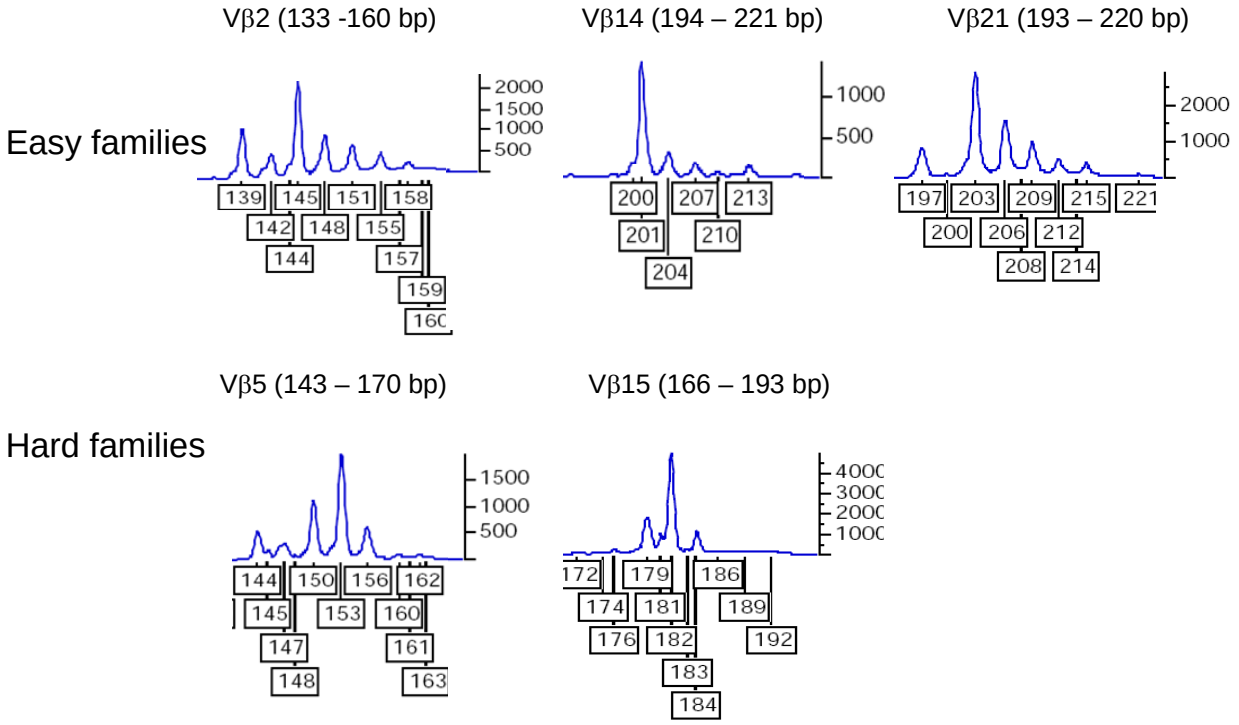


Figure 3-7. CD8^{+} CD45RO cells amplification of five Vβ families using 1 μL cDNA derived from 0.25 μg RNA. Perturbations are observed which is expected since these are effector and memory cells.

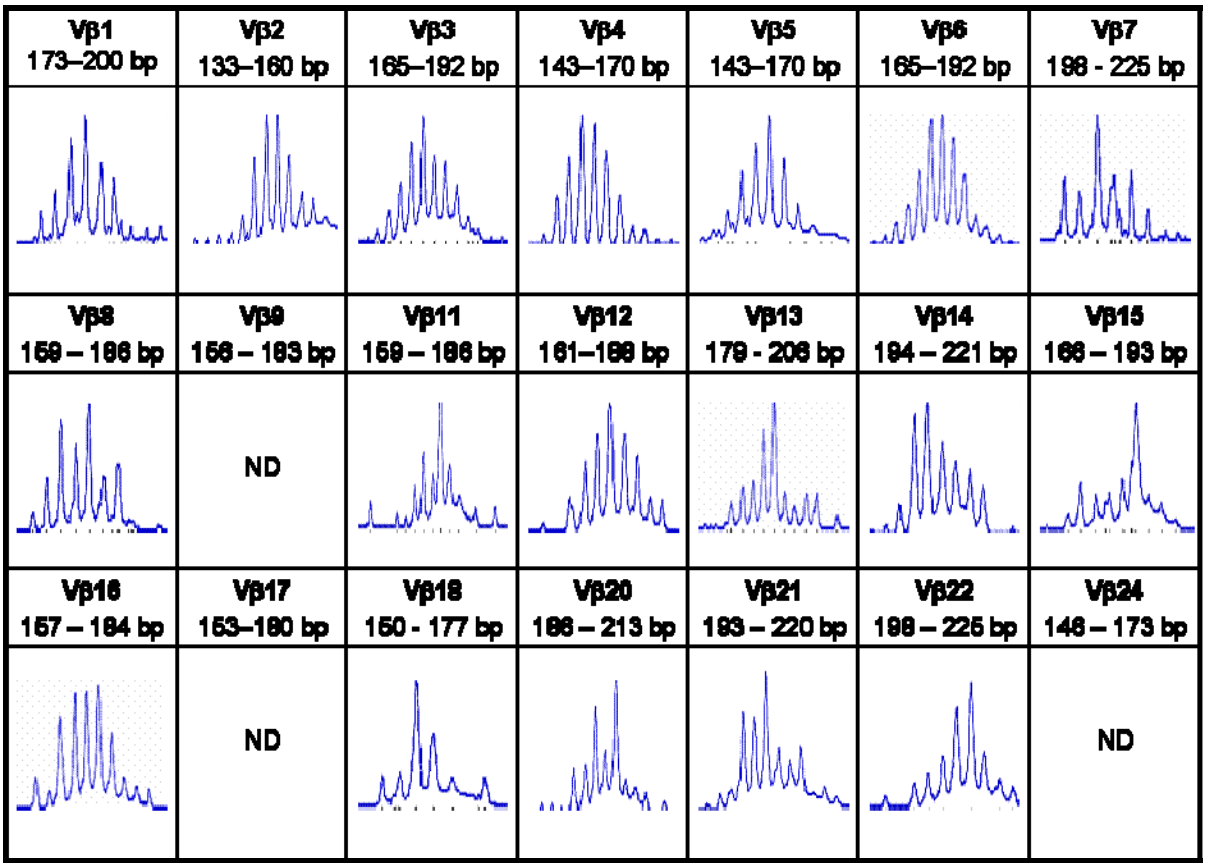


Figure 3-8. $CD8^+$ CD45RA T cells amplification of 18 Vβ families using 1 µL cDNA derived from 0.25 µg RNA. Normal length distribution is observed which is expected since these are naïve cells.

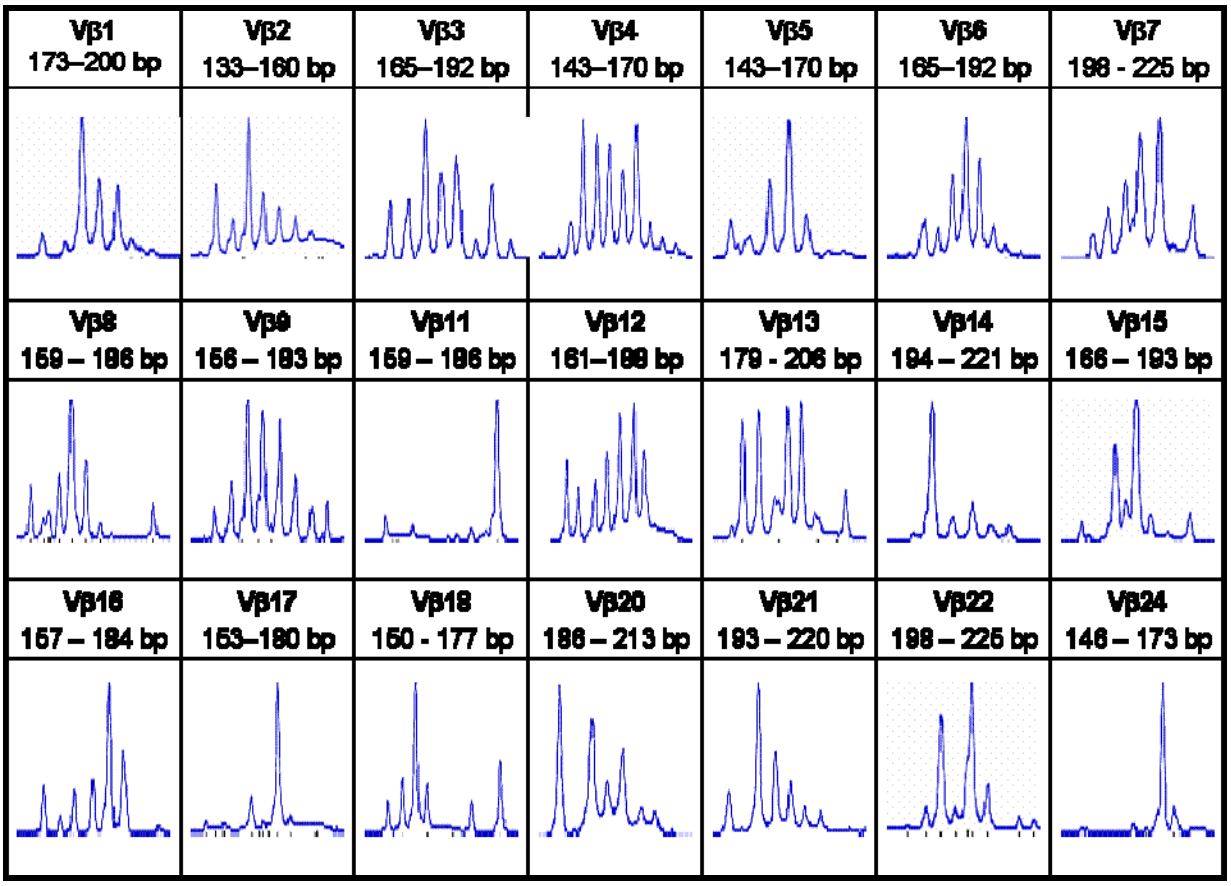


Figure 3-9. $CD8^+$ CD45RO T cells amplification of 21 Vβ families using 1 μL cDNA derived from 0.25 μg RNA. Perturbations are observed which is expected since these are effector and memory cells.

Table 3-2. Summary of optimal conditions for spectratyping

	Cord Blood	CD8 CD45RA	CD8 CD45RO	PBMC
Starting Cell Number	0.8×10^6	$0.3\text{-}0.5 \times 10^6$	$0.3\text{-}0.5 \times 10^6$	$5\text{-}10 \times 10^6$
RNA/RT	0.5 μg	0.25 μg	0.25 μg	N/A
cDNA per Vβ family	1 μL	1 μL	1 μL	N/A

Table 3-3. Concentrations of RNA for influenza vaccinated subject samples

Subject #	Cell Type	Cell Number (x 10^6)	RNA	
			Concentration (μg/μL)	Total amount (μg)
	PBMC	0.5	0.015	0.30
1	$CD4^+$	0.5	0.059	1.18
	$CD4^-$	2.1	0.089	1.78
	PBMC	1.0	0.108	2.16
2	$CD4^+$	0.5	0.039	0.78
	$CD4^-$	1.5	0.050	1.00

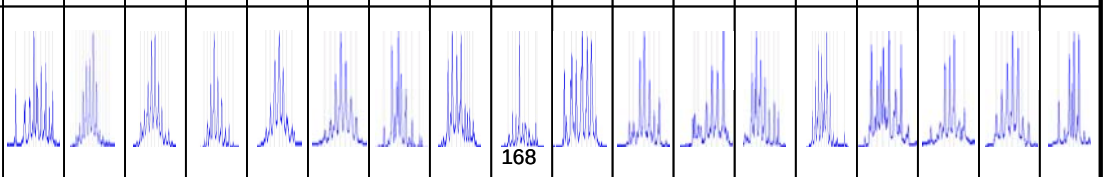
CD4	1	2	3	4	5	6	7	8	9	11	12	13	14	17	20	21	22	24
Histograph																		
Perturbation by statistics	P	N	N	N	P	N	P	P	P	P	P	P	P	P	P	P	N	P

Figure 3-10. Spectratyping results for subject 1. Eighteen Vβ families were studied with thirteen families showing perturbations as shown by the letter P. One family (Vβ9) displayed monoclonal expansion. Three families could not be amplified (Vβ15, 16, and 18) which may be due to primer inefficiency or minor populations.

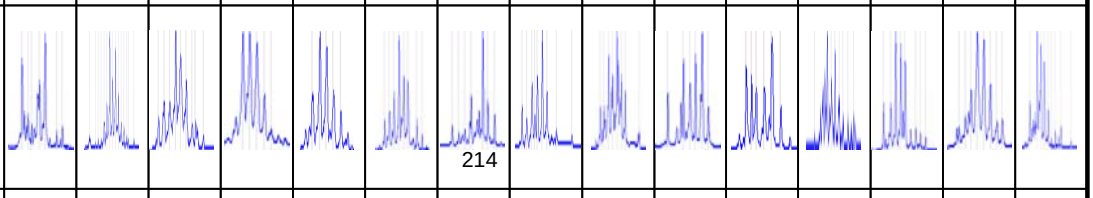
CD4	1	2	3	4	5	6	7	8	9	11	12	13	16	17	22
Histograph															
Perturbation by statistics	P	P	N	P	P	P	P	P	P	P	P	P	P	P	P

Figure 3-11. Spectratyping results for subject 2. Fifteen Vβ families were studied with fourteen showing perturbations denoted by the letter P. One family (Vβ7) displayed monoclonal expansion. Five families could not be amplified (Vβ14, 15, 18, 20, 21, and 24) due to primer efficiency or minor populations.

Vβ	1	2	3	4	5	6	7	8	9	11	12	13	14	15	16	17	18	20	21	22	24
P1	P	N	N	N	P	N	P	P	P	P	P	P	P	---	---	P	---	P	P	N	P
P2	P	P	N	P	P	P	P	P	P	P	P	P	---	---	P	P	---	---	---	P	---

Figure 3-12. Summary of results for both influenza vaccinated subjects. Nine families were perturbed in both patients (shaded in yellow). Only Vβ3 was normal in both patients (shaded in green). Two families (Vβ15 and 18) did not amplify for either patient (shaded in blue).

CHAPTER 4
CONCLUSION

Conclusion for Aim 1: The Representation of TCR Can Be Assessed in Small Numbers of Cells from T Cell Subsets

Through experiments performed in specific aim 1, it was found that 1 μL (or 2.5% of the total amount of a 40 μL reaction) cDNA derived from 0.25 μg of RNA extracted from approximately 0.3 to 0.5 x 10^6 CD45RA or CD45RO $CD8^+$ T cells separated from 5 to 10 x 10^6 PBMC was sufficient to amplify each TCR Vβ family. If using cord blood, then the amount of RNA used for one RT reaction should be scaled up to 0.5 μg (equivalent to approximately 0.8 x 10^6 cord blood PBMC) because the cord blood PBMC is a mixed population of cells and contains only 17% T cells. These conditions help to conserve the subject's sample and can be applied to CD45RA and CD45RO $CD4^+$ T cells.

Conclusion for Aim 2: Perturbations of TCR Can Provide a Surrogate Marker for Immune Response.

In comparing spectratyping results for both influenza vaccinated subjects, it was found that nine Vβ families were perturbed in both. In addition, one family, Vβ9 in subject 1 and Vβ7 in subject 2, showed monoclonal expansion. If no perturbations were observed in the post-vaccination sample, then no pre-vaccination sample would be needed. Since perturbations and at least one monoclonal expansion were observed in each subject, it can be concluded that in order to determine whether or not the expansion is due to influenza vaccination, a pre-vaccination sample is needed. The pilot study conducted here implies that the design of the larger study to include a pre-vaccination time point sample in the analysis was correct.

In regards to determining whether or not TCR Vβ spectratyping can be used as a surrogate marker of T cell activation due to vaccination, according to the literature, examining changes in the TCR Vβ repertoire could be used as a surrogate marker of T cell activation due to

vaccination [49-53]. In fact, several studies have used TCR Vβ usage to check response to a vaccine and to evaluate the ability of a response to a vaccine. While there are conflicting reports regarding the measurement of TCR activation in response to vaccination, the majority of literature seems to indicate that vaccination does indeed cause TCR activation that can be measured. In addition, several studies have shown that examining which Vβ genes are involved in immune response is important since these molecules are expressed on the T cell surface and can be used as targets for immune therapy [49-51, 54, 55]. In a heterogeneous cell population, T cells that recognize the same epitope may have a similar TCR structure that uses the same V segments [72].

In examining the Vβ TCR repertoire in encountering other vaccines, different Vβ families are perturbed according to what vaccine is used. For instance, studies on the tetanus vaccine define perturbations in Vβ2, 4, 6, 13, 14 families of $CD8^+$ T cells 14 days post-vaccination due to response to the tetanus toxoid as opposed to a generalized response to vaccination [51]. However, in examining three studies regarding hepatitis B vaccinations, there is no concurrence between the studies as to which Vβ families are activated [49, 52, 54]. This lack of concurrence may be due to the difference in time points at which the samples were taken (ranging from 48 hours post vaccination to 14 days post vaccination). The differences in TCR Vβ gene usage may also be due to different HLA haplotypes between individuals [56]. Haplotype refers to each individual person having different MHC molecules that present different antigens. If a study examines subjects with a specific haplotype then more families may be shared [37].

In addition, in studies done on measles, it was found that the TCR Vβ families that are perturbed differ between children who are vaccinated with a live attenuated virus as opposed to contracting a natural measles infection [50, 53]. The difference between the vaccine and the

natural infection may be due to the effects of the virus strain or the effect of the age of the children [50]. In applying these results to our study, given the wide variety of strains of the influenza virus, samples taken from naturally infected subjects should be compared with samples taken from vaccinated subjects to examine any differences in TCR Vβ activation.

In contrast to the amount of research done on TCR Vβ activation in the above vaccinations, little research has been done to date examining the TCR activation by the influenza vaccine. One study found that after stimulation with an immunodominant synthetic peptide (matrix protein of influenza A, M55-66), Vβ17 perturbed but it is not known if this same family would be perturbed with exposure to natural infection or vaccination [73]. Another study examining influenza found that vaccination does not substantially perturb $CD4^+$ T cells since no new perturbations were observed after influenza vaccination [74]. This study examined subjects who were over age 70 and vaccinated with the influenza vaccine. It compared pre-vaccination samples to 1 month post vaccination samples using a PCR-heteroduplex method. The same perturbations present pre-vaccination were found post-vaccination. Perturbations were found in different families for CD45RA and CD45RO subsets of $CD4^+$ T cells, but the perturbation was never present in both subsets. The Vβ2 family was found to be perturbed in CD45RA $CD4^+$ T cells and families Vβ1, Vβ5, or Vβ13 were found to be perturbed in CD45RO $CD4^+$ T cells [74]. This lack of perturbations due to the vaccine may be the result of the age of the subjects. It has previously been reported that the TCR Vβ $CD4^+$ T cell subset exhibits perturbations in subjects over the age of 65 while it is normally bell-shaped in healthy adults under age 65. Since the same perturbations were found in both pre and post-vaccination samples, it may be that these subjects were exposed to the influenza antigen previously and the expansions could represent

dominant usage of those particular families. Alternatively, the subjects may have been exposed to some other antigen previously and the perturbed Vβ families persisted and were carried over.

In examining the literature, there seem to be differences between the expansions of $CD4^+$ and $CD8^+$ T cells in healthy persons. There is abundant literature observing that there is little to no expansion in the $CD4^+$ T cell subset in healthy persons [56, 61, 62, 75]. Age, however, seems to increase the incidence of perturbation within the $CD4^+$ T cells of healthy subjects. It was found that 70% of healthy aged subjects (aged 65 to 85 years with a mean age of 76) had perturbed Vβ families in $CD4^+$ cells. The perturbed Vβ families varied from individual to individual and were stable over an 18 to 24 month period. Since these perturbations were persistent they did not represent a transient response to antigen. Among aged donors, perturbations were seen in $CD4^+$ T cells for families Vβ1, 3, 8, 9, 11, 14, 16, 17, 20, 21, and 23 while in adult donors (aged 22 to 34 years with a mean age of 26) dominant peaks were seen only in Vβ 14 and 23. The T cell expansions in $CD4^+$ T cells of aged persons may occur from repeated infections from viruses such as influenza or EBV. The expansions may represent dominant usage of Vβ families in response to antigen [75]. Differences in TCR V gene usage from person to person may be due to different antigen exposure or different HLA haplotypes between individuals [56].

In healthy subjects, $CD8^+$ T cells display more perturbations than $CD4^+$ T cells. "Skewing" refers to the observation that some Vβ genes react more favorably to $CD4^+$ T cells than to $CD8^+$ T cells. Skewing is thus representative of a particular V region gene product interacting more favorably with MHC class II molecules for the $CD4^+$ subset or MHC class I molecules in the $CD8^+$ subset during positive selection in thymic maturation [76]. The majority of the $CD4^+$ T cells gave Gaussian "bell-shaped" curves while there was a higher frequency of perturbations per

individual in the $CD8^+$ subset [65]. One study examining healthy subjects found that $CD4^+$ T cells were skewed in five Vβ families, including Vβ 2, 5, 13, 17, 22 [65] while another study found that in $CD4^+$ cells Vβ3, 9, 12, and 18 were skewed [76]. Still another study found that Vβ2, 5, 6, 9, and 22 were skewed towards $CD4^+$ T cells [77]. No single Vβ family was shown to be used in all three studies, however Vβ2, 5, and 9 are seen in two of the studies. This skewed usage may be the result of a selection process in the thymus that gives preferential selection of some V genes to $CD4^+$ over $CD8^+$ [65] and seems to indicate that the Vβ genes are not randomly used within the $CD4^+$ and $CD8^+$ T cell subsets [76].

In examining the present pilot study, nine Vβ families were perturbed in both subjects. These families included Vβ1, 5, 7, 8, 9, 11, 12, 13, and 17, with Vβ families Vβ7 and Vβ9 exhibiting monoclonal expansion. The perturbations seen in our subjects are not the result of insufficient template since the fluorescent intensity is higher than 400 and there is no evidence of distortions at the baseline. Given that $CD4^+$ T cells were studied and our review of the literature, we would expect not to observe any perturbations in the healthy subjects that were not due to an encounter with an antigen. However, in comparing the spectratyping results for both subjects to the literature regarding perturbations in the $CD4^+$ T cell subset of healthy individuals within the under age 65 age group, it was found that Vβ5, 8, 9, 12, 13, and 17 were perturbed both in our study and in at least one study in the literature (Figure 4-1). In accordance with the literature, the perturbations of the Vβ families in our two vaccinated healthy subjects may be carryover from previous antigen recognition or may be due to a reaction from the vaccine as opposed to carry over from previous antigen recognition. Further studies would need to be done to determine if these perturbations can be observed within a larger healthy population of subjects or are due to vaccine response.

Also, in comparing the present study to the majority of other studies and given that our subjects were in the 30 to 40 year age group, it would be expected that there would be no perturbations in the $CD4^+$ subset since perturbations normally increase in frequency with age. However, we observed a monoclonal expansion of Vβ9 in subject 1 and Vβ7 in subject 2. Monoclonal expansions have been observed Vβ14 and Vβ23 in one study [75] and Vβ12 in another study [77]. Given that monoclonal expansions in the Vβ families of the $CD4^+$ T cells are not normally present in the 30 to 40 year old age group, we believe that it is possible that this monoclonal expansion in each subject may be the result of the influenza vaccination, however without a pre-vaccination sample we are unable to absolutely conclude that the expansion is due to vaccination.

In conclusion, according to the cited literature, very few, if any, expansions are normally observed within the $CD4^+$ T cell subset of healthy adults below the age of 65. Some studies have observed perturbations in the $CD4^+$ subset of which perturbations in six Vβ families coincide with the results of our pilot study. These include Vβ5, 8, 9, 12, 13, and 17 [65, 76, 77]. Further studies should be done to pinpoint whether these perturbations are the result of previous exposure to antigen or if they correspond to exposure to the influenza vaccine.

In our results, Vβ15 and 18 did not amplify in either subject. This may be the result of these two families being underrepresented in the TCR repertoire due to being a minor population. There is little information in the literature regarding what Vβ families are minor populations in the $CD4^+$ T cell subset.

Further studies should be done which examine the $CD8^+$ T cell subset for expansions or clonalities due to the influenza vaccine since more expansions are normally observed in the $CD8^+$ subset than in the $CD4^+$ subset. In addition, a pre-vaccination sample should be taken

along with a sample at vaccination, 2 weeks post-vaccination, 1 month post-vaccination, 3 months post-vaccination, and 6 months post-vaccination. The pre-vaccination sample gives a baseline for the individual subject to which the other samples can be compared. The 2 week and 1 month post-vaccination samples will help to provide an idea as to the timing of the peak response to the influenza vaccine. The 3 month post-vaccination sample will examine if the TCR repertoire has returned to the baseline. The 6 month post-vaccination sample will help to examine the length of the immune response. Determining what TCR Vβ families are utilized during a response to influenza antigen has great implications on the development of an improved vaccine. By knowing which specific Vβ families are perturbed, a vaccine could be developed that targets those specific Vβ families thus ameliorating the risk of influenza infection by a different strain of the virus in those who were vaccinated. If each different strain of the virus activates the same Vβ families, production of a vaccine that targets those specific activated Vβ families may eliminate the need for changing the influenza strains in the vaccinations every year.

Vβ	1	2	3	4	5	6	7	8	9	11	12	13	14	15	16	17	18	20	21	22	24
P1	P	N	N	N	P	N	P	P	P	P	P	P	P	---	---	P	---	P	P	N	P
P2	P	P	N	P	P	P	P	P	P	P	P	P	---	---	P	P	---	---	---	P	---
Lit		P	P		P	P		P	P		P	P				P	P			P	

Figure 4-1. Summary of results as compared to previously published studies on healthy non-vaccinated adult subjects. Six Vβ families were perturbed in both of our subjects as well as in at least one out of three studies in the literature (shaded in red).

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BIOGRAPHICAL SKETCH

Michele Lawson was born in 1972 in Wheeling, West Virginia. She graduated from Bridgeport High School in Bridgeport, Ohio, in 1991 and headed off to Otterbein College in Westerville, Ohio. She graduated from Otterbein with a Bachelor of Science in life science and psychology in 1995 and moved to Akron, Ohio to attend law school. She graduated from the University of Akron School of Law in 1998 with a Juris Doctor and was admitted to practice in Ohio. In 2001, she passed the patent bar exam and was licensed as a patent attorney by the United States Patent and Trademark Office. After practicing in a variety of areas of the law for 7 years, she entered the joint degree Master of Science/Master of Business Administration (MS/MBA) Program at the University of Florida in 2005.