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Development of improved T cell receptor beta variable gene identification technology and its application post hematopoietic stem cell transplantation

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Development of improved T cell receptor beta variable gene identification technology and its application post hematopoietic stem cell transplantation.

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Doctor of Philosophy

In

Microbiology, Immunology, and Cell Biology

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ABSTRACT

Much has yet to be understood about the role of specific families of T lymphocytes in the post human hematopoietic stem cell (HSC) transplant environment. Prior work in the field has identified T cells based upon the expression of their T cell receptor beta variable regions (TCRBV). In this investigation we developed a comprehensive panel of oligonucleotides that can be used to determine the expression of all 91 alleles of the human TCRBV regions using real time PCR technology. Application of this technology to peripheral blood samples collected weekly from allogeneic peripheral blood stem cell transplant patients yielded the following findings: (1) specific TCRBV families are associated with the reactivation of cytomegalovirus (CMV) post HSC transplant with many of these same TCRBV families also being associated with the occurrence of GVHD, (2) the TCRBV repertoire engrafts in the recipient with a profile more similar to that found in the donor as opposed to that found in the recipient prior to transplant, and (3) the similar immunosuppressive agents, cyclosporin A (CSA) and tacrolimus (FK506), differentially alter the TCRBV repertoire with their administration, a difference which can not be attributed to a divergent inhibition of calcineurin or IL-2 production by CSA or FK506.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

CHAPTER 1

REVIEW OF LITERATURE

I. The History of Hematopoietic Stem Cell Transplantation

Many advancements in the field of hematopoietic stem cell transplantation were borne from the seemingly unrelated birth of nuclear technologies. With the development of atomic technologies and the potential of nuclear warfare, the effects of irradiation on biological systems became an area of imminent interest [1]. This resulted in a series of experiments conducted over the past fifty years and has greatly increased our understanding of hematopoietic stem cell transplant and the immunological mechanisms that are so critical to its success.

The modern epoch of bone marrow transplantation was pioneered by Lorenz in 1951 when he demonstrated that mice could be protected against lethal irradiation by intravenous infusion of bone marrow [2]. This discovery quickly brought about the notion that hematological malignancies could potentially be cured through irradiation and subsequent marrow grafting. Barnes, et al., showed that murine leukemia could be successfully treated using sub-lethal doses of irradiation followed by the infusion of healthy marrow [3]. The following year, the first reports detailing similar clinical trials in humans were made, describing the immediate rescuing from radiation-induced pancytopenia in cancer patients by marrow infusion [4,5]. In 1959, Mathe et al., attempted bone marrow transplantation in humans on a larger scale when they tried to rescue six victims of a radiation accident in Vinca, Yugoslavia, by transplanting allogeneic bone marrow [6]. This group continued trying to use bone marrow transplantation as treatment for hematologic malignant conditions, however, it was not until 1963 that they reported the first case of a patient surviving beyond a year. While this patient remained leukemia-free, severe graft-versus-host disease (GVHD) reactions were documented, the first of such, and the patient succumbed to infection 20 months post-transplant [7].

II. Types of Hematopoietic Stem Cell Transplantation

There are three primary types of hematopoietic stem cell transplants depending upon the stem cell source and the identity of the donor. These are termed autologous, allogeneic, and syngeneic transplants. The first type, autologous transplant, describes the process where an individual serves as his/her own stem cell source. In order for an autologous stem cell transplant to be successful, the individual's marrow must be virtually disease free prior to harvesting. An example of the use of this type of transplant

is one in which a patient must undergo intensive treatment directed at his/her tumor, which, as a consequence, would inadvertently cause destruction to his/her hematopoietic system. After the high-dose tumor therapy, the patient can then be infused with the previously collected stem cells to rescue his/her hematopoietic system. Autologous transplants are now most frequently used for hematologic malignancies such as lymphomas. Advantages to autologous transplantations are that there is no need to locate a suitable stem cell donor as well as a decreased risk of treatment-related mortality. Such transplants, however, have a risk of tumor cell contamination in the graft. Additionally, autologous transplants cannot be used in the treatment of inherited non-malignant hematopoietic diseases or acquired marrow failure states, such as aplastic anemia [8].

The second major type of hematopoietic stem cell transplant, termed an allogeneic transplant, is one in which the stem cell donor is separate from the recipient. The donor must be genetically matched to the recipient but the two can either be related or unrelated. Advantages of this type of transplant are that there is a low risk of the graft being contaminated with malignant cells and it can successfully be used in treating both malignant and non-malignant diseases of the hematopoietic system. An additional advantage to allogeneic transplant is the possible destruction of residual recipient tumor cells by the infused donor-derived cells, an occurrence termed graft-versus-tumor effect. Despite these advantages, however, there is often great difficulty in finding an appropriate donor and there is a greater increase, compared to autologous transplants, of post-transplant complications, such as graft-versus-host disease [8].

The third major type of hematopoietic stem cell transplant is one in which the donor and recipient are genetically identical, such as the case that occurs when the recipient and donor are identical twins, and is termed a syngeneic transplant. Post transplant complications tend to be diminished in this setting, compared to a non-identical allogeneic transplant, but while the majority of patients may have a sibling, it is relatively uncommon for the sibling to be an identical twin [8].

Traditionally, hematopoietic stem cells (HSC) have been harvested from bone marrow through extraction at the iliac crests. Recent advancements in transplantation technology, though, have permitted the mobilization of hematopoietic stem cells from

the bone marrow environment to the peripheral circulation through the use of growth factors such as granulocyte colony stimulating factor (G-CSF) [9-13]. HSC comprise only a small fraction of the total number of cells in the bone marrow, with a rate of approximately one HSC/10,000 total bone marrow cells and the number of HSC circulating in the peripheral blood is an exceedingly rare event [14]. After G-CSF administration, however, the frequency of HSC in the peripheral blood is increased two to five-fold greater than that found in the bone marrow [15]. Mobilized HSC can then be harvested from the peripheral blood by a pheresis procedure. A dose of at least five million HSC/kg recipient weight has been reported to be the ideal dose to achieve engraftment [16].

While multiple collections may be required in order to achieve the necessary number of stem cells for transplant using the G-CSF mobilization technique, there is a low toxicity profile associated with G-CSF administration in humans [10-13]. The side effects are generally limited to bone pain and general influenza-like symptoms and there have been no documented reports of termination of G-CSF administration to healthy donors due to its side effects. Thrombocytopenia, or the reduction in platelet number, is frequently observed post-pheresis in G-CSF mobilized donors. This reduction, as well as any bone discomfort, is generally reversed within 48 hours of cessation of drug administration. On the other hand, while bone marrow harvests require no mobilization therapies and can often be done in a single one-day collection, the pain associated with bone marrow harvests may take 2 to 4 weeks to subside [17,18]. In addition, various studies have reported a decreased time for reconstitution of the immune system, less transplant-related toxicities and mortalities, and a decreased rate of GVHD occurrence in peripheral blood stem cell recipients compared to bone marrow recipients [19-23].

The benefits of peripheral blood stem cell harvesting, compared to bone marrow harvesting, have resulted in peripheral blood stem cell transplantation becoming much more frequently used compared to bone marrow transplantation. HSC have also been collected from umbilical cord blood and used successfully in allogeneic transplantation. While cord blood HSC have decreased alloreactive potential compared to their counterparts isolated from more mature donors, cord blood stem cells exhibit a delayed

reconstitution of the hematopoietic system, thereby limiting their usage in hematopoietic stem cell transplantation [24-30].

In allogeneic transplants the conditioning regimen the recipient receives prior to transplant is highly dependent upon the type of disease the patient has as well as how aggressively it behaves [31]. There are three main objectives to the treatment. The primary objective is to eradicate the underlying hematological disease in the recipient. The second objective is to suppress the recipient's immune system to decrease the risk of the recipient rejecting the donor's stem cells. The third objective in using a conditioning regimen pre-transplant is to create space in which the donor's stem cells can engraft and growth can be accommodated [32]. While the amount of time required for immune reconstitution post hematopoietic stem cell transplant varies depending upon parameters such as the type of transplant, conditioning regimens, and immunosuppressive therapies, CD8^{+} T cell counts tend to recover to normal values within the first month post transplant with a prolonged deficiency of CD4^{+} T cell counts often seen six to twelve months post transplant, leading to the characteristic inversion of the CD4:CD8 ratio observed post transplant [33-37].

III. HLA

Based on experiments initially performed in mice [38] antigenic principles underlying cellular transplantation began to be established. Such experiments led to the recognition of what became termed the H2 transplantation antigen system [39] in mice and the HLA system in humans [40]. The human leukocyte antigen (HLA), also termed the major histocompatibility complex (MHC) forms a complex which is expressed on the cellular surface. The principle function of this molecule is to present peptides (from either self or non-self origin) to T lymphocytes, a vital component to the immune system. By doing so, the T lymphocytes are able to distinguish self versus non-self. An illustration of this principle is afforded by examining what occurs during a viral infection: if a cell is infected with a virus, pieces of viral proteins (non-self) are loaded into the MHC molecule and presented on the surface of the infected cell. The interaction of a circulating T cell with this (non-self) MHC molecule can activate the T cell, causing it to destroy the infected cell, thereby limiting the spread of infection. The same is true in the setting of transplantation. T cells in the donor graft can recognize cells within the

recipient's body as non-self, eliciting devastating immune reactions. This is the premise of a serious post-transplant complication called graft-versus-host disease (GVHD) [41].

There are three classes of HLA (or MHC) genes, termed class I, class II, and class III genes, with all three being located on chromosome 6 in humans. The names HLA-A, HLA-B, and HLA-C are given to the human MHC class I genes while the MHC class II genes are identified as HLA-DR, HLA-DP, and HLA-DQ. The products of these MHC class I genes are expressed on the surface of nearly all cells within the human body while the expression of the MHC class II genes is restricted to antigen presenting cells and thymic epithelium. Classically, MHC class I proteins present endogenous antigen (or peptide) to CD8$^+$ T cells while the MHC class II proteins present exogenous antigen (or peptide) to CD4$^+$ T cells. The final class of genes, those located within the MHC class III region, encode various immunologically relevant proteins such as complement and the tumor necrosis factor cytokines [42].
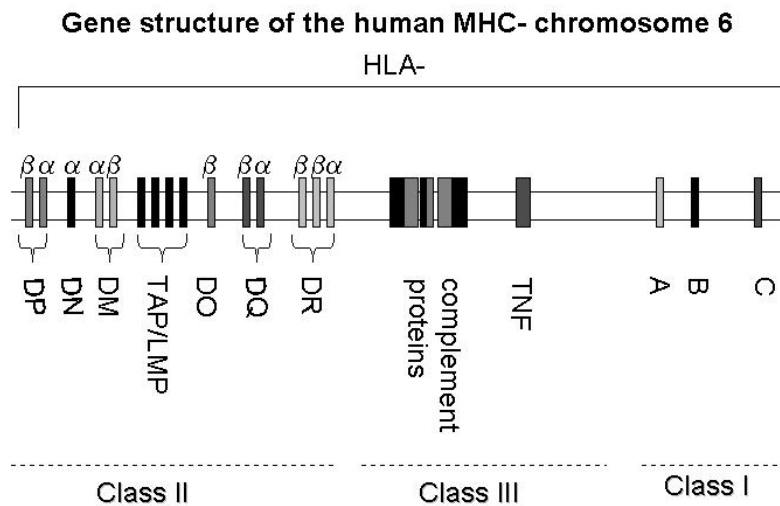
Figure 1: *Gene structure of the human major histocompatibiltiy complex (MHC)*

The HLA genes are expressed in a co-dominant fashion and are highly polymorphic [43]. In terms of their importance in hematopoietic stem cell transplantation, matching at the MHC class I HLA-A and HLA-B loci was given priority as previously there had been a lack of reagents available to accurately determine the HLA-C

genotype. Due to the advancements of molecular biology, however, such discrimination is now possible. In fact, recent reports have been made concerning the importance of HLA-C matching in hematopoietic stem cell transplantation [44-46]. As it relates to the MHC class II molecules, allelic mismatching of the donor and recipient at the HLA-DR loci was the first to be shown to be associated with an increase in graft-versus-host disease reaction [47]. The importance of HLA-DQ matching is also becoming more apparent [48]. However, like its MHC class I counterpart (HLA-C), mismatching at the HLA-DQ locus has yet to become a basis for donor exclusion. The role of HLA-DP in transplantation has remained rather controversial and is also not typically evaluated when searching for an appropriate donor [49]. In summary, the common practice of HLA matching in related donor and recipient has remained matching of the HLA-A, HLA-B, and HLA-DR loci, although typing of additional loci is commonly performed when an unrelated donor is being considered. Likewise, HLA matching is typically performed using serological methods when the donor is related and by molecular analyses when the donor and the recipient are not related.

IV. T Cells

On the converse side of the MHC molecule is the T cell, whose interaction with the MHC molecule is mediated through the T cell receptor (TCR). T lymphocytes originate in the bone marrow from a lymphoid progenitor cell and migrate to the thymus as immature cells. The thymus provides a unique microenvironment in which the T lymphocyte rearranges its receptor genes (chromosome 14, TCR alpha genes and chromosome 7, TCR beta
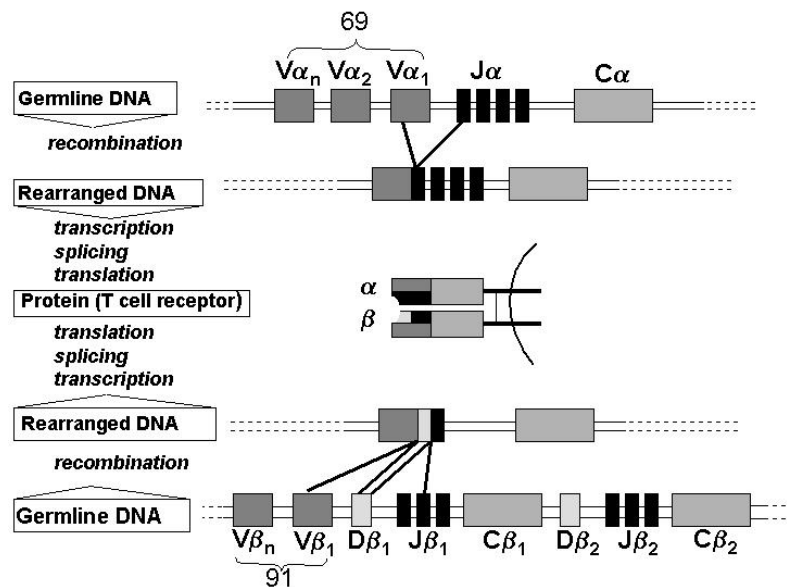
Figure 2: T cell receptor (TCR) gene rearrangement

genes [50]) and matures. T cell receptor (TCR) gene rearrangement occurs in the sub capsular region of the thymus with rearrangement of the heavy chain (or beta chain) genes occurring prior to rearrangement of the light chain (or alpha chain) genes. Within the T cell receptor beta chain locus, a diversity (D) segment is randomly paired with a joining (J) segment. This DJ unit is then rearranged with a variable (V) segment to form a VDJ segment. The constant (C) region exon is then spliced to the VDJ segment to generate the message, which is translated into the T cell receptor beta chain protein. Beta chain gene rearrangement is followed by rearrangement of the TCR alpha chain, which occurs in the same manner as for the heavy chain. However, as there is no diversity region in the light chain, rearrangement of the variable and joining segments is followed by the VJ pairing with a constant (C) segment [50-52].

After the heavy and light chain rearrangements occur, the T cell begins to undergo maturation and moves deeper into the thymus. In the cortex of the thymus the immature cells undergo positive selection where only those developing cells that recognize antigens presented by self-MHC molecules can mature. Those T cells that do not recognize antigen in the context of a self-MHC molecule are deleted by apoptosis. As the positively selected thymocytes move into the cortico-medullary junction of the thymus they undergo negative selection where those that recognize self-antigens too well are deleted. Negative selection helps to remove auto-reactive T cells from the repertoire. Finally, those thymocytes that have survived both positive and negative selection exit through the medulla of the thymus and enter the peripheral circulation where they traffic through secondary lymphoid organs monitoring the body through T cell receptor:peptide:MHC interactions [50-52]. The significance of the wide variety of MHC molecules (more than fifty alleles at each of the HLA-A, HLA-B, and HLA-DR loci identified [53]) and possible TCR rearrangements (24 million [54]) is critical to the ability of an individual's immune system to respond to a wide variety of pathogens.

With an understanding of the molecular interaction between the MHC molecule and the TCR, the importance of T cells in the post hematopoietic stem cell transplant setting becomes clearer. Investigations into these reactions and their role in graft rejection and graft-versus-host disease were made as early as 1963 [7,55].

V. GVHD

Hematopoietic stem cell transplant offers patients a potential for recovery from otherwise lethal conditions. However, associated with this possibility is the threat of graft-versus-host disease (GVHD), a reaction where the transplant recipient's tissue is attacked and destroyed by the infused donor cells. GVHD-like reactions have been observed in mice that had been treated with allogeneic marrow after irradiation. While these mice recovered from their marrow ablation, the mice died from what was termed "secondary disease", now known as graft-versus-host disease [41], a condition marked by weight loss, diarrhea, and liver and skin changes [56,57].

In 1957, such observations in mice led Billingham to establish criteria essential for the development of graft-versus-host disease. The first of these criteria is that the graft must contain immunologically competent cells. Secondly, the recipient of this graft must not be able to mount an immune response against these transplanted cells, which would lead to their ultimate destruction. Finally, the recipient must also express antigens that are not present in the donor graft [58].

Billingham's first requirement was further understood when in 1962, Gowen identified the cells responsible for GVHD as small lymphocytes [59], but it was not until 25 years after these first observations that the "immunologically competent cells" were actually identified as T lymphocytes [60]. In 1986 Kernan demonstrated a direct correlation between the severity of GVHD and the number of donor T cells transfused [67]. Attempts have been made to T-cell deplete grafts (*ex vivo*) prior to transplant which decreases the risk of GVHD, and to then use "add-backs" of donor lymphocytes post transplant in order to promote the graft-versus-tumor effect [61-79]. The complete removal of T cells from the graft, however, has been shown to cause an increase in graft failure and is, therefore, not a practical option [80,81].

Billingham's second requirement of the recipient being devoid of immuno-competent cells is typically not of great concern in hematopoietic stem cell transplantation as recipients classically receive intense immunosuppressive treatments in order to prevent the recipient-mediated rejection of the donor cells [82-84].

With the discovery of the MHC genes and the role that they play in transplantation, an increased understanding of Billingham's third requirement was achieved. As previously discussed, these MHC molecules are necessary for activation of T cells, in both autologous and allogeneic transplant settings [85]. In allogeneic transplants the matching of the donor and recipient's HLA antigens is one of the foremost criteria in selecting an appropriate hematopoietic stem cell donor. It has been observed, though, that even with matching of these antigens, GVHD reactions can still occur [86] and have been attributed to what have been termed minor histocompatibility antigens.

Minor histocompatibility antigens, or mHA, have been defined as a non-MHC locus that can elicit allogeneic tissue rejection [87,88]. Minor histocompatibility antigens are inherited and are not necessarily in close proximity to the HLA genes [89]. It has been shown that certain MHC present specific mHA [90,91] and specific mHA elicit responses from specific TCRBV [92]. There have been seventeen minor histocompatibility antigens identified thus far, with the expression of twelve of these being restricted to cells of the hematopoietic lineage [91,93,94]. At the present time, while it is known that mHA differences can contribute to GVHD, it is difficult to predict the outcome of a hematopoietic stem cell transplant based upon identification of these minor histocompatibility antigens [95,96].

One model describes the development of GVHD as occurring in two phases: the afferent and efferent phases. The afferent phase consists of the damage incurred to the recipient both by the pre-transplant conditioning regimens (chemotherapy/radiation) as well as by the activation of the donor T cells. It has been shown that the conditioning regimens cause damage to and activate tissues within the recipient, including the tissue of the gastrointestinal tract as well as the liver [97-100,]. Such damage results in the release of LPS and inflammatory cytokines, such as IL-1, tumor necrosis factor (TNF) alpha, and interferon (IFN) gamma, from the activated tissues which causes the subsequent activation of host antigen presenting cells [23,101-112]. Further complicating these circumstances is the fact that LPS and IFN gamma can act in a synergistic fashion, additionally increasing the levels of pro-inflammatory cytokines such as TNF alpha [113]. In addition it has been shown that the administration of antagonists to LPS, as well as to the receptors for these inflammatory cytokines, has ameliorated the effects of GVHD

[103,105,110,114]. The important role of LPS in GVHD is also supported by the observation that pathogen-free mice have reduced incidence of GVHD after allogeneic transplant [115,116].

The efferent stage of GVHD is comprised of the actual destruction of the recipient tissues by activated T lymphocytes responding to allogeneic antigens [117]. A combination of the afferent and efferent stages contribute to the devastation seen in GVHD.

GVHD can present itself in two forms, acute and chronic. Acute GVHD presents within the first 100 days post-transplant whereas the chronic form appears post day 100. The pathology of the two forms show similarities, however [41].

There are four grades of acute GVHD: I-IV, with IV being the most severe. While the development of GVHD grade I or II is associated with an increased risk of developing GVHD grade III or IV, little morbidity is associated with GVHD grade I. However, much greater levels of morbidity are seen with the progression of GVHD to grades II and III, and grade IV GVHD represents a life threatening condition [41].

In acute GVHD the primary target organs are the skin, gastrointestinal tract, and liver, with the skin being the most easily observed and often first diagnosed target organ. In the skin, GVHD presents as erythema and rash, commonly located on the palms and soles initially, but can spread to involve the rest of the body. In severe situations, the skin may actually become desquamated (grade IV). In terms of its effect on the gastrointestinal tract and liver functioning, symptoms include nausea, vomiting, diarrhea and hyperbilirubinemia. In each instance, with increased severity of these symptoms or worsening laboratory findings, the higher the grade of GVHD [41].

VI. Immune Suppression
Allogeneic hematopoietic stem cell transplant recipients are routinely given immunosuppressive agents to facilitate engraftment and decrease the risk of developing GVHD post transplant. Cyclosporin A (CSA), tacrolimus (FK506), and methotrexate, are examples of such agents. CSA and FK506 have similar mechanisms of action, which is to bind to cyclophilin or FK binding protein (FKBP), respectively; the resulting drug-protein complexes inhibit calcineurin activity [118-128]. The protein calcineurin is directly involved in the transcription of cytokines, such as IL-2. Through the administration of

CSA or FK506 it is possible to inhibit expansion of allo-reactive T cells and their reactions post transplant. CSA and FK506 treatment is begun one day before transplant (d-1) and continues throughout the post transplant period.

The mechanism of methotrexate, pulses of which the patients receive as part of standard GVHD prophylaxis, is through the inhibition of dihydrofolate reductase, the enzyme responsible for purine and pyrimidine synthesis, which leads to the suppression of T cell activation and adhesion molecule expression [129]. Methotrexate infusions are administered on days 1, 3, 6, and 11 post-transplant as standard GVHD prophylaxis.

While immunosuppressive agents such as these help to prevent graft rejection and GVHD, such suppression makes it difficult for the patient to mount immune responses against invading pathogens and also makes the patient more susceptible to the reactivation of latent infections, such as cytomegalovirus.

VII. Infection Susceptibility

After hematopoietic stem cell transplant patients are highly susceptible to bacterial, viral, and fungal infections. To decrease the risk of overwhelming infections post-transplant, patients receive prophylactic antibiotics. Use of prophylactic anti-fungal agents, such as fluconozole, decreases the occurrence of fungal infections, but in spite of the use of prophylactic antifungal therapy, patients can develop fungal infections including those attributed to *Candida* and *Aspergillus* species [130]. Examples of bacterial infections that are commonly observed post transplant include both gram positive (*Staphyloccocus*, *Enterococcus*, *Clostridium*, and *Corynebacterium*) and gram negative bacteria (*Haemophilus* and *Escherichia*). As previously mentioned, reactivation of latent cytomegalovirus (CMV) is a common occurrence post hematopoietic stem cell transplant [130].

VIII. Identification of T Cells Involved in GVHD Through TCRBV Analysis

In graft-versus-host disease (GVHD), the donor T cells recognize the host (recipient) MHC molecule as foreign, causing destruction of the cells expressing the allo-reactive molecule. Such reactions are also important in the recipient's defense against reactivation of latent infections such as CMV. Since it is the variable region of the TCR that is in intimate contact with the MHC molecule, determining what specific

variable families of T cells can be linked to the destructive effects of GVHD has been a focus of investigation [131-143]. Due to the prior lack of standardized nomenclature for TCR gene families, classification of the variable region data has become complicated because different investigators named the same family different names and, conversely, different families were named the same. To resolve this classification dilema an international team was formed to assign consistent and systematic names to all of the TCR variable gene segments. Bernhard Arden published the results in 1995 [144]. At present, there are 32 functional alpha TCR variable families in humans with 69 subfamily members and 25 functional beta TCR variable families in humans with 91 subfamily members. As antibodies recognizing all of these TCR variable families are not available, an alternative approach is to use RT-PCR to evaluate the expression of the messages (mRNA) used to make these surface proteins. Primer panels previously designed to detect the wide range of TCR variable families do not, however, detect all of the TCR variable families outlined in the Arden paper [138,145-189], due to the unavailability of the classification system at the time of primer development or the labor-intensive task of developing and validating a truly complete primer panel.

IX. Summary

Since its early inception, bone marrow transplantation has been used to treat conditions ranging from exposure to a radiation accident in the late 1950s to treating conditions such as leukemia and lymphoma, aplastic anemia, as well as immune-deficiency disorders such as congenital neutropenia in the twenty first century [6].

Despite advancements in the field of hematopoietic stem cell transplantation, serious complications can still occur post transplant. These include the occurrence of GVHD, severely immune-suppressed states that can lead to reactivation of latent infections such as CMV, and therapeutic agent toxicities [13,130,190].

If it is possible to determine prior to transplant the GVHD-causing T cells, those cells can be depleted or inhibited prior to blood or marrow infusion, thereby preventing the development of GVHD. Experiments performed in mice have demonstrated that when TCR variable families have been identified as being implicated in GVHD, those cells can be depleted prior to transplant, preventing development of a graft-versus-host reaction [191]. In addition, those cells can be adoptively transferred into a lethally

irradiated host and initiate a graft-versus-host disease reaction [192]. Should trials in humans yield such predictable results, blood and marrow transplantation could become a treatment modality with a much-decreased risk of morbidity and mortality and offer hope of a cure to more patients.

X. Research Objectives

The main objective of the work presented in this dissertation is to understand the role of specific groups of T cells after hematopoietic stem cell transplantation in humans. The importance of T cells post hematopoietic stem cell transplantation has long been recognized. There is a significant lack of understanding, though, as to the role specific T cells play in this setting. This project will determine the role of specific T cells, as identified by expression of their T cell receptor beta variable region, in certain post hematopoietic stem cell transplant events. This dissertation will detail the following objectives:

1. Develop a methodology to accurately detect expression of all the human TCRBV families. Many previous investigations have detailed molecular based methods used to identify the various TCRBV families in humans. These reports fail to account for the standardized classification of the TCRBV genes by the World Health Organization and often leave specific subfamilies and alleles undetected. Serological methods, such as antibody staining, have also been employed by prior studies. This method, too, lacks the ability to detect all of the human TCRBV genes. We aimed to develop a modern molecular based method that can specifically detect all alleles of the human TCRBV repertoire.

2. Utilizing technology developed in objective 1, determine the association of specific T cell with the following post hematopoietic stem cell transplant related events:

a. Graft-Versus-Host Disease (GVHD)

GVHD is a common post hematopoietic stem cell transplantation complication and we aim to determine if specific families of T cells are associated with the occurrence of this condition.

b. Cytomegalovirus (CMV) reactivation

During the immune suppressed state post transplant, patients often experience reactivation of latent infections, such as CMV, which contribute additional complications to the recovery stage. GVHD and CMV have been linked to each other in previous reports. We aim to determine (1) what specific families of T cells are associated with reactivation of CMV post transplant and (2) if there is any overlap in the T cells associated with both GVHD and CMV.

c. Immunosuppressive therapies Cyclosporin A (CSA) and tacrolimus (FK506)

Immunosuppressive therapies, such as CSA and FK506, are administered post hematopoietic stem cell transplant to curtail the development of graft rejection and graft-versus-host disease. These agents target activated T cells through their suppression of IL-2 production. We aim to determine if the effects of these agents on the TCRBV repertoire is the same with both CSA and FK506.

d. Correlation of engraftment profiles to baseline donor and recipient samples

It is not known whether the identity of the engrafting TCRBV repertoire is more like that found in the recipient prior to transplant or if it mimics that found in the donor. We aim to determine the answer to this question.

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CHAPTER 2

AN IMPROVED METHODOLOGY TO DETERMINE HUMAN TCRBV GENE EXPRESSION

ABSTRACT

Comprehensive gene expression analysis of the T cell receptor repertoire of an individual can be very useful in evaluating the immune response in a variety of conditions. Antibody based analysis methods can detect approximately sixty percent of the human T cell receptor beta variable (TCRBV) proteins, while gene expression analysis, primarily through employment of the polymerase chain reaction (PCR), has had somewhat greater success in the detection of additional TCRBV families. Many of these previous PCR methods, however, have been unable to detect all 91 alleles of the human TCRBV genes. This is primarily due to either deficiencies in the amplification of all of the variable beta families, subfamilies, and alleles, or the prior lack of a systematic classification of the TCR variable family gene segment sequences. We describe here a real time reverse-transcription polymerase chain reaction based method, which allows efficient automation and integration of amplification, detection, and analysis with sequence specific detection of all T cell receptor beta variable gene families, subfamilies, and alleles. This method, which in itself contributes significant improvements over existing technologies through its comprehensiveness and efficiency, also functions independently of variables such as sample source and sample processing and has the ability to run on multiple real-time PCR platforms, affording one the implementation of personal preferences.

INTRODUCTION

T cells constitute a component of the immune system that is able to distinguish "self" versus "non-self". This is accomplished through the interaction of their T cell receptor (TCR) with the antigen:major histocompatibility complex (a:MHC) expressed on the surface of cells. The $\alpha\beta$ T cell receptor is a heterodimer, one component of which is a beta (B) chain, consisting of both a variable (V) and a constant (C) region. It is the variable region of the chain that directly contacts the a:MHC, eliciting the T cell response. Each individual has multiple TCRBV gene segments (or families) allowing for the ability to respond to a large number of a:MHC complexes [1].

A primary way to analyze the T cell receptor repertoire in an individual is through the use of antibody-based methods. Antibody based analysis methods can detect approximately sixty percent of the human T cell receptor beta variable (TCRBV) proteins, while gene expression analysis, primarily through employment of the polymerase chain reaction (PCR) [2], has had somewhat greater success in the detection of additional TCRBV families. Many of these previous PCR methods, however, have been unable to detect all 91 alleles of the human TCRBV genes. Such a comprehensive PCR strategy, however, requires the usage of both uniform and systematic organizations of the TCRBV genetic sequences. Such a TCRBV classification system was established by the World Health Organization (WHO) and has resulted in the identification of 25 different functional B variable families (with 91 subfamily and allele members total)[3]. Based on the WHO classification of the TCRBV families, two sequences reside within the same family if there is at least 50% homology between the two sequences. Subfamily members share at least 75% sequence homology and alleles of a given TCRV gene differ at no more than a few residues [3].

Previous attempts at establishing PCR-based methods to evaluate the expression of all members of the TCR families have not taken into account the new WHO systematic classification or the wide variety of subfamilies and alleles that exist for many of the variable families [4-49]. When possible, such references were consulted for potential primer sequences. However, many of those sequences did not satisfy the requirements of amplifying all of the various subfamilies and alleles within a given variable family or they inadvertently cross-amplified the sequence of a closely related variable family. The generation of a comprehensive panel of TCRBV primer sequences presented a significant challenge as the TCRBV families all share nearly 50% homology with each other and sequences designed to detect a specific family can inadvertently cross-amplify a closely related TCRBV family. We designed primers that will specifically detect all known alleles within the identified TCRBV families and, when possible, we designed a single primer to detect all alleles of a given TCRBV family, thereby improving on previous panels that required using multiple primer sequences to amplify multiple alleles of a given TCRBV family. In addition, some of the primer sequences from previous panels, while potentially meeting our stringent specificity requirements,

had a melting temperature that fell outside of our desired ten-degree melting range of 55°C to 65°C, making it difficult to analyze expression of all of the TCRBV families in a single experiment with a single amplification cycling protocol.

We describe here how we have established a complete panel of PCR primers that can be used with sequence specific real-time PCR methodology to evaluate the expression of all the TCRBV gene families, including all subfamily and allele members. The user has the flexibility of using many sample sources and processing methods, as well as benefiting from automated and integrated amplification, detection, and analysis in a gel free environment on a wide variety of real time PCR platforms. The technology can be applied to any area of study examining T cell biology, including autoimmunity, transplantation, cancer therapy, and infectious disease.

MATERIALS AND METHODS

Primer and Probe Development

Primer sequences were developed using the TCRBV classification system described by Arden, et al. [3]. Sequences were analyzed and potential primer sequences were evaluated for cross-reactivity using GenBank's BLAST tool (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/BLAST/). Human 18S rRNA primers and TaqMan® probe were designed using Beacon Designer 2 software (PremierBiosoft International, Palo Alto, CA, USA). TaqMan® probes were analyzed for cross-reactivity using GenBank's BLAST tool (National Center for Biotechnology Information).

Peripheral Blood Samples

A volume of ~20 mL of peripheral blood was collected, via venipuncture from individual healthy donors and from a hematopoietic stem cell transplant patient, into acid citrate dextrose VACUTAINER™ blood collection tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Informed consent was obtained from participants after the nature and possible consequences of the study had been fully explained according to West Virginia University's Internal Review Board guidelines. Buffy coat layers were isolated via centrifugation at 3300 rcf for 10 minutes. Contaminating red blood cells were removed by hypotonic lysis.

Flow Cytometric Analysis

Peripheral blood composition was determined by light scatter profiling using a CELL-DYN 3500 (Abbott Diagnostics, Santa Clara, CA, USA). Additional classification of the lymphocyte population was performed by cell surface staining using antibodies specific to CD4 (Beckman Coulter, New York, NY, USA), at a volume of 20 μL antibody per 5 x 10^5 cells, CD8, and CD19 (Caltag Laboratories, Burlingame, CA, USA), both at antibody concentrations of 1 μg per 1 x 10^6 cells. Cells were blocked prior to antibody addition by incubating cells for five minutes at room temperature with human IgG at a concentration of 200 μg per 5 x 10^5 cells (Sigma Chemical Co., St. Louis, MO, USA). After antibody addition, cells were incubated 30 minutes in the dark at room temperature followed by a single wash with 1x PBS. The stained cells were then fixed in 500 μL of 1% paraformaldehyde. All prepared samples were analyzed by flow cytometric analysis using a FACScan™ (Becton Dickinson), which had been calibrated using three color Calibrite™ Beads (Becton Dickinson) and FACSCOMP™ software (Becton Dickinson). For further lymphocyte analysis of $CD19^+$, $CD8^+$, and $CD4^+$ expression 10,000 total events were collected for each sample. SSC and FSC data were acquired in the linear mode and the FL1, FL2, and FL3 parameters were collected logarithmically. Data analysis was performed using Windows Multiple Document Interface (WinMDI) version 2.8 (Joseph Trotter, The Scripps Research Institute, http://facs.scripps.edu/software.html).

RNA Isolation

Total RNA was isolated from 20-40 x 10^6 white blood cells using TRIzol® Reagent according to the manufacturer's directions (Ambion, Austin, TX, USA). RNA was dissolved in ultra-PURE™ Distilled DNAse and RNAse free water (Invitrogen Corporation, Carlsbad, CA, USA). DNase treatment was performed on isolated RNA according to the manufacturer's recommendations using DNA-free™ (Ambion). RNA purity and concentration was determined by standard 260nm:280nm spectrophotometric analysis using a Genesis 10UV Spectronic Unicam (Spectronic Instruments, Rochester, NY, USA).

RT-PCR

One Step RT-PCR was performed using the QuantiTect™ Probe RT-PCR kit (Qiagen, Valencia, CA, USA). Recommended reaction mixtures were scaled down to a total reaction volume of 20 μL using 0.04 μg RNA with the following primer and probe concentrations: 0.4 μM TCRBV primer (Biosource International, Camarillo, CA, USA), 0.4 μM TCRBC primer (Biosource International), and 0.2 μM TCRBC TaqMan® probe, 5' 6-FAM, 3' BHQ™-1 (Integrated DNA Technologies, Inc., Coralville, IA, USA). 18SrRNA control reactions were performed in parallel using 0.4 μM each of sense and anti-sense primers (Biosource International) and 0.2 μM 18SrRNA TaqMan® probe, 5' 6-FAM, 3' BHQ™-1 (Integrated DNA Technologies, Inc.).

An iCycler™ (BioRad Laboratories, Hercules, CA, USA) was used for the RT and amplification cycles. RT was performed at 50°C for 60 minutes, max ramp speed, followed by an initial Taq DNA polymerase activation step of 15 minutes at 95°C, max ramp speed. A TouchDown PCR approach [50] was used with the following cycling conditions: denaturation for 15 seconds at 95°C, max ramp speed, annealing for 30 seconds starting at 70°C decreasing by 2°C for 10 repeats, max ramp speed, and extension for 40 seconds at 72°C, min ramp speed. After this TouchDown of the annealing temperature, 50 cycles were performed as follows with the optical data collection occurring at the extension step: 15 seconds at 95°C (max ramp speed), 30 seconds at 52°C (max ramp speed), and 40 seconds at 60°C (min ramp speed). Reactions were held at 4°C upon the conclusion of the run. Amplification efficiencies using cDNA dilutions were determined using the above described cycling protocol with the deletion of the reverse transcription cycle of 50°C for 60 minutes.

cDNA isolation

PCR products were electrophoresed on a 2% agarose gel using 20 μL PCR product and 4 μL tri-color 6X loading dye (Promega, Madison, WI, USA). Promega PCR marker was loaded into a control lane at the manufacturer's specifications (Promega). Product bands were excised using the QIAquick® Gel Extraction Kit protocol according to the manufacturer's instructions (Qiagen). cDNA purity and concentration was determined by standard 260nm:280nm spectrophotometric analysis using a Genesis

10UV Spectronic Unicam (Spectronic Instruments). cDNA dilutions were performed using ultra-PURE™ Distilled DNAse and RNAse free water (Invitrogen Corporation).

Southern Blot Analysis

Nucleic acid bands were transferred from the 2% agarose gel (described above) to BioBond™ Plus Nylon Membrane (Sigma Chemical Co.) using the Alkaline Southern Breeze™ Blotting Kit (Sigma Chemical Co.). The membrane was blocked overnight at room temperature using a blocking solution of 1X Saline-Sodium Citrate (SSC), 1% Bovine Serum Albumin (BSA) (Fisher Scientific, Pittsburgh, PA, USA), and 1% Sodium Dodecyl Sulfate (SDS) (Sigma Chemical Co.). A one hour incubation at room temperature was then performed using 20pmol biotinylated primary probe directed to the TCRBC region (Integrated DNA Technologies, Inc.) per mL blocking buffer followed by three five minute washings with a wash buffer of 1X SSC and 1% SDS (Sigma Chemical Co.). A streptavidin-HRP conjugate (Amersham Biosciences, Piscataway, NJ, USA) was added at a 1:5000 dilution in blocking buffer for 1 hour at room temperature. Three final five-minute washes were performed using the wash buffer. The membrane was developed using ECL™ detection reagents (Amersham Biosciences) according to the manufacturer's instructions. The membrane was then exposed to Biomax™ MR film (Eastman Kodak Company, Rochester, NY, USA). The film was developed using a 100 Plus Automatic X-Ray Film Processor (All-Pro Imaging, Hicksville, NY, USA).

RESULTS

Primer Sequence Panel

The final TCRBV primer panel consists of fewer than 30 primers and one TaqMan® probe. The amplification of the various alleles and subfamily members for a given TCRBV family is diagrammed in Table I. As previously mentioned, each TCRBV family can have multiple subfamily members, some with additional alleles. As described by Arden, et al. [3], we have adhered to the accepted classification and nomenclature for these families and their subfamily and allele members.

Listed on the far left columns of Table 1 are the names of the primers and their respective TCRBV families, whose subfamily and allele members are listed in the adjacent column. A single primer was used for the amplification of a given TCRBV with the following exceptions: two primers were needed to amplify all BV6 (B6JLB2 and B6JLB3), BV12 (B12.1JLB and B12.2.3JLB), and BV13 (B13.1eJLB2 and B13.5JLB2) members. There is no primer to amplify BV10 or BV19 as these families only contain nonfunctional orphan or pseudogenes [3].

primer name(s)	TCR family	TCR subfamily	TCR sequence	GenBank accession #
B1H	B variable 1	BV1S1A1	PL5.2	M13836
		BV1S1A2	308C	M27904
B2Blum	B variable 2	BV2S1A1	PL2.13	M13840
		BV2S1A2	WBDP25G	D13087
		BV2S1A3	HT120	X57604
		BV2S1A4	MT1-1	M11954
		BV2S1A5	4.49	X74852
B3H	B variable 3	BV3S1	PL4.4	M13843
B4H	B variable 4	BV4S1A1	PL2.14	M13846
		BV4S1A2	HBP48	X04926
		BV4S1A3	PL5.7	M13847
B5JLB	B variable 5	BV5S1A1	PL7.16	M13849
		BV5S1A2	ph24	M14271
		BV5S2	IGRb09	X58802
		BV5S3A1	HT415.9	X57611
		BV5S3A2	HT415.3	X57612
		BV5S3A3	IGRb08	X58801
		BV5S4A1	IGRb06	X58803
		BV5S4A2	AL62.24	M97709
		BV5S6A1	HT415	X57615
		BV5S6A2	IGRb07	X58804
		BV5S6A3	AL61.270	M97707
B6JLB2/B6JLB3	B variable 6	BV6S1A1	HBP50	X04934
		BV6S1A3	4D1	M13550
		BV6S2A1	HBVT23	M27383
		BV6S2A2	VB6.3	X61441
		BV6S3A1	HBP25	X04931
		BV6S4A1	IGRb10	X58805
		BV6S4A2	WBDM28A	D13085
		BV6S4A3	L17 beta	M13552
		BV6S4A4	ph22	M14261
		BV6S4A5	1.4	X74844
		BV6S4A6	D38	L14854
		BV6S5A1	ph16	M14262
		BV6S5A2	GL-PA	X61443
		BV6S6A1	HT147	X57607
		BV6S6A2	VB6.14b	L14483
		BV6S8A1	VB6.11a	L13762
		BV6S8A2	VB6.11c	L14432
B7JLB	B variable 7	BV7S1A1	IGRB19	X58813
		BV7S2A1	PL4.19	M13856
		BV7S2A2	IGRb18	X58812
		BV7S3A1	IGRb17	X58811
		BV7S3A2	HT267.2	X57617
B8JLB	B variable 8	BV8S1	ph11	M14265
		BV8S2A1	PL3.3	M13858
		BV8S2A2	ph8	M14264
		BV8S3	(lambda)VB8.3	X07223
B9H	B variable 9	BV9S1A1	HT307	X57614
		BV9S1A2	VB9.n	L06889
B11H	B variable 11	BV11S1A1	PL3.12	M13861
		BV11S1A2	1.3	X74845
B12.1JLB/B12.2.3JLB	B variable 12	BV12S1A1	PL4.2	M13862
		BV12S2A1	IGRb13	X58808
		BV12S2A2	H18.1	L26230
		BV12S2A3	WBDM21C	D13084
		BV12S3	HT96	X57609
B13.1eJLB2/B13.5JLB2	B variable 13	BV13S1	PL4.24	M13863
		BV13S2A1	VB13.2	X61445
		BV13S3	IGRb14	X58809
		BV13S4	VB13.4	X61447
		BV13S5	IGRb15	X58810
		BV13S6A1	IGRb16	X58815
		BV13S6A2	HT165.2	X57606
		BV13S6A3	3.1	X74848
		BV13S6A4	VB13.n3	L06892
		BV13S7	H127	L26228
B14JLB2	B variable 14	BV14S1	PL8.1	M13865
B15H	B variable 15	BV15S1	ph32	M14269
B16H	B variable 16	BV16S1A1	HT370	X57723
B17Blum	B variable 17	BV17S1A1	HBVT02	M27388
		BV17S1A2	S30.10	M97725
		BV17S1A3	BV17S1	L19936
B18JLB	B variable 18	BV18S1	HBVT56	M27389
B20JLB2	B variable 20	BV20S1A1	WBDM30A	D13086
		BV20S1A3	HUT102beta	M13554
B21JLB	B variable 21	BV21S1	B17ct7	D16584
		BV21S2A1	IGRb02	X58797
		BV21S2A2	BV21.2	M33234
		BV21S2A3	V beta 21	M62377
		BV21S3A1	BV21.3	M33235
		BV21S3A2	IGRb01	X58796
B22H	B variable 22	BV22S1A1	V beta 23	M62379
		BV22S1A2	IGRb03	X58798
B23JLB2	B variable 23	BV23S1A1	V beta 22	M62378
		BV23S1A2	IGRb04	X58799
B24H	B variable 24	BV24S1A1	V beta 24	M62376
		BV24S1A2	IGRb05	X58800
		BV24S1A3	H130.1	U03115
B25JLB	B variable 25	BV25S1A1	HVB30.A	L26231
		BV25S1A3	HsVB25	L26054
CBJLB	B constant	BC1	JM	K02885
		BC2		L34740
TCRCB probe	B constant	BC1	JM	K02885
		BC2		L34740

Table 1: TCRBV primer panel organization.

The third column from the left in Table I, entitled TCR subfamily, contains all of the subfamily (S) and alleles (A) for a given TCR family, per the previously described nomenclature system [3]. The final two columns of Table I, titled TCR sequence and GenBank accession #, provide the reference sequences used in the development of our primer sequences. When available, we have provided both the sequence clone name as well as its listing in the GenBank database. Primer sequences are proprietary and are available for licensing through the Institute for Scientific Research, Inc. (Fairmont, WV)

Peripheral Blood Composition Analysis

The cellular composition of the peripheral blood from three healthy human donors is presented in Table II. Cellular composition is as expected for healthy donors. It was found that lymphocyte samples range from 30%-55% CD4$^+$ T cells, 22%-44% CD8$^+$ T cells, and 4%-11% CD19$^+$ B cells.

sample	WBC (10^3/uL)	neutrophils	eosinophils	basophils	monocytes	lymphocytes	CD19+ CD4+ CD8+
A	3.73	57.00%	1.73%	0.69%	8.98%	31.60%	4.11% 54.75% 21.65%
B	3.38	44.20%	8.08%	0.58%	6.51%	40.60%	6.32% 29.62% 43.62%
C	5.09	57.40%	1.04%	0.79%	7.21%	33.60%	10.61% 40.07% 25.16%

Table 2: *Peripheral blood composition of donor samples.*

All values are expressed as percent, except white blood cell (WBC) count, which is expressed as thousand cells/microliter. Lymphocyte counts were further differentiated into percent CD19$^+$, CD4$^+$ and CD8$^+$ as depicted in far right column.

iCycler™ fluorescence signal corresponds to Southern Blot signal

Figure 1 depicts the fluorescence readings obtained on the iCycler™ using the B1H/CBJLB primer set with the TCRCB TaqMan® probe. Fluorescence readings (RFU) are reported here as the Ct value, or the cycle at which fluorescence readings exceeded background fluorescence levels. Those samples with greater initial levels of target
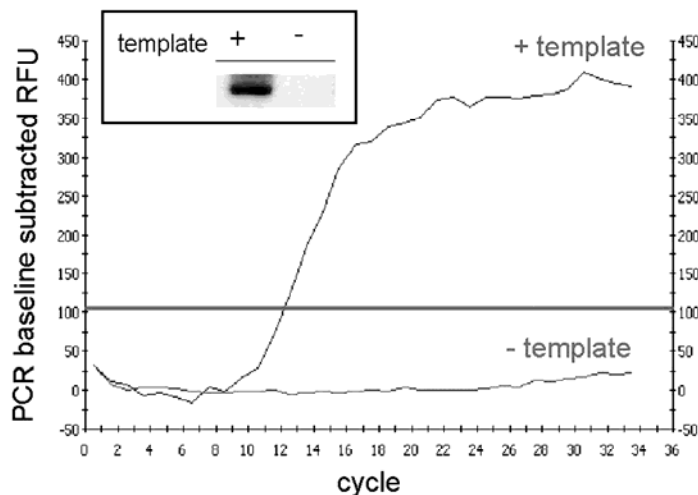
Figure 1: iCycler fluorescence readings during amplification of TCRBV1.

template have fluorescence levels that more quickly surpass these background fluorescence levels, or a lower Ct value, compared to samples with less initial copies of target template. The plus and minus template samples are indicated in Figure 1. The plus template sample has a Ct value of 12 while the no template sample has an expected Ct value >34. Plus and minus template samples were completed for all TCR variable family primers to ensure the absence of autofluorescence in the no template samples. No significant autofluorescence levels were detected with the TCRBC TaqMan® probe and any of the TCRBV primers.

In order to confirm the fluorescence readings obtained by the iCycler™, Southern Blot analysis was performed on randomly chosen TCRBV PCR products. As exampled by the B1H/CBJLB amplification product shown as the inset in Figure 1, the iCycler's™ fluorescence readings are independently confirmed by Southern Blot analysis. Again, the plus and minus template samples are labeled accordingly in the inset of Figure 1.

Amplification Efficiency Determination

cDNA was diluted into 10 fold serial dilutions and subjected to PCR amplification to determine the degree of amplification efficiency. Figure 2 depicts the fluorescence levels seen with the amplification reaction using the primer B6JLB2. The reaction using this primer was ~93% efficient, results which are typical for the other analyzed primer
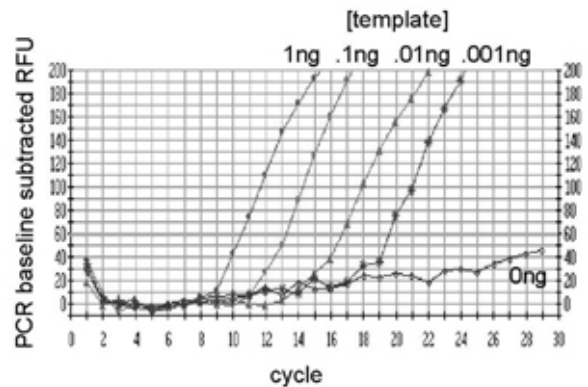
Figure 2: *Amplification efficiency analysis.*

sets. This amplification efficiency determination indicates that the PCR detection system is sensitive in response to the amount of target template initially placed into the reaction *(refer to appendix for amplification efficiency calculation equation)*.

TCRV family gene expression as determined by real-time PCR

Figure 3 depicts the TCRBV expression profiles from three human peripheral blood samples. Each data point is the mean of triplicate reactions with the standard error of the mean indicated. One way Analysis of Variance with Tukey's Post Hoc test was performed for statistical analysis. Symbols indicate significant differences between the three samples for a given primer. $$(P<0.001), #(P<0.01), and *(P<0.05). Significant differences in expression levels were seen between samples A, B, and C for TCRBV8, 12, 15, 16, 17, 18, 20, 23, and 25. Both TCRBV mRNA and protein expression levels (determined using the IOTest® Beta Mark TCR VB Repertoire Kit (Beckman Coulter)) demonstrated steady state expression levels (*refer to appendix for method and data*).

Figure 3: *Relative TCRBV mRNA expression levels of three human peripheral blood samples based on Ct values*

*(samples A, B, and C). $$ (P<0.001), # (P<0.01), * (P<0.05).*
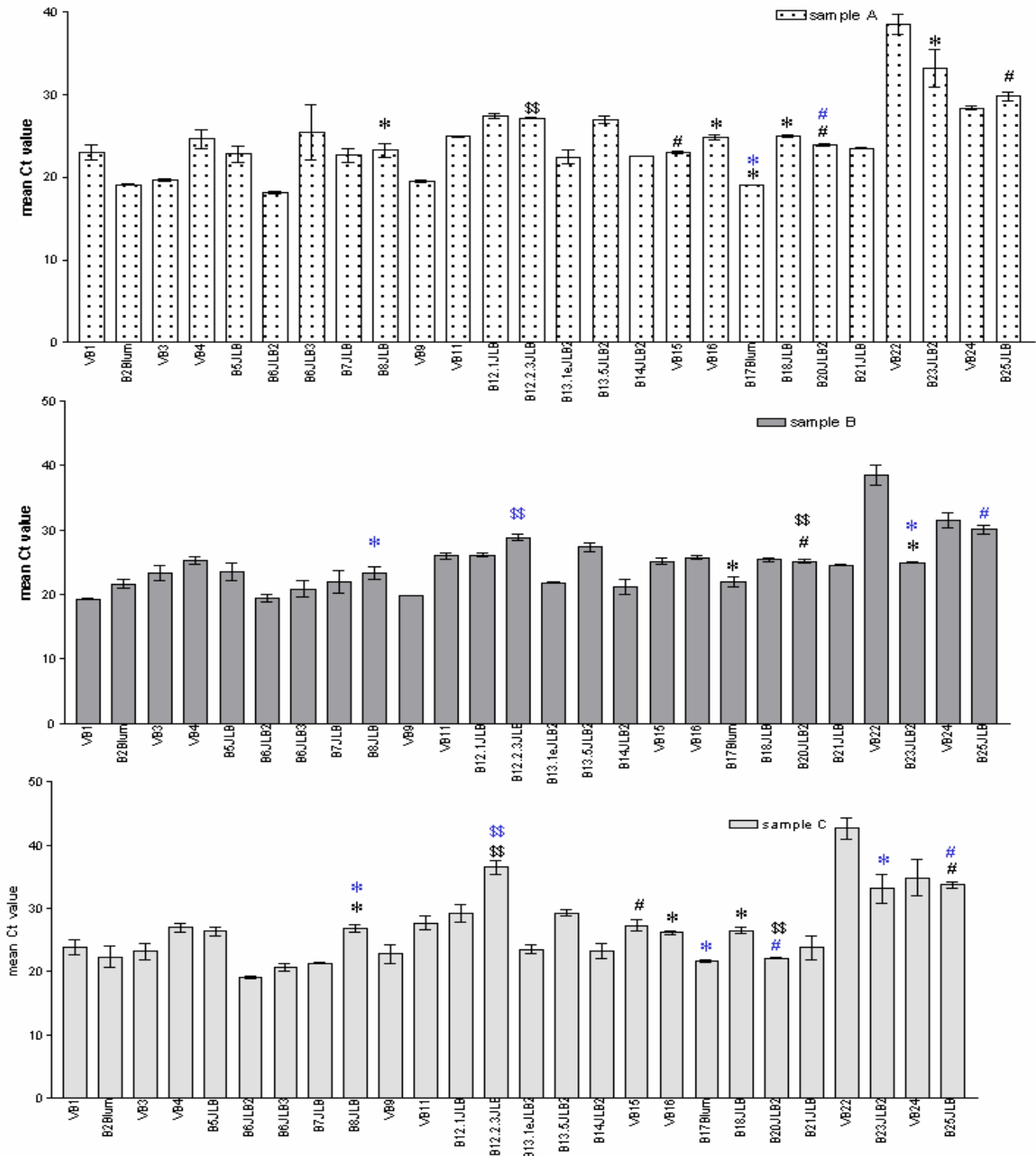
Note: A Ct difference of ~3.5 cycles was required to achieve 95% confidence, or p<0.05

Figure 4 depicts the relative TCRBV1 expression profile, as determined by Ct values, of a hematopoietic stem cell transplant patient prior to and following transplant. The patient was a 54 year-old male being treated for chronic lymphocytic leukemia and received peripheral blood hematopoietic stem cells from a matched related donor. The patient had a staphylococcus infection, as well as graft-versus-host disease and cytomegalovirus complications, immediately following transplant. These complications resolved but the patient expired at week 14 post- transplant from graft-versus-host disease complications. Significant changes in TCRBV1 expression were observed over time between weeks –1 and 3, weeks 3 and 4, weeks 7 and 8, and weeks 8 and 9.
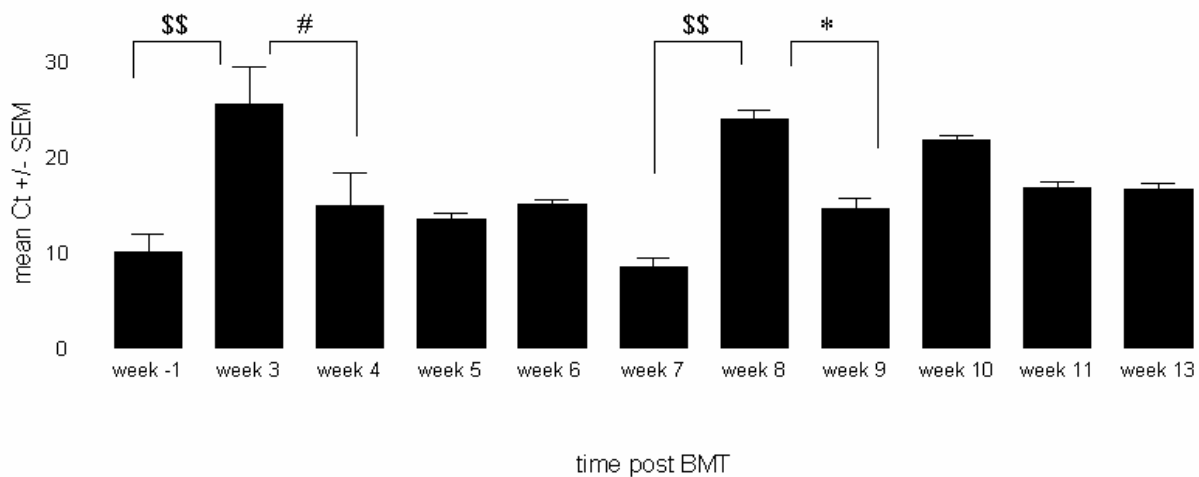
Figure 4:TCRBV1 expression level changes in a hematopoietic stem cell transplant recipient before and after transplant.

*Each data point is the mean of triplicate reactions with the standard error of the mean indicated. One way Analysis of Variance with Bonferroni's Multiple Comparison test was performed for statistical analysis to monitor changes in the TCRBV1 expression from week-to-week. $\$\$(P<0.001)$, #(P<0.01), and *(P<0.05).*

DISCUSSION

Through the employment of primer panels previously developed [4-49], many investigators have examined the usage of the human TCR beta variable genes under various biological conditions. Many of these reports, however, fall short of the desired comprehensiveness to detect all TCRBV members. For example, due to the more recent identification and classification of TCRBV families 21, 22, 23, 24, and 25, many previous reports do not include analysis of some or all of these additional five families [4-17,20-30,32-39,41-43,45,46]. Additionally many also would not amplify all subfamily and allele members within a given TCRBV family. For example, based on sequence homology analysis the panel proposed by Blumberg, et al., [7] will not amplify the subfamily member BV8.3.

Others have tried different approaches to the amplification of all TCRBV subfamily and allele members. For example, Lynas, et al., [28] describe the use of single primers to detect BV2/BV4 and BV18/BV8.3. Tsuruta, et al., [44] have five separate primers in their panel to amplify all members of the TCRBV5 family while we have been able to identify a single primer sequence that we predict will amplify all 5 subfamilies (including all 11 possible alleles). Therefore, a primer panel that will amplify all possible alleles for the TCRBV families using as few primers as possible is a noteworthy improvement in the area of T cell receptor gene expression analysis, as sample availability is often a limiting factor.

While most of the previously cited primer panels we reviewed relied on conventional PCR, followed by gel analysis and Southern Blotting or the inclusion of labeled primers or nucleotides for sequence analysis there is at least one report of the use of TCRBV primers in a SYBR green reaction [42]. While the paper by Sebille et al., yields to the same primer sequence concerns as many of the others, as it traces its primer sequence roots back to the primers described by Genevee et al., [15] and Gorski et al., [17], this does move the field of TCRBV gene expression analysis into the real-time PCR arena. We have experimented with SYBR green reactions previously but prefer the ease of analysis and additional sequence specificity offered by a TaqMan® probe.

While our study focuses on the use of peripheral blood samples, there is nothing intrinsic about our method that prevents using cells from other sources, for example cell cultures, tissue samples, or synovial fluid. We have previously used silica membrane-based extraction kits, such as GenElute™ Mammalian Total RNA Kit (Sigma Chemical Co.), Rneasy® kit (Qiagen) and PAXgene™ Blood RNA kit (PreAnalytiX, Switzerland). However, we chose to isolate RNA from our samples using phenol-chloroform extraction, due to our success with improved yields compared to the silica membrane-based technologies. While we chose to use the BioRad iCycler™ due to its availability at our facilities, its larger sample capacity, and the permitted usage of conventional PCR tubes, we have previously used the Roche Lightcycler®, demonstrating the flexibility of platform usage but not validity that the primers provide equivalent results across all platforms.

Consistent with previous reports [51-54], differences in TCRBV expression levels do exist between individuals and the level of those differences can change with time and health of the individual. Our technology has not only been demonstrated functional by the usage of sequence specific probes in real time PCR but has also been verified by Southern Blot analysis and flow cytometry utilizing available TCRBV antibodies, with the latter supporting the notion that the increased expression levels we observed with the PCR system were due to the increased number of cells expressing such receptors as opposed to only an increase in the amount of message in a given cell.

In terms of amplification efficiency, if a PCR reaction is 100% efficient, there will theoretically be a decrease in the Ct value by one each time the template amount is doubled (Roche Molecular Biochemicals, Technical Note No. LC 11/2000). Therefore, for a 10-fold dilution, a difference of 3.25 cycles should be observed between each 10-fold dilution. Our methodology is not only comprehensive but it is also efficient with a typical amplification efficiency of $\geq$ 90%.

In conclusion, we have presented here a significant improvement to existing technologies that enable one to detect all of the families, subfamilies, and alleles of the TCRBV regions classified by Arden et al., [3] using efficient, sequence specific real-time PCR methodology. The user benefits from automated and integrated amplification, detection, and analysis in a gel free environment with the choice of using various PCR

platforms, sample sources, and sample processing methods. Additionally, this PCR-based method is approximately 25-fold less expensive than comparable flow cytometry based methods. This technology can be applied to any area of study examining T cell biology, including autoimmunity, transplantation, cancer therapy, and infectious disease.

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THE PREVIOUS CHAPTER DESCRIBED THE DEVELOPMENT OF IMPROVED TECHNOLOGY CAPABLE OF ACCURATELY AND EFFICIENTLY DETECTING EXPRESSION OF ALL OF THE HUMAN TCRBV GENES.

THERE ARE MANY POTENTIAL APPLICATIONS OF SUCH TECHNOLOGY.

FOR MY PURPOSES, HOWEVER, I UTILIZED THE TECHNOLOGY TO DETERMINE WHAT SPECIFIC T CELLS ARE ASSOCIATED WITH THE FOLLOWING SPECIFIC EVENTS AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION:

1) GRAFT-VERSUS-HOST DISEASE
2) CYTOMEGALOVIRUS REACTIVATION
3) IMMUNOSUPPRESSIVE THERAPIES (CYCLOSPORIN A AND TACROLIMUS)
4) ENGRAFTMENT OF THE TCRBV REPERTOIRE

CHAPTER 3

SIMILARITY IN REPERTOIRE OF T CELLS ASSOCIATED WITH OCCURRENCES OF CMV REACTIVATION AND GVHD POST HUMAN HEMATOPOIETIC STEM CELL TRANSPLANTATION.

ABSTRACT

Cytomegalovirus (CMV), after initial infection, typically remains in a latent state and reactivates during periods of immune suppression, such as after allogeneic hematopoietic stem cell (HSC) transplant. Prior studies have indicated a restricted usage of specific TCRBV families directed against CMV antigens in otherwise healthy individuals. We monitored, on a weekly basis, the usage of the T cell receptor beta variable (TCRBV) repertoire in the peripheral blood of five HSC transplant recipients using real-time RT-PCR and found that several TCRBV families (TCRBV 1-6, 11, 12.1, 13 (excluding 13.5), 15, 16, 20, 25) were significantly associated with CMV reactivation. In addition, significant overlap exists in these families and those found to be associated with graft-versus-host disease (GVHD), a common T cell mediated post transplant complication. Our results may help to explain the common clinical association of CMV reactivation and GVHD.

INTRODUCTION

Cytomegalovirus (CMV) is a human herpes virus that infects greater than 60% of the world's adult population[1]. While this virus typically presents itself as a latent infection, it commonly reactivates during immuno-suppressed states, such as after allogeneic hematopoietic stem cell transplant (HSCT) [2]. Prior studies have indicated a restricted usage of specific T cell receptor beta variable (TCRBV) families directed against CMV antigens in otherwise healthy individuals [3-6]. We here examine the expression of specific TCRBV families associated with CMV reactivation following allogeneic hematopoietic stem cell (HSC) transplantation. In addition, we determined that overlap exists in the TCRBV families associated with CMV reactivation and graft-versus-host disease (GVHD), supporting previous reports of the clinical association of GVHD and CMV reactivation [7-9]. GVHD is a common post transplant complication where the donor-derived T cells attack and destroy the recipient's tissue based upon allogeneic disparities between the T cell receptor (TCR) and peptide:major histocompatibility complex (p:MHC) [10]. In our study peripheral blood samples were collected weekly from allogeneic HSC recipients through day 100 post-transplant. Donor

and recipient peripheral blood samples were also collected prior to pre-transplant conditioning therapies in order to serve as baseline reference samples. Despite the complexities of the post transplant environment, we found that specific subsets of T cells were significantly associated with CMV reactivation and that many of these same TCRBV families were also associated with the development of GVHD in the patient population studied.

MATERIALS AND METHODS

Patient population

Study subjects were patients undergoing allogeneic transplant treatment at West Virginia University's Blood and Marrow Transplant and Hematological Malignancy Program, Morgantown, West Virginia (Table 1). Informed consent was obtained from participants after the nature and possible consequences of the study had been fully explained according to West Virginia University's Institutional Review Board guidelines. Prior to peripheral blood stem cell (PBSC) infusion, study recipients underwent myeloblative conditioning regimens consisting of either Thiotepa (500 mg/m^2 q12h x 2 doses), Campath-1H (20mg x 2 doses), and total body irradiation (TBI) (200 cGy x 5 fractions) or Busulfan (1mg/kg p.o. q6h x 16 doses) and Cytoxan (60 mg/kg/d x 2 doses). A 20 mL peripheral blood sample was collected weekly, via venipuncture from hematopoietic stem cell transplant patients, into an acid citrate dextrose VACUTAINER™ blood collection tube (Becton Dickinson, Franklin Lakes, NJ, USA). Buffy coat layers were isolated via centrifugation at 3300 rcf for 10 minutes. Contaminating red blood cells were removed by hypotonic lysis.

recipient	sex	age	disease	donor	pretreatment regimen	CMV status recipient/donor	post tranplant complications
1	M	54	AML	MRD	TT/TBI/Campath	neg/neg	GVHD grade I bacterial pneumonia viral mouth sores *staphylococcus* infection bacterial URI/sinusitis
2	M	54	CLL	MRD	TT/TBI/Campath	pos/neg	GVHD grades II and IV CMV reactivation *staphylococcus* infection
3	M	51	NHL	MRD	TT/TBI/Campath	pos/neg	GVHD grade II CMV reactivation *staphylococcus* infection
4	F	41	CML	MRD	TT/TBI/Campath	neg/neg	GVHD grade III *C. difficile* viral mouth sores *Enterococcus faecalis*
5	F	41	CML	MUD	Bu/Cy2	pos/pos	GVHD grade II CMV reactivation bacterial gastritis *Cornybacter diptheroid*

Table 1: *Patient demographics*

All patients received peripheral blood hematopoietic stem cell transplants from an HLA-matched related donor (MRD) with the exception of recipient 5, who received stem cells from an HLA-matched unrelated donor (MUD). HLA matching was performed serologically for all MRD but was determined using molecular typing for MUD. Cytomegalovirus (CMV) status was determined in both recipient and donor prior to transplant by evaluating CMV IgG and IgM serum levels. CMV reactivation was monitored weekly by antigenemia testing for recipients at risk for CMV reactivation (donor and/or recipient with prior history of CMV exposure). Complications experienced in the recipient post transplant, as determined by standard clinical evaluation, are indicated on the far right side of the table. All patients were living at the conclusion of our study. Abbreviations: M, male; F, female; AML, acute myelogenous leukemia; CLL, chronic lymphocytic leukemia; NHL, non-Hodgkin lymphoma; CML, chronic myelogenous leukemia; TT, Thiotepa; TBI, total body irradiation; Bu, Busulfan; Cy2, Cytoxan; GVHD, graft-versus-host disease.

Donors

All donors were serologically HLA matched to the recipient at a minimum of HLA-A, B, and DR loci. The patient undergoing an unrelated donor transplants was also molecularly matched at the allele level. PBSC donors were mobilized with filgrastim (G-CSF) at 10ug/kg/day for 5 days with stem cell collection occurring on day 5. The number of PBSC collected was 3-5 x 10^6 HSC/kg of recipient body weight.

GVHD Prophylaxis and Supportive Care

Cyclosporin A (CSA) or tacrolimus (FK506) treatment started one day before transplant (d-1) as part of standard graft-versus-host disease prophylaxis and continued throughout the study period. The patients also received pulse methotrexate as part of GVHD prophylaxis. Patients were monitored bi-weekly for CSA and FK506 serum concentrations, with dosages adjusted as necessary to be within the preferred protocol serum concentrations of 200+/-20 ng/mL for CSA and 7-12 ng/mL for FK506. Additional supportive care, including anti-fungal, anti-viral, and anti-PCP prophylaxis were provided according to standard operating policies. CMV reactivation was monitored weekly by antigenemia testing (ViroMed Laboratories, Inc., Minnetonka, MN).

RNA Isolation

Total RNA was isolated from 20-40 x 10^6 white blood cells using TRIzol® Reagent according to the manufacturer's directions (Ambion, Austin, TX, USA). RNA was dissolved in ultra-PURE™ Distilled DNAse and RNAse free water (Invitrogen Corporation, Carlsbad, CA, USA). DNase treatment was performed on isolated RNA according to the manufacturer's recommendations using DNA-free™ (Ambion, Austin, TX, USA). RNA purity and concentration was determined by standard 260nm:280nm spectrophotometric analysis using a Genesis 10UV Spectronic Unicam (Spectronic Instruments, Rochester, NY, USA).

RT-PCR

One Step RT-PCR was performed using the QuantiTect™ Probe RT-PCR kit (Qiagen, Valencia, CA, USA). Recommended reaction mixtures were scaled down to a total reaction volume of 20 μL using 0.04 μg RNA with the following primer and probe concentrations: 0.4 μM TCRBV primer (Biosource International, Camarillo, CA, USA), 0.4 μM TCRBC primer (Biosource International, Camarillo, CA, USA), and 0.2 μM TCRBC TaqMan® probe, 5' 6-FAM, 3' BHQ™-1 (Integrated DNA Technologies, Inc., Coralville, IA, USA). 18SrRNA control reactions were performed in parallel using 0.4 μM each of sense and anti-sense primers (Biosource International, Camarillo, CA, USA) and 0.2 μM 18SrRNA TaqMan® probe, 5' 6-FAM, 3' BHQ™-1 (Integrated DNA Technologies, Inc., Coralville, IA, USA). Primer and probe sequences were previously described (Brewer and Ericson, *J. of Immunol. Methods,* in press). An iCycler™ (BioRad Laboratories, Hercules, CA, USA) was used for the RT and amplification cycles. RT was performed at 50^oC for 60 minutes, max ramp speed, followed by an initial Taq activation step of 15 minutes at 95^oC, max ramp speed. A TouchDown PCR approach [11] was used with the following cycling conditions: denaturation for 15 seconds at 95^oC, max ramp speed, annealing for 30 seconds starting at 70^oC decreasing by 2^oC for 10 repeats, max ramp speed, and extension for 40 seconds at 72^oC, min ramp speed. After this TouchDown of the annealing temperature, 50 cycles were performed as follows with the optical data collection occurring at the extension step: 15 seconds at 95^oC (max ramp speed), 30 seconds at 52^oC (max ramp speed), and 40 seconds at 60^oC (min ramp speed). Reactions were held at 4^oC upon the conclusion of the run.

Statistical Analyses

One-way analysis of variance with Bonferroni's Multiple Comparison Test was used to determine the TCRBV families associated with GVHD and CMV reactivation.

RESULTS

Increased expression of specific TCRBV families is associated with reactivation of cytomegalovirus post peripheral blood hematopoietic stem cell transplant.

Using serial time points, we analyzed the alterations in TCRBV expression over time to determine which TCRBV families were associated with CMV reactivation compared to periods without CMV reactivation. All patients in our study were determined to be 100% donor engrafted at day 30 post-transplant, with the exception of recipient 5, whose chimerism analysis was not performed until day 100 post transplant, at which point chimerism was also determined to be 100% donor in origin. We found an increase in the expression of TCRBV families 1-6, 11, 12.1, 13 (excluding 13.5), 15, 16, 20, and 25 with CMV reactivation compared to sampling periods in which CMV antigenemia was not detected (Table 2).

Increased expression of specific TCRBV families is associated with Graft-versus-Host Disease post peripheral blood hematopoietic stem cell transplant and has significant similarity to those families linked to CMV reactivation.

In addition to investigating the increased expression of specific TCRBV families in the setting of CMV reactivation, we also examined the expression profiles of TCRBV families associated with GVHD post HSC transplant. By weekly monitoring of the T cell repertoire, in addition to baseline sampling, we were able to circumvent some of the concerns associated with previous GVHD reports, such as the lack of baseline samples and serial time point analyses [12-17]. All five of our patients developed acute GVHD post HSCT, primarily grades I-II (Table 1). When we examined the TCRBV expression profile during periods when patients had GVHD and compared this to periods without GVHD, we found significant increases in the expression of TCRBV 1-6, 12.1, 13 (excluding 13.5), and 16, associated with GVHD grades I-II, with an additional increase in TCRBV 18 seen in a patient with GVHD grade IV (Table 2).

TCRBV increases associated with CMV reactivation	TCRBV increases associated with GVHD grade I/II
1	1
2	2
3	3
4	4
5	5
6	6
11	
12.1	12.1
13 (excluding 13.5)	13 (excluding 13.5)
15	
16	16
	*18
20	
25	

Table 2: *Increased expression of specific families of TCRBV is associated with cytomegalovirus (CMV) reactivation and Graft-versus-Host Disease (GVHD) post peripheral blood stem cell transplant.*

Specific TCRBV families were significantly increased (p<0.05) with the occurrence of CMV reactivation, compared to periods in which CMV antigenemia was not detected and also with the occurrence of GVHD grades I and II, compared to periods without GVHD complications. *In a patient with grade IV GVHD an increase in TCRBV18 was seen in addition to the increase in TCRBV families seen in GVHD grades I-II.

DISCUSSION

A number of studies have examined the role of specific T cells in the immune response to CMV antigens. While a previous study looking at the TCRBV expression during CMV reactivation in kidney and liver transplants found no preference for TCRBV usage [18], we found great similarity in our results and those examining the TCRBV expression of CMV reactive T cells found in otherwise healthy individuals [3-6]. For example, studies using CMV reactive T cell from healthy donors expressed TCRBV families 2 and 20 [6], TCRBV families 3, 6.7, 13.1, and 20 [5], and TCRBV families 1, 2, 5.1, 12, 13.1, and 16 [3]. Many of these previous studies utilized tetramer technology to remove CMV reactive T cells, which were then analyzed for TCRBV expression via antibody staining and flow cytometry. The greatest limitation of previously described

studies is that antibodies were not available to all of the TCRBV families, such as TCRBV 4, 11, 13, 15, and sometimes 25. We suspect that the reason we detected the expression of additional TCRBV families with CMV reactivation compared to these previous studies was that we used a primer panel that could detect all of the TCRBV families and their alleles.

Similarly a number of reports have described the association of specific TCRBV families with GVHD, with some of these studies not only examining peripheral blood samples but also GVHD tissue lesions [12-17,19-25]. Many of these studies, however, contained very limited numbers of sampling points; for example some studies primarily evaluated samples upon the diagnosis of GVHD and others did not contain baseline sampling [12-17]. In this aspect, our study significantly adds to the investigation of immune response after hematopoietic stem cell (HSC) transplantation by providing serial time point analyses using a comprehensive primer panel, which can detect all TCRBV families and alleles.

Several reports have detailed the clinical association of GVHD and CMV reactivation [7-9]. Larsson et al., observed a decreased risk for the development of GVHD when patients were preemptively treated with anti-viral therapy [7,8] while Vassallo et al., observed an association between skin GVHD and the presence of CMV antigens in the patient's peripheral blood [9]. A recently published study noted cross reactivity of an HLA-DR7 restricted CMV-specific T cell for an HLA-DR4 allo-antigen [7]. This could possibly account for the overlap we saw in TCRBV families associated with both GVHD and CMV reactivation.

In conclusion, the developing immune system found in hematopoietic stem cell transplant patients responds to CMV antigen exposure with the alteration of the TCRBV profile with great similarity to that described in prior non-transplant related reports. Additionally, great similarities exist in the alterations of the TCRBV profile associated with CMV reactivation and the occurrence of GVHD, supporting prior reports of such a clinical association.

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CHAPTER 4

ENGRAFTMENT OF T CELL RECEPTOR (TCR) REPERTOIRE IN MYELOABLATED ALLOGENEIC HEMATOPOIETIC STEM CELL RECIPIENTS MIMICS DONOR TCR REPERTOIRE.

ABSTRACT

Hematopoietic stem cell (HSC) transplantation is used to rescue the immune system in patients who have received myeloblative regimens targeted at eradication of various hematological malignancies. In allogeneic HSC transplantation, stem cells from a healthy donor are infused into the recipient with the intent of reconstituting the recipient's immune system. Using the most comprehensive set of TCRBV primers available, we determined through real time RT-PCR that the reconstitution of the recipient's T cell repertoire post- hematopoietic stem cell transplant was of a higher correlation to that found in the donor compared to that found in the recipient prior to transplant. Our results argue that, despite the influence of the recipient's environment, the donor stem cells mature in the recipient's body with a TCRBV repertoire reminiscent of that found in the donor.

INTRODUCTION

The major goal of allogeneic hematopoietic stem cell transplantation is to achieve complete engraftment of healthy donor hematopoietic stem cells and subsequent immune reconstitution within the new environment of the recipient. T cells are a critical component of the recipient's defense system against invading pathogens and the reactivation of latent infections through the recognition of antigen through the T cell receptor (TCR). While T cells play a critical role in the immune system's defenses, it is not known whether the maturing T cells found in the recipient display a T cell receptor beta variable (TCRBV) repertoire more like that in the donor or if they exhibit a repertoire more reminiscent of that found in the recipient prior to transplant. In this study, we used real-time RT-PCR and a comprehensive panel of primers, developed in response to the reclassification of the TCR genes [1], to monitor the profile of the engrafting TCRBV regions. In the first 100 days after transplant, despite the influence of the recipient's HSC environment on the development of the immature cells [2-4], the T cell repertoire appears to develop with a phenotype more reminiscent of that found in the donor as opposed to that found in the recipient prior to transplant.

MATERIALS AND METHODS

Patient population

Study subjects (n=4) were patients undergoing transplant treatment at West Virginia University's Blood and Marrow Transplant and Hematological Malignancy Program, Morgantown, West Virginia. Informed consent was obtained from participants after the nature and possible consequences of the study had been fully explained according to West Virginia University's Institutional Review Board guidelines. All study patients received hematopoietic stem cells collected from the peripheral blood of mobilized donors who had been primed with G-CSF. The donors were serologically HLA matched to the recipient at a minimum of HLA-A, B, and DR loci. Prior to peripheral blood stem cell (PBSC) infusion, study recipients underwent myeloblative conditioning regimens consisting of either Thiotepa (500 mg/m^2 q12h x 2 doses), Campath-1H (20mg x 2 doses), and total body irradiation (TBI) (1000 cGy total dose: given in 200cGy fractions) or Busulfan (1mg/kg p.o. q6h x 16 doses) and Cytoxan (60 mg/kg/d x 2 doses) (Table 1). Full supportive care, including GVHD prophylaxis, anti-fungal, anti-viral, and anti-PCP prophylaxis, empiric anti-microbial therapy for neutropenic febrile episodes, and transfusions were provided per standard operating policies. Peripheral blood, ~20 mL, was collected weekly, via venipuncture from hematopoietic stem cell transplant patients, into an acid citrate dextrose VACUTAINER™ blood collection tube (Becton Dickinson, Franklin Lakes, NJ, USA). Buffy coat layers were isolated via centrifugation at 3300 rcf for 10 minutes. Contaminating red blood cells were removed by hypotonic lysis.

recipient	sex	age	disease	donor	pretreatment regimen	immunosuppressive therapy	CMV status recipient/donor
1	M	54	AML	MRD	TT/TBI/Campath	CSA	neg/neg
2	M	54	CLL	MRD	TT/TBI/Campath	CSA *except for w+12 when switched to FK506 to treat GVHD IV*	pos/neg
3	M	51	NHL	MRD	TT/TBI/Campath	CSA	pos/neg
4	F	41	CML	MUD	Bu/Cy2	CSA	pos/pos

Table 1: *Patient demographics*

All patients received peripheral blood hematopoietic stem cell transplants from an HLA-matched related donor (MRD). HLA matching was performed serologically. Cytomegalovirus (CMV) status was determined in both recipient and donor prior to transplant by evaluating CMV IgG and IgM serum levels. Complications experienced in the recipient post transplant, as determined by standard clinical evaluation, are indicated on the far right side of the table. All patients were living at the conclusion of our study. Abbreviations: M, male; F, female; AML, acute myelogenous leukemia; CLL, chronic lymphocytic leukemia; NHL, non-Hodgkin lymphoma; CML, chronic myelogenous leukemia; TT, Thiotepa; TBI, total body irradiation; Bu, Busulfan; Cy2, Cytoxan; GVHD, graft-versus-host disease.

RNA Isolation

Total RNA was isolated from 20-40 x 10^6 white blood cells using TRIzol® Reagent as previously described (Brewer and Ericson, *J. of Immunol. Methods,* in press). RNA was dissolved in ultra-PURE™ Distilled DNAse and RNAse free water (Invitrogen Corporation, Carlsbad, CA, USA). DNase treatment was performed on isolated RNA according to the manufacturer's recommendations using DNA-free™ (Ambion, Austin, TX, USA). RNA purity and concentration was determined by standard 260nm:280nm spectrophotometric analysis using a Genesis 10UV Spectronic Unicam (Spectronic Instruments, Rochester, NY, USA).

RT-PCR

One Step RT-PCR was performed using the QuantiTect™ Probe RT-PCR kit (Qiagen, Valencia, CA, USA) with sequence specific detection as previously described (Brewer and Ericson, *J. of Immunol. Methods,* in press).

Statistical Analyses

Spearman rank correlation coefficient analysis was used to determine the correlation of the TCRBV engraftment phenotypes.

RESULTS AND DISCUSSION

The TCRBV repertoire engrafts with a phenotype similar to that present in the donor.

By analyzing the expression of all of the TCRBV families in the donor and recipient pre-transplant followed by weekly analysis of these families in the recipient post transplant, we could monitor the TCRBV phenotype of the recipient's engrafted cells during the early reconstitution period (Table 2). While neutrophil engraftment begins one to two weeks after transplant, complete restoration of the CD3^{+} population typically requires many months post-transplant [5]. We compared correlation parameters at weekly intervals and here report the correlation seen at baseline and at day 100. The day 100 TCRBV expression patterns obtained from patients 1, 2, and 3 showed a stronger correlation to the baseline donor TCRBV expression profile compared to the recipient's baseline TCRBV repertoires (sample 1: -0.193 vs. 0.68, sample 2: 0.330 vs. 0.510, sample 3: 0.329 vs. 0.664). At the conclusion of this study, there was a decreased correlation of recipient samples to their own pre-transplant TCRBV profile when compared to the donor's baseline TCRBV expression profile (sample 1: -0.219 vs. 0.68, sample 2: 0.289 vs. 0.510, sample 3: 0.537 vs. 0.664). These results were compared to the correlation of the TCRBV repertoire found in healthy peripheral blood donors over a period of two weeks, which exhibited a correlation to self of ~0.75 (Table 2).

sample	correlation to self t =0 (baseline)	correlation to donor t =0 (baseline)	correlation to self t =d+100	correlation to donor t =d+100
recipient 1	1	-0.192820513	-0.218803419	0.68
recipient 2	1	0.33025641	0.289230769	0.51042735
recipient 3	1	0.329230769	0.536752137	0.663931624
recipient 4	1	0.517264957	0.361367521	0.246495726

healthy volunteer donors	correlation to self t =0 (baseline)	correlation to self t =+2 weeks
donor 1	1	0.748462
donor 2	1	0.756154
donor 3	1	0.789231
donor 4	1	0.742692

Table 2: *The TCRBV repertoire engrafts with a phenotype similar to that present in the donor.*

Spearman rank correlation coefficients were determined for the TCRBV repertoire expression in both recipient and donor prior to transplant (baseline) and at the conclusion of the study (day +100). A correlation coefficient of one represents a perfect correlation. All recipients, with the exception of recipient 4, had a greater correlation to the donor than to themselves at day +100. This is compared in the context of the TCRBV repertoire observed in healthy individuals over a two-week period.

With a single exception, recipient 4, we observed the engraftment of a T cell receptor phenotype with greater correlation to baseline profiles found in the donors, as opposed to those found in the recipients, prior to transplant. Recipient number 4 was the only patient in our study who received a different pre-conditioning regimen, Cytoxan and Busulfan, compared to the TT/TBI/Campath-1H regimen. One possible explanation for this difference is the highly T-cell suppressive effect observed in patients who receive a preparative regimen containing Campath-1H (anti-CD52), an agents whose effects linger due to its persistence in patient serum several weeks after its final administration.

All patients were determined to be 100% donor engrafted at day 30 post-transplant, with the exception of recipient 4, whose chimerism analysis was not performed until day 100 post transplant, at which point chimerism was also determined to be 100% donor in origin. While these results are preliminary, they suggest that the developing TCRBV repertoire in the recipient is more closely reflecting that found in the donor as opposed to that found in the recipient prior to transplant. It is unlikely that these results are attributable to mature donor T cells found within the graft as recipients received Campath therapy (anti-CD52), which targets T cells for antibody dependent lysis [6] and samples also exhibited the formation of TRECs.

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CHAPTER 5

CYCLOSPORINE A (CSA) AND TACROLIMUS (FK506) DIFFERENTIALLY ALTER T CELL RECEPTOR (TCR) EXPRESSION *IN VIVO*.

ABSTRACT

Cyclosporin A (CSA) and tacrolimus (FK506) are two common immunosuppressive agents used post blood and marrow transplantation. They exert their effects through the inhibition of calcineurin activity and subsequent inhibition of IL-2 production. IL-2 is a cytokine needed for proliferation of activated T cells, an immune cell commonly responsible for post transplant complications such as graft rejection and graft-versus-host disease. Despite similarity in their mode of action, we observed polarized effects of CSA and FK506 on the human T cell repertoire, as monitored through the expression of the T cell receptor beta variable (TCRBV) regions. To determine the possible mechanism for this difference, *in vitro* experiments using the Jurkat human T-cell line were performed. The effects of CSA and FK506 on cell viability, cell proliferation, IL-2 production, and calcineurin inhibition were determined and no significant differences between the two agents were observed. The data suggest that a secondary mechanism of action exists for the different TCRBV repertoire induced by exposure to CSA and FK506.

INTRODUCTION

Immunosuppressive agents are widely used in the treatment of various medical conditions, in particular for allogeneic blood and marrow transplantation (BMT). Post transplant recipients are at a significant risk of rejecting their graft or developing a condition known as graft-versus-host disease (GVHD). T lymphocytes are known to be the primary immune mediators of such reactions [1,1-5]. Cyclosporin A (CSA) and tacrolimus (FK506) are two commonly employed agents used to prevent allograft rejection and to decrease the risk of developing GVHD in the BMT setting [6].
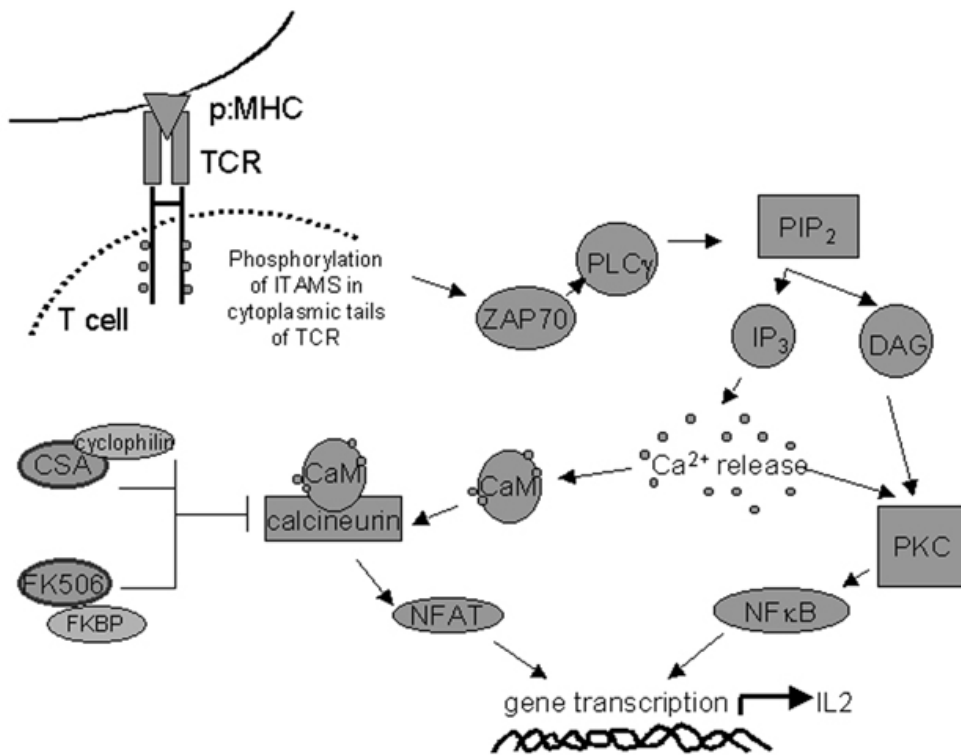
Figure 1: *Abbreviated schematic of the signaling pathway activated by engagement of the T cell receptor (TCR) and its inhibition by CSA and FK506.*

Abbreviations: p:MHC, peptide:major histocompatibility complex; TCR, T cell receptor; ITAMS, immunoreceptor tyrosine-based activation motifs; PLC, phospholipase C; PIP-2, phosphatidylinositol biphosphate; IP$_3$, inositol triphosphate; DAG, diacylglycerol; Ca^{2+}, calcium; CaM, calmodulin; PKC, protein kinase C; CSA, cyclosporin A; FKBP, FK-binding protein; NFAT, nuclear factor of activated T cells; NFκB, nuclear factor κB; IL2, interleukin 2.

85

The overall effect of CSA and FK506 is to impair the production of interleukin 2 (IL-2) by T lymphocytes. This is accomplished through the binding of CSA and FK506 to cyclophilin [7,8] and FK506-binding proteins [9-12], respectively. This binding causes the subsequent inhibition of the protein calcineurin, a protein critical to the production of IL-2 (Figure 1) [13-16]. IL-2 is produced primarily by activated T cells and is required for T cell proliferation [17]. Since extensive similarities (including mechanism of action and agent entry into cells) exist between CSA and its derivative, FK506 [18], suppression of the T lymphocyte response is expected to be comprehensive and similar between the two agents. However, we observed polarized effects of CSA and FK506 on the T cell repertoire, as monitored through T cell receptor beta variable region (TCRBV) expression patterns. FK506 suppressed the expression of the majority of TCRBV families while an increased expression in specific TCRBV families was observed with CSA. Cell viability, proliferation, IL-2 production, and calcineurin activity in response to CSA and FK506 treatments were measured *in vitro* using the Jurkat human T-cell line. In addition to their extensive usage as an *in vitro* human T cell model, Jurkat cells were selected based upon their expression of TCRBV8 [19], a TCR that showed varied responses to CSA and FK506 in our experiments. The experiments were designed to determine the potential mechanistic difference that could be responsible for the differing TCRBV expression pattern observed *in vivo*.

MATERIALS AND METHODS

Human Subjects

Study subjects (n=5) were patients who underwent allogeneic peripheral blood stem cell transplants at West Virginia University's Mary Babb Randolph Cancer Center, Morgantown, West Virginia. Informed consent was obtained from participants after the nature and possible consequences of the study had been fully explained according to West Virginia University's Internal Review Board guidelines. A volume of ~20 mL peripheral blood was collected weekly through day 100 post transplant, via venipuncture from hematopoietic stem cell transplant patients, into an acid citrate dextrose VACUTAINER™ blood collection tube (Becton Dickinson, Franklin Lakes, NJ, USA). Buffy coat layers were isolated via centrifugation at 3300 rcf for 10 minutes. Contaminating red blood cells were removed by hypotonic lysis.

Cell Lines

In vitro experiments were performed using the Jurkat human T-cell line (clone E6-1) (American Type Culture Collection, Rockville, MD). Cells were cultured at a density of 4×10^5 cells/mL in RPMI-1640 (HyClone, Logan, UT) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Hyclone), 2 mM L-glutamine (BioWhittaker, Walkersville, MD), penicillin (100U/mL) (BioWhittaker), streptomycin (100ug/mL) (BioWhittaker), and 2-mercaptoethanol (0.049 mM) (Sigma Chemical Co., St. Louis, MO), and 50 mM Hepes (BioWhittaker).

Treatments

For human studies, patients were treated with CSA and/or FK506 according to established treatment protocols at the West Virginia University Mary Babb Randolph Cancer Center's Blood and Marrow Transplant Program. CSA therapy is classically utilized when the recipient has a matched related donor (MRD) and FK506 therapy is instituted when the recipient has a matched unrelated donor (MUD) or is unresponsive post-transplant to CSA therapy. CSA and/or FK506 treatment started one day before transplant (d-1) and continued throughout the study period. Patients were monitored bi-weekly for CSA and FK506 serum concentrations. Patient dosages were adjusted, as

necessary, to be within the preferred protocol serum concentrations of 200+/-20 ng/mL for CSA and 7-12 ng/mL for FK506.

For *in vitro* cell culture studies, CSA (Bedford Laboratories, Bedford, OH) and FK506 (Fujisawa Healthcare, Inc., Deerfield, IL) were diluted in prepared media (previously described) to obtain 1/2x , 1x, and 2x of the target therapeutic dose. This resulted in the following concentrations: for CSA, 100ng/mL, 200ng/mL, and 400ng/mL; for FK506, 5ng/mL, 10ng/mL, and 20ng/mL. Cells were pretreated +/- CSA or FK506 for 30 minutes at 37°C followed by addition of the stimulating agents phorbol 12-myristate acetate (PMA) (Sigma Chemical Co.) and A23187 calcium ionophore (Sigma Chemical Co.) at 10ng/mL and 1 ug/mL, respectively.

RNA Isolation

Total RNA was isolated from 20-40 x 10^{6} white blood cells using TRIzol® Reagent according to the manufacturer's directions (Ambion, Austin, TX, USA). RNA was dissolved in ultra-PURE™ Distilled DNAse and RNAse free water (Invitrogen Corporation, Carlsbad, CA, USA). DNase treatment was performed on isolated RNA according to the manufacturer's recommendations using DNA-free™ (Ambion). RNA purity and concentration was determined by standard 260nm:280nm spectrophotometric analysis using a Genesis 10UV Spectronic Unicam (Spectronic Instruments, Rochester, NY, USA).

RT-PCR

One Step RT-PCR was performed using the QuantiTect™ Probe RT-PCR kit (Qiagen, Valencia, CA, USA). Recommended reaction mixtures were scaled down to a total reaction volume of 20 μL using 0.04 μg RNA with the following primer and probe concentrations: 0.4 μM TCRBV primer (Biosource International, Camarillo, CA, USA), 0.4 μM TCRBC primer (Biosource International), and 0.2 μM TCRBC TaqMan® probe, 5' 6-FAM, 3' BHQ™-1 (Integrated DNA Technologies, Inc., Coralville, IA, USA). The method, including primer and probe sequences, was as previously described (Brewer and Ericson, *J. Immunol. Method,* in press). In brief, 18SrRNA standardization reactions were performed in parallel using 0.4 μM each of sense and anti-sense primers

(Biosource International) and 0.2 μM 18S rRNA TaqMan® probe, 5' 6-FAM, 3' BHQ™-1 (Integrated DNA Technologies, Inc.). An iCycler™ (BioRad Laboratories, Hercules, CA, USA) was used for the RT and amplification cycles. RT was performed at 50°C for 60 minutes, max ramp speed, followed by an initial Taq activation step of 15 minutes at 95°C, max ramp speed. A TouchDown PCR approach [20] was used with the following cycling conditions: denaturation for 15 seconds at 95°C, max ramp speed, annealing for 30 seconds starting at 70°C decreasing by 2°C for 10 repeats, max ramp speed, and extension for 40 seconds at 72°C, min ramp speed. After this TouchDown of the annealing temperature, 50 cycles were performed as follows with the optical data collection occurring at the extension step: 15 seconds at 95°C (max ramp speed), 30 seconds at 52°C (max ramp speed), and 40 seconds at 60°C (min ramp speed). Reactions were held at 4°C upon the conclusion of the run. Cycle threshold (Ct) values, or the cycle at which fluorescent amplification readings exceed background level, were determined using iCycler™ iQ Optical System Software Version 3.0a (BioRad Laboratories, Hercules, CA, USA). The primer sets utilized in this study have been previously shown to yield Ct values that are directly proportional to the amount of template present in the initial reactions (Brewer and Ericson, *J. Immunol. Method,* in press).

Viability assay

3x10^6 Jurkat cells (at a density of 4x10^5 cells/mL) were plated per treatment condition. At time points 24 hours, 36 hours, and 48 hours, 1x10^6 cells were removed from the culture. Viability was determined by trypan blue exclusion (Gibco Laboratories, Grand Island, NY) and 7-AAD incorporation (BD Pharmingen, San Diego, CA). 7-AAD incorporation was performed according to the manufacturer's instructions. Stained cells were resuspended in 200 μL 1% paraformaldehyde solution. 30,000 total events were collected per sample using a Becton Dickinson FACSCalibur™ that was calibrated using Calibrite Beads™ and FACSCOMP™ software (Becton Dickinson, Franklin Lakes, NJ). Acquisition of data was performed using CellQuest™ Pro software (Becton Dickinson). Data was analyzed using Windows Multiple Document Interface (WinMDI) version 2.8

(Joseph Trotter, The Scripps Research Institute, http://facs.scripps.edu/software.html [14 December 2001]).

Cell proliferation assay

Cell proliferation was monitored by labeling cells prior to culture using the Molecular Probes Vybrant™ CFDA SE Cell Tracer Kit (Molecular Probes, Inc., Eugene, OR) according to manufacturer's instructions. $3x10^6$ CFSE labeled Jurkat cells (at a density of $4x10^5$ cells/mL) were plated per treatment condition. At time points, 24 hours, 36 hours, and 48 hours, $1x10^6$ cells were removed from the culture, spun down and washed in 1x PBS prior to being resuspend in 200uL 1% paraformaldehyde. Cells were analyzed using a Becton Dickinson FACSCalibur™ that was calibrated using Calibrite Beads™ and FACSCOMP™ software (Becton Dickinson). Acquisition of data was performed using CellQuest™ Pro software (Becton Dickinson). Results were analyzed using ModFit LT™ software (Verity Software House, Topsham, ME). A total of 30,000 events were collected and analyzed per sample.

IL-2 production

$3x10^6$ Jurkat cells (at a density of $4x10^5$ cells/mL) were plated per treatment condition. At time points, 24 hours, 36 hours, and 48 hours, $1x10^6$ cells were removed from the culture and spun down. Supernatant was harvested and used to determine IL-2 production using the BD OptEIA™ Human IL-2 ELISA Kit II (BD Biosciences, San Diego, CA) according to manufacturer's instructions. Colorimetric reactions were read on a Labsystems Multiskan MCC/340 microplate reader (Molecular Devices Corporation, Sunnyvale, CA) and data was analyzed using the Genesis Lite Version 3.0 software (Life Sciences International Ltd., Basingstoke, UK).

Calcineurin Activity

The Calcineurin Cellular Activity Assay Kit (Calbiochem, San Diego, CA) was used to determine the activity of calcineurin under various treatment condition. $4.5x10^6$ Jurkat cells (at a density of $4x10^5$ cells/mL) were pretreated with doses of CSA or

FK506 for 30 minutes at 37°C followed by stimulation with PMA and A23187 (as described above) for 0 minutes, 5 minutes, 10 minutes, 20 minutes, or 30 minutes. Cells were harvested, washed, and lysed according to kit instructions. The harvested "high speed supernatant" was desalted using Econo-Pac® 10DG Columns (Bio-Rad Laboratories). Trace contaminating salts were determined to be absent from the desalted samples using the GREEN™ reagent as described in the assay kit. Desalted "high speed supernatants" were assayed for calcineurin phosphatase activity as described by the manufacturer's instructions. Colorimetric reactions were read on a Labsystems Multiskan MCC/340 microplate reader (Molecular Devices Corporation) and data was analyzed using the Genesis Lite Version 3.0 software (Life Sciences International Ltd.).

Statistical Analyses

For TCRBV alterations in response to CSA and FK506, bivariate fit statistical analysis was employed. One-way analysis of variance with Tukey's post-hoc test was performed for all other statistical analyses.

RESULTS

Cyclosporin A and FK506 differentially impact T cells bearing specific T cell receptors.

In order to assess any alterations in the T cell receptor beta variable (TCRBV) repertoire associated with the administration of cyclosporin A (CSA) or

TCRBV	TCRBV expression change in response to increased [FK506]	p value	TCRBV	TCRBV expression change in response to increased [CSA]	p value
1	↓	0.0107	4	↑	0.0199
2	↓	0.0053	5	↑	0.0157
3	↓	0.0221	6.1, .3, .4, .6, .8	↑	0.0102
4	↓	0.0002	8	↓	0.0481
5	↓	0.0117	9	↓	0.0191
6.1, .3, .4, .6, .8	↓	0.0001	11	↑	0.0002
11	↓	0.0000	12.1	↑	0.0011
12.1	↓	0.0000	13.1-.4, .6, .7	↑	0.0018
12.2.3	↓	0.0003	15	↑	0.0009
13.1-.4, .6, .7	↓	0.0000	16	↑	0.0260
13.5	↓	0.0000	21	↑	0.0337
14	↓	0.0004	24	↓	0.0032
15	↓	0.0000	25	↓	0.0066
16	↓	0.0000			
20	↓	0.0009			
21	↓	0.0000			
22	↓	0.0100			
23	↓	0.0065			
24	↑	0.0242			

Table 1: *In vivo TCRBV expression changes in response to increases in FK506 and CSA dosages.*

Opposite trends were observed for the changes in TCRBV repertoire expression in response to the immunosuppressive agents FK506 and CSA. Results shown are derived from analysis of 5 patients, 11-14 time points/patient totaling 70 separate observations, each performed in triplicate. Reported p-values are indicative of statistical significance with a confidence interval of at least 95%, or $p<0.05$.

tacrolimus (FK506), human T cells were isolated from allogeneic peripheral (hematopoietic) stem cell transplant patients weekly up to 100 days post transplant. We hypothesized that both CSA and FK506 would have a "global" immunosuppressive

effect on the T cells within the hematopoietic stem cell recipients. Analysis of the TCRBV repertoire, however, demonstrated polarized responses of T cells to these two agents (Table 1). When TCRBV expression was analyzed in response to FK506, a predominant suppression was observed in its effects on T cells bearing specific receptors, specifically TCRBV1-6, 11-16, and 20-23. However, when samples were analyzed for the effect induced by CSA, an increase in the expression of TCRBV4-6, 11-13, 15, 16, and 21 was observed. The *in vivo* concentration of CSA and FK506 can be impacted by various factors. Calcium channel blockers, anti-fungal agents, antibiotics, and anti-inflammatory drugs are among the agents known to potentially increase the serum concentrations of CSA and FK506 (according to manufacturer). Anticonvulsants, grapefruit, and dietary supplements, such as St. John's Wort, may have the opposite effect and decrease serum concentrations of CSA and FK506 (according to manufacturer). To ensure that such metabolic alterations were not causing artificial changes in the expression of the TCRBV repertoire, serum concentrations of CSA and FK506 were also analyzed for their impact on the TCRBV expression levels. The analyses using either the actual dosage level (Table 1) or the serum level (data not shown) yielded similar results.

Cyclosporin A and FK506 affect on cell viability.

It is possible that the different TCRBV usage between CSA and FK506 *in vivo* was due to a differential effect of the two agents on T cell viability. In order to assess this potential difference in viability *in vitro* studies were performed using the T–cell derived Jurkat cell line. Jurkat cells were pretreated with a range of concentrations of CSA (0-400ng/mL) or FK506 (0-20ng/mL) prior to stimulation with PMA and A23187 and then cultured for 24 hr, 36 hr, or 48 hr at which time 7-AAD staining was done to access viability. As depicted in Figure 2, significant differences were not obtained in cell viability, despite the presence of CSA or FK506.

Figure 2: *Effects of CSA and FK506 on Jurkat cell viability as measured by 7-AAD exclusion.*

Results depicted are the means of two independent experiments. Abbreviations: no Rx, no addition of immunosuppressive agent; CSA, plus the addition of cyclosporin A; FK506, plus the addition of FK506. Experiments depicted are for target therapeutic doses of CSA (200ng/mL) and FK506 (10ng/mL).

93

Similar results were seen when cells were pretreated with any of the following concentrations of immune suppressive agents: one-half therapeutic dose (CSA: 100ng/mL, FK506: 5 ng/mL), therapeutic dose (CSA: 200 ng/mL, FK506: 10 ng/mL), or twice the therapeutic dose (CSA: 400 ng/mL, FK506: 20 ng/mL), or when viability was assessed after 24 hours or 48 hours of culture. At 24 hr, 36 hr, and 48 hr, a trend of higher cell death was observed with the pretreatment of both the therapeutic and twice the therapeutic doses of CSA compared to FK506. These difference, however, were not found to be statistically significant. Similar results were also observed when viability was assessed by trypan blue exclusion.

Treatment with Cyclosporin A and FK506 do not alter the proliferation of Jurkat cells.

With viability not noticeably altered by treatment with either CSA or FK506, the proliferative ability of the treated cells was examined next.
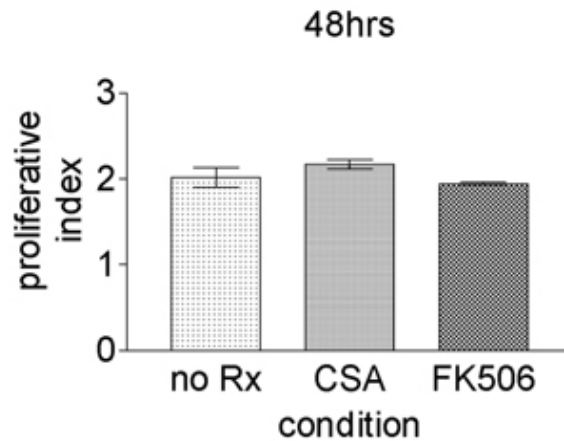
Figure 3: *Effects of CSA and FK506 on Jurkat cell proliferation as determined by CFSE cellular membrane labeling.*

The proliferative index, or the sum of the cells in all generations divided by the computed number of original parent cells present at the start of the experiment, was determined for all samples from two independent experiments with their means +/- SEM indicated. The proliferative index is a measure of the increase in cell number in the culture over the experimental period. No statistically significant differences were observed for proliferation experiments. Abbreviations: no Rx, no addition of immunosuppressive agent; CSA, plus the addition of cyclosporin A; FK506, plus the addition of FK506. Experiments depicted are for target therapeutic doses of CSA (200ng/mL) and FK506 (10ng/mL).

The proliferative index is a calculated measurement of the increase observed in the culture's cell number over the course of the experiment. As depicted in Figure 3, no statistically significant difference in cellular proliferation was observed when cells were treated with either CSA or FK506. Additionally, no statistically significant differences were detected when the cells were treated with a range of concentrations of CSA or FK506 (one-half therapeutic dose, therapeutic dose, or twice the therapeutic dose) or when cells were permitted to proliferate for 24, 36, or 48 hours.

Cyclosporin A and FK506 inhibit IL-2 production in Jurkat cells.

CSA and FK506 are known to inhibit IL-2 production by T cells [13-16]. While high IL-2 levels were produced by untreated (without the addition of CSA or FK506) stimulated Jurkat cells, significant differences were observed between the levels of IL-2 produced by treated and untreated samples, regardless of the concentration of the agents used (one-half therapeutic dose, therapeutic dose (Figure 4), or twice the therapeutic dose) or the length of the culture period (24, 36, or 48 hours). In addition, there was no statistically significant difference found in the suppression of IL-2 production between CSA and FK506 treatments. Both agents were found to inhibit IL-2 production equally and were determined to not interfere with the *in vitro* IL-2 detection assay.

Figure 4: *Effects of CSA and FK506 on IL-2 secretion by Jurkat cells as measured by ELISA.*

Data is representative of the means of two independent experiments +/- SEM. Statistical significances are noted. Dotted lines indicate assay limit of detection. Abbreviations: no Rx, no addition of immunosuppressive agent; CSA, plus the addition of cyclosporin A; FK506, plus the addition of FK506. Experiments depicted are for target therapeutic doses of CSA (200ng/mL) and FK506 (10ng/mL).

Calcineurin activity is inhibited equally by both CSA and FK506.
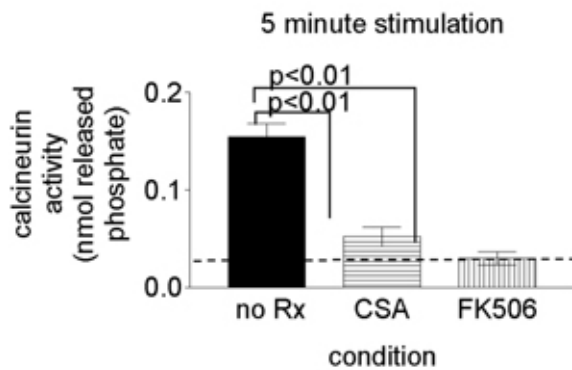
Figure 5: *Effects of CSA and FK506 on calcineurin activity in Jurkat cells.*

Data is representative of the means of two independent experiments +/- SEM. Statistical significances are noted. Dotted lines indicate assay limit of detection. Abbreviations: no Rx, no addition of immunosuppressive agent; CSA, plus the addition of cyclosporin A; FK506, plus the addition of FK506. Experiments depicted are for target therapeutic doses of CSA (200ng/mL) and FK506 (10ng/mL).

The inhibition of IL-2 production by both CSA and FK506 is known to occur by the binding of these agents to their binding partners cyclophilin and FK-binding protein, respectively, and their subsequent inhibition of calcineurin [7-12]. Therefore, the activity of calcineurin in Jurkat cells treated with CSA or FK506 was measured *in vitro*. Jurkat cells were treated with the therapeutic doses of CSA and FK506 and stimulated with PMA and A23187 calcium ionophore for 5, 10, 20, or 30 minutes prior to cellular protein collection. As anticipated, cells treated with either CSA or FK506 resulted in a significant decrease in the activity of calcineurin compared to untreated control cells (Figure 5). Significant differences, however, were only observed at the five-minute time point and no significant difference was ever observed between CSA and FK506. Additionally, it was determined that neither agent interfered with the detection of the *in vitro* calcineurin activity assay.

DISCUSSION

Despite the fact that both CSA and FK506 inhibit IL-2 production by T cells, different effects were observed on the T cell receptor repertoire in the presence of these immunosuppressive agents in peripheral blood samples from patients undergoing allogeneic peripheral blood stem cell transplant. This observation, however, cannot be attributed to differences in the viability, proliferation, IL-2 production, or calcineurin activity of Jurkat cells treated with CSA versus FK506 *in vitro*.

Since the *in vivo* setting is rather complex in experimental variables, we chose to perform *in vitro* experiments using the Jurkat human T-cell line. T helper cells have been documented to be the main target of CSA and FK506 (according to manufacturers), and this was an additional reason for using Jurkat cells (CD4^{+}) in *in vitro* experiments. An additional advantage to using this cell line is that Jurkat cells express TCRBV8 [19], a TCR that showed varied responses to CSA compared to FK506 (Table 1). A decrease in TCRBV8 was seen with CSA whereas no significant response was observed with FK506 treatment.

Two previous reports detailed the changes in the expression of TCRBV8 in response to CSA therapy. Fischer et al., described an increase in TCRBV8.5 in mice following administration of CSA therapy [21] and Severino et al., reported an increase in the expression of TCRBV8 in CSA-induced murine syngeneic graft-versus-host disease [22]. Although it has been determined that great homology exists between the TCRBV families in mice and humans [23], there is no report of TCRBV8.5 in humans [24], making these specific murine studies difficult to directly compare to our study. They do, however, suggest that CSA can exert differed effects on cells carrying a specific TCRBV.

While the inhibition of IL-2 production through the blocking of calcineurin is the accepted mechanism of action for both CSA and FK506 [13-16], a report by Marton et al., in yeast cells has alluded to the possible existence of a calcineurin-independent pathway for FK506-mediated effects [25]. Using a yeast model and various mutants, they demonstrated that many of the GCN-4 regulated genes were induced by exposure to FK506. GCN-4, a c-Jun/c-Fos homolog, is a eukaryotic transcriptional activator protein that is primarily responsible for the regulation of biosynthetic genes in *Saccharomyces*

cerevisiae [26-29]. Although different model systems, both humans and yeast are eukaryotic and a crossover in this alternative pathway may exist. There is also evidence that there is different transcriptional control in humans depending upon the TCRBV usage, with factors such as the promoter and non-coding (spacer) regions contributing to these differences [30-35].

Our results argue that a secondary mechanism of action for the altered TCRBV repertoire must exist for these immunosuppressive agents as none of the above mentioned parameters, including calcineurin activity, IL-2 production, cell viability and proliferation, exhibited differing effects in response to CSA and FK506 treatments. While the possibility still exists due to our small sample size, we do not believe that these results are attributed to donor variability as results reported were observed across the population of patient/donor pairs, not unique to any single case. Future investigation into alternative mechanisms may yield a clearer understanding of the cellular events responsible for the varying effect on T cells by the related immunosuppressive agents CSA and FK506.

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CHAPTER 6

GENERAL CONCLUSIONS AND DISCUSSION

The overall goal of this investigation was to identify and study the role of specific T lymphocytes following human hematopoietic stem cell (HSC) transplant. Current literature has stressed the importance of T lymphocytes in this setting but has thus far focused on the identification of specific families of T lymphocytes as it pertains to the occurrence of graft-versus-host disease (GVHD). Several novel findings have emerged from our investigation: (1) a panel of oligonucleotides has been developed to accurately and efficiently detect expression of all known human TCRBV regions, (2) specific TCRBV families are associated with reactivation of cytomegalovirus (CMV) post HSC transplant with many of these same TCRBV families also being associated with the occurrence of GVHD, (3) the TCRBV repertoire engrafts in the recipient with a profile more similar to that found in the donor as opposed to that found in the recipient prior to transplant, and (4) the similar immunosuppressive agents, cyclosporin A (CSA) and tacrolimus (FK506), differentially alter the TCRBV repertoire with their administration.

Prior investigations have used the TCRBV regions to identify specific T cells involved in various settings, such as with GVHD. While our study had initially planned on utilizing prior existing technology to further investigate the role of T lymphocytes in the post HSC transplant environment, we were unable to locate a method that accurately examined all of the TCRBV genes. To address this, we relied on the TCR gene classification system instituted by the World Health Organization. Using various sequence databases and strict primer sequence specifications, we were able to develop a panel of primers that could be used to accurately and efficiently identify all 91 alleles of the human TCRBV region. Using these primers and sequence specific (TaqMan® probe) real-time PCR detection, we demonstrated that our system was not only specific but also efficient and comparable to TCRBV protein expression profiles. The importance of this technology was realized and has since been protected by both U.S. and foreign patent applications.

Once a method was available to accurately identify T cells based upon their TCRBV expression, we wanted to apply this technology to our specific area of interest, the post HSC transplant setting. Many prior investigations using human HSC transplant patients have focused on identifying the T cells associated with GVHD. These studies relied on "snapshot" sampling, for example collecting a sample early post transplant and at the time of GVHD diagnosis. Many of these studies also aimed to determine if there was an association of specific T cells in the peripheral blood (PB) and in the GVHD tissue lesion, the lesion frequently being a skin biopsy. In these studies, though, such an association was rarely seen. We believed one explanation for the lack of association of PB and GVHD lesion TCR repertoires was due to the fact that PB and tissue samples were drawn simultaneously. This timing of sample procurement would not accounting for the fact that (1) the development of GVHD is not an instantaneous process and, (2) the T cells expanded in the PB would traffic to the lesion after expansion, resulting in an efflux of these cells from the PB and an influx of these cells into the lesion. In our study, we instituted a serial sampling schedule where baseline samples were collected from both the donor and recipient prior to transplant, with recipient sample collection continuing on a weekly basis post HSC transplant, up to day 100. Day 100 was chosen as our final time point since acute GVHD, by definition, occurs no later than day 100 post transplant. The chronic form of the disease may not appear for several months to years after transplant, yielding a sampling schedule not conducive to our time frame. While our initial plans of obtaining a GVHD lesion sample at time of diagnosis was not fulfilled, we were able to collect PB samples from our study patients on a regular basis. This led us to analyze not only what TCRBV families were involved in GVHD but also those families involved in other post HSC transplant events.

Our HSC transplant center here at West Virginia University has frequently observed the association of CMV reactivation post transplant and the subsequent development of GVHD. Although there are several studies that support the notion that a link does indeed exist between CMV and GVHD, no such study has looked at the similarity in TCRBV expression with these two complications post HSC transplant. We not only determined which TCRBV families were associated with CMV reactivation in this setting, but were also able to draw parallels between many of these TCRBV families and those also associated with GVHD. Further, many of the families we determined to be associated with CMV reactivation have been previously shown to be CMV reactive in what are considered otherwise healthy human donors. Not only is our study the first to identify those TCRBV families associated with CMV reactivation post HSC transplant, we were also the first to show an overlap in the TCRBV families associated with CMV and GVHD.

Since we had serial TCRBV analysis of our patients, we utilized our data sets to determine if the transplanted donor HSCs developed with a phenotype more reminiscent of that found in the recipient prior to transplant or with greater similarity to the mature profile found in the donor. We expected that the influence of the recipient's environment on these developing HSC would consequently lead to the development of a TCRBV repertoire more reminiscent of that found in the recipient prior to transplant. However, our samples exhibited an engraftment profile with a greater correlation to that found in the donor prior to transplant compared to that initially found in the recipient. While we had a small sample population (n=4) these are the first results we can find to investigate this question. Furthermore, we do not believe these results are attributable to analysis of mature donor T cells initially found within the graft as the patients that exhibited this trend all received Campath-1H therapy that specifically targets the destruction of any residual mature donor T cells found within the graft. The one patient that exhibited a greater correlation to herself than to that found in the donor at the conclusion of the study was the sole patient in our population that did not receive the Campath-1H regimen, but received an alternate myeloblative regimen. These results are preliminary but do inspire further thoughts into the contribution of the recipient environment on the development of the transplanted donor cells.

As a consequence of serial time point analysis and the treatment of all of our study participants with immunosuppressive agents, we observed that the highly similar immunosuppressive agents CSA and FK506, which are both calcineurin inhibitors, had polarized effects on the TCRBV repertoire. FK506 administration resulted in an overall suppression of TCRBV expression while the opposite effect was observed with CSA therapy. Interesting to this observation is the fact that in the HSC transplant setting, FK506 administration is customarily reserved for situations in which greater genetic disparity exists between the recipient and donor, with CSA administration typically being utilized for those transplants with fewer mismatch-related complications anticipated. Potentially our observation could help to explain the immunology behind such pharmacological strategies. To try to determine if there was some facet of the known immunosuppression mechanism of these two agents responsible for contributing to our observed difference, we examined the effect of these two agents *in vitro* using the human Jurkat T cell line. We determined that while both of these agents did decrease the activity of calcineurin and, subsequently inhibited the production of IL-2, they did so equally well.

Taken together, we feel we have contributed to the understanding of the role of specific T cells in the post HSC transplant environment. While our sample population was large compared to prior similar investigations, we hope that future studies will confirm our results using larger sample populations. Not only has our work contributed to the field of HSC transplantation but also to the field of immunology as a whole. Our TCRBV expression technology vastly improves upon that which existed in the field prior to our entry, and we anticipate the application of our technology to many areas of immunological interest, including the usage of specific TCRBV families during infectious disease, autoimmune disease, graft rejection, and even cancer therapy and graft-versus-leukemia effect. Future work will hopefully elucidate a greater understanding of the association of CMV reactivation and GVHD with the identification of specific antigens driving these T cell responses. The possible existence of a secondary mechanism of action for CSA and FK506 will also be investigated in the future and will help to encourage a re-thinking of present treatment strategies. Should all patients not

be given FK506 as opposed to the present directing of patients into treatment groups based upon the relation of the donor?

In conclusion, one of the most interesting facets of this project was the identity it took over time. As data was generated and the novelties of patient sampling became more apparent, areas that were initially anticipated to be of less interest became increasingly curious and of interest. It reasons that one of the facets to being a good scientist is to set aside pre-conceived notions and approach the data with neutrality. By these means truths are discovered and the field of science marches onward. After all, "the true scientist never loses the faculty of amazement".

APPENDIX

Amplification efficiency analysis

Amplification efficiency analysis for TCRBV primers was determined using the following formula:

Efficiency (E) = $(10^{-1/slope})-1$

Where the slope is determined by plotting the Ct value (Y-axis) vs log [cDNA] (X-axis) using 10-fold dilutions of template

Example of equation usage:

slope of amplification plot is determined to be -3.59

$E = (10^{-1/-3.59})-1$

$E = 10^{0.28}-1$

$E = 1.90-1$

$E = 0.90$, or 90% efficient

Reference: Rasmussen, R. (2001) Quantification on the LightCycler. In Meuer, S., Wittwer, C. and Nakagawara, K. (eds), Rapid Cycle Real-time PCR, Methods and Applications. Springer Press, Heidelberg, pp. 21-34.

IOTest™ Beta Mark TCR VB Repertoire Kit

The IOTest™ Beta Mark TCR VB Repertoire Kit (Beckman Coulter, Inc., Fullerton, CA) was used for TCRBV protein expression analysis. Samples were prepared according to the manufacturer's directions using 5×10^5 buffy coat white blood cells per sample, prepared as previously described. To identify CD3$^+$ cells for TCR analysis an anti-CD3 PC5 antibody and an IgG1 PC5 isotype antibody, each at a volume of 10μl/sample (Beckman Coulter, Inc.) were used as described in the IOTest™ kit specifications. Stained samples were then washed in 1X PBS prior to fixation in 500 μl of 1% paraformaldehyde. All prepared samples were analyzed by flow cytometric analysis using a FACScan (Becton Dickinson, Franklin Lakes, NJ), which had been calibrated using three color Calibrite Beads (Becton Dickinson) and FACSCOMP software (Becton Dickinson). A total of 5,000 live-gated CD3$^+$ cells were collected for analysis of each IOTest™ TCR BV expression sample. SSC and FSC data were acquired in the linear mode and the FL1, FL2, and FL3 parameters were collected logarithmically. Data analysis was performed using Windows Multiple Document Interface (WinMDI) version 2.8 (Joseph Trotter, The Scripps Research Institute, http://facs.scripps.edu/spftware.html).
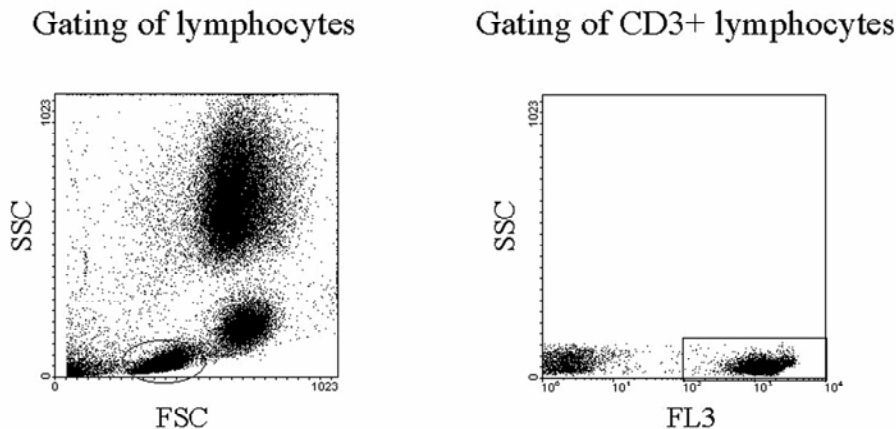
Gating of CD3$^+$ lymphocytes for IOTest TCRVB antibody analysis.
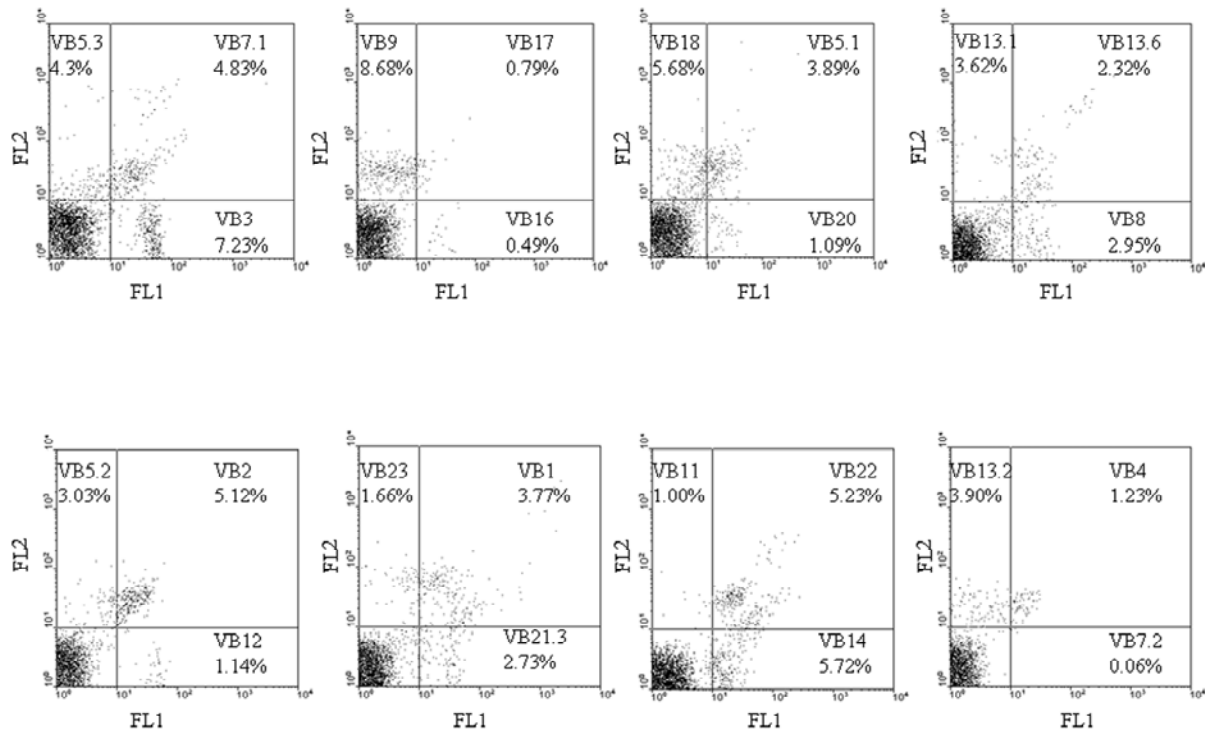
IOTest TCRVB antibody flow cytometric results expressed as percent CD3⁺ cells.

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1998	Johns Hopkins Medical Institute Immunology laboratory; studied HIV vaccine development under the direction of Dr. Robert F. Siliciano
1998	National Institutes of Health Study Group (top national applicant to accompany Colgate University study team); studied HIV pathogenesis under the direction of Dr. Michael J. Lenardo
1999	West Virginia University Health Sciences Center Cancer Center; studied AFAP-110, a cytoskeletal protein, and its structure under the direction of Dr. Daniel C. Flynn
2000	National Institute of Occupational Safety and Health (NIOSH), Centers for Disease Control, Morgantown, West Virginia; studied T cell cryopreservation under the direction of Dr. Daniel C. Lewis

2000-2005   West Virginia University Blood and Marrow Transplantation
             Laboratory; studying T cell recovery (including biological and
             therapeutic interactions) in patients after blood and bone marrow
             transplantation under the direction of Dr. Solveig G. Ericson

2001-2005   obtained and maintain Internal Review Board approval and
             certification for research project

current   certified training in the following: chemical safety, handling of blood
             borne pathogens, handling of patient related samples and
             information (HIPPA), as well as NIH sponsored training for the
             usage of human research subject (HPPERT)

Scientific Affiliations

1995-present Beta Beta Beta Biological Honorary
             1996-1997 treasurer of Eta Theta chapter
             1997-1998 vice president of Eta Theta chapter
             1997-1998 outstanding member of the year

1995-1999   American Chemical Society

1996-2002   West Virginia Academy of Sciences

1997-present Association for Women in the Sciences

1999   *Discover* Magazine reader advisory panel

1999-2001   American Society of Microbiologists

External, competitive funding

1997   Association for Women in the Sciences/National Science
             Foundation grant recipient ($2,000.00)

1998   Association for Women in the Sciences/National Science
             Foundation grant recipient ($2,000.00)

1998   Burnside Academic Scholarship recipient ($2,000.00)

1999   Burnside Presidential Scholarship recipient ($3,000.00)

2001   Burnside Presidential Scholarship recipient ($1,000.00)

Publications

1. **Brewer, J.**, Frankenberry, M., Sheil, J. Important features of class I major histocompatibility complex (MHC) molecules for alloreactive T cell recognition. WV Academy of Sciences 70(1):6, 1998.

2. Ericson, S., Henderson, A., **Brewer, J.**, Kavanaugh, K., Blobaum, A., Gibson, L. Anti-thymocyte globulin treatment of neutrophils in vitro can induce inflammatory responses as well as cell death. Experimental Hematology 29(8) Supp. 1:62, 2001.

3. **Brewer, J.**, Ericson, S. Developing a methodology to detect human T cell receptor variable family gene expression patterns. WV Academy of Sciences, accepted, 2002.

4. **Brewer, J.**, Ericson, S. Developing a methodology to detect human T cell receptor variable family gene expression patterns in graft-vs-host disease patients. International Society for Experimental Hematology, accepted, 2002.

5. Brewer, J., Henderson, A., Kavanaugh, K., VanDyke, B., Blobaum, A., and Ericson, S. The effects of antithymocyte globulin (ATG) on human neutrophil functioning. *Manuscript in preparation for submission.*

6. **Brewer, J.,** and Ericson S. *U.S. and Foreign Patents Pending*: Development of a methodology to detect expression of human T cell receptor variable family gene expression patterns.

7. **Brewer, J.,** and Ericson S. An Improved Methodology to Detect Human T cell Receptor Beta Variable Family Gene Expression Patterns. *In press, 2005, Journal of Immunological Methods*

8. **Brewer, J.,** and Ericson S. Similarity in repertoire of T cells associated with the occurrence of Cytomegalovirus reactivation and Graft-versus-Host Disease post human hematopoietic stem cell transplantation. *Manuscript submitted.*

9. **Brewer, J.,** and Ericson S. Cyclosporine A (CSA) and tacrolimus (FK506) differentially alter T cell receptor (TCR) expression *in vivo. Manuscript submitted.*

10. **Brewer, J.,** and Ericson S. Engraftment of T cell receptor (TCR) repertoire in myeloablated allogeneic hematopoietic stem cell recipients mimics donor TCR repertoire. *Manuscript submitted.*

Laboratory Technique Experience

RNA, DNA, and protein isolation
gel electrophoresis and SDS-PAGE
Southern Blotting
Western Blotting
Flow cytometry and FACS analysis
PCR, RT-PCR, Realtime PCR
DNA sequencing
Primer and Taqman probe design
Genomic analysis
ELISA
Cell separation: columns, density, and magnetic separation
CsCl gradient ultracentrifugation
radioactive isotype labeling
chromium release assay
dissection and organ harvesting
intraperitonial injections
sterile cell culture
bacterial culture, including selective and differential media culturing
viral culture
HPLC
NMR
Gas Chromatography
spectroscopy
Infrared Analysis (IR)
Ultraviolet Analysis (UV)
Atomic Absorption (AA)
titrimetric analysis
potentiometric analysis
DNA Methylation
cloning
recombination and transformation procedures
restriction mapping
genomic library construction
DNA sequencing
TUNEL
caspase assays
annexin analysis
cell proliferation analysis (CFSE tracking)
confocal microscopy
electron microscopy
transwell assays
venipuncture techniques and peripheral blood cell isolation
P3 laboratory experience

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