2011

DISTINCT T CELL CLONES ARE ASSOCIATED WITH GRAFT-VERSUS-HOST DISEASE (GVHD), AND POTENTIALLY GRAFT-VERSUS-TUMOR (GVT), RESPONSES FOLLOWING ALLOGENEIC STEM CELL TRANSPLANTATION

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A dissertation submitted in partial fulfillment of the requirements for the degree of Master's of Science at Virginia Commonwealth University.

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April 2011

Acknowledgement

First and foremost, I'd like to thank my parents for their unwavering love and support in all of my endeavors. I'd like to thank Maciej Kmieciak for teaching me how to be efficient with my experiments, and I'd like to thank my advisor, Dr. Masoud Manjili, for his guidance throughout my time in the lab. I also offer thanks to Michelle Detwiler and Chris Borrelli at Roswell Park Cancer Institute in New York for all of their work and help with my spectratyping data.

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HSCT: hematopoietic stem cell transplantation

HSCs: hematopoietic stem cells

MHC: major histocompatibility complex

HLA: human leukocyte antigen

GVHD: graft-versus-host disease

mHAgs: minor histocompatibility antigens

KIR: killer cell immunoglobulin-like receptors

OS: overall survival

BM: bone marrow

PBSC: peripheral blood stem cells

aGVHD: acute graft-versus-host disease

cGVHD: chronic graft-versus-host disease

MUD: matched unrelated donor

MRD: matched related donor

APC: antigen-presenting cell

TCR: T cell receptor

CTL: cytotoxic T lymphocyte

ISP: immunosuppression

TCD: T cell depletion

GVT: graft-versus-tumor

ECP: extracorporeal photophoresis

ATG: anti-thymocyte globulin

DLI: donor lymphocyte infusion

TAA: tumor-associated antigen

CDR: complementarity-determining region

NHL: Non-Hodgkin's Lymphoma

MM: multiple myeloma

CLL: chronic lymphocytic leukemia

PLL: prolymphocytic leukemia

CR: complete response

PR: partial response

MMF: mycophenolate mofetil

Tac: Tacrolimus

HPE: homeostatic peripheral expansion

TRM: treatment related mortality

SNPs: single nucleotide polymorphisms

CIBMTR: Center for International Blood and Marrow Transplant Research

Abstract

DISTINCT T CELL CLONES ARE ASSOCIATED WITH GRAFT-VERSUS-HOST DISEASE (GVHD), AND POTENTIALLY GRAFT-VERSUS-TUMOR (GVT), RESPONSES FOLLOWING ALLOGENEIC STEM CELL TRANSPLANTATION

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In patients undergoing hematopoietic stem cell transplantation (HSCT) with HLA-identical donors, genetic polymorphisms result in a mismatch between donors and recipients in their minor histocompatibility antigens (mHAgs), and tumors may also express tumor-associated antigens (TAA) that may not be abundantly present in the donors. Donor T cells can recognize such mHAgs and TAAs as foreign antigens and generate an objective response against hematologic malignancies in a graft-versus-tumor (GVT) effect. However, a major side effect of HSCT occurs when donor T cells are alloreactive against the recipients' normal cells, leading to graft-versus-host disease (GVHD). The ability to identify T cell clones that are exclusively involved in the GVT or GVHD responses remains elusive. In this study, we looked at clonally-driven $CD3^+$ T cells in patients with hematologic malignancies prior to and after transplantation. We identified Vβ families of increased expression

involved in GVHD or GVT responses, with Vβs 4, 11, and 23 being associated with GVHD, Vβs 9, 16, and 20 being associated with GVT, and Vβs 2, 3, 7, 8, 12, 15, and 17 being involved in GVHD and/or GVT. We were also able to identify some of the Vβ families that were increased in the peripheral blood at the site of GVHD. Furthermore, one of our twelve patients had donor lymphocyte infusions (DLIs) for treatment of relapse, from which we were able to observe oligoclonal T cells that emerged at the time of post-DLI remission and re-establishment of GVHD.

Introduction

Hematologic cancers

It was estimated that over 1.5 million people were diagnosed with cancer in 2010, and that over 500,000 died from their disease.[1] Of these diagnoses, over 43,000 were with leukemia, roughly 74,000 with lymphoma, and about 20,000 with myeloma.[1] Leukemia, lymphoma, and myeloma are all blood cancers that derive from hematopoietic stem cells (HSCs). Leukemias are defined by their cell precursors—either lymphoid or myeloid. Lymphomas are derived from lymphocytes, and can be further classified as Hodgkin's or Non-Hodgkin's Lymphoma. Myelomas are derived from plasma cells. Regardless of which cell types these cancers arise from, they are each a result of a dysregulation and malignant state of the hematopoietic system. The treatment of many blood cancers requires high doses of chemotherapy and/or radiation treatments that destroy not only cancer cells, but also the patient's healthy bone marrow.

Hematopoietic stem cell transplantation

Hematopoietic stem cell transplantation (HSCT) involves intravenous infusion of hematopoietic stem and progenitor cells to restore normal hematopoiesis and/or treat blood malignancies. The clinical application of HSCT originated in the clinical observations of severe myelosuppressive effects from radiation among nuclear bomb survivors at Nagasaki and Hiroshima.[2] In the 1950s and 1960s, research efforts were made to develop means of reversing myelosuppressive effects, including the infusion of bone marrow. Subsequent determination and understanding of the major histocompatibility complex (MHC) and human leukocyte antigens (HLA) as the major determinant of graft rejection helped to

advance the clinical application of HSCT.[2] Since the first successful transplantations in the 1960s and 1970s, HSCT is now a standard treatment, and is performed in patients with various malignant and non-malignant disorders in order to restore lymphohematopoiesis, replace diseased marrow, and reconstitute lymphohematopoiesis after marrow-ablative conditioning regimens.[2,3]

Prior to transplantation, patients undergo preparative regimens that are either myeloablative or non-myeloablative. The three major purposes of myeloablative conditioning regimens are to: eradicate malignant disease, suppress the recipient's immune system (to decrease the chance for graft rejection), and create space in the bone marrow microenvironment to allow engraftment of the donor stem cells.[3] The donor HSCs, also referred to as the graft, may be autologous (from the patient him- or herself), syngeneic (from an identical twin), or allogeneic (from any person other than the patient or an identical twin.[2]

The success of HSCT in treating hematological malignancies is continually limited by acute complications such as infectious conditions, veno-occlusive disease of the liver, and acute and chronic graft-versus-host disease (GVHD).[3] Due to preparative regimens with chemotherapy and/or radiation before HSCT, recipients suffer deficiencies in their cellular and humoral aspects of their immune system for a variable duration.[3] The cellular immunodeficiency involves decreased T cell response to alloantigens and mitogens, decreased helper $CD4^+$ T cell function, and decreased activity to intradermal skin tests. Due to the thymic involution in recipients, T cell function is dependent upon the peripheral expansion of the few donor T cells that are present in the graft.[3] Humoral immunodeficiency consists of a decrease in IgG isotype switching and antigen-specific responses, resulting in an impaired production of antibodies to pathogens.[3] Recovery of the immune system occurs within the first six months to year after HSCT. However, immunodeficiency is prolonged and more profound in patients with GVHD who

are under immunosuppressive therapy, and recovery of the immune system for these patients occurs after the discontinuation of their GVHD therapies.[3]

Graft-versus-host disease

Graft-versus-host disease develops when immunocompetent donor T cells are alloreactive against recipient tissues and organs.[4,5] In 1966, Billingham outlined three general criteria for GVHD: 1) the donor cells must be immunologically competent; 2) the recipient must be immunocompromised, or incapable of rejecting the graft; and, 3) the recipient must express tissue antigens that are not present in the donor, such that they may be recognized as non-self.[4-6] The genetic basis for the third criterion, that GVHD is a result of donor cells recognizing host antigens as non-self, is derived from genetic polymorphisms of the HLA and non-HLA systems (minor histocompatibility antigens (mHAgs), killer cell immunoglobulin-like receptors (KIRs), cytokines, and NOD2 genes).[7,8] As a result of alloreactive T cells recognizing these genetic incompatibilities, there is poorer overall survival (OS) in those individuals who develop GVHD.[9]

Risk factors for GVHD include: donor-recipient match at MHC loci (mismatches in HLA class I HLA-A, -B, -C or class II HLA-DRB1), donor stem cell source (bone marrow (BM) or peripheral blood stem cells (PBSC)), T cell dose, and other factors including donor and recipient age, donor-recipient sex mismatch (male recipient of female graft), donor parity and allosensitization, disease stage, and intensity of conditioning.[10] General associations have been made with certain allele-level mismatches in the HLA genes, with mismatching of class I alleles being associated with increased risk of GVHD or graft rejection, whereas mismatching of the class II alleles has been associated with increased incidence of GVHD with no impact on graft rejection.[9,10]

Today, GVHD is broadly categorized as chronic or acute. Historically, GVHD was classified as chronic if it presented after 100 days post-HSCT, regardless if it was clinically indistinguishable from

acute GVHD (aGVHD).[11] This classification was not satisfactory because acute and chronic GVHD have distinct clinical features that may sometimes present concomitantly and/or independent of the actual duration after transplant. The NIH consensus now includes classification of GVHD as late-onset aGVHD (after day 100) and an overlap syndrome that has features of both acute and chronic GVHD.[8,11]

Acute GHVD

Acute GVHD is graded (I-IV) based on the extent and severity of the involvement of the three major affected organs—the skin, liver, and gastrointestinal (GI) tract.[3] Grades II to IV occur in roughly 20-50% of patients who receive stem cell grafts from an HLA-matched sibling and in approximately 50-80% of patients who receive grafts from an HLA-mismatched sibling or an HLA-matched unrelated donor (MUD).[3] Long-term survival of patients with aGVHD is roughly 50% for those with grades 0-I and is as low as 11% for those with grade IV.[10] Acute GVHD is characterized by dermatitis, hepatitis, and enteritis. The onset of aGVHD is usually marked by a maculopapular rash involving the face, trunk, extremities, palms, soles, and ears.[10]

There is a general three phase model of the development of aGVHD: 1) activation of antigen-presenting cells (APCs); 2) donor T cell activation, proliferation, differentiation, and migration; and 3) destruction of target tissue.[4,5,7,8] The immune response is primed during phase one, in which APCs are activated by underlying disease and conditioning regimens.[7,8,12] Conditioning regimens cause tissue damage, resulting in the production of "danger signals," including pro-inflammatory cytokines and chemokines, microbial products (i.e. – LPS, CpG) that enter systemic circulation from damaged intestinal mucosa, and necrotic cells.[4,5,8,13,14] The inflammatory response promotes the activation and maturation of APCs, in which there is an increased expression of MHC, adhesion, and costimulatory molecules on host APCs, leading to an enhanced ability of donor T cells to recognize alloantigens, and subsequently become activated and proliferate.[8,15] Phase two represents the core of the GVHD reaction

in which donor T cells are activated in response to host APC. T cell activation is induced when T cell receptors (TCRs) recognize peptide:MHC complexes on APCs, and further requires a second signal which is generated by ligation of costimulatory molecules on APCs with their cognate receptors on T cells.[7] These activated alloreactive T cells expand and differentiate into Th1/Tc1 or Th2/Tc2 cells, each of which has been associated with differences in the manifestation of GVHD,[7] though Th1- and Tc1-type cytokines (IL-1, IL-6, and TNF-α produced by macrophages and monocytes; IFN-γ and IL-2 produced by CD4 Th1 and CD8 Tc1 cells) are predominantly associated with development of aGVHD.[16] Once activated, these T cells migrate to target tissues and subsequently recruit other effector leukocytes. The homing of these T cells is regulated by adhesion, addressin, and chemokine receptor molecules in response to the production of chemokines in the injured target tissue, which is initiated by the conditioning regimen and is later amplified by the disease process.[7] The effector, and third phase, is a complex cascade of both cellular mediators (such as cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells) and soluble inflammatory mediators (such as TNF-α, IFN-γ, IL-1, and NO) that cause destruction of target tissue.[8,15] With regards to cellular effectors, CTLs that preferentially use the Fas/FasL pathway of target lysis appear to predominate in GVHD damage of the liver (as hepatocytes express large amounts of Fas), whereas CTLs that use the perforin/granzyme pathways are more important in GVHD of the GI tract and skin.[8] This effector stage of GVHD is perpetuated by the increased inflammatory signaling that is caused by tissue damage.[7] Inflammatory effectors may be secreted as a result of stimulation of toll-like receptors (TLRs) by microbial products (such as LPS) that leak through intestinal mucosa or skin that is damaged from conditioning regimens.[4,8] For this reason, it is thought that Peyer's patches in the gut are initial sites of donor T cell activation by host APCs.[7,8]

Chronic GVHD

Chronic GVHD can occur in up to 60% of patients who survive beyond 100 days after allogeneic

HSCT, and is a complication that develops more frequently 3 to 6 months after engraftment.[10,17,18] In roughly 20% of patients with cGVHD, there is no evidence of previous aGVHD, suggesting that the two disease types have different pathogenesis. Chronic GVHD is clinically manifested in a manner similar to that of autoimmune disorders, particularly scleroderma,[3] and in contrast to aGVHD, is often characterized by fibrosis of the affected tissue or organ.[8] There are a multitude of manifestations of cGVHD, including: sicca syndrome (drying of the mouth and eyes), skin lesions (hypo- or hyperpigmentation, decreased elasticity, and loss of hair follicles and sweat glands), oral mucositis, esophageal strictures, kerato-conjunctivitis, malabsorption, hepatic involvement with hyperbilirubinemia, and suppressed hematopoietic reconstitution.[3] Ten to twenty percent of patients with cGVHD develop bronchiolitis obliterans, which is associated with hypogammaglobulinemia and poor outcome.[3]

Like aGVHD, cGVHD is also thought to be induced by donor T cells, but the nature of relevant antigens and T cell subsets, as well as the mechanisms of cGVHD, are less well understood.[3,8] There is an absence of appropriate experimental models that mimic all of the features of cGVHD, and this might be due to differences between the human and experimental species.[8,17] Current animal models for cGVHD are difficult to relate to patient outcomes since chronic models more closely mimic non-myeloablative transplants than full ablative allogeneic transplants, thus making the pathophysiology of cGVHD still poorly defined.[17] Another difference is that, in contrast to murine studies, the kinetics of clinical cGVHD is slower and only observed after prophylaxis and/or treatment for aGVHD. Even if clinical cGVHD arises *de novo* and in the absence of active immunosuppression (ISP), it is not possible to definitively rule out the impact of either GVHD prophylaxis and/or subclinical aGVHD on the subsequent development of cGVHD.[8] Some experimental models have shown that T cells from animals with cGVHD are specific for a common (shared between donor and host) determinant of MHC class II

molecules and are consequently considered "autoreactive."[8] Such autoreactive T cells are associated with a damaged thymus and the inability to delete autoreactive clones.[17] It has also been shown that autoreactive T cells can interact with IFNγ to produce the increased collagen deposition seen in chronic GVHD of the skin.[17] Many experimental models of cGVHD have been characterized by Th2-type responses involving upregulation of Th2 cytokines (IL-4, IL-5, IL-10, TGF-β) and antibody-mediated damage.[7,15-17] The association of antibodies with cGVHD is also supported by the clinical observation in female to male HSCT in which antibodies specific to Y chromosome-encoded mHAgs are present.[8] Aside from the clinical correlation of antibodies with cGVHD, both Th1 and Th2 responses have been implicated in humans post-HSCT.[8,19]

Despite experimental evidence and clinical resemblance to autoimmune diseases, there is a lack of clear clinical data on the isolation of donor-derived T cell clones that recognize non-polymorphic antigens from both the recipients and donors.[8] Instead, emerging clinical data show a strong correlation between the presence of immune responses against ubiquitously expressed mHAgs and cGVHD.[8,20] Acute GVHD is recognized more as a generalized state of donor-derived inflammation, in which tissue damage is mediated by activated donor effector cells and inflammatory cytokines.[16] On the other hand, cGVHD is more representative of chronic antigen-stimulated specific cellular and humoral immune responses directed against specific epithelial tissues and hematopoietic cells.[8,16]

Current prophylaxis of GVHD includes immunosuppressive therapies (i.e.- cyclosporine, methotrexate) that have led to reductions in the incidences of GVHD, yet these therapies suppress T cell responses not only involved in GVHD, but also the immune system as a whole.[3] Other preventative methods have attempted to reduce the incidence of GVHD by depleting T cells from the graft (TCD), but these treatments also led to increased risk of graft rejection, opportunistic infection, and relapse secondary to decreased graft-versus-tumor (GVT) effect.[3,21] Established GVHD is predominantly

treated with high-dose corticosteroid treatments (i.e. - methylprednisolone), though this treatment fails for some patients who then must undergo salvage therapy. Second line treatments include extracorporeal photophoresis (ECP), anti-thymocyte globulin (ATG), cytokine blockades (i.e. – TNFα antibodies), anti-CD3 antibodies, and chemotherapeutic agents (i.e. – nucleoside analogs), among others.[3,10,22,23] These current broad spectrum treatments are not ideal because they render patients susceptible to relapse and infectious complications.

Graft-versus-tumor effect

Beyond restoring lymphohematopoiesis, it was eventually found that donor cells were capable of providing an immunological response against malignant and diseased recipient cells, independent of chemotherapy and radiation treatments. In 1956, Barnes *et al.* demonstrated that irradiated mice that received allogeneic marrow transplants, but not syngeneic transplants, showed remission of leukemia.[24] This murine experiment was the first to suggest that donor cells may provide a graft-versus-leukemia (GVL) or graft-versus-tumor (GVT) effect. The implications of this murine experiment were realized in human studies showing that relapse rates in patients were decreased in those who developed some form of GVHD, suggesting that alloreactive donor cells were involved not only in GVHD but also in a GVT effect.[9] It is because of experimental and clinical validations of the importance of donor alloreactivity that HSCT is becoming more of an immunotherapy, as opposed to an initial means of delivering high dose therapies and replacing damaged hematopoietic cells with normal stem cells.[9,21]

It has also been shown that relapse rates are higher in patients who receive TCD grafts or who receive grafts from identical twins (syngeneic),[25] suggesting the role for T cells in alloreactivity of GVT responses. The significant role of immunocompetent donor cells has been further shown in patients who received donor lymphocyte infusions (DLI) post-transplant to treat recurrence of disease.[25-27] Many experimental and human models focus on CTLs and NK cells, with $CD8^+$ T cells being the predominant

T cell subset implicated in the GVT effect.[28-33] The dominance of one subset over another likely varies with degree of genetic disparities between donors and recipients, both with regards to HLA and mHAgs.[34] Furthermore, there are three general ideas as to how GVT effects are mediated: 1) through direct killing of malignant cells by perforin- and granzyme-mediated attack from CTLs ($CD8^+$ and $CD4^+$ T cells, and NK cells); 2) by apoptotic cell death via the Fas/FasL pathway ($CD8^+$ and $CD4^+$ T cells); or 3) by cytokine-mediated malignant cell death (CTLs) or control of proliferation (predominantly $CD4^+$ T cells) [reviewed in Barrett, 1997].[34] Aside from helper roles in augmenting $CD8^+$ CTL responses, $CD4^+$ T cells have also been associated with cytotoxic function in a class II-restricted fashion.[29,34-37]

Just as in GVHD, mHAgs are key players in the GVT effect in HLA-matched allogeneic HSCT. There has been an association of cGVHD with lower relapse rates, but it isn't known if the GVT effect relies on cGVHD or if it independently persists once cGVHD is resolved.[17, 25] There are two general types of antigens that may be targeted in a GVT capacity – polymorphic mHAgs or autologous antigens that are specifically associated with a malignant phenotype (tumor-associated antigens, or, TAAs).[9,28,38]

Minor histocompatibility antigens

Minor histocompatibility antigens are peptide epitopes that are derived from the degradation of normal cellular proteins, and are presented on either MHC class I or class II molecules.[15] mHAgs are a result of genetic variation, though the means of this variation is diverse. Genomic variations may occur via gene deletions, inverted gene sequences, multiple copy gene duplications, segmental duplications, large-scale copy number variations (CNVs) and single nucleotide polymorphisms (SNPs).[15] Nonsynonymous coding SNPs and deletion polymorphisms are crucial players in the generation of potentially immunogenic transplantation antigens as they have been identified most in autosomal mHAgs, and SNPs cause differences in the amino acid sequences of homologous proteins between recipient and donors cells. A single amino acid substitution can cause altered proteosomal cleavage,

peptide transport, HLA binding or TCR contact, all of which may trigger donor T cells to recognize these peptides as non-self.[15]

Some of the most common mHAgs studied with regards to HSCT are from genes on the Y chromosome, which have shown polymorphisms with the homologous regions of the X chromosome. In the HSCT setting, this is relevant in sex-mismatched transplants in which male patients are recipients of female stem cell grafts. It has been shown that male recipients of female grafts have a higher risk of developing GVHD and a lower risk of relapse, suggesting that some mHAgs encoded on the Y chromosome may be immunodominant.[15,29,39] HA-Y mHAgs are ubiquitously expressed,[40,41] and some of the mHAgs encoded on the Y chromosome include UTY, SMCY, and DBY.[42] SMCY and DBY are highly expressed on epithelial cells, suggesting a role for T-cell-mediated responses against these mHAgs in either GVHD or GVT effects.[42] UTY is only moderately expressed on epithelial cells, suggesting that UTY-specific T cells may promote GVT effects without augmenting GVHD.[42]

Other mHAgs that are commonly studied are HA-1, -2, and -3. HA-1 and HA-2 are preferentially expressed on hematopoietic tissues, therefore allowing them to be targeted by HA-1 and HA-2-specific T cells in a GVT effect without augmenting GVHD (though HA-1 expression has also been implicated in solid tumors).[16,20,32,38] HA-3 is preferentially expressed on non-hematopoietic tissues, suggesting it may be a target of GVHD more so than GVT effects.[42] Overall with regards to mHAgs, those that are ubiquitously expressed are prime targets for GVHD, whereas those that are restricted to the hematopoietic system may fulfill their role in eliciting a GVT response without increasing the risks of GVHD.[16,32,43] However, it has also been shown that hematopoietic cells at the site of GVHD may have a role in the development of low grade GVHD, even though the mHAgs themselves are not ubiquitously expressed on the actual tissues involved in GVHD.[20]

The HLA System

The immunological reactions that are involved in the transplantation process—GVHD and GVT effects—develop primarily from the HLA system, which is essential in T cell activation through the HLA presentation of peptides to T cells.[9] The HLA genes are located within the MHC on the short arm of chromosome 6, and includes over 200 genes.[44] The HLA class I genes code for the α polypeptide chain of the class I molecule, whereas the β chain is encoded by the β_2-microglobulin gene on chromosome 15. There are five domains in the α chain: two peptide-binding (α1 and α2), one immunoglobulin-like (α3), a transmembrane region, and a cytoplasmic tail. Of 20 HLA class I genes, three are the major components of the immune system: HLA –A, –B, and –C.[44] Unlike class I, the class II genes for both the α and β polypeptide chains are located on chromosome 6. The class II genes are designated by three letters: the first represents the class (D), the second represents the family (M, O, P, Q, or R), and the third represents the chain (A or B, which corresponds to α or β, respectively). There are four domains in each of the α and β chains: peptide-binding (α1 or β1), immunoglobulin-like (α2 or β2), the transmembrane region, and the cytoplasmic tail.[44] These two classes contain the major influential genes that are matched between donor and recipient in HSCT—HLA-A, -B, and –C genes from class I, and HLA-DRB1, -DQB1, and –DPB1 genes from class II.[9]

Class I genes are expressed on most all nucleated cells, though their level of expression is tissue-dependent.[9,44] Class II gene expression is restricted to hematopoietic cells, and are expressed predominantly on immune cells (APCs—including B cells, dendritic cells, and macrophages; and also thymic epithelial cells).[25,44] Furthermore, the expression of class II HLA molecules can be induced during inflammatory responses, such as in the presence of IFN-γ.[9,44] Class I molecules are recognized by $CD8^+$ T cells, and class II molecules are recognized by $CD4^+$ T cells.[8,9]

Class I and class II molecules present peptides to the TCR, and hence are involved in the activation of T cells associated with both GVHD and GVT effects. Traditionally, class I molecules present endogenous proteins, whereas class II molecules present exogenous proteins, though these designations are not absolute.[45] Class I molecules may present exogenous proteins, such as bacterial proteins, and class II molecules may present endogenously produced proteins such as viral proteins, though the mechanisms of these exceptions to the general rule are not well understood.[44]

Endogenous proteins are primarily marked by ubiquitin for degradation in the proteosomes, though some proteins may be degraded by soluble enzymes in the cytosol. The degraded peptides are either further degraded into amino acids in the cytosol or are transferred to the endoplasmic reticulum (ER). Of endogenously degraded peptides, some are picked up with transporters associated with antigen processing (TAPs), which are encoded by TAP1 and TAP2 genes. TAP1 and TAP2 proteins form a channel on the endoplasmic reticulum that allows peptides to enter the endoplasmic reticulum, the luminal side of which has class I molecules. Once a suitable peptide is bound to the class I molecule, the peptide-bound HLA molecule then moves to the plasma membrane and presents itself to other cells.[44]

The processing of and peptide-loading of exogenous proteins on class II molecules is primarily restricted to B cells, dendritic cells, and macrophages. Exogenous proteins are endocytosed into vesicles that fuse with primary lysosomes which then acquire proteolytic enzymes and form endosomes in which the proteins are degraded.[44] Though they are also assembled on the luminal surface of the endoplasmic reticulum, unlike class I molecules, class II molecules do not bind with peptides in the ER, but rather with a protein that prevents premature binding of peptide—the invariant chain. Class II molecule-invariant chain complexes enclosed in membranous vesicles meet their exogenous proteins upon interaction with endosomes in the cytosol.[44] These two vesicles fuse to form the MHC class II

compartment, in which exogenous proteins and most of the invariant chain are degraded by proteases. To separate the part of the invariant chain that is bound to the class II molecule, other class II molecules (HLA-DM) dislodge the remaining part and free the peptide-binding groove. Once a peptide binds to the class II molecule, these complexes are then transported to the surface of the cell.[44] Cell surfaces become decorated with as many as 100,000 to 300,000 of each of the class I and class II MHC-peptide bound complexes. As such, each normal, or uninfected, cell displays self peptides on the order of hundreds or thousands on its surface. Most peptides are displayed with around 100 copies, whereas others may present only a few or thousands of copies.[44]

During T cell development and maturation in the thymus, the immune system develops a tolerance to "self" proteins and thus has the ability to distinguish between "self" and "non-self" proteins and peptides that are displayed in the context of MHC. In HSCT, it is from the recognition of "non-self" proteins that: grafts may be rejected by host cells reacting against donor cells, alloreactive donor cells may target normal tissues of the host, causing GVHD, and/or donor cells may react against host antigens that are associated with malignancy and disease, conferring a GVT effect.[9] Even with a "perfect" HLA match, due to extensive polymorphism, an immense number of non-HLA antigens, primarily mHAgs, may be presented to T cells, and subsequently induce alloreactivity involved in either GVHD or GVT effects.

T cell Development

Pluripotent hematopoietic stem cells in the bone marrow give rise to common lymphoid and myeloid progenitors. It is the common lymphoid progenitors that, after leaving the bone marrow, enter the thymus and give rise to thymocytes. In the thymus, differentiation of immature thymocytes is mediated by the integration of intrinsic signals, which are generated downstream of the TCR, and extrinsic signals, which are obtained from numerous interactions of T cell progenitors with stromal cells and other thymocytes.[46] T cell development in the thymus can be separated into three broad steps: the first

spanning thymic colonization to T cell commitment; the second proceeding to the divergence of αβ and γδ lineages; and, the third consisting of αβ and γδ lineage cells completing their differentiation and acquiring immunological properties.[47]

The two distinct T cell lineages—αβ and γδ—arise from common precursor cells termed double-negative (DN) thymocytes (because they lack expression of CD4 and CD8 co-receptors).[46] DN cells are further divided into subsets based on their developmental potential (DN1(a-e), DN2, and DN3(a,b)). Transition from the DN1 to DN2 stage marks the initiation of gene rearrangement at the TCRβ, TCRγ, and TCRδ gene loci. Successful rearrangement of the *Tcrd* and *Tcrg* genes promotes the assembly of γδ TCRs. The αβ TCR is preceded by a pre-TCR that is formed when successful recombination of the *Tcrb* gene promotes the assembly of a TCRβ-chain, which then pairs with a surrogate TCRα-chain (the invariant pre-Tα protein).[46] The expression of a functional pre-TCR represents the controlled developmental transition beyond the DN3 stage, and is referred to as the β-selection checkpoint.[46] The β-selection checkpoint is crucial in identifying proper TCR gene rearrangement at the DN3 stage because nucleotide deletions and additions may be introduced during RAG-mediated recombination, with most of the rearrangements being out-of-frame and incapable of producing functional protein.[47] During the DN3 stage, those cells which express either a pre-TCR or a γδ TCR are rescued from apoptosis and subsequently undergo thymocyte proliferation and differentiation.[46,48] Along with these β-selection and γδ-selection events, CD5 and CD27 are upregulated and the cells increase in size, marking the transition from pre-selection DN3a to post-selection DN3b. Next, the αβ T cell lineages downregulate CD25 and upregulate CD4 and CD8, thus becoming characterized as double-positive (DP) $CD4^{+}CD8^{+}$ cells.[46] In contrast to the αβ T cell lineage, most of the γδ lineage remain DN.[46] At this stage in DP αβ T cells, *Rag* genes are re-expressed, initiating TCRα gene rearrangement and promoting the assembly of the αβ TCR heterodimers.[46,48] DP thymocytes are programmed to undergo apoptosis unless

they receive a "rescue signal" (positive selection) through the ability of their TCR to bind an MHC ligand with mild avidity.[49] During a three day window before programmed cell death, receptor editing occurs in which sequential rounds of rearrangement of the TCR-α locus occurs, increasing the chance of successful self-MHC restriction. For DP thymocytes whose TCR interacts poorly with self-peptide:MHC ligands and are subsequently unable to generate intracellular signaling needed to sustain viability, they undergo "death by neglect."[50] Some thymocytes bind to self-peptide:MHC with high avidity and as a result, apoptosis is promoted to prevent an autoimmune pathology that would result if these cells were to exit the thymus and enter the periphery.[50] This strong interaction is referred to as "negative selection," or clonal deletion. In the end, approximately 95% of DP cells fail positive selection and undergo "death by neglect." For the roughly 5% of DP cells that are positively selected, DP maturation to the single-positive (SP) stage ($CD4^+CD8^-$ or $CD4^-CD8^+$) is induced.[49]

The alloreactivity that characterizes both GVHD and GVT effects represents the cross-reactivity of TCRs for non-self peptide:non-self MHC complexes. This interaction is influenced by both the bound peptide and the MHC molecule, and may be categorized into three types: peptide:MHC dependent in which alloreactive T cells interact strongly with one specific peptide:MHC complex, but not with the same MHC bound to different peptides; peptide-dependent in which the peptides presented by non-self MHC differ from the peptides of the host; and peptide-independent in which alloreactive T cells recognize non-self MHC without a strict peptide requirement.[51] Because the majority of our patients received stem cell grafts from matched HLA-donors, we are primarily interested in the peptide-dependent reactivity of TCRs.

TCR rearrangement and the CDR3 region

The somatic assembly of the TCR genes generates a diverse T cell repertoire during thymocyte development. The four TCR genes—*Tcra, Tcrb, Tcrg, Tcrd*—are assembled through V(D)J

recombination, which is a site-specific recombination process that is directed by the lymphoid-specific recombinase (RAG, comprised of RAG1 and RAG2) and the ubiquitously expressed DNA repair proteins.[48] The RAG proteins generate double-strand breaks at recombination signal sequences (RSSs) that flank the TCR variable (V), diversity (D) and joining (J) gene segments. These breaks are subsequently resolved by non-homologous end joining.[48] The alpha and gamma loci undergo recombination between the variable (V) and joining (J) segments. The beta and delta loci undergo recombination between V and J segments, and further include one of two short diversity (D) segments.[52] There are 52 functional Vβ, 2 Dβ and 13 Jβ segments that undergo VDJ rearrangements to generate Vβ regions.[53] The αβ T cells constitute the bulk of T cell populations in lymphoid organs and normally react to peptides that are presented in the context of MHC class I or class II molecules.[47] In contrast, the γδ T cells are typically not MHC-restricted and seem to be involved in the surveillance of microbial and non-microbial tissue stress. The specificity of clonally distributed αβ TCRs is diversified through the random rearrangement of V and J genes at the TCRα locus and V, D, and J genes at the TCRβ locus of developing thymic T cells. Diversity is further created through random nucleotide insertion and deletion of a variable number of nucleotides at the VDJ junctional sites (N-regions).[53, 54]

Greater than 95% of T cells have αβ TCRs.[55] The TCR's recognition specificity for diverse peptide:MHC complexes is determined by its three complementarity-determining regions (CDRs): CDR-1, -2, and -3. CDR1 and CDR2 are coded by germline sequences (Vα and Vβ genes), whereas CDR3 is derived from the V-(D)-J and N-regions made during somatic recombination.[52-53] The TCR interaction with peptide:MHC complexes suggests a common diagonal docking mode, whereby the CDR1 and CDR2 loops contact the MHC α helices of the antigen binding groove and the more diverse CDR3 α and β loops interact primarily with the peptide. The CDR3 is the highly polymorphic recognition site of the TCR that recognizes antigen specificity, and it is for the additional diversity

generated at the CDR3 recombination junctions that the CDR3 is capable of recognizing such a vast number of peptide:MHC complexes.[52]

By analyzing the variable region of the TCR CDR3 region, we can determine the clonality of T cell responses, as different CDR3 sizes correspond to different T cell clones.[55] Due to the imprecise nature of combinatorial diversity and the random addition of nucleotides, each T cell clones expresses a unique CDR3 region of the TCR Vβ.[56] Healthy individuals have highly diverse and polyclonal TCR repertoires that typically express 8 to 10 different sizes for each Vβ CDR3 region separated by 3 nucleotides.[53] Oligoclonal and monoclonal TCR repertoires are associated with strong immune responses.[52] Oligoclonal expansion of TCR Vβ families is common and often correlates with GVHD.[53]

Hypothesis/Aims

Because treatment of GVHD is dominated by broad immunosuppression that leaves patients at greater risk for complications from infections and abrogation of the GVT effect, there is still a need to develop a method to directly target GVHD-causing T cells while sparing those involved in host defense and immunosurveillance.[57] As GVHD and GVT are both predominantly T-cell-mediated processes, analysis of patients' T cell repertoires may elucidate T cell populations involved in the pathogenesis of these processes. In a cohort of twelve patients who have undergone allogeneic HSCT for various hematologic malignancies, we performed quantitative RT-PCR (qRT-PCR) and CDR3 spectratyping of peripheral T cells to identify antigen-driven T cell responses associated with GVHD and/or GVT effects. Furthermore, by extracting DNA from GVHD biopsy samples, we are able to not only detect T cell clones at the site of GVHD, but also correlate those at the site of disease with those identified in the peripheral blood.

Materials and Methods

Patients

Twelve patients with hematological malignancies (4 with Non-Hodgkin's Lymphoma, 5 with multiple myeloma, 2 with chronic lymphocytic leukemia, and 1 with prolymphocytic leukemia) who underwent allogeneic hematopoietic stem cell transplantation (HSCT) were included in this study. Table 1 outlines patient characteristics and Figure 1 depicts the treatment schedule. Also included in the study was data from the patients' respective donors in order to compare the T cell repertoire of the patients relative to their donors at various time points post-HSCT. Donor peripheral blood $CD34^+$ stem cells (D) were collected at the time of transplant, from which we collected samples, and peripheral blood samples from recipients were collected prior to and at various time points after transplantation: R (recipient) denotes pre-transplant before the start of the conditioning regimen; R2 denotes 3 months after HSCT for all patients, except for Patients 5, 11, 13, and 14, in which R2 denotes time of GVHD onset; R3 denotes one year post-transplantation for the four patients without GVHD (Patients 3, 4, 7, and 12) and either the time of GVHD onset (Patients 6, 8, 9, 10) or 3 months post-transplantation (Patients 11, 13, and 14) for the patients who developed GVHD; R4 denotes one year post-transplantation in the GVHD group (Patients 6, 8, 9, and 11), times of active GVHD for Patients 13 (6 months post-transplant) and 14 (4 months post-transplant), and time of relapse for Patient 10 (10 months post-transplant); R5 denotes one-year post-transplant for Patient 13 and Patient 10 (R5 is also post-DLI for Patient 10);

Patients 5 and 14 do not have one-year samples, as both expired prior to this time. Patient 10 also has an R6 sample that is roughly 22 months post-HSCT and represents time of remission and re-establishment of GVHD post-DLI treatments that were roughly 2 and 11 months prior to R6. Patient outcomes are outlined in Table 2, with regards to onset and treatment of GVHD. All patients were informed of the purpose for the study and agreed to participate. This study was approved by the VCU Institutional Review Board for the collection of donor $CD34^+$ stem cells and peripheral blood samples from patients undergoing HSCT at VCUHS, at the time points aforementioned.

Figure 1:

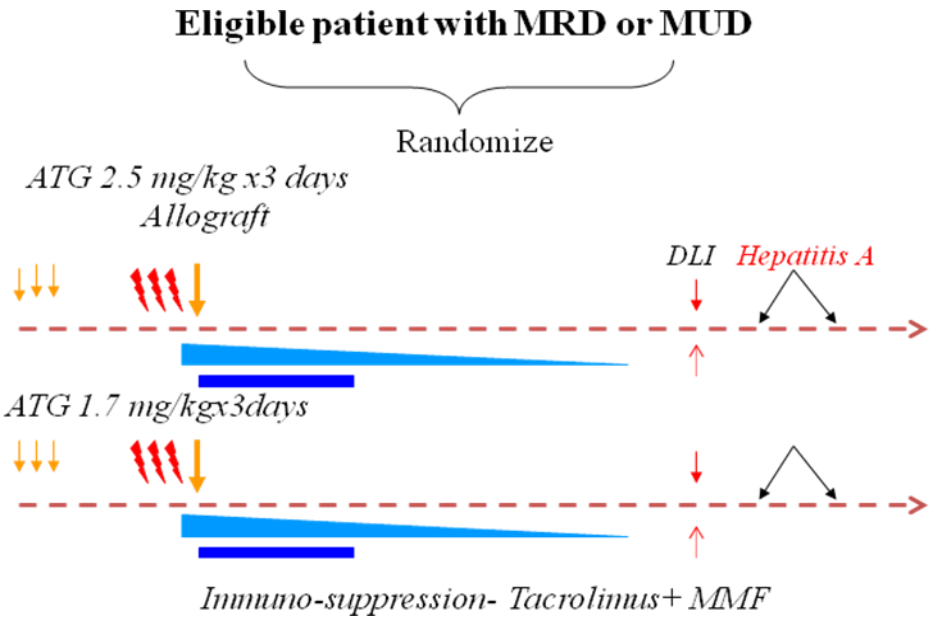


Figure 1. Treatment regimen for patients undergoing allogeneic HSCT. The treatment regimen consisted of rabbit anti-thymocyte globulin (ATG) (doses between days -9 and -7) and 450 cGy total body irradiation in three fractions on days -1 and 0. Patients were randomized as to which ATG dose they would receive. GVHD prophylaxis with tacrolimus is denoted by the light blue taper (day -3 to 120) and with mycophenolate mofetil (MMF) denoted by the dark blue bar (day 0-30). MRD: matched related donor; MUD: matched unrelated donor.

Table 1. Patient characteristics.

Patient	Age/ sex	Disease	Disease status at HSCT	Donors' age/sex	Donors	HLA-A	HLA-B	HLA-C	Donors' non-identical HLA alleles		Recipients' non-identical HLA alleles	
									Class I	Class II	Class I	Class II
3	44/F	NHL	CR	32/M	MUD	0301, 1101	0702, 5501	0303, 0701	none	DQB1 -0503, 0602	none	DQB1 0602, -
4	46/F	MM	PR	52/F	MRD	2301, 3002	1502, 440301	0210, 0718	none	DQB1- 02GM	none	DQB1- 0201
7	56/F	NHL	CR	46/M	MUD	010101, 0201	440201, 5501	0303, 0501	none	DQB1- 0301, 0501	none	DQB1- 0302, 0501
12	62/M	MM	PR	20/M	MUD	020101	070201, 1302	060201 0702	none	none	none	none
5	58/M	CLL	CR	36/M	MUD	010101, 0301	0702, 570101	0701 0702	none	none	none	none
6	68/M	CLL	CR	63/M	MRD	0101	0801, 4402	0501, 07WTR	none	none	none	none
8	57/M	PLL	CR	40/M	MUD	010101, 240	0702, 5501	0303, 0702	none	none	none	none
9	55/M	NHL	CR	65/F	MUD	0301	0702,	0702	none	none	none	none
10	57/F	MM	PR	24/M	MUD	0101, 0301	080101, 4001	0304, 0701	none	none	none	none
11	58/F	MM	PR	70/M	MRD	0201	4001, 5101	0304, 1502	none	none	none	none
13	59/F	NHL	CR	22/M	MUD	020101, 3001	1302, 440201	0501, 060201	none	DQB1- 0302, 0603	none	DQB1-0301, 0603
14	56/M	MM	PR	43/F	MUD	110101, 3201	490101, 15GYF	0102, 0701	B-5601, 490101	none	B-15GYF, 490101	none

MM: multiple myeloma; NHL: non-Hodgkin lymphoma; CLL: chronic lymphocytic leukemia; PLL: prolymphocytic leukemia; PR: partial response; CR: complete response; MRD: matched related donor; MUD: matched unrelated donor; HLA code translation for WTR: 01/06/18

Table 2. **Patient outcomes following HSCT.**

Patients	*T cell chimerism		Days post-HSCT R2/R3/R4	Patients' outcome/day of GVHD post-HSCT	Treatment for GVHD	Days post-HSCT death
	R2	R3				
3	11%	0	88/ 365/ NA	No relapse/ No GVHD	NA	
4	51%	8%	90/ 322/ NA	No relapse/ No GVHD	NA	
7	52%	63%	85/ 298/ NA	No relapse/ No GVHD	NA	
12	9%	0%	94/ 356/ NA	No relapse/ No GVHD	NA	
5	0	0	89/ NA/ NA	No relapse GVHD/89	Steroids/ ECP	162
6	0	0	90/ 148/ 372	No relapse GVHD/148	Tac/steroids/ ECP	
8	2%	3%	79/ 123/ 363	No relapse GVHD/123	Tac/steroids	404
9	2%	1%	81/ 120/ 362	No relapse GVHD/120	Tac/steroids	
10	0	0	102/ 172/ 291	Relapse/291 GVHD/160	Steroid topical	
11	0	0	54/ 87/ 364	No relapse GVHD/54	Tac/steroids/rituxan	
13	0	0	65/ 96/ 141	No relapse GVHD/65	Steroid topical	
14	0	0	66/ 97/ 129	No relapse GVHD/66	Tac/steroids/rituxan	171

Percentages indicate the proportion of T cells that are not from the engrafted donor T cells (recipient T cells). ECP: extracorporeal photophoresis; Tac: tacrolimus.

Table 3. Cell counts of donor stem cell grafts.

Patient	CD3 x 10^8/kg	CD34 x 10^6/kg
3	3.54	6.52
4	2.31	6.8
7	5.54	6.81
12	1.26	7.99
5	5.68	2.05
6	1.66	5.16
8	3.72	7.45
9	2.86	6.23
10	3.53	3.43
11	11.35	5.13
13	0.43	1.9
14	1.5	4.96

$CD3^+$ counts are 10^8/kg of recipient weight. $CD34^+$ counts are 10^6/kg recipient weight. Patients 3,4, 7, and 12 are GVHD-free and Patients 5, 6, 8-11, 13 and 14 developed GVHD.

T cell isolation and mRNA Extraction

Recipient blood samples were taken at the time points described above (R-R5), diluted with a 1:1 salt solution, and subsequently layered on Ficoll-Paque™ Premium (GE Healthcare) for density gradient centrifugation and isolation of mononuclear cells (according to manufacturer's protocol). These cells, after washing, were cultured in RPMI medium containing 0.1% autologous serum for 2 hours at 37°C and non-adherent cells were collected. $CD3^+$ cells were isolated using the EasySep® Negative Selection Human T cell Enrichment Kit (Stem Cell Technologies) according to manufacturer's protocol. Subsequently, isolated $CD3^+$ T cells were used for RNA extraction using Trizol (Invitrogen). For donors, RNA was extracted from peripheral blood $CD34^+$ stem cells. We determined that un-fractionated $CD34^+$ stem cells still had a high yield of $CD3^+$ T cells in comparison to $CD3^+$ fractions isolated using a T cell enrichment kit (Figure 2). Therefore, the T cell enrichment kit was only used for recipient samples, and we proceeded directly to culturing donor $CD34^+$ stem cells in serum-free medium at 37^oC for 2 hours and collected non-adherent cells.

Figure 2

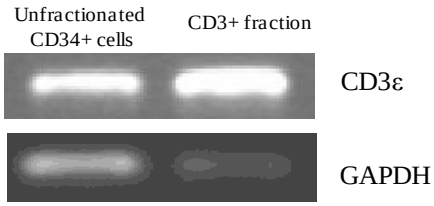


Figure 2. CD3+ fractions in un-fractionated $CD34^+$ stem cells and in isolated $CD3^+$ cells. PCR products on a 1% agarose gel showing $CD3^+$ cells in un-fractionated $CD34^+$ cells and in T cells isolated using a negative selection T cell enrichment kit ($CD3^+$ fraction). GAPDH is shown as a control.

RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Next, DNase treatment was performed using RQ1 RNase-free DNase (Promega, according to manufacturer's protocol) to digest DNA contamination. Double-stranded cDNA was prepared from 1μg of total RNA using the SuperScript™ II reverse transcriptase (Invitrogen) with a dT18 oligonucleotide primer. The cDNA synthesis was completed at 42°C for 2 hours.

Quantitative RT-PCR

Using reverse primers for 23 functional TCR Vβ[58] families and one forward primer for the constant region of the TCR Vβ (Table 4), qRT-PCR was performed for amplification of the CDR3 region using SensiMix™ SYBR & Fluorescein Kit (BIOLINE) and BioRad's CFX96 Real-Time PCR Detection System. Reaction mixtures of 20μl were prepared according to manufacturer's protocol and 5μl of 30-times diluted cDNA were used as a template. Thermo-cycle conditions consisted of initial denaturation at 95°C (10 min. each), followed by 40 cycles at 95°C (15 s) and 60°C (1 min.). The fluorescent data was acquired during each extension phase. After 40 cycles, a melting curve was generated by slowly increasing the temperature from 60°C to 95°C, while the fluorescence was measured. Amplification of CD3ε fragments with specific primers (forward 5'-CGTTCAGTTCCCTCCTTTTCTT-3', reverse-5'-GATTAGGGGGTTGGTAGGGAGTG-3'),[59] was used as a normalization control to quantify the relative expression of different TCR Vβ families. The expression of each Vβ family in recipients was compared to that of their donors to demonstrate the change in the level of mRNA. A constant donor sample was used as a control to be able to compare multiple experiments.

Statistical consideration

A 1-fold increase in expression of the recipients' Vβ over their donors' Vβ was used as an arbitrary cutoff used to distinguish meaningful increases in TCR Vβ. Since the CD3ε subunits were used for normalization and there are two CD3ε subunits and one CDR3 region associated with each TCR, the amount of CD3 template was twice as much as that of each TCR Vβ template. Therefore, a 1-fold increased expression of a given Vβ over CD3 may indicate T cell activation.

Spectratyping analysis of the CDR3 region

PCR products were prepared using 5µl 30-times diluted cDNA and a 20µl PCR master mix containing 1X PCR Buffer, 1.5 mM $MgCl_2$, 2.5 units of Velocity DNA Polymerase (Bioline), 1 µM Vβ-specific primer, and 1 µM of a FAM-tagged primer specific for the constant region of TCR (Table 4). PCR conditions were run as follows: 94°C (5 min.), 94°C (30s), 60°C (30s), 72°C (1 min.) for 35 cycles followed by 10 min. extension at 72°C.

PCR products were analyzed at Roswell Park Cancer Institute using an ABI Prism 3130 capillary sequencer followed by fragment length analysis of the CDR3 region with GeneMapper® software. Spectratyping results were analyzed with PeakScanner™ Software v1.0 from Applied Biosystems.

Table 4. TCR Vβ primers.

Vβ2	5'-TCTCATGCTGATGGCAACTTCCAAT-3'
Vβ3	5'-CGCTTCTCCCTGATTCTGGAGTCC-3'
Vβ4	5'-TTCCCATCAGCCGCCCAAACCTA-3'
Vβ5	5'-CTGAGMTGAATGTGARCRCCTTG-3'
Vβ6	5'-MAGRATGTARMKCTCAGGTGTGAT-3'
Vβ7	5'-TAAGAAGTCTTTGAAATGTGAACAAC-3'
Vβ8	5'-TGAAGATCCAGCCCTCAGAACCC-3'
Vβ9	5'-AACAGGACTCTAAGAAATTTCTGAAG-3'
Vβ10	5'-CCACGGAGTCAGGGGACACAGCAC-3'
Vβ11	5'-TGCCAGGCCCTCACATACCTCTCA-3'
Vβ12	5'-GAGAATTTCCTCCTCACTCTGG-3'
Vβ12	5'-GACCTCCCCCTCACTCTGG-3'
Vβ13	5'-CACTGAGATGTACCCAGGATATGA-3'
Vβ14	5'-GGGCTGGGCTTAAGGCAGATCTAC-3'
Vβ15	5'-CAGGCACAGGCTAAATTCTCC-3'
Vβ16	5'-GATGAGTCCGGTATGCCCAACAATC-3'
Vβ17	5'-CCCCAAAGTACCTGTTCAGAAA-3'
Vβ18	5'-TTTCTGCTGAATTTCCCAAAGAGG-3'
Vβ19	5'-TCTCAATGCCCCAAGAACGCAC-3'
Vβ20	5'-AGGTGCCCCAGAATCTCTCAG-3'
Vβ21	5'-GAYGATTCACAGTTGCCTAAGGA-3'
Vβ22	5'-AAGTGATCTTGCGCTGTGTCCCCA-3'
Vβ23	5'-GCAGGGTCCAGGTCAGGACCCCCA-3'
Vβ24	5'-ATCCAGGAGGCCGAACACTTC-3'
constant V region	5'-CGTAGAACTGGACTTGACAGCGG-3'

Degenerate bases: M = A/C; Y = C/T; R = A/G; K = T/G.

DNA Extraction

Paraffin-embedded biopsy samples from the site of GVHD were available for Patients 5, 6, 8, and 11. DNA extraction methods were previously described by Fan and Gulley.[60] From five 5µm thick sections, paraffin was dissolved using xylene washes. Xylene was was removed with ethanol washes and ethanol was evaporated in a dry heat block at 55°C. A Proteinase K solution (20mg/mL) in TEN buffer was added to the samples (100-150µL) and the samples were incubated at 55°C for at least 3 hours. To inactivate the Proteinase K, samples were incubated at 95°C for 10 minutes. Tissues were pelleted in eppendorf tubes and the supernatant was removed for use as template in PCR reactions. In order to check quality of DNA, PCR using primers specific for GAPDH (forward 5'-ATTGCCCTCAACGACCACTTTG-3', reverse-5'-TTGATGGTACATGACAAGGTGCGG-3')[61] was run. PCR reactions were run for those Vβ families in which both increased expression and oligoclonality were demonstrated in qRT-PCR and spectratyping, respectively. The primers used are the same as those outlined in Table 3, without a FAM-tag on the primer for the constant region of the TCR. PCR products were run on a 1% agarose gel to identify those Vβ families that could be identified at the site of GVHD.

Results

TCR Vβ families 4, 11, and 23 are associated with GVHD.

Using qRT-PCR, we looked at the expression of 23 TCR Vβ families in patients relative to that of their donors in order to identify changes in the T cell repertoire following allogeneic HSCT. As shown in Figure 3, we identified TCR Vβ families 4, 11, and 23 as those which demonstrated increased expression in multiple patients with GVHD (with the exception of increased expressions in Patient 12, who did not develop GVHD). TCR Vβ4 was increased in recipients, relative to their respective donors, at the time of GVHD in Patients 5, 6, 8, and 10. TCR Vβ11 was increased in Patients 5, 8, 9, 10 and 13, and TCR Vβ23 was increased in Patients 5, 9, 10, 11, and 13, all at their respective times of GVHD. In a few instances, an increased expression was also seen at the time point prior to the clinical manifestation of GVHD, suggesting that T cell clones involved in the pathophysiology of GVHD may be detectable in the blood prior to diagnosis of the disease. This was seen in Patients 8 and 10 at R2 in Vβ4, in Patients 8 and 9 at R2 in Vβ11, and in Patient 9 at R2 in Vβ23.

Increased expression of T cells is due to either antigen-specific responses or homeostatic proliferation. In order to determine if TCR Vβ families 4, 11 and 23 were antigen-driven, and potentially temporally associated with the incidence of GVHD, we performed spectratyping analysis of the CDR3 region. Correspondingly, for Vβ4, spectratyping analysis demonstrated oligoclonality in Patient 5 and monoclonality in Patients 6, 8, and 10 at their respective times of GVHD (Figure 4a).

Oligoclonality was also seen at R2 in Patients 8 and 10, as expected by their corresponding increases in qRT-PCR at both R2 and R3 (Figure 3, 4a), which suggests that clonotypes involved in GVHD may be detectable prior to clinical diagnosis. Patient 6 demonstrated monoclonality at R2 when there was not an increased expression of Vβ4, and this clone also mirrored that seen in the donor and at R4, suggesting that it may have been an antigen-experienced T cell population (Figures 3, 4a). The clone seen at time of clinical diagnosis of GVHD (R3) was different from these other timepoints, suggesting that it may have emerged in a temporal fashion in association with the onset of GVHD (Figure 4a). Patient 6's GVHD was still being treated at one year post-HSCT (R4), so it was expected that the same clonotype size would persist, though the clone at this time coincided with that seen in the donor as well as in the recipient prior to GVHD. A possible explanation could be that the clonal population that emerged at R3 did coincide with onset of acute gut GVHD, but that the severity of the disease was greatly diminished at R4, such that the clonal population was no longer actively proliferating. With regards to Patient 10, there was a continuation of clonality after the timepoint of GVHD (R4), and since GVHD was not wholly resolved at this time (10-months post-HSCT), the continuation of a GVHD-associated clone was expected.

Spectratyping anaylsis of TCR Vβ11 showed monoclonality at the time of GVHD in Patients 5, 8, 9, and 10 (Figure 4b). (Due to sample limitations there is no spectratyping analysis of Patient 13.) Patients 8 and 9 also demonstrated increased expression and monoclonality at the timepoint prior to GVHD-onset (Figures 3, 4b), and since both patients were still on GVHD prophylactic immunosuppression at R2, the oligoclonality may be due to the emergence of the GVHD-specific clonotypes early-on during lymphopenia in which peripheral expansion is promoted in efforts to re-establish the T cell repertoire. This further suggests that the alloreactive clonotypes may be detected prior to clinical manifestation of GVHD, or they may represent restoration of a limited T cell

population. For Patient 10, there was oligoclonality prior to GVHD (R2), which mirrored the donor's repertoire and there was no increased expression of Vβ11 at this time, so it may be representative of delayed thymopoiesis as the thymus may be damaged by conditioning regimens and its function is greatly reduced after the involution of the thymus during puberty.[62] A different clonotype emerged at the time of GVHD in Patient 10 (R3), suggesting the temporal association of the clonotype with the onset of GVHD (Figure 4b). However, Patient 10's GVHD was not wholly resolved at R4, and polyclonality was restored at this time. This suggests that if the clonal population at R3 was GVHD-specific, it may have been one of a group of Vβ families involved in GVHD pathogenesis in this patient, as there were no other clinically noted infections or complications at R3 with which to associate the clonal population.

Similarly for Vβ23, oligoclonality was also detected at the time of GVHD in Patients 5, 9, 10, and 11 (Figure 4c). For Patients 5, 10, and 11, oligoclonal populations emerged at the time of GVHD in parallel with the increased expression of Vβ23 at these times. Neither Patient 10 nor 11 had complete resolution of GVHD at R4, and there was not a complete restoration of polyclonality at these times, further suggesting the potential role of these clonal populations in mediating GVHD. In Patient 9, an increased expression of Vβ23 started to emerge prior to GVHD (R2) and continually increased at the time of GVHD (R3) and at one-year post-HSCT (R4), with monoclonality at all times. Interestingly, Patient 9's GVHD was clinically resolved at R4, suggesting that this clonotype may not have been involved in GVHD as it's highest expression was seen after disease occurrence was no longer clinically noted, or that the emergence of another clone was detected over that of the GVHD-associated clone. It is also possible that this clonal population could have been involved in GVT effect since the increased expression persisted beyond temporal association with GVHD. Since a continued increase in expression was not common beyond the clinical resolution of GVHD in our patients, this may be an

example of how to exclude certain clonal populations from their potential roles in the pathophysiology of GVHD. Because qRT-PCR only amplifies CDR3 regions irrespective of VDJ rearrangements, we will use high throughput sequencing to determine the specificity of the T cell clones. With regards to Patient 11, the oligoclonality that was seen at the onset of GVHD (R2) persisted through one-year post-HSCT (R4), and as GVHD was not clinically resolved at R4, the persistence of a GVHD-associated population was expected. Though the qRT-PCR expression of Vβ23 was lower at R4, the severity of the disease may have been minimal at this point. Furthermore, Donor 11 also demonstrated oligoclonality, and the clonal populations seen in Patient 11 mirrored those of the donor, suggesting another possible explanation for the oligoclonality being that the clonotypes may be antigen-experienced with clonality persisting due to delayed thymopoiesis.

For controls, we also performed spectratyping for patients who did not demonstrate increased expression relative to their donors in order to demonstrate that oligo- and mono-clonality was associated with antigen-driven T cell responses. The controls used were: Patient 9 for Vβ4, Patient 11 for Vβ11, and Patient 6 for Vβ23 (Figures 3, 4a-c). Polyclonality is seen as a Gaussian distribution of CDR3 lengths, and is a result of the random insertion of nucleotides during VDJ rearrangement in T cell development.[62] Though spectratyping of control samples was not always perfectly Gaussian (may have looked somewhat aberrant, particularly earlier-on after HSCT), there was still a distinction to be made based on sharp oligo- and mono-clonal peaks that were antigen-driven versus non-antigen-driven T cell populations. Because thymopoiesis is delayed after HSCT, restoration of normal polyclonal T cell repertoires is often greatly delayed until naïve $CD4^+$ subsets are restored.[62]

From the cohort of GVHD patients, 5 out of 8 had GVHD defined as acute—Patients 5, 6, 9, 13, and 14—with Patients 8, 10, and 11 having cGVHD. Based on qRT-PCR and spectratyping data above for Vβs 4, 11, and 23, we saw no difference in these Vβ families being more representative of chronic or

acute GVHD, as clonally-driven T cell populations were identified in both GVHD types. Patients 5 and 13 had GVHD of the skin and gut, Patient 6 had delayed acute onset of the gut, and Patient 9 had acute onset for GVHD of the skin (chronic after day 100). Patient 14 had aGVHD of the skin and late-onset acute grade IV gut GVHD. Patients 8, 10, and 11 had cGVHD of the skin. Patient 6 only demonstrated increased expression and oligoclonality of Vβ4, suggesting that Vβs 11 and 23 may be more specific to GVHD of the skin, as all other patients with increased expressions and oligoclonality for these Vβ families had skin GVHD.

Figure 3:

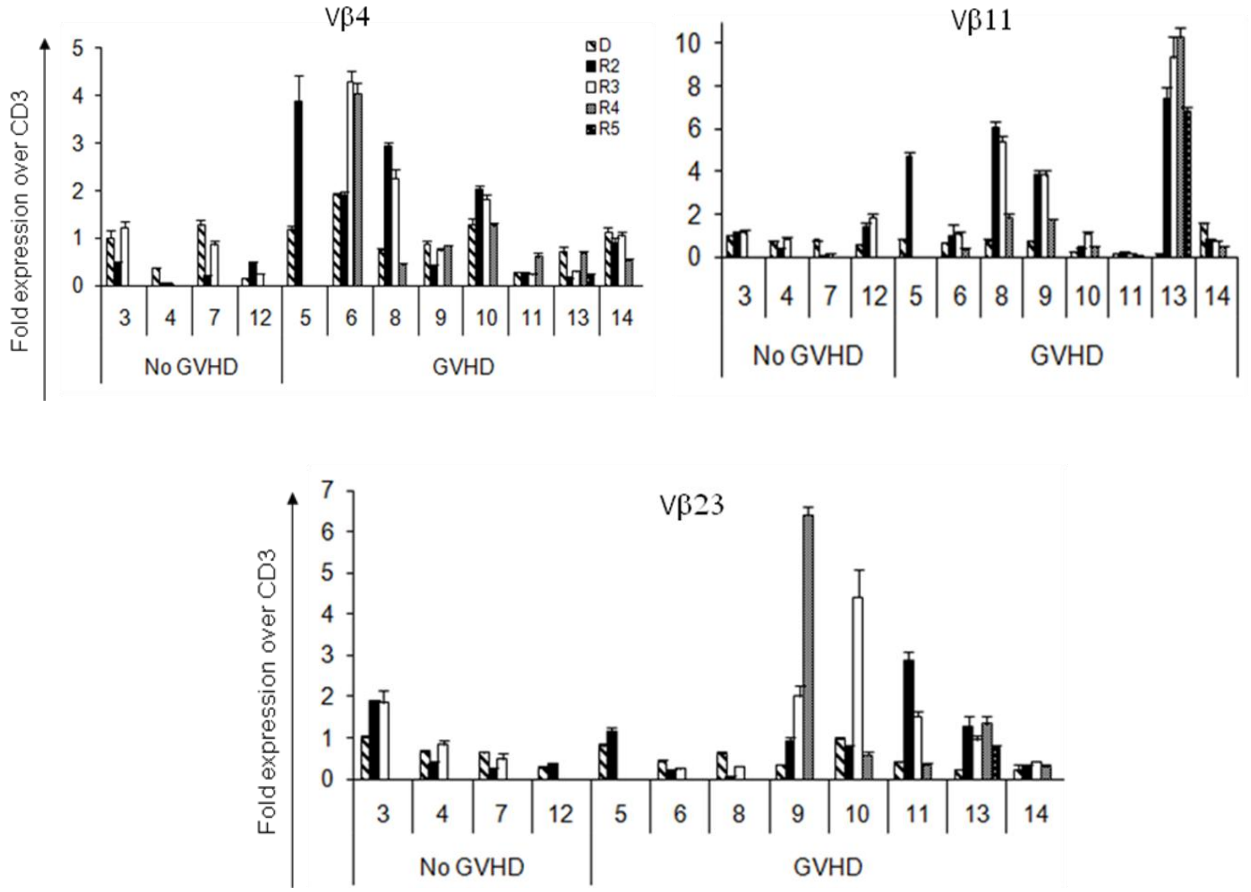


Figure 3. **qRT-PCR analysis demonstrates increased expression of TCR Vβ families 4, 11, and 23 in more than one patient with GVHD.** Peripheral T cells from recipients (R2-R5) were compared to their respective donors (D) at 90 days after HSCT (R2 for No GVHD group, and GVHD Patients 6, 8-10), time of GVHD (R2 for Patients 5, 11, 13, 14; or R3 for Patients 6, 8-10), one-year post-HSCT (R3 for No GVHD group, R4 for GVHD group except for Patients 10 and 13; R5 for Patients 10 and 13), or at time of relapse (R4 for Patient 10). Expression of all TCR Vβ families was normalized to CD3.

Figure 4:

a)

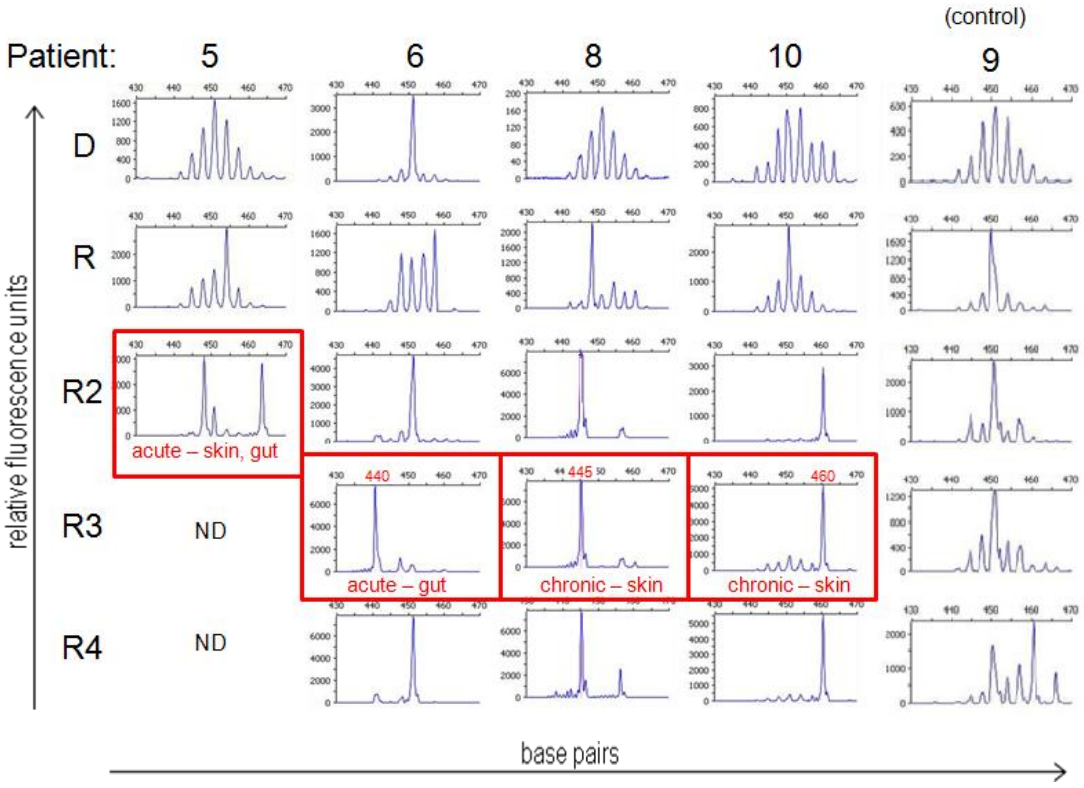


Figure 4. **Spectratyping analysis of the CDR3 region of TCR Vβ families with increased expression in more than one patient with GVHD.** a) Spectratyping analysis of Vβ4 - Patients 5, 6, 8, 10. D and R are donor, and recipient, respectively, before HSCT. R2 denotes 90 days post-HSCT for all patients except for Patients 5 and 11, for which R2 denotes time of GVHD. R3 is time of GVHD (Patients 6, 8-10) or 90 days post-HSCT (Patient 11). R4 is one-year post-HSCT, with the exception of Patient 10, for which R4 denotes time of relapse (10 months post-HSCT). A control is also shown (based on low qRT-PCR expression) to demonstrate that increased expression corresponds to the mono- or oligoclonality that is seen at the time of GVHD. ND: no data.

Figure 4b:

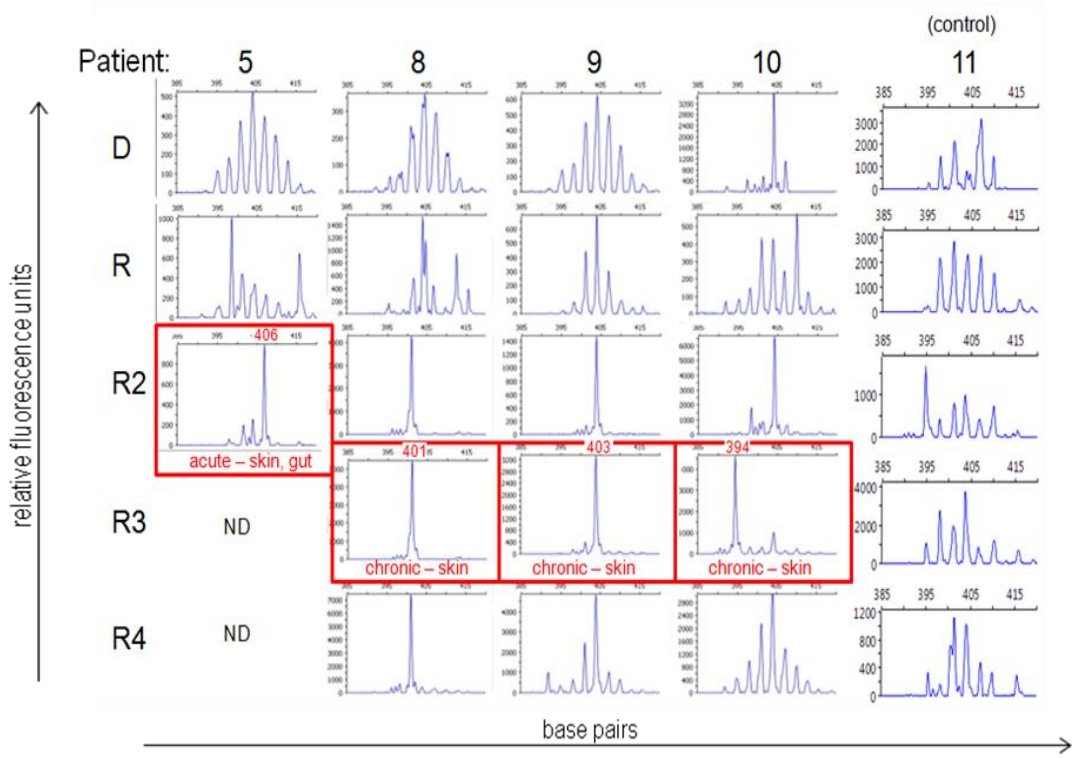


Figure 4b) Spectratyping analysis of Vβ11 - Patients 5, 8-10. D and R are donor, and recipient, respectively, before HSCT. R2 denotes 90 days post-HSCT for all patients except for Patients 5 and 11, for which R2 denotes time of GVHD. R3 is time of GVHD (Patients 6, 8-10) or 90 days post-HSCT (Patient 11). R4 is one-year post-HSCT, with the exception of Patient 10, for which R4 denotes time of relapse (10 months post-HSCT). A control is also shown (based on low qRT-PCR expression) to demonstrate that increased expression corresponds to the mono- or oligoclonality that is seen at the time of GVHD. ND: no data.

Figure 4c:

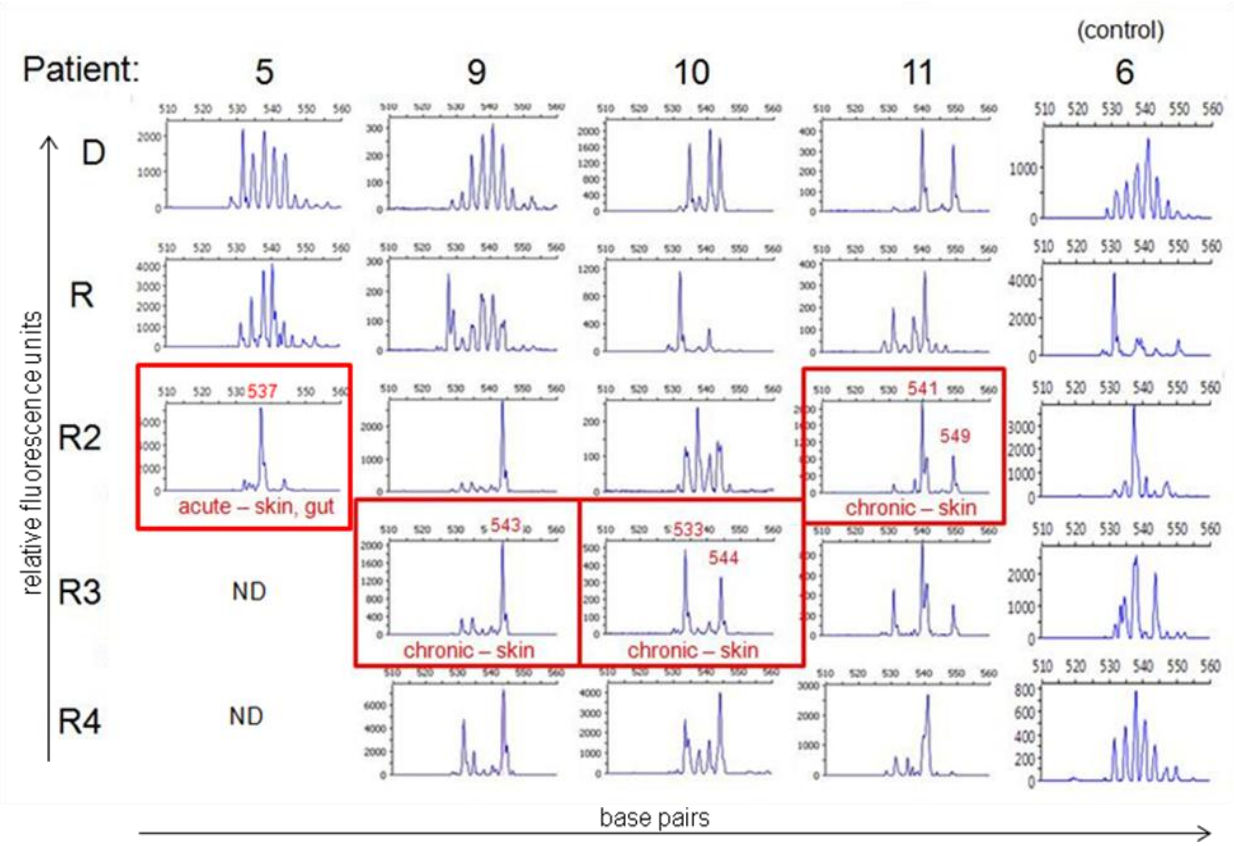


Figure 4c) Spectratyping analysis of Vβ23 – Patients 9-11. D and R are donor, and recipient, respectively, before HSCT. R2 denotes 90 days post-HSCT for all patients except for Patients 5 and 11, for which R2 denotes time of GVHD. R3 is time of GVHD (Patients 6, 8-10) or 90 days post-HSCT (Patient 11). R4 is one-year post-HSCT, with the exception of Patient 10, for which R4 denotes time of relapse (10 months post-HSCT). A control is also shown (based on low qRT-PCR expression) to demonstrate that increased expression corresponds to the mono- or oligoclonality that is seen at the time of GVHD. ND: no data.

TCR Vβ families 9, 16, and 20 are commonly associated with GVT response in GVHD-free patients.

Since GVT effects are predominantly recognized as T-cell-mediated processes in which donor T cells recognize genetic disparities within recipients, we wanted to see if Vβ families of increased expression in patients without GVHD were antigen-driven, and subsequently potentially involved in GVT responses. As shown in Figure 5, TCR Vβ9 was associated with an increased expression in all four patients without GVHD (3, 4, 7, and 12). Correspondingly, oligoclonality was seen in Patients 3, 4, and 7, at three months (R2) and one year (R3) post-HSCT for Patients 3 and 4, and at one year post-HSCT for Patient 7 (Figure 6a). (Due to sample limitations, there is no spectratyping analysis for Patient 12.) Patient 6 was used as a control for Vβ9 (Figures 5, 6a), which showed oligoclonality at R2, though this was not at the time of GVHD and polyclonality emerged in subsequent timepoints, suggesting that the increased expression and clonality seen at R2 may be associated with either homeostatic proliferation of a limited T cell population or reactivity with residual disease. Patient 8 is also shown as a control for low expression of Vβ9, but this patient demonstrated monoclonality from R-R4, which was expected as a result of the continued immunosuppressive treatments this patient was receiving (this trend was also seen for Vβs 2, 3, 7, and 17 in this patient).

TCR Vβ16 showed increased expression in Patients 3 and 4 (Figure 5) and oligoclonality at three months (R2) and one year (R3) post-HSCT in both patients (Figure 6b). Patients 7 and 9 were used as controls from the GVHD-free and GVHD groups, respectively. Though Patient 7 demonstrated low expression of Vβ16 relative to the donor, oligoclonality was seen at three months (R2) and one year (R3) post-HSCT, suggestive of the delayed thymopoiesis that is common in older patients, as there were minor clonal populations in addition to the major clonotype, and since an antigen-driven response should have shown an increased expression in qRT-PCR (Figures 5, 6b). Patient 9 demonstrated more

polyclonal expression, which was expected as a control (Figure 6b), and though the clonality was not a "perfect" Gaussian distribution, it was still reasonably so considering that complete return of normal T cell repertoires is delayed by the slower rate of the re-establishment of thymopoiesis after HSCT.

TCR Vβ20 expression was increased in Patients 3, 4, and 12 at three months (R2) and one year (R3) post-HSCT (Figure 5). Patient 3 demonstrated oligoclonality at three months and one year post-HSCT, and Patient 4 demonstrated monoclonality at one year post-HSCT (Figure 6c). The polyclonality that was seen at three months in Patient 4 was likely due to homeostatic proliferation, as there was an increased expression of Vβ20 at this time. Patients 7 and 10 were used as controls for Vβ20, from the GVHD-free and GVHD groups, respectively. As shown in Figure 6c, Vβ20 was not detected in Patient 7 at time R just prior to transplant, which was not surprising as evidenced by the extremely low qRT-PCR expression (Figure 5). Polyclonality was seen at three months post-HSCT (R2) in Patient 7, as expected by low expression. Oligoclonality was seen in Patient 7 at one year post-HSCT (R3), and though the qRT-PCR expression was lower than that of the donor, this clonality could have been due to an antigen-driven response that happened some time prior to R3. Such instances in which oligoclonality is detected without a corresponding increased expression demonstrate the limitations of qRT-PCR in amplying CDR3 regions without being able to distinguish between different VDJ rearrangements. For the other control, Patient 10 demonstrated a skewed repertoire at R2 (Figure 6c), but this could be due to delayed thymopoiesis as the patient was still under immunosuppression. Also, there was an increased expression in Patient 10 at R3 (Figure 5), though it could have been due to homeostatic proliferation as the T cell population at R3 was polyclonal.

Moreover, with regards to the GVHD-free patients, Patients 4 and 7 had mismatches of the DQB1 allele: Patient 4 (0201)/Donor 4 (02GM); Patient 7 (0302)/Donor 7 (0301) (Table 1). It is possible that GVT-responses were alloreactive against these mismatches, though the increased expression and

oligoclonality of Vβs 9, 16, and 20 were not exclusively seen in these two patients. Patient 3 was homozygous for the DQB1 0602 allele, which Donor 3 also had, though Donor 3 also had a DQB1 0503 allele. However, because the patient does not express the 0503 allele, it would be considered a mismatch in a host-versus-graft response and not in a GVT response since the donor expresses the DQB1 0602 allele as well.

Figure 5:

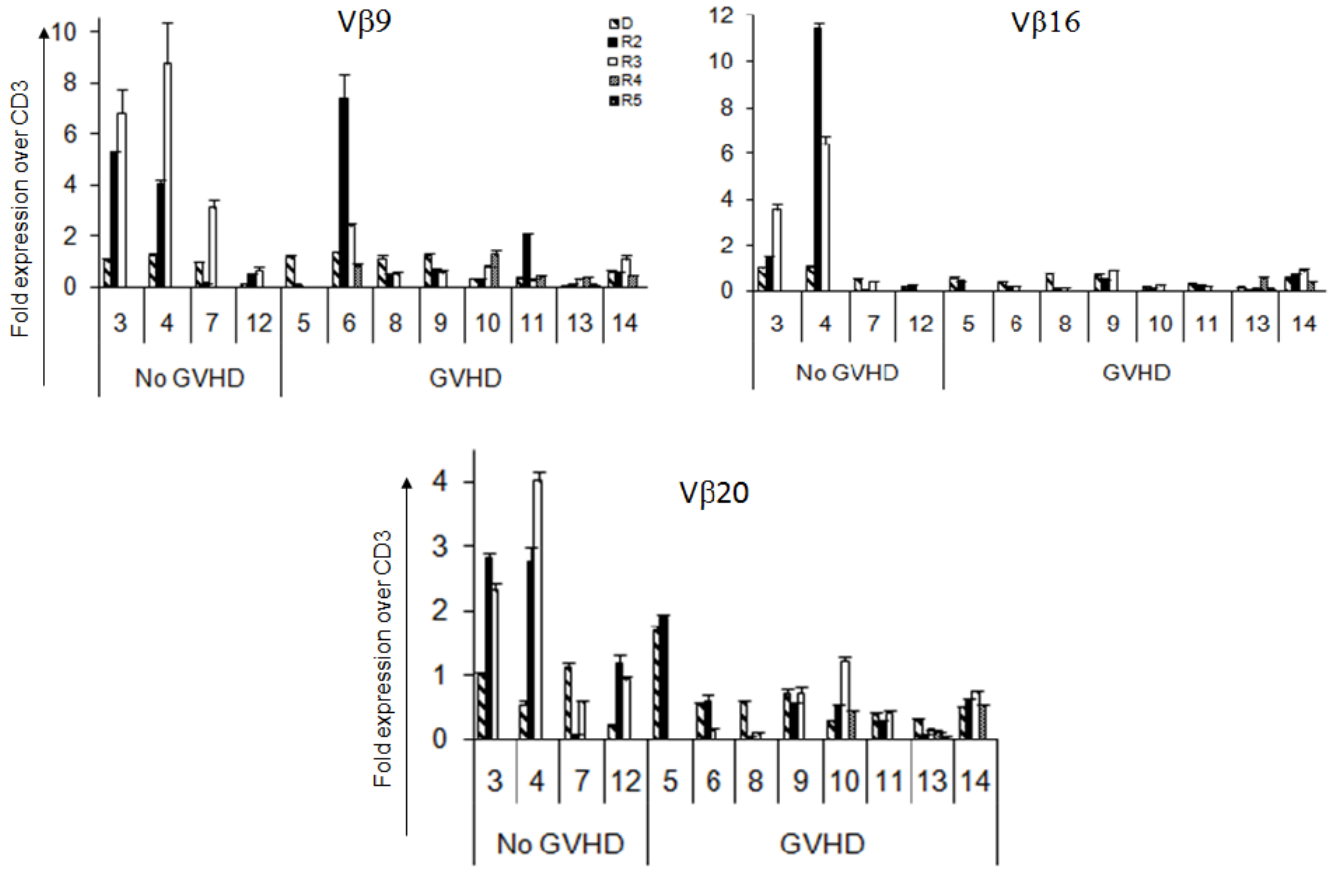


Figure 5. **qRT-PCR analysis demonstrates increased expression in TCR Vβ families 9, 16, and 20 in more than one patient without GVHD**. Peripheral T cells from recipients (R2-R5) are compared to their respective donors (D), as previously described, with expression of all TCR Vβ families normalized to CD3.

Figure 6:

6a)

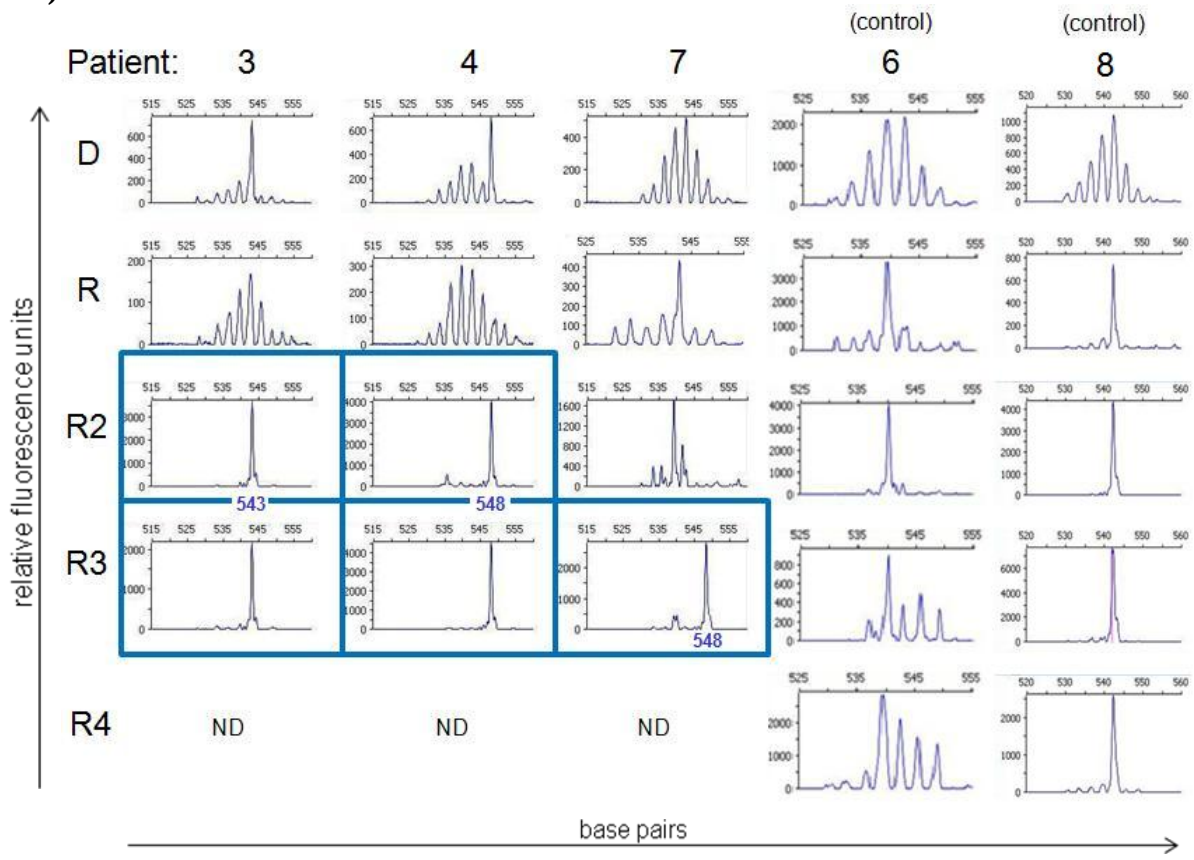


Figure 6. Spectratyping analysis of the CDR3 region of TCR Vβ families with increased expression in more than one patient without GVHD. **a)** Spectratyping analysis of Vβ9 - Patients 3, 4, and 7 show monoclonality at the times of increased qRT-PCR expression (at R2 and R3 for Patients 3 and 4, and at R3 for Patient 7); Patient 6 is shown as a control (low qRT-PCR expression). D and R are donor, and recipient, respectively, before HSCT. R2 denotes 90 days post-HSCT. R3 is one year post-HSCT in Patients 3, 4, and 7, and R3 also denotes the time of GVHD in controls 6, 9, and 10. R4 is one-year post-HSCT for Patients 6 and 9, and ten months post-HSCT for Patient 10. ND: no data.

Figure 6b:

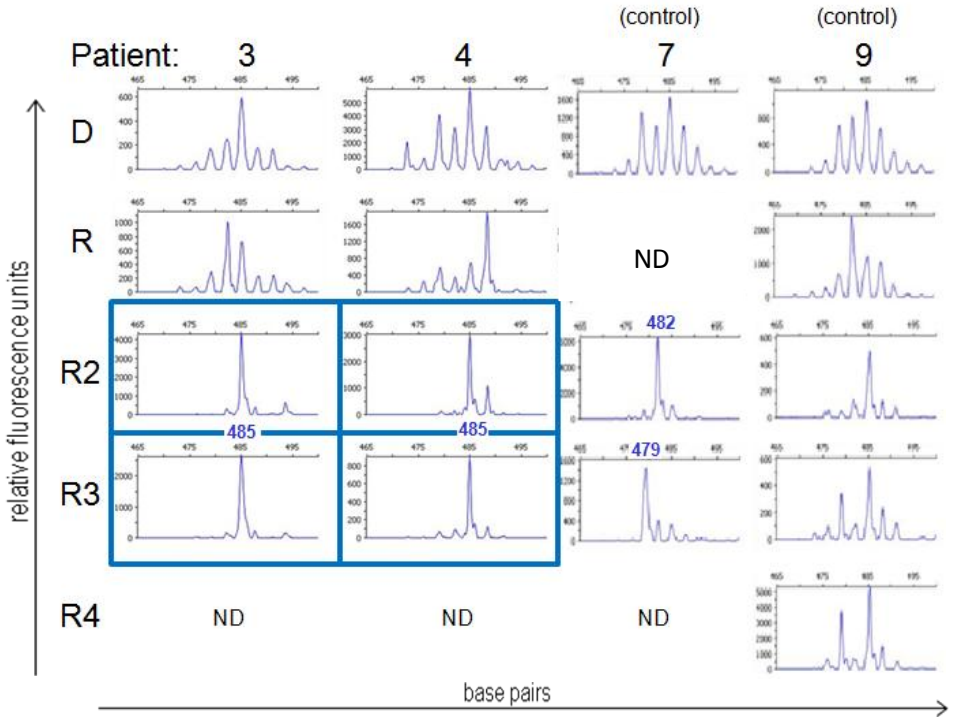


Figure 6b. Spectratyping analysis of Vβ16 - Patients 3 and 4 demonstrate monoclonality at the time of increased expression (R2 and R3). Patients 7 and 9 are shown as controls of patients without and with GVHD, respectively. D and R are donor, and recipient, respectively, before HSCT. R2 denotes 90 days post-HSCT. R3 is one year post-HSCT in Patients 3, 4, and 7, and R3 also denotes the time of GVHD in controls 6, 9, and 10. R4 is one-year post-HSCT for Patients 6 and 9, and ten months post-HSCT for Patient 10. ND: no data.

Figure 6c:

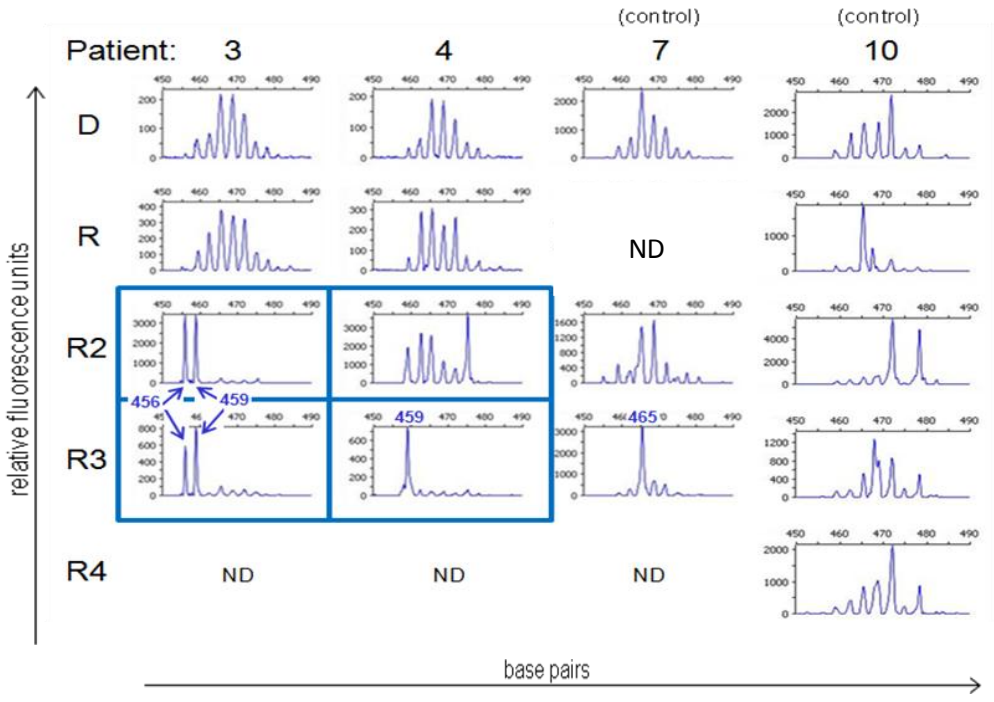


Figure 6c. Spectratyping analysis of Vβ20 - Patient 3 shows oligoclonality at R2 and R3, and Patient 4 shows monoclonality at R3. Patients 7 and 10 are controls, without and with GVHD, respectively. D and R are donor, and recipient, respectively, before HSCT. R2 denotes 90 days post-HSCT. R3 is one year post-HSCT in Patients 3, 4, and 7, and R3 also denotes the time of GVHD in controls 6, 9, and 10. R4 is one-year post-HSCT for Patients 6 and 9, and ten months post-HSCT for Patient 10. ND: no data.

TCR Vβ families 5 and 24 are privately associated with GVT response in Patient 3.

TCR Vβ families 5 and 24 demonstrated increased expression in Patient 3, suggesting a potential role in a GVT response in this patient. As shown in Figures 7a and 7b, there was an increased expression and a skewed clonal population of Vβ5 in Patient 3 at one year post-HSCT (R3). The spectratyping at R3 mirrored that at three months post-HSCT (R2), yet R2 did not have a corresponding increased expression, which may have been due to delayed thymopoiesis. The spectratyping at R3 may then have been due to an increased expression of the dominant clone (noted at 435bp in Figure 7b) with a corresponding reconstitution of the T cell repertoire. Since R3 represents one year post-transplant, the increased expression was less likely due to homeostatic proliferation as this predominantly occurs during lymphopenia early-on after transplantation. Regardless, caution should still be used with skewed populations like the one seen in Patient 3, because the distinction between oligoclonal antigen-driven responses and skewed repertoires is less clear than if there is a strong monoclonal peak with a greater increase in qRT-PCR expression (less than 1-fold increase in Vβ5 at 3R3 if considering the lower end of the error bar). Figure 7a also shows an increased expression of Vβ5 in Patient 12, but conclusions as to what this expression might correspond to will be determined in future studies (for limitations previously addressed). Patient 4 was used as a control for Vβ5 and showed polyclonality as expected.

As shown in Figures 7c and 7d, Patient 3 also showed increased expression and oligoclonality in Vβ24, though the dominant clones seen (at R2 and R3) in Figure 7d mirrored the major clonotype of Donor 3's T cell population.

Figure 7:

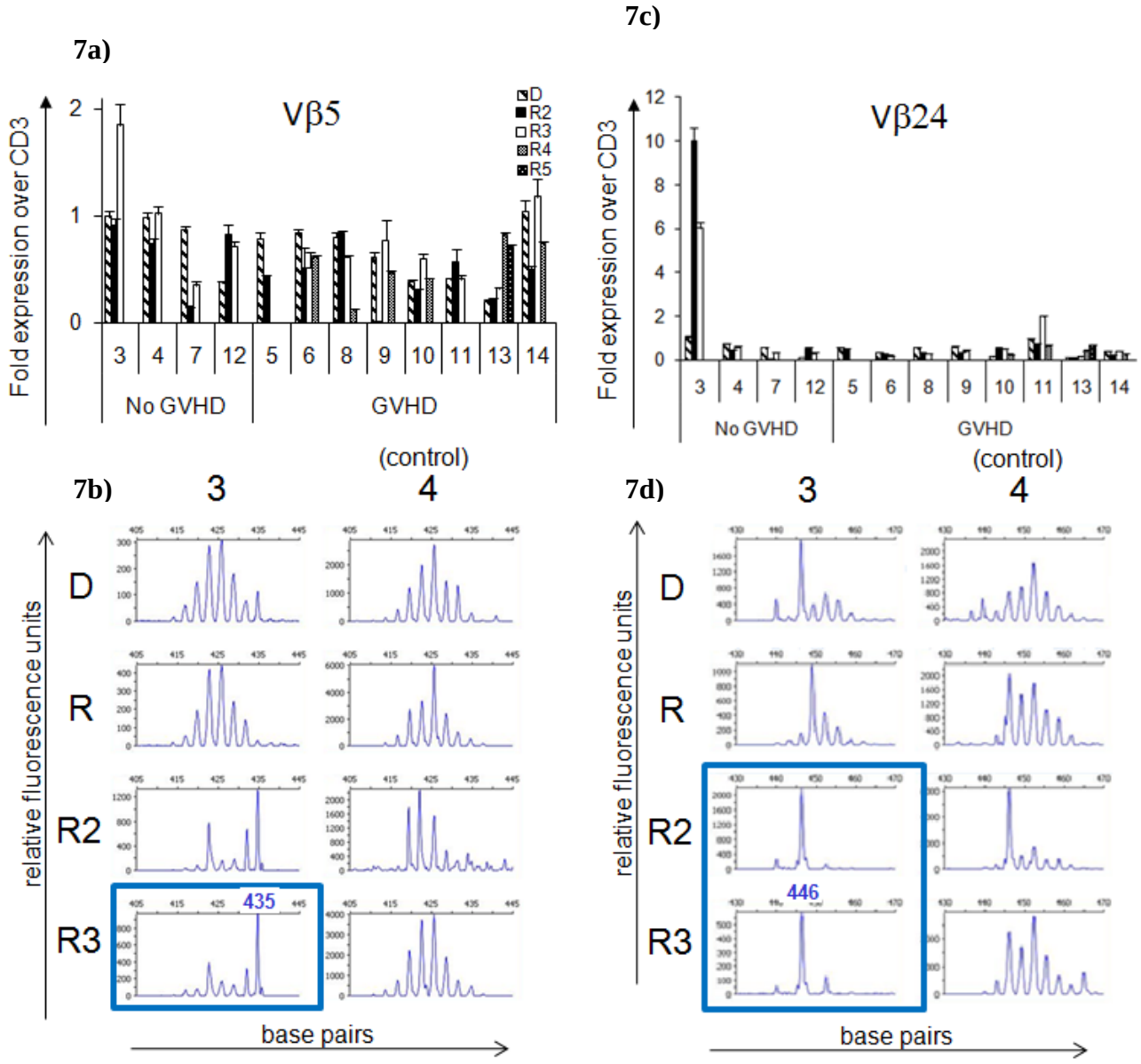


Figure 7. TCR Vβ families 5 and 24 are associated with GVT response in a patient-specific fashion. **a)** qRT-PCR analysis shows an increased expression of Vβ5 in GVHD-free Patients 3 and 12. **b)** Spectratyping analysis of Vβ5 for Patient 3 corresponds to oligoclonality. Patient 4 is shown as a control. **c)** qRT-PCR analysis of Vβ24 shows increased expression of Vβ24 in Patient 3. **d)** Spectratyping analysis of Vβ24 shows monoclonality in Patient 3 at R2 and R3. Patient 4 is shown as a control. Time points are as previously described.

Increased expression of TCR Vβ families 2, 3, 7, 8, 12, 15, and 17 are shared by both GVHD and GVHD-free patients.

In addition to identifying Vβ families that were increased in either the GVHD or GVHD-free patient groups, we also wanted to see if certain Vβ families were commonly associated with patients in both groups. As shown in Figure 8, TCR Vβ families 2, 3, 7, 8, 12, 15, and 17 show increased expression in multiple recipients, respective to their donors, in both the GVHD-free and GVHD groups.

TCR Vβ2 was increased in all GVHD-free patients as well as in GVHD Patients 6, 9, 10, 11, and 13. As shown in Figure 9 with regards to Vβ2, GVHD-free Patients 3 and 7 showed oligoclonality at one year post-HSCT (R3), as expected based on their respective qRT-PCR data. The clonality in Patient 3 followed a similar trend to that seen in this patient for Vβ5 in which the same clonality was seen at R2 and R3 though there was only a corresponding increased expression at R3. Also, the oligoclonality was borderline skewed, again demonstrating the fine line between antigen-driven peaks and the typical aberrant restoration of normal T cell repertoires in patients following HSCT. The clone seen at one year post-HSCT (R3) in Patient 7 mirrored that seen in the patient before transplant, which may represent continuation or reactivation of that antigen-experienced population, as Patient 7 had 63% recipient T cell chimerism at R3. Patients 6, 10, and 11 all showed a major clone over a polyclonal background, demonstrating restoration of the Vβ2 repertoire during homeostatic proliferation. Because there was no retention of the oligoclonality from the time of GVHD onset (R2) at R3 in Patient 11, the skewed population at R2 could also be explained by homeostatic proliferation with delayed thymopoiesis since a GVHD-associated clone should have continued from R2 to R4, as GVHD was not wholly resolved at R4. Though Patient 9 demonstrated increased expression at R3, there was a polyclonal population, suggesting that the increased expression was due to homeostatic proliferation. An oligoclonal population emerged at R4, though there was no corresponding increased expression,

demonstrating the limitations of qRT-PCR in being unable to determine the proportions of specific CDR3 sequences, as opposed to amplifying individual CDR3 sequences. Patients 4 and 8 were run as controls for Vβ2. Patient 4 demonstrated polyclonality, as expected. Patient 8 demonstrated oligoclonality from R2-R4, which as aforementioned was common, as this patient was still being treated with immunosuppressive therapies that hindered reconstitution of a normal T cell repertoire.

For TCR Vβ3, GVHD-free Patients 3, 7, and 12 and GVHD Patients 6, 11, and 13 showed increased expression (Figure 8). Patient 3 demonstrated oligoclonality at three months (R2) and one year (R3) post-HSCT (Figure 10) with minor clones in addition to the major clone, representing an oligoclonal expansion over a polyclonal background. Patient 7 demonstrated oligoclonality only at R2, which may be due to the mixed chimerism (52% recipient T cells), as this clone mirrored that of the patient at time R prior to HSCT (Figure 10). Though there was also increased expression at one year post-HSCT (R3) in Patient 7 (Figure 8), the clone mirroring that from R2 was less dominant as the spectratyping was more polyclonal (Figure 9), which may be explained by homeostatic proliferation and initial re-establishment of thymopoiesis. Retention of the clone from R2 at R3, along with the emergence of other clones at R3, may also demonstrate mixed chimerism showing a major clone from either the patient or donor and a polyclonal population from the other (Patient 7 had 63% recipient T cell chimerism at R3). With regards to the GVHD patients, oligoclonality was seen in Patients 6 and 11 at their respective times of GVHD (Figure 10). Because Patient 6 was still being treated for GVHD at one year post-HSCT (R4), the continuance of the clonal population from the time of GVHD onset (R3) at R4 was expected. Patient 11 showed oligoclonality of three dominant clones at R2 and R3, as expected based on increased expression (Figures 8, 10), and though the onset of GVHD is at R2, the patient was still being treated for GVHD at R3 and R4, so the continued clonality was expected. Furthermore, that there are potentially three antigen-associated clonal populations in Patient 11, it is possible that they

were related to GVHD and/or GVT. Patients 4, 8, and 9 are shown in Figure 8 as controls. Patients 4 and 9 demonstrated polyclonality, as expected. Patient 8 showed oligoclonality, which, as aforementioned, was resultant of continued GVHD treatment, thus hindering the reconstitution of a diverse T cell repertoire.

Increased expression of TCR Vβ7 was seen in GVHD-free Patient 3 and in GVHD Patients 6, 9, 10, 11, 13, and 14 (Figure 8). As shown in Figure 11, Patient 3 showed oligoclonality at R2 and R3, suggesting that the increased expression may have been due to an antigen-driven response. Patients 4 and 7 are shown as controls. Though a skewed repertoire was seen at R2 in Patient 4, it may have been due to delayed thymopoiesis. Patient 7 showed a dominant clone at R2, which mirrored that of the pre-transplant clone seen at R, suggesting retention of that clonal population from the patient as there was 52% recipient T cell chimerism at R2. Because this clone was still the major clone seen over the polyclonal population at R3, this clone may again represent the patient's mixed chimerism, with the dominant clone being from either the donor or recipient and a polyclonal population being due to the other. Patient 6 demonstrated polyclonality at the time of GVHD onset (R3), which was likely a result of homeostatic proliferation. Furthermore, Patient 6 demonstrated oligoclonality at R2 and R4, though there were not corresponding increases in expression at these times. This suggests that these clonal populations may have been involved in GVT responses at either time. Patients 9, 10, and 11 all showed oligoclonality corresponding to their increased expressions of Vβ7 (Figure 11). The clonality seen at the time of GVHD onset in Patients 10 (R3) and 11 (R2) continued at R3 and R4, which was not unexpected, as neither patient had complete resolution of GVHD at R4. Patient 8 was used as a control from the GVHD group and again demonstrated oligoclonality as a result of immunosuppression. The donors for Patients 10 and 11 had somewhat skewed T cell populations for Vβ7 at the time of transplant, which may be representative of antigen-experienced T cell populations. Patients 9, 10, and

11, as well as Patients 3, 7, and 8, all demonstrated oligoclonality at time R prior to transplant. This may indicate that the Vβ7 family is associated with common viral or pathogen immunity, as a skewed repertoire was also seen in Donors 7, 10, and 11. Furthermore, the Vβ7 family may have a more restricted repertoire based solely on base pair size, as the skewed repertoires were seen throughout the spectratyping data for Vβ7 both in patients with and without increased expression. This is also implied in the broader bases of the peaks, which shows a saturation of clones within a given base pair region, unlike the Gaussian distribution seen in polyclonal populations with major peaks separated by 3 nucleotides.

The TCR Vβ8 family was increased in GVHD-free Patients 4 and 12 and GVHD Patients 5, 10, 11, and 14 (Figure 8). As shown in Figure 12, Patient 4 showed oligoclonality at times R2 and R3, indicative of an antigen-driven response. Donor 4 also showed a dominant peak around the same base pair size as that seen at R2 and R3 in the recipient, indicating that this clone may have been antigen-experienced. If this donor clone was represented in the clonal population demonstrated at R2 and R3, it may demonstrate the ability for antigen-experienced T cells to recognize non-self peptide:MHC complexes.[63] The human memory T cell population is dominated by reactivities to common DNA viruses (i.e. – CMV, EBV, HSV), and the possibility of cross-reactivity of antigen-experienced T cells with foreign peptide:MHC complex is high, despite the rarity of individual cross-reactivities.[63] As shown by Melenhorst *et al.*, antigen-experienced $CD4^+$ and $CD8^+$ T cell subsets are able to recognize MHC-mismatched APCs.[63] Since Patient 4 had an HLA-DQB1 mismatch (Table 1), this clonally-driven population may have been involved in a GVT response through either cross-reactivity of an antigen-experienced T cell or from direct HLA-dependent recognition. Patient 3 is shown as a GVHD-free control, and though there was slight oligoclonality at R2, it was likely due to the patient still being under immunosuppressive treatment at R2, as the repertoire became more polyclonal at R3. GVHD

Patients 5, 10 and 11 showed oligoclonality, as expected by increased expression (Figures 8, 12). Patient 10 showed a dominant clonotype over a skewed background at the time of GVHD onset (R3). This clonal population was also seen at R2 and R4, indicating the potential for a GVHD-associated clone to be detected prior to clinical diagnosis, and the continuance of the clone at R4 was expected as this patient's GVHD was not wholly resolved at R4. Patient 11 demonstrated dramatic clonality that mirrored that of Donor 11. Though Patient 11 did not have any HLA mismatches, these clonotypes were likely antigen-experienced, and may have been involved in a GVT or GVHD response against non-self peptide:MHC in a peptide-dependent manner. Patient 6 showed a slight increase in expression at R2, but the aberrant clonality fits with homeostatic proliferation as opposed to an antigen-driven response. Patient 8 is shown as a control from the GVHD group, and was predominantly polyclonal as expected.

As shown in Figure 8, expression of the TCR Vβ12 family was increased in GVHD-free Patients 4 and 12 and GVHD Patients 6, 8, 10, and 13. Patient 4 showed a somewhat oligoclonal population at R2 (Figure 13), but this was likely due to delayed thymopoiesis and homeostatic proliferation, as polyclonality was completely restored at R3. Patients 6, 8, and 10 all demonstrated oligoclonality at the time of GVHD as expected based on increased qRT-PCR expression (Figures 8, 12). However, GVHD was not clinically resolved at R4, and clonality only persisted in Patient 6 at R4, indicative of continuance of a GVHD-associated clone. Furthermore, the major clonal populations seen in Patients 6 and 10 were the same size (433bp; Figure 12), suggesting a role for a GVT effect to a mHAg that is restricted to the hematopoietic system. Patients 3 and 11 were used as controls, and both demonstrated polyclonality as expected.

Continuing through Figure 8, expression of TCR Vβ15 was increased in GVHD-free Patients 3, 4, and 12 and GVHD Patients 10, 11 and 13. Figure 14 shows oligoclonality in Patient 3 at R2 and R3,

indicating an antigen-driven response, and Patient 4 was predominantly polyclonal, indicating homeostatic proliferation. Patients 10 and 11 also demonstrated oligoclonality at the time of GVHD, as expected. The continuance of clonality from R2 through R4 in Patient 10 may depict the ability to detect a GVHD-associated clone prior to clinical diagnosis (R3), and since GVHD was not wholly resolved at R4, this better supports the possibility of this clonal population being associated with GVHD. The sharp monoclonality seen in Patient 11 mirrored the clonotypes seen in both donor and recipient before transplant, suggesting that this population may have been antigen-experienced to a common virus or pathogen. If this clonal population was antigen-experienced, it may have been able to cross-react with non-self peptide:MHC complexes in a peptide-dependent manner (as there are no HLA mismatches in Patient 11). Patient 7 is shown as a GVHD-free control and depicted oligoclonality, which may have been due to both mixed T cell chimerism and retention of the recipient's clonotype from R (as discussed previously for this patient) and delayed thymopoiesis. Patient 8 is shown as a control from the GVHD group, and though there was slight oligoclonality at R2, it was consistent with delayed thymopoiesis as polyclonality was more evident at R3 and R4.

For the last TCR Vβ family shared by both GVHD-free and GVHD patients in Figure 8, expression of Vβ17 was increased in GVHD-free Patients 3 and 12 and GVHD Patients 10, 11, and 13. Patient 3 showed oligoclonality at R3 (Figure 15), as expected based on increased qRT-PCR expression. However, the clonality seen at R3 mirrored that seen at R2 when there was not a corresponding increase in expression, reiterating the caution in defining certain oligoclonal populations as either oligoclonally antigen-driven responses or as skewed repertoires resultant of delayed thymopoiesis and restoration of a normal T cell repertoire. Patient 4 is shown as a control from the GVHD-free group and demonstrated polyclonality as expected. Patients 10 and 11 demonstrated oligoclonality at their times of GVHD, R3 and R2, respectively. The clonality demonstrated in Patient 10 persisted from R2-

R4, suggesting the ability to potentially identify GVHD-associated T cell populations prior to clinical diagnosis, and as this patient's GVHD was not completely resolved at R4, the continuance of this clonal population was expected. The clonality seen at R2 and R3 in Patient 11 may have been involved in a GVHD or GVT response. Since Patient 11's GVHD was still being treated at R4, if the R2 and R3 clonal populations were GVHD-associated, they should have persisted at R4, though they may have been less prominent if the GVHD was very minimal at that time. Patient 8 is shown as a control from the GVHD group and demonstrated the same monoclonal trend that was evident in previous Vβ families, as this patient was under continued immunosuppression. There was also no clonal population detected in the Vβ17 family for Patient 8 at time R prior to transplant, and as the qRT-PCR expression was very low at this time, this result was not surprising.

Figure 8:

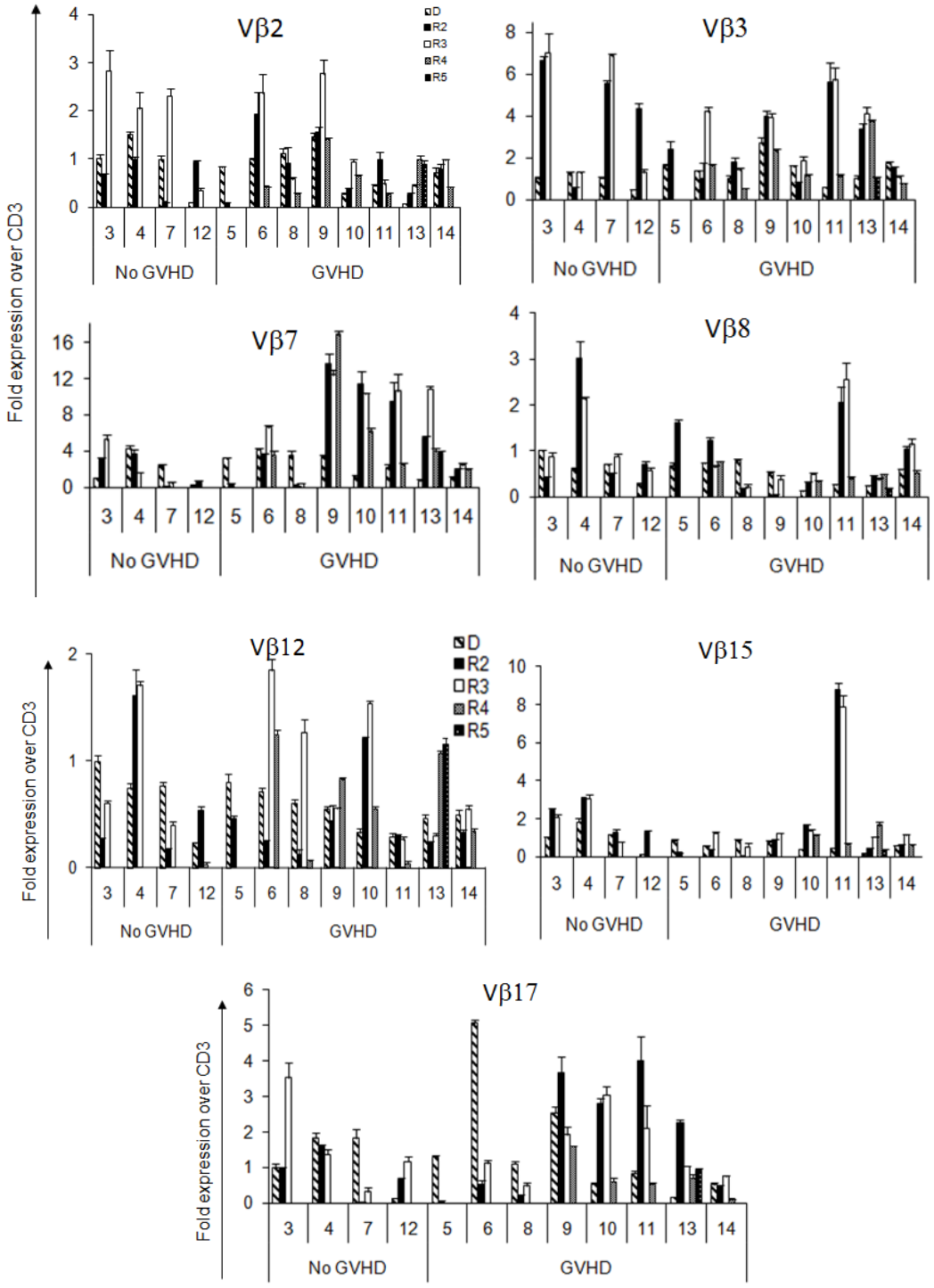


Figure 8. TCR Vβ families with increased expression in patients with and without GVHD. qRT-PCR analysis of TCR Vβ families 2, 3, 7, 8, 12, 15 and 17 in all patients. Times points are as previously described.

Figure 9:

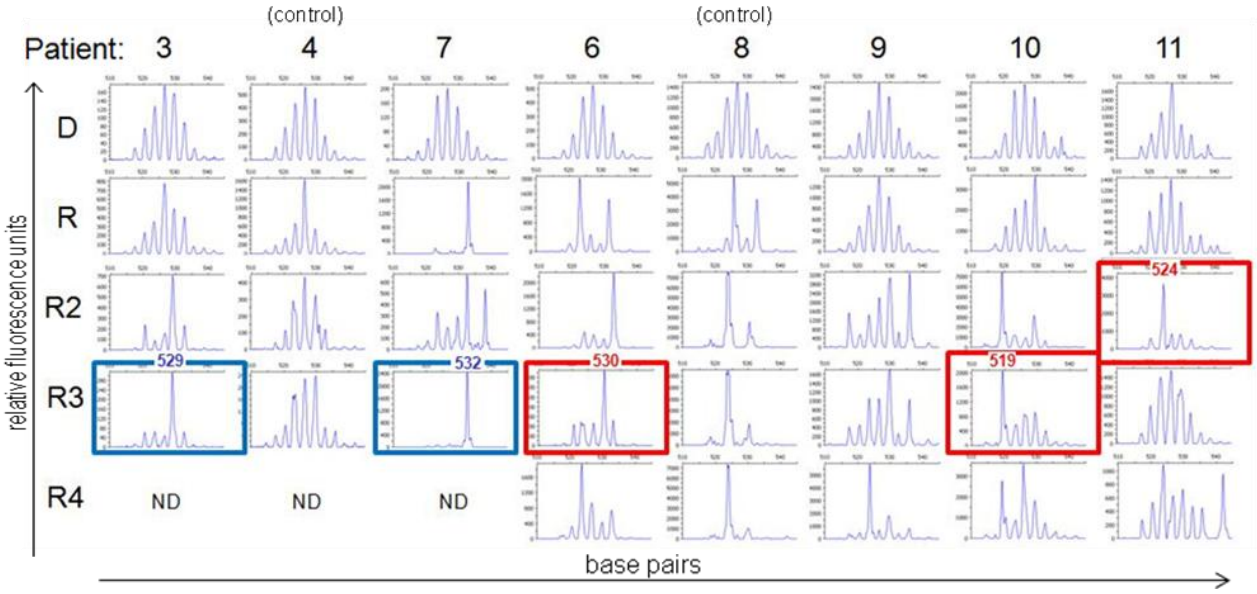


Figure 9. Spectratyping analysis of TCR Vβ2 in patients with and without GVHD. Blue boxes denote oligoclonality in patients without GVHD at the time of increased TCR Vβ2 expression (R3). Red boxes denote oligoclonality in patients with GVHD, at the time of GVHD (R3 for Patients 6, 10; and, R2 for Patient 11). Patients 4 and 8 are negative controls based on low qRT-PCR expression of Vβ2. Time points are as previously described. ND: no data.

Figure 10:

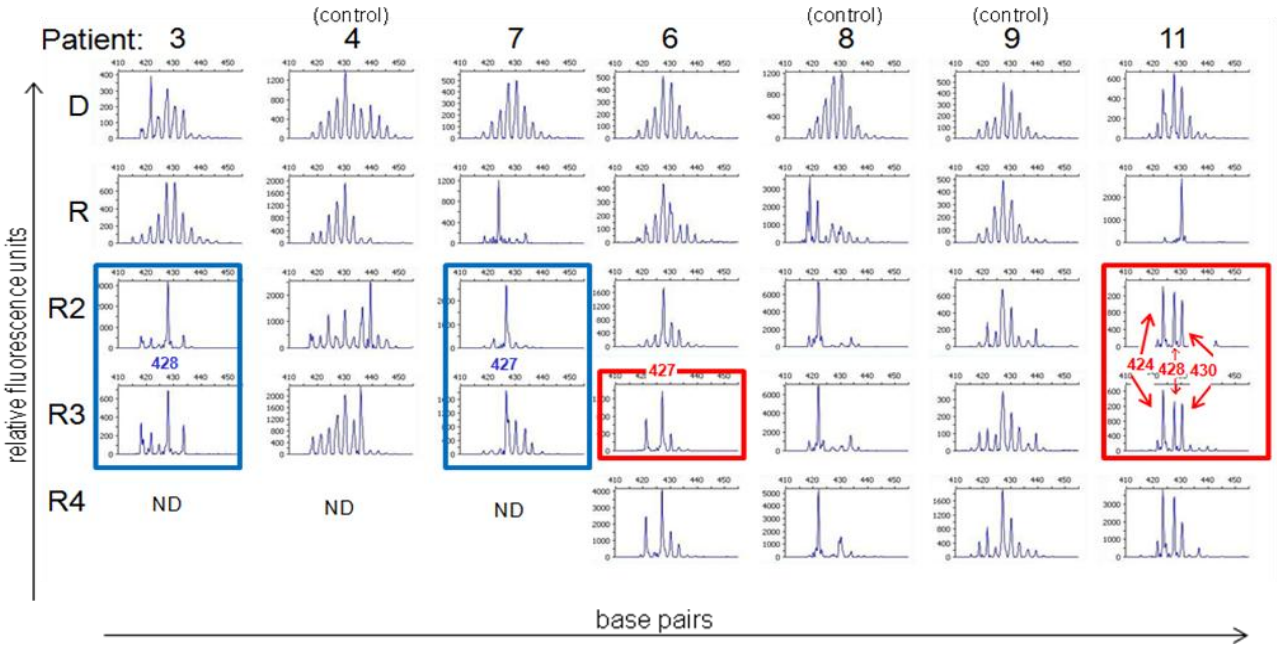


Figure 10. Spectratyping analysis of TCR Vβ3 in patients with and without GVHD. Blue boxes denote oligoclonality in patients without GVHD at the time of increased TCR Vβ3 expression (R2 -R3). Red boxes denote oligoclonality in patients with GVHD, at the time of GVHD (R3 for Patients 6, R2 for Patient 11). Patients 4, 8, and 9 are controls. Time points are as previously described. ND: no data.

Figure 11:

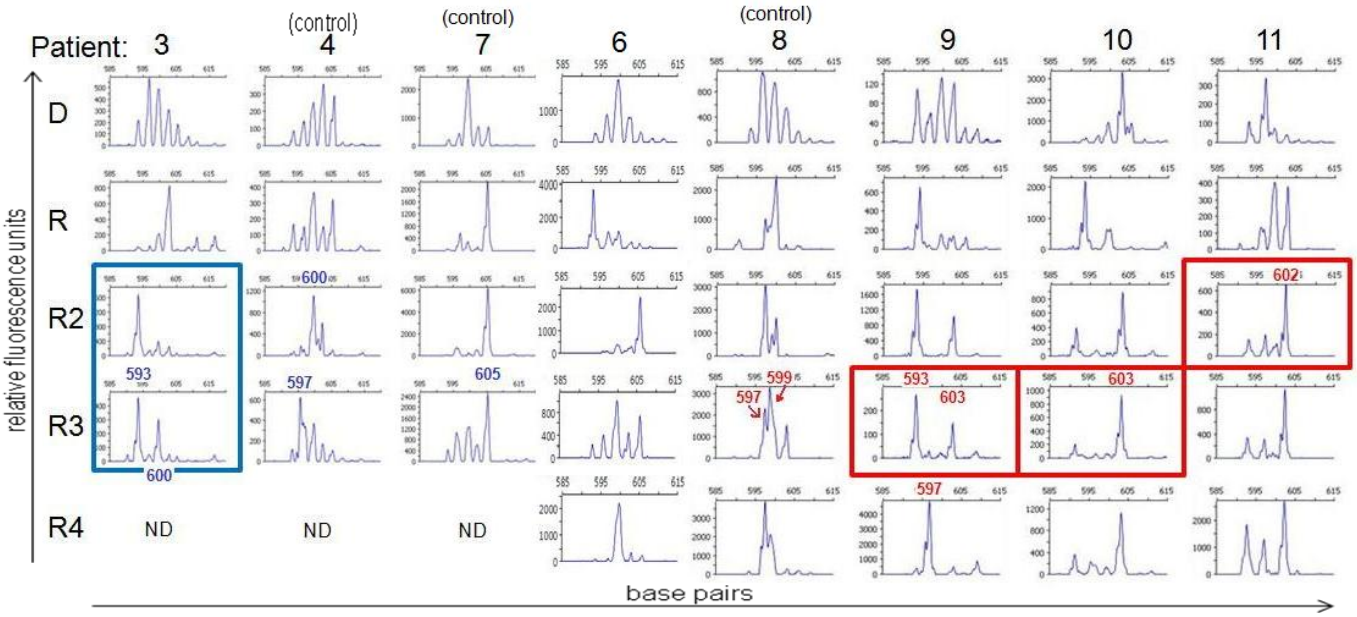


Figure 11. Spectratyping analysis of TCR Vβ7 in patients with and without GVHD. Blue boxes denote oligoclonality in patients without GVHD at the time of increased TCR Vβ7 expression (R2 -R3). Red boxes denote oligoclonality in patients with GVHD, at the time of GVHD (R3 for Patients 9, 10; and, R2 for Patient 11). Patients 7 and 8 are controls. Time points are as previously described. ND: no data.

Figure 12:

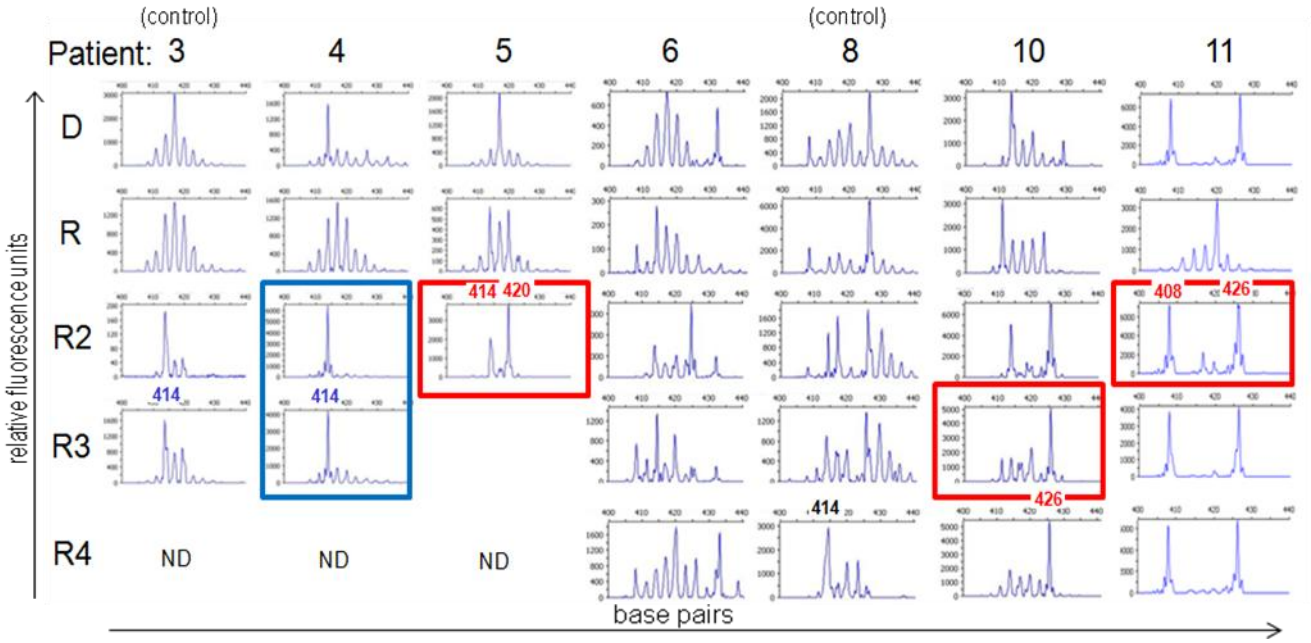


Figure 12. Spectratyping analysis of TCR Vβ8 in patients with and without GVHD. Blue boxes denote oligoclonality in patients without GVHD at the time of increased TCR Vβ8 expression (R2 -R3). Red boxes denote oligoclonality in patients with GVHD, at the time of GVHD (R3 for Patient 10; and, R2 for Patients 5 and 11). Patients 3 and 8 are controls. Time points are as previously described. ND: no data.

Figure 13:

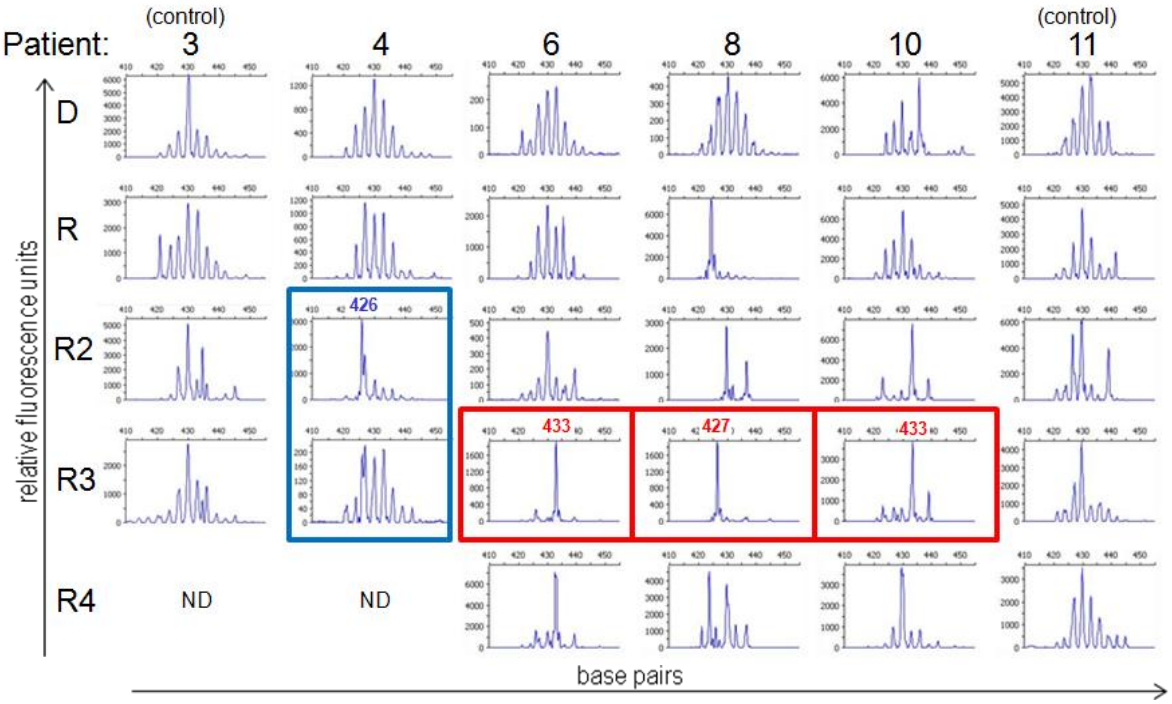


Figure 13. Spectratyping analysis of TCR Vβ12 in patients with and without GVHD. Blue boxes denote oligoclonality in patients without GVHD at the time of increased TCR Vβ12 expression (R2 - R3). Red boxes denote oligoclonality in patients with GVHD, at the time of GVHD (R3 for Patients 6, 8, and 10). Patients 3 and 11 are controls. Time points are as previously described. ND: no data.

Figure 14:

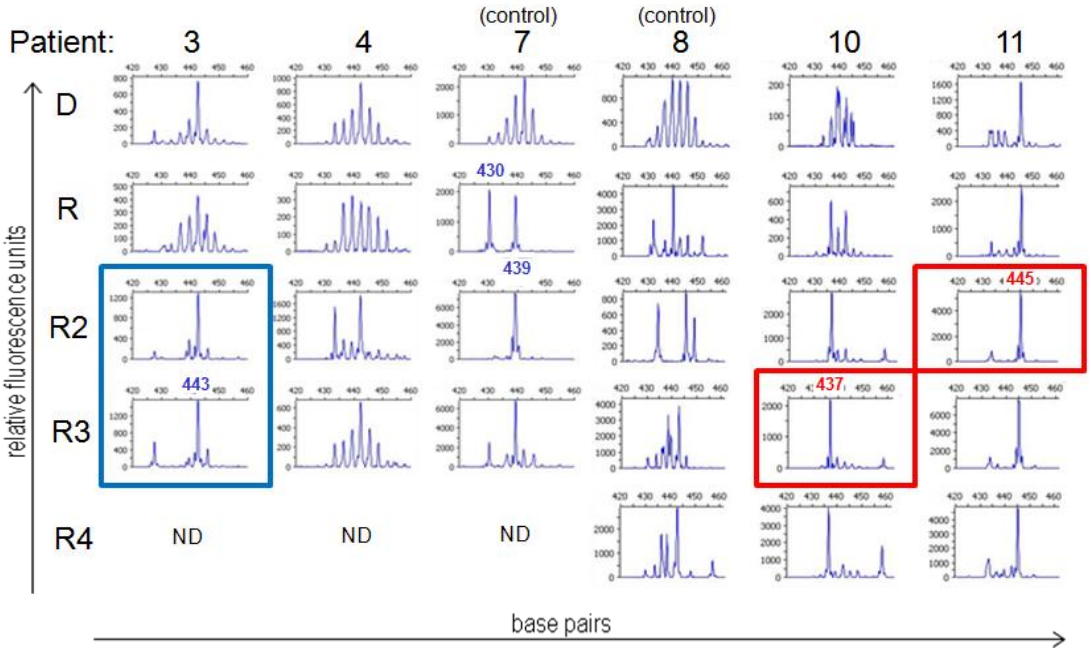


Figure 14. Spectratyping analysis of TCR Vβ15 in patients with and without GVHD. Blue boxes denote oligoclonality in patients without GVHD at the time of increased TCR Vβ15 expression (R2 - R3). Red boxes denote oligoclonality in patients with GVHD, at the time of GVHD (R3 for Patient 10, R2 for Patient 11). Patients 7 and 8 are controls. Time points are as previously described. ND: no data.

Figure 15:

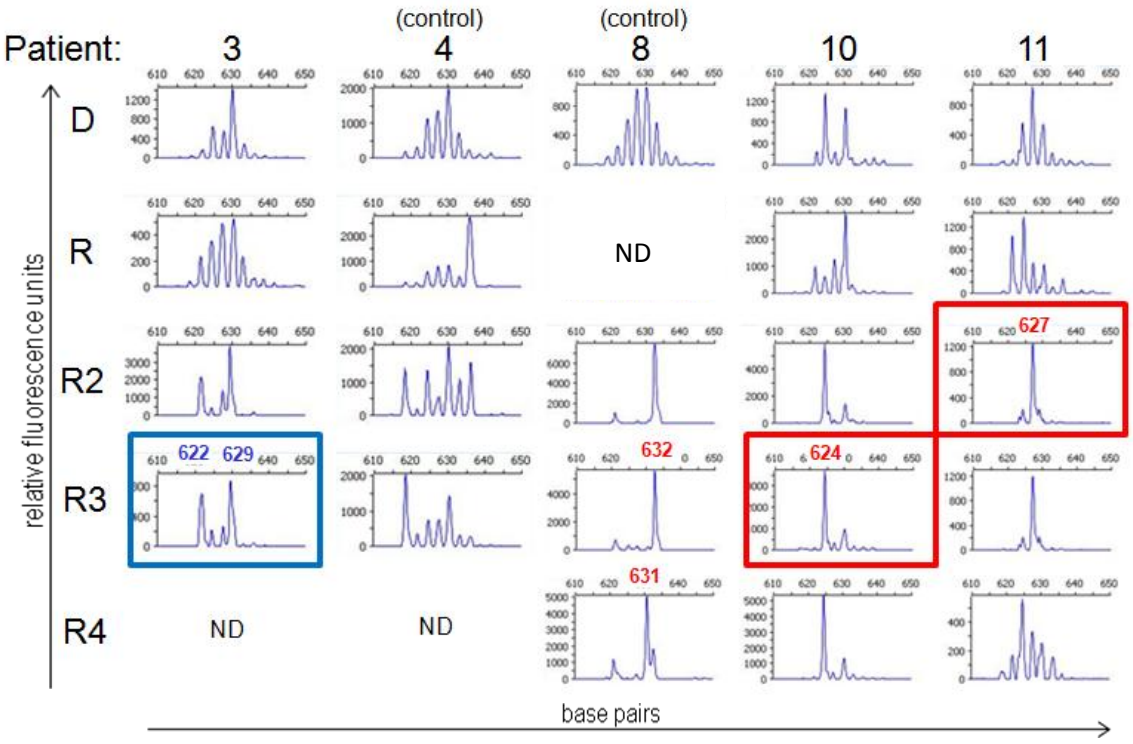


Figure 15. Spectratyping analysis of TCR Vβ17 in patients with and without GVHD. Blue boxes denote oligoclonality in patients without GVHD at the time of increased TCR Vβ17 expression (R3). Red boxes denote oligoclonality in patients with GVHD, at the time of GVHD (R3 for Patient 10, R2 for Patient 11). Patients 4 and 8 are controls. Time points are as previously described. ND: no data.

DNA extraction from GVHD-biopsy tissues identified specific Vβ families at the site of GVHD.

We next wanted to determine if there was a direct correlation between the levels of T cell clones in the circulation with those at the site of GVHD. GVHD Patients 5, 6, 8, and 11 had available paraffin-embedded biopsy samples from which we were able to extract DNA. We used GAPDH primers to run PCR reaction to confirm the extraction of DNA from these samples. Figure 14 shows the results of these PCR reactions for GAPDH, with all products showing bands on a 1% agarose gel around the 300bp region. Patients 5, 6, and 11 had one sample, corresponding to the time of GVHD, which was R2 for Patients 5 and 11, and R3 for Patient 6. Patient 8 had two samples, Sample A corresponding to the first clinical indications of GVHD onset (R3), and Sample B, corresponding to three months after Sample A, during active chronic GVHD. We also used a control sample from non-GVHD Patient 4 who had a biopsy taken around 4 months post-HSCT, which was classified as folliculitis (an infection in the hair follicles, often caused by bacteria). Based on qRT-PCR and spectratyping, we ran PCR using primers for those TCR Vβ families that demonstrated both increased expression and oligoclonality in order to see if we could detect these Vβ families at the site of GVHD. We also ran negative control PCR reactions based on Vβ families that had low qRT-PCR expression.

Patient 5 demonstrated increased expression and oligoclonality in Vβ families 4, 8, 11, and 23 (Figures 3, 4, 8, 12). PCR products for Vβs 4, 11, and 23 were detected at the site of GVHD, as shown by the PCR product bands on a 1% agarose gel (Figure 17). Control reaction for Vβ2 was detected at the site of GVHD, though there was polyclonality at this time in the peripheral T cells (Figures 17a,b). For Patient 6, PCR reactions were run for Vβs 2, 3, 4, and 12 because they demonstrated both increased expression and oligoclonality (Figures 3, 4, 8, 10, 13). From these reactions, Vβs 2 and 4 were detected at the site of GVHD (Figure 18). Vβs 6 and 9 were run as negative controls and did not show bands on

the agarose gel, as expected (Figures 5, 6a, 21a,b).

For Patient 8, because Sample A corresponds to R3 and Sample B is from 3 months after R3 and therefore does not have a corresponding peripheral T cell sample, we ran PCR products for Sample B based on our predictions for Sample A. Patient 8 demonstrated increased expression and oligoclonality in Vβ families 2, 3, 4, 11, 12, 17 (Figures 3, 4, 8-10, 13, 15). From these, Vβs 2 and 4 were detected in both Samples A and B, and Vβ11 was detected in Sample B. Vβ9 was run as a negative control based on low qRT-PCR expression (Figures 5), though it was detected at the correct size (539bp), and a smaller second band was detected around the 400bp region (Figure 19) (the second band could be due to primers binding to homologous sequences in the DNA or to amplification of non-transcribed DNA with stop codons). Though Patient 8 demonstrated oligoclonality for Vβ9 at all times (R-R4), this could have been a result of his continued immunosuppression, as the oligoclonality was seen in multiple Vβ families of low expression throughout his course of treatment. Furthermore, Vβ9 may merely represent non-clonal T cell populations at the site of GVHD. Since the clonality of the Vβ families detected at the site of GVHD is not known from our data, we cannot eliminate the various possibilities for these T cells.

For Patient 11, PCR was run for those Vβ families of both increased expression and oligoclonality: 2, 3, 7, 8, 11, 17, and 23 (Figures 3, 4, 8-12, 15). Vβ6 was also run due to its increased expression in the peripheral blood (Figure 21a). As shown in Figure 20, Vβs 2, 3, 6, 15 and 17 were detected at the site of GVHD. Vβ11 was run as a negative control, and correspondingly no band was detected.

It has been shown that Vβs 2 and 6 are common in the healthy skin of adults, so though these Vβ families may have low expression in the peripheral blood, they may also be common in the skin.[64] We detected Vβ2 at the site of disease in each of our samples that were from skin GVHD (Patients 8 and 11), in which Patient 11 had increased expression and a skewed repertoire (Figure 8, 9), and Patient 8

demonstrated low expression (Figure 8) and oligoclonality (Figure 9) in the peripheral blood. Low expression with corresponding oligoclonality was expected for Patient 8, as continued immunosuppressive treatments hindered the re-establishment of a normal T cell repertoire. With regards to Vβ6, it was only detected at the site of GVHD in Patient 11, and though there was a slight increase in qRT-PCR expression in the peripheral blood, polyclonality was seen at this time (Figure 21a,b). To compare our results to those of Menssen *et al*. in which Vβs 2 and 6 were detected in the skin of normal healthy adults,[64] we ran PCR reactions for the control folliculitis sample, Patient 4, in which Vβ2 was identified, but not Vβ6 (Figure 22). There was both low expression (Figures 8, 21a) and polyclonality (Figures 9, 21b) of Vβ2 and 6 in Patient 4, demonstrating that these Vβ families were not actively involved in antigen-driven responses in the peripheral blood. However, since Vβ2 was identified in the folliculitis sample from Patient 4, our results suggest that Vβ2 may have been involved in pathogenesis of folliculitis. This further suggests that Vβ2 may be commonly found in the skin, where it is activated by local inflammation and/or infections, as it was found in each of our skin GVHD samples as well as our non-GVHD control. Furthermore, Vβ2 was detected at the site of gut GVHD in Patient 6, in which there was an increased expression and skewed repertoire (Figures 8,9), and in Patient 5 who demonstrated low expression and polyclonality (Figures 8, 17b) in the blood. As evidenced in Patients 5 and 6, even if Vβ2 is commonly found in the skin, it may be commonly associated with the T-cell-mediated inflammatory response in GVHD, regardless of the tissue involved.

Overall, Vβ4 was identified in three, Vβ11 was identified in two, and Vβ23 was identified in one, out of the four patients with GVHD biopsies. Patients 5 and 6 had acute GVHD of the gut, whereas Patients 8 and 11 have chronic GVHD of the skin. There was no unique correlation of Vβ families between

patients with chronic versus acute, or gut versus skin, GVHD.

Figure 16:

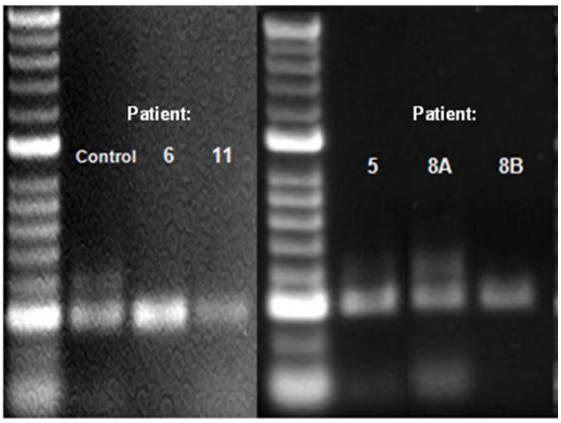
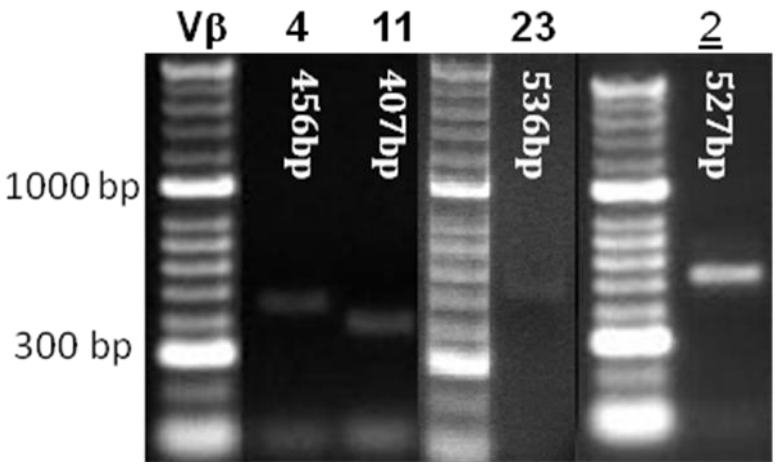


Figure 16. Extraction of DNA confirmed by GAPDH control. DNA was extraction from paraffin-embedded biopsy samples from the site of GVHD, at the time of GVHD. PCR was run with GAPDH primers as a control to verify extraction of DNA. Patients 5, 6, and 11 have one sample, corresponding to the time of GVHD, which is R2 for patients 5 and 11, and R3 for Patient 6. Patient 8's Sample A corresponds to the first clinical indications of GVHD onset (R3) and Sample B corresponds to three months after R3, during active chronic GVHD. A control sample was also used from non-GVHD Patient 4 who had a biopsy about 4 months post-HSCT, which was classified as folliculitis.

Figure 17:

17a)



17b)

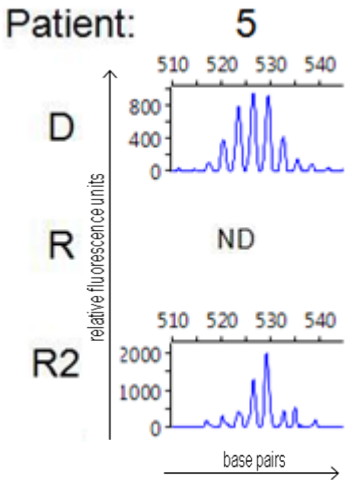


Figure 17. Patient 5 DNA extraction. a) PCR products yielded positive results for TCR Vβs 4, 11, and 23 (indicated in bold) which showed increased qRT-PCR expression and oligoclonality. Control sample Vβ2 (underlined) was also detected at the site of GVHD, even though there was low expression and polyclonality in the peripheral blood at this time. Expected sizes of products are shown in each well corresponding to the respective Vβ families. Results are shown on a 1% agarose gel. **b)** Spectratyping of Vβ2 in Patient 5's peripheral blood sample shows polyclonality at the time of GVHD (R2). ND: no data.

Figure 18:

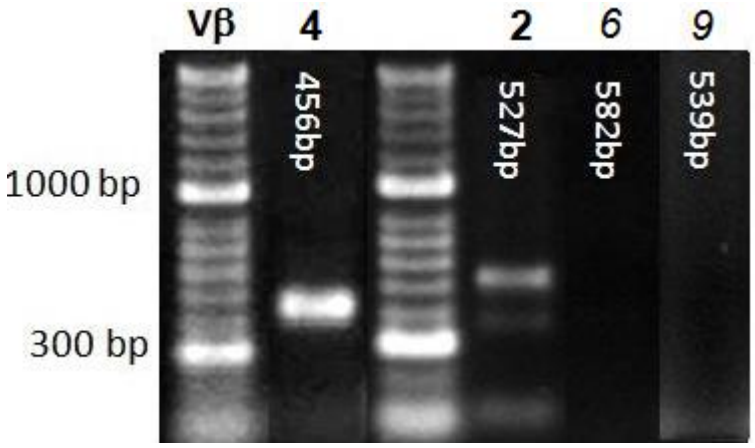


Figure 18. Patient 6 DNA extraction. PCR products yielded positive results for TCR Vβs 4 and 2 (indicated in bold), which showed high qRT-PCR expression and oligoclonality. Control sample Vβs 6 and 9 were negative (italicized), as expected based on low qRT-PCR expression at the time of GVHD. Expected sizes of products are shown in each well corresponding to the respective Vβ families. Results are shown on a 1% agarose gel.

Figure 19:

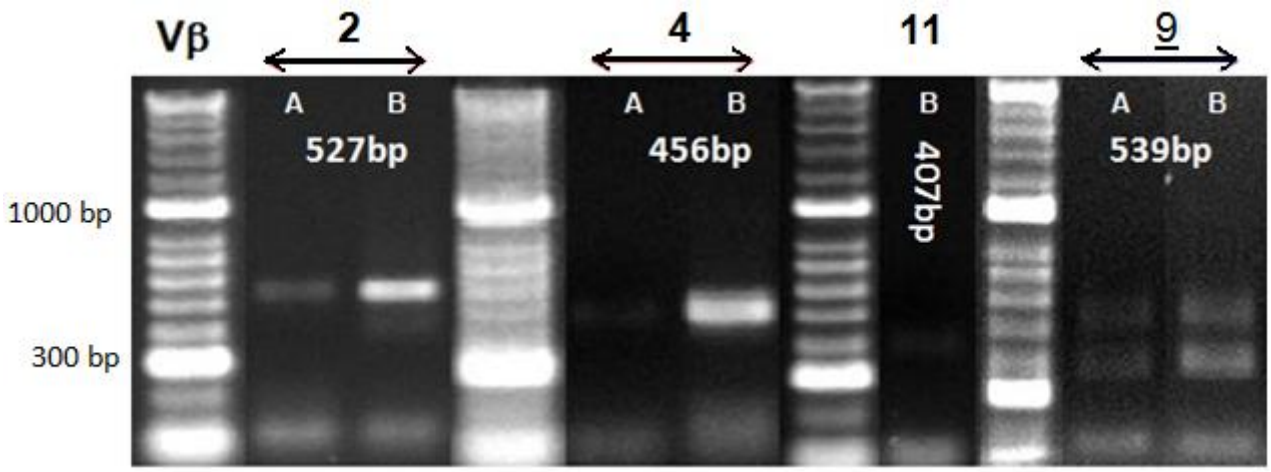


Figure 19. Patient 8 DNA extraction. The letters A and B correspond to time point R3 and three months after R3, both are times of GVHD. There is no corresponding peripheral T-cell sample for the B sample, so the PCR reactions were chosen for both A and B sample based on qRT-PCR and spectratyping analyses at the time of R3. PCR products yielded positive results for TCR Vβs 2 and 4 for both A and B and Vβ11 for B (indicated in bold). Control reactions for Vβ9 gave positive results for both samples A and B (underlined). Expected sizes of products are shown in each well corresponding to the respective Vβ families. Results are shown on a 1% agarose gel.

Figure 20:

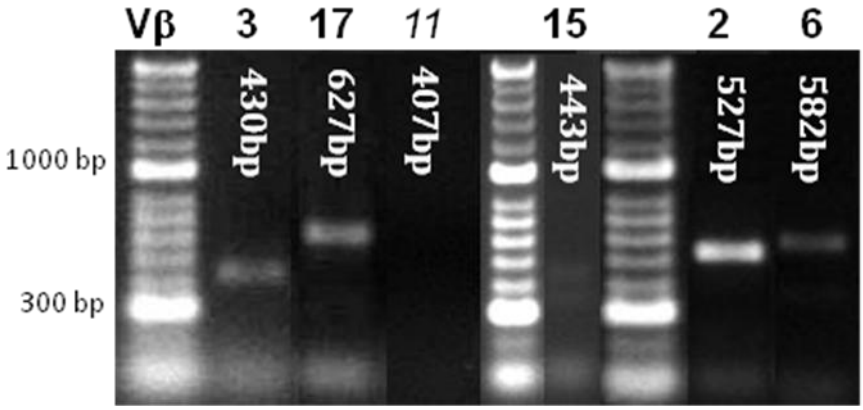
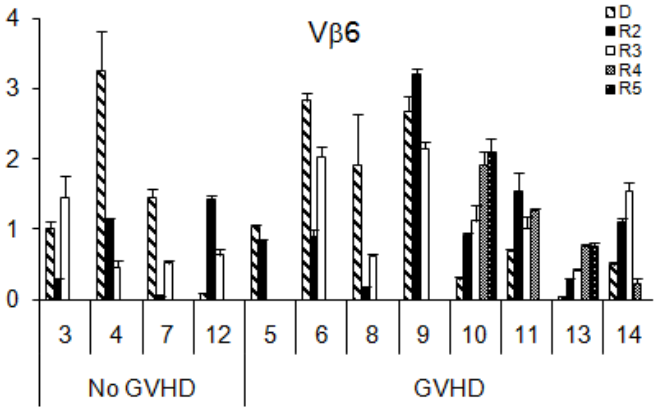


Figure 20. Patient 11 DNA extraction. PCR products yielded positive results for TCR Vβs 3, 17, 15, 2, and 6 (indicated in bold). Vβ11 was run as a negative control (italics). Expected sizes of products are shown in each well corresponding to the respective Vβ families. Results are shown on a 1% agarose gel.

Figure 21:

21a)



21b)

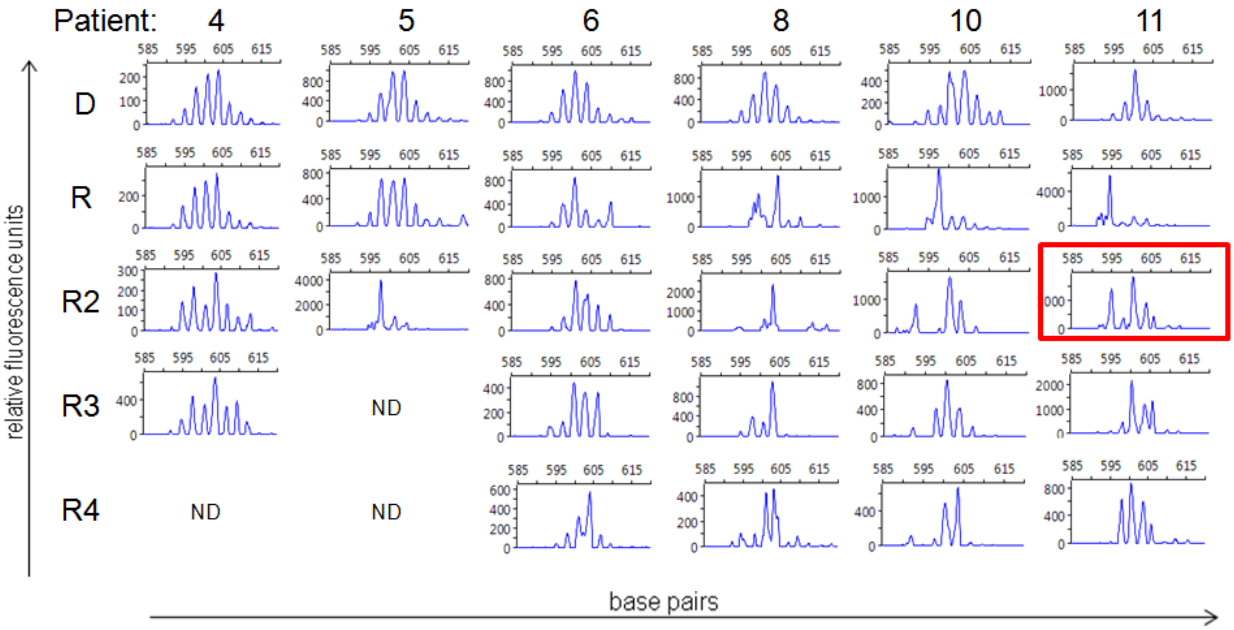


Figure 21. qRT-PCR and spectratyping analysis of Vβ6. a) qRT-PCR. **b)** Spectratyping. The red box denotes the time in which Vβ6 was detected at the site of GVHD in Patient 11. ND: No data.

Figure 22:

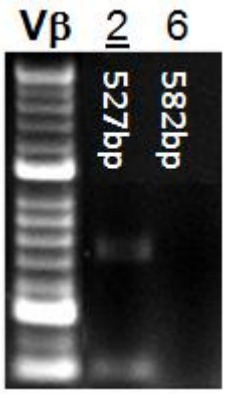


Figure 22. DNA extraction from control sample for Vβs 2 and 6. Vβ2 yielded positive results (underlined) and Vβ6 was negative. Results are shown on a 1% agarose gel.

Patient 10 – Relapse followed by remission and re-onset of GVHD post-DLI treatments

Patient 10 is a unique example of TCR Vβ repertoire at onset of GVHD, resolvement of GVHD, relapse, and post-DLI remission and GVHD. The course of her clinical events in which we obtained peripheral blood samples were: 3 months post-HSCT (R2), GVHD-onset 6 months post-HSCT (R3), relapse 10 months post-HSCT (R4), clinical resolvement of GVHD at one year post-HSCT (R5), remission and re-onset of GVHD (R6), and DLI treatments one month prior to R5 or two months prior to R6. DLI is used to induce remission in patients who have relapsed, demonstrating the powerful GVT effects of donor allorecognition of malignant cells in recipients.[16,27,65,66] Furthermore, use of DLI also carries a risk for post-DLI GVHD,[16,27,65,66] as evidenced in Patient 10.

We looked at qRT-PCR and spectratyping data in Patient 10 in order to identify T cell clones that coincided with relapse (disappearance of GVT-associated clones) and post-DLI remission and GVHD (emergence of clones). We first looked at Vβ families that were commonly associated with GVHD—4, 11, and 23 (Figures 23, 24a). Vβ4 demonstrated less than 1-fold increase in expression at R2, R3, and R5, with the same oligoclonality persisting from R2-R4, as previously mentioned, that was likely associated with cGVHD that may have been detectable prior to clinical diagnosis at R3. After the first DLI treatment, a second major Vβ4 clone emerged at R5, but was less prominent at R6, suggesting that it was not involved in GVT or GVHD as clones involved in either effect should have persisted or emerged at R6. With regards to Vβ11, the strong clonotype at the onset of GVHD (R3) was no longer evident at R4 suggesting that it may not have been GVHD-specific, as clinical resolution of GVHD was not identified until R5. Since polyclonality was restored at time of relapse (R4), the disappearance of the clone from R3 may have been GVT-associated. Also, a new clonotype emerged at R6, suggesting a role for this T cell population in either GVHD or GVT, as both remission and GVHD occurred post-DLI. However, there was low expression of Vβ11 at R6, further suggesting that this clonotype may

have been site-specific and less detecable in the peripheral blood. For Vβ23, we saw a skewed repertoire that persisted from R2-R5 that slightly shifted in size at R6, indicating a change in antigen-specificity. Since Patient 10's first course of cGVHD was clinically resolved at R5, it may be that the continued clonal population was below clinical detection of GVHD.

We next wanted to look at Vβs from the group of Vβ families that were increased in patients with and without GVHD (Figures 23, 24b). For Vβ2, there was an oligoclonal population over a polyclonal background from R2-R4, suggesting a role for a GVHD-specific clone. At R5, this clone was no longer evident, suggesting a role in a GVHD response, as clinical resolution was around R5. At R6, a new oligoclonal population emerged that may have had a role in either GVHD or GVT. In Vβ7, the peristent clone from R2-R5 was diminished at R6, at which time a new clone emerged. This clone at R6 mirrored that of the one in the recipient prior to transplant, suggesting a role in anti-myeloma response. In Vβ8, the major clonotype that was seen from R2-R4 was no longer present at R5 or R6, suggesting that it may have been specific for the patient's first bout of GVHD. Vβ12 showed a similar trend to that of Vβ8. Vβ15 showed a dominant clone that persisted from R2-R6, though a polyclonal background didn't emerge until R5. Since the skin was involved with both the first and second course of GVHD, it may be that this clone was GVHD-specific. Also, Vβ17 followed a similar trend to that seen in Vβ15.

Lastly, we looked at Vβs 9 and 20 from the group of Vβs commonly associated with GVHD-free patients to see if there was a correlation with GVT response, as well as at Vβ6 since there was also an increase in qRT-PCR expression for this Vβ family (Figures 23, 24c,d). With regards to Vβ9, an oligoclonal population emerged over at polyclonal background at R4. This clone wa not likely involved in a GVT response, since it was at the time of relapse (R4) and prior to DLI treatments (prior to and after R5), at which a GVT-specific clone should have disappeared by R4 or appeared after R5. Vβ20

demonstrated polyclonality from R3-R6, demonstrating that it was not involved in an antigen-driven response. In Vβ6, there was a skewed repertoire from R3-R5 that became more oligoclonal at R6, suggesting a clonally-driven response involved in either GVHD or GVT effects.

Figure 23:

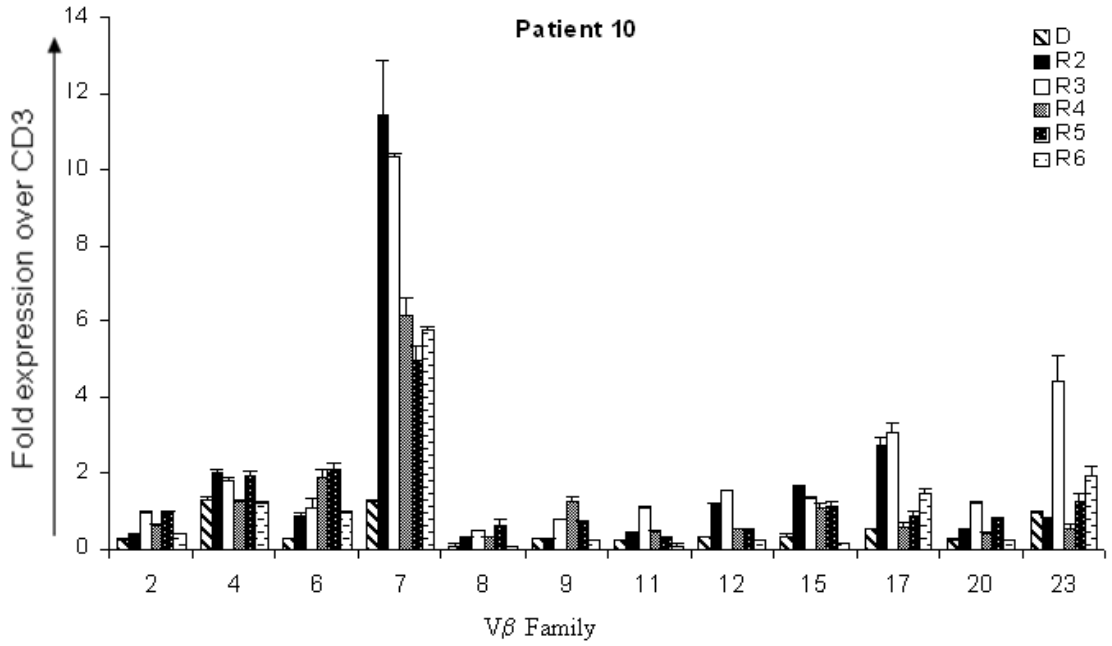


Figure 23. Expression of Vβ families in Patient 10. D: donor sample at time of transplantation; R2: recipient sample 3 months post-transplant; R3: time of GVHD onset at 6 months post-HSCT; R4: time of relapse at 10 months post-HSCT; R5: 1-year post HSCT, one month after 1st DLI treatment; R6: 22 months post-HSCT, two months after 2nd DLI treatment, time of remission and re-onset of chronic skin GVHD.

Figure 24

24a)

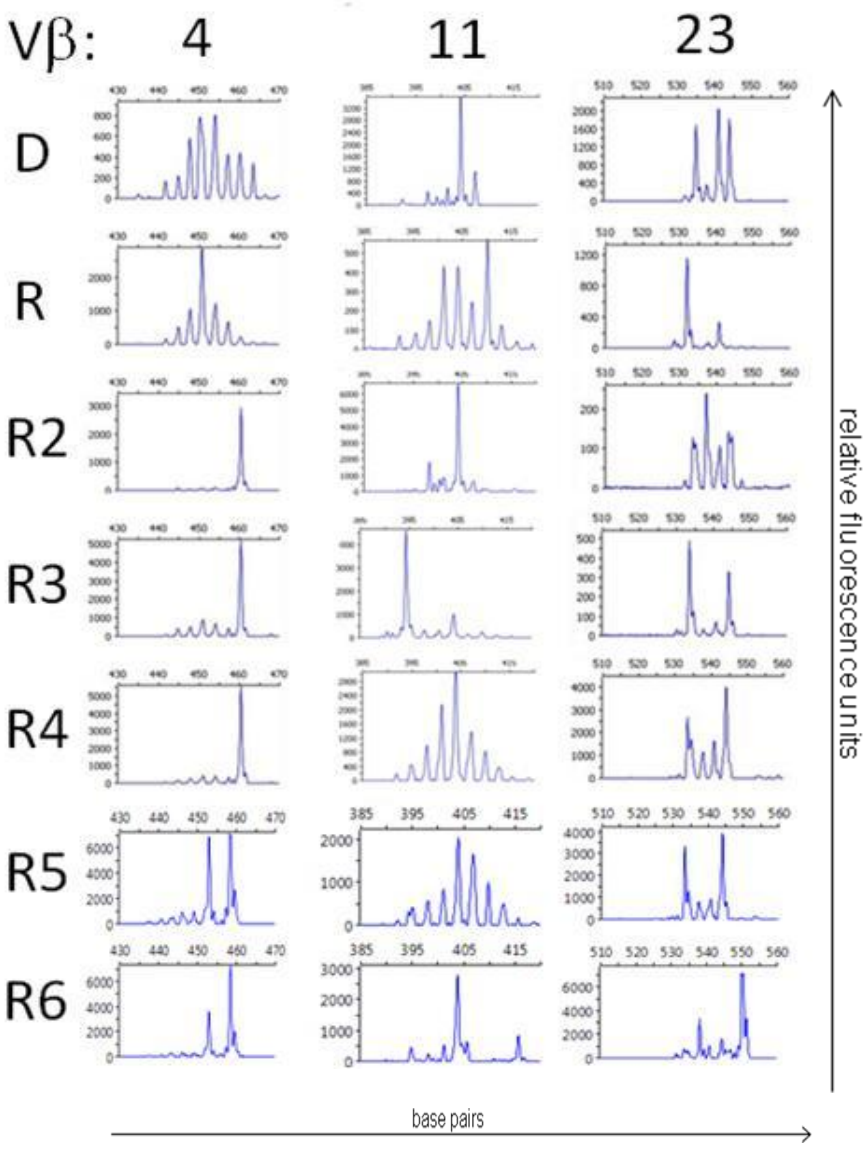


Figure 24. Spectratyping analysis for Patient 10. a) Spectratyping of Vβ families 4, 11, and 23 in Patient 10. D: donor sample at time of transplantation; R2: recipient sample 3 months post-transplant; R3: time of GVHD onset at 6 months post-HSCT; R4: time of relapse at 10 months post-HSCT; R5: 1-year post HSCT, one month after 1st DLI treatment; R6: 22 months post-HSCT, two months after 2nd DLI treatment, time of remission and re-onset of chronic skin GVHD.

Figure 24b:

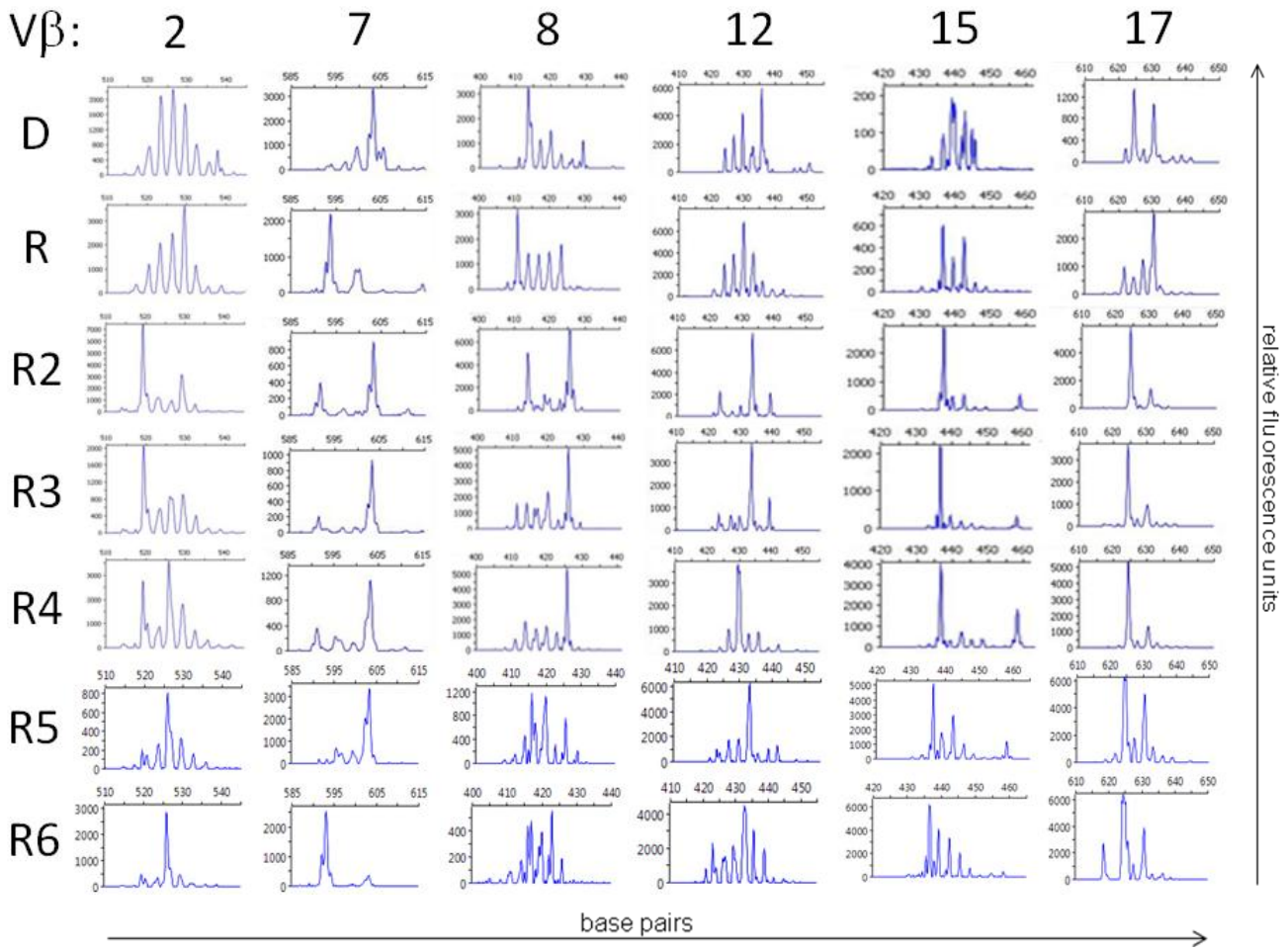


Figure 24b) Spectratyping analysis of Vβ families 2, 4, 7, 8, 12, 15, and 17 in Patient 10. D: donor sample at time of transplantation; R2: recipient sample 3 months post-transplant; R3: time of GVHD onset at 6 months post-HSCT; R4: time of relapse at 10 months post-HSCT; R5: 1-year post HSCT, one month after 1st DLI treatment; R6: 22 months post-HSCT, two months after 2nd DLI treatment, time of remission and re-onset of chronic skin GVHD.

Figure 24c:

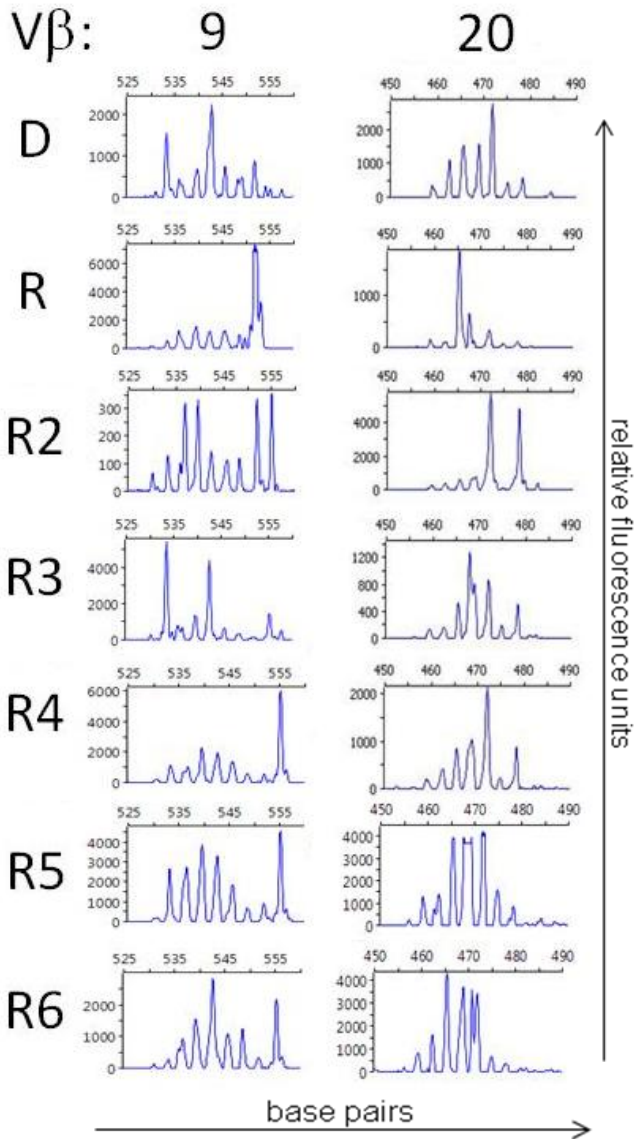
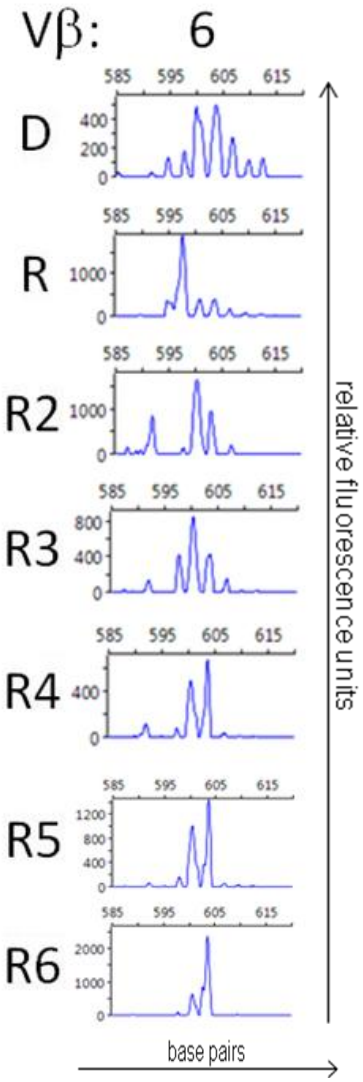


Figure 24d:



Figures 24: c) Spectratyping of Vβ families 9 and 20in Patient 10; **d)** Spectratyping of Vβ6 in Patient 10. D: donor sample at time of transplantation; R2: recipient sample 3 months post-transplant; R3: time of GVHD onset at 6 months post-HSCT; R4: time of relapse at 10 months post-HSCT; R5: 1-year post HSCT, one month after 1st DLI treatment; R6: 22 months post-HSCT, two months after 2nd DLI treatment, time of remission and re-onset of chronic skin GVHD.

Discussion

Our identification of specific T cell clonotypes commonly associated with GVHD or GVT supports the dominant characterization of GVHD as a T-cell-mediated inflammatory disease and the role for T-cell-mediated alloreactivity in both GVHD and GVT responses.[7,28,31] Current treatment of GVHD, both prophylactic and established, consists of broad immunosuppression, which not only mediates GVHD, but also increases the risk for opportunistic infection and incidence of relapse secondary to decreased GVT responses. The ability to identify T cell clones involved not only in GVHD but also in GVT would allow targeted depletion of GVHD-associated clones and augmentation of those involved in GVT responses.

By isolating RNA rather than DNA from peripheral T cells, our results are more representative of those TCR Vβ that were transcribed to mRNA for protein translation. Since oligoclonal and monoclonal CDR3 patterns are associated with strong immune responses, looking at mRNA expression by qRT-PCR and spectratyping of the CDR3 region allowed us to identify T cell clonotypes involved in antigen-driven responses.[52] By identifying Vβ families that met both of these criteria, we were able to make temporal associations of T-cell-mediated responses at the time of GVHD, as well in association of GVT effects in the absence of any clinically noted infections.

GVHD

Though we isolated RNA from un-separated T cells, several studies have noted the association of skewed, oligoclonal T cell populations with $CD8^+$ subsets and polyclonal populations with $CD4^+$ subsets.[67,68] In one such study characterizing T cell expansions in patients with myelodysplastic

syndromes (MDS), Fozza *et al*. observed that increased frequencies of expanded β−variable subfamilies in $CD4^+$ and $CD8^+$ T cells were mostly Gaussian, or skewed and oligoclonal, respectively, within the CDR3 regions.[67] In the general characterizations of GVHD and GVT as T-cell-mediated responses, $CD8^+$ CTLs damage cells bearing class I MHC, typically in the skin, gut, and liver in GVHD, and in myeloid or lymphoid tissues in GVT.[31,69] Our spectratyping results coincide with these observations, as oligoclonality has been associated with the $CD8^+$ subset.

Most studies characterize GVHD as a Th1- and Tc1-type cytokine mediated process, though this characterization is not absolute. For example, though Th1- and Tc1-type cells are abundant sources of IFN-γ, which primes macrophages and monocytes for pro-inflammatory cytokine secretion, IFN-γ has also been shown to induce apoptosis in lymphocytes.[70] Th1-type cytokines have been correlated with amplifying the development of aGVHD, whereas Th2 cells have been shown to reduce aGVHD.[10,41] Furthermore, though the alloreactive T-cell-mediated inflammatory response characterizes GVHD, this pathophysiology is broadly accepted with regards to aGVHD, as cGVHD is still less well understood.[3,17] Since cGVHD is represented as more of an antigen-specific cellular and humoral immune response,[16] the role for $CD4^+$ T cells is more plausible for chronic rather than acute GVHD. It is also thought that antigenic spread and exposure may lead to the generation of autoreactive T cells, which have been implicated in the pathogenesis of cGVHD.[3,17,57] Even if we accept the Th1- and Tc1- cytokine profiles as being restricted to aGVHD only, five out of eight GVHD patients had aGVHD. As such, the argument for $CD8^+$ CTL function is concrete for those five patients, yet entirely plausible for the three patients whose GVHD was defined as chronic, as their clonality profiles were similar to those with aGVHD. Furthermore, though $CD8^+$ subsets are the major cytotoxic effectors in GVHD, $CD4^+$ T cells have been shown to have cytotoxic functions in a MHC class II restricted manner.[34,71] Since our patients who developed GVHD did not have class I or II mismatches (with the exceptions of Patients 13 and 14

(Table 1)), we can reasonably suggest that GVHD-specific CTL function is peptide-dependent in the context of mHAgs.

Furthermore, because the thymus and bone marrow environments are susceptible to damage by pre-conditioning treatments, regeneration of lymphoid-derived T cells is limited after HSCT.[72] Though *de novo* generation of T cells from progenitors occurs via thymopoiesis, peripheral expansions of residual mature T cells are also a major contributing pathway in T cell reconstitution.[62] After HSCT while patients are lymphopenic, any remaining residual host T cells and the donor T cells transferred within the graft undergo homeostatic peripheral expansion (HPE). These early HPEs are typically characterized by oligoclonal and skewed repertoires, as peripheral expansions of mature lymphocytes are driven by antigen stimulation and/or homeostatic cytokines.[62] Under the premise that HPE augments the environment for alloreactive T cell clones, it may be possible to identify GVHD-associated clonal populations prior to clinical diagnosis, especially in instances in which GVHD onset is around the time of tapering or ending of prophylactic immunosuppression, as in our Patients 5, 6, 8, 9, and 10. The onset of GVHD for Patients 11, 13, and 14 occurred while they were still on immunosuppression, showing the ability of HPE to augment expansion of alloreactive T cell clones, even if to a lesser degree, as immunosuppression does not abrogate the allorecognition itself.

It has been shown that HPE of $CD8^+$ T cells is more efficient than HPE of $CD4^+$ cells, resulting in reduced $CD4^+$ T cell counts and inverted CD4/CD8 ratios for months to years following HSCT in hosts with limited thymic function.[62] This supports the likelihood of GVHD-associated clones representing $CD8^+$ T cells, as $CD4^+$ T cell number and function highly depends upon thymic-dependent T cell regeneration, which only occurs in a small fraction of adults who undergo HSCT.[72] Furthermore, there has been an association of Th2 cytokine subsets and Tregs with lower incidence/severity of aGVHD.[37,41] With delayed restoration of the $CD4^+$ subset after HSCT, the ability to downregulate T cell proliferation

is greatly hindered,[36] thus allowing the continued expansion of alloreactive T cell populations involved in the pathogenesis of GVHD.

Diverse T cell repertoires may not be restored until thymopoiesis is restored, and although the thymus is involuted after puberty, it is still capable of generating new T cells, albeit at a much slower rate.[73] In adults, each successive decade reduces the incidence of re-establishment of thymopoiesis, thus reducing the level of naïve cells produced and exported into the peripheral blood.[74] Over the age of 45 (as in all of our patients at the time of HSCT except for one at age 44), the frequency of significant renewal of thymopoiesis is severely reduced and normal levels of naïve $CD4^+$ T cells may not be reached until after 2 years.[74] In many older adults, this recovery of a normal level may take 3-5 years, and in some, may remain below normal levels for decades [reviewed in Williams].[62] Because of delayed thymopoiesis, we are not surprised to find that some patients who were used as controls for their low qRT-PCR expression of certain TCR Vβ families demonstrated aberrant T cell repertoires, as a normal Gaussian distribution may take years to be re-established. Furthermore, as Patient 8 was under immunosuppression throughout his course of treatment (R2-R4), it was not surprising to see oligoclonality in many Vβ families that had low expression, as the reconstitution of his T cell repertoire was continually suppressed. Similarly, early increases in expression of a Vβ family showing an aberrant or skewed repertoire (at a non-disease time) followed by decreased expression and polyclonality may merely represent the typical T cell reconstitution seen in patients after HSCT—HPE driven by homeostatic cytokines during a time of insufficient thymopoiesis before restoration of diverse naïve $CD4^+$ T cells (which are characterized by polyclonal Gaussian spectratyping of the CDR3 region).[62]

Without any other clinically noted infections or complications at the same times as those of our GVHD samples, we can reasonably propose that the clonotypes we identified may be the alloreactive agents of GVHD within these patients. Our results (Vβs 4, 11 and 23 in the GVHD group and Vβs 2, 3,

7, 8, 12, 15, 17 in the "shared" GVHD/GVT group) are consistent with previous studies that have shown that oligoclonal expansions of Vβ families is common and often correlates with GVHD.[53] In addition to identifying T cell clones in the circulation of patients with GVHD, we were also able to identify some of these Vβ families at the site of GVHD. Other studies have shown that overall T cell repertoires in skin lesions involved in GVHD differed from that in peripheral blood.[57,75,76] We, however, were able to identify some Vβ families that were increased in the peripheral blood in an antigen-driven manner (oligoclonality) that were also detectable at the site of skin and gut GVHD. From those Vβ families which we identified as being commonly associated with patients with GVHD (Vβ4 –Patients 5, 6, 8, 10; Vβ11 – Patients 5, 8, 9, 10, 13; Vβ23 – Patients 5, 9, 10, 11, 13), we were able to identify all Vβs at the site of GVHD in at least one patient: Vβ4 –Patients 5, 6, 8; Vβ11 – Patients 5, 8; Vβ23 – Patient 5. Aside from our GVHD-specific group of Vβ families, some of the Vβ families whose expression was increased and also demonstrated oligoclonality in the peripheral blood in patients both with and without GVHD (Vβs 2, 3, 7, 8, 12, 15, 17) were detected at the site of GVHD: Vβ2 – Patients 6, 11; Vβs 3, 15, 17 – Patient 11.

Additionally, Liu *et al*. observed that GVHD-specific clones may be difficult to detect in peripheral blood as the degree of expansion away from the site of disease is variable.[53] We noticed this for a few Vβ families that were detected at the site of GVHD, though their expression was not increased in the peripheral blood: Vβ2 – Patients 5, 8; Vβ9 – Patient 8; Vβ23 – Patient 5. For Patient 5, the expression of Vβ2 was almost 9-fold lower than that of Donor 5, suggesting that this Vβ family may in fact be detected at the site of GVHD, but not by its level of expression in the blood. For Patient 8, both Vβs 2 and 9 were roughly 2-fold lower than that of the donor, again suggesting that some GVHD-associated clones may be detected at the site of disease but not in the peripheral blood. For all other Vβs detected

at the site of GVHD, their respective expressions in the blood were at least 2-fold higher in the GVHD samples than in the donor samples. On the other hand, Vβ23 had less than 0.5-fold increased expression over Donor 5, suggesting that it may be difficult to establish a baseline fold-increase that can be used as a predictor of GVHD-specific clonotypes. We did not note a difference in the ability to detect clones with regards to chronic or acute manifestation of GVHD, as we were able to identify at least half of the Vβ families that were oligoclonally increased in the periphery at the site of GVHD in patients with both chronic (Patients 8, 11) and acute (Patients 5, 6) GVHD. There was also no strong difference in those Vβs that were detected in skin (Patients 8, 11) or gut (Patients 5, 6), as Vβ2 was found in all patients and Vβs 4 and 11 were detected in both gut and skin GVHD. Furthermore, we were able to compare our results with other groups that identified Vβ families at the site of GVHD: C. Liu *et al.* detected Vβs 2, 6, 17, and 23;[53] Hirokawa, *et al.* identified Vβ6;[76] X. Liu *et al*. identified Vβ2,[77] and Beck *et al*. identified Vβs 3 and 17,[57] all in skin GVHD.

In addition to Vβ families detected at the site of GVHD, certain Vβ families have been detected in the skin of healthy adults, such as Vβs 2, 3, 6, and 23,[64,78] each of which we identified at the site of GVHD (Vβ2 – Patients 5, 6, 8, 11; Vβs 3, 6, 23 – Patient 11). For Vβ2, expression in the peripheral blood was lower than that of the donors in Patients 5 and 8, but higher than the donors for Patients 6 and 11. We also detected Vβ2 in our control sample (folliculitis), though there was a lack of both increased expression and oligoclonality in the blood. If future studies detect Vβ2 at the site of disease without an increased expression or oligoclonality in the peripheral blood, then it may follow with observations made by Menssen *et al*. that Vβ2 commonly resides in the skin.[64] Furthermore, Vβ6 was detected at the site of GVHD in Patient 11. Though there was a roughly 2-fold increase in expression of Vβ6 relative to Donor 11, the clonality was more suggestive of HPE than an antigen-driven response. For Patient 11,

the two possibilities are that Vβ6 may commonly reside in the skin or is detectable at the site of GVHD without increased expression or oligoclonality in the blood. With future studies involving more patients, we will be able to see if there is an association of Vβs 2 and 6 at the site of GVHD with either high or low expression in the blood. Using more control samples will also help to identify Vβ families that commonly reside in the skin or gut. If skin-resident Vβ families are involved in the pathogenesis of GVHD, then it suggests that conditioning regimen damage to host tissues causes activation of such T cells at the site of GVHD, whereas those Vβ families not common in the skin of healthy individuals are recruited to the site of disease.

Though we isolated RNA from our peripheral blood samples, we isolated DNA from the GVHD-biopsy samples. Using DNA, those TCR Vβ families identified at the site of GVHD were representative of one copy per rearranged TCR per cell.[57] We expected to detect Vβ families at the site of GVHD that were also detected in the peripheral blood. Exceptions to this were seen in instances in which Vβ families were detected at the site of GVHD without a corresponding increased expression in the blood, with either polyclonal (Vβ2 – Patient 5) or oligoclonal (Vβ9 – Patient 8) repertoires in the blood. We plan to use high throughput sequencing in order to determine whether higher copies of certain VDJ recombination for a given TCR Vβ may predict GVHD or GVT at a molecular level.

Furthermore, other studies have noted $CD8^+$ T cell infiltrates at the site of GVHD,[20] and the absence of $CD8^+$ infiltrates in normal, healthy skin.[78] Future spectratyping analysis of the T cell clones detected at the site of disease will help us determine which T cells are active in the disease (oligoclonal) and which are bystanders common to the disease tissue (polyclonal).

GVT

Studies have shown a correlation with aGVHD and improved disease-free survival, and that patients with cGVHD from matched sibling donors correlate with fewer relapses (though also increased TRM).[17,25,79] For these reasons, the question then remains as to whether the mechanisms and effectors for GVHD and GVT are fundamentally different.[53] This question is especially prominent when we consider the augmentation of antigen-driven T cell responses early-on after transplantation when homeostatic cytokines promote T cell expansion, which primes the environment for both GVHD and GVT responses. It has also been shown that GVT effects can occur in the absence of GVHD, which implies that certain mHAgs that are expressed by malignancies are not expressed on the non-hematopoietic-restricted tissues that are targeted in GVHD.[9,15] However, with clinical observations that associate GVT with GVHD, there may be an overlap of mHAg expression, though those mHAgs that are only expressed on hematopoietic cells have become the target of interest for their potential roles in augmenting GVT effects.[43] Though our patients are heterogeneous with regards to disease, the potential to identify T cells involved in GVT effects is plausible in the context of mHAgs whose expression is limited to the hematopoietic system.

Within the group of GVHD-free patients (3, 4, 7, 12), Vβs 9, 16, and 20 demonstrated both increased expression and oligoclonality, suggesting a GVT-role for these T cells. Patient 3 also demonstrated increased expression and clonality in Vβs 5 and 24, which implies that GVT-specific T cells recognize mHAgs that are unique to that individual. Of the Vβs we identified in either our GVHD-free or GVHD patients, a few other studies have associated some of these Vβ families in the peripheral blood with various diseases: Tan *et al*. noted oligoclonal expansions of Vβs 3 and 15 in patients with diffuse large B-cell lymphoma (DLBL)[80] and Tanaka-Harada *et al*. observed biased usage of Vβs 9 and 15 in acute myeloid leukemia (AML), Vβs 4 and 12 in myelodysplastic syndrome (MDS),

and Vβs 5 and 20 in patients with AML and MDS (as well as in healthy donors),[81] suggesting the ability to identify Vβ families that recognize mHAg in a hematopoietic-restricted manner.

GVT responses are generally thought to involve T-cell-mediated cytotoxicity, with $CD8^+$ subsets being the predominant CTLs.[82] However, $CD4^+$ subsets are also recognized as having cytolytic effector functions,[28, 34-36] suggesting their potential role in cytotoxic-mediated GVT responses as well. With the exception of Patient 10, all of our patients were relapse-free, which broadens the possibility of GVT-associated T cells being identified in more than just those four patients who did not develop GVHD. Furthermore, three of our patients had HLA-DQB1 mismatches (Patients 4, 7, 13). Since class II genes are restricted to hematopoietic antigen presenting cells, these mismatches may promote a GVT effect in an mHAg-independent or mHAg:MHC-dependent manner.[9,25,44]

Lastly, Patient 10 allowed us to look for GVT-associated clones that disappear at the time of relapse. Vβ11 was the only TCR family that met this criterion, suggesting a role for Vβ11 in GVT, as opposed to GVHD, as GVHD was not clinically resolved at the time of relapse. We also expected to see new oligoclonal populations emerge at R6, which was defined as post-DLI remission and GVHD. New clones did emerge in Vβs 2, 7, 11, and 23, suggesting potential roles for these Vβs in either GVHD or GVT responses. Patient 10's remission after DLI treatments demonstrates the GVT effect in eradicating her disease.[32] Since GVHD is also common after DLI treatments, [16,27,65,66] the specificity of these clones will help distinguish which clones may be involved in which process.

Clinical Correlations

Overall, when we look at the patients in this study and consider major risk factors (HLA-mismatch, female donor to male recipient, PBSC vs. BM) that are associated with development of GVHD, we were able to make a few observations that will be better validated with a larger patient cohort in the future. With regards to HLA-matching, a mismatch at any of the HLA- A, -B, -C, and –DRB1 alleles

has been associated with worse overall survival (OS).[83] All patients were 8/8 matches for these four HLA-alleles, with the exception of Patient 14, who had an HLA-B mismatch (Table 1). Patient 14 developed late-onset acute GVHD of both skin and the GI system, and also died six months after HSCT, showing a direct correlation with the increased risk factors associated with the HLA-B mismatch. Also, Patient 14's GVHD was likely due to HLA-disparity, since the prevalence of aGVHD is upwards of 60-80% in recipients of grafts with one HLA allele mismatch.[4] Mismatches of the DQB1 allele have not been shown to have an impact on OS or increased incidence of GVHD.[83] Two of our three patients with an HLA-DQB1 mismatch were GVHD-free and are still alive over two years post-HSCT, and the one who developed aGVHD is still alive over one year post-HSCT.

Many studies have noted an increased risk of GVHD when a male recipient receives a graft from a female donor, due to H-Y antigens.[15,28,42] Two patients, 9 and 14, were male recipients of female grafts, and both developed aGVHD. Antibodies specific for H-Y mHAgs have been associated with cGVHD,[84] whereas each of these two patients developed aGVHD and also demonstrated increased expression and oligoclonality in their T cell repertoires, supporting the role of alloreactive T cells in their GVHD responses.

Donor grafts from PBSC have been associated with a greater incidence of GVHD, though grafts from BM have been associated with increased risk of relapse.[85] All of our patients received PBSC, with the exception of Patients 10 and 13. Patient 10 was the only patient in our study to relapse, which resolved after DLI treatments. Interestingly, after Patient 10 went into remission, she developed GVHD again, showing the role of donor lymphocytes in promoting GVT effects as well as augmenting GVHD. A study by the CIBMTR showed that $CD34^+$ cell doses from PBSC grafts greater than 6 x 10^6/kg were associated with decreased risk of relapse.[86] The average $CD34^+$ cell dose in our GVHD-free group was 7.03 x 10^6/kg, whereas in our GVHD group, it was 4.54 x 10^6/kg (Table 3). All of the

patients in the GVHD-free group had cell doses greater than 6 x 10^6/kg, whereas only two patients in the GVHD-group did (Patients 8 and 9). This suggests that higher $CD34^+$ cell doses help facilitate better reconstitution of the immune system, as pointed out in Ringdén *et al.* 2009,[69] and thus increase GVT effects. Furthermore, two of our patients received BM grafts, Patients 10 and 13, with cell doses of 3.43 and 1.9 x 10^6/kg, respectively (Table 3). Bone marrow grafts are associated with greater incidence of relapse, as opposed to PBSC grafts, which was evidenced by Patient 10's relapse.[85]

Furthermore, the higher amount of $CD3^+$ T cells in PBSC grafts is also associated with increased incidence of GVHD.[7] Interestingly, Patient 11 had the highest $CD3^+$ T cell dose (11.35 x 10^8/kg) and developed GVHD faster than all other patients, though only by a few days (Tables 2 and 3). However, Patient 13 had the lowest $CD3^+$ dose (4.3 x 10^7/kg, from a BM graft) and also developed GVHD (Table 3). Overall, there was no significant correlation of cell dose with increased incidence of GVHD in this cohort of patients. Furthermore, early donor T cell chimerisms are also associated with increased risk of GVHD, which was observed in our patients (Table 2).

Conclusion

In summary, we were able to identify three groups of TCR Vβ families that were associated with either GVHD or GVT effects, or were commonly associated with both GVHD and GVT. Our findings suggest that GVHD-associated T cell clones can be identified at the time of GVHD and used for targeted therapy of GVHD by means of TCR Vβ-specific antibodies or small molecules that can target the associated T cell clones. For those Vβ families that were common between GVHD and GVT groups, we need a larger number of patients to determine whether these Vβ families may be associated with GVHD and/or GVT. For those patients with GVHD, if the mHAg:MHC complexes are expressed on cells both in the hematopoietic system and at the site of GVHD, it may well be that the same T cell populations are involved in both GVHD and GVT responses. Future molecular analysis may also

reveal that there are CDR3 sequences common across TCR Vβ families, which would suggest that CTLs from various Vβ families may be able to recognize the same antigen with different avidities.[30]

Though the majority of our patients do not have any HLA-mismatches, analysis of T cell subsets ($CD8^+$ versus $CD4^+$) may help us correlate the HLA-DQB1 mismatches with $CD4^+$ recognition, as well as better understand the cytokine profiles of both acute and chronic GVHD. Regardless of $CD8^+$ or $CD4^+$ subset, we observed clonal expansions of TCR Vβ families that are indicative of antigen-driven responses. For oligoclonal populations that emerge before the time of clinical diagnosis of GVHD, we may be able to detect GVHD-associated clones before clinical manifestation of the disease, demonstrating a potential role for T cell clonality in prognostics. On the other hand, clonal populations that emerge prior to clinical diagnosis of GVHD may be involved in GVT responses. We need to determine the fine specificity of GVHD- and GVT-associated TCR Vβs in order to develop targeted therapies. In this regard, high-throughput sequencing will allow us to identify the specificity of these clonotypes based on their CDR3 sequences.

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