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Contributing Factors to Immune Cell Repertoire and Effects on Alloreactivity

By

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Science

Immunology and Molecular Pathogenesis

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B.A., University of Southern California, 2009

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Abstract

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Memory T cells, and their ability to generate an anamnestic response, are critical to protective immunity, but contribute to allogeneic organ transplant rejection. Allospecific memory can be generated through heterologous cross-reactivity and homeostatic proliferation, as well as through prior allogeneic antigen exposure. Inhibitors of calcineurin and mTOR effectively, but nonspecifically, impede memory T-cell responses. Blocking costimulation, although effective at inhibiting responses to *de novo* alloantigen, less effectively inhibits allospecific memory responses. CD28:CD80/86 pathway costimulation blockade (CoB) with CTLA4-Ig is known to prevent T cell-mediated allograft rejection in non-allosenstized mice. Clinical use of belatacept, which inhibits the same pathway in humans, provides improved patient and graft survival, improved graft function, and fewer off target side effects compared to calcineurin inhibitors. However, use of CoB in human transplantation has been hampered by the occurrence of CoB resistant rejection (CoBRR), which has been attributed in part to pathogen-driven T cell repertoire maturation and resultant heterologous alloreactive memory. We identified a population of CD57+PD1- CD4 T cells present prior to transplantation that correlated with CoBRR. Contrary to data recognizing CD57 as a marker of senescence on CD8 T cells, we discovered a non-senescent, cytolytic phenotype associated with CD57+ CD4 T cells. To study factors that contribute to the development of these CoB-resistant cells, we developed a mouse model to evaluate the effects of specific viruses on alloreactivity. Prior murine studies demonstrating virus-induced heterologous alloimmunity have been conducted using acute viral pathogens atypically encountered in humans and CoB regimens not used clinically. We therefore developed a model of virus-induced memory differentiation using murine homologues of clinically prevalent persistent and latent viruses in the presence of maintenance CoB regimens like those used clinically. While infected mice developed a significant and sustained increase in effector memory CD4 and CD8 T cells consistent with that seen in humans, infected mice did not develop heterologous alloreactivity, and were not able to reject primarily vascularized heterotopic heart transplants under treatment with CoB. Thus, memory acquisition alone is insufficient to provoke CoBRR, and latent or persistent viruses may have limited capacity to stimulate a heterologous alloimmune response.

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Chapter 1. Introduction

Transplantation is today widely used as a life-saving and life improving therapy for tens of thousands of people each year. In 2016, more than 33,500 organ transplants were performed in the United States. The success of this unique field relies heavily on our growing understanding of individuals' immune cell repertoires and the development of targeted therapies to manipulate immune responses. Over the past 65 years, the pioneering work of dedicated scientists and physicians has taken us from an era of drug deficiency to one of sufficient immunosuppressive agents, and led to revolutionary advances in the life-saving field of transplantation.

In 1953, Sir Peter Medawar published that the introduction of genetically distinct donor cells *in utero*, conferred protection of skin allografts at 8 weeks of age. This demonstrated in mice that it was possible to manipulate an organism so it would be able to receive a graft from a genetically distinct organism and not reject it, establishing that transplantation was immune driven.[1] Though he was working with inbred mice, Medawar still observed a spectrum of outcomes and what he coined as 'tolerance' was "not an all or none phenomenon". However, this was emblematic of the rapidly growing interest in transplantation such that the very next year Joseph Murray performed the first kidney transplant between identical twins.[2] Without the need for immune suppression, this established that an organ from one individual could function properly in a different individual. Following this proof of concept, the focus shifted to controlling the host's immune response.

In the following years, a number of incremental steps were made with non-specific immune suppression. Roy Calne, Gertrude Elion, George Hitchings and Joe Murray collaborated to develop and use azathioprine to successfully prolong kidney allograft survival in canines.[3,4]

Azathioprine is metabolized to 6-mercaptopurine and ultimately incorporated into a dividing cell's DNA to inhibit growth and immune responses.[5] Quickly following these observations, Thomas Starzl developed protocols combining the use of azathioprine with steroids to improve kidney allograft outcomes[6] and went on to perform the first successful liver transplant utilizing the same immunosuppressant permutation. When used in combination with antilymphocyte serum or plasma to deplete lymphocytes at the time of transplantation, there was further prolonged allograft survival.[7,8] With these developments, it became increasingly evident that T cells were involved in transplant rejection, but it was not until the development of the calcineurin inhibitors (CNIs), like cyclosporine A and tacrolimus, that rejection rates significantly decreased and one year survival rates began to increase.[9-12]

CNIs prevent proliferation and cytokine production in T cells by inhibiting the calcineurin-calmodulin signaling complex and subsequent signaling by nuclear factor of activated T cells (NFAT); this effectively suppresses TCR-mediated activation regardless of the cell's maturation state.[13] Indeed, even at low doses, CNIs effectively prevent the activation and maturation of naive cells as well as simultaneously inhibiting the activation of, and cytokine production from, memory cells.[14] This broad, but highly effective, mode of immunosuppression has made CNIs a front-line therapy in conventional immunosuppression regimens, but also significantly hinders protective immunity. Moreover, the effects of CNIs are reversible and adherence to chronic use of CNIs is critical to prevent rejection, and are unfortunately coupled with a multitude of infectious, malignant, toxic and economic side effects. CNIs[10,15-17] and inhibitors of mechanistic target of rapamycin (mTOR), like rapamycin and sirolimus, form complexes with FK506-binding proteins

(FKBP) to inhibit lymphocyte activation pathways, but have distinct effects on T cells of varying states of differentiation.

Rapamycin was approved for use in transplantation in 1997 due to its potent inhibition of T cell activation.[18,19] It is now appreciated that mTOR signaling is quite complex and inhibiting the activation of mTOR fundamentally alters T cell metabolic programming,[20] including specific effects in $CD4^+$ and $CD8^+$ memory T cells formed via homeostatic repopulation that are essential for preserving effector function.[21,22] Memory $CD4^+$ T cells, in particular, depend on glycolysis for activation and effector function through phosphatidylinositol 3-kinase signaling downstream of mTOR,[23,24] whereas memory $CD8^+$ T cells are more reliant on lipid metabolism for their activation and function.[25] Reliance on fatty acid oxidation could explain why sirolimus might cause an apparent increase in the formation of pathogen-specific memory $CD8^+$ T-cells[26-28] while concurrently abating T-cell responses to alloantigen.[29] This metabolic shift could also explain how mTOR inhibition might have tolerance-inducing effects by augmenting the formation of regulatory T cells.[30] Thus, mTOR inhibition, through complex mechanisms, seems to divert normal T-cell development and memory formation by altering metabolic priority, potentially towards a phenotype that is more amenable to long-term allograft tolerance.

Owing to their capacity to broadly suppress T-cell responses, CNIs and mTOR inhibitors have been readily incorporated into immunosuppressive regimens. Today, there are numerous approaches to immunosuppressive therapies following transplantation that include anti-proliferative agents, steroids, depletion strategies, CNIs and mTOR inhibitors. Due to the advances in surgical techniques, immunosuppression development and organ

matching, patients are living longer with allografts. Now, a main issue is the consequence of chronic immune suppression, and agents with a more discriminating effect on donor specific memory T cells would be preferable, provided that they exhibit a comparable efficacy in preventing rejection. The desire for more-targeted therapeutics has driven the development of numerous monoclonal antibodies and fusion proteins that show a high degree of specificity for molecules relevant to the differentiation of memory cells.

Naïve T cells require numerous signals to enter a program of differentiation. This requirement has likely evolved as a mechanism to avoid promiscuous or unnecessary immune activation. Signal 1, the interaction between the antigen-presenting MHC and the T cell receptor (TCR), provides the specificity of the response. Signal 2, or costimulation, such as the interaction between B7 on antigen-presenting cells (APCs) and CD28 on T cells, provides confirmation of appropriate context for the response.[31,32] This second co-stimulatory signal on naïve T cells is required for the production of IL-2, which helps to promote T-cell differentiation, and TCR ligation in the absence of costimulation can result in antigen-specific T-cell anergy.[33] Following differentiation, the expression of CD28 is markedly downregulated and the resulting memory T cells no longer require costimulation for secondary immune responses. Thus, the loss of CD28 is an important hallmark of an antigen-experienced cell in humans and non-human primates.[34]

The requirement for costimulation in T-cell differentiation forms the basis of the development of therapeutic agents designed to block the interaction between B7 and CD28, with the aim of rendering cells anergic that would respond to *de novo* alloantigen. One such agent, belatacept, a fusion protein that specifically blocks the co-stimulatory CD28–B7

interaction, has elevated the profile of memory T cells in transplant immunotherapeutics as memory cells from prior antigen exposure that no longer require costimulation may be cross-reactive to alloantigen.

Blockade of co-stimulatory molecules has been a clinical focus in transplant immunosuppression and contributed to the FDA approval in 2011 of belatacept for use in transplantation.[35] Belatacept was developed as a potential replacement for CNIs and has led to improved organ function, improved patient and graft survival, and fewer off target side effects compared to calcineurin inhibitors for up to 7 years post-transplant.[36] Its selective targeting of *de novo* allospecific responses has the advantage of sparing previously acquired protective immunity, but this selectivity has perhaps unveiled the importance of previously underappreciated allospecific memory—specifically, treatment with belatacept has proven to be ineffective in a significant minority of patients, which has presented a hurdle in its clinical use[37]. Studies to define the biological difference between patients who experience belatacept-resistant rejection (BRR) and those who do not are imperative for belatacept's future success.

Both effector and memory phenotypes downregulate CD28 expression and upregulate adhesion molecule expression; however, the precise triggers that induce these differentiation pathways remain incompletely defined. Moreover, the distinction in the literature between effector and memory phenotypes in the context of allograft rejection remains unclear. Lakkis and colleagues[38-40] have elegantly demonstrated the dependence of naive T cells on interactions with APCs in secondary lymphoid organs for the initiation of alloimmune responses. Without these organized interactions, the immune system remains ignorant of

alloantigens, resulting in peripheral tolerance. By contrast, memory T cells in the periphery can expand and effectively reject an allograft independent of secondary lymphoid organs. Similarly, the presence of heterologously alloreactive memory T cells can induce rejection despite treatment with otherwise effective immune regimens.[41]

Regardless, costimulation promotes the differentiation of naïve cells into memory cells, and the lack of requirement for further costimulation combined with the increased expression of adhesion molecules allows these cells to quickly activate and infiltrate inflamed tissues in the periphery to exert their effector function after encountering the same antigen again. However, by this very nature, effector memory T cells are potent mediators of rejection— their lower threshold requirement for activation and higher levels of adhesion molecules facilitate access to the transplanted tissue[42]— and clinical observations have revealed that increased levels of donor-specific, cytokine-producing memory cells positively correlate with high instances of rejection and poor long-term allograft survival.[43,44]

Indeed, effector cells expressing low levels of CD28 have been implicated in CD28–B7 costimulation blockade-resistant allograft rejection .[45,46] Other subsets of CD28-negative T cells that also express high levels of CD2, LFA-1 and VLA-4, are associated with rejection mediated by resistance to belatacept in human studies.[47] These data indicate that costimulation blockade, although effective at inhibiting responses to *de novo* alloantigen, is inadequate at inhibiting pre-existing allospecific memory responses.

Exposure to alloantigens, as might occur through pregnancy, blood transfusions or prior allograft transplantation, has the potential to generate populations of donor-reactive memory

T cells. Furthermore, memory cells from prior viral or bacterial infections also have the potential to cross-react with alloantigens.[48,49] This cross-reaction exemplifies the biological concept of heterologous immunity—memory responses derived from nonidentical but similar antigens—and is likely to have a dynamic role in shaping an individual's alloimmune repertoire.[50] Indeed, rapidly expanding populations of heterologous, cross-reactive memory cells confer a survival advantage over non-cross-reactive cells, such that heterologously-reactive cells become immune dominant. This occurs especially in the context of viral infection where heterologously-reactive memory cells from past immune experience respond quickly to new infections, allowing for the expansion and continued selection of memory cells that yield superior protection compared to non-cross-reactive cells.[51] Notably, a breadth of alloreactivity exists in the immune cell repertoire in humans, such that T cells that respond to donor antigen are present in naïve and memory compartments.[13,52] This phenomenon is not observed in laboratory mice due to their relatively limited antigen experience, and their lack of heterologous reactivity is a considerable difference to consider when utilizing experimental mice to model the human condition.

So, although the ability of memory cells from prior infections to cross-react with different antigens confers an evolutionary benefit by providing a broader spectrum of immunity,[53] thereby generating a heterologous memory that can fend off a greater variety of pathogens with a less diverse TCR repertoire, this ability becomes detrimental in the case of allotransplantation. Adams *et al.*[41] elegantly showed that memory T cells from mice that received multiple infections could cross-react with alloantigens and mediate the rejection of skin allografts in the setting of costimulation blockade. Specific pathogens such as *Listeria monocytogenes*,[54] gamma-herpes virus,[55] and cytomegalovirus[56,57] have been mechanistically

defined in heterologous alloimmune processes. Indeed, in mice, these infections drive T-cell differentiation and some CD28–B7 costimulation blockade indifference, though the reduction of CD28 expression in mice is modest relative to the stark downregulation seen in primates.[46] This type of direct alloresponse might be a more acute, rather than chronic, problem in transplants, and understanding the spectrum of phenotypes and activation criteria of memory T cells could help to elucidate a means of specifically targeting alloreactive cells without compromising host protective immunity.

Moving forward, improved outcomes will best be achieved by using the available immunosuppressive agents, which are now many, efficiently, to manipulate immune responses on a patient-by-patient basis, minimizing broad toxicities. Each individual's immune cell repertoire is unique at the time of transplantation based on prior antigen exposure, primary diagnosis and other potential comorbidities. Elucidating a potential phenotype or immune cell profile, prior to transplantation, that associates with an increased risk of BRR is key in order to anticipate the immunosuppression needs of each patient. Ultimately, this will guide the field through a new era of transplantation and hopefully lead to the implementation of individualized, targeted immunosuppression regimens in order to sufficiently suppress a patient's immune system and protect the transplanted organ from rejection while minimizing off target side effects of the drugs.

Chapter 2. CD57+ CD4 T Cells Underlie Belatacept-Resistant Allograft Rejection

Introduction

Kidney transplantation is a standard, life-saving therapy, but organ survival necessitates the use of immunosuppressive drugs. These drugs render a transplant recipient immune compromised, the degree of which in large part determines the clinical risk of the procedure. As such, clinical immunosuppression has evolved through the development of increasingly targeted drug therapies to successfully manipulate the immune response toward an allograft without overly impairing the recipient's protective immune capacity. Following kidney transplantation most patients receive calcineurin inhibitors (CNIs: e.g. tacrolimus), which suppress T-cell function through inhibition of a ubiquitous intracellular signaling pathway. This leads to very effective, non-specific, T-cell immunosuppression and a substantial decrease in acute rejection rates that comes at the expense of impaired protective immunity, particularly to viruses and fungi [13,58-60]; it also precipitates chronic CNI-associated non-immune nephrotoxic and metabolic side effects [61,62]. This dilemma has impelled the development of more specific, targeted therapeutics to prevent rejection without the complications observed with CNIs; the most prominent of which is belatacept.

Belatacept, a fusion protein targeting a specific extracellular costimulation pathway (the CD28-B7 receptor ligand pair), was developed as a potential replacement for CNIs. Belatacept binds to CD80 and CD86 with high affinity, preventing their binding to the critical T cell costimulatory receptor CD28; a mechanism now referred to as costimulation blockade (CoB)[63]. TCR ligation in the absence of costimulation is generally ineffective in activating naïve, antigen-specific T cells, such that CoB substantially impairs *de novo* alloimmune responses. Antigen experienced T cells often have reduced requirements for

costimulation, and thus, CoB can have a sustained inhibitory effect on new immune encounters without impairing previously established protective immunity.

Clinical trials evaluating the efficacy of belatacept-based immunosuppression demonstrated improved side effect profiles, graft function and patient and graft survival up to five years post-transplant compared to patients receiving CNI-based immunosuppression[37,64,65]. Unfortunately, patients treated with belatacept experienced more severe and higher rates of acute cellular rejection (ACR) compared to patients treated with CNIs[64,65]. This CoBRR has limited the clinical use of belatacept irrespective of its substantially reduced side-effect profile. Given the highly specific nature of belatacept's action, we sought to investigate whether the immune cell profile of an individual at the time of transplant, particularly the degree to which they have progressed from a naïve to antigen experienced cell phenotype, can be assessed to identify patients at risk for CoBRR. We find that a particular cell type, the CD57+ CD4 T cell, which is atypical in healthy adults but frequent in patients with kidney failure, is associated with belatacept-resistant rejection (BRR) and worthy of further study.

Materials and Methods

Sample acquisition

Kidney allograft recipients receiving belatacept or tacrolimus according to labeled indication were enrolled in an IRB-approved tissue acquisition protocol at the Emory Transplant Center and Duke University Abdominal Transplant Repository. Collection and use of patient blood samples for laboratory analysis was approved by the Institutional Review Board at Emory University (Approval No. IRB00006248) and Duke University (Pro00030485). Written informed consent was received from participants prior to inclusion in each study. Peripheral blood mononuclear cells (PBMCs) were collected prior to transplantation and at multiple time points post-transplantation. Patients were followed clinically and segregated by outcome for analysis. For this study, we performed a retrospective analysis of stored patient samples. All belatacept-treated patients enrolled that that had not received a prior transplant and had baseline samples available for analysis were included in this study. Within 7 months of transplantation, of the 14 patients receiving belatacept-based therapy, 9 patients experienced ACR and 5 were rejection-free. 10 tacrolimus-treated patients, 5 that experienced ACR and 5 that were rejection-free, were selected with similar demographics to the belatacept-treated patients for comparison. Longitudinal studies were performed on 50 kidney transplant recipients treated with non-depletion, non-belatacept based therapies with a stable outcome at least 1 year post transplant. Patient demographics and additional supporting information can be found in Table 2.1.

Flow cytometry and intracellular cytokine staining

PBMCs obtained prior to drug administration were analyzed by flow cytometry to characterize the immune cell repertoire at baseline, interrogating for markers of memory, differentiation, activation, exhaustion and senescence. Antibodies were used against CD2 (BD #562300), CD3 (BD #557943), CD4 (BioLegend #317435), CD8 (eBioscience #47-00878-42), LFA1 (BD #563936), CD28 (BioLegend #302930), VLA4 (BioLegend #304314), CD57 (BD #555619) and PD1 (BioLegend #329906). Intracellular staining for Ki67 (BD #556026), granzyme B (BD #562462) was done using BD Biosciences Fixation/Permeabilization Solution Kit. Intracellular staining for IFNγ (BD #562392), and TNFα (BD #340534) was done following a four-hour stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin in the presence of brefeldin A (Sigma-Aldrich). All samples were run using a BD Biosciences LSRFortessa and analyzed using FlowJo software (Treestar).

PCR

Analysis of patient telomere length was performed on QuantStudio (Life Technologies) with iTaq Sybr (Biorad). Reference DNA was generated by the combination of five healthy controls and used to produce a standard curve to determine relative telomere/single copy gene ratio [66,67].

Microarray

Peripheral blood obtained from 5 healthy controls was processed and sorted on a BD FACSAria cell sorter into CD3+CD4+CD8-CD57+PD1- and CD3+CD4+CD8-CD57-PD1- populations. Antibodies were used against CD3 (BD #560366), CD4 (BD #557852),

CD8 (eBioscience #47-0088-42), CD57 (BD #555619) and PD1 (BioLegend #329906). Total RNA was extracted from the sorted populations and quality assessed. cDNA synthesis and amplification was performed, and fragmented and biotinylated samples were hybridized to the Affymetrix Human U133 plus 2.0 probe array. The arrays were scanned and probe intensity measurements were normalized across the samples using the robust multichip average (RMA) algorithm. GEO accession GSE64805.

Immunohistochemistry

Kidney allograft biopsies were obtained per protocol time point or suspected rejection. 36 biopsies were dual stained for anti-CD57 (DAKO, Carpinteria, CA) and anti-CD4 (DAKO, Carpinteria, CA). Blinded analysis was performed to determine cell density and localization.

Allogeneic mixed lymphocyte reaction

PBMCs were obtained by cytapheresis of healthy volunteers who gave their informed written consent. PBMCs were isolated by Ficoll Paque density-gradient centrifugation (GE Heathcare), monocytes and lymphocytes were isolated by positive CD14 or CD3 selection (Miltenyi Biotec, purity > 95%). Monocytes were then plated and cultured as previously described with 25 ng/ml recombinant human IL-4 (hIL-4; ImmunoTools) and 1000 IU/ml recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF; Peprotech). At day 6, immature DCs were characterized by staining for CD14 and CD209 and stimulated for an additional 48 hr with lipopolysaccharide (LPS; 50 ng/ml, Sigma-aldrich) (activated DC). For proliferation assay, lymphocytes were stained with 1 μM Violet Proliferation Dye 450 (VPD450; BD Biosciences) in DPBS for 10 min at 37°C and washed 2 times in complete medium and added to activated allogeneic DC (DC/$CD3^+$ T cells ratio :

1:3) with or without belatacept (25μg/ml). After 7 days of co-culture, VPD450 dilution and phenotype were analyzed by FACS. Antibodies were purchased from BD Biosciences (V450-labeled anti-CD14, APC-labeled anti-CD209, APC-Cy7-labeled anti-CD3, BUV395-labeled anti-CD4, PerCP-Cy5.5-labeled anti-CD8, BV605-labeled anti-CCR7, PE-labeled anti-CD57, APC-labeled anti-27, PE-Cy7-labeled anti-CD38) or from Miltenyi Biotec (FITC-labeled anti-CD45RO). Flow cytometry data were recorded with an LSR Fortessa cell analyser (BD Biosciences) and analyzed with Diva software (BD Biosciences).

Statistics

Differences in percentage of CD57+PD1- CD4 T cells were calculated using Mann-Whitney test due to the bimodal distribution of this cell subset. For additional flow cytometry analyses, *P* values were calculated using unpaired *t* tests, linear regressions and one-way and two-way ANOVA, and r values were computed using Pearson correlation coefficients. For the microarray, a paired *t* test was employed on the normalized expression values to find differentially expressed genes in CD57+ compared to CD57- cells. For immunohistochemistry, significant differences between stable and rejection outcomes were calculated using an unpaired *t* test. *P* values of less than 0.05 were considered statistically significant.

Results

High prevalence of CD57+PD1- CD4 T cells in peripheral blood prior to transplant is associated with belatacept-resistant rejection.

It is known that the activation of antigen-experienced T cells is less costimulation-dependent relative to that of naïve cells[68,69], and molecules associated with antigen experience have been related to CoBRR[70-72]. We therefore explored pre-transplant expression of surface markers indicative of antigen experience and terminal differentiation, specifically PD1 and CD57. PD1 is upregulated following activation and maintains expression on exhausted T cells[73,74], and CD57 is a carbohydrate epitope present on replicative senescent cells[75-78]. While this has been well described in the context of CD8 T cells[79,80] our study identified a population of CD4 T cells in the peripheral blood of transplant patients receiving belatacept-based immunosuppression that were CD57+PD1- and correlated with BRR, regardless of memory status (Figure 2.1B). No significant differences in outcome were observed in any subset of PD1 or CD57 expressing CD8 T cells (Figure 2.1C).

To distinguish whether this was specific to BRR, or characteristic of rejection in general, we studied patients treated with conventional tacrolimus-based immunosuppression. Using the same parameters, no significant difference between outcome and the expression of CD57 or PD1 was elucidated in CD4 or CD8 T cells (Figure 2.1D, E). Furthermore, there was no artificial segregation of CD57+PD1- CD4 or CD8 T cells prior to transplantation in patients that went on to receive belatacept compared to patients that went on to receive tracrolimus (Figure 2.2). These data demonstrate that CD57 expression on CD4 T cells in peripheral blood prior to transplant associates with rejection specifically in belatacept-treated patients.

We found this population of cells to be rare in healthy controls, but significantly increased in patients with kidney failure (Figure 2.3), increasing its unique relevance to kidney transplantation.

CD57+ CD4 T cells are not senescent by traditional indices.

CD57 has been described as a marker of immune cell senescence, a phenotype that would be unlikely to segregate with an active process like allograft rejection. Therefore, we employed independent assays to ascertain whether this was true in our cell population of interest. First we performed PCR analysis of relative telomere length in the same patient cohort described above. Terminally differentiated cells that have undergone multiple divisions have shortened telomeres [81], which should correlate with increased CD57 expression. In the patients treated with belatacept, expression of CD57 on CD4 T cells did not correlate with relative telomere length (Figure 2.4A). This lack of correlation indicated a possible non-traditional role of CD57 on CD4 T cells. Secondly, we looked at expression of Ki67, a protein strictly associated with cell proliferation [82]. Increased expression of CD57 on CD4 T cells in belatacept-treated patients significantly correlated with increased Ki67 expression ($p < 0.01$) (Figure 2.4B), also inconsistent with cell senescence. The non-senescent phenotype of CD57+ CD4 T cells in this setting contradicts the paradigm of CD57 expression and senescence.

The gene profile of CD57+PD1- CD4 T cells is highly associated with allograft rejection.

To gain insight into the function of CD57+PD1- CD4 T cells, we performed a microarray to examine their gene expression profile. PBMCs from healthy controls were sorted into CD57+PD1- and CD57-PD1- CD4 T cells and analyzed by microarray. A heat map of

normalized gene expression revealed a distinct transcriptome associated with CD57+ compared to CD57- CD4 T cells (Figure 2.5A). The most upregulated genes were indicative of a highly cytotoxic profile and included molecules involved in activation, adhesion, cell trafficking and cytotoxicity. We then imported the gene expression profiles into an independent Ingenuity Pathway Analysis (IPA), an unsupervised analysis of over 600 biological pathways. IPA remarkably deemed 'Allograft Rejection Signaling' the most closely associated signaling pathway with the genes upregulated in CD57+ CD4 T cells (Figure 2.5B).

CD57+ cells have increased expression of adhesion molecules.

Adhesion molecules, such as CD2 and LFA1, have also been implicated in CoBRR. Indeed, their selective elimination has been shown to substantially reduce the risk of CoBRR in animal models[70-72,83]. To evaluate the relationship between expression of CD57 and CD2, LFA1, and VLA4, we performed additional phenotyping on baseline PBMCs from transplant patients. $CD2^{hi}$CD4 T cells were shown to express high levels of LFA1 and VLA4, with CD57 present on cells that expressed high levels of these adhesion molecules (Figure 2.6A). Importantly, while all CD57+ cells expressed high levels of CD2, LFA1 and VLA4, the converse was not true, such that CD57 was a unifying marker of increased adhesion molecule expression (Figure 2.6B). These results suggested that CD57+PD1- CD4 T cells may not only serve as a marker of rejection risk in the periphery, but may play an active role in mediating rejection, prompting us to determine whether these cells were present in rejecting allografts.

There is an increased density of CD57+ T cells in rejecting kidney allografts.

Clinical kidney allograft biopsies obtained either for protocol surveillance or at the time of suspected rejection and were dual stained for CD4 and CD57 (Figure 2.7A). CD57+ CD4 T cells were present in rejecting kidney allografts at a higher density compared to stable kidney allografts (Figure 2.7B). In fact, patients with a higher percentage of circulating CD57+PD1- CD4 T cells prior to transplantation also had an increased density of CD57+ CD4 T cells in rejecting allografts. Thus, this population of cells is not only present in the peripheral blood, but they are capable of infiltrating the graft, likely due to the increased expression of adhesion molecules.

CD57+ CD4 T cells are CD28- and exhibit cytolytic properties.

Multiple groups have shown that CD28 loss is associated with CoBRR[84,85], but loss of CD28 did not statistically associate with BRR in our study. While we found an inverse correlation between CD57 and CD28 expression (Figure 2.8A), not all CD28- cells were CD57+ (Figure 2.8B). *In vitro* studies revealed that CD57+PD1- CD4 T cells are almost exclusively polyfunctional, producing both IFNγ and TNFα following stimulation with PMA and ionomycin (Figure 2.8C), while CD28-PD1-CD4 T cells were largely incapable of producing either IFNγ or TNFα (Figure 2.8D). Furthermore, all CD57+ CD4 T cells expressed high levels of Granzyme B compared to CD57- CD4 T cells, which express low levels of Granzyme B (Figure 2.8E). As such, while loss of CD28 perhaps identified cells indifferent to CD28-B7 blockade by belatacept, CD57 expression identified those CD28 negative cells capable of actively mediating BRR.

CD57+ CD4 T cells are resistant to the immunosuppressive effects of belatacept.

To determine whether CD57+ CD4 T cells are indeed resistant to the immunosuppressive effects of belatacept, CD3 T cells from healthy volunteers were labeled with Violet Proliferative Dye 450 and co-cultured with activated allogeneic dendritic cells (DCs). After 7 days in culture in the presence or absence of belatacept, cell subset proliferation was assessed by flow cytometry. Both CD57+ and CD57- CD4 T cells were able to proliferate in response to activated DCs in the absence of belatacept (Figure 2.9A). While proliferation of CD57- CD4 T cells was significantly inhibited in the presence of belatacept, proliferation of CD57+ CD4 T cells remained uninhibited (Figure 2.9B, C), confirming that CD57+ CD4 T cells are activated in a costimulation independent fashion. With mounting evidence that CD57+ CD4 T cells underlie BRR, we sought to examine the stability of these cells in patients following transplantation on non-belatacept based therapies, with the thought that patients with a high percentage of CD57+PD1- CD4 T cells at the time of transplantation, who may be more likely to reject on belatacept based therapies, may be better candidates for initial immunosuppression with CNIs, and later be converted to belatacept, to avoid the long term side effects of chronic CNIs.

CD57+PD1- CD4 T cells persist in the periphery following transplantation.

To assess the stability of patients' peripheral blood phenotypes over time, we performed a longitudinal analysis of 50 patients who received kidney transplants and were on non-depletional, non-belatacept based therapies with a stable outcome at least one year post transplant. Remarkably, the percentage of CD57+PD1- CD4 T cells was seen to persist in the periphery of patients following transplantation (Figure 2.10A), particularly in the CD4 TEMRA population (Figure 2.10B). Furthermore, CD57+ cells maintained high levels of

expression of adhesion molecules CD2, LFA1 and VLA4 (Figure 2.10C), revealing additional molecules to potentially target and inhibit the activation of CD57+ CD4 T cells.

Discussion

The search for a suitable alternative to CNI-based immunosuppression has become increasingly relevant as long-term outcomes and toxicities have gained prominence in clinical transplantation. Early results with belatacept, though encouraging with regard to graft function and off-target side effects, have been disappointing with regard to the ability to prevent early acute rejection. This has impelled an explicit look for mechanistic correlates of belatacept resistance and in particular a means of risk stratification for patients to guide immunosuppressive regimen choice. We chose to investigate variability in cell surface phenotype recognizing that individuals' lymphocyte repertoire evolves considerably over time as a result of environmental pathogen exposure and that these changes markedly alter cells' costimulation requirements including, but not limited to, expression of and dependence on CD28 [86-89]. These characteristics have experimentally been shown to mollify the efficacy of CoB-based regimens. In this series of experiments, we show this to be potentially relevant to humans, and characterize a particular cell type that is prevalent in renal failure patients, the CD57+PD1- CD4 T cell, the presence of which correlates with the risk of CoBRR, is present within rejecting allografts, and whose characteristics relate to the known mechanisms of cellular allograft rejection and costimulation resistance. It is important to note that there are several mechanisms of allograft rejection and the prevalence of CD57+PD1- CD4 T cells demonstrates a potential additional, and not exclusive, mechanism of CoBRR to be aware of in this population.

Although CD57+ CD4 T cells have previously been associated with cell senescence in HIV infected patients [78,90], we have demonstrated that CD57 is a unique, unifying marker of

human CD4 T cells that are enriched in patients with kidney failure and have a cytolytic profile characterized by high levels of adhesion molecules previously implicated in CoBRR. CD57+CD4 T cells are capable of infiltrating allografts and producing multiple cytokines upon stimulation. In this context, CD57 appears not to be a marker of senescence, but rather a potential surrogate indicator of patients' immune history as it relates to BRR. This population is consistent with that described previously as being prevalent in the kidney transplant population[91]. Their enrichment in patients with renal failure is an additional novel observation of these studies, the mechanism of which deserves further study, as this cell subset is not associated with routinely documented patient demographics (Table 1). Similarly, additional studies are necessary to completely define the mechanism by which these cells mediate CoBRR. Nevertheless, their association with and proximity to rejecting kidneys is clearly demonstrated in our studies.

CD57+ CD4 T cells have been previously described in the context of transplantation. Legendre, et al described a higher percentage of CD57+ (then, Leu7+) CD4 T cells in patients that experienced rejection compared to stable patients[91]. This study, predating the tacrolimus era, combined with studies reported herein, strongly implicates this cell type as worthy of attention. Collectively, these data support the finding that CD57+ CD4 T cells play a role in allograft rejection, particularly in the absence of CNIs, and that prospective assessment of this population could be used to identify patients at particular risk for ACR, especially in costimulation blockade-based regimens.

Graft survival rates have improved in the past 20 years largely due to the development of targeted immunosuppressant drugs. Traditional immunosuppression management consists

of universalized algorithms of serum drug levels and dosing regimens that are generally applied to transplant populations. With increasingly specific forms of immune manipulation, the opportunity for more personalized immune management becomes more evident and indeed, may become increasingly required. This is particularly true when selectively targeting a molecule that progressively diminishes in expression over a lifetime of immune perturbation. Though the increased risk of rejection associated with belatacept-based immunosuppression has proved a significant challenge for its clinical implementation, CD57+PD1- CD4 T cells represent a potential therapeutic target, as well as a practical screening tool to identify patients at higher risk for acute cellular rejection on belatacept-based therapy. The ability to clearly and accurately define markers of risk of rejection will help tailor immunosuppression therapies on an individual basis.

We acknowledge that these studies have been performed on a small group of patients, albeit one that has been very carefully monitored and controlled. Clinical intolerance for high rejection rates has prevented us from expanding the clinical cohort substantially. As our clinical goal has been to provide adjuvant therapies to mitigate BRR, expanding this cohort has not been feasible. The persistence of CD57+PD1- CD4 T cells in the periphery up to one year post transplant emphasizes the need for additional studies to elucidate associations between CD57 and other costimulation molecules to potentially target and inhibit the activation of CD57+ CD4 T cells, as well as phenotype these cells following transplantation. Nevertheless, we believe these observations to be sufficiently statistically supported and mechanistically plausible to warrant their reporting and to stimulate further study.

In summary, we find that expression of CD57 identifies a population of polyfunctional CD28 negative cells with cytolytic potential. While CD28 loss is intuitively necessary, it does not appear to be sufficient for BRR. Thus, CD28 loss may define belatacept indifference, but CD57 expression appears to identify cells mediating belatacept resistance. Importantly, this phenotype is present in the peripheral blood of patients awaiting transplant and assessing the prevalence of CD57+PD1- CD4 T cells prior to transplantation may identify patients not suited for belatacept-based therapy.

Figures

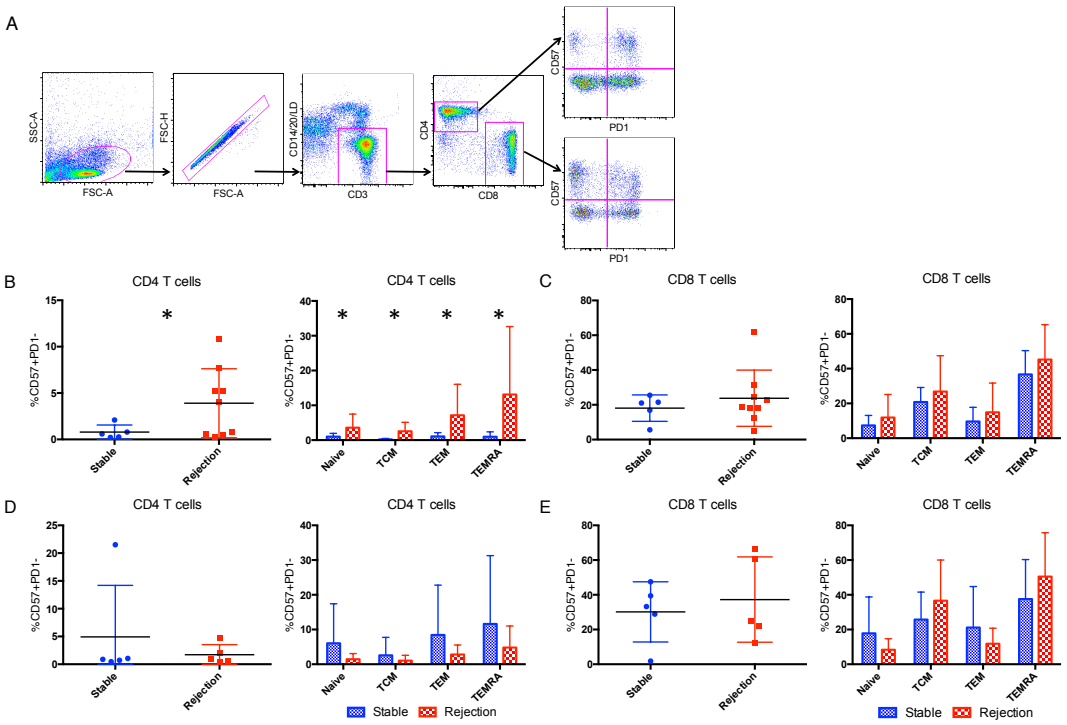

Figure 2.1: CD57+PD1- CD4 T cells in peripheral blood associate with belatacept-resistant rejection. (A) Representative flow plot of the gating strategy to identify CD57+PD1- T cells. (B) The left panel shows baseline PBMCs from patients treated with belatacept who went on to experience acute cellular rejection had a significantly higher prevalence of CD57+PD1- CD4 T cells compared to patients with a stable outcome ($p<0.03$). The right panel shows CD57+PD1- CD4 T cells were not segregated to terminally differentiated effector memory cells, but were significantly increased in patients with rejection compared to patients with a stable outcome in all memory compartments based on CD45RA and CCR7 expression ($p<0.03$). (C) The left panel shows that the percentage of CD57+PD1- CD8 T cells did not segregate with outcome in belatacept-treated patients. The right panel demonstrates the lack of association is true regardless of memory subset. (D, E) Analysis of baseline PBMCs from patients treated with tacrolimus reveals no correlation between outcome and prevalence of CD57+PD1- CD4 or CD8 T cells.

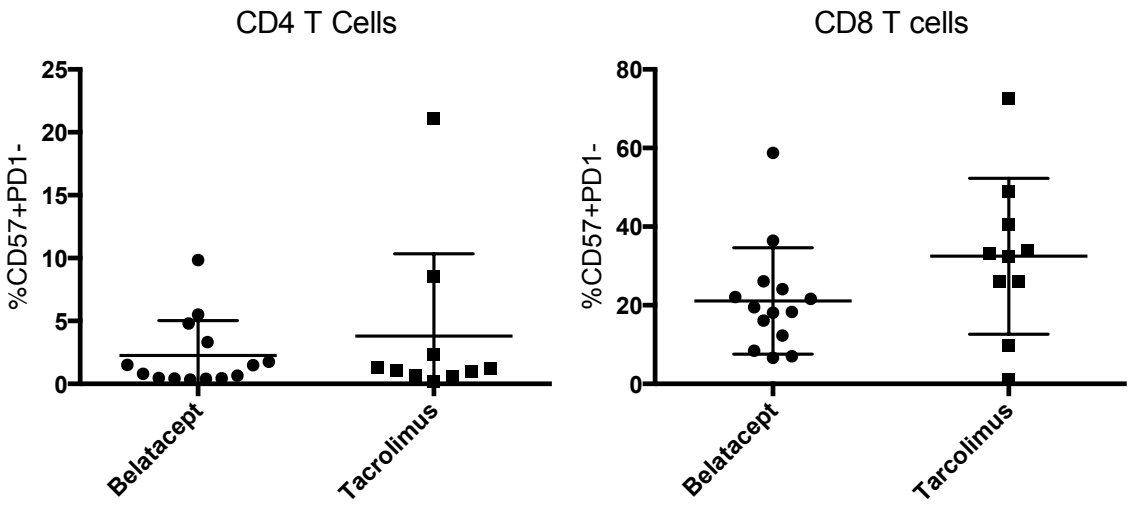

Figure 2.2: No artificial segregation of CD57+PD1- T cell prior to transplantation. Neither CD57+PD1- CD4 T cells ($p<0.74$, left) nor CD8 T cells ($p<0.11$, right) were unevenly distributed prior to transplantation in patients that went on to receive belatacept compared to patients that went on to receive tacrolimus.

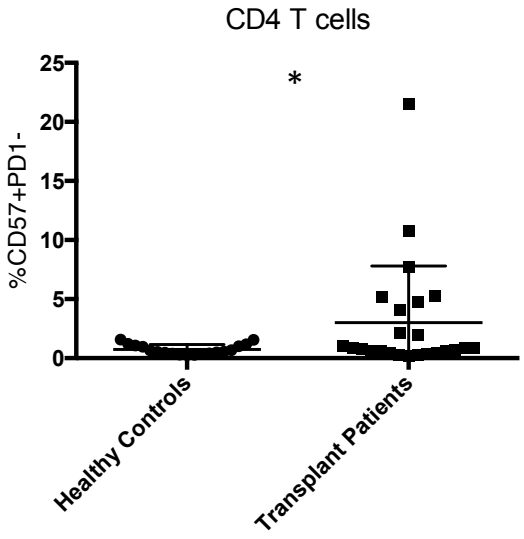

Figure 2.3: CD57+PD1- CD4 T cells are atypical in healthy controls. The frequency of CD57+PD1- CD4 T cells is significantly increased in PBMCs of patients with kidney failure awaiting transplant compared to PBMCs of healthy controls ($p<0.05$), highlighting their unique relevance to kidney transplantation.

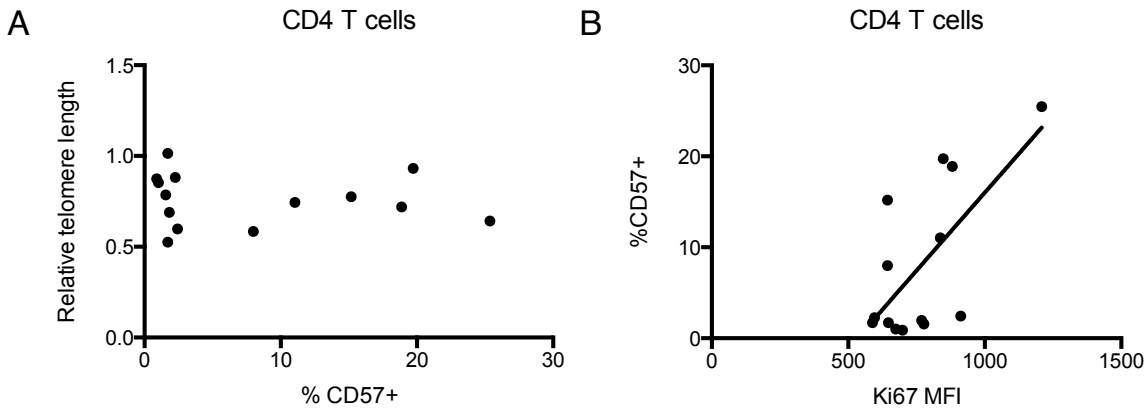

Figure 2.4: CD57+ CD4 T Cells are not senescent based on traditional indices. (A) Relative telomere length of belatacept-treated patients was determined by PCR and did not correlate with CD57 expression on CD4 T cells in the same belatacept-treated patients. (B) Ki67 expression was significantly correlated with increased expression of CD57 on CD4 T cells in belatacept-treated patients ($p<0.01$, $r=0.6704$).

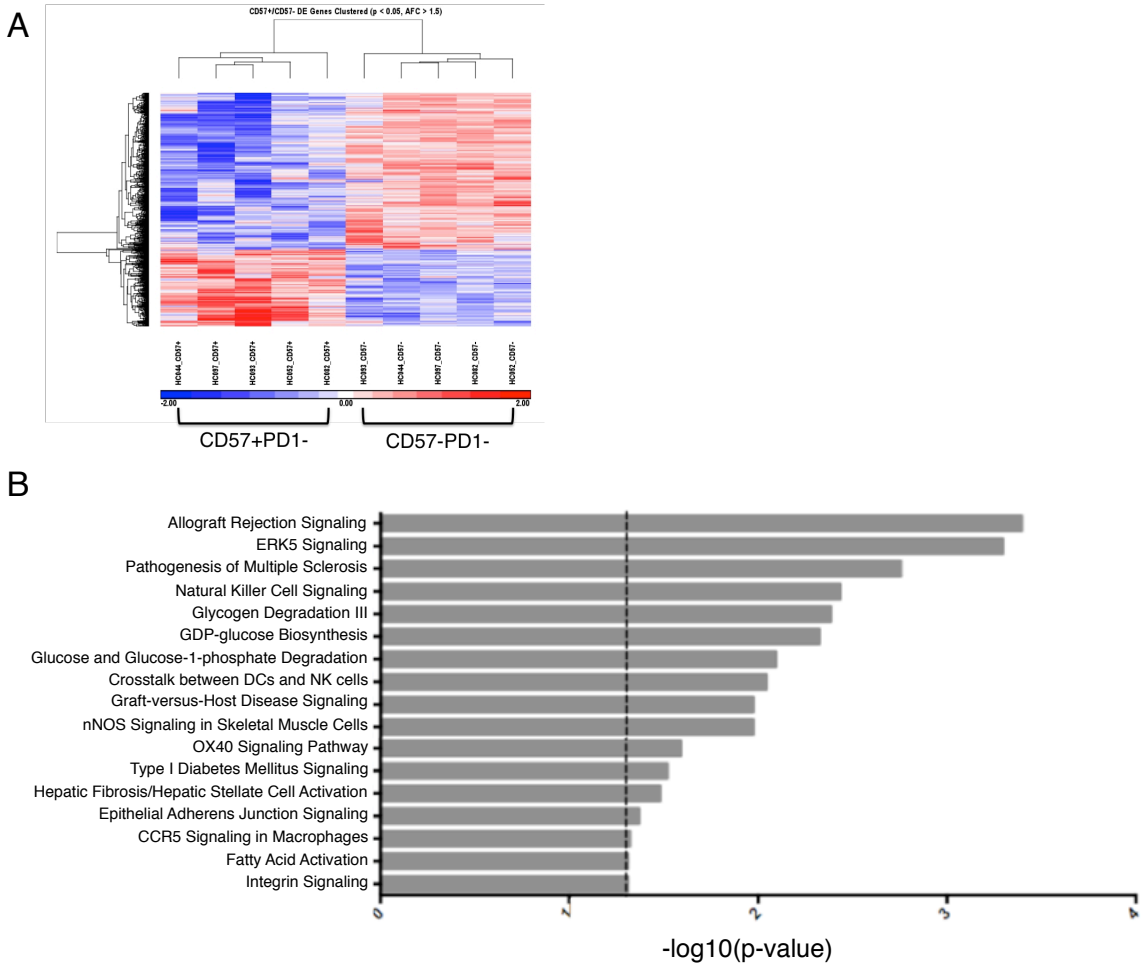

Figure 2.5: Genes Involved in Allograft Rejection are upregulated in CD57+ CD4 T cells. (A) PBMCs from healthy controls (n=5) were sorted into CD57+PD1- and CD57-PD1- CD4 T cells. RNA was extracted, cDNA synthesis and amplification was performed and samples were hybridized to the Affymetrix Human U133 plus 2.0 probe array. Clear segregation of gene expression was observed in CD57 positive compared to CD57 negative CD4 T cells. (B) Ingenuity pathway analysis deemed 'Allograft Rejection Signaling' the most closely associated signaling pathway with the genes upregulated in CD57+ compared to CD57-CD4 T cells.

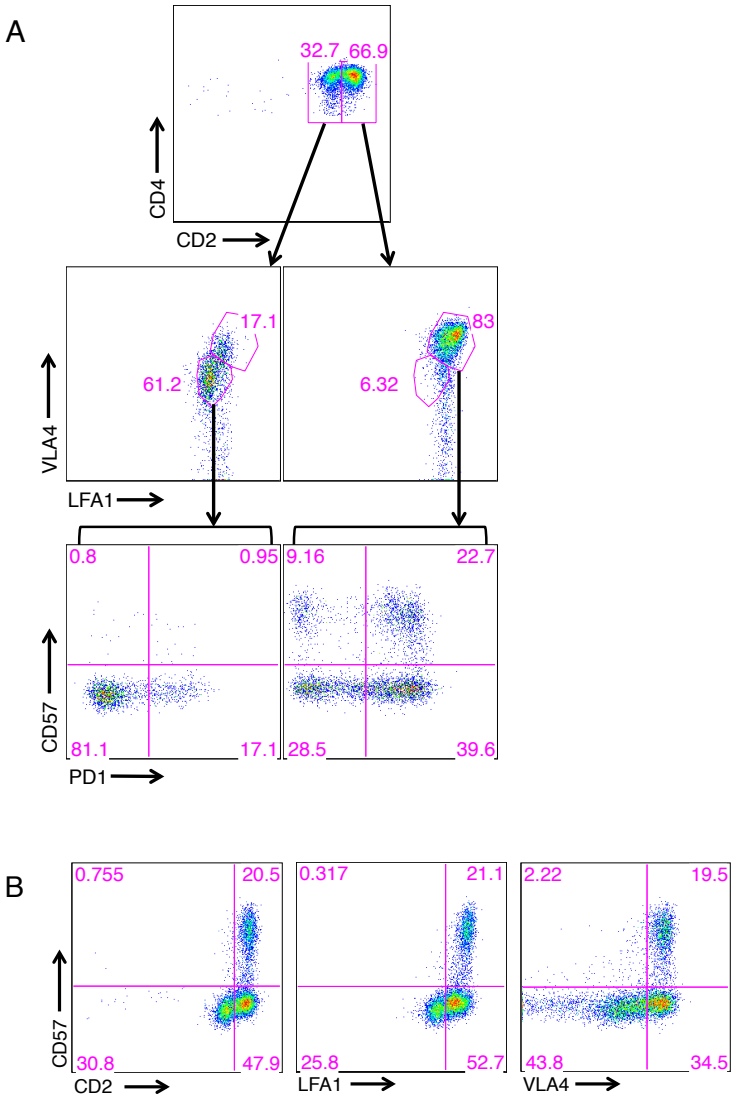

Figure 2.6: CD57 is present on cells with increased expression of other adhesion molecules implicated in CoBRR. (A) Representative flow cytometry plots of PBMCs from patients prior to transplantation demonstrate CD2hi CD4 T cells also express high levels of adhesion molecules LFA1 and VLA4, whereas CD2lo CD4 T cells express low levels of LFA1 and VLA4. CD57+ CD4 T cells are found exclusively in the CD2hi LFA1hi VLA4hi subset. (B) Representative flow cytometry plots of PBMCs from the same patients prior to transplantation validate that all CD57+ CD4 T cells express high levels of CD2, LFA1 and VLA4.

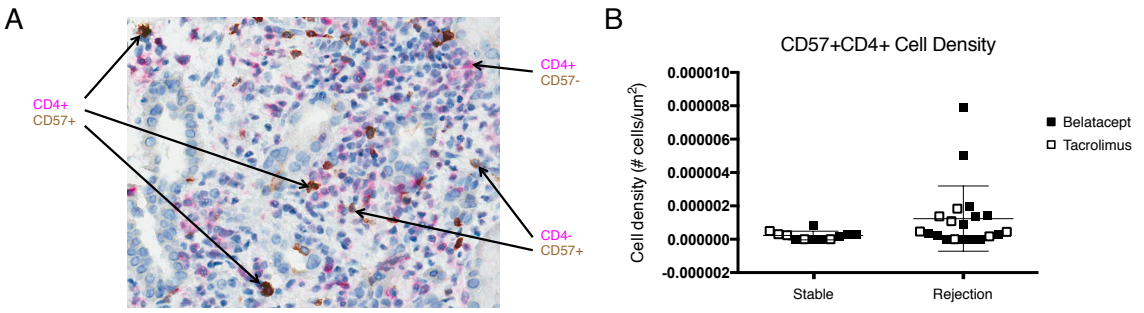

Figure 2.7: Increased density of CD57+ T cells in rejecting allografts. (A) A representative kidney allograft biopsy dual stained to show CD4 (in pink) and CD57 (in brown) expression demonstrates that CD57+ CD4 T cells are able to infiltrate kidney allografts. (B) Summary data of all biopsies from patients treated with belatacept (closed squares) and patients treated with tacrolimus (open squares) demonstrate the density of CD57+ CD4 T cells in kidney biopsies collected at the time of rejection is greater compared to patient biopsies obtained at a stable protocol time point ($p=0.08$).

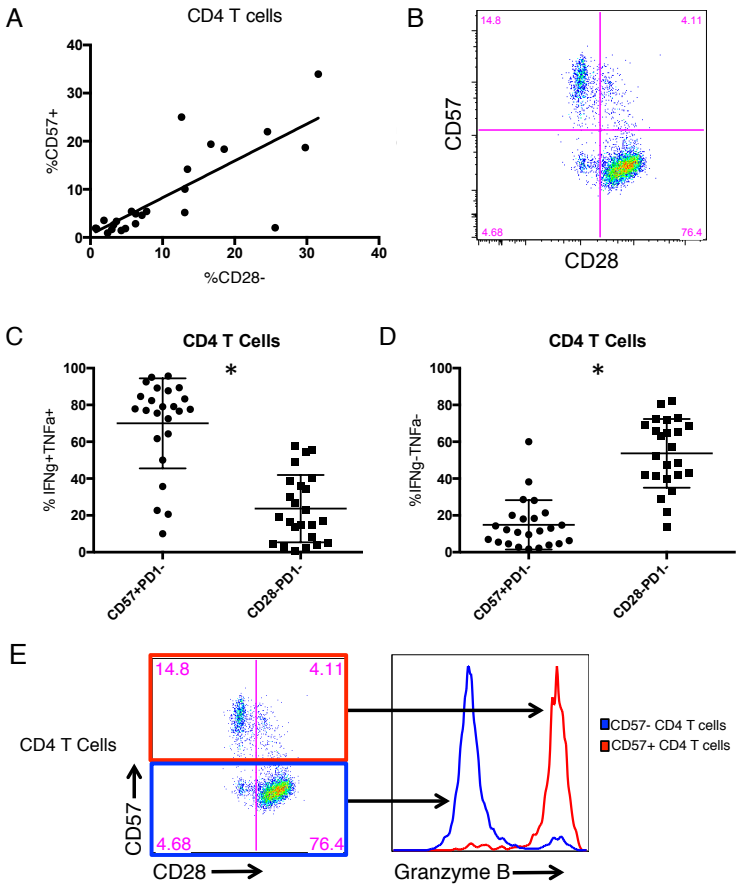

Figure 2.8: CD57+ CD4 T cells are polyfunctional with a cytolytic profile. (A) Summary data for all patient samples, n=24, show an inverse correlation of CD57 and CD28 expression on CD4 T cells at baseline, prior to transplantation ($p<0.01$, $r= 0.7759$). (B) Representative flow plot of patient samples at baseline demonstrate CD57 and CD28 are not expressed in a mutually exclusive manner. (C) Baseline PBMCs from the same patients were stimulated with PMA and ionomycin. Following stimulation, most CD57+PD1- CD4 T cells produce both IFNγ and TNFα ($p<0.01$). (D) Following stimulation with PMA and ionomycin, most CD28+PD1- CD4 T cells from the same patients' baseline PBMCs are incapable of producing either IFNγ or TNFα ($p<0.01$). (E) Representative flow and histogram of patient baseline PBMCs demonstrating CD57+ CD4 T cells express high levels of Granzyme B (in red) while CD57- CD4 T cells express low levels of Granzyme B (in blue).

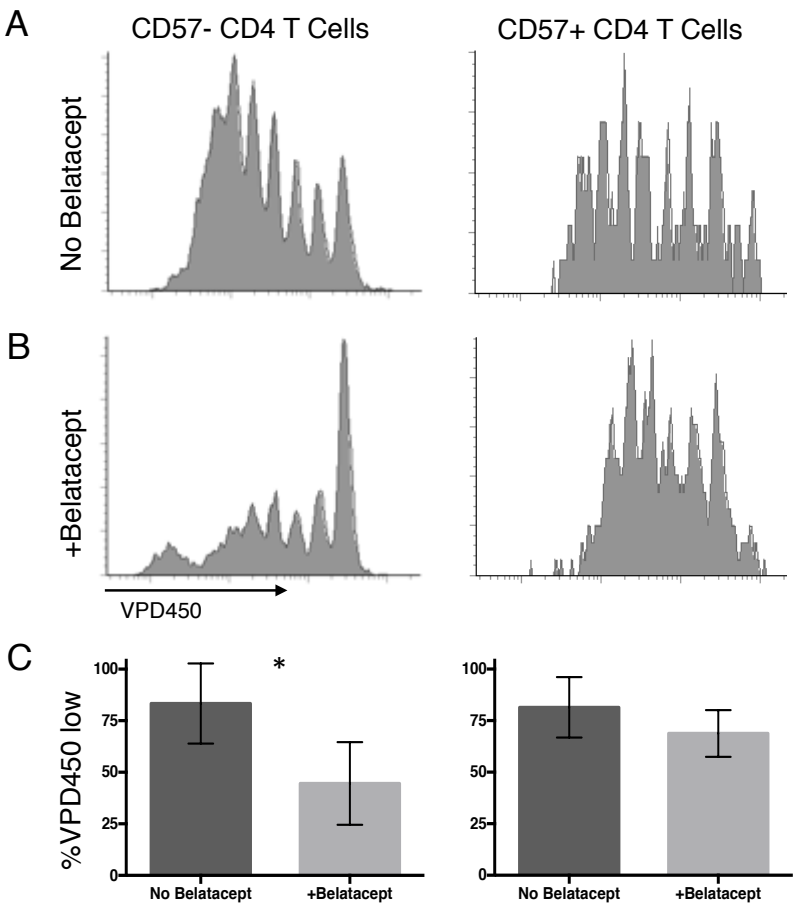

Figure 2.9: CD57+ CD4 T cells are resistant to in vitro immunosuppression with belatacept. (A) CD57- (left panels) and CD57+ (right panels) CD4 T cells from healthy controls are able to proliferate in response to activated allogeneic dendritic cells in the absence of belatacept. (B) Proliferation of CD57- CD4 T cells is inhibited by belatacept while CD57+ CD4 T cells retain the ability to proliferate in the presence of belatacept. (C) Summary data shows proliferation of CD57- CD4 T cells is significantly inhibited ($p<0.03$) with the addition of belatacept, while no significant change in proliferation is observed in CD57+ CD4 T cells ($n=4$).

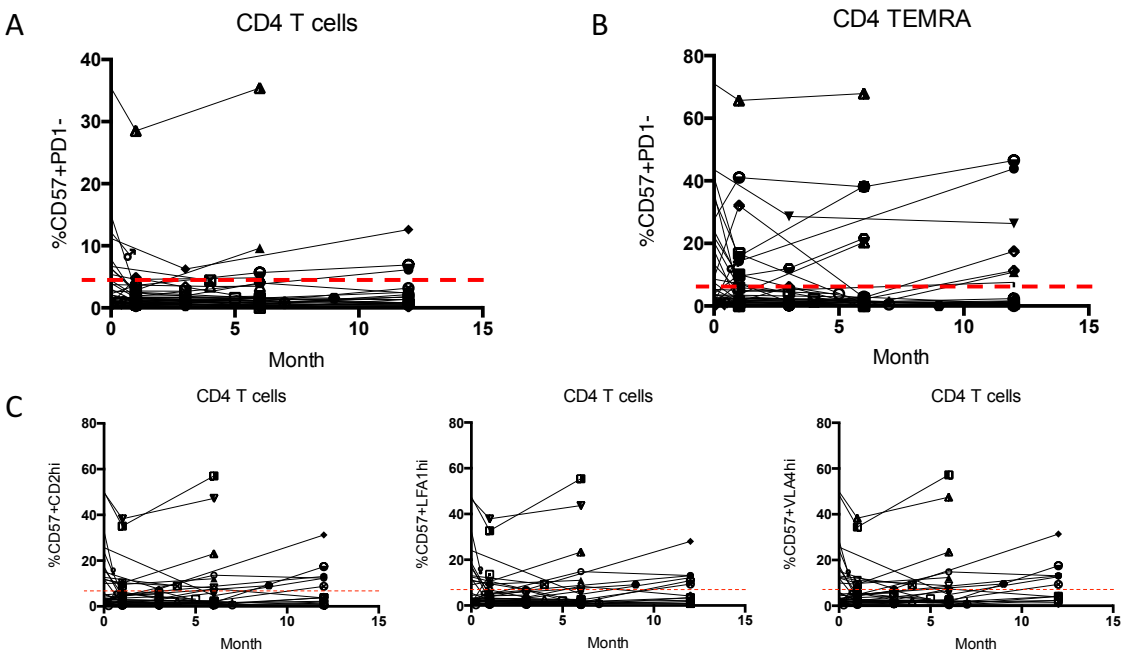

Figure 2.10: CD57+PD1- CD4 T cells persist in the periphery and maintain high levels of adhesion molecule expression following kidney transplant. (A) The percentage of CD57+PD1- CD4 T cells persists in the periphery of patients following transplantation. (B) CD4 TEMRA population maintains the highest percentage of CD57+PD1- cells. (C) CD57+ CD4 T cells maintain high levels of expression of adhesion molecules CD2, LFA1 and VLA4.

Patient #	Age at TX	Gender	Ethnicity	Cause of ESRD	Dialysis	Duration of dialysis (days)	EBV	CMV	%CD57+PD1- CD4 T cells	Donor	Treatment	Outcome
1	59	M	AA	Tuberous sclerosis	HD	2135	pos	neg	1.07	n/a	Tacrolimus	Rejection
2	40	M	n/a	HTN	n/a		pos	neg	0.988	DCD	Tacrolimus	Stable
3	64	M	AA	FSGS	HD	1470	pos	pos	2.31	n/a	Tacrolimus	Rejection
4	37	M	W	Alport's	HD	4327	pos	neg	0.216	LRD	Tacrolimus	Rejection
5	64	M	W	DMII	PD	700	pos	pos	21.1	DCD	Tacrolimus	Stable
6	58	M	AA	DMII	n/a		pos	neg	1.31	DCD	Tacrolimus	Stable
7	36	F	Hispanic	FSGS	PD	80	pos	pos	0.684	DCD	Tacrolimus	Rejection
8	52	M	W	Wegener	HD	1296	pos	neg	1.27	DCD	Tacrolimus	Stable
9	49	F	AA	HTN	PD	1136	pos	neg	0.561	DCD	Tacrolimus	Stable
10	56	F	AA	HTN/DMII	HD	2581	pos	pos	8.54	DCD	Tacrolimus	Rejection
11	66	M	W	IgA nephropathy	none		pos	pos	0.429	LUD	Belatacept	Stable
12	55	M	W	Adult PKD	HD	341	n/a	n/a	0.348	DCD	Belatacept	Stable
13	55	M	AA	HTN	HD	1569	pos	n/a	1.49	DCD	Belatacept	Rejection
14	68	M	AA	DMII	HD	2000	pos	pos	3.32	DCD	Belatacept	Rejection
15	68	M	AA	n/a	HD	1921	pos	neg	0.449	DCD	Belatacept	Rejection
16	35	M	AA	FSGS	HD	3572	pos	pos	0.469	DCD	Belatacept	Rejection
17	n/a	n/a	n/a	Adult PKD	n/a		pos	pos	0.399	DCD	Belatacept	Stable
18	62	F	AA	DMII	none		pos	neg	4.81	DCD	Belatacept	Rejection
19	26	F	Hispanic	DMII/reflux UTIs	HD	1997	pos	neg	9.84	DCD	Belatacept	Rejection
20	36	F	n/a	HTN	none		pos	pos	5.5	DCD	Belatacept	Rejection
21	53	M	n/a	HTN/glomerulonephritis	HD	1679	pos	pos	1.5	DCD	Belatacept	Stable
22	66	M	W	DMII, HTN	HD	109	pos	pos	0.807	DCD	Belatacept	Rejection
23	43	M	W	n/a	HD	3937	pos	pos	0.658	LUD	Belatacept	Stable
24	59	M	AA	HTN	PD	743	pos	neg	1.77	n/a	Belatacept	Rejection

Table 2.1: No significant correlation between percentage of CD57+PD1- CD4 T cells and other patient demographics. This table illustrates patient demographics, cause of end stage renal disease (ESRD), the type and duration of pre-transplant dialysis received, EBV and CMV serology, percentage of CD57+PD1- CD4 T cells, whether they received a kidney from a deceased or living donor, whether patients received belatacept- or tacrolimus-based immunosuppression following transplantation and whether they experienced a rejection or stable outcome. In this sample set, there was no significant correlation between percentage of CD57+PD1- CD4 T cells and other patient demographics. HTN = hypertension, FSGS = focal segmental glomerulosclerosis, DMII = type 2 diabetes mellitus, PKD = polycystic kidney disease, HD = hemodialysis, PD = peritoneal dialysis, EBV = Epstein-Barr virus, CMV = Cytomegalovirus, DCD = deceased donor, LRD = living related donor, LUD = living unrelated donor, n/a = not available.

Chapter 3. Manipulating Mice to Model the Human Condition

Introduction

T cells with a memory phenotype mediate costimulation blockade resistant rejection (CoBRR), and early CoBRR has been seen in patients on belatacept-based immunosuppression. In a study of renal allograft recipients, we found that patients who experienced acute cellular rejection had a markedly higher percentage of terminally differentiated CD57+PD1- CD4 T cells at the time of transplantation compared to those without rejection.[92] In order to study this cell population in a controlled environment, we moved toward an *in vivo* mouse model to specifically manipulate individual factors that may contribute to allograft rejection.

Inbred mice are an invaluable tool in immunology research due to genetic homogeneity of each strain, which provides opportunities for highly controlled and reproducible experiments. This is crucial when investigating mechanism of action and contributing factors to an array of human conditions. However, mice are not humans, and the characteristics of laboratory mice differ greatly from the human condition.[93,94] Three important differences of relevance to the immune repertoire between transplant patients and experimental mice are secondary complications of organ failure, age and environmental antigen exposure.

Mice are generally transplanted from a healthy baseline state. As such, they do not experience secondary complications of organ failure or transplantation, many of which have potential immune implications. As an example, patients presenting with renal failure often have concomitant hyperparathyroidism–a result of parathyroid gland dysregulation in the face of altered renal excretion of phosphorous leading to excess parathyroid hormone (PTH)

in the blood stream and perturbed calcium homeostasis. Parathyroid glands maintain calcium levels within the blood, and low calcium levels trigger the parathyroid glands to release PTH in order to activate osteoclasts, which break down bone and release calcium.[95,96] PTH, bone and calcium metabolism are increasingly associated with the immune function of T cells.[97,98] Indeed, in kidney transplant patients, postoperative hyperparathyroidism (termed tertiary hyperparathyroidism) coincides with the period of greatest risk for rejection following transplantation. Thus, the lack of immune impact of altered PTH in mice may serve as an example of a condition explaining a differential response to CoB compared to humans following transplant.

In most allograft transplant models, mice are generally used between 8 and 10 weeks of age. When taking into account the difference in lifespan between mice and humans,[99] experimental mice are far younger than the average age of kidney transplant recipients, the majority of whom are over 40 years of age at the time of transplant.[100] Thus, it is important to account for differences that may occur as a result of aging, or more specifically, aging in a non-gnotobiotic environment. Determining whether the immune cell repertoire shifts to a more memory-like phenotype with age and its obligatory exposure to environmental and microbiomic flora, particularly interrogating for markers of activation, memory and terminal differentiation, has relevance in considering CoBRR.

Regarding environmental exposure, experimental mice are housed in a relatively clean and environmentally controlled housing facilities, such that their antigen exposure is extremely limited. This is very different from humans who are continuously engaging antigenic insults in addition to being infected with several latent and persistent viruses. To better model this,

we chose to infect mice with murine homologs of highly prevalent latent and persistent viruses that are routinely problematic in immunosuppressed transplant recipients; polyomavirus (PyV), murine cytomegalovirus (mCMV) and gammaherpesvirus 68 (HV68). Mice that harbor persistent or latent viral infections may, in turn, develop a higher precursor frequency of potentially heterologously alloreactive cells. In these studies, we aimed to meticulously study the effects of age, PTH and viral infections on murine immune cell repertoires to develop a reliable mouse model more representative of the human condition.

Materials and Methods

Animals

C57BL/6 (H-2^b) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice 12 months of age and older were ordered from the NIH NIA (Bethesda, MD). All mice were housed in a specific pathogen-free barrier facility.

PTH administration

C57BL/6 mice were treated with either continuous or intermittent human PTH1–34 (Bachem California Inc., Torrance, CA). Continuous PTH (cPTH) or vehicle control was administered at 80 μg/kg/day for 4 weeks by implantation of an ALZET osmotic pump model-1002 (DURECT corporation Cupertino, CA).[101,102] Intermittent PTH (iPTH) or vehicle control was injected at 80 μg/kg/day subcutaneously for 4 weeks.[97]

Viral infections

C57BL/6 mice were mock infected with sterile PBS, infected with $1x10^6$ PFU/mL of polyomavirus (PyV), $1x10^5$ PFU/mL of murine cytomegalovirus (mCMV), $1x10^5$ PFU/mL of murine gammaherpesvirus 68 (HV68), or all three. PyV was administered via footpad injection in 50ul sterile PBS per foot. MCMV and HV68 were administered via intraperitoneal injection in 250ul of sterile PBS. Mice that received all three infections sequentially were bled on day 0, prior to viral exposure, injected with PyV, and bled at peak (day 7) and memory (day 21) time points to track changes in PBMCs. On day 21, mice were injected with mCMV, and bled on day 7 and 21 post infection. On day 42, mice were injected with HV68 and bled on day 7 and 21 post infection. Mice that received single

infections were injected on the same day the mice that received all three were infected: PyV on day 0, mCMV on day 21 and HV68 on day 42. Mice that received mock infections, or mice that were only infected with one virus were injected with sterile PBS as a control for potential activation due to the injection.

Flow cytometry

For aging studies, spleens were harvested and the splenocytes were analyzed by flow cytometry, interrogating for markers of memory (CD62L, CD44), activation (CD69), proliferation (Ki67), exhaustion (PD1) and senescence (KLRG1).

For PTH studies, spleens and bone marrow were harvested and cells were analyzed for markers of memory (CD62L, CD44).

For viral infection studies, blood was collected and PBMCs were analyzed for markers of memory (CCR7, CD44) and costimulation (CD28) as previously described[85].

Results

Murine immune cell profiles change significantly with age

We investigated the immune cell profiles of mice ranging from 8 days to 30 months of age to determine whether T cell phenotypes change with age in a controlled, specific pathogen free environment. We found that as mice age, they move from a predominantly naïve immune cell repertoire toward a more memory-like immune cell repertoire (Figure 3.1A). Specifically, there was a significant increase in both CD4 and CD8 effector memory T cells (Figure 3.1B).

Markers of immune cell activation also increased in mice with age. CD69, one of the earliest markers of activation,[103] was significantly increased in older mice on both CD4 and CD8 T cells across all memory subsets (Figure 3.2). We were also interested in the expression of PD1 and KLRG1, since PD1 is a common marker of exhausted cells as a result of constant antigen stimulation,[104] and KLRG1 has been described as a marker of senescent cells in mice,[73,105] akin to CD57 in humans, which has also been associated with increasing age.[106,107] There was a significant increase of PD1+ CD4 and CD8 T cells in mice with age (Figure 3.3A), as well as a significant increase of KLRG1+ CD4 and CD8 T cells with age (Figure 3.3B).

To determine whether the increased activation and differentiation observed was due to constant cell turnover, we investigated the expression of Ki67, a marker expressed only in proliferating cells.[108] We found increased proliferation in both CD4 and CD8 T cells in mice at 8 days and 6 weeks of age, with a consistent basal level of proliferation reached by 5 months of age (Figure 3.4). The sustained, low level of proliferation could be a contributing

factor to the persistent activation and differentiation of cells as mice age. Aside from aging, transplant patients also experience secondary complications of organ failure and transplantation, one of which is dysregulated PTH.

Treatment with PTH does not significantly alter murine immune cell profiles

It has previously been reported that PTH induces immune cell production of TGFβ, IL-6 and TNFα,[95,102,109] which can lead to Th17 differentiation of naïve T cells, and T cells play a central role in PTH-induced bone resorption[97]. With the function of PTH and T cells thus intertwined, we sought to determine whether specific T cell memory subsets would be altered by intermittent or continuous administration of PTH compared to vehicle control. Intermittent PTH (iPTH) has been shown to activate osteoblasts and increase bone density while cPTH has been shown to activate osteoclasts and decrease bone density, both of which rely on T cell interactions. Thus, while cPTH is more relevant to tertiary hyperparathyroidism experienced by transplant recipients, we chose to evaluate the effect of both iPTH and cPTH on T cell differentiation. Interestingly, in over 4 weeks of treatment, we observed no difference in memory subsets of CD4 nor CD8 T cells in the spleen (Figure 3.5A), or in the bone marrow (Figure 3.5B) as a result of either intermittent or continuous administration of PTH. Though previous reports have described changes in Th17 cells as a result of treatment with PTH, we did not observe any difference in mice treated with PTH for 4 weeks compared to vehicle control (Figure 3.5C).

Infection with latent and persistent viruses permanently alters murine immune cell profiles

Another major contributing factor to immune cell differentiation in humans is viral infection. Therefore, we chose to infect mice with murine homologs of highly prevalent

latent and persistent viruses that are routinely problematic in immunosuppressed transplant recipients. Mice sequentially infected with PyV, mCMV and HV68 experienced a shift in memory subsets characterized by a sustained increase in both CD4 and CD8 effector memory cells (Figure 3.6). To determine whether the increased effector memory cells may also be indifferent to CoB, we assessed the cells for expression of CD28.

Multiple viral infections can lead to CD28 downregulation in mice

Sequential, heterologous infections with PyV, mCMV and HV68 induced slight CD28 downregulation, but never complete loss of CD28. Infection with HV68 was the primary driver of CD28 downregulation on CD4 TEM (Figure 3.7A), while single infections with each virus resulted in significant CD28 downregulation on CD8 TEM compared to uninfected mice (Figure 3.7B). Though viral infection clearly results in sustained memory formation and CD28 downregulation, the lack of CD28 loss may still leave mice permissible to CoB.

Discussion

Utilizing animal models to study specific cell subsets and their mechanisms of action is key to the development and use of clinical agents. As such, it is imperative to establish feasible models that reflect the human condition as closely as possible. Here, we have explored three major differences between mice and humans to identify specific variables that may be manipulated to better model the human condition in the setting of transplantation: age, secondary complications of organ failure, and viral infections.

Though we observed increased activation and a significant shift toward memory in the immune cell repertoire of mice as they aged, simply using older mice for transplant experiments may not be a practical model. There are notable challenges and associated costs in keeping mice alive for years until they are significantly aged. Importantly, mice over the age of 8 months are scarcely available for purchase and waiting for a year until mice reach an appropriate age for experiments is neither time nor cost effective. As such, age may not be a feasible factor to focus on manipulating in future studies. However, these data do elucidate important differences between young and old mice and establish a baseline reference for future long-term murine studies. As these changes may simply reflect ongoing environmental antigen exposure, provocative viral exposure may be an appropriate approach toward achieving this state.

In addition to age, there are many secondary complications of organ failure and transplantation that patients experience. In these studies, we focused on hyperparathyroidism, a common disorder in patients with chronic kidney failure. While

previous studies have focused on the relationship between T cell function and bone loss as a consequence of PTH administration, we sought to determine whether PTH administration specifically altered T cells phenotypes to make them less susceptible to costimulation blockade. The lack of changes observed in T cell repertoire coupled with the additional effects of the surgery required to implant the osmotic pump to administer the PTH does not make this a feasible mode of manipulation to better model the human condition. Many other potential differences exist, such as chronic uremia in kidney failure, and these may be worthy of future study.

The development of mature immune cell repertoires in humans is largely influenced by viral exposure. We have demonstrated that mice infected with common latent and persistent viruses, PyV, mCMV and HV68, develop an immune cell repertoire consistent with antigen exposure and maturation observed in humans. Heterologous immunity has already been shown to play a role in costimulation blockade resistant rejection (CoBRR), though early studies utilized acute viral infections rarely of consequence clinically.[41,110] Thus, moving forward, a model of memory differentiation as a result of latent and persistent viruses that are pervasive and problematic in transplant recipients may yield further insight into the mechanism of early CoBRR observed in patients.

Figures

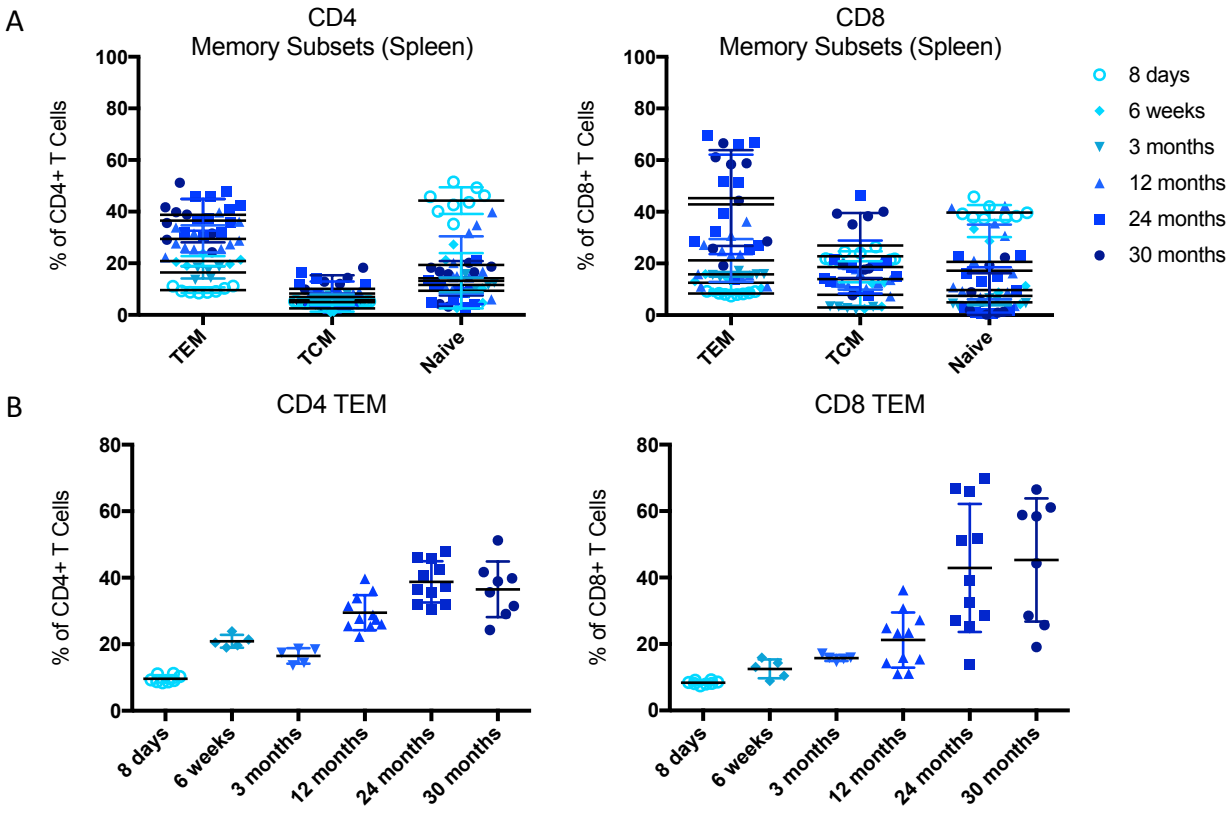

Figure 3.1: Changes in memory compartments in mice with age. (A) As mice age, they move from a more naïve immune cell repertoire toward a more memory-laden immune cell repertoire. (B) Significant increase in both CD4 ($p<0.0001$) and CD8 ($p<0.0001$) effector memory T cells (TEM).

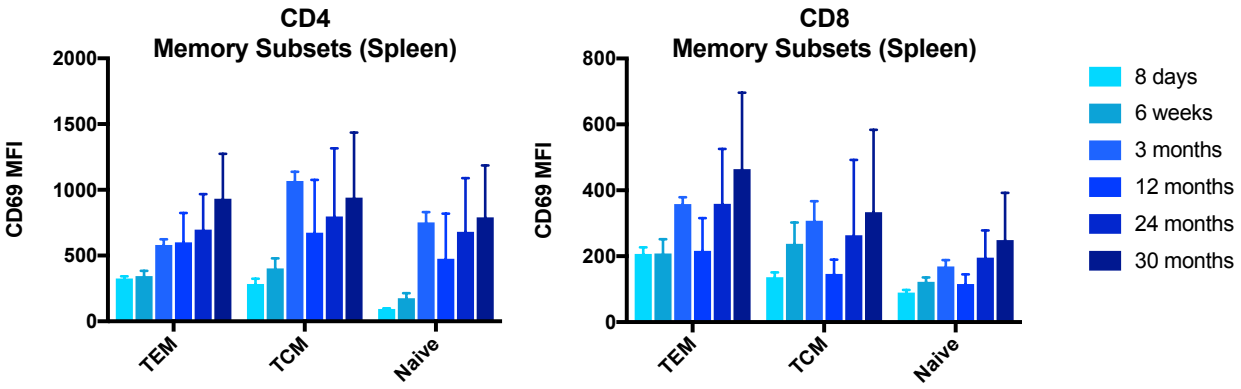

Figure 3.2: Increased activation in mice with age. CD69, a marker of immune cell activation, is significantly increased in mice as they age on both CD4 ($p<0.0001$) and CD8 ($p<0.0001$) T cells across all memory subsets.

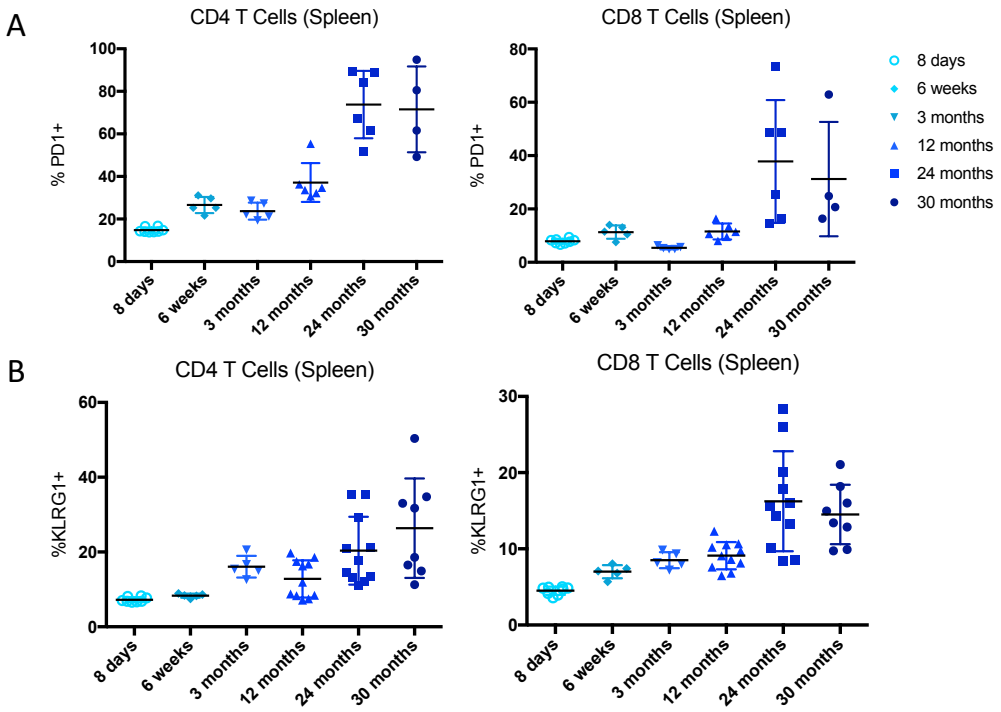

Figure 3.3: Increased expression of PD1 and KLRG1 on T cells in mice as they age. (A) Significant increase of PD1+ CD4 ($p<0.001$) and CD8 ($p=0.0003$) T cells in mice as they age. (B) Significant increase of KLRG1+ CD4 ($p<0.0001$) and CD8 ($p<0.0001$) T cells in mice as they age.

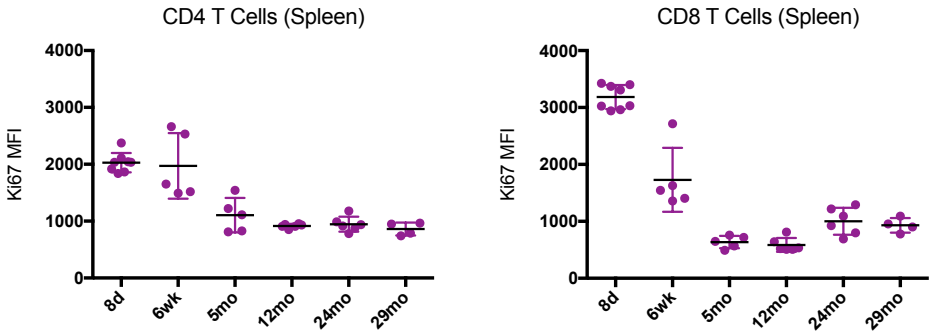

Figure 3.4: Homeostatic proliferation plateaus after 5 months of age. Homeostatic proliferation of both CD4 and CD8 T cells is increased in mice at 8 days and 6 weeks of age, but reaches a consistent baseline after 5 months of age as evidenced by changes in Ki67 expression.

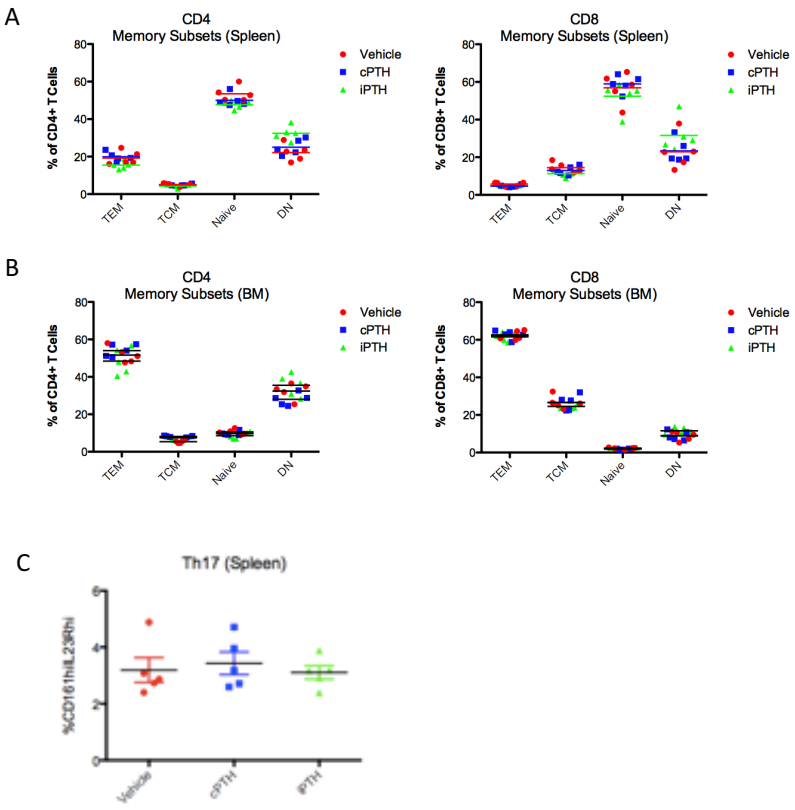

Figure 3.5: No significant difference in T cell subsets following treatment with PTH. No significant impact on memory subsets of CD4 nor CD8 T cells was observed in the spleen (A) or the bone marrow (B) following treatment with continuous or intermittent doses of PTH compared to vehicle control. (C) Percentage of Th17 cells in the spleen were not affected by PTH administration.

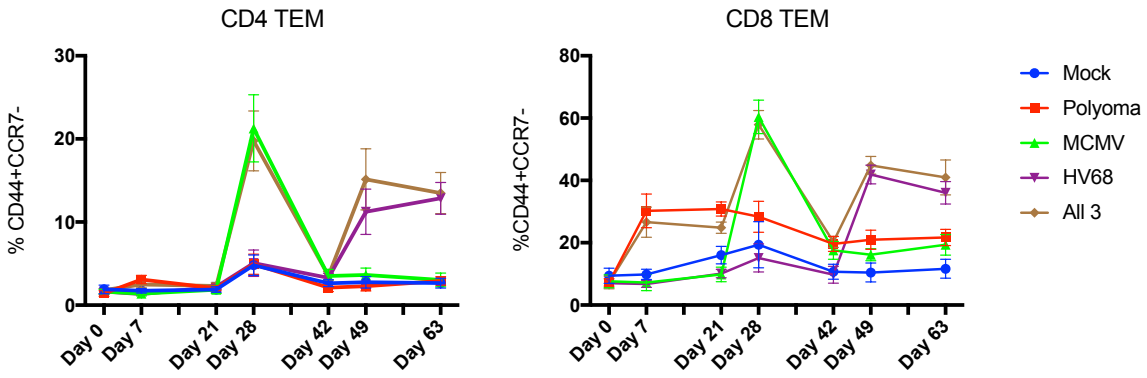

Figure 3.6: Sustained increase in effector memory cells following sequential infection with heterologous viruses. Mice sequentially infected with polyomavirus, murine cytomegalovirus and gammaherpesvirus 68 display a sustained increase in both CD4 ($p<0.0001$) and CD8 ($p<0.0001$) effector memory cells.

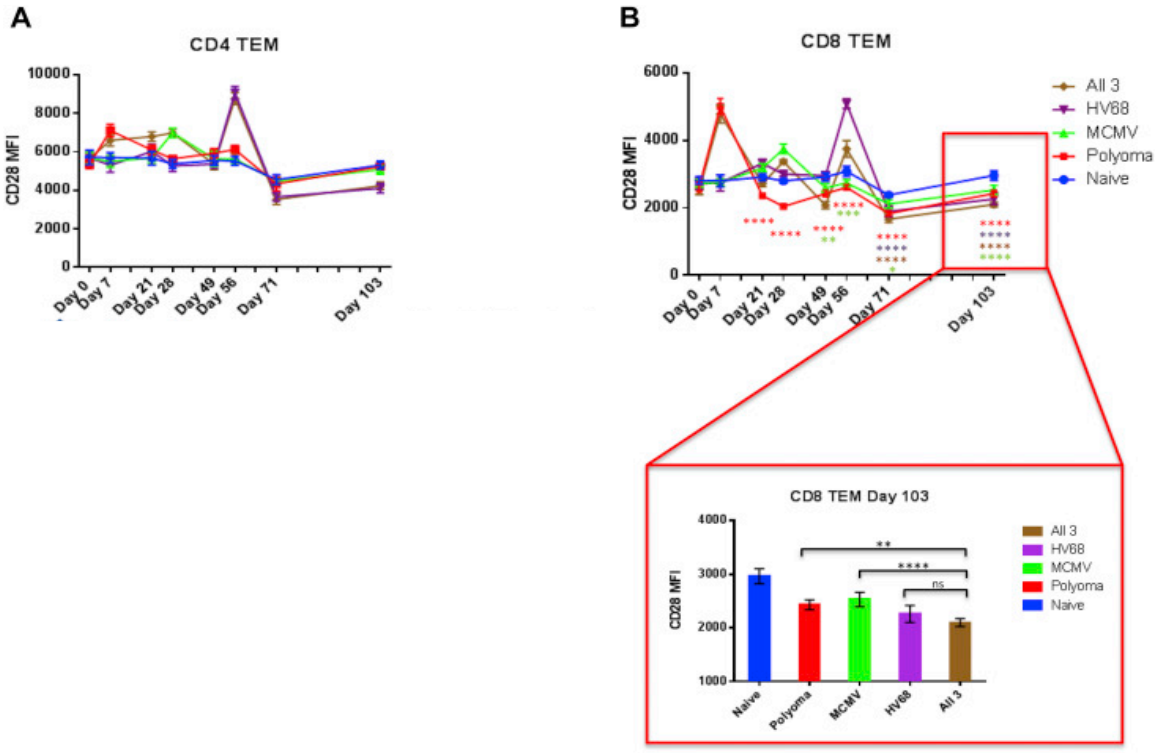

Figure 3.7: Sequential, heterologous infections induce CD28 downregulation. (A) Infection with HV68 is the primary driver of CD28 downregulation on CD4 TEM. (B) Single infection with each virus results in significant CD28 downregulation on CD8 TEM compared to uninfected mice.

Chapter 4. T Cell Repertoire Change Induced by Persistent and Latent Viral Infection is Insufficient to Induce Costimulation Blockade Resistant Organ Allograft Rejection in Mice

Introduction

Costimulation blockade (CoB) with the CD80/86-specific fusion protein CTLA4-Ig has long been known to prevent T cell-mediated allograft rejection in mice.[111-113] Dramatic and lasting anti-rejection effects have been achieved with short-term CTLA4-Ig treatments, and appropriately spurred substantial translational work to develop CoB-based therapies for use in clinical organ transplantation.[114,115] Indeed, as CoB with CTLA4-Ig, or a higher affinity analogue, belatacept, has transitioned into the clinic, patients treated with belatacept have significantly better long term graft function and survival compared to patients treated with standard calcineurin inhibitors.[36,116] Despite the long term benefits of belatacept, pervasive acute CoBRR has hampered its clinical usage. As such, studies into the nature of CoBRR hold importance in realizing the beneficial impact of CoB in human transplantation.

The prevailing mechanism thought to give rise to CoBRR has been T cell repertoire maturation driven by ongoing pathogen exposure.[117-119] Young, specific pathogen free laboratory mice are immunologically naïve, particularly when compared to nonhuman primates and humans presenting for organ transplantation. Humans are exposed to innumerable antigens throughout life, and this growing antigen experience results in a phenotypic shift of the lymphocyte repertoire so as to decreases the repertoire's general dependence on costimulatory pathways, like the CD28:CD80/86 pathway, for activation. Teleologically, this enables cells to more rapidly mount potent recall responses upon subsequent antigen encounters.[120-124] Indeed, seminal pre-clinical studies investigating the relationship of pathogen exposure to CoBRR have shown that CoBRR can be induced by

exposure to some viruses associated with resultant heterologous alloreactive memory.[41,57,125,126] Recent clinical data have, however, found no correlation between prior viral infection and acute allograft rejection in patients on conventional immunosuppressive medications,[127] and no overt association has been seen in early studies involving CoB.[64,92,128,129] Thus, processes that drive the immune cell repertoire toward costimulation-independence are areas of active investigation to elucidate the mechanism of CoBRR and identify better targets in the future.

While heterologous alloimmunity and CoB resistance can clearly be provoked in mice through acute viral exposure, the changes fostered by chronic viral exposure remain undefined, and the degree to which these mechanisms carry over into the clinic has not been established. Specifically, numerous characteristics of prior experiments used to investigate CoBRR in mice have differed markedly from conditions encountered in the clinic. Viruses used in many mouse models of memory such as lymphocytic choriomeningitis virus and vaccinia virus are rarely of consequence clinically,[130-134] and the viral-specific effects of these pathogens have not been segregated from the more general effects of viral exposure such as bulk T cell repertoire maturation. Many studies have combined CD28:CD80/86 blockade with CD40:CD154 blockade,[55,72,111,112,114,135-137] though the later agent is not available clinically. Furthermore, the type of allograft used in mouse studies has often been secondarily vascularized skin or bone marrow chimeras,[41,57,72,135] both of which are convenient and have rapid readout, but differ substantially from vascularized organ grafts. Lastly, many mouse studies use a short course of CoB rather than an indefinitely administered maintenance regimen used clinically. While each of these differences are valid experimental techniques that provide unique, well-controlled options for understanding aspects of CoBRR, we sought

to design a model to determine whether viral exposure consistent with that known to occur in humans leads to CoBRR using a CoB regimen used clinically.

CoBRR in humans has been associated with a terminally differentiated subset of CD57 expressing CD4 T cells.[92] Unfortunately, CD57 is not expressed on lymphocytes in mice, so we used a comparable molecule to identify a subset of potentially similar, terminally differentiated cells CD4 T cells.[107,138,139] In mice, KLRG1 has commonly been described as a surrogate for CD57 in humans, though largely in the context of terminally differentiated, senescent CD8 T cells. Thus, we developed a murine model utilizing persistent and chronic viruses that are common and routinely problematic in the transplant population to induce a more terminally differentiated immune profile in mice. Moreover, we employed a primarily vascularized heterotopic heart transplant model, so vasculopathy could be observed and assessed, along with immunosuppressive agents already approved for clinical use to facilitate translation of experimental findings to the clinic. We find that despite substantial repertoire change following viral exposure, CoBRR is not readily induced by latent or persistent viral infection in the presence of chronic immunosuppression.

Materials and Methods

Animals and viral infections

C57BL/6 (H-2^b) and Balb/c (H-2^d) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were housed in a specific pathogen-free barrier facility and were 8-12 weeks of age at the beginning of the experiment. C57BL/6 mice were mock infected with sterile PBS, infected with $1x10^6$ PFU/mL of polyomavirus (PyV), $1x10^5$ PFU/mL of murine cytomegalovirus (mCMV), $1x10^5$ PFU/mL of murine gammaherpesvirus 68 (HV68), or all three. PyV was administered via footpad injection in 50ul sterile PBS per foot. MCMV and HV68 were administered via intraperitoneal injection in 250ul of sterile PBS. Mice that received all three infections sequentially were bled on day 0, prior to viral exposure, injected with PyV, and bled at peak (day 7) and memory (day 21) time points to track changes in PBMCs. On day 21, mice were injected with mCMV, and bled on day 7 and 21 post infection. On day 42, mice were injected with HV68 and bled on day 7 and 21 post infection. Mice that received single infections were injected on the same day the mice that received all three were infected: PyV on day 0, mCMV on day 21 and HV68 on day 42. Mice that received mock infections, or mice that were only infected with one virus were injected with sterile PBS as a control for potential activation due to the injection (Figure 2).

Human samples

Patients with renal failure were enrolled in an IRB-approved immune monitoring protocol at Emory University (Approval No. IRB00006248). Peripheral blood mononuclear cells (PBMCs) were collected prior to transplantation and banked for batched analysis. For analysis, aliquots were thawed and interrogated for markers of memory by flow cytometry.

Heterotopic heart transplantation and immunosuppression

Balb/c donor hearts were transplanted into the abdomen of C57BL/6 recipients using a modified version of the methods previously described by Corry et al[140]. Mice that received CTLA4-Ig were given 250ug/dose on days 0, 2, 4, 7 and weekly following transplantation. Mice that received rapamycin were given 2ug/dose. Mice treated with rapamycin during infection were given one dose/day on days -2 through 7 post infection. Mice that received rapamycin following transplantation were given 3 doses/week every Monday, Wednesday and Friday. Allografts were monitored by palpation, and mice were sacrificed upon rejection (beating cessation) or at the end of the study.

Flow cytometry

Blood was collected serially from mice via maxillary vein and processed for PBMCs in order to track changes in the immune cell profile over time. PBMCs were interrogated for markers of memory (CCR7, CD44), and terminal differentiation. Mouse gating strategy: Samples are first gated on general lymphocytes, then single cells, followed by CD3+CD19- T cells. We then gated on CD4 or CD8 positive cells, and finally on memory subsets defined by expression of CD44 and CCR7: naïve (CD44-CCR7+), TCM (CD44+CCR7+) and TEM (CD44+CCR7-), or KLRG1 as previously described[85].

Human blood samples were procured, stained and analyzed as previously described[71,92]. Samples are first gated on general lymphocytes, then single cells followed by live CD3+CD14-CD20- T cells. We then gated on CD4 or CD8 positive populations and finally

on memory subsets based on expression of CCR7 and CD45RA: naïve (CCR7+CD45RA+), TCM (CCR7+CD45RA-), TEM (CCR7-CD45RA-), and TEMRA (CCR7-CD45RA+).

Histology

Donor heart allografts were harvested upon sacrifice and placed in 10% buffered formalin for histology. Hematoxylin and eosin staining was performed to visualize allograft damage and cellular infiltrate. Stained slides were de-identified and sent to a cardiothoracic pathologist for blinded assessment of rejection.

DNA extraction and viral PCR

DNA was extracted from donor heart allografts per protocol 'Purification of Total DNA from Animal Tissues' with Qiagen kit #69504. PCR was performed to determine whether viral DNA had infiltrated the grafts using TaqMan primers and probes. PyV primer sequences: CGCACATACTGCTGGAAGAAG and TCTTGGTCGCTTTCTGGATAC. PyV probe sequence: 6FAMATCCTTGTGTTGCTGAGCCCGATGAMGBNFQ. MCMV primer sequences: AGGGCTTGGAGAGGACCTACA and GCCCGTCGGCAGTCTAGTC. MCMV probe sequence: 6FAMAGCTAGACGACAGCCAACMGBNFQ. HV68 primer sequences: GGCCGCAGACATTTAATGAC and GCCTCAACTTCTCTGGATATGCC. HV68 probe sequence: 6FAMATTTGGGCGCAATGTGTTGGATGAATAMRA.

Results

Infection with latent and persistent viruses permanently alters the murine immune cell repertoire

Most mouse transplant models utilize relatively young and immune-naïve mice, whereas human kidney transplant recipients, the majority of whom are over the age of 50 at the time of transplantation[141], have considerable immune experience. Figure 4.1A portrays the wide range of memory subsets in both CD4 and CD8 T cells from 24 patients with renal failure based on expression of CCR7 and CD45RA. In stark contrast, Figure 4.1B shows that CD4 and CD8 T cells from 50 C57BL/6 mice are almost exclusively naïve with minimal memory populations. Thus, a mouse model that more accurately reflects the outbred human population is necessary to assess immune responses to allotransplantation.

In order to establish a more human-like model with a more memory-laden immune cell repertoire, we infected mice with latent and/or persistent virus homologues known to be common in the human population and precarious in the setting of transplantation. We sequentially infected mice with polyomavirus (PyV, BK virus homologue), murine CMV (mCMV, CMV homologue), and murine gammaherpesvirus 68 (HV68, EBV homologue). The experimental design is depicted in Figure 4.2. Each infection resulted in an increase in both CD4 and CD8 TEM cells ($p < 0.001$), with the biggest increase in CD8 TEM in mice that received all three infections (Figure 4.3A).

Next, we sought to determine whether all three infections could be given on the same day and yield a similar change in CD4 and CD8 TEM while condensing the experimental timeline. Mice were bled on day 0, then received either mock or all three infections and were

bled on day 7, 14, 21, 42 and 69 post infection to track changes in immune cell repertoire. Simultaneous infection with all three viruses resulted in a comparable sustained increase in TEM ($p < 0.001$) in both the CD4 and CD8 T cell compartments compared to mice that received mock infections (Figure 4.3B). This established a model of murine memory differentiation due to infection with clinically pertinent persistent and latent viruses that more closely resembles the diversity observed in human patients (Figure 4.1C).

In addition to memory formation, evaluating expression of other markers of differentiation and inhibition, like KLRG1, PD1and CTLA4, is also important to identify antigen-experienced cells that may be implicated in clinical CoBRR[73,74,139,142,143]. CTLA4 is particularly of interest in the context of CoB, since CTLA4-Ig inhibits both CD28 costimulation as well as CTLA4 coinhibition[33]. In our murine model of memory formation, the expression of KLRG1 was significantly upregulated ($p < 0.0001$) on CD4 and CD8 T cells in mice that received all three infections compared to mock infected mice (Figure 4.4). This is consistent with previous studies that have shown KLRG1 upregulation in mice in response to chronic viral antigen exposure[105,138]. Expression of PD1 was largely unchanged, and no long-term difference in expression of CTLA4 was observed on antigen-experienced CD4 or CD8 cells in mice that received all three infections compared to mice that received mock infections (data not shown).

Latent and persistent viral infection does not augment IFNg production in response to allogeneic antigen

To determine whether the resulting phenotypic changes had an effect on the cells' ability to respond to allogeneic antigen, we performed in vitro mixed lymphocyte reactions and measured interferon gamma (IFNg) production. C57BL/6 mice were infected with either

mock, PyV, mCMV, HV68 or all three viruses. Mice were sacrificed 40 days post infection and splenocytes were harvested for *in vitro* stimulation with either C57BL/6 syngeneic or Balb/c allogeneic stimulators. No difference in IFNg production was observed in response to syngeneic or allogeneic stimulation between any of the mock or infected mice (Figure 4.5). Stimulation with PMA and ionomycin was used as a control to demonstrate the potential cytokine production in response to TCR-independent stimulation. Though no difference in IFNg production was observed in response to *in vitro* stimulation with allogeneic or syngeneic antigen, we sought to determine whether there may be a differential *in vivo* effect on allograft survival.

Latent and persistent viral infections do not impact allograft survival in the setting of costimulation blockade

Following infection with either mock or all three viruses, mice received heterotopic heart transplants and were treated with CTLA4-Ig alone (Figure 4.6A), or CTLA4-Ig with rapamycin (Figure 4.6B). No significant difference in graft survival was observed in mice that received all three infections compared to uninfected mice in the presence of CTLA4-Ig alone or CTLA4-Ig with rapamycin. Based on previous reports that rapamycin augments CD8 T cell responses to pathogens while inhibiting graft-specific responses[125], we sought to investigate whether the presence of rapamycin in our infection model would alter either the expansion or quality of memory cells and thus affect allograft survival.

The presence of rapamycin during infection does not alter the kinetics of TEM expansion or allograft survival

To determine whether rapamycin would have an effect on viral responses, memory formation, and ultimately allograft survival, C57BL/6 mice were either mock infected or infected with PyV, mCMV and HV68 in the presence or absence of rapamycin. Intriguingly,

mice that received infections in the presence of rapamycin demonstrated almost identical kinetic expansion of both CD4 and CD8 TEM as mice that did not receive rapamycin during infection (Figure 4.7A). Though the percentage of memory cell formation was unchanged in the presence of rapamycin, it was pertinent to assess the ability of those cells to respond to allogeneic antigen *in vivo*. Therefore, mice went on to receive a heterotopic heart transplant to determine whether there would be an impact on allograft survival when treated with CTLA4-Ig. Intriguingly, there was no graft loss in mice infected in the presence or absence of rapamycin (Figure 4.7B). So, while treatment with rapamycin did not inhibit responses to these persistent and latent viruses, we cannot conclude that rapamycin was more effective at prolonging graft survival since neither group experienced a rejection.

Evidence of chronic rejection in mock infected mice and mice that received all three infections

Though no difference in graft loss was observed in these studies, there was a decrease in beating quality of the allografts over time. Thus, upon sacrifice, donor hearts were excised for immunohistochemistry and viral titers to assess cellular infiltrates and determine whether they may be due to viral- or allo-responses. Remarkably, there was no appreciable difference in grades of rejection between the groups at the experimental endpoint, though all did show evidence of chronic rejection. Figure 4.8A shows representative histology slides from C57BL/6 mice mock infected with PBS or infected with all three viruses that received fully mismatched heterotopic heart transplants from Balb/c mice and CTLA4-Ig on days 0, 2, 4, 7 and weekly until rejection or sacrifice at day 80 post-transplant. Figure 4.8B shows representative slides from mock infected or infected C57Bl6 mice that received fully mismatched heterotopic heart transplants from Balb/c mice and were treated with CTLA4-Ig on days 0, 2, 4, 7 and weekly and rapamycin every Monday, Wednesday and Friday until

sacrificed at day 75 post-transplant. Figure 4.8C shows representative slides from mock infected or infected C57Bl6 mice that received fully mismatched heterotopic heart transplants from Balb/c mice and were treated with CTLA4-Ig on days 0, 2, 4, 7 and sacrificed on day 14 post-transplant. Furthermore, there was no viral DNA present in the heart allografts at the time of sacrifice, signifying lymphocytes that trafficked into the allograft were unlikely to be responding to viral antigen (data not shown).

Discussion

Through these studies, we have established a reliable murine model of memory differentiation using persistent and latent viral homologues to those common in the human population: PyV, mCMV and HV68. Both sequential and simultaneous infection with these viruses resulted in significant and permanent expansion of CD4 and CD8 TEM to levels consistent with those seen in adult humans, as well as an increase in KLRG1 expression. Though heterologous alloreactivity with atypical acute infections has been shown to significantly decrease allograft survival in the context of CoB, we have observed no increase in CoBRR in mice infected with PyV, mCMV and HV68 compared to mock infected mice under varying chronic immunosuppression regimens. Thus, the type of infection, degree of allograft vascularization and immunosuppression regimen likely play a large role in the heterologous response to allogeneic antigen.

These results highlight a possible distinction between true CoBRR (rejection occurring despite ongoing costimulation blockade) and the stability of tolerance established by CoB but persisting after drug withdrawal. Although the latter has been shown to be perturbed by viral infection, the former has not been formally tested in mice until this set of experiments. These data make clear that the mere presence of memory cells as a result of multiple chronic infections is insufficient to induce overt rejection. This is an important distinction. Prior studies have modeled "heterologous" immunity by creating antigen identity between a pathogen and an allograft[126,144]. Although antigen identity studies show, intuitively, that antigen identity between a pathogen and an allograft can lead to cross-sensitization, such identity is clinically unlikely. The more common scenario relates to a situation where

exposure to one antigen improves the avidity of clones that are both specific to that antigen and which have modest alloantigen binding capacity. General allospecific binding is achieved through thymic selection that was appropriate for the host, but not for the donor MHC. This improved avidity, for example by increased adhesion molecule expression and a lowering the activation threshold of a cell, could be expected to be spurred forward by any immune maturation. In this case, despite broad repertoire maturation, a more aggressive phenotype of rejection could not be provoked. Similarly, the emergence of an increasingly differentiated repertoire is not, in and of itself, synonymous with the development of a CoB resistant repertoire, as evidenced by the lack of CoBRR when Cob is dosed continuously, as it is in humans. This is not to say that memory acquisition is inconsequential as it relates to alloimmunity, but rather that the effect is insufficient to overwhelm ongoing therapy. These effects may be more impactful, or less well controlled, in the absence of ongoing CoB. Thus, CoB induced tolerance (or perhaps anergy), may be more mercurial and easily perturbed. We would thus suggest that prior studies are perhaps better considered models of tolerance disruption than true CoB resistance.

These data are consistent with recent clinical observations whereby humans that were well controlled on belatacept monotherapy or belatacept and rapamycin, despite routine viral exposures, were weened successfully from immunosuppression, but underwent rejection when faced with an acute viral illness off drug.[129] These data are also consistent with observational human studies that fail to demonstrate any increased risk of allograft rejection conferred by prior latent herpesvirus exposure,[64,127,128] and indicate that repertoire maturation alone is insufficient to provoke a capacity for CoBRR under ongoing therapy. Specific effector populations that are truly resistant to CoB may emerge prior to transplantation and

may play a role in CoBRR, much like we have observed in kidney transplants recipients,[92] but it seems unlikely that these cells derive from the viruses evaluated in this study. Furthermore, within the parameters of these experiments, KLRG1 on murine CD4 T cells cannot be used as a surrogate for CD57 expressing human CD4 T cells, which limits the ability to manipulate parameters in vivo that will directly transplant to the patient population.

Development of murine models that meticulously reflect the patient population and build on established mechanistic studies is imperative, not only to determine which cells mediate CoBRR, but also to understand their clinical mitigation. Here, we have shown that memory cells derived from persistent and latent viral infections do not impact allograft survival in the presence of CoB. Further studies are needed to define the differential impact of acute and latent infections on allograft survival, as well as the distinction between tolerance established by CoB and the development of cells that are truly resistant.

Figures

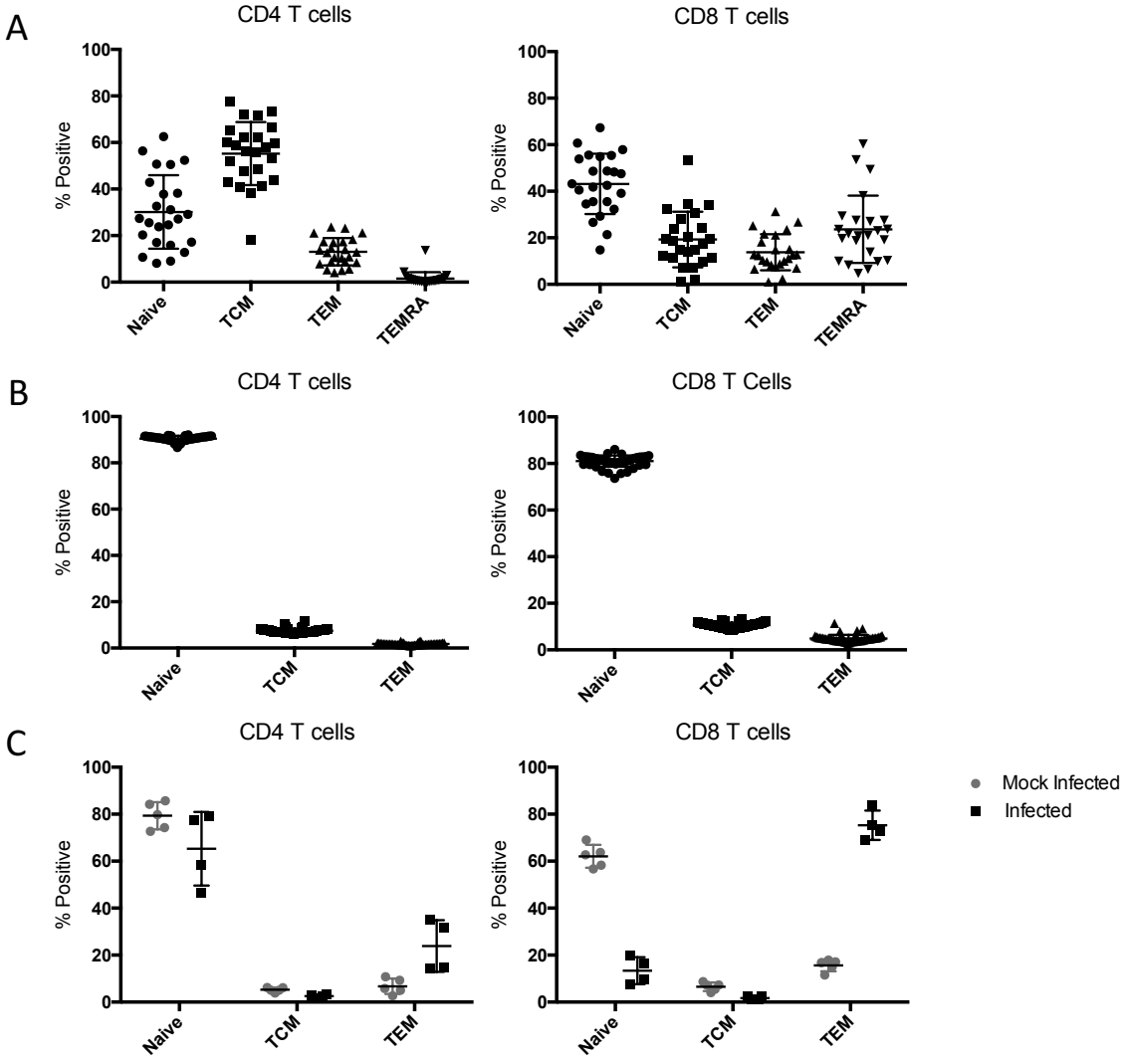

Figure 4.1: Human vs murine memory repertoires. (A) Peripheral blood samples from 24 patients with renal failure have highly diverse immune cell repertoires in both the CD4 and CD8 T cell compartments. (B) In stark contrast, peripheral blood samples from 50 C57BL/6 mice have an almost exclusively naïve immune cell repertoire in both the CD4 and CD8 T cell compartments. (C) Mice infected with PyV, mCMV and HV68 exhibit a more diverse immune cell repertoire compared to mock infected mice.

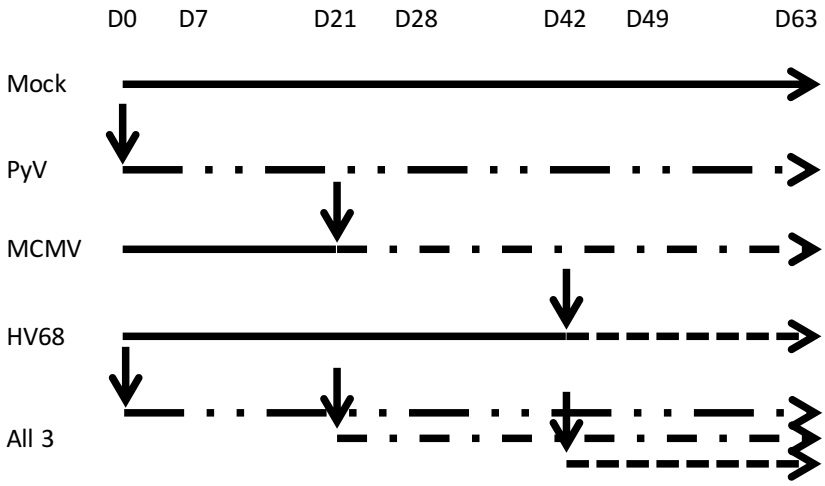

Figure 4.2: Experimental design for sequential viral infections of C57BL/6 mice. Mice that received PyV were infected on day 0, all other groups received sterile PBS injections. Mice that received mCMV were infected on day 21; all other groups received sterile PBS injections. Mice that received HV68 were infected on day 42, all other groups received sterile PBS injections.

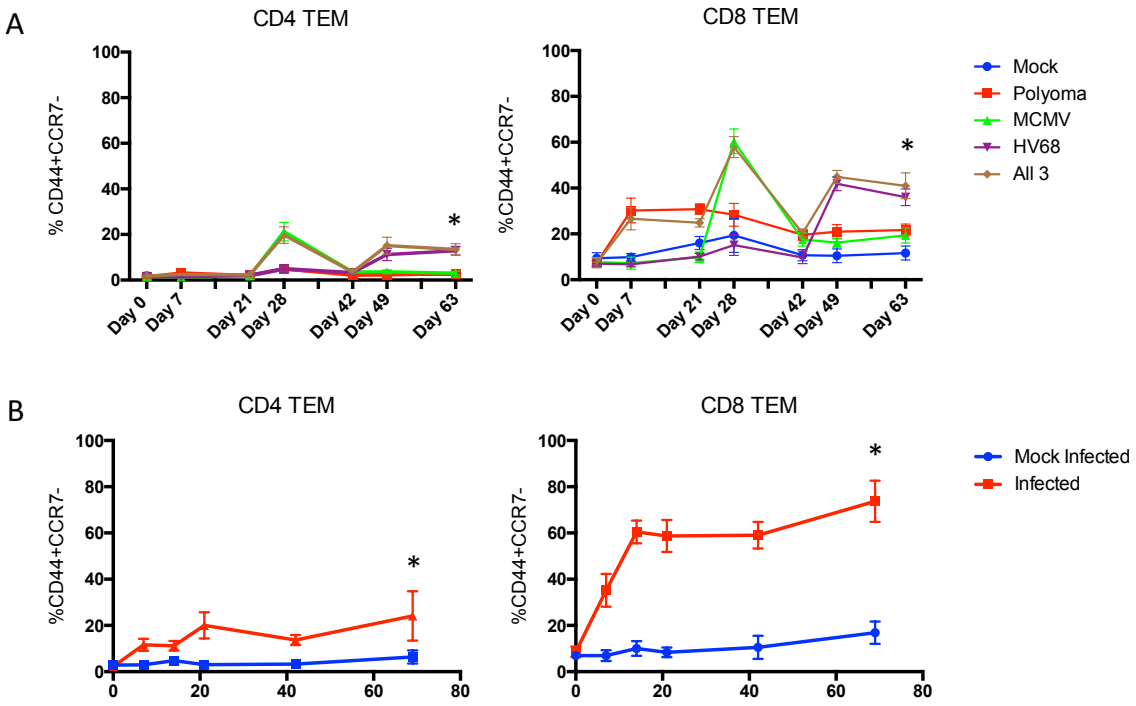

Figure 4.3: Time course of the change in peripheral blood phenotype in C57BL/6 mice following infection with PyV, mCMV and HV68. (A) Sequential and (B) simultaneous infections with multiple viruses result in a significant and sustained increase in TEM cells in both the CD4 and CD8 T cell compartments compared to mice that received mock infections ($p < 0.001$).

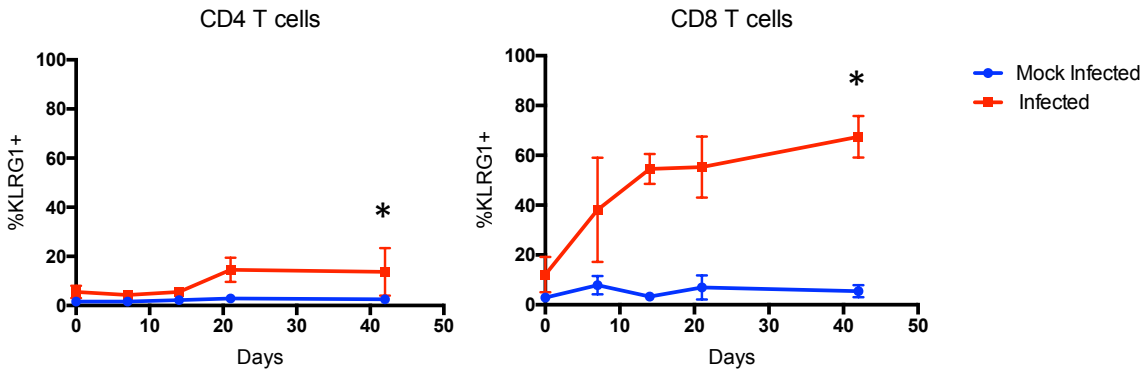

Figure 4.4: Significant upregulation of KLRG1 in infected compared to non-infected mice. Expression of KLRG1 was significantly upregulated on CD4 and CD8 T cells of mice that received all three infections compared to mice that received mock infections ($p < 0.0001$).

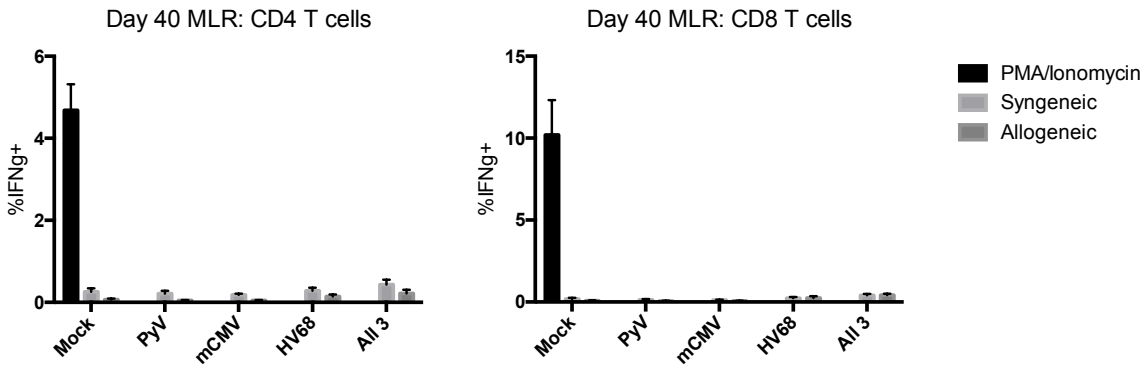

Figure 4.5: No difference in IFNg production in response to syngeneic or allogeneic stimulation. 40 days after infection with either mock, PyV, mCMV, HV68 or all three viruses, C57BL/6 mice were sacrificed and splenocytes were harvested for in vitro stimulation with either C57BL/6 syngeneic or Balb/c allogeneic stimulators. Stimulation with PMA and ionomycin was used as a control to show potential cytokine production.

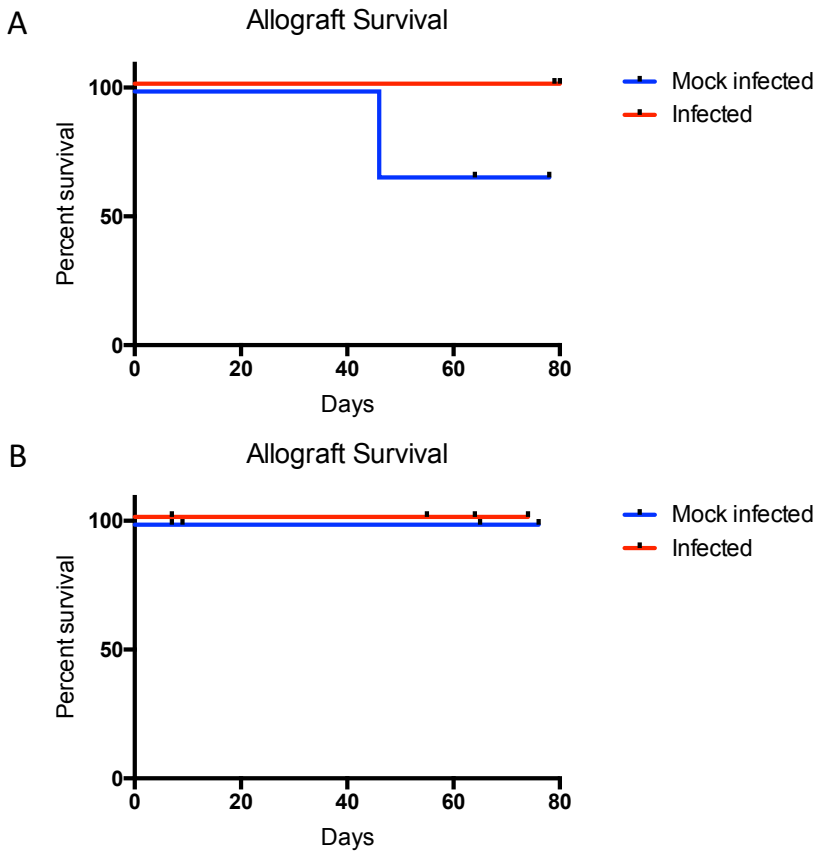

Figure 4.6: No difference in allograft survival when treated with CTLA4-Ig with or without rapamycin. C57BL/6 mice were simultaneously infected with PyV, mCMV and HV68. At least 60 days post infection, mice were given a heterotopic heart transplant from Balb/c donors and beating quality was monitored for survival. (A) No difference in graft survival in mice treated with CTLA4-Ig days 0, 2, 4, 7, weekly. (B) No difference in graft survival in mice treated with CTLA4-Ig days 0, 2, 4, 7, weekly and rapamycin 3x per week. Decrement survival indicates immunosuppressive death with a functioning allograft.

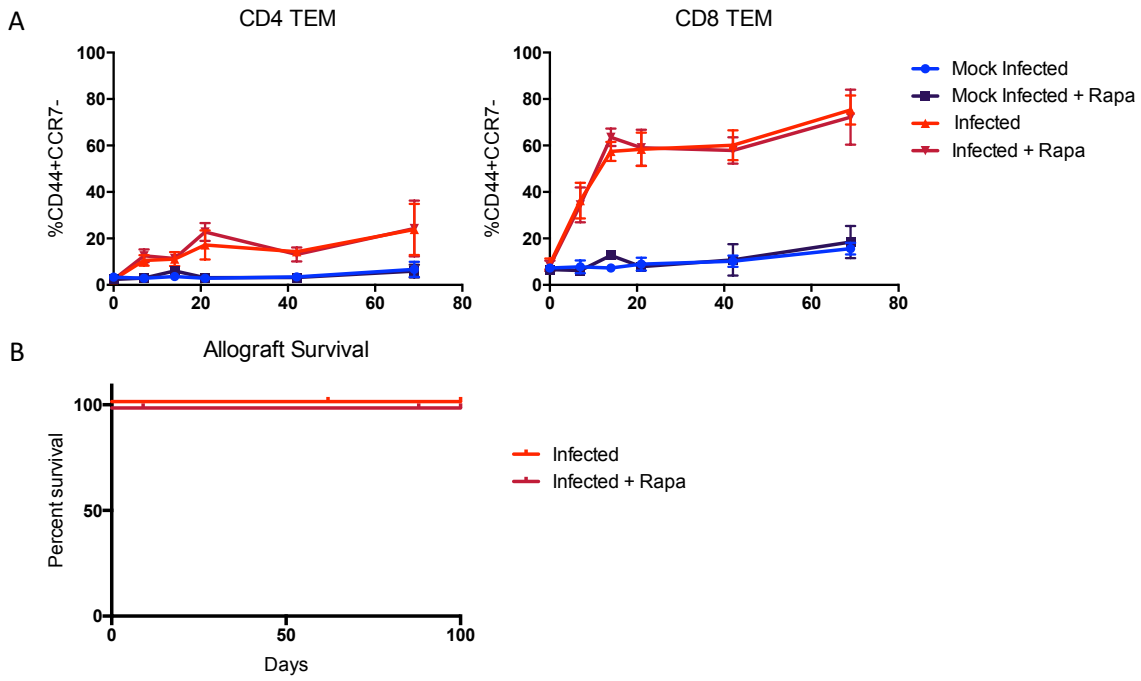

Figure 4.7: Presence of Rapamycin during infection does not impact TEM expansion or graft survival. C57BL/6 mice were simultaneously infected with PyV, mCMV and HV68 in the presence or absence of rapamycin. (A) The kinetic expansion of CD4 and CD8 TEM is almost identical in the presence or absence of rapamycin during primary infection. At least 60 days post infection, mice were given a heterotopic heart transplant from Balb/c donors and beating quality was monitored for survival. (B) No difference in graft survival was observed in mice treated with CTLA4-Ig on days 0, 2, 4, 7, weekly.

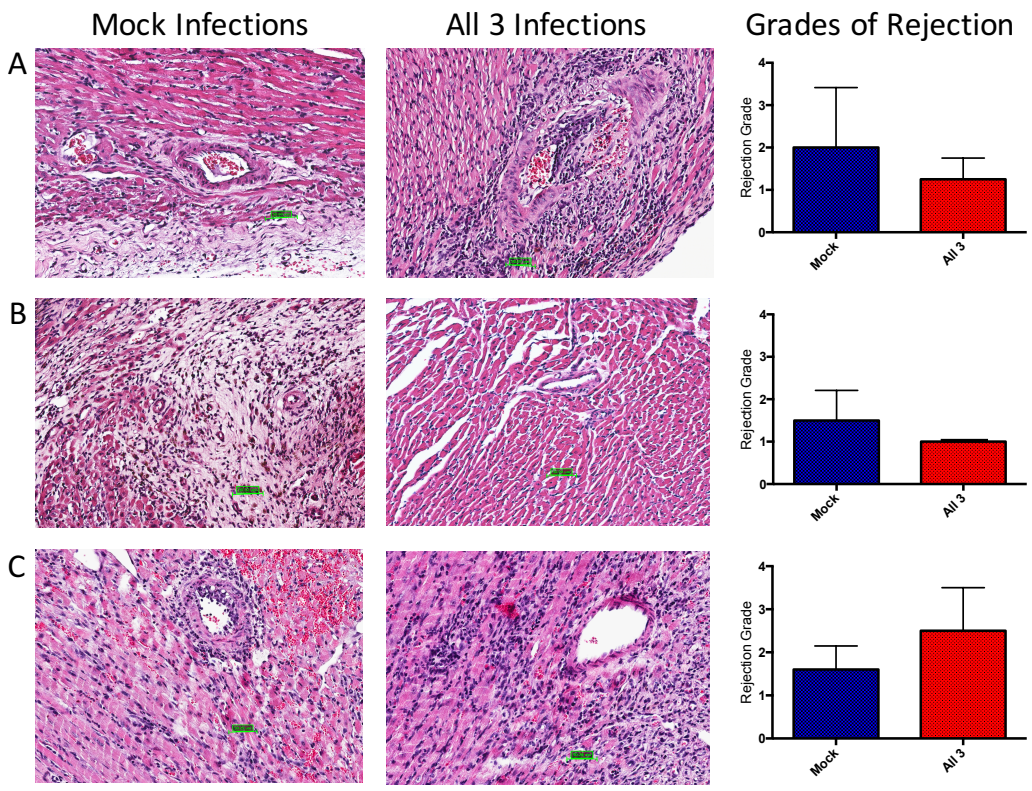

Figure 4.8: No difference in grades of rejection between mock infected mice and mice that received all three infections. Donor Balb/c hearts were excised from C57BL/6 recipients at the time of sacrifice. H&E staining was performed and slides were sent to a cardiac pathologist for blinded analysis. (A) No difference in grades of rejection in mice treated with CTLA4-Ig days 0, 2, 4, 7, weekly at day 80 post-transplant. (B) No difference in grades of rejection in mice treated with CTLA4-Ig days 0, 2, 4, 7, weekly + Rapa 3x per week at day 75 post-transplant. (C) No difference in grades of rejection in mice treated with CTLA4-Ig days 0, 2, 4, 7 at day 14 post-transplant.

Chapter 5. Discussion

The technical success of transplantation has led to challenges with chronic immune suppression

Surgical advances in the past 60 years have made transplants highly successful technically and continue to propel the field forward. Indeed, in addition to life-saving organ transplants, vascularized composite allotransplantation (VCA)- transplantation of a functional unit composed of muscle, bone, nerve and skin- is now a life-improving treatment option for patients suffering from limb loss or severe disfigurement.[145] With a number of agents available to alter and inhibit immune responses, immunosuppression regimens used in organ transplantation have been largely successful and patients are living longer than ever with transplanted organs, such that chronic immune suppression is now the issue.

To date, more than 697,700 people have received transplants in the United States,[146] the vast majority of whom rely on chronic immunosuppression to elude rejection. CNI-based immunosuppression is the standard therapy prescribed post-transplant due to the ability to effectively suppress all T cell responses. Their effective but broad immunosuppressive capabilities not only inhibit rejection, but impede other necessary immune responses, including existing protective immunity. Furthermore, CNIs must remain present in the system for continued T cell inhibition, imposing a life-long burden of medication adherence on transplant recipients. What's more, chronic use of CNIs, though necessary to protect the organ from rejection, also comes with a host of problematic side effects, not limited to susceptibility to opportunistic infections and cancers, risk of viral reactivation and nephrotoxicity. Thus, the development of belatacept as a replacement therapy for CNIs was a major advance in the development of immunosuppression.

Because belatacept inhibits costimulation, naïve T cell responses are inhibited while protective immunity remains intact, thus avoiding many of the non-specific side effects of standard immunosuppression therapy. An added benefit to belatacept's mode of action is the resulting anergy of T cells inhibited by belatacept, such that immunosuppression can potentially be weaned without the risk of rejection. Furthermore, patients treated with belatacept have improved long term graft function and survival compared to patients treated with CNIs.[147] However, the increased rates of early acute rejection in patients initially treated with belatacept have hampered its clinical use and spurred additional studies to investigate what may account for the clinical difference.

Development of assays to screen patients at risk for belatacept resistant rejection

There are many tools available to phenotype patients to elucidate markers of risk for belatacept resistant rejection (BRR), and the development of a diagnostic assay to screen patients prior to transplantation and determine whether they may be good candidates for belatacept-based therapies is paramount to its clinical success. It is important that such an assay utilize samples and techniques that are easily accessible and yield quick and accurate results. For this reason, our finding that CD57+PD1- CD4 T cells in the peripheral blood of transplant recipients, prior to transplantation, are associated with BRR, is truly exciting and propelled us through a series of experiments investigating CD57+ CD4 T cells and their potential mechanisms of action.

Currently, the biology of CD57 remains largely undefined.[77,148] It was first characterized as a general marker of NK cells, though we now know that it's on a subset of functionally distinct, or mature, NK cells.[149,150] It is also expressed on central and peripheral nervous

tissue, associated with the neural crest and related to neural cell movement and development.[151-154] In humans, chronic viral infections like CMV and HIV have been shown to expand CD57+ T cells,[155,156] likely in response to persistent immune stimulation, with decreased proliferative capacity. Arguably, most studies of CD57 have focused on investigating its role as a marker of replicative senescence on CD8 T cells,[75] but the description of CD57 on CD4 T cells has not been detailed other than its association with follicular helper T cells.[157,158] In our studies, we did not find CD57+ CD4 T cells to be significantly correlated with prior viral infection or any patient demographics other than renal failure, though our power to formally assess this was limited. Thus, while CMV infection may lead to an increase in CD57+CD4 T cells, we cannot say that CD57+ T cells arise solely from CMV infection, and CMV infection alone does not correlate with risk of belatacept-resistant rejection.

Notably, CD57 binds P-selectin and L-selectin,[159] both of which mediate interactions between leukocytes and endothelial cells to aid in leukocyte trafficking.[160] We have shown that CD57+ CD4 T cells also express increased levels of other adhesion molecules CD2, LFA1 and VLA4. The association of CD57 with increased adhesion molecule expression, combined with the inverse correlation of CD57 and CD28 expression, highlights the potential to target other molecules associated with leukocyte:endothelial cell interactions and memory acquisition in combination with belatacept to inhibit CoBRR.

Targeting allospecific memory by combining costimulation blockade with other immunosuppressive agents

Cells undergo many changes following antigen encounters in the presence of CD28 costimulation, and the resulting memory cells express specific molecules that offer potential

targets for additional immunosuppression. Specifically, as a cell progresses from a naïve phenotype, it loses CD28 expression and gains the expression of adhesion molecules such as CD2, leukocyte function-associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4). CD2 aids in T-cell activation and adhesion by binding to LFA-3 on antigen-presenting cells and inflamed tissues.[123,161] LFA-1 and VLA-4 bind intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) respectively on activated peripheral vascular endothelium at sites of inflammation, and are involved in cell migration and extravasation required for effector functions.[162-164]

The distinct surface molecule expression and co-stimulatory requirements of memory cells[165,166] have been exploited experimentally in investigations combining blockade of costimulation with suppression of adhesion molecule expression. For example, the LFA-3-Ig fusion protein, alefacept, targets memory T cells in psoriasis by inducing their apoptosis,[167] prompting investigation into its potential use in the setting of transplantation. The results from initial *in vitro* studies using alefacept in combination with costimulation blockade supported this strategy, showing the depletion of $CD8^+$ memory T cells that were otherwise indifferent to belatacept.[71] The combined use of alefacept and belatacept in non-human primate models of kidney transplantation resulted in the depletion of both $CD4^+$ and $CD8^+$ effector memory T cells and improvement in allograft survival compared to costimulation blockade alone.[70] However, continued preclinical studies in kidney, islet and vascularized composite allograft tissue transplantation have—not unexpectedly—demonstrated a loss of protective immunity,[168-170] emphasizing the critical balance of memory-directed therapy required for protective immunity.

Similarly, studies of rodent heterotopic heart transplants showed that blockade of LFA-1 prevented the early infiltration of alloreactive memory T cells into the donor allograft.[171] These results correlate with those from other studies combining costimulation and adhesion molecule blockade to augment allograft survival. For example, the use of efalizumab, an LFA-1 antagonist, in islet transplantation in type 1 diabetics improved long-term allograft survival and rates of single-donor insulin independence in conjunction with costimulation blockade.[172-174] Targeting VLA-4 in conjunction with costimulation blockade has also shown increased survival in rodent transplant models compared to costimulation blockade alone.[175] Thus, inhibition of LFA-1 or VLA-4 in combination with costimulation blockade might have a synergistic effect, particularly in the setting of heterologous memory, whereby inhibition of integrin binding might selectively target cells that are resistant to costimulation blockade.[57,176]

This memory-focused therapeutic strategy, although efficacious, seems to have a narrow therapeutic window that will require additional investigation before a clinical strategy becomes accepted. As an example, efalizumab and alefacept both showed promise in abrogating memory-specific responses, prompting clinical trials and receiving initial commercial success for psoriasis, but both were subsequently withdrawn from the market, citing loss of endogenous viral control as the main cause.[169,177] Their risk:benefit balance in transplantation remains to be defined. Similarly, VLA-4 antagonists such as natalizumab require further preclinical and clinical studies to determine their potential in a clinical transplant setting.

Mechanistic studies have begun to reveal how homeostatic repopulation can be manipulated to favor an allotolerant repertoire.[178] Indeed, depletion therapy can induce donor-specific hyporesponsiveness, although the mechanisms might depend on the adjuvant immunosuppression being used at the time of depletion.[179-181] In general, depletion might present a unique opportunity to shape the immune cell repertoire. Studies of alemtuzumab-mediated depletion in combination with belatacept and sirolimus (which inhibits IL-2 production) have shown that the burst of homeostatic activation is countered by a corresponding burst of regulatory T and B cell generation, and that this phenomenon is associated with exceptionally low rates of rejection.[129] Furthermore, recent data from our lab suggests sirolimus not only has a regulatory effect on lymphocytes, but induces expression of inhibitory markers on endothelial cells as well. This approach might offer an opportunity to specifically target the nutritional requirements of memory T cells in order to shape the immune-cell repertoire and aid in the elimination of allospecific effector memory T cells.

Inherent species differences prove challenging when developing animal models

Animal models have played a key role in drug development and have been instrumental in understanding cell-specific pathways, but it is important to remain cognizant that they are not humans and translating results from bench to bedside and back has unique challenges. For instance, outside of the neural crest, CD57 does not appear to be expressed on lymphocytes in mice, which has limited the utility of mouse models to study CD57+ CD4 T cells in this context. We have developed a rigorous model of clinically relevant viral-induced memory formation with the hope of stimulation the development of a subset of similarly differentiated cells in mice, but thus far have been unsuccessful in replicating the difference in acute rejection rates observed clinically in the setting of costimulation blockade. This is

not to say that viral-induced memory cells do not play a role in transplant rejection, but rather latent and persistent viral infection may not have the same effect on allograft rejection as an acute immune perturbation.

For example, in experimental models of transplantation where immune tolerance has been achieved in the setting of costimulation blockade by CTLA4-Ig, the introduction of donor-specific memory cells has overcome this tolerance.[41,182,183] Furthermore, Th17 memory cells, a proinflammatory subset of $CD4^+$ T cells, express high levels of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), a co-inhibitory molecule that binds B7 molecules. Thus, B7 inhibition by belatacept might augment the potency of Th17 cells by preventing ligation of their co-inhibitory receptors; indeed, an increase in the frequency of Th17 cells was associated with acute rejection in rodent models.[184] These data warrant further investigation of Th17 cells in transplant patients. Specifically, whether they might themselves express CD57, or are a distinct subset also capable of mediating CoBRR.

Individualized immunosuppression should be the goal

Each patient presents for transplantation with a unique immune cell repertoire based on prior antigen experience, yet we continue to prescribe the same post-transplant standard of care. It is thus not surprising that we observe a spectrum of outcomes following transplantation. We now have the remarkable capacity to take a sample of peripheral blood from a patient, phenotype their immune cell profile, and stratify patients better suited for CNI- or CoB-based immunosuppression. The future lies in exploiting the diverse immunosuppressive agents available, and the continued development of novel agents, to inhibit specific cell subsets known to mediate rejection. We must move away from treating

each post-transplant patient with the same immunosuppressive regimen, and instead moved toward an era of individualized treatment.

Continued progress will always inspire new questions and superior methods to improve transplant outcomes, with the ultimate goal of immunosuppression-free transplant tolerance. Dedicated studies of human immune cell function will lead to further development of novel agents to be used in conjunction with existing regimens. Implementation of individualized immunosuppression therapy based on the immune cell profile of transplant patients prior to transplantation will not only improve transplant outcomes, but also lend further insight to make transplant tolerance a reality.

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