ABSTRACT

Transfusion of platelets is a potentially life-saving therapy to maintain hemostasis in patients with severe thrombocytopenia of various etiologies. One of the main impediments to ongoing platelet transfusions is humoral immunization to platelet alloantigens, predominantly HLA antigens (MHC class I). Once alloantibodies have been formed, HLA incompatible platelets can be rapidly cleared by the recipient, resulting in little or no increase in platelet counts and thus essentially no therapeutic benefit. If a patient generates alloantibodies against multiple HLA epitopes, then finding compatible platelets can become difficult, and in many cases impossible. With alloimmunization to multiple HLA types, platelets can cease to be a viable therapy for thrombocytopenia. For patients who only need transient support with platelet transfusion, alloimmunization is not typically a serious problem; however, in patients who require ongoing therapy, anti-HLA antibodies can eliminate a life-saving therapy for which there is typically no viable substitute. In addition to rendering platelet transfusions ineffective, the induction of anti-HLA antibodies can also complicate subsequent transplantation. As platelet transfusions are given to some patient populations as support while they are awaiting organ transplantation, induction of anti-HLA antibodies has an additional negative impact on this population, both from the standpoint of complicating the transplant and also potentially moving the patient down the waiting list for donor organs. The implementation of filter leukoreduction has decreased rates of alloimmunization to platelet transfusion, but residual rates remain at approximately 20%, with 1 in every 5 transfusion recipients becoming alloimmunized. Thus, additional interventions are required to reduce alloimmunization rates. Co-stimulatory blockade is a strategy to prevent alloimmunization, which has now come to fruition with the FDA approval of CTLA4-Ig for prevention of solid organ transplantation. This grant application is a pre-clinical effort to begin translation of CTLA4-Ig for use in preventing alloimmunization to transfused platelets. This approach would not be used in the general population, but focused on select patient populations who are particularly vulnerable to HLA alloimmunization (patients requiring ongoing platelet transfusion and/or awaiting transplantation). We have developed a murine model of alloimmunization to transfusion of filter leukoreduced platelets. In this system, CTLA4-Ig abrogates alloimmunization to platelet transfusion. The model system has been engineered to have sufficient tools to allow detailed mechanistic elucidation of CTLA4-Ig function and also side effects on systemic immunity. The proposed studies are a hypothesis driven effort designed to both generate basic understanding of the immunology of platelet alloimmunization and CTLA4-Ig mechanisms of action, but with a distinct focus on pre-clinical issues necessary for subsequent human trials of CTLA4-Ig therapy to prevent alloimmunization to transfused platelets.

PROJECT NARRATIVE

Platelets are necessary to help prevent bleeding and transfusion of platelets is a life-saving therapy for a number of diseases that result in low platelet counts. In some patients, the immune system recognizes platelets from other people as foreign and prevents platelet transfusion from working. This grant is focused on a novel therapeutic approach to prevent the immune system from interfering with platelet transfusion and has the potential to benefit any patient with a disease requiring platelet transfusion.

SPECIFIC AIMS: A wide variety of pathologies lead to thrombocytopenia, and in many instances, platelet counts can drop to dangerously low levels. In such cases, platelet transfusion therapy is an essential treatment, both to stop ongoing bleeding and also as a prophylaxis for new hemorrhagic sequelae (1). Because platelets express MHC I, they carry Human Leukocyte Antigens (HLA), which are polymorphisms in MHC proteins. Accordingly, transfusion of platelets can lead to humoral alloimmunization against HLA. Anti-HLA Alloantibodies can lead to serious problems in ongoing treatment with platelet transfusions, as platelets carrying HLA antigens against which a recipient is immunized can be rapidly cleared, essentially eliminating any efficacy of transfusion (2, 3). In cases where patients become immunized to multiple common HLA antigens, finding compatible and/or HLA matched units can become challenging, and at times impossible, thus depriving severely thrombocytopenic patients of a life-saving therapy (2, 3). For patients being supported with transfusion therapy while awaiting a transplant, alloimmunization to HLA has an additional and very serious risk, as having anti-HLA antibodies can knock someone down the list for receiving a donor organ, in some cases rendering a patient practically ineligible for a life-saving transplant (4). Thus, in aggregate, alloimmunization to HLA has serious sequelae for patients requiring ongoing platelet transfusions.

A number of approaches have been developed to decrease alloimmunization to platelets, each of which focuses on modifying the platelet unit, including leukoreduction or UV irradiation (5); currently, the only standard intervention is filter leukoreduction. Whereas leukoreduction certainly decreases alloimmunization, the residual rates are approximately 20% (5). It is important to note that not all patients who are alloimmunized become refractory; however, given the large number of patients who receive platelet transfusions each year, this equates to a substantial quantity. Thus, alloimmunization to platelet transfusions remains a significant problem, with limited technologies to intercede in the process.

Central Hypothesis: Administration of CTLA4-Ig to transfusion recipients will significantly decrease alloimmunization to MHC alloantigens in units of transfused platelets.

Costimulatory blockade in general, and CTLA4-Ig in particular, have been developed over the last decade with the goal of preventing solid organ transplantation without causing the severe immunosuppression seen with traditional regimens (6, 7). CTLA4-Ig has now been FDA approved for use in humans, and has been shown to be safe when used alone, and not in combination with other immunosuppressant (8) (see section C1 below). However, the potential utility of CTLA4-Ig to decrease alloimmunization to transfused platelet products has not been tested. In this context, we propose the following specific aims to test the central hypothesis (and a series of sub-hypotheses) focused on translation of CTLA4-Ig into use for the subset of human platelet transfusion recipients for whom alloimmunization to HLA would represent a serious medical problem (i.e. patients requiring extended therapy or awaiting transplantation (bone marrow or solid organ)).

Specific aim 1: Cellular Mechanisms of CTLA4-Ig Prevention of Alloimmunization to Transfused Platelets. Our preliminary data indicate that CTLA4-Ig abrogates alloimmunization to transfused PLTs in a murine model (see sections C2.1 & C2.2). This observation is equally consistent with either a simple temporary blockade of the immune response or sustained immunological tolerance. In this aim, we will make use of sophisticated murine tools and reagents to elucidate the cellular mechanisms by which CTLA4-Ig prevents alloimmunization to transfused platelet products.

Specific aim 2: Effects of Heterologous Immunity and Antecedent Pathogen Exposure on CTLA4-Ig Efficacy. In the context of solid organ transplantation, the efficacy of CTLA4-Ig has been shown to be affected by the repertoire of cross-reactive memory T cells as a function of previous virus infection (called heterologous immunity) (9, 10). In this aim, the effect of heterologous immunity on CTLA4-Ig efficacy for preventing alloimmunization to transfused platelet units will be studied.

Specific aim 3: Potential Infectious Sequelae of CTLA4-Ig Use in the Context of Transfusion Medicine. CTLA4-Ig has proven (in animals and humans) to be a less dangerous immunosuppressant that traditional pharmacological anti-rejection drugs (8), likely because established immunological memory is little effected by CTLA4-Ig (see section C1). However, transfusion represents a unique landscape, as patients may be exposed to new pathogens by the transfusion itself. Although the blood supply is screened for the most dangerous common transfusion transmitted infections, transfusions may transmit all manner of viral infections that are typically not pathological, but which may represent a dangerous infection in a patient immunosuppressed with CTLA4-Ig. In this aim, platelet units tainted with cytomegalovirus (CMV), both from acutely infected or latently infected donors, will be transfused in the context of CTLA4-Ig therapy. In this way, the potential danger of infectious disease during CTLA4-Ig administration for platelet transfusion will be investigated.

RESEARCH STRATEGY:

A. Significance: Platelet transfusion support is widespread predominantly in the treatment of disorders of platelet production of both natural (malignancy and bone marrow failure syndromes) and also iatrogenic causes (bone marrow transplantation, chemotherapy, and other drugs causing thrombocytopenia) (1). Platelet transfusions are also essential to maintaining adequate hemostasis in cases of platelet dysfunction despite normal platelet numbers (e.g. after extracorporeal membrane oxygenation, in uremic platelet syndrome after dialysis, and a number of rare disorders of platelet function such as Glanzsman's Thrombasthenia and Bernard-Soulier Syndrome). Alloimmunization to HLA antigens on transfused platelets can render subsequent platelet transfusions ineffective, thus removing the ability to maintain hemostasis (2, 3). Resulting bleeding diatheses can result in substantial morbidity and/or mortality. In addition, alloantibodies to HLA can complicate organ transplantation and may push a candidate down the waiting list (4). Finally, in addition to expressing HLA, platelet specific glycoproteins carry a number of natural polymorphisms that generate targets for antibody binding (Human Platelet Antigens 1-15 (HPA 1-15)) (11). Immunization to HPA variants is less of a problem than anti-HLA, in the context of providing compatible platelets for transfusion; however, anti-HPA antibodies can cross the placenta and destroy fetal platelets, leading to neonatal alloimmune thrombocytopenia (12).

Despite progress due to leukoreduction, alloimmunization rates to platelet transfusion remain significant (19% in patients with acute myelogenous leukemia [exact frequency unknown in other patient populations]) (5). This application proposes to study a novel approach to preventing alloimmunization to transfused platelets. We have now demonstrated in a pre-clinical murine model that CTLA4-Ig completely ablates alloimmunization to MHC I alloantigens on transfused platelet units (see preliminary data, section C2.1). CTLA4-Ig would not find an application for patients receiving limited numbers of platelet transfusions over shorter periods of time. However, for patients requiring longer-term platelet transfusion, and for whom HLA alloimmunization may render platelet transfusion ineffective or complicate subsequent transplantation, successful translation of CTLA4-Ig to prevent HLA alloimmunization would be of very high significance.

B. Innovation: This application combines several sophisticated technologies and model systems to allow an in depth mechanistic and translational analysis of the use of CTLA4-Ig for alloimmunization to platelet transfusion. None of the tools or technologies is completely novel from the standpoint that they have never previously been described. However, the adaptation of these tools to platelet transfusion immunology is unique and the application of CTLA4-Ig to the problem of alloimmunization to platelet transfusion is a novel approach. A brief list of the innovations in this grant is provided here, with expanded details provided below.
1) The vast majority of platelet units are filter leukoreduced in the United States. We have gone to great lengths to precisely model this process in the generation of units of murine platelets, including use of the same filters as utilized for human platelets (13). The resulting platelet units function and circulate normally after transfusion (13). **2)** The use of TCR75 transgenic mice (14) allows the visualization of early events in activation and expansion of CD4+ T cells specific for allo-MHCI antigens on transfused platelets (15)(see C2.2); to the best of our knowledge, this represents the first time that TCR transgenic technologies have been applied to the study of alloimmunization to transfused platelets. **3)** This is the first attempt to apply co-stimulatory blockade in general, and CTLA4-Ig in particular, to platelet alloimmunization, and **4)** To the best of our knowledge, the proposed studies are the first integrated animal model that analyzes alloimmunization to platelets in the context of antecedent viral infections. These studies have a practical utility regarding CTLA4-Ig function, but are also relevant to understanding alloimmunization to platelet transfusion in general.

C. Approach:
C1. Background on Co-stimulatory blockade and CTLA4-Ig: It is now appreciated that for a naïve T cell to activate and differentiate into an effector T cell, at least two separate and distinct signals are required (16). Signal 1 is recognition of cognate antigen by the T cell receptor (TCR). For the TCR, antigen is in the form of peptide presented by MHC on the surface of an antigen presenting cell (APC). Signal 2 is sufficient expression of a "co-stimulatory molecule" on the APC and ligation of its receptor on the T cell. In the absence of signal 1, there is no effect on the T cell (with or without signal 2), as the TCR ligation is the gatekeeper of T cell activation. However, signal 1 in the absence of signal 2 results in an abortive activation of the T cell; activation begins, but the cell is rendered apoptotic or anergic (i.e. the cell persists but fails to respond to subsequent stimuli, even with both signal 1 and 2 that would have activated a naïve T cell). In some settings, this "anergic" cell differentiates into a Treg that actively suppresses immune responses. This basic understanding of T cell activation led to the concept that if one could pharmacologically deprive T cells of

signal 2 at the same time they encounter signal 1, then one could convert a scenario of T cell immunization into one of anergy and/or tolerance. There have now been a whole series of different co-stimulatory molecules described; however, the most predominant and major pathway is expression of B7.1 or B7.2 on an APC and ligation of CD28 on the responding T cells (17, 18). In addition to CD28, T cells also express an additional B7.1/B7.2 ligand called CTLA4, which serves as a negative feedback for CD28 activation (19). Costimulatory blockade for the B7-CD28 pathway is achieved by fusing the extracellular domain of CTLA4 to the constant region of IgG1, thus generating a dimeric and very stable soluble CTLA4 (CTLA4-Ig). As CTLA4 has a higher affinity for B7 than does CD28, it effectively competes for B7 binding, and when given in sufficient quantity, deprives T cells of signal 2 (see figure 1 for diagram of process).

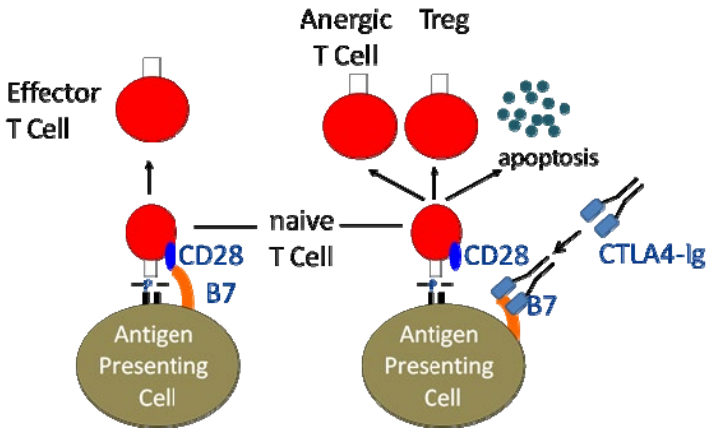

Figure 1. Theorteical Model of CTLA4-Ig function.

Unlike naïve T cells, which have never encountered the antigen they recognize, memory T cells are formed after a successful immune response. Memory T cells do not have the same requirements for signal 2 that naïve T cells have, which is part of the reason that memory responses are so much more sensitive and rapid than a primary response (20). The functional effect of this is that CTLA4-Ig treatment does not inhibit memory responses, but blocks only responses to antigens not yet encountered (21, 22). Thus, unlike many other pharmacological immunosuppressants (e.g. cyclosporine), CTLA4-Ig has less of an effect upon existing immune status from vaccines and previous infections. Therefore, existing immunity to all manner of opportunistic, commensal, and routine pathogens is less affected than new responses to previously unencountered antigens.

CTLA4-Ig has now been used in a large number of humans and is FDA approved (named Orencia) for long-term treatment of rheumatoid arthritis. A higher affinity form has also been recently FDA approved for solid organ transplantation (called Nulojix). The version of CTLA4-Ig used in the preliminary data and proposed experiments is Orencia, which has fewer side effects and less risk of infection [referred to as CTLA4-Ig in this application]. In the tested human patient populations, treatment with CTLA4-Ig has been shown to be a safe form of immunosuppression when used as a monotherapy, with no increase in significant infectious complications (8). This is not to imply that CTLA4-Ig treatment is without concern, and increased frequency of upper respiratory tract infections and nasopharyngitis have been observed (23). Such infections typically are transient and not serious, but if they do become problematic, therapy can be discontinued. When combined with other immunosuppressants, more serious infections were infrequently observed, including herpes zoster (0.7%) and bronchitis (0.7%) (23). However these patients were concomitantly on additional and powerful immunosuppresants (i.e. NSAIDS, corticosteroids, TNF blocking agents, azathioprine, chloroquine, gold, hydoxychloroquine, leflunomide, sulfasalazine, and anakinra) (23). In contrast, when given as a monotherapy (as proposed in the current approach), CTLA4-Ig induced complications from infection are infrequent and mild. Nevertheless, because new exposure to viral pathogens can be encountered during transfusion, and thus co-incident with CTLA4-Ig administration, the issue of newly encountered viruses is given special consideration in the current application. This issue is specifically investigated experimentally in aim 3.

Specific Issues Surrounding Application of CTLA4-Ig to Alloimmunization to Platelets: In the context of the current proposal, it is essential to note that single agent co-stimulatory blockade (i.e. use of CTLA4-Ig alone) has had limited success in the field of sold organ transplant. Thus, it is a fair question as to why it is our prediction that such an approach will have efficacy in preventing alloimmunization to platelet transfusion. Predictably, in the context of solid organ transplantation, use of CTLA4-Ig alone has had much greater efficacy as the strength of the immunogen has decreased. For example, CTLA4-Ig alone has little benefit to transplanted skin (amongst the most immunogenic transplants (24)), but shows substantial benefit in the context of cardiac transplantation (less immunogenic than skin) (25, 26). Platelet transfusion is clearly less immunogenic than any solid organ transplant. It is a fair statement that essentially 100% of solid organ transplants will reject without some form of pharmacological immunosuppression of the recipient. In contrast to

this, leukoreduced platelet transfusions only induce alloantibodies in a minority of recipients, despite the lack of any external immunosuppression (5). The reasons platelets have decreased immunogenicity compared to solid organs is unclear, but several potential factors include 1) potentially less activation of innate immunity due to no inflammation of anastomosis or necrotic tissue, 2) restriction of platelets to circulation and exclusion from lymphatics, thus no antigen to dendritic cells and draining lymph nodes, and 3) although platelet units do contain some cytokines, if collected and transfused properly, no microbial products will be present and thus less activation of pattern recognition receptors will occur. Finally, whereas CTLA4-Ig must overcome both CD8+ T cell immunity (and in some cases also antibodies) to prevent rejection of solid organs, only antibody responses must be overcome to prevent humoral alloimmunization to platelets. Of additional relevance is that from a practical standpoint, efficacy of CTLA4-Ig need not be 100% to be useful in the setting of platelet transfusion. Clearly, rejection of a transplanted solid organ is of extreme significance and must be avoided essentially at all costs; however, while alloimmunization to transfused platelets is clearly a problem, it is not a clinical disaster that leads to rapid death. Thus, if CTLA4-Ig had a partial effect (e.g. 50% decrease) it would still be of considerable utility. As above, it is not envisioned that CTLA4-Ig would be applied to all patients that receive CTLA4-Ig, but would be focused on the especially vulnerable patients who required long-term platelet transfusion therapy and those being supported while awaiting organ transplantation.

C2. Experimental Model and Preliminary Studies:

C2.1. CTLA4-Ig Abrogates Alloimmunization to Transfused Filter Leukoreduced Platelets in Mice. To test the effects of CTLA4-Ig on humoral alloimmunization to transfused leukoreduced platelets (LR-PLT), CTLA4-Ig was administered to B6 mice (i.p. injection) several hours prior to a transfusion of leukoreduced platelets (LR-PLT) from BALB/c donors. LR-PLT were prepared as previously described, under conditions that decrease leukocytes to the same degree as human platelet units and result in platelets with normal function and circulation post-transfusion (13). The H-2 complex in mice is the equivalent of the HLA in humans and contains 3 MHCI genes (K, D, and L). The superscript after H-2 indicates genetically diverse haplotypes. As B6 mice are H-2^b and BALB/c mice are H-2^d, humoral alloimmunization to MHC antigens will consist of an antibody response to the three MHCI molecules contained within H-2^d (K^d, D^d, and L^d). Anti-H-2^d antibodies are measured by indirect immunofluorescence, using BALB/c splenocytes as targets mixed with serum from test animals. The targets are then incubated with a fluorescently tagged anti-Ig antibody, and analyzed by flow cytometry. The mean fluorescence intensity (MFI) is determined on BALB/c targets. For all samples, background MFIs from B6 targets are subtracted from BALB/c MFIs (adjusted MFI). An adjusted MFI that is greater than 2 standard deviations above signal from naïve mice is considered a positive alloimmunization.

Serum was obtained from the mice 21 days post-transfusion and tested for antibodies. Additional LR-PLT transfusions were given on days 21, 28, 35, and 42 post-transfusion and serum was collected at the indicated time-points.

In control mice that did not receive CTLA4-Ig, alloimmunization first became obvious after the second transfusion (day 28) and increased with additional transfusions. By day 49, 88% of mice had strong anti-H-2^d alloantibodies (7/8 mice). In contrast there were no detectable anti-H-2^d alloantibodies in mice that received the single dose of CTLA4-Ig at time point zero. The observed alloantibodies were not a non-specific effect of

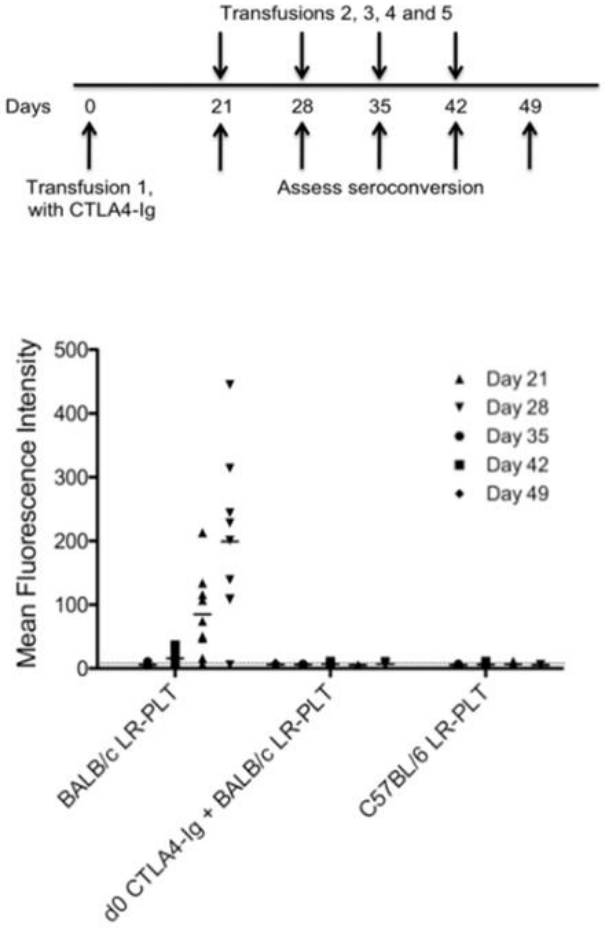

Figure 2. CTLA4-Ig prevents humoral alloimmunization to transfused LR-PLT.

platelet transfusion, as no alloantibodies were detected in mice that received B6 LR-PLT. Together, these data indicate that CTLA4-Ig abrogates humoral alloimmunization to LR-PLT in mice and that a single dose provides sustained protection.

C2.2. CTLA4-Ig Prevents Activation and Expansion of CD4+ T Cells Specific for MHCI Antigen from Donor LR-PLT Presented by Recipient MHCII. The strategy of costimulatory blockade focuses on depriving T cells of the necessary "signal 2" to augment TCR ligation. This strategy predicts that CTLA4-Ig will prevent activation and proliferation of CD4+ T cells specific for immunogens encountered when the CTLA4-Ig is present. The early stages of an immune response to a previously unencountered antigen (naïve response) are notoriously difficult to detect and analyze, because the precursor frequency of T cells specific for a given antigen is extremely low (several hundred T cells in the whole animal). To circumvent this obstacle, immunologists have made extensive use of mice that are transgenic for a T cell receptor of a given specificity, such that every T cell in the entire animal is of a known specificity. Experimenting on the TCR-transgenic animal itself is not useful, but the animal serves as a source of naïve T cells of known specificity. Small numbers of TCR transgenic T cells can then be adoptively transferred into a wild-type animal, creating an easily observable population of naïve antigen specific T cells that can be analyzed during the early stages of an immune response.

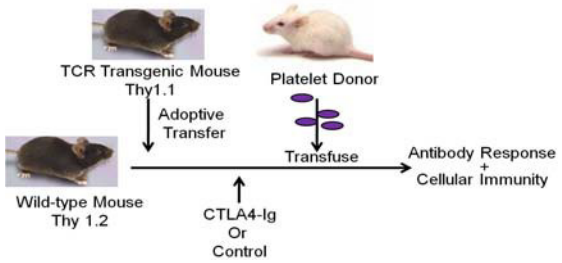

Figure 3. Experimental design for use of TCR transgenic T cells to study CTLA4-Ig effects on immune response to LR-PLTs

To adapt this approach to the study of humoral alloimmunization and the study of CTLA4-Ig, we have made use of the TCR75 mouse. The TCR75 mouse recognizes a peptide from an MHCI alloantigen on BALB/c platelets (K^d peptide 54-68) when processed and presented by recipient MHCII (I-A^b from B6 mice) (14). We have bred the TCR75 mouse so as to carry a natural variant of the Thy1 T cell marker as a congene. TCR75 mice are Thy1.1 whereas B6 mice are Thy1.2. As we have antibodies specific to Thy1.1 and Thy1.2, this allows visualization of the donor TCR75 T cells in the recipient mice (Figure 4A, left panel). This results in an easily identifiable population with a gating strategy that detects essentially no background noise in animals that don't receive adoptive transfer (see figure 4A, right panel).

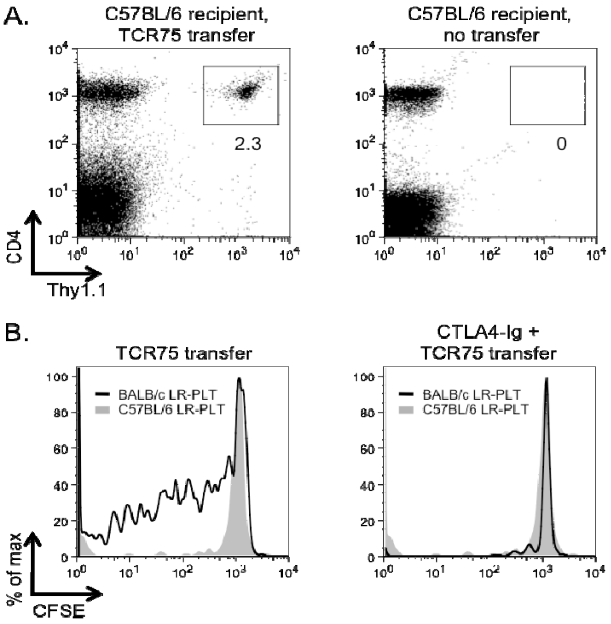

Figure 4 CTLA4-Ig blocks CD4+ T cell division in response to antigen on transfused LR-PLTs. Visualization of TCR75 T cells after adoptive transfer into B6 recipients **(A)**. Analysis of antigen specific CD4+ T cell proliferation (TCR75) in response to transfusion of LR-PLT in the presence or absence of CTLA4-Ig **(B).**

To assess the effects of CTLA4-Ig on the CD4+ T cell response to foreign MHC on a LR-PLT transfusion, B6 mice were adoptively transferred with small numbers of TCR75 T cells, which had been labeled with CFSE. CFSE is a fluorescent dye that will label cells in a fairly uniformed peak. If the cells divide, then the CFSE is equally distributed to daughter cells, resulting in a decrease of fluorescence by ½. Thus, division can be assessed in a gated population by observing progressive decrease in CFSE signal. Gating on adoptively transferred TCR75 cells (with anti-CD4 and anti-Thy1.1), and assessing CFSE dilution demonstrated that TCR75 CD4+ T cells undergo vigorous proliferation upon exposure to LR-PLT from BALB/c donors that express the K^d antigen (Figure 4B, left panel). This proliferation was antigen-specific and not a non-specific effect or LR-PLT transfusion, as no division was observed in response to transfusion of syngeneic (B6) LR-PLTs. These data demonstrate that the TCR75 system is a viable approach to visualize early events in activation of CD4+ T cells specific for antigen on transfused LR-PLT. Injection of CTLA4-Ig into these mice prior to LR-PLT transfusion abrogated proliferation of the TCR75 CD4+ T cells (Figure 4B, right panel), indicating that the mechanism of CTLA4-Ig function is consistent with its predicted effect of preventing T cell

activation by blocking co-stimulatory signals. Additional animals that received TCR75 CD4+ T cells were allowed to progress to 2 weeks post-transfusion to assess the effects upon anti-H-2d antibody induction in the presence of TCR75 T cells. Much like unmanipulated B6 mice, antibodies to H-2^d were detected in recipients that received BALB/c LR-PLTs and injection of CTLA4-Ig abrogated this response (data not shown). These data demonstrate that the addition of the TCR75 T cells, which does increase the precursor frequency of CD4+ T cells specific for Kd, does not alter the underlying biological outcomes observed in unmanipulated B6 mice. Together, these data not only demonstrate the TCR75 system for analyzing antigen-specific CD4+ T cells responses to MHCI alloantigen on transfused LR-PLTs, but elucidate mechanistic insight to CTLA4-Ig function in the context of LR-PLT transfusion, in particular, the inhibition of CD4+ T cell activation/proliferation.

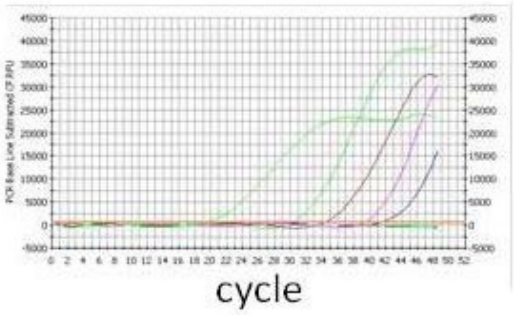

Figure 5 Quantitative real-time PCR for MCMV viral DNA.

C2.3 Monitoring Viral Infection with murine cytomegalovirus (MCMV): In order to quantify the viral loads of MCMV during infection, we developed a quantitative real-time PCR assay for detection of MCMV DNA. The quantity of viral CMV DNA was determined based upon the cycle number at which signal was first detected. The positive control (light green) was first detected at 20 cycles (Figure 5), where as the negative control (orange) never amplified despite 48 rounds. To establish the quantitative nature of the assay, a titration of specimens taken from an infected mouse was carried out. Specimens at dilutions of undiluted (green), 1:10 (brown) ,1:100 (pink),or 1:1000 (blue) gave signal after 31, 34, 39, and 42 cycles, respectively. This assay is highly reproducible, as the same specimen amplified in 4 separate reactions, resulted in detection after 31 cycles in all 4 reactions (data not shown). Together, these data demonstrate that we have developed a real-time PCR assay for MCMV viral load, which is both quantitative and reproducible.

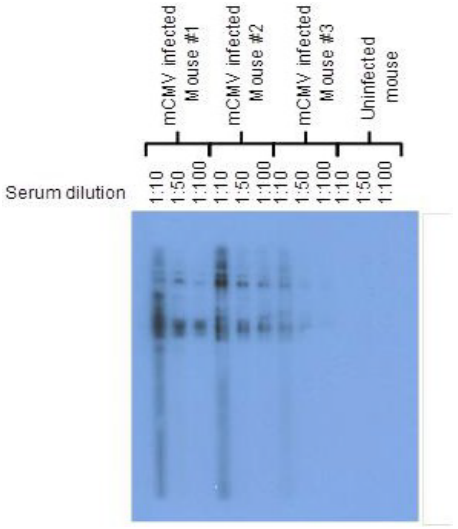

Figure 6 Development of Western Blot assay to measure anti-CMV serology in infected mice.

C2.4 Monitoring immune responses to MCMV:

C2.4.1 humoral immunity: To develop a specific assay to measure anti-MCMV serology, we utilized a Western blot in which protein extracts from MCMV infected cells were resolved by SDS-Page and transferred to nitrocellulose membranes. To allow the screening of multiple serum samples on the same blot, SDS-page gels with a single well extending across the whole gel were loaded with extracts of MCMV infected cells. After electrophoresis, the resolved proteins were transferred to nitrocellulose and the membrane was then mounted in a Mini-Protean II multiscreen apparatus (BioRad), allowing analysis of up to 40 samples at a time. Titration of serum demonstrates the positive titers of anti-MCMV antibodies in 3 out of 3 infected mice, whereas no bands are detected in uninfected mice (Figure 6).

C2.4.2 Cellular immunity: CD8+ MCMV specific T cells are required for the elimination of MCMV infection from most tissues. To monitor the expansion of MCMV specific CD8+ T cells, we utilized intracellular cytokine staining following stimulation with an immunodominant MCMV peptide, and tetramer reagents constructed from the same viral peptide. Splenocytes from infected mice were stained with anti-CD8 and the HGIR tetramer, which recognizes an immunodominant peptide in MHC I. A distinct CD8+ tetramer+ population was visible in

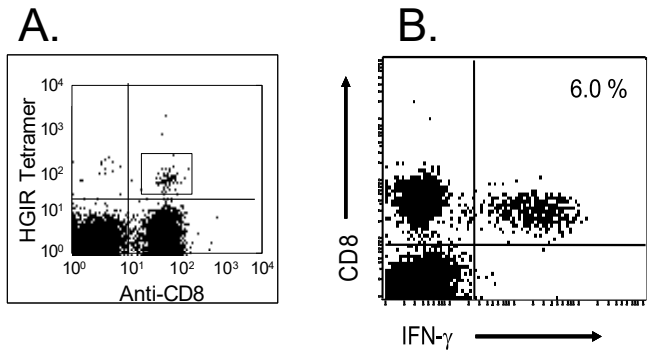

Figure 7 Tetramer staining to determine generation of CD8+ T cells specific for MCMV in infected mice **(A)**. Intracellular cytokine staining for IFN-γ of CD8+ T cells from MCMV infected mice stimulated with MCMV antigen **(B)**.

mice infected with MCMV (figure 7A). No tetramer positive population was visible in uninfected mice (data not shown). To further characterize the CD8+ T cell anti-MCMV response, splenocytes from infected mice were restimulated *in vitro* with splenocytes pulsed with MCMV peptide, followed by intracellular cytokine staining for IFN-γ. A distinct population of CD8+ T cells expresses IFN-γ upon restimulation with MCMV peptide, indicating differentiation into effector CD8+ T cells (Figure 7B). Together, the above data indicate that we have the systems in place to monitor progression of viral infection, antibody response of the recipient, and cellular immunity to MCMV infection.

C3. Long-term Goal of Project and Team of Investigators Assembled: The primary focus of this application is laying the preclinical groundwork to translate CTLA4-Ig into use in humans for the prevention of alloimmunization to transfused platelets. Sufficient analytic tools have been included and experiments have been focused on discrete hypotheses (see aims below), such that basic understanding will be generated regarding the mechanism by which CTLA4-Ig inhibits alloimmunization to transfused platelets (e.g. immune inhibition vs. immune deviation vs. tolerance (deletional or active Treg mediated suppression)). Basic understanding will also be generated regarding the effects of heterologous immunity (due to antecedent infections) on CTLA4-Ig effects. Finally, safety in the context of newly encountered viral infections will be analyzed. Although each of these studies will generate basic understanding, which is of great value in its own right, the experiments have been focused on practical knowledge that is required to proceed into non-human primate studies and/or human trials. The P.I. on this application (Dr. Zimring) has extensive expertise in murine modeling of transfusion immunology. To add the necessary additional expertise needed for a focus on translation into humans, three experts in co-stimulatory blockade in rodents, non-human primates, and humans have been recruited. Drs. [redacted] are world renowned leaders in the field of costimulatory blockade in the context of solid organ transplantation. Work in the laboratories of these investigators was the first to define issues of heterologous immunity, and have involved extensive use of co-stimulatory blockade in non-human primates and in human trials. Indeed, Dr. [redacted] has been a long-term leader in co-stimulatory blockade, whose efforts have contributed substantially to the successful translation of CTLA4-Ig into FDA approved use in humans. Dr. [redacted] is a world leader in translational research on alloimmunization to platelets and has successfully carried out large human clinical trials, with substantial impact on the practice of transfusion medicine. Finally, Dr. [redacted] is an expert in the study of transfusion-transmitted CMV, both in murine systems and human trials. Thus, in aggregate, the expertise in basic murine models of transfusion immunology (Dr. Zimring), expertise in transfusion-transmitted CMV (Dr. [redacted]), translation of basic findings into human trials in platelet transfusion (Dr. [redacted]), and study of costimulatory blockade from mice to non-human primates to humans (Drs. [redacted]) forms a fundamental research team with the ability to interpret and direct the proposed studies towards future translational studies.

C4. Experimental Design:

C4.1. Specific aim 1: Cellular Mechanisms of CTLA4-Ig Prevention of Alloimmunization to Transfused Platelets.

C4.1.1. Rationale: Costimulatory blockade was conceived as a strategy to prevent the activation of T cells by depriving them of signal 2. However, over the past several years, detailed mechanistic studies have demonstrated that the effects of CTLA4-Ig are much more complicated than originally conceived, and may include: **1)** simple blocking of T cell activation, **2)** induction of anergy, and/or **3)** induction of regulatory T cells (T_{reg}); different outcomes are observed in different transplant settings (27). Exposure to alloantigen on transfused platelets represents a somewhat distinct immunological stimulus compared to solid organ transplant, and the mechanisms of CTLA4-Ig in this context have not been studied. In this aim, the cellular mechanism(s) by which CTLA4-Ig affects alloimmunization to transfused platelets will be dissected.

C4.1.2. Hypothesis: Prevention of humoral alloimmunization to platelet transfusion by CTLA4-Ig involves more than simple prevention of T cell activation.

C4.1.3. Experimental Design: We will make use of two distinct model systems to dissect the cellular immunology by which CTLA4-Ig prevents alloimmunization LR-PLT. As detailed in the preliminary data (see section C2.2), use of the TCR75 mouse allows analysis of CD4+ T cells specific for alloantigen during the very early points of alloimmunization, where the critical effects of co-stimulatory blockade take place. In addition to the central role that CD4+ T cells play in providing help to B cells, it has also been shown that CD8+ T cells play a special regulatory role in the process of humoral alloimmunization to PLTs (28). Thus, we have adapted the system to also allow analysis of CD8+ T cells that are specific to alloantigens on PLTs. In this case, we

make use of mOVA mice, which express a membrane bound form of Ovalbumin (OVA) (29). This mouse expresses mOVA on leukocytes and PLTs, but not on RBCs (data not shown); thus, the pattern of expression is the same as is seen with MHC alloantigens. PLTs from the mOVA mouse will be harvested, leukoreduced, and transfused into wild-type recipients (data not shown to due space limitations). In this case, we make use of the OT-I mouse, which analogous to the TCR75 mouse, expresses a transgenic TCR that recognizes the OVA SIINFEKL peptide when presented by recipient MHCI (Kb). Much like TCR75 CD4+ T cells in response to BALB/c platelets, OT-I CD8+ T cells proliferate robustly in response to LR-PLTs from mOVA mice but not wild-type control platelets (data not shown due to space limitations). To allow simultaneous analysis of CD4+ and CD8+ T cell responses to the same platelets, we have bred the mOVA mouse with B6 mice congenic for the MHC from BALB/c mice (B6.H2d). These mice have platelets that express all H-2^d MHCI alloantigens (including the K^d seen by TCR75) and also express the mOVA seen by OT-I mice (data not shown).

C4.1.4 Monitoring early activation events of platelet specific $CD4^+$ and $CD8^+$ T cells: In this subaim, small numbers of TCR75 and/or OT-I T cells will be adoptively transferred into wild-type B6 mice prior to their transfusions with LR-PLTs from B6.$H2^d$-mOVA donors. Control groups will receive no treatment or control IgG1 whereas experimental animals will receive CTLA4-Ig. Transferred TCR75 and/or OT-I T cells will be visualized by staining with anti-CD4, anti-CD8, and anti-Thy 1.1. This staining strategy will allow us to distinguish CD4+ T cells specific for antigens presented by MHCII (TCR75; CD4+CD8-Thy1.1+) and CD8+ T cells specific for antigens presented by MHCI (OT-I; CD4-CD8+Thy1.1+). In some cases, cells will also be stained with the MHCII and MHCI tetramers for which TCR75 and OT-I T cells are specific, respectively (I-A^b-Kd 54-68 and K^b-SIINFEKL). Staining with tetramer will confirm antigen specificity and eliminate problems of background recombination in the TCR transgenic animals. In addition, tetramer staining will give the added advantage of being able to observe the background response by endogenous T cells at later time points when they are numerous enough to observe (tetramer +, Thy 1.2+).

C4.1.5 Localization of PLT specific T lymphocytes: Over a 21 day time course (but focusing on early time points), mice will be sacrificed and tetramer +,Thy 1.1 + cells will be enumerated in spleen, peripheral lymph nodes, liver, lung, and bone marrow by flow cytometry. Frozen sections will also be prepared and stained with anti-Thy 1.1. Anti-B220 will be included on a separate color to allow orientation to B cell zones. This staining is up and working in our lab and an example is shown in figure 8. Figure 8 has a section of spleen from a B6 mouse (Thy1.2) that was adoptively transferred with T cells from a Thy1.1 mouse, and stained with anti-Thy1.1 (green) and anti-B220 (red). B cell zones are seen in red. The Thy1.1 T cells are seen in the T cell zone, and specifically indicate transferred T cells, as only minimal particulate background is seen in mice that didn't receive adoptive transfer (data not shown). This will allow a localization of PLT specific T cells in tissues and also allow a determination of location relative to lymphatic architecture (i.e. location in cortex, paracortex, germinal centers etc.). Together, the data generated here will demonstrate the kinetics of expansion and the location (both anatomically and histologically) of $CD4^+$ and $CD8^+$ T cells in response to transfusion of platelets carrying the recognized antigen.

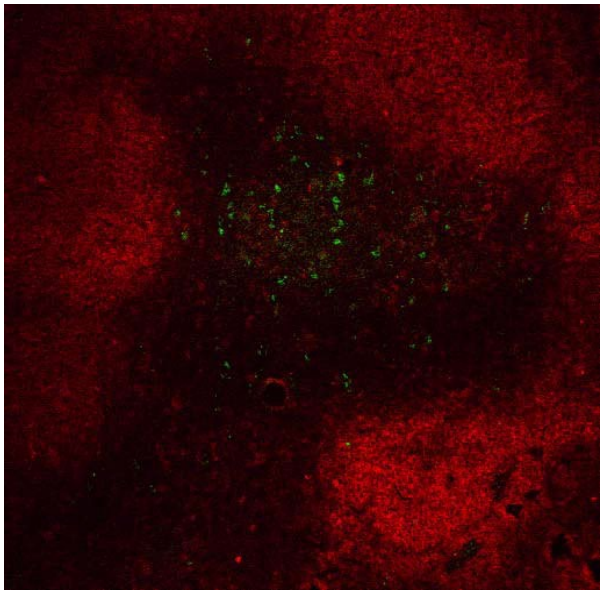

Figure 8. Immunofluorescent histology on frozen sections from spleen of mouse adoptively transferred with Thy1.1+ T cells. B cell zones are in red, transferred T cells are seen in green.

C4.1.6. Extent of proliferation of lymphocytes: T cells typically undergo clonal expansion upon activation. The extent to which this occurs, with regards to both kinetics and location, is an important component of understanding T cell immunization. The enumeration above will determine accumulation of T cells, which may be due to proliferation but can also be a function of trafficking or rates of cell death. To distinguish proliferation, TCR75 or OT-I T cells will be labeled with CFSE prior to adoptive transfer. Over a 14 day time course, mice will be sacrificed and the extent of proliferation of TCR75 and OT-I T cells in spleen, peripheral lymph nodes, liver, lung, and bone marrow will be determined flow cytometrically by gating on the transferred T cells and measuring decrease of CFSE fluorescence (see section C2.2 for example). For later time points

when CFSE is too dilute to measure, we will make use of an approach using BrdU as has been described precisely to allow analysis of cells after 10 divisions, when CFSE will be diluted out (30).

C4.1.7. State of activation and differentiation of platelet specific T cells: The above studies will determine the expansion and proliferation of T cells in response to platelet transfusion. However, it is also essential to determine the phenotype of the T cells. Over a 14 day time course, mice will be sacrificed and the phenotype of TCR75 and OT-I T cells will be determined in spleen, peripheral lymph nodes, liver, lung, and bone marrow by flow cytometric methods.

1. *T cell activation:* Specific multicolor antibody cocktails will be utilized containing anti-CD69, anti-CD44, anti-CCR7 and anti CD62L. After gating on TCR75 or OT-I T cells early activation and later activation/differentiation will be monitored by staining with anti-CD69 and anti-CD44, respectively. Conversion to a memory phenotype will be assessed, including a distinction between central memory ($CCR7^{high}/CD62L^{high}$) vs. effector-memory ($CCR7^{low}/CD62L^{low}$).
2. *Th1 vs. Th2 vs. T_{FH} cells in the $CD4^+$ T cell compartment:* Expression of cytokines by T cells is an essential component of their ability to provide help to and promote class switching in B cells. However, expression of Th1 type cytokines (i.e. IL-2, IFN-γ,) tend to favor promotion of cellular over humoral immunity, whereas expression of Th2 type cytokines (i.e. IL-4, IL-10) tend to promote humoral immunity. Intracellular cytokine staining using commercially available systems will be utilized to determine the cytokine profiles being expressed by TCR75 T cells. In addition to direct cytokine staining (which may lack sensitivity), some cells will be restimulated in the presence of Breveldin A, to allow accumulation of intracellular cytokines. We have recently published a peer-reviewed paper using this approach (31) and an example of this type of staining can be seen in figure 6.

   Recently, it has been appreciated that a special subset of follicular T cells (T_{FH}) play a special role in the germinal center in the process of providing T cell help for B cell differentiation into antibody secreting plasma cells (32). As the primary effect being studied in the current application is antibody synthesis to alloantigen, a logical sub-hypothesis is that T_{FH} are being altered by CTLA4-Ig treatment. The effects of CTLA4-Ig on T_{FH} will be determined by gating on TCR75 T cells and staining with antibodies to CXCR5 and PD-1 (32). In combination with histological localization of TCR75 T cells that are responding to transfused LR-PLT (see section C4.1.5), a detailed understanding of the effects of CTLA4-Ig on T_{FH} will be generated.
3. *Tc1 vs. Tc2:* In the case of $CD8^+$ T cells, cytokine staining (as above for Th1 and Th2) will be performed on OT-I cells to distinguish Tc1 vs. Tc2 profiles. In addition, staining for other effector molecules known to be expressed in $CD8^+$ T cells will be performed (perforin and granzyme B). We have recently published a peer-reviewed paper using this approach (31).
4. *Tregs:* The possibility that $CD4^+$ T cells and/or $CD8^+$ T cells may differentiate into Tregs, which have the capacity to promote immune tolerance, will be analyzed by staining with a commercially available kit for Fox-P3, as Fox-P3 expression correlates strongly to the Treg phenotype. Furthermore, intracellular cytokine staining will also be carried out using antibodies to tolerogenic cytokines such as TGF-beta and IL-10 that are typically expressed by Tregs. As above, restimulations in the presence of Breveldin A will also be carried out. We have recently published a peer-reviewed paper that analyzed Fox-P3 positive CD4+ T cells and are well experienced at this technique (31).
5. *Analysis of Anergy using TCR75 and OT-I T cells:* We have demonstrated that treatment with CTLA4-Ig prevents proliferation of CD4+ T cells specific for MHCI alloantigen on transfused PLTs (see section C2.2). However, lack of proliferation does not necessarily equate to lack of differentiation. It is likely that the TCR75 T cells are still seeing signal 1 (cognate peptide presented by MHCII) but not receiving signal 2. Signal 1 without signal 2 can lead to an anergic state. Anergy is classically defined as T cells that are not deleted but do not activate and differentiate into effectors upon subsequent delivery of signals 1 and 2 that would result in full activation of naïve T cells (33). Importantly, anergic T cells can be suppressive in some settings and anergy can also be reversed by some stimuli. To test for anergy, after exposure to CTLA4-Ig and LR-PLT transfusion, TCR75 T cells will be recovered from the mouse and restimulated by 2 separate approaches: **1)** exposure to C57BL/6 splenocytes that have been pulsed with the cognate peptide recognized by TCR75 [Kd-54-68] (in vitro stimulation), and **2)** adoptive transfer into naïve C57BL/6 mice followed by transfusion with LR-PLT from BALB/c donors (in vivo stimulation). In either case, the re-isolation procedure will remove any CTLA4-Ig in the system and allow restimulation in the absence of any lingering co-stimulatory blockade to assess the resulting phenotype of the T cells

C4.1.8. Sample size and statistical analysis: These experiments will require large numbers of mice, as analysis involving tissues will be terminal, and based upon our previous experience, at least five mice will be required for each time point. The exact number is difficult to predict, as the precise time points utilized may change based upon initial observations. However, we will be sure to incorporate sufficient animals in the experiments to have ample specimens at each time point. The mean values from each group will be collected and standard deviations determined. For any observed differences a p value of ≤ 0.05 will constitute significance. Experiments will be reproduced a minimum of three times.

C4.1.9. Predicted Outcome and Potential Problems: Each of the techniques required to complete the proposed studies are currently up and running in our lab; thus, we do not anticipate any unusual technical difficulties. Our preliminary data demonstrate that CTLA4-Ig prevents alloimmunization to platelets (sections C2.1 & C2.2). As cellular mechanisms and outcomes of CTLA4-Ig blockade can vary based upon the immunogen, and platelets have distinct immune responses compared to solid organ transplantation, these studies will provide novel information in the context of a previously unexplored area. Given that signal 1 will still be present, it is our prediction that CD4 and/or CD8 T cells specific for platelet antigens will become anergized, and may acquire negative regulatory function. This would be consistent with the prolonged protection that CTLA4-Ig provides (see section C2.1). Understanding if tolerance is achieved in this way is of fundamental importance in practical design of treatment regimens. In either case, the experimental design has sufficient analytic power to allow rigorous testing of the hypothesis and determination of different outcomes; thus, it is our opinion that useful knowledge will be obtained regardless of the experimental results.

C4.2. Specific aim 2: Effects of Heterologous Immunity and Antecedent Pathogen Exposure on CTLA4-Ig Efficacy:

C4.2.1. Rationale: As a general principle, co-stimulatory blockade has been a highly successful story of translation from murine models to human medicine. However, the enthusiasm to which this success gives rise was greatly tempered by the observation that CTLA4-Ig shows much better efficacy in mice than in either humans or non-human primates (34). However, Dr. [redacted] group (a collaborator on this project) has recently demonstrated that the differences in CTLA4-Ig efficacy is actually due to differences in how we treat rodents and primates, not due to differences in their biology. Unlike humans or non-human primates, experimental mice are typically kept in pathogen free environments and do not suffer the normal cycle of infection and convalescence from environmental pathogens. Therefore, experimental mice do not have the normal reservoir of memory T cells against third-party antigens. Because memory T cells can cross-react with alloantigens, and are more resistant to CTLA4-Ig than naïve T cells, previous infections can give low level priming for subsequent alloimmune responses. The data supporting this conclusion is that purposefully exposing mice to a series of pathogens, which their immune systems cleared after illness, results in converting CTLA4-Ig responses in mice to a very similar profile as what is seen in non-human primates and humans (9, 10). Second, it has now been shown that the efficacy of CTLA4-Ig in preventing transplant rejection in a non-human primate (not previously exposed to alloantigen) correlates with the number of memory T cells that are cross-reactive with alloantigen (35). Together, these data indicate that in the context of solid organ transplant, pre-existing memory T cells (as a result of previous infection), which are cross-reactive with alloantigen affect the efficacy of CTLA4-Ig.

As discussed in section C1, alloimmunization to transfused platelets is a substantially weaker immunological stimulus than solid organ transplantation. Moreover, even though the target of antibodies is MHCI, the T cell antigen is peptides from MHCI processed and presented on MHCII, and thus not direct allorecognition of the foreign MHC. It is thus unclear if pre-existing memory T cells against pathogens will confer the same effects upon efficacy of CTLA4-Ig in preventing alloimmunization to transfused PLTs. In this aim, this issue will be tested in our murine model.

C4.2.2. Hypothesis: Exposure to a series of infections that model normal exposure to environmental pathogens will prime memory CD4+ T cells with sufficient cross reactivity to alloantigens so as to decrease efficacy of CTLA4-Ig in preventing alloimmunization to transfused LR-PLTs.

C4.2.3 Experimental Design: Heterologous immunity will be induced in C57BL/6 mice through a series of infections with viruses that cause transient illness, followed by robust immune responses that clear the pathogen and result in long term immunological memory. Mice will be sequentially infected with lymphocytic choriomeningitis virus (LCMV), vaccinia virus (VV), and vesicular stomatitis virus (VSV). This is the same approach used by Dr. [redacted] (who is a collaborator on this grant, see letter of support) that showed

the effects of heterologous immunity upon CTLA4-Ig efficacy for solid organ transplants (10). After these animal have convalesced from their infections, the following four groups will be studied.

Group	Transfusion	Treatment	Purpose
Naïve B6 mice	BALB/c LR-PLT	IgG1 control	Positive control group for alloimmunization to PLTs
Naïve B6 mice	BALB/c LR-PLT	CTLA4-Ig	Control for CTLA4-Ig potency/efficacy
Sequentially Infected	BALB/c LR-PLT	IgG1 control	Control effects of sequential infection upon baseline alloimmunization response to LR-PLTs
Sequentially Infected	BALB/c LR-PLT	CTLA4-Ig	Experimental group to test effects of sequential infection upon CTLA4-Ig efficacy

Groups 1 and 2 will serve as controls that a given set of LR-PLT transfusions are immunogenic and that the preparation of CTLA4-Ig we are using is biological active. Group 3 will establish a baseline for the effects of heterologous immunity on humoral alloimmunization to platelets. The data obtained from group 3 will in of itself constitute novel understanding of factors that influence antibody responses to platelets and may help explain why some patients (but not others) become alloimmunized. In the context of groups 1-3, group 4 will allow a vigorous test analysis of the role that heterologous immunity plays in the efficacy of CTLA4-Ig for preventing alloimmunization to LR-PLT.

After the basic effects are understood, adoptive transfer of TCR75 T cells will be used (prior to viral infection) and mice from each group will be analyzed over a time course by each of the techniques described in aim 1. To avoid redundancy and out of space considerations, the reviewer is referred to aim 1 above. A synopsis of the techniques to be used includes: 1) Analysis of antibodies to H-2d MHC alloantigens on transfused LR-PLT, 2) Effects upon activation, expansion, enumeration, location, phenotype (Th1, Th2, or Treg), memory status, deletion and/or anergy of CD4+ T cells specific for PLT derived alloantigen.

C4.2.4. Predicted Outcomes and Potential Problems: It is our prediction that the outcome of these experiments will be to reject the hypothesis; in particular, that heterologous immunity will be less of an impediment to CTLA4-Ig blockade for humoral alloimmunization to PLTs than it has been shown to be for solid organ transplantation. The basis for this opinion is that despite normal exposure to environmental pathogens, CTLA4-Ig maintains efficacy in humans (albeit not as good as mice) for solid organ transplants and that transfused LR-PLTs represent a weaker alloimmunogen than do solid organ transplants. Moreover, humoral alloimmunization to MHCI antigens on LR-PLTs is more akin to responding to a minor antigen, as CD4+ T cells are not likely to be directly activated by donor tissues, but rather respond to recipient APCs that have processed and presented peptides from donor antigens (indirect pathway). In this way, the frequency of anti-viral memory T cells that cross-react with alloantigen is likely lower than in solid organ transplant. This is the basis for our prediction that the effects of memory T cells from previous infections will have less of a neutralizing effect upon the efficacy of CTLA4-Ig in the context of LR-PLT transfusion. Of course, these predictions are just speculation and may not be correct; previous infection may have a large effect upon CTLA4-Ig. In either case, the experimental design in this aim has the power to distinguish between the two outcomes and determine the effects of previous infections on CTLA4-Ig efficacy on PLT transfusion induced alloimmunization. Moreover, the use of the TCR75 T cells allows detailed mechanistic analysis to understand the cellular basis for whatever phenomenology is observed.

In the event that heterologous immunity does substantially decrease the efficacy of CTLA4-Ig, then the data will question whether trials to test CTLA4-Ig on human patients receiving PLT are reasonable to carry out. However, such a finding will also provide an experimental platform with which to test maneuvers to circumvent heterologous immunity. Currently, one of the most promising approaches in solid organ transplantation is the addition of blockade of the LFA-1 pathway to CTLA4-Ig, which has been shown to overcome heterologous immunity in non-human primates (36). The developers of this approach are Dr. [redacted]; each of whom are key personal on this grant (see letters of support). Thus, we have considerable expertise in the application of this and other modifications of costimulatory blockade to circumvent heterologous immunity, should it prove to be a problem in blocking alloimmunization to LR-PLT transfusion.

C4.3. Specific aim 3: Potential Infectious Sequelae of CTLA4-Ig Use in the Context of Transfusion Medicine.

C4.3.1. Rational: CTLA4-Ig has proven (in animals and humans) to be a less dangerous immunosuppressant than traditional pharmacological anti-rejection drugs. In particular, the risk of opportunistic infection, which is typically encountered with immunosuppressants, is very low in the case of CTLA4-Ig (see section C1). Even with chronic use, CTLA4-Ig has proven a safe drug, based mostly upon experience with its primary FDA approved indication, rheumatoid arthritis (8). The very reason that CTLA4-Ig is safe is likely because established immunological memory is little affected by CTLA4-Ig (the same reason that heterologous immunity in aim 2 is a theoretical concern). Thus, unlike pharmacological immunosuppression that indiscriminately inhibits and/or eliminates lymphocytes (e.g. cyclosporine, methotrexate, etc.), CTLA4-Ig has a lesser effect upon established immunity. Thus, existing protective responses that an individual acquires through routine exposure vaccines, pathogens, and opportunistic microbes remains intact. However, of great importance, is the special vulnerability that CTLA4-Ig may cause for microbes not previously encountered by a patient. If a pathogen is encountered for the first time during CTLA4-Ig therapy, immune responses may be significantly decreased. Clinical experience with CTLA4-Ig in humans demonstrates that in the context of routine environmental exposure to seasonal viruses and minor pathogens, this appears to result in a potential prolongation of infection or exacerbation of symptoms, but is typically not dangerous. However, there is special concern in the context of transfusion, where patients may be transfused with blood containing previously unencountered viruses.

One obvious candidate is cytomegalovirus (CMV). Because it is so prevalent, the blood supply is not routinely screened for CMV. It is for this reason that transfusion recipients who have not previously been infected by CMV are at particular risk for primary infection as a result of blood transfusion (37, 38). Deferring clinically ill volunteers from blood donation likely minimizes units with active CMV viremia; however, most CMV is transmitted by latent virus in leukocytes, which is reactivated upon transfusion. Leukoreduction has considerably decreased such transmission, but has not eliminated the problem (37, 38). In particularly susceptible patients, CMV negative blood can often be provided, but not in all circumstances. While adults with new CMV infection can have moderate illness with subsequent convalescence, immunosuppressed patients can have severe manifestations of CMV infection leading to substantial morbidity, and in some cases mortality.

C4.3.2. Hypothesis: Administration of CTLA4-Ig will decrease the strength and/or kinetics of a primary immune response to mCMV infection but will not affect a memory recall response.

C4.3.3. Experimental Design: We will make use of a murine model of transfusion-transmitted CMV developed by Dr. John Roback, who is a collaborator on this grant (39, 40). In collaboration with Dr. [redacted], Dr. Zimring (P.I. on this application) has added immune response analysis to the system (see section C2.4). We will utilize the murine CMV (MCMV [Smith Strain]), which is homologous to human CMV and causes similar illness. BALB/c mice will be infected with $1x10^6$ pfu of mCMV by i.p. injection and will be allowed to mount an immune

Group	Treatment	Transfusion	Purpose
1	None	LR-PLT from acutely viremic BALB/c	Positive control for transmission of mCMV from acutely viremic LR-PLTs
2	None	LR-PLT from convalescent BALB/c	Positive control for transmission of mCMV from latent donors
3	IgG1 control	LR-PLT from acutely viremic BALB/c	Control for non-specific effects of IgG administration
4	IgG1 control	LR-PLT from convalescent BALB/c	Control for non-specific effects of IgG administration
5	CTLA4-Ig	L LR-PLT from acutely viremic BALB/c	Experimental group to test effects of CTLA4-Ig on Immune Response to viremic LR-PLT
6	CTLA4-Ig	LR-PLT from convalescent BALB/c	Experimental group to test effects of CTLA4-Ig on Immune Response to LR-PLT from chronically infected donor

response, clear the mCMV viremia, and progress to a healthy state with latent mCMV (convalescent mice). After convalescent mice are established, additional animals will be infected and viral loads will be monitored by

quantitative real-time PCR on peripheral blood (see section C2.3). LR-PLT from both convalescent mice and from acutely infected mice will be collected (at the peak viremia for the latter). Recipient B6 mice will be treated with CTLA4-Ig or control IgG and will then be transfused with LR-PLTs from convalescent or viremic donors; the above groups will be utilized. A separate series of experiments will be performed on B6 recipients who have already convalesced from a MCMV infection to assess CTLA4-Ig affects on established memory.

Peripheral blood will be obtained on days 2,4,7,14,21,and 28 days and analyzed for viral loads and immune response (described below). These initial time points are chosen as they cover the known kinetics of MCMV infection and adaptive immunity; however, after initial experience with CTLA4-Ig treated animals, the sampling schedule may be adjusted accordingly to capture any alterations in kinetics due to CTLA4-Ig. The following additional parameters will be studied in each group.

1. *Overall health:* General health or sickness of mice will be assessed daily by taking weights of the mice, observing ambulation, and looking for hunched posture and/or piloerection of fur. Average weight is a good indicator of disease progress; normal mice infected with MCMV undergo progressive weight-loss during the acute stage of the infection and then gain weight back as the virus is cleared. However, animals who succumb to the virus have ongoing weight loss from which they don't recover. Standard IACUC guidelines will be utilized to terminate mice after over 25% weight loss has occurred.
2. *Viral Loads:* Quantitative Real-Time PCR will be utilized to establish viremia.
3. *Humoral Immunity to mCMV:* Serum will be tested for anti-mCMV antibodies using the Western blot assay.
4. *CD8+ T cell response to mCMV:* Peripheral blood leukocytes will be stained with anti-CD8 and a panel of tetramers containing immunodominant epitopes of MCMV in MHCI. An example of tetramer staining is showed in section C2.4.2. A detailed phenotypic and functional analysis of the CD8+ T cells will also be performed. To avoid redundancy, and due to space limitations, the reviewer is referred to the analysis of T cell responses described in aim 1, using the same approach (gating on tetramer + cells and analyzing phenotype).

Terminal Analysis: Circulating blood gives a good picture of overall viral loads and immune response; however, blood does not assess pathology of target organs of infection. Thus, additional mice from each group will be sacrificed over a range of time points and will be subjected to the following analysis.

1. Clinical metabolic profile. We currently utilize a veterinary clinical analyzer (Abaxis VetScan VS2) to obtain the following parameters on mouse blood: ALB, ALP, ALT, AMY, total bilirubin, BUN, Ca, Phos, creatinine, glucose, sodium, potassium, an total protein.
2. Immunohistology for CMV pathology: CMV infection can cause substantial disease in a variety of anatomical locations, including spleen, liver, GI track etc. A full necropsy will be carried out on all tested animals, fixed sections will be prepared, and will be analyzed by both standard H&E staining and immunohistology with antibodies to CMV proteins. Please see figure 9 for an example of staining that we performed on infected and uninfected mice.
3. DNA will be extracted from the tissues and quantitative real-time PCR will be performed to analyze the viral load for a given organ.

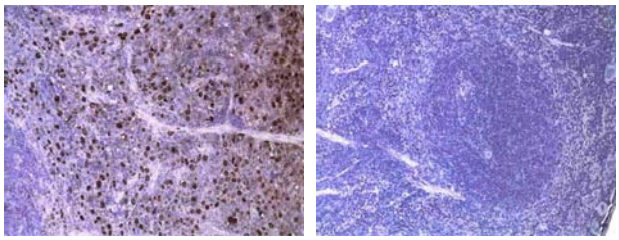

Figure 9 Immunohistochemistry with anti-MCMV

C4.3.4. Predicted Outcome and Potential Problems: We have experience with all of the assay systems in this aim, and in collaboration with Dr. [redacted] (collaborator on his grant; please see attached letter of support), we have generated the preliminary data presented herein. Thus, we do not anticipate any unusual technical difficulties with the proposed studies. It is our prediction that administration of CTLA4-Ig concurrent with mCMV infection will delay the kinetics of the immune response, and may exacerbate disease; however, as viral infections (e.g. mCMV) are much more potent immunogens than LR-PLTs, we predict that alloimmunization to platelets will be prevented while anti-mCMV responses will just be delayed. It is much more likely that blood from acutely viremic donors will cause disease than latent donor. As CMV transmission in humans is almost exclusively from latent donors, then this may not present a problem. Nevertheless, if mCMV disease is severe and leads to substantial illness in the mice, then we will titrate down the CTLA4-Ig dose to determine if there is a level of CTLA4-Ig that prevents alloimmunization to platelets without inducing severe defects in anti-mCMV responses. As our study will provide a detailed analysis of the effects of CTLA4-Ig on the anti-mCMV response, we will derive mechanistic understanding that will allow modification of the approach, if necessary.

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