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Regulation of Humoral Immunity to Allogeneic Platelet Transfusion

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Doctor of Philosophy

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Abstract

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Platelet transfusion is an essential treatment for thrombocytopenia, which can result from a wide range of conditions including acute hemorrhage, hematological malignancies, hematopoietic stem cell transplantation, and congenital platelet disorders. A life-saving therapy, transfusion may also carry significant risks. Anti-donor antibodies can be developed and abrogate the efficacy of transfused platelets. Increasingly sophisticated methodologies, including leukoreduction and irradiation of platelet products have decreased the incidence of anti-donor antibodies by limiting the recipient's exposure to antigenic white blood cells. In spite of these efforts, alloimmunization following platelet transfusion still occurs in a significant percentage of patients.

We began our studies by addressing a commonly held assumption in the transfusion literature, which states that donor MHC class II is required for the humoral response to allogeneic platelet transfusion. To test this hypothesis, we carried out immunization experiments by transfusing MHC class II deficient allogeneic whole blood. We observed that anti-donor MHC class I specific antibodies were of equal magnitude when comparing transfusion of blood from wild type vs. MHC class II null donors. However, there were qualitative differences in the IgG subtype response. These data indicate that whatever is responsible for the immunogenicity of leukocytes regarding MHC class I alloantigens, it is not the expression of donor MHC class II.

To study the role of the $CD4^+$ T cell in shaping the humoral immune response to allogeneic platelets, we developed a tractable T cell receptor transgenic mouse model of platelet transfusion. The TCR75 mouse is specific for a peptide derived from the BALB/c MHC class I molecule presented by the C57BL/6 MHC class II. The adoptive transfer of TCR75 cells into C57BL/6 recipients enhanced the normally undetectable antibody response to a single BALB/c leukoreduced platelet-rich plasma transfusion. Division of the TCR75 cells was restricted to the spleen, and did not occur in the lymph nodes or liver. Splenectomy or treatment with the costimulatory blockade reagent CTLA4-Ig abrogated

both $CD4^+$ T cell division and alloantibody production. These data support the critical role of $CD4^+$ T cell help and establishes a mouse model for exploring T cell-specific modalities for the prevention of platelet refractoriness.

Although we have developed a suitable model of platelet isolation and transfusion, our system lacks the aspect of platelet storage. Multiple physical and biochemical effects are incurred during platelet storage, which may not only impact post-transfusion survival but also alloimmunization. As a precursor to a platelet system, we developed and characterized a mouse red blood cell storage model using a transgenic mouse strain expressing enhanced green fluorescent protein. We demonstrated that the post-transfusion survival of stored mouse red blood cells was relatively similar to that reported for human red blood cells when indexed for their respective normal lifespan. Furthermore, mouse red blood cells developed phenotypic changes comparable to those described for humans. Together, these results demonstrate the potential utility of a mouse system to study the effects of storage and transfusion of red blood cells and provide some insight for the development of an analogous murine platelet storage model.

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Introduction

Clinical significance of platelet alloimmunization

Platelets are anucleate discoid shaped cells, averaging roughly 2 – 5 μm in diameter in humans (1). Despite this relatively small size, platelets play an essential role in the maintenance of hemostasis. In response to vascular injury platelets rapidly change shape and adhere to the sites of damage. This minimizes blood loss, provides a scaffold for the arrival of accessory cells, and facilitates the healing process. Patients with low platelet counts, a condition referred to as thrombocytopenia, can be at an increased risk for bleeding episodes. Uncontrolled bleeding can lead to consequences ranging from mild petechiae and bruising, to intracranial hemorrhage and possibly death.

The first successful attempt at blood transfusion to correct uncontrolled bleeding due to a low platelet count was described in 1910 (2). During the 1950's, platelet transfusion achieved significant clinical utility when applied to patients undergoing chemotherapy; the percentage of deaths due to hemorrhage would be reduced from 67% to 37% with the novel application of platelet transfusion support (3). The utilization and scope of platelet transfusion has since grown and is now a staple of the modern transfusion medicine service. An estimated ten million platelet units were transfused in the United States in 2006 for the prevention and treatment of thrombocytopenia due to a plethora of conditions including hematologic malignancies, solid tumors, infection, surgery, trauma, as well as congenital and acquired platelet dysfunctions (4).

Although platelet transfusions are often effective in ameliorating the risk of bleeding due to thrombocytopenia, many questions remain about the optimal use of platelets and how to deal with some of the significant adverse events associated with transfusion; among these include alloimmunization. Although difficult to determine

exactly, due to the complex pathophysiology often found in transfusion recipients, it has been estimated in a large clinical trial of acute myeloid leukemia patients that roughly 45% of recipients will develop anti-donor antibodies following transfusion of pooled-random donor platelets (5). These anti-donor antibodies have the potential to bind platelets and facilitate their clearance. Immunization across multiple allotypes can make it difficult (and at times impossible) to obtain sufficient units of compatible platelets to meet the hemostasis needs of sensitized patients. In addition, these anti-donor antibodies may exacerbate chronic rejection of transplanted organs, including decreasing the survival of transplanted lungs, hearts, and kidneys (6-8), as well as complicating hematopoietic bone marrow transplantation (9), and mediating hemolytic transfusion reactions due to anti-human leukocyte antigen (HLA) antibodies reacting with red blood cell associated Bennett-Goodspeed antigens (10).

A brief retrospective of transfusion medicine

The first documented animal to human blood transfusion was administered by Jean-Baptiste Denis in 1667 (11). He transfused several patients with a single dose of sheep's blood, without complication. However, those given multiple transfusions lead to what is now appreciated as a hemolytic transfusion reaction: abdominal pain, fever, chills, and dark red urine. Although these observations were consistent with Denis's hypothesis that the procedure was flushing evil humors from the body, the practice of transfusion would fall out of favor for the next 150 years. It would not be until 1818 that James Blundell, a British obstetrician, would perform the first efficacious human blood transfusion, to treat a women with postpartum hemorrhage (12). However,

transfusion was not dependable therapy; the donor blood tended to coagulate and recipients were likely to suffer severe adverse events.

The etiology of these transfusion reactions would begin to be elucidated by the work of Karl Landsteiner. In 1901, he noted that red blood cells agglutinate when incubated with the sera from certain donors but not others. These observations would become the experimental basis for the ABO blood group system (13). As other blood groups were described over the ensuing years it became possible to identify compatible donor and recipient blood types, which greatly improved the safety and reliability of transfusion. By 2009, in excess of 300 different human blood group antigens have been identified (14).

During the early 1900's, direct transfusion from donor to recipient remained a necessity, as no acceptable method to preserve blood outside the body had yet to be developed. However, various chemical approaches to anticoagulation were developed over the following decades. In 1914, Albert Hustin reported the first human transfusion using sodium citrate as an anticoagulant (15), which allowed blood to be refrigerated safely for several days. It was found that the addition of dextrose would extend storage to up to two weeks. Acid citrate dextrose (ACD), developed in 1943 by Loutit and Mollison, allowed blood to be stored for up to 4 weeks (16). Citrate phosphate dextrose was subsequently adopted after studies showed blood could be stored for up to 28 days with superior red cell survival compared to ACD, which lacked phosphate (17). Although rudimentary blood donor services were established as early as 1921 (18), the advances in storage techniques ushered in the establishment of the modern "blood bank"

(19) where blood and blood components such as packed red blood cells or platelets could be routinely stored and saved for future use.

Development of platelet transfusion as a therapeutic modality

Max Schultze published one of the first reports of the platelet as a distinct component of blood in 1865, describing them as "colorless little spherules" (20). William Osler would observe in 1874 that platelets make up the bulk of clots formed on damaged blood vessels (21). However, Giulio Bizzozero, circa 1881, is widely regarded as the first to clearly appreciate the platelet's importance in hemostasis (22). He showed that platelets were a distinct cell subset of the blood and were the first component to adhere to damaged vessel walls.

The transfusion of whole blood was first shown to reverse the course of severe bleeding in thrombocytopenic patients by William Duke in 1910. He observed that after correcting the platelet count by whole blood transfusion, bleeding time would decrease. Although these observations established an appreciation for the correlation between platelet count and hemostasis, technical limitations at the time precluded the ready availability of purified platelets. Several decades would elapse before platelet transfusions themselves would become routine.

Research was hastened during the 1950's when it was found that thrombocytopenia was a major cause of death after radiation exposure as well as a serious side effect from emerging chemotherapeutic agents. In 1950, plastic bags replaced glass bottles for collection and storage of blood (1). This allowed differential centrifugation methods that could easily separate cellular components based on density

in a sterile setting. Room temperature storage with gentle agitation would be recognized as an optimal setting for platelet storage. Novel plastic materials that facilitated increased oxygen exchange would be developed as well as synthetic storage and additive solutions to help maintain a proper pH. The development of apheresis techniques allowed the collection of therapeutic doses of platelets from a single donor. Furthermore, many of the lessons learned from red blood cell transfusion, such as ABO matching, overlapped to define similar rules for platelet donor and recipient compatibility.

General platelet physiology

The average healthy adult produces roughly 1×10^{11} platelets each day. Platelets are produced in the bone marrow from megakaryocyte precursors, which can each give rise to roughly 2000 - 5000 new platelets (23). Serum levels of thrombopoietin serve as the primary growth factor for the megakaryocytes and their precursors. The constitutive hepatic production of thrombopoietin is cleared by receptors on platelets, resulting in normal levels when the platelet production is normal, and elevated levels when the platelet production is reduced. Thus, although exogenous addition of platelets by transfusion increases platelet counts, it also may also transiently suppress *de novo* platelet production in the recipient. Two-thirds of platelets are normally present in the circulation while the spleen will sequester the remaining third. Splenomegaly, arising from infection, trauma or medication, may lead to a thrombocytopenia due to increased platelet sequestration (24).

When the vascular endothelium remains biochemically and physically intact, platelets are preset in the circulation in a quiescent state as relatively small discoid cells (1). Its shape is maintained by a network of actin filaments near the plasma membrane and a circumferential band of microtubules (25). A prominent feature is the involuted external plasma membrane. This forms an extensive canalicular system that allows serum components to enter the platelet space, facilitates the release of granular contents, and greatly increases the cell's surface area. Platelets contain lysosomes, peroxisomes, and mitorchondria as well as specialized dense bodies and granules. Dense bodies contain storage pools of compounds such as ADP, ATP, serotonin and calcium. α-granules and house a variety of contents including von Willebrand factor, fibrinogen, fibronectin, thrombospondin and P-selectin, as well as natural anticoagulants, coagulation factors and growth factors important for angiogenesis and wound repair (26). Although mature platelets lack a nucleus, they do contain mRNA and have the ability to translate some proteins.

The vascular endothelium normally acts to inhibit platelet activation and the coagulation cascade, proteins of which circulate in an inactive form while brisk blood flow helps to remove any activated proteins that may transiently form. The luminal surface of the endothelium, unlike the subendothelium, is devoid of thrombogenic molecules such as collagen, fibronectin, and tissue factor. Endothelial products such as nitric oxide and endothelial-ADPase serve to directly inhibit platelet activation. During vascular injury, exposure of normally sequestered molecules in the subendothelium triggers a complex cascade of events leading to the activation and adherence of platelets and activated factor Xa. Factor Xa will then complex with factor Va and convert

prothrombin to thrombin. Thrombin cleaves activation peptides from circulating fibrinogen monomers, which then polymerizes to form an insoluble fibrin clot near the site of injury. The fibrin clot, along with thrombin, further activates surrounding platelets to seal the breach in the vessel wall. Activated platelets will also secrete granular contents that recruit accessory cells to aid in the healing process (27).

In addition to their critical role in hemostasis, platelets are prominently involved in both innate and adaptive immunity. Platelets can have been shown to exhibit direct antimicrobial effects against pathogens such as bacteria, viruses, fungi, and helminthes (28-31). Activated platelets may secrete a host of inflammatory mediators such as platelet factor 4, IL-1α, IL-7, IL-8, RANTES, MIP-1α, TGF-β, serotonin, histamine, and prostaglandins (32). Platelet can express several immune related surface molecules such as major histocompatibility complex (MHC) class I, Fc receptors, and complement binding proteins (33-35). Adhesion molecules such as CD62P on platelets are involved in trafficking of immune cells to sites of inflammation and injury (36). Both murine and human platelets express functional Toll-like receptors -1, -2, -4 and -9, which enable them to detect a variety of pathogen-associated molecular patterns, which potentiates platelet aggregation and mediates the secretion of granule contents (33, 37-39). Furthermore, activated platelets can be a major source of CD154. Platelet derived CD154 has been shown to activate endothelial cells (40, 41), induce the maturation of dendritic cells (42), and play a prominent role in providing stimulation for the B cell production of antibody (35, 43).

Platelets that do not participate in the formation of thrombi or the inflammatory response are eventually cleared from the circulation following a life span of roughly

seven days in humans (44), and four days in mice (45). By tracking the fate of radiolabeled autologous platelets the spleen, and to a lesser extent the liver, has been identified as the predominant site for platelet clearance (46-48). Patients with impaired reticuloendothelial function have shown a defect in their ability to clear platelets even when elevated levels of platelet-specific antibodies are present (49). Furthermore, in select clinical settings, splenectomy is a treatment option to raise platelet counts for autoimmune diseases such as immune thrombocytopenic purpura (50). Whether the efficacy of splenectomy is due to the removal of Fc receptors expressing splenic macrophages or by impairing the T cell – B cell interactions involved in the synthesis of antibody is not clear (51).

Senescent platelets are identified and removed from circulation through a process that is not completely understood. This may possibly involve a novel form of programmed cell death, the expression of scavenger receptor ligands, loss of membrane asymmetry, shedding of vital membrane components, or morphological changes (52, 53). Splenic macrophages have been shown to phagocytose and present platelet antigens *in vitro* (54). However, very little has been done to characterize what cells *in vivo* are necessarily responsible for platelet clearance. Whether the cells responsible for normal clearance of senescent platelets are also the same cells responsible for autoimmune or allogeneic responses is not known.

Clinical platelet storage and transfusion

Platelet products destined for clinical use are derived from two general methods; isolation from whole blood collection or purified by apheresis. Random-donor platelet

units are isolated from whole blood units collected in an anticoagulant-preservative solution. The platelet-rich plasma (PRP) is separated from the red blood cell fraction by low speed centrifugation. This method is predominantly utilized by the United States, while elsewhere platelets are prepared from whole blood collection by buffy coat isolation. Apheresis platelets are isolated by passing the donor blood through a closed apparatus that separates the PRP by centrifugation and returns the remainder of the blood back to the donor. Typically, most apheresis products contain far more platelets than those isolated from whole blood and may be split into several individual units. In the United States, approximately 75% of platelet transfusions are collected by apheresis (4).

In the United States, platelet collection and storage are governed by the Food and Drug Administration's guidelines. Platelet products are stored at 20 - 24°C with continuous gentle agitation in plasma, as no artificial platelet storage solutions are currently approved. Plasma may not be the optimal storage solution, as donor antibodies and serum proteins are associated with adverse events such as allergic reactions, transfusion-related acute lung injury and ABO-incompatible hemolysis (55). Platelets must be stored in specially designed oxygen-permeable plastic bags, as anoxic conditions favor metabolic pathways that lead to the build up of harmful lactic acid (56). Storage time is limited to 5 days under these conditions due to the risk of bacterial contamination (57). Cold storage of platelet units, which would inhibit bacterial growth, is precluded by the fact that chilled platelets are rapidly removed from circulation upon transfusion (58). It has been estimated that between 1 in 1000 to 1 in 3000 platelet products contain detectable levels of bacterial contamination; the transfusion of which

may result in fever, sepsis, shock, or death (59, 60). Currently, there are several standards and methods employed to evaluate the presence of bacterial contamination, which have served to decrease the rates of septic reactions (61). However bacterial contamination remains a significant concern.

In adults, the typical platelet dose is a single apheresis product or 4-6 whole blood derived platelet units pooled, which will represent a platelet dose of roughly 3-4 x 10^{11} platelets. Responses to platelet transfusion are assessed by observing improvements in bleeding or by measuring a post-transfusion platelet increment. In general, platelet units are selected based only on ABO compatibility. However, supplies are sometimes suboptimal and the use of non-ABO matched platelets is not uncommon.

Platelet refractoriness

Platelet transfusion refractoriness is a clinical term used to describe the failure to achieve an expected incremental response to a platelet transfusion. The precise definition of platelet refractoriness varies, but it is generally defined as two ABO-compatible transfusions that yield a corrected platelet count increment on consecutive days of less that 5000 (5). If patients do not respond adequately to transfusions, cross-matched or HLA matched platelets may be necessitated. However, this may not be an easy task. Due to the diversity of HLA antigen in the human population, it may be logistically impossible to obtain platelets to meet every need.

Refractoriness is normally due to non-immune mediated factors. In a study of cancer and hematological disease patients, non-immune factors were estimated to be present in 72–88% of refractory cases (62-65). Non-immune factors include bleeding,

fever, sepsis, splenomegaly, disseminated intravascular coagulation, graft-versus-host disease, as well as the use of antibiotics and antifungal drugs (63, 66-68). Quality of the platelet concentrate, handling and transportation may also play a role (69). Non-immune refractoriness often resolves in a transient fashion.

Immune mediated refractoriness predominantly affects patients with high transfusion requirements, such as those with congenital bleeding disorders or undergoing chemotherapy (70-73). Multiparous women and minorities, who tend to have HLA phenotypes that are not well represented in the donor pool, are also at a higher risk (72, 74). Before the widespread use of leukoreduced blood components to reduce HLA alloimmunization rates, an estimated 45–70% of chronically transfused patients developed refractory antibodies to donor MHC class I antigens (5, 75, 76). This antibody-mediated process is most often directed against HLA antigens and, to a lesser extent, human platelet antigens (HPA) (68, 77). Due to the platelets expression of MHC class I, these anti-HLA antibodies can bind platelets and facilitate their clearance. Immune mediated refractoriness is not normally a transient condition due to the presence of immunological memory.

Allospecific T cell activation

Optimal T cell activation generally requires two signaling events (78). 'Signal 1' is initiated by the antigen specific interaction between the T cell receptor and the MHC molecule loaded with peptide. 'Signal 2' is provided by what are broadly referred to as costimulatory signals, such as those provided by the CD28 and CD40 signaling pathways. Antigen presenting cells (APCs) up-regulate the expression of costimulatory

molecules in response to a wide variety of inflammatory stimuli including tissue damage, cytokines and conserved pathogen-associated molecules, such as LPS or double stranded RNA (79-82). In the absence of an inflammatory milieu, APCs remain in a quiescent state and express relatively low levels of costimulatory molecules (83). Under these conditions, T cell activation is inhibited by the absence of 'signal 2' leading to anergy, apoptosis, or possibly a regulatory T cell like phenotype (84-87). This natural process may be mimicked artificially by the administration of costimulatory blockade reagents; fusion proteins or monoclonal antibodies designed to physically block costimulatory ligand and receptor binding between the T cell and APC.

MHC molecules provide a scaffold with which to display a sampling of what is going on both inside and outside the cell. MHC class I presents peptides mainly derived from proteins synthesized inside the cell and are recognized mostly by T cells expressing the CD8 co-receptor. MHC class II normally presents peptides derived from antigens taken up from outside the cell and are generally recognized by T cells expressing the CD4 co-receptor. Immature T cells undergo a dynamic process in the thymus in which the developing T cells must recognize self MHC within a certain range of affinity to receive signals for continued survival, in what is termed positive selection (88). Autoreactive T cells with too high of an affinity for self peptides are deleted before leaving the thymus by a variety of cells including thymic epithelial cells and bone marrow derived dendritic cells (89).

Exposure to genetically distinct donor MHC via transfusion presents a unique situation for the recipient's T cells. The donor MHC may be taken up by recipient APCs, treated as a foreign peptide, and presented on its own MHC class II. Donor-

specific recipient $CD4^+$ T cell can then ligate this MHC-peptide complex to become activated, a process referred to in this setting as indirect recognition. However, due to structural differences and similarities between the donor and self MHC molecules, recipient T cells can interact with donor cells in such a way that T cell receptor signaling occurs independent of the particular peptide being presented by the MHC (90). This process is referred to as direct allorecognition, since recipient T cells can directly recognize donor MHC without further processing (91, 92). Up to 10% of the total T cell compartment in a given individual may be alloreactive, thus, allogeneic responses tend to be quite potent (93).

Humoral response to platelet transfusion

The antibody response to allogeneic platelet transfusion is predominantly directed against donor MHC class I epitopes (94). Although platelets contribute roughly 70% of the total MHC class I molecules found in whole blood (95), there is considerable controversy in the transfusion medicine field as to the identity of the antigen responsible for the anti-donor humoral response. Animal studies have reported that removal of leukocytes from transfused platelets eliminates the primary alloantibody response to donor MHC class I (96, 97). Furthermore, clinical trials have shown that the use of leukoreduced platelet units correlates with a decrease in alloantibody production (98). The prevailing explanation in the transfusion literature for a leukocyte requirement is that it is the exposure to donor MHC class II expressing leukocytes that stimulates recipient anti-donor antibody production. Whereas platelets normally express MHC class I but not MHC class II (99), select leukocyte subsets including dendritic cells,

monocytes, B cells and some T cell populations express both MHC class I and II (100, 101).

Following transfusion of donor leukocytes as contaminants in the platelet unit, donor MHC class II can interact with and activate allospecific recipient $CD4^+$ T cells via direct recognition. Although the antigen specificity of these donor reactive T cells is conceptually unclear, they may be sufficient to provide help to recipient B cells specific for anti-donor antigens. Indeed, there are substantial data to indicate an essential role for the direct pathway in various models of solid organ and bone marrow transplantation rejection (102-104). In a mouse model of peripheral blood leukocyte transfusion, both antibody depletion of MHC class II positive donor cells and use of MHC class II null donors greatly reduced the recipient anti-MHC class I antibody response (105). Together, with the efficacy of leukoreduction in decreasing alloimmunization in humans, these findings present compelling evidence for the interpretation that donor MHC class II positive leukocytes are required for primary alloimmunization to MHC class I. This interpretation is widely circulated and stated as fact in much of the transfusion literature (106-108).

In addition to the direct pathway, the indirect allorecognition pathway has also been identified as a mechanism of humoral alloimmunization, and consists of consumption of donor cells by recipient APCs, followed by the processing and presentation of donor MHC class I molecules on recipient MHC class II. These APCs can then activate recipient $CD4^+$ helper T cells, which then provide signals to B cells to produce anti-donor antibodies. If as is generally held, that MHC class II expressing donor leukocytes are indeed required for alloimmunization to transfused cells, then it

would indicate that the indirect pathway was not alone sufficient for alloimmunization in the context of platelet transfusion.

Solid organ transplantation models, including skin and heart grafts, have demonstrated that antibody and cytotoxic T cell responses can be elicited through the indirect pathway (92, 109, 110). Furthermore, it has been reported that allogeneic platelets phagocytosed *in vitro* by recipient adherent splenic macrophages could stimulate the proliferation of previously sensitized splenocytes under certain cell culture conditions (54). Similar methods were used to show that these *ex vivo*-manipulated macrophages were sufficient to initiate an anti-donor antibody response when adoptively transferred to naïve recipients (111). Analogous strategies have been used in efforts to augment the often ineffective immune response against cancer by *ex vivo* loading of tumor specific antigens to APCs, with varying success (112). These data do not necessarily demonstrate that the indirect pathway can stimulate a primary response to allogeneic platelets, but they do support processing and presentation of donor MHC class I by recipient APCs, and indicate that the indirect pathway is intact, if not sufficient. This raises the question as to whether the direct pathway really is required, and thus the role of donor MHC class II. Also of relevance is the observation that patients can become alloimmunized to platelet specific antigens, such as HPA, which are not expressed on donor leukocytes (106). Finally, additional animal data have challenged the historical experiments demonstrating that platelets alone are not immunogenic, as extreme leukoreduction of platelets failed to eliminate alloimmunization in a separate study with mice (113).

Much ado about platelets

The appreciation of the biological role of platelets has progressed significantly since their description by Giulio Bizzozero over a century ago (20). Platelet transfusion is currently a critical component of the maintenance of hemostasis for many clinical conditions. In addition to their role in the prevention of bleeding, platelets are prominently involved in both the innate and adaptive immune response. However, the physiological characteristics and host interactions that contribute to the platelet's role in immunity, such as the expression of MHC class I and bioactive CD154, the secretion of inflammatory cytokines, adherence to lymphocytes through the externalization of adhesion molecules, and the ability to aggregate into an immobilized clot, may exacerbate the development of refractoriness when present in the setting of allogeneic platelet transfusion. In the United States, platelets are stored on average for 2 – 3 days prior to transfusion (4), during which time platelets may develop an array of biochemical and physical alterations, which may complicate their post-transfusion efficacy and immunogenicity. It is difficult to explore these issues in the setting of human clinical transfusion; however, these are issues that may potentially have profound clinical significance for the development of the anti-donor response. This thesis explores several aspects of the issue of platelet alloimmunization in a controlled mouse experimental setting.

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Chapter 2

MHC Class II on Transfused Murine Blood is Not Required for Alloimmunization Against MHC Class I

Abstract

Transfusion of allogeneic platelet products can result in antibodies against donor MHC class I antigens, leading to a refractory state to subsequent platelet transfusions. However, there is disagreement in the field regarding the molecular mechanisms of humoral alloimmunization. One hypothesis states that donor MHC class II is a requirement for alloimmunization. However, other studies have suggested that donor MHC class I is alone sufficient and MHC class II is not required. We utilized a mouse model of anti-MHC class I alloimmunization to transfused blood, which employed donors with a complete deletion of all MHC class II genes. BALB/c (H-2^d) recipients were transfused with blood from either C57BL/6 (H-2^b) or MHC class II null donors on a C57BL/6 background. Anti-MHC class I alloimmunization was monitored by indirect immunofluorescence. Recipients of either wild type or MHC class II null blood produced equivalent humoral responses against donor MHC class I antigens. However, there was variation in the relative amounts of IgG subclasses. These data reject the hypothesis that donor MHC class II expression is required for alloimmunization to MHC class I antigens.

Introduction

The major histocompatibility complex (MHC) I is both highly polymorphic and expressed on a wide variety of hematopoietic cells. Thus, it is not surprising that transfusion frequently results in humoral alloimmunization in the form of anti-HLA antibodies. Leukoreduction of platelet products has substantially decreased HLA alloimmunization. In one study, the transfusion of allogeneic platelet products to acute myelogenouse leukemia patients induce anti-HLA antibodies in approximately 45 percent of recipients while leukoreduction decreases the incidence to 18 percent [1]. The most obvious therapeutic sequelae of anti-HLA antibodies is platelet refractoriness, which can result in difficulty or even the inability to treat thrombocytopenia by transfusion of platelets in patients immunized to multiple allotypes [1-3]. In addition, anti-HLA antibodies can promote chronic rejection of transplanted organs, including decreasing survival of transplanted lungs [4], hearts [5], and kidneys [6].

Significant investigation has been carried out regarding the cellular and molecular mechanisms involved in HLA alloimmunization. The substantial decrease in HLA alloimmunization by filter leukoreduction of platelets is not due to elimination of the offending antigen, as platelets themselves account for roughly 70% of the total MHC class I molecules found in whole blood [7]. In the context of leukoreduction efficacy, it has been concluded that leukocytes are more immunogenic than platelets regarding MHC class I antigens. Because MHC class II is expressed on leukocytes, but generally absence from platelets, it has been hypothesized that MHC class II expression is responsible for the increased immunogenicity of leukocytes.

Donor cells co-expressing MHC class II along with MHC class I has been widely circulated as a requirement for alloimmunization and stated as fact in the platelet transfusion immunology literature [8-11]. However, there are conflicting data as to the validity of this conclusion. Kao et al. used a mouse model to demonstrate that induction of MHC class I antibodies by transfusion of leukocytes was profoundly diminished after depletion of MHC class II positive cells from the donor cells[11]. These data serve as the most definitive experimental basis for concluding that MHC class II expression is responsible for the increased immunogenicity of leukocytes. However, Semple et al. have reported that recipient antigen presenting cells (APCs) can phagocytose, process, and present donor cell derived MHC class I molecules on recipient MHC class II. These APCs can then activate recipient $CD4^+$ T cells [12,13]. The capacity of the indirect pathway to induce immunity does not directly test a role for donor MHC class II, but it does raise the question, if recipient APCs can present donor MHC class I antigens on their own MHC class II, then by what mechanisms would donor MHC class II be required?

In aggregate, the separate observations of Kao and Semple appear mutually exclusive in the context of the normal paradigms of humoral alloimmunization; indeed, the data seem to necessitate the generation of a hypothesis by which donor MHC class II is required through mechanisms other than activation of $CD4^+$ T cells. Alternatively, the reported data are not generalizable phenomena outside the confines of the experimental systems used to generate them. Kao et al. utilized highly purified and manipulated leukocyte populations that do not necessarily reflect the composition or status of transfused leukocyte populations. To address this issue we carried out alloimmunization

experiments with MHC class II null donors, using minimally manipulated blood products for transfusion.

Herein, we report that anti-MHC class I alloantibody responses were of equal magnitude comparing transfusion of blood from wild type vs. MHC class II null donors. This observation indicates that whatever is responsible for the increased immunogenicity of leukocytes regarding MHC class I alloantigens, it is not co-expression of MHC class II. Together, these data resolve an apparent paradox in the field by rejecting the current belief that donor MHC class II is responsible for increased immunogenicity of donor leukocytes.

Materials and Methods

Mice: 6 to 8 week old B6.129S2-H2$^{dlAb1\text{-}Ea}$/J (MHC class II null, H-2^{b}) [14], C57BL/6 (H-2^{b}), BALB/c (H-2^{d}), and BALB.B (H-2^{b}) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). H2-K$^{b-/-}$D$^{b-/-}$ (K^{b}D^{b} null, H-2^{b}) mice were generously provided by Dr. Aron Lukacher (Emory University, Atlanta, GA) [15]. All studies and procedures were carried out in accordance with Emory University's Institutional Animal Care and Use Committee guidelines.

Blood preparation and transfusion: For each experiment, fresh blood from two donor mice was collected by retroorbital enucleation into 200μL of acid citrate dextrose solution and washed twice with Dulbecco's phosphate buffered saline (DPBS). Recipient animals were transfused with 100μL of washed whole blood resuspended to 500μL in DPBS by tail vein injection.

Indirect antibody test and Ig subclass determination: Serum was collected at the indicated time points and frozen at -80°C until use. To quantify the anti-donor antibodies and their Ig subclass, sera were diluted as indicated and incubated with 1x10^{6} splenic target cells for 30 minutes at 4°C. The cells were washed in FACs buffer (DPBS supplemented with 0.5% bovine serum albumin, 1mM EDTA and 50mM Hepes buffer, pH 7.2) and then incubated with FITC-anti-CD19, PE-anti-CD3, and either allophycocyanin-goat anti-mouse Ig, allophycocyanin-anti-IgG$_1$ (BD Pharmingen, San Jose, CA) or allophycocyanin-anti-IgM (Southern Biotech, Birmingham, AL) for 30 minutes at 4°C. Additional secondary antibodies included horseradish peroxidase

(HRP) conjugated goat anti-mouse IgG_{2a}, IgG_{2b}, or IgG_3 (Bethyl Laboratories, Montgomery, TX) followed by Cy5 conjugated goat anti-HRP (Jackson ImmunoResearch, West Grove, PA). Determination of bound anti-donor antibodies utilized a $CD3^+$ $CD19^-$ parent gate to avoid interference from anti-Ig binding to B cells or Fc receptor binding. Samples were acquired by a FACScan flow cytometer (BD Pharmingen) and analyzed using FlowJo (Treestar, Ashland, OR).

Statistical Analysis: Statistical significance was determined by two-way ANOVA with a Bonferroni post-test at an alpha level of 0.05. Analysis was performed using the GraphPad Prism Software Suite (GraphPad Software, Inc., La Jolla, CA).

Results

Absence of donor MHC class II does not decrease the humoral response to allogeneic MHC class I

Washed whole blood from wild type C57BL/6 or MHC class II null donors (B6.129S2-H2$^{dlAb1\text{-}Ea}$/J) was transfused into BALB/c recipients. The B6.129S2-H2$^{dlAb1\text{-}Ea}$/J strain has a complete genetic deletion of the entire MHC class II locus, resulting in the absence of MHC class II. The phenotype of the MHC class II null mice was confirmed by flow cytometry with antibodies to MHC class II; no MHC class II was present on blood cells from B6.129S2-H2$^{dlAb1\text{-}Ea}$/J mice (data not shown).

The anti-donor antibody response was quantified by indirect immunofluorescence against donor targets and syngeneic control targets (Figure 2.1A). The adjusted mean fluorescent intensity (MFI) reported was the fluorescence signal from experimental sera minus the background signal from naïve BALB/c control sera. The difference in the anti-donor antibody response was not statistically significant between transfusion of wild type and MHC class II null blood from day-21 post-transfusion sera (Figure 2.1B). Furthermore, titration of sera from day 21 showed essentially overlapping curves (Figure 2.1C).

A panel of splenocyte targets was used to analyze the specificity of the antibody response. Antibody binding was similar using either C57BL/6 or BALB.B targets, congenic for the C57BL/6 MHC haplotype H-2^{b} on a BALB/c genetic background (Figure 2.2). Because the BALB.B targets are genetically identical to the recipient mice, with the exception of encoding the donor C57BL/6 MHC genes, these data indicate that most of antibodies recognized antigens encoded by the donor MHC. To further

characterize the specificity, targets were used from C57BL/6 donors with a complete deletion of MHC class I genes (K^dD^b null mice [15]). The majority of the antibody binding was lost with MHC class I null targets, indicating that the alloantibodies were predominantly specific for MHC class I (Figure 2.2). However, low levels of fluorescence above that seen with syngeneic targets suggest some minor binding to elements outside of the MHC class I locus, possibly minor antigenic variants between the donor and recipients. Together, these data indicate that recipient mice make quantitatively similar antibody responses specific for MHC class I antigens regardless of whether the donor cells express MHC class II.

Qualitative analysis of alloimmunization to MHC class II null and wild type whole blood

Kinetics and Ig subtypes were analyzed by testing serum from 3, 7, 14, and 21 days post-transfusion with a panel of secondary reagents. Consistent with the above data, total Ig and IgM responses were similar for recipients transfused with either wild type or MHC class II null blood (Figure 2.3). However, analysis of IgG subtypes revealed that the relative levels of IgG_1 predominated for responses against wild type blood whereas IgG_{2a}, IgG_{2b}, and IgG_3 predominated for responses against blood from MHC class II null donors (Figure 2.3).

Discussion

Our data demonstrate that transfusion of blood from donor mice with a complete genetic deletion of MHC class II stimulates an anti-MHC class I alloantibody response of similar magnitude to transfusion of blood from wild type donors. These data reject the hypothesis that donor MHC class II expression is required for recipient anti-MHC class I alloantibody formation. Our findings are in disagreement with similar studies previously reported by Kao et al. [11]. The exact reasons are unclear, but there were substantial methodological variations between the two studies. Kao et al. transfused highly purified and manipulated leukocytes; however, these cells were not clearly characterized as to their composition prior to transfusion and may have lacked significant immunogenic subsets [16-18]. Furthermore, the viability of the cells transfused was not reported. Purification and manipulation can lead to apoptosis of leukocytes, and apoptotic cells are known to be poorly immunogenic, and in some cases tolerogenic [19]. Kao et al. did define conditions under which MHC class II null leukocyte transfusions are not immunogenic. However, the positive immunological responses in the current study reject the hypothesis that it is a general biological principle that donor MHC class II is a requirement for transfusion induced alloimmunization to MHC class I.

Although the current findings are in contradiction to the studies by Kao et al., our findings are consistent with the logical prediction of published data in reductionist systems that suggest the indirect pathway is alone sufficient for alloimmunization to donor MHC class I [12,13]. Semple et al. demonstrated that allogeneic platelets phagocytosed *ex vivo* by recipient adherent splenic macrophages could stimulate the proliferation of previously sensitized splenocytes under certain cell culture conditions [12].

Further studies by this group reported that these *ex vivo*-manipulated macrophages were sufficient to initiate an anti-donor antibody response when adoptively transferred to naïve recipients [13]. Although these data do not address the requirements of donor MHC class II in the process of alloimmunization to donor MHC class I, they nevertheless provide a biological pathway (Indirect recognition, see Figure 2.4), which is a plausible scenario for immunization that does not involve donor MHC class II.

Semple et al. also demonstrated a relationship between allogeneic platelets and contaminating leukocytes in modulating the recipient immune response, showing that platelet transfusions remain immunogenic even with extreme leukoreduction [20]. The donor mice used in this study were of a severe combined immunodeficiency, or SCID, phenotype. Lymphocytes were absent, including MHC class II positive B cells, however the authors showed that MHC class II expressing monocytes persist in SCID donors at twice the frequency as wild-type mice. Although this study assessed the requirements and regulatory roles of lymphocytes, it did not specifically address the requirement of donor MHC class II. In contrast, the current study formally tests and rejects the hypothesis that donor MHC class II is required as a modality of alloimmunization to MHC class I.

While our data demonstrate that donor MHC class II is not required, this does not necessarily mean that it is not affecting alloimmunization. We observed significant differences in the relative amounts of IgG subtypes in mice responding to wild type vs. MHC class II null donors. The increase in IgG_1 suggests a T_H2-like polarization when MHC class II was present on donor cells while the increase in IgG_{2a}, IgG_{2b} and IgG_3 suggests a T_H1-like polarization in its absence. This may be due to bystander effects

from the direct pathway contributing cytokines such as IL-4 and TGF-β to the local milieu. However, the altered IgG subtypes may also be an epiphenomenon of the relative absence of $CD4^+$ T cells and regulatory T cells in the MHC class II null transfusion.

One might question the use of unfractionated blood as opposed to leukoreduced units of platelets in the current experiments. However, this design was utilized because the hypothesis being tested was not concerned with platelets themselves, as platelets do not express MHC class II; the hypothesis was focused on the requirements for MHC class II expression on leukocytes contained in a transfused blood product. So as to avoid unnecessary manipulation, which may lead to alterations in biology, unfractionated blood was utilized. Due to this approach, it is essential to note that the current data have no bearing on the question of whether or not leukocytes are required for alloimmunization to transfused platelets, only the extent to which expression of MHC class II is required. The current data indicate that if leukocytes are required, their necessity is not a function of expression of MHC class II inducing the direct pathway of $CD4^+$ T cell activation (Direct recognition, see Figure 2.4), as is widely held in the transfusion literature [8-11]. Thus, in addition to providing a clear answer in the context of a mouse model, these findings lay the rational basis for re-examining the role of donor MHC class II in human alloimmunization to MHC class I.

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Figure Legends

Figure 2.1. Quantification of the anti-donor antibody response following transfusion from wild type or MHC class II null donors. (A) Representative histograms of the anti-donor antibody response by indirect immunofluorescence at a serum dilution of 1:30 using C57BL/6 allogeneic targets (left panel) or BALB/c syngeneic targets (right panel). **(B)** Total Ig anti-donor antibody response by indirect immunofluorescence using C57BL/6 targets. **(C)** Titration of day-21 sera against C57BL/6 targets. Combined data from 3 independent experiments with 5 mice per group (n=15). Error bars indicate standard error of the mean.

Figure 2.2. Anti-donor antibody epitope specificity. Sera from BALB/c recipients were diluted 1:30 and incubated against a panel of splenocyte targets. Combined data from 3 independent experiments with 5 mice per group (n=15). Error bars indicate standard error of the mean.

Figure 2.3. Kinetics and Ig subclass determination of the anti-donor antibody response. Sera from BALB/c recipients were diluted 1:30 and incubated against C57BL/6 targets. Ig specific secondary reagents were then used to determine the particular Ig subclass of the bound antibody at the indicated time points. Combined data from 3 independent experiments with 5 mice per group (n=15). Error bars indicate standard error of the mean. (* $p < 0.05$)

Figure 2.4. Direct and indirect antigen presentation pathways in the context of whole blood transfusion. For direct recognition, transfusion of MHC class II expressing donor leukocytes (shown in maroon) as contaminates in the platelet unit can interact with and activate recipient $CD4^+$ T cells (shown in blue), which then may provide help to recipient B cells (shown in yellow) specific for anti-donor antigens. With indirect recognition, donor MHC class I molecules (shown in red) from donor cells, i.e. platelets, red blood cells, leukocytes, etc., can be phagocytosed by recipient APCs (shown in green) then processed and presented on recipient MHC class II. These recipient APCs can then activate a $CD4^+$ helper T cell response to provide signals to B cells for the production of anti-donor antibody.

Figure 2.1

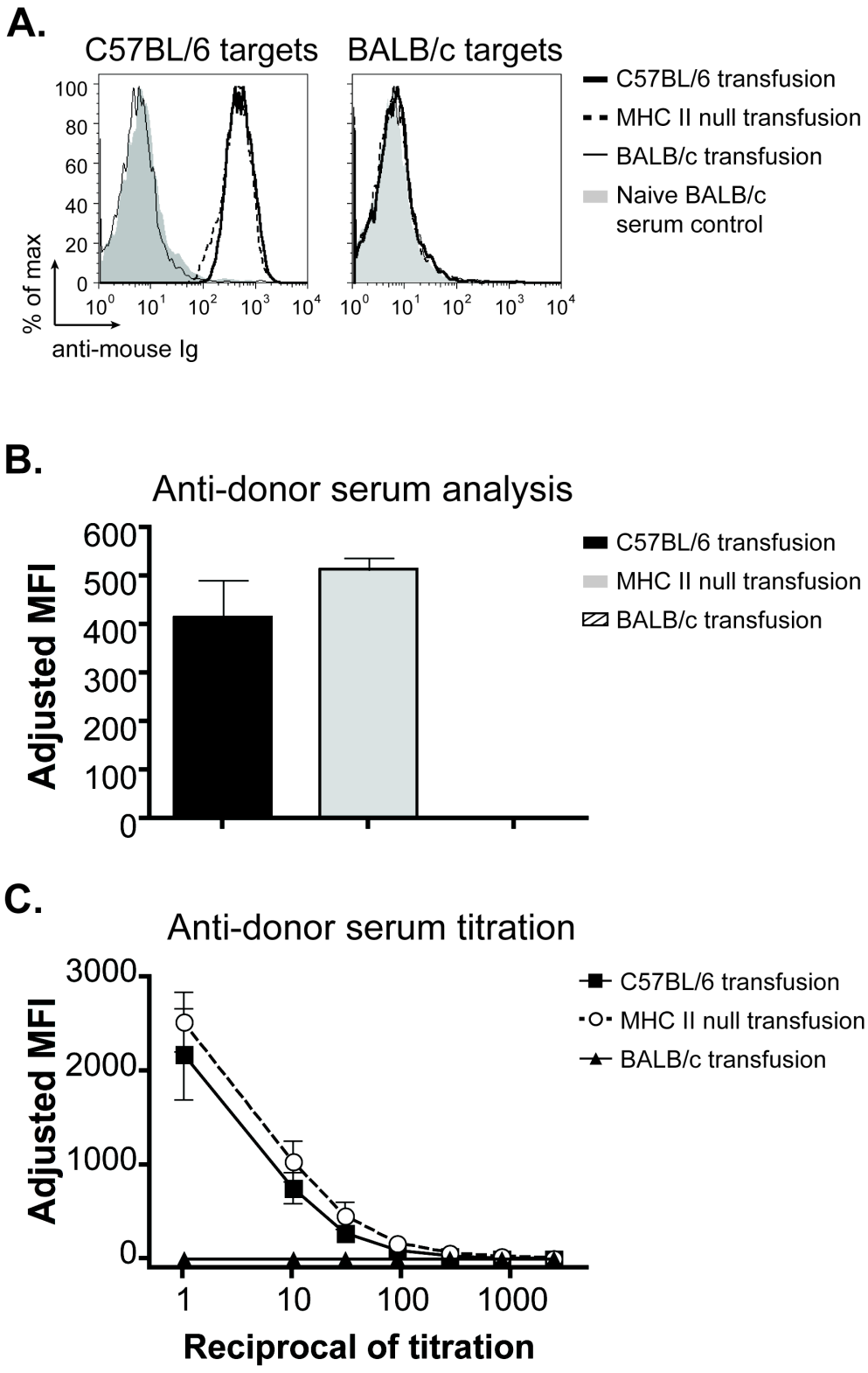

A.
C57BL/6 targets
BALB/c targets
C57BL/6 transfusion
MHC II null transfusion
BALB/c transfusion
Naive BALB/c serum control
% of max
anti-mouse Ig
B.
Anti-donor serum analysis
Adjusted MFI
C57BL/6 transfusion
MHC II null transfusion
BALB/c transfusion
C.
Anti-donor serum titration
Adjusted MFI
C57BL/6 transfusion
MHC II null transfusion
BALB/c transfusion
Reciprocal of titration

Figure 2.2

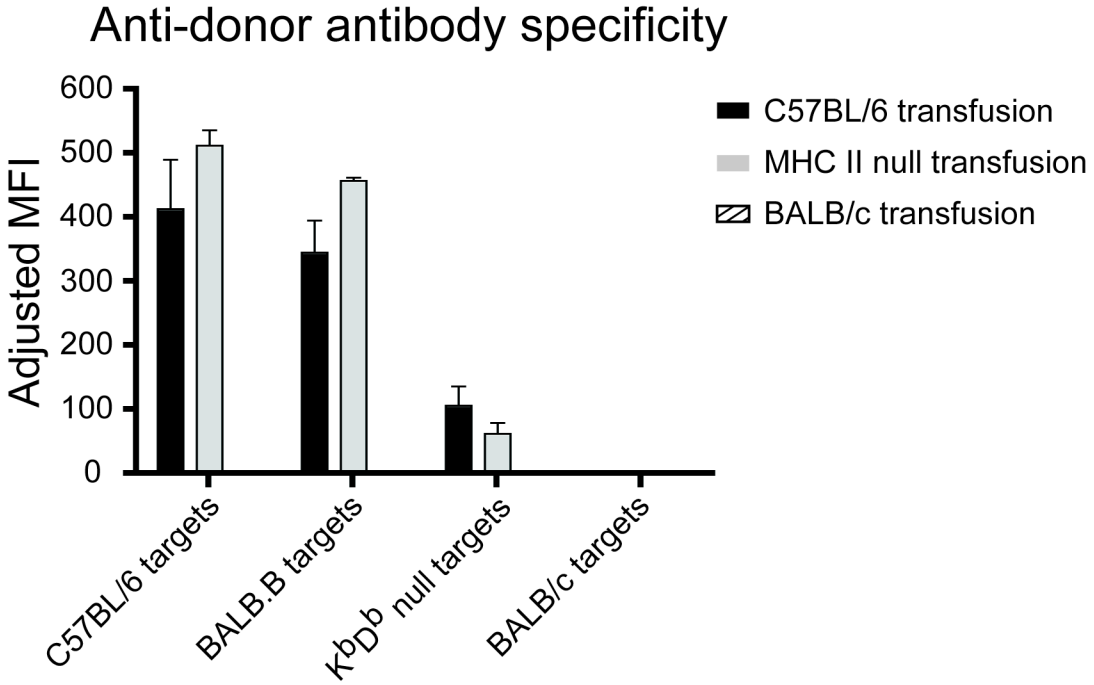

Anti-donor antibody specificity
Adjusted MFI
600
500
400
300
200
100
0
C57BL/6 transfusion
MHC II null transfusion
BALB/c transfusion
C57BL/6 targets
BALB.B targets
KbDb null targets
BALB/c targets

Figure 2.3

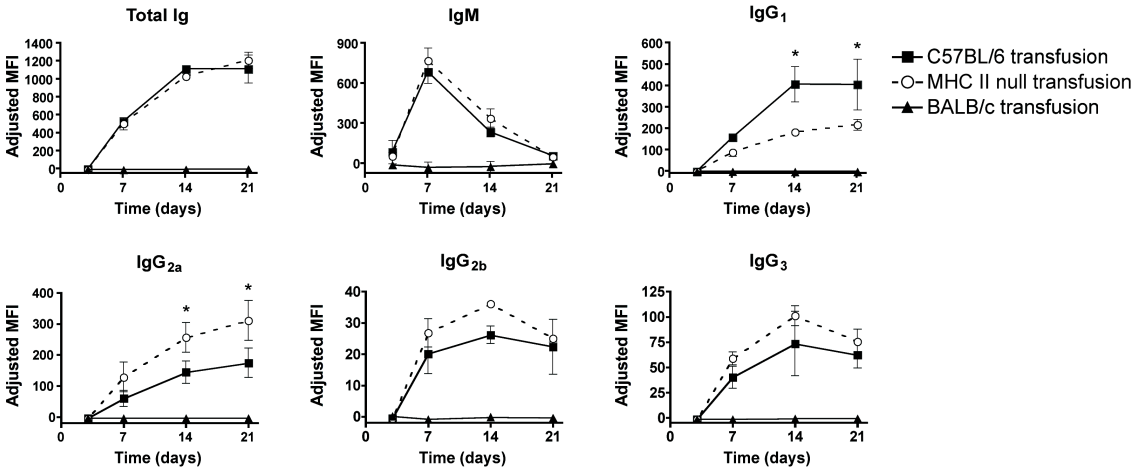
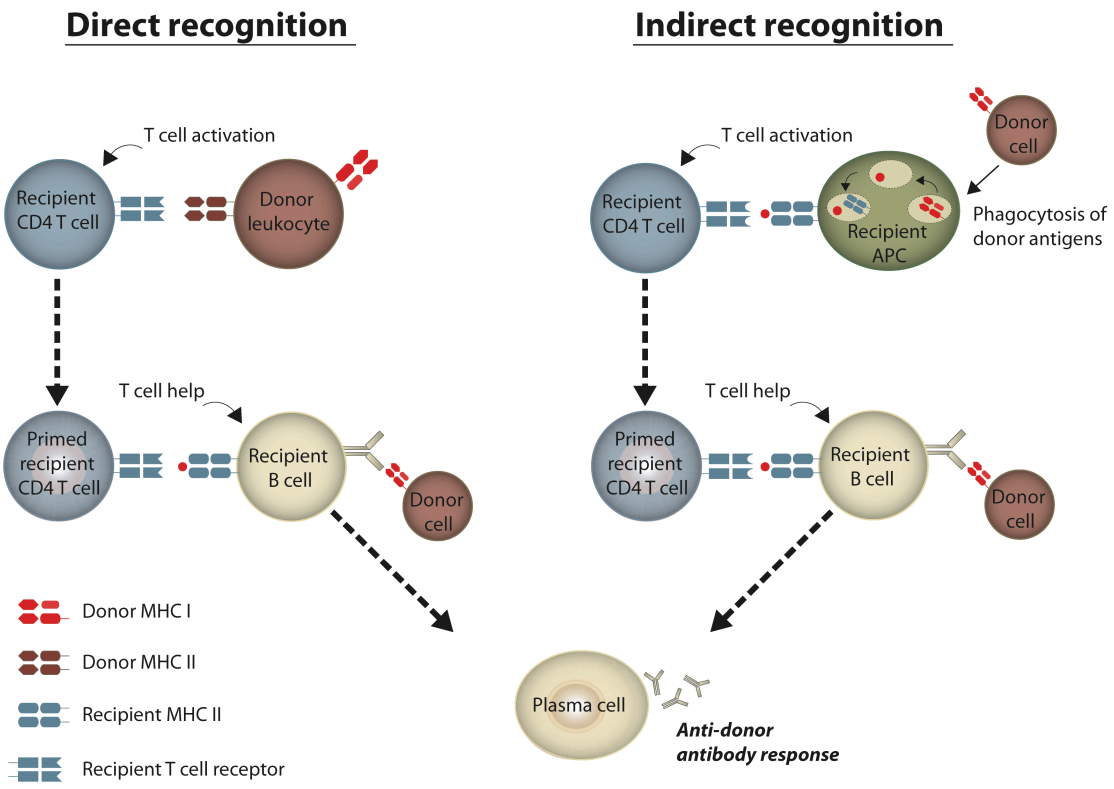

Total Ig
IgM
IgG1
IgG2a
IgG2b
IgG3
Adjusted MFI
Time (days)
C57BL/6 transfusion
MHC II null transfusion
BALB/c transfusion

Figure 2.4


Direct recognition
Indirect recognition
T cell activation
Recipient CD4 T cell
Donor leukocyte
T cell help
Primed recipient CD4 T cell
Recipient B cell
Donor cell
Donor MHC I
Donor MHC II
Recipient MHC II
Recipient T cell receptor
T cell activation
Donor cell
Recipient CD4 T cell
Recipient APC
Phagocytosis of donor antigens
T cell help
Primed recipient CD4 T cell
Recipient B cell
Donor cell
Plasma cell
Anti-donor antibody response

Chapter 3

Characterization and Visualization of the Allogeneic Platelet Specific $CD4^+$ T cell Response

Abstract

Alloantibodies are a clinically significant sequela of HLA mismatched platelet transfusion, potentially rendering patients refractory to ongoing transfusion support. The response is often IgG class switched, suggesting a role for $CD4^+$ T cell help; however, the requirement for $CD4^+$ T cells has never formally been tested. We hypothesized that anti-donor antibody production following platelet transfusion is critically dependent upon the $CD4^+$ T cell response. To test this hypothesis we developed a mouse system that allows the discrimination of an alloantigen specific $CD4^+$ T cell response against an authentic platelet antigen. We made use of the TCR75 $CD4^+$ T cell receptor transgenic mouse, which is specific for a single peptide derived from the BALB/c H-$2K^d$ MHC class I molecule presented by C57BL/6 MHC class II I-A^b ($K^d_{54\text{-}68}$/I-A^b). While depletion of $CD4^+$ T cells from C57BL/6 recipients prior to leukoreduced platelet rich plasma transfusion ablated the anti-donor antibody response, the adoptive transfer of TCR75 cells enhanced the response to a single transfusion. Division of the TCR75 cells was restricted to the spleen, and not the lymph nodes or liver. Splenectomy or blockade of the CD28 costimulatory pathways completely inhibited both $CD4^+$ T cell division and alloantibody production. These data support a critical role for $CD4^+$ T cell help in the humoral response to platelet transfusion and establish an effective mouse model for exploring $CD4^+$ T cell-specific modalities for preventing platelet alloimmunization.

Introduction

Roughly 1.5 million patients receive platelet transfusions each year in the United States alone. Although often lifesaving, transfusion also carries certain complications, including the development of donor-specific platelet alloantibodies. The exact incidence of platelet alloimmunization is difficult to determine, but it has been reported from a large clinical trial of acute myeloid leukemia patients that roughly 18% of recipients transfused with leukoreduced random donor platelets developed alloantibodies (1). These anti-donor antibodies have the potential to bind platelets expressing the donor antigen and mediate their clearance, rendering some immunized recipients refractory to subsequent transfusions. Furthermore, immunization across multiple allotypes can make it difficult and at times impossible to obtain sufficient units of compatible platelets to support the hemostasis needs of these alloimmunized patients.

The presence of $CD4^+$ T cells reactive to platelet antigens was first described in patients with chronic idiopathic thrombocytopenic purpura (ITP) (2-4). Platelet glycoprotein GP-IIb/IIIa peptide specific $CD4^+$ T cells were subsequently isolated from ITP patients (5). Recently, several groups have reported the identification and isolation of human platelet antigen (HPA)-1a–specific T cells (6, 7), which have been implicated in the development of HPA-1a–induced neonatal alloimmune thrombocytopenia (8). However, relatively little is known about the underlying cellular responses that result in anti-donor antibody production following allogeneic platelet transfusion, particularly with regards to the antigen specific T cell response.

Immune mediated platelet refractoriness is primarily characterized by an IgG response directed against donor human leukocyte antigens (9). Donor reactive $CD4^+$ T cells are considered to play a prominent role in the pathogenesis due to their capacity to provide B cell help for the production of a class-switched antibody. The potential isolation of alloreactive $CD4^+$ T cells for study from refractory patients is complicated and logistically challenging due to the lack of common antigenic epitopes. Murine models, however, offer the advantage of readily available genetically identical strains on well-defined inbred genetic backgrounds. Using mouse models of leukoreduced platelet transfusion, alloreactive $CD4^+$ T cells have been shown to be elicited coincident to the generation of anti-donor antibody (9-11). However, there is currently no mouse model that allows the visualization of a defined platelet-specific $CD4^+$ T cell alloresponse.

We developed a tractable mouse model to study the allospecific $CD4^+$ T cell response to platelet transfusions. We utilized a $CD4^+$ T cell receptor (TCR) transgenic mouse, TCR75, which is specific for a single peptide derived from the H-$2K^d$ MHC class I molecule presented by the MHC class II, I-A^b ($K^d_{54\text{-}68}$/I-A^b) (12). The adoptive transfer of TCR75 cells into C57BL/6 recipients enhanced the antibody response to a single BALB/c leukoreduced platelet-rich plasma (LR-PRP) transfusion. Division of the TCR75 cells was restricted to the spleen, and not the lymph nodes or liver. Furthermore, splenectomy or treatment with the costimulatory blockade reagent CTLA4-Ig ablated both $CD4^+$ T cell division and alloantibody production. These data support the critical role for T cell help and establish an effective mouse model for exploring $CD4^+$ T cell-specific modalities for preventing platelet alloimmunization.

Materials and Methods

Mice: Female C57BL/6 (H-2^b) mice were used at 6 to 8 weeks of age for recipients and C57BL/6 or BALB/c (H-2^d) were used at 8 to 12 weeks of age as donors (The Jackson Laboratory, Bar Harbor, ME). The Emory University Department of Animal Resources bred the 3A9 x B6.PL-Thy1.1 (H-2^k x H-2^b) and TCR x Thy1.1 mice. The TCR75 x Thy1.1 (H-2^b) mice were a generous gift from Dr. Pat Bucey (12). All studies and procedures were carried out in accordance with Emory University's Institutional Animal Care and Use Committee guidelines.

Platelet rich plasma preparation and transfusion: Donors were exsanguinated by orbital enucleation and whole blood was collected at a ratio of 1:8 in acid citrate dextrose. 2 mL of Dulbecco's phosphate buffered saline (DPBS) was added to each 1 mL of whole blood, mixed by inversion, and centrifuged at 80 x *g* for 10 minutes. Platelet-rich plasma (PRP) was collected, mixed by inversion, and re-spun at 80 x *g* for 10 minutes. Isolated PRP was pooled, and passed over a Neonatal Purecell PL High Efficiency Leukocyte Reduction Filter (Pall Corporation, Port Washington, NY).

To enumerate platelets and residual red blood cells, aliquots were stained with anti-CD41 PE or isotype control rat IgG_1 PE for platelets and anti-TER119 PE or isotype control rat IgG_{2b} PE for red blood cells (BD Pharmingen, San Jose, CA). FACS buffer was used in all staining (DPBS supplemented with 0.5% bovine serum albumin, 1 mM EDTA and 50 mM Hepes buffer, pH 7.2). Residual leukocytes were enumerated by staining with propidium iodide (Invitrogen, Carlsbad, CA) in a 1 g/L sodium citrate dihydrate solution with 0.7% Zap-oglobin II reagent (Beckman Coulter, Brea, CA) and

0.7% RNAse cocktail (New England Biolabs, Ipswich, MA). APC beads were added to determine absolute numbers (BD Pharmingen). Samples were run on a FACSort and analyzed using FlowJo (Treestar, Ashland, OR).

Following enumeration, the LR-PRP was normalized to 2 x 10^8 platelets/mL by pelleting at 1942 x g for 10 minutes and resuspended in Tyrode's buffer (1 mM HEPES, 137 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, and 5.5 mM glucose in Milli Q water, pH 7.4). 500 μL of LR-PRP suspension (10^8 total platelets) was transfused via tail vein and contained, on average, 5.8×10^4 RBCs (116 RBCs/μl), and fewer than 500 leukocytes (1/μl).

$CD4^+$ T cell depletion: $CD4^+$ T cells were depleted by intra-peritoneal (i.p.) injection with 250 μg of the monoclonal antibody GK1.5 at 4 and 2 days prior to each LR-PRP transfusion (Bio X Cell, West Lebanon, NH). Depletion was confirmed by staining representative animals for $CD4^+$ T cells in the blood, spleen and lymph nodes using the anti-CD4 antibody RM4-5 (BD Pharmingen), a monoclonal antibody with a binding site that does not compete with the epitope recognized by GK1.5.

Adoptive transfer of transgenic donor reactive cells: To track cell division, TCR75 whole splenocytes were labeled with 5 μM CFSE according to the manufacture's protocol (Invitrogen, Eugene, OR). 1 x 10^6 labeled cells in DPBS were adoptively transferred via tail-vein injection 24 hours prior to transfusion of LR-PRP.

Surgical splenectomy: Mice were anesthetized with ketamine and xylazine, and given buprenorphine for post-operative analgesia. A small incision was made in the left subcostal abdominal wall, the spleen was exteriorized, and removed by cauterizing the connecting vessels. The incision was closed using wound clips. Mice were kept on a heating pad and monitored for recovery. Control groups underwent sham surgery and were maintained under similar conditions. Wound clips were removed after two weeks and animals were allowed an additional week of recovery prior to use.

Seroanalysis: Sera from immunized animals was diluted 1:10 and incubated with $1x10^6$ BALB/c splenocyte target cells at 4°C for 30 minutes in FACS buffer. The cells were washed twice in FACS buffer and then incubated with anti-CD19 FITC, anti-CD3 PE, and goat anti-mouse Ig APC at 4°C for 30 minutes (BD Pharmingen). Cells were washed twice in FACS buffer and samples were run on a FACSort (BD) and analyzed using FlowJo (Tree Star, version 9.0.1). Determination of bound anti-donor antibodies utilized a $CD3^+CD19^-$ parent gate to avoid interference from anti-Ig binding to the BCR on B cells or Fc receptor binding. The adjusted mean fluorescent intensity (MFI) reported was the fluorescence signal from experimental sera minus the background signal from naïve C57BL/6 control serum. A positive response was determined if an adjusted MFI was greater than two standard deviations above the mean of sera from syngeneic transfused recipients.

Isolation of spleen, liver, and lymph node single cell suspensions: Spleen and lymph nodes were processed in DPBS by grinding the tissues between frosted glass slides. Red

blood cells were lysed with ammonium chloride (Sigma-Aldrich, St. Louis, MO). Lymphocytes were isolated from the liver as previously described (13), with minor modifications. Animals were euthanized by cervical dislocation before laparotomy. The portal vein was cut and the heart was perfused with 5-10 ml of 1% (w/v) collagenase D (Sigma-Aldrich) in DPBS. The liver was removed, dissevered and incubated in 1% (w/v) collagenase D in RPMI for 15 min at 37°C before being passed through a 100 μm nylon cell strainer. Hepatocytes were removed by discarding the pellets from a series of four centrifugations in RPMI at 20 x *g* for 4 min. The non-parenchymal cell suspension was pelleted at 300 x *g* for 10 min and then resuspended in a 44% percoll solution (Sigma-Aldrich). 5 mL of the cell suspension was underlaid with 3 mL of 67% percoll in a 15 mL conical tube and centrifuged at 863 x *g* for 20 min. The buffy coat interface was drawn off, washed once with RPMI, and the purified lymphocytes were resuspended in FACS buffer.

Results

Kinetics of the murine humoral response to multiple allogeneic platelet transfusions

To determine the kinetics of the anti-donor antibody response, C57BL/6 mice were transfused weekly with BALB/c LR-PRP. Platelets were collected using a soft-spin method similar in principle to that used for human platelet isolation in the United States and previously reported and characterized by our laboratory (14). Naïve C57BL/6 recipients did not mount a detectable anti-donor antibody response, to a single BALB/c LR-PRP transfusion monitored out to 28 days (Figure 3.1). However, successive weekly transfusions showed a compounding effect and all recipients were consistently alloantibody-positive following four transfusions.

Depletion of $CD4^+$ T cells prior to allogeneic LR-PRP transfusion ablates the anti-donor antibody response

To confirm previous studies suggesting that $CD4^+$ T cell help is required for the anti-donor antibody response (9, 11), we depleted $CD4^+$ T cells from recipient mice prior to LR-PRP transfusion. C57BL/6 recipients were treated with the monoclonal antibody GK1.5 at 4 days and 2 days prior to each LR-PRP transfusion. As shown by the representative flow plots, this antibody effectively eliminated $CD4^+$ T cells throughout the four weeks of our platelet transfusion protocol (Figure 3.2A). We measured a strong anti-donor antibody response in control animals receiving four weekly allogeneic LR-PRP transfusions. However, in GK1.5 treated recipients that lack $CD4^+$ T cells, we failed to detect an anti-donor antibody response above that seen for the syngeneic transfused controls (Figure 3.2B).

There is the potential that the CD4 depleting antibody also removed CD4 expressing non-T cell subsets that may be critical for the anti-platelet response (15-20). To control for this possibility, we performed transfusion experiments into $CD4^+$ T cell receptor transgenic recipients specific for an irrelevant peptide to the allogeneic donors. The 3A9 mouse is a TCR transgenic specific for the HEL_{48-62} peptide from hen-egg lysozyme presented by the MHC class II, $I\text{-}A^k$ (21). Greater than 95% of the $CD4^+$ T cell compartment of these mice express the transgenic T cell receptor (data not shown). In comparison to the strong humoral response by the non-transgenic littermates, the 3A9 recipients did not mount a detectable anti-donor antibody response following four weekly BALB/c LR-PRP transfusions (Figure 3.2C).

Humoral response to allogeneic platelets is augmented by increased precursor frequency of $CD4^+$ T cell help

To allow *in vivo* visualization of an allospecific $CD4^+$ T cell response during the development of the anti-donor antibodies we utilized the TCR75 mouse, which expresses a transgenic T cell receptor that reacts with a defined peptide derived from the $H\text{-}2K^d$ MHC class I molecule presented by the MHC class II, $I\text{-}A^b$ ($K^d_{54-68}/I\text{-}A^b$) (22). On a Thy1.1 congenic background, the TCR75 cells can be readily discriminated following adoptive transfer into Thy1.2 C57BL/6 recipients by flow cytometry (Figure 3.3A).

CFSE labeled TCR75 cells were adoptively transferred into C57BL/6 recipients, which were then transfused with LR-PRP 24 hours later. Proliferation was assessed by the dilution of the cytoplasmic CFSE dye. By day 5, the TCR75 cells had gone through

multiple rounds of division in response to BALB/c LR-PRP. No significant division was noted for animals transfused with either syngeneic C57BL/6 LR-PRP or BALB.B LR-PRP [congenic for the C57BL/6 MHC haplotype H-2^b on a BALB/c genetic background] (Figure 3.3B). Furthermore, the increased precursor frequency of allospecific $CD4^+$ T cells augmented the otherwise undetectable anti-donor antibody response to a single BALB/c LR-PRP transfusion as early as day seven (Figure 3.3C).

The spleen is the required site for the alloantigen specific $CD4^+$ T cell response to transfused platelets.

We next determined where anatomically this $CD4^+$ T cell activation was localized. C57BL/6 recipients were surgically splenectomized or sham-operated prior to the adoptive transfer of CFSE labeled TCR75 cells and given a single LR-PRP transfusion 24 hours later. TCR75 cells proliferated robustly in the spleen of intact recipients following transfusion with BALB/c LR-PRP (Figure 3.4A). However, division was observed in only trace numbers in the liver and lymph nodes of these intact animals. The activation of these antigen specific splenic $CD4^+$ T cells correlated with a strong anti-donor antibody response (Figure 3.4C).

Although the TCR75 cells were present in detectable numbers in splenectomized recipients, we failed to observe significant division in either the liver or lymph nodes (Figure 3.4B). Furthermore, in spite of the high precursor frequency of alloreactive cells, these splenectomized recipients did not mount a detectable anti-donor antibody response (Figure 3.4C). In addition, the strong endogenous anti-donor antibody response detected in sham-operated recipients was also severely reduced for

splenectomized recipients given four weekly BALB/c LR-PRP transfusions (Figure 3.4D).

T cell costimulatory blockade effectively inhibits the response to allogeneic LR-PRP transfusion

To test the efficacy of inhibiting the anti-platelet antibody response by blocking the CD28/B7 signaling pathway, we utilized CTLA4-Ig, which also known as Abatacept. Abatacept is an FDA approved drug for the treatment of rheumatoid arthritis that selectively binds to CD80 and CD86 on antigen presenting cells, thus inhibiting CD28 mediated T cell activation (23). CFSE labeled TCR75 cells were adoptively transferred to C57BL/6 recipients and 24 hours later these animals were transfused with BALB/c LR-PRP, with or without the administration of CTLA4-Ig. As shown by the representative histograms, TCR75 cells in the spleen go through multiple rounds of division in response to BALB/c LR-PRP by day 5 (Figure 3.5A) and these recipients mount a rapid anti-donor antibody response (Figure 3.5B). However, when CTLA4-Ig was administered at the time of BALB/c LR-PRP transfusion, TCR75 division was inhibited (Figure 3.5A) and the anti-donor antibody response was undetectable (Figure 3.5B). Furthermore, the endogenous C57BL/6 anti-donor antibody response was similarly ablated when CTLA4-Ig was given concurrently with each of four weekly LR-PRP transfusions (Figure 3.5C).

Discussion

We have developed a novel mouse model to visualize and characterize the alloantigen specific $CD4^+$ T cell response to fully mismatched LR-PRP transfusion. We utilized the TCR75 transgenic mouse, which expresses a transgenic $CD4^+$ T cell receptor specific for an authentic alloantigen derived from the BALB/c MHC class I molecule presented by the C57BL/6 MHC class II. Adoptive transfer of the TCR75 cells augmented the normally undetectable anti-donor antibody response to a single BALB/c LR-PRP transfusion. The $CD4^+$ T cell response was localized to the spleen, and not the lymph nodes or liver. Furthermore, the anti-donor antibody response was dependent upon the presence of the spleen and the CD28 T cell costimulatory pathway.

In contrast to antigens from transplanted solid organs or microbial infections that frequently drain to the local lymphatics, platelets are largely sequestered to the peripheral circulation for which the spleen is the primary organ responsible for surveying and promoting immune responses to blood born antigens. Clearance of radiolabeled platelets is localized predominantly in the spleen (24, 25), and splenectomy is performed in select clinical situations in efforts to raise platelet counts (26, 27). Thus, it is not unexpected that the spleen would also be involved in alloimmunization following platelet transfusion. It has been reported that allogeneic platelets phagocytosed *in vitro* by recipient adherent splenic macrophages could stimulate the proliferation of previously sensitized splenocytes under certain conditions (9). Similar methods were used to show that these ex vivo-manipulated macrophages were sufficient to initiate an anti-donor antibody response when adoptively transferred to naïve recipients (11). Analogous strategies have been used in efforts to augment the often

ineffective immune response against cancer antigens by ex vivo loading of tumor specific peptides to APCs, with varying success (28). These data do not necessarily demonstrate that the spleen is the site of $CD4^+$ T cell priming *in vivo*, but they do support the capacity of splenic APCs to process and present platelet derived antigens. Previous studies in our laboratory have shown that the spleen is required for the allogeneic response to red blood cells (29), however a similar requirement for platelets has not been described.

Our data suggest that the splenic microenvironment is necessary for $CD4^+$ T-cell priming and proliferation in response to allogeneic LR-PRP transfusion. This may be due to unique aspects of the splenic architecture (30), specialized splenic APC populations (30-32), or the spleens role in the maintenance of extrasplenic B-1a cells (33). The spleen may also be required for priming of naïve antigen-specific B cells, however monitoring the B cell response was beyond the scope of the current manuscript. Splenectomized recipients did not suffer from a general defect in their ability to respond to alloantigen, as splenectomized recipients were capable of mounting an anti-donor antibody response to whole blood transfusion, although with slightly delayed kinetics and of lower magnitude than sham-operated recipients (data not shown).

In addition to the spleen, the liver may also serve as a site of platelet clearance (34). The liver is characterized as a relatively tolerogenic organ due to its distinct intrahepatic lymphocyte populations (35, 36). In the splenectomized recipients described herein, donor platelets may have been phagocytosed by liver APCs, however the TCR75 cells present in the hepatic environment remained undivided (Figure 3.4B). It is possible that the conditions of hepatic alloantigen presentation not only lead to a

lack of immunity, but also to active tolerance. Additional experimentation is required to investigate the patterns of platelet consumption by liver APC subsets, as well as the tolerogenic potential of antigen presentation to $CD4^+$ T cells in this setting.

Although we have demonstrated that $CD4^+$ T cell help is required for the anti-donor antibody response to allogeneic LR-PRP transfusion, this does not necessarily exclude the involvement of the donor platelet itself in modulating the response. As an antigen, platelets share several similarities with T cell independent antigens. Type 2 T independent antigens are molecules containing polyvalent B cell epitopes capable of cross-linking the B cell receptor to an extent that $CD4^+$ T cell help is not required for antibody production. With the expression of donor-MHC class I on their surface, particularly in the context of an immobilized thrombus, platelets may also provide a similar scaffold for cross-linking the B cell receptor. Furthermore, platelets can secrete many inflammatory mediators such as IL-1α, IL-8, RANTES, and TGF-β, as well as CD154 (37, 38). Platelet derived CD154 has been shown to directly stimulate B cell proliferation and antibody production (39). Thus, platelets have the potential to provide both B cell receptor cross-linking and costimulation to induce alloantibody production in the absence of $CD4^+$ T cell help in some settings. However, the current findings indicate that MHCI on platelets is not a T cell independent antigen for fresh platelets transfused into healthy recipients.

There are important caveats to our experimental design that diverge from what is typically found in the clinical setting. First, these experiments were performed under relatively non-inflammatory conditions. Patients receiving platelet transfusions often have significant underlying pathology, such as trauma, infection, or disease. Previous

studies in our laboratory have shown that the inflammatory status of the recipient alters the particular APC subsets consuming RBC and modulates the antibody response (40, 41); a similar effect may also occur following platelet transfusion. Second, platelet units are stored for up to 5 days in the US prior to transfusion. It is possible that storage may activate platelets and cause the release of inflammatory cytokines, thus, increasing the antigenicity of the transfusion. A similar phenomenon has recently been described for stored RBCs (42). Third, platelet refractoriness is typically defined in the clinical setting as a failure to raise platelet counts following two consecutive transfusions (43). Although we assessed the emergence of the anti-donor antibody response, we did not determine if these anti-donor antibodies were impacting platelet post-transfusion survival. Future experiments, potentially using GFP-platelets, similar to what has been published by our laboratory for tracking RBC post-transfusion survival (44), may be used to address this issue.

In summary, we have developed a novel mouse system to study the critical role of $CD4^+$ T cells in the allogeneic platelet antibody response. Anti-donor antibody production was found to be $CD4^+$ T cell-dependent, as well as requiring an intact spleen and CD28 costimulation. These data are important for providing a thorough understanding of the mechanisms behind platelet alloimmunization, which may lead to novel immunomodulatory therapeutic approaches, potentially including strategies such as costimulatory blockade. Actively preventing immune mediated refractoriness rather than managing patients when anti-donor antibodies have already been elicited may prove to be a more efficient course of care. This could potentially avoid bleeding

complications in multiply transfused patients, as well as preserving precious platelet resources, which are chronically in short supply.

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Figure Legends

Figure 3.1. ***Kinetics of the anti-donor antibody response following LR-PRP transfusion.*** C57BL/6 recipients were transfused weekly, as noted, with BALB/c LR-PRP. The total Ig anti-donor antibody response was quantified by indirect immunofluorescence against BALB/c splenocyte targets. Combined data from three independent experiments with three mice per group (n=9).

Figure 3.2. ***Antigen specific CD4$^+$ T cell help is required for the anti-donor antibody response to transfused LR-PRP.*** **(A)** Analysis of CD4$^+$ T cell levels following treatment with the CD4-depleting antibody GK1.5. Recipients were injected i.p. at 4 and 2 days prior to each LR-PRP transfusion. CD4$^+$ T cells levels are shown from the spleen of representative animals at days 0 and 21. CD4$^+$ T cells levels in treated animals (center panels) were comparable to isotype control (right panels) throughout the time of LR-PRP transfusion. Flow plots are representative of two independent experiments with two mice per group. **(B)** C57BL/6 recipients were either untreated or injected i.p. with GK1.5, then received four weekly BALB/c or syngeneic LR-PRP transfusions. The total Ig anti-donor antibody response was quantified by indirect immunofluorescence against BALB/c splenocyte targets. **(C)** Non-transgenic littermates of 3A9 TCR transgenic mice received four weekly BALB/c LR-PRP transfusions. The total Ig anti-donor antibody response was quantified by indirect immunofluorescence against BALB/c splenocyte targets. Combined data from three independent experiments with three mice per group (n=9). Error bars indicate standard error of the mean.

Figure 3.3. ***Adoptive transfer of TCR75 cells augments the anti-donor antibody response.*** **(A)** 1 x 10^6 CFSE labeled TCR75 x Thy1.1 splenocytes were adoptively transferred into C57BL/6 recipients. The TCR75 cells can be readily discriminated from the Thy1.2$^+$ recipient cells by the expression of the Thy1.1 congenic marker. **(B)** C57BL/6 recipients were adoptively transferred with 1 x 10^6 CFSE labeled TCR75 splenocytes. 24 hours later, these mice received a single BALB/c (dark line), BALB.B (dashed line) or syngeneic C57BL/6 LR-PRP (shaded histogram) transfusion. TCR75 division was assessed in the spleen at day 5 by dilution of the CFSE dye on the CD4$^+$ Thy1.2$^+$ cells. Representative histograms are from three independent experiments with three mice per group (n=9). **(C)** Sera was collected from the adoptively transferred C57BL/6 recipients 7 and 14 days post-LR-PRP transfusion. The total Ig anti-donor antibody response was quantified by indirect immunofluorescence against BALB/c splenocyte targets. Combined data from three independent experiments with three mice per group (n=9). Error bars indicate standard error of the mean.

Figure 3.4. ***The splenic microenvironment is required for antigen-specific CD4$^+$ T cell division and the anti-donor antibody response.*** **(A)** Intact and **(B)** splenectomized recipients were adoptively transferred with 1 x 10^6 CFSE labeled TCR75 splenocytes, followed by a single LR-PRP transfusion 24 hours later. Five days later division was assessed in the spleen, liver, and lymph nodes by dilution of the CFSE dye on the CD4$^+$ Thy1.2$^+$ cells. In all panels, dark lines represent mice transfused with BALB/c LR-PRP and gray shaded histograms represent control mice transfused with syngeneic LR-PRP.

Representative histograms are from three independent experiments, with three mice per group (n=9). **(C)** Intact and splenectomized recipients were adoptively transferred with 1 x 10^6 CFSE labeled TCR75 splenocytes, followed by a single BALB/c or C57BL/6 LR-PRP transfusion 24 hours later. The total Ig anti-donor antibody response was quantified by indirect immunofluorescence against BALB/c splenocyte targets 7 and 14 days post-LR-PRP transfusion. **(D)** Intact and splenectomized C57BL/6 recipients received four weekly BALB/c or C57BL/6 LR-PRP transfusions. The total Ig anti-donor antibody response was quantified by indirect immunofluorescence against BALB/c splenocyte targets 7, 14, 21, and 28 days post-transfusion. Combined data from three independent experiments with three mice per group (n=9). Error bars indicate standard error of the mean.

Figure 3.5. ***CTLA4-Ig inhibits both the CD4$^+$ T cell and humoral response to allogeneic LR-PRP transfusion.*** (A) C57BL/6 recipients were adoptively transferred with 1 x 10^6 CFSE labeled TCR75 splenocytes, followed by a single LR-PRP transfusion 24 hours later with or without i.p. injection of 250µg CTLA4-Ig. Five days later TCR75 division was assessed in the spleen by dilution of the CFSE dye on the CD4$^+$ Thy1.2$^+$ cells. In both panels, dark lines represent mice transfused with BALB/c LR-PRP and gray shaded histograms represent control mice transfused with syngeneic LR-PRP. Representative histograms are from three independent experiments, with three mice per group (n=9). **(B)** C57BL/6 recipients were adoptively transferred with 1 x 10^6 CFSE labeled TCR75 splenocytes, followed by a single BALB/c or C57BL/6 LR-PRP transfusion 24 hours later with or without i.p. injection of 250µg CTLA4-Ig. The total

Ig anti-donor antibody response was quantified by indirect immunofluorescence against BALB/c splenocyte targets 7 and 14 days post-transfusion. **(C)** C57BL/6 recipients received four weekly BALB/c or C57BL/6 LR-PRP transfusions with or without i.p. injection of 250μg CTLA4-Ig concurrent with each transfusion. The total Ig anti-donor antibody response was quantified by indirect immunofluorescence against BALB/c splenocyte targets 7, 14, 21, and 28 days post-LR-PRP transfusion. Combined data from three independent experiments with three mice per group (n=9). Error bars indicate standard error of the mean.

Figure 3.1

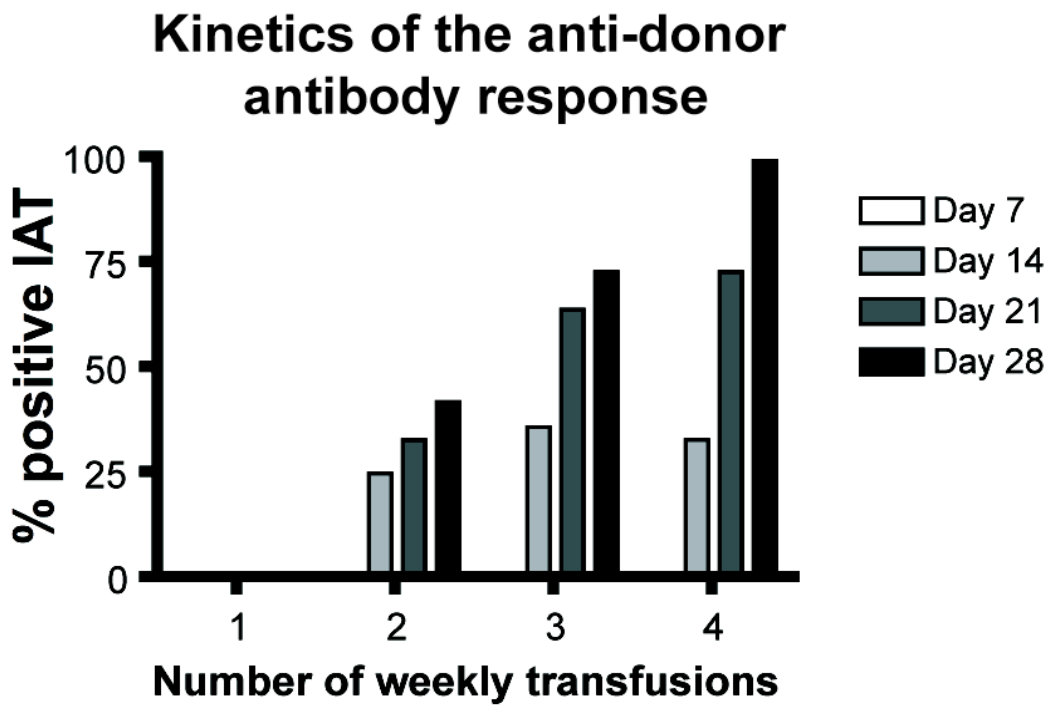

Kinetics of the anti-donor antibody response
% positive IAT
100
75
50
25
0
1
2
3
4
Number of weekly transfusions
Day 7
Day 14
Day 21
Day 28

Figure 3.2

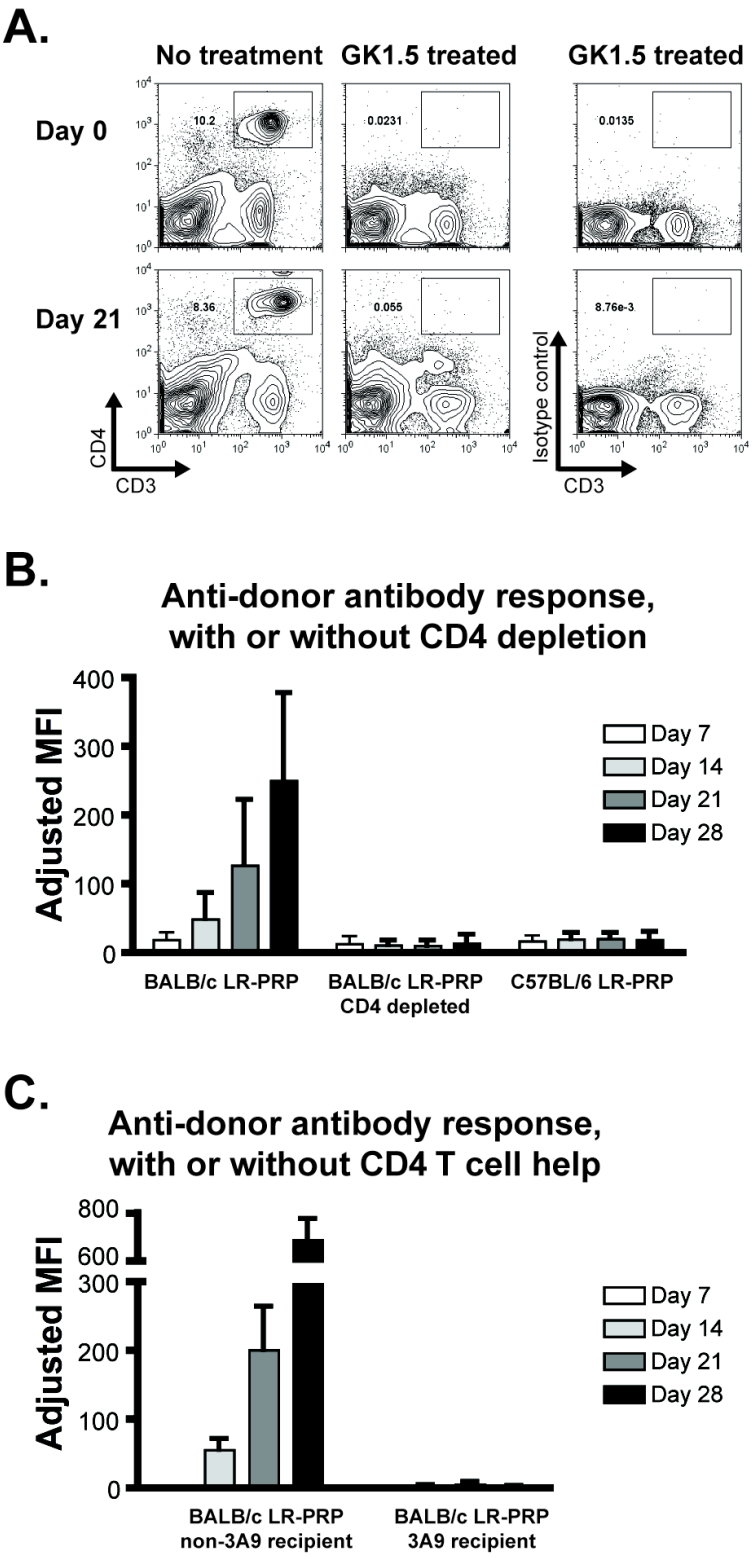

A.
No treatment
GK1.5 treated
GK1.5 treated
Day 0
Day 21
10.2
0.0231
0.0135
8.36
0.055
8.76e-3
CD4
CD3
Isotype control
CD3
B.
Anti-donor antibody response,
with or without CD4 depletion
Adjusted MFI
400
300
200
100
0
Day 7
Day 14
Day 21
Day 28
BALB/c LR-PRP
BALB/c LR-PRP
CD4 depleted
C57BL/6 LR-PRP
C.
Anti-donor antibody response,
with or without CD4 T cell help
Adjusted MFI
800
600
300
200
100
0
Day 7
Day 14
Day 21
Day 28
BALB/c LR-PRP
non-3A9 recipient
BALB/c LR-PRP
3A9 recipient

Figure 3.3

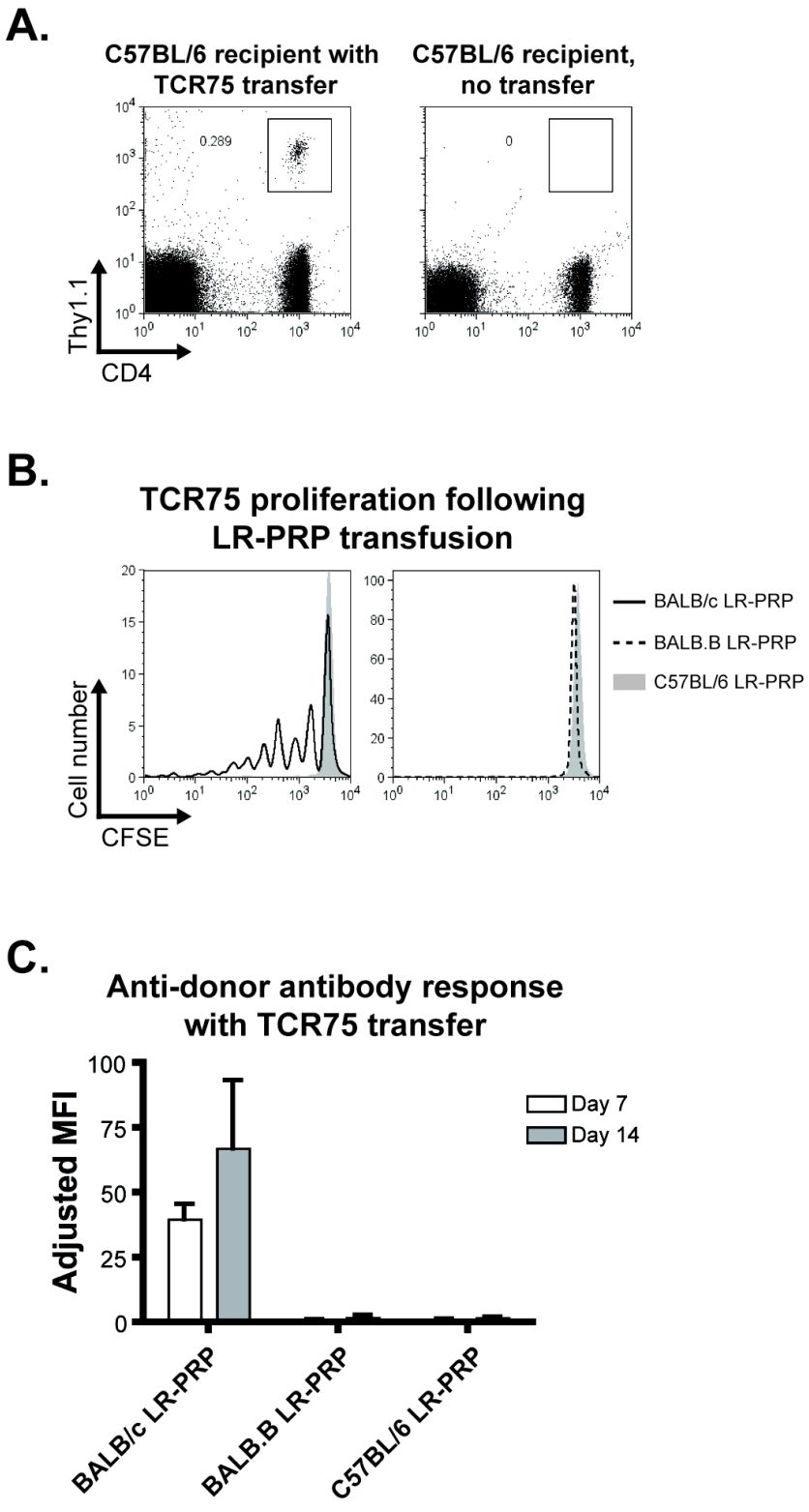

A.
C57BL/6 recipient with TCR75 transfer
C57BL/6 recipient, no transfer
0.289
0
Thy1.1
CD4
B.
TCR75 proliferation following LR-PRP transfusion
Cell number
CFSE
BALB/c LR-PRP
BALB.B LR-PRP
C57BL/6 LR-PRP
C.
Anti-donor antibody response with TCR75 transfer
Adjusted MFI
Day 7
Day 14
BALB/c LR-PRP
BALB.B LR-PRP
C57BL/6 LR-PRP

Figure 3.4

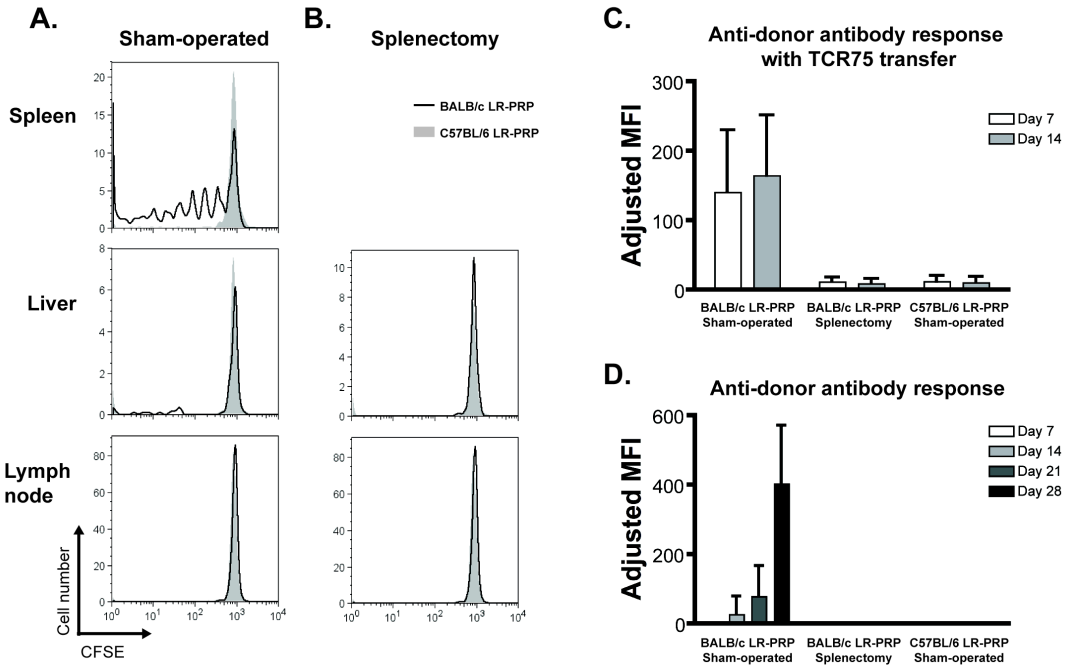
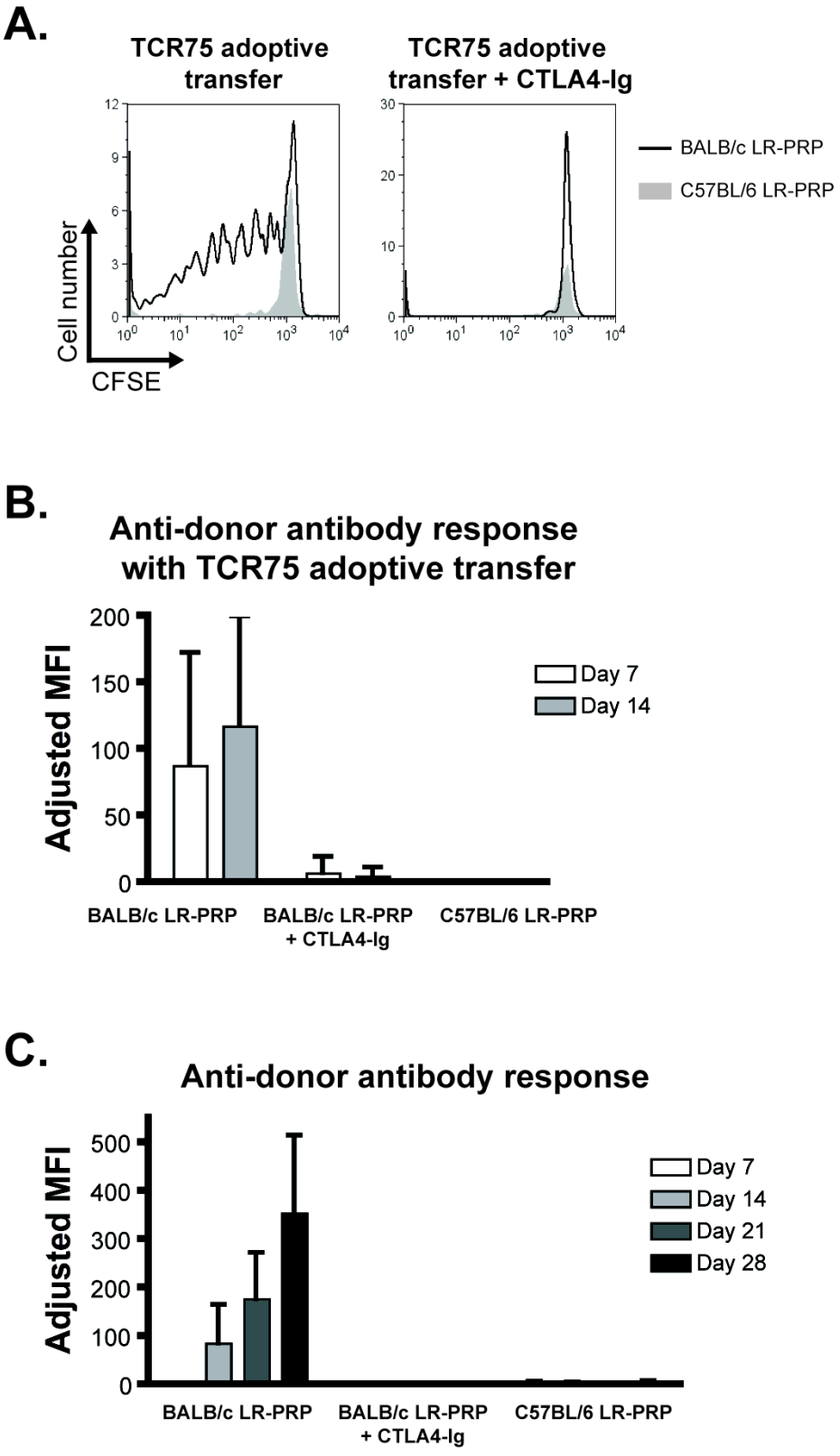

A.
Sham-operated
B.
Splenectomy
BALB/c LR-PRP
C57BL/6 LR-PRP
Spleen
Liver
Lymph node
Cell number
CFSE
C.
Anti-donor antibody response with TCR75 transfer
Adjusted MFI
Day 7
Day 14
BALB/c LR-PRP Sham-operated
BALB/c LR-PRP Splenectomy
C57BL/6 LR-PRP Sham-operated
D.
Anti-donor antibody response
Adjusted MFI
Day 7
Day 14
Day 21
Day 28
BALB/c LR-PRP Sham-operated
BALB/c LR-PRP Splenectomy
C57BL/6 LR-PRP Sham-operated

Figure 3.5


A.
TCR75 adoptive transfer
TCR75 adoptive transfer + CTLA4-Ig
BALB/c LR-PRP
C57BL/6 LR-PRP
Cell number
CFSE
B.
Anti-donor antibody response with TCR75 adoptive transfer
Adjusted MFI
Day 7
Day 14
BALB/c LR-PRP
BALB/c LR-PRP + CTLA4-Ig
C57BL/6 LR-PRP
C.
Anti-donor antibody response
Adjusted MFI
Day 7
Day 14
Day 21
Day 28
BALB/c LR-PRP
BALB/c LR-PRP + CTLA4-Ig
C57BL/6 LR-PRP

Chapter 4

A Novel Mouse Model of Red Blood Cell Storage and Post-Transfusion in Vivo Survival

Abstract

Storage of RBCs is necessary for an adequate blood supply. However, reports have identified potential negative sequelae of transfusing stored RBCs. An animal model would be useful to investigate the pathophysiology of transfusing stored RBCs. However, it has been reported that storage of rat RBCs in CPDA-1 resulted in an unexpected sudden decline in post-transfusion survival. We developed a mouse model of RBC storage and transfusion to assess survival kinetics of mouse RBCs. RBCs expressing green fluorescent protein were collected in CPDA-1, filter leukoreduced, adjusted to a 75% hematocrit, and stored at 4ºC. At weekly intervals, stored RBCs were transfused into C57BL/6 recipients. RBC survival was measured by flow cytometry and ^{51}Chromium labeling. Phosphatidylserine externalization and CD47 expression was also evaluated. Mean 24-hour survival of transfused RBCs was 99%, 91%, 64%, 54%, 30%, and 18% following 0, 7, 14, 21, 28, and 35 days of storage, respectively. Stored RBCs showed an initial rapid clearance with subsequent extended survival. Increased surface phosphatidylserine and decreased CD47 expression was also observed. Mouse RBCs showed a progressive decline in survival, as a function of storage time, unlike the precipitous loss of viability previously reported for rat RBCs. Moreover, changes in the measured surface markers were analogous to trends reported for human RBCs. Together, these findings provide an initial characterization of a novel mouse model of RBC storage with the potential to serve as an experimental platform for studying the pathophysiological consequences of transfusing stored RBCs.

Introduction

In order to ensure an adequate supply of transfusable blood, red blood cells (RBCs) are maintained at 4°C for various times depending on the particular storage solution. CPDA-1, an FDA-approved solution, allows for the storage of human RBCs for up to 35 days. FDA standards currently require that following transfusion, an average of >75%, SD ± 9% of RBCs circulate for at least 24 hours [1]. Clinical use of RBCs stored under these or similar conditions has been the standard of care for decades. RBC transfusions can rapidly reverse the symptoms of anemic patients suffering from clinically evident hypoxia. However, there are studies to suggest that transfusion of stored RBCs may have deleterious effects in certain patient populations [2,3]. Although these studies are of potential importance, cohort studies may contain confounders, such as severity of illness in the study population. Thus, whether transfusing stored RBCs directly leads to adverse outcomes remains controversial [4].

The potential negative sequelae of blood transfusion may be explained by several hypotheses, including accumulation of molecules that prime leukocyte inflammation [5], oxidized proteins and lipids [6], and perturbations of nitric oxide biology [7]. To test these and other hypotheses under controlled and ethically permissible settings, an appropriate animal model of RBC storage and transfusion would be of great utility. Murine models would be particularly valuable, due to the availability of numerous immunological tools, strains, and genetically manipulated variants. However, murine models involve several challenges, the most important of which is whether the biology of stored murine RBCs accurately reflects that of human RBCs. This is particularly relevant in light of published data reporting that rat RBCs stored in CPDA-1 rapidly deteriorate with only a

26% 24-hour survival after 15 days [8]. In contrast, a published abstract reported a 24-hour post-transfusion survival of 80% for rat RBCs stored for 28 days in CPDA-1 [9]. The reason for this difference is unclear.

Given the considerable advantages that a murine model would provide, and in the context of the discrepancy regarding stored rat RBCs, we tested the properties of mouse RBCs processed and stored under similar conditions to human RBC storage. A transgenic mouse strain expressing enhanced green fluorescent protein ($H2K^b$-eGFP) [10] in RBCs was utilized to allow for detailed post-transfusion analysis of stored RBCs. We demonstrate that 24-hour post-transfusion survival of stored mouse RBCs showed relatively similar kinetics to that reported for human RBCs, when indexed for the normal lifespan of mouse RBCs. We also report that mouse RBCs developed changes in membrane molecules with a trend comparable to those described for human RBCs. Together, these results demonstrate the potential utility of a mouse system to study the effects of storage and transfusion of RBCs.

Methods

Mice: Adult 6- to 8-wk-old C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). $H2K^b$-eGFP mice [10] were a generous gift from Dr. Derek A. Persons (St. Jude Children's Research Hospital, Memphis, TN), and were bred by the Emory Division of Animal Resources Animal Husbandry service. All studies and procedures were carried out in accordance with Emory University and/or Columbia University Institutional Animal Care and Use Committee guidelines.

Red blood cell harvest and storage: For each experiment, eight to ten donor $H2K^b$-eGFP mice were exsanguinated by orbital enucleation into a 50 mL conical tube containing 2.8 mL of CPDA-1, yielding roughly 8 to 10 mL of blood. The total volume was then brought up to 20 mL with additional freshly obtained blood from wild-type C57BL/6 donors resulting in a final CPDA-1 concentration of 14%. The total collection time was approximately 20 to 30 minutes. Blood was passed through a Neonatal High Efficiency Leukocyte Reduction Filter for Red Blood Cells (Pall Biomedical Products Company, East Hills, NY). The leukoreduced blood was centrifuged for 10 minutes at 1350 rpm and supernatant was partially removed to obtain a hematocrit of approximately 75%. The blood was then transferred to multiple 500 μL Eppindorf tubes, leaving a small residual air space, and stored in the dark at 4°C until use. The CPDA-1 in these studies was obtained directly from DEHP-PVC human blood storage bags.

Phenotypic analysis: At the indicated time points stored RBCs were assessed for CD47 expression and Annexin V binding. Freshly collected blood was used as a baseline control. Eryptotic RBCs were generated by incubating freshly isolated RBCs for 2 hours at 37°C in RPMI-1640 containing 0.4 mM $CaCl_2$, then stimulated for an additional 30 minutes with 8 μM ionomycin. All RBC suspensions were washed three times with PBS and diluted 1:1000 in FACs buffer (DPBS supplemented with 0.5% bovine serum albumin, 1 mM EDTA and 50 mM Hepes buffer, pH 7.2) for anti-CD47 staining or binding buffer (10 mM Hepes buffer, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) for Annexin V staining. For CD47 staining, diluted RBCs were incubated with biotinylated anti-mouse CD47 or biotinylated goat IgG (isotype matched control) (R&D Systems, Minneapolis, MN) at 4°C for 30 minutes. RBCs were washed twice with FACs buffer and then incubated with streptavidin APC (BD Pharmingen, San Jose, CA) at 4°C for 30 minutes. RBCs were washed twice with and re-suspended in 500 μL FACs buffer prior to analysis. For Annexin V staining, 100 μL of diluted RBCs were incubated with 5 μL Annexin V-APC (BD Pharmingen) at room temperature in binding buffer for 20 minutes. An additional 400 μL of binding buffer was then added prior to analysis. Anti-CD47 and Annexin V binding were assessed by flow cytometry and analyzed using FlowJo Software (Treestar, Ashland, OR).

Assessment of RBC survival by flow cytometry: Following 0, 7, 14, 21, 28, and 35 days of storage, RBCs were diluted 1:5 in PBS and 500 μL was transfused by tail vein injection into each recipient mouse (3-4 mice per experimental group). At 10 and 30 minutes, 1, 2, 18 and 24 hours, and weekly post transfusion time points, recipients were

retro-orbitally bled into heparinized tubes and 3 μL of blood was diluted into 1 mL of FACs buffer. The percentage of donor eGFP$^+$ RBCs was determined flow cytometry. The percentage of donor RBCs at time 0 was calculated based on a best-fit regression curve over the initial 24 hours (GraphPad Software, La Jolla, CA). 24-hour survival was determined by the ratio of the percentage of eGFP$^+$ RBCs at 24 hours divided by the percentage of eGFP$^+$ RBCs estimated for time 0.

Assessment of RBC survival by ^{51}Chromium labeling: Fresh RBCs and RBCs stored for 14 days in CPDA-1 were each resuspended to a 33% hematocrit with endotoxin-free normal saline and 1 mL aliquots were then incubated with 140 μL of a 1 mCi/mL solution of ^{51}Chromium (GE Healthcare, Piscataway, NJ) for 30 minutes at room temperature. Labeled RBCs were washed 3 times with normal saline and re-suspended to a 50% hematocrit. C57BL/6 mice were transfused with 100 μL of ^{51}Chromium-labeled RBCs by tail vein injection. Blood was collected by retro-orbital bleed immediately following transfusion and at 1, 2 and 24 hours post-transfusion. The collected blood was centrifuged and the height of the packed RBC column was measured. Percent survival was calculated as 100 multiplied by the ratio of counts per minute per millimeter of the RBC column height at each time point versus the immediate post-transfusion time point.

Statistical Analysis

To determine the significance of the flow cytometry survival data, we utilized a two way ANOVA analysis with a Bonferroni post-test for individual value comparison. For

Annexin V and CD47 data analysis, a non-parametric test (Kruskal-Wallis) was utilized with Dunn's post-test for individual value comparison. A two-tailed unpaired Student's t test was used for ^{51}Chromium survival data. A level of significance of a=0.05 was set for all tests. All analysis was performed using the GraphPad Prism Software Suite (GraphPad Software).

Results

Use of eGFP transgenic RBC donors allows tracking of RBC survival in vivo

In the current study, it was imperative to minimize deviations from the collection and storage conditions used with human RBCs. Moreover, it was important to avoid possible biochemical alterations to the RBCs. Therefore, we developed a method to track transfused RBCs, using $H2K^b$-eGFP donor mice [10].

To establish detection parameters, $eGFP^+$ RBCs from freshly obtained blood were characterized by flow cytometry. An RBC gate was established using forward and side scatter properties. Cells in the RBC gate were uniformly positive when stained for the mouse RBC surface marker Ter-119, as compared to an isotype matched control antibody (Figure 4.1A). Essentially all RBCs from $H2K^b$-eGFP mice express a high, uniform level of eGFP fluorescence; no eGFP is detected in C57BL/6 control mice (Figure 4.1B). Transfusion of $H2K^b$-eGFP RBCs into wild-type mice resulted in a distinct and traceable population of $eGFP^+$ donor RBCs. These events are not due to non-specific background, as significant numbers of $eGFP^+$ events are not detected in untransfused recipients (Figure 4.1C).

$H2K^b$-eGFP donors were exsanguinated and whole blood was collected to a final concentration of 14% CPDA-1 by volume, to mimic the conditions used for human RBC storage. Prior to storage, the blood was leukoreduced using a Pall neonatal leukoreduction filter. To decrease the number of $H2K^b$-eGFP mice required, the total blood volume was increased by mixing C57BL/6 and $H2K^b$-eGFP blood prior to leukoreduction. This did not interfere with the subsequent analysis, as eGFP-negative RBCs are excluded by our flow cytometry gating methods as described above (Figure

4.1C). After leukoreduction, the blood was centrifuged and supernatant was removed to adjust the final hematocrit to approximately 75%. The blood was then aliquoted into 500 μL Eppendorf tubes, allowing a small pocket of air at the top. These "units" were sealed and kept at 4°C for up to 35 days.

Analysis of phosphatidylserine externalization and CD47 expression

Increased surface expression of phosphatidylserine [11,12] and decreased CD47 expression occur with stored human RBCs [12,13]. To analyze these parameters following storage of mouse RBCs, levels of anti-CD47 and Annexin V staining were assessed on Ter-119$^+$ eGFP$^+$ events within the established RBC gate and were quantified as mean fluorescence intensity. Each stained sample was compared to freshly isolated C57BL/6 RBCs and a fold change was calculated. Analysis of the combined data of 4 independent experiments demonstrated that the stored eGFP$^+$ RBCs did not exhibit a statistically significant increase in Annexin V binding until a 2-fold increase by day 28 and a 3-fold increase by day 35 (Figure 4.2A). A representative histogram is shown displaying the shift that occurred during storage. *In vitro* induced eryptotic RBCs are presented in the shaded histogram as a positive control for Annexin V staining.

There was a progressive decline in CD47 expression that reached statistical significance by 28 days and continued through day 35 (Figure 4.2B). The representative histogram shows the gradual loss of surface CD47 levels on the RBC population following storage. The decline in CD47 was not an artifact of a nonspecific decrease in cell size, as no decrease in the intensity of Ter-119 staining was observed on this same population (data not shown). The induced eryptotic RBCs did not show a significant

decrease in CD47 expression, suggesting that the loss of this molecule is due to other mechanisms than Ca^{2+} flux.

Survival in vivo of transfused stored mouse RBCs.

$H2K^{b}$-eGFP RBCs stored for the indicated times were transfused into wild-type recipients and survival was monitored by enumerating $eGFP^{+}$ RBCs at 10 and 30 minutes, and at 1, 2, 18, and 24 hours post-transfusion. Long term survival was assessed at weekly intervals post-transfusion. Representative survival curves are shown in Figure 4.3. Similar to humans, the clearance of transfused stored RBCs occurs in two distinct phases: an initial rapid clearance (Figure 4.3A) followed by an extended survival to at least 50 days (Figure 4.3B). Combined analysis of 4 independent experiments demonstrated that the mean 24-hour survival of transfused $eGFP^{+}$ RBCs was approximately 99%, 91%, 64%, 54%, 30%, and 18% following 0, 7, 14, 21, 28, and 35 days of storage in CPDA-1 (Figure 4.3C).

To compare flow cytometry to the clinical method used in human patients, additional studies were performed using ^{51}Chromium-labeled freshly isolated RBCs and 14-day stored RBCs. Like flow cytometry, this method detected a rapid initial clearance of stored RBCs immediately following transfusion (Figure 4.4A). The 24-hour survival of 14-day stored RBCs was found to be comparable as measured by either flow cytometry (i.e. 64% SD ± 5.0%) (Figure 4.3C) or ^{51}Chromium labeling (i.e. 65% SD ±7.7%) (Figures 4.4B).

Discussion

RBC transfusion is a common treatment in a variety of clinical settings with over 14 million units of RBCs collected and distributed per year in the United States alone. Recent evidence suggests that transfusion of RBCs stored for greater than 14 days may increase the risk of medical complications[2,3]. For these reasons, a tractable animal model for studying the adverse effects of transfusing stored RBCs would be of great utility. Because the storage of rat RBCs in CPDA-1 was reported to result in highly accelerated aging *in vitro* [7], which differs from stored human RBCs, rodent models have been considered to be of only limited utility. However, our current results demonstrate that, in contrast to what was reported for rats, stored mouse RBCs do not undergo a similar precipitous decline is post-transfusion survival.

In evaluating the significance of our findings, we interpreted mouse RBC storage in the context of their normal lifespan. Mouse and human RBCs have an average normal survival of 55 and 120 days *in vivo*, respectively. Therefore, 35-day storage of human RBCs in CPDA-1 represents approximately 29% of the normal RBC lifespan. The analogous 29% of the normal mouse RBC lifespan is 16 days. One might argue that this thinking is inappropriate, and that absolute time of preservation at 4°C ought to apply equally to either population. However, as RBCs continue to age *ex vivo*, it is our position that it is appropriate to assess the properties of stored mouse RBCs as a function of their normal rate of aging *in vivo*. By these criteria, our data indicate that mouse RBCs age somewhat more quickly in CPDA-1 than do human RBCs. However, the general pattern of progressive decline is similar between the two species. The decreased survival of mouse RBCs may represent a qualitative difference in biology. Alternatively

differences in survival may be due to quantitative variables such as the fact that CPDA-1 has been optimized to storage of human RBCs, and known differences in mouse blood chemistry (e.g. osmolarity) [14]. Moreover, the absence of plasticizer, present in human blood bags and reported to extend human RBC survival, may also be responsible [15].

It is important to note that the FDA requirement for 75% post-transfusion survival at 24 hours represents an average. Indeed, some units of RBCs stored under approved conditions for the appropriate length of time have substantially worse survival [16]. Such differences can be donor specific. For example, in a study by Mishler et al., RBCs collected on two separate occasions from the same donor had a 24-hour survival of 33% and 41%, respectively [17]. This was in contrast to RBCs from other donors that had a mean survival of 74% and 75%. Thus, survival appears to be based upon donor specific factors. Similar to the above observations in humans, it would be reasonable to predict that genetic background and/or environmental factors may likewise affect survival of transfused mouse RBCs. This was not evaluated in the current study because the eGFP transgene is not yet backcrossed onto different strains.

Although phosphatidylserine externalization increased with storage, it does not statistically differ until after day 28. Likewise, the loss of CD47 expression does not reach significance until day 28. However, the 24-hour post-transfusion survival data clearly indicate that the *in vivo* survival is significantly decreased as early as day 14. These results demonstrate that neither phosphatidylserine nor CD47 expression is significantly altered at a storage time when the post-transfusion survival is decreased, suggesting that clearance of the transfused RBCs is due to other factors. However, one cannot rule out a sudden alteration in cell surface expression immediately after

transfusion that may not be apparent in the stored RBC unit itself. A description of an apoptotic-like pathway in RBCs (i.e. eryptosis) that induces sudden changes of this nature has been previously described [18].

Substantial efforts were made to model human RBC storage conditions accurately. For example, the ratio of CPDA-1 to blood, the use of filter leukoreduction, and the final hematocrit were all the same as those used with human blood. However, the storage conditions are not completely ideal. In the current study, RBC units are stored in 500 μL Eppendorf tubes. Clearly, the plastic of the Eppendorf tubes differs from the storage bags used for human RBCs. As plasticizer continuously leaks from the bag material itself, our attempt to introduce the plasticizer into our stored mouse units by obtaining CPDA-1 from DEHV-PVC bags did not lead to the same concentrations present in stored human units. Because plasticizer has been shown to increase the post-transfusion survival of stored human RBCs, this may have a significant effect [19,20]. In addition, collection time from mice necessitated a longer exposure of initial RBCs with undiluted CPDA-1, which is known to have a potential to shock the early collected RBCs. Also of note is the fact that mouse blood has a slightly higher osmolarity as compared to human blood. Thus, a preservative such as CPDA-1, designed for human use, may not be optimal for mouse RBC storage.

The current studies focus on 24-hour post-transfusion survival and alterations in surface phosphatidylserine and CD47. Although these parameters reflect a few of the known properties of stored human RBCs, several other well characterized biochemical changes exist, including alterations in ATP, 2,3-DPG, changes in shape, alterations in membrane deformability, increased osmotic fragility, and changes in hemoglobin (e.g.

altered S-nitrosohemoglobin). Ongoing evaluation of this model would benefit from analysis of these parameters.

The current findings indicate that when stored under similar conditions to those used for humans, mouse RBCs do not display a precipitous decrease in post-transfusion survival as reported for rats [7]. Use of the $eGFP^+$ transgenic mouse allows for characterization of RBCs during storage as wells as tracking following transfusion. Together, these data suggest that this is a valid model that may be a useful platform to study pathophysiological effects of transfusing stored RBCs.

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Figure Legends

Figure 4.1. Mouse RBCs expressing transgenic eGFP provide a traceable donor population following transfusion into wild-type recipients. Fresh whole blood from $H2K^b$-eGFP and C57BL/6 mice was analyzed for the expression of eGFP on Ter-119^+ RBCs. A distinct forward and side scatter RBC gate was established **(A, left panel).** The validity of the RBC gate was assessed by staining with anti-Ter-119 or isotype matched control **(A, right panel)**. Using the forward and side scatter RBC gate established in panel A, eGFP fluorescence was measured in $H2K^b$-eGFP RBCs as compared to the background fluorescence in wild-type C57BL/6 RBCs **(B)**. When $H2K^b$-eGFP RBCs were transfused into C57BL/6 recipients, donor RBCs were clearly discernable from recipient RBCs; only minimal background fluorescence was seen in untransfused recipients **(C)**.

Figure 4.2. Storage of mouse RBCs in CPDA-1 induces a progressive increase in phosphatidylserine externalization and decrease in CD47 expression. At weekly intervals stored mouse RBCs were stained with Annexin V **(A)** or anti-CD47 **(B)**. The analysis was restricted to Ter-119^+ $eGFP^+$ events within the RBC gate (see Figure 1C). Representative histograms are shown to the right. The data is presented as a fold change between the indicated stored RBC specimen and fresh RBCs. Anti-CD47 staining was calculated by subtracting the mean fluorescence intensity of an isotype matched control stain from the mean fluorescence intensity of staining with anti-CD47 for each specimen. The presented data represents a combination of 4 separate experiments. Error bars indicate standard deviation. ($*$ p value < 0.05)

Figure 4.3. Survival of stored mouse RBCs transfused into wild-type recipients. Donor RBCs were monitored by flow cytometry using eGFP fluorescence as a means to distinguish donor from recipient RBCs. Survival was monitored as a percent of donor cells within the RBC gate. Representative results from one experiment are shown from 14-days of storage. An initial rapid clearance occurred within the first several hours **(A)** followed by markedly slower clearance of the remaining donor RBCs over 50 days **(B)**. At the indicated time of storage, $eGFP^+$ RBCs were transfused into wild-type C57BL/6 recipients and 24-hour survival was monitored by flow cytometry **(C)**. The presented data is the compilation of four independent experiments. ($*$ p value < 0.05)**.**

Figure 4.4. ^{51}Chromium 24-hour survival of transfused mouse RBCs. 14-day stored RBCs were labeled with ^{51}Chromium and transfused into wild-type C57BL/6 recipients. Survival of transfused RBCs was determined using a gamma counter to measure radioactivity. An initial rapid clearance of stored RBCs was observed compared to freshly isolated RBCs **(A)**. The 24-hour survival was 65% ($*$ p value <0.05), which was comparable to the flow based determination (64%), (see Figure 4.3C).

Figure 4.1

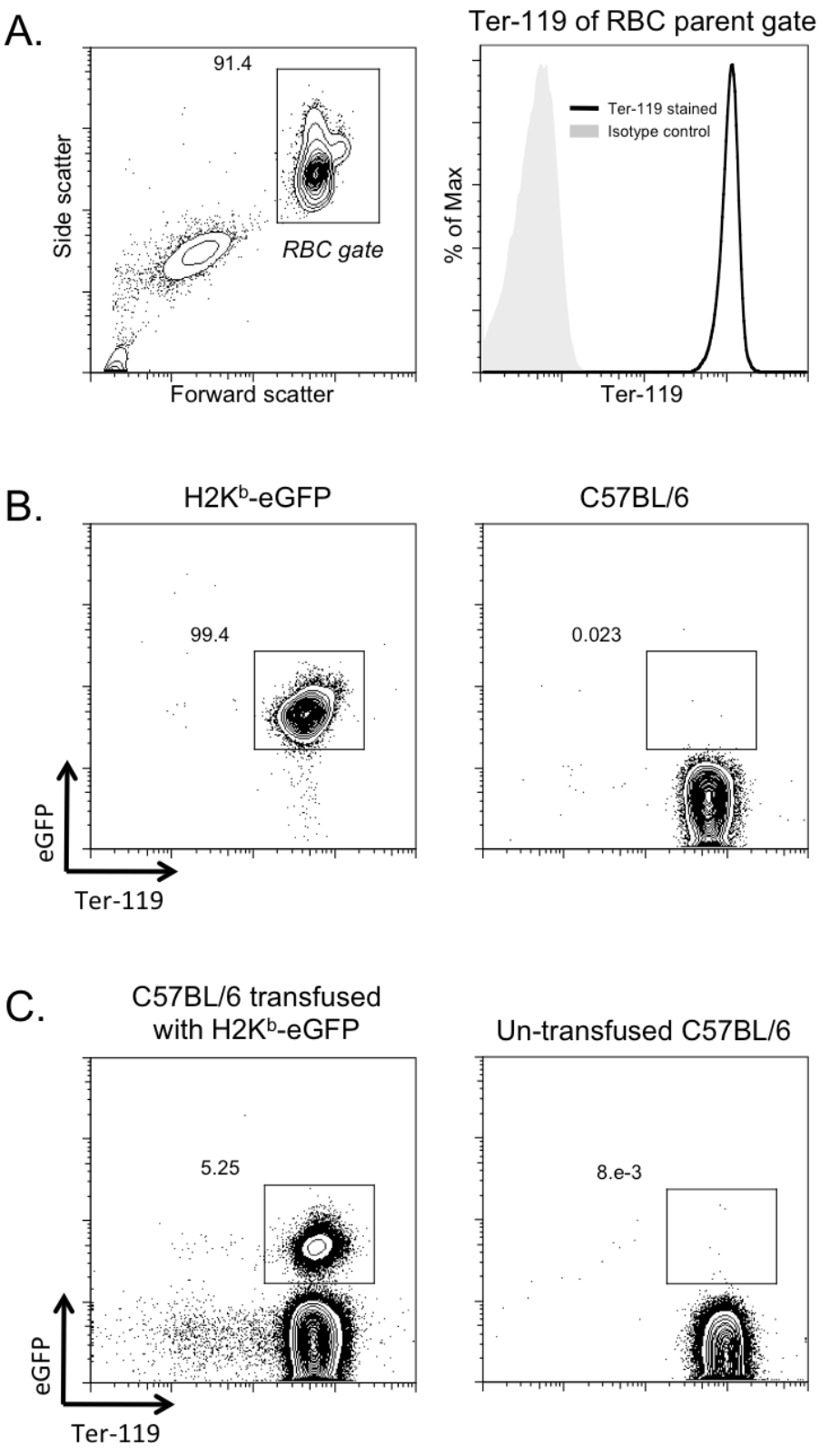

A.
91.4
Side scatter
RBC gate
Forward scatter
Ter-119 of RBC parent gate
Ter-119 stained
Isotype control
% of Max
Ter-119
B.
H2K^{b}-eGFP
C57BL/6
99.4
0.023
eGFP
Ter-119
C.
C57BL/6 transfused with H2K^{b}-eGFP
Un-transfused C57BL/6
5.25
8.e-3
eGFP
Ter-119

Figure 4.2

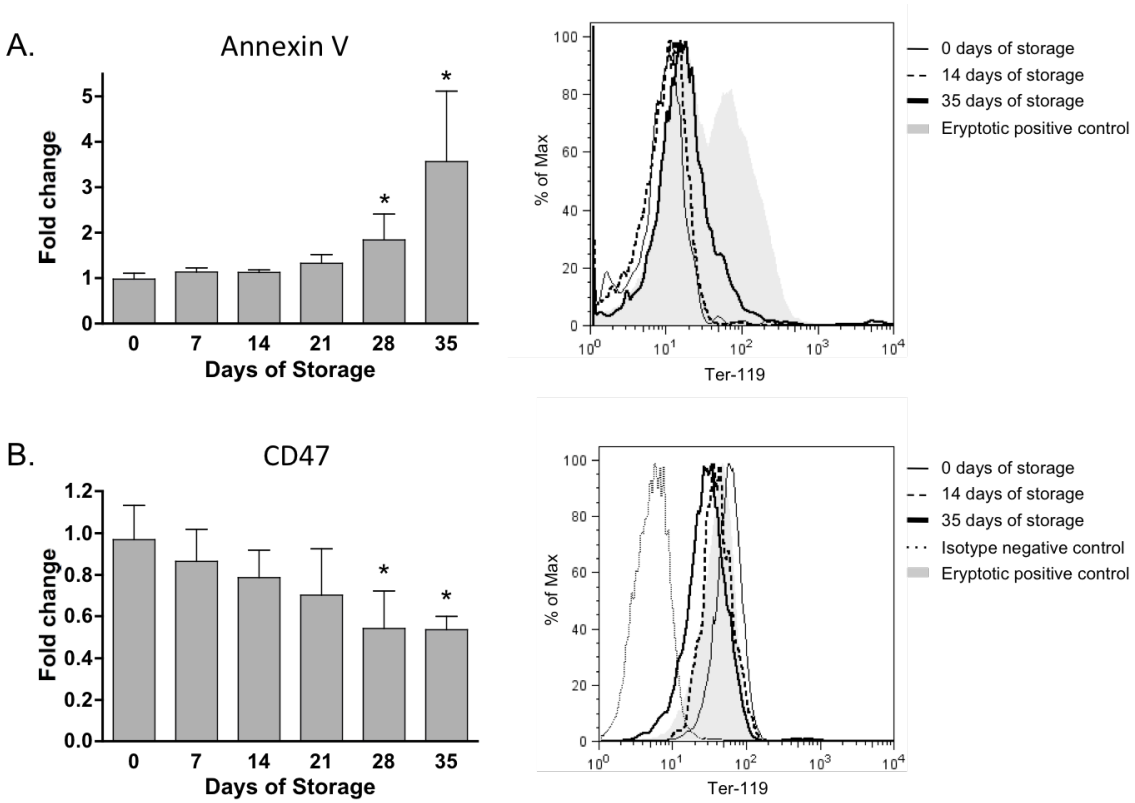

A.
Annexin V
Fold change
Days of Storage
0
7
14
21
28
35
% of Max
Ter-119
0 days of storage
14 days of storage
35 days of storage
Eryptotic positive control
B.
CD47
Fold change
Days of Storage
% of Max
Ter-119
0 days of storage
14 days of storage
35 days of storage
Isotype negative control
Eryptotic positive control

Figure 4.3

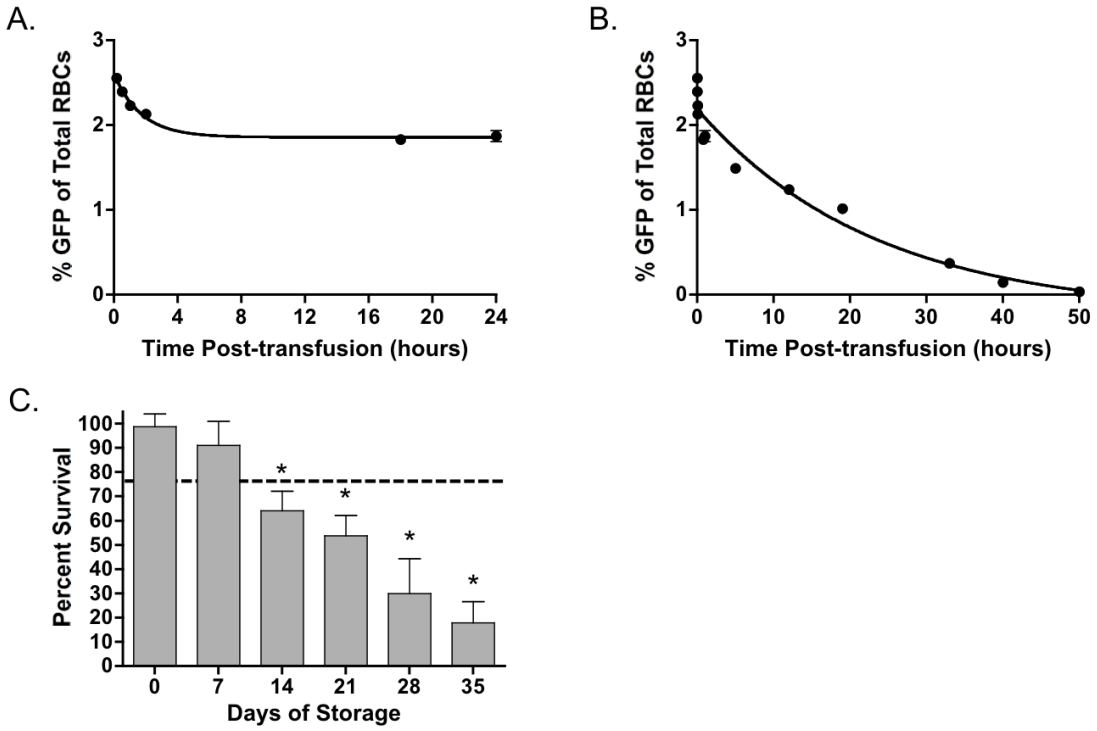
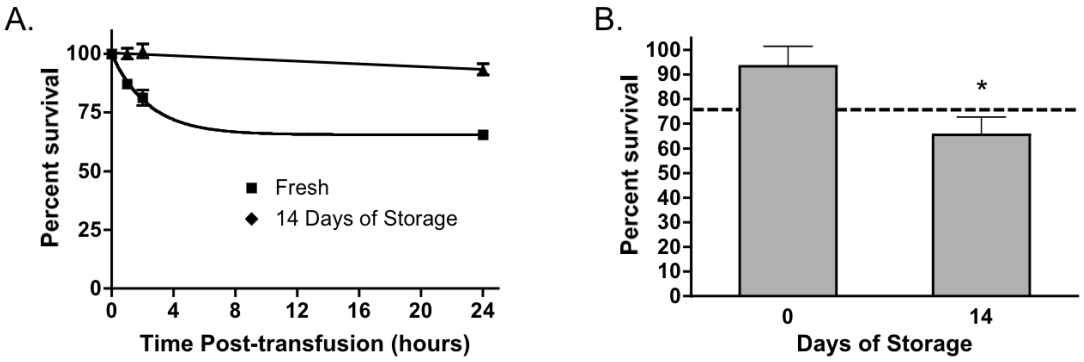

A.
% GFP of Total RBCs
Time Post-transfusion (hours)
B.
% GFP of Total RBCs
Time Post-transfusion (hours)
C.
Percent Survival
Days of Storage

Figure 4.4


A.
Percent survival
100
75
50
25
0
0
4
8
12
16
20
24
Time Post-transfusion (hours)
Fresh
14 Days of Storage
B.
Percent survival
100
90
80
70
60
50
40
30
20
10
0
*
0
14
Days of Storage

Chapter 5

Discussion

Summary

Platelet transfusion is an essential treatment for thrombocytopenia, which can result from a wide range of conditions including acute hemorrhage, hematological malignancies, hematopoietic stem cell transplantation, and congenital platelet disorders. Although it is a life-saving therapy, transfusion may also carry significant risks. Anti-donor antibodies can be developed and abrogate the efficacy of transfused platelets. Increasingly sophisticated methodologies, including leukoreduction and UVB irradiation of platelet products have decreased the incidence of anti-donor antibodies by limiting the recipient's exposure to antigenic white blood cells. In spite of these efforts, alloimmunization following platelet transfusion still occurs in a significant percentage of patients.

We began our studies by addressing an apparent incongruity in the transfusion literature, which states:

"*Therefore, in patients receiving multiple platelet transfusions, it is the exposure to donor leukocytes that is responsible for primary HLA alloimmunization*." AABB Technical Manual (1)

"*Although foreign antigen is presented to helper T cells by the subject's own HLA class II-positive antigen-presenting cells, the induction of a primary immune response to foreign class I antigens must be presented by class II-positive APCs of the donor." "Thus alloimmunization against class I antigens should be prevented when class II-*

positive cells have been either removed from red cell or platelet concentrates or inactivated." Blood Transfusion in Clinical Medicine, (2)

Similar statements with regard to the requirement for donor MHC class II can be found throughout the leading transfusion textbooks (3). Due to the fact that platelets express the offending MHC class I antigen, it seems counterintuitive that donor MHC class II would also be required. To test the hypothesis that donor MHC class II is required, we carried out alloimmunization experiments by transfusing MHC class II deficient allogeneic whole blood. We observed that anti-donor alloantibody responses were of equal magnitude when comparing transfusion of blood from wild type vs. MHC class II null donors. This indicates that whatever is responsible for the immunogenicity of platelet transfusion it is not the expression of MHC class II on donor leukocytes.

To study the role of the $CD4^+$ T cell in shaping the humoral immune response to allogeneic platelets, we developed a tractable mouse model of platelet transfusion. We made use of the TCR75 T cell receptor transgenic mouse, which is specific for a single peptide derived from the $H\text{-}2K^d$ MHC class I molecule presented by $I\text{-}A^b$. The adoptive transfer of TCR75 cells into C57BL/6 recipients enhanced the normally undetectable antibody response to a single BALB/c leukoreduced platelet-rich plasma transfusion. Division of the TCR75 cells was restricted to the spleen, and not observed in the lymph nodes or liver. Furthermore, splenectomy or treatment with the costimulatory blockade reagent CTLA4-Ig ablated both $CD4^+$ T cell division and alloantibody production. These data support the critical role for $CD4^+$ T cell help and establish a mouse model for

exploring T cell-specific modalities for the prevention of alloimmunization to platelet antigens.

Although we have developed a suitable model of platelet isolation and transfusion, which closely mirrors the clinical setting, our system lacks the potentially critical aspect of platelet storage. Multiple effects are incurred during the storage of human platelets that may have drastic effects not only on post transfusion survival but also alloimmunization. As a precursor to a platelet model, we developed and characterized a murine red blood cell model of storage and post transfusion survival similar to the conditions of human red blood cell storage. A transgenic mouse strain expressing enhanced green fluorescent protein ($H2K^b$-eGFP) was utilized to allow for detailed post-transfusion analysis. We demonstrated that 24-hour post-transfusion survival of stored mouse red blood cells showed relatively similar kinetics to that reported for human red blood cells, when indexed for the normal lifespan of mouse red blood cells. We also reported that mouse red blood cells developed changes in membrane molecules with a trend comparable to those described for humans. Together, these results demonstrate the potential utility of a mouse system to study the effects of storage and transfusion of red blood cells and provide some insight for the development of an analogous murine platelet storage model.

Discussion

To leukoreduce or not to leukoreduce

Transfusion medicine has undergone dramatic changes through the centuries. The description of the ABO blood group system in the early 1900's began to shed light on the nature of immediate severe transfusion reactions. The development of collection and storage methods would be hastened by the demands for blood products brought on by World War I. The utilization of stored blood would only increase as operative procedures became technically more complex and medical specialties such as solid organ transplantation and chemotherapy emerged. By the 1970's screening for hepatitis B became standard and tests HIV, hepatitis C, and HTLV would later follow to safeguard the blood supply. To reduce the growing concern over alloimmunization rates, Canada, Britain, and France adopted universal leukoreduction in the late 1990s and in 1998, the FDA unanimously voted in favor of universal leukoreduction of blood components for the United States. However, it was cited that sufficient evidence-based data in favor of universal leukoreduction were lacking. Both leukoreduced and non-leukoreduced blood components currently remain approved in the United States.

Leukoreduction is a process in which the white cells, ordinarily present in collected blood components, are intentionally reduced in number prior to transfusion into the recipient (4). This is achieved either coincident to the collection process, such as with apheresis or buffy coat methods, or by filtration, as is done for platelets isolated from whole blood collection. It is hypothesized that transfusions containing donor leukocytes cause adverse effects by multiple mechanisms. Infectious pathogens such as cytomegalovirus, herpes viruses, or Epstein–Barr virus, are normally more concentrated

in leukocytes than the rest of the blood product (5). Donor leukocytes may have suppressive effects on the recipient immune system, which may exacerbate postoperative infection and complicate cancer surveillance (6). During storage, leukocytes may release inflammatory mediators into the platelet units that may mediate acute febrile transfusion reactions (7). Furthermore, contaminating leukocytes are perceived as the primary mediator of alloimmunization and platelet refractoriness (1-3).

There are disadvantages to leukoreduction as well. The implementation of leukoreduction across the United States would add significant upfront cost to platelet preparation and may place additional financial burden on the health care system (8). The added manipulation required may increase the risk of bacterial contamination. Platelet loss due to trapping during filtration is also a concern for the chronically low supplies of platelets. Finally, although leukoreduction decreases alloimmunization rates, it does not completely eliminate it and a substantial number of platelet recipients still become HLA sensitized (9).

Leukoreduction of platelet units

An important aspect to deciding whether universal leukoreduction should be implemented and how this should be undertaken is to clearly understand the mechanisms behinds the efficacy. Our data in Chapter 2 addresses on of these mechanisms by testing the hypothesis that the anti-donor antibody response is due to the exposure of the recipient to donor MHC class II.

The prevailing explanation states that the exposure to donor MHC class II expressing leukocytes stimulates recipient anti-donor antibody production via direct

recognition by $CD4^+$ T cells. Following transfusion of donor leukocytes as contaminants in the platelet unit, donor MHC class II can interact with and directly activate allospecific recipient $CD4^+$ T cells, which then may provide help to recipient B cells specific for anti-donor antigens (10). In addition to the direct pathway, the indirect pathway has also been identified as an important mechanism of alloimmunization (11, 12). In the context of the anti-donor antibody response, this pathway consists of the consumption of donor cells by recipient antigen presenting cells, followed by the processing and presentation of peptides derived from donor MHC class I molecules on recipient MHC class II. These APCs can then activate recipient $CD4^+$ T cells, which provide signals to B cells to produce an analogous anti-donor antibody response. If, as it is generally held, MHC class II expressing donor leukocytes are indeed required for alloimmunization to transfused cells, then this would indicate that the indirect pathway was not able to lead to alloimmunization in the context of platelet transfusion.

In aggregate, the above findings present conflicting data concerning the relative requirements of the direct and indirect pathways, and thus the basis for MHC class II expressing donor cells being needed for alloimmunization. We tested the requirements for MHC class II on donor cells by carrying out a detailed comparison of wild-type donors vs. donors with a complete genetic deletion of the entire MHC class II region. We reported that strong alloantibody responses were observed in recipients transfused with blood from MHC II null donors, thus demonstrating that MHC II on donor cells is not required for alloimmunization in this murine model. These findings suggest that designing leukoreduction filters with the sole aim of removing all of the donor MHC class II positive cells may not be the optimal strategy to reduce alloimmunization.

Alternative interpretations for the efficacy of leukoreduction should also be considered. Leukoreduction may not just decrease the immunogenicity of the platelet transfusion it may actively create an immunosuppressive dose. Experimental leukodepletion in mouse models to levels well below what is achieved in humans resulted in a paradoxical increase in immunogenicity of transfused platelets (13). The conclusion that an immunosuppressive population has been removed is supported by the observation that adding back small numbers of leukocytes into these extreme leukodepleted platelets can decrease alloimmunization up to a point, after which immunogenicity returns. Different leukocyte populations in peripheral blood may have enhancing or inhibiting effects on HLA alloimmunization (14). This possibility is supported by the observation that the effects of contaminating leukocytes appear to depend upon the type of leukoreduction method used (15-17).

Defining the relative effects of different peripheral leukocyte subsets on humoral HLA alloimmunization would have substantial medical significance. While attempts to purify subsets to effective homogeneity have generated useful information, they cannot rule out effects of contaminating populations or variation incurred by the isolation process. Our lab is currently engineering a genetic approach to selectively express allogeneic MHC class I on different tissues. This novel mouse system will circumvent the existing experimental limitations of traditional cellular fractionation experiments and allow the assessment of the immunoregulatory properties of different peripheral leukocyte subsets based upon the selective expression of MHC class I. These experiments will be described in detail in the future directions section.

Modeling platelet isolation and collection in a murine setting

For our mouse platelet collection protocol, we adapted the soft-spin method used predominantly by the United States. As detailed in Chapter 3, platelet-rich plasma was isolated by differential centrifugation from whole blood and then passed over a commercially available neonatal leukoreduction filter. Following enumeration, the LR-PRP was resuspended in Tyrode's buffer prior to transfusion. Only low amounts of residual red blood cells were observed and contaminating leukocytes were consistently below the level of detection (less than 500 leukocytes per transfusion) for our quantification assay. Transfusion of less than 1000 leukocytes has been shown to be alone insufficient to elicit an anti-donor antibody response (18, 19).

Platelets are a delicate cell subset to work with experimentally. They tend to activate *in vitro*, leading to clumping and loss of viability. Thus, we took measures to isolate and transfuse the platelets as quickly and consistently as possible. Furthermore, the quality and functionality of the platelet units collected by our protocol was assessed by several methodologies. First, the platelets were tested by aggregometry, a routine clinical test used to assess the platelet's ability to aggregate in response to collagen. Second, the platelet morphology was confirmed by flow cytometry during enumeration. Platelets typically fall within a specific forward by side scatter gate (20). Deviation from this profile may indicate platelets that had gone through significant activation. Third, during the development of our isolation protocol, the post-transfusion survival of murine platelets was examined by transfusing eGFP platelets into naïve syngeneic recipients. $eGFP^+$ platelets were detectable for at least 72 hours after transfusion, consistent with published data of murine platelet survival (21). Lastly, the platelet units

were assessed using a routine blood bank method known as the 'swirl' test (22). The quality of the platelets can be roughly determined by the presence or absence of 'swirling', which can be observed visually when the unit is gently rocked and held up to a light source. Platelets with an altered morphology due to damage or activation do not 'swirl'.

Several animal models have reported data demonstrating that platelets alone are not immunogenic. Early experiments in canines and rhesus macaques showed that platelet transfusion failed to induce cytotoxic antibodies and actually improved solid organ transplant survival (23-25). Welsh, et al. reported in 1977 that purified allogeneic rat platelets failed to induce either humoral or cell-mediated immune responses, and multiple transfusions led to specific non-reactivity after challenge with donor splenocytes (26). In 1981, Claas, et al. showed that purified mouse platelets were not able to induce a primary antibody response despite repeated injections (18). However, when the platelet suspensions were spiked with at least 10^3 leukocytes, an antibody response was induced.

It is not entirely clear why these early studies failed to report a significant antibody response following platelet transfusion, while in contrast our experiments have. There are several methodological differences to consider. As noted previously, platelets are notoriously sensitive and *ex vivo* manipulation can easily perturb their biology. It is unclear in these older studies what the viability of the transfused platelets was or if any morphological or functional assays were performed prior to transfusion. Our murine system has the advantage of animals with defined genetic background, which may not have been the case with mongrel canines and non-human primates (23-25). An

important distinction was the methods used to detect the anti-donor antibody response. Lymphocytotoxicity, as used in the previously mentioned animal studies (18, 23-26), only have the ability to detect lyric antibody subtypes. The higher sensitivity of the indirect immunofluorescent techniques may have allowed our assays to detect the low levels of anti-donor antibodies induced by our allogeneic platelet transfusions.

While we have developed a model of platelet isolation and transfusion, there is an important component absent from our model that is present in the human setting. The isolated mouse platelets were immediately transfused in the recipients with minimal time *ex vivo*. However, this is almost never the case clinically as platelets are normally stored for several days prior to use (27).

Recipient T cell response to allogeneic platelet transfusion

As an antigen, platelets conceptually share several similarities with T cell independent antigens. Type 2 T independent antigens are molecules containing polyvalent B cell epitopes capable of cross-linking the B cell receptor to an extent that $CD4^+$ T cell help is not required for antibody production (28). With the expression of donor MHC class I on their surface, particularly in the context of an immobilized thrombus, platelets may provide a sufficient scaffold for cross-linking the B cell receptor and inducing B cell activation. Furthermore, platelets express a variety of inflammatory mediators such as IL-1β, RANTES, TNFα, TGFβ, and notably for the generation of the antibody response, CD154 (29-32). Platelet derived CD154 has been shown *in vitro* to directly stimulate B cell proliferation and antibody production (30, 33-35). In studies of the antibody responses to adenovirus, *in vivo* evidence suggests that platelet derived

CD154 is sufficient to support B cell isotype switching in CD154 deficient recipients (36). Platelets have also been shown to enhance germinal center formation (37). Thus, platelets have the potential to provide both B cell receptor cross-linking and costimulation to induce alloantibody production independent of $CD4^+$ T cell help.

In chapter 4, we directly tested if $CD4^+$ T cell help was required for the humoral response to allogeneic platelet transfusion. We used two approaches to test this requirement. First, $CD4^+$ T cells were depleted by monoclonal antibody from recipients prior to transfusion. Second, CD4 T cell receptor transgenic recipients specific for an irrelevant peptide with respect to the alloantigen were used as antigen specific help deficient recipients. In both methods, the absence of T cell help abrogated the recipients' ability to mount an anti-donor antibody response. Thus, under these experimental conditions, allogeneic platelets cannot serve as T independent antigens. However, there are several variables to consider. The recipient mice were housed under sterile conditions, generally healthy, and free of any obvious signs of pathology. Furthermore, the platelet units were transfused immediately following collection, and thus did not incur the platelet storage lesion. Danger signals provided by TLR agonists have been shown to increase the immunogenicity of red blood cell transfusion (38), and platelets are known to undergo several biochemical and physical alterations during storage that may impact their post-transfusion efficacy and immunogenicity (7, 39).

Thus, although we have described conditions where platelets require $CD4^+$ T cell help to induce a humoral response, this does not necessarily prove that allogeneic platelets are incapable of directly stimulating B cells independent of $CD4^+$ T cell help in some settings. This distinction has important implications for the implementation of

strategies directed at preventing platelet refractoriness by inhibiting the $CD4^+$ T cell response.

Modeling blood component storage in murine systems

The availability of blood components is a critical aspect to the care of many hospitalized patients. In the United States, blood transfusion is the most common reported discharge code and it is estimated that one out of every three Americans will require transfusion of a blood product at some point in their life (27). Retrospective cohort studies have reported a correlation between blood storage duration and morbidity and mortality rates following transfusion (3–5). Despite these observations, no large controlled clinical trials have been conducted to evaluate the relationship between storage and clinical outcomes; ethical, logistic, and financial factors all contribute to the difficulties of performing these studies. Non-human primates have been used as test subjects, but the expense and approvals required for these types of studies may exceed those for human.

We established a novel murine system to assess the impact of red blood cell storage on post-transfusion survival. We modeled the general method used for human blood storage and set up what we referred to as a mouse blood bank. Stored mouse red blood cells showed a progressive decline in survival as a function of storage time and changes in surface markers were analogous to trends reported for human red blood cells. This murine system is not a perfect representation of the human setting, due in part to the inherently shorter life span of mouse red blood cells (~ 55 days) as compared to human red blood cells (~ 120 days) and the fact that the storage solutions used were

originally optimized for human biochemistry. However, these findings establish a novel mouse model with the potential to serve as an experimental platform for studying the pathophysiological consequences of transfusing stored red blood cells.

One potential consequence of the changes in biological properties incurred during storage is alteration of the cells immunogenicity. It is challenging to isolate factors in humans that regulate red blood cell immunogenicity due to the large number of variables such as antigenic differences between donor and recipient, recipient HLA type, dose and duration of antigen exposure, and the status of the recipient at the time of transfusion. Published studies from our laboratory and our collaborators have built upon the mouse blood bank system presented herein and tested the hypothesis that red blood cell alloimmunization is regulated by biologic changes in the stored unit that accumulate as a function of storage time (40, 41). It was reported that storage significantly increases the humoral immune response after transfusion (40), which may be related to increased levels of iron deposition in the tissues (41). These studies suggest that red blood cell storage length is a factor influencing alloimmunization and provide a rational basis to test the hypothesis that a similar effect is present in the human setting.

The experience gained from modeling red blood cell storage will directly translate to an important future direction with regards to the platelet work described herein. Currently, no murine model of human platelet storage has been described. Developing an analogous system to what we have presented for murine red blood cells presents several unique challenges that will be discussed in the future directions.

Prevention of platelet alloimmunization

Transfusion of blood products, in particular the transfusion of platelets, can lead to induction of anti-HLA antibodies in the recipient. These anti-HLA antibodies can lead to significant clinical problems. First, preformed anti-HLA antibodies can rapidly induce clearance of transfused platelets. Second, anti-HLA antibodies can promote chronic rejection of transplanted organs, including decreased survival of lungs (42), hearts (43), and kidneys (44). Lastly, the use of blood products from donors with antibodies to HLA antigens may lead to acute lung injury in recipients who express the recognized HLA. Leukoreduction has substantially decreased the percentage of patients who become HLA alloimmunized to transfused platelets, however, as over four million units of platelets are transfused each year in the United States alone, platelet transfusion induced anti-HLA antibodies remains a problem.

In Chapter 4 we provided evidence to support the hypothesis that $CD4^+$ T cell help is required for the anti-donor antibody response to allogeneic platelet transfusion. If donor specific $CD4^+$ T cell are indeed required, then this cell type may be an effective target to inhibit platelet refractoriness. The field of solid organ transplantation has a large body of work dedicated to the goal of inhibiting the donor reactive T cell responses (45-50). These studies may serve as a valuable resource for the transfusion community. Several broad-spectrum immunosuppressive agents, such as cyclosporine, rapamycin, and corticosteroids, are in wide use to prevent graft rejection (51-53). However, these pharmaceuticals often have serious side-effect profiles that may not be acceptable for use in the transfusion setting.

Costimulatory blockade reagents offer the benefits of minimal side effects and specific suppression of activate T cells. Murine studies have demonstrated that long-term solid graft survival can be achieved by inhibition of the CD28 signaling, in some cases even as the sole therapy (54, 55). Blockade of the CD28 pathway is commonly achieved using the recombinant fusion protein CTLA4-Ig, which consists of the extracellular domain of the CTLA4 molecule fused to the Fc portion of IgG_1 (55, 56). This fusion protein functions as a competitive inhibitor, as CTLA4 binds with a higher affinity to CD80 and CD86 expressed on APCs than does CD28 (57). It is believed to primarily impact naïve cells, based on the role of CD28 in the production of IL-2 and the initiation of T cell activation (58). CTLA4-Ig has been shown to inhibit the humoral response to both sheep red blood cells and allogeneic rat whole blood transfusion in murine models (55, 59). A humanized form of CTLA4-Ig, known as abatacept, is FDA approved for the treatment for psoriasis and rheumatoid arthritis (60, 61).

When given concurrently with each platelet transfusion, CTLA4-Ig was found to effectively inhibit the anti-donor antibody response to allogeneic platelet transfusion. BALB/c specific TCR75 cells failed to divide in response to BALB/c platelets when CD28 signaling was blocked by CTLA4-Ig. Furthermore, a single dose of CTLA4-Ig given with a priming dose of BALB/c platelets inhibited the formation of the anti-donor antibody response to four weekly platelet transfusions given 21 days later. Several interpretations may be gleaned from these data. First, CTLA4-Ig may have remained at a high enough serum concentration throughout the transfusion protocol to sufficiently inhibit the anti-donor response. Second, in the absence of CD28 signaling donor reactive cells may have been deleted from the periphery through activation induced cell

death. Third, donor specific cells may have converted to a regulatory cell like phenotype, which may have suppressed any newly activated antigen specific cells. Although we observed that the antigen specific CD4 T cell response was absent in the presence of CTLA4-Ig, we cannot exclude the role of direct inhibition of the donor specific B cell by CTLA4-Ig with our current experimental setup.

Our data suggest that the splenic microenvironment is necessary for $CD4^+$ T-cell priming and proliferation in response to allogeneic platelet transfusion. This may be due to unique aspects of the splenic architecture required for the sequestration of platelets (62), specialized splenic APC populations (63, 64), or the spleens role in the maintenance of extrasplenic B-1a cells (65). The spleen may also be required for priming of naïve antigen-specific B cells to platelet antigens (66). Splenectomized recipients did not suffer from a general defect in their ability to respond to alloantigen, as splenectomized recipients were capable of mounting an anti-donor antibody response to whole blood transfusion, although with slightly delayed kinetics and of lower magnitude than sham-operated recipients (data not shown). However, the spleen may not necessarily be required for the maintenance of the anti-platelet memory response.

In addition to the spleen, the liver may also serve as a site of platelet clearance (67). The liver is characterized as a relatively tolerogenic organ due to its distinct intrahepatic lymphocyte populations (68). In our splenectomized recipients, donor platelets may have been phagocytosed and presented by liver APCs. However the TCR75 cells in the liver of splenectomized animals cells remained undivided. It is possible that the conditions of hepatic alloantigen presentation not only lead to a lack of immunity, but also to active tolerance. Additional experimentation is required to

investigate the patterns of platelet consumption by liver APC subsets, as well as the possibility of tolerance induced by platelet antigen presentation in this setting.

Future directions

The work presented herein can be taken in many different directions over both the short and long term. A brief discussion follows of experiments that could be undertaken to follow up the research presented herein.

Short term future directions

Previous studies in our laboratory have tested the hypothesis that the inflammatory status of the transfusion recipient influences the immunization to transfused red blood cells (38). It was found that recipients treated with poly(I:C), a synthetic molecule that mimics viral double stranded RNA, have an enhancement of humoral immunization to transfused alloantigens on mouse red blood cells. In contrast, inflammation with LPS, a component of the gram-negative bacterial cell wall, decreased humoral immunization. Similar studies to determine if recipient inflammation impacts platelet alloimmunization have not been explored.

A separate, but similar issue is that of contamination of the platelet unit itself during storage. While transfusion transmission of viral infections have dramatically decreased as a result of donor testing at the time of collection, bacterial contamination has proved more difficult to address and remains the most prevalent transfusion-associated infectious risk factor (69). Platelet components are stored at room temperature, which is permissive to bacterial proliferation. Reports from clinical centers

utilizing culture-based bacterial testing have reported rates of contamination between 1:1000 to 1:2000 (70, 71). Bacteria are most often introduced into platelet units from skin flora at the time of collection or, less frequently, through asymptomatic donor bacteremia or during processing of the unit. Gram positive staphylococci are the most common organisms implicated in platelet bacterial contamination, while Gram negative organisms such as *Klebsiella pneumoniae*, *Escherichia coli*, *Serratia* species, and *Enterobacter* species, although less common, are more likely to result in clinically significant reactions (69).

Intravenous transfusion of any bacteria is obviously undesirable and may lead to symptoms ranging from mild febrile reactions to severe septic shock and organ failure. However, there may be sequelae beyond this as well. The platelets within the contaminated unit may become activated during storage. Toll-like receptors are known to be exquisitely sensitive to very low concentrations of their ligands (72, 73). Thus, bacterial contamination below what provokes an obvious clinical response in the patient or that can be picked up by current detection methods may induce platelet activation and these activated platelets may be more immunogenic as a result.

Platelets are undoubtedly metabolically active during room temperature storage, which may lead to diminished platelet function over time (74, 75). Metabolic byproducts accumulate during room temperature storage, leading to a fall in pH (76). pH levels below 6.0–6.2 have been associated with severely diminished platelet viability (77-79). Mediators of thrombosis stored in platelet granules as well as platelet derived CD154 accumulate in the storage medium over time (80). Moreover, platelet surface

CD62P expression increases during storage indicating that platelets continuously release their granule content and become activated (81).

As previously described, a significant deviation of our murine platelet alloimmunization model from the clinical setting is the absence of storage prior to transfusion. Although, we have concluded from our $CD4^+$ T cell depletion experiments that platelets are a T-dependent antigen, these transfusions were performed with relatively quiescent donor platelets. The same may not necessarily be the case for activated platelets following prolonged storage. 'Mouse sizing' platelet storage may prove more difficult than our efforts to scale down storage of mouse red blood cells. Mouse platelets have a much shorter normal life span (~4 days) as compared to humans (~ 7 days) as well as the obvious volume limitations (82, 83). Platelets require continuous, gentle agitation in gas permeable bags to ensure oxygen transfer. Room temperature storage in plasma will likely encourage bacterial contamination as it does in the human setting. However, a reliable and accurate mouse platelet storage model would be invaluable for investigating the platelet storage lesion and the sequela of transfusing stored platelets under controlled conditions.

In Chapter 4, we presented data to support the hypothesis that the spleen is required for the humoral response to allogeneic platelets. This observation presents several potential avenues for future study. First, what factors unique to the spleen promote the humoral response? Immunohistochemistry could be utilized to track $eGFP^+$ platelets to identify where anatomically platelets are sequestered in the spleen and what particular antigen presenting cell populations are phagocytosing them. It is currently not known what cell populations are responsible for senescent platelet clearance, and

whether this population differs for the clearance of allogeneic platelets. The particular antigen presenting cell subsets may also be distinctly sensitive to inflammatory conditions, as has been reported for red blood cell antigen presentation (38, 84). Secondly, the non-responsiveness in splenectomized recipients may be due to active mechanisms tolerance due to donor antigen presented by the liver milieu. Following platelet transfusion, TCR75 cells could be isolated from the liver of splenectomized recipients and compared to those from intact animals for the expression of markers such as PD-1, CTLA4, or FOXP3. These cells could be adoptively transferred into naïve intact recipients to test for their ability to mediate suppression to a subsequent donor antigen challenge. Third, the spleen may be required for the primary response, but not the memory response. Antigen experienced TCR75 cells could be isolated from intact mice and adoptively transferred to splenectomized recipients. Subsequent challenge with donor platelets could test if the spleen were required for the maintenance of the memory response.

Long term future directions

It cannot be definitively concluded from our platelet transfusion experiments that platelets alone are immunogenic and that the humoral response we observed was not simply due to contaminating leukocytes. To test this hypothesis, we will generate a novel transgenic mouse with tissue specific expression of the MHC class I alloantigen. DNA encoding the K^d MHC class I molecule will be introduced by homologous recombination directly into the K^b locus of a C57BL/6 mouse. Cre-lox technology will be utilized to allow conditional expression of the K^d gene by the introduction of a Lox-

STOP-Lox cassette upstream of the K^d start codon (85). By breeding this transgenic animal with existing commercially available transgenic strains that have specific expression of CRE in different cell subsets, experimental donors can be generated with restricted expression of the K^d MHC class I alloantigen. This system will allow us to not only test if delivery of MHC class I on platelets is alone allogeneic, but also to determine the potential immunoregulatory effects when allogeneic MHC class I is present on other cell subsets as well.

The majority of the experiments presented herein focus on the role of the $CD4^+$ T cell response, which presumably provides help to donor specific B cells. However, the experiments lacked the ability to focus directly on the donor specific B cell response. To look at the allospecific B cell response, we could utilize B cell receptor transgenic animals. The 3-83 B cell receptor transgenic mouse would be, in most aspects, an ideal candidate for these studies (86, 87). Greater than 95% percent of the B cells in these mice express the transgenic BCR. The 3-83 BCR has dual alloreactive specificity for H-$2K^k$ with high affinity and H-$2K^b$ with low affinity, which affords this model a level of sophistication not achieved in many other BCR systems. The 3-83 B cells can be traced by flow cytometry or immunohistochemistry using the 3-83-idiotypic antibodies or by either K^k or K^b tetramers.

Clinical trials could be envisioned pairing costimulation blockade treatment with platelet transfusions for patients at high-risk for refractoriness or where the induction of alloimmunization would be detrimental to future solid organ transplantation or bone marrow replacement therapy. In addition to CTLA4-Ig, blockade of the CD40 signaling pathway through the use of anti-CD40 monoclonal antibodies may be particularly suited

for preventing alloimmunization following platelet transfusion (50, 88). Platelets are a major source of CD154, which has been shown in many settings to modulate the antibody response (30, 31). Treatment with an appropriately designed anti-CD40 blocking antibody could potentially inhibit the alloantibody response and neutralize the additive effects of platelet derived CD154.

General conclusions

Platelet transfusion is an essential treatment for thrombocytopenia, which can result from a wide range of conditions including acute hemorrhage, hematological malignancies, and hematopoietic stem cell transplantation. A valuable life-saving therapy, transfusion also carries significant risks. Alloantibodies can be developed against donor antigens present on the platelet surface. As a result, an alloimmunized patient may become refractory to subsequent transfusions. Increasingly sophisticated methodologies, including leukoreduction and irradiation of platelet products, have decreased the incidence of anti-platelet antibodies by limiting the recipient's exposure to antigenic white blood cells. In spite of these efforts, alloimmunization following platelet transfusion still occurs in a percentage of patients and leukoreduction remains a controversial topic.

Many questions remain about the optimal use of platelets and how some of the significant adverse events associated with platelet transfusion arise and how best to deal with them. A broader appreciation of the interplay between the recipient immune response and donor platelets is needed to elucidate these issues. Hopefully, the current findings in this work and the future utility of the model systems developed within this dissertation will contribute to this understanding.

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