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Major and Minor Alloimmunization Induced by Platelet Transfusions: Mechanisms, Consequences, and Interventions

By

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

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Major and Minor Alloimmunization Induced by Platelet Transfusions: Mechanisms, Consequences, and Interventions

By

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B.S., University of Georgia, 2004

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In response to vasculature injury, anucleated cell fragments, referred to as platelets, adhere and aggregate to damaged blood vessels to preserve hemostasis through clot formation and wound healing. Platelet disorders resulting in low platelet counts or dysfunctional platelets can require aggressive platelet transfusion support to aid in maintaining proper hemostasis levels. Although a vital therapy, platelet transfusions are associated with long-term clinical consequences, such as development of donor specific alloimmunity and consequential refractoriness to subsequent platelet transfusions. When successful, bone marrow transplantations are a potential cure for the above patients. In a non-malignant setting, eligible patients can receive Human Leukocyte Antigen (HLA)-matched bone marrow transplant. Due to the absence of neoplasia in such patients, it is difficult to justify the toxic effects of stringent conditioning regimens that are otherwise beneficial in eradicating cancerous cells and promoting engraftment. Thus, many physicians choose to transplant under reduced intensity conditions. However, under these conditions, it is observed that chronically transfused patients reject the HLA-matched bone marrow transplant at higher rates than minimally or un-transfused patients. Because the transplants are HLA-matched or -identical, it is predicted that rejection is due to minor histocompatibility antigens. Therefore, the goal of the current dissertation is to test and characterize the role of prior alloimmunization against minor histocompatibility antigens, expressed by transfused platelet units, in the rejection of an MHC-identical, unrelated donor bone marrow transplant. The data

presented herein demonstrate that minor histocompatibility antigen reactive cellular alloimmunity induced in response to allogeneic leukocyte reduced platelet transfusions can induce rejection of a subsequent MHC-matched bone marrow transplant expressing the target minor histocompatibility antigens under reduced intensity conditions. To the extent that the observed murine biology reflects human physiology, these findings additionally indicate potential clinical interventions to overcome refractoriness to platelet or red blood cell transfusions and rejection of an HLA-matched transplant under reduced intensity conditions in allogeneic platelet transfused patients.

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Chapter 1

Introduction

In response to vasculature injury, anucleated cell fragments, referred to as platelets, adhere and aggregate to damage blood vessels to preserve hemostasis through clot formation and wound healing. Patients with blood disorders resulting in low platelet counts, referred to as thrombocytopenia, or dysfunctional platelets may require aggressive platelet transfusion support to aid in maintaining proper hemostasis. Although a vital supportive therapy, platelet transfusions are associated with adverse reactions that result in clinical consequences, such as development of humoral alloimmunity to donor specific alloantigens and consequent refractoriness to subsequent platelet transfusions expressing the target antigens.

Because platelet transfusions are a transient therapy for such patients and disease severity can surpass beneficial effects of supportive therapies, bone marrow transplantations are also performed as a potential cure (1-7). In a non-malignant setting, eligible patients receive Human Leukocyte Antigen (HLA)-matched or -identical bone marrow transplantations. Due to the absence of neoplasia, it is difficult to clinically justify utilizing stringent conditioning regimens that in a malignant setting have beneficial toxicity effects in killing cancerous cells in addition to promoting engraftment. Thus, many physicians choose to transplant patients with non-malignant blood disorders (also referred to as hematological disorders) under reduced intensity conditioning regimens (3, 7, 8). Under the reduced intensity conditions, however, it is observed that chronically transfused patients reject HLA-matched bone marrow transplantations at higher rates than minimally or un-transfused patients (9-14).

Because the transplants are HLA-matched but minor histocompatibility antigen mismatched, it is predicted that rejection occurs across minor histocompatibility antigen barriers (discussed further in *Minor Histocompatibility Antigens* section). Presentation of minor histocompatibility antigens to recipient T cells can occur through four distinct allorecognition pathways: 1) direct presentation, 2) semi-direct presentation, 3) indirect presentation, and/or 4) cross presentation (discussed further in *Alloimmunization to minor histocompatibility antigens* section). Using murine models, it has been demonstrated that donor reactive antibodies against alloantigens expressed on transfused blood products can occur through the indirect presentation pathway (15, 16). However, because there are residual leukocytes, referred to as "passenger" leukocytes, in platelet products, others hypothesize that the direct allorecognition pathway can additionally, if not alone, induce donor reactive alloantibodies in response to platelet transfusions. It is speculated that donor "passenger" leukocytes express endogenous polymorphic peptides, albeit HLAs or minor histocompatibility antigens, that can be directly presented to recipient T cells.

To prevent alloantibody responses through the predicted direct presentation pathway, the U.S. Federal Food and Drug Administration currently and routinely requires platelet products to be leukocyte reduced to decrease the quantity of "passenger" leukocytes in the platelet concentrates. Although, leukocyte reduction substantially decreases the rate of alloantibody production (17-19), it does not prevent alloimmunization; thus, suggesting that the predicted direct pathway alone is not required for alloantibody production to occur in

response to platelet transfusions or that the induction of alloantibody responses to platelet transfusions only require a few “passenger” leukocytes. Moreover, the failure to inhibit alloimmunization through leukocyte reduction methodology suggests the possibility of transfused platelets themselves as a source of minor histocompatibility antigens.

As demonstrated by the abundance of research on alloantibody responses to platelet transfusions, the dominant immunological sequela and effect of leukocyte reduction on platelet transfusions is alloantibody responses that can result in refractoriness to subsequent platelet transfusions. Because very little to no research is performed on cellular responses to platelet transfusions, the immunological sequelae and effects of leukocyte reduction on cellular alloimmune responses remains undefined. Thus, the current dissertation tests the hypothesis that $CD8^+$ T cell alloresponses are primed in response to leukocyte reduced platelet transfusions through the indirect presentation pathway and that this cellular alloresponse is required to induce rejection of a subsequent Major Histocompatibility Complex (MHC)-matched bone marrow transplantation across minor histocompatibility antigen barriers. The clinical significance of the present studies is the speculation that leukocyte reduced platelet transfusions act as an additional rejection vector relevant to bone marrow transplantations. Moreover, to the extent that a solid organ graft shares minor histocompatibility antigens with platelet products, the current dissertation extends the potential clinical relevance of platelet transfusions to rejection of solid organ transplantations, as many transplant candidates receive platelet transfusions as

part of their pre- and peri-operative care (20, 21). Because observations made in this dissertation are in a murine system, conclusions on the clinical implications of these data cannot be carried out without testing the general hypothesis in a human setting. Nonetheless, the data presented in this current dissertation introduces potential and previously unappreciated immunological sequelae in transfusion medicine and transplantation.

Transfusion Medicine

A brief historical overview

Combination of William Harvey's research on blood circulation in 1628 (22, 23) and Sir Christopher Wren's development of intravenous injections 30 years later inspired the first attempts at blood transfusion (24, 25). In 1665 the first successful transfusion was performed when English anatomist and physiologist Richard Lower transfused nearly exsanguinated dogs by connecting the recipient's jugular vein to a healthy donor dog's carotid artery. The blood transfusions prolonged the exsanguinated dogs' lives; leading Richard Lower to conclude that blood transfusions can be utilized to treat ailments resulting in lost or corrupt blood (26).

A few years later, in 1667, French physician Jean-Baptiste Denis performed the first successful transfusion to a human by transfusing a young, agitated man with lamb blood (24, 27). Therein, Denis recorded the first hemolytic transfusion reaction, characterized by abdominal pain, fever, chills and passing of dark 'soot' colored urine containing excessive amounts of iron from

lysed red blood cells. Although the young man was symptomatic for the now appreciated hemolytic transfusion reaction, he survived and transfusion of animal blood to a human proved to be triumphant. However, it was in the course of Denis's fourth transfusion patient that hemolytic transfusion reactions consequential of animal-to-human transfusions revealed to be dangerous and deathly; thus, resulting in the prohibition of animal-to-human transfusions and transfusion practice falling out of favor for the next 150 years (24, 27).

It was not until 1818, when British obstetrician James Blundell performed the first efficacious human-to-human blood transfusion in a woman suffering from post-partum hemorrhage (28, 29), that transfusion practice would be resurrected. However, despite James Blundell's advancement, transfusion of animal blood to humans was nonetheless performed and increasingly associated with hemolytic transfusion reactions that frequently resulted in morbidity and/or mortality. The etiological concept for the above transfusion reactions unfortunately was unidentified until the early 20th century when the science of transfusion medicine began to rapidly advance.

In 1901, Karl Landsteiner performed the earliest form of cross-matching blood by mixing recipient sera with donor blood to assess donor and recipient compatibility, with incompatibility measured as donor blood agglutination. The experimental observations made by Karl Landsteiner stated "the serum of healthy men will agglutinate not only the red cells of animals, but also often those of other individual humans." It was this experimental observation that prompted Karl Landsteiner's research and discovery of the ABO blood group system, for

which he received the Nobel Prize for Medicine in 1930 (30, 31). Soon thereafter Sturli and DeCastello (1902), and Landsteiner and Weiner (1940) discovered the fourth ABO blood group antigen AB and the Rhesus blood group system, respectively. With the amalgamation of blood group antigen knowledge and applicable cross-matching blood technology in transfusion medicine, the incidence of hemolytic transfusion reactions significantly decreased in adults and neonates; thus, finally allowing for safer and more dependable transfusions.

Moreover, with the above methodologies implemented to generate more reliable transfusions, the transfusion medicine community focused attention to the lack of a critical innovation that would resolve the inability to transfuse without both donor and recipient present. In 1914, the necessity of donors and recipients to be in the same room was removed when Albert Hustin performed the first human-to-human transfusion using refrigerated donor blood stored in sodium citrate, an anti-coagulant, for several days (32). This successful experimental observation paved the way for U.S. army officer Oswald Robertson to generate the earliest version of a modern transfusion blood bank. In 1916, while serving in France during World War I, Oswald Robertson discovered that addition of dextrose to anti-coagulant storage solutions extended blood storage up to two weeks. Using this dextrose and citrate solution, Oswald Robertson developed the first blood bank, or 'blood depot'; consisting of cross-matched blood collected into glass tubes containing the citrate and dextrose mixture, and then stored on ice for up to 26 days (33). While Oswald Robertson is credited with inventing the

first blood bank, the Soviet Union in 1932 was the first nation to develop a true national blood bank in Leningrad.

Further advancement in the science of blood banking occurred in 1943 with Loutit and Mollison's research demonstrating that acidification of anti-coagulant storage solutions permitted autoclaving of such solutions to prevent bacterial growth during storage. With this knowledge, Loutit and Mollison generated the now exceedingly utilized acid-citrate-dextrose anti-coagulant storage solution that allows blood to be stored at 4°C for up to four weeks (34). A few years later, in 1957, an extension and more progressive version of acid-citrate-dextrose were generated with the addition of phosphate to create the now universally utilized citrate-phosphate-dextrose anti-coagulant storage solution. In comparison to acid-citrate-dextrose, citrate-phosphate-dextrose extended blood storage up to 28 days with greater red blood cell survival (35). In 1979, the addition of adenine to citrate-phosphate-dextrose anti-coagulant storage solution to generate the modern age storage solution citrate-phosphate-dextrose-adenine-1 extended the shelf life of whole blood and red blood cell unit survival up to 35 days.

Simultaneous to the development of blood storage technology, Carl Walter and W.P. Murphy, Jr., in 1949, introduced plastic bags to the blood banking community for blood collection and storage. Breakable glass bottles, originally utilized to collect and store blood, were replaced with durable plastic bags; which allowed for sterile differential centrifugation methods that fractionate blood components based on density. Using these adapted plastic bags, Joseph Edwin

Cohn performed the first separation of three main blood products, red blood cells, platelets and plasma; which opened up doors for the foundation of various individual blood component transfusion therapies such as red blood cells and platelets.

Development of platelet transfusion medicine

As the majority of milestone findings in science are made, the discovery of platelets was a sheer accident. In 1865, the initial aim of Max Schultze's research was to test the effects of temperature on the protoplasm of blood cells. However, upon observing live blood samples under a microscope, Max Schultze uncovered a unique cell population accurately portrayed as loosely clumped, 'colorless little spherules' that were smaller than red blood cells in size and under certain circumstances had the appearance of rays covered with fibrous material (36). It was therein that platelets were unearthed and acknowledged for the first time as a new distinct blood cell population.

The physiological function of these 'colorless little spherules' was slowly unveiled a few years later, beginning with William Osler's observation that a clot formed on a damaged blood vessel primarily consisted of these 'colorless little spherules' (37). In 1882, Giullio Bizzozero tested the role of the 'colorless little spherules' in clot formation, also referred to as a thrombus, and hemostasis when in response to vasculature damage. Using a needle, Giullio Bizzozero exerted light pressure on the wall of an artery found in the mesenteric tissue of a live guinea pig and observed that the 'colorless little spherules' were the first

component in the blood to adhere to the damaged blood vessel and also were the main structural integrity of the subsequent thrombus (36, 38). Furthermore, in this report, Giullio Bizzozero named the 'colorless little spherules' blutplättchen in German; which in English translates to platelets.

Albeit the physiological role of platelets in maintaining hemostasis was concluded by the end of the 19th century, the value of platelets in transfusion medicine remained to be unveiled. It was in 1910 when William Duke reported for the first time the usefulness of platelets in transfusion medicine. When presented with three bleeding (also referred to a hemorrhaging) patients symptomatic of thrombocytopenia, William Duke transfused the patients with platelet containing fresh whole blood to reverse the hemorrhaging. Upon transfusion, William Duke observed an inverse correlation in whole blood transfusion and hemorrhaging time (39-41); thus, for the first time demonstrating the importance of transfusing platelets as a therapeutic modality to prevent hemorrhaging.

It was not until the 1960s, a decade subsequent to differential centrifugation technology permitting platelet fractionation from whole blood, that the first isolated platelet transfusions were performed. In 1964 investigators at the National Cancer Institute noted that in multiple retrospective studies of mortality in leukemia patients hemorrhaging was the major cause of death (41). More importantly, in these early studies the investigators observed an inverse correlation in platelet transfusions and hemorrhaging episodes. As platelet transfusions were given to hemorrhaging leukemia patients, a reversal and

decrease in the frequency of hemorrhaging occurred. This inverse correlation led to the current establishment of isolated platelet transfusions as an efficient supportive therapy to maintain hemostasis.

Unfortunately, the inability to store viable platelet rich plasma prevented the widespread use of platelet transfusions. Nevertheless, in 1971 the lack of proper platelet storage conditions was overcome when Scott Murphy and Frank Gardner's research demonstrated that storage of platelet rich plasma at 22°C with a gentle agitation, in contrast to the previous 4°C storage, extended the shelf life of platelet rich plasma to three to four days (42, 43). Under these conditions, though, platelet viability decreased 15 to 20% per day (43); suggesting that optimization of platelet storage conditions remained. In 1975 platelet viability during storage enhanced when Scott Murphy and Frank Gardner demonstrated that platelet rich plasma products require storage in a gas permeable plastic bag in a 10% CO_2 atmosphere, so as to not to disrupt platelet metabolism and to prevent the pH from decreasing below 6 (44). With the ability to finally store platelets, modern era platelet transfusion practice was achieved.

Clinical platelet transfusions

Approximately 1.5 and 2.9 million platelet transfusions are administered in the United States and Europe, respectively, as prophylactic or corrective therapy for patients with thrombocytopenia or platelet dysfunction (20). Imbalances in hemostatic equilibrium, due to thrombocytopenia or loss in platelet function, can transpire as an end product of a plethora of congenital and acquired conditions,

including malignant and non-malignant hematopathologies (i.e. aplastic anemia and von Willebrand disease), trauma, liver or kidney diseases, and medications (41, 45). In the United States, platelet transfusions are administered to patients actively hemorrhaging, or demonstrating symptoms of petechia, easy bruising, mucosal membrane bleeding and/or a decline in platelet count to roughly 10,000 – 100,000 platelets per µl (45, 46).

Currently, two methodologies exist in the United States to isolate platelet concentrates for clinical transfusions, platelet fractionation from pre-collected whole blood or via apheresis (20, 45-47). Although the practices differ slightly in technique, both systems utilize differential centrifugation technology to harvest platelets. To harvest platelet rich plasma from whole blood, collected in an anti-coagulant solution in a closed plastic bag system, whole blood undergoes a low speed centrifugation that fractionates platelets rich plasmas from red blood cells and leukocytes. Harvested platelet rich plasma then undergoes a fast speed centrifugation to pellet the platelets, followed by re-suspension in 50 to 70 mL of the original donor plasma. Platelet rich plasma isolated through this technique is also referred to as random donor platelet concentrates, as multiple donor platelet rich plasma units are combined to generate one platelet transfusion dose. In apheresis, platelet rich plasma is isolated through a similar technique; however, in real time, donor blood is passed through a mechanical apparatus that separates platelet rich plasma from the whole blood while simultaneously returning remaining blood components to the donor.

Succeeding fractionation through the whole blood methodology, platelet rich plasma is passed over a leukocyte reduction filtration system to significantly diminish the quantity of “passenger” leukocytes in the platelet unit. The predominate goal of leukocyte reduction technology is to potentially abolish deleterious risks associated with transfusing “passenger” leukocytes, such as viral transmission, febrile non-hemolytic transfusion reactions, and alloimmunization against donor HLA molecules (45). In the blood, leukocytes can carry viruses such as cytomegalovirus (CMV) and human immunodeficiency virus (HIV); thus in lessening the amount of transfused “passenger” leukocytes, the risk of blood borne viral transmission is also decreased. Furthermore, because pro-inflammatory cytokines (i.e. IL-6 and IL-1β) that promote febrile reactions upon transfusion can be derived from activated “passenger” leukocytes during storage, platelet products are leukocyte reduced prior to storage to diminish the build up of pro-inflammatory cytokines in the stored platelet unit and thus the frequency of febrile non-hemolytic transfusion reactions (48).

Platelet units are additionally leukocyte reduced prior to storage or transfusion in an attempt to significantly reduce the magnitude of alloimmunization against donor HLA molecules. Before leukocyte reduction technology, roughly 45 - 70% of aggressively transfused patients developed antibodies reactive to donor HLA Class I antigens that consequentially resulted in refractoriness to subsequent platelet transfusions in roughly 30% of these patients (17, 49). However, upon adaptation of leukocyte reduction, a number of human and murine studies have convincingly demonstrated a positive correlation

in transfusion of leukocyte reduced platelet units, and an extensively decreased rate of donor specific alloantibody production and refractoriness to platelet transfusions (17, 19, 20, 46, 50, 51). The most prevalent multicenter clinical trial testing the beneficial effects of leukocyte reduction in reducing alloimmunity was the TRAP trial performed in 1997. In this clinical trial, alloantibody production in a cohort of apheresis or leukocyte reduced random donor platelet transfused acute myeloid leukemia patients were assessed. While 45% of acute myeloid leukemia patients transfused with non-leukocyte reduced single apheresis units (control group) developed donor HLA specific alloantibodies, roughly 18% of acute myeloid leukemia patients transfused with leukocyte reduced random donor platelet units generated alloantibodies against donor HLA molecules (17). Moreover, the reduction in donor HLA reactive alloimmunization rates in leukocyte reduced platelet transfused patients positively correlated to a decline in rate of refractoriness; 13% of the control group developed refractoriness in comparison to approximately 3% in the leukocyte reduced platelet transfused group. Though these data demonstrate the beneficial effects of leukocyte reduction in reducing the rate of alloimmunization and refractoriness, decreasing the quantity of “passenger” leukocytes in platelet units does not eliminate the ability to generate an alloresponse to donor alloantigens expressed by transfused platelet products. Nevertheless, because leukocyte reduction significantly reduces the rate of alloimmunization, the U.S. Federal Food and Drug Administration mandates that platelet units contain fewer than or equal to 1 - 5 x 10^6 “passenger” leukocytes (20, 46). Unfortunately, because alloantibody

responses upon leukocyte reduction are not eliminated and fiscal limitations hinder leukocyte reduction in numerous cities, states, blood banks, hospitals, and countries inapplicable, alloimmunization and resultant refractoriness persist as a dilemma in transfusion medicine.

In addition to regulating the quantity of “passenger” leukocytes in a transfused platelet unit, the U.S. Federal Food and Drug Administration standardizes platelet unit storage. Platelet rich plasma is stored in plasma at 20 - 24°C for up to 5 days. Platelets are stored in an oxygen-permeable plastic bag, as anoxic conditions induce metabolic pathways that result in lactic acid build up and consequently platelet death (20, 45). To assure that oxygen is accessible to all platelets, the units are continuously gently agitated to prevent platelet sedimentation during storage. Because plasma and storage at 20 - 24°C increases risks of bacterial growth over time, platelet units are stored for only up to 5 days (45). It is possible that platelet units can be stored at colder temperatures similar to red blood cell units; however, cold storage of platelets induces platelet glycoprotein receptor (i.e. GP1b) clustering that upon transfusion is recognized by Kupffer cells (liver macrophages) in the liver that rapidly clear the transfused chilled platelets from circulation (20, 52). Furthermore, platelet storage does not exceed 5 days, as a number of studies demonstrate that approaching 7 days of storage platelets activate, undergo morphological alterations, lose aggregation function *in vivo*, and become contaminated with bacterial pathogens (46, 53, 54).

Because a number of modifications can occur during storage, quality control assays are performed on the stored platelets prior to transfusion. Prior to transfusion, platelet units are tested for morphological transformations via the "swirl" test (assessment of discoid morphology of platelets based on the ability to refract light), tested for pathogen contamination (bacterial and viral), and tested for ABO and Rhesus blood group antigen compatibility (20, 47). Because the short lived shelf life of platelet units renders the supply of platelet units less than demand, platelet units are typically only ABO and Rhesus matched to further prevent alloimmunization against donor antigens and subsequent refractoriness to both platelet and red blood cell transfusions. HLA-matching platelets to recipients typically is performed for patients who are refractory to subsequent random donor platelet transfusions due to the existing HLA reactive alloimmunization.

Depending on platelet counts and the underlying physiological causation for prophylactic platelet transfusion therapy, patients with thrombocytopenia or dysfunctional platelets will typically receive 3 - 4 x 10^{11} compatible, high quality platelets (45). Because whole blood derived platelet units result in approximately 0.5 x 10^{11} platelets per unit, 4 - 8 units of matched whole blood derived platelet units are pooled to generate one random donor platelet dose of 1 - 3 x 10^{11} platelets. In contrast, platelet apheresis yields 1 - 3 x 10^{11} platelets per unit, which equates to one or more therapeutic platelet doses. Roughly 75% of transfused platelet units in the United States are apheresis products, as platelet apheresis generates one platelet dose simultaneous to reducing the probability of

pathogen transmission, alloimmunization against multiple donor alloantigens, and the rate of platelet refractoriness (45, 55). However, because the effect of platelet dosing on immunogenicity of a platelet transfusion is unknown, it is debated that the higher dose of platelets in an apheresis unit is actually more immunogenic than the smaller dose of platelets in a single pooled random donor platelet unit.

Because alloimmunity based physiological factors can induce immediate clearance of transfused platelets, and thus defeat the purpose of a platelet transfusion, platelet transfusion efficacy is measured at 10 or 60 minutes and 18 - 24 hours post transfusion. Responses to platelet transfusions are measured as (1) improvement in hemorrhaging, (2) absolute platelet increment counts (post-transfusion minus pre-transfusion platelet count with platelet transfusion effectiveness measured as a 10,000 platelet/μl increase post-transfusion), (3) corrected count increments (ratio of body surface area by platelet increment count to number of platelets transfused), and/or (4) percent platelet increments (ratio of observed to expected platelet count increment) (20, 45-47). When successful, platelet transfusions are a vital supportive therapy that restores hemostasis and prevents future hemorrhaging.

Although an imperative and life saving therapy, platelet transfusions can be associated with a wide array of potential clinical adverse effects that are categorized as 1) infectious and 2) non-infectious complications. Infectious by-products traditionally associated with platelet transfusions include CMV, Hepatitis B, Hepatitis C, HIV, Human T-Lymphotropic Retrovirus I and II (HTLV-I and –II),

malaria, and bacterial contaminants (56, 57). Recently, the Advancing Transfusion and Cellular Therapies Worldwide (AABB) Transfusion Transmitted Disease committee confirmed emerging pathogens Babesia, Trypanosoma, Dengue, and the prion responsible for resulting in variant Creutzfeldt-Jakob disease as additional agents of high concern to transfusion medicine (56, 57). However, with the advent of nucleic acid testing for pathogens and alternate sophisticated technology to screen donors and the blood supply for pathogens and prions, the risk of transfusion-transmitted infections has significantly decreased by roughly 10,000-fold (58, 59).

With the incidence of transfusion-transmitted infections approaching low levels (i.e. 1 in 2.3 million for HIV), the current concerns in platelet transfusion medicine are more focused on non-infectious reactions to platelet transfusions (59, 60); which are referred to as non-infectious serious hazards of transfusion and subcategorized into 1) non-immune and 2) immune mediated complications. Transfusion-associated circulatory overload (TACO) is an example of a non-immune mediated non-infectious serious hazard of transfusion that more often occurs in response to an increase in plasma transfusion rate, but can additionally result from an increased platelet and/or red blood cell transfusion rate. Patients with underlying cardiopulmonary complications, renal failure, and infants are at a greater risk of developing transfusion-associated circulatory overload, and may develop physiological symptoms including dyspnea, cough, tachycardia, hypertension, and widened pulse pressure (59, 60). The most common immune mediated non-infectious serious hazards of transfusion that can occur after

platelet transfusions include hemolytic transfusion reactions, febrile non-hemolytic transfusion reactions, post-transfusion purpura, transfusion related acute lung injury (TRALI), transfusion related immunomodulation (TRIM), allergic/anaphylactic reactions, transfusion associated graft versus host disease, and alloimmunization against donor alloantigens (59, 60). Of the immune mediated non-infectious serious hazards of transfusion reactions, alloantibody responses against donor alloantigens expressed on transfused platelet concentrates, such as HLA, ABO, and Human Platelet Antigens (HPAs), can result in hemolytic transfusion reactions, febrile non-hemolytic transfusion reactions, post-transfusion purpura, and refractoriness to subsequent platelet and red blood cell transfusions (20, 59, 60). Moreover, it is speculated that transfusion induced alloimmunization against such donor alloantigens is an additional immunological obstacle in bone marrow and solid organ transplantation (10-14, 61-63). Rates of allergic/anaphylactic reactions and alloimmunization induced immune complications can be substantially decreased upon platelet concentrate irradiation (which can additionally reduce rates of transfusion associated graft versus host disease), leukocyte reduction prior to storage of the platelet units, and by testing patients serologically for donor reactive alloantibodies prior to a subsequent transfusion and/or transplantation.

Alloantibody responses to platelet transfusions

Because donor specific alloantibodies induced in response to platelet transfusions can result in numerous adverse immunological reactions such as

those mentioned in the *Clinical platelet transfusion* section, the focal point of immunity in transfusion medicine is humoral alloimmunization. Clinically, humoral alloimmunization is defined as the generation of antibodies reactive against a tissue alloantigen derived from a genetically dissimilar individual of the same species through a transfusion and/or transplantation. In the context of platelet transfusions, patients can become alloimmunized against antigens expressed on the cell surface of the platelets, and/or the residual red blood cells and "passenger" leukocytes in the transfused platelet concentrate. Clinically, alloantibody production in response to platelet transfusions is predominately directed against HLA Class I molecules; approximately 20 - 40% of transfused patients become alloimmunized (17, 49, 64-67).

Conversely, 2 - 10% of allogeneic transfused patients develop donor reactive antibodies to platelet specific polymorphisms, such as HPAs 1 - 15 (59, 64, 66, 67). ABO and Rhesus D compatible platelet units are sometimes but not always administered, and thereby can limit the generation of alloantibodies against these blood group antigens when matched (20, 46, 47). However, platelet units are not matched for additional polymorphisms including HPAs expressed on platelet specific glycoproteins. The majority of clinically detectable HPA reactive alloantibodies are specific to the HPA-Ib and HPA-5b alloantigens, and to a lesser extent to HPAs carried on platelet specific glycoproteins GPIb/IX/V, GPIIb/IIIa, GPIba/IIa, and GPIb/IX (59, 64, 67). While the clinical incidence of HPA alloimmunization is unaltered in patients that receive leukocyte

reduced platelet transfusions (59, 64, 67), the probability of HLA Class I alloimmunization significantly decreases (17, 19, 20, 46, 51).

The reduction in the incidence of HLA alloantibody responses in leukocyte reduced platelet transfused patients is widely hypothesized to be due to a significant diminishment in donor MHC Class II expressing leukocytes required to initiate T cell dependent alloantibody responses. In the absence of significant inflammation and high interferon γ, platelets express HLA Class I but not HLA Class II molecules, whereas leukocytes including antigen presenting dendritic cells, macrophages, and B cells express both low levels HLA Class I and II molecules (68, 69). It is reasoned that through direct allorecognition (discussed further in *Alloimmunization to minor histocompatibility antigens* section) donor "passenger" leukocytes expressing HLA Class II molecules can activate recipient $CD4^+$ T cells to provide help to recipient B cells specific for donor HLA Class I molecules. Inhibition of detectable MHC Class I reactive alloantibodies in murine recipients transfused with allogeneic platelet units lacking leukocytes further contend that "passenger" leukocytes are required for the direct induction of humoral alloresponses to platelet transfusions (50, 70).

However, because HLA and HPA alloimmunization is not eliminated in patients transfused with platelet concentrates significantly reduced in "passenger" leukocyte quantity, it is argued that the induction of humoral alloimmunization in response to platelet transfusions additionally can be elicited through the alternate indirect allorecognition pathway (discussed further in *Alloimmunization to minor histocompatibility antigens* section). Through the

indirect presentation pathway, recipient antigen presenting cells can present consumed and processed donor HLA Class I peptides on recipient HLA Class II molecules to recipient $CD4^+$ T cells that upon activation provide help to recipient B cells reactive against donor HLA Class I molecules. A number of *in vitro* murine studies have reported the proliferative capacity of previously sensitized splenocytes in response to recipient antigen presenting cells pulsed with allogeneic platelets only when the indirect presentation pathway is intact (15, 71, 72). These findings suggest that humoral alloimmunization in response to platelet transfusions can occur through the indirect presentation pathway. However, whether humoral alloresponses to platelet transfusions necessitate the direct and/or indirect pathways still remains uncertain. Thus, the focal point of platelet transfusion medicine continues to be understanding the immunological mechanisms of humoral alloimmunization against clinically relevant alloantigens expressed on transfused platelet concentrates, and thereby to develop efficacious antigen specific modalities to prevent platelet transfusion induced humoral alloimmunization.

Indeed, fundamental in generating therapies to prevent alloantibody production in response to platelet transfusions, such studies do not consider the less appreciated environmental and/or genetic factors as explanations for the inability to clinically prevent humoral alloimmunization. Inflammation of murine recipients at the time of the transfusion has been documented to have an effect on red blood cell alloimmunization, and thus suggest a potential function of environmental factors in the generation and modulation of platelet transfusion

induced humoral alloimmunity (73-76). Currently, the effects environmentally induced recipient inflammation has on humoral alloresponses to platelet transfusions is not well understood, as there are little to no murine models available to directly test these effects in a platelet transfusion setting.

Likewise, there are few studies directly assessing the role of genetic predispositions in regulating humoral alloresponses to platelet transfusions. It has been reported that donor MHC Class I alloantibody responses to platelet transfusions in murine recipients correlate with the expression of MHC Class II I-A and I-E (77). Recipient expression of I-E has been demonstrated to correlate with generation of high donor MHC Class I reactive alloantibody titers, whereas the lack of recipient I-A expression correlates with less, if not undetectable, alloantibodies specific for donor MHC Class I molecules. Based on crystallography, the murine I-A molecules are analogous to the human HLA-DQ, whereas the murine I-E molecules are counterparts of the human HLA-DR molecules; thus, indicating a potential role of genetics in alloresponses. A recent clinical study additionally has suggested that expression of immunomodulatory genes, such as TRIM21, play a critical role in modulating alloantibody responses to red blood cell transfusions in sickle cell disease patients (78). The TRIM21 protein has been described to negatively regulate immune activation through ubiquination and degradation of the interferon regulatory factors (79). This clinical study observed a correlation in the age in which sickle cell disease patients becomes alloimmunized following red blood cell transfusions and the TRIM21 gene; which was interpreted to regulate rates of recipient immune

maturation and tolerance during the immature stage of immunological development. Regardless of the regulatory factors, the heart of platelet transfusion medicine is centered on humoral alloimmunization because of its immediate clinical adverse effects.

Cellular responses to platelet transfusions

The evident clinical consequences of humoral alloimmunization account for the firmly rooted studies characterizing antibody responses to platelet transfusions; however, because there are two arms to adaptive immunity, it is certainly legitimate to inquire as to the clinical relevance of the less apparent and perhaps potentially under appreciated cellular alloimmunity in platelet transfusion medicine. The cellular arm of immunity consists of both adaptive $CD4^+$ or $CD8^+$ T cells, and innate NK, NKT, or in some instances γσ T cells. Of the murine studies characterizing the induction of cellular alloresponses to platelet transfusions, the predominant objective of each study has been to test the role of donor reactive $CD4^+$ or $CD8^+$ T cells and NK cells in the regulation of humoral alloimmunity to platelet transfusions (15, 80).

However, outside the impact cellular alloimmunity has on the induction of humoral alloimmunization, little to no research is focused on the potential immediate outcome cellular alloimmunization has on subsequent transfusions. Likewise, there is currently little to no research focused on the long-term effects of cellular alloimmunization on the disease being treated. Aggressive platelet transfusion support is an integral component in the treatment of non-malignant

hematologies resulting in thrombocytopenia and/or dysfunctional platelets. As a potential cure, some patients with non-malignant bone marrow failure syndromes can receive an HLA-matched bone marrow transplant (discussed further in *Bone marrow transplantations for non-malignant hematological disorders* section). The success rates of bone marrow transplantations in general are dependent on established donor reactive alloimmunity at the time of transplantation. Moreover, it has recently been demonstrated that alloimmunization against blood group antigens can contribute to rejection of a solid organ transplantation (81). Thus, it is necessary to characterize the mechanisms of cellular alloresponses induced in response to platelet transfusions and to question the long-term effects of platelet transfusion induced cellular alloimmunization on patients receiving a subsequent bone marrow or solid organ transplantation.

Bone marrow transplantations for non-malignant hematological disorders

Non-malignant hematological disorders are a group of diseases that are occur as a result of a deficiency in bone marrow function. The bone marrow dysfunction can arise from a genetic mutation and/or immunological disequilibrium, and can affect one or more of the three cellular components of blood (red blood cells, platelets, and leukocytes). Of the extensive family of non-malignant blood disorders, aplastic anemia, Fanconi anemia, and Gaucher's disease are a few examples of non-malignant hematologies that can result in thrombocytopenia. Whereas aplastic anemia is an autoimmune non-malignant hematological disorder, Fanconi anemia and Gaucher's disease are both due to

autosomal recessive mutations (5, 6, 82, 83). In addition to a deficiency in platelet output, some non-malignant blood disorders can result in the production of dysfunctional platelets. A few examples of these disorders are Bernard–Soulier syndrome, Glanzmann's thrombasthenia, and pseudo von Willebrand disease. All three disorders can be due to an autosomal mutation in an essential glycoprotein required for optimal platelet function; however, Glanzmann's thrombasthenia and von Willebrand disease have also be reported to occur as a result of autoimmunity (84, 85).

Because the defects in non-malignant hematologies arise from a disconnect in the bone marrow function, bone marrow transplantations are performed in an effort to restore endogenous hematopoiesis through replacing defective hematopoietic stem cells with healthy stem cells (1-7, 86). Though bone marrow transplantation is currently the only effective cure for these patients, not all patients with non-malignant hematological disorders qualify for the bone marrow transplantation. Because the bone marrow transplantation process is high risk, patients with severe and high-risk disease that do not predispose the patient to transplantation related morbidity or mortality are only eligible for the bone marrow transplantation (86). Moreover, bone marrow transplantations are performed more often in children than adult patients, as the rate of transplant related morbidity and mortality is higher in adults than children (86). Lastly and most importantly, it is preferred that patients have an HLA-matched, sibling donor (86). Only a small fraction of patients have matched sibling donors, and thereby is the main barrier to bone marrow transplantation for

patients with non-malignant hematological disorders. HLA-matched, unrelated donors can be utilized under certain circumstances; however, the frequency of engraftment and survival rates using HLA-matched, unrelated donors is poor as a result of an increase in major and minor alloantigenic differences (86).

In the malignant setting, bone marrow transplantation rejection is rare due to the use of stringent conditioning regimens that eliminate recipient immunity and therefore rejection. Although beneficial in promoting engraftment and killing neoplastic cells, the toxic effects of stringent regimens can result in morbidity and mortality. Because patients with non-malignant bone marrow failure syndromes lack neoplasia, it is difficult to justify the use of stringent conditions. Thus, patients with non-malignant hematological disorders receive an HLA-matched bone marrow transplantation under reduced intensity conditions with the idea that partial chimerism will cure the disease (3, 7, 8, 87, 88). However, under reduced intensity conditions, engraftment of the HLA-matched bone marrow transplantation occurs less efficiently; roughly 15% of the transplanted patients reject the bone marrow transplantation (1, 9, 89).

Traditionally, a high dose of cyclophosphamide was utilized to condition patients with non-malignant bone marrow failure syndromes undergoing HLA-matched bone marrow transplantation. However, under these reduced intensity conditions, approximately 25 - 60% of transplanted patients rejected the HLA-matched bone marrow transplantation (2, 4, 5, 90). Thus, in an effort to promote engraftment, the high dose of cyclophosphamide conditioning regimen was intensified with the inclusion of irradiation. Addition of irradiation improved

engraftment; however, an increase in mortality rates resulting from graft-versus-host disease, interstitial pneumonitis, and/or secondary malignancies was also observed (5, 6, 9). Thus, to promote engraftment in the presence of decreased toxicity, chemotherapy based conditioning regimens were then investigated. Such regimens include cyclophosphamide, fludarabine, busulfan, and/or anti-thymocyte globulin. Although, under conditions including one or more of the above immunosuppressants the rate of survival increased as a result of a correlated decrease in adverse conditioning regimen related complications, the frequency of HLA-matched bone marrow transplantation rejection slightly increased in comparison to conditioning regimens that included irradiation (5, 8, 9, 90-92).

There are currently three working hypotheses that may not be mutually exclusive to explain HLA-matched bone marrow transplantation rejection in patients with non-malignant hematological disorders. One hypothesis is that the more advanced the disease the greater perturbation the disease has on the bone marrow microenvironment, and thereby the less supportive the marrow environment is to a bone marrow transplantation. Analogous, it is posited that bone marrow microenvironment injury due to adverse non-immune mediated effects of the transfusion treatments result in the inability to stably engraftment a bone marrow transplantation. Conversely and more widely accepted is the hypothesis that some patients with a non-malignant blood disorder reject the HLA-matched bone marrow transplantation as a result of the failure to completely ablate donor reactive immunity established in the patient prior to transplantation.

Though donor reactive immunity in patients with non-malignant blood disorders can additionally arise from heterologous immunity or pregnancy, we hypothesize that it is more likley that alloimmunity against donor antigens in patients with non-malignant hematologies is derived from prior red blood cell and/or platelet transfusions that are routinely administered to these patients as an essential component of their treatment. It is clinically observed that chronically transfused patients reject an HLA-matched bone marrow transplantation at a greater frequency than minimally or un-transfused patients (9-14); which is consistent with the idea that blood transfusions sensitize patients with non-malignant blood disorders against a subsequent HLA-matched bone marrow transplantation. Though the bone marrow patients with non-malignant hematologies receive is matched at the HLA loci, polymorphic differences in proteins outside the HLA loci, referred to as minor histocompatibility antigens (discussed further in *Minor Histocompatibility Antigens* section), persist. To the extent that the prior transfused blood products and donor hematopoietic stem cells express some of the same minor histocompatibility antigens, it is hypothesized that blood transfusions elicit a minor histocompatibility antigen specific alloresponse that consequentially induces rejection of an HLA-matched bone marrow transplantation expressing the target minor histocompatibility antigens. If such is the case, then rejection could be resolved by offering the bone marrow transplantation to patients with non-malignant hematologies prior to aggressive transfusion support.

Role of minor histocompatibility antigens in transplantation

Minor histocompatibility antigens

The discovery of minor histocompatibility antigens stem from tumor transplantation studies in which transplanted tumors grew in inbred murine recipients but failed to grow in unrelated strains (93). It was suggested that genetic differences between the unrelated recipients and donors were responsible for rejection of the transplanted tumor. These genetic disparities influencing the acceptance or rejection of murine transplanted tissues were later discovered to be encoded in the histocompatibility 1, 2, and 3 loci (94). Using congenic murine recipients that were identical at the histocompatibility 1 and 3 loci but were disparate at the histocompatibility 2 locus, rapid rejection of transplanted tumor and skin grafts was observed (95). Conversely, it was reported that slower and more chronic rejection of tumor and skin grafts occurred in congenic murine recipients that were identical at the histocompatibility 2 locus but differed at the histocompatibility 1 and 3 loci. Together, these findings demonstrated each histocompatibility loci, and thereby the encoded proteins MHC (in the histocompatibility 2 locus) and minor histocompatibility antigens (within the histocompatibility 1 and 3 loci), as diverse functional entities in transplantation.

It was not until a little over a decade later that the potential involvement of minor histocompatibility antigens in human transplantation was observed in a patient that rejected an HLA-identical, sibling skin graft (96, 97). The attribution of minor histocompatibility antigens in the failure to engraft an HLA-identical

transplant was shortly thereafter supported by *in vitro* clinical analysis of cytolytic T lymphocytes isolated from an aplastic anemia female patient that rejected a male HLA-identical, sibling bone marrow transplantation. In this analysis, the isolated cytolytic T lymphocytes (CTLs) from the rejecting female patient lysed male targets that were HLA-identical to the female patient (98, 99); thus, further indicating the entailment of minor histocompatibility antigens in rejection of a human graft. Such studies also opened doors for numerous *in vitro* clinical analyses that defined a variety of human minor histocompatibility antigens, including the male H-Y antigens, through characterizing the peptide reactivity of *in vivo* primed recipient T lymphocytes derived from transplanted or transfused individuals.

It is now well known that minor histocompatibility antigens are allelic polymorphic variant peptides that differ by one or more amino acids (100-102). Traditionally, minor histocompatibility antigens were characterized as processed peptide fragments originating from intracellular proteins. However, with recent murine studies demonstrating that polymorphic extracellular proteins can be cross presented onto MHC Class I molecules (103), it is likely that minor histocompatibility antigens can additionally be derived from polymorphic extracellular proteins such as blood group antigens. Despite the origin of the minor histocompatibility antigen, both cellular and/or humoral alloresponses can be induced (further discussed in the *Alloimmunization to minor histocompatibility antigens* section). To elicit a cellular alloresponse to either intracellular or extracellular protein derived minor histocompatibility antigens, the protein

carrying the minor histocompatibility antigen is processed and the resulting minor histocompatibility antigen peptide is presented on HLA Class I and/or II molecules to $CD8^+$ and/or $CD4^+$ T cells, respectively. If the minor histocompatibility antigen is carried on an extracellular protein, both the cellular and humoral adaptive arms of immunity collaborate to elicit an optimal humoral alloresponse. In addition to the activation of $CD4^+$ T cells, the minor histocompatibility antigen epitope in the native structure of the polymorphic protein necessitates recognition by an alloreactive B cell that upon receiving help from the already activated $CD4^+$ T cell differentiates into a plasma cell generating antibodies recognizing the minor histocompatibility epitope in its three dimensional native configuration.

Though it is known that humoral and cellular responses can be elicited against minor histocompatibility antigens, the exact mechanism in which each immunological adaptive arm mediates rejection of HLA-matched transplantations is poorly understood. It is well known that minor histocompatibility antigen reactive $CD8^+$ T cells mediate rejection of HLA-matched bone marrow and solid organ transplantations. Though minor histocompatibility antigen reactive $CD4^+$ T cells are detected in recipients rejecting an HLA-matched transplant, the exact immunological role of these $CD4^+$ T cells in mediating graft rejection is unclear (102, 104, 105). Murine transplantation studies have demonstrated that rejection of an MHC-matched bone marrow transplant requires both $CD4^+$ and $CD8^+$ T cells (105-107). The observed alloreactive $CD8^+$ T cell response mediating graft rejection was more profound when in conjunction with alloreactive $CD4^+$ T cells;

thus, raising the question of the functional role of $CD4^+$ T cells in rejection of MHC-matched bone marrow transplantations (107). It is postulated that the $CD4^+$ T cells are either required to provide help to 'license' antigen presenting cells to fully activate $CD8^+$ T cells or the $CD4^+$ T cell compartment are effectors that can directly mediate graft rejection through undefined effector mechanisms (102, 104-108). Moreover, though it is well understood that minor histocompatibility antigen reactive antibodies can mediate rejection of solid organ transplantations and MHC-mismatched bone marrow transplantations through complement and antibody dependent cellular cytotoxicity activation, it is unclear if alloreactive antibodies can mediate rejection of HLA-matched bone marrow transplantations across minor histocompatibility antigen barriers (109, 110). Albeit the immune mediated mechanisms of rejection of HLA-matched transplantations remain unclear, the barrier alloimmunization against minor histocompatibility antigens play in engraftment of HLA-matched transplantations remain a significant clinical impasse.

In addition to graft rejection, alloresponses to minor histocompatibility antigens generate adverse clinical complications. One such potentially fatal example is graft versus host disease in which donor alloreactive T cells mediate rejection of any recipient tissue expressing the target host minor histocompatibility antigen. In graft versus host disease, donor mature $CD4^+$ and/or $CD8^+$ T cells in transplanted bone marrow are activated in the recipient in response to recognition of host minor histocompatibility antigen peptides presented on host HLA Class I and/or II molecules (111). Though graft versus

host disease under certain circumstances is fatal, it has recently been manipulated into a curative therapy for some patients with malignancies. In this therapy, patients with malignancies, such as leukemia, receive allogeneic bone marrow transplantations in hopes of eliciting a graft versus malignancy, or graft versus leukemia, effect (111). The bone marrow transplantation cures the patient by not only restoring healthy endogenous hematopoiesis, but also inducing donor T cell alloreactivity against the neoplastic cells.

Because all polymorphic proteins within a host can potentially give rise to minor histocompatibility antigens, it is quite surprising that graft rejection and/or graft versus host disease do not occur in all HLA-matched transplantations. It is reasoned that several checkpoints restrict the number of minor histocompatibility antigens that can fulfill criteria to be a risk for graft versus host disease and graft rejection. For a minor histocompatibility antigen peptide to elicit a cellular response, the peptide must be processed and stably presented on HLA Class I and/or II molecules to $CD8^+$ and/or $CD4^+$ T cells, respectively. Similar to altered peptide ligands that can act as T cell antagonist depending on the amino acid alterations in the loaded peptide (112), it is hypothesized that polymorphisms within a given protein can alter the anchor amino acid residues of a minor histocompatibility antigen peptide such that the peptide is no longer stably presented in HLA molecules to respective alloreactive T cells; and thus, results in the inability of the recipient T cells to elicit a strong cellular response against the minor histocompatibility antigen (100, 102).

Immunogenicity of minor histocompatibility antigens is additionally limited by immunodominance. It has been reported that in the presence of multiple known minor histocompatibility antigen disparities $CD8^+$ T cells isolated from rejecting murine recipients responded to only a few minor histocompatibility antigen peptides *in vitro*; thus, suggesting a hierarchy in minor histocompatibility antigen immunogenicity (100, 102). It is hypothesized that immunodominance of a minor histocompatibility antigen is dependent on its ability to generate a strong synergistic minor histocompatibility antigen specific collaboration between alloreactive cytolytic and helper T cells. Because the strength of a cellular response is reliant on the affinity of the combinatorial minor histocompatibility antigen peptide and HLA molecule complex, it is also proposed that immunodominance of a minor histocompatibility antigen is regulated by the affinity of the minor histocompatibility antigen peptide to the HLA molecule. Likewise, the avidity of the TCR and minor histocompatibility antigen peptide:HLA complex that is regulated by the number of minor histocompatibility antigen peptide and HLA molecule complexes expressed on antigen presenting cells can determine the immunodominance of the minor histocompatibility antigen. Generation of an optimal cellular response necessitates a threshold that allows for an appropriate avidity between the T cell and the minor histocompatibility antigen peptide:HLA molecule complex. It is equally consistent that the immunogenicity of a minor histocompatibility antigen is independent of the minor histocompatibility antigen peptide, but as an alternative, dependent on the available T cell repertoire in the periphery of the transplant recipient.

Similarly, the immunogenicity and dominance of a minor histocompatibility antigen can depend on the precursor frequency of minor histocompatibility antigen reactive lymphocytes. Minor histocompatibility antigens are generally a weaker immunogen than HLA molecules. Thus, for a minor histocompatibility antigen reactive alloresponse to mediate rejection of an HLA-matched transplantation, a recipient might necessitate prior immunization to the minor histocompatibility antigen. Through prior exposure, the precursor frequency of alloreactive lymphocytes in the recipient increases to a level that is sufficient to induce rejection of tissues expressing the target minor histocompatibility antigens. Exposure and subsequent immunity against minor histocompatibility antigens can arise through pregnancy, transfusions, and/or heterologous immunity. Clinical studies have demonstrated that multiparity can result in the induction of alloresponses to minor histocompatibility antigens including the H-Y, HA-1, and HA-2 (113, 114); thus, implicating a natural source for alloresponses to minor histocompatibility antigens that can consequentially result in rejection of a bone marrow transplantation expressing the target alloantigens. Previous canine and murine studies have additionally demonstrated that red blood cell transfusions administered prior to transplantation correlated with rejection of Dog Lymphocyte Antigen (DLA)- or MHC-matched bone marrow transplantations (canine and murine, respectively) expressing identical minor histocompatibility antigens as the transfused blood products (63, 115). Although undefined, it is reasonable to question the influence transfusions might have on the status of solid organ transplantations, as alloimmunization against blood group antigens

Kidd and Duffy have been correlated with rejection of renal transplants that expressed these blood group antigens (81). Moreover, as a number of patients receiving bone marrow and solid organ transplantations require transfusion support pre- and peri-transplantation (20, 21), it is potentially worth testing the ability of alloresponses against minor histocompatibility antigens expressed on transfused blood products to mediate rejection of an HLA-matched transplantation expressing the target minor histocompatibility antigens.

Alloimmunization to minor histocompatibility antigens

In the setting of a transfusion and transplantation, recipients can elicit an adaptive cellular and/or humoral alloresponse against minor histocompatibility antigens expressed on donor tissue. Allorecognition of minor histocompatibility antigens for both the cellular and T cell dependent humoral adaptive arms of immunity can occur through four different cross-talking pathways. The four currently characterized pathways known to play a role in allorecognition of minor histocompatibility antigens are the 1) direct presentation, 2) semi-direct presentation, 3) indirect presentation, and 4) cross presentation pathways.

In the direct recognition pathway, donor antigen presenting cells present minor histocompatibility antigen peptides on HLA Class I and/or II molecules to recipient $CD8^+$ and/or $CD4^+$ T cells, respectively. The presented minor histocompatibility antigen peptides can be derived from endogenous intracellular polymorphic proteins, as well as exogenous polymorphic proteins acquired from consumption of donor necrotic and/or apoptotic particles. Because T cells

undergo thymic positive selection that biases the survival of T lymphocytes capable of recognizing such minor histocompatibility antigen peptides on self HLA molecules, it is obvious that alloresponses against minor histocompatibility antigens in an HLA-matched setting can occur through the direct presentation pathway.

Because not all transplantations and very few transfusions are intentionally matched at the HLA loci, it is legitimate to address how alloreactivity against minor histocompatibility antigens can be induced through the direct presentation pathway in an HLA-mismatched setting. To accept the requirement of the direct recognition pathway in an HLA-mismatched setting, it is necessary to transgress from the concept of HLA restriction. Despite thymic positive selection for HLA restricted T cells, T cell receptors have a certain degree of promiscuity that permits recognition of minor histocompatibility antigen peptides presented on allogeneic HLA molecules. This plasticity in HLA restriction can be explained by the 'multiple binary complex' hypothesis. In this hypothesis it is posited that minor histocompatibility antigen peptides bound to allogeneic HLA molecules alter the configuration of peptide:allogeneic HLA molecule complexes such that the amino acid residues recognized by the T cell receptors mimic the T cell receptor recognizing amino acid residues of the target peptides presented on self HLA molecules (116, 117); thus, allowing for the induction of cellular alloresponses against minor histocompatibility antigens presented on allogeneic HLA molecules, as well as the generation of a dominant alloreactive T cell

response against the minor histocompatibility antigen as opposed to the naturally more immunogenic allogeneic HLA molecules.

Using $CD4^+$ T cell transgenics that recognize a peptide derived from ovalbumin (OVA), it has been observed that both H-2^d restricted DO11.10 and H-2^b restricted OT-II transgenic T cells can recognize the $OVA_{323-339}$ peptide presented on I-A^b (118). Though this cross-reactivity is more commonly observed with $CD4^+$ T cell responses to minor histocompatibility antigens, similar observations have additionally been made with $CD8^+$ alloreactive T cells. Thus, demonstrating that cellular alloimmunity against minor histocompatibility antigens can be elicited through direct presentation of the alloantigen peptide on either identical or allogeneic HLA molecules to recipient alloreactive T cells.

In addition to activating cellular alloresponses, allorecognition of extracellular minor histocompatibility antigens can elicit a T cell dependent humoral alloresponse. For a T cell dependent humoral response to occur, recipient B cells that express an immunoglobulin receptor reactive to the minor histocompatibility antigen necessitate help from recipient $CD4^+$ T cells. Antigen presenting B cells specific for the minor histocompatibility antigen can present antigenic peptides on HLA Class II molecules to recipient $CD4^+$ T cells. Through co-stimulatory factors and cytokines, the $CD4^+$ T cells provide help to the alloreactive B cells to undergo affinity maturation and class switching. The now activated B cells terminally differentiate into plasma cells that secrete high affinity and class switched immunoglobulins reactive to the target minor

histocompatibility antigen epitope in its natural cell membrane associated three-dimensional configuration.

Cellular and T cell dependent humoral alloresponses against minor histocompatibility antigens can additionally occur through the semi-direct presentation pathway, which shares similarities to the direct allorecognition pathway. In the semi-direct presentation pathway, recipient antigen presenting cells acquire allogeneic HLA molecules presenting minor histocompatibility antigen peptides that are shed from donor cells, and thereby act similarly to donor antigen presenting cells in the direct presentation pathway (116, 119). Recipient alloreactive $CD8^+$ and $CD4^+$ T cells can subsequently recognize and respond to acquired donor HLA Class I and/or II molecules presenting minor histocompatibility antigens, respectively. How the donor HLA molecules are transferred to recipient antigen presenting cells is not fully understood. However, it is hypothesized that donor HLA molecules can be transferred to recipient antigen presenting cells through cell to cell contact and/or the uptake of donor HLA molecule containing exosomes (small vesicles) that were released from donor cells (116, 120, 121).

Sharing similarities with the induction of alloresponses to minor histocompatibility antigens through the semi-direct recognition pathway, the activation of an alloresponse through the indirect presentation pathway necessitates recipient antigen presenting cells acquiring only minor histocompatibility antigens from the periphery. In the indirect recognition pathway, recipient antigen presenting cells consume, process, and present minor

histocompatibility antigens on HLA Class II molecules to recipient alloreactive $CD4^+$ T cells, respectively. Because B cells can act as antigen presenting cells and require $CD4^+$ T cell help for T cell dependent antigens, it is more likely that T cell dependent humoral alloresponses against minor histocompatibility antigens can occur through the indirect rather than the direct presentation pathway. The minor histocompatibility antigen peptides presented to recipient alloreactive $CD4^+$ T cells in the indirect pathway are derived from exogenous polymorphic proteins acquired from consumption of donor particles or proteins shed from transplanted or transfused grafts. Because generally consumed exogenous antigens presented on HLA Class II molecules result in $CD4^+$ T cell responses and intracellular antigens presented on HLA Class I molecules induce $CD8^+$ T cell immunity, the dominant alloimmune response induced against minor histocompatibility antigens through the indirect presentation pathway is theoretically a $CD4^+$ T cell response.

Although the indirect allorecognition pathway does not favor the induction of a $CD8^+$ T cell response to exogenous antigens, a fourth pathway, referred to as the cross presentation pathway, is physiologically available to allow for the activation of $CD8^+$ T cell responses to exogenous antigens. The cross presentation pathway converges with the indirect recognition pathway and enables recipient antigen presenting cells to present minor histocompatibility antigen peptides on self HLA Class I molecules to recipient alloreactive $CD8^+$ T cells (108). The cross presentation pathway was originally described by Michael Bevan to propose a mechanism for the activation of $CD8^+$ T cell responses

against viruses that do no infect antigen presenting cells (122). The mechanisms involved in cross presentation remain poorly understood; however, it is known that endocytosed antigens are redirected to cytoplasmic proteasomes that generate HLA Class I presentable antigenic peptides that are then loaded onto HLA Class I molecules through an undefined mechanism. Though dendritic cells are the predominant professional antigen presenting cells capable of activating alloreactive $CD8^+$ T cells through cross-presentation, B cells can additionally activate $CD8^+$ T cell responses through this pathway (108, 123). However, to activate $CD8^+$ T cells through the cross presentation of antigenic peptides, B cells also necessitate activation through the engagement of Toll-like receptors. Moreover, although macrophages, neutrophils, granulocytes, and mast cells can cross present exogenous antigenic peptides, these cell subsets are weaker at inducing the activation of alloreactive $CD8^+$ T cell responses (108, 124).

Because the indirect and cross presentation pathways necessitate *de novo* antigen processing and presentation at the time of transplantation or transfusion, alloresponses against minor histocompatibility antigens are delayed in comparison to alloimmunity induced through the direct allorecognition pathway. As a consequence, alloimmunization elicited through the indirect pathway is described to be the dominating immunological rejection vector in the later phase of graft rejection. Conversely, alloimmunity induced through the direct allorecognition pathway is characterized to play a critical role in the early acute phase of graft rejection, as direct recognition of readily presented minor histocompatibility antigen peptides on donor antigen presenting cells can occur

immediately upon encounter of the donor antigen presenting cells with recipient alloreactive T cells (125). The role of the semi-direct presentation pathway in induction of alloresponses against minor histocompatibility antigens derived from a transplantation or transfusion remains undetermined. However, murine studies characterizing alloimmunization against donor alloantigens expressed on transfused red blood cell or platelet units have suggested that immunization occurs through the direct, indirect, and/or cross presentation pathways (15, 103).

CTLA4-Ig co-stimulation blockade therapy in transplantation

Because alloreactive T cells are critical mediators of graft rejection, the current immunosuppressive therapies utilized in transplantation to promote engraftment are focused on inhibiting T cell activation. To generate an optimal T cell response, a T cell necessitates three activating signaling events (126, 127). The first signal, 'Signal 1', is delivered via T cell receptor recognition of its cognate ligand, composed of an antigenic peptide and HLA molecule expressed on professional antigen presenting cells. 'Signal 2' is provided through interaction of co-stimulatory ligands and receptors that promote T cell activation, differentiation, and proliferation. Lastly, as a down stream effect of 'Signal 2', 'Signal 3' is supplied as soluble factors, such as cytokines, to propagate and augment the T cell response in the form of clonal expansion and differentiation.

Ligation of co-stimulatory molecules activate intracellular signaling cascades that synergize with the signaling events of the T cell receptor to promote expression of downstream transcription factors and genes essential for

generating optimum cellular responses. Examples of co-stimulatory pathways involved in the activation of cellular responses include CD28/B7, CD40/CD40L, OX40/OX40L, LFA-1/ICAM, LFA-3/CD2, ICOS/ICOSL, 4-1BB/4-1BBL, LIGHT/HVEM, CD27/CD70, and CD30/CD30L. Because the CD28/B7 co-stimulatory pathway is the most critical for T cell activation, it constitutes the most thoroughly studied co-stimulatory pathway in transplantation (126). The CD28 co-stimulatory receptor is constitutively expressed on naïve and memory T cells. There are two co-stimulatory ligands, B7.1 (CD80) and B7.2 (CD86), for the CD28 co-stimulatory receptor. Whereas the co-stimulatory ligand B7.1 is constitutively expressed at trace levels on antigen presenting cells and immediately up regulated upon activation, the co-stimulatory ligand B7.2 is expressed after activation of the antigen presenting cells. In the presence of an inflammatory milieu, activated professional antigen presenting cells up regulate the B7 co-stimulatory ligands to promote T cell activation through engagement of the CD28 co-stimulatory receptor. Signaling through the CD28 co-stimulatory receptor results in an increase in transcription and messenger RNA stability of the interleukin 2 (IL-2) cytokine, elevation in the expression of anti-apoptotic molecules, T cell differentiation through the synthesis of cytokines, subsequent reduction in the threshold level of T cell receptors, and enhancement in B cell activation and isotype class switching (126, 128, 129). The enhancement in IL-2 synthesis promotes activation and proliferation of the target T cell, as well as indirectly the proliferation of previously activated T cells.

Because co-stimulation through the CD28/B7 pathway results in activation and augmentation of T cell responses as a whole, negative signals are crucial to regulate the magnitude and duration of an immune response. In the context of the CD28/B7 co-stimulatory pathway, cytolytic T lymphocyte antigen 4 (CTLA4) is a co-inhibitory receptor that is up regulated on activated T cells to negatively modulate cellular responses (126, 130). The CTLA4 co-inhibitory receptor is structurally homologous to CD28; however, CTLA4 has a higher avidity to the B7.1/B7.2 co-stimulatory ligands. Engagement of the CTLA4 co-inhibitory receptor with the B7 molecules results in decreased T cell proliferation as a result of IL-2 synthesis inhibition, and a block in effector cytokine production and cell cycle through the impediment of G_1 to S phase progression (126). The mechanism by which CTLA4 inhibits co-stimulation, and thereby T cell activation, remains undefined. It is unknown if CTLA4 regulates co-stimulation and T cell responses through competitive binding to the B7.1/B7.2 co-stimulatory ligands, through the induction of immunosuppressive cytokines such as transforming growth factor-β (TGF-β), and/or by directly antagonizing the T cell receptor and CD28 mediated signal transductions (126, 130). Nonetheless, ligation of CTLA4 negatively regulates T cell responses through the potential induction of peripheral T cell tolerance or anergy, and is a promising therapeutic target for manipulation of alloimmune responses during transplantation.

Traditionally, engraftment of bone marrow and solid organ transplantations was clinically promoted using immunosuppressive reagents that non-specifically regulate T cell alloresponses at various stages of T cell activation. A few

examples of such immunomodulatory therapies include calcineurin inhibitors (i.e. cyclosporine), DNA synthesis inhibitors (i.e. cyclophosphamide and fludarabine), corticosteroids, mTOR inhibitors (i.e. rapamycin), and irradiation. Though effective in inhibiting T cell alloresponses and rejection of a transplanted graft, such immunosuppressants are strongly associated with adverse clinical consequences including hepatic failure, nephrotoxicity, metabolic dysfunction, and hypertension. The above immunosuppressants additionally non-specifically inhibit the entirety of the recipients' T cell repertoire, and thereby significantly provoke the likelihood of acquiring opportunistic infections. Thus, to reduce the burden of toxic side effects without sacrificing efficacy in promoting engraftment, co-stimulatory blockades were developed as a new approach to antigen specific immunosuppression, replacing traditional non-antigen specific immunosuppressants that require therapeutic monitoring as a result of chronic toxicity effects.

Because the CD28/B7 co-stimulatory pathway is the most crucial co-stimulatory pathway for T cell activation, pharmacological reagents inhibiting the CD28/B7 co-stimulatory pathway has been developed. In the absence of sufficient CD28/B7 co-stimulation, T cells that engage peptide:HLA molecule complexes on quiescent antigen presenting cells fail to receive optimal signaling to activate, and thereby become tolerant or anergic (126, 128, 130). As an additional consequence, the tolerant or anergic T cells can undergo apoptosis as a result of abortive activation, decreased IL-2 production, down regulation of the IL-2 receptor, and reduction in expression of anti-apoptotic molecules (128).

Blockade of the CD28/B7 co-stimulation pathway is achieved pharmacologically via using a recombinant fusion protein that combines the extracellular domain of the human co-inhibitory protein CTLA4 with a modified portion of the crystallized fragment (Fc) of human IgG1 (CTLA4-Ig). In addition to the CTLA4-Ig fusion protein, there are currently three alternate co-stimulatory blockade regimens of clinical interest: anti-LFA-1 (LFA-1/ICAM pathway), anti-CD40L (CD40/CD40L pathway), and LFA3-Ig (LFA-3/CD2 pathway) (131). Animal studies using all four of these co-stimulatory blockade regimens have demonstrated efficacy in prolonging renal, cardiac, pancreatic, and skin allograft survival (131-134). However, of these co-stimulatory blockades, CTLA4-Ig and LFA3-Ig are the only regimens approved by the U.S. Federal Food and Drug Administration for autoimmune prophylaxis, and clinical Phase II and/or III trials for prolonging survival of renal and hepatic transplants (131, 134-136).

Similar to the unknown inhibitory mechanisms of the naturally occurring CTLA4 co-inhibitory protein, the mechanisms in which CTLA4-Ig inhibits T cell alloresponses remains undefined. It was originally hypothesized that CTLA4-Ig inhibits T cell activation by blocking interaction between the CD28 co-stimulatory receptor and the B7 co-stimulatory ligands. However, recent studies have demonstrated that CTLA4-Ig interaction with the B7 co-stimulatory ligands on dendritic cells can enhance the production of indoleamine 2,3-dioxygenase (IDO) which inhibits T cell activation either through the catabolism and depletion of tryptophan, an essential amino acid for T cell activation, or the generation of tryptophan catabolites 3-hydroxyanthranilic and quinolinic acids which have been

demonstrated to induce selective thymocyte and/or T helper cell apoptosis (130, 137). Despite the unknown mechanisms in which CTLA4-Ig inhibits T cell activation, it is of clinical relevance as CTLA4-Ig has demonstrated efficacy in autoimmune and solid organ transplantation settings.

Though co-stimulatory blockade therapy has effectively demonstrated prolongation of allograft survival, recent animal studies have demonstrated that the efficacy of CTLA4-Ig is dependent on the recipient precursor frequencies of alloreactive $CD4^+$ and $CD8^+$ T cells. In the presence of a high precursor frequency of donor reactive naïve or memory $CD4^+$ and $CD8^+$ T cells, co-stimulatory blockade regimens are less potent at overcoming immunological barriers to allograft transplantations and tolerance induction (128, 138-140). Memory alloimmunity against donor antigens can arise from *a prior* transplantation, pregnancy, platelet or red blood cell transfusions, and exposure to environmental pathogens. Animal studies have additionally demonstrated that donor reactive memory elicited in response to intentional donor (i.e. skin graft) and/or pathogenic (i.e. viral or bacterial infections) antigen exposure can resist co-stimulatory blockade and consequentially mediate graft rejection (128, 140). In contrast to the naïve counterpart, memory responses in general are resistant to immunosuppressive therapeutic interventions due to a lower activation threshold that requires less 'Signal 1' and 'Signal 2'. Because 'co-stimulation blockade resistance' has thus far been observed in MHC disparate settings in which the MHC alloreactive T cell precursor frequency is higher than minor histocompatibility antigen specific T cells, it is reasonable to question the efficacy

of co-stimulatory blockade in preventing rejection of an HLA-matched graft across minor histocompatibility antigen barriers. Moreover, as ‘co-stimulatory blockade’ additionally occurs in the presence of memory alloresponses, it is justifiable to test the efficacy of co-stimulatory blockade in preventing memory alloresponses from being established.

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Chapter 2

Transfusion of Minor Histocompatibility Antigen-Mismatched Platelets Induces Rejection of Bone Marrow Transplants in Mice

Abstract

Bone marrow transplantation (BMT) is most commonly used in the treatment of malignancy, but also represents a cure for non-malignant hematological disorders. In the context of malignancy, stringent conditioning regimens are utilized to both kill the neoplasia and to facilitate BMT engraftment; BMT rejection is uncommon under these conditions. However, morbidity and mortality can result from sequelae of stringent conditioning, making its use difficult to justify in BMT for non-malignant disorders. Reduced intensity conditioning and HLA-matched BMT has limited toxicity concerns, but also leads to significantly higher rates of BMT rejection, presumably due to an intact immune system. The relevant patient population typically receives transfusion support, often including platelets, and frequency of rejection correlates with frequency of transfusion. In this context, we hypothesized that immunization to transfused platelets contributes to subsequent BMT rejection. Immunity to transfused platelets is best characterized regarding anti-HLA antibodies; however, such antibodies are unlikely to play a role in BMT rejection that is HLA matched. However, transfused platelets also carry minor histocompatibility antigens. Using a murine system, we report that transfusion of minor antigen mismatched platelets induces subsequent BMT rejection. These findings suggest previously unappreciated sequelae of immunization to platelets in the context of transplantation.

Introduction

Patients who have bone marrow failure syndromes are typically given supportive care including transfusions of red blood cells (RBCs) and platelets (PLTs). Some marrow failure patients subsequently undergo bone marrow transplantation (BMT) in an effort to restore endogenous hematopoiesis (1-4). In general, rejection of a BMT is now a rare event due to the use of stringent conditioning regimens that substantially decreases recipient immunity. However, such regimens have considerable toxicity that can lead to morbidity and mortality. This toxicity is an acceptable risk when treating a patient with a malignancy, as the toxic effects have the benefit of killing cancerous cells in addition to promoting engraftment. However, for patients receiving BMT for marrow failure syndromes that do not involve malignancy, the toxic side effects of stringent conditioning regimens are difficult to justify. Accordingly, reduced intensity conditions have been developed to allow BMT without substantial toxicity (3, 5, 6). However, engraftment is less efficient under these conditions; up to 15% of the transplanted patients reject the BMT (4, 7, 8).

Several causes for the increased BMT rejection rates under reduced intensity regimens have been proposed, one of which is immunological rejection of transplanted marrow. It has been noted that the frequency of BMT rejection correlates to the number of transfusions received (9-13) and PLTs are routinely given as part of transfusion support to some marrow failure patients. Clinical concern regarding immune responses to PLT transfusions are typically focused on the development of anti-HLA antibodies, and consequent refractoriness to subsequent PLT transfusions. However, anti-HLA antibodies are unlikely to participate in BMT rejection, as the BMT is typically HLA

matched in this setting. In contrast, bone marrow is not matched for minor histocompatibility antigens (mHAs). mHAs are polymorphic proteins that differ between individuals by one or more amino acids (14-16), and can elicit humoral responses to their native structure or T cell responses when peptides containing the variant amino acids are presented by recipient MHCs.

Traditionally, leukocytes in transfused blood products have been thought to be the main source of immunization. Accordingly, PLT products are now routinely stringently leukoreduced prior to transfusion; indeed, removal of leukocytes substantially decreases anti-HLA antibody responses (17-19). However, PLTs also carry mHAs, and it is possible that the transfused PLTs themselves are a source of mHA antigen sensitization. To test this hypothesis, we developed methodologies for collecting, filter leukoreducing, and transfusing murine PLTs in a fashion that models human PLT transfusion. Using this approach, we tested the hypothesis that transfusion of leukoreduced PLT concentrates (LR-PLT) primes recipients for rejection of subsequent MHC-matched BMT across mHA barriers. We developed an MHC-matched:mHA-mismatched BMT model in a murine system, using reduced intensity conditioning. Rejection was observed in recipients transfused with mHA-disparate LR-PLT products, whereas engraftment was observed in recipients that were naïve or transfused with syngeneic (B6) LR-PLTs. These findings demonstrate that PLT transfusions are capable of inducing rejection of MHC-matched BMT across mHA differences, and describe a previously unappreciated potential immunological sequelae of PLT transfusion in the context of transplantation.

Materials and Methods

Mice

Female C57Bl/6J (H-2^b), BALB.B (C.B10-H-2^b/LiMcdJ (H-2^b)) and BALB/c (H-2^d) mice were used as donors at 8-12 weeks of age. B6.PL-*Thy1*^{a}/CyJ (H-2^b) female mice were used as recipients at 6-8 weeks of age. Mice were purchased from the Jackson Laboratories (Bar Harbor, ME). C57Bl/6J EGFP mice were a generous gift from Dr. Derek A. Persons [St. Jude Children's Research Hospital, Memphis, TN (20)] and were bred by the Emory Division of Animal Resources Animal Husbandry service. All mice were housed in Emory University Department of Animal Resources facilities and procedures were performed according to approved IACUC protocols.

Antibodies for Flow Cytometry

Antibodies were purchased from BD Pharmingen (anti-CD41-PE, PE anti-TER119/erythroid-PE, Rat $IgG2_b$-PE, APC goat anti-mouse Igs, FITC anti-mouse CD3, PE anti-mouse CD90.2 and FITC anti-mouse CD19) and eBioscience (Rat IgG1 and APC anti-mouse CD90.1).

Platelet Preparation

Donors (C57Bl/6J, BALB.B and BALB/c) were exsanguinated and whole blood was collected in 1:8 acid-citrate-dextrose (ACD; BD Vacutainer). 2 ml of DPBS was added to each ml of whole blood in a 15 ml conical tube, samples were mixed through gentle inversion, and centrifuged at 80 x g for 10 minutes. Platelet rich plasma (PRP) was collected, mixed by gentle inversion, and re-spun at 80 x g for 10 minutes. Isolated

PRP was pooled and approximately 20 ml were passed over a Neonatal Purecell PL High Efficiency Leukocyte Reduction Filter (Pall Corporation). PLTs were enumerated as described below. After enumeration, PLT concentration was normalized to 2 x 10^8 PLT/mL by pelleting at 3,000 rpm (1,942 x g) for 10 minutes and re-suspending 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, 5.5 mM glucose in Milli Q water at pH=7.4). 500 µl of re-suspended LR-PLT (10^8 PLT total) was transfused through the tail-vein. All PLT handling was performed at room temperature. A "swirl" test was performed on all LR-PRP concentrates to test the quality of PLTs in solution (21).

Platelet, Red Blood Cell and Leukocyte Enumeration

To count PLTs and residual RBCs, 20 µl of LR-PRP was diluted in 100 µl FACS buffer (DPBS with 0.2% bovine serum albumin and 0.24 mM EDTA) and centrifuged at 3,000 rpm (1,942 x g) for 2 minutes at room temperature. The samples were stained with anti-CD41-PE or isotype control rat IgG1-PE for PLTs, and anti-TER119-PE or isotype control rat $IgG2_b$-PE for RBCs. All antibodies were used at a dilution of 1:100 in FACS buffer. Samples were incubated in the dark at room temperature for 30 minutes and then washed once with FACS buffer. Samples were re-suspended in 100 µl FACS buffer. 10 µl of each sample was mixed with 940 µl FACS buffer and 2.5 x 10^5 APC-beads (BD Pharmingen) were added to allow counting of absolute numbers. Samples were run on a FACS Caliber and analyzed using FlowJo 8.8.2. 12,500 events of APC-beads were collected resulting in 50 mL of analyzed sample.

Residual leukocytes were enumerated by flow cytometry using the method previously described (22). Briefly, a 25µl aliquot of LR-PRP was stained using 150 ng/ml Propidium Iodide (PI) in a 1 g/l sodium citrate dihydrate solution with 0.7% Zap-oglobin II reagent (Beckman Coulter), 0.7% RNAse cocktail (New England Biolabs), effectively staining all nucleated cells. Nuclei were enumerated on a BD FACSort flow cytometer. A known quantity of APC labeled beads was added to each sample to allow acquisition of absolute counts (BD Biosciences).

Isolation of Whole Blood

Whole blood was collected in 1:8 ACD. The blood was then washed three times with mouse PBS (23); centrifuging at 1,350 rpm (394 x g) for 15 minutes. While aspirating the supernatant, the buffy coat was not disrupted. The whole blood was re-suspended in 1x DPBS at a 1:4 dilution and 500 µl transfusions were given by tail vein.

Functionality of LR-PLT products

Prior to transfusion, PLT concentrates in Tyrode's buffer were exposed to collagen and monitored for percent aggregation over time using a clinical aggregometer. Aggregation was monitored as a function of light transmission. Tyrode's buffer was used as a blank and control PLT without collagen was used to monitor spontaneous aggregation.

In vivo Survival of LR-PLT concentrates

Transgenic C57Bl/6J female mice expressing enhanced green fluorescence protein (EGFP) on PLTs were utilized as LR-PLT donors. LR-PLT concentrates were isolated

and transfused as above. The presence of EGFP LR-PLTs was measured by enumerating EGFP positive events in a platelet gate based on forward and side scatter at indicated time points post transfusion. Identity of the events as PLTs was confirmed with anti-CD41 staining at terminal time points.

Seroanalysis

Serum was diluted 1:10 in FACS buffer and incubated with BALB.B SPLs or PLTs for 30 minutes, washed three times with FACS buffer, and incubated 30 min in a 1:100 dilution of APC goat anti-mouse Igs. B cells were excluded by staining with anti-mouse CD19-FITC (1:50 in FACS buffer), and PLT targets were visualized with anti-mouse CD41-PE (1:100 in FACS buffer). Samples were analyzed by flow cytometry; fluorescence of APC was used to indicate the presence of antibody on targets.

In vivo Survival of BALB.B Splenocyte Targets

Female C57Bl/6J and BALB.B SPLs were isolated and washed in 1x RPMI, 10% fetal bovine serum. SPLs were incubated in RBC lysis buffer (Sigma-Aldrich) (2 mL/spleen) for 5 minutes at room temperature. The cells were washed three times in 1x DPBS. During the third wash, cells were enumerated using a hemocytometer. C57Bl/6 and BALB.B SPL were incubated with $CFSE^{hi}$ (6 uM) or $CFSE^{lo}$ (5 nM), respectively at 37^0C for 5 minutes. Samples were mixed at equal volumes and injected into tail veins (total dose of $5x10^6$ per target).

Bone Marrow Transplantation

Bone marrow was harvested from femurs and tibias by standard techniques. BM cells were enumerated using a hemocytometer, and re-suspended at 10 x 10^6 cells/mL in 1x DPBS. 500 µl (5 x 10^6 BM cells) of BM was injected into the tail vein all recipients, which were previously conditioned with sub-lethal gamma irradiation treatments (700 rads), 24 hours prior to BMT. Engraftment was monitored by staining peripheral blood with anti-CD3-FITC (1:50), anti-Thy1.1-APC (1:2,500) and anti-Thy1.2-PE (1:2,500) in FACS buffer for 30 minutes at 4^0C. The cells were washed three times and analyzed by flow cytometry. Engraftment was assessed six weeks post BMT by percent donor Thy-1.2^+ T cells.

Statistics

Statistical analysis was performed using one-way ANOVA with Dunnett's post-test and column statistics. Significance was determined by a P value less than 0.05.

Results

Transfusion of LR-PLT products induces BMT rejection.

To test the hypothesis that transfusions of LR-PLT products induce rejection of MHC-matched BMT across mHA differences, an MHC-matched:mHA-mismatched BMT model was developed (Figure 1A). In this system, B6 Thy-1.1 (H-2^b) recipients were transfused twice with BALB.B (H-2^b; mHA-mismatched) or BALB/c (H-2^d; MHC- and mHA-mismatched) LR-PLT concentrates. One week after the second transfusion, recipients received a BALB.B BMT under reduced intensity conditions. Six weeks later, BMT engraftment was assessed using the T cell congeneic markers Thy-1.1 (recipient) and Thy-1.2 (donor). All donors and recipients were females. Engraftment was defined as a percentage of donor cells that exceeded two standard deviations above the mean of positive control mice that were known to reject (transfused with BALB/c whole blood). Because the recipients and BM donors were MHC-matched in this system, alloreactivity against MHC is not available as a rejection vector. In contrast, rejection across mHA barriers remains possible due to mHA mismatches between donors and recipients. As the BM and PLT donors are on the same genetic background, and thus express the same mHAs, BMT rejection due to sensitization against mHAs in LR-PLT products is possible.

The combined results of three experiments demonstrated that 13/15 (86%) of the recipients transfused with BALB.B LR-PLT products rejected the BALB.B BMT (Figure 1B). We hypothesized that immunization to mHAs occurred through processing and presentation of donor BALB antigens by recipient antigen presenting cells (indirect pathway). However, as BALB.B donors and Thy-1.1 recipients both express H-2^b MHC,

direct presentation by donor cells may also have occurred. To examine this question, BALB/c PLT donors were used. Since the BALB/c LR-PLT concentrates were MHC-mismatched to the recipients (Figure 1B), direct presentation of BALB mHAs to recipient lymphocytes, in the context of the recipients' self-MHC (H-2^b), was not available. Although it is possible that there is some cross-reactivity of H-2^b restricted mHA specific T cells with similar peptides presented by H-2^d MHC, this approach minimizes the direct presentation vector. 12/15 (80%) of the recipients transfused with BALB/c LR-PLT concentrates rejected the BALB.B BMT (Figure 1B), suggesting that indirect presentation of mHAs was sufficient to induce rejection of the BMT.

The observed rejection was not due to insufficient recipient conditioning and/or lack of bone marrow viability, as the naïve recipients uniformly engrafted the BALB.B BMT (Figure 1B). Likewise, rejection was not due to non-specific effects of the PLT transfusion process since engraftment was observed in 14/15 (93%) of the recipients receiving syngeneic (B6) LR-PLT products (Figure 1B); the single mouse that did not have engraftment also did not have anti-BALB immunity post-transplant (see below) and thus likely represents failure of the BMT injection, not immunological rejection. Together, these findings demonstrate that LR-PLT product transfusions induce rejection of MHC-matched BMT across mHA barriers.

Assessment of murine LR-PLT concentrates.

Although PLT isolation procedures vary between the United States, Canada, and Europe, most units are passed over leukoreduction filters to remove the vast majority of leukocytes. To model this process in our murine system, mouse blood was centrifuged

at a slow speed, and PLT rich plasma (PRP) was isolated by collecting the supernatant (as is the method used in the United States), and the PRP was then passed over the a brand of leukoreduction filters used to process human PLTs (Pall Corporation). To assess the quality of murine PLT products processed by this method, for each preparation that was transfused, PLTs, RBCs and leukocytes were enumerated using lineage specific markers CD41 (PLTs), TER119 (RBCs) or nucleic acid binding propidium iodide (nucleated leukocytes) (Figure 2A-C). Whole blood was used to establish scatter and staining gates for RBCs and PLTs (Figure 2A). The composition of the LR-PLT products was approximately 97% PLTs (Figure 2B), and only low amounts of RBCs were observed. Background staining was established using isotype controls and unstained samples (Figure 2B). Leukocyte gates from propidium iodide staining were established on whole blood (Figure 2C) and leukocytes were undetectable in all but one LR-PRP transfusion (Figure 2C and TABLE 1). Titrations of a known concentration of leukocytes in LR-PRP demonstrated that the leukocyte assay detected as few as 500 leukocytes per transfused unit (data not shown). Thus, LR-PLT units contained approximately $1x10^8$ PLT, $5x10^4$ RBCs and $\leq$ 500 leukocytes. $1x10^8$ PLTs were transfused as this is a volume adjusted equivalent to the transfusion of 10^{11} human PLTs, which is the content of one human PLT unit (21, 24).

The quality and functionality of the PLTs themselves was assessed using a PLT aggregometer routinely used in the clinical labs for assessment of PLT function. Upon exposure to a physiological substance (collagen), PLTs in the LR-PLT concentrates aggregated normally (Figure 2D), indicating that the murine LR-PLTs retain the ability to aggregate in response to a natural agonist. Post-transfusion survival of murine LR-

PLTs was tested by transfusing into naïve B6 (H-2^b) recipients. To allow visualization of the transfused PLTs, donors consisted of transgenic mice expressing enhanced green fluorescence protein (EGFP) in their PLTs. $EGFP^+$ $CD41^+$ PLTs were easily detectable in the peripheral blood for at least 72 hours post-transfusion (Figure 2E).

PLTs were also assessed using a routine blood bank method, the "swirl" test (21). In this test, the quality of the PLT morphology was determined by the presence or absence of "swirling" when gently rocked while held up to a light. Prior to each transfusion, the processed PLTs "swirled" (data not shown); indicating that the PLTs were discoid in shape (21). PLTs with an altered morphology (i.e. spherical) due to damage or activation do not "swirl" (21).

Together, the above data demonstrate that the processing of murine LR-PLT products by methodologies that model human PLT processing (i.e. differential centrifugation and filter leukocyte reduction) results in a product that is similar to human PLT products with regards to cellular composition, swirling properties, agonist based aggregation, and post-transfusion survival.

Analysis of BALB specific immunity in platelet transfused recipients.

Although it has previously been demonstrated that MHC-mismatched PLT transfusions elicit anti-MHC humoral and cellular alloresponses (25, 26), it is unclear if similar responses are induced against mHAs expressed by the transfused PLT products, in the MHC-matched setting. To test the hypothesis that the PLT transfusions induced anti-mHA immunity, recipients were analyzed for anti-BALB antibodies and for the ability to eliminate cells expressing the same mHAs as the BALB LR-PLT

concentrates. Naïve and BALB/c whole blood transfused recipients were used as negative and positive controls, respectively.

The presence of anti-BALB antibodies was tested by an indirect immunofluorescence assay using BALB.B PLT and splenocyte (SPL) targets. Using BALB.B SPL targets, increased assay signal was detected in recipients transfused with BALB LR-PLT products (Figure 3A) compared to control syngeneic (B6) LR-PLT transfused recipients; however, this difference did not achieve statistical significance. No difference in anti-BALB antibodies were detected using BALB.B PLT targets, which is not due to inability of the assay to detect PLT bound antibodies, as both BALB.B PLT and SPL target bound antibodies were detected using positive control sera. Thus, no significant levels of anti-BALB antibodies were detected in any of the groups prior to BMT.

PLT induced immunity against BALB mHAs was also tested through an in vivo survival assay using cellular targets expressing the relevant mHAs. As the assay is terminal, representative mice from each group were utilized and injected intravenously with BALB.B SPL targets. As an internal negative control, B6 SPL targets were co-injected along with the BALB.B SPL targets. To allow target visualization and enumeration, BALB.B and B6 SPL targets were labeled with different concentrations of the fluorescent dye CFSE. Twenty-four hours after infusion, recipients were sacrificed and targets were enumerated in the spleen. Numbers of BALB.B SPL targets were normalized to B6 SPL targets to control for differences in injection and splenic processing. Immunity was defined as a specific absence of BALB.B SPL targets (normalized to B6 targets) to an extent that exceeded two standard deviations above

the mean of the control mice receiving syngeneic (B6) LR-PLT products. Substantial immunity was observed in recipients transfused with BALB.B or BALB/c LR-PLT concentrates (8/9 (88%) and 6/9 (66%) respectively) (Figure 3B).

Rejecting recipients demonstrate BALB specific immunity in vivo in the absence of detectable anti-BALB antibodies.

Given that the process of BMT can substantially alter the immune system and responses to transplant antigens, transplanted recipients were tested for BALB mHA specific immunity after the outcome of BMT was determined (see Figure 1A). Serum was collected to test for antibodies by indirect immunofluorescence (as above); recipients were then injected with CFSE labeled BALB.B and B6 SPL targets, 24-hour target survival was assessed. No anti-BALB antibody signal was detected above background (naïve mice) in any groups, using either SPL or PLT targets (Figure 4A). In contrast, BALB.B SPL targets were eliminated in 100% of the recipients that had rejected the BMT, with normal target survival in control animals (Figure 4B). The few animals that had received BALB LR-PLTs and did not reject the BMT were the same mice that did not eliminate the BALB.B SPL targets. Of note, the percent clearance of BALB.B SPL targets observed in two of the engrafting recipients previously transfused with BALB.B LR-PLT concentrates was roughly 20-30% above the background of syngeneic (B6) LR-PLT transfused recipients (Figure 1B, 4B). This raises the possibility that rejection would have subsequently occurred in the two engrafting BALB.B LR-PLT transfused recipients. As the in vivo survival assay is terminal, the ongoing status of the BMT was not further assessed. Together, these data indicate the presence of anti-

BALB specific immunity that correlates with rejection of the BMT. The exact nature of the immunity is unclear, but the data suggest that anti-BALB antibodies are not the effector mechanism.

Discussion

It has been clinically observed that patients who receive multiple transfusions prior to transplantation have a greater frequency of rejecting HLA-matched BMT under reduced intensity conditioning (7, 9-13). Due to HLA-matching, any rejection across immune barriers is likely to involve mHAs. Because many of the patients are transfused with LR-PLT products as part of their transfusion support, we hypothesize that immune responses to mHAs on the transfused PLTs contribute to the increased rates of BMT rejection by immunizing to mHAs that are also expressed by subsequent stem cells in a BMT. However, the correlation between multiple transfusions and BMT rejection may not be causal, as increased transfusion might simply reflect more advanced disease with greater perturbation of the bone marrow microenvironment. Our data indicate that transfusions of LR-PLTs induce BMT rejection. As the animals have no damage to the bone marrow due to underlying hematological disease, the most likely explanation is rejection induced by LR-PLT transfusion. These findings in no way exclude that when present, bone marrow disease may also contribute to rejection; however, this study does demonstrate that transfusion of mHA-mismatched LR-PLTs is sufficient to induce rejection of MHC-matched BMT with reduced intensity conditioning.

The transplantation marker utilized in these studies (Thy1.1/Thy1.2) is a T cell specific epitope; thus, the engraftment data do not unequivocally rule out the possibility of lineage specific engraftment. CD45.1/CD45.2 congenic markers, which are expressed broadly on leukocytes, are often used in BMT studies to avoid the issue of lineage specific engraftment. However, they were avoided in the current studies

because it has been reported that CD45.1/CD45.2 can serve as mHAs that contribute to rejection under reduced conditioning regimens (27).

Although substantial PLT enrichment procedures were utilized, there are very low levels of RBCs and leukocytes (see TABLE 1). Since leukocytes can be professional antigen-presenting cells (APCs), they could theoretically stimulate recipient T cells as part of the direct pathway. However, when we purposely mismatched the PLT and BMT for MHC, while maintaining common mHAs (BALB/c PLT, BALB.B marrow), BMT rejection still occurred at approximately the same frequency (see Figure 1B). We interpret these findings to indicate that the indirect pathway is sufficient to induce BMT rejection. However, one must acknowledge that some T cell epitopes, (e.g. $OVA_{323-339}$), can be presented by both H-2^b and H-2^d MHCs, and T cells generated on one MHC background may cross-react with the other MHC background (28). Thus, although these data suggest indirect presentation, we cannot unequivocally rule out persistence of the direct pathway. However, if this were the case, it is likely that the number of T cells capable of cross-reacting would be substantially fewer, and rejection would be less robust. An additional concern in interpretation is that it is also possible that rejection in the recipients transfused with BALB/c LR-PLT concentrates was due to anti-H-2^d alloresponses cross-reacting with mHA/MHC complexes on the BALB.B BMT. However, we reject this possibility, as we have recently demonstrated that recipients transfused with whole blood from B6 mice congenic for H-2^d (B6.H-2^d), which isolates the anti-$H2^d$ component, do not reject BALB.B BMT in spite of strong anti-H-2^d alloresponses (29). Thus, while there are alternate interpretations, the most likely implication is that the direct pathway is not required and that the indirect pathway is

sufficient to sensitize recipients against mHAs expressed by the transfused PLT products.

Experimentally, the presence of residual RBCs and leukocytes is a concern regarding the identification of the precise transfusion component responsible for inducing rejection. However, this is irrelevant from the standpoint of modeling human transfusion and testing the hypothesis that PLT transfusions induce BMT rejection, as human PLT units have similar contamination with RBCs and leukocytes. Indeed, the residual RBCs and leukocytes are required to accurately model human PLT transfusions. Thus, the data suggest that transfusions of "PLT units" induce BMT rejection. However, ultimately identifying the exact component responsible will be important in circumventing the problem. For example, if it is indeed indirect presentation of mHAs on PLTs themselves by recipient APCs that is responsible, then immunomodulation (i.e. immunosuppressants or altered induction regimens) will be required, as one cannot withhold the PLT transfusions due to hemostasis needs. However, if the residual RBCs or leukocytes are responsible, then better PLT enrichment procedures may solve the problem. In addition to examining the immunological mechanism of rejection, future studies should address the component of the unit responsible for immunization.

The observed rejection occurred in the presence of anti-BALB immunity as indicated by selective elimination of BALB targets during in vivo target survival assays, which correlated strongly with BMT rejection (see Figure 3 and Figure 4). This outcome could be due to either specific cellular immunity (i.e. cytotoxic T cells) or humoral responses (i.e. antibody dependent cellular cytotoxicity). However, the absence of

detectable anti-BALB antibodies in LR-PLT recipients, either before or after BMT (see Figure 3 and 4) suggests that humoral responses are not likely involved. Therefore, the combined findings suggest that cellular immunity against mHAs expressed by transfused LR-PLT concentrates induces rejection of MHC-matched BMT expressing the same mHAs.

Substantial efforts were taken to model processing of human PLT products and assessing the quality of the enriched PLTs. However, one variable of the PLT transfusion process that was not controlled for was the transfusion of stored versus fresh PLTs. Typically, patients receive PLT products that have been stored up to 5 days. When PLTs are stored for a long period of time, they release soluble CD154 and can shed MHC (30). In addition, during storage, residual leukocytes and/or PLTs in the PRP concentrates can release various pro-inflammatory mediators (31-33). In theory, these storage based changes may alter the immune responses to transfused PLTs.

Currently, the main appreciated immunological sequelae of PLT transfusion is the generation of anti-HLA antibodies, which can make patients refractory to subsequent PLT transfusions. The relevancy of the current findings to human medicine is that they suggest an additional immunological vector pertinent to BMT; in particular, PLT transfusion induced BMT rejection across mHA barriers. Moreover, while the current studies focus on BMT, to the extent that solid organ transplants share mHAs with PLTs, it is also possible that PLT transfusions promote rejection in other transplant settings. Of course, the current findings are in a mouse model, and testing the hypothesis in a human setting would be required before any clinical conclusions can be drawn. Nevertheless, this raises a potential new issue in transfusion medicine and

organ transplantation. Currently, neither mHA disparities nor anti-mHA immunity is measured clinically.

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Figure Legends

Figure 1: *Transfusion of LR-PLT products induces MHC-matched BMT rejection.* **(A)** Experimental Model of MHC-matched BMT. Proteins carrying mHAs are indicated by diamonds, with color differences distinguishing polymorphisms. BALB.B donors were MHC-matched:mHA-mismatched, while BALB/c donors were MHC- and mHA-mismatched. Indicated recipients received two PLT transfusions, after the second transfusion, mice were then given BMT and engraftment was monitored. Seroanalyses and in vivo survival of BALB targets was also performed both prior to and after BMT (see figures 3 and 4). **(B)** BMT engraftment results. Engraftment is represented by % Thy-1.2 cells in peripheral blood, the mean of each group is represented as a horizontal line. Statistics were generated using one-way ANOVA with Dunnett's post-test. Illustrated is the combined data from three independent experiments.

Figure 2: *Quality of isolated LR-PLT concentrates.* **(A-C)** The quantity of PLTs, RBCs and leukocytes was assessed using cell surface markers CD41, TER119 **(B)** or nucleic acid binding propidium iodide **(C)**, respectively. Isotype controls (Rat IgG1 for PLTs and Rat $IgG2_b$ for RBCs) and unstained samples were also enumerated to control for nonspecific signal **(B and C,** respectively). Whole blood samples **(A+C)** were included for gating purposes. **(D)** Functionality of isolated PLTs. B6, BALB.B and BALB/c PLTs were exposed to collagen and measured for percent aggregation over time using an aggregometer. PLTs without collagen exposure were also included to control for spontaneous aggregation. **(E)** in vivo Survival of LR-PLT products. PLTs from EGFP transgenic mice were transfused into naïve B6 recipients, and tracked over time.

Representative flow cytometry is presented; the left flow plot utilizes an un-transfused mouse to establish background, while the right plot illustrates $EGFP^+$ $CD41^+$ PLTs from a transfused recipient. Figures in (A-C) demonstrate the gating strategy used for quantifying each cell subset. Panels A-E are each representative data from experiments reproduced at least three times.

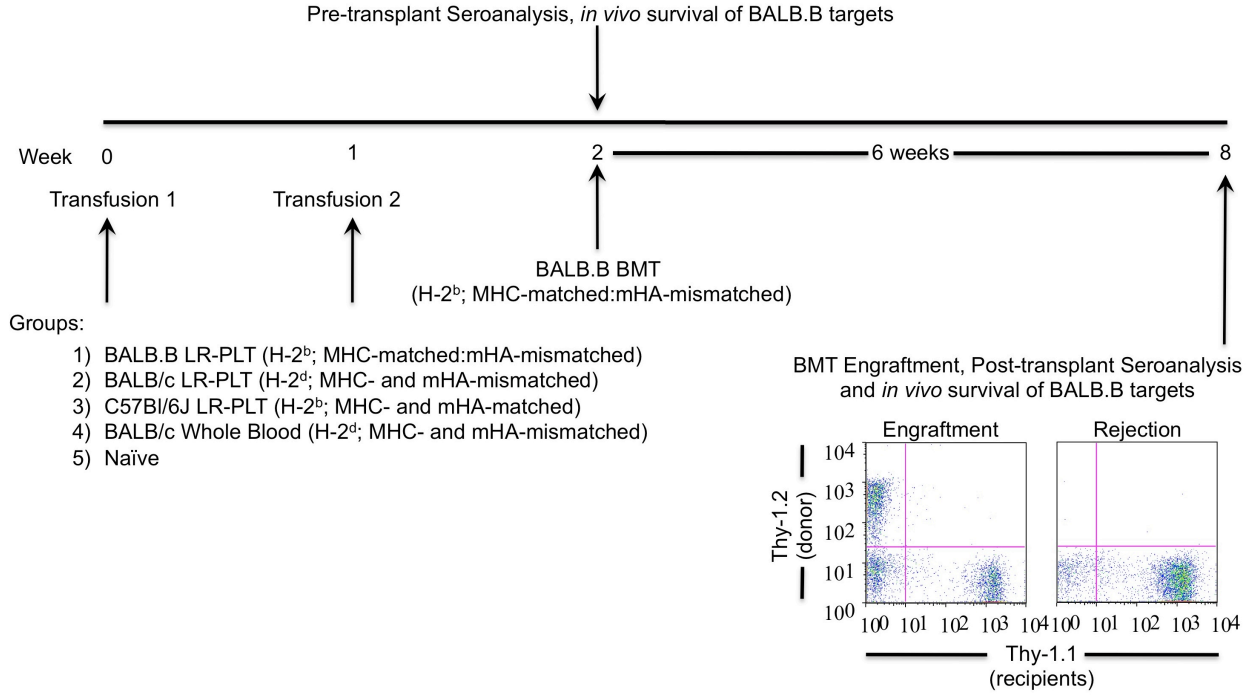
Figure 3: *Analysis of Alloimmunization against BALB mHAs before BMT*. **(A)** Indirect immunofluorescence staining was used to assess the presence of anti-BALB antibodies using BALB.B SPL (white) and PLT (grey) targets. **(B)** BALB specific immunity was assessed by *in vivo* survival of BALB.B SPL targets. Error bars in (A) represent the mean $\pm$ SEM. The mean of each group in (B) is represented as a horizontal line. Statistics were generated using one-way ANOVA with Dunnett's post-test. The data shown for both panels A and B is the combined data from three separate experiments.

Figure 4: *Alloimmunization against BALB mHAs after BMT.* **(A)** Indirect immunofluorescence staining was used to assess the presence of anti-BALB antibodies using BALB.B SPL (white) and PLT (grey) targets. **(B)** BALB specific immunity was assessed by in vivo survival of BALB.B SPL targets. Error bars in (A) represent the mean $\pm$ SEM. The mean of each group in (B) is represented as a horizontal line. Statistics were generated using one-way ANOVA with Dunnett's post-test. The data shown in both panels A and B is the combined data from three separate experiments.

TABLE 1. *Assessment of leukocyte and RBC contamination in LR-PLT units*. In the experiments described in this manuscript, every preparation of LR-PRP was evaluated for residual leukocyte and RBC contamination, as described in materials and methods. Data illustrated in this table are a mean of numbers from all three experiments (n=6).

Figure 1

A.


Pre-transplant Seroanalysis, *in vivo* survival of BALB.B targets
Week 0
1
2
6 weeks
8
Transfusion 1
Transfusion 2
BALB.B BMT
(H-2b; MHC-matched:mHA-mismatched)
Groups:
1) BALB.B LR-PLT (H-2b; MHC-matched:mHA-mismatched)
2) BALB/c LR-PLT (H-2d; MHC- and mHA-mismatched)
3) C57Bl/6J LR-PLT (H-2b; MHC- and mHA-matched)
4) BALB/c Whole Blood (H-2d; MHC- and mHA-mismatched)
5) Naïve
BMT Engraftment, Post-transplant Seroanalysis
and *in vivo* survival of BALB.B targets
Engraftment
Rejection
Thy-1.2 (donor)
Thy-1.1 (recipients)

B.

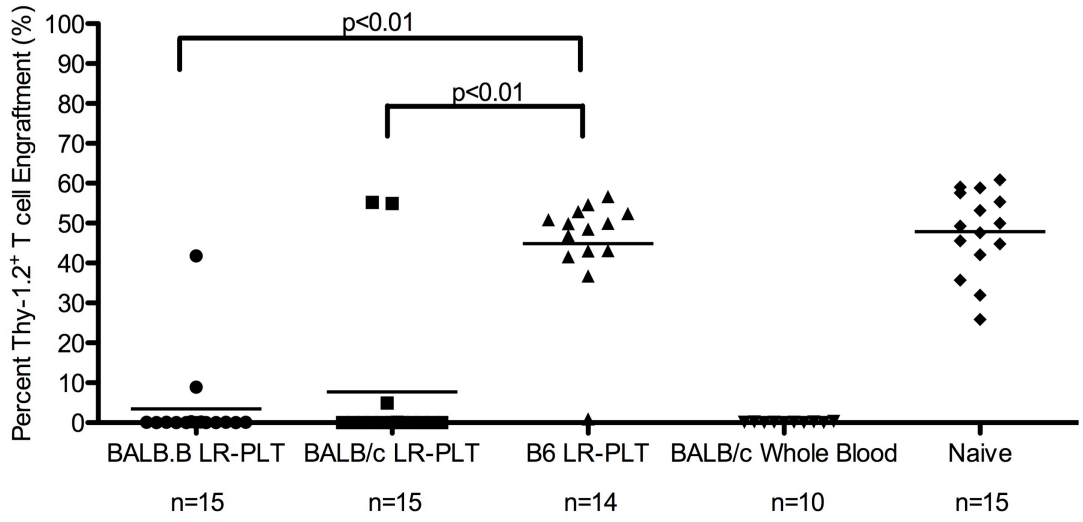

Percent Thy-1.2+ T cell Engraftment (%)
p<0.01
p<0.01
BALB.B LR-PLT
BALB/c LR-PLT
B6 LR-PLT
BALB/c Whole Blood
Naive
n=15
n=15
n=14
n=10
n=15

Figure 2

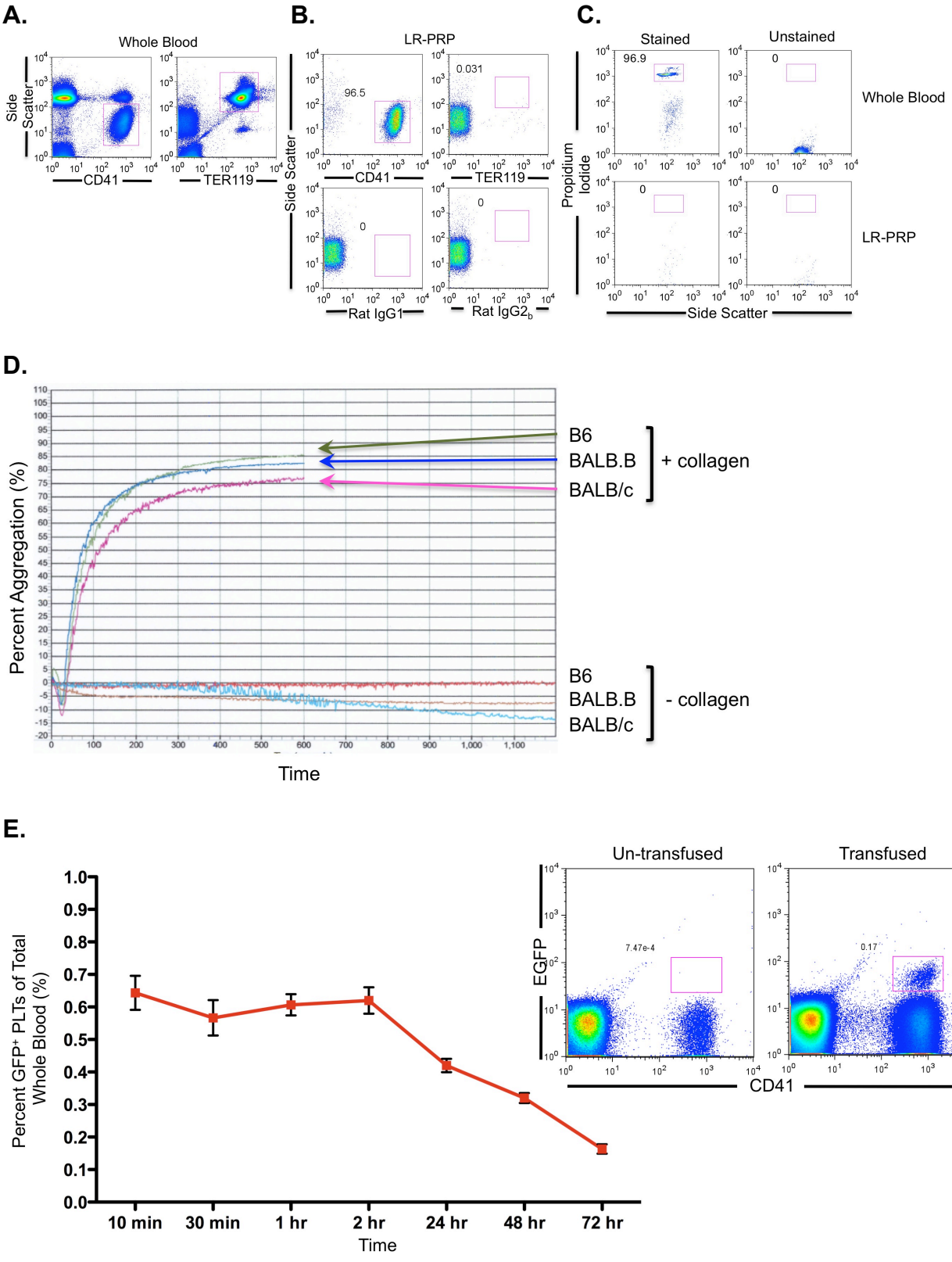
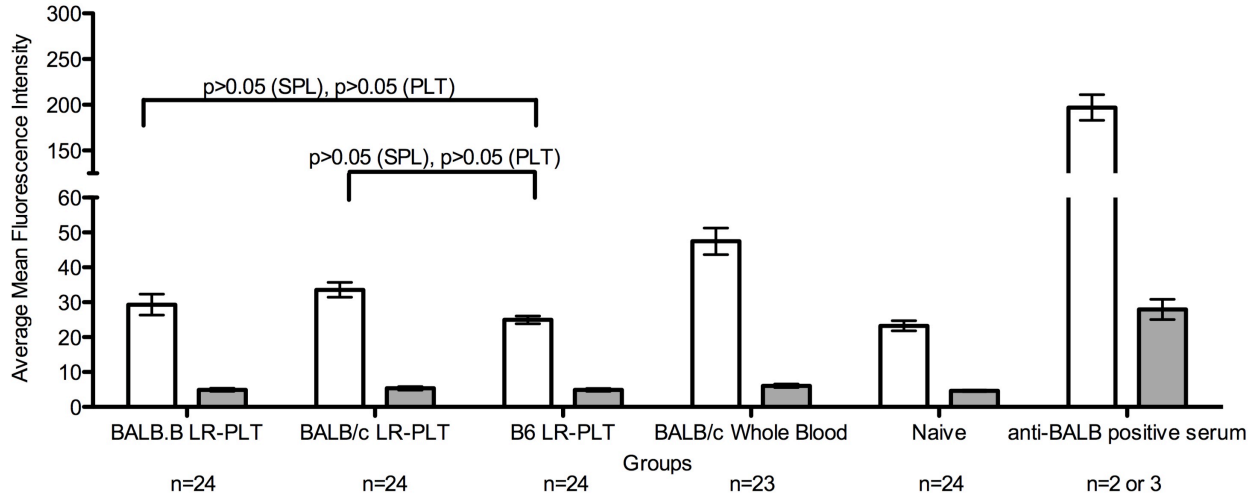

A.
Whole Blood
Side Scatter
CD41
TER119
B.
LR-PRP
96.5
0.031
0
0
Side Scatter
CD41
TER119
Rat IgG1
Rat IgG2b
C.
Stained
Unstained
96.9
0
0
0
Propidium Iodide
Whole Blood
LR-PRP
Side Scatter
D.
Percent Aggregation (%)
B6
BALB.B
BALB/c
+ collagen
B6
BALB.B
BALB/c
- collagen
Time
E.
Percent GFP+ PLTs of Total Whole Blood (%)
10 min
30 min
1 hr
2 hr
24 hr
48 hr
72 hr
Time
Un-transfused
Transfused
7.47e-4
0.17
EGFP
CD41

Figure 3

A.


Average Mean Fluorescence Intensity
300
250
200
150
60
50
40
30
20
10
0
p>0.05 (SPL), p>0.05 (PLT)
p>0.05 (SPL), p>0.05 (PLT)
BALB.B LR-PLT
BALB/c LR-PLT
B6 LR-PLT
BALB/c Whole Blood
Naive
anti-BALB positive serum
Groups
n=24
n=24
n=24
n=23
n=24
n=2 or 3

B.

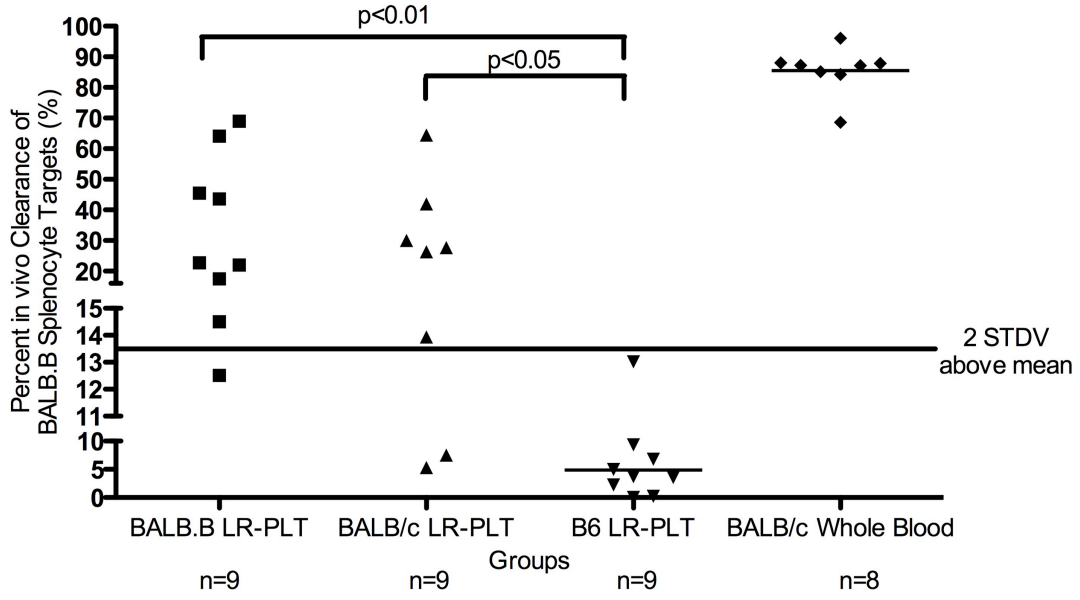
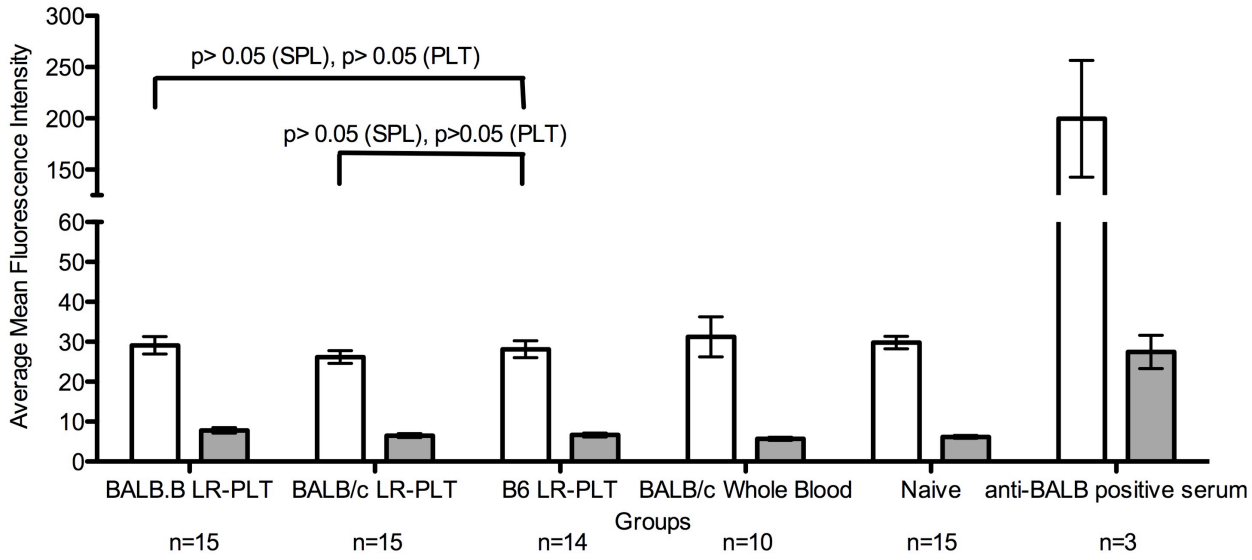

Percent in vivo Clearance of BALB.B Splenocyte Targets (%)
100
90
80
70
60
50
40
30
20
15
14
13
12
11
10
5
0
p<0.01
p<0.05
2 STDV above mean
BALB.B LR-PLT
BALB/c LR-PLT
B6 LR-PLT
BALB/c Whole Blood
Groups
n=9
n=9
n=9
n=8

Figure 4

A.


Average Mean Fluorescence Intensity
300
250
200
150
60
50
40
30
20
10
0
p> 0.05 (SPL), p> 0.05 (PLT)
p> 0.05 (SPL), p>0.05 (PLT)
BALB.B LR-PLT
BALB/c LR-PLT
B6 LR-PLT
BALB/c Whole Blood
Naive
anti-BALB positive serum
Groups
n=15
n=15
n=14
n=10
n=15
n=3

B.

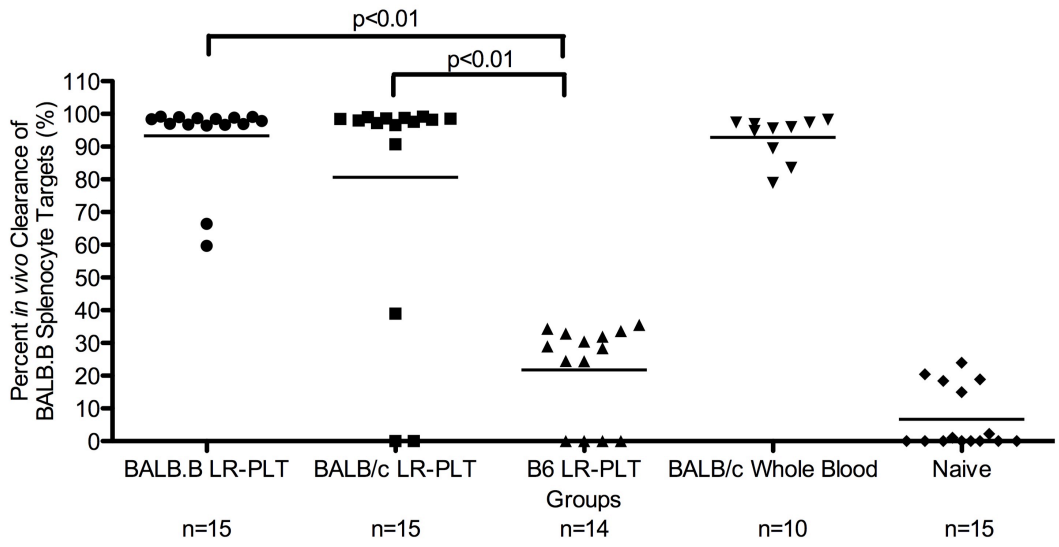

Percent *in vivo* Clearance of BALB.B Splenocyte Targets (%)
110
100
90
80
70
60
50
40
30
20
10
0
p<0.01
p<0.01
BALB.B LR-PLT
BALB/c LR-PLT
B6 LR-PLT
BALB/c Whole Blood
Naive
Groups
n=15
n=15
n=14
n=10
n=15

Table 1

Assessment of leukocyte and rbc contamination in LR-PLT units

Groups	Average leukocytes per transfusion unit	Average rbc per transfusion unit
BALB.B LR-PRP	Undetectable ($n = 5$); 118.6 (.24/μl) ($n = 1$)	5.8×10^4 (116 rbc/μl)
BALB/c LR-PRP	Undetectable	4.4×10^4 (88 rbc/μl)
B6 LR-PRP	Undetectable	5.7×10^4 (114 rbc/μl) ($n = 5$); Undetectable ($n = 1$)

Chapter 3

Mechanisms of Alloimmunization and Subsequent Bone Marrow Transplantation Rejection Induced by Platelet Transfusion in a Murine Model

Abstract

For many non-malignant hematological disorders, HLA-matched bone marrow transplantation (BMT) is curative. However, due to lack of neoplasia, the toxicity of stringent conditioning regimens is difficult to justify, and reduced-intensity conditioning is thus utilized. Unfortunately, current reduced-intensity regimens have high rates of BMT rejection. We have recently reported in a murine model that minor antigens (mHAs) on transfused platelet products induce subsequent BMT rejection. Most non-malignant hematological disorders require transfusion support prior to BMT and the rate of BMT rejection in humans correlates to the number of transfusions given. Herein, we perform a mechanistic analysis of platelet transfusion induced BMT rejection and report that unlike exposure to alloantigens during transplantation, platelet transfusion primes alloimmunity but does not stimulate full effector function. Subsequent BMT is itself an additional and distinct immunizing event, which does not induce rejection without antecedent priming from transfusion. Both $CD4^+$ and $CD8^+$ T cells are required for priming during platelet transfusion, but only $CD8^+$ T cells are required for BMT rejection. In neither case are antibodies required for rejection to occur. These findings provide novel mechanistic insight to immunization to mHAs on transfused platelets, vectors of transfusion induced BMT rejection, and provide a rational basis for development of therapeutic interventions.

Introduction

Bone marrow transplantation (BMT) is currently the only effective cure for a variety of non-malignant hematological disorders including aplastic anemia, β-thalassemia, sickle cell disease, fanconi anemia and others (1-6). Overall, rejection of BMT is now a rare event; however, this is because the vast majority of BMT is given in the setting of malignancy where the need to kill neoplasia justifies the use of stringent conditioning regimens that effectively eliminate functional alloimmunity. Although beneficial in promoting engraftment and killing neoplastic cells, the toxic effects of stringent regimens can result in morbidity and mortality. Because patients with non-malignant bone marrow (BM) failure syndromes do not have cancerous cells, the toxicity of stringent conditions is difficult to justify. Thus, patients with non-malignant hematological disorders typically receive an HLA-matched BMT under reduced intensity conditions (2, 6, 7). However, under a variety of reduced intensity conditions, rejection of HLA-matched BMT still occurs at a frequency of up to 15%, depending upon the patient population and conditioning used (3, 4, 7-10).

Why some patients reject the HLA-matched BMT under the above reduced intensity conditions and others do not remain unclear. However, it has been observed that chronically transfused patients reject HLA-matched BMT at a greater frequency than minimally or un-transfused patients and that rate of BMT rejection correlated to the number of transfusions given (11-15). These findings suggest that transfusion may induce alloimmunity capable of rejecting a subsequent BMT. Because these BMTs are HLA matched or HLA identical (in

the case of matched siblings), minor antigens (mHAs) are the most likely cause of rejection. Utilizing a murine model, we have recently reported that transfusion of leukoreduced allogeneic platelets (LR-PLT) induces rejection of a subsequent MHC-matched BMT across mHA barriers (16). This was the first direct demonstration that exposure to mHAs on transfused PLTs could induce BMT rejection.

In the current report we elucidate mechanisms of alloimmunization and BMT rejection due to mHAs on transfused PLTs. We report that whereas PLT transfusion is required to induce subsequent BMT rejection, the PLTs do not induce a full effector response to mHAs. In contrast, transfusion primes the immune system to alloantigens and the subsequent BMT is an additional immunizing event that pushes the system to full effector function. Both $CD4^+$ and $CD8^+$ T cells are required for the priming event to occur, but only $CD8^+$ T cells are required for rejection of the BMT. In neither case are antibodies required. Together, these findings provide novel insight into the nature of alloimmunization to mHAs on transfused PLTs and distinguish a difference from what has been observed in the context of other alloantigen exposure (i.e. solid organ transplantation).

Materials and Methods

Mice

Female BALB/c (H-2^d), BALB.B [C.B10-H-2^b/LiMcdJ (H-2^b)], and C57BL/6J (H-2^b), mice were purchased from Jackson Laboratories (Bar Harbor, ME). BALB/c and BALB.B mice were used as LR-PLT and/or whole blood donors at 8 - 12 weeks of age. Female BALB.B, (BALB.B x C57BL6/J) F_1, and (BALB.B x B6 Thy1.1) F_1 BM donors were utilized at 6 - 8 weeks of age. Seroanalyses and *in vivo* clearance target donors, BALB.B and C57BL/6J were utilized at 8 - 12 weeks of age. Female C57BL/6J recipients were utilized at 6 - 8 weeks of age. (BALB.B x C57BL/6J) F_1, (BALB.B x B6 Thy1.1) F_1, and μMT (H-2^b) mice were bred at the Emory Division of Animal Resources Animal Husbandry Service. All mice were housed in Emory University Department of Animal Resources facilities and procedures were performed according to approved IACUC protocols.

Antibodies for Flow Cytometry

Antibodies were purchased from BD Pharmingen (PE anti-mouse CD41, PE anti-TER119/erythroid, PE Rat $IgG2_b$, APC goat anti-mouse Igs, FITC anti-mouse CD19, FITC anti-mouse CD4 (clone: RM-45), FITC anti-mouse CD8α (clone: 53-6.7), APC anti-mouse CD3ε, FITC Rat $IgG2_a$, κ, FITC anti-mouse CD3ε, FITC anti-mouse CD229.1/Ly9.1, and PE anti-mouse CD90.2/Thy1.2) and eBioscience (PE Rat IgG1, APC anti-mouse CD229/Ly9, and APC anti-mouse CD90.1/Thy1.1).

Leukocyte Reduced Platelet Product Preparation

LR-PLT products were harvested as previously described (16). Briefly, donor BALB/c and C57BL/6J whole blood was collected in 1:8 acid-citrate-dextrose (ACD; BD Vacutainer). 2 mL of 1x DPBS was added to each mL of whole blood. Samples were centrifuged at 80 x g for 10 minutes, followed by collection of PLT rich plasma (PRP) and re-centrifugation 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). PLTs were enumerated as described below. After enumeration, PLTs were pelleted at 3,000 rpm (1,942 x g) for 10 minutes and re-suspended 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, 5.5 mM glucose in Milli Q water at pH = 7.4) to 2 x 10^8 PLT/mL. 500 µl of re-suspended LR-PLT (10^8 PLT total) was transfused through the tail-vein. All PLT handling was performed at room temperature.

Enumerating Platelets, and Residual Red Blood Cells and Leukocytes

PLTs and residual RBCs were enumerated using a 20 µl LR-PRP sample diluted in 100 µl FACS buffer (1x DPBS with 0.2% bovine serum albumin and 0.24 mM EDTA). Samples were then centrifuged at 3,000 rpm (1,942 x g) for 2 minutes at room temperature. The samples were stained with PE anti-mouse CD41 or isotype control PE Rat IgG1 for PLTs, and PE anti-mouse TER119 or isotype control PE Rat $IgG2_b$ for RBCs. All antibodies were diluted at 1:100 in FACS buffer. Samples were incubated for 30 minutes in the dark at room temperature

and then washed once with FACS buffer. After washing, samples were re-suspended in 100 µl FACS buffer, followed by the transfer of 10 µl of each sample to 940 µl FACS buffer. Cells were enumerated by adding 2.5 x 10^5 APC-beads (BD Pharmingen) to each sample. Samples were run on a BD FACS Caliber and analyzed using FlowJo 9.1. 12,500 events of APC-beads were collected. All recipients transfused with BALB/c and C57BL/6J LR-PLT units received on average 3.5 x 10^4 and 3.6 x 10^4 RBCs total, respectively (data not sown).

25 µl LR-PRP was stained with 150 ng/mL Propidium Iodide (PI) in a 1 g/L sodium citrate dihydrate solution with 0.7% Zap-oglobin II reagent (Beckman Coulter), 0.7% RNAse cocktail (New England Biolabs) to effectively stain all nucleated cells and enumerate residual leukocytes in the LR-PRP concentrate. 100,000 APC-beads were added to each sample for absolute counts. Nuclei were enumerated on a BD FACS Caliber and analyzed using FlowJo 9.1. 10,000 events of APC-beads were collected. Based on previously described titration studies, BALB/c and C57BL/6J LR-PLT transfused recipients received approximately less than 500 total leukocytes (data not shown).

Isolation of Whole Blood

Whole blood was collected in 1:8 ACD, followed by washed three times with 1x DPBS and centrifugation at 1,350 rpm (394 x g) for 15 minutes at 4°C. While aspirating the supernatant, the buffy coat was not disrupted. The whole blood

was re-suspended in 1x DPBS at a 1:5 dilution and 500 µl transfusions were given by tail vein.

CD4 and CD8 depletion

$CD4^+$ T cells were depleted using anti-CD4 (clone: GK1.5) depleting antibody purchased from Bio X Cell (West Lebanon, NH), while $CD8^+$ T cell depletion was performed utilizing anti-CD8β depleting antibody (clone: H35) generously gifted by Dr. Aron Lukacher (Emory University, GA). Isotype control antibodies for each depleting antibody were also administered to parallel groups. Isotype control antibodies Rat $IgG2_b$ and whole molecule Rat IgG were purchased from Bio X Cell and Jackson Laboratories, respectively. Antibodies were intra-peritoneal (i.p.) injected with 250 µg of CD4 depleting antibody or Rat $IgG2_b$ (CD4 depleting antibody's isotype control), or 1 mg of CD8β depleting antibody or Rat IgG (CD8β depleting antibody's isotype control). All antibodies were diluted in 1x DPBS and antibody treatment regimen was twice, a day apart.

Assessing CD4 and CD8 depletion

Depletion of $CD4^+$ and $CD8^+$ T cells was confirmed 24 hours after the second treatment in the peripheral blood of transplant recipients, and in the peripheral blood, spleen, peripheral lymph nodes (inguinal, axillary, brachial and jugular), and BM of representative recipients. Spleen, peripheral lymph nodes, and BM were harvested in 1x RPMI, 10% fetal bovine serum (FBS). Peripheral blood leukocytes and leukocytes from BM were isolated through lysis of RBCs using

RBC lysis buffer (Sigma). Samples were washed three times in FACS buffer and stained for $CD3^+$ $CD4^+$ or $CD8^+$ T cells. Samples were incubated for 30 minutes at 4°C in the dark and then washed three times with FACS buffer. Following the third wash, the cells were re-suspended in 100 µl FACS buffer and transferred to 200 µl FACS buffer. Samples were run on a BD FACS Caliber and analyzed using FlowJo 9.1.

Bone Marrow Transplantation

24 hours prior to BMT, all recipients were conditioned with sub-lethal gamma irradiation treatments (700 rads). BM was harvested from femurs and tibias in 1x RPMI, 10% FBS. Cells were washed three times in 1x DPBS. During the third wash, BM cells were enumerated using a hemocytometer, and then re-suspended at 10 x 10^6 cells/mL in 1x DPBS. 500 µl (5 x 10^6 BM cells) transfusions were injected into the tail veins of all recipients. Engraftment was monitored six weeks post-BMT by staining peripheral blood leukocytes. To assess engraftment in recipients transplanted with BALB.B or (BALB.B x C57BL6/J) F_1 BM, peripheral blood leukocytes were stained with APC anti-mouse CD229/Ly9 (1:100), followed by PE anti-mouse CD3ε (1:100) and FITC anti-mouse CD229.1/Ly9.1 (1:50) in FACS buffer for 30 minutes at 4°C. Engraftment was measured as percent donor $CD229.1^+$ cells (BALB.B BMT) or $CD229.1^+$ T cells [(BALB.B x C57BL/6J) F_1 BMT]. For recipients transplanted with (BALB.B x B6 Thy1.1) F_1 BM, engraftment was determined by staining recipient peripheral blood leukocytes with FITC anti-mouse CD3ε (1:50), APC

anti-mouse CD90.1/Thy1.1 (1:2500), and PE anti-mouse CD90.2/Thy1.2 (1:2500). 10,000 $CD3^+$ events were collected and engraftment was measured as percent donor $Thy1.1^+$ T cells. All samples were run on a FACS Caliber and analyzed by FlowJo 9.1.

Seroanalysis: Indirect Immunofluorescence Staining

Sera was diluted 1:10 in FACS buffer and incubated with BALB.B SPL or PLT targets for 30 minutes at 4°C for SPLs and room temperature for PLTs. Samples were washed three times with FACS buffer, and re-suspended in APC goat anti-mouse Igs diluted in FACS buffer 1:100 for 30 minutes in the dark at the appropriate temperatures previously mentioned. B cells in the SPL targets were excluded using FITC anti-mouse CD19 diluted in FACS buffer 1:50, and PLT targets were visualized with PE anti-mouse CD41 diluted 1:100 in FACS buffer. Samples were run on a FACS Caliber and analyzed on FlowJo 9.1; MFI of APC was used to indicate the presence of antibody bound targets. 20,000 $CD19^+$ (for SPLs) or $CD41^+$ (for PLTs) events were collected.

In vivo Survival of BALB.B Splenocyte Targets

Female C57BL/6J and BALB.B SPLs were harvested in 1x RPMI, 10% fetal bovine serum (FBS). SPLs were incubated in RBC lysis buffer (2 mL/spleen) for 5 minutes at room temperature. Cells were then washed three times in 1x DPBS. During the third wash, cells were enumerated using a hemocytometer. C57BL/6 SPL targets were labeled with $CFDA^{hi}$ (3 μM), while BALB.B SPL

targets were labeled with $CFDA^{lo}$ (2.5 nM) at 37°C for 10 minutes. Samples were mixed at equal volumes to generate 10 x 10^6 cells/mL per target population. 500 µl transfusions were given through tail veins (total dose of 5 x 10^6 cells per target). Recipient SPLs were harvested roughly eighteen hours post transfusion and 10,000 events of $CFDA^{hi}$ C57BL/6J targets were collected. Samples were run on a FACS Caliber and analyzed on FlowJo 9.1. Percent *in vivo* clearance was calculated as the ratio of $CFDA^{hi}$:$CFDA^{lo}$ events in experimental recipients normalized to the average ratio of $CFDA^{hi}$:$CFDA^{lo}$ events in naïve recipients.

Statistics

Statistical analysis was performed using one-way ANOVA with Dunnett's post-test and column statistics. Significance was determined by a P value less than 0.01.

Results

Both $CD4^+$ T cells and $CD8^+$ T cells are required for alloimmunization to mHAs on transfused PLTs.

To test the respective roles of $CD4^+$ and $CD8^+$ T cells in rejection of an MHC-matched BMT in LR-PLT transfused recipients, we made use of our previously described model of MHC-matched:mHA mismatched BMT (16). In this system, C57BL/6J (H-2^b) recipients are transfused twice, a week apart, with BALB/c (H-2^d; MHC- and mHA-mismatched) LR-PLT concentrates (Figure 1A). One week after the second transfusion, recipients are given a BALB.B (H-2^b; mHA-mismatched) BMT under reduced intensity conditions. The BM donors and recipients are MHC-matched (both H-2^b) but mHA-mismatched (C57BL/6 vs BALB backgrounds) and the PLT donors share mHAs (but not MHC) with the BM donors. Rejection does not occur unless BMT recipients have been previously transfused. Thus, rejection is likely due to antecedent exposure to mHAs on the PLT inducing alloimmunity against mHAs on the marrow.

Experimental groups consisted of injecting antibodies that deplete $CD4^+$ cells (clone: GK1.5) or $CD8\beta^+$ cells (clone: H35) after LR-PLT transfusions but prior to BMT. To assess non-specific effects of antibody injection, control animals received the corresponding Rat $IgG2_b$ or Rat IgG isotype control antibodies. Because the CD4 and CD8β depleting antibodies can potentially mask the epitope binding site utilized to visualize the cell subsets by flow cytometry, and thus can result in an artificial detection of $CD4^+$ or $CD8^+$ T cell depletion, anti-CD4 (clone: RM4-5) and anti-CD8α (clone: 53-6.7) antibodies

recognizing epitopes distinct from the depleting antibodies were utilized, respectively. Almost complete deletion of $CD4^+$ or $CD8^+$ cells was observed with no depletion observed using control antibodies (representative animals shown in Figure 1B). Some animals were sacrificed, and there were no detectable $CD4^+$ or $CD8^+$ T cells in the spleen, BM, or peripheral lymph nodes, thus indicating that monitoring depletion by peripheral blood reflects elimination in secondary lymphoid and hematopoietic compartments (data not shown).

Engraftment was assessed six weeks post BMT using the CD229 congenic markers ([CD229.1-donor] and [CD229.2-recipient]). The results from three combined experiments demonstrated that 13/15 (86%) of the BALB/c LR-PLT transfused recipients treated with CD8β depleting antibody engrafted the BALB.B BMT (Figure 1C). In addition, the combined data of three experiments showed that 15/15 (100%) of the BALB/c LR-PLT transfused recipients treated with the CD4 depleting antibody engrafted the BALB.B BMT (Figure 1C). Prevention of rejection by $CD4^+$ or $CD8^+$ depletion was not the result of non-specific effects, as BALB.B BMT rejection was observed in 13/15 (86%) of recipients treated with the Rat $IgG2_b$ or Rat IgG control antibodies (Figure 1C). Likewise, the failure to reject the BALB.B BMT was not due to excessive conditioning, as 13/15 (86%) of the naïve recipients engrafted the BALB.B BMT (Figure 1C). Together, these data indicate that both $CD4^+$ and $CD8^+$ T cells are required for PLT transfusion induced BMT rejection.

Both $CD4^+$ and $CD8^+$ Cells are Required for Generation of mHA Specific Alloimmunity

mHA specific alloimmunity was analyzed in the above defined experimental groups. The presence of donor reactive antibodies was tested through an indirect immunofluorescence staining using BALB.B splenocyte (SPL) and PLT targets and flow cytometry. Responders to BALB mHAs were defined as having a mean fluorescence intensity (MFI) exceeding two standard deviations above the mean of the background naïve recipients. No statistically significant anti-BALB alloantibodies were detected above the background naïve recipients using either BALB.B SPL or PLT targets (Figure 2A). Likewise, no significant differences were observed between recipients treated with CD4 or CD8β depleting antibodies, or the corresponding isotype control antibody treated groups (Figure 2A). The absence of anti-BALB antibody detection was not due to the inability to detect target bound antibodies using the indirect immunofluorescence staining assay, as anti-BALB antibodies were detected in the sera from positive control animals that were humorally immunized against BALB mHAs (Figure 2A).

The ability of alloimmune effector mechanisms to directly kill target cells expressing BALB mHAs was tested using an *in vivo* survival assay. Target cells (BALB.B SPLs) were labeled with the fluorescent dye CFDA, injected into recipient mice, and survival was monitored by enumerating surviving cells in the recipient spleen by flow cytometry approximately eighteen hours post infusion. To control for non-specific clearance of the infused targets, syngeneic C57BL/6J

SPL targets were labeled with a different concentration of CFDA and were co-injected with BALB.B SPL targets. BALB.B SPL targets were normalized to C57BL/6J SPL targets to control for differences in injection and splenic processing.

The combined results from three experiments demonstrated that neither the CD4 depleted (0/13 mice) nor the CD8 depleted animals (1/15 mice) that had failed to reject BMT had significant clearance of mHA targets. In contrast, the control animals receiving non-specific antibody injection, and which rejected the BMT, had strong clearance of the mHA expressing targets (12/13 mice and 13/13 mice) (Figure 2B). Together, these data indicate that both $CD4^+$ and $CD8^+$ cells are required for the generation of mHA specific alloimmunity capable of eliminating mHA expressing targets.

$CD8^+$ T Cells but not $CD4^+$ T Cells are required for BMT Rejection.

Because the above measures of alloimmunity were performed after exposure to mHAs both during PLT transfusion and during BMT, it was unclear to what extent each process contributed to the alloimmunity that develops. Exposure to mHAs on transfused PLTs is clearly required, as neither naïve mice nor mice receiving syngeneic transfusions reject BMT (16). However, we have also previously reported that *in vivo* clearance of mHA expressing targets is variable when measured after PLT transfusion (but prior to BMT) and does not statistically predict BMT rejection (16). In contrast, the current findings and our previous experiments demonstrate that *in vivo* clearance of donor targets

approaches roughly 100% after BMT rejection (16). Combined, these data suggest that LR-PLT transfusions prime recipients for a donor mHA alloresponse that differentiates into to a full effector alloresponse during the BMT.

In the above context, the lack of either rejection or *in vivo* clearance after $CD4^+$ depletion (see figure 2) is equally consistent with either $CD4^+$ T cells being direct lytic effectors or $CD4^+$ T cells providing help for development of $CD8^+$ cytolytic T lymphocytes (CTL). To distinguish between these two scenarios, we generated an experimental design that allowed re-transplantation studies. C57BL/6J ($H\text{-}2^b$) recipients were transfused two times with BALB/c ($H\text{-}2^d$; MHC- and mHA-mismatched) LR-PLT concentrates and were then given a BMT from (BALB.B x C57BL/6J) F_1 donors under reduced intensity conditions (Figure 3A). Engraftment was measured six weeks later using the CD229 congenic markers. The results from three independent experiments demonstrated that 64/76 (84%) of the BALB/c LR-PLT transfused recipients rejected the (BALB.B x C57BL/6J) F_1 BMT (Figure 3B). Failure to engraft was not due to insufficient conditioning or poor BMT as 15/15 (100%) of the naïve recipients engrafted the (BALB.B x C57BL/6J) F_1 BMT (Figure 3B).

The mice were then subjected to depletion studies as above (see Figure 3A for experimental design). One day after the second treatment, $CD4^+$ or $CD8^+$ T cell depletion was confirmed by measuring depletion in the peripheral blood (Figure 3C). In addition, representative animals were sacrificed and essentially complete depletion was observed in the spleen, lymph nodes and bone marrow (data not shown). After depletion, recipients then received a second BMT with

(BALB.B x B6 Thy1.1) F_1 donors, differing from the first donors only by the Thy1.1 antigen. Six weeks later, BMT engraftment was assessed using the T cell congenic markers Thy1.2 (recipient) and Thy1.1 (donor). In this way, both BMT carried the relevant mHAs of the BALB background, but engraftment of the second transplant ($Thy1.1^+$) could be distinguished from any residual hematopoietic activity from the first BMT.

The data from three combined experiments demonstrated that 15/17 (88%) of the BALB/c LR-PLT transfused recipients that rejected the first MHC-matched BMT engrafted a second (BALB.B x B6 Thy1.1) F_1 BMT upon treatment with the CD8β depleting antibody (Figure 3D). This was similar to control naïve mice, in which 15/15 (100%) mice that engrafted the first (BALB.B x C57BL/6J) F_1 BMT also engrafted the second (BALB.B x B6 Thy1.1) F_1 BMT. The inability to reject the second (BALB.B x B6 Thy1.1) F_1 BMT in the CD8β depleting antibody treated recipients was not due to non-specific immunomodulating effects of the depleting antibody, as 12/13 (92%) of the BALB/c LR-PLT transfused recipients that rejected the first BMT also rejected the second (BALB.B x B6 Thy1.1) F_1 BMT in the presence of the Rat IgG isotype control antibody (Figure 3B and 3D). In contrast to CD8 depletion, 17/17 (100%) of the recipients that rejected the first BMT also rejected the second (BALB.B x B6 Thy1.1) F_1 BMT despite depletion of $CD4^+$ T cells (Figure 3D). This is also in contrast to depletion after transfusion but prior to BMT (see figure 1).

BALB mHA specific alloimmunity was tested in recipients after the outcome of the second BMT was determined by measuring both donor reactive

antibodies and *in vivo* survival of BALB.B SPL targets. Donor reactive antibodies were detected through indirect immunofluorescence staining using BALB.B SPL and PLT targets. Responders were defined as having a MFI exceeding two standard deviations above the mean of the background naïve recipients. No statistically significant differences in anti-BALB alloantibodies were observed in any groups above the background naïve recipients (Figure 4A). Likewise, no statistically significant differences were observed between recipients treated with the CD4 or CD8β depleting antibodies, and the corresponding isotype control treated groups (Figure 4A).

In vivo target survival studies were performed as above using BALB.B SPL targets and control C57BL/6J SPL targets. The combined results from three independent experiments demonstrated that 15/17 (88%) of the recipients that engrafted the second MHC-matched BMT upon treatment with the CD8β depleting antibody failed to clear the BALB.B SPL targets *in vivo* (Figure 3D and 4B). In contrast, *in vivo* clearance of BALB.B SPL targets was detected in 16/17 (94%) of the CD4 depleting antibody treated recipients that rejected the second MHC-matched BMT (Figure 3D and 4B).

The absence of detectable *in vivo* clearance of BALB.B SPL targets in the engrafting CD8β depleting antibody treated recipients was not due to non-specific effects of the depleting antibody, as 12/13 (92%) of the isotype control Rat IgG antibody treated recipients that rejected the second MHC-matched BMT cleared the BALB.B SPL targets *in vivo* (Figure 3D and 4B). Together, these data demonstrate that a change in immunity occurs as a result of the BMT itself,

and that in the final matured immune response, $CD8^+$ T cells but not $CD4^+$ T cells are required as effectors mediating rejection of an MHC-matched BMT across mHA barriers in LR-PLT transfused recipients.

Neither B cells nor antibodies are required for LR-PLT transfusion induced BMT rejection.

The lack of detectable antibodies by indirect immunofluorescence suggests that humoral immunity is not important in BMT rejection in this context; however, to formally test the role of antibodies, the MHC-matched BMT model was adapted to include recipients that lack B cells as a result of a genetic mutation in the μ immunoglobulin chain gene (16, 17). In this system, C57BL/6J or μMT recipients ($H\text{-}2^b$) were subjected to the LR-PLT transfusion/BMT system described above. The results from three combined experiments demonstrated that 13/17 (77%) of the μMT recipients that were transfused with BALB/c LR-PLT products rejected the BALB.B BMT (Figure 5A). This did not reflect an intrinsic difference in the ability of μMT to engraft, as 9/9 (100%) of the μMT recipients transfused with C57BL/6J LR-PLT products engrafted the BALB.B BMT (Figure 5A). Likewise, the failure to engraft the MHC-matched BMT in the BALB/c LR-PLT transfused μMT and C57BL/6J recipients was not due to insufficient conditioning of the recipients, as 7/9 (78%) and 15/15 (100%) of the naïve μMT and C57BL/6J recipients, respectively, engrafted the BALB.B BMT (Figure 5A).

Anti-donor immunity was measured through an *in vivo* survival assay using BALB.B SPL targets and control C57BL/6J SPL targets as above. The

results from three combined independent experiments demonstrated that 13/13 (100%) of the rejecting BALB/c LR-PLT transfused μMT recipients cleared the BALB.B SPL targets *in vivo* (Figure 5B). The few BALB/c LR-PLT transfused μMT recipients that engrafted the BALB.B BMT had no detectable *in vivo* clearance of the BALB.B SPL targets (Figure 5B). Together, these findings demonstrate that B cells are not required for rejection of an MHC-matched BMT in LR-PLT transfused recipients to occur.

Discussion

Patients with non-malignant hematological diseases can undergo an HLA-matched BMT under reduced intensity conditions in an effort to promote normal hematopoiesis (1-6). However, under such conditions, roughly 15% of the transplanted patients reject the HLA-matched BMT (1, 10, 18). It has been appreciated that the likelihood of rejection increases with the number of transfusions received (11-15). Although this could be a simple effect that worse disease both requires more transfusion and results in poor marrow environment, we have previously reported that LR-PLT transfusion induces BMT rejection in a murine model (16). As patients with non-malignant hematological disorders typically require aggressive red blood cell (RBC) and/or PLT transfusion support to maintain hemostasis, it is likely that anti-donor immunity in these patients arises from the transfusion support. Using this system, the current report tests both the nature of the alloimmunization as a result of LR-PLT transfusion and also the immunological vectors involved in BMT rejection.

Neither antibodies nor *in vivo* clearance are consistently observed after LR-PLT transfusion, and yet the LR-PLT transfusion clearly results in subsequent BMT rejection. These data are consistent with two separate scenarios: 1) PLT transfusion can prime an immune response, but not generate full effector function until a subsequent exposure to antigen is encountered during BMT and 2) PLT transfusion induces a full effector response but the precursor frequency is so low that it is not detected by *in vivo* clearance assays but it expands upon subsequent BMT rejection. Depletion of $CD4^+$ T cells prevents BMT rejection if

performed post-transfusion/pre-BMT but has no effect upon retransplantation after mice have undergone one BMT rejection. These data thus support the model in which the nature of immunity is altered by the BMT itself and favor model 1 in which LR-PLT transfusion primes an immune response but does not induce full effector function.

Because depletion of $CD8^+$ cells prevents rejection regardless of the time of depletion, $CD8^+$ T cells are likely the main effector cells in this system. Nevertheless, $CD4^+$ cells are still required early on, most likely to give help to $CD8^+$ T cells. This is in some contrast to what has been observed in some viral infections, where $CD8^+$ T cells are capable of initial differentiation into full effectors without $CD4^+$ T cells. Such $CD8^+$ T cells can have altered memory recall (e.g. “helpless cells”); however, they nevertheless can differentiate into cytolytic effectors early on with some control for viral infection (19). It is unclear if the difference observed herein is due to a qualitative difference in transfused antigen (e.g. the relative lack of innate immune activation and no toll like receptor ligation) or is a quantitative difference due to fewer antigenic differences. Regardless, these findings suggest that the nature of transfusion induced alloimmunity to mHAs and its effect upon BMT may be a distinct landscape in which interventions that target helper $CD4^+$ T cells may be efficacious if employed pre-BMT even if exposure to mHAs on transfused cells has taken place. However, after BMT, directly shutting down effector CTLs would seem to be required in the current model.

It is worth noting that CD4 and CD8 are expressed on other cell types in addition to T cells (i.e. dendritic cells for CD8 and macrophages for CD4). Thus, one must always consider potential alternate interpretations of depletion studies. However, because the CD8 depleting antibody used in this study recognizes the CD8β chain and $CD8^+$ dendritic cells exclusively express the CD8α homodimer, it is unlikely that engraftment was due to the removal of $CD8\alpha^+$ dendritic cells.

In the current system, we reject the hypothesis that B cells or humoral immunity are required vectors of rejection. The μMT recipients are genetically modified knockouts that do not generate B cells as a result of a targeted gene mutation in the immunoglobulin μ chain gene (17), and yet they undergo LR-PLT transfusion induced BMT rejection. Likewise, no antibodies to mHAs are detected in LR-PLT transfused wild-type recipients that undergo rejection. This does not rule out the potential for antibodies to participate in transfusion induced BMT rejection in other settings; indeed, there are a number of mHAs on PLTs that constitute antibody epitopes (HPA 1-15) (20), which if expressed during hematopoiesis, could be a target for rejection. However, the current studies do demonstrate that antibodies are not required for transfusion induced BMT rejection, and at least in this model, are not likely to be involved.

Because the current findings are in a murine system, testing the hypothesis in a human setting would be required prior to drawing clinical conclusions. Nevertheless, the current findings suggest that while LR-PLT transfusion primes BMT rejection, the BMT itself is a required immunizing event. Thus, despite multiple transfusions, specific targeting of CD4 and/or CD8 T cells

prior to BMT may be of use in this population, whereas after the BMT has been given, targeting of $CD8^+$ T cells may be more important.

Currently, the reduced intensity conditioning regimens patients with non-malignant hematological disorders receive for an HLA-matched BMT have demonstrated to be inadequate in preventing rejection in all transplant recipients. The relevancy of these current findings to human medicine is the clinical implication of an alternate conditioning regimen that directly targets the $CD8^+$ T cell response induced in response to LR-PLT transfusions; in particular, the development of a novel $CD8^+$ T cell specific regimen and/or the sole administration of a high dose of anti-thymocyte globulin (ATG). Indeed, it has been observed clinically that the rate of HLA-matched BMT engraftment improves under a high dose of ATG in patients with non-malignant BM failure syndromes (2, 7, 9).

In aggregate, the studies in this report indicate that immunization to mHAs by LR-PLT transfusions has specific properties compared to other alloimmunization scenarios. In particular, LR-PLT transfusions appear to prime full alloimmunity upon exposure to BMT, but do not in of themselves progress all the way to effector function. In addition to providing a rational basis to modify therapeutic interventions and conditioning regimens, these findings provide insight into the nature of transfusion as an immune stimulus in the cellular compartment.

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Figure Legends

Figure 1: *Depletion of $CD4^+$ or $CD8^+$ T cells prevents rejection of a BALB.B BMT in BALB LR-PLT transfused recipients.* **(A)** Experimental model testing requirement of $CD4^+$ and $CD8^+$ depletion in PLT transfusion induced BMT rejection. While BALB/c donors were MHC- and mHA-mismatched, BALB.B BM donors were MHC-matched:mHA-mismatched to the recipients. Designated recipients received two PLT transfusions a week apart. After the second transfusion, indicated recipients were treated intraperitoneal (i.p.) with anti-CD4 (clone: GK1.5) or anti-CD8β (clone: H35) depleting antibodies, or isotype control antibodies Rat $IgG2_b$ or Rat IgG, respectively. Depletion of $CD4^+$ or $CD8\beta^+$ T cells was monitored in the peripheral blood, spleen, peripheral lymph nodes, and BM. Twenty-four hours after the second treatment, designated recipients received a BALB.B BMT under reduced intensity conditions. Seroanalysis and *in vivo* survival of BALB.B targets was also performed after BMT (see figure 2). **(B)** Depletion analysis of $CD4^+$ and $CD8^+$ T cells in BMT recipients. Peripheral blood leukocytes were harvested from BMT recipients and stained for $CD4^+$ or $CD8^+$ T cells using anti-CD4 (clone: RM4-5) and anti-CD8α (clone: 53-6.7) antibodies. **(C)** BALB.B BMT engraftment results. Percent $CD229.1^+$ cells in the peripheral blood represent engraftment; the mean of each group is represented as a horizontal line. Statistics were generated using column statistics and a one-way ANOVA with Dunnett's post-test. Illustrated is the combined data from three independent experiments.

Figure 2: *BALB specific Alloimmunization after depletion and BMT.* **(A)** BALB specific alloantibody production. anti-BALB antibodies in sera of transfused and transplanted recipients were assessed using BALB.B SPL (white) and PLT (grey) targets in an indirect immunofluorescence staining. **(B)** *In vivo* survival of BALB.B targets. Immunity against BALB expressing targets was assessed by *in vivo* survival of BALB.B SPL targets labeled with CFDA. Error bars in (A) represent the mean ± SEM. The mean of each group in (B) is represented as a horizontal line. Statistics were generated using column statistics and a one-way ANOVA with Dunnett's post-test. The data shown for both panels A and B is the combined data from three separate experiments.

Figure 3: *Rejection of a BALB.B BMT is mediated by a $CD8^+$ cellular response that requires the presence of $CD4^+$ cells prior to BMT.* **(A)** Experimental model testing the requirement of $CD4^+$ and $CD8^+$ T cells as the rejection vectors. Using the depletion model described in Figure 1A, BALB/c PLT donors were MHC- and mHA-mismatched, whereas (BALB.B x C57BL/6J) and (BALB.B x B6 Thy1.1) F_1 BM donors were MHC-matched but mHA-haplomismatched. Recipients were transfused twice, a week apart. One week after the second transfusion, recipients received a (BALB.B x C57BL/6J) F_1 BMT. Six weeks later, indicated recipients were treated i.p. with anti-CD4 or anti-CD8β depleting antibodies, or isotype control antibodies Rat $IgG2_b$ or Rat IgG, respectively. Depletion of $CD4^+$ or $CD8^+$ T cells was monitored in the peripheral blood. Recipients were then given a (BALB.B x B6 Thy1.1) F_1 BMT. Seroanalysis and *in vivo* survival of

BALB.B targets was performed after BMT (see figure 4). **(B)** (BALB.B x C57BL/6J) F_1 BMT engraftment results. Engraftment was assessed in the peripheral blood of transplant recipients. BMT engraftment was measured as a percentage of CD229.1$^+$ T cells two standard deviations above the rejecting BALB/c whole blood transfused recipients. All four BALB/c LR-PLT transfused recipients in (B) were treated the same. The BALB/c LR-PLT transfused recipients were split into the four treatment groups to compare engraftment during the first and second transplantation in panels (B) and (D), respectively. The symbol for each group in panel (B) corresponds with that in panel (D). **(C)** Analysis of CD4$^+$ and CD8$^+$ T cell depletion in re-transplant recipients. Peripheral blood leukocytes were harvested from the peripheral blood of recipients receiving the second BMT and were stained for CD4$^+$ or CD8$^+$ T cells using anti-CD4 (clone: RM4-5) and anti-CD8α (clone: 53-6.7) antibodies. **(D)** Engraftment results for (BALB.B x B6 Thy1.1) F_1 BMT recipients. Engraftment was defined as having a percentage of Thy1.1$^+$ T cells two standard deviations above the mean of the rejecting recipients treated with the corresponding isotype control antibody. The mean of each group in (B) and (D) is represented as a horizontal line. Statistics were generated using column statistics and a one-way ANOVA with Dunnett's post-test. Illustrated is the combined data from three independent experiments.

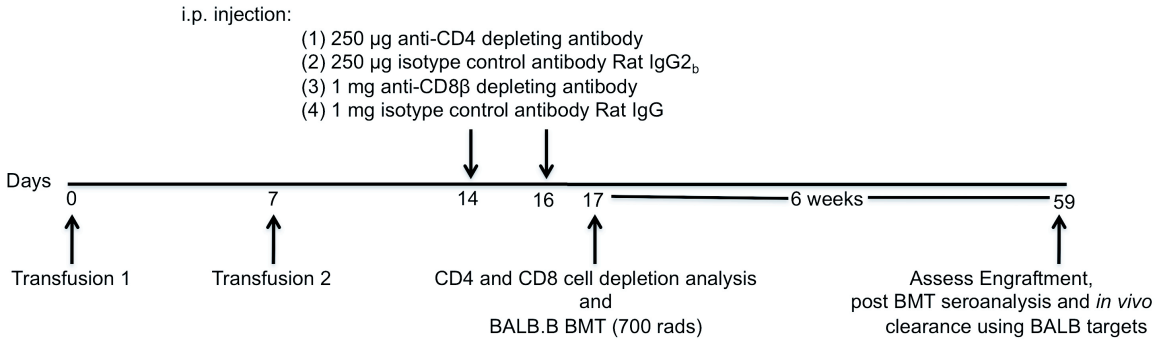
Figure 4: *Alloimmunization against BALB mHA(s) after depletion and re-transplantation.* **(A)** BALB specific alloantibody production. anti-BALB antibodies

in the sera of transfused and transplanted recipients were assessed using BALB.B SPL (white) and PLT (grey) targets in an indirect immunofluorescence staining. **(B)** *In vivo* survival of BALB.B targets. Immunity against BALB expressing targets was assessed by *in vivo* survival of BALB.B SPL targets labeled with CFDA. Error bars in (A) represent the mean ± SEM. The mean of each group in (B) is represented as a horizontal line. Statistics were generated using column statistics and a one-way ANOVA with Dunnett's post-test. Illustrated are data from three combined independent experiments.

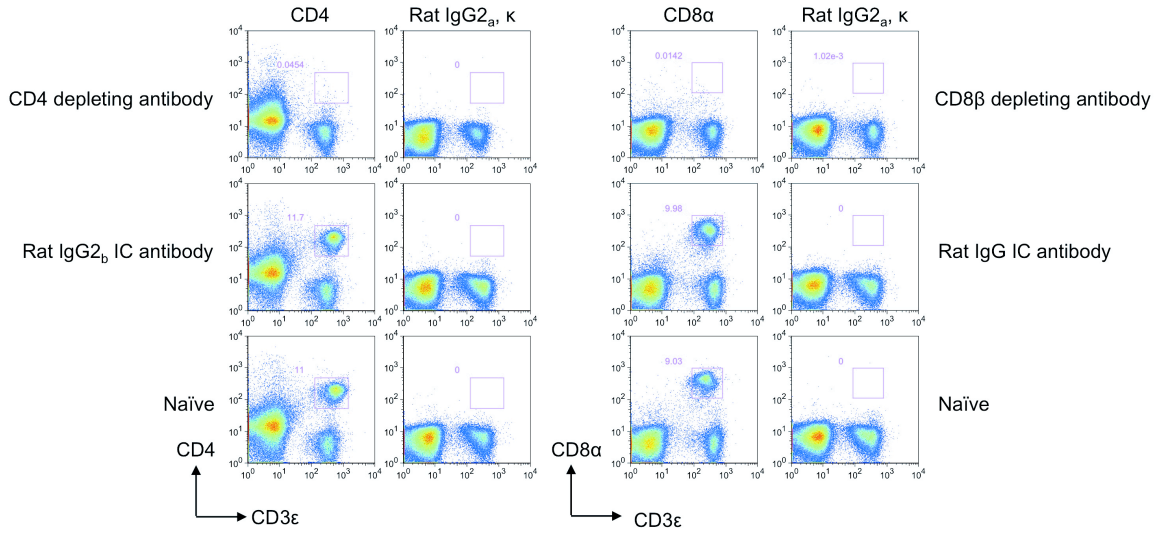
Figure 5: *B cells are not required for BALB LR-PLT transfusions to induce rejection of a BALB.B BMT.* **(A)** Engraftment results. Engraftment is measured as a percentage of $CD229.1^+$ cells in the peripheral blood of transplant recipients. **(B)** Alloimmunization to BALB mHAs. BALB specific immunity was measured by *in vivo* survival of BALB.B SPL targets. The mean of each group in (A) and (B) is represented as a horizontal line. Statistics were generated using one-way ANOVA with Dunnett's post-test. The illustrated results in (A) and (B) for BALB/c LR-PLT transfused μMT and C57BL/6J recipients are the combined data from three independent experiments. The data depicted for μMT and C57BL/6J recipients transfused with C57BL/6J LR-PLT concentrates in (A) and (B) are the combined results from two independent experiments.

Figure 1

A.


i.p. injection:
(1) 250 µg anti-CD4 depleting antibody
(2) 250 µg isotype control antibody Rat IgG2b
(3) 1 mg anti-CD8β depleting antibody
(4) 1 mg isotype control antibody Rat IgG
Days
0
7
14
16
17
6 weeks
59
Transfusion 1
Transfusion 2
CD4 and CD8 cell depletion analysis
and
BALB.B BMT (700 rads)
Assess Engraftment,
post BMT seroanalysis and in vivo
clearance using BALB targets

B.


CD4
Rat IgG2a, κ
CD8α
Rat IgG2a, κ
CD4 depleting antibody
CD8β depleting antibody
Rat IgG2b IC antibody
Rat IgG IC antibody
Naïve
Naïve
CD4
CD3ε
CD8α
CD3ε

C.

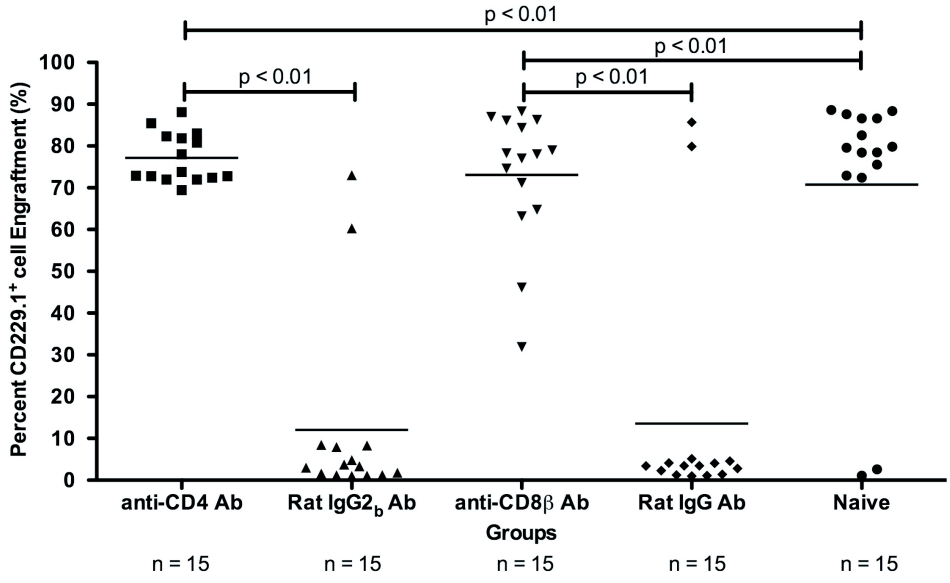
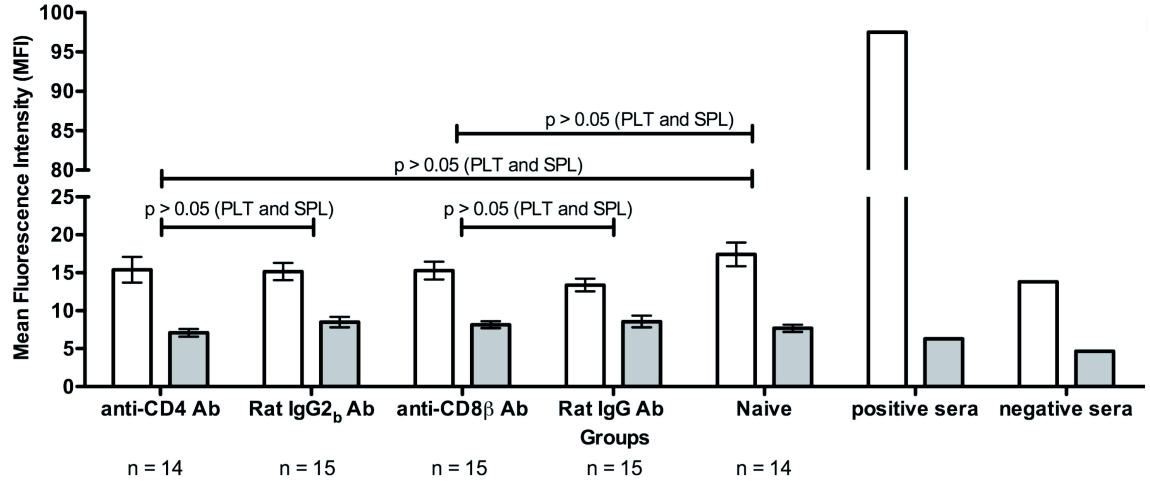

p < 0.01
p < 0.01
p < 0.01
p < 0.01
Percent CD229.1+ cell Engraftment (%)
100
90
80
70
60
50
40
30
20
10
0
anti-CD4 Ab
Rat IgG2b Ab
anti-CD8β Ab
Rat IgG Ab
Naive
Groups
n = 15
n = 15
n = 15
n = 15
n = 15

Figure 2

A.


Mean Fluorescence Intensity (MFI)
100
95
90
85
80
25
20
15
10
5
0
p > 0.05 (PLT and SPL)
p > 0.05 (PLT and SPL)
p > 0.05 (PLT and SPL)
p > 0.05 (PLT and SPL)
anti-CD4 Ab
Rat IgG2b Ab
anti-CD8β Ab
Rat IgG Ab
Naive
positive sera
negative sera
Groups
n = 14
n = 15
n = 15
n = 15
n = 14

B.

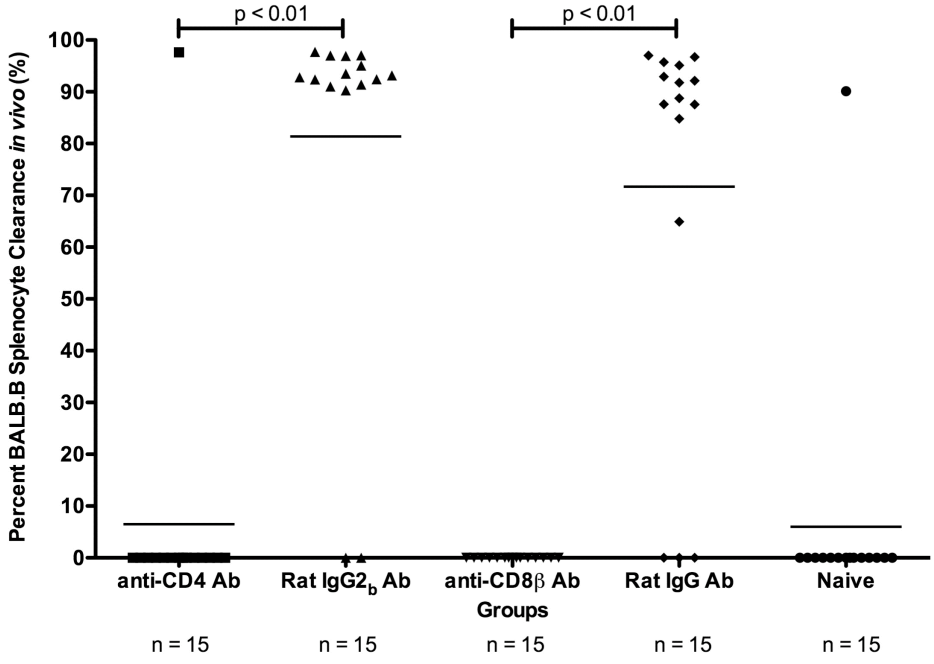

Percent BALB.B Splenocyte Clearance in vivo (%)
100
90
80
70
60
50
40
30
20
10
0
p < 0.01
p < 0.01
anti-CD4 Ab
Rat IgG2b Ab
anti-CD8β Ab
Rat IgG Ab
Naive
Groups
n = 15
n = 15
n = 15
n = 15
n = 15

Figure 3

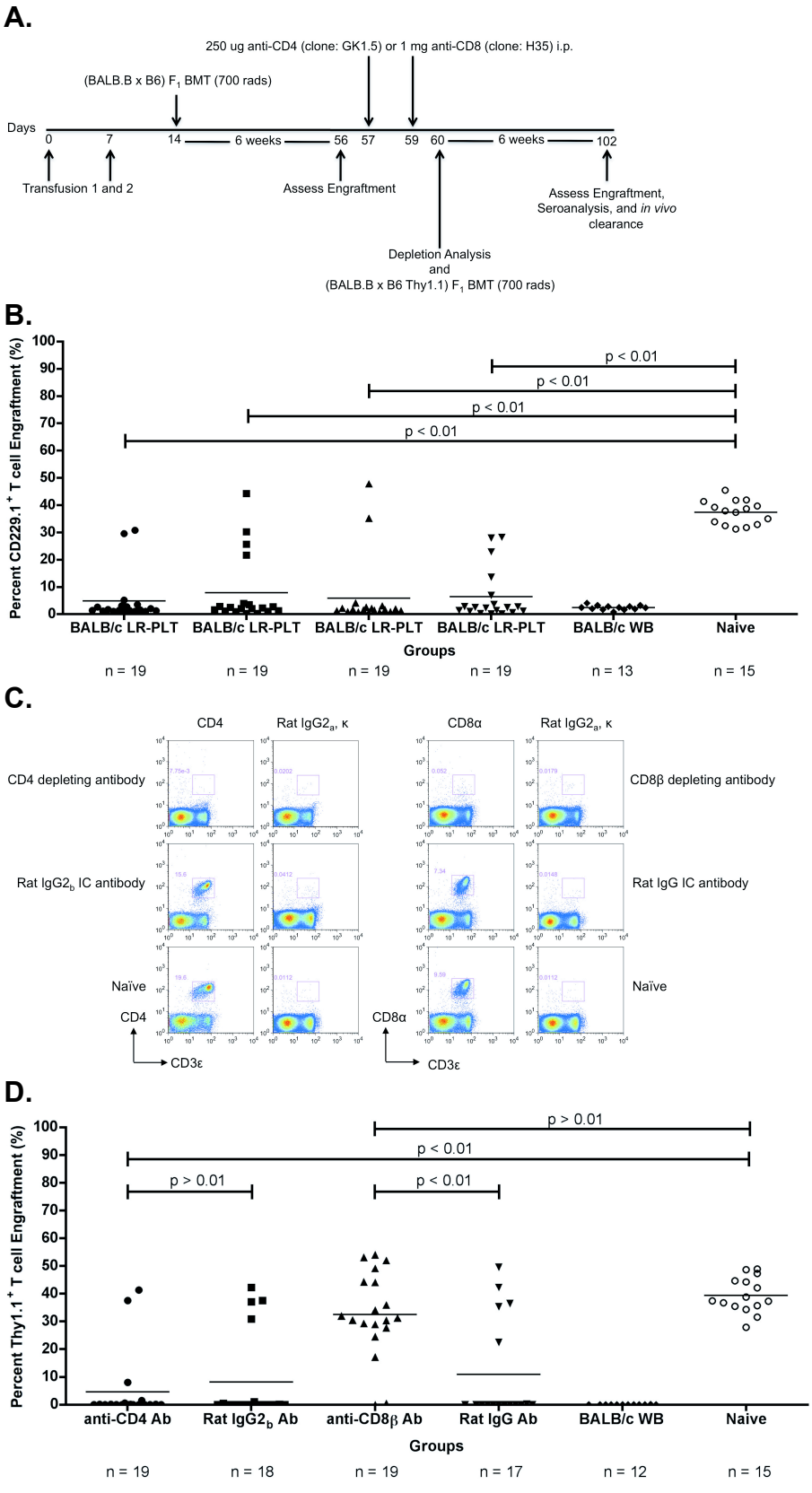

A.
250 ug anti-CD4 (clone: GK1.5) or 1 mg anti-CD8 (clone: H35) i.p.
(BALB.B x B6) F1 BMT (700 rads)
Days
0
7
14
6 weeks
56
57
59
60
6 weeks
102
Transfusion 1 and 2
Assess Engraftment
Assess Engraftment, Seroanalysis, and in vivo clearance
Depletion Analysis and (BALB.B x B6 Thy1.1) F1 BMT (700 rads)
B.
Percent CD229.1+ T cell Engraftment (%)
p < 0.01
p < 0.01
p < 0.01
p < 0.01
BALB/c LR-PLT
BALB/c LR-PLT
BALB/c LR-PLT
BALB/c LR-PLT
BALB/c WB
Naive
Groups
n = 19
n = 19
n = 19
n = 19
n = 13
n = 15
C.
CD4
Rat IgG2a, κ
CD8α
Rat IgG2a, κ
CD4 depleting antibody
CD8β depleting antibody
Rat IgG2b IC antibody
Rat IgG IC antibody
Naïve
Naïve
CD4
CD3ε
CD8α
CD3ε
D.
Percent Thy1.1+ T cell Engraftment (%)
p > 0.01
p < 0.01
p > 0.01
p < 0.01
anti-CD4 Ab
Rat IgG2b Ab
anti-CD8β Ab
Rat IgG Ab
BALB/c WB
Naive
Groups
n = 19
n = 18
n = 19
n = 17
n = 12
n = 15

Figure 4

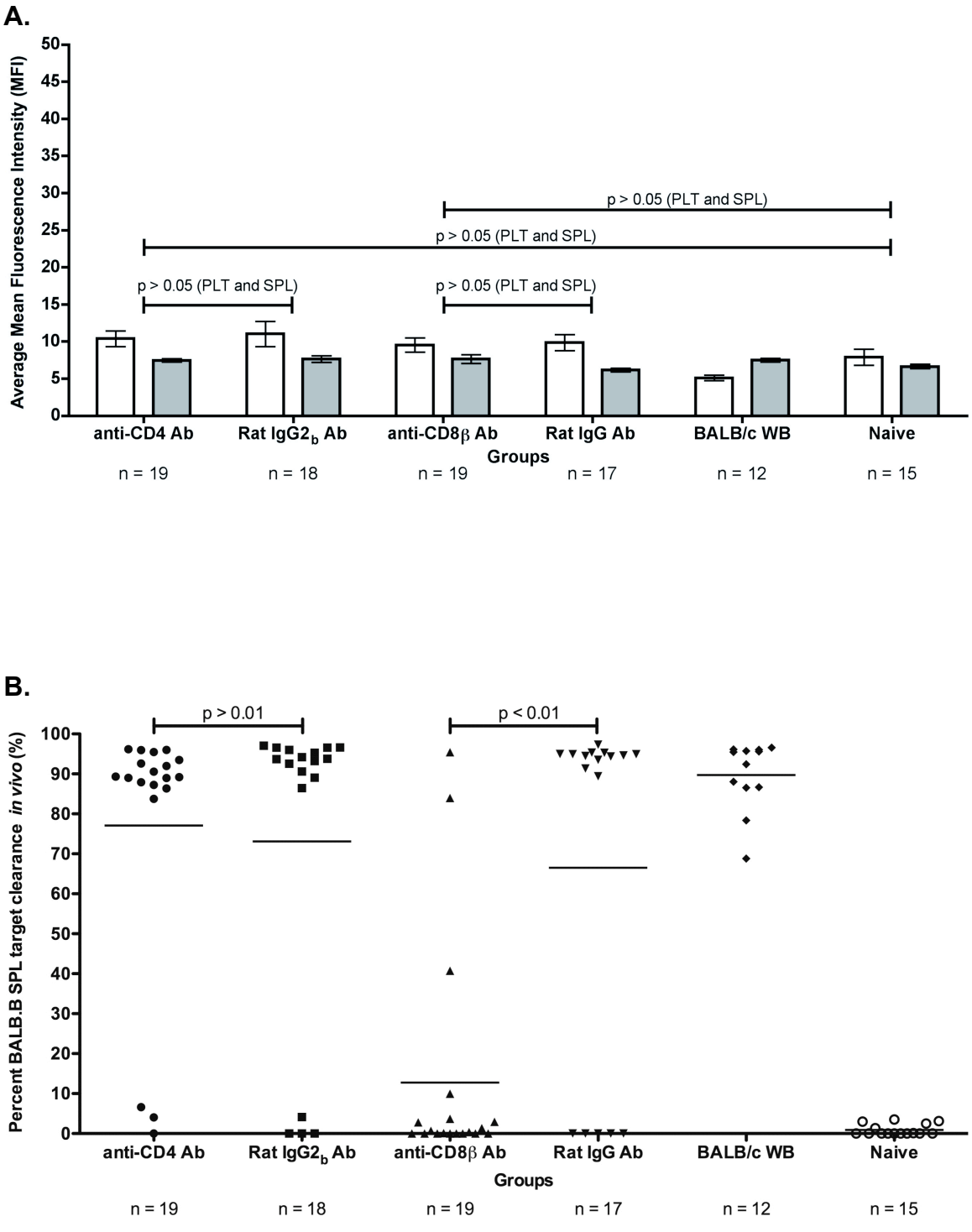
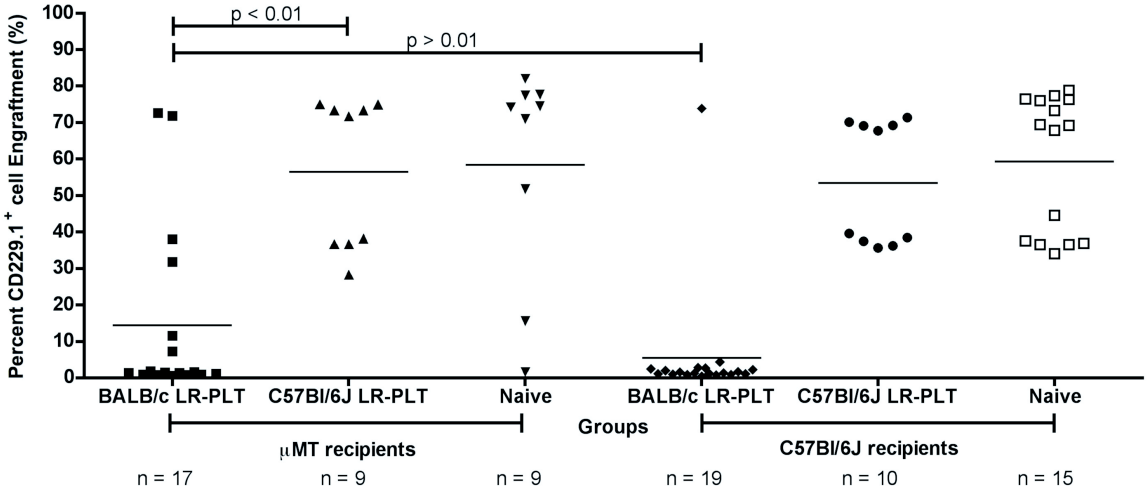
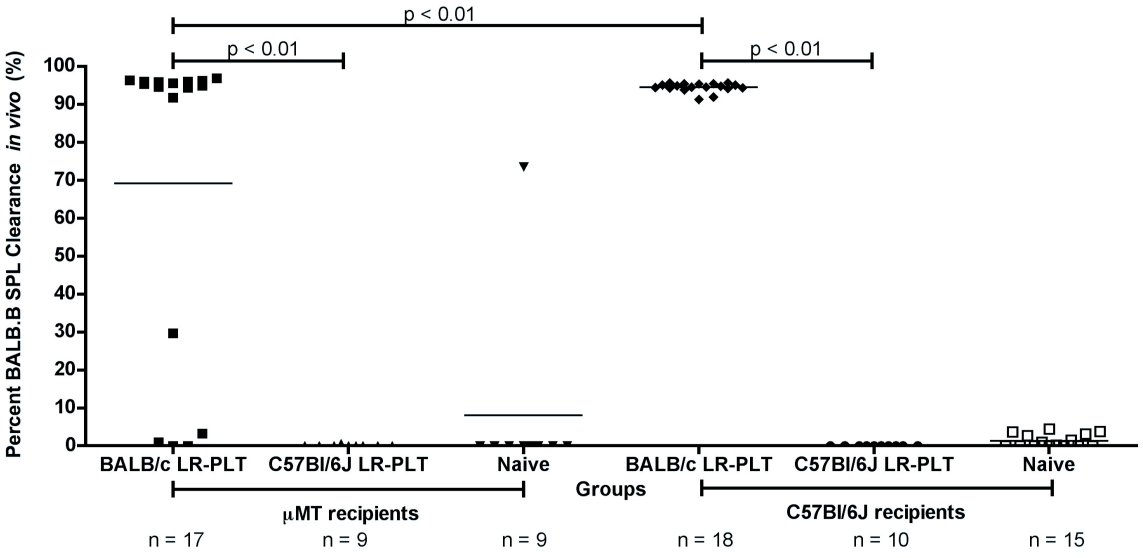

A.
Average Mean Fluorescence Intensity (MFI)
50
45
40
35
30
25
20
15
10
5
0
p > 0.05 (PLT and SPL)
p > 0.05 (PLT and SPL)
p > 0.05 (PLT and SPL)
p > 0.05 (PLT and SPL)
anti-CD4 Ab
Rat IgG2b Ab
anti-CD8β Ab
Rat IgG Ab
BALB/c WB
Naive
Groups
n = 19
n = 18
n = 19
n = 17
n = 12
n = 15
B.
Percent BALB.B SPL target clearance in vivo (%)
100
90
80
70
60
50
40
30
20
10
0
p > 0.01
p < 0.01
anti-CD4 Ab
Rat IgG2b Ab
anti-CD8β Ab
Rat IgG Ab
BALB/c WB
Naive
Groups
n = 19
n = 18
n = 19
n = 17
n = 12
n = 15

Figure 5

A.


p < 0.01
p > 0.01
Percent CD229.1+ cell Engraftment (%)
100
90
80
70
60
50
40
30
20
10
0
BALB/c LR-PLT
C57Bl/6J LR-PLT
Naive
BALB/c LR-PLT
C57Bl/6J LR-PLT
Naive
Groups
μMT recipients
C57Bl/6J recipients
n = 17
n = 9
n = 9
n = 19
n = 10
n = 15

B.


p < 0.01
p < 0.01
p < 0.01
Percent BALB.B SPL Clearance in vivo (%)
100
90
80
70
60
50
40
30
20
10
0
BALB/c LR-PLT
C57Bl/6J LR-PLT
Naive
BALB/c LR-PLT
C57Bl/6J LR-PLT
Naive
Groups
μMT recipients
C57Bl/6J recipients
n = 17
n = 9
n = 9
n = 18
n = 10
n = 15

Chapter 4

Alloimmunization against Red Blood Cell or Platelet Antigens is Independent of TRIM21 Expression in a Murine Model

Abstract

Generation of alloantibodies to transfused RBCs can be a serious medical problem for patients who require chronic RBC transfusion therapy. Patients with sickle cell disease have a substantially increased rate of alloimmunization compared to other chronically transfused populations. A recent study has forwarded the hypothesis that a polymorphism in an immunoregulatory gene in close proximity to beta-globin (TRIM21 rs660) plays a role in the increased rates of RBC alloimmunization in sickle cell patients. In particular, it was hypothesized that rs660C/T decreases expression of TRIM21, resulting in loss of a negative feedback pathway in immune responses and increased RBC alloimmunization. To test the effects of TRIM21 expression on alloimmunization, we analyzed antibody responses to alloantigens on RBCs and platelets transfused into wild-type and TRIM21 KO mice. No significant increases were seen in the frequency or magnitude of humoral immunization to alloantigens on transfused RBCs or platelets in adult or juvenile TRIM 21 KO recipients compared to wild-type controls. Moreover, recipient inflammation with poly (I:C) enhanced RBC alloimmunization to similar degrees in both TRIM21 KO and wild-type control recipients. Together, these data rule out the hypothesis that decreased TRIM21 expression enhances transfusion induced humoral alloimmunization, in the context of a reductionist murine model.

Introduction

Although RBC transfusion can be lifesaving, it is not without risk. One such risk includes the development of antibodies against foreign antigens on transfused donor RBCs (RBC alloimmunization). When a recipient mounts an antibody response against a given RBC antigen, subsequent transfusion with RBCs expressing the same antigen is typically contraindicated due to the risk of hemolysis of the transfused RBCs. Besides leading to costly and time consuming evaluations in the blood bank, RBC alloantibodies also put a patient at risk of delayed hemolytic transfusion reactions. Furthermore, RBC alloimmunization may also lead to hemolytic disease of the fetus and newborn in women of childbearing age. Thus, RBC alloimmunization is a significant hazard of transfusion.

In the general population of transfusion recipients, rates of RBC alloimmunization approximate 3%. However, certain patient populations have substantially higher rates; most notably, those with sickle cell disease (SCD). Overall, the RBC alloimmunization rate in SCD patients is approximately 25% (1), with a range of reported rates between 8 and 47% (1-7). This high rate of alloimmunization can lead to substantial difficulty and cost in identifying sufficient units of RBCs compatible for the needs of SCD patients, and in some cases, can lead to significant morbidity and/or mortality due to difficulty or inability to locate compatible units of RBCs.

The underlying mechanisms responsible for increased RBC alloimmunization in SCD patients remain unclear. It has been argued that

increased rates of transfusion are responsible, as many SCD patients receive chronic transfusion for years. Furthermore, it has been argued that SCD patients encounter more foreign antigens than other groups, due to donor/recipient disparities in ethnic origin. The majority of blood donors in the United States are Caucasian, whereas the majority of SCD patents are of African descent; there are significant differences in antigen expression between Caucasians and African Americans (especially with regards to C, E, and K antigens). Alternatively, is has been argued that the sickle cell pathophysiology itself is responsible, due to increased inflammation and activation of innate immunity, which promotes adaptive responses upon encountering foreign antigens. To add to these existing hypotheses, Tatari-Calderone *et al* have recently advanced a novel hypothesis that the immune systems of SCD patients are indeed more prone to RBC alloimmunization, due not to the biological changes as a result of SCD, but rather due to inheritance of an immunoregulatory gene that co-segregates with the sickle globin gene (6).

TRIM21 (Ro52;SSA1) is a known immunoregulatory gene that is in very close proximity to β-globin, and it has recently been reported that the rs660 polymorphism in TRIM21 correlates with the age at which SCD patients become alloimmunized following RBC transfusion (6). A major role of TRIM21 appears to be as a negative feedback to immune activation by ubiquitinating and promoting the destruction of interferon regulatory factors (8). The rs660 polymorphism is the result of a single base alteration in the sequence ". . . AGAGAT{C/T}TTTG . . . " Because the rs660 polymorphism is not within the

coding region of the TRIM21 gene, it has been proposed to play a role in regulating levels of TRIM21 expression (6, 9). Thus, the overall hypothesis advanced by Tatari-Calderone *et al* is that rs660C/T leads to lower levels of TRIM21 expression than rs660T/T, resulting in less negative feedback to immune activation, and higher rates of alloimmunization to RBC transfusion.

To test the hypothesis that levels of TRIM21 expression influence RBC alloimmunization, we have utilized a murine model with a well defined RBC antigenic difference between donor and recipient. Herein we report that there is no difference in frequency or magnitude of RBC alloimmunization between TRIM21 KO (10) and wild-type recipients. We further report no difference in alloimmunization to a foreign MHC on transfused leukoreduced platelets (LR-PLT) between TRIM21 KO and wild-type mice. In aggregate, these studies demonstrate that TRIM21 expression is not an independent variable in the frequency or magnitude of alloimmunization to transfused RBCs or platelets in a mouse model.

Materials and Methods

Mice

Female C57BL6/J (B6) and BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). TRIM21 knock out (TRIM21 KO; H-2^b) mice on a B6 background and HOD RBC donor mice were generated as previously described (10, 11) on a FVB background. TRIM21 KO and B6 recipients were used at 10-16 weeks (adults) or 3 weeks of age (juveniles). TRIM21 KO, HOD, and FVB mice were bred at the Emory Division of Animal Resources Animal Husbandry Service. All mice were housed in Emory University Department of Animal Resources facilities and procedures were performed according to approved IACUC protocols.

Treatment of mice to induce inflammation

A subset of mice were injected i.p. with 100 micrograms of poly (I:C) (Amersham Biosciences, Sweden) dissolved in Dulbecco's Phosphate Buffered Saline (DPBS), 4 hours prior to transfusion. Control mice received the same volume of DPBS.

Isolation and transfusion of donor RBC and platelet units

Whole blood from transgenic HOD donors was collected in a 1:8 ratio of RBC to ACD as previously described (11). The blood was washed three times with DPBS and centrifuged at 1,350 rpm (394 x g) for 10 minutes. The packed RBCs were re-suspended in 1x DPBS at a 1:5 dilution (100 µl packed RBCs in a total

volume of 500 µl DPBS and 60 µl of packed RBCs in a total volume of 300 µl DPBS were given to adults and juveniles, respectively). In some experiments, whole blood was leukocyte reduced (LR) using a RBC specific Pall Neonatal Leukoreduction filter prior to washing. Leukocyte reduced platelet units were isolated as previously described (12); briefly, platelet rich plasma was isolated by centrifugation and was passed over a Neonatal Purecell PL High Efficiency Leukocyte Reduction Filter (Pall corp.) The leukoreduced platelets were centrifuged and resuspended at a concentration of $2x10^8$ platelets/ml, followed by transfusion of 10^8 platelets per recipient. All transfusions were given by tail vein.

Analysis of humoral alloimmunization.

Antibody responses to transfused HOD RBCs or BALB/c LR-PLT were evaluated by indirect immunofluorescence using flow cytometry (FACS). Serum was diluted as indicated in FACS buffer and incubated with HOD RBCs or BALB/c splenocyte targets. Control targets consisting of FVB RBCs or, B6 splenocytes (SPLs) were used for HOD or BALB/c targets, respectively. All incubations were for 30 minutes at 4°C. Samples were then washed three times with 100 µl FACS buffer, and were incubated for 30 minutes in a 1:100 dilution of allophycocyanin (APC) conjugated goat anti-mouse Igs in FACS buffer. B cells in the BALB/c or B6 SPL targets were excluded by staining with FITC anti-mouse CD19 (BD Pharmingen, 1:50 in FACS buffer). All samples were run on a FACS Caliber and analyzed on FlowJo 8.8.2. Mean fluorescence intensity (MFI) was used to

indicate the presence of antibody bound to cell targets, with adjusted MFI being defined as the mean fluorescence intensity of serum cross matched with control RBCs or splenocytes subtracted from the signal of serum crossmatched with target RBCs or splenocytes.

Statistical Analysis

Adjusted mean fluorescence intensity was compared between groups using a two way ANOVA with a Bonferroni post-test (Graphpad Prism), with a statistically significant value being $p<0.05$.

Results

Rate and magnitude of RBC alloimmunization is independent of TRIM21 expression

To test the hypothesis that TRIM21 expression is an independent variable in alloimmunization to an RBC antigen, HOD RBCs were transfused into either TRIM21 KO mice or wild-type recipients (HOD RBCs express hen egg lysozyme, a portion of ovalbumin, and human Duffy^{b}). Anti-HOD antibodies were assayed two weeks following transfusion by indirect immunofluorescence using HOD RBCs as targets and antigen negative wild-type FVB RBCs to control for background staining. Responders to HOD RBCs were defined as having an MFI of two standard deviations above the mean of sera cross-matched to control FVB RBCs. Combined results of three experiments demonstrated alloimmunization to HOD in 13/14 TRIM21 KO mice (93%) and 15/15 B6 recipients (100%), (Figure 1A). Although there were several outliers, overall there was no increase in the magnitude of anti-HOD alloantibody responses in recipients lacking TRIM21 compared to wild type controls; in contrast, TRIM21 KO recipients made lower levels of anti-HOD ($p=0.02$).

To test the effects of TRIM21 on RBC alloimmunization in juvenile mice, 3 week old TRIM21 KO or B6 recipients were transfused with a weight adjusted volume of HOD RBCs. Combined data from three experiments demonstrated low levels of detectable anti-HOD antibody in 8/15 TRIM21 KO mice (53%) and 10/15 B6 mice (67%). There was no significant difference in the magnitude of anti-HOD response between the two groups ($p=0.6$) (Figure 1B). Overall, the

frequency and magnitude of RBC alloimmunization in juvenile mice were substantially lower than similar data from adult-immunized mice, consistent with the blunted response of the young and/or developing immune system. Together, these data demonstrate that within the juvenile populations, the absence of TRIM21 does not alter the frequency or magnitude of alloimmunization against the HOD antigen on transfused RBCs.

Inflammation with poly (I:C) does not alter relative rates or magnitude of alloimmunization to RBCs in TRIM21 KO vs. wild-type mice

We hypothesized that transfusion in the context of ongoing inflammation involving interferon regulatory pathways would be a sensitive approach to measure biological changes in alloimmunization due to decreased TRIM21. We tested this hypothesis utilizing poly (I:C), a double stranded RNA that has previously been shown to enhance RBC alloimmunization (13, 14).and that induces the same IRF family members down regulated by TRIM21 (8). Adult TRIM21 KO mice or wild-type B6 mice were treated with poly (I:C) 4 hours prior to transfusion with HOD RBCs. Data from three combined experiments demonstrated that 13/15 TRIM21 KO (87%) and 10/10 B6 (100%) recipients had detectable levels of anti-HOD alloantibodies, with no difference in magnitude of alloimmunization between the groups (Figure 2, p=0.1).

Alloimmunization against transfused PLTs is not altered in TRIM21 KO mice

Similar to RBC transfusions, transfusions of PLTs can result in humoral alloimmunization, most commonly against MHC I alloantigens. To test the role of

TRIM21 in this process, TRIM21 KO or wild-type B6 mice were transfused with leukoreduced PLT products (LR-PLT) from BALB/c donor mice. Because BALB/c mice have an H-2^d MHC haplotype and B6 mice have a H-2^b MHC haplotype, anti-$H2^d$ is the dominant antibody response in this setting. Mice received three transfusions, each a week apart, and the presence of anti-BALB/c alloantibodies in recipient sera was assessed by indirect immunofluorescence using BALB/c splenocytes as targets and B6 splenocytes as negative control cells to establish background staining. Responders were identified as having a MFI of two standard deviations above the mean of sera cross-matched to B6 spleen cell targets.

Data from three combined experiments demonstrated a statistically significant response rate of 7/15 (47%) and 5/15 (33%) in TRIM21 KO and B6 recipients, respectively. In addition, each group had 4 strong responders (Figure 3). This BALB/c into B6 strain combination is known to result in moderate responses to platelet antigens, as observed here, which is optimal for detecting increases or decreases. Thus, analogous to adult and juvenile recipients of HOD RBC transfusions, no statistically significant differences were observed in the frequency and magnitude of PLT alloimmunization between TRIM21 KO and B6 recipients.

Discussion

The described studies were undertaken in response to the novel hypothesis proposed by Tatari-Calderone *et al* suggesting genetic polymorphisms in the TRIM21 gene of transfused patients with SCD influenced the generation of RBC alloantibodies, particularly in patients under the age of 5 years at the time of transfusion. Our data comparing TRIM21 KO and wild type mice do not support this hypothesis. Specifically, there was no observable difference in kinetics or magnitude of humoral alloimmunization to transfused RBCs in juvenile or adult recipients with a targeted deletion of the TRIM21 gene (10). Furthermore, there was no difference observed in the enhancement of alloimmunization in response to inflammation with poly (I:C), an agent known to activate IRF family members, upon which TRIM21 exerts its immunoregulatory effects. Finally, we did not detect a difference using different antigenic stimuli (RBCs as well as platelets). Together these data reject the hypothesis that decreased TRIM 21 expression alone increases RBC or platelet alloimmunization in the described model systems.

The initial basis for Tatari-Calderone *et al* hypothesizing an effect of TRIM21 upon RBC alloimmunization was the physical proximity of the TRIM21 immunoregulatory gene to the β-globin gene. Their data, suggesting an impact of a TRIM21 C/T gene polymorphism on RBC alloimmunization only in children under 5 years of age, were interpreted as suggesting a potential role for TRIM21 in affecting the rate at which the immune system matures. This polymorphism was suggested to decrease TRIM21 expression, thus resulting in less negative

feedback on IRF pathways and enhanced immune maturation. If TRIM21 plays such a role in mice, we did not detect it in 3 week old recipients, which were demonstrably still in the midst of immune system development, due to decreased immune responses compared to adult mice.

The importance of our studies is that they allow a reductionist isolation of a single variable that might influence alloimmunization following RBC or platelet transfusions. The data lend support to rejection of the human data as representing a causal effect and suggest a correlation. However, there are also limitations to the current approach and numerous caveats that must be considered in the interpretation. First, one must always consider the degree to which murine biology reflects human biology. Second, one must consider that the TRIM21 KO mice utilized in these studies did not have sickle cell disease, nor were they multiply transfused. Third, it must be considered that unlike the reduced levels of TRIM21 seen in humans, these murine recipients were completely deficient in TRIM21. Finally, although it is known that the TRIM21 KO strain utilized in these studies has immune dysfunction and dysregulated cytokine expression, it cannot be assumed that similar results would necessarily be seen in a different strain of TRIM21 KO mice (developed in parallel by Espinosa et al) (15).

Conclusions

In summary, the data presented herein isolate TRIM21 expression as an independent variable and test the impact of its absence on alloimmunization to RBCs and PLTs in juvenile and adult recipients. Although we cannot rule out complications due to compensatory pathways in the TRIM21 KO mice, the presence of dysregulated cytokines indicates that the knockout mice have an altered phenotype (10) and that TRIM21 has a non-redundant role in immune regulation. In this context, we conclude that decreased expression of TRIM21 is not alone sufficient to regulate alloimmunization to RBCs or platelets in otherwise healthy animals or in animals exposed to an inflammatory stimulus prior to transfusion.

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Figure Legends

Figure 1: *Adult and juvenile TRIM21 KO mice do not have increased antibody responses to alloantigen on transfused RBCs.* Wild-type (B6) mice or TRIM21 KO mice were transfused with donor RBCs expressing the HOD antigen. 2 weeks post-transfusion, serum was collected and anti-HOD responses were monitored using indirect immunofluorescence (1:5 dilution). Antibody binding to HOD RBC targets was measured by flow cytometry and background was subtracted using wild-type FVB control RBCs (adjusted MFI). No increased immunization was seen in either adult (1A) or juvenile (1B) TRIM21 KO mice compared to wild-type B6 controls. In contrast, a trend towards decreased immunization was seen in adult TRIM21 KO mice. The data are the combined results of three experiments (n = 14-15 mice total/group).

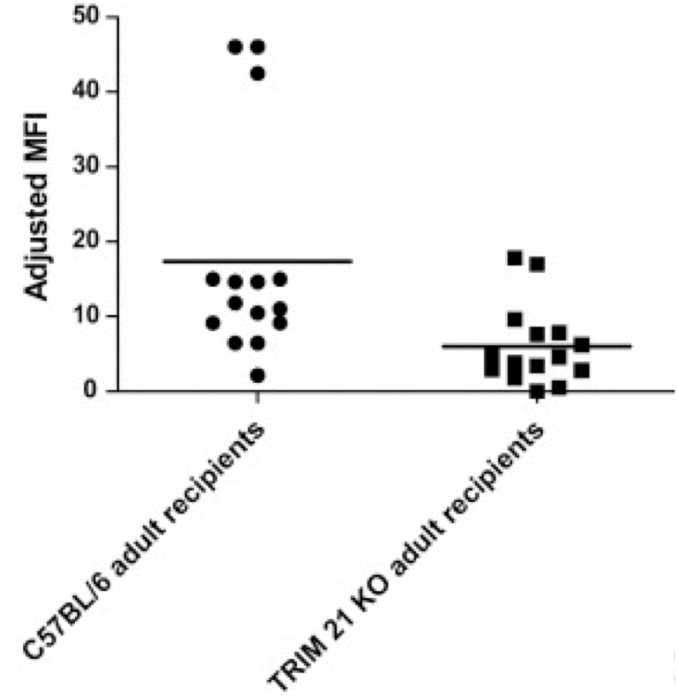
Figure 2: *TRIM21 KO and wild-type mice have similar responses to RBC alloimmunization in the context of poly (I:C) induced inflammation.* Wild-type (B6) mice or TRIM21 KO mice were injected with poly (I:C), followed 4 hours later by transfusion with donor RBCs expressing the HOD antigen. 2 weeks post-transfusion, serum was collected and anti-HOD responses were monitored using indirect immunofluorescence (1:5 dilution). Antibody binding to HOD RBC targets was measured by flow cytometry and background was subtracted using wild-type FVB control RBCs (adjusted MFI). No increased immunization was seen in adult TRIM21 KO mice compared to wild-type B6 controls. The data are

the combined results of three experiments (n = 15 TRIM21 KO mice and 10 B6 wild-type mice).

Figure 3: *TRIM21 KO and wild-type mice have similar rates and magnitude of humoral alloimmunization to transfused platelets.* Wild-type (B6) mice or TRIM21 KO mice were transfused with platelets from BALB/c donors. Three sequential transfusions were given (weekly) and serum was collected 2 weeks after the third transfusion. Antibody binding to BALB/c splenocyte targets was measured by flow cytometry (1:5 dilution of serum) and background was subtracted using wild-type B6 control splenocytes (adjusted MFI). No significant increase in frequency or magnitude of immunization was observed between TRIM21 KO and wild-type mice. The data are the combined results of three experiments (n = 15 mice/group).

Figure 1

A.


Adjusted MFI
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40
30
20
10
0
C57BL/6 adult recipients
TRIM 21 KO adult recipients

B.

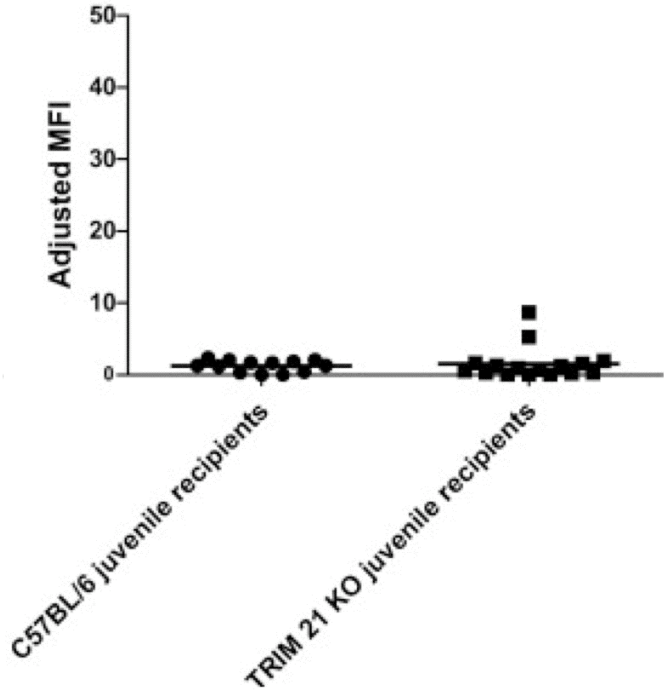

Adjusted MFI
50
40
30
20
10
0
C57BL/6 juvenile recipients
TRIM 21 KO juvenile recipients

Figure 2

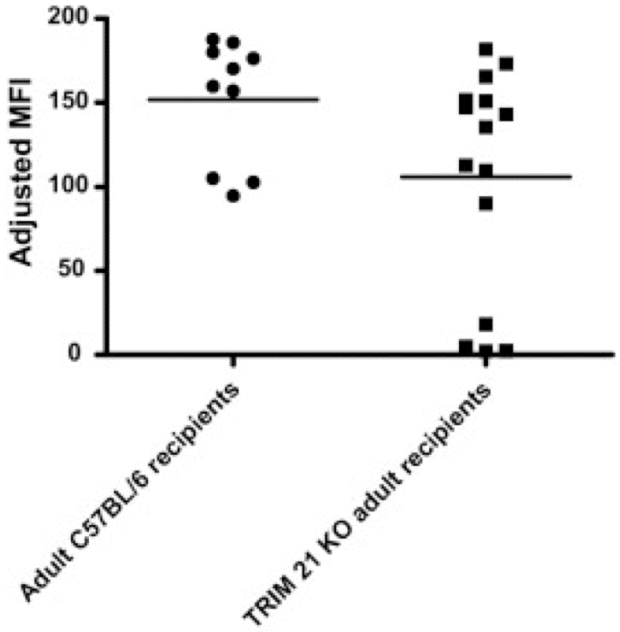
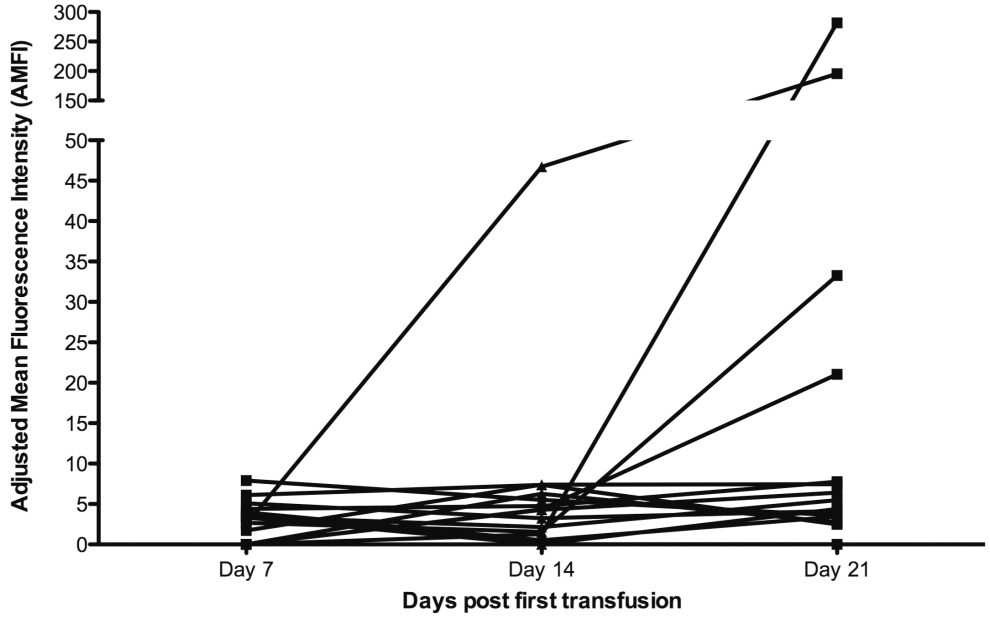

200
150
100
50
0
Adjusted MFI
Adult C57BL/6 recipients
TRIM 21 KO adult recipients

Figure 3

A.


Adjusted Mean Fluorescence Intensity (AMFI)
300
250
200
150
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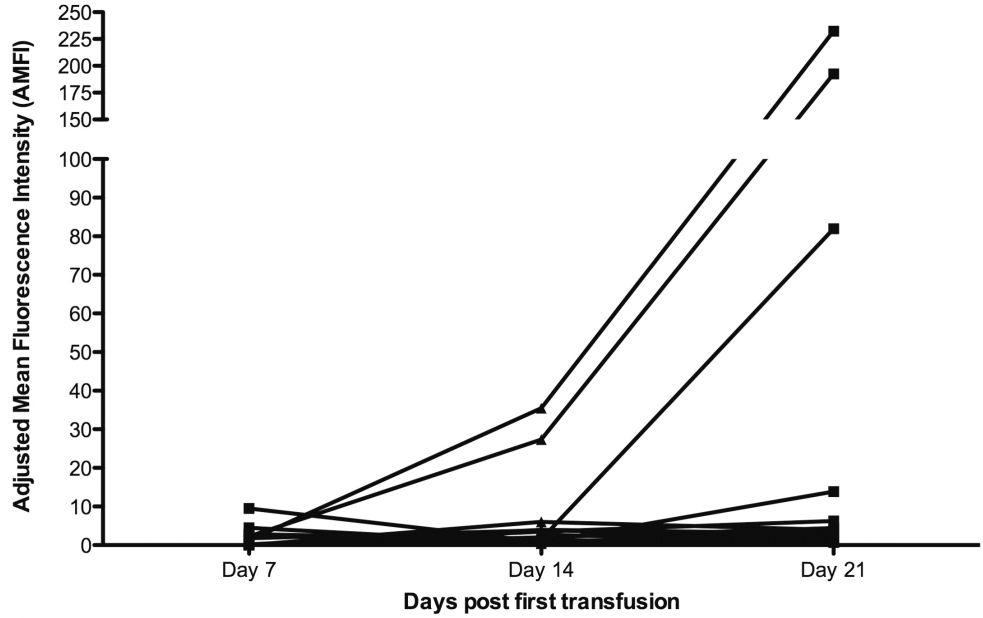
40
35
30
25
20
15
10
5
0
Day 7
Day 14
Day 21
Days post first transfusion

B.


Adjusted Mean Fluorescence Intensity (AMFI)
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225
200
175
150
100
90
80
70
60
50
40
30
20
10
0
Day 7
Day 14
Day 21
Days post first transfusion

Chapter 5

Co-stimulatory Blockade Prevents Rejection of MHC-matched Bone Marrow Transplants in Platelet Transfused Mice

Abstract

Transfused platelets are a life saving therapy for patients with a variety of medical conditions leading to thrombocytopenia. However, humoral alloimmunization to antigens on transfused platelets, most typically MHC Class I antigens, can render patients refractory to ongoing transfusion therapy, thereby precluding a life sustaining treatment. In addition, recent murine studies have demonstrated that alloimmunity to donor minor histocompatibility antigens on transfused platelets can induce rejection of a subsequent MHC-matched bone marrow transplant. As many patients are transfused with platelets prior to bone marrow transplantation, this is a clinical concern. Humoral and cellular alloimmunization to platelet antigens can be difficult to manage, and there are currently no interventions available to prevent immunization to alloantigens on transfused platelets. Co-stimulatory blockade is an approach that has shown the ability to mitigate alloimmunization in contexts outside of transfusion. Using murine systems, we report herein that treating recipients with CTLA4-Ig at time of transfusion prevents both humoral and cellular immune responses to major and minor antigens. Commercial formulations of CTLA4-Ig are U.S. Federal Food and Drug Administration approved for use in humans outside of the context of transfusion. Thus, the current findings have the potential to lead to rapid translation of this approach into humans.

Introduction

Severe thrombocytopenia can result as a component of a wide variety of diseases, and in many cases, transfusion of platelets is required to prevent hemorrhaging. A percentage of patients receiving platelet transfusions mount an antibody response against alloantigens, most notably HLA antigens but also human platelet antigens. When such antibodies arise, it can lead to a refractory state, which in extreme cases eliminates platelet transfusion therapy as a viable treatment modality. This can result in substantial morbidity and mortality. Thus, humoral alloimmunization to platelet antigens can be a significant problem, for which there is currently no medical prophylaxis.

Of additional concern are patients with non-malignant bone marrow failure syndromes resulting in thrombocytopenia or platelet dysfunction (1-4). For these patients, bone marrow transplantation is a potential cure. Because stringent conditioning regimens beneficial in promoting engraftment and killing neoplastic cells can result in morbidity and mortality, it is clinically difficult to justify transplanting patients with non-malignant hematologies under such conditions. As an alternative, patients with non-malignant bone marrow failure syndromes are administered an HLA-matched bone marrow transplant under reduced intensity conditions (3, 5, 6). However, under these conditions, roughly 15% of transplanted patients reject the HLA-matched bone marrow transplantation (1, 7, 8). Because the bone marrow transplantation is matched at the MHC loci, the most likely immunological vector mediating rejection in these patients is alloreactivity to minor histocompatibility antigens expressed on the donor bone

marrow. Consistent with this hypothesis, we have recently reported in a murine system that transfusion of leukocyte-reduced platelets induces rejection of a subsequent MHC-matched bone marrow transplant across minor histocompatibility antigen differences (9). As with humoral alloimmunization, there are currently no medical interventions to prophylax against immunization to minor histocompatibility antigens on transfused platelets.

Activation and generation of an optimal T cell response requires: 1) signals delivered via the T cell receptor after recognition of the peptide:MHC complex on antigen-presenting cells and 2) accessory signals in the form of co-stimulation (10). Early murine studies have demonstrated that allograft survival can be extended through the specific inhibition of the CD28-B7.1/B7.2 signaling pathway, the first described and most thoroughly characterized T cell co-stimulatory pathway (11, 12). Blockade of the CD28-B7.1/B7.2 signaling pathway can be achieved pharmacologically using a recombinant fusion protein that combines the extracellular domain of the human cytotoxic T-lymphocyte associated antigen 4 (CTLA4) with a modified Fc region of the human IgG1 (CTLA4-Ig). CTLA4 is a T cell surface receptor that negatively regulates T cell activation in part by competing with CD28 for binding to the ligands B7.1 and B7.2, which are expressed on antigen presenting cells (13). CTLA4-Ig, known commercially as Abatacept, is currently approved by the U.S. Federal Food and Drug Administration for the treatment of psoriasis and rheumatoid arthritis (14). Ongoing clinical trials using a second-generation derivative, known commercially as Belatacept, are in Phase III for human renal allografts (15). Because CTLA4-

Ig has shown clinical success in inhibiting autoimmunity and alloimmunization, we hypothesized that the immunosuppressive effects of CTLA4-Ig would extend to the prevention of alloimmunization to transfused platelets.

Herein, we report that CTLA4-Ig prevents both humoral alloimmunization to MHC Class I alloantigens and also transfusion induced bone marrow transplantation rejection across minor histocompatibility antigen barriers, in a mouse model. These findings indicate that blockade of the CD28-B7.1/B7.2 co-stimulatory pathway during initial alloantigen exposure is alone sufficient to prevent humoral and cellular immunization to major and minor alloantigens. Although the findings are in a murine model, the outcome suggests a clinical strategy to allow transfusion of allogeneic platelets while prophylaxing against both humoral and cellular alloimmunization.

Materials and Methods

Mice

Female C57BL/6J (H-2^b), BALB/c (H-2^d), and BALB.B [C.B10-H-2^b/LiMcdJ (H-2^b)] mice were purchased from Jackson Laboratories (Bar Harbor, ME). BALB/c mice were used as leukocyte reduced platelet and whole blood donors at 8 - 12 weeks of age, while BALB.B bone marrow donors and C57BL/6J recipients were utilized at 6 - 8 weeks of age. BALB.B, BALB/c, and C57BL/6J donors for seroanalysis and/or *in vivo* clearance assays were utilized at 8 - 12 weeks of age. Female TCR75 x Thy1.1 (H-2^b) mice were a generous gift from Dr. Pat Bucey (16). All mice were housed in Emory University Department of Animal Resources facilities and all studies and procedures were carried out in accordance with Emory University's Institutional Animal Care and Use Committee guidelines.

Antibodies for Flow Cytometry

Antibodies were purchased from BD Pharmingen (PE anti-mouse CD41, PE anti-TER119, PE Rat $IgG2_b$, APC goat anti-mouse Igs, FITC anti-mouse CD19, FITC anti-mouse CD4, FITC anti-mouse CD8α, PE anti-mouse CD3ε, APC anti-mouse CD3ε, FITC Rat $IgG2_a$, k, and FITC anti-mouse CD229.1/Ly9.1) and eBioscience (PE Rat IgG1 and APC anti-mouse CD229/Ly9).

Leukocyte Reduced Platelet Rich Plasma Preparation

Leukocyte reduced platelet products were harvested as previously described (9). Briefly, donor whole blood was collected in 1:8 acid-citrate-dextrose (ACD; BD Vacutainer). 1 mL of whole blood in ACD was added to 2 mL of 1x DPBS. Samples were centrifuged at 80 x g for 10 minutes, followed by collection of the platelet rich plasma and centrifugation at 80 x g for 10 minutes. Isolated platelet rich plasma was pooled and passed over a Neonatal Purecell PL High Efficiency Leukocyte Reduction Filter (Pall Corporation Port Washington, NY). Platelets were enumerated as described below. Following enumeration, platelets were pelleted at 1,942 x g for 10 minutes and re-suspended 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, 5.5 mM glucose in Milli Q water, pH 7.4) to 2 x 10^8 platelet/mL. 500 µl of re-suspended leukocyte reduced platelet (10^8 platelet total) was transfused through the tail-vein. All platelet handling was performed at room temperature. A "swirl" test was performed on all leukocyte reduced platelet concentrates to test the quality of platelets in solution (17).

Isolation of Whole Blood

Whole blood was collected in 1:8 ACD and washed three times with 1x DPBS at 394 x g for 15 minutes. The washed whole blood was re-suspended in 1x DPBS at a 1:5 dilution and 500 µl transfusions were given by tail vein.

Adoptive Transfer of Donor Specific $CD4^+$ T cells

$CD4^+$ T cell alloresponses to donor leukocyte reduced platelet transfusions were tested utilizing the TCR75 transgenic T cell, which is specific for the $H\text{-}2^d_{54\text{-}68}$ peptide presented by MHC Class II, $I\text{-}A^b$ (16). TCR75 whole splenocytes were liberated by mechanical disruption and incubated in Red Blood Cell lysis buffer (2 mL/spleen) for 5 minutes at room temperature. The cells were washed once in 1x DPBS, washed three times with warm 1x RPMI, and then incubated for 20 minutes with 5 μM CFSE (Invitrogen, Eugene, OR) in a 37°C water-bath. Cells were then washed two times with cold 1x RPMI supplemented with 10% fetal bovine serum (FBS), and washed twice with 1x DPBS prior to re-suspension in 1x DPBS. 1 x 10^6 CFSE labeled TCR75 whole splenocytes were adoptively transferred via tail vein injection and allowed to circulate for 24 hours prior to experimentation.

CTLA4-Ig treatment

The CD28-B7.1/B7.2 costimulatory pathway blocking fusion protein CTLA4-Ig was a generous gift from the laboratory of Dr. Christian P. Larsen (Emory University, GA). In the bone marrow transplantation and TCR75 studies, C57BL/6J recipients were administered 500 μg of CTLA4-Ig i.p. two hours prior to the first leukocyte reduced platelet transfusion. To control for this treatment process in the bone marrow transplantation experiments, parallel groups were treated with 500 μg of human IgG1 isotype control antibody (BioXCell, West Lebanon, NH) i.p.

Bone Marrow Transplantation

Twenty-four hours prior to bone marrow transplantation, all recipients were conditioned with sub-lethal gamma irradiation treatments (700 rads). Bone marrow was harvested from femora and tibiae in 1x RPMI with 10% FBS. Cells were washed three times in 1x DPBS. During the third wash, bone marrow cells were enumerated using a hemocytometer, and then re-suspended at 10 x 10^6 cells/mL in 1x DPBS. 500 µl (5 x 10^6 bone marrow cells) transfusions were injected into the tail veins of all recipients. Engraftment was monitored six weeks post-bone marrow transplantation by staining peripheral blood leukocytes with APC anti-mouse CD229/Ly9 (1:100) for 30 minutes at 4°C. The samples were washed three times in FACS buffer and then stained with FITC anti-mouse CD229.1/Ly9.1 (1:50) and PE anti-mouse CD3ε (1:100) in FACS buffer for 30 minutes at 4°C. The cells were washed three times and re-suspended in FACS buffer. Samples were run on a FACS Caliber and analyzed by FlowJo. 10,000 $CD3^+$ events were collected. Engraftment was measured as percent donor $CD229.1^+$ cells.

Seroanalysis: Indirect Immunofluorescence Staining

Sera was diluted 1:10 in FACS buffer and incubated with BALB.B or BALB/c splenocyte targets for 30 minutes at 4°C. The samples were then washed three times and stained with APC goat anti-mouse Ig (1:100), FITC anti-mouse CD19 (1:100) and PE anti-mouse CD3ε (1:100) in FACS buffer for 30 minutes at 4°C, in the dark. Samples were run on a FACS Caliber and analyzed on FlowJo. A

$CD19^-$ $CD3^+$ parent gate was utilized to avoid nonspecific background signal due to the B cell receptor and Fc receptor expressing cells. A positive response was defined as two standard deviations above the mean of sera from naïve C57BL/6J mice.

Statistics

Statistical analysis was performed using one-way ANOVA with Dunnett's post-test and column statistics. Significance was determined by a P value less than 0.05.

Results

Anti-donor antibody response following leukocyte reduced platelet transfusions is prevented by administration of co-stimulatory blockade

To test the efficacy of CTLA4-Ig in inhibiting humoral alloimmunization to transfused platelets, C57BL/6J (H-2^b) recipients were treated intraperitoneal (i.p.) with 500 µg of CTLA4-Ig two hours prior to each of four weekly transfusions from either BALB/c (H-2^d; MHC- and minor histocompatibility antigen-mismatched) or syngeneic C57BL/6J leukocyte reduced platelet donors. Sera were collected one week after each transfusion and tested for anti-donor antibodies through indirect immunofluorescence staining using BALB/c splenocyte targets. Positive anti-donor antibody responses were defined as a mean fluorescence intensity (MFI) two standard deviations above the mean MFI of naïve C57BL/6J mice. The results from three combined experiments demonstrated that control mice transfused with BALB/c leukocyte reduced platelet mounted progressively stronger anti-BALB antibody responses (Figure 1A). In contrast, CTLA4-Ig treated recipients had no detectable alloantibodies; indeed, the MFI of CTLA4-Ig treated mice transfused with BALB/c leukocyte reduced platelet was equivalent to control groups receiving syngeneic platelet transfusions (Figure 1A).

Additional studies were carried out to assess the kinetic requirements of CTLA4-Ig efficacy. A single dose of CTLA4-Ig was given at the time of the first transfusion and then no further CTLA4-Ig was given with subsequent transfusions. The combined data from three independent experiments demonstrated that the single initial dose was sufficient to prevent subsequent

alloimmunization over 28 days, as no alloantibodies were detected in mice receiving CTLA4-Ig (Figure 1B). Together, these data demonstrate that CTLA4-Ig effectively inhibits the humoral response to transfused allogeneic platelets, and that repeat dosing with every transfusion is not required.

CTLA4-Ig targets platelet-specific $CD4^+$ T cell division

The rational basis for CTLA4-Ig development was the prediction that it would interfere with co-stimulation to T cells. In the context of humoral alloimmunization, the effect upon $CD4^+$ helper T cells is most relevant. To test the effects of CTLA4-Ig on $CD4^+$ T cells specific for PLT antigens, a $CD4^+$ T cell transgenic murine system was utilized. The TCR75 transgenic mouse expresses a T cell receptor on $CD4^+$ T cells that reacts with a peptide derived from the BALB/c H-$2K^d$ MHC class I molecule presented by the C57BL/6J MHC class II, I-A^b ($K^d_{54\text{-}68}$/I-A^b) (18). Thus, $CD4^+$ T cells from the TCR75 mouse are specific for a peptide on donor PLTs that is processed and presented on recipient MHC Class II molecules. TCR75 mice were bred onto a C57BL/6J background congenic for the Thy1.1 allele, such that adoptive transfer of TCR75 cells into wild-type C57BL/6J (Thy1.2) results in an easily identifiable population (Figure 2A). This staining strategy does not detect any significant background $CD4^+$ T cells in C57BL/6J that have not received adoptive transfer (Figure 2A). In these experiments, all recipients received adoptive transfer of TCR75 splenocytes labeled with CFSE to allow subsequent assessment of proliferation.

Sera was collected at days seven and fourteen post transfusion and tested for anti-donor antibodies through indirect immunofluorescence staining using BALB/c splenocyte targets. Responders were defined as having an MFI two standard deviations above the mean MFI of naïve C57BL/6J mice. BALB/c leukocyte reduced platelet transfused recipients mounted a robust antibody response to BALB/c alloantigen (Figure 2B). In contrast, no significant antibody response to BALB/c alloantigens was detected in mice treated with CTLA4-Ig. Thus, the addition of TCR75 cells as an experimental maneuver did not alter the effects of CTLA4-Ig.

Five days post transfusion of BALB/c or C57BL/6J leukocyte reduced platelet products, mice were sacrificed and TCR75 cell were visualized through flow cytometry by gating on the $CD4^+$ $Thy1.1^+$ population. In three out of three experiments, TCR75 cells progressed through multiple rounds of division in response to a single BALB/c leukocyte reduced platelet transfusion (representative histogram Figure 2C). This proliferative response was specific to the BALB/c leukocyte reduced platelet transfusion, as no division was noted in recipients transfused with syngeneic C57BL/6J leukocyte reduced platelet products (Figure 2C). Administration of CTLA4-Ig two hours prior to the BALB/c leukocyte reduced platelet transfusion prevented the proliferative response of the platelet specific $CD4^+$ T cells (Figure 2C). Together, these data demonstrate that CTLA4-Ig not only inhibits the donor specific antibody response, but also the donor specific helper $CD4^+$ T cell response to allogeneic platelet transfusions.

CTLA4-Ig treatment at the time of transfusion prevents leukocyte reduced platelet transfusion induced bone marrow transplantation rejection

In addition to being an immunological impediment to subsequent platelet transfusions through antibody responses, we have recently reported that alloimmunization to platelet transfusion can also induce rejection an MHC-matched bone marrow transplant across minor histocompatibility antigen barriers (9). To test the hypothesis that CTLA4-Ig has an effect upon platelet transfusion induced bone marrow transplantation rejection, we utilized a model system in which C57BL/6J (H-2^b) recipients were transfused weekly for four weeks with BALB/c leukocyte reduced platelet concentrates (H-2^d: MHC- and minor histocompatibility antigen-mismatched), with or without a single dose of 500 μg CTLA4-Ig (Figure 3A). One week after the last transfusion, recipients received a BALB.B (H-2^b; MHC-matched and m minor histocompatibility antigen-mismatched) bone marrow transplant under reduced intensity conditions. Six weeks later, bone marrow transplantation engraftment was assessed using CD229 congenic markers; BALB.B donor cells are CD229.1^+ and C57BL/6J cells are CD229.2^+ (Figure 3B). Engraftment was defined as a percentage of donor CD229.1^+ cells that exceeded two standard deviations above the mean of rejecting recipients transfused with BALB/c leukocyte reduced platelet products. To control for non-specific effects of the CTLA4-Ig, a control group received human IgG1 of the same isotype as the Fc portion of CTLA4-Ig.

The combined results of three experiments demonstrated that 14 out of 15 (93%) of the BALB/c leukocyte reduced platelet transfused recipients treated with

a single CTLA4-Ig dose engrafted the BALB.B bone marrow transplantation (Figure 3D). In contrast, only 1 out of 15 (7%) of the BALB/c leukocyte reduced platelet transfused recipients treated with the human IgG1 isotype control antibody engrafted the BALB.B bone marrow transplantation (Figure 3D). Moreover, failure to reject the bone marrow transplantation was not due to excessive conditioning of the recipient, as 14 out of 14 (100%) of the untreated naïve recipients engrafted the BALB.B bone marrow transplantation (Figure 3D). Together, these observations demonstrate that a single dose of CTLA4-Ig at the time of the initial antigen exposure prevents alloimmunization to leukocyte reduced platelet transfusions and the subsequent rejection of a MHC-matched bone marrow transplant across minor histocompatibility antigen barriers.

Alloimmunization against BALB minor histocompatibility antigens in platelet transfused engrafting recipients treated with CTLA4-Ig

To distinguish the different effects of alloantigen on transfused leukocyte reduced platelet concentrates compared to alloimmunization as a result of the bone marrow transplantation procedure, alloimmunity was assessed before and after bone marrow transplantation. Indirect immunofluorescence staining was used to assay for anti-MHC antibodies and antibodies against non-MHC derived alloantigens by using BALB/c or BALB.B splenocyte targets, respectively. Responders were defined as having an MFI of two standard deviations above the mean MFI of the background naïve recipients. Sera from BALB/c whole blood

transfused and naïve recipients were utilized as positive and negative controls, respectively.

Alloantibodies to BALB/c antigens were detected in all positive control mice receiving BALB/c whole blood transfusions, but not in naïve animals (Figure 4A). Transfusion of BALB/c leukocyte reduced platelet products induced anti-BALB/c antibodies in 16 out of 25 (64%) of the control mice treated with the human IgG1 isotype control antibody, but in only 5 out of 25 (20%) of the recipients' receiving CTLA4-Ig (Figure 4A). Antibodies to minor antigens on BALB.B splenocyte targets were detected at lower levels and in fewer animals (Figure 4B). Similar to BALB/c whole blood immunization, 11 out of 25 (44%) of the recipients treated with the human IgG1 isotype control antibody had detectable anti-BALB.B antibodies, compared to only 1 out of 25 (4%) of the CTLA4-Ig treated animals. Together, these data confirm that CTLA4-Ig suppresses humoral alloimmunity to both major and minor antigens.

In vivo survival assays were also performed using targets expressing the BALB minor histocompatibility antigens. Because the assay is terminal, representative recipients were utilized. C57BL/6J splenocyte targets, an internal negative control for non-specific clearance, were co-infused along with donor BALB.B splenocyte targets. Each population was labeled with different concentrations of the fluorescent dye CFSE to allow visualization and enumeration of the targets. Eighteen hours later, the survival of BALB.B cells was determined; C57BL/6J syngeneic targets were used as a negative control to normalize BALB.B target survival. The ratio of BALB.B to C57BL/6J splenocyte

targets was also normalized to naïve recipients to control for injection and splenic processing.

The overall results from three combined experiments demonstrate that prior to bone marrow transplantation, there is no detectable *in vivo* killing activity in mice transfused with BALB/c leukocyte reduced platelet products, either in the CTLA4-Ig or human IgG1 isotype control antibody treated groups (Figure 4C). The assay had the ability to detect cytotoxic alloimmunity, as 15 out of 15 (100%) of the positive control mice transfused with BALB/c whole blood had detectable *in vivo* killing activity (Figure 4C). In stark contrast, 100% of mice that rejected the MHC-matched bone marrow transplantation (either transfused with BALB/c whole blood or transfused with BALB/c leukocyte reduced platelet products and treated with the control human IgG1 isotype control antibody) had specific *in vivo* killing of allogeneic targets (Figure 5C). Only 1 out of 15 (7%) of mice treated with CTLA4-Ig had detectable *in vivo* killing (Figure 5C). These data indicate that the *in vivo* kill assay correlates with rejection after bone marrow transplantation, but does not predict rejection prior to transplantation; suggesting that the leukocyte reduced platelet transfusions prime the immune response but do not lead to detectable effector function.

Discussion

Platelet transfusion support is an integral component of the treatment of thrombocytopenia of marrow failure etiology. However, alloimmunization to platelets can complicate and in some cases prohibit platelet transfusion, thus rendering a life-saving therapy ineffective. Moreover, we have recently identified additional and less appreciated potential sequelae of platelet transfusion; in particular, that alloimmunization to minor histocompatibility antigens on platelets may induce subsequent bone marrow transplantation rejection (9). Currently, an HLA-matched bone marrow transplant under reduced intensity conditions is the only cure for a variety of non-malignant hematological disorders (1-4). Prevention of bone marrow transplantation by immunization due to antecedent platelet transfusions effectively prevents a viable cure for these illnesses. Thus, in aggregate, alloimmunization to platelet transfusions is a serious complication of platelet transfusion therapy. As severely thrombocytopenic patients require platelet transfusion support to avoid life-threatening bleeding episodes, withholding platelet transfusions is not a viable option. Currently, there is no medical therapy to prophylax against alloimmunization to platelet. We describe the efficacy of an U.S. Federal Food and Drug Administration approved drug in preventing both antibody responses to MHC and preventing the induction of bone marrow transplantation rejection across minor histocompatibility antigen barriers in a murine model.

The presented data suggest that CTLA4-Ig therapy may have efficacy in preventing alloimmunization to platelets in humans. However, the extent to

which the efficacy of CTLA4-Ig in the murine models will translate into the human setting is as of yet untested. The general mechanisms and effects of CTLA4-Ig have been fairly parallel between rodents and humans; however, CTLA4-Ig has shown greater efficacy in animal models of solid organ transplantation than in non-human primates. There are considerable data to argue that this is because primates treated with CTLA4-Ig often have existing memory T cells whereas experimental mice are typically naïve. However, the landscape of platelet transfusion is different than other scenarios, as the strength of the stimulus is substantially smaller than in a solid organ transplant. Moreover, we know exactly when the patient is exposed to antigens, as it is the platelet transfusion itself that is the immunogen. Thus, CTLA4-Ig may not suffer the same limitation in the case of platelet transfusion as in other human settings.

It is worth noting that *in vitro* studies looking at human patients with chronic autoimmune thrombocytopenic purpura (AITP) have investigated the effects of CTLA4-Ig on the platelet-specific autoimmune T cells. Similar to previous reports (19), the investigators found that cells from patients with AITP had more robust responses to antigen presenting cells pulsed with platelets than healthy controls. This response was significantly decreased by the addition of CTLA4-Ig and cyclosporine A to the *in vitro* culture. Although these studies are *in vitro* responses and use autoimmune rather than alloimmune pathways, they do show effects of CTLA4-Ig on human T cells specific for platelet derived antigens.

In addition to the ability to induce bone marrow transplantation rejection, it has been posited that alloimmunization to platelet antigens may induce rejection of solid organs. Patients with hepatic and renal failures often receive aggressive platelet transfusion support prior to transplantation. This exposure to donor antigen may contribute to rejection of the transplant if donor minor histocompatibility antigen alloimmunity occurs in response to the platelet transfusions. Thus, it may also be possible to extend the use of CTLA4-Ig in preventing platelet transfusion induced alloimmunization for solid organ transplantation under reduced intensity conditions.

As with all immunosuppressive regimens, one must worry about the possibility of opportunistic infections. However, compared to general pharmacological immunosuppressants this is one potential advantage of CTLA4-Ig, in that CTLA4-Ig selectively prevents activation of naïve T cells but is much less potent for memory T cells. Thus, existing immunity and immunological memory to previously encountered microbes remains mostly intact. Hence, this is likely a much more gentle approach than full pharmacological immunosuppression.

In summary, alloimmunization to platelet antigens is a serious sequelae of platelet transfusion therapy, which is a life saving maneuver for a wide variety of human diseases. In addition to rendering platelet transfusion therapy ineffective in some patients, alloimmunization has the potential to promote rejection of subsequent transplants. Currently, there is no therapeutic maneuver to prevent or even mitigate platelet alloimmunization, other than leukoreduction of the

platelet units. However, even under the best current technologies, alloimmunization still occurs in at least 19% of patients (20). Herein we demonstrate that a drug approved by the U.S. Federal Food and Drug Administration for treatment of humans with rheumatoid arthritis, also has substantial efficacy in preventing platelet alloimmunization in mice. This represents a potential intervention to overcome the sequelae associated with alloimmunization to transfused platelet concentrates.

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Figure Legends

Figure 1: *Humoral responses to platelet transfusions in recipients treated with or without CTLA4-Ig.* **(A)** Alloantibody responses to leukocyte reduced platelet (LR-PLT) transfusions with concurrent CTLA4-Ig treatment. C57BL/6J recipients were administered a 500 μg dose of CTLA4-Ig i.p. two hours prior to each weekly transfusion of MHC- and minor histocompatibility antigen-mismatched BALB/c leukocyte reduced platelet concentrates. Sera was collected one week after each transfusion and tested for anti-donor antibodies using BALB/c splenocyte targets by indirect immunofluorescence staining. **(B)** BALB specific antibodies to leukocyte reduced platelet transfusions in the presence of a single CTLA4-Ig treatment. On day -21, C57BL/6J recipients were administered a single 500 μg dose of CTLA4-Ig i.p. two hours prior to a BALB/c leukocyte reduced platelet transfusion. Recipients received four subsequent transfusions a week apart, on days 0, 7, 14, and 21. Sera was collected one week after each transfusion and tested for anti-BALB antibodies by indirect immunofluorescence staining using BALB/c splenocyte targets. Responders in (A) and (B) were defined as having an MFI two standard deviations above the mean MFI of the background sera from naïve C57BL/6J mice (dotted line). Error bars represent the mean $\pm$ SD. Illustrated is the combined data from three independent experiments.

Figure 2: *Donor specific $CD4^+$ T cell responses to platelet transfusions with or without CTLA4-Ig treatment.* **(A)** Representative dot plots illustrate the ability to readily discriminate adoptively transferred TCR75 x Thy1.1 splenocytes from

C57BL/6J x Thy1.2 recipients five days post transfer using the Thy T cell congenic marker. **(B)** Division of adoptively transferred TCR75 cells in response to a leukocyte reduced platelet (LR-PLT) transfusion in the presence or absence of CTLA4-Ig. CFSE labeled TCR75 splenocytes were adoptively transferred into C57BL/6J recipients. Twenty-four hours later, recipients received a BALB/c (dark line) or syngeneic C57BL/6J (shaded histogram) leukocyte reduced platelet transfusion with or without 500 µg of CTLA4-Ig i.p two hours prior to transfusion. TCR75 division was measured in the spleen as dilution of the CFSE dye in $CD4^+$ $Thy1.1^+$ cells five days post transfusion. The illustrated histograms are representative of the trend observed in three independent experiments. **(C)** Anti-donor antibody responses to leukocyte reduced platelet transfusions in adoptively transferred recipients. TCR75 splenocytes were adoptively transferred into C57BL/6J recipients. Twenty-four hours later, recipients received a BALB/c or syngeneic C57BL/6J leukocyte reduced platelet transfusion with or without 500 µg of CTLA4-Ig i.p two hours prior to transfusion. Sera were collected at seven and fourteen days post transfusion and tested for anti-donor antibodies by indirect immunofluorescence staining using BALB/c splenocyte targets. Responders were defined as having an MFI two standard deviations above the mean MFI of the background sera from naïve C57BL/6J mice (dotted line). Error bars represent the mean $\pm$ SD. The data shown are the combined data from three independent experiments.

Figure 3: *Engraftment of an MHC-matched bone marrow transplantation in leukocyte reduced platelet transfused recipients treated with CTLA4-Ig.* **(A)** Experimental model testing the ability of CTLA4-Ig to prevent platelet induced rejection of an MHC-matched bone marrow transplantation (BMT). BALB/c platelet donors were MHC- and minor histocompatibility antigen-mismatched, whereas BALB.B bone marrow donors were MHC-matched but minor histocompatibility antigen-mismatched with respect to the C57BL/6J recipients. Recipients received a single 500 µg dose of CTLA4-Ig or human IgG1 i.p. two hours prior to the first transfusion. Recipients received four leukocyte reduced platelet transfusions, a week apart. After the fourth transfusion, recipients received a BALB.B bone marrow transplant under reduced intensity conditions. Seroanalysis and *in vivo* survival of BALB.B targets was performed before and after BMT (see Figures 4 and 5). **(B)** The $CD229^+$ congenic markers. Representative dot plots illustrate the ability to detect C57BL/6J recipient $CD229^+$ (left panel) and BALB.B donor $CD229.1^+$ (right panel) cells by flow cytometry. **(C)** $CD229^+$ cell engraftment. The representative dot plots demonstrate engraftment as the presence of a double positive $CD229.1^+$ $CD229^+$ population (left panel) and rejection as the absence of this double positive population (right panel). Chimerism is indicated by the presence of both the double positive $CD229.1^+$ $CD229^+$ population and the single positive $CD229^+$ population. **(D)** BALB.B bone marrow transplantation engraftment results. Engraftment is represented as percent $CD229.1^+$ cells in the peripheral blood; the horizontal lines denote the mean of each group. Rejection was measured as having a

percent CD229.1^{+} cells engraftment two standard deviations above the mean of the positive control group known to reject, recipients treated with isotype control antibody human IgG1. Statistics were generated using column statistics and a one-way ANOVA with Dunnett's post-test. Illustrated is the combined data from three independent experiments.

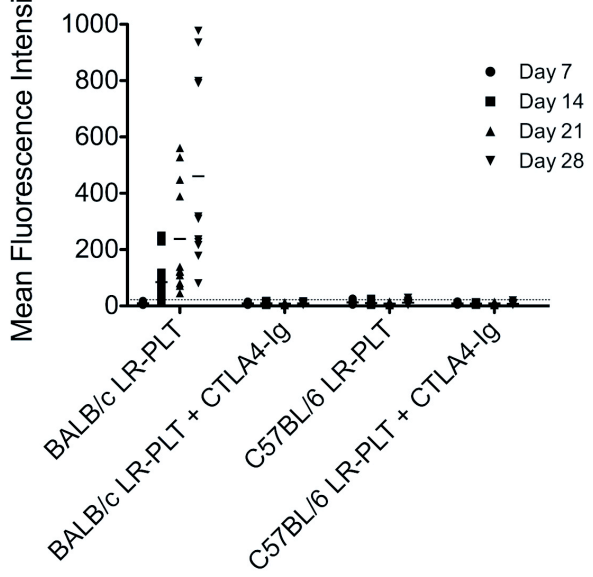
Figure 4: *Effects of CTLA4-Ig on alloimmunization in platelet transfused recipients before transplantation.* **(A)** and **(B)** BALB alloantibody responses before bone marrow transplantation. Indirect immunofluorescence staining was used to assess the presence of anti-BALB antibodies using BALB/c (A) and BALB.B (B) splenocyte targets. Responders were defined as having a MFI two standard deviations above the mean MFI of the background naïve recipients (dotted line). **(C)** *In vivo* survival of BALB expressing targets. BALB specific immunity was assessed *in vivo* as survival of BALB.B splenocyte targets in representative leukocyte reduced platelet transfused recipients. The horizontal line denotes the mean of each group. Statistics were generated using column statistics and a one-way ANOVA with Dunnett's post-test. The data shown in both panels is the combined data from three separate experiments.

Figure 5: *Alloimmunization in CTLA4-Ig treated bone marrow transplantation recipients transfused with leukocyte reduced platelet products.* **(A)** and **(B)** Alloantibody production after bone marrow transplantation. The presence of anti-BALB antibodies was tested six weeks after transplantation using BALB/c (A) or

BALB.B (B) splenocyte targets by indirect immunofluorescence staining. Responders were determined as having a MFI two standard deviations above the mean MFI of the background naïve recipients (dotted line). **(C)** Post transplantation survival of BALB expressing targets *in vivo.* BALB specific immunity was assessed by *in vivo* survival of BALB.B splenocyte targets in bone marrow transplantation recipients. The horizontal line denotes the mean of each group. Statistics were generated using column statistics and a one-way ANOVA with Dunnett's post-test. The data shown in both panels is the combined data from three separate experiments.

Figure 1

A.


Mean Fluorescence Intensity
1000
800
600
400
200
0
Day 7
Day 14
Day 21
Day 28
BALB/c LR-PLT
BALB/c LR-PLT + CTLA4-Ig
C57BL/6 LR-PLT
C57BL/6 LR-PLT + CTLA4-Ig

B.

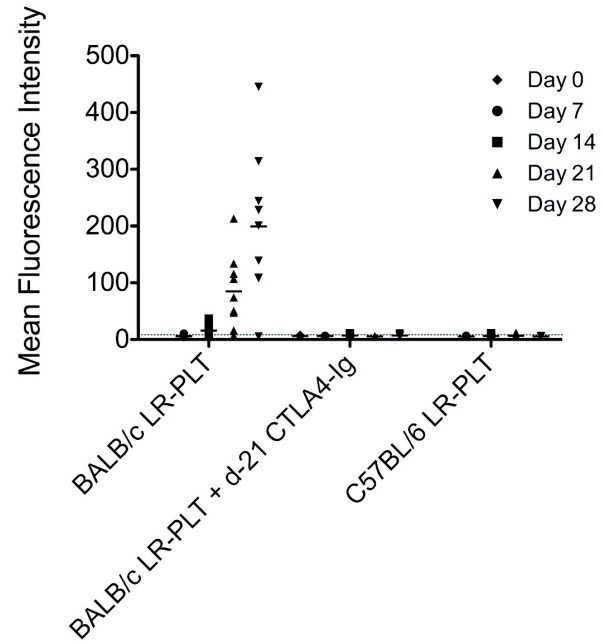
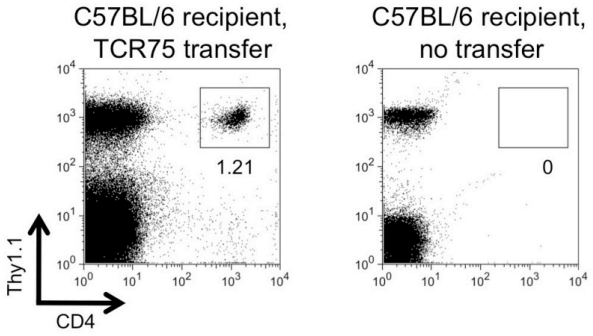

Mean Fluorescence Intensity
500
400
300
200
100
0
Day 0
Day 7
Day 14
Day 21
Day 28
BALB/c LR-PLT
BALB/c LR-PLT + d-21 CTLA4-Ig
C57BL/6 LR-PLT

Figure 2

A.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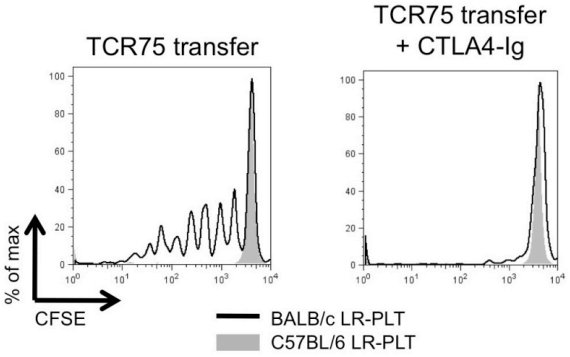
C57BL/6 recipient,
no transfer
1.21
0
Thy1.1
CD4

B.


TCR75 transfer
TCR75 transfer
+ CTLA4-Ig
% of max
CFSE
BALB/c LR-PLT
C57BL/6 LR-PLT

C.

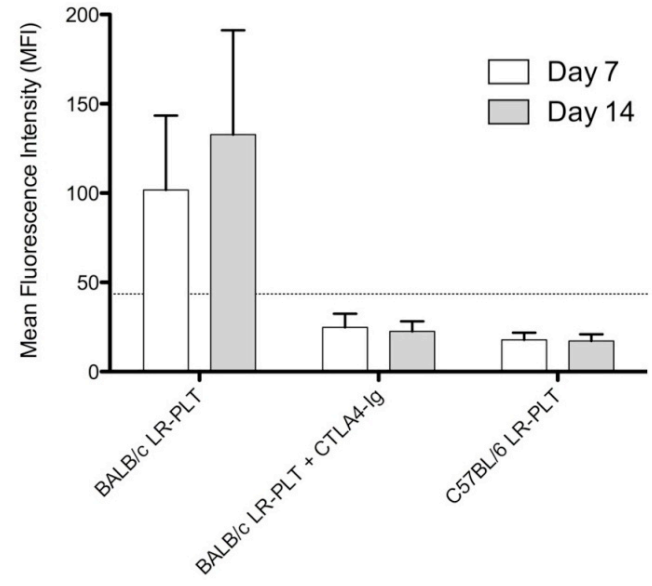
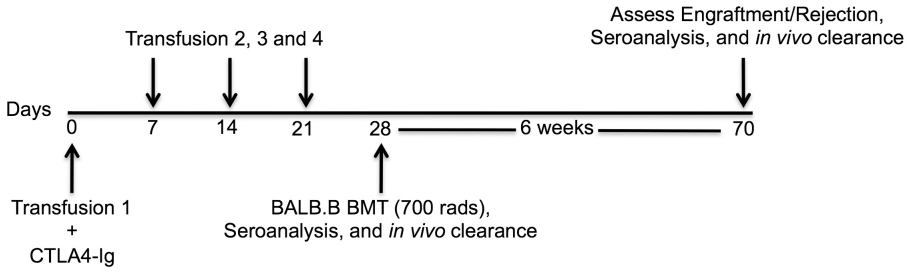

Mean Fluorescence Intensity (MFI)
200
150
100
50
0
Day 7
Day 14
BALB/c LR-PLT
BALB/c LR-PLT + CTLA4-Ig
C57BL/6 LR-PLT

Figure 3

A.



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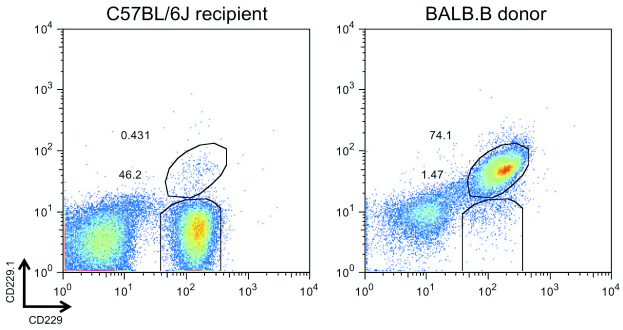

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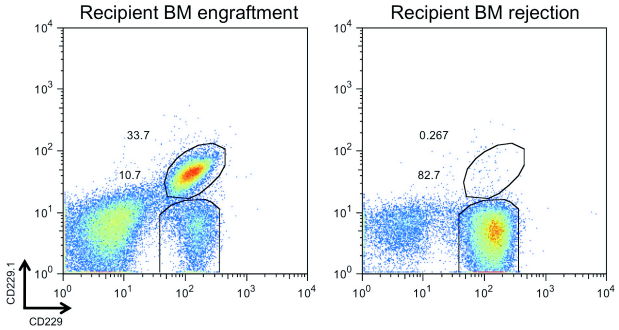


D.

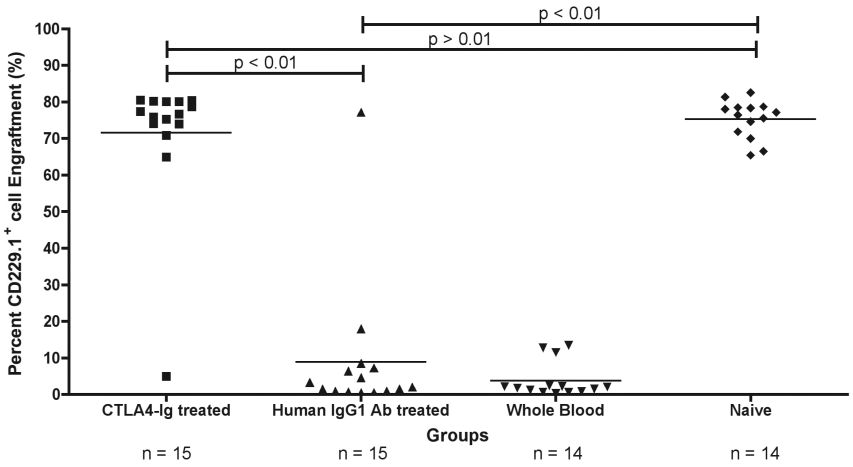


Figure 4

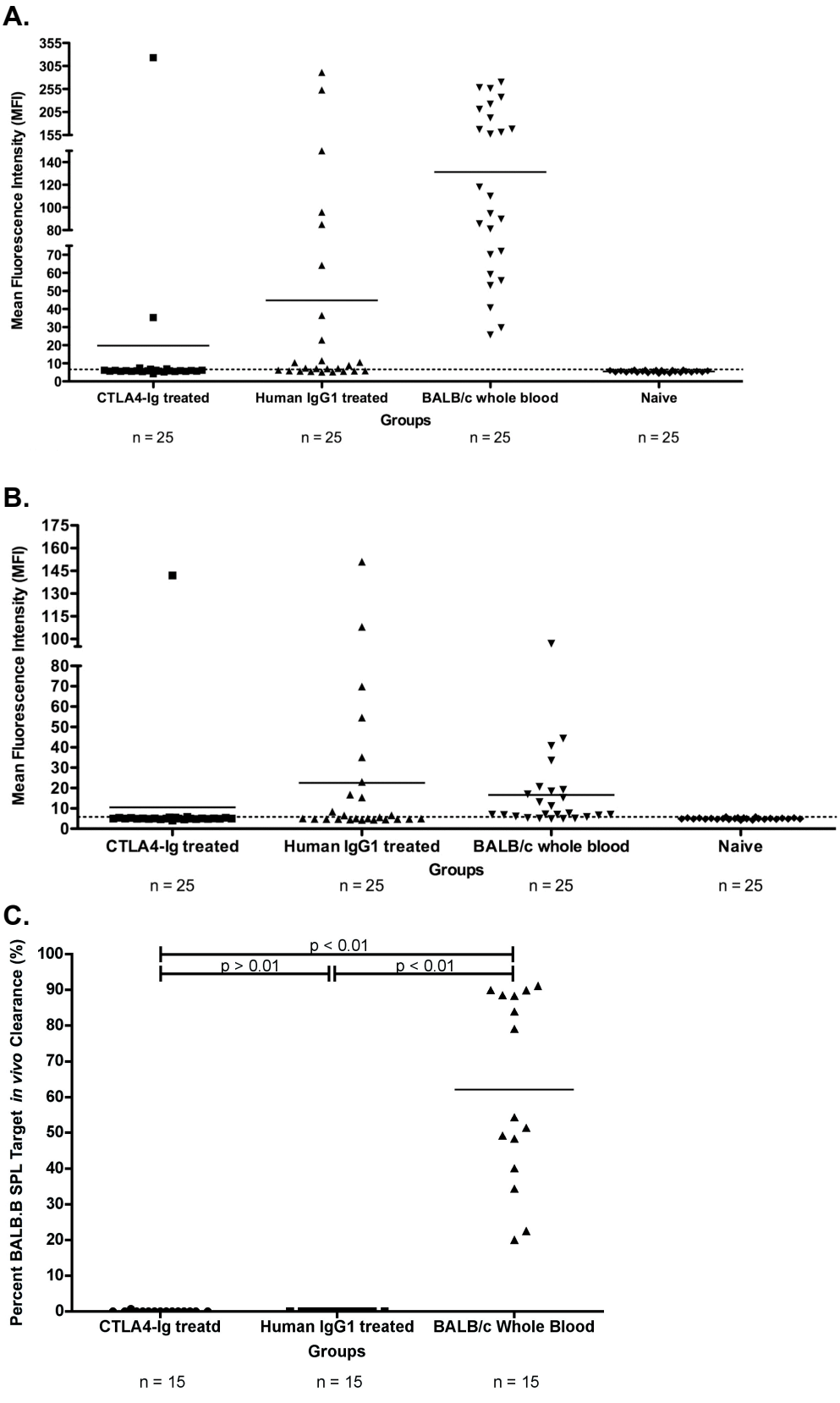
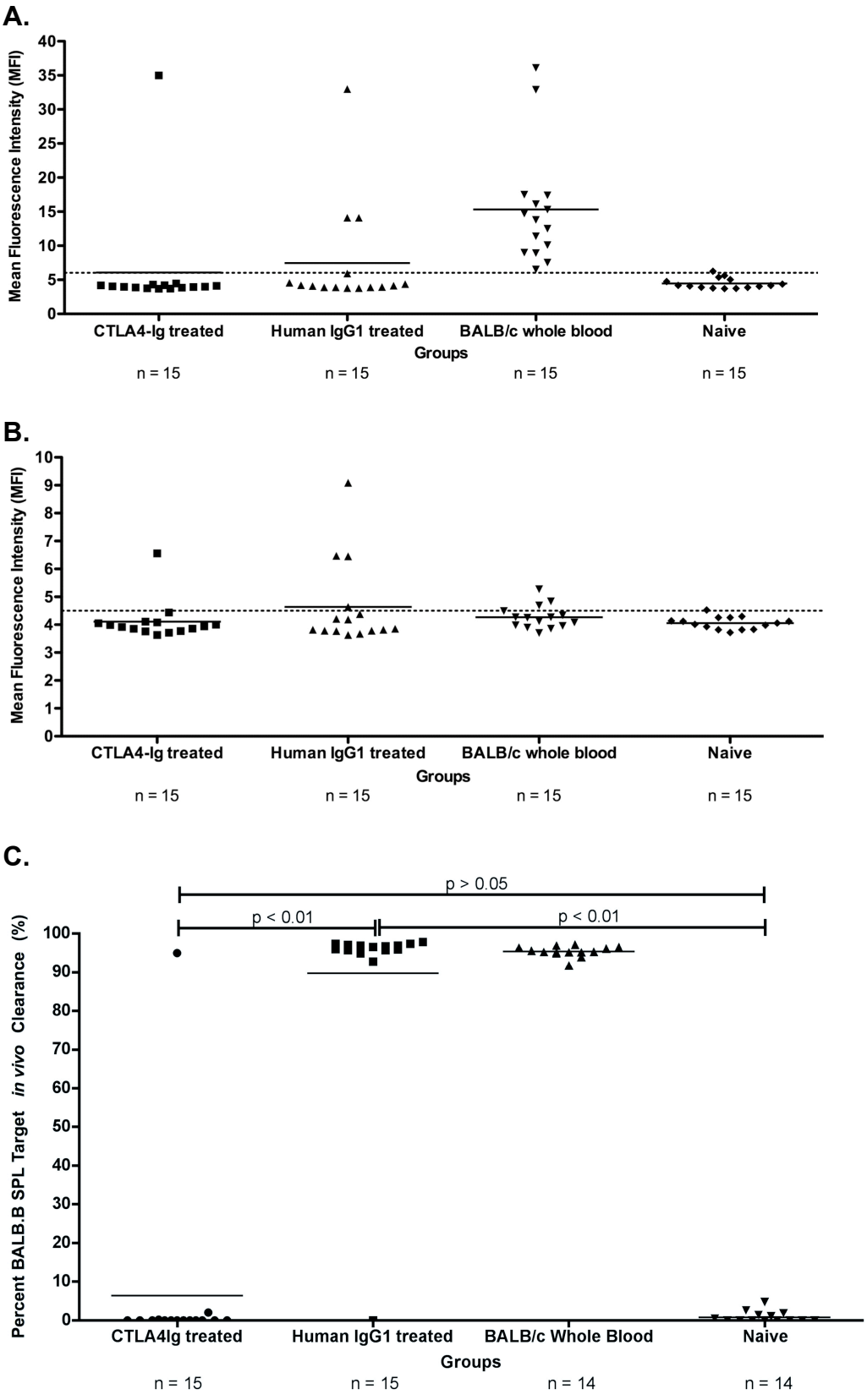

A.
Mean Fluorescence Intensity (MFI)
355
305
255
205
155
140
120
100
80
70
60
50
40
30
20
10
0
CTLA4-Ig treated
Human IgG1 treated
BALB/c whole blood
Naive
Groups
n = 25
n = 25
n = 25
n = 25
B.
Mean Fluorescence Intensity (MFI)
175
160
145
130
115
100
80
70
60
50
40
30
20
10
0
CTLA4-Ig treated
Human IgG1 treated
BALB/c whole blood
Naive
Groups
n = 25
n = 25
n = 25
n = 25
C.
Percent BALB.B SPL Target in vivo Clearance (%)
p < 0.01
p > 0.01
p < 0.01
100
90
80
70
60
50
40
30
20
10
0
CTLA4-Ig treatd
Human IgG1 treated
BALB/c Whole Blood
Groups
n = 15
n = 15
n = 15

Figure 5


A.
Mean Fluorescence Intensity (MFI)
CTLA4-Ig treated
Human IgG1 treated
BALB/c whole blood
Naive
Groups
n = 15
n = 15
n = 15
n = 15
B.
Mean Fluorescence Intensity (MFI)
CTLA4-Ig treated
Human IgG1 treated
BALB/c whole blood
Naive
Groups
n = 15
n = 15
n = 15
n = 15
C.
p > 0.05
p < 0.01
p < 0.01
Percent BALB.B SPL Target in vivo Clearance (%)
CTLA4Ig treated
Human IgG1 treated
BALB/c Whole Blood
Naive
Groups
n = 15
n = 15
n = 14
n = 14

Chapter 6

Discussion

Summary

Alloimmunization against minor histocompatibility antigens is a widely accepted immunological barrier to engraftment of bone marrow and solid organ transplantations. It has been hypothesized that sensitization against these polymorphic antigens occurs in response to exposure to minor histocompatibility antigens expressed on fetal tissue(s) or transfused blood products. Though correlative, clinical observations suggest that transfusions are an immunological barrier to engraftment of bone marrow transplantations under reduced intensity conditions (1-7); the long-term effects of alloimmunization against alloantigens expressed on transfused platelet products have on the engraftment of HLA-identical bone marrow transplantations remain undefined.

Thus, we developed a murine model to test the role of allogeneic platelet transfusions in engraftment of MHC-matched bone marrow transplantations under reduced intensity conditions. The data from these studies demonstrate that minor histocompatibility antigen reactive alloimmunity is primed in response to allogeneic leukocyte reduced platelet transfusions. In the context of MHC restriction, the observed alloimmunity did not require the direct presentation of minor histocompatibility antigen peptides to sufficiently elicit donor reactive T cell alloresponses. Variability in the percent *in vivo* elimination of donor targets expressing the minor histocompatibility antigens, but the complete absence of donor reactive antibodies after transfusions and before transplantation was observed in recipients transfused with allogeneic leukocyte reduced platelet concentrates. Collectively, these data suggest that minor histocompatibility

antigens expressed on transfused allogeneic leukocyte reduced platelet products elicit a cellular alloresponse.

Under reduced intensity conditions, this observed alloimmunity strongly correlated with rejection of a subsequent MHC-matched bone marrow transplantation expressing the target minor histocompatibility antigens. Rejection of the MHC-matched bone marrow transplantation occurred in the presence of *in vivo* clearance of minor histocompatibility antigen expressing targets, but in the absence of donor reactive alloantibodies. With the combined before and after transplantation results characterizing alloimmunization, these findings suggest that allogeneic leukocyte reduced platelet transfusions prime for a cellular alloresponse that subsequently is pushed to full effector function during the bone marrow transplantation.

Further characterization of the alloresponses demonstrated the requirement of both $CD4^+$ and $CD8^+$ T cells (after transfusion and before transplantation) during the priming phase of alloimmunization. However, only $CD8^+$ T cells were found to be required to mediate rejection of the MHC-matched bone marrow transplantation. Collectively, these findings indicate that donor reactive $CD4^+$ T cells are required to help generate effector $CD8^+$ T cell responses capable of mediating rejection of a subsequent MHC-matched bone marrow transplantation in recipients transfused with allogeneic leukocyte reduced platelet products. Moreover, these data suggest that allogeneic leukocyte reduced platelet transfusions are a single type of immunization that primes for a $CD8^+$ T cell alloresponse and the bone marrow transplantation expressing the

target minor histocompatibility antigens itself is an additional immunization event that pushes the primed $CD8^+$ T cells to develop into full functional effectors.

Because clinically not all platelet transfused patients reject an HLA-matched bone marrow transplantation under reduced intensity conditions, it is hypothesized that differential expression of recipient immunomodulatory proteins regulate alloresponses to transfused allogeneic leukocyte reduced platelet concentrates; and thus, influence the outcome of a subsequent bone marrow transplantation. One immunomodulatory protein clinically suggested to play a critical role in the modulation of alloimmunization induced in response to transfusions is TRIM21 (8). Using a TRIM21 knockout mouse (9), we found that the TRIM21 protein is not required to regulate the rate or magnitude of alloimmunization against alloantigens expressed on transfused leukocyte reduced platelet products. However, as there are numerous immunomodulatory proteins, we cannot unequivocally rule out the general role of recipient immunomodulatory proteins in the immunogenicity of allogeneic platelet transfusions and subsequent transplantation rejection.

There are, at the present time, no available clinical interventions effective in inhibiting the establishment of alloimmunization and preventing immediate or long-term effects of alloimmunization, such as refractoriness (immediate reaction of humoral alloimmunity) and graft rejection (downstream reaction of cellular alloimmunization). However, one potential approach is the use of immunomodulatory pharmacological reagents; of particular interest is the U.S. Federal Food and Drug Administration approved co-stimulatory blockade,

CTLA4-Ig, utilized to prolong solid organ graft survivals. A single dose of CTLA4-Ig during the first of three subsequent murine allogeneic leukocyte reduced platelet transfusions was found to block the induction of donor reactive alloimmunization against polymorphic major and minor histocompatibility antigens expressed on the transfused platelet concentrates. Rejection of subsequent MHC-matched bone marrow transplantation was additionally prevented in the CTLA4-Ig treated recipients that were transfused with allogeneic platelet products.

Currently, the main clinical focus of platelet transfusion medicine is humoral alloimmunization against HLA molecules expressed on transfused platelet products. However, the relevance of the current findings to human platelet transfusion practice is the introduction of a previously and surprisingly unappreciated immunological sequela of platelet transfusion medicine. The results in the current dissertation additionally indicate a significant barrier pertinent to HLA-matched bone marrow transplantation. Though the current studies focus on bone marrow transplantation, to the extent that solid organ transplantations share minor histocompatibility antigens with transfused leukocyte reduced platelet concentrates, it is possible that alloimmunization induced in response to platelet transfusions can influence the outcome of a solid organ transplantation expressing the target alloantigens. However, because the observations made are in a murine system, conclusions on the clinical implications of these data cannot be carried out without testing the general hypothesis in a human setting. To the extent that these results reflect human

biology, the findings presented herein indicate potential clinical interventions to overcome refractoriness to platelet transfusions and HLA-matched bone marrow or solid organ transplantation rejection under reduced intensity conditions in patients that are transfused with allogeneic platelet concentrates.

Discussion

Bone marrow transplantation in patients with non-malignant hematologies

Patients with non-malignant hematological disorders resulting in thrombocytopenia or platelet dysfunction can undergo HLA-matched bone marrow transplantation under reduced intensity conditions as a potential cure (10-16). However, under reduced intensity conditions, approximately 15% of transplanted patients reject the bone marrow transplantation (12, 15, 17). Although intensifying the conditioning regimens can potentially enhance engraftment, it is difficult to justify the additional increase in toxicity effects associated with more stringent conditions. There are currently three leading hypotheses addressing the failure to engraft HLA-identical bone marrow transplantations in patients with non-malignant hematological disorders.

One hypothesis is that the severity of an underlying disease perturbs the bone marrow microenvironment such that the marrow compartment cannot support a bone marrow transplant. It has clinically been observed that patients with a variety of non-malignant hematologies develop bone marrow injury as a result of perivascular fibrosis of blood vessels in the bone marrow and hematopoietic stem cell replacement with adipose tissue (14, 18). Likewise, it is hypothesized that the failure to engraft HLA-matched bone marrow transplantations in patients with non-malignant hematologies is due to non-immune mediated clinical consequences associated with the aggressive transfusion support many of these patients require to maintain hemostasis. Clinical observational studies have reported a correlation in the presence of iron

overload in heavily red blood cell transfused sickle cell disease patients and the failure to engraft an HLA-matched bone marrow transplantation (19, 20); suggesting that non-immune mediated transfusion related complications can influence the engraftment of a subsequent bone marrow transplantation through disruption of the recipients' bone marrow compartment.

An additional and equally consistent hypothesis is that donor bone marrow reactive immunity mediates rejection of a subsequent HLA-matched bone marrow transplant. In an attempt to decrease the incidence of graft rejection across the more immunogenic HLA barriers, bone marrow transplantations for patients with non-malignant hematologies are matched at the HLA loci as closely as possible. Conversely, no genotyping methodologies are currently clinically available to match recipients and donor bone marrow for minor histocompatibility antigens. Thus, minor histocompatibility antigens represent an additional immunological barrier to engrafting HLA-matched bone marrow transplantations in patients with non-malignant hematological disorders.

Alloimmunization against minor histocompatibility antigens can arise from a prior pregnancy, red blood cell or platelet transfusion, and/or heterologous immunity. Alloresponses against the minor histocompatibility antigens H-Y, HA-1, and HA-2 have been observed in multiparious females (21, 22). Though a likely source for sensitization against alloantigens, our data strongly support the hypothesis that alloimmunity against minor histocompatibility antigens in patients with non-malignant blood disorders is derived from the transfusion support that is an essential component of these patients treatment. It has clinically been

observed that the incidence of HLA-matched bone marrow transplantation rejection increases with the number of transfusions patients with non-malignant hematological disorders receive prior to the transplantation (1-6).

We acknowledge the possibility that this observed correlation between transfusions and bone marrow transplantation rejection may not be causal, as the increase in number of required transfusions might reflect the severity of disease and thereby greater perturbation of the bone marrow microenvironment. However, because many patients with non-malignant hematologies receive platelet transfusions before transplantation to prevent severe bleeding episodes, we hypothesize that rejection is more than likely due to alloimmunization against minor histocompatibility antigens derived from the transfusions. Thus, we generated a murine model in which recipients lacked an underlying hematological disorder and bone marrow injury to directly test the effect of allogeneic leukocyte reduced platelet transfusions on the efficiency to engraft an MHC-matched bone marrow transplant under reduced intensity conditions. The data presented herein indicate that alloresponses against minor histocompatibility antigens are induced in response to allogeneic leukocyte reduced platelet transfusions (Chapter 2).

Because the transfused leukocyte reduced platelet products were MHC-mismatched to the recipient, these results additionally suggest that direct allorecognition of minor histocompatibility antigen peptides expressed on transfused “passenger” leukocytes in the allogeneic platelet concentrates is not required to induce donor reactive alloimmunity (Chapter 2). However, we are

aware that alloimmunity can be induced through the direct or semi-direct allorecognition of an antigenic peptide presented on an allogeneic MHC molecule (23-27). Murine studies have demonstrated that both MHC restricted H-2^b (OT-II) and allogeneic restricted H-2^d (DO11.10) $CD4^+$ T cell transgenics can recognize and respond to an identical cognate antigenic ligand ($OVA_{323-339}$) presented on self MHC H-2^b (28). Because the induction of alloresponses can contravene MHC restriction, we cannot unequivocally rule out that alloimmunization occurred through the direct and/or semi-direct allorecognition of minor histocompatibility antigen peptides presented on allogeneic MHC molecules. However, if it is the case that presentation of minor histocompatibility antigens transgressed from HLA restriction in this system, than we predict that the quantity of cross-reactive recipient T cells would be too inadequate to robustly induce rejection.

These data additionally do not exclude the possibility that the platelets themselves act directly as antigen presenting cells or the source for semi-direct presentation of minor histocompatibility antigen peptides by recipient antigen presenting cells. Platelets express both the required 'Signal 1' and 'Signal 2' molecules for $CD8^+$ T cell activation, the antigenic peptide:MHC Class I molecule complexes and co-stimulatory molecules, both of which are secreted during storage (29, 30). Though we predict unlikely, we cannot rule out platelets directly acting as antigen presenting cells or donors of peptide:MHC Class I molecule complexes for semi-direct presentation using the current system.

Nevertheless, characterization of donor reactive alloimmunity after transfusions and before transplantation is indicative of the induction of adaptive

cellular alloresponses against minor histocompatibility antigens expressed on transfused platelet products, as *in vivo* selective elimination of alloantigen expressing targets but not donor reactive alloantibodies was detected (Chapter 2). In contrast to humoral alloimmunity, there is currently little to no understanding in the transfusion community of the induction of cellular alloimmunization in response to allogeneic platelet transfusions, as no known immediate immune mediated adverse transfusion related complications are directly due to the adaptive cellular arm of alloimmunity. Moreover, the long-term potential consequences of alloimmunization against alloantigens derived from transfused leukocyte reduced platelet products on bone marrow and solid organ transplantations are poorly understood. As murine allogeneic platelet transfusions are characterized to elicit $IgG2_a$ alloantibodies that correlate with T helper 1 (T_H1) alloresponses (31), it is not completely unexpected that cellular alloimmunity against minor histocompatibility antigens on transfused allogeneic platelet products would induce bone marrow transplantation rejection (Chapter 2); which additionally tends to be correlative with T_H1 responses (32, 33).

Alloimmunity mediating bone marrow transplantation rejection

Characterization of donor reactive alloimmunity once the outcome of the bone marrow transplantation was determined further indicate that adaptive cellular alloresponses elicited in response to allogeneic leukocyte reduced platelet concentrates can mediate rejection of a graft expressing the target alloantigens, as *in vivo* selective elimination of minor histocompatibility antigen

expressing targets but donor reactive alloantibodies were not observed (Chapter 2). Depletion of $CD4^+$ or $CD8^+$ T cells in allogeneic leukocyte reduced platelet transfused recipients prior to transplantation prevented rejection of the MHC-matched bone marrow transplantation (Chapter 3). Considering the observed cellular alloimmunity was inconsistently measurable before transplantation (Chapter 2), and yet subsequent rejection occurred in allogeneic leukocyte reduced platelet transfused recipients, we interpret these data to suggest that platelet transfusions prime a cellular alloresponse or stimulate a low effector T cell precursor frequency in which immunological effects of such effector responses are too small to be detected through the utilized *in vivo* survival and serological methodologies. Rejection of the MHC-matched bone marrow transplantation was not prevented upon depletion of $CD4^+$ T cells in allogeneic leukocyte reduced platelet transfused recipients that had previously rejected a bone marrow transplant expressing the target alloantigens (Chapter 3). Because $CD8^+$ T cell depletion prevented rejection of either the first or second MHC-matched bone marrow transplantation (Chapter 3), we predict that $CD8^+$ T cells are the most likely rejection vectors in this system.

It is well established that minor histocompatibility antigen reactive $CD8^+$ T cells can mediate rejection of bone marrow and solid organ transplantations; however, the immunological role of alloreactive $CD4^+$ T cells in rejection of an HLA-matched transplant is less evident (34-36). Animal transplantation studies demonstrating the requirement of both $CD4^+$ and $CD8^+$ T cells in rejection of MHC-matched bone marrow transplantations additionally indicate a bi-functional

role of alloreactive $CD4^+$ T cells in rejection of MHC-matched bone marrow transplantations (37). In the context of an MHC (HLA)-matched transplant, alloreactive $CD4^+$ T cells are hypothesized to be required for graft rejection as providing help to alloreactive $CD8^+$ T cells and/or effectors that directly mediate graft rejection (34-39). Although $CD8^+$ T cell responses have been demonstrated to occur in the absence of $CD4^+$ T cell help during a viral infection, the recall response of the $CD8^+$ T cell memory compartment is defective and of low quality (40). However, because platelets are less immunogenic than viruses, the findings herein suggest that the $CD4^+$ T cells induced in response to the allogeneic leukocyte reduced platelet transfusions is most likely required to give help to $CD8^+$ T cells.

Collectively, we interpret these data to indicate that $CD8^+$ T cells are primed but not stimulated to full effector function in response to minor histocompatibility antigens expressed on allogeneic leukocyte reduced platelet products. These findings suggest that the bone marrow transplantation in it self is an additional immunizing event that in the presence of $CD4^+$ helper T cells stimulates terminal differentiation of primed $CD8^+$ T cells into full cytolytic effectors capable of mediating graft rejection. Moreover, we propose that if this is the case, targeting helper $CD4^+$ T cells and/or $CD8^+$ T cells before transplantation as an alloimmunity specific conditioning regimen may be efficacious in preventing the establishment of an optimal $CD8^+$ T cell effector alloresponse and subsequent graft rejection. However, for immunosuppressive therapies after bone marrow transplantation, immunosuppressants that directly

target $CD8^+$ effector T cells may be advantageous to promote and maintain engraftment.

In contrast to cellular alloimmunization, these findings indicate that humoral alloimmunity against minor histocompatibility antigens is not induced in response to allogeneic leukocyte reduced platelet transfusions, as donor alloreactive antibodies were not detected before and after transplantation (Chapter 2). This interpretation was supported by rejection of the MHC-matched bone marrow transplantation in B cell deficient allogeneic leukocyte reduced platelet transfused recipients (Chapter 3). Combined, we interpret these data to suggest that humoral alloimmunity against minor histocompatibility antigens is not induced in response to allogeneic leukocyte reduced platelet transfusions, and thereby are not required to mediate rejection of subsequent MHC-matched bone marrow transplantations expressing the target minor histocompatibility antigens.

However, a concern in our interpretation is that the minor histocompatibility antigens shared between the platelet and bone marrow donors in the present experimental system are unknown. Thus, it is possible that in the current model the minor histocompatibility antigens responsible for cellular alloimmunization are expressed on intracellular polymorphic proteins. Indeed, if the minor histocompatibility antigens shared between the transfused platelet concentrates and hematopoietic stem cells are expressed on intracellular proteins that in theory are unable to elicit humoral alloresponses, we cannot unequivocally rule out that had donor reactive alloantibodies established in

response to leukocyte reduced platelet transfusions been present at the time of transplantation rejection of a graft expressing the target alloantigens would have occurred.

Approximately 2 - 10% of allogeneic platelet transfused patients develop donor reactive antibodies to platelet specific polymorphisms such as HPAs (41-45). Clinically, recipient and donor platelet products, similar to donor bone marrow, are not genotypically matched at minor histocompatibility antigen loci. At present time, the only minor histocompatibility antigens serologically matched to recipients are those expressed on the blood group antigens ABO and Rhesus (46-48). Though leukocyte reduction of platelet concentrates is found to decrease rates of donor reactive HLA alloantibodies (47-51), the incidence of alloantibodies against platelet specific polymorphic antigens is unaltered in patients that receive leukocyte reduced platelet transfusions (41-43). Thus, it is justifiable to question if humoral alloimmunity induced in response to alloantigens expressed on platelet transfusions can mediate rejection of a subsequent HLA-matched bone marrow transplantation expressing the target minor histocompatibility antigens.

Experimental studies utilizing the membrane bound ovalbumin (mOVA) transgenic murine system, in which humoral and/or cellular alloresponses are known to be elicited in response to the mOVA protein, are currently ongoing to test the hypothesis that alloantibodies induced in response to platelet transfusions mediate rejection of MHC-matched bone marrow transplantations under reduced intensity conditions. Thus far, the findings from these ongoing

studies suggest that donor reactive alloimmunity [indicated as variably detectable alloantibodies and selective *in vivo* clearance of donor targets (data not shown)] against an extracellular polymorphic protein expressed on transfused leukocyte reduced platelet products induces rejection of a subsequent MHC-matched bone marrow transplantation under reduced intensity conditions (Figure 1).

Depletion of $CD4^+$ or $CD8^+$ T cells after transfusions and prior to the bone marrow transplantation for the majority did not prevent rejection in recipients transfused with the allogeneic leukocyte reduced platelet products (Figure 2A). Conversely, the majority of platelet transfused recipients that lack B cells engrafted the MHC-matched bone marrow transplantation (Figure 2B). Collectively, these findings suggest that humoral alloimmunity against extracellular minor histocompatibility antigens induced in response to leukocyte reduced platelet transfusions can mediate rejection of an MHC-matched bone marrow transplantation under reduced intensity conditions. However, because the findings from two out of three experiments testing the requirement of B and T cells were inconsistent, significant conclusions from these results cannot currently be made.

The mechanism of antibody mediated bone marrow transplant rejection across minor histocompatibility antigen barriers is poorly understood, if at all known to occur. Thus, to our knowledge it would be a mechanistically novel and clinically relevant finding for transfusion and transplantation medicine if minor histocompatibility antigen reactive B cells were found to mediate rejection of MHC-matched bone marrow transplantations in platelet transfused recipients.

However, this might be unlikely, as it has clinically been demonstrated that HLA-identical bone marrow transplantations mismatched for HPAs 1 - 5, which are known to elicit humoral alloantibody responses, are not a minor histocompatibility antigen barrier to HLA-identical bone marrow transplantations (45, 52). Though we are aware that this clinical study does not directly assess the influence of HPA reactive alloantibodies on bone marrow transplantations and does not reflect the biology of all potential humoral alloimmunity inducing minor histocompatibility antigens, these observations do imply that alloantibody responses against a platelet specific alloantigen does not affect the outcome of a subsequent HLA-identical bone marrow transplant mismatched HPAs 1 - 5. Indeed, if humoral alloimmunity induced in response to allogeneic platelet transfusions are found to mediate MHC-matched bone marrow transplantation rejection, we postulate that the addition of B cell depletion therapy (i.e. rituximab) at the time of transplantation and thereafter can be a potential resolution to antibody mediated bone marrow transplantation rejection in chronically platelet transfused patients. However, because the study is ongoing and up to now inconclusive, the question: can humoral alloimmunity induced in response to an extracellular polymorphic protein expressed on leukocyte reduced platelet transfusions mediate rejection of HLA-matched bone marrow transplantations expressing the target extracellular alloantigen, remains un-answered.

These findings by no means reject the hypothesis that non-immune mediated complications of the underlying disease or treatment contribute to rejection of an HLA-matched bone marrow transplant in patients with non-

malignant hematological disorders. On the contrary, we interpret these data to demonstrate and support the hypothesis that adaptive cellular alloimmunity against minor histocompatibility antigens induced in response to allogeneic platelet transfusions are alone sufficient, if not an additional factor, to influence the status of HLA-matched bone marrow transplantations under reduced intensity conditions. Because immune mediated rejection in humans is hypothesized to be due to insufficient immunosuppression of established donor reactive alloimmunity and platelet transfusions are routinely administered pre- and/or peri-operative during transplantations performed under reduced intensity conditions (48, 53), we extend our hypothesis and current findings to rejection of HLA-matched solid organ transplantations.

Rejection of solid organ transplantations have been reported to occur as a result of alloimmunization against donor blood group antigens expressed on the graft (54). Thus, to the extent that minor histocompatibility antigens are shared on peripheral blood cells and transplanted solid organ grafts, we hypothesize that donor reactive adaptive cellular alloimmunization induced in response to allogeneic platelet transfusions can mediate rejection of a subsequent solid organ transplantation expressing the target alloantigens under reduced intensity conditions. Overall, the findings presented in the current dissertation clinically implicate platelet transfusions as additional and previously unappreciated immunological sequelae in transplantation.

Resolving platelet transfusion induced bone marrow transplantation rejection

To the extent that these murine findings reflect human biology, there are currently no effective therapies available to inhibit the induction of alloresponses to allogeneic platelet transfusions and subsequent HLA-matched transplantation rejection under reduced intensity conditions. Although leukocyte reduction methodology has been implemented in an attempt to decrease the rate of donor alloimmunization, decreasing the quantity of "passenger" leukocytes in transfused allogeneic platelet units does not eliminate alloresponses against minor histocompatibility antigens expressed on the transfused platelet products (47-51). Moreover, because it is clinically observed that the incidence of HPA alloimmunization is unaltered in patients that receive allogeneic leukocyte reduced platelet transfusions (41-43), it is hypothesized that alloimmunization against minor histocompatibility antigens is not due to "passenger" leukocytes in the platelet enriched transfused concentrates.

Alternatively and though we predict less likely, it is hypothesized that the inadequacy of leukocyte reduction technology to completely abrogate alloimmunization is due to the loss of regulatory leukocytes that consequentially render allogeneic platelet products more immunogenic. Murine studies testing alloantibody responses against MHC Class I molecules have demonstrated that alloantibody responses are exacerbated upon transfusion of allogeneic extremely leukocyte reduced platelet units (31). This murine study additionally has demonstrated that the addition of MHC Class II expressing B cells back to allogeneic extremely leukocyte reduced platelet products substantially diminishes the rate and magnitude of alloantibody responses; thus, suggesting that

immunogenicity of transfused allogeneic leukocyte reduced platelet concentrates is due to a loss of regulatory leukocytes during the leukocyte reduction process. However, we reject this interpretation, as the rate of alloimmunization increases in patients transfused with non-leukocyte reduced platelet products (49).

An alternate and strongly advocated interpretation is that alloimmunization against minor histocompatibility antigens on transfused allogeneic leukocyte reduced platelet concentrates is due either to the platelets themselves or the residual red blood cells in the platelet enriched transfused products. Recent murine studies have demonstrated that allogeneic leukocyte reduced red blood cell concentrates can induce rejection of an MHC-matched bone marrow transplant across minor histocompatibility antigen disparities (55). However, upon utilizing a reductionist system in which the alloantigen is red blood cell specific, rejection of a subsequent MHC-matched bone marrow transplant ubiquitously expressing the target alloantigen was not observed (Desmarets, M. *et al* in submission). Moreover, because the transfused leukocyte reduced red blood cell products were not devoid of platelets (55), we interpret these combined findings to indicate that the platelets in the transfused leukocyte reduced red blood cell concentrates were the immunogen responsible for induction of alloresponses against minor histocompatibility antigens.

Although less likely, we must additionally consider the possibility that the immunogenicity of the transfused blood products is independent of the cell subsets transfused, but indeed due to genetic differences in expression of immunomodulatory proteins. Recent clinical studies report that the rate and

magnitude of alloimmunization against transfused red blood cell products in sickle cell disease patients is due to the differential expression of the immunomodulatory protein, TRIM21 (8). On the contrary, when reduced to a murine system in which the expression of TRIM21 is completely removed (9), our data indicate that alloimmunization against alloantigens expressed on transfused red blood cell or platelet products is independent of the immunomodulatory protein TRIM21 (Chapter 4). Though there are numerous immunomodulatory proteins, for the studies presented in the current dissertation we hypothesize that the platelet themselves are immunogenic. Moreover, because we consider the red blood cells or platelets to be the likely the immunogens, we predict that no extent of leukocyte reduction will completely abrogate alloimmunization to red blood cell or platelet transfusions. Thus, it is necessary to investigate alternate clinical interventions to resolve alloimmunization induced in response to transfusions either before the alloantigen is introduction (at the time of transfusion) or once alloimmunity is established in the patient (conditioning regimens for transplantation or prophylaxis after transplantation).

One clinical approach to preventing HLA-matched graft rejection in patients sensitized against minor histocompatibility antigens expressed on allogeneic leukocyte reduced platelet products, and potentially allogeneic leukocyte reduced red blood cell transfusions, is to avoid chronic transfusion as much as possible preceding a transplantation. This strategy would require the introduction of bone marrow or solid organ transplantation earlier on in the

disease. However, this is not logistically feasible as many patients do not have HLA-matched, sibling donors.

An alternate and currently impractical clinical intervention is to match for minor histocompatibility antigens. This approach can be tackled at the donor to donor or donor to recipient fronts; donor platelet or graft can be matched to recipients, or donor platelet can be matched to the graft for minor histocompatibility antigens. Because not all minor histocompatibility antigens are known, the present clinically available genotyping methodologies to match for minor histocompatibility antigens are of limited use. There are serological techniques available to match for minor histocompatibility antigens; however, the serological assays matching for minor histocompatibility antigens are restricted to well known and clinically relevant alloantigens, such as blood group antigens.

Though serologically recipients can be tested for donor reactive humoral alloimmunity, this would only be applicable if alloantibodies induced in response to allogeneic leukocyte reduced platelet or red blood cell transfusions are found to be capable of mediating graft rejection across minor histocompatibility antigen barriers. Conversely, there are currently no immunological assays available to test recipients for donor specific cellular alloimmunization. Moreover, such immunological assays may not be clinically applicable if as in our murine studies human allogeneic leukocyte reduced platelet transfusions prime for alloresponses, and thereby render immune mediated rejection un-predictable prior to transplantation.

If the therapeutic target is inhibiting the establishment of alloimmunity at the time of transfusion, then the immunomodulatory costimulatory blockade CTLA4-Ig may be clinically employed. A single dose of CTLA4-Ig at the time of the initial transfusion was found to block the induction of major and minor alloantigen specific alloimmunization in response to allogeneic leukocyte reduced platelet transfusions in mice (Chapter 5). The inhibition of alloimmunization correlated with engraftment of a subsequent MHC-matched bone marrow transplantation under reduced intensity conditions (Chapter 5). Thus, these results support investigation of the use of CTLA4-Ig as a pharmacological means to block the induction of alloimmunization in response to allogeneic leukocyte reduced platelet transfusions in humans, as well as the immediate resolution to red blood cell or platelet transfusion refractoriness and long-term solution to HLA-matched transplantation rejection in recipients aggressively transfused with allogeneic leukocyte reduced platelet products.

These findings do not address if CTLA4-Ig can inhibit rejection of an MHC-matched bone marrow transplant once alloimmunity is established in response to allogeneic leukocyte reduced platelet transfusions. However, it is worth noting that recent animal studies have demonstrated that in the presence of a high precursor frequency of donor reactive memory T cells, co-stimulatory blockade regimens are less potent at overcoming immunological barriers to allograft transplantations and tolerance induction (56-58). Animal studies have additionally demonstrated that donor reactive memory elicited in response to a previous transplantation, pregnancy, transfusion, and/or pathogenic antigen

exposure can resist co-stimulatory blockade and consequentially mediate graft rejection (57, 59). In contrast to naïve T cells, memory T cells in general are resistant to immunosuppression due to a lower activation threshold, and thus requiring less 'Signal 2'.

Moreover, the effects of CTLA4-Ig have thus far been mostly analogous between rodents and humans in solid organ transplantation settings. However, to the extent that our data are clinically translatable and human platelet transfusions prime a patient against an alloantigen, we hypothesize that co-stimulatory blockade resistance would not be a clinical issue. Moreover, this could easily be tested in patients, as it is known exactly when a patient is exposed to an alloantigen in the context of a platelet transfusion. Thus, though possible, we predict that memory alloresponse limitations will not be a barrier to CTLA4-Ig as an immunomodulatory intervention utilized to prevent HLA-matched bone marrow transplantation in platelet transfused patients. However, if memory alloimmunity induced in response to platelet transfusions do render patients resistant to CTLA4-Ig, various co-stimulatory blockades including anti-CD40L, LFA3-Ig, and anti-LFA-1 could potentially be added to the CTLA4-Ig regimen to overcome resistance to co-stimulatory blockade therapy. Such combinatorial co-stimulatory blockade regimens have been utilized in murine systems and have demonstrated efficacy in prolonging graft survival (60); however, of note high memory precursor frequencies have also been found to play a critical role in the resistance of co-stimulatory blockade regimens that include CTLA4-Ig and anti-CD40L (56, 58, 59).

Parallel to utilizing CTLA4-Ig prior to transplantation to prevent rejection of an HLA-matched bone marrow transplant, we additionally submit that T cell specific therapies can be utilized to prevent rejection in patients transfused with platelet products. Rejection of an MHC-matched bone marrow transplant in allogeneic leukocyte reduced platelet transfused recipients was observed upon depletion of $CD4^+$ or $CD8^+$ T cells before transplantation (Chapter 3). Thus, novel T cell depletion therapies specific for $CD4^+$ or $CD8^+$ T cells, or general T cell specific anti-thymocyte globulin can be considered conditioning regimens to prevent rejection of a HLA-matched graft in platelet transfused patients. However, if the window of clinical intervention is during transplantation or the goal is to maintain engraftment, then a novel $CD8^+$ T cell specific regimen would likely be the better therapeutic modality.

Future Directions

To expand our understanding of alloimmunization against platelet transfusion induced MHC-matched transplantation rejection, there are many logistical questions that remain to be tested. Thus, the following sections include descriptions of short-term experiments to further our knowledge on platelet alloimmunogenicity and subsequent rejection, as well as long-term clinical translation assays and the therapeutic modalities indicated by our findings.

Short-Term Future Directions

Characterization of alloimmunity induced in response to allogeneic leukocyte reduced platelet products indicate the induction of $CD4^+$ and $CD8^+$ T cell responses, in which $CD4^+$ T cells provide help to $CD8^+$ T cells that are necessary for graft rejection (Chapter 2 and 3). However, our findings do not elucidate the location of priming, kinetics of the alloresponse, and effector requirements of $CD4^+$ and $CD8^+$ T cells in eventually resulting in graft rejection. Due to the unknown target minor histocompatibility antigen(s) in the current murine system, such mechanistic studies currently cannot be performed. However, there are a battery of alternate approaches that could prove useful to define alloimmunization in response to allogeneic platelet transfusions and rejection of a subsequent MHC-matched graft.

One potential approach is to utilize a transgenic system (i.e. mOVA) in which there are readily available T cell transgenics, peptides, and tetramers. Experiments utilizing these immunological tools would include 1) kinetic assays

of the alloresponse, 2) effectors present before transplantation and after rejection through intracellular cytokine straining (i.e. granzyme B, perforin, IFN-γ, IL-2, TNFα), and 3) location of priming and activation through flow cytometry and immunofluorescence staining in various secondary lymphoid organs. An alternate approach to test the effectors mediating graft rejection would be to give allogeneic leukocyte reduced platelet transfused recipients that lack the particular effector of interest (i.e. granzyme B knockout and FasL knockout) an MHC-matched bone marrow transplant under reduced intensity conditions. *In vivo* survival assays after transfusion/before transplantation and after rejection may suffice useful in functionally testing each of the effectors. This approach of course works only under the assumption that the knockout does not alter the immunogenicity of the transfused platelet products, as well as solely affects the function of the effectors capable of generating the effectors.

Alternatively, isolating the natural target minor histocompatibility antigen responsible for alloimmunization and rejection can be useful to allow the generation of subsequent immunological tools, including T cell transgenics and tetramers, which would subsequently permit mechanistic characterization of the natural alloresponses to allogeneic platelet transfusions and rejection of an MHC-matched graft. To segregate the target minor histocompatibility antigen in the current experimental system, (BALB x C57BL/6J) F_1 mice would be backcrossed with the parental BALB.B strain. The resulting F_2 generation would be utilized as recipients for BALB/c leukocyte reduced platelet transfusions and subsequent BALB.B bone marrow transplantation. The rejecting F_2 recipients would be

backcrossed onto the parental BALB.B background. This process would continue until rejection was no longer observed. The DNA from the previous backcrossed generation that lastly rejected the bone marrow transplantation would be isolated and sequenced. Single nucleotide polymorphism analysis of the resulting sequence would theoretically identify genes carrying the target minor histocompatibility antigens responsible for MHC-matched bone marrow transplantation rejection in murine recipients transfused with allogeneic leukocyte reduced platelet products. Subsequent deep sequencing and experimental testing would additionally be required.

In theory, solely backcrossing the rejecting recipients would allow for the isolation of the minor histocompatibility antigen responsible for alloimmunization and subsequent rejection. Of course, this methodology functions under the assumption that there are just a few alloantigenic differences; however, if there are multiple minor histocompatibility antigen disparities with each encoded on different chromosomes, then this proposed experimental approach might not succeed. Thus, it might be advantageous to first perform frequency analyses.

In contrast to the induction and requirement of cellular alloresponses, rejection of the MHC-matched bone marrow transplantation in allogeneic leukocyte reduced platelet transfused recipients that lack B cells appear to indicate that B cells are not required for rejection in platelet transfused recipients (Chapter 3). However, ongoing experiments utilizing a transgenic system known to elicit humoral alloimmunity appear to indicate that alloantibody responses can be elicited in response to extracellular polymorphic proteins expressed on

transfused leukocyte reduced platelet products. Given the inconsistency in these findings, though, the requirement of humoral alloimmunity induced in response to allogeneic platelet transfusions in mediating graft rejection necessitates further formal testing. Transferring sera from allogeneic leukocyte reduced platelet transfused recipients into naïve recipients that subsequently undergo MHC-matched bone marrow transplantation can be performed to directly assess the necessity of alloantibodies in graft rejection. However, it might prove useful to additionally transfer sera from rejecting recipients. Our data indicate that the bone marrow transplantation in itself serves as an additional immunizing event that can potentially skew the primed immunological response, as qualitatively the transfused leukocyte reduced platelet products and bone marrow transplantation differ (Chapter 3). As a functional assay of immune mediated rejection due to the alloantibodies transferred, *in vivo* survival assays of donor targets can additionally be performed before and after transplantation.

Though our findings suggest the use of potential clinical therapies (i.e. CTLA4-Ig or a novel CD8 T cell specific depleting antibody) to prevent alloimmunization and rejection, such therapeutic modalities may not be necessary if residual “passenger” leukocytes are the immunogen resulting in alloimmunization. If the “passenger” leukocytes are the required cell subset, then more stringent leukocyte depleting methodologies need to be developed. Thus, determining the immunogenic cell subset(s) is important to simplify assessment of which clinical modality is necessary to abrogate alloimmunization in response to platelet or red blood cell transfusions. In combination with recent murine

findings [(55); Desmarets, M. *et al* in submission], our data suggest that platelets are the immunogenic cell subset in transfused blood products. However, because platelet and leukocyte specific transgenic mice are currently unavailable, we at the present time cannot investigate the cell subset required for the induction of an alloresponse and subsequent MHC-matched graft rejection. Although the use of better fractionation technology in theory would be equally effective in solely isolating platelets, leukocytes, or red blood cells, it is difficult to knowingly completely enrich for one cell subset.

Thus, the requirement of platelets, red blood cells, and/or leukocytes as the accountable cell subset(s) expressing the target minor histocompatibility antigens responsible for alloimmunization can be tested using the CRE-loxP system to allow for tissue specific expression of the alloantigen. Using the CRE-loxP system, we are currently generating a tissue specific conditional knockin transgenic mouse that will express the fusion protein HOG consisting of Hen-Egg-Lysozyme (HEL), Ovalbumin (OVA), and Enhanced green fluorescence protein (GFP). Insertion of a transcriptional stop element, flanked by loxP sites (Lox-stop-Lox) in the 5' un-translated region of the HOG protein, would inhibit HOG expression and allow the resulting transgenics to be used in generating cell specific knockin donor strains. The non-transcriptional HOG transgenic and various CRE strains (platelet, leukocytes, red blood cells, and ubiquitous) would additionally be backcrossed onto a congenic background that differs at the MHC loci to prevent direct presentation of HOG peptides in the context of self MHC.

The resulting non-transcriptional HOG transgenics and CRE strains would then be bred to generate mice ubiquitously or only expressing the HOG protein on platelets, leukocytes, or red blood cells (Figure 3). The CRE strain encoded downstream of a ubiquitous promoter would additionally be bred with the original non-transcriptional HOG transgenic mice to generate MHC-matched bone marrow transplantation donors that differ from the recipients only at the HOG loci. The tissue specific HOG knockin mice would be utilized as leukocyte reduced platelet donors to directly test the cell subset(s) in transfused allogeneic platelet concentrates responsible for minor histocompatibility antigen exposure.

Long-Term Future Directions

Because our findings are developed in a murine system, the clinical implications of these data are unclear. To translate the induction of cellular alloimmunity in response to platelet transfusions to the human setting, it would be of potential interest to use available tetramer reagents to test platelet transfused recipients' peripheral blood for minor histocompatibility antigen reactive $CD8^+$ and/or $CD4^+$ T cells. However, one shortcoming to this approach is that not all minor histocompatibility antigens expressed on platelet units are known.

Moreover, the use of tetramers would only suggest the induction of a cellular alloresponse, but would not test the effects of the induced cellular alloimmunity on a subsequent transplantation. Thus, one additional approach that would suggest a potential role of $tetramer^+$ minor histocompatibility antigen reactive $CD8^+$ and/or $CD4^+$ T cells in the peripheral blood of platelet transfused

patients on subsequent HLA-matched bone marrow transplantations would be to utilize anti-thymocyte globulin or a $CD8^+$ T cell specific depleting antibody as conditioning regimens. Simultaneous to testing our hypotheses in a clinical setting, this would allow for the clinical trial of anti-thymocyte globulin or a $CD8^+$ T cell specific depleting antibody, as a safer reduced intensity regimen that would promote engraftment with minimal toxicity effects.

On a similar note, the role of minor histocompatibility antigen reactive $CD8^+$ T cells in rejection of an HLA-matched bone marrow transplant in platelet transfused patients would be to utilize anti-thymocyte globulin or a $CD8^+$ T cell specific depleting antibody after transplantation. This approach would additionally test anti-thymocyte globulin or a $CD8^+$ T cell specific depleting antibody as prophylaxis treatment to prevent subsequent rejection of HLA-matched bone marrow transplantations in platelet transfused patients. It might additionally be of clinical interest to test this approach in patients receiving a solid organ transplantation that require pre- and peri-operative platelet transfusion support (48, 53), as well as mild pharmacological immunosuppression that would potentially allow for induction a cellular alloresponse against minor histocompatibility antigens shared between the graft and transfused platelet units. However, because the impact alloimmunization elicited in response to platelet transfusions has on solid organ transplantations has yet to be tested in an animal system, it would be beneficial to initially test the influence alloimmunization has on solid grafts prior to clinical translation.

Because our findings demonstrate that CTLA4-Ig at the time of transfusion blocks alloimmunization in response to platelet transfusions and subsequent MHC-matched bone marrow transplantation rejection (Chapter 5), it is possible to additionally have a trial to test CTLA4-Ig as a clinical prophylaxis therapy to prevent alloimmunization and subsequent refractoriness to blood transfusions or transplantation rejection. However, the kinetics of how long a single dose of CTLA4-Ig remains biologically effective is unclear. Because it may be that CTLA4-Ig clinically is required during multiple platelet transfusions to prevent alloimmunization from occurring, CTLA4-Ig at the time of transfusions may not be the most logistically and economically feasible methodology to block alloimmunization and subsequent refractoriness or transplant rejection. Thus, it may be important to initially test rejection of an MHC-matched bone marrow transplant in platelet transfused murine recipients in the presence of CTLA4-Ig administered prior to transplantation but after transfusion. If the results from this murine study demonstrate engraftment of the MHC-matched bone marrow transplantation, it is important to test the ability of CTLA4-Ig in promoting engraftment of HLA-matched bone marrow or solid organ transplantations in platelet transfused patients.

General Conclusions

Platelet transfusion support is an integral component of the treatment for patients suffering with thrombocytopenia and/or platelet dysfunction. Although a vital therapy, platelet transfusions are associated with adverse clinical consequences, such as alloimmunization. Leukocyte reduction therapy has been implemented in an attempt to prevent alloimmunization. However, it has become evident that current leukocyte reduction techniques are alone insufficient to prevent alloimmunization, and its subsequent immediate and long-term effects. The findings presented in this thesis suggest that in addition to humoral alloimmunity, cellular alloresponses can be induced in response to platelet transfusions, and the resulting donor reactive cellular alloimmunity can result in rejection of a transplant under conditions in which the recipient's immune system remains largely intact.

Although, the current model used in this thesis has a certain degree of sophistication, it does not allow for extensive investigation of the immunological consequences associated with platelet transfusion therapy. Thus, the development of new advanced model systems is necessary to further our knowledge of the effects of alloimmunization induced in response to platelet transfusions on transplantation. Platelet transfusion support is a life saving therapy; and thus, it is imperative to pursue this line of research to provide safer transfusions and transplantations.

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Figure Legends

Figure 1: *Rejection of mOVA bone marrow transplantations in mOVA leukocyte reduced platelet transfused recipients.* **(A)** Engraftment results. Engraftment is measured as percent OVA^+ T cells in the peripheral blood. **(B)** OVA Specific Alloantibody Production. mOVA reactive antibodies in sera of transfused and transplanted recipients were assessed using mOVA splenocyte targets in an indirect immunofluorescence staining. **(C)** Alloimmunization in mOVA bone marrow transplantation recipients. Immunity to mOVA targets was measured as *in vivo* survival of mOVA splenocyte targets. The mean of each group in all the panels is represented as a horizontal line. Statistics were generated using column statistics and a one-way ANOVA with Dunnett's post-test. Illustrated is the combined data from three independent experiments.

Figure 2: *$CD4^+$ and $CD8^+$ T cell, and B cell requirement to reject mOVA bone marrow transplantation in mOVA leukocyte reduced platelet transfused recipients.* **(A)** mOVA bone marrow transplantation engraftment results. Engraftment is represented by percent OVA^+ T cells in peripheral blood. **(B)** Engraftment results. Engraftment is measured as percent OVA^+ T cells in the peripheral blood. Statistics were generated using column statistics and a one-way ANOVA with Dunnett's post-test. The data shown in all panels is the combined data from three separate experiments.

Figure 3: *Experimental design to generate the diverse tissue specific conditional knockins and the subsequent utility of them.* Tissue specific conditional knockin transgenic mice are currently being generated utilizing the CRE-loxP system. The transgenic mice will express a novel fusion protein HOG, containing Hen-Egg-Lysozyme (HEL), Ovalbumin (OVA), and Enhanced green fluorescence protein (GFP). The HOG transgenic mice will be generated on a C57BL/6J background and subsequently backcrossed onto a B6 H-2^d congenic background. All CRE strains will be purchased on a C57BL/6J background and additionally backcrossed onto a B6 H-2^d background. Platelet and bone marrow donors will be tissue specific transgenic HOG mice on a B6 H-2^d background, whereas bone marrow donors will be on a C57BL/6J background.

Figure 1

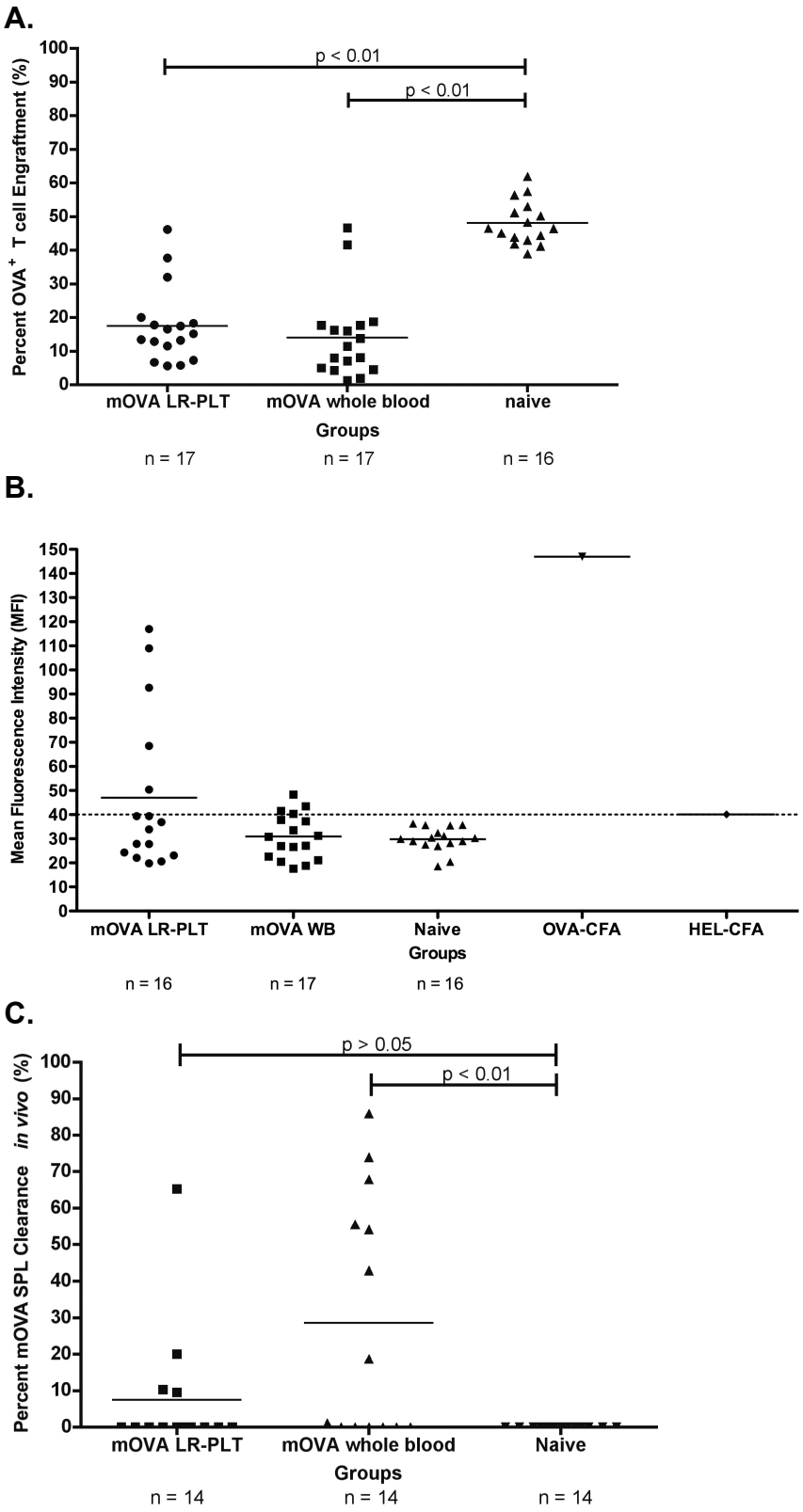
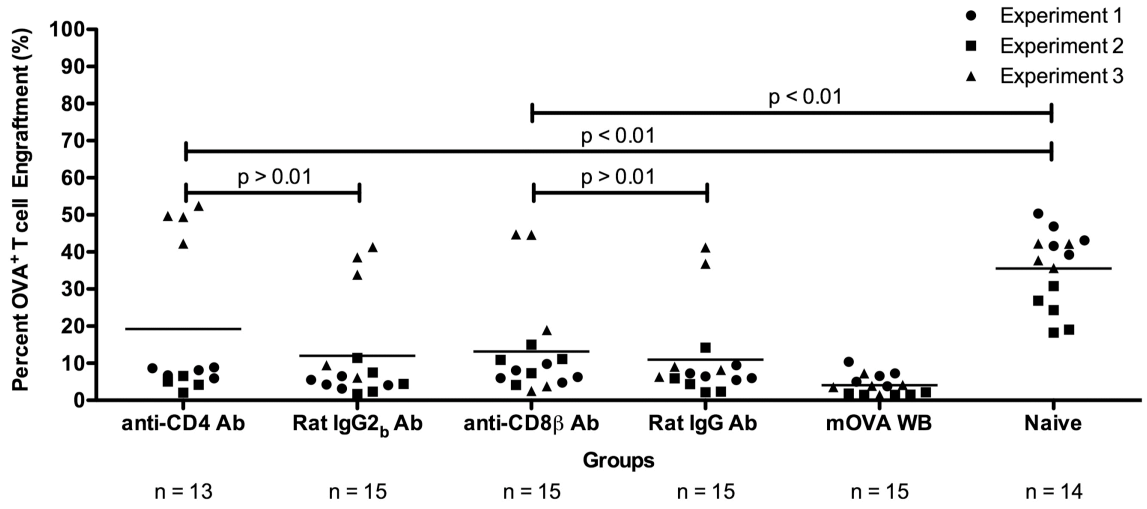

A.
p < 0.01
p < 0.01
Percent OVA+ T cell Engraftment (%)
mOVA LR-PLT
mOVA whole blood
naive
Groups
n = 17
n = 17
n = 16
B.
Mean Fluorescence Intensity (MFI)
mOVA LR-PLT
mOVA WB
Naive
OVA-CFA
HEL-CFA
Groups
n = 16
n = 17
n = 16
C.
p > 0.05
p < 0.01
Percent mOVA SPL Clearance in vivo (%)
mOVA LR-PLT
mOVA whole blood
Naive
Groups
n = 14
n = 14
n = 14

Figure 2

A.


Experiment 1
Experiment 2
Experiment 3
Percent OVA+ T cell Engraftment (%)
100
90
80
70
60
50
40
30
20
10
0
p < 0.01
p < 0.01
p > 0.01
p > 0.01
anti-CD4 Ab
Rat IgG2b Ab
anti-CD8β Ab
Rat IgG Ab
mOVA WB
Naive
Groups
n = 13
n = 15
n = 15
n = 15
n = 15
n = 14

B.

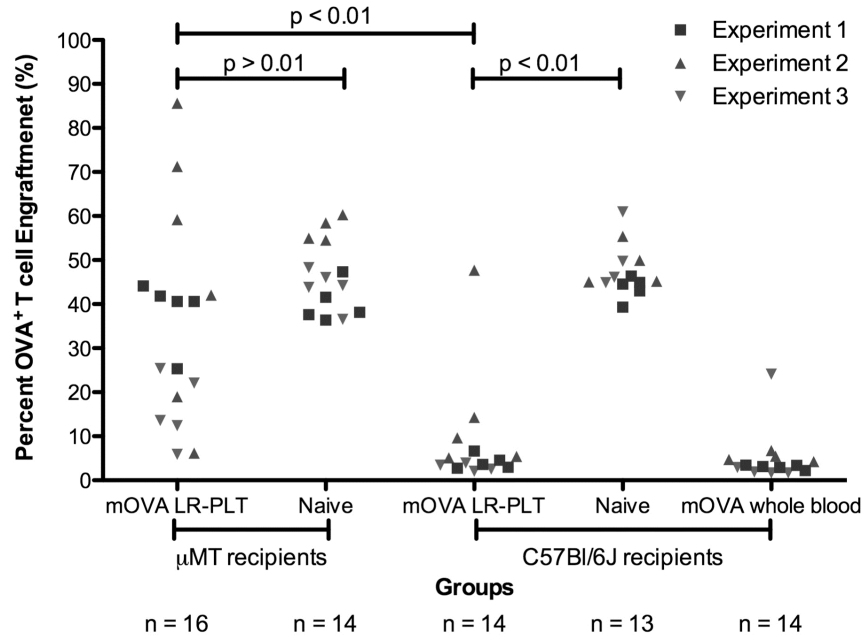
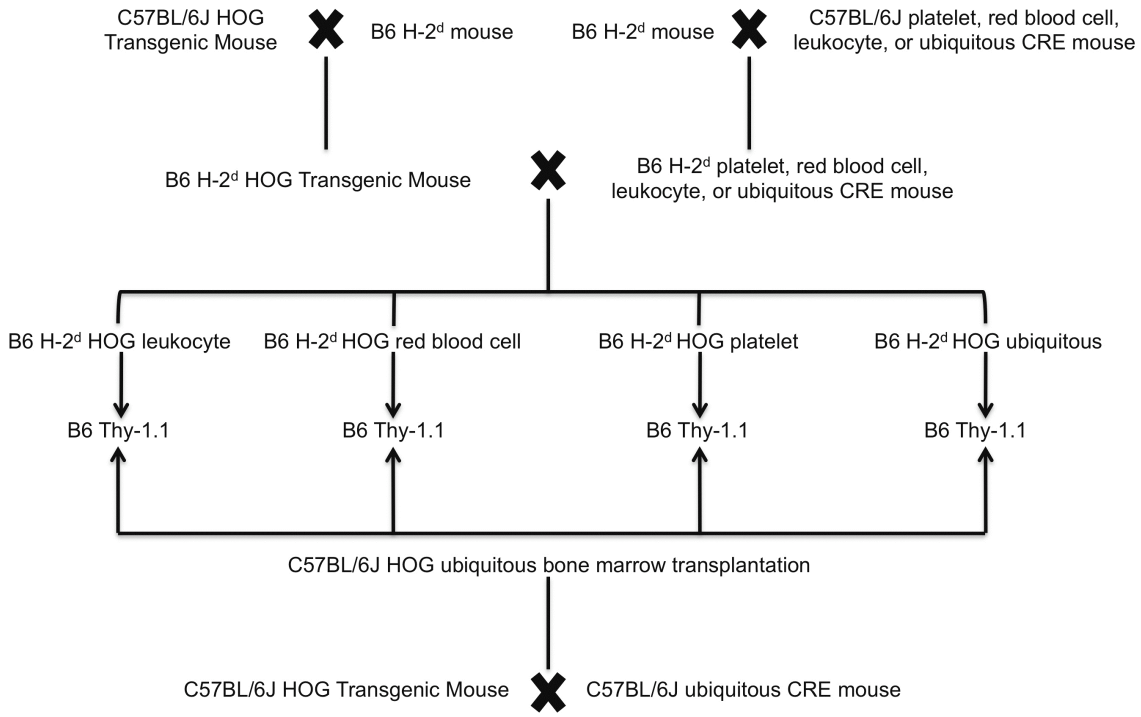

p < 0.01
p > 0.01
p < 0.01
Experiment 1
Experiment 2
Experiment 3
Percent OVA+ T cell Engraftmenet (%)
100
90
80
70
60
50
40
30
20
10
0
mOVA LR-PLT
Naive
mOVA LR-PLT
Naive
mOVA whole blood
μMT recipients
C57Bl/6J recipients
Groups
n = 16
n = 14
n = 14
n = 13
n = 14

Figure 3


C57BL/6J HOG Transgenic Mouse
B6 H-2d mouse
B6 H-2d mouse
C57BL/6J platelet, red blood cell, leukocyte, or ubiquitous CRE mouse
B6 H-2d HOG Transgenic Mouse
B6 H-2d platelet, red blood cell, leukocyte, or ubiquitous CRE mouse
B6 H-2d HOG leukocyte
B6 H-2d HOG red blood cell
B6 H-2d HOG platelet
B6 H-2d HOG ubiquitous
B6 Thy-1.1
B6 Thy-1.1
B6 Thy-1.1
B6 Thy-1.1
C57BL/6J HOG ubiquitous bone marrow transplantation
C57BL/6J HOG Transgenic Mouse
C57BL/6J ubiquitous CRE mouse