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Minor Antigens on Transfused Red Blood Cells in Immunity and Tolerance

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An abstract of

A dissertation submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

Immunology and Molecular Pathogenesis

2010

Abstract

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By Maxime Desmarets

HLA matched bone marrow transplantation (BMT) with reduced intensity conditioning is a cure for several non-malignant hematological disorders that require chronic transfusion, such as sickle cell disease and aplastic anemia. However, there are unusually high BMT rejection rates in these patients. Rejection correlates with the number of pre-BMT transfusions, and it has been hypothesized that pre-immunization to antigens on transfused blood primes BMT rejection. Moreover, the implementation of leukoreduction in many transplantation centers has not decreased the rejection rates for solid or bone marrow grafts. Leukoreduction reduces greatly the number of transfused leukocytes, but does not eliminate them. Therefore, the goal of this thesis is to investigate the effects of red blood cells on immune responses in the context of transplantation.

Using a novel mouse model of RBC transfusion and MHC matched BMT; we showed that transfusion of RBC products induced BMT rejection across minor histocompatibility antigen (mHA) barriers. While leukoreduction decreases the immunogenicity of RBC transfusion, it is insufficient to prevent BMT rejection. To study the immunogenicity of the RBC themselves, we generated a novel transgenic mouse with RBC specific expression of a model mHA: the HOD mouse. Using this model we demonstrated that transfusion of HOD RBCs does not induce rejection of mHA expressing BMT. On the contrary, adoptively transferred CD8+ T cells were activated upon stimulation by HOD RBCs but quickly decreased in number in the spleen, suggesting a deletional tolerance mechanism. Together, these data suggest that mHAs on RBCs themselves are not capable of inducing BMT rejection under the current conditions but nevertheless suggest a potential for involvement in modulating CD8+ T cell response.

Cellular immunization to mHAs is neither monitored nor managed by current transfusion medicine practice; however, the current data suggest that immune responses to mHAs on RBCs may represent an unappreciated and significant consequence of transfusion.

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“Blut ist ein ganz besonderer Saft”

– Mephistopheles in Goethe’s Faust

Acknowledgements

I would like to thank Jim Zimring, for being a wonderful advisor and a good friend during my time at Emory University. Thanks to the member of the Zimring lab for making the long hours spent in the lab so much fun. I would like to thank all of my family for their unwavering support, even in times of doubt. Unfortunately, my grandfathers were not able to witness the fruit of my and their labor; this work is dedicated to them. I would like to thank Patrick Mahoney, without whom none of this work would have been possible and for never letting me take myself too seriously. Special thanks go to Dominique Weber and Peter Jensen for believing in me when I needed it the most.

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Chapter 1

Introduction

Our knowledge of red blood cell (RBC) transfusion is extensive. It is built upon empirical data accumulated through centuries of practice. Every step of the process is understood in great detail. The collection of the blood is regulated by protocols to make the blood supply safer and more available for the recipients. Blood products are processed with strict procedures to separate each of its components, so as to extend the use of each blood unit. Hundreds of blood group antigens have been extensively characterized. Their structure and genetic variations are known. For many, the function of the protein is even known. Sophisticated methods of cross-matching have been developed to prevent hemolytic transfusion reactions. In some cases, immunization can be prevented as well. Yet, our understanding of why and how red blood cell antigens influence the immune responses in the recipient remain somewhat limited. Only a small fraction of transfused patients mount an antibody response against most blood group antigens, but the reasons why are still unclear. Transfusions have an immunosuppressive impact for some transplants and yet appear to induce rejection of others. What causes these differences?

During blood transfusions two incredibly complex systems collide, and the outcome of this encounter is somewhat uncertain. Ground rules have been discovered, but some of the most fundamental ones remain unknown. Some in the field think of blood transfusion as a liquid transplant. Indeed, tissues from a donor interact with the recipient immune system. Transfusion medicine research is now adopting the research methodologies of transplantation immunology research as well. Transplantation has benefitted from research in immunology based on animal models for decades, and great discoveries have been made. However, animal models present limitations as well. The

work presented herein is based on such animal models, and therefore should not be interpreted as more than what it is (i.e. it does not necessarily predict human biology). However, it is capable of modeling some of the aspects of the issues addressed, in particular, the influence of blood transfusion on subsequent bone marrow transplants, and whether red blood cell antigens can induce CD8+ T cell responses. A large population in the world is afflicted with non-malignant blood disorders. Currently, the only cure for these patients is bone marrow transplantation (BMT). Yet, it is becoming increasingly evident that the transfusion support that the disease requires may also hinder the chances of a successful subsequent transplant. With this work, we attempted to model the clinical situation in a mouse model and gain some insights in the cellular responses to red blood cell antigens.

Transfusion medicine

Historical considerations

William Harvey's research on blood circulation in the early 17th century led to the first attempts at blood transfusion, however all of them proved fatal. The first successful transfusion to a human was performed by French physician Jean-Baptiste Denis by transfusing two pints of lamb blood to a young man. The practice proved of course terribly dangerous and transfusions from animal to humans were prohibited about 10 years later, and the practice of transfusion fell out of favor for the next century.

In 1818, obstetrician James Blundell performed the first human to human blood transfusion between a wife and her husband to treat post-partum hemorrhage (1). Blundell also became the inventor of the first instruments dedicated to transfusion. A

transfusion using Blundell's 'gravitator' is shown in figure 1. The practice of blood transfusion remained relatively unchanged until the end of the century, but continued to be dangerous and sensational. A transfusion in Paris still made the news in the United States in 1874 (figure 2).

The science of transfusion medicine began to develop in the early 20th century with the discovery of the concept of separate blood types, and development of early forms of cross-matching blood by mixing the donor's and recipient's bloods. The year 1901 was a milestone in transfusion medicine with the discovery by Karl Landsteiner of the ABO blood group system, then called ABC system, for which he subsequently received the Nobel Prize for Medicine in 1930. His discovery paved the way for safer blood transfusions (2). In 1907 Ludvig Hektoen devised of an early form of cross-matching by mixing the blood of the donor and the recipient to detect 'incompatible mixtures.'

Modern era transfusion medicine unfolded with the invention of blood banking, which removed the need for donor and recipients to be in the same room. In 1916, United States Army officer Oswald Robertson is credited with creating the first blood bank or 'blood depot' while serving in France during World War I (3). In the 1930s however, the Soviet Union developed a system of national blood banks with the first true blood bank created in Leningrad in 1932.

In 1940, Joseph Edwin Cohn was the first to perform the separation of the three main blood products, Red Blood Cells, Platelets and Plasma, allowing the full use of each component. Albumin and fibrinogen also became available. At about the same time, Landsteiner *et al*. discovered the Rhesus system. It was then recognized to be responsible

for the majority of the transfusion-associated reactions. This was the second most important discovery in the history of blood transfusion immunology, and paved the way for the identification of many other blood group antigens in the future.

Advances in blood banking followed quickly thereafter, improving the storage and safety of blood products. A good example is the use of CPDA-1, which prolongs the duration of possible storage of blood products up to 35 days. The use of blood transfusions during surgeries and blood disorders has expanded dramatically since the end of World War II; and in 2006, 15 million units of blood were transfused in the United States alone.

Indications of RBC transfusion

The main indication for RBC transfusion is of course anemia. Most RBC transfusions are performed in preoperative care, and for patients in critical care units. Conditions associated with chronic anemia also require long-term transfusion support.

The 'trigger' for transfusion (i.e. the factors that motivate a physician to order blood) remains controversial. In the 1980s, it was common practice to transfuse patients whose hemoglobin had fallen below 10 g/dL and/or with a hematocrit below 30%, but it is becoming increasingly clear that these numbers are not a universal rule and specific conditions require modifications to the transfusion protocols. For example, it has been shown that intensive care patients are less at risk of myocardial infarction and congestive heart failure if hemoglobin levels are maintained slightly below 9 g/dL rather than above 10 g/dL(4). On the contrary, the risk of stroke in sickle cell disease children is reduced when using a more aggressive transfusion protocol (5). In recent years, research indicates

that, in most situations, patients benefit from a conservative transfusion trigger (i.e. hemoglobin lower than 9 g/dL) than from aggressive transfusion therapy (6).

Other factors impact the use of RBC transfusion. In the 1990s, the infectious risks associated with transfusion motivated a reduction of its indications. Thanks to technical advances in pathogen detection as well as leukoreduction, these risks have been greatly reduced. Because of this and of population ageing (percentages of transfused people increase with age), as well as the risks associated with insufficient transfusion, RBC transfusion use has been steadily increasing since the early 2000s (7).

Adverse effects of RBC transfusion

There are numerous potential adverse effects associated with blood transfusions. They fall into two main categories: 1) Infectious complications, and 2) noninfectious complications also called non-infectious serious hazard of transfusion (NISHOT). Infectious complications include Hepatitis B, HTLV I and II, Hepatitis C, HIV 1 and 2, Malaria, and bacterial contaminations (6). Screening of the blood supply for these pathogens has decreased the risk of transmission greatly, and infectious complications of transfusion are now much less prevalent than NISHOTs in developed nations. In recent years however, emerging pathogens such as Babesia, Trypanosoma, and Dengue, are becoming a source of concern (8). Finally, the prion transmitted variant Creutzfeldt-Jakob disease has been confirmed as a possible risk of transfusion (9).

NISHOTs cover a wide array of adverse effects of various natures, but can be divided into immune reactions and non-immune reactions. For example, a major non-immune adverse effect is caused by the iron overload associated with transfusion.

Chronically transfused patients are at high risk for iron overload, and may develop, endocrine dysfunction (growth retardation, diabetes, hypothyroidism, and hypogonadism), and cardiac disease (6, 10). Iron overload has also been shown to correlate with a higher risk of bone marrow transplant failure (11, 12). As the incidence of infectious adverse effects have been decreasing over the last decades, immune adverse reactions to blood transfusions have attracted more attention. They include hemolytic transfusion reactions (HTR), febrile nonhemolytic transfusion reactions (FNHTR), allergic/urticarial/anaphylactic transfusion reactions, transfusion-related acute lung injury (TRALI), posttransfusion purpura , transfusion-associated graft versus host disease (TA-GvHD), microchimerism, transfusion-related immunomodulation (TRIM), and alloimmunization. FNHTRs, allergic reactions and alloimmunization are by far the most common of these reactions (6, 10). Alloimmunization occurs in 2-8% percent of all transfused patients (13-15) and has been identified as an obstacle to bone marrow transplantation (16-21), and more recently a role for alloimmunization has been suggested for solid organ transplant rejection (22).

Humoral alloimmunization to RBC transfusion

Humoral alloimmunization is the production of antibodies against alloantigens expressed by transfused components. In the case of red blood cell transfusions, patients can become alloimmunized to blood group antigens. The most immunogenic antigen is Rhesus (Rh) D. Transfusion with D positive blood induces anti-D antibodies in 30 to 80% of D negative recipients (alloimmunization rates are much lower than these numbers thanks to the implementation of blood phenotyping and cross-matching techniques) (23-

25). Repeat transfusions with incompatible blood can lead to potentially lethal hemolytic transfusion reactions. Overall, humoral alloimmunization is one of the most studied aspects of transfusion medicine research because of its high clinical significance.

The mechanisms that regulate alloimmunization are not well understood. Until recently the lack of murine models has limited our analysis of alloimmunization mechanisms to mostly retrospective studies. It is known that there is some level of HLA restriction in particular for antigens in the Duffy (Fy), Kell (K) and Kidd (Jk) systems (26, 27). Also, the basic immunologic steps in developing an antibody to red blood cells are well understood, but the determinants that govern the emergence of antibodies to RBC antigens are still poorly understood. Nevertheless, the recent development of murine models, and their popularization in the transfusion research community, is now poised to change this. Indeed, clusters of novel findings have recently emerged. It has been demonstrated that even in genetically identical and gender matched recipient mice, exposure to a model blood group antigen results in some animals that make antibodies and some that don't (i.e. responder vs non-responder just as in humans). This suggests that there are also environmental factors involved in alloimmunization (28, 29). One such environmental factor emerging in current research is the inflammation of the recipient at the time of transfusion (30-33). Overall, humoral alloimmunization is one of the most studied aspects of transfusion medicine research because of its high clinical significance.

Cellular alloimmunization to RBC transfusion

In the eyes of transplantation biologists, RBC antigens represent potential minor histocompatibility antigens and it is known that transplant rejection driven by minor

antigens rely on antibody and cellular responses (see minor antigens in transplantation section below). Although the importance of immunity to transfused blood is well appreciated as a factor of transplant rejection (16-21), the transfusion research community has perhaps surprisingly under-appreciated cellular responses to red blood cells. This is likely due to the fact that RBC antibodies are what makes locating antigen negative RBC units to transfuse to the immunized patient difficult and antibodies are the cause for hemolytic reactions, not cellular immunity. These two situations represent major concerns in the practice of transfusion medicine. On the other hand, RBC induced cellular immunity in general, and CD8+ T lymphocyte responses have not been clearly demonstrated to occur and, even if they did, they would have no known consequences in the field of transfusion medicine.

Even though the transfusion research community does not fully appreciate the importance of cellular immunity to RBC transfusion, in the context of transplantation it is reasonable to investigate whether cellular responses to RBC transfusion occur; indeed, the potential for such responses has recently been demonstrated. Zimring *et al*. have indicated the possibility for cross-presentation of RBC associated antigens to recipient CD8+ T cells (34).

Leukoreduction

White blood cells (WBC) are removed from blood products through a process called filter leukoreduction. In the United States, most blood products are leukoreduced before storage of the unit. Prestorage leukoreduction started being implemented at the instruction of the Blood Products Advisory Committee (BPAC) to the US Food and Drug

Administration (FDA) in 1998 (35). However, both leukoreduced and non-leukoreduced blood products remained FDA approved, so more scientific data about both types of products can be acquired. Since then, implementation of leukoreduction has been expanding in the United States. As of today, most Western European nations have chosen to implement universal leukoreduction of blood products. Modern leukoreduction filters remove 4-5 log of the leukocytes from the blood; however, some residual WBCs remain. The American Association of Blood Banks requires that no more than 5×10^6 residual be present per unit transfused (36). In Europe, this limit was set at 1×10^6 per unit (37).

Convincing data has shown that leukoreduction of blood products reduces the numbers of FTRs (38, 39), HLA immunization rates (40), and transfusion related CMV transmission (41). However it is unclear whether leukoreduction has modified the rates of alloimmunization to blood group antigens or platelet antigens (HPA) (40), and it is not effective in preventing transfusion associated graft versus host disease (6).

Evidence suggests that leukoreduction has not reduce the incidence of graft rejection in solid organs (42) or bone marrow transplantation (18, 43).

Bone marrow transplantation in non-malignant blood disorders

Non-malignant blood disorders are a disparate group of diseases that involve deficient bone marrow function. Their origin is genetic, autoimmune, or can be multifactorial. Sickle cell anemia and thalassemia, in which the synthesis of hemoglobin is impaired, are the most common single gene disorders (44, 45). Autoimmune non-malignant blood disorders are mostly represented by aplastic anemia (46). Diamond-Blackfan anemia is a rare congenital erythroid specific disorder with a poorly understood

pathophysiology (47, 48). Finally, lysosomal storage disorders, such as Tay-Sachs disease and Gaucher disease, are a group of rare genetic diseases that cause anemia because of a metabolic defect that affects RBCs, in addition to many other systems (49, 50).

Bone marrow transplantation is currently the only known cure for the non-malignant blood disorders mentioned above, as it replaces the defective bone marrow with healthy hematopoietic stem cells. Until recently the risk associated with BMT for non-malignant blood disorders (sickle cell disease in particular) was too high. Recent advances in conditioning regimens and supportive care have reduced this risk, and the long-term survival rates in thalassemia and sickle cell disease after BMT is about 90% (11, 45). This has renewed the interest in bone marrow transplant for sickle cell anemia. Nevertheless, bone marrow transplants have largely remained reserved for patients with severe or high-risk disease. Very few transplants have been performed in adults with these disorders because of the higher transplant morbidity and mortality compared to children.

The main barrier to BMT in sickle cell disease is that only a small fraction of patients have access to a matched sibling donor (51), and results using HLA mismatched donors are poor, with increased rates of GvHD, infections and graft rejection (45). Recently, some have sought to increase the accessibility of BMT for sickle cell disease patients by using unrelated cord blood stem cells with some success (52). This was made possible because cord blood is known to decrease the risk of GvHD. However, these successes have been dampened by the increased risk of early fatal complications, because

of the lower engraftment rate, delayed kinetics of neutrophil recovery, and the lack of transfer of pathogen-specific memory T cells (53).

Reduced intensity condition regimens are now increasingly used to reduce transplant related mortality, and under the rationale that only partial chimerism is sufficient to cure the underlying disease (54, 55). However, this has been met with mixed successes because of high graft rejection rates. It appears that putting the focus on myeloablation results in transient chimerism. Some also have suggested that the high rejection rates were due to pretransplant transfusion exposure, and that more intense conditioning regimens would be required for these patients. It is hoped that future studies putting the emphasis on more immunosuppression will result in sustained chimerism and maintain the low mortality risk (45). If that is the case, bone marrow transplantation could be offered to patients with lower risk disease and possibly prior to transfusion exposure.

Minor histocompatibility antigens in transplantation

Minor histocompatibility antigens

The concept of minor histocompatibility antigen (mHA) was discovered after graft rejection occurred during transplants between MHC identical mice (56) and later, in humans, after transplants between MHC identical siblings (57, 58). It was hypothesized that non-MHC molecules, were capable of inducing transplant rejection, albeit at a slower pace than MHC molecules. It was demonstrated that proteins that are polymorphic within a species could become minor antigens in a transplant situation. Peptides from these proteins once processed by the antigen presentation machinery of APCs can be presented

to CD8+ and CD4+ T cells by MHC class I and II molecules, respectively. Immune responses to mHAs are carried out by cytolytic CD8+ T lymphocytes but CD4+ T cell help is required to mount a response (59). Immune responses to mHAs are generally weaker than MHC responses and require prior immunization to be measured in vitro (MHC disparate mixed lymphocytes reactions do not require this), the immunization increases the precursor frequency of mHA specific T cells to a level sufficient to be measured in vitro. A prior immunity to mHAs also accelerates graft rejection.

Theoretically all polymorphic proteins could give rise to mHAs, but in practice there are several mechanisms that limit the numbers of mHAs that can induce a significant response *in vivo*. During a transplant with many minor mismatches, only a few immunodominant antigens induce CD8+ T cell clones to proliferate selectively, while others appear briefly or not at all. The rules of immunodominance are poorly understood, but the phenomenon gives hope that mHA mismatched transplant rejection could be controlled by focusing efforts of tolerance induction on the few immunodominant mHAs involved in the particular donor/recipient pair. Many mHAs have already been identified and well characterized in mice and in humans and it is already possible to predict some of the mismatches that exist between donor and recipient.

One of the most dramatic effects of mHAs is the GvHD that they cause during BMT. GvHD can be fatal. mHAs also mediate the beneficial graft versus leukemia effect in malignant blood disorders. mHA driven BMT rejection is less well documented in humans, but it is well established in mice.

Effector mechanisms of bone marrow graft rejection differ depending on previous immunization to mHA. In naïve mice, CD8+ T cells reject bone marrow transplants in a

Fas/FasL-Granzyme-Perforin dependent manner. In previously immunized mice, CD8+ T cells can reject mHA-mismatched bone marrow by a separate mechanism that does not require Perforin or Fas, but this mechanism remains poorly understood (60). While it is clear that antibodies are an important mediator in MHC mismatched BMT (61, 62), several studies of mHA mismatched BMT rejection have failed to detect antibodies to mHAs in MHC matched BALB.B into B6 transplants (60, 63) and underline the importance of CD8+ T cells responses in the rejection of such grafts.

Pregnancies and transfusion can induce immunization to mHAs. Studies have shown that multiparity in mice and humans can induce immunization to HY or male antigens, human studies have also shown immunity to HA-1 and HA-2 (64, 65). However, the implications on bone marrow transplantation are still unclear (66). While studies have shown that transfusion prior to MHC matched bone marrow transplantation influence rejection in canine models and in humans, it is not known which specific mHAs are the target of the immunity causing BMT rejection. Blood group antigens from the ABO system have been shown to have great influence on the outcome of kidney, liver and heart transplants, but appear to be less important in BMT. A role for other blood groups such as Duffy and Kidd has been also suggested (22). As mentioned above, the recent implementation of leukoreduction for blood products is another confounding factor in the understanding of immunity to RBC transfusion and its consequences on transplantation.

Allorecognition of minor histocompatibility antigens

Immune responses to minor antigens are a two-step process. First the antigen needs to be recognized by the alloreactive T cell before activating and mounting an alloresponse. Allorecognition of minor antigens can occur following two different mechanisms: 1) direct presentation, and 2) indirect presentation. A third recently described presentation pathway combines some of the characteristic of both pathways (67).

The direct presentation pathway is the recognition of the MHC-peptide complex presented by a donor antigen-presenting cell (APC). Foreign minor antigen derived peptides from intracellular proteins as well as from exogenous proteins acquired by taking up necrotic and/or apoptotic cellular material from donor cells are presented into MHC class I and II. In the context of a transplant direct recognition can occur at the time of the first contact of the T cell and the APC as peptides have already been processed and are readily presented to the T cells. As a consequence, the direct pathway of antigen presentation tends to dominate in the early phase of the transplant.

It is important to note that in MHC disparate transplant situations, minor antigens cannot be directly presented to recipient T cells. In this situation, the allorecognition of the MHC component of the MHC-peptide complex will take precedence and most of the alloresponse will be directed against the MHC molecules. However, this statement must be nuanced by the fact that T cell receptors have a certain degree of promiscuity and may be capable of recognizing the same peptide although they are restricted to different MHC molecules. It is particularly true for MHC II molecules. As an example, it has been shown

that both the H-2^d restricted DO11.10 T cells and the H-2^b restricted OT-II T cells can recognize the $OVA_{323-339}$ peptide presented by I-A^d (68).

Indirect presentation is defined by the recognition of foreign peptide presented by the MHC molecules of the recipient on its own APCs. In this situation, foreign cellular debris from donor cells must be taken up and degraded by the recipient APCs into peptides loaded onto MHC molecules. In general, intracellular proteins are presented into MHC I and exogenous antigens onto MHC II. In theory, indirect allorecognition can only occur with CD4+ T cells and in a transplant situation CD4+ T cell response should dominate. However, a process known as cross-presentation enables APCs to present exogenous peptides into MHC I and therefore to induce a CD8+ T cell alloresponse also. The indirect allorecognition pathway requires antigen processing and presentation *de novo* that begins after the transplant has occurred. As a consequence, responses driven by allorecognition are slightly delayed compared to direct allorecognition and the indirect pathway of antigen presentation tends to dominate later during the transplant, after the donor APC have been eliminated by the early, direct presentation driven alloresponse. Direct and indirect allorecognition of mHAs are illustrated in figure 1.3.

A third pathway has also been recently identified: semi-direct presentation. Recent publications have shown that full MHC molecules can be transferred from donor APCs to recipient APCs, and the cells that have received the MHC molecules can now stimulate T cells to generate an alloresponse (69). The mechanisms of this presentation pathway are not fully understood but likely involve cell-cell contacts (70), and may also occur as a consequence of uptake of small vesicules or exosomes released by the donor APCs (71). The significance of the semi-direct pathway is still being determined, and it is

not know whether mHAs carried by transfused red blood cells can be presented in such a way. It is also not known which pathway contributes more to the alloresponse to minor antigen carrying blood transfusions, and it is likely that the relative contribution of each pathway will be different during the transfusion of whole blood and that of leukoreduced blood since the numbers of donor APCs are reduced by a 4 to 5 log.

Cross-priming

Intracellular antigens are usually presented onto MHC I, and exogenous antigens on MHC II. These classical presentation pathways however do not explain how it is possible to mount an immune response to a virus that does not infect APCs. Michael Bevan proposed a 'cross-presentation' mechanism by which exogenous antigens can be redirected to be presented into MHC I molecules. Cross-priming is the activation of CD8+ T cell by antigens cross-presented by recipient APCs (72). Professionals APCs, such as dendritic cells are the main cell type capable of cross-presentation, but macrophages, B cells, and liver sinusoidal endothelial cells are also capable of cross-presenting antigen. However, the outcome of cross-presentation by liver endothelial cells is antigen-specific T cell tolerance (73-75).

The significance of this mechanism was not immediately apparent, and it was first thought to be an obscure mechanism that had little importance in CTL activation. It is now well understood, and cross-presentation is perceived as a major pathway involved in the immunity to infectious diseases as well as an essential mechanism in monitoring tissues for the expression of foreign antigens. In transfusion, however, its role is largely

unappreciated. This is consistent with the lack of research related to CD8+ T cells responses to red blood cell transfusions.

Cross-tolerance

Cross-presentation is not only essential in mounting CTL responses to foreign antigens it is also involved in the induction of tolerance. Kurts *et al*. first observed that cross-presentation of self-antigens induces the deletion of CD8+ T cells in the periphery and they named the mechanism 'cross-tolerance' (76, 77). The mechanism has been found to be essential in maintaining CD8+ T cell tolerance to peripheral tissues. However, all of these models were based on the use of transgenic T cells. Luckashenak *et al*. recently demonstrated, in a model that lacks TCR transgenic cells, that mice deficient in cross-presentation accumulate autoreactive CD8+ T cells and are more susceptible to autoimmune responses than wild type mice (78).

Recently, Griffith *et al*. have observed that the cross-tolerance is not limited to self-antigens and autoimmunity. Injections with apoptotic cells can also induce tolerance to foreign antigens under some circumstances (79). It is important to note that injections with donor apoptotic cells do not always result in tolerance, as Li *et al*. observed that treatment with apoptotic leukocyte could limit the levels of engraftment of bone marrow after reduced intensity conditioning regimen in mice (80). Cross-priming is a double-edged sword, and a better understanding of the requirements for tolerance and immunity is necessary.

The field of transfusion is no stranger to double-edged sword phenomena, as it is well known in the transfusion community that transfusion can not only lead to immunity

(in the form of antibodies to blood group antigens) but also to tolerance. Donor specific transfusion has been used since the 1970s to induce tolerance in kidney transplants (81). Transfusion related immune modulation is a highly controversial topic in the transfusion community but suggests that at least some of the time blood transfusions can induce tolerance in the recipient (82, 83). Is it possible the Jekyll/Hyde nature of cross-priming itself provides an explanation for this dichotomy?

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Figure Legends

Figure 1.1. A page from the June 13, 1829 issue of the Lancet with the article "Observations on transfusion of blood" by James Blundell (1790-1877) with illustration showing a blood transfusion using the Gravitator an instrument of his invention.

Figure 1.2. Hand colored engraved image titled "The transfusion of Blood – An operation at the Hôpital de la Pitié, at Paris." drawn by Miranda from Harper's Weekly. Shows scene of doctors performing a blood transfustion. Harpers Weekly, July 4, 1874.

Figure 1.3. Pathways of allorecognition involved in RBC transfusion and alloresponse effector mechanisms involved in minor histocompatibility antigen driven transplant rejection. Minor antigens on transfused RBC units can be presented to the recipient's CD4+ and CD8+ T cells after processing and presentation in the MHC II presentation pathway as well as the MHC I compartment through cross-presentation. In leukoreduced RBC units, direct presentation of minor antigens is possible for residual leukocytes cells carrying at least MHC I. However, professional antigen presenting cells such as dendritic cells are required for direct presentation of minor antigens to CD4+ T cells. Theoretically, platelets and RBCs may be involved in direct presentation as they have been demonstrated to carry some level of MHC I, but their exact contribution is unknown. Once activated CD4+ T cells provide the necessary help for efficient B and CTL response. B cells specific for the protein from which the minor antigen derives may receive help from CD4+ T cells through linked recognition.

THE LANCET.

Vol. II.] LONDON, SATURDAY, JUNE 13. [1828-9.

OBSERVATIONS ON TRANSFUSION OF BLOOD.

By Dr. Blundell.

*With a Description of his Gravitator.**

States of the body really requiring the infusion of blood into the veins are probably rare; yet we sometimes meet with cases in which the patient must die unless such operation can be performed; and still more frequently with cases which seem to require a supply of blood, in order to prevent the ill health which usually arises from large losses of the vital fluid, even when they do not prove fatal.

In the present state of our knowledge respecting the operation, although it has not been clearly shown to have proved fatal in any one instance, yet not to mention possible, though unknown risks, inflammation of the arm has certainly been produced by it on one or two occasions; and therefore it seems right, as the operation now stands, to confine transfusion to the first class of cases only, namely, those in which there seems to be no hope for the patient, unless blood can be thrown into the veins.

The object of the Gravitator is, to give help in this last extremity, by transmitting the blood in a regulated stream from one individual to another, with as little exposure as may be to air, cold, and inanimate surface; ordinary venesection being the only operation performed on the person who emits the blood; and the insertion of a small tube into the vein usually laid open in bleeding, being all the operation which it is necessary to execute on the person who receives it.

The following plate represents the whole apparatus connected for use and in action:—

* The instrument is manufactured by Messrs. Maw, 55, Aldermanbury.

Tab. 1.

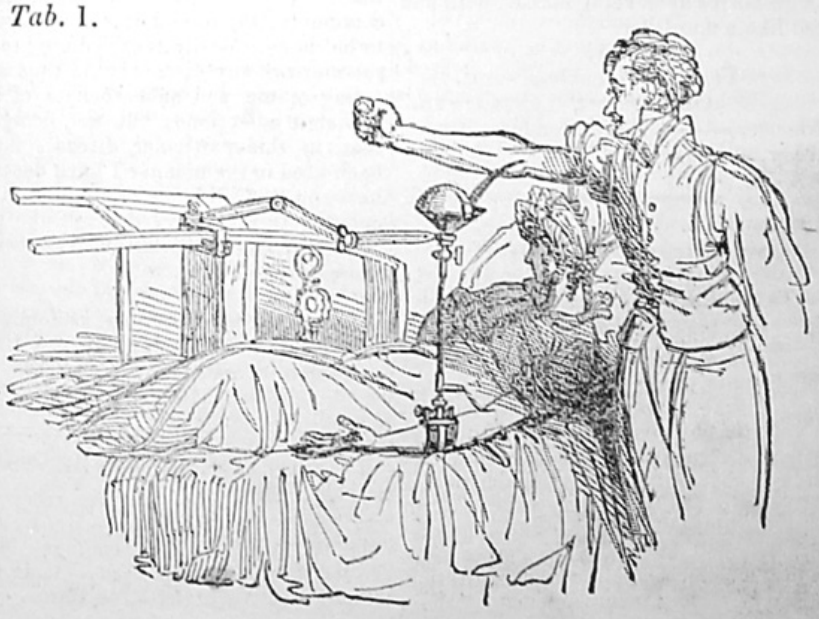

Figure 1.1

Figure 1.2

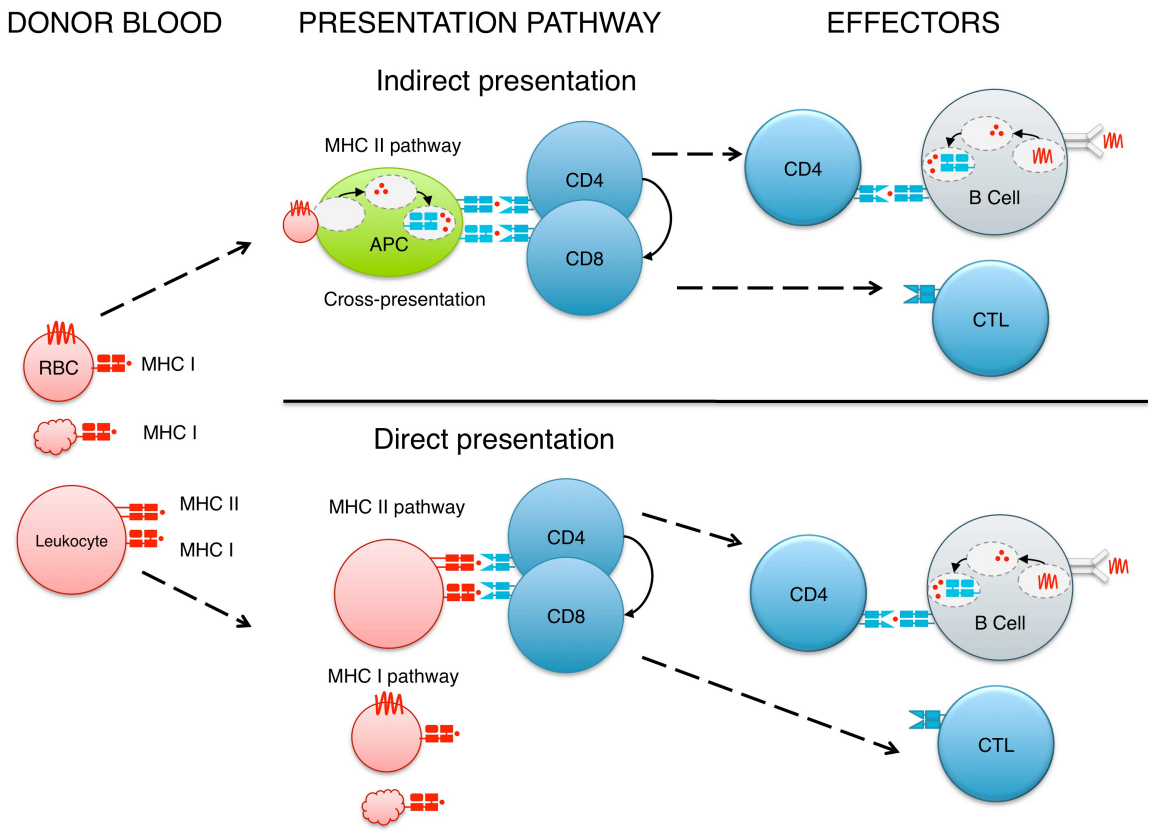
DONOR BLOOD
PRESENTATION PATHWAY
EFFECTORS
Indirect presentation
MHC II pathway
APC
CD4
CD8
Cross-presentation
CD4
B Cell
CTL
RBC
MHC I
MHC I
Leukocyte
MHC II
MHC I
Direct presentation
MHC II pathway
CD4
CD8
MHC I pathway
CD4
B Cell
CTL

Figure 1.3

Chapter 2

Minor Histocompatibility Antigens On Transfused Leukoreduced Units of Red Blood Cells Induce Bone Marrow Transplant Rejection in a Mouse Model

Abstract

When successful, HLA matched bone marrow transplantation (BMT) with reduced intensity conditioning is a cure for several non-malignant hematological disorders that require chronic transfusion, such as sickle cell disease and aplastic anemia. However, there are unusually high BMT rejection rates in these patients. Rejection correlates with the number of pre-BMT transfusions, and it has been hypothesized that pre-immunization to antigens on transfused blood may prime BMT rejection. Using a novel mouse model of RBC transfusion and MHC matched BMT, we report that transfusion of RBC products induced BMT rejection across minor histocompatibility antigen (mHA) barriers. It has been proposed that contaminating leukocytes are responsible for transfusion-induced BMT rejection; however, filter leukoreduction did not prevent rejection in the current studies. Moreover, we generated a novel transgenic mouse with RBC specific expression of a model mHA, and demonstrated that transfusion of RBCs induced a CD8+ T cell response. Together, these data suggest that mHAs on RBCs themselves are capable of inducing BMT rejection. Cellular immunization to mHAs is neither monitored nor managed by current transfusion medicine practice; however, the current data suggest that mHAs on RBCs may represent an unappreciated and significant consequence of RBC transfusion.

Introduction

When successful, bone marrow transplantation (BMT) is a cure for non-malignant hematological disorders such as sickle cell disease (SSD), aplastic anemia, Diamond-Blackfan anemia, and Fanconi anemia (1-8). Currently, most BMT is performed in the context of treating neoplasia, and stringent recipient conditioning is used to attack the malignancy. In this context, BMT rejection is now a rare event, as the conditioning regimens essentially eliminate recipient immunity. However, for disorders in which no malignancy is being treated (e.g. SSD), it is difficult to justify the use of stringent myeloablative conditioning regimens, which can lead to substantial morbidity and mortality from toxic side effects. Accordingly, it is highly desirable to develop reduced-intensity BMT conditioning regimens that minimize toxic side effects.

Unfortunately, current reduced-intensity regimens result in significant rates of BMT rejection in patients with non-malignant hematological disorders (9-12). One factor that potentially contributes to rejection is the requirement for chronic transfusion therapy. There is a strong correlation between the number of transfusions a patient has received and the likelihood of BMT rejection (13-15). Moreover, canine studies have demonstrated that transfusion of peripheral blood from bone marrow donors prior to transplant can induce BMT rejection in otherwise healthy animals (16-18). Together, these data are most consistent with the hypothesis that transfusion primes the recipient to reject bone marrow through immunization to donor antigens. Because the donors and recipients are MHC matched (both in the human trials and animal studies), immunization against minor histocompatibility antigens (mHAs) is most likely responsible for the rejection (19, 20).

Leukocytes present in the transfused blood are traditionally believed to be the source of immunization against both HLA antigens and mHAs. However, implementation of universal leukoreduction in multiple centers has failed to decrease the rates of BMT rejection in multiply transfused patients (21, 22). Transfusion standards require that leukoreduced red blood cells (RBCs) contain fewer than 5×10^6 leukocytes per unit in the United States (23) and fewer than 1×10^6 leukocytes per unit in Europe (24). Hence, leukoreduction decreases but does not eliminate leukocytes; thus, it remains possible that the residual leukocytes are responsible for BMT rejection. However, an alternate hypothesis is that exposure to mHAs on the RBCs themselves can lead to recipient immunization through processing and presentation of mHAs by recipient APCs, leading to BMT rejection. To test this hypothesis, we engineered a mouse model of leukoreduced blood transfusion and MHC identical–mHA mismatched BMT.

Herein, we report that transfusion of leukoreduced RBC units between MHC identical–mHA mismatched mice induces subsequent BMT rejection. Although such rejection could be due to direct presentation of mHAs on residual leukocytes, we demonstrate that leukoreduction is sufficient to prevent transfusion-induced BMT rejection due to antigens expressed on leukocytes but not RBCs. Using a novel transgenic mouse with RBC specific expression of a model antigen, the HEL-OVA-Duffy mouse (HOD mouse), we report that mHAs on transfused RBCs undergo crosspresentation with subsequent expansion of recipient CD8+ T cells specific for the transfused mHA. Together, these findings demonstrate the potential of mHAs on RBCs to induce sufficient immunity to reject a subsequent BMT.

Materials and Methods

Mice

C57BL/6 (B6), BALB/c, B6.PL-Thy1^a (B6.Thy1.1), C.B10-H2b (BALB.B) and B6.C-H2d (B6.H2d) mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and housed in Emory University Department of Animal Resources facilities. Mice were 8 to 12 weeks of age, and all procedures were performed according to approved IACUC protocols. HOD mice were created as described. HOD and mHEL (25) mice were bred by the Emory Division of Animal Resources Animal Husbandry service (mHEL mice are available from Jackson Laboratories).

Generation and characterization of the HOD mouse

The whole open reading frame for HEL (including the N terminal signal sequence) was amplified by PCR. Primers were designed to insert a BamHI site on the 5' end, engineer a Kozak consensus sequence upstream of the start codon, remove the stop codon, and insert a SmaI site at the 3' end (5'- TCG GAT CCG CCA CCA TGA GGT CTT TGC TAA TCT T-3'; 5'- GGC CCG GGC AGC CGG CAG CCT CTG ATC C -3'). The portion of the OVA open reading frame (ORF) encoding amino acids 251-349 was amplified with primers that inserted a SmaI site on the 5' end (in frame with the HEL ORF), removed the stop codon, and inserted an XhoI site on the 3' end (5'-GGC CCG GGG GCC TTG AGC AGC TTG AGA G -3'; 5'- GGC TCG AGC ACT CCA GCC TCT GCT GAC CC -3'). Finally, the complete ORF for Duffy was amplified with primers designed to insert an XhoI site on the 5' end in frame with the start codon and insert an XbaI site on the 3' end after the stop codon (5'- TGA ACT CGA GAT GGC

CTC CTC TGG GTA TGT CC-3; 5-CTC TAG ACT AGG ATT TGC TTC CAA GGG TGT -3'). Plasmids containing cDNA templates for amplification for HEL and OVA were generous gifts from Dr. Judith Kapp (University of Alabama at Birmingham) and Dr. Emil Unanue (Washington University, St. Louis, MO). Human Duffy was amplified from a Quick-Clone cDNA library synthesized from human bone marrow (Clontech, Mountain View, CA). PCR was carried out with high fidelity pfu enzyme (Stratagene, Cedar Creek, TX), the amplified fragments were ligated into pGEM-T Easy (Promega, Madison WI), and the inserts were sequenced in both directions. The HOD cDNA was assembled through sequential ligation in pGEM-T Easy. The complete ORF was reamplified with the 5' HEL primer and a new 3' Duffy primer designed to introduce a BglII site in the 3' end, and complete sequencing was carried out. The completed HOD ORF was excised by digestion with BamHI and BglII and was ligated into (μ'LCR β pr delta Nco Bgl) that was digested with BglII. Recombinants were isolated and orientation was confirmed through sequencing. The final construct was linearized and bacterial elements were removed through digestion with ClaI and SacII. The linearized construct was purified and submitted to the Emory Core Transgenic Facility for pronuclear injection. Founder pups were screened by PCR and subsequently characterized by flow cytometry.

HOD expression was assessed by staining peripheral blood with polyclonal murine antisera to HEL or OVA generated through immunizing B6 mice with HEL or OVA protein. Expression of Duffy antigens was assessed using human antisera to Fy^a or Fy^b, or monoclonal anti-Fy^3 (MIMA-29, generous gift from Marion Reid and Greg Halverson at the New York Blood Center). APC conjugated allospecific anti-$IgG1^b$ or

anti-Human Ig (BD Biosciences, San Jose, CA) was used as secondary antibodies. Expression on leukocytes or platelets was analyzed by also staining with anti-CD45, anti-CD41, and anti-TER-119 (BD Biosciences). Leukocytes were defined as $CD45^{+}TER\text{-}119^{-}$ events and PLT as $CD41^{+}TER\text{-}119^{-}$ events. Flow cytometry was carried out using a BD FACSort and data was analyzed using FlowJo software (TreeStar, Ashland, OR).

Leukoreduction of mouse blood

Mouse blood was obtained by retro-orbital bleeding into anticoagulant containing adenine, citrate, and dextrose (ACD Solution A, BD, Franklin Lakes, NJ). RBCs were washed with phosphate buffered saline (PBS) adjusted to 340 mOsm with NaCl (modified PBS or mPBS) and leukoreduced over a neonatal leukoreduction filter (Purecell Neo; Pall Medical, East Hills, NY) previously primed with 12 ml of mPBS. The blood was then washed 3 times with mPBS to remove platelets. Each transfusion consisted of 100 μl of packed RBCs diluted with 400 μl PBS injected in the tail vein.

Enumeration of residual WBCs

Residual WBCs in the leukoreduced blood were enumerated by flow cytometry using the method previously described (26). Briefly, a 25 μl aliquot of blood was stained using 150 ng/ml Propidium Iodide (PI) (Invitrogen, Carlsbad, CA) in a 1 g/l sodium citrate dihydrate solution with 0.7% Zap-oglobin II reagent (Beckman Coulter, Fullerton, CA), 0.7% RNAse cocktail (New England Biolabs, Ipswich, MA), 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).

Bone marrow transplantation

Whole bone marrow was prepared from the femur and tibia of donor mice. For BALB.B bone marrow transplantation, B6.Thy1.1 recipients were irradiated with a dose of 650 cGy, on the same day 5 x 10^6 whole bone marrow cells from BALB.B donor were injected intravenously. For HY mismatched bone marrow transplantation, female B6.Thy1.1 recipients were irradiated with a dose of 500 cGy and injected on the same day with 5 x 10^6 whole bone marrow cells from male B6 donors.

Bone marrow engraftment analysis

Bone marrow engraftment was monitored by flow cytometry. Peripheral WBCs were stained with FITC labeled anti-CD3, PE labeled anti-Thy1.2 (BD Biosciences), and APC labeled anti Thy1.1 (eBioscience, San Diego, CA) antibodies. Percent donor engraftment was calculated using $Thy1.1^+$ events / ($Thy1.1^+$ events + $Thy1.2^+$ events) x 100. Engraftment was defined as a sustained chimerism of greater than 5%, 6 weeks after transplant. The use of CD45.1/CD45.2 markers was purposely avoided in our system, as CD45.1/CD45.2 has been reported to be an mHA capable of inducing rejection (27).

In vivo killing assay

In vivo killing assays were performed according to a method adapted from Barber D, et al (28). Briefly, RBC lysed spleen cells from B6 and B6.H2d mice were labeled with a

different concentration of CFSE: 3 μM (B6 control population) or 0.25 μM (B6.H2d cells). 5 x 10^6 cells of each population were mixed and injected into experimental groups. 12 to 15 hours after injection, spleens from recipient mice were harvested and remaining CFSE labeled target cells were analyzed by flow cytometry. Percent specific removal of CFSE labeled donor target cells was calculated as follows: [1 – (B6.H2d / B6) / (B6.H2d $_{\text{in a naïve mouse}}$ / B6 $_{\text{in a naïve mouse}}$)] x 100.

In vivo cross-presentation to OT-I T cells

On day –1, 15 x 10^6 splenocytes from OT-I donors were labeled with 3μM CFSE (Invitrogen) and adoptively transferred i.v. into 8-12 week old B6.Thy1.1 recipients and left to circulate for 24 hours. On day 0, whole blood from FVB and HOD donor mice was collected and anticoagulated with ACD. Some of the HOD blood was leukoreduced as described above. The blood was then washed 3 times in mPBS and 100 μl of packed blood diluted with 400 μl PBS was injected i.v. into the recipient mice. On day 4, expansion of the OT-I T cells in the spleen of the recipient mice was measured by staining with an anti-CD8 antibody (BD Biosciences) and OT-I specific MHC tetramer Kb/SIINFEKL. After gating on CD8+CFSE$^+$ cells, the proliferation of the OT-I T cells was measured by CFSE dilution. Samples were analyzed on a BD FACSort flow cytometer.

Statistical analyses

For BMT studies, the significance of the rejection rate was analyzed with a Fisher's exact test on the contingency table formed by the engraftment and rejection events in the

groups of mice transfused with mismatched leukoreduced blood and transfused with syngeneic leukoreduced blood. For the crosspresentation to OT-I studies, the significance of the expansion of the OT-I T cells was analyzed with a one-way ANOVA with Dunnett's post test comparing the HOD and HOD LR group to the FVB control group. Analysis was performed using GraphPad Prism version 5.0a for Macintosh, GraphPad Software, San Diego California USA, www.graphpad.com.

Results

Transfusing leukoreduced units of RBCs induces BMT rejection across mHA barriers

We engineered a murine system in which processing of RBC units models current methodologies of manufacturing human RBCs units, including collection solution and filter leukoreduction (as described in materials and methods). To assess the degree of leukoreduction, residual white blood cells (rWBC) were quantified in each RBC preparation using a PI based flow cytometric method adapted from a previously described technique (29). As a positive control for visualization of rWBCs, whole blood was stained and PI^+ events corresponding to WBCs were enumerated (figure 2.1A). rWBCs were easily detectable in whole blood (figure 2.1A, middle panel), and this was not due to background signal, as no events were observed in unstained samples (figure 2.1A, left panel). Filter leukoreduction resulted in a considerable decrease in rWBCs (figure 2.1A, right panel). Enumeration of rWBCs demonstrated that on average there were fewer than 1000 rWBCs per 100μl unit transfused. At the human scale, this would represent 2.5 x 10^6 rWBCs per 250ml unit of packed RBCs. Although volumes of RBCs per human unit vary, this constitutes less the 5 x 10^6 residual leukocytes per unit, which is the standard for blood transfusion in the USA (23).

B6.Thy1.1 mice were given weekly intravenous transfusions of 100 μl of packed, leukoreduced RBCs from BALB.B donors. This amount of blood approximates the volume of a human RBC unit in the context of mouse whole blood volume. Negative control mice were transfused with 100 μl packed leukoreduced RBCs from B6 donors. Both groups received 4 weekly transfusions. One week after the last blood transfusion, mice were conditioned and transplanted with bone marrow from BALB.B donors.

Engraftment of the BMT was evaluated 6 weeks post transplant by staining peripheral WBCs of transplanted mice with antibodies against the Thy1.1 (recipient) and Thy1.2 (donor) congenic markers. Representative plots of mice with BMT engraftment and rejection are shown (figure 2.1B).

Whereas 100% of naïve mice displayed BMT engraftment, transfusion of leukoreduced BALB.B blood into B6.Thy1.1 recipients induced BMT rejection (figure 2.2A). Combined analysis of three separate experiments with 17 total recipient mice demonstrated a rejection rate of 94% ($p<0.0001$) after transfusion with leukoreduced BALB.B blood. This effect was not due to non-specific factors in the transfused RBCs, as no rejection was observed in recipients receiving B6 RBCs (figure 2.2A). In this model system, mice displaying engraftment had a range of T cell chimerism from 18-81% and 48-70% for untransfused and B6 transfused mice, respectively (figure 2.2B). In mice that rejected BMT, essentially no donor Thy1.2 positive cells were detectable (figure 2.2B; see figure 2.1B for representative flow cytometry data). Early experiments showed that BALB.B whole blood was more potent than leukoreduced blood in inducing BMT rejection; a single transfusion of whole blood induced 100% rejection whereas rejection was variable with fewer than 3 transfusions of leukoreduced RBCs (data not shown). Together, these data demonstrate that transfusion of mHA mismatched RBCs induces BMT rejection, and that while leukoreduction decreases the immunogenicity of RBC transfusion compared to whole blood, it is insufficient to prevent transfusion-induced BMT rejection.

Direct and indirect pathways in mHAs based transfusion-induced BMT rejection

To study the contribution of the indirect pathway of antigen presentation in transfusion-induced BMT rejection, we modified our model by using BALB/c mice instead of BALB.B mice as RBC donors, while keeping BALB.B mice as BMT donors. As both B6 recipients and BALB.B BMT donors encode the H-2^b MHC haplotype, any rejection of BALB.B marrow across mHA barriers will occur in response to peptides presented on MHC encoded by H-2^b. BALB.B and BALB/c mice are congenic and only differ at the MHC locus (BALB/c mice express H-2^d MHC molecules). Thus, BALB/c APCs are incapable of directly presenting mHAs peptides in MHC molecules encoded by H-2^b. Accordingly, BALB/c cells cannot induce an immune response to BALB mHAs on H-2^b MHC through the direct pathway. However, the indirect presentation pathway remains unaltered and mHAs on donor cells may be processed and presented on H-2^b MHC by the B6.Thy1.1 recipient's APCs.

B6.Thy1.1 mice were transfused weekly with 100 μl of packed leukoreduced RBCs from BALB/c donors. 4 weekly transfusions were given; one week after the last blood transfusion all mice were conditioned and transplanted with BALB.B bone marrow. Whereas control mice receiving no transfusion or autologous B6 RBCs had 100% engraftment, 93% of mice receiving leukoreduced BALB/c RBCs rejected the BMT (figure 2.2C). These data suggested that the indirect pathway was sufficient for transfusion-induced BMT rejection; however, it remained possible that the major alloresponse against H-2^d MHC on BALB/c blood cross-reacted with mHAs on BALB.B to the extent that it induced BMT rejection. To assess this possibility, we utilized B6 mice congenic for the H-2^d MHC as RBC donors (B6.H2d), thus isolating the H-2^d

component of the immune response. To enhance the alloresponse, recipient mice were transfused up to 3 times with packed whole blood (not leukoreduced). One week following the last transfusion, mice were conditioned and BALB.B marrow was transplanted. No rejection was observed in any of the transfused animals (figure 2.2E). There was a trend to lower levels of engraftment in mice receiving H-2^d blood (figure 2.2F), but this trend did not reach statistical significance; no difference was seen between H-2^d transfused and untransfused groups. Lack of BMT rejection was not due to lack of alloimmunization, as *in vivo* killing assays demonstrated elimination of the H-2^d targets (figure 2.2G). These data demonstrate that a robust anti-H-2^d response does not cause rejection of BALB.B marrow, and rules out that the anti-H-2^d response caused BMT rejection after transfusion of BALB/c RBCs. It is worth noting that despite their differences in MHC molecule sequence, a fraction of mHA peptides may be presented by MHC from both H-2^d and H-2^b haplotypes. This may particularly be the case for MHC class II, as the MHC II molecules I-A^b and I-A^d have relatively few differences and are known to present some of the same peptides (30). Thus, we cannot unequivocally rule out that a low level of direct presentation is possible to the extent that a T cell specific for an H-2^d/mHA complex may crossreact with an H-2^b/mHA complex. Together, these findings suggest that the direct pathway is not required and suggest that the indirect presentation of mHAs on transfused leukoreduced blood is sufficient to induce an immune response that causes BMT rejection.

Leukoreduction prevents BMT rejection induced by HY mHAs mismatched blood

The above data demonstrate that transfusion of RBC units induces BMT rejection. Although the units are stringently leukoreduced by the same methodologies used on human blood, leukocytes are nevertheless not completely eliminated. Thus, two hypotheses are equally consistent with the observed data: 1) mHAs on RBCs induce BMT rejection and 2) mHAs on the low number of rWBCs are sufficient to induce rejection. To assess the contribution of rWBCs, we narrowed the transplantation barrier to a known minor antigen system, the male antigen (HY) (20). We specifically chose this system, because the HY antigens, Smcy and Uty in H-2^b animals (31), are predicted nuclear proteins (http://www.uniprot.org/uniprot/Q62240, http://www.uniprot.org/uniprot/P79457). As RBCs are anucleate cells, one would not predict Smcy or Uty to be present in RBCs; indeed, the male antigens have not been detected in RBCs (32, 33). We utilized this system to assess if the extent of leukoreduction was sufficient to prevent BMT rejection to an mHA present on leukocytes but not RBCs.

Female B6.Thy1.1 mice were transfused with RBCs from male B10.BR mice, followed by BMT with male B6 donor marrow. B10.BR mice, which are H-2^k, were utilized to minimize the effects of direct presentation analogous to the BALB/c–BALB.B combination, as any rWBCs would not present Smcy or Uty in the context of H-2^b MHC. Whereas a single transfusion of whole blood induced BMT rejection in 100% of mice, 4 weekly transfusions of leukoreduced male RBCs induced BMT rejection in only 13% of mice (figure 2.3). These data demonstrate that the extent of filter leukoreduction is generally sufficient to eliminate transfusion-induced BMT rejection in response to an

antigen on leukocytes, which is not on RBCs. This also suggests that residual leukocytes are not sufficient to cause BMT rejection in the previous experiments transfusing BALB.B or BALB/c blood into B6.Thy1.1 recipients. This supports the hypothesis that mHAs on RBCs are contributory.

Generation of a novel transgenic donor mouse with RBC specific expression of defined mHAs

Although the above data suggest a role for mHAs on RBCs in transfusion-induced BMT rejection, an RBC specific mHA was required to focus our analysis on the role of RBCs to the exclusion of WBC antigens. In addition, one would need sufficient analytic tools to study the immune response to the RBC specific mHA. To achieve these criteria, we chose to generate a novel transgenic mouse with RBC specific expression of existing model antigens for which a wide array of analytic tools already existed. We engineered a fusion protein of a model humoral antigen (HEL) and a model cellular antigen (OVA). To allow linkage to the RBC by natural means, the HEL-OVA protein was further fused in frame to the human Duffy blood group antigen (the Duffy b (Fy^b) variant was used). The open reading frame codes for the triple fusion protein HOD (figure 2.4A). To provide RBC specific expression, the HOD open reading frame was ligated into an expression vector containing the human β-globin gene promoter, β-globin locus control region (μ'LCR), and the β-globin gene intron 2 and 3' enhancer (figure 2.4B). This construct has previously been reported to confer RBC specific expression in a transgenic mouse (34).

A founder mouse that had germ-line integration of the transgene was identified and bred to generate a working colony. The transgene was transmitted with Mendelian segregation and litter sizes were normal, suggesting no deleterious effects of transgene integration (data not shown). Using RBCs from wild-type mice as a negative control, HOD RBCs stained positive with anti-HEL, anti-OVA, anti-Fy^b and anti-Fy^3 (figure 2.4C). The staining with anti-OVA was not an artifact of HEL contamination of the OVA used to generate anti-OVA antiserum, as anti-OVA did not stain cells from a mouse with ubiquitous expression of surface HEL (mHEL mouse (25), figure 2.4C). HOD RBCs stained negative with control mouse serum (data not shown). HOD RBCs also stained negative with anti-Fy^a (as predicted since the Fy^b Duffy variant was used in the HOD construct).

Expression on non-erythroid cells was assessed by gating on $CD45^+$ leukocytes or $CD41^+$ platelets and assessing anti-OVA staining. No HOD expression was detected on $CD45^+TER\text{-}119^-$ leukocytes (figure 2.4D). This was not an artifact of the stain not working, as leukocytes from the mHEL mouse were strongly positive. Likewise, no expression of HOD was detected on $CD41^+TER\text{-}119^-$ platelets (figure 2.4E). Together, these data indicate that the HOD antigen is expressed in a conformation that preserves the different epitopes from the fusion partners and in an RBC specific fashion.

mHAs on transfused RBCs are crosspresented on MHC I of recipient APCs

Based upon the ability of leukoreduced RBCs to induce BMT rejection, we hypothesized that mHAs on transfused RBCs were capable of inducing anti-mHA immunity after processing and presentation by recipient APCs. In the field of transfusion

medicine, it is well established that RBC antigens can induce activation of recipient CD4+ T cells and antibody secreting B cells. Here, we further hypothesized that mHAs on transfused RBCs could be crosspresented into recipient MHC class I and stimulate CD8+ T cells. To test this hypothesis, we utilized OT-I transgenic mice, which express a T cell receptor that recognizes a peptide from OVA (SIINFEKL) presented by H-2K^b MHC-I. OT-I splenocytes were adoptively transferred to wild-type B6.Thy1.1 recipient mice. Transferred OT-I T cells were visualized by a K^b-SIINFEKL tetramer reagent. To allow an assessment of proliferation, OT-I T cells were labeled with CFSE prior to adoptive transfer. Transfusion of HOD RBCs induced a substantial expansion of OT-I T cells compared to negative control mice that received wild-type FVB blood (figure 2.5A,B). Expansion of OT-I T cells was not significantly decreased by leukoreduction of HOD RBCs. The expansion was a function of CD8+ T cell proliferation, as CFSE was diluted out in mice that received HOD whole blood or leukoreduced HOD RBCs, but not wild-type FVB RBCs (figure 2.5C). These data indicate that mHAs expressed on transfused RBCs can be processed by recipient APCs and crosspresented on MHC class I in a fashion that leads to expansion of mHA specific CD8+ T cells.

Discussion

Currently, the primary immunological focus of transfusion medicine is to monitor the generation of antibody responses against blood group antigens on transfused RBCs. Indeed, providing crossmatch compatible RBCs to patients is essential to avoid hemolytic transfusion reactions, which may lead to substantial morbidity, and in some cases, mortality. However, several clinical observations have demonstrated a correlation between the number of transfused units and the likelihood that a patient will reject BMT (13, 14). This observation is equally consistent with two distinct hypotheses. First, repeat transfusion is causal for subsequent BMT rejection. Second, the severity of the underlying illness both necessitates more frequent transfusion and increases likelihood of BMT rejection (i.e. transfusion is correlative to rejection but not causal). Although severity of illness may certainly play a role in the likelihood of BMT rejection, the data presented herein demonstrate a causal link between transfused RBC units and BMT rejection across mHA barriers in a murine model. This defines a novel immunological sequela of RBC transfusion, one that is currently neither monitored nor addressed by clinical transfusion immunology.

It is our hypothesis that exposure to mHAs on RBCs is alone sufficient to mediate transfusion-induced BMT rejection, as rejection occurred despite stringent leukoreduction. Of course, rWBCs cannot be entirely eliminated, thus making it impossible to unequivocally rule out that trace levels of rWBCs contribute to the effect. From the standpoint of establishing the transfusion-induced BMT rejection phenomenon, this issue is not practically relevant, as like our murine model, units of human leukoreduced RBCs likewise have rWBCs present. However, identifying the immunizing

component of the RBC unit is central to devising a strategy to address the problem. If rWBCs are in fact responsible, then efforts should focus on developing better leukoreduction methods. However, if mHAs on RBCs are sufficient, then improving leukoreduction will not remedy the problem. The observation that leukoreduction is sufficient to prevent transfusion-induced BMT rejection in response to a leukocyte specific antigen (HY antigen; see figure 2.3) indicates that the number of rWBCs after leukoreduction is insufficient to cause BMT rejection, suggesting that mHAs on a non-WBC component contribute to inducing rejection in leukoreduced transfusion-induced BMT rejection in the BALB.B → B6 strain combination. However, it is important to consider that the BALB.B → B6 strain combination has numerous antigenic differences and represents a greater immunological barrier than the HY system. Thus, it is also possible that rWBCs are more potent in the BALB.B → B6 system.

We further tested the hypothesis that exposure to RBC mHAs is sufficient to induce immunity by creating the HOD mouse and observing that exposure to an RBC specific mHA can induce a cellular response in the CD8+ compartment. These results build on our previous observations that CD8+ T cells responded to OVA chemically crosslinked to leukoreduced RBCs (35). However, in these previous experiments, as OVA was crosslinked to rWBCs, the crosslinking procedure may have altered the biology of RBCs. By using an RBC specific genetic approach in the HOD mouse, the current data circumvent these caveats and provide substantial additional support to the notion that processing and presentation of mHAs on RBCs stimulates CD8+ T cells in the absence of contaminating donor WBCs. It is worth acknowledging that we cannot rule out that trace levels of HOD expression on WBCs, which are below our limits of

detection, are responsible for activating CD8+ T cells. However, given the lack of detectable HOD on WBCs by flow cytometry, and the similar expansion of OT-I T cells with leukoreduced RBCs and whole blood, we consider this possibility unlikely.

By minimizing the direct antigen presentation pathway in the BALB/c–C57BL/6 strain combination, our data suggest that the indirect pathway of antigen presentation is sufficient for transfusion-induced BMT rejection. The direct pathway was likewise minimized in the induction of OT-I activation by HOD RBCs, as the HOD mouse is on an FVB background and does not express H-2^b. Together, these data indicate that processing of the blood by the recipient's APCs and presentation to the recipient's T cells is sufficient to activate an immune response against an mHA present on RBCs. However, the OT-I data only represent activation of CD8+ T cells, not established immunity, and thus represent a potential afferent mechanism of induction of immune responses by allogeneic RBCs. The effector mechanism of transfusion-induced BMT rejection has yet to be determined. However, the known activation of CD4+ T cells and B cells by transfused RBCs, and the current HOD data indicating activation of CD8+ T cells provides several potential pathways of rejection.

In aggregate, the above evidence strongly suggests that exposure to mHAs present on RBCs is capable of inducing cellular immunity and BMT rejection. Of course, the applicability of these findings to the human setting depends upon the parallels between mouse and human biology. Nevertheless, these findings may explain the increased rates of BMT rejection in multiply transfused patients. Moreover, to the extent that mHAs are shared on RBCs and solid organs, these data predict increased rejection of solid organs in multiply transfused patients, as it has been suggested in recent years (36). Finally, as

transfusion may induce memory cells specific for donor antigens, multiple transfusions may decrease efficacy of immunomodulatory approaches such as co-stimulatory blockade, which are less effective at inhibiting memory vs. naïve T cells. Extension of these studies into the human setting may consist of analyzing chronically transfused patients for T cell immunity against known human mHAs through tetramer analysis of freshly isolated or restimulated peripheral T cells.

In the context of the above data indicating that exposure to mHAs on RBCs can induce immunity, it is our prediction that no extent of leukoreduction will prevent transfusion-induced BMT rejection. Exposure clearly cannot be eliminated, as patients need the therapeutic effect of the transfused RBCs. At first consideration, it seems unlikely that matching donor and recipient mHAs during transfusion of patients likely to receive a transplant will be feasible, as the involved mHAs may be undefined and/or too varied to allow matching. However, it is possible that the specific mHAs are limited to antigen present on both mature RBCs and expressed by hematopoietic stem cells. In such a case, the number of relevant antigens may be limited, and matching may ultimately become feasible. In the absence of matching, development of immunomodulation strategies and/or improved conditioning regimens that can overcome existing immunity while limiting toxicity will likely be required.

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Tables

	RBC Donor	Recipient	BMT Donor	Main conclusion
Figure 2A,2B				
Strain	BALB.B	B6	BALB.B	Exposure to mHAs in leukoreduced RBC units induce BMT rejection.
MHC	H-2^b	H-2^b	H-2^b	
mHA	BALB	B6	BALB	
Figure 2C,2D				
Strain	BALB/c	B6	BALB.B	The indirect presentation pathway is sufficient to induce BMT rejection.
MHC	H-2^d	H-2^b	H-2^b	
mHA	BALB	B6	BALB	
Figure 2E-G				
Strain	B6.H2d	B6	BALB.B	Immunization against H-2^d is not responsible for mHA mismatched BMT rejection.
MHC	H-2^d	H-2^b	H-2^b	
mHA	B6	B6	BALB	
Figure 3				
Strain	Male B10.BR	Female B6	Male B6	Removal of leukocyte-associated mHAs by filtration leukoreduction is sufficient to prevent BMT rejection.
MHC	H-2^k	H-2^b	H-2^b	
mHA	HY	-	HY	

Table 2.1. *Summary of strains used in the research. Grayed out cells emphasize MHC and mHA mismatches to the recipient.*

Figure Legends

Figure 2.1. Flow cytometric enumeration of residual WBCs in leukoreduced blood and measurement of T cell chimerism in transplanted mice. Each leukoreduced unit of blood was analyzed for residual WBCs. A representative analysis is shown. Leukoreduction resulted in fewer than 1000 rWBCs on average **(A)**. Peripheral blood from transplanted and control mice was stained with antibodies against Thy1.1 and Thy1.2 and analyzed by flow cytometry after gating on $CD3^+$ events. Representative plots of a mouse with engraftment and a mouse displaying rejection of BMT are shown **(B)**.

Figure 2.2. Transfusion of mHA mismatched leukoreduced blood induces rejection of subsequent BMT. Transfusion with mHA mismatched leukoreduced blood induces rejection of BMT from BALB.B donors. The number of mice with engraftment was significantly reduced after 4 transfusions with mHA mismatched blood (group BALB.B LR) as compared to mice transfused with syngeneic blood (group B6 LR) ($p<0.0001$, Two-tailed Fisher's exact test) **(A)**. Chimerism in engrafted animals was somewhat variable from experiment to experiment but resulted in a clear determination of engraftment vs. rejection **(B,D,F)**. Use of BALB/c RBC donors induces rejection of BALB.B bone marrow, suggesting the indirect pathway is active for donor RBCs ($p<0.0001$, Two-tailed Fisher's exact test) **(C)**. Transfusion-induced mHA mismatched BMT rejection is not the result of alloimmunization against $H\text{-}2^d$ MHC molecules, as no rejection is seen in mice transfused with whole blood from B6.H2d mice (group B6.H2d WB) **(E)**. Specific lysis against target cells from B6.H2d donor mice measured in an in vivo killing assay, indicating that anti-H2d alloimmunization occurred **(G)**. Data shown

are the combined results of at least three separate experiments in each figure. The data corresponding to the no transfusion and syngeneic transfusion groups were combined for all the experiments, and are presented in multiple panels (indicated with *).

Figure 2.3. Leukoreduction prevents transfusion-induced rejection of HY+ BMT. Transfusion of whole blood but not leukoreduced blood from HY+ (Male) donors into female B6.Thy1.1 causes rejection of HY+ BMT. Injection with male whole blood induced rejection in 93% of the recipients when compared to untransfused mice or mice transfused with syngeneic blood (p=0.0004, Two-tailed Fisher's exact test). Transfusions with Male LR blood did not significantly affect the number of mice displaying BMT engraftment **(A)**. Chimerism in engrafted animals was variable with the observation of microchimerism in some animals. **(B)**. Data shown are the compounded results of two separate experiments.

Figure 2.4. RBC specific expression of a model fusion antigen by the HOD mouse. Predicted structure of the HOD antigen **(A)**. Structure of the HOD construct and amino-acid sequence of the HOD antigen **(B)**. The HOD antigen is under control of the RBC specific μ'LCR promoter and is a fusion of the full sequence of HEL, a fragment of OVA containing both the OT-I T cell specific epitope (SIINFEKL) and the OT-II specific epitope (ISQAVHAAHAEINEAGR), and the human Duffy blood group antigen. Additional features are indicated including the 'Duffy b' polymorphism (D), the first transmembrane domain of the molecule FFILTSVLGILASSTVLFMLF), and the epitope for the Duffy 3 specific MIMA 29 monoclonal antibody (ALDLLL). The above features

are underlined in the figure. Two amino-acids were added at each fusion junction due to introduction of restriction sites Sma I and Xho I (PG and LE, in black). Staining of HOD blood demonstrates the predicted epitopes on RBCs, with no detectable HOD on $CD45^{+}TER\text{-}119^{-}$ leukocytes or $CD41^{+}TER\text{-}119^{-}$ **(C-E)**. While only the OVA antigen was utilized in the course of the presented research, the HEL and Duffy antigens were included for alternate research purposes outside the scope of the current report.

Figure 2.5. mHAs on transfused leukoreduced HOD blood are cross-presented into MHC class I by recipient APCs. Expansion of OT-I T cells in the spleen of recipient mice after transfusion with HOD whole blood or HOD LR blood, control mice were transfused with blood from FVB donors **(A)**. Representative flow cytometry plots **(B)**. CFSE dilution of OT-I T cells in response to transfusion of HOD blood **(C)**. Data shown are representative of three separate experiments with similar results.

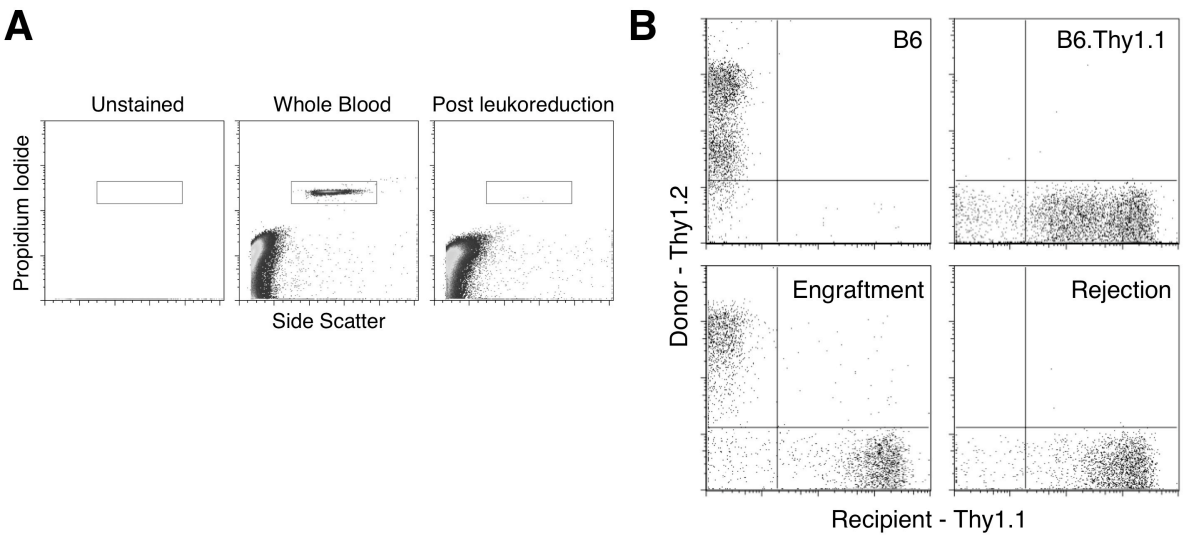
A
Unstained
Whole Blood
Post leukoreduction
Propidium Iodide
Side Scatter
B
B6
B6.Thy1.1
Engraftment
Rejection
Donor - Thy1.2
Recipient - Thy1.1

Figure 2.1

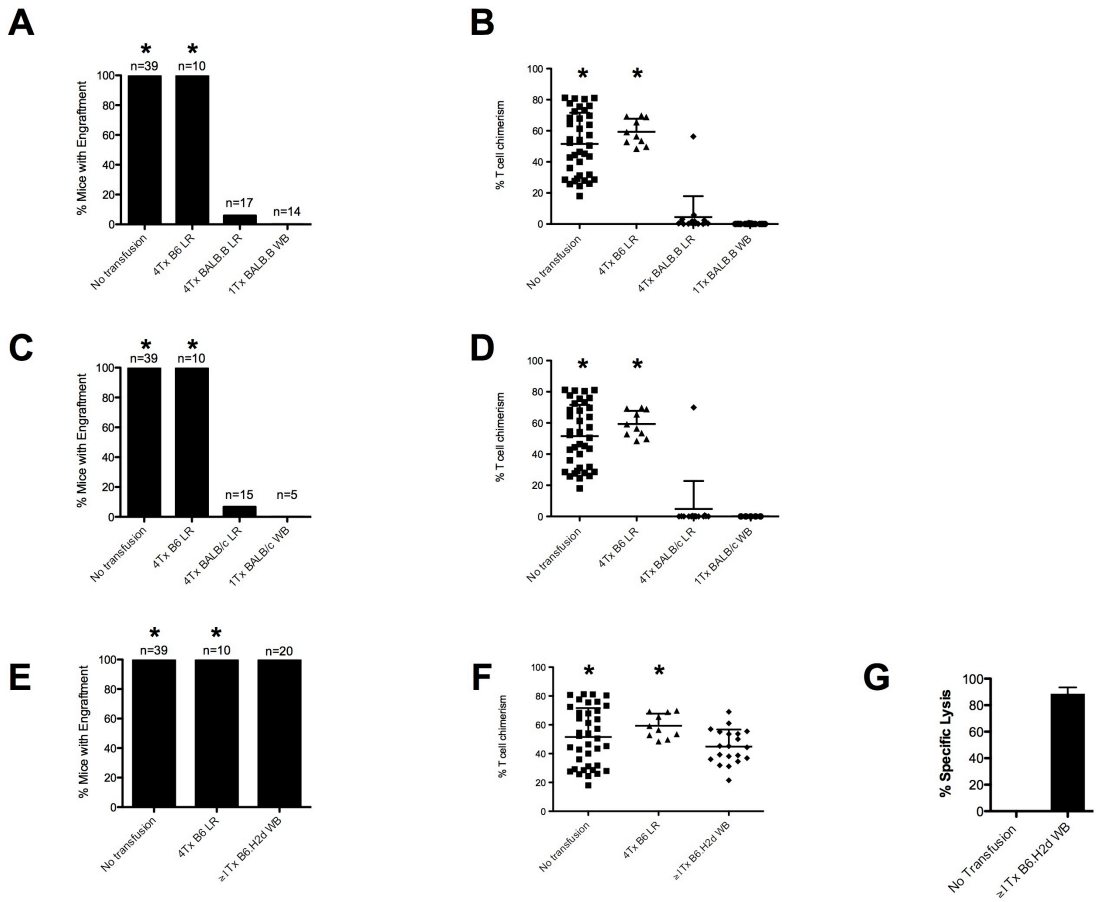
A
B
C
D
E
F
G
% Mice with Engraftment
% T cell chimerism
% Specific Lysis
n=39
n=10
n=17
n=14
n=15
n=5
n=20
No transfusion
4Tx B6 LR
4Tx BALB.B LR
1Tx BALB.B WB
4Tx BALB/c LR
1Tx BALB/c WB
≥1Tx B6.H2d WB
No Transfusion
* same data shown in multiple panels for clarity

Figure 2.2

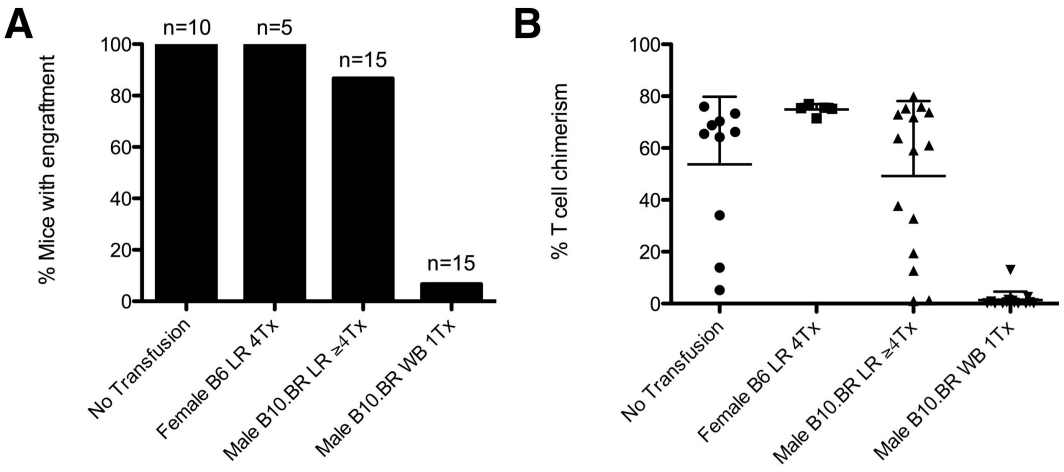
A
% Mice with engraftment
100
80
60
40
20
0
n=10
n=5
n=15
n=15
No Transfusion
Female B6 LR 4Tx
Male B10.BR LR ≥4Tx
Male B10.BR WB 1Tx
B
% T cell chimerism
100
80
60
40
20
0
No Transfusion
Female B6 LR 4Tx
Male B10.BR LR ≥4Tx
Male B10.BR WB 1Tx

Figure 2.3

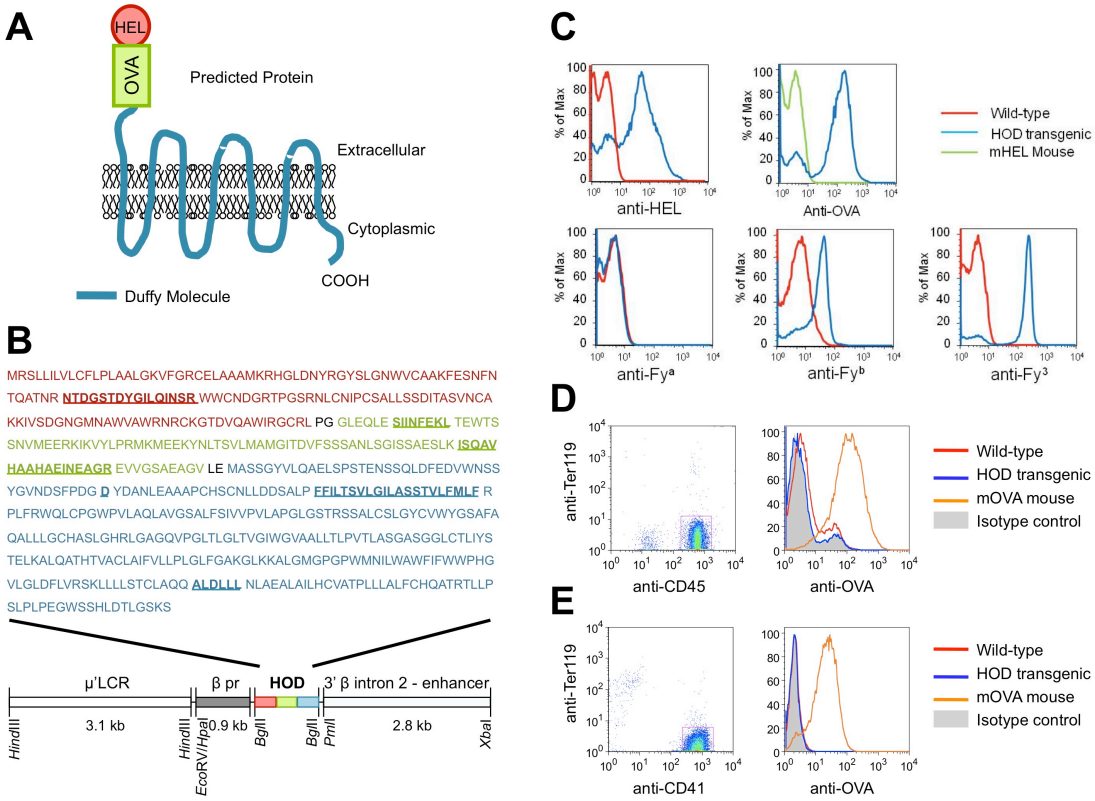
A
HEL
OVA
Predicted Protein
Extracellular
Cytoplasmic
COOH
Duffy Molecule
B
MRSLLILVLCFLPLAALGKVFGRCELAAAMKRHGLDNYRGYSLGNWVCAAKFESNFN
TQATNR NTDGSTDYGILQINSR WWCNDGRTPGSRNLCNIPCSALLSSDITASVNCA
KKIVSDGNGMNAWVAWRNRCKGTDVQAWIRGCRL PG GLEQLE SIINFEKL TEWTS
SNVMEERKIKVYLPRMKMEEKYNLTSVLMAMGITDVFSSSANLSGISSAESLK ISQAV
HAAHAEINEAGR EVVGSAEAGV LE MASSGYVLQAELSPSTENSSQLDFEDVWNSS
YGVNDSFPDG D YDANLEAAAPCHSCNLLDDSALP FFILTSVLGILASSTVLFMLF R
PLFRWQLCPGWPVLAQLAVGSALFSIVVPVLAPGLGSTRSSALCSLGYCVWYGSAFA
QALLLGCHASLGHRLGAGQVPGLTLGLTVGIWGVAALLTLPVTLASGASGGLCTLIYS
TELKALQATHTVACLAIFVLLPLGLFGAKGLKKALGMGPGPWMNILWAWFIFWWPHG
VLGLDFLVRSKLLLLSTCLAQQ ALDLLL NLAEALAILHCVATPLLLALFCHQATRTLLP
SLPLPEGWSSHLDTLGSKS
μ'LCR
β pr
HOD
3' β intron 2 - enhancer
3.1 kb
0.9 kb
2.8 kb
HindIII
HindIII
EcoRV/HpaI
BglII
BglII
PmlI
XbaI
C
% of Max
anti-HEL
Anti-OVA
anti-Fy^a
anti-Fy^b
anti-Fy^3
Wild-type
HOD transgenic
mHEL Mouse
D
anti-Ter119
anti-CD45
anti-OVA
Wild-type
HOD transgenic
mOVA mouse
Isotype control
E
anti-Ter119
anti-CD41
anti-OVA
Wild-type
HOD transgenic
mOVA mouse
Isotype control

Figure 2.4

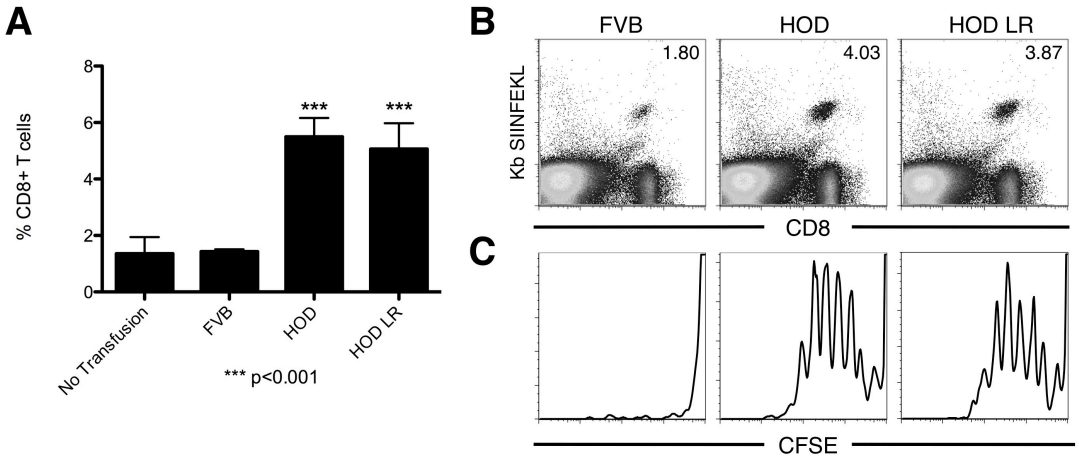
A
% CD8+ T cells
8
6
4
2
0
***
***
No Transfusion
FVB
HOD
HOD LR
*** p<0.001
B
FVB
HOD
HOD LR
1.80
4.03
3.87
Kb SIINFEKL
CD8
C
CFSE

Figure 2.5

Chapter 3

Cross-Presentation of a Minor Antigen on Transfused Red Blood Cells Leads to Deletion of Alloreactive CD8 T cells

Abstract

HLA-matched bone marrow transplantation (BMT) is a cure for nonmalignant hematological disorders; however, rejection rates are high and correlate with the number of antecedent transfusions. Recently, using murine models, we reported that minor antigens (mHAs) in transfused leukoreduced RBC or platelet units induce rejection of subsequent BMT. To study RBCs as an immunogen, we utilized transgenic donors that express a model mHA selectively on RBCs (HOD mouse). Transfusion of HOD blood did not induce BMT rejection of marrow that shared mHAs with the HOD RBCs. Likewise, no endogenous anti-HOD CD8+ T cell response was detected with antigen-specific tetramer reagents. Adoptively transferred OT-I T cells rapidly expanded after HOD blood transfusion; however, only a semi-effector phenotype was observed (TNF-α and IFN-γ secretion, but minimal Granzyme B). After initial expansion, OT-I T cells contracted rapidly to very low levels. A similar trend was observed by in vivo CTL assay, with only transient lytic activity. Together, these data indicate that RBCs may not be the component of RBC units that induces BMT rejection, and suggest that contaminating platelets or leukocytes may be responsible. Moreover, these data suggest that RBCs themselves may act as a deletional tolerogen regarding CD8+ T cell responses.

Introduction

When successful, bone marrow transplantation (BMT) is a cure for nonmalignant hematologic disorders such as sickle cell disease, aplastic anemia, Diamond-Blackfan anemia, and Fanconi anemia (1-8). For such disorders, where no malignancy is present, it is difficult to justify the use of myeloablative conditioning regimens, which can lead to morbidity and mortality. Accordingly, it is highly desirable to develop reduced-intensity BMT conditioning regimens that minimize toxic side effects. Unfortunately, current reduced-intensity regimens result in significant rates of BMT rejection in patients with nonmalignant hematologic disorders (9-12).

As the frequency of rejection correlates with the number of transfusions given prior to BMT in humans (13-15) and in canine transplantation models (16-19), it has been hypothesized that minor antigens (mHAs) on donor blood induce immunity to mHAs expressed on subsequently transplanted marrow, resulting in immunological rejection. In support of this hypothesis, we have previously reported that transfusion of stringently leukoreduced RBCs induces rejection of MHC matched:mHA mismatched BMT under reduced-intensity conditioning. In other settings, it has been reported that BMT rejection across minor antigen barriers and rejection of mOVA transplants are mediated by CD8+ cytotoxic T lymphocytes (20-24). Consistent with this, we also previously reported that mHAs on transfused RBCs underwent cross-presentation and induced early expansion of antigen-specific CD8+ T cells (25, 26).

In aggregate, the above findings led us to propose that mHAs on RBCs themselves induce anti-mHA immunity leading to BMT rejection. However, leukoreduced RBC units are a complex product, and although filter leukoreduction removes the vast majority of leukocytes, a small number persist in the unit. Moreover, leukoreduction filters do not specifically remove

platelets, which contaminate RBC units at variable levels. We have recently reported that like RBCs, transfusion of mHA mismatched platelet products can induce BMT rejection (27). These platelets products were filter leukoreduced and had only trace levels of RBCs; however, one cannot unequivocally conclude platelets themselves are responsible, due to contaminating entities.

Although it is possible that both platelets and RBCs can induce BMT rejection, we cannot rule out that BMT rejection due to transfusion of RBC units was due to contaminating platelets, and vice-versa. To further dissect this issue and test the role of RBCs themselves as an immunogen for mHAs, we engineered a system in which transfusion donors expressed an RBC specific mHA and marrow donors expressed the same mHA on hematopoietic stem cells. Using this approach, we report herein that transfusion of blood expressing an RBC specific mHA does not induce BMT rejection. Moreover, it induces only a transient expansion of mHA specific CD8+ T cells with a semi-effector phenotype, followed by a rapid contraction and deletion to essentially undetectable levels. These findings do not contradict the observation that transfusion of RBC units induces BMT rejection; however, they suggest that the RBCs themselves may not the component of the unit that is responsible. Moreover, these data suggest that mHAs on RBCs can act as a vector for deletional tolerance in the CD8+ T cell compartment. Together, these studies provide a novel further characterization of the properties of transfused RBCs as an immunogen in the context of cross-priming into the MHC I pathway and effects upon CD8+ T cell responses.

Materials and methods

Mice

C57BL/6 and B6.PL-Thy1^a (B6.Thy1.1) mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and housed in Emory University Department of Animal Resources facilities. Mice were 8 to 12 weeks of age, and all procedures were performed according to protocols approved by Emory's Institutional Animal Care and Use Committee. The HOD (28) and mOVA (23) transgenic mice were previously described. FVB, HOD and mOVA mice were bred by the Emory Division of Animal Resources Animal Husbandry service.

Viruses

The wild type mouse polyoma virus (PyV) strain A2 and recombinant PyV.OVA-I were prepared in baby mouse kidney cells, as previously described (41). Briefly, recombinant PyV.OVA-I was generated through the insertion of the SIINFEKL coding sequence in frame at a unique BlpI restriction site in the coding region of middle T antigen (42). Mice were injected subcutaneously (s.c.) in hind footpads with 1 x 10^6 PFU of virus.

Synthetic peptides

The SIINFEKL and LT359-368Abu (SAVKNY[Abu]SKL) peptides were synthesized by the solid-phase method using F-moc chemistries (Emory University Microchemical Core Facility) and high-pressure liquid chromatography purified to more than 90% purity. For simplicity, the LT359-368Abu peptide, in which the cysteine residue at position 7 was replaced with the thiol-less cysteine analog residue α-aminobutyric acid, is referred to as LT359 peptide (43). Peptide

stocks were solubilized in water or DMSO and stored at –20°C. Peptides were diluted in assay medium immediately before use.

Blood collection and transfusion

Mouse blood was obtained by retro-orbital bleeding into ACD solution A (BD, Franklin Lakes, NJ). The blood was then washed 3 times with phosphate buffered saline (PBS) adjusted to 340 mOsm with NaCl (modified PBS or mPBS) to remove platelets. Each transfusion consisted of 100 μl of packed RBCs diluted with 400 μl PBS injected in the tail vein.

Bone Marrow Transplantation

B6.Thy1.1 recipients were conditioned by total body irradiation with 650 cGy. The next day, whole bone marrow was prepared from the femur and tibia of donor mOVA mice and 5 x 10^6 whole bone marrow cells from were injected intravenously into the irradiated recipients. Engraftment was measured 6 weeks after transplant by flow cytometry using the Thy1.1 (recipient) and Thy1.2 (donor cells) congenic markers.

Flow cytometry

All antibodies used were purchased from BD Biosciences (San Jose, CA) except where noted. Cells were stained in PBS containing 0.2% bovine serum albumin and 3mM ethylenediaminetetraacetic acid. Antibody staining was performed at 4°C or room temperature for 30 min; allophycocyanin-conjugated Db-LT359 and Kb-SIINFEKL tetramer staining was performed at room temperature for 30 min. Cells were surface stained with a fluorescein isothiocyanate-conjugated anti-CD8, phycoerythrin-conjugated monoclonal antibodies (MAb)

specific for CD25, CD44, CD107a with matching isotype controls, and phycoerythrin-Cy5 conjugated MAbs specific for CD69, CD62L, with matching isotype controls. Intracellular staining was performed using the Cytofix/Cytoperm kit (BD Biosciences) in conjunction with a PE conjugated Granzyme B specific MAb (Clone GB12, Invitrogen). PE conjugated Annexin-V and 7AAD staining was performed using the PE Annexin V Apoptosis Detection Kit I (BD Biosciences). All flow cytometry data was acquired on a FACSort flow cytometer (Beckton Dickinson, San Jose, CA) and analyzed with FlowJo software (TreeStar, Ashland, OR).

Intracellular Cytokine Staining

Spleens from recipient mice were harvested 4 days after transfusion or infection with polyoma virus. 2 x 10^6 splenocytes were incubated with or without SIINFEKL peptide at a 10μM concentration in standard growth media (SGM: RPMI with 10% FBS, L-Glutamine, 2 mercapto-ethanol) for 5 hours at 37°C in the presence of GolgiPlug with Brefeldine A (BD Biosciences, San Jose, CA) used according to manufacturer's recommendations. Excess peptide was washed away with SGM. OT-I T cells were surface stained with phycoerythrin-Cy7 conjugated anti CD8 antibody, APC conjugated Kb-SIINFEKL tetramer, and with antibodies against intracellular cytokines: FITC conjugated anti IFNγ, and PE conjugated anti TNFα.

In vivo cytotoxicity assay

In vivo cytotoxicity assays were performed according to a method adapted from Barber D, *et al.* (32). Briefly, RBC lysed spleen cells from B6 mice were pulsed with SIINFEKL peptide (10μM), LT359 peptide (10μM) or no peptide in SGM for 30 min at 37°C. Excess peptide was removed by washing with PBS. Each cell population was then labeled differently: no peptide

cells with 3μM CFDA-SE (CFSE, Invitrogen); SIINFEKL pulsed cells with 0.25μM CFSE; and LT359 pulsed cells with 2μM FarRed DDAO-SE (FarRed, Invitrogen). 5 x 10^6 cells of each peptide pulsed populations were mixed and injected into transfused/infected mice or naive mice. 12 to 15 hours after injection, spleens from recipient mice were harvested and the remaining labeled target cells were analyzed by flow cytometry. Percent specific lysis of labeled donor target cells was calculated as follows: [1-(# peptide pulsed events / # no peptide events) / (# peptide pulsed events $_{\text{in a naive mouse}}$ / # no peptide events $_{\text{in a naive mouse}}$)] x 100.

Statistical analyses

Intracellular cytokine staining experiments were analyzed with a two-way ANOVA with Bonferroni post-tests to compare specific groups. Analysis was performed using GraphPad Prism version 5.0b for Macintosh, GraphPad Software, San Diego California USA, www.graphpad.com.

Results

Transfusion with HOD blood does not induce BMT rejection

To test the hypothesis that mHAs on the RBCs themselves are capable of inducing BMT rejection, we engineered a model system utilizing HOD and mOVA donor mice. HOD mice express a fusion protein containing OVA specifically on RBCs, with no detectable expression on leukocytes or platelets (26). mOVA mice express OVA under an actin promoter, with wide tissue distribution (23). To test if mOVA is expressed on hematopoietic stem cells, we analyzed bone marrow from mOVA mice. Surface OVA expression was detected on CD34+ bone marrow cells, suggesting that mOVA could serve as a target antigen for BMT rejection (figure 1A). Thus, by transfusing recipients with RBC units from HOD mice, and then subsequently performing a BMT with mOVA donors, the immunological effects of exposure to a single mHA on RBCs and its subsequent effects on BMT can be assessed.

Despite four transfusions with HOD blood, BMT rejection was not observed (Figure 1B). Moreover, the percent chimerism in HOD blood recipients was equivalent to chimerism in control mice receiving wild-type FVB RBCs. In our previous studies, four transfusions of RBCs processed in the same manner were sufficient to induce BMT rejection in an MHC matched:mHA mismatched model (BALB.B $\rightarrow$ C57BL/6) (28). This negative finding was not due to the inability of mHAs from OVA to serve as a vector for rejection of mOVA bone marrow, as control mice infected with polyomavirus that expresses the SIINFEKL peptide from OVA (PyV.OVA-I) had robust BMT rejection.

Transfusion of sterile RBCs introduces a foreign antigen in the absence of an obvious activator of innate immunity. As innate immune activation is required for responses to many antigens, additional mice were infected with wild-type polyomavirus (PyV), followed by

transfusion and subsequent BMT rejection. No rejection was induced by HOD transfusion despite systemic infection with PyV. Additional activators of innate immunity were also studied, to control for potential idiosyncratic properties of PyV. Poly (I:C) is a toll-like receptor 3 agonist that induces viral-like inflammation; and we have previously reported that poly (I:C) injection substantially increases humoral responses to transfused RBCs (29). However, in the current situation, administration of poly (I:C) did not modify the outcome of the BMT (data not shown).

To assess the extent of immunization in the CD8+ T cell compartment, recipient mice were bled weekly and cells were stained with Kb-SIINFEKL tetramer, which represents an immunodominant CD8+ T cell epitope for OVA. Despite 4 transfusions with HOD blood, no SIINFEKL specific CD8+ T cells were observed in the absence or presence of PvV infection (Figure 3.1C). Since circulating lymphocytes may not reflect the biology in the lymphatics, representative mice were sacrificed and splenocytes were also analyzed; SIINFEKL specific T cells were likewise undetectable in the spleen (data not shown). This was not due to lack of capacity to respond, or due to poor tetramer reagents, as a robust SIINFEKL tetramer specific population was observed in control mice infected with PyV.OVA-I. Likewise, failure of PyV to enhance the response to HOD RBCs was not due to the virus failing to infect, as strong tetramer responses to the immunodominant peptide from PyV (Db-LT359) was observed (Figure 3.1D). Together, these data indicate that a single mHA on transfused RBCs neither induces BMT rejection nor detectable CD8+ T cell expansion, either on its own or in the context of inflammation induced by a viral infection.

CD8+ T cells expand and contract rapidly upon cross-priming by RBC associated mHAs.

We have previously reported that RBC associated antigens can be cross-presented to adoptively transferred CD8+ T cells and as a consequence induce early proliferation of the CD8+ T cells (28). However, in the current study, we did not detect expansion of endogenous SIINFEKL specific CD8+ T cells upon transfusion with HOD blood (in the absence of adoptive transfer). Our initial analysis of adoptively transferred cells focused only at early time points (28); thus, both of the above observations could be explained by the hypothesis that CD8+ T cells specific for RBC mHAs undergo initial proliferation followed by a subsequent deletion. In the absence of adoptively transferred CD8+ T cells, such an expansion and contraction would likely be undetectable due to the low precursor frequency of CD8+ T cells specific to a given antigen in naïve mice. To circumvent this limitation, we performed detailed kinetics analysis of OT-I splenocytes adoptively transferred into B6 mice followed by transfusion with HOD blood. As in the previous experiments, some of the mice were infected with PyV also in order to activate innate immunity. Positive control mice were infected with PyV.OVA-I.

Consistent with our previous findings (30), we found that OT-I T cells displayed a 5-fold expansion in the spleen of recipient mice after transfusion with HOD blood 4 days after transfusion (Figure 3.2A). However, this expansion did not result in a stable population, and numbers rapidly declined by 8 days post transfusion and remained at very low levels out to 14 days. Activation of innate immunity by infection with wild-type PyV enhanced the initial response to about a 20-fold expansion, but did not prevent the rapid contraction to very low levels by 8 days post-transfusion. The failure of OT-I T cells to establish a persistent response after HOD transfusion was not due to their inability to do so, as positive control mice infected with PyV.OVA-I had only moderate post-peak contraction and settled into a stable populations,

as is typical during viral infection. The observed responses were antigen-specific, as no significant expansion was seen in mice transfused with FVB blood regardless of infection with PyV (figure 3.2A).

Together, these data confirm that mHAs on RBCs can be cross-presented by recipient APCs and activate CD8+ T cells. Direct presentation is not likely in these studies because 1) the mHA is RBC specific and 2) the donor mice are on an FVB background, which does not express the K^b molecule to which OT-I T cells are restricted. The findings further indicate that whereas CD8+ T cells responding to their cognate antigen expressed by a virus rapidly expand and then establish a stable population, exposure to the same antigen on RBCs induces initial expansion and then dramatic contraction to only very low levels of persisting CD8+ T cells.

CD8+ T cells responding to RBC mHAs display partial activation and effector molecule expression.

To assess the phenotype of CD8+ T cells responding to RBC antigens, OT-I T cells were stained for several markers known to correlate to activation and effector function. FVB transfusion and PyV-OVA-I infection were used as negative and positive controls, respectively. OT-I T cells displayed an increased expression of both CD69 and CD44 in response to HOD blood, indicating cellular activation. As is typical for these molecules, CD69 expression was transient whereas CD44 remained elevated (figure 3.2B). Unlike positive control cells stimulated with PyV-OVA-I, transfusion of HOD blood induced only minimal increases in CD25 at early time points and only a slight decrease in CD62L.

Analysis of molecules associated with effector function demonstrated that little or no expression of CD107a was found in the OT-I T cells of mice transfused with HOD at day 2 when

compared to FVB control. In contrast, PyV.OVA-I infected mice displayed robust staining for CD107a at day 2. Similarly, only very slight levels of Granzyme B expression were observed in OT-I T cells responding to HOD transfusion whereas high levels were induced by PyV-OVA-I. CD107a or LAMP-1 is a marker of degranulation only found at the surface of cells after the fusion between the membrane of granules and the cytoplasmic membrane has occurred (31). GzB is one of the classical mediators of lytic activity for CD8 T cells. Interestingly, mice infected with PyV immediately before transfusion with HOD blood showed increased expression of both CD107a and Granzyme B, compared to either PyV or HOD blood alone, suggesting a synergistic effect of viral infection on the CD8+ T cell differentiation in response to RBC associated mHAs.

Although OT-I T cells cross-primed by the RBC associated HOD antigen only express lytic molecules (GzB) over a short period of time (see figure 3.2B), we wanted to know whether they were capable of other effector functions such as cytokine secretion. We measured the frequency of cytokine secreting OT-I T cells upon stimulation with SIINFEKL peptide *ex vivo* at day 4, which corresponds to the peak of the response in the OT-I T cells. Representative data plots are shown in figure 3.3A.

The expression of additional effector molecules was assessed by intracellular cytokine staining. Transfusion of HOD blood induced expression of both IFNγ and TNFα in a significant number of OT-I T cells, and about 20% of the OT-I T cells simultaneously secreted IFNγ as well as TNFα. This effect was significantly enhanced when the mice were also infected with PyV. This effect was specific, as no increase was seen in mice receiving control FVB blood or PyV in the absence of HOD transfusion (figure 3.3B).

CD8+ T cells that activate in the absence of some effector molecule induction (e.g. Granzyme B), have been associated with regulatory functions. Thus, we performed intracellular staining on the OT-I T cells for Foxp3 expression. In the mice transfused with HOD blood we did not observe any expression of Foxp3 when compared to the FVB control group. A discrete FoxP3+ population was observed in control CD4+ T cells, and not seen with isotype matched control, indicating that the assay was functioning (data not shown).

Together, the above data demonstrate that whereas CD8+ T cells exposed to their cognate antigen expressed by a viral infection express the full array of effector molecules, exposure to the same antigen on RBCs results in development of a partial effector phenotype with expression of effector cytokines, but decreased changes in surface molecules and only minimal signs of degranulation or expression of granzyme B.

Transient lytic activity is observed in CD8+ T cells responding to mHAs on RBCs

Because only partial effector molecule expression was observed, we tested the lytic activity of CD8+ T cells stimulated by mHAs on RBCs. To address this issue we adapted a previously described *in vivo* CTL assay (32) to the HOD antigen where SIINFEKL or LT359 peptide pulsed spleen cells were used as targets. Representative plots are shown in figure 3.4A. Four days after transfusion and/or infection mice transfused with HOD blood displayed a relatively strong lytic activity specific for the SIINFEKL epitope (figure 3.4B). Consistent with the contraction of CD8+ T cells seen in mice transfused with HOD RBCs, the lytic activity rapidly decreases and becomes undetectable at day 12. Similar to the enhancement of effector molecule expression, transfusion of HOD blood coincident with PyV infection prolongs the window of lytic activity temporarily, followed by a rapid decline. As a control for infection with

PyV.OVA-I virus we measured lytic activity against the LT359 epitope (the immunodominant epitope for this virus). We observed a strong and robust lytic activity against LT359 at later time points in all the groups that were infected with PyV virus (figure 3.4C).

Differential apoptosis is not the cause of altered outcomes in CD8+ T cell populations.

We hypothesized that the rapid contraction phase seen in CD8+ T cells responding to mHAs on RBCs was due to increased induction of apoptosis, analogous to activation induced cell death. To test this hypothesis, we assayed levels of phophatidylserine on the cell surface by staining with Annexin V. Cell viability was determined by co-staining with 7-AAD, a dye that is excluded from live cells and taken up by dead cells. A representative flow panel is shown in figure 3.5A. A baseline level of apoptosis was seen in control mice receiving FVB blood, and this was only slightly increased by infection with wild-type PyV. In contrast, there was a significant increase in Annexin V positive cells after transfusion with HOD RBCs, and this was slightly enhanced by coincident infection with PyV. Interestingly, the highest levels of apoptosis are seen in mice infected with PyV.OVA-I. Kinetic analysis demonstrates that the percentages of apoptotic cells are progressively declining in mice that received HOD RBCs (with or without PyV). In contrast, percentages of apoptotic cells are increasing both in control mice that did not receive antigen (FVB or FVB+PyV) and mice that received PyV-OVA. Given that activated CD8+ T cells are simultaneously undergoing proliferation and apoptosis, these data must be considered in the context of cellular expansion. We conclude that some CD8+ T cells are induced to undergo apoptosis when exposed to antigen either on RBCs or a virus, and that this apoptosis likely contributes to the contraction phase in response to either stimulus. However, taken together with enumeration data (see figure 3.2A), net deletion predominates in response to

RBCs whereas persistence predominates in response to PyV-OVA I. As even a greater percentage of cells undergo apoptosis in the latter case, we conclude that differential apoptosis is not responsible for the ultimate persistence or deletion of these populations (figure 3.5B).

Discussion

Herein, we report the first detailed analysis of activation, expansion, and effector functions of CD8 T cells responding to a single RBC-associated mHA. We confirmed that RBC associated antigens are capable of cross-priming adoptively transferred OT-I T cells leading to a proliferative response. However, we proceed to demonstrate that expansion is only short lived, followed by a rapid contraction phase, dropping to very low levels. Phenotypic analysis during activation demonstrates acquisition of a partial-effector function, including expression of IFNγ and TNFα, but not Granzyme B. Recent work from our group indicates that recipient inflammation enhanced humoral alloimmunization to antigens on RBCs (33). Therefore, we hypothesized that infection with a virus immediately before transfusion would impact the CD8+ T cell response to mHAs carried by the RBCs. Indeed, it appears that infection with a virus changed the phenotype of the cross-primed T cells, at least temporarily. We observed an expression of GzB and CD107a two days after transfusion with HOD blood only in the mice that were also infected with PyV. Lytic activity against the SIINFEKL epitope was also enhanced. However, even transfusion of HOD blood combined with PyV infection did not lead to a sustained CD8+ T cell response; thus, there appear to be additional factors required in order to obtain a full CD8+ T cell response (34).

In a previous report, we demonstrated that transfusion of RBC units induced rejection of subsequent BMT when the marrow donor shared minor antigens with the transfusion donor, which were foreign to the recipient. We interpreted this rejection as being due to mHAs, as the bone marrow donor and recipients were fully MHC matched. In the current report, we now demonstrate that when the system is reduced to a single mHA shared by RBC donor and BM donor (i.e. ovalbumin), then the transfusion of RBC units is insufficient to induce BMT rejection.

There are several hypotheses that are consistent with all of the observed data. First, it may be an issue of antigenic difference and dose, as in the previous report showing rejection, the mice were fully mismatched for mHAs at multiple loci (i.e. BALB – C57BL/6). Second is the fact that while the RBC units in our previous report were stringently leukoreduced, residual leukocytes still remained. Thus, if contaminating leukocytes are responsible, then genetically restricting the mHA to RBCs in the current study may remove immunization. Third, contaminating platelets in the leukoreduced RBC units in our previous report may have been responsible; indeed, we have recently reported that leukoreduced platelet units can also induce BMT rejection. Thus, it remains possible that RBCs themselves are responsible for inducing BMT rejection in the BALB – C57BL6 model, and that crossing multiple mHA barriers are required for the effect. However, the current data bring into question if RBCs are immunogenic and raise the issue that contaminating leukocytes or platelets may be responsible.

Whether RBCs themselves or contaminating leukocytes/platelets are required is a central issue to future blood product preparation. In the event that it is RBCs, but the extent of mHA mismatch is critical, then even partial matching for RBC mHAs may decrease effects on subsequent BMT rejection. However, if contaminating leukocytes or platelets are responsible, then more stringent depletion protocols may prevent the induction of BMT rejection. The development of better experimental depletion methodologies and/or generation of additional animals with genetically restricted antigen expression may help to address this issue.

Within the current report, the failure of transfusion of HOD RBCs to induce rejection of mOVA marrow is entirely consistent with both the lack of observed endogenous tetramer positive cells and the deletional effects of HOD transfusion on adoptively transferred CD8+ OT-I T cells. Although a transient activation of OT-I T cells was observed, their effector function was

only partially developed with a lack of detectable CD107a or Granzyme B expression. During the expansion phase, the OT-I T cells nevertheless had observable lytic function; however, lytic activity was lost coincident with the contraction of the OT-I T cells to very low levels. In contrast, infection with PyV-OVA-I induces a sustained OT-I population with expression of the full complement of effector molecules. The contraction thus appears to be somewhat specific to RBC associated mHA and occurs even if HOD RBCs are transfused coincident with wild-type PyV infection, albeit with altered kinetics. The data presented here suggest that RBC associated mHAs are capable of inducing the deletion of alloreactive CD8+ T cells in the periphery. This is to our knowledge the first demonstration of such a phenomenon. Cross-presentation of cell associated antigens has been shown to induce tolerance in the thymus (35) as well as in the periphery (36) but in both cases, self-antigens were deleted. We propose that the phenomenon of cross-tolerance can be extended to foreign antigens as well. Whether exposure to HOD RBCs actually induces a tolerogenic state to subsequent exposure to OVA antigens is currently under investigation. Nevertheless, the data indicate that RBC transfusion has the potential to induce tolerance to mHAs in the periphery. This may represent an unappreciated mechanism underlying the immunomodulatory effect of RBC transfusion. It is well known by transplant immunologists that donor specific transfusion of whole blood induces tolerance to solid organ transplants (37, 38). Many mechanisms have been proposed to explain the phenomenon including clonal deletion of alloreactive T cells. The importance of deletion of alloreactive CD8+ T cells in induction of tolerance is well understood (39). Overall, the data presented here may explain why donor specific transfusion is particularly effective in kidney transplants. It has been recently suggested that blood group antigens may be relevant to kidney transplantation (37). The clonal deletion of

alloreactive T cells to antigens shared by RBCs in the current studies suggests a possible tolerance pathway in this regard.

It must be acknowledged that the OT-I TCR has especially high affinity for the Kb-SIINFEKL complex, and that the observed biology may be biased towards behavior of high affinity CD8+ T cells. Indeed, it has been shown in some systems that OT-I T cells are deleted by encountering presented SIINFEKL but expand in response to lower affinity peptides (40). However, as the biology using adoptively transferred OT-I T cells (deletion of reactive cells) reflects the observed patterns of lack of BMT rejection in mice not receiving adoptive transfer, there is no basis to reject the OT-I findings as inconsistent with biology involving a range of TCR affinities.

In summary, the current report demonstrates that a single model mHA with restricted expression to RBCs does not induce BMT rejection, and instead appears to induce deletion of reactive CD8+ T cells. Transfusion of RBC products by us and others, in both mice and in dogs, have been shown to induce subsequent BMT rejection. However, RBC products are never 100% pure, and it has not been technically feasible to rule out the contribution of contaminating leukocytes and/or platelets. Using a genetic approach to restrict an mHA to RBCs, these studies bring into question whether it is the RBCs themselves that are responsible or if contaminating leukocytes and/or platelets contributing to the effect.

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Figure Legends

Figure 1. Multiple transfusions with HOD blood do not induce rejection of mOVA BMT or mHA specific CD8+ T cells. Bone marrow from mOVA mice was stained for OVA expression on $CD45^{dim}$-$CD34^{+}$ hematopoietic stem cells and $CD45^{hi}$-$CD3^{+}$ T cells **(A)**. C57BL/6 mice were given with four weekly transfusions of HOD or FVB blood followed by BMT from mOVA donors. Some mice were infected with PyV or PyV.OVA-I as indicated. Engraftment was assessed 6 weeks after BMT **(B)**. Peripheral blood of animals from each group was stained with anti-CD8 and Kb/SIINFEKL tetramer or Db/LT359 tetramer at the indicated timepoints **(C + D)**. Symbols for panel C, D: FVB ●, HOD ■, PyV + FVB ○, PyV + HOD □, PyV.OVA-I △. Data shown are representative of 3 separate experiments with 2 to 3 mice per group. Error bars represent the standard deviation for the data point.

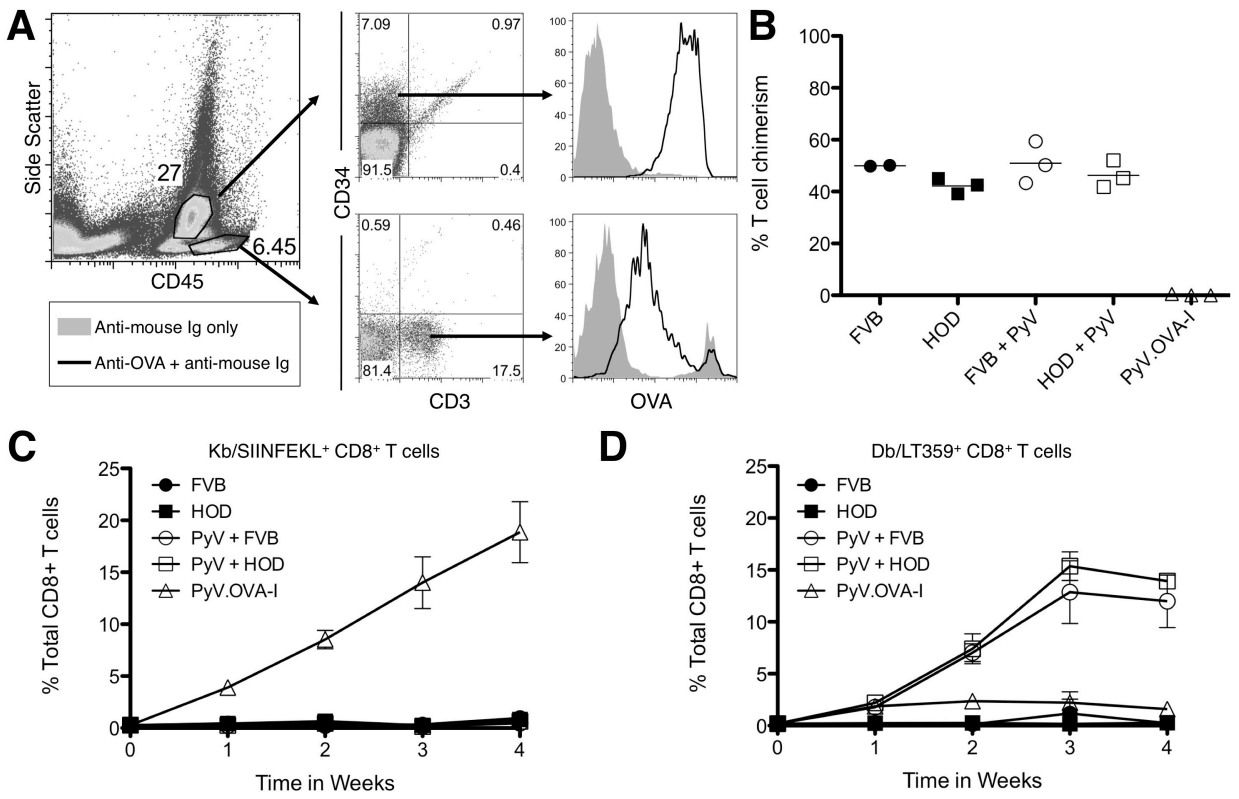
Figure 2. Transfusion with HOD blood induces transient activation and expansion of adoptively transferred OT-I T cells followed by profound contraction. Mice received adoptively transfer of OT-I CD8 T cells followed by transfusion with HOD or FVB blood and/or infection with PyV or PyV-OVA-I as indicated. Spleens were harvested and Kb/SIINFEKL+CD8+ T cells were enumerated **(A)** and were also stained with the indicated antibodies **(B)**. Data shown is the compounded results of 3 separate experiments with 2 to 3 mice per group. Symbols: FVB ●, HOD ■, PyV + FVB ○, PyV + HOD □, PyV.OVA-I △. Error bars represent the standard deviation for the data point.

Figure 3. Transfusion with HOD blood induces expression of IFNγ and TNFα in responding OT-I cells. IFNγ and TNFα secretion was measured by intracellular cytokine staining after ex vivo peptide restimulation of splenocytes from recipient mice. Representative flow cytometry plots of the staining with or without SIINFEKL peptide restimulation for the different experimental groups are shown. Plots are gated on $CD8^+$ Kb/SIINFEKL $tetramer^+$ events. Combined data shown are the compounded results of 3 separate experiments with 2 mice per group **(B).** Statistical analysis was performed with two-way ANOVA analysis with Bonferroni post-tests, * = $p<0.05$, *** = $p<0.001$. Error bars represent the standard deviation for the data point.

Figure 4. Transfusion with HOD blood induces transient cytolytic activity against OVA in adoptively transferred OT-I T cells. Cytolytic activity against SIINFEKL or LT359 peptide pulsed targets was measured in an in vivo cytolytic assay. Representative flow cytometry plots for the different experimental groups are shown **(A)**. Combined data at the indicated timepoints are shown **(B+C)**. Data shown are the compounded results of 3 separate experiments with 2 mice per group. Symbols: FVB ●, HOD ■, PyV + FVB ○, PyV + HOD □, PyV.OVA-I △. Error bars represent the standard error of the mean for the data point.

Figure 5. Measurement of apoptosis in OT-I T cells stimulated with HOD RBCs. Apoptosis in live adoptively transferred OT-I cells was measured by staining splenocytes of recipient mice with Kb/SIINFEKL tetramer, 7-AAD, and Annexin V. Representative flow cytometry plots gated on the $CD8^+$ KbSIINFEKL $tetramer^+$ events for the different experimental groups at day 4 after transfusion/infection are shown **(A)**. Data shown are the compounded results of 2 separate

experiments with 2 mice per group. Symbols: FVB ●, HOD ■, PyV + FVB ○, PyV + HOD □, PyV.OVA-I △. Error bars represent the standard error of the mean for the data point **(B)**.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FVB
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FVB + PyV
HOD + PyV
PyV.OVA-I
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Db/LT359+ CD8+ T cells
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Time in Weeks
FVB
HOD
PyV + FVB
PyV + HOD
PyV.OVA-I

Figure 3.1

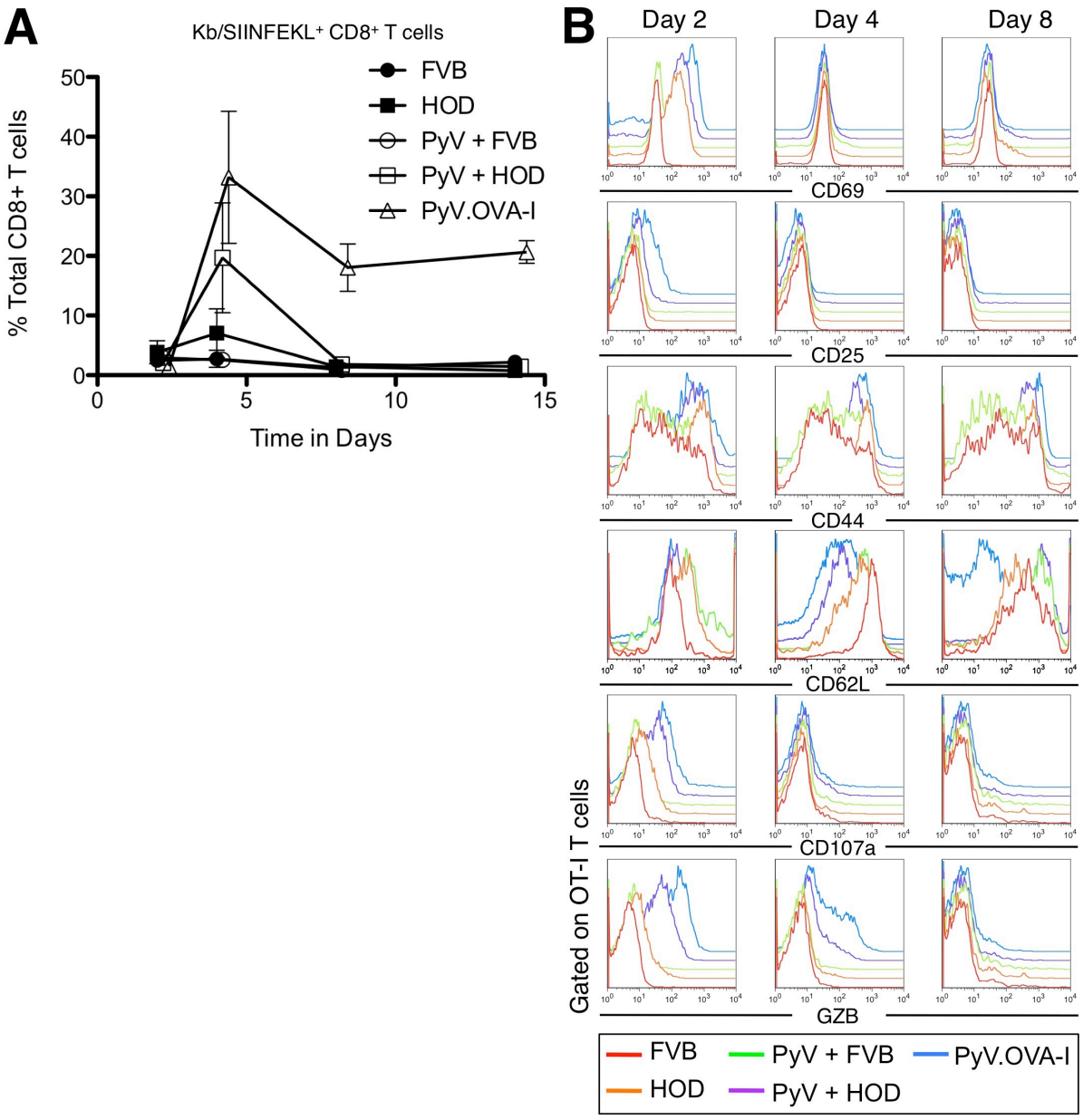
A
Kb/SIINFEKL+ CD8+ T cells
FVB
HOD
PyV + FVB
PyV + HOD
PyV.OVA-I
% Total CD8+ T cells
Time in Days
B
Day 2
Day 4
Day 8
CD69
CD25
CD44
CD62L
CD107a
GZB
Gated on OT-I T cells
FVB
HOD
PyV + FVB
PyV + HOD
PyV.OVA-I

Figure 3.2

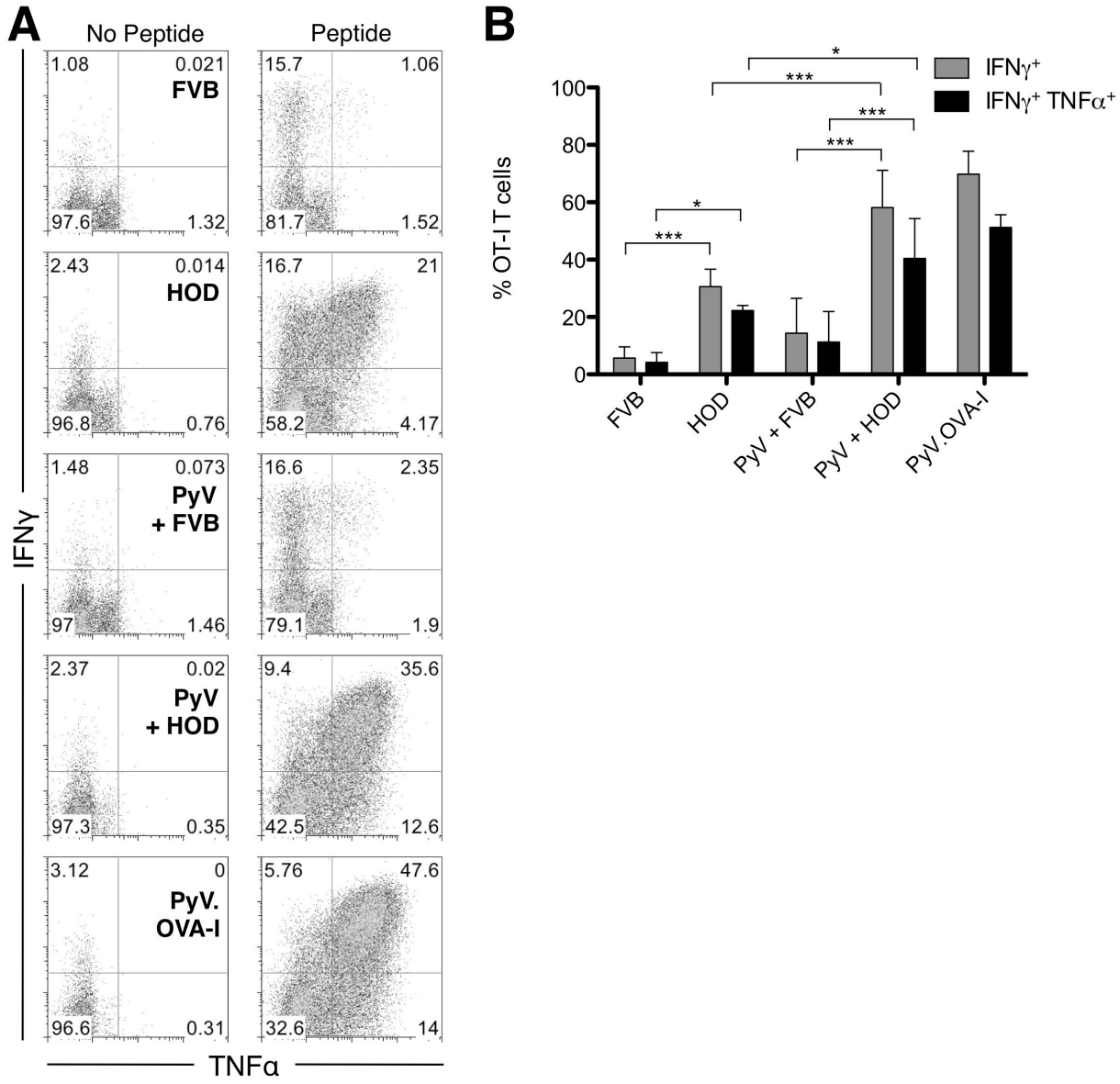
A
No Peptide
Peptide
1.08
0.021
FVB
97.6
1.32
15.7
1.06
81.7
1.52
2.43
0.014
HOD
96.8
0.76
16.7
21
58.2
4.17
1.48
0.073
PyV
+ FVB
97
1.46
16.6
2.35
79.1
1.9
2.37
0.02
PyV
+ HOD
97.3
0.35
9.4
35.6
42.5
12.6
3.12
0
PyV.
OVA-I
96.6
0.31
5.76
47.6
32.6
14
IFNγ
TNFα
B
% OT-I T cells
IFNγ+
IFNγ+ TNFα+
100
80
60
40
20
0
*
***
***
***
*
***
FVB
HOD
PyV + FVB
PyV + HOD
PyV.OVA-I

Figure 3.3

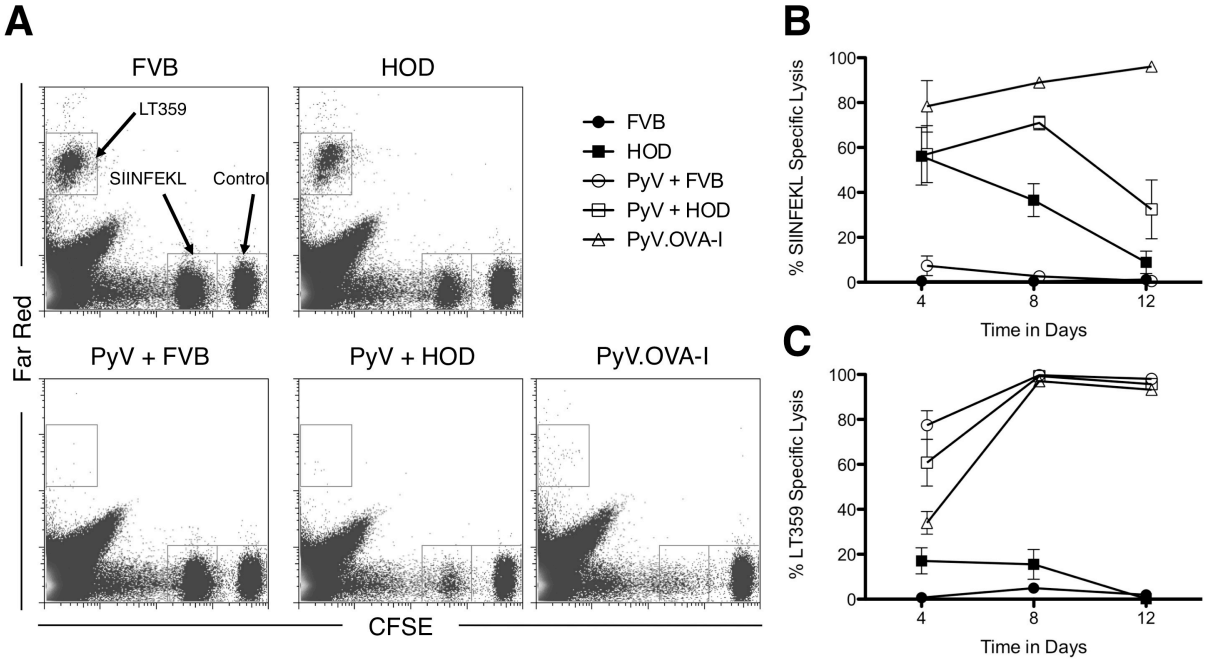
A
FVB
HOD
LT359
SIINFEKL
Control
FVB
HOD
PyV + FVB
PyV + HOD
PyV.OVA-I
Far Red
PyV + FVB
PyV + HOD
PyV.OVA-I
CFSE
B
% SIINFEKL Specific Lysis
100
80
60
40
20
0
4
8
12
Time in Days
C
% LT359 Specific Lysis
100
80
60
40
20
0
4
8
12
Time in Days

Figure 3.4

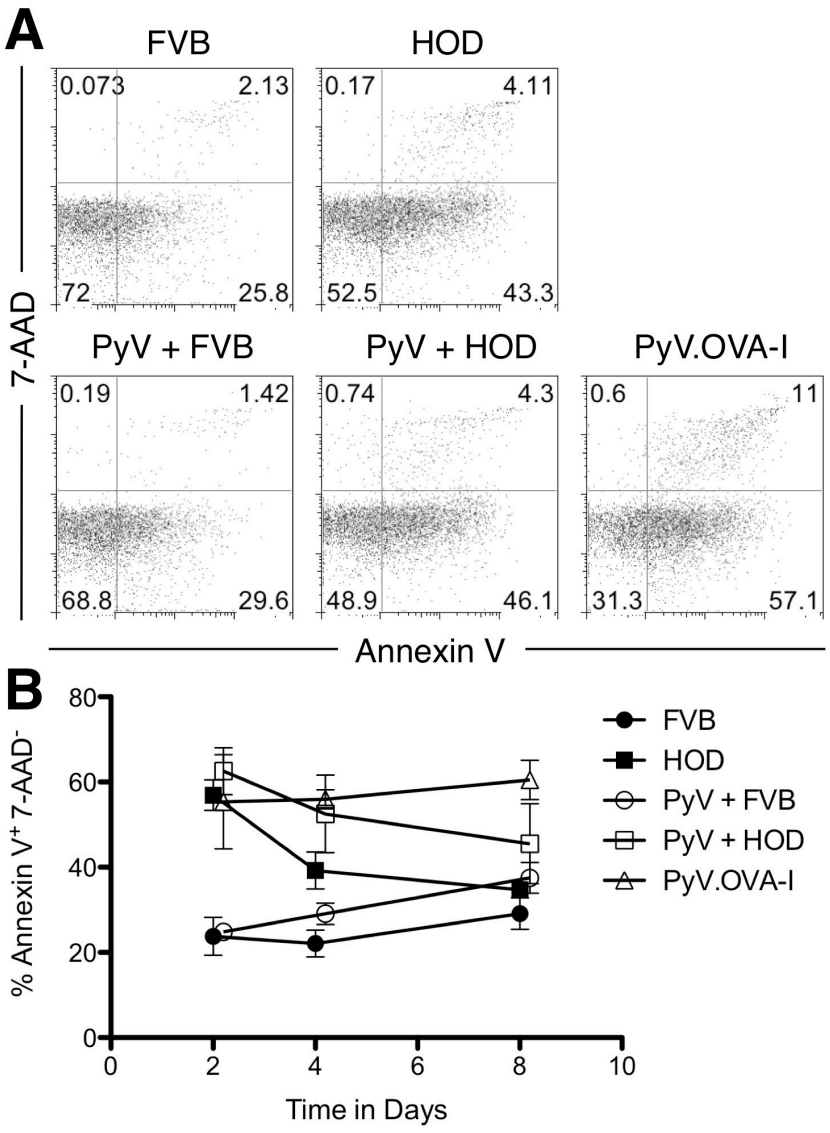
A
FVB
HOD
0.073
2.13
72
25.8
0.17
4.11
52.5
43.3
PyV + FVB
PyV + HOD
PyV.OVA-I
0.19
1.42
68.8
29.6
0.74
4.3
48.9
46.1
0.6
11
31.3
57.1
7-AAD
Annexin V
B
% Annexin V+ 7-AAD-
80
60
40
20
0
0
2
4
6
8
10
Time in Days
FVB
HOD
PyV + FVB
PyV + HOD
PyV.OVA-I

Figure 3.5

Chapter 4

Modeling Bone Marrow Transplant Rejection by Minor Histocompatibility Antigen Mismatched Red Blood Cell Units

Abstract

HLA matched bone marrow transplantation (BMT) with reduced intensity conditioning is a cure for several non-malignant hematological disorders that require chronic transfusion, such as sickle cell disease and aplastic anemia. However, there are unusually high BMT rejection rates in these patients. Rejection correlates with the number of pre-BMT transfusions, and it has been hypothesized that pre-immunization to antigens on transfused blood may prime BMT rejection. Using a novel mouse model of RBC transfusion and MHC matched BMT, we showed that transfusion of RBC products induced BMT rejection across minor histocompatibility antigen (mHA) barriers. While leukoreduction decreases the immunogenicity of RBC transfusion, it is insufficient to prevent BMT rejection. To study the immunogenicity of the RBC themselves, we generated a novel transgenic mouse with RBC specific expression of a single model mHA: the HOD mouse. Using this model we demonstrated that transfusion of HOD RBCs does not induce rejection of mHA expressing BMT. Rather, HOD RBC transfusion leads to transient expansion followed by rapid contraction of antigen-specific CD8 T cells. Together, these data suggest that mHAs on RBCs themselves are not capable of inducing BMT rejection under the current conditions but nevertheless suggest a potential for CD8+ T cell response. Here we discuss the development of the transfusion/transplantation models and begin to investigate which component of the transfused RBC units are involved in BMT rejection Additionally, we explore the effector mechanisms of rejection.

Introduction

Bone marrow transplantation (BMT) from HLA matched donors with a reduced conditioning regimen is a cure for non-malignant hematological blood disorders such as sickle cell anemia or aplastic anemia. Patients with these conditions require transfusion support in the course of their disease. In severe cases of sickle cell anemia this can represent as much as hundreds of red blood cell (RBC) units during a lifetime. It has been demonstrated that multiple transfusions are associated with more frequent bone marrow rejection in a canine model as well as in humans. It was generally accepted that leukocytes present it the RBC units were responsible for the increased rejection, but the implementation of universal leukoreduction for blood products in some medical centers has not decreased the rates of rejection of bone marrow or solid organ transplants. Therefore, we hypothesized that transfusion of RBCs themselves is capable of inducing BMT rejection. Testing of this hypothesis requires the development of models of minor histocompatibility antigen (mHA) mismatched (but MHC matched) blood transfusion followed by BMT. In chapter 2, we demonstrated that mHAs on transfused leukoreduced RBC units were capable of inducing rejection of BMT from a donor matched to the RBC donor. We present here the development of additional models and discuss their characteristics. Moreover, these studies beg two additional questions. First, what components of the RBC units are involved in inducing immunity? Second, what is the nature of immune response that rejected BMT engraftment? We begin to address both issues here. To address the first question, we hypothesized that residual WBCs (rWBCs) remaining after leukoreduction do not induce BMT rejection. To test this hypothesis we removed RBC from the leukoreduced BALB.B blood using a hypotonic RBC lysis buffer

and transfused B6.Thy1.1 recipient mice with the resulting preparation. The mice then received a BMT from BALB.B donors. We found that after multiple transfusions under certain conditions rWBC preparations could also induce BMT rejection and discuss the implications of this finding.

To address the question of the nature of the immune response that causes BMT rejection, we measured immunity to mHAs after transfusions using in vivo killing assays and assessed the presence of antibodies against BALB mHAs using a flow cytometry based indirect immunofluorescence assay.

In parallel, we hypothesized that blood from HOD donor mice could induce rejection of OVA expressing mOVA bone marrow. In HOD blood, only RBCs express HOD antigen, a fusion protein made of HEL, OVA and Duffy b. WBCs and platelets do not carry the protein (see figure 2.4). We found that multiple transfusions with HOD blood did not induce mOVA BMT rejection (see figure 3.1), but peptide epitopes derived from the HOD protein are capable of being cross-presented to adoptively transferred OT-I T cells. Moreover, injection with poly (I:C) prior to transfusion enhanced the response in the cross-primed OT-I T cells. These data lead us to conclude that RBC have the potential to induce CD8+ T cells responses, and, provided that CD8+ T cells are the main mediator of rejection, BMT rejection. However, in the HOD model the conditions necessary for this effect are not present.

Materials and Methods

Mice

C57BL/6 (B6), BALB/c, B6.PL-Thy1^a (B6.Thy1.1), C.B10-H2b (BALB.B), B10.BR-H2k (B10.BR) and FVB/NJ (FVB) mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and housed in Emory University Department of Animal Resources facilities. Mice were used at 8 to 12 weeks of age, and all procedures were performed according to protocols approved by Emory's Institutional Animal Care and Use Committee. The HOD (1), mHEL (2) and Act-mOVA.B6 (mOVA) (3) mice were previously described and were bred by the Emory Division of Animal Resources Animal Husbandry service (mHEL mice are available from Jackson Laboratories).

Blood collection, leukoreduction and transfusion

Mouse blood was obtained by retro-orbital bleeding into ACD solution A (BD, Franklin Lakes, NJ). The blood was then washed 3 times to remove platelets, with phosphate-buffered saline (PBS) adjusted to 340 mOsm with NaCl (modified PBS or mPBS). Some units were leukoreduced using a neonatal leukoreduction filter (Purecell Neo; Pall Medical, East Hills, NY) previously primed with 12 ml of mPBS. Each transfusion consisted of 100 μl of packed RBCs diluted with 400 μl DPBS injected in the tail vein.

Bone marrow transplantation

Except where indicated, B6.Thy1.1 recipients were irradiated with a dose of 650 cGy for BALB.B and mOVA transplantation, or 500 cGy for HY transplantation. The next day, donor whole bone marrow was prepared from the femur and tibia in PBS and 5 x 10^6

whole bone marrow donor cells were injected intravenously. Bone marrow engraftment was monitored by flow cytometry. Peripheral WBCs were stained with FITC labeled anti-CD3, PE labeled anti-Thy1.2 (BD Biosciences), and APC labeled anti Thy1.1 (eBioscience, San Diego, CA) antibodies. Percent donor engraftment was calculated using $Thy1.1^+$ events / ($Thy1.1^+$ events + $Thy1.2^+$ events) x 100. Engraftment was defined as a sustained chimerism of greater than 5%, 6 weeks after transplant. The use of CD45.1/CD45.2 markers was purposely avoided in our system, as CD45.1/CD45.2 has been reported to be an mHA capable of inducing rejection (4).

In vivo killing assays

In vivo killing assays were performed according to a method adapted from Barber D, et al. (5).

BALB killing assay. RBC lysed splenocytes from B6 and BALB.B mice were labeled with a different concentration of CFDA-SE (CFSE, Invitrogen): 3 μM (B6 control population) or 0.25 μM (BALB.B cells). 5×10^6 cells of each population were mixed in PBS and injected into experimental groups. 12 hours after injection, spleens from recipient mice were harvested and remaining CFSE labeled target cells were analyzed by flow cytometry. Percent specific removal of CFSE labeled donor target cells was calculated as follows: $[1 - (\text{BALB.B}_{\text{experimental mouse}} / \text{B6}_{\text{experimental mouse}}) / (\text{BALB.B}_{\text{naïve mouse}} / \text{B6}_{\text{naïve mouse}})] \times 100$.

HY killing assay. RBC lysed spleen cells from female B6, Female B10.BR and male B6 mice were labeled as follows: female B6 control population with 3 μM CFSE, male B6 population with 0.25 μM CFSE, and female B10.BR population with 2 μM PKH26

(PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labeling used according to manufacturer's recommendations, Cat# PKH26GL, Sigma-Aldrich, St. Louis, MO), 5×10^6 cells of each population were mixed in PBS and injected into experimental groups. 12 hours after injection, spleens from recipient mice were harvested and remaining labeled target cells were analyzed by flow cytometry. Female B6 (syngeneic) cells were used as an internal control population. Percent HY specific killing was calculated as follows: $[1 - (\text{male B6}_{\text{experimental mouse}} / \text{female B6}_{\text{experimental mouse}}) / (\text{male B6}_{\text{naïve mouse}} / \text{female B6}_{\text{naïve mouse}})] \times 100$. Percent H2-k specific killing was calculated as follows: $[1 - (\text{female B10.BR}_{\text{experimental mouse}} / \text{female B6}_{\text{experimental mouse}}) / (\text{female B10.BR}_{\text{naïve mouse}} / \text{female B6}_{\text{naïve mouse}})] \times 100$.

In vivo crosspresentation to OT-I T cells.

On day –1, 15×10^6 splenocytes from OT-I donors were labeled with 3 μM CFSE (Invitrogen) and adoptively transferred i.v. into 8-12 week old B6 recipients and left to distribute for 24 hours. On day 0, whole blood from FVB and HOD donor mice was collected and anticoagulated with ACD. The blood was then washed 3 times in mPBS and 100 μl of packed blood diluted with 400 μl PBS was injected i.v. into the recipient mice. Expansion of the OT-I T cells in the spleen of the recipient mice was measured by staining with an anti-CD8 antibody (BD Biosciences) and OT-I specific K^bSIINFEKL MHC tetramer at the indicated time points.

Flow cytometry

All antibodies used were purchased from BD Biosciences (San Jose, CA) except where noted. Cells were stained in phosphate-buffered saline containing 0.2 % bovine serum albumin and 3 mM ethylenediaminetetraacetic acid (FACS buffer). Antibody staining was performed at 4°C or room temperature for 30 min; allophycocyanin-conjugated $K^bSIINFEKL$ tetramer staining was performed at room temperature for 30 min. Cells were surface stained with a fluorescein isothiocyanate-conjugated anti-CD8 or a phycoerythrin-conjugated anti-CD107a. Control staining with isotype matched antibodies was also performed. Intracellular staining was performed using the Cytofix/Cytoperm kit (BD Biosciences) in conjunction with a PE conjugated anti Granzyme B (Clone GB12, Invitrogen). All flow cytometry data was acquired on a FACSort flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, OR).

Flow cytometric analysis of leukoreduced blood.

Staining of rWBCs in the leukoreduced blood was adapted from a reviously described method (6). Briefly, a 25 μl aliquot of blood was stained using 150 ng/ml Propidium Iodide (PI) (Invitrogen, Carlsbad, CA), anti-CD19 FITC, and anti-CD3 APC in a 1 g/l sodium citrate dihydrate solution with 0.7% Zap-oglobin II reagent (Beckman Coulter, Fullerton, CA), 0.7% RNAse cocktail (New England Biolabs, Ipswich, MA), effectively staining all nucleated cells.

Seroanalysis by indirect immunofluorescence.

Serum was diluted 1:10 in FACS buffer and incubated with BALB.B splenocytes for 30 minutes, washed 3 times with FACS buffer, and incubated with a 1:100 dilution of APC goat anti-mouse immunoglobulins. B cells were excluded by staining with anti-mouse CD19-FITC. Samples were analyzed by flow cytometry; Mean fluorescence intensity of APC was used to indicate the presence of antibody on targets.

Viruses

The wild type mouse polyoma virus (PyV) strain A2 and recombinant PyV.OVA-I were prepared in baby mouse kidney cells, as previously described (7). Briefly, recombinant PyV.OVA-I was generated through the insertion of the SIINFEKL coding sequence in frame at a unique BlpI restriction site in the coding region of middle T antigen (8). Mice were injected subcutaneously (s.c.) in hind footpads with 1 x 10^6 PFU of virus.

Intracellular Cytokine Staining

Spleens from recipient mice were harvested 4 days after transfusion or infection with polyoma virus. 2×10^6 splenocytes were incubated with or without SIINFEKL peptide at a 10 μM concentration in standard growth media (SGM: RPMI with 10% FBS, L-Glutamine, 2 mercapto-ethanol) for 5 hours at 37°C in the presence of GolgiPlug with Brefeldine A (BD Biosciences) used according to manufacturer's recommendations. Excess peptide was washed away with SGM. OT-I T cells were surface stained with phycoerythrin-Cy7 conjugated anti-CD8 antibody, APC conjugated K^bSIINFEKL

tetramer, and with antibodies against intracellular cytokines: FITC conjugated anti-IFNγ, and PE conjugated anti-TNFα.

Synthetic peptides

The SIINFEKL peptide was synthesized by the solid-phase method using F-moc chemistries (Emory University Microchemical Core Facility) and purified by high-pressure liquid chromatography to greater than 90%. Stocks were solubilized in water and stored at –20°C. The peptide was diluted in SGM immediately before use.

Effect of TLR agonists

On day –1, mice were adoptively transferred with OT-I splenocytes. On day 0, mice were transfused via the lateral tail vein with 100 μL of HOD blood diluted 400 μL with PBS. One hour before transfusion, a subgroup of mice were pretreated intraperitoneally (i.p.) with 100 μg of poly(I:C) (Amersham, Piscataway, NJ) or 100 μg of LPS from Escherichia coli 0127:B8 (Sigma, St Louis, MO) in 500 μL PBS.

Results

Development of a reduced intensity conditioning regimen for BALB.B – B6 BMT

We hypothesized that transfusion of leukoreduced blood units from mHA mismatched BALB.B donors is capable of inducing rejection of bone marrow from BALB.B donors. To test this hypothesis we needed a murine model of BMT that mimics the clinical situation, which consists of reduced conditioning regimens. We needed to find a conditioning regimen that wouldn't be lethal to recipient mice but would allow engraftment of a BMT from BALB.B donor mice. The conditioning regimen consisted solely of irradiation of the mice. A dose titration was carried out over a range of 200 to 700 cGy (figure 4.1A). We compared engraftment of control B6 bone marrow (all recipients were B6 mice congenic for Thy1.1) to that of mHA mismatched BALB.B bone marrow, and found that the minimal conditioning regimen for engraftment of BALB.B bone marrow was 600 cGy (figure 4.1B). For subsequent experiments, a dose of 650 cGy was utilized, as it represented the lowest dose that consistently provided engraftment. Chimerism with Thy1.2 congenic bone marrow (B6 donors) occurred at doses as low as 200 cGy. These data indicate that mHAs on the BALB.B bone marrow represent a significant immunological barrier to engraftment that can be overcome by increasing the dose administered to the recipient.

In an effort to better characterize our BMT model, the source bone marrow was analyzed for T cell and hematopoietic stem cell content by flow cytometry. We found that CD34+ hematopoietic stem cells represented 6-8% of the $CD45^{dim}$ population known to contain the true hematopoietic stem cells in humans (9) and CD3+ T cells represented 2-3% of the $CD45^{hi}$ population (figure 4.1C). These numbers were sufficient for

consistently successful transplantation in mice. Of note, these numbers represent about 7 x 10^6 CD34+ cells / kg, which is significantly lower than the amount of CD34+ cells usually used in humans (0.5 to 3 x 10^8 cells / kg). The T cell content of the harvested bone marrow was also significantly lower than that observed in humans (12% of total nucleated cells in non T cell depleted grafts).

Mice that displayed engraftment showed no sign of graft versus host disease (GvHD), with no apparent weight loss, normal posture and behavior, and no piloerection. This result may be explained by the low T cell content of the transplanted bone marrow, but the outcome was expected as GvHD after bone marrow transplant from whole bone marrow is usually rare in mice. Murine models of GvHD usually require the addition of T cell to the donor bone marrow preparation. As a model, this represent a significant departure from the usual outcome of BMT in humans in which mHAs can mediate devastating GvHD (10).

Blood leukocyte composition changes before and after leukoreduction

Although leukoreduction removes most of the WBCs present in the blood (~3 log reduction in WBCs), residual leukocytes remain after leukoreduction and may be involved in the induction of immunity to mHAs present in the blood. The composition of the blood leukocytes before and after leukoreduction was analyzed. It is important to note that we performed the analysis on samples that were not used for injection into mice because they contained more than 1000 rWBCs per 100 μL injection. It is otherwise technically difficult to analyze the leukocyte composition for lack of sufficient event numbers to delineate a population. Attempting analysis with blood that had optimal

leukoreduction would have required the use of an inordinate number of animals, and that is difficult to justify. We found that the cellular composition of leukocytes was significantly altered by the leukoreduction process. In particular there was a relative enrichment in CD3+ T cells (73% post vs. 26% pre) and a decrease in CD19+ B cells (5% post vs 35% pre), other nucleated cells were also depleted but to a lesser degree (21% post vs 37% pre) (figure 4.2). These results are not consistent with data observed in humans. Multiple studies from Roback et al. and others have indicated a large predominance of granulocytes representing up to 90% of rWBCs and also monocytes (6%) and a preferential removal of lymphocytes (11-13). Our study focused on T and B cells and did not differentiate between granulocytes and monocytes. The reasons for these inconsistent results are not known to us, but we can speculate that differences were observed because the study was performed on what was considered a failed leukoreduction attempt.

Residual WBCs preparations are capable of inducing BMT rejection

Although we have evidence that mHAs in leukoreduced RBC units can induce BMT rejection (1) it is unclear what cell type or types in the leukoreduced unit are involved in the induction. To address this issue we sought to isolate the leukocyte component of leukoreduced RBC units by lysing RBCs. The cells remaining after RBC lysis of leukoreduced units were injected into B6 mice. Therefore, the injections consisted only of the rWBCs present in leukoreduced blood. However, the purity of rWBCs prepared using this method is about 65-70% with the majority of contaminating events consisting of residual RBCs fragments (figure 4.3A). Each rWBCs preparation

was analyzed to enumerate the WBCs present at each step of the process (i.e. after leukoreduction and after RBC lysis). Leukoreduction filters were capable of reducing the number of WBCs by a factor ranging from 5×10^2 to 1×10^3. After RBC lysis, volumes were adjusted in an attempt to normalize the rWBCs concentration to unlysed units; however some variation could not be avoided, in particular during the final transfusion (figure 4.3B). After 3 transfusions with the prepared rWBCs and transplantation with BALB.B bone marrow, 2 out of 5 mice (40%) had BMT rejection. No rejection was observed in mice given 1 or 2 transfusions prior to BMT. As expected, 100% of control mice transfused with BALB.B whole blood rejected the BMT (figure 4.3C,D). When analyzing these mice with an in vivo killing assay, we found that immunization was undetectable before BMT, even in the mice that subsequently rejected BMT. However, the mice that rejected the BMT showed strong lytic activity against BALB.B targets after BMT (figure 4.3E).

In vivo killing but not anti-BALB antibodies are detectable in BALB.B BMT rejecting recipients

BMT can substantially alter the immune system and responses to transplant antigens. To gain some insights into the effector mechanism of BMT rejection, transplanted recipients were tested for BALB mHA–specific immunity after the outcome of BMT was determined (figure 4.4A,B). Serum was collected to test for antibodies by indirect immunofluorescence (see methods); recipients were then injected with CFSE-labeled BALB.B and B6 splenocyte targets; 12-hour target survival was assessed. Pre BMT, splenocyte targets were eliminated in some of the mice injected with BALB.B LR

blood but undetectable in mice injected with BALB/c blood. The reasons for the difference in lysis observed in BALB.B and BALB/c mice is unclear but may be due to skewing of the response in BALB/c mice toward the H-2^d MHC. Nevertheless, BALB.B target lysis was a poor predictor of BMT rejection in our model as in many occurrences lytic activity in the in vivo killing assay test group was completely undetectable although most of the transplanted mice displayed rejection. It is important to mention that in vivo killing assay are a terminal assay and lytic activity and BMT engraftment cannot be determined in the same animals. Post BMT, BALB.B splenocyte targets were eliminated in 100% of the recipients that had rejected the BMT, with normal target survival in control animals. The few animals that had received BALB.B LR RBCs and did not reject the BMT were the same mice that did not eliminate the BALB.B targets. (figure 4.4C). No anti-BALB antibody signal was detected above background (B6 LR transfused mice) in any of the groups transfused with leukoreduced blood (figure 4.4D). 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 and suggest a cellular immune mechanism. However, we cannot rule out that the indirect immunofluorescence assay is not sensitive enough to detect the BALB antibodies. Other more sensitive techniques (ELISA) may be required.

HY BMT reduced intensity conditioning regimen

Similar to the BALB.B BMT model, a reduced intensity conditioning regimen was required for the HY transplantation model that would allow engraftment of HY+

bone marrow. This was established by exposing female B6.Thy1.1 recipients to increasing doses of radiation ranging from 200 to 600 cGy. The lowest amount of radiation necessary to allow HY+ bone marrow engraftment was 400 cGy; however, a 500 cGy dose was required for consistent engraftment in all mice (Figure 4.5). Thus, a dose of 500 cGy was used for all subsequent experiments involving HY^+ BMT. Similar to titrations carried out to optimize the BALB.B BMT model, control female congenic bone marrow engrafted with a dose as little as 200 cGy. The difference in engraftment threshold between male and female bone marrow indicates that HY mHAs represent an immunological barrier to BMT that can be overcome by increasing the irradiation dose.

Analysis of immunization to HY mHAs and H-2^k MHC after transfusion with leukoreduced Male B10.BR blood

Although HY+ bone marrow was not rejected after transfusion with male B10.BR leukoreduced blood (see figure 2.3), it was important to understand whether immunity to HY antigens occurred at all in our model. Moreover, because HY+ was rejected after transfusion of whole blood from male B10.BR donors, it was also necessary to establish, as it was done for transfusion with BALB/c blood in chapter 2 (figure 2.2), that immunization to H-2^k MHC is not sufficient to induce Male B6 (H-2^b, HY+) BMT rejection, because of a bystander lytic effect .

We repeated some of the BMT experiments performed for chapter 2 with an additional group of mice transfused with female B10.BR blood (figure 4.6A,B). None of the mice transfused with female B10.BR blood displayed rejection of BMT from male B6 donors. As previously observed, the positive control group transfused with male B10.BR

whole blood did induce rejection of male B6 BMT marrow. This control is performed in all experiments to indicate that rejection of HY+ marrow is possible in the experiment and that mice were conditioned properly.

Immunity to Male B6 (HY+) and female B10.BR (H-2^k) splenocytes was measured in an in vivo killing assay. Mice transfused with H-2^k whole blood (female B10.BR whole blood or Male B10.BR whole blood) had a strong response against H-2^k targets before and after BMT. Mice transfused with leukoreduced H-2^k blood did not display significant immunity. Immunity to HY antigens could only be detected in mice transfused with male whole blood. Mice transfused with male leukoreduced B10.BR blood did not display significant immunity. Together these data indicate that immunization to H-2^k MHC is not sufficient to induce rejection of H-2^b HY+ BMT which further supports that rejection of HY+ marrow by male whole blood is antigen specific.

Interestingly, transfusion with male B10.BR whole blood did not induce detectable immunity before BMT. The robust immunity against H-2^k targets observed after BMT indicate as we observed in the BALB model that leukoreduction reduces the immunogenicity of the blood but is insufficient to prevent immunization. Moreover, the data suggest that killing assays are a poor predictor of HY+ BMT outcome (figure 4.6C,D).

HEL expression on HOD blood

In chapter 2, it was demonstrated that HOD RBCs express all the elements of the HOD fusion protein: HEL, OVA and the human blood group Duffy in its b variant. Because the research presented do not involve HEL, the expression of HEL on HOD

leukocytes and platelets was not assessed. Nevertheless, in an effort to characterize the newly created HOD mouse, it is important to verify that HEL also displays the same expression pattern as OVA on HOD blood, that is to say RBC specific expression with lack of detectable expression on other blood lineages (leukocytes and platelets). To analyze expression on leukocytes, HOD blood was subjected to RBC lysis followed by staining with anti-CD45, anti-TER-119, and a monoclonal anti-HEL antibody (4B7) conjugated to Alexa Fluor 647 (14). To analyze HEL expression on platelets, HOD whole blood was stained with conjugated anti-CD41, anti-TER119, and APC conjugated 4B7. While 4B7 staining was high on the leukocytes of mHEL mice (positive control), HEL expression was not detected on $CD45^+$ $TER\text{-}119^-$ leukocytes (figure 4.7A). Likewise, it was not detected on $CD41^+$ $TER\text{-}119^-$ platelets. The experiment failed to detect HEL on platelets from a mHEL mouse, which raises the question of the sensitivity of this assay for platelets. However, the lack of detection was not due to a deficient antibody reagent as experiments on leukocytes and platelets were performed together.

Poly (I:C) enhances the cross-priming efficiency of CD8+ T cells by RBC derived antigens

We showed in chapter 3 that inflammation of the recipient enhances the efficiency of the cross-priming of OT-I T cells by RBC derived mHAs. When recipient mice were infected with polyoma virus (PyV), expansion of the OT-I T cells in the spleen was increased, survival of the cells was prolonged, and expression of the effector molecules Granzyme B and the marker of degranulation CD107a were increased (figure 3.2B), a higher percentage of OT-I T cells also expressed effector cytokines (figure 3.4B).

However, infection with PyV along with transfusion of HOD blood did not induce rejection of mOVA BMT (in the absence of adoptive transfer of OT-I T cells). We hypothesized that the use of more potent mediators of inflammation, such as LPS and poly (I:C), would enhance the cross-priming efficiency of the APCs and induce a more potent response in the cross-primed OT-I T cells. After adoptive transfer of the OT-I splenocytes, but immediately before the transfusion with HOD blood, we injected 100 μg of poly (I:C) or LPS intraperitoneally into recipient B6 mice.

Similar to infection with PyV, injection of poly (I:C) enhanced the expansion of OT-I T cells in the spleen of the recipients upon cross-priming with HOD RBCs, but the OT-I T cells do not maintain a sustained response and are quickly removed from the spleen after about 8 days (figure 4.8A). Consequently, activation of innate immunity by poly (I:C) was insufficient induce mOVA BMT rejection (figure 4.8B). This was not due to the inability of mHAs from OVA to serve as a vector for rejection of mOVA bone marrow, as control mice infected with SIINFEKL expressing polyomavirus (PyV.OVA-I) had robust BMT rejection.

CD107a or LAMP-1 is a marker of degranulation only found at the surface of cells after the fusion between the membrane of granules and the cytoplasmic membrane has occurred (15). Granzyme B is one of the classical mediators of lytic activity for CD8 T cells. Expression of CD107a and Granzyme B by cross-primed OT-I T cells was also increased by injecting the recipient mice with poly(I:C) (figure 4.8C). More of the cross-primed OT-I T cells expressed IFNγ, and TNFα expression was also enhanced unpon injection with poly (I:C) (figure 4.8D). However, these enhancements were insufficient to produce a sustained OT-I CD8+ T cell response and induce mOVA BMT rejection.

Discussion

We hypothesized that mHAs in leukoreduced RBCs units are capable of inducing immunity. We needed to develop a model capable of testing the hypothesis. The clinical situation that we were trying to model consists in an MHC matched BMT with a reduced intensity conditioning regimen. In this situation, mHAs are likely to represent a significant barrier to engraftment. Because of the great variability in human genetics, in all likelihood multiple mHAs will be different between donor and recipient. Therefore, we chose the BALB model because it modeled the clinical situation of multiple minor antigen mismatched more closely. Because it was unknown which antigens would be shared by the transfused RBCs and the donor bone marrow, having multiple minor antigen mismatches, and as a consequence a more polyclonal response to the transfusions, also favored the likelihood of BMT rejection in the model. Our first set of experiments determined that mHAs were indeed a significant immunological barrier to engraftment that could be overcome by a non-lethal or reduced conditioning regimen (figure 4.1). Throughout our research we used the minimal conditioning regimen that allowed consistent engraftment of the donor bone marrow. These conditioning regimens were different for the BALB model and the HY model (figures 4.1 and 4.5). The advantages of using such minimal conditioning regimens is that it sensitizes the model toward rejection. This raises the question of whether this model is too artificial in its sensitivity to BMT rejection, and indeed we found that low levels of immunity was required to induce rejection (figures 4.4). We think that the system in this situation models precisely the clinical situation. The goal of reduced intensity conditioning regimens is to establish consistent engraftment but not to completely replace the

hematopoietic compartment of the patient. In sickle cell anemia and thalassemia, a mixed chimerism is sufficient to remove the symptoms of the disease (16, 17). It is well established that the half-life of sickle cell RBCs is greatly reduced compared to healthy RBCs. This causes the healthy RBCs to overtake the sickle cell RBCs in the blood simply because of their extended half-life. A small proportion of healthy hematopoietic stem cells in the marrow are sufficient to reconstitute a normal RBC compartment in the blood. Therefore, as is the case in our model, patients are at high risk of rejection as their own immune system is mostly preserved, which may explain why rejection is a high frequency event in these patients, just as it is in our model.

In chapter 2, we demonstrated that mHAs in leukoreduced RBC units were capable of inducing rejection of BMT possessing the same mHAs. However, it remained unclear which component of the RBC units are involved in and/or required for this process. The data presented here helped us better understand the model of BALB.B BMT, as well as the determinants for rejection. We saw that leukoreduction not only decreases dramatically the numbers of leukocytes in the blood but also affects the relative composition of the WBCs present in the blood with a relative enrichment in T cells. This may have an impact in the capacity of residual WBCs to induce immunity against the mHAs in the blood since T cells, although considered non-professional APCs, are capable of processing and presenting mHAs on MHC I. However, one needs to consider that presentation of antigens by T cell can lead to tolerance rather than immunity for lack of proper costimulation (18). In order to gain a better understanding of the effectiveness of leukoreduction in reducing alloimmunization, it may be useful to further investigate

the exact content of the residual WBCs and the capacity of specific lineages to induce immunity after transfusion.

Even though leukoreduction in our model was able of reducing the number of leukocytes by about 3 log, we found that after multiple transfusions with very small numbers of residual WBCs, BMT rejection could occur. It is interesting to note that the rejection only occurred after the third transfusion with residual WBCs. That particular transfusion contained significantly more (1900 vs. less than 400 in the two other transfusions) and one must wonder whether there is a threshold under which the rWBCs remain unnoticed by the recipient's immune system. It may be possible that there is a cumulative effect, and that immunity occurs after the immune system encounters a certain number of foreign cells, or that boosting multiple times is required. In any case, this threshold appears to be very low.

When studying the type of immunity induced by transfusion with leukoreduced RBCs, we found that although immunity against BALB mHAs was detectable in some cases before BMT (figure 4.4C), it is a very poor predictor of rejection. Most of the mice had undetectable immunity in an *in vivo* killing assay, but then rejected subsequent BMT. Transfusion with leukoreduced RBCs also does not induce detectable antibodies against mHAs in the recipient. Overall, even though transfusions with leukoreduced RBCs do not generate measurable immunity, they appear to act as a priming event for subsequent exposure to the foreign antigen.

Our understanding of the mechanisms involved in the rejection of the BMT is limited. However, the data presented herein with the BALB model indicate that after BMT immunity against BALB targets is robust in the mice that have rejected the BMT,

and yet no antibodies against BALB mHA carrying targets are detected by our methods. Although we cannot exclude the possibility of the presence of low-level antibody production involved in antibody dependent cell cytotoxicity (ADCC) type of events, we can speculate that humoral immunity is not the main effector mechanism involved in BMT rejection. More likely cellular immunity is involved, and we predict a role for $CD8^+$ and potentially $CD4^+$ cytolytic T lymphocytes (CTL).

One significant exception to this phenomenon is evidenced by another mHA mismatched transfusion/BMT model. That model is based on the HY mHAs carried by male mice. When female mice are transfused with male leukoreduced blood multiple times they do not reject a subsequent BMT from male donors. We have discussed some of the reasons why this may be in chapter 2. In particular, we hypothesized that the lack of rejection was due to the absence of HY antigens from the RBCs because they lack a nucleus. This hypothesis was supported by the fact that there is no measurable immunity in the recipient after transfusion with leukoreduced male RBCs (figure 4.6). We are lacking an analysis of antibodies against HY. We attempted to measure anti-HY antibodies by western blot, but did not have the required positive controls to confirm our assay was working, as we were unable to identify antibodies that accurately detect the nuclear HY proteins Smcy and Uty (19). The only reagents available detect what was believed to be the serological HY antigen of which the molecular basis in mice has yet to be found (20).

The multi mHA BALB model is a powerful model to study a clinically relevant phenomenon, due to its more clinically relevant polyclonal response to mHAs on transfused RBCs. However, the lack of key immunological tools such as TCR transgenic

mice limits its usefulness to study the early activation events after transfusion with RBCs, as well as the effector mechanisms and molecules involved in rejection of BALB bone marrow.

Another key issue in our research was the inability to restrict expression of an mHA to a particular lineage in the blood (RBCs, or leukocytes). To address this, we developed a reductionist model through the generation of the HOD mouse, and concurrently utilizing the mOVA mouse. By using HOD mice as donors, we are able to transfuse recipients with blood where the HOD model antigen is expressed only on RBCs. Similarly, using mOVA mice as BMT donors allowed us to focus our study on a single antigen target to induce BMT rejection (figure 4.8). Utilizing these mice in this manner, we have developed a model that maintains most of the significance of the BALB model with a much-increased analytic capacity and control over the variables involved in the studied phenomenon. In this model, the adoptive transfer of OT-I and OT-II cells can also provide us with a better understanding of the interplay between the players involved in the transfusion induced BMT rejection. In chapter 3, we begin to explore the determinants of immunity in RBC induced BMT rejection. We found that the requirements are quite complex, and that we are only beginning to understand them through this research and in other related research (6, 21). Emerging as one of these determinants is the inflammation of the recipient. This can be caused by many factors, and we explored one of them in chapter 3: viral infections. We found that infection with a virus immediately before transfusion can affect the quantity and quality of the response in the recipient.

In this chapter, we hypothesized that the changes associated with the viral infection were due to toll-like receptors (TLR) agonists such as LPS and poly (I:C). Although treatment with poly (I:C) did not induce rejection of mOVA BMT, it did enhance the response to transfused HOD blood in the adoptively transferred OT-I T cells (figure 4.9). These data indicate that while poly (I:C) may regulate cross-priming of RBC mHA specific antigens, the changes are not alone sufficient to result in BMT rejection. This also suggests that the effect of PyV infection on the cross-priming of CD8+ T cells by RBC antigens may at least in part be due to TLR ligation.

Finally, we focused our efforts on a better characterization of the models used in our research. We explained the rationale behind the conditioning regimens used in the different BMT models. We evaluated the quality of the transfused or transplanted tissues. First, we analyzed the composition of rWBCs after filter leukoreduction and the composition of the transplanted marrow. We better characterized the expression of the HOD model minor antigen on leukocytes and platelets of HOD blood. We provided a better understanding of the effector mechanisms involved in transfusion induced rejection of mHA mismatched BMT. Additionally we evaluated the effects of a potent TLR ligand on the cross-priming of CD8+ T cells by RBC associate antigens and its effect or lack thereof on BMT.

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Figure Legends

Figure 4.1. BALB.B bone marrow transplantation. Increasing doses of total body irradiation were given to recipient mice prior to injection with whole bone marrow cells to determine a reduced (non lethal) intensity conditioning regimen that would allow for engraftment of BALB.B bone marrow. Engraftment was evaluated by measuring the proportion of donor T cells in the recipient. Representative flow cytometry data is shown in the top panel **(A)**. Graphical representation with 3 mice per group. Error bars represent the standard deviation for the data point **(B)**. Flow cytometric characterization of donor BALB.B bone marrow with $CD34^+$ hematopoietic stem cells and $CD3^+$ T cells **(C)**.

Figure 4.2. Leukocyte composition of mouse blood before and after leukoreduction. Nucleated residual WBCs were stained with propidium iodide and counterstained with anti-CD3 and anti-CD19 to analyze the composition in B cells, T cells, and other leukocytes before **(B)** and after leukoreduction **(C)**. There is no background noise when staining with propidium iodide as evidenced by the total absence of signal when unstained **(A).**

Figure 4.3. Multiple transfusions with mHA-mismatched rWBCs induce rejection of subsequent BMT. Purity of the prepared rWBCs was evaluated by flow cytometry **(A)**. Numbers of WBCs before and after leukoreduction (**B**). Engraftment of BALB.B BMT in B6.Thy1.1 recipients as average T cell chimerism **(C)**. Engraftment as percentage of mice with more than 5% T cell chimerism **(D)**. Analysis of immunization to BALB.B before and after BMT. BALB-specific immunity was assessed by in vivo

survival of BALB.B splenocyte targets. Error bars represent the standard deviation for the data point **(E)**.

Figure 4.4. Analysis of immunization against BALB mHAs before and after BMT. Engraftment of BALB.B BMT in B6.Thy1.1 recipients as average T cell chimerism **(A)**. Engraftment as percentage of mice with more than 5% T cell chimerism **(B)**. Analysis of immunization to BALB.B before and after BMT **(C, D)**. BALB-specific immunity was assessed by in vivo survival of BALB.B splenocyte targets. The horizontal line represents the mean of each group **(C)**. Indirect immuno-fluorescence staining was used to assess the presence of anti- BALB antibodies using BALB.B splenocytes targets **(D)**.

Figure 4.5. HY+ bone marrow transplantation. Increasing doses of total body irradiation were given to female B6 recipient mice prior to injection with whole bone marrow cells to determine a reduced (non lethal) intensity conditioning regimen that would allow for engraftment of HY^+ (Male) bone marrow. Engraftment was evaluated by measuring the proportion of donor T cells in the recipient. Representative flow cytometry data is shown in the top panel **(A)**. Graphical representation of engraftment levels at 6 weeks. Error bars represent the standard deviation for the data point **(B)**. Engraftment of HY+ bone marrow over time **(C)**.

Figure 4.6. Analysis of immunization against HY mHAs before and after BMT. Engraftment of HY^+ BMT in B6.Thy1.1 recipients as average T cell chimerism **(A)**. Engraftment as percentage of mice with more than 5% T cell chimerism **(B)**. Analysis of

immunization to HY mHAs and H-2k MHC before and after BMT **(C, D)**. HY and H-2k specific immunity was assessed by in vivo survival of Male B6 and Female B10.BR splenocyte targets, respectively, before BMT **(C)** and after BMT **(D)**. Error bars represent the standard deviation for the data point.

Figure 4.7. HEL is undetectable on leukocytes and platelets of the HOD mouse. Staining of HOD blood shows with the HEL specific monoclonal antibody 4B7 shows no detectable HEL on $CD45^+$ $TER\text{-}119^-$ leukocytes **(A)**, $CD3^+$ $TER\text{-}119^-$ T cells **(B)**, or $CD41^+$ $TER\text{-}119^-$ platelets **(C)**.

Figure 4.8. Poly (I:C), but not LPS enhances cross-priming efficiency of OT-I CD8+ T cells by HOD on RBCs. Expansion of adoptively transferred OT-I CD8+ T cells after transfusion with HOD blood, in the presence of LPS or Poly(I:C) in the spleen of recipients B6 mice **(A)**. Staining of splenic adoptively transferred OT-I $CD8^+$ T cells with anti CD107a and anti-Granzyme B after transfusion with HOD blood into B6 recipient mice previously injected with LPS or Poly(I:C) **(B)**. Intracellular cytokine staining with anti-IFNγ and anti-TNFα antibodies of splenic adoptively transferred OT-I T cells after transfusion with HOD blood into B6 recipient mice previously injected with LPS or poly (I:C) **(C)**. Error bars in A and C represent the standard deviation for the data point.

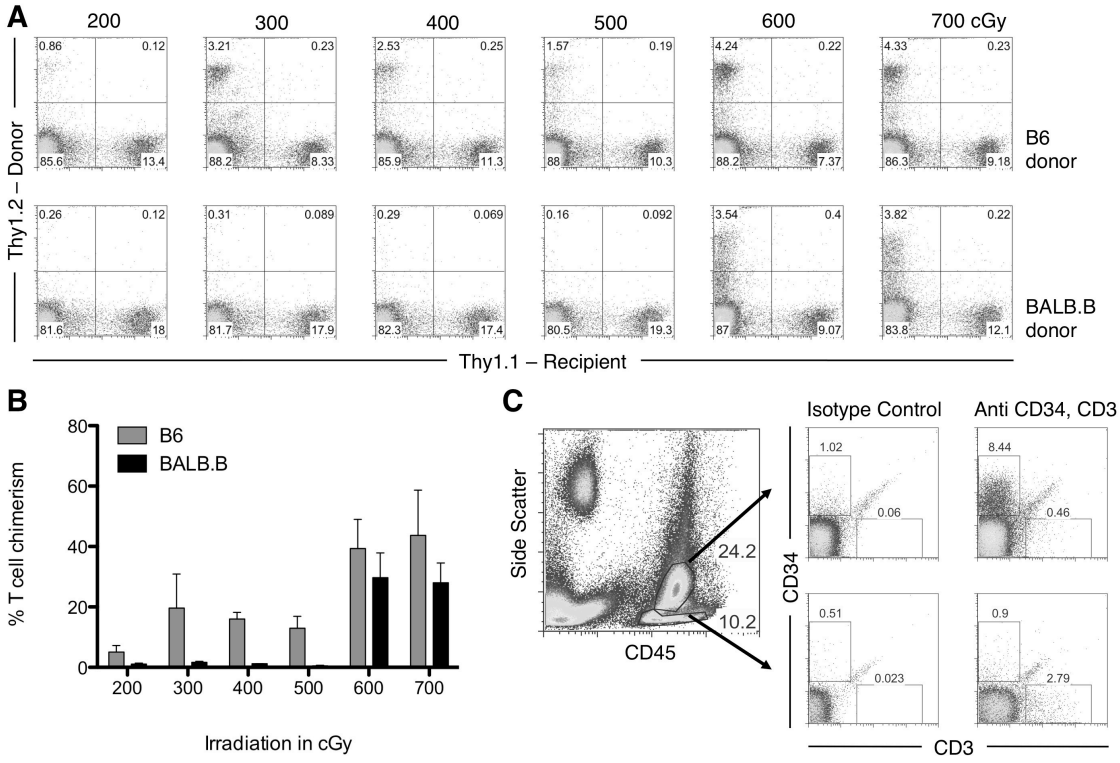
A
200
300
400
500
600
700 cGy
Thy1.2 – Donor
B6 donor
BALB.B donor
Thy1.1 – Recipient
B
% T cell chimerism
B6
BALB.B
Irradiation in cGy
C
Side Scatter
CD45
24.2
10.2
CD34
CD3
Isotype Control
Anti CD34, CD3

Figure 4.1

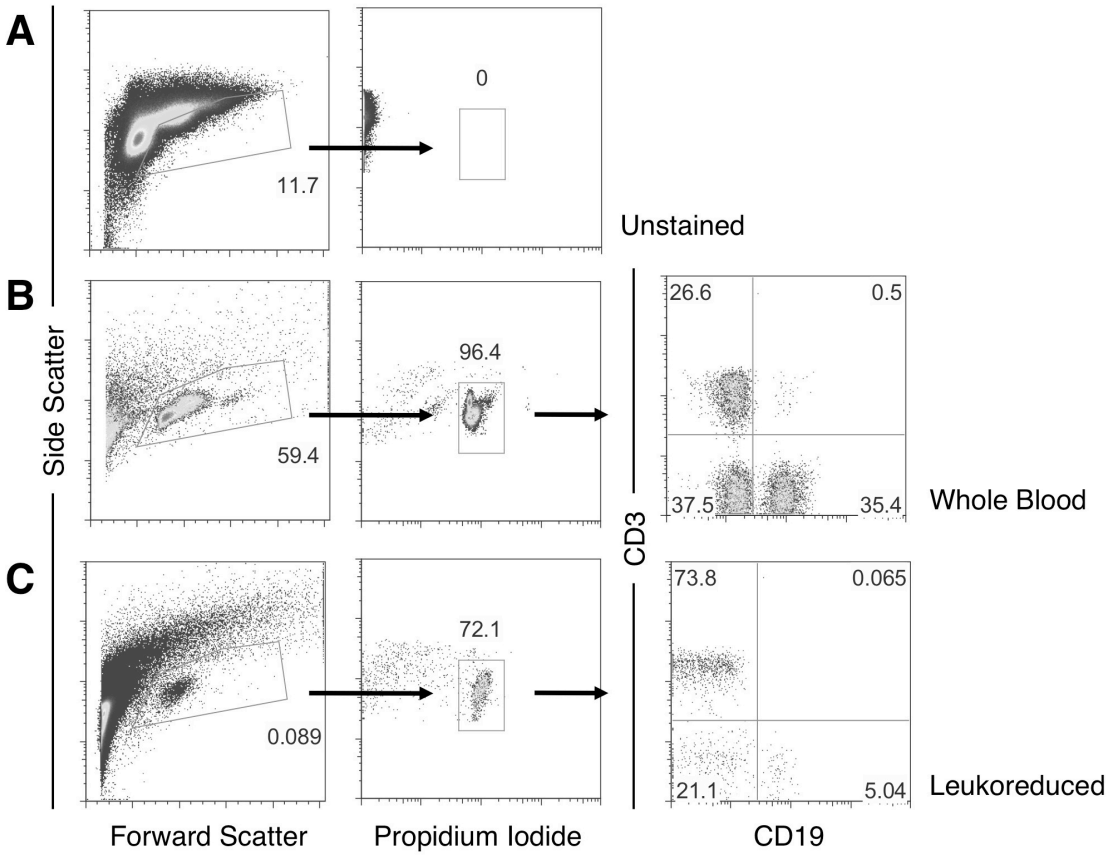
A
B
C
Side Scatter
11.7
0
Unstained
59.4
96.4
26.6
0.5
37.5
35.4
Whole Blood
0.089
72.1
73.8
0.065
21.1
5.04
Leukoreduced
CD3
Forward Scatter
Propidium Iodide
CD19

Figure 4.2

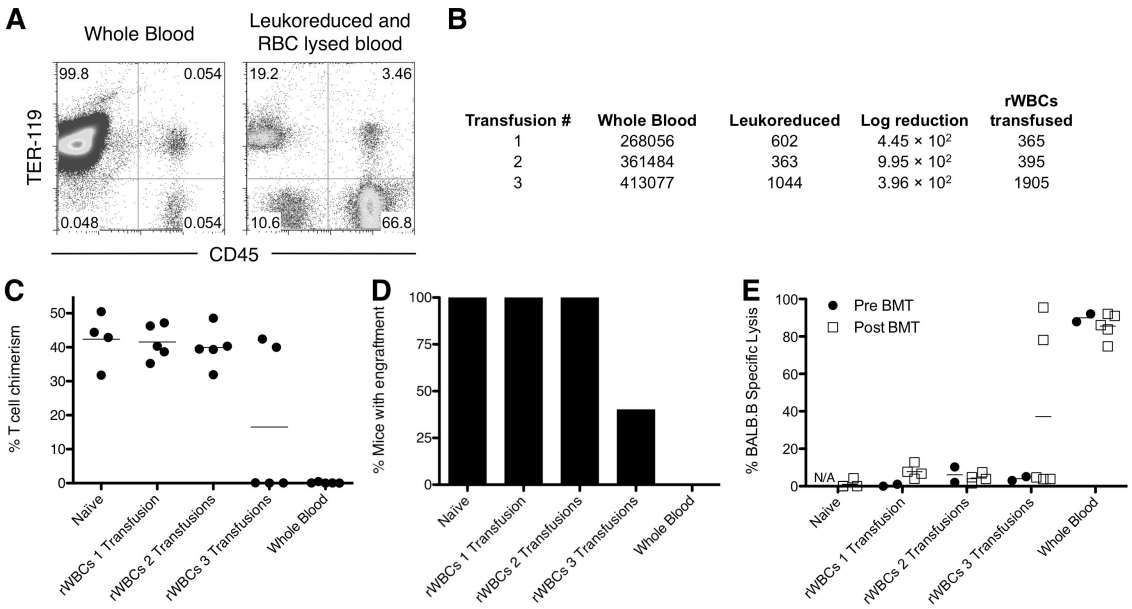
A
Whole Blood
Leukoreduced and RBC lysed blood
99.8
0.054
0.048
0.054
19.2
3.46
10.6
66.8
TER-119
CD45
B
Transfusion # | Whole Blood | Leukoreduced | Log reduction | rWBCs transfused
1 | 268056 | 602 | 4.45 × 10² | 365
2 | 361484 | 363 | 9.95 × 10² | 395
3 | 413077 | 1044 | 3.96 × 10² | 1905
C
% T cell chimerism
Naive
rWBCs 1 Transfusion
rWBCs 2 Transfusions
rWBCs 3 Transfusions
Whole Blood
D
% Mice with engraftment
E
% BALB.B Specific Lysis
Pre BMT
Post BMT
N/A

Figure 4.3

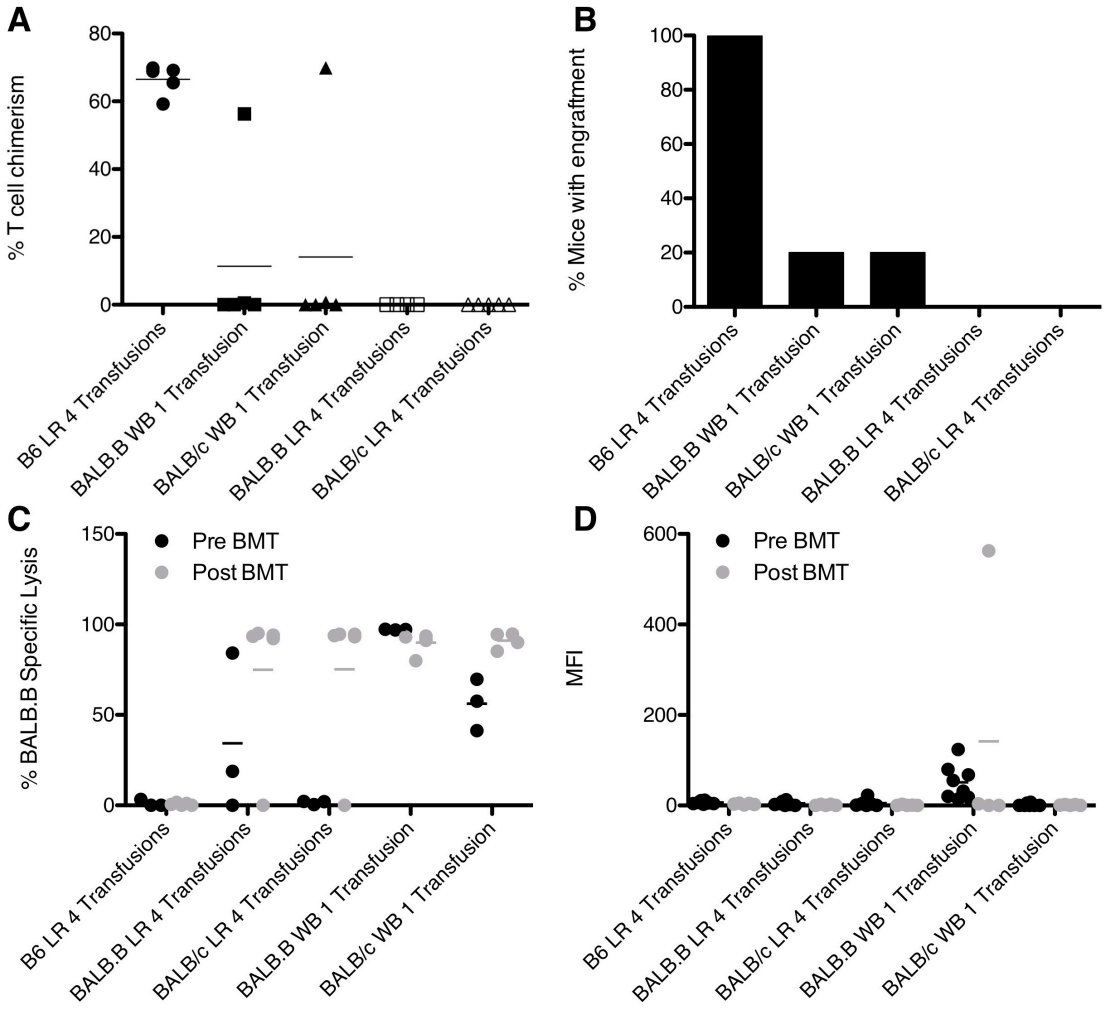
A
% T cell chimerism
80
60
40
20
0
B6 LR 4 Transfusions
BALB.B WB 1 Transfusion
BALB/c WB 1 Transfusion
BALB.B LR 4 Transfusions
BALB/c LR 4 Transfusions
B
% Mice with engraftment
100
80
60
40
20
0
B6 LR 4 Transfusions
BALB.B WB 1 Transfusion
BALB/c WB 1 Transfusion
BALB.B LR 4 Transfusions
BALB/c LR 4 Transfusions
C
% BALB.B Specific Lysis
150
100
50
0
Pre BMT
Post BMT
B6 LR 4 Transfusions
BALB.B LR 4 Transfusions
BALB/c LR 4 Transfusions
BALB.B WB 1 Transfusion
BALB/c WB 1 Transfusion
D
MFI
600
400
200
0
Pre BMT
Post BMT
B6 LR 4 Transfusions
BALB.B LR 4 Transfusions
BALB/c LR 4 Transfusions
BALB.B WB 1 Transfusion
BALB/c WB 1 Transfusion

Figure 4.4

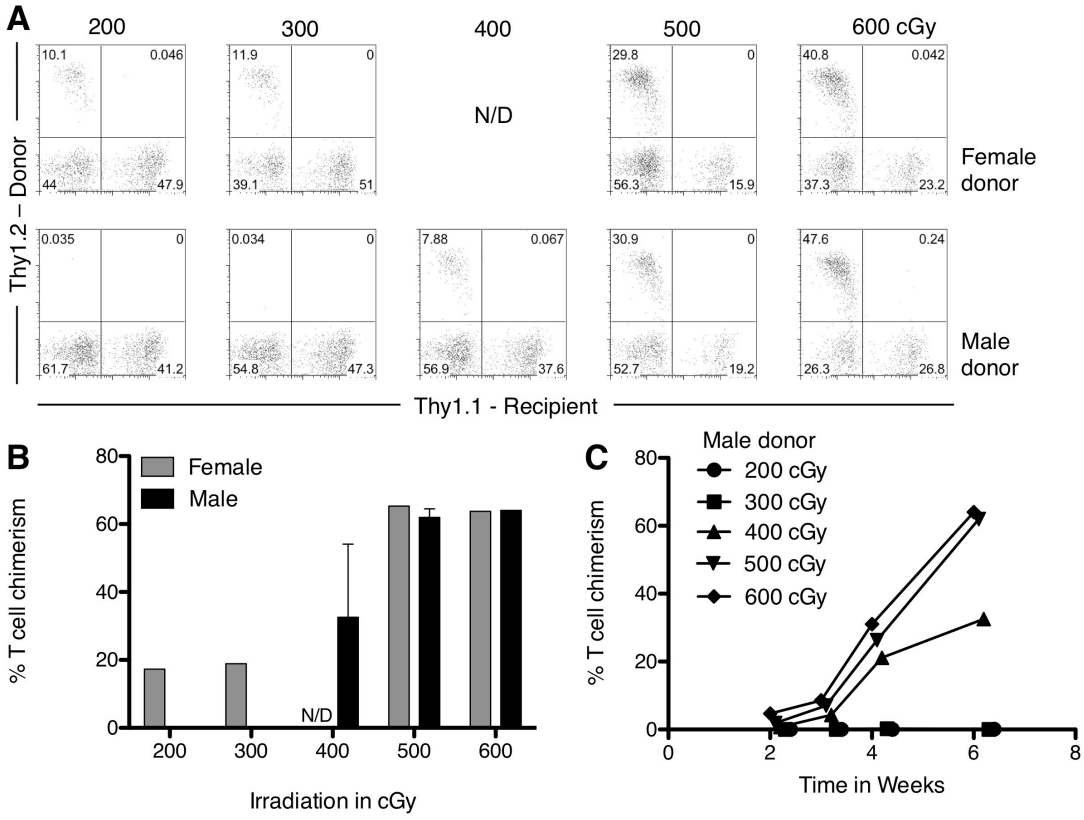
A
200
300
400
500
600 cGy
N/D
Female donor
Male donor
Thy1.2 – Donor
Thy1.1 - Recipient
B
Female
Male
% T cell chimerism
Irradiation in cGy
C
Male donor
200 cGy
300 cGy
400 cGy
500 cGy
600 cGy
% T cell chimerism
Time in Weeks

Figure 4.5

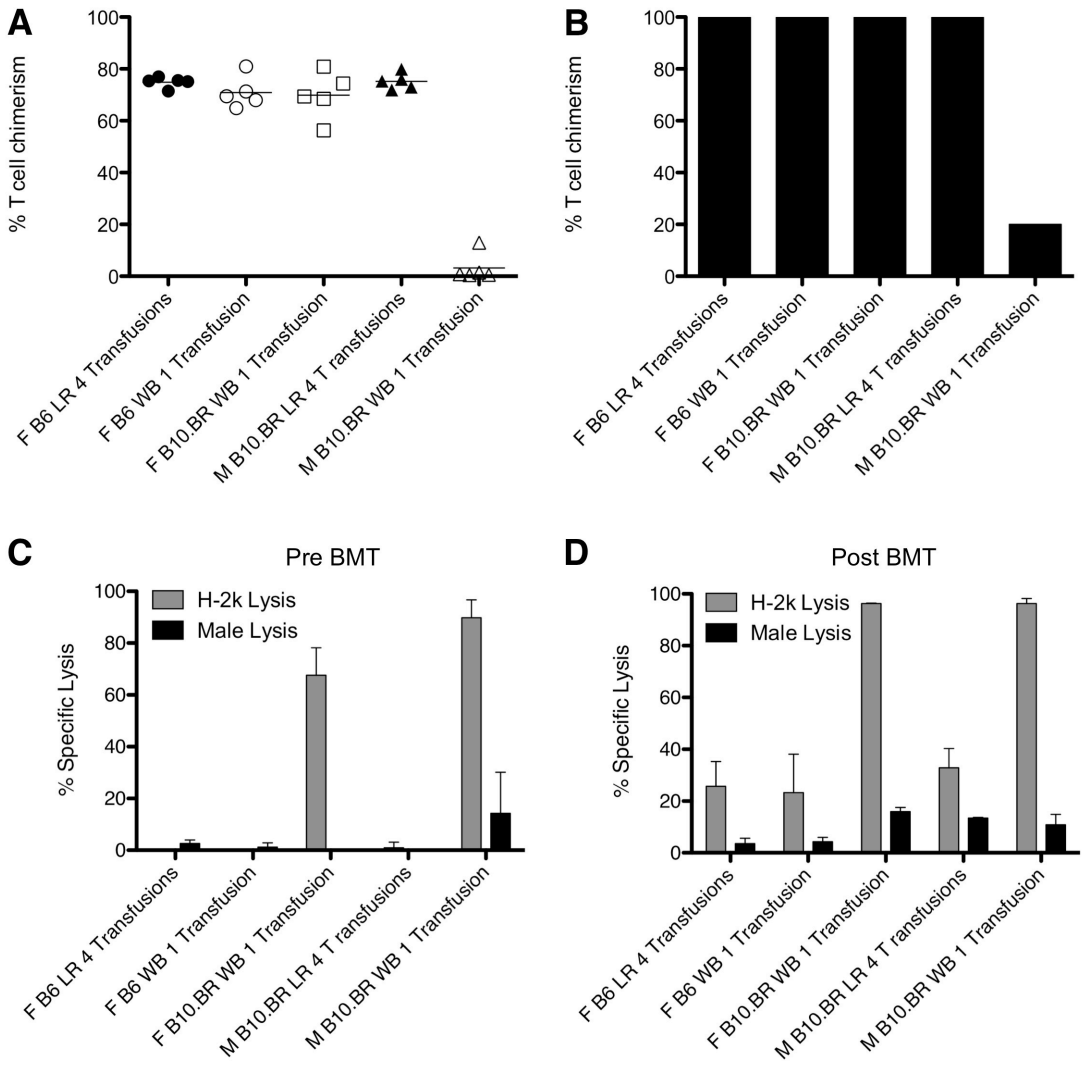
A
% T cell chimerism
100
80
60
40
20
0
F B6 LR 4 Transfusions
F B6 WB 1 Transfusion
F B10.BR WB 1 Transfusion
M B10.BR LR 4 T ransfusions
M B10.BR WB 1 Transfusion
B
% T cell chimerism
C
Pre BMT
H-2k Lysis
Male Lysis
% Specific Lysis
D
Post BMT

Figure 4.6

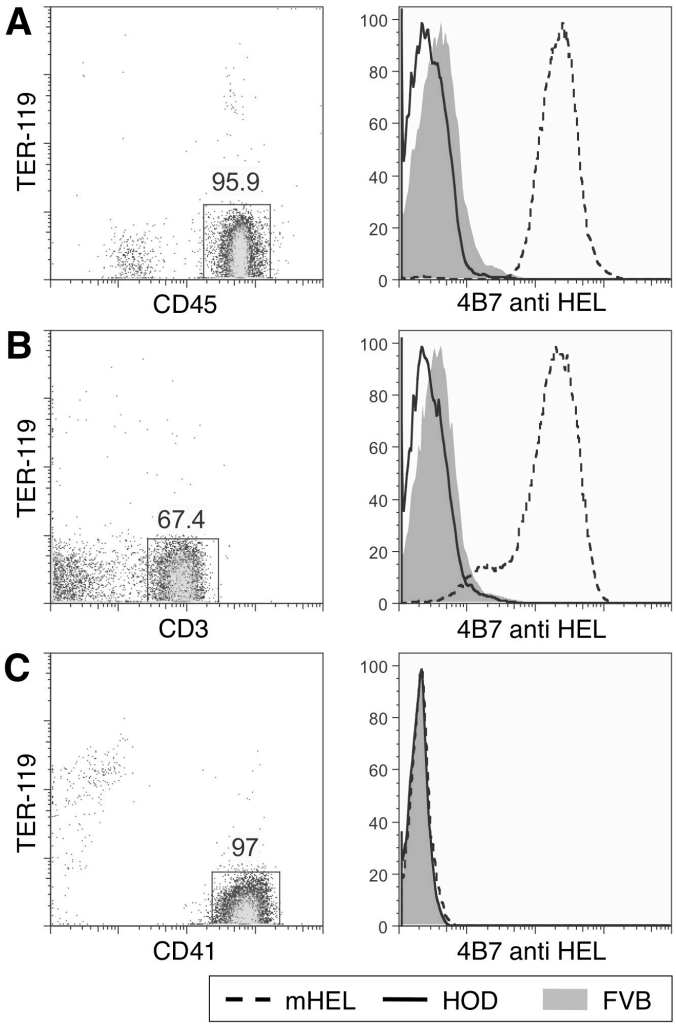
A
TER-119
95.9
CD45
4B7 anti HEL
B
TER-119
67.4
CD3
4B7 anti HEL
C
TER-119
97
CD41
4B7 anti HEL
mHEL
HOD
FVB

Figure 4.7

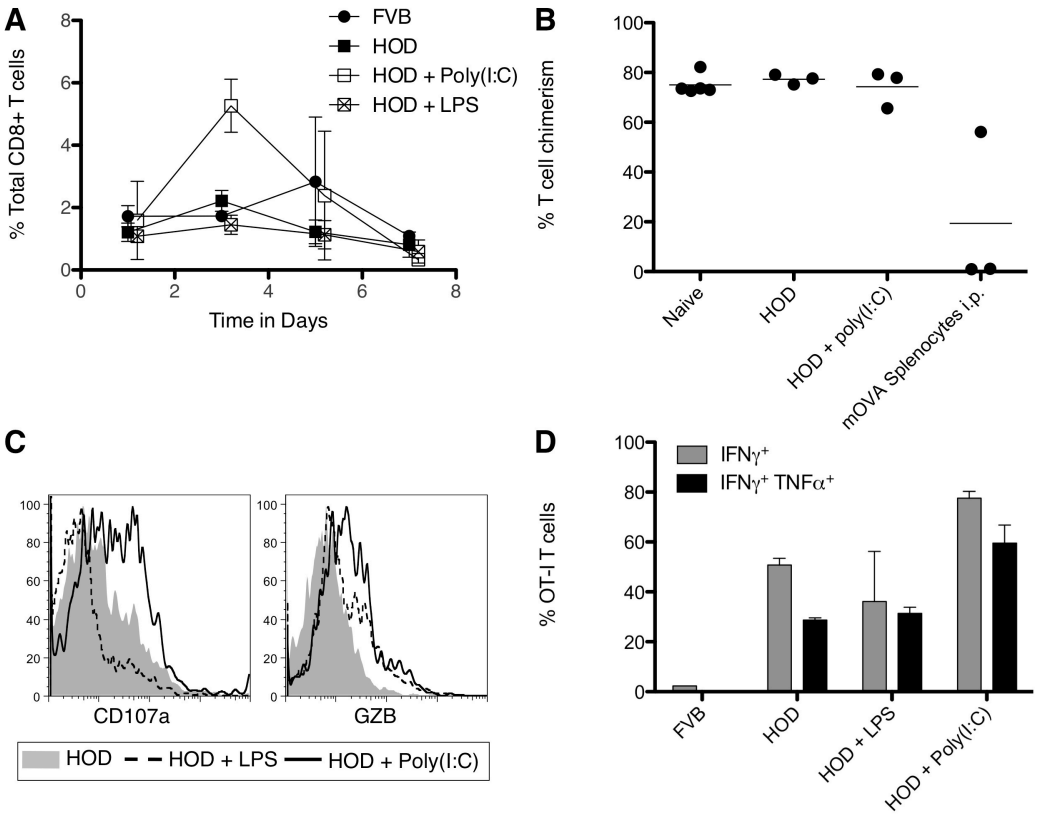
A
% Total CD8+ T cells
Time in Days
FVB
HOD
HOD + Poly(I:C)
HOD + LPS
B
% T cell chimerism
Naive
HOD
HOD + poly(I:C)
mOVA Splenocytes i.p.
C
CD107a
GZB
HOD
HOD + LPS
HOD + Poly(I:C)
D
% OT-I T cells
IFNγ+
IFNγ+ TNFα+
FVB
HOD
HOD + LPS
HOD + Poly(I:C)

Figure 4.8

Chapter 5

Discussion

Summary

The relevance of minor antigens in bone marrow transplantation is now well established in the field of bone marrow transplantation. It is well understood that mHAs are responsible for rejection of HLA matched bone marrow grafts as well as debilitating GvHD. Sensitization to mHAs is now perceived as a risk of graft rejection and two vectors of exposure have been identified: pregnancy and transfusion. However, it was believed that the implementation of leukoreduction would remedy this problem. The research presented here is an important correction to this idea. Minor antigens on transfused leukoreduced RBC units also have the potential to induce transplant rejection, in this case, bone marrow transplant rejection.

We developed a murine model to test this potential, and began to analyze the underlying mechanisms including which components in the RBC units are involved. We found that exposure to minor antigens via the indirect presentation pathway was sufficient to induce bone marrow transplant rejection. We also found that in the HY model, leukoreduction was sufficient to prevent BMT rejection. These results suggested that RBCs themselves are involved in the immunization process. However, we saw that residual WBCs themselves can also induce BMT rejection, and that the threshold number of transfused rWBCs required to induce rejection appears to be extremely low. However, these data do not reject the hypothesis that RBCs are involved in the immunization process. The only way to study the mechanisms involved in immunization to RBC associated antigen was to create a model with expression of an RBC specific antigen.

The HOD mouse was created specifically for that purpose. Because we studied a phenomenon related to transplantation, we focused on cellular immunity and the CD8+ T

cell compartment, the main effector mechanism involved in minor antigen driven BMT rejection (outside of the context of transfusion) (1, 2). The HOD mouse allowed us to test if RBC specific minor antigens are capable of being cross-presented by recipient APCs to CD8+ T cells. These data are a key argument in favor of RBCs as an immunogenic stimulus. However, when we studied the consequences of cross-priming by RBC associated antigens, we found that in the conditions of our model (which do not necessarily reflect the reality of the practice of RBC transfusion), the response induced in the CD8+ T cells specific for the HOD model antigen is one of tolerance, with deletion of allospecific CD8+ T cells from the spleen. We also found that this response could be transformed by factors other than the contents of the transfusion itself (i.e.inflammation and infection with pathogens). While the data found in the HOD model may appear discordant from the initial findings of transfusion induced BMT rejection, it merely underlines the complexity of the requirements for immune responses to RBC antigens. These data also indicate that RBCs may provide an exciting new avenue of research in the field of transplantation tolerance, and the HOD mouse may prove to be a valuable tool for these purposes.

Discussion

Bone marrow transplantation in non-malignant blood disorders

In the research presented herein we sought to address an issue that was brought to our attention by clinicians. Hematologists in charge of treating sickle cell patients are struggling with the fact that the few attempts of treating sickle cell anemia had an unusual rate of BMT failure, even in medical centers that have implemented universal leukoreduction of blood products (3, 4). There are several hypotheses to explain this phenomenon. Theses hypotheses fall into two main categories: immune and non-immune explanations. The non-immune explanations are related to the nature of the underlying condition or the treatment of the disease. In particular, perturbations in the marrow microenvironment due to ischemic marrow injury can occur in sickle cell anemia (5-7). Iron overload associated with the transfusion support necessary during the care of sickle cell patient to alleviate vaso-occlusive episodes can also perturb bone marrow function and has been documented to be associated with bone marrow failure during transplants (8, 9). Other hypotheses involve enhanced immunization to donor minor antigens either because of sensitization to mHAs (10) or because of heterologous immunity after infections (usually viral infections) (11, 12).

Recently, a number of studies have been focusing on sensitization to mHAs and its implications for BMT. In particular, it has been demonstrated in humans that donor specific transfusion induces mHA specific CTLs (13-15). Pregnancy has also been identified as a mechanism for sensitization to mHAs (16). These data give credibility to the hypothesis that transfusion is a cause of the immunity to mHAs. However, it was unknown whether leukoreduction was sufficient to prevent this effect. The data presented

herein suggest that RBC transfusion even after extreme filter leukoreduction can still sensitize the recipient to donor specific minor antigens and therefore support the sensitization through transfusion hypothesis. However, our research does not reject non-immune hypotheses and it is important to keep them in mind for future research on bone marrow transplantation for non-malignant blood disorders.

In the course of our research we sought to characterize the immunity induced by RBC transfusion. We found that in many cases, antibodies to mHAs as well as lytic activity against donor targets was difficult to measure and that BMT rejection occurred despite undetectable antibodies and killing activity. Of course this may be due to a lack of sensitivity of our flow cytometry based assays, but it also suggests that these assay are poor predictor of the BMT outcome. This may complicate the localization of mHA compatible bone marrow, as there appear to be no good marker of sensitization.

Considering that our data suggest that RBCs themselves are immunogenic, we predict that no extent of leukoreduction will prevent transfusion induced BMT rejection. Potential approaches to prevent sensitization of transfused patients may include matching mHA from donor and recipient, provided that only a few mHAs from the blood and the marrow are shared. Other approaches include tolerance induction using immunomodulatory techniques (such as costimulation blockade). However, the best strategy may be to avoid transfusion as much as possible to prevent all sensitization and proceed with BMT at an early age. This strategy may become more relevant as new reduced conditioning regimens make BMT transplants safer than before (17).

Transfusion medicine

As seen in introduction (chapter 1), transfusion medicine specialists have been focusing their efforts on monitoring humoral alloimmunization to blood group antigens and finding ways to understand the mechanisms involved in alloimmunization to RBC antigens with the ultimate goal to reduce the incidence of alloimmunization. There are benefits to the practice of leukoreduction of blood products. In particular, HLA immunization is reduced when using leukoreduced RBC units as well as platelet units (18-22). It is clear, in light of our data as well as work from other groups (19), that these benefits are limited and that leukoreduction does not prevent alloimmunization completely. However, few groups have studied mHA alloimmunization as opposed to HLA alloimmunization. Our model indicates that for mHAs too, leukoreduction decreases alloimmunization but cannot prevent it entirely, while a single transfusion of whole blood was sufficient to induce immunity and BMT rejection in our model, multiple transfusions of leukoreduced blood gave the same result.

It is important to note that the mechanism for BMT rejection does not appear to be antibody mediated, as no antibodies could be detected after transfusions. This suggests the possibility of CD8+ cytolytic T lymphocyte (CTL) responses by RBC transfusion. This side effect to transfusion receives little or no appreciation in the transfusion community, and yet our model shows that they can have long term unsuspected consequence on subsequent bone marrow and potentially solid organ transplants (23). The notion that blood transfusions induce transplant rejection challenges one of the well-established paradigms in the transfusion community: that RBC transfusions skew immune responses toward a Th2 phenotype (24-26). Transplant rejections tend to be Th1

mediated and Th2 responses tend to be associated with better graft survival (although these concepts are being reevaluated and chronic graft rejection can also be mediated by Th2 responses) (27, 28). While it is true that Th2 responses dominate after whole blood transfusions it does not preclude the possibility of Th1 responses. The advent of universal leukoreduction has profoundly modified the types of products that are being transfused to patients and some have raised the possibility that extreme leukoreduction enhances the immunogenicity of platelets (29, 30). It is possible that the immune deviation changes because of the leukoreduction process. This hypothesis would be congruent with the data presented in chapter 1.

The work by John Semple's group on extreme platelet leukoreduction and enhanced immunogenicity (29, 30) as well as recent data from Patel *et al*. (31) also support the hypothesis that no amount of leukoreduction will ever completely abrogate immunization to RBC transfusions, and other strategies will likely have to be developed to solve the problem of alloimmunization. The matching of donor blood group antigens had tremendous results in reducing the frequency of allo-immunization and immune mediated hemolytic reactions. Immunomodulatory strategies are already available thanks to the extensive research on the induction of tolerance to donor antigens in transplantation. Some of them are already being investigated: regulatory T cells (32, 33), the anti CD20 monoclonal antibody rituximab (34), for example. Others, like costimulation blockade (35) may prove invaluable too, in some specific situations. One can imagine that if BMT becomes more prevalent and safer for patients with sickle cell anemia or other nonmalignant blood disorders, only a few transfusions would be required before the patient becomes eligible for BMT. If the number of transfusions is limited

before transplant, administrating the costly but effective anti CD154 antibody at the time of transfusion may represent a way to prevent alloimmunization, and increase the chances of BMT engraftment.

As discussed in chapter 3, it appears that mHAs on transfused RBCs do not induce BMT rejection through cross-priming, as we had hypothesized. On the contrary RBC specific CD8+ T cells appear to be deleted upon cross-priming by the recipient APCs. Nevertheless, interesting data suggested that the immunological milieu at the time of transfusion could slightly influence the outcome of the CD8+ response. We observed that viral infections shortly before transfusion could transiently enhance the response of the RBC specific CD8+ T cells. This effect was not able to overcome the deletion of the CD8+ T cells, but it represents an interesting model of what some already suspect to be true; that the immunologic milieu at the time of transfusion may influence the outcome of the immune response in the recipient (33, 36-40). This model may prove invaluable in understanding the complex requirement for immunity to RBC antigens.

Tolerance Immunology

During our efforts to evaluate which cell type in the leukoreduced RBC units is involved in immunization, we discovered that the RBC specific HOD antigen did not induce an OVA specific CD8+ T cell response or induce mOVA BMT rejection. On the contrary, HOD specific CD8+ T cells were rapidly removed from the spleen after partial activation. These data suggest that this is a form of deletional tolerance in the periphery, observed directly for the first time with RBC associated antigens. The tolerance mechanism appears similar to cross-tolerance to cell associated self-antigens first

described by Kurts *et al*. (41, 42) with upregulation if CD69 and CD44 but little or no CD25 upregulation and partial CD62L downregulation described by Hernandez *et al*. (43). However some differences emerge when we compare effector functions in the model of cross-tolerance to self-antigens and our model with foreign RBC antigens. Hernandez *et al*. saw little or no IFNγ secretion and no CTL activity. The reason why these differences occurred is unknown but may be due to the intrinsic differences in the transgenic CD8+ T cells that were adoptively transferred. Nevertheless, it is important to note that under the conditions that we use it appears likely that tolerance to a foreign antigen was induced by the RBCs.

The tolerance phenomenon observed here may also represent one of the mechanisms involved in maintaining tolerance to RBC antigens in the periphery and the HOD/OT-I model may be of interest in the study of how this tolerance can be maintained but more interestingly how it is broken in situations such as autoimmune aplastic anemia.

Implications concerning the immunomodulatory effect of blood transfusion

In the 1970s, it was observed that RBC transfusion could prolong renal allograft survival (44), but in addition to this beneficial effect, research suggested that detrimental consequences existed also. Some studies suggested that increased rates of cancer recurrences/metastasis, and more common perioperative infections are also a consequence of the immunomodulatory effect of transfusion (45, 46). However, these studies are difficult to interpret because of the difficulty to find comparable transfused and untransfused groups.

WBCs appear to be important for the immunomodulatory effect of transfusion, but many other mechanisms to explain the phenomenon have been proposed: soluble MHC I (46), and platelets (47). Some have proposed that dendritic cells (48) are required for the effect. It is reasonable to hypothesize that some of the immunomodulatory effect, at least in the CD8 compartment is explained by the mechanism of cross-tolerance discussed above (49, 50).

Future directions

As we have suggested above, the work presented herein can be taken in many different directions over the long term; however, we will discuss some of the experiments that could be performed as the next logical steps to our research.

Short term future directions

The effector mechanisms of the transfusion induced BMT rejection evoked in chapter 2 remain largely unknown, and although they do not appear to involved humoral immunity, this remains to be formally tested. The usual battery of experiments required in these situations could prove useful to determine whether the immunity is transferrable to otherwise naïve mice. These experiments would include the transfer of serum, to test for involvement of antibody responses; and the transfer of CD4+ and/or CD8+ T cells of transfused animals. T cell depletion experiments could also be performed to further establish the requirements for BMT rejection in our model. CD4+ T cell depletion, using GK1.5 antibody; and CD8+ T cells depletion, using clone 53-6.7 antibody before BMT would help investigate the hypothesis that CD8+ T cells are the main effector mechanism. Depletion prior to transfusion would not likely be a fruitful approach, as it would not be possible to distinguish between alterations in immune induction vs. effector response for any observed alterations in rejection.

In chapter 3, we described what appears to be deletional tolerance of RBC specific CD8+ T cells. Increased apoptosis in the OT-I T cells of HOD transfused mice suggests that the deletion is apoptosis mediated. This needs to be formally demonstrated

by repeating the experiments in apoptosis deficient mice (caspase deficient mice), or as a preliminary approach using the Z-VAD-FMK peptide, a known caspase inhibitor (51).

We have not formally demonstrated that the deletion of the alloreactive CD8+ T cells truly confers tolerance to a subsequent antigenic exposure. This can be tested by adoptive transfer of OT-I CD8+ T after transfusion with HOD RBCs or after injection with irrelevant mHEL RBCs for example, followed by an mOVA BMT. There is a potential obstacle to performing these experiments. Koehn *et al*. have observed in a model of mOVA bone marrow chimeric mice that adoptive transfer of either OT-I or OT-II T cells alone was insufficient to induce mOVA bone marrow rejection. Both OT-I and at least a small number OT-II were necessary to observe rejection (52). The experiments may have to be repeated in the presence of OT-II T cells. Moreover, Koehn *et al*. described a bone marrow chimera model that also represents an excellent system to test the induction of tolerance by RBC antigens since it provides a way to avoid having to subject the adoptively transferred T cells to the conditioning regimen (53).

One of the caveats to our model of cross-tolerance is the disproportion between the number of OT-I T cells injected into the recipient and the number of endogenous OVA CD4 T cells. Kurts *et al*. have demonstrated that CD4 T cell help can overcome the deletion of CD8+ T cell induced by cross-priming (54). This emphasizes the importance of performing these experiments in order to understand whether tolerance to RBC antigens is a physiological possibility or simply an artifact of our model.

Long term future directions

In chapter 2, we showed that transfusion with leukoreduced RBC units was sufficient to induce BMT rejection. We investigated this issue because of a trend in human bone marrow transplantation showing high rates of BMT rejection. To extend our findings to the human setting, and to support the hypothesis further, it would be desirable to establish whether RBC transfusion in fact induces mHA specific responses in the recipients. These experiments are possible due to the many mHAs characterized, as well as available tetramer reagents specific for these mHAs (10). A possible approach would be to measure mHA specific CD8+ T cells in the peripheral blood of transfused patients transfused with leukoreduced units using available tetramer reagents.

As advances are made in the field of transplantation, it is likely that the use of BMT in non-malignant blood disorders will increase; therefore, it is important to investigate methods of preventing transfusion induced transplant rejection. One such approach using FDA approved drugs is to use Belatacept to block CD28 B7 signaling. In addition, anti-CD154 could be used at the time of transfusion to prevent immunization to leukoreduced RBC units from occurring; and, as a consequence, prevent BMT rejection in our mouse model.

In chapter 3, we showed that the RBC associated HOD antigen was capable of inducing deletion of cross-primed OT-I T cells. We hypothesized that it was due to activation induced cell death (AICD). AICD has been shown to be a Fas–FasL dependent mechanism (55, 56) and the analysis of FasL expression on the HOD stimulated OT-I T cells is one of the next steps in studying this phenomenon. However, the phenotype of the cross-primed OT-I T cells is more consistent with activation-induced non-

responsiveness (AINR) as described by Mescher *et al*., which differs from anergy. In AINR, CD8+ T cells do become IFNγ producing effector cells capable of lytic activity but fail to produce IL-2, which in turns limits there proliferation (57). This phenotype can be overcome by in vitro culture of the CD8+ T cell with IL-2 for a few days. In AINR there are also signaling alterations in the MAP-kinase pathway (58), which could be explored to further test the hypothesis. Finally, Haspot *et al*. in a model of peripheral tolerance to allo-antigens in CD8+ T cells have found a role for the PD-1–PD-L1 pathway (59). Although the phenotype of the OT-I T cells in our model is not entirely consistent with the model of peripheral deletion of CD8+ T cells observed by Haspot *et al*., it would be worthwhile to explore this pathway in the RBC induced deletion phenomenon since RBCs represent a continuous source of antigen which may cause exhaustion in the stimulated CD8+ T cells.

General conclusions

Alloimmunization to transfused blood, represents a major public health and economic issue because of the large numbers of transfusions performed every day and the cost of matching units for compatibility. Leukoreduction is the main method adopted to reduce its incidence, but it has become clear that it is not sufficient to prevent immunization and its consequences for transplantation. The work of this thesis suggests that there is a complex interplay between the different components of blood products, and that consequences are not simply related to how many leukocytes are present in the blood. RBC associated antigens may also induce immunologic responses under proper conditions. However, these remain poorly understood.

Blood transfusion is a practice that saves lives daily but the development of new models and further research would significantly improve our understanding of its immunologic consequences. It is therefore imperative to pursue this line of research further, in order to provide safer transfusions, potential new applications in transplantation medicine, and new insights in the mechanisms underlying the loss of peripheral tolerance.

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