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Alloimmunization to transfused platelets requires priming of CD4⁺ T cells in the splenic microenvironment in a murine model

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Abstract

Background—Alloantibodies are a clinically significant sequelae of platelet transfusion, potentially rendering patients refractory to ongoing platelet transfusion support. These antibodies are often IgG class switched, suggesting the involvement of CD4⁺ T cell help; however, platelet specific CD4⁺ T cells have not been visualized *in vivo* and specifics of their stimulation are not completely understood.

Study Design and Methods—A murine model of alloimmunization to transfused platelets was developed to allow *in vivo* assessment and characterization of CD4⁺ T cells specific for platelet MHC alloantigen. Platelets were harvested from BALB/c mice, filter leukoreduced, and transfused into C57BL/6 recipients. Platelet specific CD4⁺ T cell responses were visualized by using a TCR transgenic mouse that detects peptide from donor MHC I presented on recipient MHC II. Antibody responses were determined by indirect immunofluorescence using BALB/c donor targets.

Results—C57BL/6 recipients of BALB/c leukoreduced platelet transfusions produced anti-BALB/c antibodies, with proliferation of antigen specific CD4⁺ T cells seen in the spleen but not lymph nodes or liver. Depletion of recipient CD4⁺ cells or splenectomy independently abrogated the alloantibody response.

Conclusion—We report a novel model to study antigen-specific CD4⁺ T cells during alloimmunization to platelet transfusion. The presented data support a critical role for CD4⁺ T cell help in the humoral response to platelet transfusion and establish the spleen as a required microenvironment for effective CD4⁺ T cell priming against donor platelet derived MHC I.

Introduction

Roughly 1.5 million patients receive platelet (PLT) transfusions each year in the United States alone. Although potentially lifesaving, PLT transfusions also carry certain risks, including the development of donor-specific PLT alloantibodies. The exact incidence of PLT alloimmunization varies depending upon the clinical situation; however, it has been reported from a large clinical trial of acute myeloid leukemia patients that roughly 18% of recipients developed alloantibodies following transfusion of leukoreduced PLTs (LR-

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PLTs)¹. Once induced, anti-donor antibodies have the potential to bind PLTs expressing the donor antigen and mediate their clearance, rendering some immunized recipients refractory to subsequent PLT transfusions. For certain patients who become immunized to multiple alloantigens, finding sufficient units of compatible PLTs becomes difficult and at times impossible. In such cases, PLT transfusion ceases to be a viable therapy for thrombocytopenia.

Immune mediated PLT refractoriness is typically observed in the presence of an IgG response directed against donor human leukocyte antigens (HLA)². Donor reactive CD4⁺ T cells are considered likely to play a prominent role in the pathogenesis due to their capacity to provide help to B cells and promote the production of a class-switched antibody. The presence of CD4⁺ T cells reactive to PLT antigens has been described in patients with chronic idiopathic thrombocytopenic purpura (ITP)^{3–6}. Moreover, several groups have reported the identification and characterization of human PLT antigen (HPA)-1a-specific T cells in the context of HPA-1a-induced neonatal alloimmune thrombocytopenia^{7–9}. However, less is known about the underlying cellular responses that result in anti-donor antibody production following allogeneic PLT transfusion, particularly with regards to the initiation of the antigen specific T cell response.

Using mouse models of LR-PLT transfusion, alloreactive CD4⁺ T cells have been shown to be elicited coincident to the generation of anti-donor antibody^{2,10,11}. However, no mouse model has been described that allows the characterization of the initial CD4⁺ T cell response to a defined PLT alloantigen. Herein, we describe a tractable mouse model to study the immune responses of C57BL/6 recipients to BALB/c LR-PLTs, utilizing a CD4⁺ T cell receptor (TCR) transgenic mouse (TCR75), which is specific for a single peptide derived from the H-2K^d MHC I molecule presented by the MHC II, I-A^b (K^d_{54–68}/I-A^b)¹². Depletion of CD4⁺ T cells eliminated alloantibody responses to transfused LR-PLTs, whereas adoptive transfer of TCR75 cells into C57BL/6 recipients substantially enhanced alloantibody production. Division of the TCR75 cells was restricted to the spleen, and was not observed in the lymph nodes or liver. Splenectomy abrogated both CD4⁺ T cell division and alloantibody production. These data support the critical role of the splenic microenvironment for initial priming of CD4⁺ T cell help in response to alloantigen on transfused LR-PLTs, without which, humoral alloimmunization does not occur.

Materials and Methods

Mice

C57BL/6 (H-2^b), BALB/c (H-2^d), and BALB.B [C.B10-H-2^b/LiMcJ (H-2^b)] mice were purchased from Jackson Laboratories (Bar Harbor, ME). BALB/c and BALB.B mice were used as LR-PLT donors at 8–12 weeks of age. C57BL/6 mice were used as PLT transfusion recipients at 6–8 weeks of age. BALB/c donor splenocytes were used for seroanalysis at 8–12 weeks of age. TCR75 × Thy1.1 (H-2^b) mice were a generous gift from Drs. Pat Bucy and Judith Kapp¹³. Both TCR75 and 3A9 × B6.PL-Thy1.1 (H-2^k × H-2^b) were bred by the Emory University Department of Animal Resources. In all studies, only female mice were used. All studies and procedures were carried out in accordance with Emory University's Institutional Animal Care and Use Committee guidelines.

Antibodies for Flow Cytometry

Antibodies purchased from BD Pharmingen include PE anti-mouse CD41, PE anti-TER119/erythroid, PE rat IgG2b isotype control, PE rat IgG1 isotype control, APC goat anti-mouse IgG, FITC anti-mouse CD19, PE anti-mouse CD3e, APC anti-mouse CD4, and PerCP anti-mouse Thy1.1.

Leukocyte Reduced PLT Rich Plasma and Whole Blood Preparation

LR-PLT products were harvested as previously described¹⁴. Briefly, donor whole blood was collected in 1:8 acid-citrate-dextrose (ACD; BD Vacutainer). 1 mL of whole blood in ACD was added to 2 mL of 1x DPBS. Samples were centrifuged at $80 \times g$ for 10 minutes, followed by collection of the PLT rich plasma and centrifugation at $80 \times g$ for 10 minutes. Isolated PLTs were pooled and passed over a Neonatal Purecell PL High Efficiency Leukocyte Reduction Filter (Pall Corporation Port Washington, NY). PLTs were enumerated as described below. Following enumeration, PLTs were pelleted at $1,942 \times g$ for 10 minutes and re-suspended in Tyrode's buffer (1 mM HEPES, 137 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.4 mM Na_2HPO_4 , 5.5 mM glucose in Milli Q water, pH 7.4) to 2×10^8 PLTs/mL. 500 μ L of re-suspended LR-PLTs (10^8 PLT total) were transfused through the tail-vein. All PLT handling was performed at room temperature. A "swirl" test was performed on all LR-PLT concentrates to test the quality of PLTs in solution¹⁵; PLTs that did not swirl properly were not used. For whole blood transfusion, donor whole blood was collected as above, and washed 3 times in DPBS prior to transfusion.

Enumerating PLTs, Residual Red Blood Cells, and Contaminating Leukocytes

To count PLTs and residual RBCs, 20 μ L of LR-PLTs was diluted in 100 μ L of FACS buffer (1x DPBS with 0.2% bovine serum albumin and 0.24 mM EDTA) and centrifuged at $1,942 \times g$ for 2 minutes at room temperature. The samples were stained with PE anti-mouse CD41 or isotype control PE Rat IgG1 for PLTs, and PE anti-mouse TER119 or isotype control PE Rat IgG2b for RBCs. All antibodies were diluted at 1:100 in FACS buffer. Samples were incubated for 30 minutes in the dark at room temperature and then washed once with FACS buffer. Samples were re-suspended in 100 μ L FACS buffer and 10 μ L of each sample was mixed with 940 μ L FACS buffer. To count absolute numbers, 2.5×10^5 APC-beads (BD Pharmingen) were added to each sample. Samples were run on a BD FACS Scan and analyzed using FlowJo (Treestar, Portland OR). 12,500 events of APC-beads were collected.

To enumerate contaminating leukocytes, 25 μ L of LR-PLTs were stained with 150 ng/mL Propidium Iodide (PI) in a 1 g/L sodium citrate dihydrate solution with 0.7% Zap-oglobin II reagent (Beckman Coulter), 0.7% RNase cocktail (New England Biolabs). 1×10^5 APC-beads were added to each sample for absolute counts. PI positive nucleated cells were enumerated on a BD FACS Scan and analyzed using FlowJo. 10,000 total APC-beads were collected.

CD4⁺ T cell depletion

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

Adoptive Transfer of Donor Specific CD4⁺ T cells

CD4⁺ T cell alloresponses to donor LR-PLT transfusions were tested utilizing the TCR75 transgenic T cells, which are specific for the H-2^d₅₄₋₆₈ peptide presented by MHC II, I-A^b¹³. TCR75 whole splenocytes were isolated by mechanical disruption and were incubated in RBC lysis buffer (Sigma Aldrich, St. Louis, MO) for 5 minutes at room temperature. The cells were washed once in 1x DPBS, washed three times with warm 1x RPMI, and then

incubated for 20 minutes with 5 μ M CFSE (Invitrogen, Eugene, OR) in a 37°C water-bath. Cells were then washed two times with cold 1x RPMI supplemented with 10% fetal bovine serum (FBS) (Mediatech, Manassas, VA), and washed twice with 1x DPBS prior to re-suspension in 1x DPBS. 1×10^6 CFSE labeled TCR75 whole splenocytes were adoptively transferred via tail vein injection and allowed to circulate for 24 hours prior to experimentation.

Seroanalysis: Indirect Immunofluorescence Staining

Sera were diluted 1:10 in FACS buffer and incubated with 1×10^6 BALB/c splenocyte targets for 30 minutes at 4°C. The samples were then washed three times and stained with APC goat anti-mouse Ig (1:100), FITC anti-mouse CD19 (1:100) and PE anti-mouse CD3 ϵ (1:100) in FACS buffer for 30 minutes at 4°C, in the dark. Samples were run on a FACS Scan and analyzed on FlowJo. A CD19⁻ CD3⁺ parent gate was utilized to avoid nonspecific background signal due to the B cell receptor and Fc receptor expressing cells. A positive response was defined as two standard deviations above the mean of sera from naïve C57BL/6J mice.

Surgical splenectomy

Mice were anesthetized with ketamine and xylazine, and given buprenorphine for postoperative analgesia. A small incision was made in the left subcostal abdominal wall, the spleen was exteriorized, and removed by cauterizing the connecting vessels. The incision was closed using wound clips. Mice were kept on a heating pad and monitored for recovery. Control groups underwent sham surgery (incision followed by clips, but no splenectomy) and were maintained under similar conditions. Wound clips were removed after two weeks and animals were allowed an additional week of recovery prior to experimentation.

Isolation of spleen, liver, and lymph node single cell suspensions

Lymphocytes were isolated from the spleen and lymph nodes by mechanical disruption in 1x DPBS. Red blood cells were lysed prior to resuspension in FACS buffer. Lymphocytes were isolated from the liver as described¹⁶, with minor modifications. The portal vein was cut and the heart was perfused with 5–10 ml of 1% (w/v) collagenase D (Sigma-Aldrich) in DPBS. The liver was removed, dissected and incubated in 1% (w/v) collagenase D in RPMI for 15 min at 37°C before being passed through a 100 μ m nylon cell strainer. Hepatocytes were removed by discarding the pellets from a series of four centrifugations in RPMI at $20 \times g$ for 4 min. The non-parenchymal cell suspension was pelleted at $300 \times g$ for 10 min and then resuspended in a 44% percoll solution (Sigma-Aldrich). 5 mL of the cell suspension was underlaid with 3 mL of 67% percoll in a 15 mL conical tube and centrifuged at $863 \times g$ for 20 min. The buffy coat interface was drawn off, washed once with RPMI, and the purified lymphocytes were resuspended in FACS buffer.

Statistics

Statistical analysis was performed PRISM software and significant alloimmunization was determined as a signal that exceeded two standard deviations above background.

Results

Recipient CD4⁺ T cells are required for humoral alloimmunization to LR-PLT transfusion

The observed IgG response to antigens on transfused PLTs in humans demonstrates class switching, a process that typically requires that B cells receive CD4⁺ T cells help. In addition, the presence of CD4⁺ T cells specific for PLT antigens have been observed to correlate with alloimmunization in animal models^{2,11}. However, to the best of our

knowledge, a causal requirement of CD4⁺ T cells has never been formally tested in the context of PLT transfusion. To test the hypothesis that CD4⁺ T cells are required for IgG responses to alloantigens on transfused PLTs, we depleted CD4⁺ T cells from recipient mice. C57BL/6 recipients were treated with the monoclonal antibody GK1.5 at 4 days and 2 days prior to transfusion with LR-PLTs. As shown by the representative flow plots, GK1.5 injection eliminated detectable CD4⁺ T cells throughout our PLT transfusion protocol [21 days] (Figure 1A).

CD4⁺ depleted mice and control animals were each transfused with LR-PLTs from BALB/c donors, weekly for four weeks (days 0,7,14, and 21). Transfusions were given by tail vein and consisted of 1×10^8 LR-PLTs per recipient. LR-PLTs were prepared as previously described, and under conditions where PLTs circulate normally post-transfusion and aggregate normally when exposed to collagen ¹⁴. For each unit of 1×10^8 LR-PLTs, there were on average, $5.8 \times 10^4 \pm 5.5 \times 10^4$ RBCs (95% C.I.) and fewer than 500 total leukocytes (limit of detection for assay). MHC antigens in BALB/c mice are encoded by the H-2^d haplotype whereas C57BL/6 mice encode H-2^b; therefore, an anti-H-2^d antibody response represents humoral alloimmunization.

Anti-donor antibody responses were observed in mice not depleted of CD4⁺ T cells starting after the second transfusion (day 14) and increased in intensity at days 21 and 28 (Figure 1B). As in human transfusions, a distribution of strong responders, moderate responders, and non-responders was observed. In contrast, no significant antibody response was observed in any mice depleted of CD4⁺ T cells, even after 4 weekly transfusions (Figure 1B). The observed alloimmunization was specific to exposure to allogeneic LR-PLT transfusions, as no antibodies were detected in control animals receiving syngeneic (C57BL/6) LR-PLTs (Figure 1B).

Although lack of response after depletion of CD4⁺ T cells is consistent with the interpretation that CD4⁺ T cells are required, there is the potential that the CD4 depleting antibody exerted its effect by other mechanisms. First, there are CD4 expressing non-T cell lineages that may be critical for the anti-PLT response ¹⁷⁻²². Second, the mechanisms of CD4⁺ T cell depletion may interfere with the normal way transfused LR-PLTs are processed (e.g. by saturating macrophages or inducing cytokines). To control for these possibilities, we utilized a separate experimental approach to assess CD4⁺ T cell requirements. The 3A9 mouse expresses a transgenic TCR specific for the HEL₄₈₋₆₂ peptide from hen-egg lysozyme presented by the MHC II, I-A^k ²³. Greater than 95% of the CD4⁺ T cell compartment of these mice express the transgenic T cell receptor (data not shown). Thus, 3A9 mice have normal development of CD4⁺ T cells, but the CD4⁺ T cell specificity is fixed on a target other than H-2^d alloantigen. In contrast to the strong humoral response by the non-transgenic littermates, the 3A9 transgenic recipients did not mount a detectable anti-donor antibody response following four weekly BALB/c LR-PLT transfusions (Figure 1C). Together, these data provide two lines of evidence that CD4⁺ T cells are required for an IgG response to MHC alloantigens on transfused LR-PLTs.

Direct characterization of CD4⁺ T cell response specific for MHC alloantigen on transfused LR-PLTs

To allow direct characterization of an allospecific CD4⁺ T cell response during the development of anti-donor antibodies we utilized the TCR75 mouse, which expresses a transgenic TCR that recognizes peptide from donor MHC I presented on recipient MHC II; specifically, TCR75 recognizes a peptide from K^d presented by I-A^b (K^d₅₄₋₆₈/I-A^b) ¹³. The TCR75 donor mice were bred onto a homozygous Thy1.1 C57BL/6 background, such that TCR75 cells can be readily discriminated following adoptive transfer into wild-type C57BL/6 recipients that express the Thy 1.2 gene. Staining with anti-CD4 and anti-Thy1.1 identifies

the transferred TCR75 population (Figure 2A). This gating strategy is specific for TCR75 cells, as the gate is empty in C57BL/6 mice that did not receive adoptive transfer (Figure 2A).

To monitor the response of TCR75 cells to antigen, CFSE labeled TCR75 cells were adoptively transferred into C57BL/6 recipients followed by transfusion with LR-PLTs 24 hours later. By day 5, TCR75 CD4⁺ T cells in the spleen had undergone multiple rounds of division in response to BALB/c LR-PLTs. This response was antigen-specific, as no significant division was observed in recipients of syngeneic C57BL/6 LR-PLTs. Moreover, this response was not due to BALB background genes outside the MHC locus, as transfusion of LR-PLTs from BALB mice congenic for the C57BL/6 MHC (BALB.B) induced no response (Figure 2B). Finally, the increased precursor frequency of CD4⁺ T cells specific for H-2^d MHC antigen augmented the otherwise undetectable anti-donor antibody response to a single BALB/c LR-PLT transfusion as early as day 7, suggesting that the proliferating TCR75 CD4⁺ T cells had differentiated into a helper phenotype (Figure 2C).

The spleen is required for the initiation of the alloantigen specific CD4⁺ T cell response to transfused PLTs

Transfused PLTs represent circulating cellular antigens that are cleared after normal senescence by phagocytes in the spleen and liver. In general, PLTs are not thought to enter the lymphatics in considerable numbers. Thus, we hypothesized that the initiation of the immune response to transfused LR-PLTs would take place in the splenic microenvironment. To test this hypothesis, a cohort of mice underwent surgical splenectomy, and control animals underwent sham surgery. After a three week recovery period, the animals were transfused with LR-PLTs. CFSE labeled TCR75 CD4⁺ T cells were adoptively transferred to mice prior to transfusion of LR-PLTs.

TCR75 cells proliferated robustly in the spleen of intact recipients following transfusion with BALB/c LR-PLTs (Figure 3A). However, only trace amounts of division were observed in the liver and lymph nodes of these intact animals. The activation of these antigen specific splenic CD4⁺ T cells correlated with a strong anti-donor antibody response in sham operated animals (Figure 3C). In the absence of a spleen, there was neither division of TCR75 cells (in lymph nodes or liver), nor was there a detectable antibody response. (Figure 3B and C).

To control for any alterations in biology that may have occurred as a result of transferring TCR75 cells into the recipient groups, additional studies compared humoral alloimmunization to transfused LR-PLTs in splenectomized or control animals without any transfer of TCR75 cells. The sham operated animals had normal alloimmunization, with increasing frequency and titer over 28 days (4 weekly transfusions). In contrast, no antibodies were detectable, despite 4 transfusions, in splenectomized mice (Figure 4A). To control for the possibility that splenectomy renders recipients categorically incapable of becoming immunized to alloantigen, additional groups of sham or splenectomized mice were transfused with a single transfusion of BALB/c whole blood, and monitored for alloantibody formation 7 and 14 days later. In contrast to what was observed for LR-PLTs, no significant difference was seen in humoral alloimmunization between sham and splenectomized recipients (Figure 4B). In aggregate, the above findings indicate that a spleen is required for the initial alloimmunization to MHC antigens on transfused LR-PLTs, and that CD4⁺ T cells specific for donor alloantigen do not proliferate outside the splenic microenvironment (in the liver or lymph nodes).

Discussion

We report herein, that in the absence of a spleen, humoral alloimmunization to MHC I antigens does not occur in response to transfusion with LR-PLTs in a mouse model. We further report that coincident with loss of alloantibody formation, CD4⁺ T cells specific for donor peptide presented by recipient MHC II do not proliferate in the liver or lymph nodes of splenectomized mice. Our interpretation of these data, taken in the context of our demonstration that CD4⁺ T cells are required for alloimmunization, is that CD4⁺ T cell activation and differentiation into a helper phenotype requires the presence of a spleen, and that without the splenic microenvironment, insufficient CD4⁺ T cell help is generated to allow humoral alloimmunization. Such may be due to unique aspects of the splenic architecture²⁴ or specialized splenic antigen-presenting cell (APC) populations^{24–26}. Although the current findings focus on CD4⁺ T cell responses because CD4⁺ T cells are typically responsible for B cell help, it is also important to note that the MHCI antigen can directly stimulate recipient CD8⁺ T cells, which may have distinct effects upon the immune response, but are outside the scope of the current report.

The current findings are consistent with previous reports that allogeneic PLTs phagocytosed *in vitro* by recipient adherent splenic macrophages stimulated the proliferation of previously sensitized splenocytes under certain conditions². Similar methods were used to show that these *ex vivo*-manipulated macrophages were sufficient to initiate an anti-donor antibody response when adoptively transferred to naïve recipients¹¹. The data in the current report demonstrate the first *in vivo* exploration of this issue (to our knowledge), and establish the essential nature of the spleen in a primary alloimmunization to transfused LR-PLTs in mice.

As the current studies are carried out in a murine model, which may not reflect biology of other species or human biology, it is unclear to what extent our findings are a generalizable principle. Repeated transfusion of C57BL/6 platelets into BALB/c recipients also fails to induce antibody responses in splenectomized recipients (Personal Communication from John Semple, unpublished data), indicating that our findings are not idiosyncratic to one strain combination. However, in a study by Shlichter et al. investigating platelet transfusion in baboons, alloimmunization was in fact observed in 2 out of 2 baboons that had been previously splenectomized²⁷. However, alloimmunization of the splenectomized baboons required more transfusions than did control animals. Moreover, the platelet units were not leukoreduced, and contained on average 7.5×10^5 leukocytes²⁷. In the context of our whole blood data in splenectomized mice, it is thus difficult to determine if the murine biology reflects or differs from that seen in baboons.

It is difficult to assess, based upon existing clinical data, if a human spleen is required for alloimmunization to HLA antigens on transfused PLT products. Surgical splenectomy is a therapeutic maneuver for the forms of ITP that are refractory to other treatments. However, in such cases, antibody responses to PLT antigens have already been formed at the time of splenectomy, and in the altered context of whatever immune dysregulation led to autoimmunity. Moreover, and of great importance, is the fact that the current data pertain only to initial exposure to MHC antigens on LR-PLTs. Canonically, after an initial activation, T cells undergo a diaspora to different tissues as memory T cells. It is well described that memory populations have substantially less stringent requirements for activation than do naïve T cells. It is thus possible that extra-splenic compartments, while not capable of supporting primary activation of CD4⁺ T cells, may be able to stimulate a recall response. If such were the case, then only human patients splenectomized prior to any transfusion or pregnancy would be meaningful subjects in comparison to the present findings. Since splenectomy is only performed in the context of existing pathology that has not responded to antecedent therapy (typically also involving transfusion), such a human

population is difficult to isolate. It is also important to note that an accessory spleen is present in about 10% of humans²⁸. Furthermore, patients can develop post-splenectomy splenosis in which hundreds of diffuse splenic nodules in the bowel, mesentery, and omentum can serve the full hematological and immunological functions of an intact spleen, but are not detectable except during exploratory surgery²⁹. Thus, to test the hypothesis that a spleen is required for primary alloimmunization to transfused products in a human, one would need to have a highly selected patient population with considerable controls and ancillary studies to assess the above variables. Moreover, given that a spleen is not required for alloimmunization to leukocytes in the current study, highly sensitive assays of leukocyte contamination and leukoreduction filter failure would need to be carried out in human trials.

In addition to the spleen, the liver may also serve as a site of PLT clearance³⁰. The liver is characterized as a relatively tolerogenic organ due to its distinct intrahepatic lymphocyte populations and APCs^{31,32}. In the splenectomized recipients described herein, donor PLTs may have been phagocytosed by liver APCs, however the TCR75 cells present in the hepatic environment remained undivided (Figures 3A and B). It is possible that the conditions of hepatic alloantigen presentation not only lead to a lack of immunity, but also to active tolerance. Additional experimentation is required to investigate the patterns of PLT consumption by liver APC subsets, as well as the tolerogenic potential of antigen presentation to CD4⁺ T cells in this setting.

Although we have demonstrated that CD4⁺ T cell help is required for the anti-donor antibody response to allogeneic LR-PLT transfusion, this does not necessarily exclude CD4⁺ T cell independence in all settings. As an antigen, PLTs share several similarities with T cell independent antigens. Type 2 T independent antigens are molecules containing polyvalent B cell epitopes capable of cross-linking the B cell receptor to an extent that CD4⁺ T cell help is not required for antibody production. With the expression of donor-MHC I on their surface, particularly in the context of an immobilized thrombus, PLTs may also provide a similar scaffold for cross-linking the B cell receptor. Furthermore, PLTs can secrete many inflammatory mediators such as IL-1 α , IL-8, RANTES, and TGF- β , as well as CD154^{33,34}. PLT derived CD154 has been shown to directly stimulate B cell proliferation and antibody production³⁵. Thus, PLTs may have the potential to provide both B cell receptor cross-linking and costimulation to induce alloantibody production in the absence of CD4⁺ T cell help in some settings. The current findings indicate that MHC I on PLTs is not a T cell independent antigen for fresh PLTs transfused into healthy recipients; however, it is unclear if the fundamental biology will be altered in thrombocytopenic animals with or without hemostatic challenge and thrombus formation.

Human PLT units may be stored for up to 5 days in the US prior to transfusion, and the PLTs in the present experiments were all collected and transfused on the same day. Thus, although we took considerable efforts to model collection, filter leukoreduction, and transfusion, it is possible that changes during PLT storage may alter the immune properties of transfused PLTs. Indeed, we have described such changes for stored RBCs in an analogous murine system³⁶.

An additional consideration is that our experiments were carried out without intentionally inducing inflammation in the recipient mice. Previous studies in our laboratory have shown that the inflammatory status of the recipient alters the particular APC subsets consuming RBCs and modulates the alloantibody response^{37,38}; a similar effect may also occur following PLT transfusion. Patients receiving PLT transfusions often have significant underlying pathology, such as trauma, infection, or disease. It is also interesting to note that there were considerable differences amongst responses in the mice tested herein. Although genetically identical, the recipients each have potential environmental differences that may

affect their immune response, including baseline alterations in inflammation. Thus, while we did not intentionally inflame any animals, environmental factors may nevertheless play a role.

In summary, we have utilized a murine system to assess the role of the splenic micro-environment in primary alloimmunization to MHC antigens on transfused LR-PLTs and the role of CD4⁺ T cells in this process. In the absence of a spleen, neither synthesis of alloantibodies nor activation of alloantigen specific CD4⁺ T cells occurs in response to allogeneic LR-PLT transfusion. We interpret these findings to indicate that the reason a spleen is required is to allow an environment in which CD4⁺ T cells can become activated to transfused antigens. These data support the critical role for CD4⁺ T cell help and provide a rational basis for exploring CD4⁺ T cell modalities for preventing alloimmunization to transfused PLTs.

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Figure 1. Antigen specific CD4⁺ T cell help is required for the anti-donor antibody response to transfused LR-PLTs

(A) Analysis of CD4⁺ T cell levels following treatment with the CD4-depleting antibody GK1.5. Recipients were injected i.p. at 4 and 2 days prior to each LR-PLT transfusion. CD3⁺CD4⁺ T cells are shown from the spleen of representative animals at days 0 and 21. Numbers of CD4⁺ T cell numbers in treated animals (center panels) dropped to baseline signal seen with isotype control (right panels) throughout the time of LR-PLT transfusion. Flow plots are representative of two independent experiments with two mice per group. (B) C57BL/6 recipients were either untreated or injected i.p. with GK1.5, and then received four weekly BALB/c or syngeneic LR-PLT transfusions. The total Ig anti-donor antibody response was quantified by indirect immunofluorescence against BALB/c splenocyte targets. (left panel: individual animals, right panel: combined data). (C) 3A9 TCR transgenic mice and non-transgenic littermates received four weekly BALB/c LR-PLT transfusions.

The total Ig anti-donor antibody response was quantified by indirect immunofluorescence against BALB/c splenocyte targets. (left panel: individual animals, right panel: combined data). In panels B and C, the horizontal dashed line marks two standard deviations above the mean signal for mice receiving syngeneic LR-PLTs, and thus represents the cut-off for a statistically significant response. Combined data from three independent experiments are shown in panels B and C, with three mice per group per experiment (n=9 mice total/group). Error bars indicate standard deviation.

Figure 2. Characterization of in vivo CD4⁺ T cell response to alloantigen on transfused LR-PLTs (A) 1×10^6 CFSE labeled TCR75 $\times$ Thy1.1 splenocytes were adoptively transferred into wild-type C57BL/6 (Thy1.2) recipients and were visualized by staining with anti-CD4 and anti-Thy1.1 (B) C57BL/6 recipients were adoptively transferred with 1×10^6 CFSE labeled TCR75 splenocytes and were then given a single transfusion of LR-PLT 24 hours later from either BALB/c (dark line), BALB.B (dashed line) or syngeneic C57BL/6 (shaded histogram) donors. Representative histograms are from three independent experiments with three mice per group (n=9). (C) Sera were collected from the TCR75 adoptively transferred C57BL/6 recipients 7 and 14 days post-LR-PLT transfusion. The total Ig anti-donor antibody response was quantified by indirect immunofluorescence against BALB/c splenocyte targets (left panel: individual animals, right panel: combined data). The horizontal dashed line marks two standard deviations above the mean signal for mice receiving syngeneic LR-PLTs, and thus represents the cut-off for a statistically significant response. Combined data from three

independent experiments with three mice per group (n=9). Error bars indicate standard deviation.

Figure 3. The splenic microenvironment is required for antigen-specific CD4⁺ T cell division and humoral alloimmunization

(A) Sham-operated and (B) splenectomized recipients were adoptively transferred with 1×10^6 CFSE labeled TCR75 splenocytes, followed by a single LR-PLT transfusion 24 hours later. Five days after transfusion, division was assessed in the spleen, liver, and lymph nodes by gating on CD4⁺ Thy1.1⁺ cells and assessing CFSE dilution. Representative histograms are from three independent experiments, with three mice per group (n=9). (C) Intact and splenectomized recipients were adoptively transferred with 1×10^6 CFSE labeled TCR75 splenocytes, followed by a single BALB/c or C57BL/6 LR-PLT transfusion 24 hours later. The total Ig anti-donor antibody response was quantified by indirect immunofluorescence against BALB/c splenocyte targets 7 and 14 days post-LR-PLT transfusion. (left panel: individual animals, right panel: combined data). The horizontal dashed line marks two

standard deviations above the mean signal for mice receiving syngeneic LR-PLTs, and thus represents the cut-off for a statistically significant response.

Figure 4. A spleen is required for the anti-donor antibody response to LR-PLTs but not whole blood

Sham-operated and splenectomized C57BL/6 recipients received four weekly BALB/c or C57BL/6 LR-PLT transfusions (**A**) or a single transfusion of whole blood (**B**). The total Ig anti-donor antibody response was quantified by indirect immunofluorescence against BALB/c splenocyte targets at the indicated time-points post-transfusion. (left panel: individual animals, right panel: combined data). The horizontal dashed line marks two standard deviations above the mean signal for mice receiving syngeneic transfusions, and thus represents the cut-off for a statistically significant response. Combined data from three independent experiments with at least three mice per group are shown for all panels. Error bars indicate standard deviation.