TRANSFUSION MEDICINE

Allogeneic platelet transfusions prevent murine T-cell–mediated immune thrombocytopenia

Li Guo,[1-3] Lei Yang,[1,2] Edwin R. Speck,[1,2,4] Rukhsana Aslam,[1,2,4] Michael Kim,[1,2] Christopher G. J. McKenzie,[1,2,4] Alan H. Lazarus,[1-6] Heyu Ni,[1-3,5,6] Ming Hou,[7] John Freedman,[1,5,6] and John W. Semple[1-6,8]

[1]The Toronto Platelet Immunobiology Group, [2]Keenan Research Centre for Biomedical Sciences, St. Michael's Hospital, and [3]Institute of Medical Science, University of Toronto, Toronto, ON, Canada; [4]Canadian Blood Services, Toronto, ON, Canada; [5]Department of Medicine, and [6]Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada; [7]Cancer Center, Qilu Hospital, Shandong University, Jinan, China; and [8]Department of Pharmacology, University of Toronto, Toronto, ON, Canada

Key Points

- Allogeneic platelet MHC class I transfusions can both prevent and/or alleviate anti-CD61 (GPIIIa) T-cell–mediated ITP.
- The transfusions reverse abnormal bone marrow megakaryocyte histology and inhibit CD61-induced cytotoxicity.

Platelet transfusions are life-saving treatments for many patients with thrombocytopenia; however, their use is generally discouraged in the autoimmune disorder known as immune thrombocytopenia (ITP). We examined whether allogeneic platelet major histocompatibility complex (MHC) class I transfusions affected antiplatelet CD61-induced ITP. BALB/c CD61 knockout mice (CD61^{-}/H-2^{d}) were immunized against platelets from wild-type syngeneic BALB/c (CD61^{+}/H-2^{d}), allogeneic C57BL/6 (CD61^{+}/H-2^{b}), or C57BL/6 CD61 KO (CD61^{-}/H-2^{b}) mice, and their splenocytes were transferred into severe combined immunodeficient (SCID) mice to induce ITP. When nondepleted splenocytes were transferred to induce antibody-mediated ITP, both CD61^{+} platelet immunizations generated immunity that caused thrombocytopenia independently of allogeneic MHC molecules. In contrast, when B-cell–depleted splenocytes were transferred to induce T-cell–mediated ITP, transfer of allogeneic MHC-immunized splenocytes completely prevented CD61-induced ITP development. In addition, allogeneic platelet transfusions into SCID mice with established CD61-induced ITP rescued the thrombocytopenia. Compared with thrombocytopenic mice, bone marrow histology in the rescued mice showed normalized megakaryocyte morphology, and in vitro CD61-specific T-cell cytotoxicity was significantly suppressed. These results indicate that antibody-mediated ITP is resistant to allogeneic platelet transfusions, while the T-cell–mediated form of the disease is susceptible, suggesting that transfusion therapy may be beneficial in antibody-negative ITP. (*Blood*. 2014;123(3):422-427)

Introduction

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder characterized by an isolated thrombocytopenia defined as a peripheral blood platelet count of less than 100×10^9/L.[1,2] The pathogenesis of ITP is complex but appears to be primarily due to immunoglobulin G (IgG)-mediated peripheral platelet destruction in the spleen and/or bone marrow megakaryocyte inhibition and destruction.[3-7] A second mechanism of ITP has been suspected; however, as the famous 1951 Harrington experiments showed, only 16 of the 26 (62%) ITP plasma infusions into healthy volunteers caused thrombocytopenia. Subsequently, the cumulative results of antibody studies in patients with ITP revealed that antiplatelet antibodies can only be identified in approximately 60% of patients with ITP.[8-13] In those patients with no identifiable antibodies, Olsson et al[14] elegantly demonstrated that cytotoxic T lymphocyte (CTL) cytotoxicity was responsible for the thrombocytopenia, and this was subsequently confirmed by other studies in both humans and animals with ITP.[15-17] The latter ITP animal model also demonstrated that compared with antibody-mediated ITP, the T-cell–mediated form of the disorder was not sensitive to platelet-sparing therapies such as intravenous gammaglobulin (IVIg).[17]

Therapy for patients with ITP generally includes steroids and/or IVIg, and if those fail, other treatment options such as rituximab, thrombopoietin (TPO) receptor agonists, and/or splenectomy are available.[1,2] Of interest, despite the benefit of platelet transfusions for many thrombocytopenic conditions such as the thrombocytopenia secondary to leukemia,[18-20] the use of allogeneic platelets in patients with ITP have been generally withheld.[21-24] Allogeneic platelet transfusions for chronic ITP are recommended only as emergency treatment and should be used in combination with other treatments such as IVIg.[1,2] The reason for avoiding platelet transfusions in ITP concern the ineffectiveness of the treatment due to autoantibody-induced platelet clearance and the potential for triggering adverse immune responses when transfused platelets are recognized by autoantibodies.[21] However, very few reports published support the lack of benefit for platelet transfusions in ITP, and those published are mostly case reports where more than half actually show a benefit from platelet transfusions (satisfactory corrected count increment) in patients with ITP.[21-24]

Our murine model of active CD61-specific ITP demonstrates both antibody- and T-cell–mediated thrombocytopenia. Because

Submitted August 23, 2013; accepted November 18, 2013. Prepublished online as *Blood* First Edition paper, November 20, 2013; DOI 10.1182/blood-2013-08-523308.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

the T-cell–mediated form of thrombocytopenia was resistant to IVIg,[17] we examined whether allogeneic platelet transfusions may have a differential effect on the two immunopathologic forms of ITP. The results showed that although antibody-mediated ITP was not affected by allogeneic platelet transfusions, the T-cell–mediated form of the disorder was alleviated by the transfusions. This suggests that platelet transfusions may be beneficial in individuals suffering from antiplatelet antibody-negative ITP.

Methods

Mice

Female BALB/c (CD61$^+$/H-2^d), C57BL/6 (CD61$^+$/H-2^b), and B6.129S2-Itgb3^{tm1Hyn}/J CD61 (CD61$^-$/H-2^b) knockout (KO) mice, 8 to 12 weeks of age were used as platelet donors and were obtained from The Jackson Laboratory. BALB/c CD61 KO mice (CD61$^-$/H-2^d) were bred in the laboratory of Dr Heyu Ni and used as platelet recipients and were the source of immune splenocytes. Female CB.17 severe combined immunodeficient (SCID) (H-2^d, CB17/Icr-*Prkdc*^{scid}/IcrIcoCrl) mice (8 to 12 weeks of age) were used as spleen-cell transfer recipients for induction of ITP and were obtained from the Charles River Laboratories. All mice were housed in the Li Ka Shing Knowledge Institute's Research Vivarium and the St. Michael's Hospital Animal Care Committee approved all animal studies.

Platelet preparation and immunization of CD61 KO mice

Leuko-reduced platelets from the indicated donor mice (see supplemental Table 1 on the *Blood* Web site) were prepared as previously described.[17] Blood was collected from the indicated donor mice, diluted with 1× phosphate buffered saline (PBS) containing 10% citrate-phosphate-dextrose with adenine (CPDA) (or PBS/CPDA buffer) and centrifuged at 120*g*. Platelet-rich plasma was then collected and washed at 450*g*. The washed platelets were resuspended in PBS, adjusted to 1×10^9 cells/mL, and 100 μL was transfused into BALB/c CD61 KO (CD61$^-$/H-2^d) mice weekly for 4 weeks.

Serum anti-CD61 and anti-major histocompatibility complex (MHC) class I antibody production

Serum IgG anti-CD61–specific or antiplatelet MHC class I–specific antibodies were detected by flow cytometry. Briefly, 1×10^6 leuko-reduced platelets from the indicated donor mice were incubated with titrations of serum from the indicated immune BALB/c CD61 KO mice for 30 minutes at room temperature (RT). The platelet-serum mixtures were washed once with PBS/CPDA buffer and then labeled with a fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody (Fc-specific; Caltag Laboratories, Mississauga, ON, Canada) in the dark at RT for 30 minutes. The mixture was washed with the PBS/CPDA buffer, and acquired and analyzed by flow cytometer (BD FACSort; Becton Dickinson, Mississauga, ON, Canada).

Preparation of splenocytes and cell depletion

Immune CD61 KO mice (CD61$^-$/H-2^d) were euthanized and their spleens removed, homogenized in RPMI 1640 (medium), and washed twice by centrifugation at 400*gg* for 15 minutes. Splenocyte suspensions were further treated with ammonium-chloride-potassium red blood cell (RBC) lysing solution and washed with 1× PBS to remove lysed RBC. Cell depetion studies were carried out using the STEMCELL Technologies EasySep Mouse CD19 Positive Selection kit (catalog no. 18754), according to the manufacturer's instructions. Splenocytes were combined with the labeling reagent and phycoerythrin selection cocktail, and magnetic particles were added and placed within a magnetic field to isolate CD19$^+$ cells. The unbound CD19$^-$ cells were resuspended in RPMI 1640 (medium) at the concentration of 1.5×10^5/mL and CD19-depletion efficiencies were determined by flow cytometry. CD19$^+$ cells were less than 1% of all splenocytes after depletion.

ITP induction

SCID mice were prescreened for the presence of serum IgG by an enzyme-linked immunosorbent assay and any mouse with a serum IgG concentration greater than 20 μg/mL was deemed "leaky" and excluded from study. ITP was induced as previously described.[17] On day −1, CB.17 SCID mice were bled via the saphenous vein, and pretreatment platelet counts were measured using a Beckman Coulter Counter LH750 hematology analyzer. Natural killer cells were depleted in the mice by an intraperitoneal infusion of 50 μL of a rabbit anti-asialo GM1 antibody (Wako Pure Chemical Industries, Ltd). On day 0, the mice were sublethally γ irradiated (200 cGy) and received 100 μL (1.5×10^4 cells total) of the nondepleted or CD19-depleted immune splenocytes (intraperitoneal) from the indicated immune CD61 KO mice. Peripheral blood of SCID mouse recipients was collected weekly from the saphenous vein and platelet counts were measured. The protocol for ITP induction and expected immune responses are shown in supplemental Figure 1 and supplemental Table 1, respectively.

Bone marrow histology and megakaryocyte counts

Engrafted SCID mice were euthanized on day 28, their femurs removed and dissected of muscle tissue, the epiphyses cut off, and the bones shafts placed in fixative (B+ fixative; BBC Biochemical) for 12 hours. The bones were further decalcified with 10% nitric acid for 2 hours and then processed and embedded in paraffin. Sections were stained with hematoxylin and eosin. All images were taken with cellSens Standard software under a ×40 objective lens (Olympus BX50), with the numerical aperture of objective lens at 0.75. The megakaryocyte count of each mouse was calculated as the mean value of 10 counts from 10 random fields (450 μm × 320 μm/field) throughout each slide.

Cytotoxicity assay

Anti-CD61 cytotoxic activity of splenocytes from the transfused CD61 KO mice and engrafted SCID mice was measured using a flow cytometric kit (7-AAD/CFSE Cell-Mediated Cytotoxicity Assay Kit, item no. 600120; Cayman Chemical Company), and the murine macrophage cell line PU5-1.8 (H-2^d) was used as a target cell (ATCC, TIB-61) because this cell line expresses both syngeneic MHC class I and CD61 antigens (determined by flow cytometry, not shown). Target cells were stained with 5-(6)-carboxy-flourescein diacetate succinimidyl ester (CFSE) for 15 minutes at RT, washed once with RPMI-1640 (medium), and 10^4 cells were incubated in 96 well V-bottom plates with the indicated splenocytes (effectors) for 4 hours at 37°C at different Effector:Target ratios (1.25:1, 2.5:1, 5:1, and 10:1). The plates were then centrifuged and the cells resuspended in 7-AAD staining solution to label dead cells. The samples were analyzed by flow cytometry (MACSQuant Analyzer, Miltenyi Biotec) and cytotoxic activity was evaluated by analyzing the percentage of dead cells within the CFSE-labeled target cell population. To address the effects of platelets on splenic cytotoxicity, 10^5 splenocytes were first incubated with either BALB/c wild-type (WT) (CD61$^+$/H-2^d) or C57BL/6 WT (CD61$^+$/H-2^d) platelets at a splenocyte/platelet ratio of 1:15 in V-bottom plates for 4 hours at 37°C, washed twice with RPMI 1640 (medium), and then used as effectors in the cytotoxicity assay with labeled PU5-1.8 target cells.

Statistical analysis

Differences between means were analyzed with Student *t* test, and $P < .05$ was considered significant.

Results

Platelet MHC class I and CD61 antigens generate high titred IgG antibody responses

When BALB/c CD61 KO mice were immunized with either BALB/c (CD61$^+$/H-2^d), C57BL/6 (CD61$^+$/H-2^b), or C57BL/6 CD61 KO (CD61$^-$/H-2^b) platelets, significant levels of antiplatelet IgG antibodies against the corresponding CD61 and/or allogeneic MHC class I antigens were detected in the sera after the fourth platelet

transfusion (that is, anti-CD61 antibodies, anti-CD61 and MHC antibodies, and anti-MHC antibodies were seen, respectively [Figure 1]).

Platelet allogeneic MHC class I antigens do not affect anti-CD61 antibody-mediated ITP

The effect of allogeneic MHC class I molecules on CD61-specific IgG-mediated ITP was determined by transferring nondepleted splenocytes from CD61 KO mice immunized against the 3 platelet populations into SCID mice, and then monitoring platelet counts weekly. As described previously,[17] irradiation-induced thrombocytopenia occurred at day 7 in all mice (Figure 2A-B). Compared with SCID mice transferred with naïve mouse splenocytes, nondepleted splenocytes from CD61 KO mice immunized against either $CD61^+/H\text{-}2^d$ or $CD61^+/H\text{-}2^b$ platelets induced significant thrombocytopenia throughout the 28-day protocol (day 21, 28, $P < .05$) (Figure 2A).

Platelet allogeneic MHC class I antigens prevent anti-CD61 T-cell–mediated ITP

To determine whether allogeneic MHC class I molecules have an effect on CD61-specific T-cell–mediated ITP, splenocytes from the CD61 KO mice immunized with the 3 platelet populations were first depleted of $CD19^+$ B cells and then transferred into SCID mice. SCID mice transferred with CD19-depleted immune splenocytes against $CD61^+/H\text{-}2^d$ platelets developed thrombocytopenia throughout the 28-day protocol (Figure 2B). However, SCID mice transferred with CD19-depleted immune splenocytes against $CD61^+/H\text{-}2^b$ platelets were rescued from the T-cell–mediated thrombocytopenia at days 21 and 28 ($P < .01$) (Figure 2B).

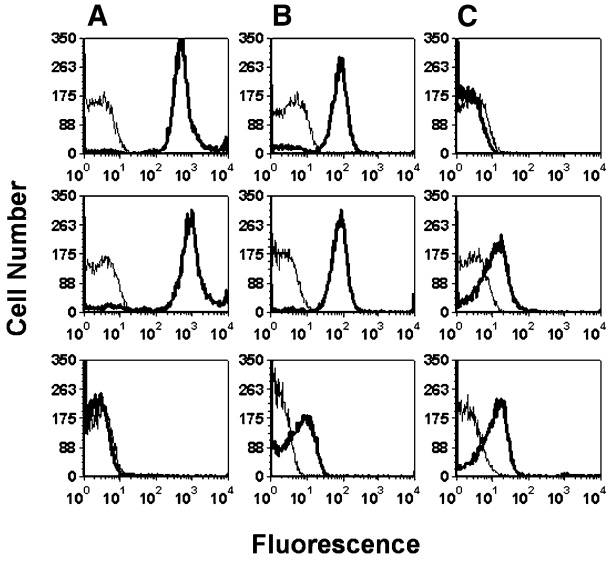

Figure 1. Generation of antiplatelet IgG antibodies in CD61 KO mice. Representative flow cytometric histograms of anti-CD61 and/or anti-MHC class I serum reactivity from immunized BALB/c CD61 KO mice. BALB/c CD61 KO mice were transfused weekly (×4) with platelets from either (A) BALB/c ($CD61^+/H\text{-}2^d$) mice; (B) C57BL/6 ($CD61^+/H\text{-}2^b$) mice; or (C) C57BL/6 CD61 KO ($CD61^-/H\text{-}2^b$) mice. Serum was prepared and incubated (1:400 dilution shown) with either BALB/c platelets (top panels), C57BL/6 platelets (middle panels), or C57BL/6 CD61 KO platelets (lower panels), and then incubated with a fluorescein isothiocyanate-conjugated goat anti-mouse IgG secondary antibody, and fluorescence was analyzed by flow cytometry. The thin lines in each histogram indicate the pretreatment serum reactivity, while the thick lines show serum reactivity after 4 platelet transfusions. Splenocytes from the immune mice were then used in the indicated experiments.

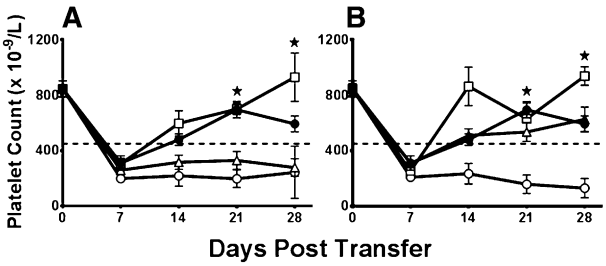

Figure 2. Allogeneic platelet MHC class I antigens inhibit T-cell–mediated ITP. Platelet counts in SCID mice transferred with either (A) nondepleted, or (B) CD19-depleted splenocytes from either naïve BALB/c mice (●; N = 18), BALB/c CD61 KO mice immunized against platelets from BALB/c ($CD61^+/H\text{-}2^d$) mice (○; N = 11), C57BL/6 ($CD61^+/H\text{-}2^b$) mice (△; N = 18), or C57BL/6 ($CD61^-/H\text{-}2^b$) CD61 KO mice (□; N = 8). Results are presented as weekly platelet counts ($\times 10^9$/L) after splenocyte transfer. The dotted horizontal line represents the mouse platelet cutoff counts for thrombocytopenia. In (A) $^*P < .05$ for □ vs △ or ○, and (B) $^*P < .05$ for △ vs ○.

Allogeneic platelet transfusions prevented T-cell–mediated ITP in transferred SCID mice

To ensure that allogeneic platelet transfusions would prevent an established T-cell–mediated ITP, SCID mice were first transferred with ITP-inducing, CD19-depleted, CD61 KO immune splenocytes against BALB/c ($CD61^+/H\text{-}2^d$) platelets, and then transfused weekly with either 10^8 BALB/c syngeneic or C57BL/6 allogeneic platelets. Compared with control nontransfused mice, platelet counts in ITP SCID mice transfused with allogeneic C57BL/6 platelets were significantly elevated (day 28; $P < .05$) (Figure 3).

Allogeneic platelet MHC class I molecules reverse abnormal bone marrow morphology and numbers in anti-CD61 T-cell–mediated ITP

Compared with SCID mice transferred with naïve splenocytes (Figure 4A), CD19-depleted splenocytes transferred from CD61 KO mice immunized against BALB/c ($CD61^+/H\text{-}2^d$) platelets caused significant abnormalities in the SCID mouse bone marrow megakaryocytes (Figure 4B). Megakaryocytes in these mice had increased size, pyknotic nuclei, and irregular membranes (Figure 4B). In contrast, however, megakaryocytes in the SCID mice receiving CD19-depleted CD61 KO immune splenocytes against either C57BL/6 ($CD61^+/H\text{-}2^b$) or C57BL/6 CD61 KO ($CD61^-/H\text{-}2^b$) platelets showed no difference in bone marrow histology when compared with the control mice (Figure 4C-D). Cumulatively, the numbers of megakaryocytes were significantly increased in mice exhibiting antibody-mediated ITP, whether induced by splenocytes from CD61 KO mice immune against $CD61^+/H\text{-}2^d$ or $CD61^+/H\text{-}2^b$ platelet populations (Figure 4E). However, in mice with T-cell–mediated thrombocytopenia (or the SCID mice that received CD19-depleted immune splenocytes), the presence of MHC antigens on platelets significantly reduced the number of megakaryocytes to normal levels (Figure 4E).

Allogeneic platelet MHC class I molecules inhibit CD61-specific T-cell cytotoxicity in vitro

To further examine the relationship between platelet counts and the anti-CD61 T-cell response, the cytotoxic activity of splenocytes from immunized CD61 KO mice and the transferred SCID mice were examined using an in vitro cytotoxicity assay against syngeneic $CD61^+$ target cells (PU5-1.8) (Figure 5A). Splenocytes from CD61 KO mice immunized with BALB/c ($CD61^+/H\text{-}2^d$) platelets showed significantly elevated anti-CD61 cytotoxicity at an Effector:Target ratio of 10:1, as compared with control naïve splenocytes ($P < .01$)

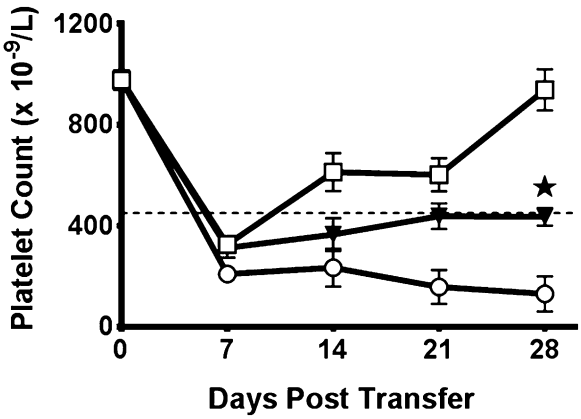

Figure 3. Allogeneic platelet transfusions inhibit T-cell–mediated ITP. Platelet counts in SCID mice transferred with CD19-depleted CD61 KO immune splenocytes against BALB/c ($CD61^+/H\text{-}2^d$) platelets and then transfused weekly with either nothing (○; N = 5), syngeneic BALB/c platelets (▼; N = 5), or allogeneic C57BL/6 platelets (□; N = 5). Results are presented as the weekly platelet counts ($\times 10^9$/L) after splenocyte transfer (*$P < .05$ for □ vs ○).

(Figure 5B). This cytotoxicity was also observed in the spleens of SCID mice receiving those immune CD61 KO splenocytes (Figure 5C). In contrast, the presence of allogeneic platelet MHC molecules ($CD61^+/H\text{-}2^b$) significantly inhibited the anti-CD61 T-cell–mediated cytotoxicity to levels similar to control naïve splenocytes in both CD61 KO mice and their SCID recipients at an Effector:Target ratio of 10:1 ($CD61^+/H\text{-}2^b$ vs $CD61^+/H\text{-}2^d$; $P < .05$) (Figure 5B-C). To directly address the modulatory effect of allogeneic platelets on the anti-CD61 T-cell–mediated cytotoxicity, BALB/c CD61 KO mice splenocytes ($CD61^-/H\text{-}2^d$) immunized against syngeneic WT platelets ($CD61^+/H\text{-}2^d$) were first incubated with either syngeneic ($CD61^+/H\text{-}2^d$) or allogeneic ($CD61^+/H\text{-}2^b$) platelets, and then examined for their cytotoxic activity against PU5-1.8 cells. Compared with splenocytes incubated with syngeneic platelets, those with allogeneic platelets showed significantly decreased cytotoxic activity ($P < .05$) (Figure 5D).

Discussion

Platelet transfusions are generally not indicated as a treatment for ITP, although the data supporting this contention is rather scarce in current medical literature. Because ITP is caused by at least two distinct immune effector processes (for example, antibody- and/or T-cell–mediated thrombocytopenia), it is possible that these immunopathologies may have differential responses to therapy. This is supported by an animal model of ITP which demonstrated that while antibody-mediated thrombocytopenia was sensitive to IVIg treatment, T-cell–mediated thrombocytopenia was not.[17] In the current murine ITP study, we have further elucidated the effect of allogeneic platelet transfusions on antibody and T-cell–mediated ITP. Immunization by platelet transfusions containing both allogeneic MHC class I molecules and CD61 molecules ($CD61^+/H\text{-}2^b$) rendered the spleens of CD61 KO mice ($CD61^-/H\text{-}2^d$) incapable of mediating anti-CD61–specific T-cell–mediated ITP in SCID mice. This was also true in SCID mice with established T-cell–mediated ITP and were transfused with allogeneic platelets. These responses correlated with a normalization of bone marrow megakaryocytes and the inhibition of in vitro T-cell–mediated cytotoxicity. The results suggest that platelet transfusions may be a beneficial therapy in those antiplatelet, antibody-negative individuals who exhibit T-cell–mediated ITP.

In 2003, Olsson et al showed that in patients with active ITP who did not have any identifiable antiplatelet antibodies, there was a significantly increased cytotoxic activity by peripheral blood T cells against self-platelet antigens.[14] This activity was reduced in those patients whose platelet counts increased either due to therapy or spontaneously, and this has also been reproduced in a larger clinical trial, as well as in animal studies.[15-17] In our current murine study, analogous results were observed in that CD19-depleted CD61 KO immune splenocytes against $CD61^+/H\text{-}2^d$ platelets induced significant thrombocytopenia when transferred into SCID mice (Figure 2). This correlated to enhanced T-cell cytotoxicity against $CD61^+$ syngeneic target cells in vitro (Figure 5B-C). This indicates that murine platelet CD61 antigens can effectively trigger CTL responses, as in patients with ITP.[14-17]

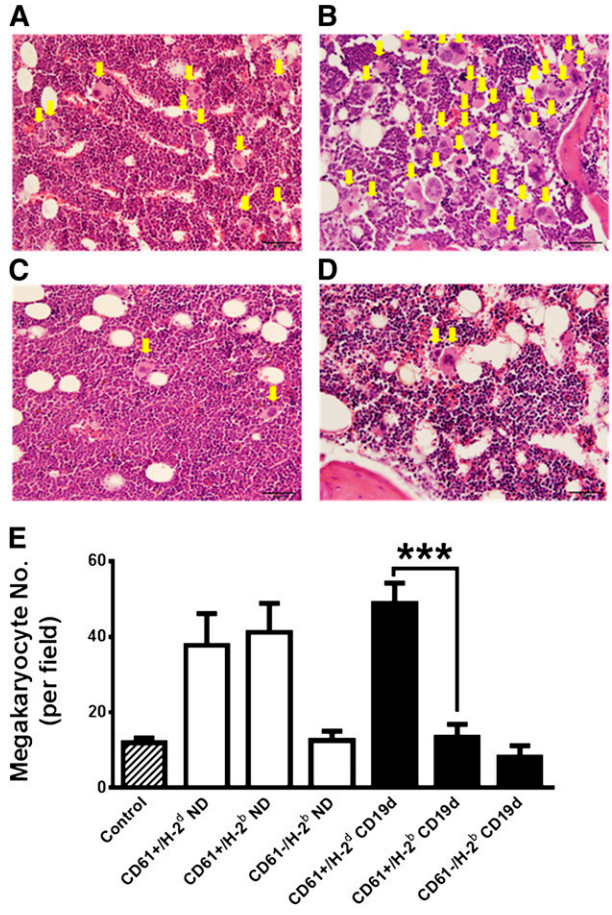

Figure 4. Allogeneic platelet MHC class I antigens rescue megakaryocyte abnormalities in T-cell–mediated ITP. Representative hematoxylin and eosin stained bone marrows of SCID mice transferred with CD19-depleted splenocytes from (A) a control BALB/c naïve mouse or BALB/c CD61 KO mice immunized against (B) BALB/c ($CD61^+/H\text{-}2^d$) platelets; (C) C57BL/6 ($CD61^+/H\text{-}2^b$) platelets; or (D) C57BL/6 CD61 KO ($CD61^-/H\text{-}2^b$) platelets. The bars in each panel represent 50 μm. The yellow arrows point to megakaryocytes. (E) Cumulative results of megakaryocyte enumeration in the bone marrow of control naïve SCID mice (hatched column), SCID mice transferred with either nondepleted splenocytes (antibody-mediated ITP) from the indicated platelet donors (white columns), and SCID mice transferred with CD19-depleted splenocytes (T-cell–mediated ITP) from the indicated platelet donors (black columns). At least 10 fields in each bone marrow were counted and the results are presented as the mean megakaryocyte number per field (***$P < .001$). Images of all panels were taken at ×40 original magnification with a 0.75 numerical aperture of the objective lens.

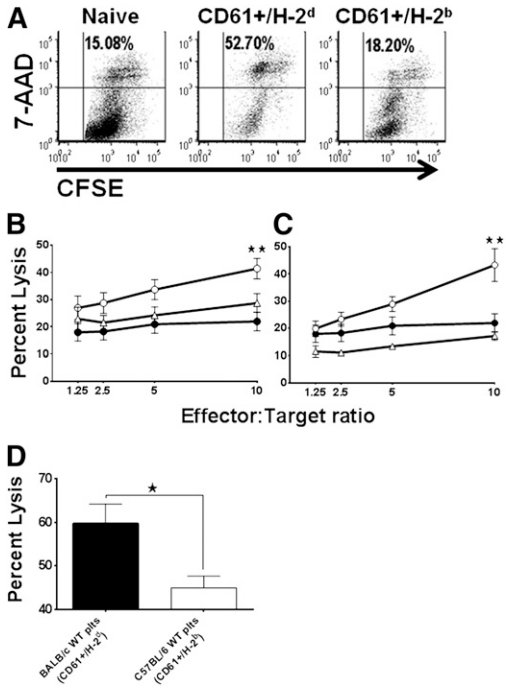

Figure 5. Effect of allogeneic platelet MHC class I antigens on in vitro cytotoxicity in antibody- and T-cell–mediated ITP. (A) Representative flow cytometric dot plot analysis of cytotoxic killing against $CD61^+/H\text{-}2^d$ PU5-1.8 target cells by nondepleted splenocytes from either a naïve BALB/c mouse (left panel), or from CD61 KO mice immunized either against BALB/c ($CD61^+/H\text{-}2^d$) platelets (middle panel), or against C57BL/6 ($CD61^+/H\text{-}2^b$) platelets (right panel). Splenocytes were incubated with CFSE-labeled $CD61^+/H\text{-}2^d$ PU5-1.8 cells for 4 hours in vitro, then stained with the vital dye 7-AAD and acquired on a flow cytometer. Results are shown at an Effector:Target ratio of 10:1. (B) Cumulative cytotoxicity of splenocytes from naïve BALB/c mice (●; N = 19), CD61 KO mice transfused weekly with 10^8 platelets from either BALB/c ($CD61^+/H\text{-}2^d$ [○; N = 10]), or C57BL/6 ($CD61^+/H\text{-}2^b$ [△; N = 10]) mice. (C) Cumulative cytotoxicity of splenocytes from naïve BALB/c mice (●; N = 19) or SCID mice transferred with the corresponding CD61 KO immune splenocyte populations as in (B) at 4 weeks post-transfer. Results in (B) and (C) are expressed as Percent Lysis at the indicated Effector:Target ratios (**$P < .01$ for ○ vs △). (D) Splenocytes from BALB/c CD61 KO mice immunized against platelets from BALB/c ($CD61^+/H\text{-}2^d$) mice were incubated with either syngeneic BALB/c WT platelets ($CD61^+/H\text{-}2^b$) or allogeneic C57 BL/6 WT platelets ($CD61^+/H\text{-}2^b$) for 4 hours in vitro, and then examined for their cytotoxicity against PU5-1.8 target cells. Results in (D) are expressed as Percent Lysis at an Effector:Target ratio of 5:1 (N = 3) (*$P < .05$).

SCID mice transferred with CD19-depleted splenocytes from CD61 KO mice ($CD61^-/H\text{-}2^d$) that were immunized with allogeneic $CD61^+/H\text{-}2^b$ platelets did not develop thrombocytopenia (Figure 2). This correlated with a loss of in vitro cytotoxicity against $CD61^+$ target cells (Figure 5). Furthermore, in SCID mice transferred with CD19-depleted splenocytes from CD61 immune KO mice and then transfused weekly with allogeneic platelets ($CD61^+/H\text{-}2^b$), their platelet counts were found to be significantly rescued compared with control ITP mice (Figure 3). The mechanism of how cytotoxic T-cell inhibition and a rescue of platelet counts occur is unknown, but some possibilities exist. For example, CTL functions by inducing target cell cytolysis via apoptosis that is initiated by the interaction between their T-cell receptor complex and MHC class I/peptide complexes on target cells.[25,26] Platelet MHC class I molecules are primarily adsorbed from plasma and consist of truncated heavy chains that are unstable, supported by the finding that more than 80% can be eluted from the platelet surface by chloroquine diphosphate without affecting platelet membrane integrity.[27-32] Moreover, platelet MHC class I molecules can be partially stabilized by the addition of exogenous β_2-microglobulin in vitro, indicating a reduction of the β_2-microglobulin molecule on the platelet surface.[33] Therefore, because of these features, allogeneic platelet MHC class I molecules may evoke a direct faulty interaction with the CTL T-cell receptor, thereby anergizing the effector cell. This is supported by studies that show allogeneic platelet MHC class I molecules cannot activate CTLs on their own,[33] as well as our previous work demonstrating that allogeneic platelet transfusions can significantly enhance donor-specific skin graft survival, an immune process that is almost exclusively elicited by CTL.[34] Figure 5D shows that in vitro CTL cytotoxicity could be prevented by allogeneic platelets, whereas syngeneic platelets had no effect in supporting the concept that T-cell–mediated ITP may be inhibited by the direct interaction of T cells with allogeneic platelets. On the other hand, the denatured platelet MHC class I molecules may be processed within antigen-presenting cells in a manner that does not facilitate proper cross presentation of platelet allogeneic MHC peptides to the CTL. We, the authors, are currently investigating this.

The ITP in mice, whether induced by antibodies or T cells, showed abnormal bone marrow histology; megakaryocytes were increased in number and showed evidence of apoptosis (for example, pyknotic nuclei [Figure 4]). This data supports human studies which have shown that patients with ITP have elevated numbers of megakaryocytes that exhibit apoptosis-like qualities,[35-37] and this concurs with in vitro assays which show that antiplatelet antibodies significantly inhibit megakaryocyte growth.[6,7] The increased number of megakaryocyte in this model may be a compensation response to the thrombocytopenia and immune attack causing apoptosis of megakaryocytes. Perhaps more intriguing, however, is the observation that when mice were rescued of T-cell–mediated ITP by allogeneic platelet transfusions, their bone marrow megakaryocyte histology was similar to that of control mice (Figure 4). This suggests that inhibiting the antiplatelet T-cell cytotoxic response by allogeneic transfusions may relieve the megakaryocytes from T-cell attack and enable them to maintain platelet production.

Future applications of these murine findings in humans will require the prerequisite screening for antiplatelet antibodies; this may however, pose obstacles such as the requirement for quick turnaround times and potential assay sensitivity issues. Nonetheless, the results presented here suggest that, in at least a proportion of individuals who are antibody negative, allogeneic platelet transfusions may have a beneficial role in alleviating ITP.

In summary, our study shows that anti-CD61–specific T-cell–mediated immune responses can induce thrombocytopenia in SCID mice recipients, mimicking the observation made in antibody-negative ITP patients. The cell-mediated ITP could be abolished by transfusions of allogeneic platelet MHC class I antigens indicating a potential immunomodulatory benefit of allogeneic platelet transfusions in ameliorating T-cell–mediated ITP.

Acknowledgments

This work was supported by a Canadian Blood Services research and development grant. L.G. is a recipient of an Ontario Trillium scholarship. C.G.J.M is a recipient of a Canadian Blood Services post-doctoral fellowship.

Authorship

Contribution: L.G. designed research, performed and supervised all experiments, collected, analyzed, and interpreted data, performed

statistical analysis, and wrote the first draft of the manuscript; L.Y., E.R.S., R.A., M.K., and C.G.J.M performed experiments, collected, analyzed, and interpreted data; A.H.L., M.H., H.N., and J.F. designed research, interpreted data, edited the manuscript, and contributed animal specimens; and J.W.S. provided financial resources, designed research, analyzed and interpreted data, and edited the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

The current affiliation for L.Y. is Department of Hematology, Wuxi Peoples's Hospital, Wuxi, Jiangsu, China.

Correspondence: John W. Semple, St. Michael's Hospital, 30 Bond St, Toronto, ON M5B 1W8, Canada; e-mail: semplej@smh.ca.

References

1. Rodeghiero F, Stasi R, Gernsheimer T, et al. Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. *Blood.* 2009;113(11): 2386-2393.
2. Provan D, Stasi R, Newland AC, et al. International consensus report on the investigation and management of primary immune thrombocytopenia. *Blood.* 2010;115(2):168-186.
3. Cines DB, Bussel JB, Liebman HA, Luning Prak ET. The ITP syndrome: pathogenic and clinical diversity. *Blood.* 2009;113(26):6511-6521.
4. Harrington WJ, Minnich V, Hollingsworth JW, Moore CV. Demonstration of a thrombocytopenic factor in the blood of patients with thrombocytopenic purpura. *J Lab Clin Med.* 1951;38(1):1-10.
5. Shulman NR, Weinrach RS, Libre EP, Andrews HL. The role of the reticuloendothelial system in the pathogenesis of idiopathic thrombocytopenic purpura. *Trans Assoc Am Physicians.* 1965;78: 374-390.
6. Chang M, Nakagawa PA, Williams SA, et al. Immune thrombocytopenic purpura (ITP) plasma and purified ITP monoclonal autoantibodies inhibit megakaryocytopoiesis in vitro. *Blood.* 2003; 102(3):887-895.
7. McMillan R, Wang L, Tomer A, Nichol J, Pistillo J. Suppression of in vitro megakaryocyte production by antiplatelet autoantibodies from adult patients with chronic ITP. *Blood.* 2004;103(4):1364-1369.
8. Brighton TA, Evans S, Castaldi PA, Chesterman CN, Chong BH. Prospective evaluation of the clinical usefulness of an antigen-specific assay (MAIPA) in idiopathic thrombocytopenic purpura and other immune thrombocytopenias. *Blood.* 1996;88(1):194-201.
9. Kiefel V, Freitag E, Kroll H, Santoso S, Mueller-Eckhardt C. Platelet autoantibodies (IgG, IgM, IgA) against glycoproteins IIb/IIIa and Ib/IX in patients with thrombocytopenia. *Ann Hematol.* 1996;72(4):280-285.
10. McMillan R. Antiplatelet antibodies in chronic adult immune thrombocytopenic purpura: assays and epitopes. *J Pediatr Hematol Oncol.* 2003; 25(Suppl 1):S57-S61.
11. McMillan R, Wang L, Tani P. Prospective evaluation of the immunobead assay for the diagnosis of adult chronic immune thrombocytopenic purpura (ITP). *J Thromb Haemost.* 2003;1(3):485-491.
12. Fabris F, Scandellari R, Ruzzon E, Randi ML, Luzzatto G, Girolami A. Platelet-associated autoantibodies as detected by a solid-phase modified antigen capture ELISA test (MACE) are a useful prognostic factor in idiopathic thrombocytopenic purpura. *Blood.* 2004;103(12): 4562-4564.
13. Najaoui A, Bakchoul T, Stoy J, et al. Autoantibody-mediated complement activation on platelets is a common finding in patients with immune thrombocytopenic purpura (ITP). *Eur J Haematol.* 2012;88(2):167-174.
14. Olsson B, Andersson PO, Jernås M, et al. T-cell-mediated cytotoxicity toward platelets in chronic idiopathic thrombocytopenic purpura. *Nat Med.* 2003;9(9):1123-1124.
15. Zhang F, Chu X, Wang L, et al. Cell-mediated lysis of autologous platelets in chronic idiopathic thrombocytopenic purpura. *Eur J Haematol.* 2006; 76(5):427-431.
16. Zhao C, Li X, Zhang F, Wang L, Peng J, Hou M. Increased cytotoxic T-lymphocyte-mediated cytotoxicity predominant in patients with idiopathic thrombocytopenic purpura without platelet autoantibodies. *Haematologica.* 2008;93(9): 1428-1430.
17. Chow L, Aslam R, Speck ER, et al. A murine model of severe immune thrombocytopenia is induced by antibody- and CD8+ T cell-mediated responses that are differentially sensitive to therapy. *Blood.* 2010;115(6):1247-1253.
18. Slichter SJ. Controversies in platelet transfusion therapy. *Annu Rev Med.* 1980;31:509-540.
19. Hirsch EO, Gardner FH. The transfusion of human blood platelets with a note on the transfusion of granulocytes. *J Lab Clin Med.* 1952; 39(4):556-569.
20. Han T, Stutzman L, Cohen E, Kim U. Effect of platelet transfusion on hemorrhage in patients with acute leukemia. An autopsy study. *Cancer.* 1966;19(12):1937-1942.
21. Thachil J. The myths about platelet transfusions in immune-mediated thrombocytopenias. *Br J Haematol.* 2010;150(4):494-495.
22. Carr JM, Kruskall MS, Kaye JA, Robinson SH. Efficacy of platelet transfusions in immune thrombocytopenia. *Am J Med.* 1986;80(6): 1051-1054.
23. Salama A, Kiesewetter H, Kalus U, Movassaghi K, Meyer O. Massive platelet transfusion is a rapidly effective emergency treatment in patients with refractory autoimmune thrombocytopenia. *Thromb Haemost.* 2008; 100(5):762-765.
24. Spahr JE, Rodgers GM. Treatment of immune-mediated thrombocytopenia purpura with concurrent intravenous immunoglobulin and platelet transfusion: a retrospective review of 40 patients. *Am J Hematol.* 2008;83(2):122-125.
25. Kaech SM, Ahmed R. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naïve cells. *Nat Immunol.* 2001;2(5):415-422.
26. Bousso P, Robey E. Dynamics of CD8+ T cell priming by dendritic cells in intact lymph nodes. *Nat Immunol.* 2003;4(6):579-585.
27. Blumberg N, Masel D, Mayer T, Horan P, Heal J. Removal of HLA-A,B antigens from platelets. *Blood.* 1984;63(2):448-450.
28. Kao KJ, Cook DJ, Scornik JC. Quantitative analysis of platelet surface HLA by W6/32 anti-HLA monoclonal antibody. *Blood.* 1986;68(3): 627-632.
29. Kao KJ. Plasma and platelet HLA in normal individuals: quantitation by competitive enzyme-linked immunoassay. *Blood.* 1987;70(1):282-286.
30. Kao KJ. Selective elution of HLA antigens and beta 2-microglobulin from human platelets by chloroquine diphosphate. *Transfusion.* 1988; 28(1):14-17.
31. Neumüller J, Tohidast-Akrad M, Fischer M, Mayr WR. Influence of chloroquine or acid treatment of human platelets on the antigenicity of HLA and the 'thrombocyte-specific' glycoproteins Ia/IIa, IIb, and IIb/IIIa. *Vox Sang.* 1993;65(3):223-231.
32. Ghio M, Contini P, Mazzei C, et al. Soluble HLA class I, HLA class II, and Fas ligand in blood components: a possible key to explain the immunomodulatory effects of allogeneic blood transfusions. *Blood.* 1999;93(5):1770-1777.
33. Gouttefangeas C, Diehl M, Keilholz W, Hörnlein RF, Stevanović S, Rammensee HG. Thrombocyte HLA molecules retain nonrenewable endogenous peptides of megakaryocyte lineage and do not stimulate direct allocytotoxicity in vitro. *Blood.* 2000;95(10):3168-3175.
34. Aslam R, Speck ER, Kim M, Freedman J, Semple JW. Transfusion-related immunomodulation by platelets is dependent on their expression of MHC Class I molecules and is independent of white cells. *Transfusion.* 2008;48(9):1778-1786.
35. Houwerzijl EJ, Blom NR, van der Want JJ, et al. Ultrastructural study shows morphologic features of apoptosis and para-apoptosis in megakaryocytes from patients with idiopathic thrombocytopenic purpura. *Blood.* 2004;103(2): 500-506.
36. Li S, Wang L, Zhao C, Li L, Peng J, Hou M. CD8+ T cells suppress autologous megakaryocyte apoptosis in idiopathic thrombocytopenic purpura. *Br J Haematol.* 2007;139(4):605-611.
37. Yang L, Wang L, Zhao CH, et al. Contributions of TRAIL-mediated megakaryocyte apoptosis to impaired megakaryocyte and platelet production in immune thrombocytopenia. *Blood.* 2010; 116(20):4307-4316.