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Platelets Contribute to Allograft Rejection through Glutamate Receptor Signaling

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Platelets recruit leukocytes and mediate interactions between leukocytes and endothelial cells. Platelets have been long described as markers of transplant rejection, but the contribution of platelets to transplant rejection has not been critically examined. We demonstrate in this study that following T cell initiation of allograft rejection, platelets contribute to T cell recruitment and increased plasma inflammatory mediators and accelerate T cell-mediated skin graft rejection. Prior work from our laboratory has shown that platelets secrete glutamate when activated, which then induces platelet thromboxane production by signaling through platelet-expressed ionotropic glutamate receptors. Glutamate receptor antagonists therefore represent, to our knowledge, novel inhibitors of platelet-accelerated inflammation. We have found that plasma glutamate is increased in mice that receive skin grafts and that mice treated with glutamate receptor antagonists have improved graft survival and decreased plasma thromboxane, platelet factor 4 (CXCL4), and IFN-γ. Taken together, our work now demonstrates that subsequent to T cell initiation of skin graft rejection, platelets contribute to further T cell recruitment and that by blunting glutamate-mediated platelet activation, graft survival is improved. The Journal of Immunology, 2010, 185: 000–000.

A ccording to the National Organ Procurement and Transplantation Network, ∼23,000 Americans received an organ transplant in 2008. Current immune suppression protocols have greatly improved transplant survival, but despite many therapeutic advances, allograft rejection continues. Platelets have key roles in the recruitment of immune cells and can accelerate vascular inflammatory diseases (1–5). We have implicated platelets in amplifying alloantibody-mediated transplant inflammation (6). However, the role of platelets in T cell-directed immune responses in general, and transplant rejection in particular, has not been explored fully.

Early pathological descriptions of transplants recognized the presence of platelets in kidney transplants (7) and a number of studies followed in which platelets were labeled with indium to track their accumulation in renal allografts (8, 9). These studies led to the general observation that platelets accumulate in renal allografts and may be markers of graft rejection. Recent studies have also identified the presence of prominent intravascular platelet aggregates in experimental and clinical transplants that undergo Ab-mediated rejection (10–13). Kirk and colleagues (14) have gone beyond these more observational studies and made a critical finding that CD154 (CD40 ligand) shed from platelets can serve as a co-stimulatory molecule remote from the transplant to induce rejection of murine cardiac allografts. Using a skin transplant model, we demonstrated that platelets have a key role in increasing leukocyte trafficking and graft vascular inflammation (6). These studies set the stage for a deeper investigation into platelets not only as markers of transplant vascular injury, but also as mediators contributing to the pathogenesis of graft rejection. Platelets can recruit lymphocytes to the site of the inflammation through contact-dependent and independent mechanisms. T cells express P-selectin glycoprotein ligand 1 that interacts with P-selectin expressed by activated platelets. Platelets may also interact with T cells through CD154 on platelets. In experiments of ischemia-reperfusion injury to the liver, platelets augmented the recruitment of CD4+ T cells to hepatic sinusoids (15). Activated platelets also enhance the intrahepatic accumulation of cytotoxic T lymphocytes in murine models of viral hepatitis (16). Platelet and T cell interactions may do more than just localize T cells; they may also augment T cell immune responses. CD154 on platelets can augment the help delivered by low levels of CD4+ T cells for germinal center development and isotype switching to IgG Ab production (17). We have found that the platelet-derived chemokine platelet factor 4 (PF4/CXCL4) can increase T cell CXCR3 expression and T cell trafficking in a model of neurovascular inflammation (18). These studies provide a rationale to consider the poorly explored role of platelets in T cell-mediated allograft rejection.

Cyclooxygenase (COX) products, such as PGs and thromboxane, have important roles in stimulating and shaping acquired immune responses. Platelets are a major source of these proinflammatory molecules. Receptors for COX products are expressed by many immune cells, including T cells. Recent work has demonstrated that some PGs, such as PGE2, acting on T cells facilitate Th1 cell differentiation and amplify IL-23–mediated Th17 cell expansion.
(19). The same investigators had found earlier that the binding activity for thromboxane receptors (TPs) is high in T cells, but not in B cells, suggesting that thromboxane–TP signaling may modulate peripheral T cell immune responses (20, 21). Studies specific to transplantation have demonstrated that TP−/− mice had reduced immune-mediated tissue injury following cardiac transplantation, showing that thromboxane augments cellular immune responses to transplants and inflammatory tissue injury (22).

We have recently demonstrated that platelets have an important role in alloantibody-induced innate immune responses to skin grafts (6). Platelets support leukocyte and endothelial cell interactions and contribute proinflammatory molecules that sustain leukocyte recruitment (23). We have also demonstrated that platelets contain and release large concentrations of glutamate upon activation. Platelets express ionotrophic glutamate receptors including 2-amino-5-hydroxy-5-methyl-4-isoxazolepropion acid (AMPA) and kainate type receptors (24). Glutamate promotes platelet activation by signaling via platelet glutamate receptors, leading to COX activation, the production of thromboxane, and amplification of platelet activation (25). Glutamate-induced platelet thromboxane production may do more than just support platelet activation and thrombus growth; it may also promote immune activation, including T cell-driven transplant rejection.

We demonstrate in this study that platelets have a role in transplant rejection. Using a skin graft model, we found that following T cell initiation of graft rejection, platelets are activated, leading to increased plasma glutamate and platelet thromboxane production. Glutamate receptor antagonists demonstrate therapeutic potential in reducing T cell-mediated transplant rejection.

Materials and Methods
Reagents
PF4, TNF-α, and IFN-γ ELISA kits were purchased from R&D Systems (Minneapolis, MN). Thromboxane B2 (TBX2) ELISA kits were purchased from Cayman Chemical (Ann Arbor, MI). Glutamate concentration was determined using a Glutamate Oxidase kit from Invitrogen (Carlsbad, CA). The 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) was purchased from Sigma-Aldrich (St. Louis, MO).

Platelet-depletion Ab and its control IgG were purchased from Emfret Analytics (Würzburg, Germany). The depleting Abs are a mixture of rat mAbs directed against mouse GPIb (CD42b). This is a platelet-specific receptor and results in the depletion of only platelets. At the concentrations we used (1 μg/ml), platelet depletion is sustained for more than 3 d (Supplemental Fig. 1). mAbs to the TCR β-chain (clone H57-597), CD4, CD8, and CD14 were purchased from BD Pharmingen (San Diego, CA).

Transplants
All mouse studies were performed under protocols approved by the University of Rochester Animal Care and Use Committee using procedures we have published (6). Briefly, B6 nude mice (Taconic Farms, Germantown, NY) were anesthetized with ketamine and xylazine (80 and 13 mg/kg, respectively) and transplant beds prepared by removal of the epidermis and subcutaneous fat. The 6-cyano-7-nitroquinoidaline-2,3-dione (CNQX) was purchased from Sigma-Aldrich (St. Louis, MO). Platelet-depletion Ab and its control IgG were purchased from Emfret Analytics (Würzburg, Germany). The depleting Abs are a mixture of rat mAbs directed against mouse GPIb (CD42b). This is a platelet-specific receptor and results in the depletion of only platelets. At the concentrations we used (1 μg/ml), platelet depletion is sustained for more than 3 d (Supplemental Fig. 1). mAbs to the TCR β-chain (clone H57-597), CD4, CD8, and CD14 were purchased from BD Pharmingen (San Diego, CA).

Immunohistochemistry
Harvested transplants were fixed in methanol/water/acetic acid (60:30:10%). Tissue was then embedded and sectioned and immunohistochemistry performed using protocols and procedures described previously with Abs to CD3 (26, 27). T cell isolations
T cells were isolated from the spleen and lymph nodes of control C57BL/6 mice using a negative selection T cell enrichment kit (StemCell Technologies, Vancouver, British Columbia) i.v. via the retro-orbital plexus into graft recipients.

To quantify T cell infiltrates in the skin grafts, we harvested skin from the center of the transplant tissue and placed it in 3 ml RPMI media with 5% FBS for 1 h. The base of the graft was then gently scraped with a razor blade, the skin then sectioned into small pieces, passed in and out of an 18-gauge needle, and vortexed vigorously to dissociate cells. The cells were transferred into a 15-ml tube, 1 ml Percoll added, mixed, and centrifuged at 1300 × g for 30 min to isolate a mononuclear cell layer at the interface. Mononuclear cells were then washed before incubating with a mAb and the number of cells as percent total mononuclear cells determined by flow cytometry.

Statistical methods
Data are expressed as means ± SD. Statistical comparisons between two groups were performed using Student t test. Graft survival was analyzed using a log-rank test.

Results
To determine the role of platelets in T cell-dependent transplant rejection, we used a skin transplant model in which the thin skin from an ear of a B10.A mouse (H-2Kk) is grafted onto the flank of a C57BL/6 nude mouse (H-2Kb). After allowing 7 d for graft healing and establishment of vascular connections, bandages were removed, and mice were reconstituted with 1 × 106 T cells isolated from naive wild-type C57Bl6 mice. Using this model in our prior published work, we have demonstrated that the skin grafts are vascularized, and we have imaged both platelet and WBC perfusion of the grafts (23). Other have also demonstrated that vessels of the host dermis and the skin graft connect by 7 d post-transplantation and that endothelial cells within the graft are of donor origin until ~21 d (28). The time of T cell reconstitution is referred to as study day 0.

To establish that platelets were activated following T cell reconstitution and T cell initiation of graft rejection, plasma was isolated from control ungrafted nude mice, mice that received skin grafts but no T cells, and mice that were given skin grafts and were T cell reconstituted. PF4 is a platelet-specific chemokine and was used as a plasma marker of platelet activation. Mice that received skin grafts but no T cells did not have significantly increased plasma PF4 compared with control nude mice (Fig. 1A, white versus gray bars). However, T cell-reconstituted mice had a large increase in plasma PF4 that was sustained over 4 d after T cell reconstitution (Fig. 1A, black versus white bars), indicating that T cells are needed to initiate platelet activation. T cell reconstitution in the absence of a skin allograft had no effect on platelet activation (Supplemental Fig. 2).

To determine whether platelet activation plays a role in graft rejection, mice that had received skin grafts were T cell reconstituted, and on study day 0, mice were either treated with control IgG or a mixture of mAbs (1 μg/g) against mouse GPIb (CD42b) to deplete platelets. This treatment greatly reduces platelet numbers (Supplemental Fig. 1) but has no effect on other cell populations (18, 23, 29). Skin graft survival was then evaluated on day 7 using visual parameters. Total rejection was manifested by scab formation and graft retraction (Fig. 1B). Approximately 50% of control IgG-treated mice had visible evidence of graft rejection, whereas <20% of platelet-depleted mice had rejected skin grafts (Fig. 1C). To look at graft survival over a longer time, we also followed the grafts for 10 d after T cell reconstitution. In contrast to Fig. 1C, to achieve sustained platelet depletion, mice were treated with control IgG or platelet-depleting Ab on days 0 and 4. By day 10, control graft survival was only 11% (Fig. 1D, white diamonds). In contrast, mice made platelet deficient had 50% graft survival on day 10 (Fig. 1E, black squares). These data demonstrate that following initiation of skin graft inflammation by T cells, platelets have a significant role in accelerating rejection.
Platelets have well-described roles in promoting monocyte trafficking across sites of vascular inflammation (1–3), but the role of platelets and platelet-derived inflammatory mediators in supporting T cell trafficking is less well understood. On study day 7, a large number of CD3-positive cells were noted infiltrating skin grafts (Fig. 2A, left panels). However, the number of CD3+ cells was decreased in platelet-depleted mice (Fig. 2A, right panels). To determine whether platelets increase T cell trafficking into transplanted tissue, skin grafts from control and platelet-depleted mice were collected on study day 5, and mononuclear cell infiltrates were isolated from the graft followed by density centrifugation. T cells were quantified by flow cytometry with Abs to TCR (Supplemental Fig. 3). Approximately 35% of the mononuclear cells isolated from the control skin grafts were TCR positive compared with only 25% in platelet-depleted mice (Fig. 2B). These data demonstrate that platelets increase T cell infiltration into skin grafts.

Not only may platelets augment T cell recruitment, they may also influence cytokine production. To examine this, plasma from control and platelet-depleted mice was collected on study day 5 and TNF-α and IFN-γ measured using an ELISA for each. Mice that were skin grafted and T cell reconstituted had greatly increased plasma TXB2 compared with control ungrafted mice (Fig. 4A, black bar versus white bar). Nude mice with skin grafts but not T cell reconstituted have the same plasma TXB2 as control mice (Supplemental Fig. 4). Platelet depletion abrogated the increased plasma TXB2 found in T cell reconstituted and skin-grafted mice (Fig. 4A, gray bar versus white bar), indicating that platelets are an important source of thromboxane during skin graft rejection.

Platelets release glutamate upon activation (24, 30), and we have shown that glutamate increases platelet reactivity and thromboxane production through platelet ionotropic glutamate receptors (24, 25). Our recent work demonstrated that this is in part due to glutamate-mediated stimulation of platelet thromboxane receptors (24, 25). To determine whether plasma glutamate is increased in skin graft recipients, plasma was isolated from control ungrafted nude mice, mice that were skin grafted but not T cell reconstituted, and mice that were skin grafted and T cell reconstituted. Using an enzymatic colorimetric assay, glutamate concentration was determined. Similar to plasma PF4, a skin graft alone did not increase plasma glutamate; however, with initiation of rejection, plasma glutamate was significantly increased 2 and 5 d after T cell reconstitution (Fig. 4B).

Because glutamate drives platelet thromboxane production, glutamate receptor antagonists, such as CNQX, may be beneficial in improving graft survival. Our past work has demonstrated that
AMPA receptor antagonists decrease platelet activation, in part by blunting agonist-induced thromboxane production (31, 32). Based on these data, glutamate-driven platelet thromboxane production may be very important in graft rejection, and CNQX is a potentially valuable compound to ameliorate these effects. To test this, we treated skin graft recipients with either control PBS or 2 mg/kg CNQX daily by i.p. injection, and graft survival was monitored for 7 d. Similar to our preceding experiments, control PBS-treated mice had ∼65% graft rejection (Fig. 5A, white bar). In contrast, mice treated with CNQX had much improved graft survival, with ∼35% of skin grafts rejecting (Fig. 5A, black bar). Graft rejection was also observed over a longer time, and mice treated with CNQX had significantly improved graft survival (Fig. 5B).

We have reported previously that glutamate receptor signaling helps augment platelet activation and that CNQX is an effective platelet antagonist (24). To determine whether the protective effect of CNQX is in part due to platelet inhibition, plasma was isolated from control mice, platelet-depleted mice, and CNQX-treated mice. All of these mice were skin grafted and T cell reconstituted. As expected, at early (day 2) and late (day 6) time points after T cell reconstitution, control mice had elevated plasma PF4 concentrations (Fig. 5C). Platelet-depleted mice had greatly reduced plasma PF4 at day 2, and as platelet numbers rebound, the PF4 concentration increased by day 6 (Fig. 5C). CNQX-treated mice had significantly reduced plasma PF4 compared with control mice, indicating that its protective effect may in part be due to platelet inhibition (Fig. 5C).

Similar to platelet depletion studies, the delay in transplant rejection was also reflected in decreased T cell infiltrates. Staining

FIGURE 2. Platelets promote graft inflammation. A, Immunohistochemistry. Skin grafts were immunostained with anti-CD3 Ab. Left panels represent control mice and right panels platelet-depleted mice. B, T cell recruitment. On study day 5, mononuclear cells were isolated from control and platelet-depleted skin grafts and the number of TCR-positive cells quantified by FACS analysis. *p < 0.03.

FIGURE 3. Platelets promote cytokine production. A, Plasma TNF-α. Plasma was isolated from control and platelet-depleted mice on day 5, and TNF-α was measured by ELISA (n = 5 ± SD). *p < 0.01. B, Plasma IFN-γ. Plasma was isolated from control and platelet-depleted mice on day 5, and IFN-γ was determined by ELISA (n = 5 ± SD). *p < 0.05.
for CD3-positive T cells demonstrated fewer T cells in CNQX-treated skin grafts compared with grafts on untreated control mouse grafts (Fig. 6A). Mononuclear cells were also isolated from control and CNQX-treated mouse skin grafts and TCR-positive cells as the percent of total mononuclear cells was determined by flow cytometry. Approximately 25% of isolated mononuclear cells from control mice were TCR positive (Fig. 6B).

The number of infiltrating T cells was greatly reduced in mice treated with CNQX (Fig. 6B). We also further defined the types of cellular infiltrates by quantifying CD4, CD8 T cell subsets and macrophages (CD14 positive) by flow cytometry. Platelet-depleted mice had reduced numbers of CD4-, CD8-, and CD14-positive cells, but only CD8+ cells were significantly reduced compared with control skin graft mice (Fig. 6C, gray bar versus white bar). All types of cells were significantly reduced in mice treated with CNQX (Fig. 6C, black bar versus white bar).

If AMPA receptor antagonists increase graft survival in part by blunting glutamate-mediated platelet thromboxane production and immune stimulation, we predicted that CNQX-treated mice would have reduced plasma thromboxane and T cell-derived cytokines. To determine this, we isolated plasma from control and CNQX-treated mice and measured TXB2 and IFN-γ. CNQX significantly reduced plasma TXB2 (Fig. 6D) and IFN-γ (Fig. 6E) compared with control mice. These data indicate that inhibition of platelet glutamate receptors increased graft survival in part by directly or indirectly reducing plasma proinflammatory mediators.

Discussion
Platelets are dynamic cells with important hemostasis and immune regulatory functions. Our past work used a model in which alloantibody initiated inflammation in skin grafts to demonstrate an important role for platelets in leukocyte recruitment (6). The present experiments demonstrate that platelets themselves do not initiate skin graft inflammation and rejection, but with T cell reconstitution and T cell-initiated graft rejection, platelets are activated, leading to an increase in plasma thromboxane, IFN-γ, augmented T cell infiltrates, and graft rejection. Whether platelets are activated by T cell-driven graft vascular inflammation or by direct T cell interactions is not clear in this study, but our data support a role for platelets in sustaining T cell recruitment and graft rejection. We also demonstrated that plasma glutamate is increased in mice that received skin grafts and that glutamate receptor antagonists improve graft survival and blunt T cell responses. These data point to platelet-mediated mechanisms of transplant rejection and glutamate-mediated platelet COX activation and a potential clinical utility for glutamate receptor antagonists in prolonging graft survival.

Platelet interactions with innate immune cells are much better defined than platelet and T cell interactions. However, platelet interactions with T cells may be just as important. Platelets express numerous surface receptors for contact-dependent interactions with T cells, and platelets also secrete a large number of chemokines, cytokines, and other inflammatory molecules with the potential to increase T cell responses in a contact-independent manner. Our data indicate that platelets, perhaps via glutamate-mediated
thromboxane release, may contribute to allograft rejection. It is clear that platelet-depleted mice and mice treated with glutamate receptor antagonists have reduced plasma IFN-γ. This may indicate an important role for platelets in sustaining T cell recruitment and activation and a role for platelets in enhancing IFN-γ production in the cycle of allograft inflammation (33, 34). IFN-γ results in endothelial cell activation and, with it, more platelet activation, T cell recruitment, and continued IFN-γ production. Therefore, by blunting platelet activation, this cycle with IFN-γ as a central player may be broken.

We have found that as early as 2 d post-reconstitution with unsensitized T cells, there is vascular inflammation and platelet activation. Studies have indicated that within 48 h posttransfer, T cells may undergo as many as three rounds of division (35), and as many as 20% of T cells may recognize alloantigen (36). Fairchild and coworkers (37) have demonstrated that endogenous CD8 memory T cells in nonsensitized recipients infiltrate cardiac allografts within 24 h of reperfusion. In addition, the nude mouse can make IgM alloantibodies that may cross-link MHC and activate complement prior to T cell reconstitution, helping to rapidly localize T cells to the graft.

In this work, we present data to demonstrate a potential role for platelet glutamate receptor signaling stimulation of thromboxane production in transplant-recipient mice. This in turn may assist in T cell recruitment, activation, and transplant rejection. Glutamate has long been known to be an important signaling molecule in the CNS, but its role in the periphery is becoming better appreciated.

Glutamate receptors are expressed on non-CNS tissue including platelets, and we have shown that platelet glutamate receptors have a role in platelet activation and thromboxane production (24, 25, 30, 38). We have also demonstrated that in a large cohort of platelet donors, a block of polymorphisms in the kainate-type glutamate receptor is associated with a decrease in patient plasma thromboxane and platelet aggregation (25). These data demonstrate a potentially important clinical relevance for this class of receptors.

COX enzymes are expressed by many cells, but platelets are a major source of COX-derived inflammatory molecules that contribute to vascular inflammatory diseases such as atherosclerosis. TP signaling has also been shown to be important in graft rejection, as TP receptor knockout mice have improved heart allograft survival (22, 39).

Thromboxane represents a single platelet-derived inflammatory molecule that may have a role in further T cell recruitment. Other platelet-derived inflammatory molecules may be just as important. For example, PF4 alone or in concert with other chemokines such as CCL5 (RANTES) might also increase T cell infiltrates into the skin grafts. Platelet alpha-granules also contain numerous cytokines such as IL-1β, TGF-β, and TNF-α. These cytokines have multiple effects in transplants, and of particular relevance to platelets are the effects of these cytokines on vascular endothelial cells. For example, TNF-α stimulates the exocytosis of von Willebrand factor and P-selectin from Weible-Palade storage granules in endothelial
cells (40, 41). The increased expression of these adhesion molecules promotes platelet activation and attachment that may then increase T cell recruitment and trafficking.

Based on these data, we propose that in our model, T cells initiate vascular inflammation resulting in platelet recruitment, platelet activation, elaboration of glutamate, platelet thromboxane production, and sustained platelet activation and T cell recruitment. Despite some attention in the 1970s and early 1980s, the role of platelets in transplant rejection has not been closely examined. Platelets are much more numerous than other immune cells and, when stimulated, release or produce large quantities of proinflammatory molecules. Because of their vital role in hemostasis, this important function has been greatly overlooked. There are few platelet antagonists currently in clinical use. These are used mainly for people at risk for heart attack or stroke, and platelet inhibitors are now a major part of reducing the risk of cardiovascular events. Our work demonstrates a potential role for platelet-derived thromboxane in T cell recruitment and graft rejection, further underscoring the possible utility of common drugs like aspirin and ibuprofen and perhaps glutamate receptor antagonists to block COX upstream signaling. Thromboxane and other COX products promote platelet activation and attachment that may then increase T cell recruitment and trafficking.

As an example of the potential of these molecules, during acute systemic inflammation, thromboxane A2 is the key mediator that is increased in the circulation. Thromboxane A2 receptor is highly expressed in most immune thymocytes and mediates DNA fragmentation and apoptosis. J. Exp. Med. 178: 1825–1830.


Supplemental Figure 1: Time Course for Platelet Depletion.
Supplemental Figure 2: T-cell reconstitution of nude mice does not increase platelet activation. Plasma was isolated from control mice or mice 2 days after T-cell reconstitution (N=5).
Supplemental Figure 3: Examples of FACS analysis of TCR positive infiltrates in skin grafts of control and AMPAR antagonist treated mice.
Supplemental Figure 4: Skin Graft Procedure Does Not Increase Plasma Thromboxane. Plasma was isolated from control nude mice or mice that received skin grafts (no T-cells) 7 days post graft procedure (N=4).