Thrombopoiesis, the process by which circulating platelets arise from megakaryocytes, remains incompletely understood. Prior studies suggest that megakaryocytes shed platelets in the pulmonary vasculature. To better understand thrombopoiesis and to develop a potential platelet transfusion strategy that is not dependent upon donors, of which there remains a shortage, we examined whether megakaryocytes infused into mice shed platelets. Infused megakaryocytes led to clinically relevant increases in platelet numbers. The released platelets were normal in size, displayed appropriate surface markers, and had a near-normal circulating half-life. The functionality of the donor-derived platelets was also demonstrated in vivo. The infused megakaryocytes mostly localized to the pulmonary vasculature, where they appeared to shed platelets. These data suggest that it may be unnecessary to generate platelets from ex vivo grown megakaryocytes to achieve clinically relevant increases in platelet numbers.
Infusion of mature megakaryocytes into mice yields functional platelets

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Introduction

While the number of platelet donors is increasing, there is still a significant donor shortage due to the growing population of patients with serious illnesses associated with thrombocytopenia and hemorrhage (1). The use of donor-derived platelets raises the following concerns: variability of quality and quantity, risk of infectious transmission, short lifespan of stored platelets, bacterial contamination during storage, and development of alloantibodies in multi-transfused patients. These problems highlight a need for new strategies to generate platelets for infusion therapy.

Thrombopoiesis, the process by which circulating platelets arise from megakaryocytes remains incompletely understood. In vitro studies suggest that platelets form nodes at tips of proplatelet strands (2). However, direct visualization of live calvaria marrow using multiphoton intravital microscopy suggests that megakaryocytes release large cytoplasmic fragments into the vasculature (3), which must then undergo reorganization into platelets. Studies based on morphologic analysis and quantification of megakaryocyte-like polyploid nuclei in the pulmonary venous system suggested that megakaryocytes release platelets in the lungs (4). Derivation of platelets from megakaryocytes in culture was first reported in 1995 (5) but has been difficult to quantitatively upscale. To date, the best published result from infused FL small cells enriched with proplatelets using a 2-step density gradient to produce “large cells,” with more than half possessing a diameter greater than 50 μm and with only approximately 2.5:1 proplatelets/cell (Figure 1A). The remaining “small cells” had approximately 10:1 proplatelets/cell (Figure 1A). Ploidy analysis showed FL small cells with low DNA ploidy relative to FL large cells (Figure 1B).

Positive control WT platelets were isolated and infused into huIlb+ recipient mice, with only human αtIIb on their platelet surface (8), to allow flow cytometric detection of infused platelets using species-specific anti-αtIIb (CD41) Abs. After infusion, WT platelets were detected in huIlb+ recipient mice immediately (i.e., 5-minute time point), with an overall half-life of approximately 36 hours (Figure 1, C, E, and F). In contrast, infused FL large cells resulted in delayed platelets, with a peak at approximately 90 minutes (Figure 1, D and E). These platelets had a shorter overall half-life of approximately 20 hours. Based on the number of cells infused, the peak increase in platelet count, and the recipient mouse blood volume of approximately 2 ml, we calculated 100–200 platelets from each large cell, assuming all cells gave rise to platelets. Infused FL small cells enriched with proplatelets gave rise to an immediate peak similar to infused WT platelets; however, these platelets had a truncated half-life of approximately 2 hours (Figure 1E). Infusing adult BM megakaryocytes resulted in a similarly delayed appearance of platelets as with FL-derived megakaryocytes, but with a slightly longer half-life of 24 hours.
Figure 1
Characterization and infusion of megakaryocytes. (A) Representative fields of small and large cells. Scale bars: 100 μm. (B) Representative analysis of DNA content of FL small and large cells. (C) Flow cytometry from recipient mouse before and after infusion of 10^8 WT platelets or (D) 10^6 FL large cells. (E) Flow cytometric percentage of 10^6 infused FL large cells and (F) 10^6 infused adult BM cells. (G) Percent platelet rise in irradiated thrombocytopenic mice after infusion. n = 5 per arm. Mean ± 1 SD are shown. Initial platelet counts (10^8/ml) in the 3 groups were: CATCH buffer, 1.8 ± 0.2; platelets, 1.9 ± 0.3; large cells, 1.0 ± 0.2. (H) Size determination of circulating recipient (blue) and infused platelets (red) by forward versus side scatter analysis. (I) Representative flow cytometric analysis of infused and FL-derived platelets comparing P-selectin, GPIbα, and GPIX.
To simulate clinical thrombocytopenia, we irradiated mice and infused CATCH buffer (1× PBS, 1.5% BSA, 1 mM adenosine, 2 mM theophylline, and 0.38% sodium citrate), WT platelets, or FL large cells near the induced platelet nadir and observed that both platelets and megakaryocytes significantly increased the platelet count relative to CATCH buffer over a time course of more than 24 hours (Figure 1G).

To understand the shortened half-life of platelets derived from infused large cells, we examined their size distribution and microparticles compared with infused WT platelets and found no differences (Figure 1H). Another indicator of platelet activation is the expression of surface P-selectin (9). Flow cytometric analysis showed that surface P-selectin levels were similar in platelets derived from infused FL cells and infused WT platelets (Figure 1I). ADAM17 is a metalloproteinase found in cultures that shortens platelet half-life (10) and cleaves the glyocalcin extracellular portion of GPIbα, inactivating the GPIb/IX receptor without altering receptor density (11). Platelets derived from infused FL cells and infused WT platelets displayed similar ratios of extracellular GPIb to GPIX (Figure 1I). Thus, the slightly shortened half-life of the platelets derived from infused large cells does not appear to be due to ADAM17 activity.

Clinically, platelets are transfused not to increase platelet number, but to reverse or prevent a bleeding diathesis due to thrombocytopenia. In the laser-induced cremaster arteriole injury model, we looked at incorporation of infused WT platelets and platelets derived from infused FL cells into growing clots. In all settings, the platelets readily become incorporated into the developing clots (Figure 2A–C). Representation images of platelets incorporating into clots after infusion of platelets or indicated cells. (A) Donor WT platelets detected using a labeled anti–mouse αIIb Ab. (B and C) Same as in A, but after infusion of FL cells. (D) Sequential stills from left to right noting a recirculating mouse αIIb+ cell (arrowheads) after small cell infusion. Scale bars: 30 μm. (E) Summation of donor platelets incorporated into growing thrombi after infusion of either WT platelets or FL cells. Twenty movies were evaluated per group.
Thrombi. WT platelets and platelets derived from FL large cells were similarly incorporated (Figure 2, A and B). Platelets derived from FL small cells incorporated to a lesser extent, with a distinct population of CD41⁺ cells that recirculated and rarely incorporated into the clot (Figure 2, C and D, and Supplemental Video 1). This phenomenon was rarely seen after infusion of FL large cells (Figure 2B and Supplemental Video 2), and never with infused WT platelets (Figure 2A and Supplemental Video 3).

We also examined platelet function using an FeCl₃ carotid artery injury model after infusing either CATCH buffer or FL large cells into the hcdFlb⁻ recipient mice, which have a mild bleeding diathesis likely because of low CD41 surface density (12). Infusion of FL large cells significantly shortened time to development of stable occlusion (Table 1). We also infused FL large cells from mUK mice, which ectopically express and store urokinase in their α-granules (13) and are resistant to thrombosis in the FeCl₃ carotid artery injury model. Transfusion of mUK platelets into WT mice blocked clot development (14). We reasoned that if infused mUK platelets from FL large cells are functional, they would interfere with clotting in the hcdFlb⁻ recipient mice. Indeed, infused mUK large cells failed to form stable occlusions (Table 1).

To begin to address where infused megakaryocytes shed platelets, we labeled megakaryocyte nuclei with BrdU. Various organs were isolated up to 36 hours after infusion. Virtually all of the BrdU-stained nuclei were in the lungs (Figure 3A). Stained nuclei were visible for up to 24 hours (Figure 3B and data not shown). A few nuclei were visible in the red pulp of the spleen (Figure 3C). None were present in the liver, heart, brain, or BM (data not shown). The BrdU-labeled large cells appear to retain their cytoplasm for up to 30 minutes after infusion in pulmonary microvessels (Figure 3, B and D), consistent with the delay in peak platelet count after infusion of FL large cells. To confirm this observation, we stained the tissues with Stained nuclei were visible for up to 24 hours (Figure 3B and Table 1).

In summary, we have addressed whether infused megakaryocytes can release platelets within the pulmonary bed and demonstrated that biologically active platelets can be formed in vivo from infused modified megakaryocytes. By targeting urokinase delivery to sites of injury (20), this approach may allow ectopic protein delivery in vivo from mUK mouse megakaryocytes (Table 1).
generate 10^9 mature megakaryocytes to achieve a 10% rise in platelet count in an average 70-kg patient.

**Methods**

**Characterization of the mice studied.** Donor cells and platelets were derived from C57BL/6 WT mice (The Jackson Laboratory) or mUK-transgenic mice, which ectopically express murine urokinase within megakaryocytes (14). Recipient mice were homozygously transgenic for hαIIb and null for the expression of platelet mouse αIIb (mαIIb–/–) (8), designated hαIIb+ mice, and expressed 20% of the level of CD41 seen on human platelets (12). All animal studies were done with approval of the Institutional Animal Utilization Committee at the Children’s Hospital of Philadelphia.

Isolation of platelets and megakaryocytes ex vivo. FL megakaryocytes were obtained from E14 FL cells homogenized and cultured as previously described (7). Adult BM cells were obtained from femurs and tibiae of C57BL/6 mice (21). Mature megakaryocytes were isolated using a 2-step density gradient (21). Washed platelets derived from the inferior vena cava of C57BL/6 mice in acid-citrate-dextrose were prepared as previously described (22). Platelet counts were determined using a HemaVet counter (Triad Associates). Platelets and/or megakaryocytes were infused into recipient mice retro-orbitally or by tail vein; the two approaches gave similar outcomes (Supplemental Figure 3).

**Characterization of the megakaryocytes.** Megakaryocytes to proplatelet number was determined visually with a hemocytometer. DNA ploidy was assessed by flow cytometry after staining with propidium iodide using a FACScan (BD) as described previously (23).

Flow cytometric studies in infused hαIIb+ mice. Retro-orbital blood samples from recipient mice were double stained with monoclonal FITC-conjugated mouse anti-human CD41 Ab (eBioscience) and monoclonal phycoerythrin-conjugated (PE-conjugated) rat anti–mouse CD41 Ab (BD Biosciences) for 30 minutes and analyzed by flow cytometry.

Activation of infused platelets was assessed by 3-color whole blood flow cytometry, using monoclonal mouse anti–human CD41 (PerCP-Cy5.5), PE-rat anti–mouse CD41, and monoclonal FITC–rat anti–mouse P-selectin Abs (all from BD Biosciences). To examine the relative expression of membrane receptors in recipient versus donor platelets, whole blood was stained with mouse anti–human CD41 (PerCP-Cy5.5), PE-rat anti–mouse CD41, and either monoclonal FITC-labeled rat anti–mouse GPIbα or rat anti–mouse GPIX Ab (Emfret Analytics).

**Infusion studies in thrombocytopenic hαIIb+ mice.** Mice were subjected to a high dose of irradiation (1,000 centigrays total; two sessions, 24 hours apart). Platelet counts were initially monitored daily to determine a temporal platelet profile. Animals included in these studies had platelet counts between 1 × 10^8 to 2 × 10^8/ml on day 7 after irradiation and immediately before infusion of the cells in 200 μl CATCH buffer. Additional counts were done at 4, 24, and 48 hours after infusion.

**Cremaster laser injury functional studies.** One hour after cell infusion, recipient hαIIb+ male mice were studied in the cremaster laser injury model (24). Anti–mouse CD41 Fab fragments labeled with Alexa Fluor 488 (Pierce Biotechnology) were injected intravenously 5 minutes before injury. Data were collected over a course of 2.5 minutes at 5 frames per second.

**FeCl₃ carotid artery injury functional studies.** FeCl₃-induced arterial injury was induced in hαIIb+ mice 1 hour after infusion. Studies were done as previously described (20), but the 20% FeCl₃ injury was for 3 minutes. Total flow was recorded for 30 minutes.

**Infused megakaryocyte fate studies.** FL-derived cells were cultured for 5 days and then exposed to 10 μM BrdU (Sigma-Aldrich) for 48 hours.
brief report

prior to infusion. Following infusions, recipient mice were sacrificed and organs isolated, then fixed in formalin at predetermined time points. Detection of BrdU-labeled nuclei was performed with a rat polyclonal anti-BrdU Ab and a biotinylated rabbit polyclonal anti-rat IgG as the secondary Ab (Abcam), followed by a DAKO detection kit (EnVision). Megakaryocyte cytoplasms in the lungs were similarly studied using a goat polyclonal Ab against murine c-fos (Santa Cruz Biotechnology Inc.) and a secondary biotinylated rabbit polyclonal anti-goat IgG Ab (Abcam).

Statistics. Differences between groups were compared using 2-tailed Student’s t test. Statistical analyses were performed using Microsoft Excel. Differences were considered significant when P values were 0.05 or less.

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