Hemostasis vs. homeostasis: Platelets are essential for preserving vascular barrier function in the absence of injury or inflammation

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Platelets are best known for their vasoprotective responses to injury and inflammation. Here, we have asked whether they also support vascular integrity when neither injury nor inflammation is present. Changes in vascular barrier function in dermal and meningeal vessels were measured in real time in mouse models using the differential extravasation of fluorescent tracers as a biomarker. Severe thrombocytopenia produced by two distinct methods caused increased extravasation of 40-kDa dextran from capillaries and postcapillary venules but had no effect on extravasation of 70-kDa dextran or albumin. This reduction in barrier function required more than 4 h to emerge after thrombocytopenia was established, reverting to normal as the platelet count recovered. Barrier dysfunction was also observed in mice that lacked platelet-dense granules, dense granule secretion machinery, glycoprotein (GP) VI, or the GPVI signaling effector phospholipase C (PLC) γ2. It did not occur in mice lacking α-granules, C type lectin receptor-2 (CLEC-2), or protease activated receptor 4 (PAR4). Notably, although both meningeal and dermal vessels were affected, intracerebral vessels, which are known for their tighter junctions between endothelial cells, were not. Collectively, these observations 1) highlight a role for platelets in maintaining vascular homeostasis in the absence of injury or inflammation, 2) provide a sensitive biomarker for detecting changes in platelet-dependent barrier function, 3) identify which platelet processes are required, and 4) suggest that the absence of competent platelets causes changes in the vessel wall itself, accounting for the time required for dysfunction to emerge.

platelets | vascular integrity | homeostasis | GPVI signaling | dense granules

The vascular endothelium forms a continuous barrier whose permeability helps to maintain normal tissue homeostasis by impeding or permitting the passage of plasma molecules and cells across the vessel wall (1). Severe disorders of barrier function can allow erythrocytes as well as solutes to escape and, if the loss exceeds the capacity of the lymphatic system to compensate, will result in edema and organ dysfunction. Well-described extreme examples include sepsis, systemic inflammatory disorders, and the cytokine release syndrome. Disorders of barrier function can, however, also be relatively subtle, allowing solutes, but not erythrocytes, to escape and, in the process, revealing much about the ways that basal barrier function is normally maintained.

Here, we have focused on understanding the role that platelets play when neither injury nor inflammation is present. Platelets have a long-established role in the hemostatic response to injury and a more recently established role in preventing the escape of erythrocytes and plasma proteins in the setting of inflammation (2, 3). The idea that platelets also support vascular homeostasis dates back to studies on frogs (4) and isolated organs (5), and to electron micrographs that showed thinning of the endothelium and increased microvascular permeability in thrombocytopenic rabbits and humans (6, 7). Collectively, these older observations suggest that platelets have a role in maintaining barrier function but reveal little about the mechanisms involved (2, 3, 8–11). In hemostasis, platelets are activated by agonists such as collagen, thrombin, adenosine diphosphate (ADP), and thromboxane A_2 (TxA_2), triggering events that include and depend on granule secretion. In inflammatory conditions, platelets primarily rely on signaling through GPVI and CLEC-2 to prevent bleeding (3), but granule secretion and platelet activation by thrombin, ADP, and TxA_2 have been shown to be dispensable (8, 12).

To better understand the role of platelets in maintaining vascular homeostasis, we began by asking whether severe thrombocytopenia causes vascular leakage in vivo and, if so, which platelet processes are normally involved in preventing it. Since detection methods like the Miles assay that are based on albumin loss proved to be insufficiently sensitive for measuring homeostatic vascular barrier function, we have used intravital microscopy and the differential extravasation of fluorescently tagged dextran molecules from capillaries and postcapillary venules to detect barrier dysfunction. An initial comparison of probes confirmed a previous report that there is a slow

Significance

The human circulatory system includes the heart, arteries, veins, and capillaries, all of which are lined by endothelial cells to prevent the escape of vascular contents into surrounding tissues. Platelets are blood cells that are best known for their essential role in preventing blood loss after injury. Here, we employed sensitive methods to measure changes in vascular barrier function in vivo and used them to identify a role for platelets in maintaining vascular homeostasis. The results highlight the role of platelet collagen receptors and dense granules, show which vessels are particularly dependent on platelets for this purpose, and point to an underappreciated interchange between platelets and endothelial cells that is lost in severe thrombocytopenia.

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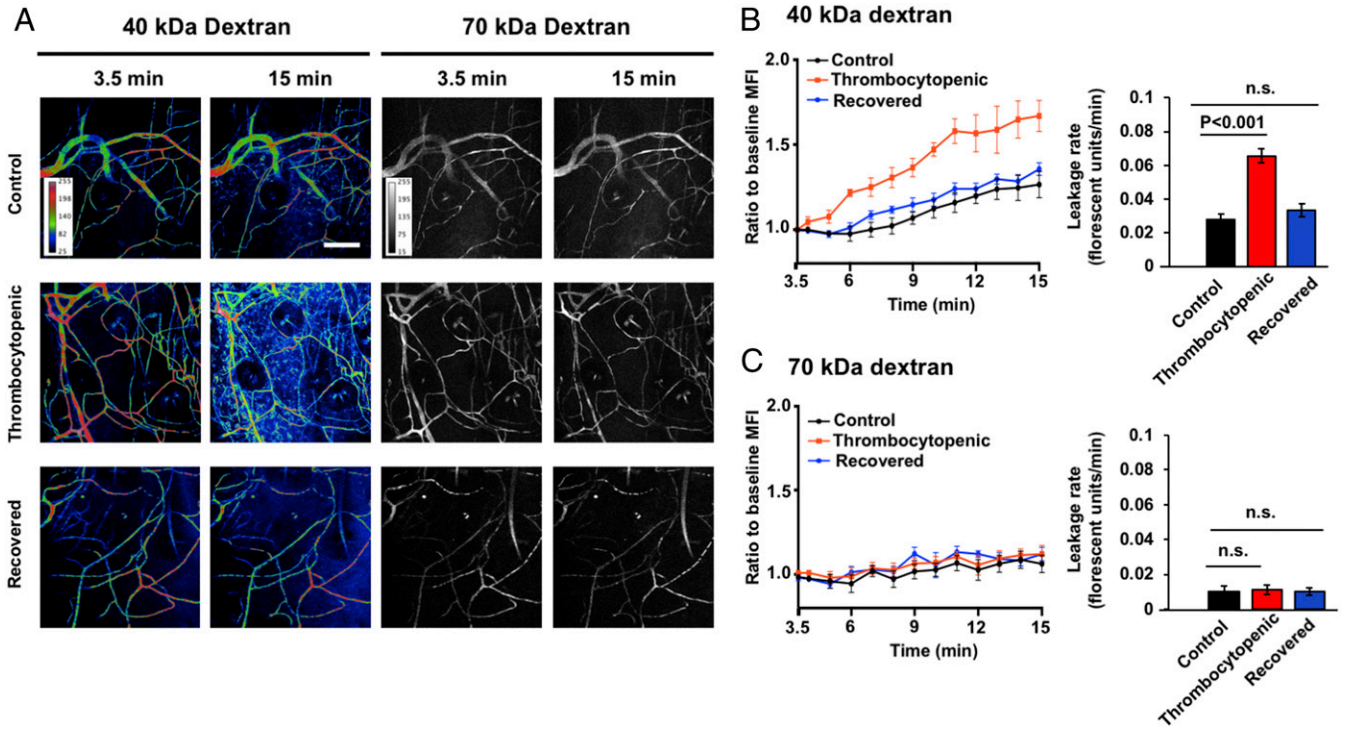

Fig. 1. Severe thrombocytopenia produces a reversible defect in homeostatic vascular barrier function. (*A*) Representative images of the skin microcirculation on the dorsal side of the ear captured at 3.5 and 15 min after i.v. infusion of fluorescent 40-kDa or 70-kDa dextran into either control mice that received polyclonal nonimmune IgG (*Top*), thrombocytopenic mice that were studied 24 h after injection of 0.4 μg/g anti-GPIbα (*Middle*, platelet count ≤5% of normal), and partially recovered mice that were studied 24 h after injection of 0.3 μg/g anti-GPIbα (*Bottom*, platelet count at least 30% of normal). (Scale bar: 100 μm.) The intensity bar shown was used to assign a value to each pixel based on the intensity of the fluorophore. Brighter pixels are assigned to higher intensity values and darker areas with lower intenisty values correspond to weaker fluorophore positive pixels. Rainbow intensity scale was used to denote 40 kDa dextran and grey scale was used to for 70 kDa dextran. (*B*) Increased escape of 40-kDa dextran from thin-walled capillaries and post-capillary venules. On the *Left* is shown the ratio over time of the MFI in the interstitial tissue to the MFI at 3.5 min. On the *Right* is shown the leakage rate. (*C*) Same as *B*, but with 70-kDa dextran. Data points indicated as mean ± SEM. $n = 5$ to 7 mice in each group. n.s., nonsignificant.

extravasation of 40-kDa, but not 70-kDa, dextran from healthy vessels (13). We now show that the rate of 40-kDa dextran extravasation is sensitive to the circulating platelet count, increasing in the setting of severe thrombocytopenia and then reverting to normal as the platelet count recovers. This defect in barrier function is detectable in the dermal and meningeal vascular beds, but not in the intracerebral vasculature, and requires >4 h to emerge after thrombocytopenia is established. Preventing it requires platelet GPVI, PLCγ2, and dense granule secretion, loss of any of which recapitulates the effects of thrombocytopenia. In contrast to the responses to injury and inflammation, preservation of barrier function does not require the presence of platelet thrombin receptors, CLEC-2, or α-granule secretion. Collectively, these results demonstrate a role for platelets in maintaining vascular homeostasis, characterize the limits of the role, and establish some of the underlying mechanisms that are involved.

Results

Severe Thrombocytopenia Produces a Reversible Defect in Vascular Barrier Function. We began by observing the extravasation of fluorophore-conjugated 40-kDa and 70-kDa dextran from capillaries and postcapillary venules in the skin microcirculation on the dorsal

Table 1. Complete blood counts of control mice treated with polyclonal nonimmune IgGs, mice rendered thrombocytopenic with 0.4 μg/g anti-GPIbα antibody, and mice recovered from thrombocytopenia by ≥30% platelets after anti-GPIbα antibody (0.3 μg/g) treatment

	IgG-treated control	Thrombocytopenic mice	Platetet-recovered mice
Red blood cells, M/μL	9.23 ± 0.11	9.40 ± 0.40	9.30 ± 0.21
Hemoglobin, g/dL	14.67 ± 0.17	14.92 ± 0.66	14.71 ± 0.31
Hematocrit, %	43.38 ± 0.64	44.12 ± 2.22	42.65 ± 0.21
Platelets, K/μL	897 ± 23	49 ± 10***	390 ± 50**
Mean platelet volume, fL	6.0 ± 0.06	7.43 ± 0.09	6.59 ± 0.07
White blood cells, K/μL	9.62 ± 0.56	10.38 ± 0.43	8.96 ± 0.42
Neutrophil, %	33.21 ± 2.27	39.31 ± 2.13	32.56 ± 2.14
Lymphocyte, %	61.46 ± 2.32	52.27 ± 1.96	60.09 ± 2.28
Monocyte, %	3.33 ± 1.63	6.30 ± 2.05	5.51 ± 0.23

All groups of mice were tested at 24 h post-antibody treatment. $n = 10$ to 15 mice in each group. $^{**}P < 0.01$, $^{***}P < 0.001$. M/μL, millions per microliter; K/μL, thousands per microliter.

Table 2. Complete blood counts of polyclonal nonimmune IgG-treated control mice and mice recovered from thrombocytopenia by ≥30% platelets at 48 h post-0.4 μg/g anti-GPIbα antibody treatment

	IgG-treated control	Platelet-recovered mice after 0.4 μg/g antibody treatment
Red blood cells, M/μL	9.17 ± 0.20	9.5 ± 0.25
Hemoglobin, g/dL	14.47 ± 0.33	14.93 ± 0.41
Hematocrit, %	41.38 ± 1.64	43.15 ± 1.27
Platelets, K/μL	944 ± 34	368 ± 6.0**
Mean platelet volume, fL	5.9 ± 0.06	6.67 ± 0.08
White blood cells, K/μL	8.46 ± 0.70	9.19 ± 0.54
Neutrophil, %	32.67 ± 3.35	35 ± 3.65
Lymphocyte, %	63.23 ± 3.54	57.57 ± 3.69
Monocyte, %	2.54 ± 1.11	3.87 ± 0.44

The counts were performed at 48 h after antibody administration. $n = 5$ to 7 mice in each group. $^{**}P < 0.01$.

side of the ear in mice with normal platelet counts. Under basal conditions 70-kDa dextran was retained in the blood stream while 40-kDa dextran leaked slowly into the interstitial space (Fig. 1 *A*, *Top* and Fig. 1 *B* and *C*). Similar studies were then performed in mice rendered severely thrombocytopenic by infusing 0.4 μg/g polyclonal rat anti-mouse GPIbα antibody (14). The antibody produced thrombocytopenia within 30 min (*SI Appendix*, Table S1) and maintained the platelet count at ≤5% of control for at least 24 h (Table 1). In the thrombocytopenic mice, the rate of 40-kDa dextran extravasation was increased compared to the controls. Loss of 70-kDa dextran extravasation was unaffected (Fig. 1 *A*, *Middle* and Fig. 1 *B* and *C*). Notably, albumin extravasation, which is a commonly used metric of barrier dysfunction in inflammatory states, was also unaffected by thrombocytopenia (*SI Appendix*, Fig. S1).

To determine whether barrier function recovers as the platelet count rises, a second cohort of mice received a smaller dose of anti-GPIbα (0.3 μg/g rather than 0.4 μg/g). At this reduced dose, platelet counts still dropped to ≤5% of normal within 2 h but recovered to ≥30% of pretreatment levels by 24 h, which is when the mice were tested (Table 1). In this cohort, there was no increase in the rate of 40-kDa dextran extravasation compared with controls (Fig. 1 *A*, *Bottom* and Fig. 1*B*). Finally, we treated a third cohort of mice with the higher antibody dose (0.4 μg/g) and allowed them to recover for 48 rather than 24 h. After 48 h, mice in this third cohort whose platelet count was at least 30% of control were tested and found to have 40-kDa dextran extravasation rates identical to the IgG controls (*SI Appendix*, Fig. S2*A* and Table 2). Neither the low dose nor the high dose antibody regimen affected the extravasation of 70-kDa dextran (Fig. 1 *A*, *Bottom* and Fig. 1*C* and *SI Appendix*, Fig. S2*B*). These results show that the barrier dysfunction caused by severe thrombocytopenia is reversible.

Increased Extravasation in Thrombocytopenic Mice Is Independent of Inflammation. Inflammation is known to increase vascular permeability and erythrocyte extravasation under thrombocytopenic conditions (2, 13). To determine whether the increased rate of 40-kDa dextran extravasation that we observed in the thrombocytopenic mice was due to the absence of platelets and not due to concomitant inflammation, plasma cytokine levels were measured. The panel included cytokines including ones that are associated with vascular barrier dysfunction: TNFα, IFNγ, and VGEF. With the exception of a small increase in IL-1α and IL-17, there were no differences in cytokine levels between the thrombocytopenic mice and those that had either never been thrombocytopenic or which had recovered from thrombocytopenia (Table 3). We cannot, of course, exclude changes in cytokines that were not included in the panel, but additional studies described below and the absence of increased 70-kDa dextran or albumin extravasation suggested that inflammation is unlikely to be a factor in the increased 40-kDa dextran leak found in thrombocytopenic mice.

Vascular Barrier Dysfunction Is Independent of the Method Used to Induce Thrombocytopenia. To assess whether the increased vascular permeability observed in thrombocytopenia is independent of the method used to produce thrombocytopenia, an additional

Table 3. Plasma levels of indicated cytokines (pg/mL) in polyclonal nonimmune IgG-treated control mice, thrombocytopenic mice, and in mice recovered from thrombocytopenia by ≥30% platelets at 24 h post–anti-GPIbα antibody treatment

Cytokine, pg/mL	IgG control mice	Thrombocytopenic mice	Platelet-recovered mice
GM-CSF	22.24 ± 3	21.69 ± 3	19.16 ± 0.02
IFN-γ	14.09 ± 10	15.95 ± 11	25.40 ± 14
IL-1a	9.84 ± 7	15.63 ± 11	18.36 ± 6.5*
IL-1b	11.84 ± 4	10.52 ± 8	15.93 ± 4
IL-2	10.02 ± 3	6.82 ± 2.1	5.83 ± 2*
IL-5	6.24 ± 3	6.41 ± 3.6	2.87 ± 1.2
IL-6	32.59 ± 35	22.74 ± 15	24.18 ± 8
IL-7	3.71 ± 2	2.13 ± 3	4.16 ± 2.6
IL-9	17.05 ± 10	16.08 ± 7	17.11 ± 7
IL-13	25.58 ± 8	37.22 ± 26	18.45 ± 5.5
IL-17	0.80 ± 0.4	1.99 ± 1.5*	1.16 ± 0.8
TNF-α	6.42 ± 3	8.01 ± 2.4	8.05 ± 4.6
VEGF	0.86 ± 0.6	0.57 ± 0.2	0.43 ± 0.04

$n = 7$ to 10 mice in each group. $^{*}P < 0.05$.

Table 4. Complete blood counts of vehicle-treated control and busulfan treatment-induced thrombocytopenic mice (single dose of 20 mg/kg i.p.) on day 13 after injection

	Vehicle-treated control mice	Busulfan treatment-induced thrombocytopenic mice
Red blood cells, M/μL	9.44 ± 0.05	8.44 ± 0.13
Hemoglobin, g/dL	15.03 ± 0.36	13.32 ± 0.16
Hematocrit, %	42.70 ± 0.28	41.72 ± 0.60
Platelets, K/μL	829 ± 79	135 ± 30.87***
Mean platelet volume, fL	5.7 ± 0.05	6.3 ± 0.10
White blood cells, K/μL	14.31 ± 8.43	5.66 ± 0.39*
Neutrophil, %	20.86 ± 7.84	13.74 ± 1.94
Lymphocyte, %	76.36 ± 7.91	82.34 ± 2.72
Monocyte, %	0.96 ± 0.28	1.16 ± 0.26

$n = 10$ mice in each group. *$P < 0.05$, ***$P < 0.001$.

cohort of mice was rendered thrombocytopenic with busulfan (15). A single dose of the drug reduced the platelet count by 85% on day 13 (Table 4) and resulted in an increase in 40-kDa, but not 70-kDa, dextran extravasation (Fig. 2 *A* and *B*).

Barrier Dysfunction Requires More Than 4 h and Less Than 24 h to Emerge. To determine how quickly changes in barrier function emerge after the onset of severe thrombocytopenia, we measured dextran extravasation 4 h rather than 24 h after injecting a standard dose of anti-GPIbα antibody (0.4 μg/g). In contrast to the results obtained after 24 h, we observed no differences in the rate of dextran extravasation between the thrombocytopenic mice and the controls (Fig. 2 *C* and *D*).

PAR4 Is Dispensable for Maintaining Vascular Barrier Function. Having established that platelets are important for protecting homeostatic vascular integrity, we next performed a series of studies to determine which aspects of platelet biology are required to maintain barrier function, starting with platelet receptors and downstream signaling molecules. The protease-activated receptor family member, PAR4, mediates the response of mouse platelets to thrombin and is crucial for achieving hemostasis after vascular injuries (16). Deletion of PAR4 receptor impairs hemostasis but has no effect on red cell extravasation during inflammation (3). To determine whether platelets utilize thrombin signaling to maintain homeostatic barrier function, dextran extravasation was measured in PAR4$^{-/-}$ mice. We found no differences in 40- and 70-kDa dextran leakage kinetics from matched controls (Fig. 3 and *SI Appendix*, Fig. S3*A*), indicating that platelet PAR4 receptors are dispensable for maintaining the homeostatic vascular barrier.

Signaling by GPVI, but Not CLEC-2, Is Required to Maintain Vascular Barrier Function. Platelets express two major immunoreceptor tyrosine-based activation motif (ITAM) receptors, GPVI and

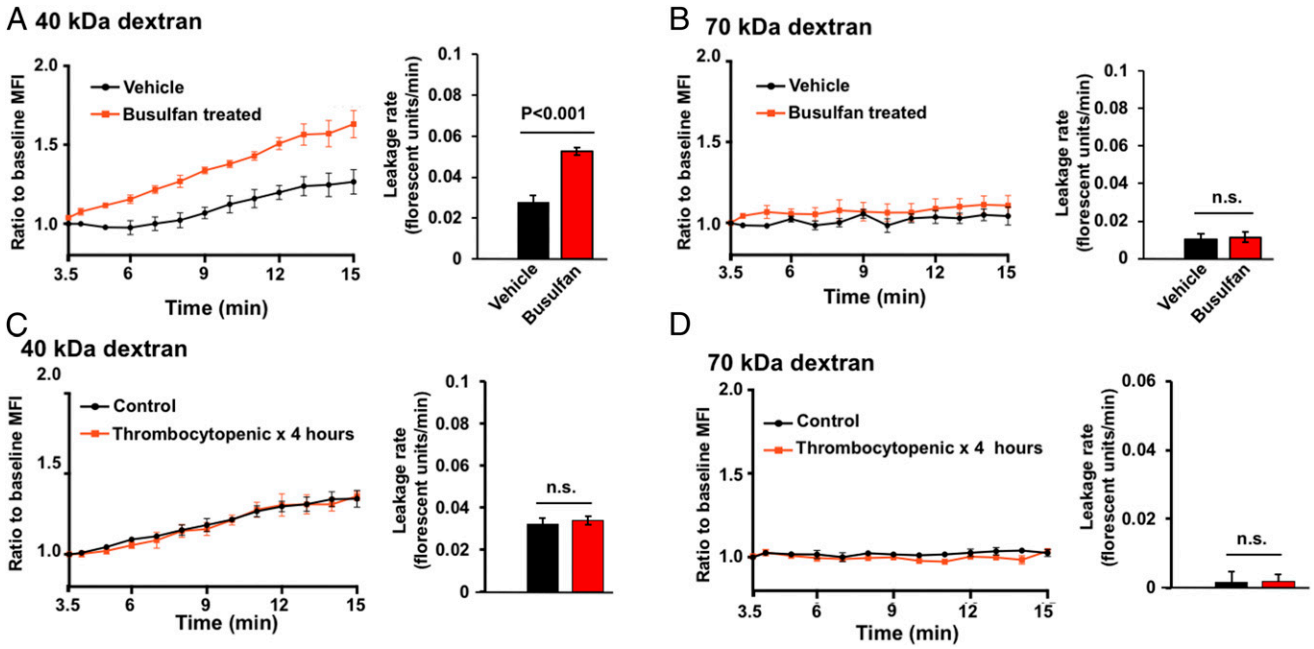

Fig. 2. Changes in barrier function are independent of the method of induction of thrombocytopenia and emerge gradually. Mice were rendered thrombocytopenic with a single dose of busulfan (20 mg/kg). Vascular permeability studies were performed on 13 d afterwards. The platelet counts in the drug treated mice at the point were 10 to 15% of normal control mice. (*A*) MFI in the interstitial space area and leakage rate following infusion of 40-kDa dextran. (*B*) Same as *A* with 70-kDa dextran. Control mice received vehicle. Data points indicated as mean ± SEM. $n = 5$ mice each group. n.s., nonsignificant. (*C*) Vascular barrier function in mice was measured 4 h after induction of thrombocytopenia with 0.4 μg/g anti-GPIbα. Circulating platelet counts fell to ∼5% of control within 30 min of antibody administration. MFI in the interstitial space area following infusion of 40-kDa dextran. (*D*) Same as *C* with 70-kDa dextran. Control mice received similar amounts of nonimmune IgG. Data points indicated as mean ± SEM. $n = 4$ mice in each group. n.s., nonsignificant.

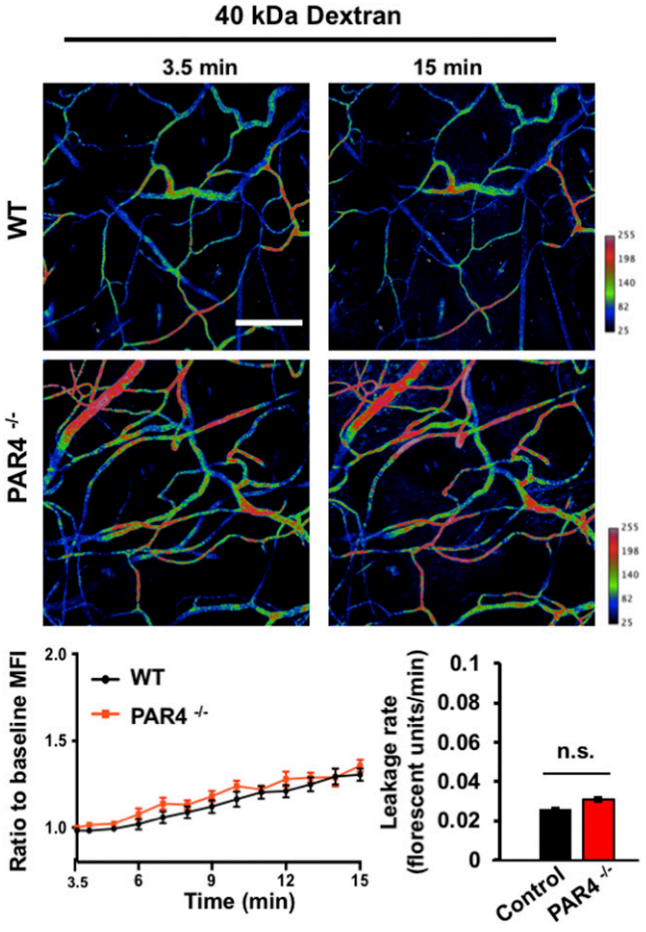

Fig. 3. Platelet PAR4 signaling is not required for maintaining homeostatic vascular barrier function. Representative images obtained 3.5 and 15 min after infusion of 40-kDa dextran into PAR4$^{-/-}$ mice and matched controls. Ratio of the MFI normalized to MFI at 3.5 min and leakage rate. Data points indicated as mean ± SEM. $n = 6$ mice each group. (Scale bar: 100 μm.) n.s., nonsignificant.

CLEC-2, both of which induce platelet activation via PLCγ2. GPVI is expressed exclusively on megakaryocytes and platelets. CLEC-2 is expressed on immune cells, megakaryocytes, and platelets. The major physiological ligand for GPVI is collagen (17, 18). The ligand for CLEC-2 is podoplanin (18). Both have been shown to support hemostasis in inflammatory settings (12). To test if GPVI is needed to sustain homeostatic barrier function, mice were injected with JAQ1 antibody, which down-regulates GPVI from the surface of circulating platelets through receptor internalization and shedding and results in a GPVI knockout-like phenotype (19, 20). Dextran extravasation was measured 72 to 96 h later, when the platelet count was normal (Table 5), but the platelets showed no detectable surface GPVI expression and failed to respond to convulxin, a snake venom toxin that activates GPVI (*SI Appendix*, Fig. S3*B*) (21). Like thrombocytopenia, the acute loss of GPVI from platelets in GPVI-depleted mice resulted in increased leakage of 40-kDa, but not 70-kDa dextran, highlighting the role of GPVI in maintaining barrier function (Fig. 4*A* and *SI Appendix*, Fig. S4*A*).

To determine the effects of chronic, rather than acute, loss of GPVI, we also tested barrier function in GPVI$^{-/-}$ mice (22). Increased extravasation of 40-kDa, but not 70-kDa dextran was observed (Fig. 4*B* and *SI Appendix*, Fig. S4*B*). Next, the role of CLEC-2 was tested by injecting the CLEC-2-depleting antibody, INU1 (23). This antibody is known to induce targeted down-regulation of CLEC-2 through Src-family kinase dependent receptor internalization in vivo, followed by intracellular degradation (24). After 120 h, the INU1-treated mice had normal platelet counts (Table 5), but their platelets were deficient in CLEC-2 surface expression and failed to respond to the CLEC-2–activating toxin, rhodocytin (*SI Appendix*, Fig. S3*C*). CLEC-2–depleted mice showed normal basal vascular barrier function, with no increase in dextran extravasation compared to the controls (Fig. 4*C* and *SI Appendix*, Fig. S4*C*).

Thus, in contrast to inflammatory settings where signaling through both GPVI and CLEC-2 is required to prevent loss of red blood cells, GPVI is required to maintain homeostatic vascular barrier function, but CLEC-2 is not. To determine whether it is a signaling-dependent function of GPVI, we also studied dextran extravasation in wild-type (WT) mice that had been rescued after lethal irradiation with hematopoietic cells from either PLCγ2$^{-/-}$ or PLCγ2$^{+/+}$ mice (25). Successful transplantation was confirmed by the recovery of blood cell counts (Table 6) and the loss of the response to convulxin in the PLCγ2$^{-/-}$ chimeras, but not the PLCγ2$^{+/+}$ chimeras (*SI Appendix*, Fig. S5*A*). Dextran extravasation measurements showed that the PLCγ2$^{-/-}$ chimeras phenocopied the GPVI knockouts, showing an increased rate of 40-kDa, but not 70-kDa dextran extravasation (Fig. 4*D* and *SI Appendix*, Fig. S5*B*).

Platelet-Dense Granules, but Not α-Granules, Are Required to Maintain Vascular Homeostasis. Downstream signaling through GPVI results in granule content release and is an important step in achieving efficient platelet response. Platelets contain α, dense, and lysosomal granules (26). α-granule proteins are derived from both synthetic and endocytic pathways (27). Dense granules are platelet-specific lysosome-related organelles (28, 29). These

Table 5. Complete blood counts of mice treated with polyclonal nonimmune IgGs, mice rendered GPVI-depleted by i.v. infusion of JAQ1 (0.5 μg/g), and mice made CLEC-2–deficient by INU1 (5 μg/g) antibody treatment

	IgG-treated control mice	GPVI-depleted mice	CLEC-2–depleted mice
Red blood cells, M/μL	9.17 ± 0.11	9.09 ± 0.10	9.06 ± 0.22
Hemoglobin, g/dL	14.19 ± 0.17	13.97 ± 0.16	13.96 ± 0.38
Hematocrit, %	42.77 ± 0.64	42.54 ± 0.58	42.75 ± 1.21
Platelets, K/μL	855 ± 28	770 ± 50	782 ± 55
Mean platelet volume, fL	5.94 ± 0.04	6.10 ± 0.03	6.13 ± 0.08
White blood cells, K/μL	10.50 ± 0.79	12.37 ± 0.72	11.09 ± 1.06
Neutrophil, %	26.29 ± 2.87	19.48 ± 4.66	21.40 ± 4.85
Lymphocyte, %	69.23 ± 2.77	77.44 ± 2.65	74.19 ± 4.68
Monocyte, %	2.00 ± 0.23	1.29 ± 0.12	1.70 ± 0.45

All blood samples were acquired within 72 to 96 h after antibody treatment. $n = 10$ to 15 mice in each group.

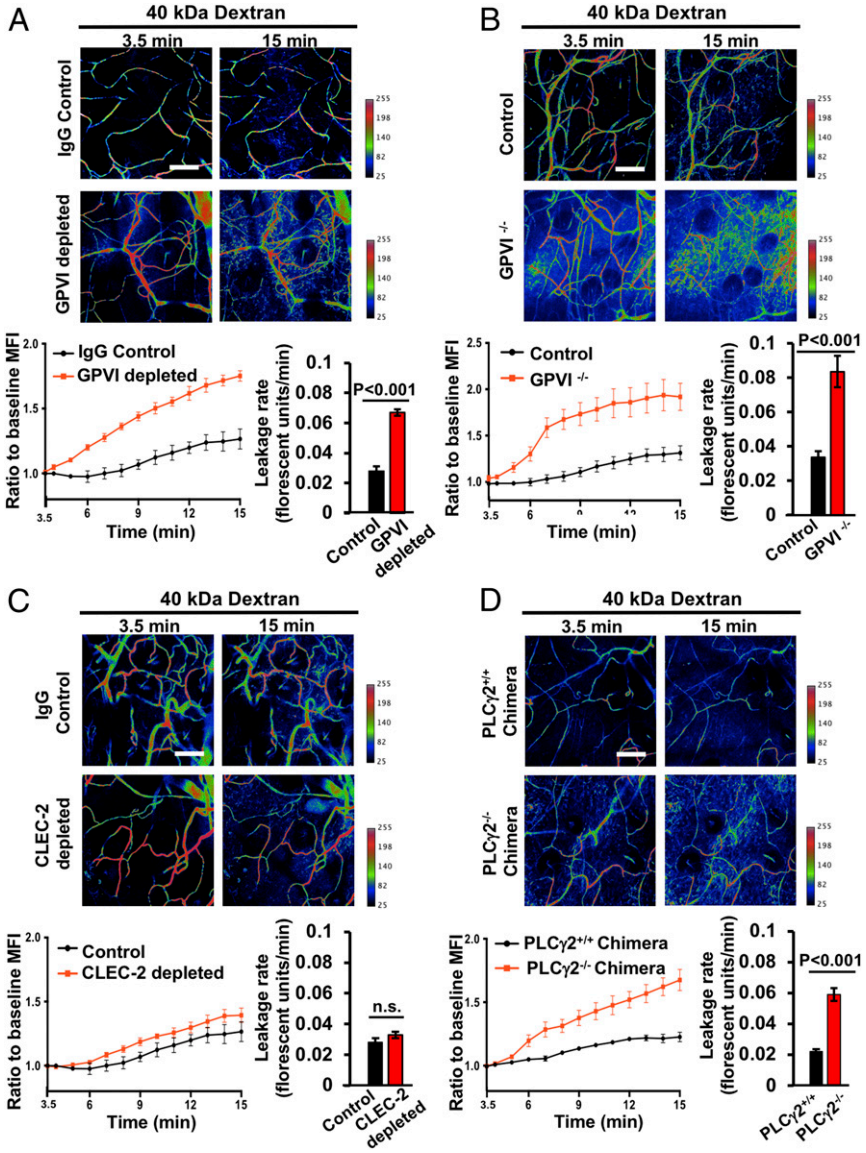

Fig. 4. Loss of GPVI and PLCγ2, but not CLEC-2, increases 40-kDa dextran extravasation. (*A*, *Top*) Images of the dermal microcirculation 3.5 and 15 min after infusion of 40-kDa Texas red dextran into mice whose platelets were rendered GPVI-deficient by injecting the anti-GPVI antibody, JAQ1. Controls received equivalent amounts of polyclonal nonimmune IgG. (*A*, *Bottom*) Ratio of the MFI normalized to MFI at 3.5 min. $n = 5$ mice in each group. (*B*) Same as *A* for GPVI$^{-/-}$ mice. $n = 6$ mice in each group. (*C*) Same as *A* but for mice depleted of CLEC-2 by administration of antibody INU1. Matched controls received nonimmune IgG. $n = 5$ mice in each group. (Scale bars: 100 μm.) Mean ± SEM. (*D*) Images of the dermal microcirculation 3.5 and 15 min after infusion of 40-kDa Texas red dextran into lethally irradiated WT mice rescued with bone marrow from WT or PLCγ2$^{-/-}$ mice. Ratio of the MFI normalized to MFI at 3.5 min. Data points indicated as mean ± SEM. $n = 6$ mice in each group. (Scale bars: 100 μm.)

granules contain serotonin, adenosine, polyphosphates, growth factors, sphingosine-1-phosphate (S1P), and angiogenic proteins that are known to regulate vascular permeability (30). To determine whether platelet granule contents contribute to vascular barrier function, we studied mice with either α-granule defects (Nbeal2$^{-/-}$) or dense granule defects (BLOC-1$^{-/-}$ and Unc13d^{Jinx}) under basal conditions. Nbeal2$^{-/-}$ mice, which recapitulate the human gray platelet syndrome (31), displayed leakage kinetics indistinguishable from controls (Fig. 5*A*). In contrast, BLOC-1$^{-/-}$ (Pallid) mice, which have defective dense granule biogenesis and lack platelet dense granules (32), showed an increase in 40-kDa dextran extravasation compared to controls, as did Unc13d^{Jinx} mice (33), which have platelet-dense granule contents but are unable to secrete them (Fig. 5 *B* and *C*). The 70-kDa dextran extravasation was negligible in all three lines (*SI Appendix*, Fig. S6). Since BLOC-1$^{-/-}$ and Unc13d^{Jinx} have normal platelet counts and α-granule contents (32, 33), these results suggest that platelets require dense granules, but not α-granules, to maintain barrier function.

GPVI Is Also Required to Maintain Vascular Homeostasis in Meningeal Blood Vessels, but Not in Cerebral Vessels. The meninges protect the brain and spinal cord from mechanical injury, infection, and inflammation. Vessels within the meningeal dura, unlike cerebral vessels, are fenestrated and potentially open to the passage of large molecules injected into the blood stream (34). To determine whether platelets regulate homeostasis in vascular beds other than in the skin, meningeal and cerebral vessels were tested for changes in vascular barrier characteristics in the absence of platelet GPVI signaling. Similar to what was observed in the skin, the rate of 40-kDa dextran extravasation was increased in GPVI-depleted mice compared to controls (Fig. 6*A*). No

Table 6. Complete blood counts of PLCγ2$^{+/+}$ chimera and PLCγ2$^{-/-}$ chimera at 4 wk posttransplantation

	PLCg2$^{+/+}$ chimera	PLCγ2$^{-/-}$ chimera
Red blood cells, M/μL	9.54 ± 0.33	9.10 ± 0.30
Hemoglobin, g/dL	14.14 ± 0.39	13.66 ± 0.47
Hematocrit, %	44.18 ± 1.13	42.44 ± 1.13
Platelets, K/μL	791 ± 79	898 ± 61
Mean platelet volume, fL	5.81 ± 0.12	5.98 ± 0.27
White blood cells, K/μL	6.07 ± 2.58	4.39 ± 1.3
Neutrophil, %	35.82 ± 16.55	32.2 ± 4.66
Lymphocyte, %	56.31 ± 19.35	59.32 ± 5.39
Monocyte, %	4.02 ± 2.4	3.48 ± 1.24

WT mice were lethally irradiated, and isolated bone marrow from PLCg2$^{+/+}$ or PLCγ2$^{-/-}$ mice was i.v. injected. $n = 7$ mice in each group.

extravasation of 40-kDa dextran was observed from deeper cerebral blood vessels in both IgG-treated control and GPVI-immunodepleted mice (Fig. 6*B*). The 70-kDa dextran was retained in both control and GPVI-depleted mice (*SI Appendix*, Fig. S7).

Discussion

An intact endothelium is essential for normal vascular function, forming a barrier that can regulate the passage of blood-borne molecules, cells, and water across the vessel wall and into nearby tissues. One of the early hallmarks of deteriorating vascular health is increased permeability which, if unchecked, can lead to hypoproteinemia and edema. The idea that platelets support vascular barrier function over and above their role in hemostasis dates back more than 75 y. The evidence includes electron micrographs demonstrating morphological changes in the endothelium in the absence of platelets (5, 6, 35). More recent studies have focused on understanding how platelets minimize the loss of erythrocytes in the setting of localized and systemic inflammation (2, 13). Those studies highlight differences in the molecular mechanisms underlying the platelet responses to inflammation and injury. In the setting of vascular injury, platelets draw on receptors for collagen, thrombin, ADP, and TxA_2 and require granule secretion. In the setting of inflammation, they depend on collagen receptors and CLEC-2, but receptors for the other platelet agonists are dispensable (3, 8). Here, we have focused on the role of platelets in maintaining barrier function under homeostatic conditions using intravital real-time fluorescence microscopy.

Our results show the following. 1) Severe thrombocytopenia is sufficient by itself to cause a reversible increase in the extravasation of 40-kDa dextran from capillaries and post-capillary venules. It does not cause an increase in the extravasation of 70-kDa dextran or albumin, which helps to define the limits of what can escape from blood vasculature when platelets are absent. 2) The thrombocytopenia-related dextran leak does not appear immediately after thrombocytopenia is established but requires >4 h to emerge. 3) The effects of thrombocytopenia can be recapitulated in mice with defects in dense granule biogenesis or secretion, or which lack platelet GPVI or PLCγ2. This helps to distinguish the homeostatic effects of platelets from those in the settings of injury and inflammation. 4) Vessels in the dura are affected by the absence of platelet GPVI, but intracerebral vessels with their tighter junctions between endothelial cells are not.

It is worth emphasizing that the selection of biomarkers proved to be critical for these studies. Vascular integrity is commonly assessed in vivo using leakage of albumin and dyes that bind to albumin as biomarkers of barrier dysfunction. The present studies show that platelet-dependent barrier protection does not affect the loss of albumin. Dextran molecules normally leak in a size-dependent manner, small dextran molecules (≤10 kDa) leaking freely under

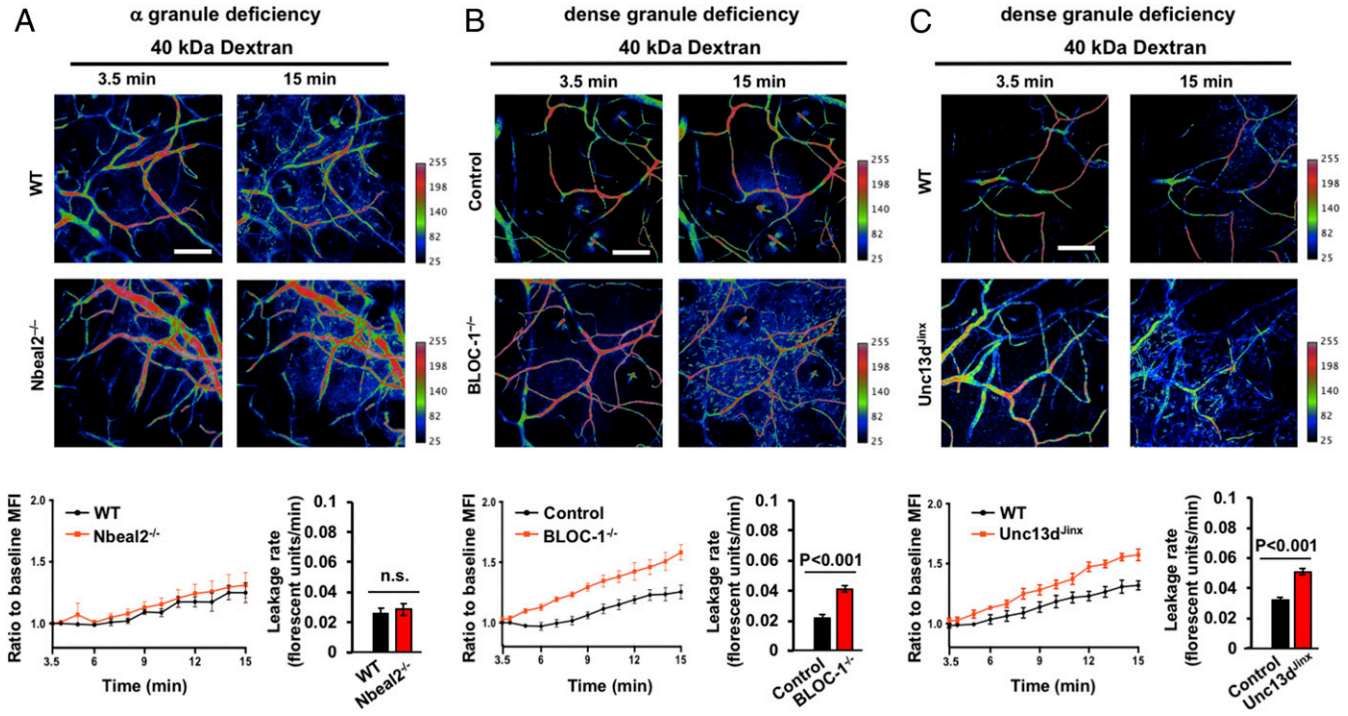

Fig. 5. Platelet-dense granules, but not α-granules, are required to maintain vascular homeostasis. (*A*, *Top*) Images of the dorsal ear skin microcirculation captured 3.5 and 15 min after infusion of 40-kDa dextran in Nbeal2$^{-/-}$ mice and matched controls. (*A*, *Bottom*) Ratio of the MFI in the interstitial tissue area over time normalized to the MFI at 3.5 min. $n = 5$ mice in each group. (*B* and *C*) Same as *A* with BLOC-1$^{-/-}$ (Pallid) mice and Unc13d^{Jinx} mice. Data points for each group indicated as mean ± SEM. $n = 6$ mice in each group. (Scale bars: 100 μm.)

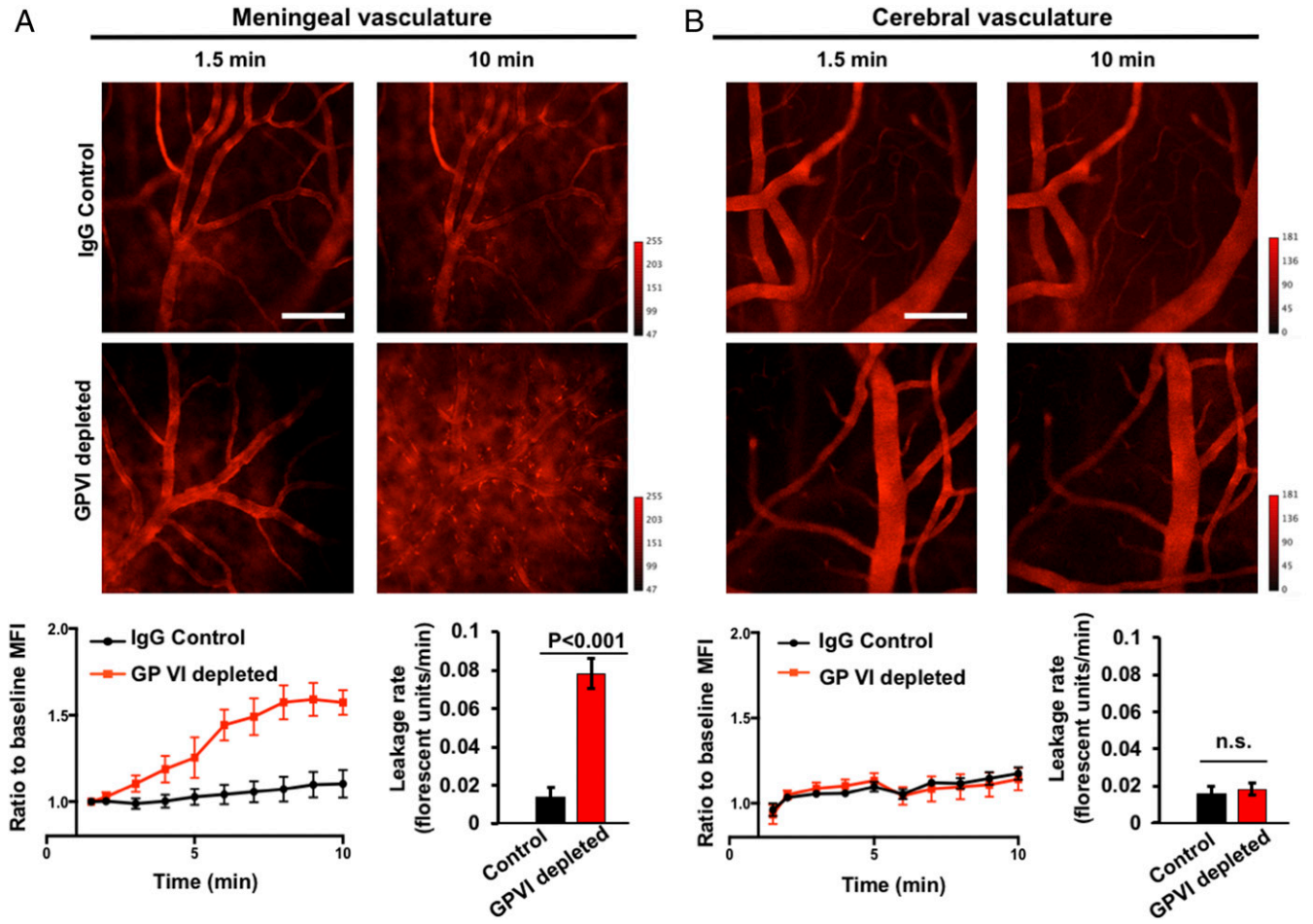

Fig. 6. GPVI depletion also reduces barrier function in meningeal vessels, but not intracerebral vessels. Representative images of (*A*) meningeal and (*B*) intracerebral vessels captured 1.5 and 10 min after i.v. infusion of 40-kDa dextran. (Scale bars: 100 μm.) MFI in the interstitial space over time normalized to MFI at 1.5 min (baseline). Meningeal and intracerebral vessels were imaged using multiphoton fluorescence microscopy through a thinned-skull cranial window as one z stack. The top surface of skull comprised of meningeal blood vessels followed by deeper cerebral vessels, separated by avascular subarachnoid space. The meningeal vessels were distinguished from cerebral vessels by their location and structure. Data points indicated as mean ± SEM. $n = 4$ mice in each group. n.s., nonsignificant.

basal conditions, while larger dextran molecules of 70 kDa or above leak little if at all (13, 36, 37). Here, we found that 40-kDa dextran provides a sweet spot on this curve, allowing us to detect the changes that occur when competent platelets are removed and then restored. To get the best in vivo snapshot of the homeostatic vascular barrier without surgical manipulation and its associated inflammation, we used multiphoton microscopy to image dermal microvasculature without surgical manipulation in the majority of our studies (13). To reduce the likelihood that the effects we observed in thrombocytopenic and GPVI-deficient mice were due to the methods used to produce thrombocytopenia and GPVI deficiency, we compared two different methods for accomplishing each. Although busulfan treatment has been reported to increase the bleeding time, it is not clear that this is necessarily due to more than the associated thrombocytopenia (38).

Given these results, what might be concluded at this point about the mechanisms by which platelets sustain homeostatic barrier function? The observation that the effects of thrombocytopenia are recapitulated by removing GPVI or PLCγ2 suggests that barrier function is normally preserved by platelet interactions with a GPVI ligand followed by PLCγ2 signaling and dense granule release (Fig. 7). Whether that ligand is collagen or something else remains to be determined. Recent evidence suggests that GPVI can also be activated by fibrin (39), but there is little reason to suspect at this point that there is ongoing deposition of fibrin throughout the vasculature under homeostatic conditions. Interactions between GPVI and glycosaminoglycans (GAGs) on the endothelial cell surface remain a possibility to be explored. Several types of collagen are prominent vessel wall components throughout the vasculature. The possibility that small amounts of collagen become exposed at intervals even in healthy individuals is not unreasonable but requires further investigation. We are not aware of reports of individual platelets or small masses of platelets adhering to the junctions between endothelial cells under homeostatic conditions. Thus, although we favor a constant "kiss and run" kind of interaction that leads to transient platelet activation, low level dense granule secretion, and positive effects on vascular integrity, the full basis for this remains to be determined (Fig. 7). Enhanced loss of 40-kDa dextran in the absence of platelets, GPVI, or dense granules provides a useful biomarker for changes in barrier function, but the absence of edema in the various mice that we studied suggests that the lymphatic system can keep up with the accelerated losses, at least under homeostatic conditions. This likely changes under conditions of additional stress, such as occurs in the presence of viral or bacterial sepsis or during states associated with cytokine storm.

If platelets are regularly releasing molecules near the vessel wall to support vascular integrity, what might those molecules be? The present studies suggest that platelet-dense granules, but not

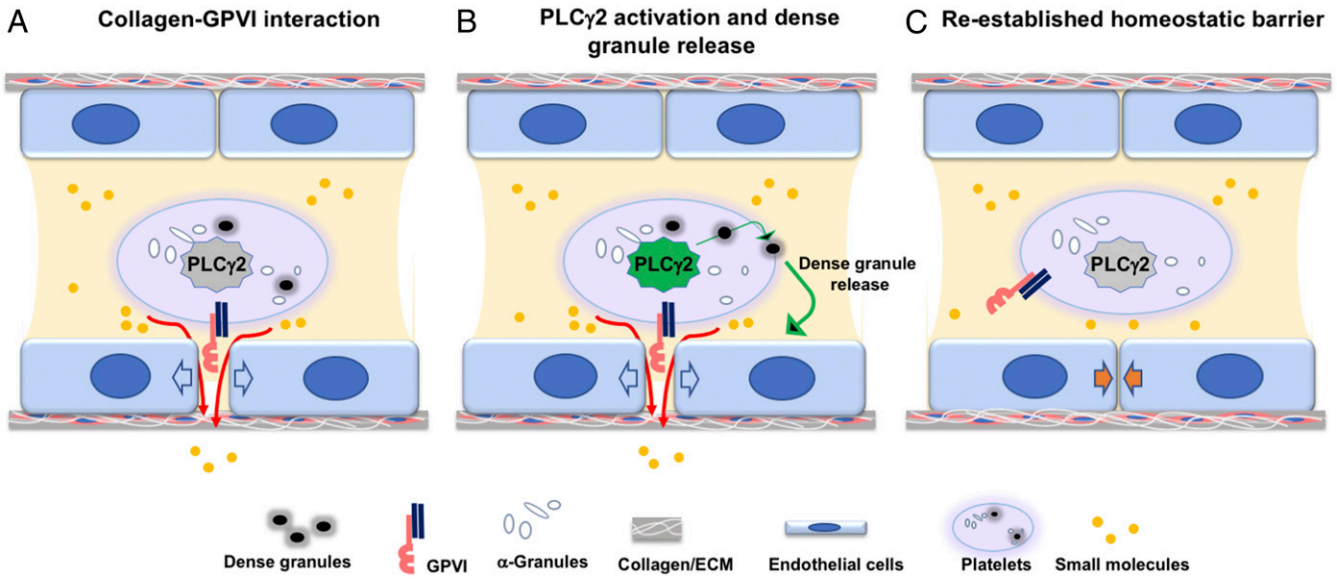

Fig. 7. Platelet are essential to maintain vascular homeostasis. Based on the results, we propose a model in which the intermittent appearance of small gaps between endothelial cells is a normal event within a healthy microvasculature. (*A*) Platelets sense exposed extracellular matrix components including collagen. (*B*) This leads to transient GPVI-dependent platelet activation and low-level dense granule secretion. Secretion products interact with endothelium and (*C*) reestablish the normal junctions between endothelial cells. Increased escape of small molecules like 40-kDa dextran from thin-walled capillaries and postcapillary venules is a marker for the persistence of these gaps in the absence of platelets, GPVI, PLCγ2, and dense granules.

α-granules, are required. The failure of the Nbeal2$^{-/-}$ mice to recapitulate the effects of thrombocytopenia not only points away from α-granule secretion, it also points away from many of the molecules with vascular effects that are carried in platelets, including VEGF and angiopoietin, both of which are believed to be stored in α-granules. What about S1P? Platelets contain two pools of S1P, a readily exchangeable, metabolically active pool, and an α-granule pool that requires platelet activation (40). S1P promotes barrier function: Mice lacking plasma S1P constitutively leak albumin. However, it has been shown that erythrocytes, not platelets, serve as the main source of plasma S1P and that loss of the platelet S1P pool does not alter vascular permeability, albeit with a less sensitive assay (41). As a result, for the moment, we favor the hypothesis that it is a dense granule constituent and not S1P that mediates the platelet effects on barrier function. Platelet-dense granules contain serotonin, adenine nucleotides, and polyphosphate. Which, if any, of these could be supporting vascular homeostasis? Serotonin is actively taken up by platelets, stored in dense granules (42, 43), and is known to increase vascular permeability (44, 45). However, we found that neither thrombocytopenia, GPVI deficiency, nor dense granule deficiency causes an increase in plasma serotonin levels. This suggests that an increase in plasma serotonin is not the cause of barrier dysfunction in thrombocytopenia.

Finally, how might something released from platelets impact the endothelium under homeostatic conditions? The observation that the effects of thrombocytopenia require more than 4 h to emerge is informative. It suggests that we are not observing the consequences of a failure of platelets to block local "mini-leaks" between endothelial cells since that ability would likely degrade as soon as the platelet count drops sufficiently. Instead, we hypothesize that there are changes within the local endothelium in the absence of platelets that involve either alterations in posttranslational modifications of junctional molecules or alterations in the transcription and/or translation of endothelial cell molecules that turn over with a half-life of a few hours at most. Which, if either, of these hypotheses is correct is a subject of ongoing investigation.

Methods

Additional methods are included in *SI Appendix*.

Mice. Animal studies were performed according to Institutional Animal Care and Use Committee of the University of Pennsylvania-approved protocols. Platelet, GPVI, and CLEC-2 immunodepletion studies were performed using 8- to 12-wk-old B6(Cg)-Tyrc-2J/J mice (stock number 000058; The Jackson Laboratory). Transgenic mouse lines were obtained or developed as noted in the text. Comparisons were made with sex, age, and, whenever possible, littermate controls. For generating bone marrow transplants, 5- to 6-wk-old C57BL/6 male mice that had been lethally irradiated with two doses of 5.5 Gy were rescued with bone marrow cells isolated from PLCγ2$^{+/+}$ or PLCγ2$^{-/-}$ mice. Animals were fed water containing sulfamethoxazole (1 g/L) and trimethoprim (0.2 g/L) for 1 wk after transplantation. Blood counts were measured 6 wk after transplantation. GPVI and CLEC-2 signaling was detected by flow cytometry using convulxin and rhodocytin, respectively. Vascular barrier function was measured 6 to 8 wk posttransplantation.

Platelet and Receptor Depletion. For the platelet immunodepletion studies, polyclonal rat anti-mouse GPIbα antibody (0.4 μg/g body weight) was administered intravenously. Blood counts were measured using a Procyte Dx Hematology Analyzer (IDEXX) 30 min, 2 h, and 24 h posttreatment. Control mice received similar amounts of nonimmune immunoglobulin. An additional set of mice received a single dose of busulfan (20 mg/kg) or vehicle by intraperitoneal (i.p.) injection. Imaging studies were performed 13 d later when the platelet count was <15% of control. For platelet recovery after depletion studies, two different doses of antibody were used. For the first set, mice received polyclonal rat anti-mouse GPIbα antibody (0.3 μg/g body weight) which led to ≥95% platelet clearance within 2 h. Thrombocytopenic mice that had recovered at least 30% of their platelet count after 24 h were imaged. In the second set, mice received polyclonal rat anti-mouse GPIbα antibody (0.4 μg/g body weight), which led to severe thrombocytopenia within 30 min that persisted for at least 24 h. Mice recovering at least 30% of their platelet count at 48 h were tested. GPVI or CLEC-2 immunodepletion was performed by intravenous (i.v.) administration of JAQ1 (0.5 μg/g) or INU1(5 μg/g) antibodies. Successful depletion of GPVI or CLEC-2 was tested as described in the text.

Time-Lapse Image Acquisition of Bloodstream and Leakage Quantification in Mouse Ear Dermis. To image the dermal microcirculation, mice were anesthetized with ketamine (100 mg/kg), xylazine (10 mg/kg), and acepromazine

(3 mg/kg) and maintained at a core temperature of 37 °C. The dorsal side of the ear to be studied was attached to an imaging platform using tissue glue. The mice were injected retroorbitally with 250 μg of 40-kDa neutral dextran-Texas red and 70-kDa dextran-fluorescein isothiocyanate (FITC). Image acquisition began 3.5 min after injection when the tracers were uniformly distributed within the circulation. For studies using bovine serum albumin (BSA)-Texas red, 1 mg of protein was injected. Imaging was performed with a Leica SP8 two photon microscope system (Leica Microsystems, Wetzlar, Germany) equipped with a femtosecond laser (Coherent, Santa Clara, CA). The fluorophores were excited at 910 nm, and images were obtained with a 20× water objective lens. Stacks of 10 to 15 images, spaced 4 μm apart, were acquired every 30 s frequently comprising of capillaries and postcapillary venules. The images obtained were converted to rainbow color-scale images according to fluorescent intensity using ImageJ (NIH). More than 50 small regions of interest in the extravascular space and adjacent to blood vessels were manually circumscribed. The mean fluorescent intensities (MFIs) of each area were measured every 30 s for 15 min post-dextran infusion. The MFIs at each time point were normalized to baseline MFI at 3.5 min. The leakage rate for each curve was determined as the rate of change of fluorescent intensity of indicated dextran in the interstitial space over time.

Statistical Analysis. All images were analyzed using Fiji ImageJ software (NIH). Statistical analysis and graphs were produced using Prism 6.0 software (GraphPad Software, San Diego, CA). The statistical significance of differences between experimental groups was determined with a parametric Student's *t* test. *P* values of less than 0.05 were considered significant.

Data Availability. All study data are included in the article and *SI Appendix*.

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