Platelets release pathogenic serotonin and return to circulation after immune complex-mediated sequestration


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There is a growing appreciation for the contribution of platelets to immunity; however, our knowledge mostly relies on platelet functions associated with vascular injury and the prevention of bleeding. Circulating immune complexes (ICs) contribute to both chronic and acute inflammation in a multitude of clinical conditions. Herein, we scrutinized platelet responses to systemic ICs in the absence of tissue and endothelial wall injury. Platelet activation by circulating ICs through a mechanism requiring expression of platelet Fcγ receptor IIA resulted in the induction of systemic shock. IC-driven shock was dependent on release of serotonin from platelet-dense granules secondary to platelet outside-in signaling by uP-selectin and its ligand fibrinogen. While activated platelets sequestered in the lungs and leaked vasculature of the blood–brain barrier, platelets also sequestered in the absence of shock in mice lacking peripheral serotonin. Unexpectedly, platelets returned to the blood circulation with emptied granules and were thereby ineffective at promoting subsequent systemic shock, although they still underwent sequestration. We propose that in response to circulating ICs, platelets are a crucial mediator of the inflammatory response highly relevant to sepsis, viremia, and anaphylaxis. In addition, platelets recirculate after degranulation and sequestration, demonstrating that in adaptive immunity implicating antibody responses, activated platelets are longer lived than anticipated and may explain platelet count fluctuations in IC-driven diseases.

Platelets are best known for their involvement in hemostasis. Their abundance in blood underlies their perfect positioning for constant surveillance of the endothelium to ensure vascular integrity. The role of platelets, however, is not restricted to the hemostatic response (1). Not only do platelets express a vast array of mediators serving wound repair but they also possess immune receptors and inflammatory molecules (1–3). Moreover, following tissue injury or infection, platelets are a central effector in driving an inflammatory process for recruiting leukocytes, principally neutrophils, to the affected site (4–6). The platelet contribution at an inflamed site also includes the prevention of bleeding (7, 8), as platelets seal the breaches formed during the extravasation of neutrophils (9).

Platelet activation occurs at the interface with the vasculature, is elicited by injury or inflammation, and is restricted to the endothelium or tissue. However, given the great number of platelets in blood and their extensive set of immune receptors, systemic immune triggers can also activate platelets while in circulation (i.e., in the absence of endothelial or tissue injury). In rheumatic diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and antiphospholipid syndrome, there is a prevalence of pathogenic antibody–antigen scaffolds, called immune complexes (ICs), in circulation in patients (10). Anaphylactic reactions due to allergens (11, 12) and i.v. administration of drugs, such as in heparin-induced thrombocytopenia (HIT) (13), also implicate ICs, thereby provoking acute platelets | immune complexes | Fc receptor | serotonin | thrombocytopenia

Immune complexes (ICs) form when antibodies encounter their antigens. ICs are present in blood in multiple pathological conditions. Given the abundance of platelets in blood and that they express a receptor for ICs, called Fcγ receptor IIA (FcγRIIA), we examined the impact of ICs in blood in a mouse model. We found that circulating ICs induced systemic shock, characterized by loss of consciousness, by activating platelet FcγRIIA. Shock was mediated by the liberation of serotonin, a molecule better known for its role in the brain, from platelet granules. During shock, platelets were sequestered in the lungs and brain and returned to the blood circulation after their degranulation. Platelets are thus crucial in response to ICs. 


Conflict of interest statement: J.E.I. has a financial interest in and is a founder of Platelet BioGenesis, a company that aims to produce donor-independent human platelets from human-induced pluripotent stem cells at scale. J.E.I. is an inventor on this patent. The authors declare no other conflict of interest.

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inflammatory responses and a rapid drop in platelet count (thrombocytopenia). ICs can also form in blood during sepsis and viremia, when antibodies in the immune host recognize microbial antigens or their toxins.

The pathological link between ICs and cellular responses is mediated by members of the Fcγ receptor (FcγR) family. The low-affinity receptor FcγRIIA is the sole FcγR expressed by platelets in humans, and is thereby the most abundantly expressed receptor in blood (12, 14, 15). Of note, it is the dominant receptor activated by Zika virus if in the presence of sera from convalescent patients infected by dengue or West Nile virus, pointing to the formation of ICs in blood in patients with preexisting antiflavivirus immunity (16). Moreover, diverse strains of bacteria and the influenza virus H1N1 trigger platelet FcγRIIA activation after forming ICs in an immune host (17, 18). In addition, studies with human platelets show that ICs from patients with SLE also activate platelets through FcγRIIA (19). However, mice do not express FcγRIIA, and murine platelets are completely devoid of any FcγRs (12). Thus, IC-mediated responses in mice strongly favor leukocytes, which express other FcγRs, while platelets play small or accessory roles in mouse models implicating ICs.

To more accurately model FcγRIIA reactions in mice, transgenic FcγRIIA (FcγRIIA<sup>TGN</sup>) mice that display FcγRIIA expression on platelets and certain leukocytes, including monocytes and neutrophils as in humans, were developed (12, 12). Notably, stimulation of FcγRIIA in transgenic mice induces a shock response that is reminiscent of anaphylaxis in humans, with a strong reliance on neutrophil activity and implicating release of platelet-activating factor (PAF) (14, 19–21).

How ICs trigger systemic responses is not completely understood, as no study has definitively determined the contribution of platelets to this response in vivo. Here, we employed FcγRIIA<sup>TGN</sup> mice to elucidate the precise mechanisms by which platelets contribute to IC-mediated shock.

Results

Platelets Are Critical During the Systemic Response. ICs injected into FcγRIIA<sup>TGN</sup> mice trigger systemic shock (22–24). Although thrombosis in the lungs and thrombocytopenia accompany systemic shock in these mice (22, 25–27), the platelet contribution to IC-induced shock has never been formally assessed. We used heat-aggregated HA-IgG (HA-IgG (160 ± 7 nm in diameter) was injected i.v. into WT mice lacking FcγRIIA (FcγRIIA<sup>null</sup>) and FcγRIIA<sup>TGN</sup> mice. Significant temperature loss (15 min) preceded by rapid significant systemic shock (3 min) in all of the FcγRIIA<sup>TGN</sup> mice was confirmed, but it was never observed in the FcγRIIA<sup>null</sup> mice (Fig. L4 and Movie S1). All of the FcγRIIA<sup>TGN</sup> mice collapsed, underwent profound immobility, and lost consciousness (Fig. S1A). Mice recovered from the shock and hypothermia within 180 min, after which no residual signs were apparent (Fig. S1B). The occurrence of shock was dependent on the concentration of ICs present in blood, not induced by monomeric IgG, similarly induced when murine ICs were used, and more profound in male FcγRIIA<sup>TGN</sup> mice compared with females (Fig. S1 C–E).

To formally assess the contribution of platelets to the systemic shock, we depleted platelets (>98%) before injection of ICs. Thrombocytopenic mice were completely protected from IC-mediated systemic responses (Fig. 1F and Fig. S1F), identifying a critical role for platelets in shock induced by circulating ICs. In platelets, efficient αIbb3 signaling relies on the FcγRIIA immunoreceptor tyrosine-based activation motif, and, accordingly, the expression of FcγRIIA by platelets enhances fibrinogen-mediated platelet activation (30). Thus, we verified the functional association of FcγRIIA and the αIbb3 receptor in vivo by comparing the response in FcγRIIA<sup>TGN</sup>/β3<sup>−/−</sup> and FcγRIIA<sup>TGN</sup>/β3<sup>+/+</sup> mice. The FcγRIIA<sup>TGN</sup>/β3<sup>−/−</sup> mice were completely resistant to IC challenge (Fig. 1C), further emphasizing the role of platelets and pointing to the requirement of αIbb3 in the mechanism of IC-mediated shock.

While αIbb3 may amplify FcγRIIA signaling through common kinases located in the platelet cytoplasm, it is predominantly known as the fibrinogen receptor, which may contribute upon binding to platelet activation (31–33). Fibrinogen also mediates platelet–neutrophil interactions by bridging αIbb3 and macrophase-1 antigen (Mac-1) (34). Hence, platelet–neutrophil aggregates formed in blood in the presence of ICs (Fig. S2A), as well as neutrophils, were also necessary in the systemic responses (Fig. S2B), in agreement with prior studies (23, 28). To verify the specific role of fibrinogen, we used mutant fibrinogen chimeric mice. We lethally irradiated fibrinogen<sup>Δ5</sup> (Fib<sup>Δ5</sup>) and fibrinogen<sup>390-396A</sup> (Fib<sup>390-396A</sup>) mutant mice, which express mutant fibrinogen unable to bind αIbb3 or Mac-1 (34, 35), respectively, and engrafted the mice with bone marrow from FcγRIIA<sup>TGN</sup> mice. After injection with ICs, we found that fibrinogen binding to αIbb3 was necessary for the systemic response, whereas bridging platelets with leukocytes through fibrinogen binding to Mac-1 was dispensable (Fig. 1D and E). Consistent with these data, the blockade of Mac-1 as well as PSGL-1, the counterreceptor of platelet P-selectin on leukocytes (36), had no effect on shock (Fig. S2C).

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Like αIbβ3, GPDH is critical in the prevention of bleeding and thrombosis, and the ablation of the gene coding for GPIb in mice leads to severe bleeding defects (37). As GPDH is localized within lipid raft membrane microdomains in physical proximity with FcγRIIA (38), we used a GPDH-blocking Fab to probe the contribution of GPDH, and found that it is dispensable in shock mediated by ICs (Fig. 1F). Furthermore, the pharmacological blockade of von Willebrand factor (vWF) binding to GPDH (ATA; aurintricarboxylic acid) or administration of recombinant tissue plasminogen activator (tPA; alteplase) to promote the lysis of any existing thrombi also had no effect on shock (Fig. 1 G and H). These findings further demonstrate that platelet activation by circulatory ICs implicates pathways distinct from those promoting vaso-occlusion and vascular injury.

Identification of the Shock Mediator. Consistent with the rapid response, an increase in platelet factor 4 (PF4) and serotonin, components stored in α- and δ-granules, respectively, was detected in blood within 10 min (at the time of mouse collapse and before hypothermia) of the IC trigger in FcγRIIA+TGN mice (Fig. 2A). We therefore hypothesized that a component released from platelet granules may be responsible for induction of systemic shock. Intravital microscopy of the microvasculature using two-photon microscopy revealed significant vessel leakage and vasodilatation in response to IC injection in FcγRIIA+TGN mice (Fig. 2 B and C). Vessel leakage was systematically observed in response to IC injection in both FcγRIIA+TGN and FcγRIIA−null mice, but was absent in FcγR−null mice, pointing to the role of other receptor(s) for ICs (Fig. S3A). It was also maintained in the absence of neutrophils, further suggesting that vascular leakage is not the key pathological feature downstream of FcγRIIA driving IC/platelet-mediated shock (Fig. S3A).

In stark contrast, vasodilatation was a response observed exclusively in FcγRIIA+TGN mice (Fig. 2C). Identified first as a serum agent mediating vascular tone, serotonin is a powerful vasoconstrictor when added to smooth muscle cells. However, it is a potent vaso dilator on endothelial cells (39). In the case of systemic activation in the absence of vascular injury, and thus an intact endothelium, we hypothesized that serotonin might be the key platelet component driving vasodilatation. This is supported by the fact that the majority of peripheral serotonin, which represents 95% of the total body serotonin pool, is stored in platelet δ-granules (40, 41). Platelets do not synthesize serotonin; they utilize their serotonin transporter (SERT) to capture circulatory serotonin, with the latter being generated by enterochromaffin cells from the digestive tract by the enzyme tryptophan hydroxylase 1 (Tph1) (42, 43). Consistent with our hypothesis, vasodilatation was abrogated in FcγRIIA+Tph1−/− mice in response to ICs (Fig. 2C). It was also maintained in absence of neutrophils, further confirming the direct role of peripheral serotonin in IC-induced vasodilatation of microvessels (Fig. S3B).

Serotonin levels in plasma were back to normal 1 h postshock (Fig. S3C). To directly verify its role in systemic shock, serotonin was injected into FcγRIIA−null mice, which resulted in a shock response reminiscent of the responses observed in IC-injected mice (Fig. 2D). We then used a selective serotonin reuptake inhibitor (SSRI), a SERT blocker used as an antidepressant, to inhibit serotonin storage by platelets. Administration of the SSRI for 3 wk to deplete the serotonin content of δ-granules (41) revealed the important contribution of serotonin uptake in IC-mediated inflammation: SSRI treatment nearly abolished the systemic shock response (Fig. 2E). Furthermore, FcγRIIA+Tph1−/− mice lacking platelet serotonin (43) showed almost complete resistance to shock (Fig. 2F), confirming the critical role of peripheral serotonin in IC-mediated shock. In addition, blockade of the 5-hydroxytryptamine 2 receptor family, which is expressed in the periphery by platelets, but also by monocytes and macrophages, dendritic cells, eosinophils, B and T lymphocytes, endothelial cells, fibroblasts, cells from the cardiovascular system, and neurons in the peripheral nervous system (40), was effective at reducing shock (Fig. 2G). In support of the potential role of αIbβ3 in degranulation, serotonin was not released in FcγRIIA+Tph3−/− mice after IC injection, in agreement with the absence of shock in those mice (Fig. S3D). Key actors in the prevention of bleeding and thrombosis, the platelet-derived mediators ADP and thromboxane A2, were dispensable for shock (Fig. S3 E and F). In addition, neutrophil extracellular traps, which can be present in thrombosis (44), were detected during shock according to quantifications of circulating nucleosomes (Fig. S3G); however, the injection of DNase did not protect mice from shock (Fig. S3H).

Together, these observations highlight the importance of platelet-derived serotonin in the systemic response to ICs in vivo, a platelet response that is distinct from that traditionally observed in hemostasis and thrombosis.

And the Platelet Count? Circulating platelets were also monitored throughout the systemic response and beyond. We observed that FcγRIIA+TGN mice rapidly underwent profound thrombocytopenia (~10% of total normal platelet count) in response to ICs, whereas FcγRIIA−null mice presented with only very minimal or...
no changes in platelet counts (Fig. 3A). In fact, thrombocytopenia occurred rapidly (<10 min), and the platelet count gradually increased, reaching ~20% by 60 min, and it was 50% resolved within 24 h (Fig. 3A). The occurrence of thrombocytopenia was critically dependent on the expression of FcγRIIA and was similarly induced in males and females (Fig. S4A). Only platelets were affected, as the levels of RBCs, monocytes, and neutrophils were unchanged in response to ICs (Fig. S4 B-D). Thrombocytopenia was as profound in native fibrinogen mice as in FibγΔΔ and Fibγ390−396A mice (Fig. 3B), suggesting that fibrinogen binding was dispensable, and that thrombocytopenia only modestly involved β3, potentially through binding to its other ligands. Conversely, thrombocytopenia was unaltered by the inhibition of GP Ib and vWF interactions, or the blockade of thromboxane A₂ synthesis and ADP (Fig. 3B). Thrombin–antithrombin complexes and D-dimers, which are evidence of coagulation activation and thrombus degradation, respectively, were not significantly elevated 24 h after shock (Fig. S4 E and F), and consistent with this, the destruction of potential thrombi by the injection of alteplase did not impact platelet count (Fig. 3B). These data further dissociate the platelet response in coagulation and thrombosis from IC-induced thrombocytopenia.

Platelet conversion to microparticles also did not explain the profound thrombocytopenia, as microparticle levels only increased 60 min after the IC trigger (Fig. S4C). The decrease in platelet number could also not be attributed to platelet interaction with leukocytes, as neutrophil depletion, or blockade of Mac-1 and PSGL-1, had no effect on thrombocytopenia (Fig. S4D). Given that FcγRIIA⁻/⁺Tph1⁻/⁺ mice also underwent profound thrombocytopenia (Fig. 3B), and that exogenous serotonin did not induce thrombocytopenia (Fig. S4E), this suggests that serotonin serves as the effector of IC-mediated shock but is not the driver of the resulting thrombocytopenia. Thus, although thrombocytopenia occurs concurrently with shock, it is not causative in the mechanism of inducing the shock.

The unanticipated rapid recovery in platelet count prompted a detailed analysis of the platelets present in blood 24 h after IC challenge. We found that platelets were not activated or apoptotic, as evidenced by the lack of phosphatidylserine (PS) and P-selectin on their surface (Fig. 3C). However, the platelet granule content was markedly reduced (Fig. 3D), as ~30% of the circulating platelets contained no detectable serotonin or PF4 (Fig. 3E), suggesting significant degranulation. Electron microscopy further confirmed that circulating platelets were frequently devoid of any granules (Fig. 3F). Notably, platelets still expressed surface FcγRIIA 24 h postshock (Fig. S5 A and B), and still underwent thrombocytopenia if challenged a second time with ICs (Fig. S5C). This is in contrast to shock, where FcγRIIA⁻/⁺ mice were resistant to shock induced by further challenges (Fig. 3G), and neither serotonin nor PF4 was induced in the blood of these mice (Fig. 3H). Thus, we hypothesized that platelets circulating 24 h postshock were, in fact, platelets that had already degranulated and had undergone temporary sequestration. To verify this, we performed fluorescent labeling of FcγRIIA⁻/⁺ platelets, which we adoptively transferred into FcγRIIA⁻/⁺ mice. As expected, 87 ± 12% of fluorescent platelets, which were negative for PS (Fig. S5D), rapidly became undetectable from the blood circulation after an IC trigger. Of particular importance, 42 ± 16% of the fluorescently labeled FcγRIIA platelets, devoid of any surface PS, were identified in blood 24 h after shock (Fig. 3I), confirming a temporary sequestration of platelets following FcγRIIA activation and their return to the blood circulation after degranulation.

Localization of Sequestration Sites. We next aimed to determine the sites of platelet sequestration in response to FcγRIIA activation. An intravital imaging system (IVIS) provided evidence of fluorescently labeled platelets in the lungs (Fig. 4A), but not in
other locations, in agreement with previous studies (22, 25–27). Platelet thrombi populated with neutrophils were also evident in the lungs (Fig. 4B), although no pulmonary edema was observed (Fig. 4C). Thrombi contribution to platelet sequestration and shock was not significant: ablation of the β3 gene, or blockade of GPIb using Fab, significantly reduced thrombus formation in FcγRIIA TG/N mice (Fig. 4D) with only a modest or no impact on thrombocytopenia, respectively (Fig. 3B). Furthermore, serotonin had no effect on thrombus formation, as the number of thrombi in the lungs remained unchanged in FcγRIIA TG/N/Tph1 knockout mice (Fig. 4D). These data identify the lungs as the apparent major site for platelet sequestration, and highlight that although thrombi are present in the lungs, these thrombi play no role in the thrombocytopenia and shock mediated by ICs.

However, we suspected that other anatomical sites of platelet sequestration might exist, as the IVIS approach may not permit an optimal distinction between immobilized platelet aggregates and circulating platelets in the microvasculature, where small platelet aggregates are to be expected. In addition, if the entire platelet population was sequestered in the lungs, it was puzzling that none of the mice died from the IC trigger. Using a quantitative approach to measure fluorescently labeled CD41+ platelets, we estimated that the lungs, in fact, contained only 16% of the total platelet load (Fig. 4E), confirming that other sites of platelet sequestration likely existed.

The presence of platelets outside the vasculature was excluded first, as no platelets were detected in the thoracic lymph (Fig. S6A). Furthermore, assessment using whole-mouse imaging of yellow fluorescent protein (YFP) in FcγRIIA TG/N/CD41-YFP mice, which constitutively express YFP in CD41-expressing cells, efficiently identified thrombi in the lungs and megakaryocytes in the bone marrow, but did not reveal platelets outside blood vessels or in other tissues (Fig. S6B). Therefore, we speculated that tracking ICs in mice might be a more efficient means of leading us to platelet sequestration sites. We found that ICs not only localized to the lungs but were also observed in the brain vasculature (Fig. 4F). Thus, the brain microvasculature was examined using two-photon microscopy in live FcγRIIA TG/N/CD41-YFP mice, with the inclusion of FcγRIIA TG/N/CD41-YFP mice for comparison. We observed profound leakage of the brain vasculature in both FcγRIIA null and FcγRIIA TG/N mice when injected with ICs (Fig. 4G and Movies S2 and S3). Of importance, in the presence of ICs, small platelet aggregates readily formed in the leaky brain microvasculature, but only if FcγRIIA was expressed by platelets (Fig. 4G and Movie S2). Thrombi were not detected in the microvasculature of the kidney, liver, or spleen of FcγRIIA TG/N mice injected with diluent or ICs (Fig. S6C), and two-photon microscopy of the femurs in live FcγRIIA TG/N/CD41-YFP mice did not reveal any platelet aggregation in sinusoids in the bone marrow (Fig. S6D). These data suggest that in the presence of ICs, platelets sequester in certain microvascular beds, notably in the lungs and brain, and that this event occurs independent of thrombosis and leakage.

Role of Platelet FcγRIIA and Serotonin in Acute Inflammatory Responses. During microbial invasion, foreign antigens are recognized by host antibodies and form ICs. Hence, incubation of human platelets in the presence of influenza virus or various strains of bacteria leads to platelet activation, which strictly requires the presence of plasma and FcγRIIA (17, 18). While these observations suggest that motifs on pathogens contribute to the formation of ICs, whether they can trigger platelet activation in vivo has not been established.

Antibodies against lipopolysaccharide (LPS), a common gram-negative pathogen-associated molecular pattern (PAMP), were detected in the blood of healthy volunteers similar to patients with septic shock due to confirmed gram-negative bacteria infection (Fig. 5A). In contrast to humans, mice housed in a facility with high standards of cleanliness present no detectable anti-LPS antibodies (nonimmune mice) (Fig. 5B). Therefore, we immunized mice with small quantities of bacterial PAMP (LPS), which had no perceptible effect on FcγRIIA null or FcγRIIA TG/N mice (Fig. 5C). FcγRIIA null and FcγRIIA TG/N mice developed equivalent anti-LPS antibody levels within 3 wk (LPS-immune mice) (Fig. 5B), and, importantly, a subsequent injection of LPS only induced serotonin release and shock in FcγRIIA TG/N mice (Fig. 5 C and D). The shock induced by LPS-containing ICs was dependent on the presence of platelets, and was greatly diminished in the absence of peripheral serotonin or β3 gene expression (Fig. 5E–G). Moreover, plasma serotonin levels were significantly reduced in FcγRIIA TG/N/β3−/− mice after LPS injection (Fig. 5D). Thrombocytopenia was dramatically increased in the presence of...
mental conditions (23, 28, 29). Of importance is that in all models, platelet FcRIIA and serotonin were involved in shock, while FcRIIA was implicated in thrombocytopenia (Fig. S7 C–E).

Thus, as in our passive model of IC-mediated immune reaction, active immunization with gram-negative PAMPs, virus, or protein antigen dominantly implicates platelet FcRIIA and serotonin, and presents with transient platelet sequestration. The mechanisms unveiled in this study are illustrated in Fig. 6.

Discussion

In this study, we examined platelet activity in IC-induced systemic inflammation in the absence of vascular insult. We confirmed a central role for platelet activation and identified serotonin as a critical platelet component mediating mechanisms of shock.

Albeit artificial, models utilizing ferric chloride, laser-induced injury, or injection of cytokines or PAMP in tissue/organ (e.g., crenameter, muscle, liver, lungs) (4–6, 9, 47) have provided crucial insights to key pathways implicated in inflammation. Herein, we used HA-IgG as a surrogate model of ICs and revealed critical components in the in vivo response to systemic ICs, which were further confirmed using active immunization with endotoxin, virus, or a commonly used antigen in the study of anaphylaxis. Our observations were only possible using FcRIIA(TGN)$^{\text{null}}$ mice, where we confirmed the functional association of FcRIIA and $\alpha$IIb$\beta$3 in platelet activation, and suggest that targeting these receptors may have clinical benefits in severe conditions involving ICs. Conversely, other molecules (i.e., P-selectin, GPIb, ADP, thromboxane) that classically play a dominant role in platelet activation in thrombosis, or favor platelet and neutrophil interactions, were dispensable. The contribution of neutrophils to the systemic response to ICs is, however, not excluded, as neutrophils have also been previously implicated, possibly through the release of PAF (12, 23, 28). Hence, although serotonin release was maintained in the absence of neutrophils, shock was dramatically reduced (Figs. S2 B and S8 A). We thus propose that the release of serotonin precedes the neutrophil contribution to shock, consistent with the reported role of serotonin in neutrophil activation (48). As vasodilation was also present in the absence of neutrophils (Fig. S3 B), these data suggest that platelets, through serotonin, orchestrate neutrophil activation and endothelial cell functions, and that these events can occur independently (Fig. 6).

Vascular leakage is another feature systematically observed when ICs were present in blood. Intriguingly, leakage and vasodilation were not connected, as leakage took place independent of FcRIIA but vasodilation critically required FcRIIA and serotonin. As such, vessel permeability likely involved smaller postcapillaries vessels and more subtle changes in endothelial cell interactions, and was also insufficient to induce significant edema in the lungs, although platelets and neutrophils accumulated in great number in the lungs. In addition, leakage critically involved IC-mediated signaling, given that it was totally absent in FcRγ(TGN)$^{\text{null}}$ mice (Fig. S3 A). While neutrophils express other IgG receptors (other than FcRIIA), they were dispensable in the process (Fig. S3 A). Therefore, leakage could be attributed to mast cells or basophils, for instance, which also express an array of receptors, such as FcRI and FcRII, capable of responding to ICs and can mediate permeability, potentially through histamine release (49).

Whereas serotonin is mostly known for its role in mood, anxiety, psychosis, or memory in the central nervous system, more than 95% of total body serotonin is present in the periphery (39). The majority of peripheral serotonin is stored in platelets, and our observations further revealed that platelets from female mice contain less serotonin than those from males (Fig. S8 B), which may explain, in part, the aggravated phenotype observed in male mice compared with females following IC challenge. Moreover,
although platelets in females and males expressed similar levels of FcγRIIA, female FcγRIIA TGN mice presented significantly lower platelet counts than males, which could also partially explain the reduced shock in females in comparison to male mice (Fig. S8 C–E). Other immune components, such as complement C5a, are also more abundant in males than in females (50). Whether the serotonin reservoir in platelets explains a fundamental gender-related dichotomy in susceptibility to inflammatory responses to ICs remains to be established.

The function of serotonin in platelets is not clear; studies suggest that it is important for the serotonylation of proteins necessary in platelet aggregation (51). However, SSRIs are typically used by patients during the perioperative period and mice lacking peripheral serotonin present only mild bleeding defects (43). Thus, the present study sheds light on a major role of platelet serotonin in response to systemic ICs are unclear. We can speculate that in response to a microbial invasion in an immune host, it might be preferable to reduce blood flow to prevent dissemination of the pathogen to vital organs and to facilitate its capture by phagocytes. As serotonin also mediates organ regeneration (52), its liberation may be pivotal to regrowth following insults caused by pathogen invasion.

Thrombocytopenia, which coincides with thrombi formation in the lungs, has been reported to occur in FcγRIIA TGN mice, and requires the expression of guanine nucleotide exchange factor CalDAG-GEF1 and 12-lipoxygenase (22, 25–27, 53). We showed here that the formation of thrombi or occlusion of blood vessels was not the primary cause of shock; thrombosis was characterized in lungs following IC administration. It implicates FcγRIIA and β3, and, in contrast to shock, it involves GPIb and not serotonin. These data support the notion that shock and thrombosis are independent events. Vascular leakage occurs independent of platelets and FcγRIIA. Molecules implicated in leakage are presented in the figure. NETosis, neutrophil extracellular traps; P-sel, P-selectin.

Fig. 6. Platelets release pathogenic serotonin and return to blood circulation after IC-mediated degranulation and sequestration. Sequential events (numbered 1–6) were observed when circulating ICs encountered platelets. Platelets are abundant in blood and in humans (not in WT mice); they express FcγRIIA, a low-affinity receptor for IgG. ICs activate FcγRIIA present on platelets (1), which change αIIbβ3 to its active conformation (2). (3) Active αIIbβ3 binds its extracellular ligand fibrinogen, which mediates outside-in signaling and granule release. In the absence of αIIbβ3, there is no granule release. It is suggested (dotted line) that serotonin engages neutrophils (4), and it is further hypothesized (dotted line) that serotonin induces vasodilatation through its action on endothelial cells (5). (6) Multiple manifestations are observed when ICs form in blood. Shock, characterized by loss of consciousness, immobility, shallow respiration, and hypothermia, strictly implicates platelets, αIIbβ3 binding to fibrinogen, and serotonin release. In the absence of neutrophils, serotonin is released but shock is abolished. It is surmised that neutrophils produce PAF in response to serotonin, which may contribute to shock downstream of serotonin release. Mediators of shock are indicated in the figure. Thrombocytopenia is due, at least in part, to platelet sequestration in certain vascular beds, notably in the lung and brain microvasculature. Sequestration implicates FcγRIIA but, in contrast to shock, occurs independent of serotonin and neutrophils, and only partially implicates β3. Thrombocytopenia is only transient, and platelets return to blood circulation with emptied granules. Microparticle release is observed in blood before return of platelets. Roles of different molecules in thrombocytopenia are indicated in the figure. Vasodilatation is implicating platelet-derived serotonin and is occurring independent of the presence of neutrophils. Thrombosis was characterized in lungs following IC administration. It implicates FcγRIIA and β3, and, in contrast to shock, it involves GPIb and not serotonin. These data support the notion that shock and thrombosis are independent events. Vascular leakage occurs independent of platelets and FcγRIIA. Molecules implicated in leakage are presented in the figure. NETosis, neutrophil extracellular traps; P-sel, P-selectin.

Table 6

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<td>FcγRIIA</td>
<td>β3</td>
<td>Serotonin X</td>
<td>FcγRIIA</td>
</tr>
<tr>
<td>GP Ib</td>
<td>Platelets X</td>
<td>Neutrophils X</td>
<td>GP Ib X</td>
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<td>Neutrophils X</td>
<td>Serotonin X</td>
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<td>Serotonin X</td>
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<td>Cox 1 X</td>
<td>ADP X</td>
<td>Fibrinogen X</td>
<td>SHT2a X</td>
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<td>Fibrinogen X</td>
<td>Thrombosis X</td>
<td>Fibrinogen X</td>
<td>NETosis X</td>
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Fig. 6. Platelets release pathogenic serotonin and return to blood circulation after IC-mediated degranulation and sequestration. Sequential events (numbered 1–6) were observed when circulating ICs encountered platelets. Platelets are abundant in blood and in humans (not in WT mice); they express FcγRIIA, a low-affinity receptor for IgG. ICs activate FcγRIIA present on platelets (1), which changes αIIbβ3 to its active conformation (2). (3) Active αIIbβ3 binds its extracellular ligand fibrinogen, which mediates outside-in signaling and granule release. In the absence of αIIbβ3, there is no granule release. It is suggested (dotted line) that serotonin engages neutrophils (4), and it is further hypothesized (dotted line) that serotonin induces vasodilatation through its action on endothelial cells (5). (6) Multiple manifestations are observed when ICs form in blood. Shock, characterized by loss of consciousness, immobility, shallow respiration, and hypothermia, strictly implicates platelets, αIIbβ3 binding to fibrinogen, and serotonin release. In the absence of neutrophils, serotonin is released but shock is abolished. It is surmised that neutrophils produce PAF in response to serotonin, which may contribute to shock downstream of serotonin release. Mediators of shock are indicated in the figure. Thrombocytopenia is due, at least in part, to platelet sequestration in certain vascular beds, notably in the lung and brain microvasculature. Sequestration implicates FcγRIIA but, in contrast to shock, occurs independent of serotonin and neutrophils, and only partially implicates β3. Thrombocytopenia is only transient, and platelets return to blood circulation with emptied granules. Microparticle release is observed in blood before return of platelets. Roles of different molecules in thrombocytopenia are indicated in the figure. Vasodilatation is implicating platelet-derived serotonin and is occurring independent of the presence of neutrophils. Thrombosis was characterized in lungs following IC administration. It implicates FcγRIIA and β3, and, in contrast to shock, it involves GPIb and not serotonin. These data support the notion that shock and thrombosis are independent events. Vascular leakage occurs independent of platelets and FcγRIIA. Molecules implicated in leakage are presented in the figure. NETosis, neutrophil extracellular traps; P-sel, P-selectin.
were observed in the lungs and brain, principally in the smaller and intertwined vessels, platelets may also be hiding in other yet-to-be-discovered vascular beds despite our careful investigations. In thrombotic thrombocytopenic purpura, profound thrombocyto-
penia is explained by the failure of ADAMTS13 to perform proteolysis of vWF attached to the endothelium (54), a mecha-
nism distinct from what is observed in IC-induced thrombocyto-
penia, as the blockade of vWF and GP Ib had no effects on thrombocytopenia in our study. As in-depth whole-mouse imaging uniquely identified megakaryocytes and thrombi, which comprise more stable and adherent platelets, it suggests that the majority of the sequestered platelets were dislodged by the perfusion pro-
cedure. Therefore, we propose that platelets might be bridged together by ICs, and that platelet–IC scaffolds may be loosely trapped in the microvasculature.

Degranulated platelets recirculate, a finding of potential sig-
ificance for elucidating mechanisms underlying thrombocyto-
penia. The absence of PS at the surface suggests that they are not procoagulant platelets, known as balloon- or zombie-like platelets (55). How platelets return to the circulation after se-
questration is unclear, but it is reasonable to speculate that disengagement of FcγRIIA after its desensitization by yet un-
known mechanisms (e.g., unidentified immunoreceptor tyrosine-
based inhibitory motif-containing receptors or phosphatases) or IC internalization by platelets (56) might release platelets from platelet–IC scaffolds and permit their liberation from the mi-
crovasculature. FcγRIIA expression was maintained, however, on recirculating platelets, suggesting that it might be recycled if the internalization of ICs is implicated. Of interest is that platelets at thrombi surfaces visualized in vascular injury models appear loosely packed and lightly activated (57, 58), and might also return to the circulation. As platelets activated in vitro with thrombin can also circulate after degranulation if transfused (59, 60), our study reveals that thrombotic and immunological trig-
gers can induce degranulation independent of platelet elimina-
tion. These models contrast with the general belief that platelets “have only one life,” and may not recirculate after undergoing activation in vivo.

The insertion of human activating (FcγRIIA/RIIb/hIbb) and
inhibitory (FcγRIIB) FcγRI into the equivalent murine locus confirmed the predominance of FcγRIIA in systemic shock in the humanized mouse (28), suggesting that our findings may well be translatable to humans as platelets from transgenic mice and humans express equivalent levels of FcγRIIA (18). While these approaches cannot fully recapitulate all of the subtleties of IC-driven inflammation in humans, it is very likely that the mechanisms revealed in our study may, at least in part, take place in disease states such as rheumatic disease, HIT, sepsis, viremia, anaphylaxis, and adverse reactions following i.v. IgG therapy.

PAMPs trigger innate immune responses through activation of pattern-recognition receptors, but the recognition of PAMPs by antibodies in adaptive immunity can modulate different re-
sponses (61). We found that LPS, a Toll-like receptor 4 (TLR-4) trigger, dominantly implicates platelets and serotonin when in-
volved in the adaptive immune response in mice that had been preexposed to LPS. All adult volunteers we examined also dis-
played antibodies directed against the endothetoxin. Interestingly, humans lacking TLR signaling molecules are extremely suscepti-
able to infections in infancy and childhood, and thereafter de-
velop significant resistance, consistent with the prevalent role of adaptive immunity in adults (62). Sepsis susceptibility is associ-
ated with FcγRIIA polymorphism (63) and is accompanied by elevated serotonin and endothelial hyperpermeability (64), which could be attributed to platelets. Furthermore, thrombo-
cytopenia measured in patients with sepsis is a strong predictor of mortality (65, 66), and our data suggest that platelets may be sequestered through FcγRIIA activation in some patients. It is thus important to note that most mouse models of sepsis or viral infection may be suboptimal, as they overlook the contribution of FcγRIIA in a nonimmune host. Observations from trials during which LPS was injected into volunteers revealed that humans responded more promptly to small doses of LPS than mice (67), pointing to the involvement of ICs and FcγRIIA in humans, a pathway absent in mice.

Circulating platelets presenting reduced granule content are
reported in different contexts, such as cardiovascular diseases, type-2 diabetes mellitus, preeclampsia, autoimmune diseases, and sepsis (68–72). In patients with severe sepsis, ADP and se-
rotonin contents in platelets were reduced by 40% and 50%, respectively (71). However, very little is known about the impact of in vivo platelet degranulation on platelet life span. In chronic conditions implicating ICs, such as rheumatic diseases, ICs constantly trigger platelet FcγRIIA, consistent with the frequent shifts in platelet count. Hence, microthrombi are recognized in these diseases, including in the lungs, and the platelet content of serotonin is reportedly reduced in patients with SLE and RA by 25% and 27%, respectively (72, 73), suggesting that platelets might indeed circulate in their degranulated form in various contexts implicating ICs (70). Neuropsychiatric SLE is the least understood, yet the most prevalent, manifestation of SLE (74). Our identification of localized platelet activation in the brain microvasculature and leakage of the blood–brain barrier may thus have implications in the important and poorly understood neurological manifestations in rheumatic diseases.

In summary, our study reveals platelet contributions to in-
flammation in reactions involving ICs. It appears that the FcγRIIA signaling and serotonin release are unique in regard to their major role in inflammation and minor roles in the prevention of bleeding, suggesting that interference in this process might be a promising avenue for further research.

Materials and Methods

Mice. C57BL/6J (FcγRIIA<sup>−/−</sup> mice), FcγRIIA<sup>−/−</sup>hemizygous mice (12, 20), and Fcγ<sup>−/−</sup> mice were obtained from The Jackson Laboratory. FcγRIIA<sup>−/−</sup> hemi-
gous mice described to express human FcγRIIA on platelets, megakaryo-
cytes, monocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, and dendritic cells (12, 20) were backcrossed to C57BL/6J more than 10 times. The β3<sup>−/−</sup> mice (30), T<sup>h</sup>1<sup>−/−</sup> mice (43), and CD41<sup>−/−</sup> YFP mice (75) were crossed with FcγRIIA<sup>−/−</sup> mice to obtain FcγRIIA<sup>−/−</sup>β<sup>3<sub>−/−</sub></sup> (30), FcγRIIA<sup>−/−</sup> T<sup>h</sup>1<sup>−/−</sup>, and FcγRIIA<sup>−/−</sup>CD41<sup>−/−</sup> YFP mice. Chimeric mice were generated by transfer of bone marrow cells of FcγRIIA<sup>−/−</sup> mice into FcγRIIA<sup>−/−</sup> (WT), F<sub>β<sub>3<sub>−/−</sub></sub></sub> and F<sub>β<sub>3<sub>−/−</sub></sub> Irradiated mice (34, 35, 76). Guidelines of the Canadian Council on Animal Care were followed in a protocol approved by the Animal Wel-
fare Committee at Laval University (2013-106-3).

HA-IgG as an IC Model. Human IgG (IC) (MP-biological, Sigma–Aldrich and Innovative Research) were i.v. injected (600 µg and 750 µg) in males and females, respectively. In some experiments, human IgG was dissolved but not neutralized (monomeric) or mouse IgG was aggregated and injected in mice (29).

Measure of Shock. Temperature was measured using a rectal probe ther-

ometer at indicated time points. Signs of apparent shock were assessed as described previously (24, 77) using a score from 0 to 3 (24, 77). A score of 3 represents completely immobilized and unconscious mice (mice collapse and do not react to sound or touch), a score of 2 represents mice with im-
paired mobility and irregular respiration, a score of 1 corresponds to mice with slow motions and shallow respiration, and a score of 0 describes normal mice. In anaphylaxis experiments implicating BSA injection in BSA-
immunized FcγRIIA<sup>−/−</sup> mice, death was sometimes observed. In those ex-
ceptional cases, shock was scored as 4. Scores measured in experimental groups were averaged and are presented as a function of time (minutes). Scores for individual mice for key experiments are provided in SI Materials and Methods (Fig. S1 A and P).

LPS Immunization Model. Mice were immunized with i.v. injection of 1 mg/kg

LPS (Escherichia coli 0111:B4; Sigma–Aldrich) at days 0, 14, 28, and 42. At the first and the last immunizations, shock and temperature were monitored for 1 h. Mouse blood was drawn by cardiac puncture 10 min after the fourth...
Flow Cytometry. Flow cytometry was performed using a BD FACSCanto II instrument with forward scatter coupled to a photomultiplier tube “small particles option” flow cytometer (BD Biosciences). Platelets, platelets interacting with neutrophils, and platelet microparticles were analyzed.

Histology. In some experiments, organs were collected at the end of the experiment. Intratracheal instillation with 1 mL of 4% paraformaldehyde in lungs was performed before collection. Brain, kidneys, spleen, heart, liver, and lungs were collected and then fixed in 4% paraformaldehyde for 24 h (lungs) or 72 h (other organs); they were then washed and stored in PBS at 4 °C before histology. After fixation, paraffin-embedded organs were cut into 5-μm sections and stained with hematoxylin and eosin. Thrombi were observed on five different spots at 400x resolution, they were counted using light microscopy (BX51, Olympus) by a blinded investigator, and the numbers of lung thrombi per square millimeter were calculated. Neutrophils were identified in lungs of i-Injected FcγRIITGON mice.

Two-Photon Intravital Microscopy. For in vivo imaging of the mouse brain, FcγRIITGON/CD41-YFP mice (8–12 wk old) were anesthetized with 1–2% isoflurane (vol/vol) and a cranial window was made to expose the vasculature of the sensorimotor cortex. Animals were imaged 2 wk after the surgery. Briefly, for the imaging session, the head of the mouse was restrained using a custom-built cranial stereotaxic apparatus (David Kopf Instruments) and placed under the microscope. For the ear imaging, mice were anesthetized and the hair recovering the ears was gently removed using Nair, a commercial depilatory lotion. One ear then was gently flattened and fixed on a Plexiglas bloc using MEl-EpidermGlu (Medisav Services). Gelseal (GE Healthcare) was applied around the tissues to form a watertight rim, and the imaging cavities were filled with sterile HBSS without Ca2+ and Mg2+ (Gibco). Body temperature was kept at 37 °C throughout all procedures with a heating pad. Four to six different vessels per mouse were analyzed at 1 and 8 min postinjection.

Patients with Septic Shock. Adult (age ≥ 21 y) patients with septic shock and sepsis (118:1113 J Thromb Haemost, 356:175 J Clin Invest) were recruited from the surgical, medicine, and cardiac intensive care units. After approval of the protocol, patients were enrolled in the study between June 2016 and March 2017, with a follow-up period of 1 year. Inclusion criteria were: age ≥ 18 y; sepsis or septic shock (118:1113 J Thromb Haemost, 356:175 J Clin Invest) defined by the Surviving Sepsis Campaign guidelines (11:264 Crit Care Med, 194:5579 JAMA, 194:5579 JAMA) and accompanied by adequate fluid resuscitation (S. 6). For identification of gram-negative bacteria, blood samples were obtained from patients upon intensive care unit (ICU) admission as part of their routine clinical care. Blood samples underwent gram staining and culturing in a clinical pathology laboratory. Gram-negative pathogens were identified from the gram stain and/or cultures by the clinical laboratory. Healthy, fasting adult (age ≥ 21 y) control subjects provided informed consent. Following informed consent, demographic data, physiological parameters, and laboratory data were recorded. Plasma was harvested by centrifugation on whole blood collected in sterile ACD vacutainer tubes. Plasma was frozen at −80 °C until used for assays. In patients with septic shock, plasma was obtained within 48 h of ICU admission.

Study Approval. Informed consent was obtained from all human subjects in the study. The study was approved by the institutional review board at University of Utah.

Statistical Analysis. Results are presented as mean ± SEM. The statistical significance for comparisons between groups was determined using one-way ANOVA, two-way repeated-measures ANOVA, an unpaired Student’s t-test, or a Mann–Whitney U test. The D’Agostino–Pearson test was used as a normality test. All statistical analysis was done using Prism software package 6 (GraphPad Software).

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