Nucleation of platelets with blood-borne pathogens on Kupffer cells precedes other innate immunity and contributes to bacterial clearance

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Through the use of intravital imaging of the liver, we demonstrate a collaborative role for platelets with Kupffer cells (KCs) in eradicating blood-borne bacterial infection. Under basal conditions, platelets, via the platelet-adhesion receptor GP Ib, formed transient ‘touch-and-go’ interactions with von Willebrand factor (vWF) constitutively expressed on KCs. Bacteria such as Bacillus cereus and methicillin-resistant Staphylococcus aureus (MRSA) were rapidly caught by KCs and triggered platelets to switch from ‘touch-and-go’ adhesion to sustained GP Ib-mediated adhesion on the KC surface to encase the bacterium. Infected GP Ibα-deficient mice had more endothelial and KC damage than did their wild-type counterparts, which led to more fluid leakage, substantial polycythemia and rapid mortality. Our study identifies a previously unknown surveillance mechanism by which platelets survey macrophages that rapidly converts to a critical host response to blood-borne bacteria.

Innate immunity is the most ancient defense system for vertebrates against microbes. The host innate immune defense is composed of an array of cells with sensitive receptors that detect microbial products and instigate a counterattack after infection. The gut is a likely portal for the entry of pathogens into the host and therefore many cells of the immune system reside in the liver, an organ that filters blood as it percolates through the vast array of vascular sinusoids. In fact, the liver is becoming increasingly known for its immunological roles, including its ability to eradicate systemic infection of the vasculature via an ongoing constitutive network of intravascular macrophages called ‘Kupffer cells’ (KCs). KCs are strategically positioned and reside as stationary macrophages in the liver sinusoids to trap, phagocytose and remove pathogens in transit through the circulation. Indeed, efficient binding of blood-borne pathogens to the KC surface constitutes the first line of host immunological defense in the circulation2,3.

The survival of a pathogen depends on its ability to evade the well-evolved host immune response. No pathogens have been more successful in this than the human-targeting pathogens Staphylococcus aureus and the spore-forming Bacillus family, which have found ways to survive the hostile intracellular environment of macrophages. S. aureus produces many toxins that cause cell lysis4–5, whereas the spores of Bacillus can germinate, multiply and escape from host cells6–9. Bacillus anthracis is highly virulent to mammals and causes anthrax10. In addition, isolates of Bacillus cereus have been linked to lethal infections with a clinical presentation similar to that of B. anthracis and thus pose a potential serious public health problem11,12. Despite the presence and accumulation of cells of the immune response at the site of infection, such infections are characterized by bacteremia and severe tissue damage that leads to death13. In fact, B. cereus contains many bacterial factors that induce phagocytic cell death14,15 and potentially cause substantial host tissue damage and compromise the antimicrobial immune response.

In addition to their role in hemostasis, the role of platelets in immunity is also garnering growing interest. Platelets express many major receptors of the innate immune system, including most Toll-like receptors, thrombin receptors, complement receptors and various adhesion molecules16. In fact, platelets are able to recognize molecular features of microbes and contain many immunomodulatory mediators essential for alerting and recruiting cells of the immune system17–19. Indeed, the appropriate interaction between, for example, monocytes and platelets leads to more production of various cytokines20, and interactions between neutrophils and platelets leads to the production of neutrophil extracellular traps18,21. Platelets have also been shown to produce various antimicrobial molecules, including defensins22, thrombocidins23 and kinocidins24, which suggests that they are able to interact with and kill bacteria directly25. Despite that, how direct contact between platelets and bacteria might occur in the main stream of blood, under shear conditions, is unclear. One possibility is that bacteria might inadvertently collide with platelets in the circulation. However, events in the immune system are usually not left to chance. Therefore, instead of a haphazard approach for platelet-bacteria

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interactions, a systematic and purposeful approach must exist to allow platelets to interact with bacteria.

Published work has shown that members of the genus *Bacillus* (such as *B. cereus*) can induce platelet aggregation within minutes of exposure to human or mouse blood in an *in vitro* flow-chamber assay. Such aggregates could either potentially shield the bacteria from cells of the immune system or be a mechanism for enlisting the help of platelets to eradicate the bacteria. If the latter is true, then it is likely that platelets patrol the vasculature in a systematic way and in some manner localize together with blood-borne bacteria. After 4 h of stimulation with lipopolysaccharide, platelets are able to bind neutrophils as well as KCs; although the former interaction is critical for the formation of neutrophil extracellular traps, it is unclear why platelets would interact with KCs. Here, through the use of a multichannel intravital spinning-disk confocal microscope, we observed, independently of any stimulus, a previously unknown patrolling mechanism of platelets in blood that involved ongoing ‘touch-and-go’ interactions of the platelet-adhesion receptor GPIb (CD42) with constitutively expressed von Willebrand factor (vWF) on KCs in liver sinusoids under basal conditions. We used *B. cereus* as a model pathogen (as well as methicillin-resistant *S. aureus* (MRSA)) and found that the surface of the KCs became the ‘battleground’ where caught bacteria encountered a swarm of platelets that converted their ‘touch-and-go’ activity to sustained GPIIb-GPIIIa-dependent adhesion and actively encased the bacteria for eradication within the first minute of encounter. This previously undescribed mechanism preceded the recruitment of all other cells of the innate immune response and was critical for host survival after infection with Gram-positive bacteria.

**RESULTS**

A platelet patrolling mechanism in liver

Intravital spinning-disk confocal microscopy of the liver microvasculature showed continuous ‘touch-and-go’ activity (briefer than 1 s) of individual circulating platelets in the sinusoids of the liver (Fig. 1a). The addition of fluorescence-tagged antibody to F4/80 (anti-F4/80) identified a vast network of KCs lining the luminal side of sinusoids, and under basal conditions, a substantial number of circulating platelets transiently touched those immobilized KCs (Fig. 1b,c). We also saw platelets touching the sinusoidal endothelium (Fig. 1b). Very few platelets attached to the KCs for an extended period, and we observed even fewer aggregates (Supplementary Video 1), which suggested little or no activation under basal conditions. Depletion of KCs through previously unknown surveillance mechanism whereby platelets interacted with bacteria.

Rapid platelet response to *B. cereus in vivo*

Platelets are essential for primary hemostasis and are becoming increasingly recognized for their role in immunity and inflammation. In uninfected wild-type (control) mice, the circulating blood platelet count was approximately $8.5 \times 10^8$ per ml; this abundance was much lower 4 h after infection with our model bacterium, *B. cereus* (Fig. 1a). Within the first 4 h of infection with *B. cereus*, most wild-type mice survived, with less than 10% mortality (Fig. 1b). In contrast, all mice that were depleted of platelets before infection died within 4 h of infection (Fig. 1b). Although the amount of *B. cereus* given was absolutely lethal to all mice depleted of platelets, some platelet-deficient mice survived and managed to survive beyond 24 h (the end of the experiment; Fig. 2b). These data indicated a fundamental and critical role for platelets in rapid host defense against and early survival during infection with the lethal pathogenic bacterium *B. cereus*. Most bacteria were trapped in the liver, with an order of magnitude less trapped in the lungs and spleen (Fig. 2c). Key clearance mechanisms must have occurred early in response to this pathogen, as within 1 h of infection, bacteremia was low and all bacteria were eliminated from blood by 4 h in platelet-deficient mice (Fig. 2d). In contrast, mice depleted of platelets had greater bacteremia at both 1 h and, to a lesser extent, 4 h after infection but before death (Fig. 2d). Collectively, these data suggested that platelets had a role in host defense against *B. cereus* and helped facilitate clearance of this pathogen by the liver.

Platelets dock on KCs after infection with *B. cereus*

Rapid trapping of *B. cereus* was mediated by KCs (within seconds) in the liver vasculature. Time-lapse videos demonstrated that immediately after pathogen capture, the ‘touch-and-go’ activity of platelets was converted to profound sustained recruitment of platelets to the KC surface (Fig. 3a and Supplementary Video 2) with subsequent encasement of the bacteria by the platelets. Those platelet aggregates

![Figure 1](https://example.com/figure1.png)

**Figure 1** Platelets form ‘touch-and-go’ interactions in the liver sinusoids. (a) Intravital microscopy of rapid and transient ‘touch-and-go’ interactions (arrowheads) of platelets (labeled with anti-CD49b; red) on KCs (labeled with anti-F4/80; blue) in wild-type mouse liver (additional images, Supplementary Video 1). Numbers in corners indicate time (in seconds). Original magnification, ×20. (b) Quantification of ‘touch-and-go’ interactions of platelets with KCs or the endothelium in the livers of wild-type mice (WT), mice depleted of KCs (KC-depl), *Gp1ba*−/− mice (Gp1ba-def) and GPIb-deficient mice (GPIb-def) in basal conditions (n ≥ 3 mice per group). *P < 0.01 (one-way analysis of variance (ANOVA)). (c) Total ‘touch-and-go’ interactions of platelets in the livers of mice as in (b) (n ≥ 3 per group). *P < 0.01 and **P < 0.001 (one-way ANOVA). (d) Total ‘touch-and-go’ interactions of platelets in the livers, brain, muscle, skin and ear of wild-type mice (n ≥ 3 per group) in basal conditions. *P < 0.001, each versus liver (one-way ANOVA). FOV, field of view; ND, not detected. Data are representative of three (a–c) or two (d) experiments (error bars (b–d), s.e.m.).
‘preferentially’ localized together with KCs that had captured B. cereus (Fig. 3a). On occasion, we also observed the formation of aggregates at a distance from any B. cereus (Fig. 3a). Quantification of aggregates by size showed that the livers of B. cereus–infected mice had significantly more small (10-µm²) and large (25- and 50-µm²) platelet aggregates than did those of sham-infected mice (Fig. 3b). That activity persisted throughout the observation period of 30 min (Fig. 3c). Heat-killed B. cereus did not induce platelet aggregation in the liver throughout the first 30 min of the observation period (Fig. 3c).

To better visualize and understand the progression from capture of B. cereus to platelet recruitment, we used high magnification and four-dimensional reconstruction (three-dimensional reconstruction in real time). In one such analysis we found a KC with no B. cereus and no large platelet aggregates (Fig. 3d, top left). Within the first minute, B. cereus bound to the KC (Fig. 3d, top middle). Within seconds after bacteria were captured, platelets began to bind to the KC and, within another minute, a substantial aggregate enveloped the B. cereus (Fig. 3d, top right) and, over the next 3 min, completely covered the B. cereus (Fig. 3d, bottom row). Thus, the four-dimensional imaging showed that platelets formed an encasement around trapped B. cereus on the surface of KCs.

Platelets dock on KCs after infection with MRSA

The encasement of bacteria by platelets was not restricted to B. cereus. A strain of community-associated MRSA (strain USA300) also induced the recruitment of platelets to KCs and their aggregation on KCs after infection (Fig. 4a). Unexpectedly, a strain of methicillin-susceptible S. aureus (MSSA; strain Xen29) did not initiate platelet aggregation (Fig. 4a), which suggested that this platelet surveillance system dealt with only a specific subset of pathogens. In a manner similar to noted for B. cereus–infected mice, platelet aggregations in the liver ‘preferentially’ localized together with KCs that had captured MSSA (Fig. 4b). In addition, mice depleted of platelets demonstrated greater mortality 8 h and 12 h after infection with MRSA (Fig. 4c), which suggested that docking of platelets and their encasement of bacteria in the liver may have contributed to rapid host defense against and early survival during infection with that blood-borne pathogen. Moreover, the mechanism of that rapid nucleation of platelets after infection seemed to be largely independent of the Toll-like receptor pathways that use the adaptor MyD88 (Supplementary Fig. 1). All of those events preceded any recruitment of neutrophils, which was entirely MyD88 dependent (Supplementary Fig. 2). Therefore, the recruitment of platelets was probably neutrophil independent.
Platelet aggregation requires capture of bacteria by KCs

We next investigated the mechanisms by which platelets rapidly accumulated on KCs after bacterial infection in vivo. To determine whether it was the KCs or the bacteria that caused the firm adhesion of platelets to the vessel wall, we treated a group of mice with chloroquine liposomes to deplete them of KCs. Mice were completely depleted of KCs by that method, whereas samples from untreated mice had at least 40 KCs per microscopic field of view (Fig. 5a). After treatment with chloroquine liposomes, there were significantly fewer B. cereus detected in the sinusoids (Fig. 5b). Notably, although all KCs were gone, a few B. cereus were detectable in the sinusoids; these bacteria were bound directly to the vessel wall (Supplementary Video 3). However, that binding of bacteria was not sufficient to induce platelet aggregation, as we did not observe platelet aggregates in the mice depleted of KCs (Fig. 5c), which indicated that the capture of bacteria by KCs was essential for the recruitment and aggregation of platelets. Indeed, even the minor aggregation seen in untreated mice was abrogated in the absence of KCs (Fig. 5c). Complement opsonization was also necessary for the clearance of B. cereus. In fact, like mice depleted of KCs, mice deficient in the complement component C3 demonstrated less capture of B. cereus (Fig. 5b) and platelet aggregation (Fig. 5d) than that of wild-type mice. Thus, the interaction of B. cereus with both KCs and complement was necessary for the induction of platelet aggregation.

GPIb, vWF and GPIIb recruit platelets to KCs

GPIb is reported to bind to vWF to allow platelet adhesion and aggregation at sites of vascular injury28. Gpiba−/− mice did not accumulate platelets on KCs after infection with B. cereus (Fig. 6a), which suggested that GPIb was critical to the bacteria-induced recruitment of platelets. To determine how the GPIb on platelets bound to KCs, we stained various proteins and unexpectedly found large amounts of vWF deposited in liver sinusoids (Fig. 6b). Notably, in addition to the staining of vWF on the endothelium, there were large clusters of vWF on the KCs (Fig. 6c). Blockade of vWF significantly inhibited platelet accumulation after capture of B. cereus by KCs (Fig. 6d). Although we had expected to find no deposition of vWF under control conditions in the sinusoids, we observed substantial staining on KCs in the absence of any bacteria (Fig. 6b,c). In fact, although there was significantly more staining of vWF after infection with B. cereus, there was considerable constitutive vWF expression in the liver vasculature (Fig. 6b). That basal expression of vWF potentially contributed to the GPIb-mediated docking of platelets on KCs as part of the surveillance mechanism described above (Fig. 1). Although vWF and GPIb were critical to the docking of platelets on the surface of KCs, this docking seemed transient, and it was only after B. cereus was caught that the platelet ‘touch-and-go’ interaction converted to sustained adhesion (Fig. 5b,c). It is well established that GPIb induces tethering, but not firm adhesion, to vWF29. In fact, GPIb-dependent aggregation has been reported only in the conditions of extremely high shear (>10,000 s⁻¹) associated with stenotic arteries and not in the low shear environment of the liver sinusoids. However, GPIb is not the only vWF receptor on platelets. In fact, vWF is able to mediate firm adhesion of platelets via a second platelet receptor, GPIb-IIIa. To assess that potential adherence mechanism, we infected GPIb-deficient

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**Figure 4** Aggregation of platelets after infection with MRSA in vivo. (a) Quantification of platelet aggregates larger than 25 µm² in liver sinusoids in wild-type mice (n ≥ 4 per group) after sham, MSSA or MRSA infection. *P < 0.05 (one-way ANOVA). (b) Three-dimensional reconstructed imagery of the capture (arrows) of MSSA or MRSA (green) by a KC (blue) and initiation of platelet aggregation (red) in liver sinusoids after 10 min of infection in vivo. Original magnification, ×20. (c) Survival of MSSA- or MRSA-infected wild-type mice (n ≥ 9 per group) pretreated with control or platelet-depleting serum. *P < 0.01 (log-rank test). Data are representative of two experiments (error bars (a), s.e.m.).

**Figure 5** Capture of B. cereus by KCs is required for platelet aggregation. (a) Intravital microscopy of KCs (labeled with anti-F4/80; red) and endothelium (labeled with anti-PECAM; blue) in livers from mice left undepleted (UD) or treated with chloroquine liposomes for depletion of KCs (KC-depl) in vivo (additional images, Supplementary Video 3). Scale bars, 50 µm. (b) Capture of B. cereus in liver sinusoids of wild-type mice left undepleted or depleted of KCs, and those of C3-deficient mice (C3-def), assessed after 10 min of infection (n ≥ 4 mice per group). *P < 0.01 (one-way ANOVA). (c) Quantification of platelet aggregates larger than 25 µm² in liver sinusoids of wild-type mice left undepleted or depleted of KCs (n ≥ 4 per group), assessed after sham or B. cereus infection. *P < 0.05 (one-way ANOVA). (d) Platelet aggregates larger than 25 µm² in liver sinusoids of wild-type and C3-deficient mice (n ≥ 4 individual mice per group), assessed after sham or B. cereus infection. *P < 0.05 (one-way ANOVA). Data are representative of two experiments (error bars (b-d), s.e.m.).
mice with *B. cereus*. Indeed, GPIIb-deficient mice had much less platelet accumulation on the surface of KCs after infection than did their wild-type counterparts (Fig. 6e), despite the lack of effect on the initial 'touch-and-go' surveillance of KCs by platelets, whereas the interaction between vWF and GPIIb-GPIIa mediated the accumulation of platelets after capture of *B. cereus* by KCs.

**Platelet recruitment protects the host after infection**

There was less than 10% mortality for wild-type mice within the first 4 h of infection with *B. cereus* (Fig. 7a). In contrast, more than 80% of *Gp1ba<sup>−/−</sup>* mice died within 4 h after infection (Fig. 7a). Similar to mice depleted of platelets, *Gp1ba<sup>−/−</sup>* mice had much more bacteremia at 1 h after infection with *B. cereus* than did their wild-type counterparts (Fig. 7b). Notably, we obtained nearly identical results with MRSA (Fig. 4c). *Gp1ba<sup>−/−</sup>* mice were also unable to clear *B. cereus* from the blood by 4 h after infection (Fig. 7b). The inability to control infection with *B. cereus* was greater in GPIIb-deficient mice than in mice depleted of platelets (probably because the latter had only 90% fewer circulating platelets after depletion) and indicated the importance of GPIIb. Most septic infections do not originate in the bloodstream but instead originate in some other infectious focus and then enter the blood. To model that process, we used intraperitoneal challenge with *B. cereus*. Again, the ability of platelets to shield the host from *B. cereus* was essential for host survival, as mice deficient in platelets (or GPIb) had diminished survival relative to that of platelet-sufficient mice (Supplementary Fig. 3). Together these data indicated that platelet accumulation and encasement of *S. aureus* and *B. cereus* were essential to the host defense against these pathogens.

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**Figure 6** GPIb and GPIIb mediate platelet aggregation after infection with *B. cereus*. (a) Quantification of platelet aggregates larger than 25 µm² in liver sinusoids of wild-type and *Gp1ba<sup>−/−</sup>* mice (n ≥ 4 per group) after sham infection or 10 min of infection with *B. cereus*. *P* < 0.05 (t-test). (b) Area of vWF staining in liver sinusoids of sham-infected wild-type mice and wild-type mice for 10 min infected with *B. cereus* (n ≥ 5 per group). *P* < 0.05 (t-test). (c) Intravital microscopy of vWF (red) on endothelium (white arrowheads) and on KCs (labeled with anti-F4/80 (blue); yellow arrows) in liver sinusoids of sham-infected wild-type mice or infected for 10 min with *B. cereus*. Scale bar, 50 µm. (d) Platelet aggregates larger than 25 µm² in liver sinusoids of sham-infected wild-type mice (Sham) and of wild-type mice given no pretreatment (*B. cereus*) or pretreated with blocking antibody to vWF (Anti-vWF *B. cereus*) and then infected with *B. cereus* (n ≥ 4 mice per group). *P* < 0.05 (one-way ANOVA). (e) Platelet aggregates larger than 25 µm² in liver sinusoids of sham- or *B. cereus*-infected wild-type and GPIIb-deficient mice (n ≥ 4 per group). *P* < 0.05 (one-way ANOVA). Data are representative of two experiments (error bars a, b, d, e, s.e.m.).

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**Figure 7** Platelet aggregation after *B. cereus* infection protects the host. (a) Survival of *B. cereus*-infected wild-type and *Gp1ba<sup>−/−</sup>* mice (n ≥ 10 per group). *P* < 0.001 (log-rank test). (b) Bacterial load in the peripheral blood of surviving wild-type and *Gp1ba<sup>−/−</sup>* mice (n ≥ 10 per group) at 1 h and 4 h after infection with *B. cereus*. *P* < 0.05 (t-test). (c) Liver damage in wild-type and *Gp1ba<sup>−/−</sup>* mice (n ≥ 6 per group) at 4 h after sham or *B. cereus* infection, assessed as concentration of alanine transaminase (ALT) in plasma. *P* < 0.05 (two-way ANOVA). (d) Intravital microscopy of necrotic cells (labeled with propidium iodide; red) and KCs (labeled with anti-F4/80; blue) in liver sinusoids from wild-type and *Gp1ba<sup>−/−</sup>* mice infected intravenously for 4 h with *B. cereus*. Scale bar, 50 µm. (e) Quantification of propidium iodide staining (PI<sup>+</sup> area) in the lungs of wild-type and *Gp1ba<sup>−/−</sup>* mice (n ≥ 4 per group) at 4 h after sham or *B. cereus* infection. *P* < 0.01 (two-way ANOVA). (f) Hematocrit of the peripheral blood of wild-type and *Gp1ba<sup>−/−</sup>* mice (n ≥ 6 per group) at 4 h after sham or *B. cereus* infection. *P* < 0.001 (two-way ANOVA). (g) Capture of microspheres by KCs in wild-type and *Gp1ba<sup>−/−</sup>* mice (n ≥ 4 per group) given sham or *B. cereus* infection and, 4 h later, given intravenous injection of inert microspheres and assessed after 10 min (for analysis of KC function). *P* < 0.01 (two-way ANOVA). (h) Myeloperoxidase (MPO) activity in the lungs of wild-type and *Gp1ba<sup>−/−</sup>* mice (n ≥ 6 per group) at 4 h after sham or *B. cereus* infection, to assess infiltration of neutrophils. *P* < 0.01 (two-way ANOVA). Data are representative of three (a, b, f) or two (c-e, g, h) experiments (error bars b, d, f, g, h, s.e.m.).
The inability to efficiently respond to infection with \textit{B. cereus} caused overt liver injury, dysfunction and cell death. Wild-type mice infected with \textit{B. cereus} had some hepatic damage, as shown by their higher concentration of alanine aminotransferase in serum after infection than before infection; however, \textit{Gp1ba}−/− mice had significantly higher concentrations of alanine aminotransferase in serum at 4 h after infection with \textit{B. cereus} than did their wild-type counterparts (Fig. 7c). There was also a significantly greater frequency of staining with propidium iodide (indicative of cell death) in the livers of \textit{B. cereus}-infected \textit{Gp1ba}−/− mice than in their wild-type counterparts, and such staining localized together with both the endothelial lining of the liver sinusoids and with KCs (Fig. 7d). Quantification of that staining confirmed only minimal cell death in wild-type mice but much (tenfold) more in the livers of \textit{Gp1ba}−/− mice after infection (Fig. 7e). We also observed cellular dysfunction in both the endothelium and KCs. We observed significantly more plasma leakage, as shown by the increase in the hematocrit of wild-type mice from 40% before infection to 50% at 4 h after infection with \textit{B. cereus} (Fig. 7f).

In the surviving \textit{Gp1ba}−/− mice, we noted greater exacerbation in the hematocrit after infection, which reached a value of nearly 70% within 4 h of infection (Fig. 7f). The amount of hemoglobin was similar in the two strains of mice (data not shown), which suggested a loss of fluid rather than an increase in the number of red blood cells. KCs from the livers of infected \textit{Gp1ba}−/− mice had impaired trapping ability, but those from infected wild-type mice did not (Fig. 7g). Catching of inert microspheres injected intravenously was the same without infection (control conditions; Fig. 7g). However, after infection with \textit{B. cereus}, significantly fewer microspheres were captured in the livers of \textit{Gp1ba}−/− mice than in their wild-type counterparts (Fig. 7g). That finding potentially explained the greater abundance of circulating \textit{B. cereus} in \textit{Gp1ba}−/− mice than in their wild-type counterparts at 4 h after infection (Fig. 7b). There were also some signs of exacerbated inflammation in other organs, as we observed more recruitment of neutrophils to the lungs of infected \textit{Gp1ba}−/− mice than to their wild-type counterparts, perhaps reflective of the enhanced bacterial dissemination in the \textit{Gp1ba}−/− mice (Fig. 7h). The failure of platelets to encapsulate the bacterium and shield the host resulted in greater cellular dysfunction and death in the liver, which led to vascular damage and the inhibition of pathogen capture by KCs (model of interactions among cell types and molecules, Supplementary Fig. 4).

DISCUSSION

Although platelets continue to be regarded as small cell fragments that participate only in hemostasis, their origin, membrane receptors and intracellular contents provide irrefutable support for the view that platelets have a central role in fighting infection. In invertebrates, the hemocyte participates both in hemostasis and in the defense against pathogens, acting as an ancient granulocyte\textsuperscript{35}. Indeed, the platelet has retained many features of the prototypical granulocyte, including the ability to respond to infection via archetypal pattern-recognition receptors such as Toll-like receptors\textsuperscript{18}; changing from a discoid shape to an amoeboid shape in the presence of infection\textsuperscript{31}, moving by chemo- taxis toward signature bacterial motifs such as formylated peptides\textsuperscript{32,33}, and releasing oxidants and potent antimicrobial proteins (such as defensins and so on)\textsuperscript{33}. In this study, we found that platelets actively patrolled the vascular bed and formed ‘touch-and-go’ interactions with KCs in the liver sinusoids. Those interactions converted rapidly to firm adhesion once specific microbes were caught by KCs. Although it has been shown that platelets can attach to and kill microbes in the confines of a test tube under static conditions\textsuperscript{34,35}, the mechanism by which platelets could possibly trap bacteria in the dynamic conditions of blood flow has remained unknown. In this study, through the use of dual-camera multichannel spinning-disk intravitral confocal microscopy, we were able to demonstrate a previously unknown mechanism of the innate immune system: platelets localized to sites of bacterial capture on KCs, where they encapsulated and helped facilitate the killing of the microbe.

Intravitral imaging showed that platelets transiently interacted predominately with KCs but also with the endothelium under basal conditions. Indeed, after depletion of KCs, the number of patrolling platelets was much lower. Moreover, tissues such as brain, skin or muscle that lack KCs had few patrolling platelets. Given the ‘catch bonds’, with slow on rates and fast off rates, formed by GPIb and vWF, the rapid ‘touch-and-go’ baseline interactions we observed in the uninfected liver would not be unexpected. KCs have been reported to clear the blood of vWF\textsuperscript{36}; however, our data suggest that such apparent clearance may actually be part of an essential surveillance mechanism of the innate immune system whereby vWF is concentrated on the KC surface, which creates ‘landing pads’ for circulating platelets. Such vWF ‘hot spots’ on KC surface became reactive for platelets, which allowed rapid, transient docking, a hallmark feature of tethering via ‘catch bonds’. Although this type of vWF clearance in the spleen has also been reported, patients who have undergone splenectomy have not shown the expected increase in vWF concentrations, which suggests a dominate role for the liver in the sequestration of vWF\textsuperscript{37}. Therefore, despite the relevance of the spleen as an important organ of the immune system, we speculate that most patrolling interactions between platelets and intravascular macrophages occur in the liver. Thus, the liver represents the critical platform for platelet surveillance and protection from highly pathogenic, blood-borne infections.

We used \textit{B. cereus} in this study, as it best demonstrated the importance of platelets in mammalian immunity against pathogens and suggested no redundancy for platelets in this system. Without platelet immunity, mammals would be far more susceptible to such infections. Our observations were not restricted to \textit{B. cereus}, as KCs also recruited platelet help in response to other strains of bacteria (such as MRSA). We are also tempted to speculate that our data hint at the possibility that ‘platelet immunity’ is needed to combat \textit{B. anthracis} and other deadly bacterial strains. Indeed, \textit{B. anthracis} is a strain closely related to \textit{B. cereus} and also directly damages endothelial cells by pathogenic factors \textit{in vitro}\textsuperscript{38}, not dissimilar the results we obtained \textit{in vivo} with \textit{B. cereus}. Isolates of \textit{B. cereus} have also been linked to lethal infection by contamination of both food and blood with a clinical presentation similar to that noted after infection with \textit{B. anthracis}. Despite the presence and accumulation of cells of the immune response at the site of infection with \textit{Bacillus}, such infections are characterized by bacteremia and severe tissue damage that leads to death\textsuperscript{12}. In fact, \textit{B. cereus} contains many bacterial factors that induce phagocytic cell death\textsuperscript{13-15} and have the potential to cause substantial host tissue damage, compromising the antimicrobial immune response. \textit{B. cereus} has been shown to induce rapid platelet aggregation after exposure to human or mouse blood in an \textit{in vitro} flow chamber\textsuperscript{39}. Our data suggest that such aggregation serves to enlist platelets to help encase bacteria and restrict the escape of bacteria from KCs.

Although the spleen is often thought to be a key site for capturing bacteria\textsuperscript{39,40}, the liver is the main site for the trapping for \textit{B. cereus} (as we have shown here) and many other pathogens\textsuperscript{31}. We found a ten- to hundred-fold greater accumulation of \textit{B. cereus} and \textit{S. aureus} in the liver than in the spleen, and imaging showed much of this capture occurred via the KC (data not shown). Capture of bacteria...
alone was not sufficient to eradicate those two specific pathogens, and in fact it was necessary that KCs recruited platelets via GPIb. As assessed by flow cytometry, platelet GPIb has been reported to bind bacteria directly, transporting bacteria to macrophages\textsuperscript{35}. However, directly tracking the KCs, platelets and bacteria in vivo, we found that platelets used GPIb to tether to the vWF constitutively expressed on the surface of KCs and then used GPIIb firmly adhere to that vWF. Such tethering and binding of platelets to KCs serves to localize the bacteria together with the platelets and increases the chances that platelets find bacteria in blood.

Notably, our data showed that platelets arrived at sites of infection in liver much faster than neutrophils did. Furthermore, because they outnumber all other leukocytes in the vasculature tenfold, platelets could be of great benefit in some vascular infections. In fact, in the absence of platelets or in the absence of GPIb (which resulted in platelets’ being unable to localize to sites of infection), we observed rapid death of both the KCs and the endothelium, more leakage of plasma out of the vasculature, and host mortality. Platelets can release many antimicrobial molecules and can participate directly in host mortality. Platelets can find bacteria in blood.

Furthermore, platelets are able to stimulate macrophages to improve their killing function. Indeed, the platelet is responsible for more than 95% of circulating CD40L, an important activator of macrophages that enhances their ability to kill ingested microbes\textsuperscript{42}. Notably, KCs were less effective at catching foreign particles in the absence of platelets, which suggested that platelets either improve KC function or, more likely, prevent the demise of KC function. Given the critical role of platelets in the ability of the host to capture and clear certain bacteria, we speculate that the observed increase in community-acquired infection with MRSA and the increased usage of aspirin, a potent inhibitor of platelet activation, may in some way be related.

Clearly, platelets have more intricate and diverse roles in immune responses than have been recognized before. Our data have identified role for rapid platelet aggregation and encasement of MRSA and \textit{B. cereus} that prevented bacteremia and pathogen-induced endothelial permeability, liver dysfunction and mortality. The ongoing surveillance activity of platelets led to a nearly instantaneous recruitment of platelets (within seconds) to the KC surface and was critical to the host response to infection with \textit{B. cereus} or MRSA. Notably, that platelet recruitment occurred long before classic effector cells of the innate immune response (neutrophils) were recruited. Our data have identified a previously unrecognized and essential sentinel program for platelets during infection with certain bacteria.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

C.H.Y.W. and C.N.J. designed and did most of the experiments; C.H.Y.W. prepared the manuscript; B.P. did all of the muscle, skin and ear experiments; N.I.C. contributed some liver-imaging experiments; C.N.J. made all of the manuscript revisions and did additional experiments; and P.K. provided overall supervision, helped design all of the experiments and prepared the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. All protocols used were in accordance with the guidelines drafted by the University of Calgary Animal Care Committee and the Canadian Council on the Use of Laboratory Animals. Wild-type and C3-deficient mice on the C57BL/6 background were from the Jackson Laboratory. Gpi1a−/− mice on the C57BL/6 background were a gift from T. Chavakis. GPIIb-deficient mice (CD41−YFPki/ki, with sequence encoding yellow fluorescent protein knocked into the locus encoding CD41) were a gift from K. McNagny. Mice were maintained in a specific pathogen–free, double-barrier unit at the University of Calgary. Mice 8–12 weeks of age with a body weight of 20–24 g were used in the studies.

Antibodies and treatments. The following antibodies were used for intravital imaging: phycoerythrin-conjugated Armenian hamster antibody to mouse CD49b (3.5 µg per mouse; HMzen2; BD Biosciences Pharmingen), Alexa Fluor 647-conjugated rat antibody to mouse F4/80 (2.5 µg/mouse; MB8; eBioscience), and rat antibody to mouse PECAM-1 (390; eBioscience) conjugated to Alexa Fluor 647 with a protein-labeling kit according to the manufacturer’s instructions (Invitrogen). Nérotic cells were visualized by intravenous administration of 5 µL 2.5 mM propidium iodide (Sigma).

Mice were depleted of platelets by intraperitoneal injection of 100 µl anti-thrombocyte serum (CL.A31440; Cedarlane) 24 h before infection. For inhibition of vWF, each mouse was given intravenous injection of 50 µg polyclonal rabbit antibody to human vWF with strong cross-reaction with mouse vWF (A0082; Dako) 30 min before infection. Normal rabbit serum was administered in the same way as a control. KC depletion was achieved by intravenous injection of 200 µl of clodronate liposomes 43,44 at 30 h before infection. For analysis of the trapping ability of KCs in vivo, 5 × 107 inert fluorescent poly-chromatic microspheres (Polysciences) were administered intravenously.

Bacteria. The B. cereus strain used in this study (UW8S 43-25; provided by R.R. Pompano and R.F. Ismagilov)26 expresses green fluorescent protein45. For each experiment, B. cereus was freshly inoculated from glycerol stocks (15%) into 5 ml Luria–Bertani medium containing 20 µg/ml chloramphenicol (EMD Chemicals). B. cereus was grown for 16 h on a rotary shaker at 37 °C. Cultures were then diluted 1:10 in fresh Luria–Bertani medium containing 20 µg/ml chloramphenicol and was grown for another 2 h until the density reached approximately 1 × 108 CFU/ml (measured as absorbance at 660 nm). Mice were infected intravenously with 5 × 107 CFU B. cereus. For experiments with heat-killed B. cereus, 5 × 107 CFU/ml B. cereus in Luria–Bertani medium were killed by incubation for 30 min at 99 °C. The heat-killed bacteria were washed with saline and resuspended for injection into mice. For intraperitoneal-infection studies, mice were infected with 2 × 107 CFU B. cereus.

The strain of MSSA (Xen29) used in this study was the 12600 strain (NCTC8532). Log-phase growth was achieved after 2 h after subculture in 5 ml Luria–Bertani medium containing 200 µg/ml kanamycin (EMD Chemicals). The MRSA strain used in this study was the USA300 strain. Log-phase growth was achieved after 1.5 h after subculture in 5 ml brain-heart–infused medium containing 100 µg/ml chloramphenicol (EMD Chemicals). Mice were infected intravenously with 5 × 107 CFU MSSA or MRSA labeled with 2.5 mM SYTO 9 green fluorescent nucleic-acid stain.

Intravital spinning-disk confocal microscopy of the liver. For all experiments, mice were anesthetized by intraperitoneal injection of ketamine (200 mg per kg body weight; Bayer Animal Health) and xylazine (10 mg per kg body weight; Bimeda-MTC). Intravital microscopy of the liver was done as described46. The microscopy settings for these tissues have been described27. Surgical preparation for intravital microscopy for each tissue is described below.

For brain microscopy, the head of the mouse was held on a stereotaxic board to prevent movement. Skin covering the parietal bone of the mouse skull was reflected, and the left parietal bone was carefully thinned with a high-speed drill (Fine Science Tools) to allow transillumination. Care was taken to not open the cranial vault to ensure that physiological pressures and blood flow were maintained. An upright microscope was used for intravital visualization of leukocyte biology in the pial microvasculature through this thinned skull tissue.

The mouse cremaster muscle was used to study neutrophil recruitment as described47. Each anesthetized mouse was placed on a special cremaster-preparation board, and body temperature was maintained with a heating pad. An incision was made in the scrotal skin to expose the left cremaster muscle, which was then carefully dissected free of the associated fascia. The cremaster muscle was cut longitudinally with a cautery. The testicle and the epididymis were separated from the underlying muscle and were moved into the abdominal cavity. The muscle was held flat on an optically clear viewing pedestal and was secured along the edges with 4–0 suture. The exposed tissue was superfused with 37 °C warmed bicarbonate-buffered saline, pH 7.4, and was covered with a coverslip. An upright microscope was used for intravital visualization of intravascular biology.

For ear microscopy, each anesthetized mouse was placed on a heating pad to maintain body temperature, and the dorsal hair from the right ear was gently removed with depilatory cream (Nair; Church & Dwight) without any irritation. The ear was carefully flattened on an elevated preparation board, was superfused with 37 °C warmed bicarbonate-buffered saline, pH 7.4, and was held in place with a coverslip applied on the dorsal side of the ear. An upright microscope was used for intravital visualization of intravascular biology.

Semiquantitative analysis of platelet aggregation. ‘Snapshots’ were generated from intravital videos, and images corresponding to the red fluorescence channel alone (platelets labeled with phycoerythrin-conjugated anti-CD49b (HMzen2; BD Biosciences Pharmingen)) were exported as tif documents. For analysis of aggregate size and number, image files were opened with ImageJ software (version 1.45; US National Institutes of Health) and image contrast was set to maximum for sharp definition of the borders of each platelet aggregate. To account for variability in background fluorescence between experiments and between antibody lots, and to eliminate fluorescence attributed to rapidly circulating platelets, the minimum brightness threshold was adjusted for each experiment. This threshold was then applied to images from all treatment groups in the experiment, which allowed direct comparison of aggregate size and number for treatment groups. After the minimum brightness threshold was set for each image, platelet aggregates 25 or 50 µm2 in size in each field of view were counted with the Analyze Particles function of ImageJ.
Semiquantitative analysis of vWF deposition. ‘Snapshots’ were generated from intravital videos, and the images corresponding to the red fluorescence channel alone (phycoerythrin-conjugated antibody to rabbit vWF (A0082; Dako) or immunoglobulin G (M1-14D12; eBioscience)) were exported as tif documents. For analysis of vWF staining, image files were opened with ImageJ software and image contrast was set to maximum for sharp definition of the borders of staining. Once the minimum brightness threshold was set for each image, vWF staining in each field of view was assessed with the Analyze Particles function of ImageJ.

Semiquantitative analysis of necrotic cells. Necrotic cells were visualized by intravenous administration of 5 µl 2.5 mM propidium iodide (Sigma). The propidium iodide was allowed to circulate for 2 min, and ‘snapshots’ of at least three fields of view per mouse were obtained between 2 min of 5 min after the administration of propidium iodide. Those images were exported as tif documents. For analysis of necrotic-cell staining, image files were opened with ImageJ software and image contrast was set to maximum for sharp definition of the borders of staining. Once the minimum brightness threshold was set for each image, the propidium iodide–positive necrotic cells in each field of view were counted with the Analyze Particles function of ImageJ.

Bacteriological analysis. Anesthetized mice were washed with 70% ethanol under sterile conditions. Blood was collected by cardiac puncture. The lungs, liver and spleen were removed after thoracotomy, weighed and homogenized. For determination of colony forming units (CFU), 10 µl of tissue homogenate or blood was serially diluted, plated onto Luria-Bertani agar plates supplemented with 20 µg/ml chloramphenicol, incubated at 37 °C for 18 h, and bacterial colonies were counted.

Assessment of liver injury. The anesthetized mice were washed with 70% ethanol under sterile conditions. Blood was collected in a heparinized syringe by cardiac puncture. Sample were then centrifuged at 400g for 10 min for the retrieval of plasma. Alanine transaminase in the plasma was measured with an Alanine Aminotransferase (ALT) Reagent Set according to the manufacturer’s protocol (Biotron Diagnostics).

Statistical analysis. Data were compared by the unpaired two-tailed Student’s t-test or one-way ANOVA with Bonferroni multiple-comparisons post-hoc test. Statistical significance was accepted at a P value of <0.05.

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