



HUMAN & MOUSE CELL LINES

Engineered to study multiple immune signaling pathways.

Transcription Factor, PRR, Cytokine, Autophagy and COVID-19 Reporter Cells
ADCC, ADCC and Immune Checkpoint Cellular Assays



The Journal of Immunology

RESEARCH ARTICLE | JANUARY 01 2017

Platelets Mediate Host Defense against *Staphylococcus aureus* through Direct Bactericidal Activity and by Enhancing Macrophage Activities **FREE**

Ramadan A. Ali; ... et. al

J Immunol (2017) 198 (1): 344–351.

<https://doi.org/10.4049/jimmunol.1601178>

Related Content

Ability of different isolates of *Staphylococcal aureus* to induce reactive metabolites in human polymorphovuclear leucocytes (P4429)

J Immunol (May,2013)

Neutrophil Effector Responses to Cystic Fibrosis Clinical Isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

J Immunol (May,2020)

Bactericidal Properties of Group IIA and Group V Phospholipases A₂

J Immunol (March,2001)

Platelets Mediate Host Defense against *Staphylococcus aureus* through Direct Bactericidal Activity and by Enhancing Macrophage Activities

Ramadan A. Ali, Leah M. Wuescher, Keith R. Dona, and Randall G. Worth

Platelets are the chief effector cells in hemostasis. However, recent evidence suggests they have multiple roles in host defense against infection. Reports by us and others showed that platelets functionally contribute to protection against *Staphylococcus aureus* infection. In the current study, the capacity of mouse platelets to participate in host defense against *S. aureus* infection was determined by assessing two possibilities. First, we determined the ability of platelets to kill *S. aureus* directly; and, second, we tested the possibility that platelets enhance macrophage phagocytosis and intracellular killing of *S. aureus*. In this study we report evidence in support of both mechanisms. Platelets effectively killed two different strains of *S. aureus*. A clinical isolate of methicillin-resistant *S. aureus* was killed by platelets (>40% killing in 2 h) in a thrombin-dependent manner whereas a methicillin-sensitive strain was killed to equal extent but did not require thrombin. Interestingly, thrombin-stimulated platelets also significantly enhanced peritoneal macrophage phagocytosis of both methicillin-resistant *S. aureus* and methicillin-sensitive *S. aureus* by >70%, and restricted intracellular growth by >40%. Enhancement of macrophage anti-*S. aureus* activities is independent of contact with platelets but is mediated through releasable products, namely IL-1 β . These data confirm our hypothesis that platelets participate in host defense against *S. aureus* both through direct killing of *S. aureus* and enhancing the antimicrobial function of macrophages in protection against *S. aureus* infection. *The Journal of Immunology*, 2017, 198: 344–351.

Platelets are the second most abundant blood cell, outnumbered only by erythrocytes in the circulation ($\sim 1.5\text{--}4.0 \times 10^{11}$ platelets per liter of blood in healthy adult humans). The classical role attributed to platelets has been in coagulation, prevention of bleeding, initiation of wound healing, and maintenance of vascular integrity. However, it is becoming increasingly clear that platelets contribute to diverse immunological processes extending beyond the traditional view of platelets as fragmentary mediators of hemostasis and thrombosis (1). Platelets are now appreciated for their contributions to both innate and adaptive immunity.

The capacity of platelets to participate in immunity is largely due to their ability to store and release a myriad of inflammatory and bioactive molecules, and express a wide range of functional immunoreceptors. Platelets store these bioactive molecules in three types of intracellular storage granules, dense (δ -), α (α -), and lysosomal (λ -) granules, the contents of which are released into the circulation or translocated to the surface upon platelet activation (1, 2). These mediators attract and modulate the effector cells of the immune system. In addition, platelets themselves demonstrate a direct effector function and should be regarded as effector immune cells (3).

Platelets appear to have multiple roles in host defense against infection. They are among the first cells to detect endothelial injury and microbial pathogens as they gain access to or invade the bloodstream or tissues (4, 5). Platelets interact with various bacterial species, viruses, fungi, and protozoa, and demonstrate antimicrobial functions (6, 7). The mechanisms of platelet-bacteria interactions are complex, reflecting the diversity of platelet receptors involved in the recognition of bacteria. These bacterial receptors include complement receptors, Fc γ RIIa, TLRs, GPIIb-IIIa, and GPIb, and the interaction of platelets and bacteria is mediated through direct or indirect binding to these and other receptors (reviewed in 8). Upon contact with certain bacteria, platelets can become activated, aggregate, and de-granulate (9–13). Activated platelets release over 300 known secretory products including anti-microbial products collectively known as platelet microbicidal proteins (14).

S. aureus is an opportunistic Gram-positive bacterium that is a main cause of skin infection in both community and hospital settings (15, 16). It is also among the most predominant endovascular pathogens and can lead to life-threatening conditions because it can efficiently disseminate from those sites of infection, causing invasive diseases including bacteremia, pneumonia, and infective endocarditis as well as organ damage and septic shock (17, 18). Thus, platelet interactions with *S. aureus* is rapidly becoming a model system, as the consequences of these interactions likely play significant roles in shaping infection and host defense.

Recent reports by us and others showed that platelets functionally contribute to protection against *S. aureus* infection (19–23). Using our novel inducible platelet-depleted mouse model, we demonstrated that platelet-depleted mice have a high bacterial burden in the kidneys, a more severe cytokine storm, and decreased survival compared with wild-type counterparts (19). However, the mechanistic detail of how platelets participate in host defense against *S. aureus* infection is not clearly understood. In the current study, the capacity of mouse platelets to participate in host defense

Department of Medical Microbiology and Immunology, University of Toledo College of Medicine and Life Sciences, Toledo, OH 43614

ORCID: 0000-0002-2626-7964 (R.G.W.).

Received for publication July 5, 2016. Accepted for publication October 27, 2016.

This work was supported by National Institutes of Health RO1 HL122401 (to R.G.W.).

Address correspondence and reprint requests to Dr. Randall G. Worth, Department of Medical Microbiology and Immunology, University of Toledo College of Medicine and Life Sciences, 3000 Arlington Avenue, MS 1021, Toledo, OH 43614. E-mail address: randall.worth@utoledo.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: DPBS, Dulbecco's PBS; DPI, diphenyleneiodonium chloride; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-sensitive *S. aureus*; ROM, reactive oxygen metabolite.

Copyright © 2016 by The American Association of Immunologists, Inc. 0022-1767/16/\$30.00

against *S. aureus* infection was determined by addressing two questions: first, can platelets directly kill *S. aureus*? Second, do platelets enhance macrophage phagocytosis and intracellular killing of *S. aureus*? Our evidence supports both defense mechanisms.

Materials and Methods

Mice

Six- to eight-week old male and female C57BL/6 (B6) mice were maintained in the animal facility at the University of Toledo Health Science Campus, in a specific pathogen-free environment. All mouse experiments were performed according to National Institutes of Health guidelines with approval of the Institutional Animal Care and Use Committee at the University of Toledo.

Platelet preparation

Mouse blood was obtained via cardiac puncture under isoflurane anesthesia. Blood was collected using a 1–3 ml syringe with 25 G needle containing anticoagulant (22.0 g/l trisodium citrate, 8.0 g/l citric acid, and 24.5 g/l dextrose), and was pooled into 1.5 ml microcentrifuge tubes. Platelets were isolated and purified by centrifugation as previously described (24).

Bacteria preparation

A derivative of a previously characterized clinical isolate of methicillin-resistant *S. aureus* (MRSA) USA300 (25) was provided by Dr. R.M. Blumenthal (University of Toledo, Toledo, OH). Methicillin-sensitive *S. aureus* (MSSA) strain NRS72 (Sanger 476) was obtained from Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA). Bacteria were grown overnight at 37°C in tryptic soy broth, centrifuged at 10,000 × *g* for 5 min and suspended in 1 ml of Dulbecco's PBS (DPBS) buffer without calcium or magnesium. Bacteria were washed with DPBS and enumerated using a hemacytometer.

Bacteria killing assay

S. aureus (1 × 10⁷/100 μl) were placed into a microcentrifuge tube and mixed with quiescent platelets or platelets activated with 0.1 U/ml thrombin (Chronolog, Havertown, PA) at a multiplicity of infection of 1. The solution was centrifuged at 300 × *g* for 1 min to maximize platelet-bacteria contact, followed by 2 h incubation in a 37°C water bath. Following incubation, a 1% saponin solution was added to lyse platelets. Solutions were serially diluted and plated on tryptic soy agar plates, incubated overnight at 37°C and CFU were tallied for each plate. When inhibitors were used, platelets were pretreated with the inhibitor for 15 min at 37°C prior to activation and mixing with bacteria. Cytochalasin D (Sigma Aldrich, St. Louis, MO) was used at a final concentration of 20 μM, and diphenyleiiodonium chloride (DPI) (Sigma Aldrich) was used at 10 μM. Each experiment was performed in triplicate and serial dilutions were plated in triplicate. Each experiment was performed at least three times on different days.

Macrophage preparation and phagocytosis/intracellular killing assays

To obtain fresh peritoneal macrophages, mice were injected i.p. with 1 ml thioglycollate (3%) and peritoneal fluids were obtained after 4 d. Peritoneal lavage was performed by injecting 10 ml DPBS, and removed using a syringe. The fluid was centrifuged to isolate peritoneal macrophages, which were resuspended in RPMI 1640 medium. A sample of collected cells was stained using fluorescently conjugated anti-CD45, CD11b, and F4/80 (BioLegend, San Diego, CA), and analyzed by flow cytometry. We observed that >90% of peritoneal cells were CD45+, CD11b+, and F4/80+, indicating that they are of the macrophage lineage (data not shown). Phagocytosis of *S. aureus* by peritoneal macrophages was performed in the presence or absence of thrombin-activated platelets or releasates from thrombin-activated platelets. *S. aureus* (10⁷ CFU) was mixed with macrophages (10⁶) in microcentrifuge tubes followed by the addition of platelets (10⁷) or an equal volume of DPBS. Thrombin (0.1 U/ml) was used to activate platelets. The suspension was then incubated for 1 h in 37°C water bath. At this time (*t* = 0 h) the cells were washed with DPBS three times by centrifugation at 200 × *g* for 1 min to clear nonphagocytosed bacteria, as well as platelets or their products. A fraction of the macrophage population was lysed with 0.2% triton X-100, serially diluted and plated to record the CFU of *S. aureus* as a measure of phagocytosis or internalization. For bactericidal assays, the remaining cells continued incubating for 3 h (*t* = 3 h) in the presence or absence of fresh thrombin-activated platelets, after which

the macrophages were washed as at *t* = 0 h. The cells were lysed with 0.2% triton X-100 and CFU were counted to determine the intracellular killing efficiency. In some experiments, macrophages were incubated with recombinant IL-1β (10 ng/ml), sCD40L (100 ng/ml), or RANTES (10 ng/ml) for this assay (all from PeptoTech, Rocky Hill, NJ).

Microscopy

S. aureus (10⁷) were stained with SYTO9 dye (1:50, Life Technologies, Grand Island, NY) for 15 min at room temperature according to the manufacturer's instructions, before washing two times at 10,000 × *g* for 10 min. Platelets (10⁷) were stained with anti CD42b-PE (1:50; BioLegend) for 20 min at room temperature. Platelets and bacteria were then mixed with or without 0.1 U/ml thrombin and incubated for 2 h at 37°C protected from light. After incubation, the platelet-*S. aureus* suspension was centrifuged at 300 × *g* for 10 min, resuspended in 10 μl of DPBS, and mounted using Fluoromount G (Southern Biotech, Birmingham, AL).

In some experiments, *S. aureus* were stained with FITC-conjugated anti-*S. aureus* polyclonal primary Ab (Abcam, Cambridge, MA) for 30 min at 37°C. Peritoneal macrophages were incubated with FITC-labeled *S. aureus* in RPMI 1640 medium in the presence or absence of 0.1 U/ml thrombin-activated platelets for 1 h (*t* = 0). Peritoneal macrophages were washed with DPBS to clear nonphagocytosed bacteria and platelets. Peritoneal macrophages were then stained with PE-conjugated anti-IgG secondary Ab, plated on coverslips, and observed under confocal microscopy to distinguish intracellular (FITC+, PE–) from extracellular (FITC+, PE+) bacteria.

Microscopy was performed using an Olympus FV1000 confocal microscope. Slides were imaged using the Olympus PlanAPO60×/1.42 oil immersion objective at 1× optical zoom (macrophages/bacteria) or 2× optical zoom (platelets/bacteria). Images were acquired using the FV10-ASW v2.1 software. The following fluorescence filter sets were used for image acquisition: FITC (Ex. 488 nm/Em. 519 nm), tetramethylrhodamine (Ex. 543 nm/Em. 578 nm).

ELISA

Peritoneal macrophages (10⁷/ml) were incubated with unstimulated or thrombin-activated platelets (10⁸/ml) in DPBS in the presence or absence of *S. aureus* (10⁷ CFU/ml) and the mixtures were incubated for 1 h. Supernatants were collected and stored at –20°C until used for ELISA analysis for IL-1β. IL-1β mini ELISA kit was purchased from PeptoTech (Rocky Hill, NJ) and the assay was performed according to the manufacturer's instructions.

Statistical analysis

Data are expressed as mean ± SE. Significant differences between two groups were determined using the two-tailed, unpaired Student *t* test (Figs. 2A, 3A). All other group comparisons were analyzed using one-way ANOVA with Tukey multiple comparison test. Bartlett's test for equal variances was also performed. Statistical significance was set at *p* < 0.05.

Results

Direct killing of *S. aureus* by platelets

Previous reports have shown that platelets can kill *S. aureus*, but the bactericidal mechanism(s) involved are not well understood. To establish the capacity of mouse platelets for direct killing of *S. aureus*, freshly washed mouse platelets were cocultured with bacteria and the bactericidal activity quantified by measuring viable bacteria CFU after 2 h incubation. Fig. 1A shows that unstimulated platelets did not reduce the number of MRSA CFUs, whereas thrombin-stimulated (0.1 U/ml thrombin) platelets effectively reduced the number of CFUs of MRSA compared with the input numbers (>40% killing in 2 h). In comparison, platelets did not require thrombin activation to kill MSSA (Supplemental Fig. 1A). These data suggest that killing different *S. aureus* species has a different dependency on platelet activation.

Platelet-mediated killing of *S. aureus* is dependent on cytochalasin D and independent of DPI

Data from previous reports suggest that *S. aureus* killing most likely occurs extracellularly due to the release of platelet microbicidal proteins and/or β-defensins (22, 23). However, *S. aureus* can be internalized by human platelets (26). To assess whether

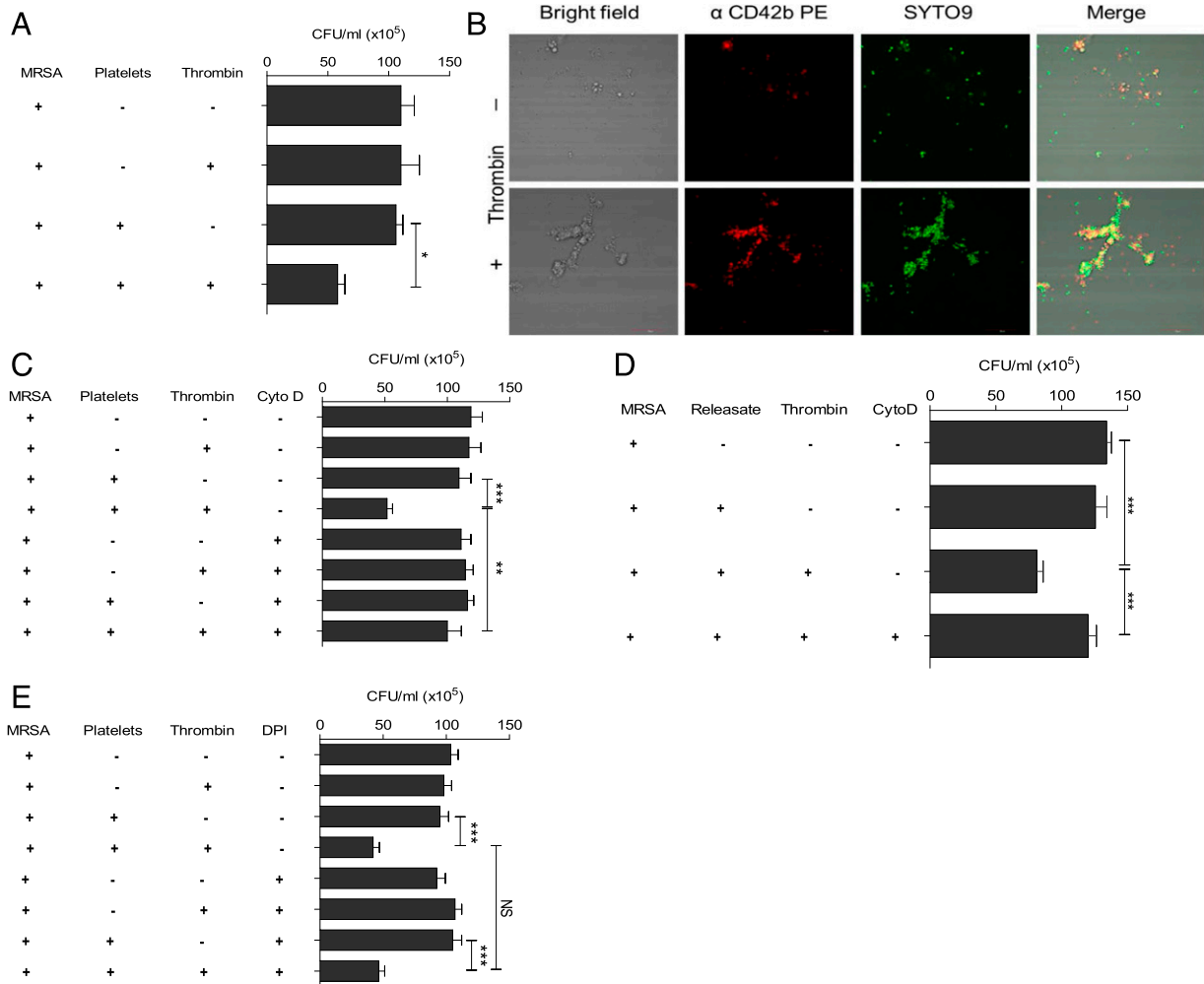


FIGURE 1. Bactericidal activity of platelets against *S. aureus*. MRSA was incubated with unstimulated or thrombin-activated platelets (multiplicity of infection = 1) or their releasate, for 2 h at 37°C in triplicate microfuge tubes. When indicated, platelets were pretreated with inhibitor for 15 min prior to incubation with bacteria. **(A)** Effect of platelet activation on MRSA survival. **(B)** Representative images of platelets binding MRSA after staining with anti CD42b PE (red, α -platelet) and SYTO9 FITC (green, bacteria) respectively. **(C and D)** Effect of cytochalasin D on bactericidal activity of platelets or platelet releasate respectively. **(E)** Effect of DPI on platelet bactericidal activity. All measures are presented as mean \pm SE ($n = 3$). * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

mouse platelet-mediated bactericidal activity depends on internalization of *S. aureus*, cocultures were directly observed using confocal imaging. Platelets could bind bacteria and appeared to form clusters (Fig. 1B), but internalized bacteria were rarely observed. Furthermore, the bactericidal activity was actin dependent, as the addition of cytochalasin D was able to block killing (Fig. 1C). Although cytochalasin D is known to inhibit phagocytosis, it also prevents secretion in platelets. Thus, because internalization of *S. aureus* by platelets was seldom observed, killing activities may be attributed to a secreted product. To further test this hypothesis, we repeated the killing assay in the presence or absence of platelet releasate. Only releasate mediated killing and no killing was observed by releasate from cytochalasin D-pretreated platelets (Fig. 1D). Collectively, these data support the notion that platelets mediate *S. aureus* killing independent of internalization.

Platelets are known to produce modest quantities of reactive oxygen metabolites (ROM) (27). Therefore, we tested the effect of ROM on platelet bactericidal activity. To do this, platelets were pretreated with DPI to inhibit ROM production for 15 min prior to incubation with *S. aureus*. Surprisingly, DPI had no effect on bacterial killing (Fig. 1E). These data suggest that mouse platelets do not require ROMs for bactericidal activity.

Platelets enhance macrophage uptake and restricted intracellular growth of S. aureus

In our previous studies, platelet-depleted mice rapidly succumbed to *S. aureus* blood infection compared with wild-type mice, which survived the infection. This observation may seem at odds with previous reports showing the importance of neutrophils in host-defense against *S. aureus* infection. However, platelets enhance many neutrophil activities, including bacterial killing and neutrophil extracellular trap production (28). These findings suggest that the roles of platelets extend beyond the direct killing of *S. aureus*, and that platelets are important for the function of other phagocytic cells for clearing *S. aureus*. There is also strong evidence of macrophage activity in *S. aureus* infection (29). To test whether platelets enhance macrophage uptake and intracellular killing of *S. aureus*, peritoneal macrophages were incubated with MRSA (1:10 ratio) for 1 h in the presence or absence of thrombin-activated platelets before lysis to assess intracellular CFU. As shown in Fig. 2A, coculture with activated platelets doubled the internalization of MRSA by macrophages, as indicated by the higher CFU per ml at time = 0 h. MSSA internalization by macrophages was also enhanced by platelets (Supplemental Fig. 1B).

To delineate whether bacteria are not just bound to macrophages but truly internalized, we labeled bacteria with FITC-conjugated anti-*S. aureus* primary Ab prior to incubation with macrophages, followed by PE-conjugated anti-IgG secondary Ab after incubation, and then observed the cells by confocal microscopy to distinguish intracellular (FITC+, PE-) from extracellular (FITC+, PE+) bacteria. We observed that most *S. aureus* were FITC+ PE-, indicating that they are internalized by macrophages (Fig. 2B). Importantly, macrophages appear to ingest more bacteria in the presence of activated platelets (lower row) as compared with nonactivated platelets (upper row). To provide a quantitative measure of internalization, CFUs were recovered from lysed macrophages, which show increased phagocytosis in the presence of activated platelets (Fig. 2E). It should also be noted that activated platelets enhanced phagocytosis and restricted intracellular growth of MSSA similar to MRSA (Supplemental Fig. 1B, 1C).

To test whether platelets play any role in enhancing *S. aureus* killing by macrophages, peritoneal macrophages from Fig. 2A time = 0 h were cocultured in the absence or presence of freshly prepared platelets for an additional 3 h ($t = 3$ h) and CFUs were enumerated. In the absence of platelets there was a 3–4-fold

increase in the CFU collected from macrophages after 3 h, suggesting bacterial replication inside macrophages (Fig. 2C, 2D). However, in the presence of thrombin-activated platelets, MRSA replication was significantly reduced, suggesting that platelet-stimulated macrophages are more efficient at restricting the intracellular growth of *S. aureus*. It should also be noted that platelet-mediated enhancement of phagocytosis and restriction of intracellular growth is thrombin dependent (Fig. 2E, 2F). This is consistent and supports our observations in Fig. 2C.

Releasates from thrombin-stimulated platelets enhance macrophage uptake and restrict intracellular growth of S. aureus

Platelets can engulf bacteria and facilitate their transport to immune effector cells (26, 30). So it is possible that platelets capture blood-borne pathogens and deliver them through direct contact to macrophages for phagocytosis and killing, consistent with observations in Fig. 2. Another possibility is that enhanced uptake and killing occurs through soluble mediators released by platelets. To determine whether platelet-enhanced uptake and killing was due to direct interaction or through releasable products, parallel

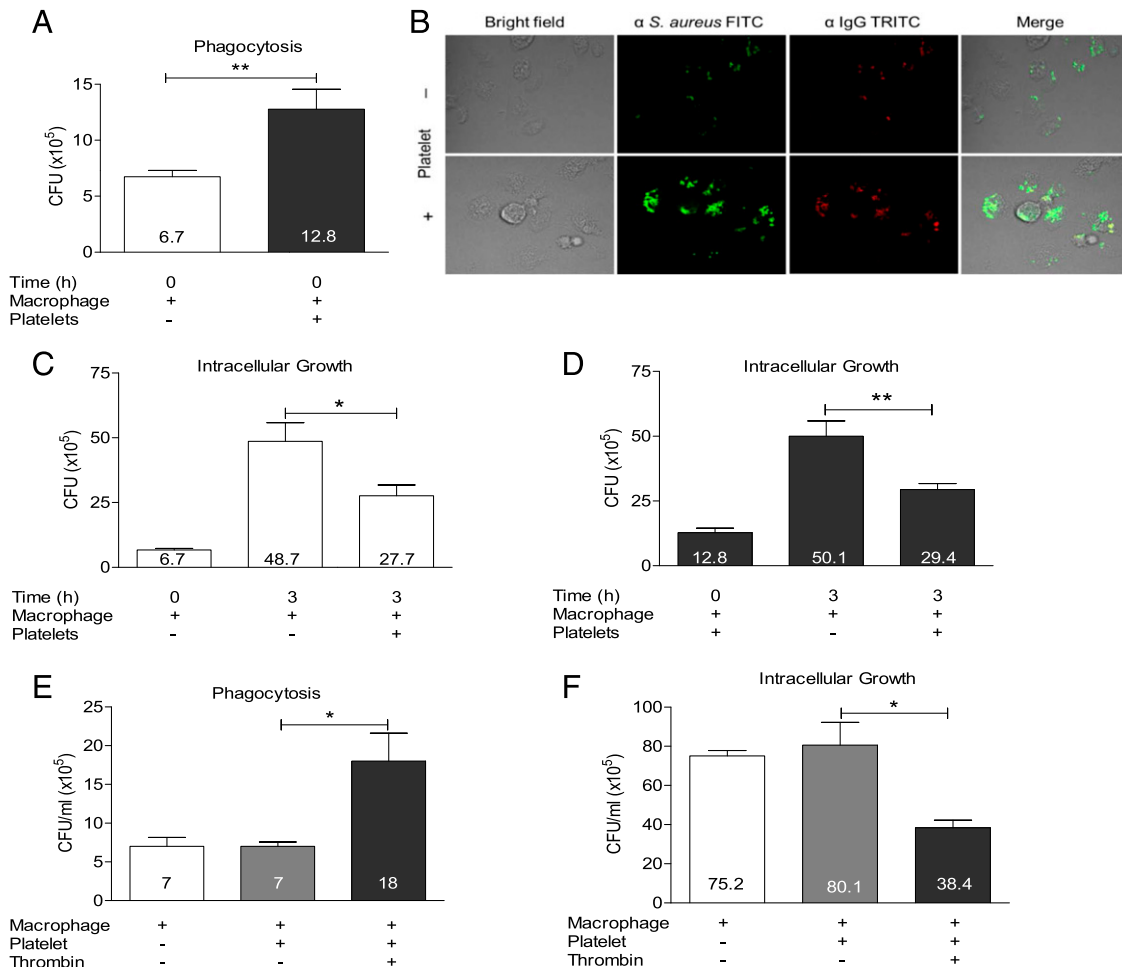


FIGURE 2. Platelets enhance macrophage phagocytosis and restrict intracellular growth of *S. aureus*. Peritoneal macrophages were incubated with MRSA in RPMI 1640 medium in the presence or absence of 0.1 U/ml thrombin-activated platelets for 1 h ($t = 0$). Peritoneal macrophages were washed with PBS to clear nonphagocytosed bacteria and platelets. **(A)** MRSA CFU collected from macrophage lysates at $t = 0$. **(B)** Representative confocal images of macrophages at $t = 0$. Peritoneal macrophages were incubated with FITC-labeled MRSA in RPMI 1640 medium in the presence or absence of 0.1 U/ml thrombin-activated platelets for 1 h ($t = 0$), washed with PBS, and stained with PE-conjugated anti-IgG secondary Ab. **(C and D)** Macrophages from white or black bars in **(A)** were further incubated for 3 h ($t = 3$) in the presence or absence of freshly activated platelets, intracellular MRSA were collected from macrophage lysates, and CFUs were determined. **(E)** MRSA CFU collected from macrophage lysates at $t = 0$. **(F)** Macrophages from white, gray, or black bars in **(E)** were further incubated for 3 h ($t = 3$) with platelets in the presence or absence of thrombin, intracellular MRSA were collected from macrophage lysates, and CFUs were determined. All measures are presented as mean \pm SE ($n = 3$). * $p < 0.05$, ** $p < 0.005$.

experiments were performed as in Fig. 2 except peritoneal macrophages were incubated with releasates collected from resting or activated platelets instead of intact platelets. Interestingly, platelet releasates alone significantly enhanced macrophage uptake of bacteria by over 60% (Fig. 3A). This uptake was supported by confocal images indicating enhanced *S. aureus* internalization (Fig. 3B). Additionally, platelet releasates restricted intracellular growth of MRSA, as indicated by significant inhibition of bacterial growth compared with control (Fig. 3C, 3D). The effect mediated by platelet releasates also appears similar to that mediated by platelets (compare Figs. 2 and 3). Collectively, these data indicate that platelets mediate this process through releasable products rather than through direct interactions with macrophages.

Platelets enhance macrophage uptake and restrict intracellular growth of *S. aureus* through IL-1 β

Activated platelets release many bioactive molecules with immune functions that affect other immune cells, including macrophages. For example, platelets are a rich source of cytokines and chemokines such as IL-1 β , sCD40L, and RANTES (14, 31–33). To assess this, phagocytosis and killing assays were performed as above except peritoneal macrophages were incubated with *S. aureus* in the presence or absence of recombinant IL-1 β , sCD40L, RANTES, or a combination of all three. Unexpectedly IL-1 β , but not sCD40L or RANTES, significantly increased the uptake of *S. aureus* by macrophages by ~33% (Fig. 4A), and the addition of all mediators combined did not add to the effect observed by IL-1 β alone (data not shown). Intracellular killing assays were also performed in the presence or absence of these recombinant cytokines to determine if they can mimic the effects of intracellular killing of *S. aureus* by macrophages. Again, only IL-1 β , but not sCD40L or RANTES, significantly inhibited intracellular growth of *S. aureus* (Fig. 4C, 4D). Dose-dependency studies determined that the optimal dose of IL-1 β was 10 ng/ml for both internalization and reducing intracellular growth (Fig. 5).

To further test this hypothesis, uptake and intracellular killing assays were repeated with platelet releasates in the presence or absence of anti-IL-1 β neutralizing Ab. Internalization of *S. aureus* by macrophages showed a modest 25% decrease in the presence of IL-1 β neutralizing Ab, which did not reach statistical significance. However, neutralization of IL-1 β from platelet releasates significantly abrogated the reduced intracellular growth of *S. aureus* by macrophages by 81% (Fig. 6).

Finally, IL-1 β levels present in the solution before and after the addition of platelet releasates were quantified. As shown in Fig. 7, neither platelets (nonactivated or thrombin-activated) nor macrophages produced detectable amounts of IL-1 β . However, in the presence of MRSA, both cell populations produce marginal amounts of IL-1 β (all mice showed increases but statistical significance was only noted in three of four mice tested). Macrophages only produced measurable amounts of IL-1 β when cocultured with MRSA and platelets. However, the addition of thrombin-activated platelets caused a robust increase in the IL-1 β produced by macrophages thus implying that thrombin-activated platelets are required for optimal IL-1 β production.

Discussion

Accumulating evidence suggests that platelets contribute to diverse immunological processes, extending beyond their classical role in hemostasis and thrombosis. Platelets engage the immune system by interacting with various immune cells (34, 35), and participate in both innate and adaptive immune responses. Our recent publication indicated that platelets provide protection to *S. aureus* infection (19). Based on this observation, the goal of the current study was to further elucidate the mechanistic details of how platelets promote protection against *S. aureus* infection and whether this occurs directly and/or through the potentiation of leukocytes antimicrobial functions.

In this study, we observed that 1) platelets kill *S. aureus* directly in an activation-dependent manner; 2) activated platelets bind, but

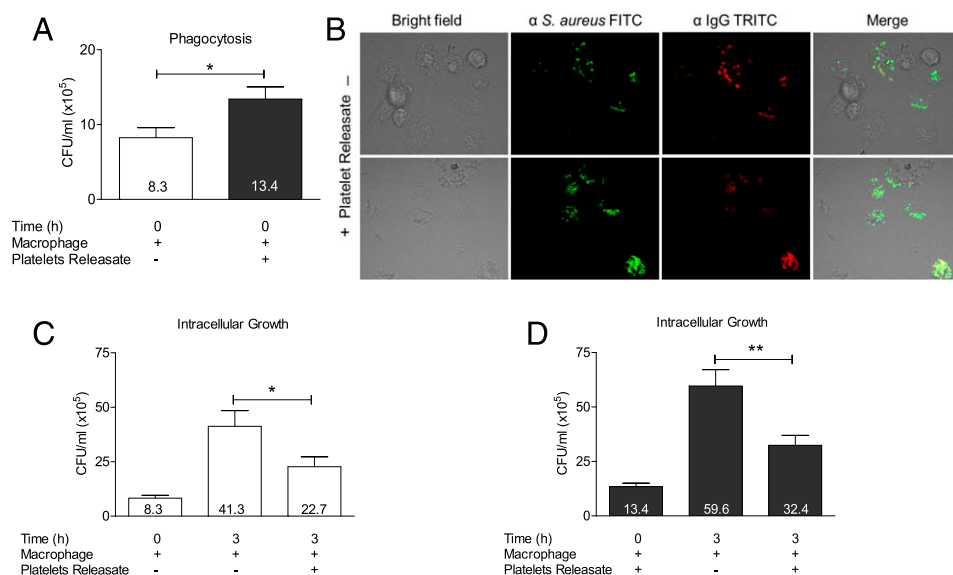
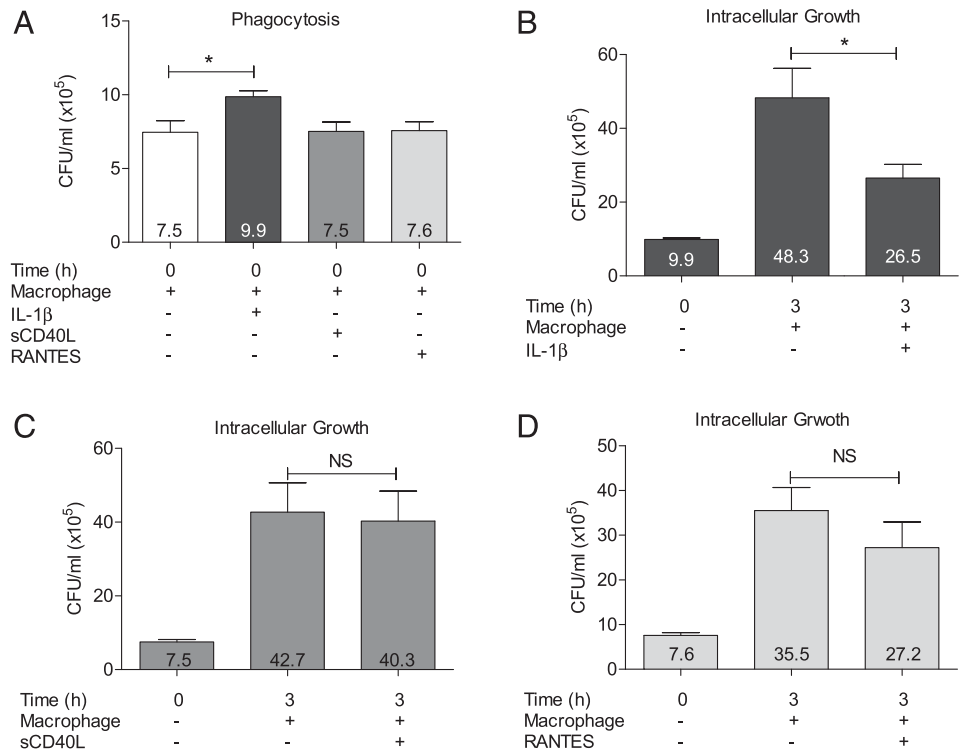


FIGURE 3. Releasates from thrombin-stimulated platelets enhances macrophage uptake and restrict intracellular growth of *S. aureus*. Peritoneal macrophages were incubated for 1 h ($t = 0$) with MRSA in RPMI 1640 medium in the presence or absence of releasates collected from 0.1 U/ml thrombin-activated platelets. Peritoneal macrophages were washed with PBS to clear nonphagocytosed bacteria. **(A)** MRSA CFU from macrophage lysates at $t = 0$. **(B)** Representative confocal images of macrophages at $t = 0$. Peritoneal macrophages were incubated with FITC-labeled MRSA in RPMI 1640 medium in the presence or absence of releasates collected from 0.1 U/ml thrombin-activated platelets for 1 h ($t = 0$), washed with PBS, and stained with PE-conjugated anti-IgG secondary Ab. **(C and D)** Macrophages from white or black bars in **(A)** were further incubated for 3 h ($t = 3$) in the presence or absence of fresh releasate collected from 0.1 U/ml thrombin-activated platelets and intracellular MRSA from macrophage lysates was determined. All measures are presented as mean \pm SE ($n = 3$). * $p < 0.05$, ** $p < 0.01$.

FIGURE 4. IL-1 β enhanced *S. aureus* phagocytosis by peritoneal macrophages and restricted their intracellular growth. Peritoneal macrophages were incubated with MRSA in RPMI 1640 medium in the presence or absence of IL-1 β , CD40L, or RANTES for 1 h ($t = 0$). Peritoneal macrophages were washed with PBS to clear nonphagocytosed bacteria. **(A)** MRSA collected from macrophage lysates at $t = 0$ were plated and CFUs enumerated. **(B–D)** Macrophages from black or gray bars in **(A)** were further incubated for 3 h ($t = 3$) in the presence or absence of IL-1 β , CD40L, RANTES, washed with PBS, then intracellular MRSA were recovered from macrophage lysates, and CFU were determined. All measures are presented as mean \pm SE ($n = 4$). * $p < 0.05$. NS, not significant.



rarely internalize, *S. aureus* and force the pathogens into clusters; 3) cytochalasin D is able to block killing; and 4) platelet bactericidal activity appears to be independent of ROM. The inhibition of killing by cytochalasin D suggests that bactericidal activity is actin dependent. Actin polymerization and rearrangement is an important process for phagocytosis as well as for fusion of intracellular granules with plasma membrane, which is common during platelet activation. Because 1) internalization of *S. aureus* was not observed, 2) we detected killing of *S. aureus* using releasates from thrombin-activated platelets, and 3) cytochalasin D inhibited killing by intact platelets and platelet releasates, we interpret these data to suggest that release of granule contents is responsible for killing *S. aureus*. Thus, our data are consistent with previous observations suggesting that *S. aureus* killing most likely occurs extracellularly (22).

Our studies also suggest an indirect role of platelets in pathogen clearance through potentiation of phagocytic and killing capacity of phagocytes. It was observed that 1) activated platelets or their releasates enhance *S. aureus* phagocytosis and restrict intracellular growth within peritoneal macrophages; 2) platelet-secreted IL-1 β , but not CD40L or RANTES, mediated enhancement of *S. aureus*

phagocytosis and restricted intracellular growth in peritoneal macrophages; and 3) IL-1 β -neutralizing Ab blocked platelet releasate-mediated enhancement of *S. aureus* phagocytosis and allowed *S. aureus* intracellular survival in peritoneal macrophages. These data suggest that the role of platelets extends beyond direct encounter of pathogens, and that platelets play a critical role in mediating phagocytic leukocyte antimicrobial functions during infection.

The IL-1 family of proteins has been implicated for its ability to regulate functional properties of many immune cell types and is an important mediator of host response to infection (36, 37). Activated platelets secrete IL-1 β and mediate inflammatory signaling by regulating IL-1 β synthesis (38). Additionally, IL-1 β is secreted by activated murine macrophages upon exposure to LPS (39). A recent study has shown that IL-1 β stimulates antimicrobial immunity in macrophages through the recruitment of other antimicrobial effector molecules (40). In that study, recombinant IL-1 β restricted intracellular replication and growth of *Mycobacterium tuberculosis* in murine and human macrophages. Our results are, collectively, consistent with these previous studies.

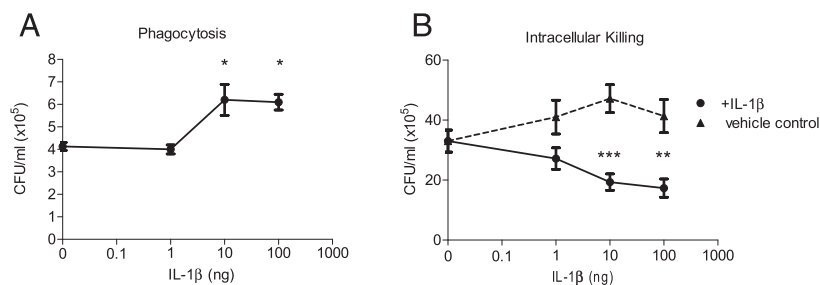


FIGURE 5. IL-1 β dose response relationship. Peritoneal macrophages were incubated with MRSA in RPMI 1640 medium in the presence of different concentrations of IL-1 β . Peritoneal macrophages were washed with PBS to clear nonphagocytosed bacteria. **(A)** MRSA CFU from macrophage lysates at $t = 0$. **(B)** Macrophages from **(A)** were further incubated for 3 h ($t = 3$) in the presence (●) or absence (▲) of IL-1 β , washed with PBS, then intracellular MRSA were collected from macrophage lysates, and CFUs were determined. All measures are presented as mean \pm SE ($n = 3$). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

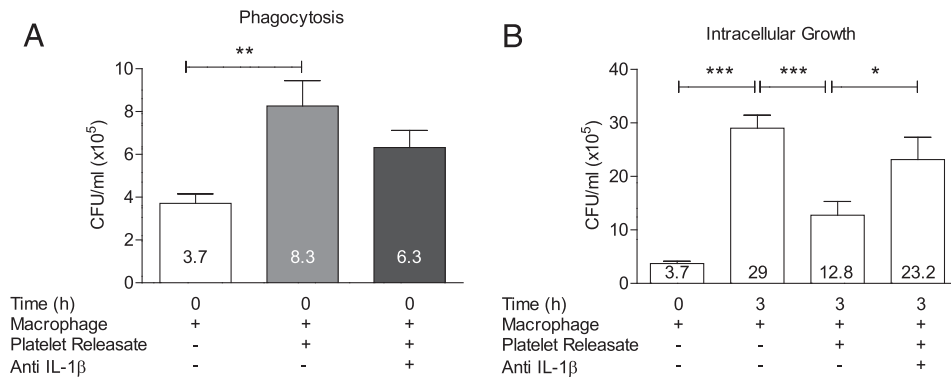


FIGURE 6. Effect of neutralizing IL-1 β on platelet releasate-mediated phagocytosis and restriction of intracellular growth of *S. aureus* by peritoneal macrophages. Peritoneal macrophages were incubated with MRSA in RPMI 1640 medium with releasate collected from 0.1 U/ml thrombin-activated platelets for 1 h ($t = 0$) in the presence or absence of anti-IL-1 β -neutralizing Ab. Peritoneal macrophages were washed with PBS to clear nonphagocytosed bacteria. **(A)** MRSA collected from macrophage lysates at $t = 0$ were plated and CFUs enumerated. **(B)** Macrophages from white bar in **(A)** were further incubated for 3 h ($t = 3$) with freshly added platelets releasate in the presence or absence of anti-IL-1 β and intracellular bacteria CFUs from macrophage lysates were determined. All measures are presented as mean \pm SE ($n = 3$). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

Our data indicate that platelet-produced IL-1 β induced macrophages to further secrete additional IL-1 β , amplifying growth restriction of *S. aureus*. Macrophages produce marginal levels of IL-1 β in response to *S. aureus*, (Fig. 7). However, upon the addition of activated platelets, IL-1 β levels increased dramatically. The effect of IL-1 β on the uptake of *S. aureus* by macrophages did not seem to be as significant as its effect on restricting intracellular growth. However, we observed that activated platelets surround *S. aureus* and force bacteria into clusters, which has been previously reported (23). The enhanced aggregation and clustering of bacteria will likely facilitate the engulfment of increased bacteria numbers by macrophages, thus enhancing phagocytosis and promoting rapid clearance of bacteria from circulation. In parallel to direct encounter of pathogens, platelets mediate bactericidal activities indirectly through enhancement of phagocytosis and restricted intracellular growth by other leukocytes.

Our work supports other recent evidence that platelets assist in clearance of *S. aureus* (and potentially other bacterial species) by liver Kupffer cells. Kupffer cells are macrophage-like cells that have been shown to interact with platelets during septic infection with *S. aureus* (30). In light of our current observations, the *S. aureus* captured by Kupffer cells and subsequently surrounded by platelets may be enhancing the clearance of *S. aureus* in vivo

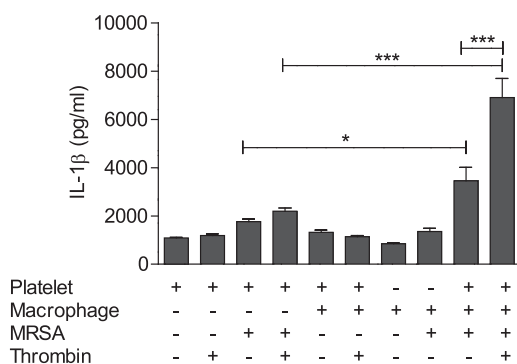


FIGURE 7. Platelets secrete IL-1 β and synergistically induce macrophages secretion of IL-1 β . Peritoneal macrophages (10^7 /ml) were incubated with unstimulated or thrombin-activated platelets (10^8 /ml) in PBS in the presence or absence of MRSA (10^7 CFU/ml) and the mixtures were incubated for 1 h. Supernatants were collected and analyzed by ELISA for quantification of IL-1 β . All measures are presented as mean \pm SE ($n = 4$). * $p < 0.05$, *** $p < 0.0001$.

through several mechanisms. First, interaction between Kupffer cells and platelets is mediated by GPIb (CD42) and GPIIb (CD41), which are known to activate platelets. This may provide an activating signal required for platelets to kill *S. aureus* localized to Kupffer cell surfaces. Secondly, platelet release of IL-1 β may enhance Kupffer cell activities (uptake and reduced intracellular survival) similarly to peritoneal macrophages in our study. Therefore, platelets and Kupffer cells work in a coordinated fashion to localize *S. aureus* to defined areas where platelets can accumulate, kill *S. aureus*, and activate Kupffer cells.

Overall, platelets appear to be an emerging central player in host-defense through direct bactericidal activities and by orchestrating innate immune cells to perform more efficiently in clearing infectious pathogens.

Disclosures

The authors have no financial conflicts of interest.

References

- Smyth, S. S., R. P. McEver, A. S. Weyrich, C. N. Morrell, M. R. Hoffman, G. M. Arepally, P. A. French, H. L. Dauerman, and R. C. Becker. 2009. Platelet Colloquium Participants. 2009. Platelet functions beyond hemostasis. *J. Thromb. Haemost.* 7: 1759–1766.
- Yeaman, M. R. 2014. Platelets: at the nexus of antimicrobial defence. *Nat. Rev. Microbiol.* 12: 426–437.
- Garraud, O., and F. Cognasse. 2015. Are platelets cells? And if yes, are they immune cells? *Front. Immunol.* 6: 70.
- Al Dieri, R., B. de Laat, and H. C. Hemker. 2012. Thrombin generation: what have we learned? *Blood Rev.* 26: 197–203.
- Gardiner, E. E., and R. K. Andrews. 2013. Platelets: envoys at the infection frontline. *J. Infect. Dis.* 208: 871–873.
- Klinger, M. H. F. 1997. Platelets and inflammation. *Anat. Embryol. (Berl.)* 196: 1–11.
- Yeaman, M. R. 1997. The role of platelets in antimicrobial host defense. *Clin. Infect. Dis.* 25: 951–968, quiz 969–970.
- Hamzeh-Cognasse, H., P. Damien, A. Chabert, B. Pozzetto, F. Cognasse, and O. Garraud. 2015. Platelets and infections - complex interactions with bacteria. *Front. Immunol.* 6: 82.
- Abdulrehman, A. Y., E. C. Jackson, and A. McNicol. 2013. Platelet activation by *Streptococcus sanguinis* is accompanied by MAP kinase phosphorylation. *Platelets* 24: 6–14.
- Kälvegren, H., C. Skoglund, C. Helldahl, M. Lerm, M. Grenegård, and T. Bengtsson. 2010. Toll-like receptor 2 stimulation of platelets is mediated by purinergic P2X1-dependent Ca $^{2+}$ mobilisation, cyclooxygenase and purinergic P2Y1 and P2Y12 receptor activation. *Thromb. Haemost.* 103: 398–407.
- Sun, D., N. I. Popescu, B. Raisley, R. S. Keshari, G. L. Dale, F. Lupu, and K. M. Coggeshall. 2013. *Bacillus anthracis* peptidoglycan activates human platelets through Fc γ RII and complement. *Blood* 122: 571–579.
- Keane, C., D. Tilley, A. Cunningham, A. Smolenski, A. Kadioglu, D. Cox, H. F. Jenkinson, and S. W. Kerrigan. 2010. Invasive *Streptococcus pneumoniae* trigger platelet activation via Toll-like receptor 2. *J. Thromb. Haemost.* 8: 2757–2765.

13. Arman, M., K. Krauel, D. O. Tilley, C. Weber, D. Cox, A. Greinacher, S. W. Kerrigan, and S. P. Watson. 2014. Amplification of bacteria-induced platelet activation is triggered by FcγRIIA, integrin αIIbβ3, and platelet factor 4. *Blood* 123: 3166–3174.
14. Coppinger, J. A., G. Cagney, S. Toomey, T. Kislinger, O. Belton, J. P. McRedmond, D. J. Cahill, A. Emili, D. J. Fitzgerald, and P. B. Maguire. 2004. Characterization of the proteins released from activated platelets leads to localization of novel platelet proteins in human atherosclerotic lesions. *Blood* 103: 2096–2104.
15. Cho, J. S., Y. Guo, R. I. Ramos, F. Hebroni, S. B. Plaisier, C. Xuan, J. L. Granick, H. Matsushima, A. Takashima, Y. Iwakura, et al. 2012. Neutrophil-derived IL-1β is sufficient for abscess formation in immunity against *Staphylococcus aureus* in mice. *PLoS Pathog.* 8: e1003047.
16. Talan, D. A., A. Krishnadasan, R. J. Gorwitz, G. E. Fosheim, B. Limbago, V. Albrecht, and G. J. Moran, EMERGENCY ID Net Study Group. 2011. Comparison of *Staphylococcus aureus* from skin and soft-tissue infections in US emergency department patients, 2004 and 2008. *Clin. Infect. Dis.* 53: 144–149.
17. Gordon, R. J., and F. D. Lowy. 2008. Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection. *Clin. Infect. Dis.* 46(Suppl. 5): S350–S359.
18. Moreillon, P., Y. A. Que, and A. S. Bayer. 2002. Pathogenesis of streptococcal and staphylococcal endocarditis. *Infect. Dis. Clin. North Am.* 16: 297–318.
19. Wuescher, L. M., A. Takashima, and R. G. Worth. 2015. A novel conditional platelet depletion mouse model reveals the importance of platelets in protection against *S. aureus* bacteremia. *J. Thromb. Haemost.* 13: 303–313.
20. Yeaman, M. R., D. C. Norman, and A. S. Bayer. 1992. Platelet microbicidal protein enhances antibiotic-induced killing of and postantibiotic effect in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 36: 1665–1670.
21. Yeaman, M. R., S. M. Puentes, D. C. Norman, and A. S. Bayer. 1992. Partial characterization and staphylocidal activity of thrombin-induced platelet microbicidal protein. *Infect. Immun.* 60: 1202–1209.
22. Trier, D. A., K. D. Gank, D. Kupferwasser, N. Y. Yount, W. J. French, A. D. Michelson, L. I. Kupferwasser, Y. Q. Xiong, A. S. Bayer, and M. R. Yeaman. 2008. Platelet antistaphylococcal responses occur through P2X1 and P2Y12 receptor-induced activation and kinocidin release. *Infect. Immun.* 76: 5706–5713.
23. Kraemer, B. F., R. A. Campbell, H. Schwertz, M. J. Cody, Z. Franks, N. D. Tolley, W. H. Kahr, S. Lindemann, P. Seizer, C. C. Yost, et al. 2011. Novel anti-bacterial activities of β-defensin 1 in human platelets: suppression of pathogen growth and signaling of neutrophil extracellular trap formation. *PLoS Pathog.* 7: e1002355.
24. Worth, R. G., C. D. Chien, P. Chien, M. P. Reilly, S. E. McKenzie, and A. D. Schreiber. 2006. Platelet FcγRIIA binds and internalizes IgG-containing complexes. *Exp. Hematol.* 34: 1490–1495.
25. McCullough, A. C., M. Seifried, X. Zhao, J. Haase, W. J. Kabat, R. Yogev, R. M. Blumenthal, and D. Mukundan. 2011. Higher incidence of perineal community acquired MRSA infections among toddlers. *BMC Pediatr.* 11: 96.
26. Youssefian, T., A. Drouin, J.-M. Massé, J. Guichard, and E. M. Cramer. 2002. Host defense role of platelets: engulfment of HIV and *Staphylococcus aureus* occurs in a specific subcellular compartment and is enhanced by platelet activation. *Blood* 99: 4021–4029.
27. Wachowicz, B., B. Olas, H. M. Zbikowska, and A. Buczyński. 2002. Generation of reactive oxygen species in blood platelets. *Platelets* 13: 175–182.
28. Clark, S. R., A. C. Ma, S. A. Tavener, B. McDonald, Z. Goodarzi, M. M. Kelly, K. D. Patel, S. Chakrabarti, E. McAvoy, G. D. Sinclair, et al. 2007. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat. Med.* 13: 463–469.
29. Flannagan, R. S., B. Heit, and D. E. Heinrichs. 2015. Antimicrobial mechanisms of macrophages and the immune evasion strategies of *Staphylococcus aureus*. *Pathogens* 4: 826–868.
30. Wong, C. H. Y., C. N. Jenne, B. Petri, N. L. Chrobok, and P. Kubers. 2013. Nucleation of platelets with blood-borne pathogens on Kupffer cells precedes other innate immunity and contributes to bacterial clearance. *Nat. Immunol.* 14: 785–792.
31. Hawrylowicz, C. M., S. A. Santoro, F. M. Platt, and E. R. Unanue. 1989. Activated platelets express IL-1 activity. *J. Immunol.* 143: 4015–4018.
32. Henn, V., J. R. Slupsky, M. Gräfe, I. Anagnostopoulos, R. Förster, G. Müller-Berghaus, and R. A. Kroczeck. 1998. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 391: 591–594.
33. Klingler, M. H., D. Wilhelm, S. Bubel, M. Sticherling, J. M. Schröder, and W. Kühnel. 1995. Immunocytochemical localization of the chemokines RANTES and MIP-1 alpha within human platelets and their release during storage. *Int. Arch. Allergy Immunol.* 107: 541–546.
34. Ghasemzadeh, M., and E. Hosseini. 2013. Platelet-leukocyte crosstalk: linking proinflammatory responses to procoagulant state. *Thromb. Res.* 131: 191–197.
35. Li, N. 2008. Platelet-lymphocyte cross-talk. *J. Leukoc. Biol.* 83: 1069–1078.
36. Oppenheim, J. J., E. J. Kovacs, K. Matsushima, and S. K. Durum. 1986. There is more than one interleukin 1. *Immunol. Today* 7: 45–56.
37. Dinarello, C. A. 2009. Immunological and inflammatory functions of the interleukin-1 family. *Annu. Rev. Immunol.* 27: 519–550.
38. Lindemann, S., N. D. Tolley, D. A. Dixon, T. M. McIntyre, S. M. Prescott, G. A. Zimmerman, and A. S. Weyrich. 2001. Activated platelets mediate inflammatory signaling by regulated interleukin 1β synthesis. *J. Cell Biol.* 154: 485–490.
39. Beuscher, H. U., C. Günther, and M. Rölinghoff. 1990. IL-1 beta is secreted by activated murine macrophages as biologically inactive precursor. *J. Immunol.* 144: 2179–2183.
40. Jayaraman, P., I. Sada-Ovalle, T. Nishimura, A. C. Anderson, V. K. Kuchroo, H. G. Remold, and S. M. Behar. 2013. IL-1β promotes antimicrobial immunity in macrophages by regulating TNFR signaling and caspase-3 activation. *J. Immunol.* 190: 4196–4204.