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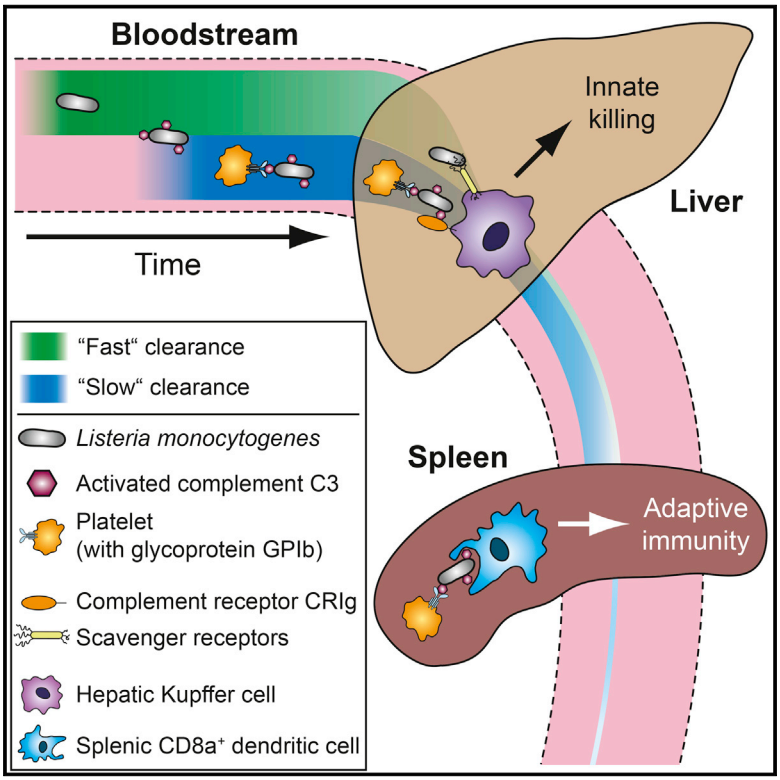
Dual-Track Clearance of Circulating Bacteria Balances Rapid Restoration of Blood Sterility with Induction of Adaptive Immunity

Graphical Abstract



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In Brief

Broadley et al. report that overall clearance of intravascular bacteria is the cumulative result of two parallel and kinetically distinct pathways. Platelet binding shifts blood-borne bacteria from "fast clearance" via scavenger receptors toward CRIg-dependent "slow clearance." Consequently, "dual-track" clearance balances rapid restoration of blood sterility with induction of specific antibacterial immunity.

Highlights

- Platelet-bound and free *L. monocytogenes* clear from circulation with distinct kinetics
- Liver macrophages mediate "fast clearance" of intravascular LM via scavenger receptors
- Platelet binding shifts LM from "fast" into CRIg-dependent "slow" clearance pathways
- "Dual-track clearance" balances innate and adaptive immunity during bacteremia

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Dual-Track Clearance of Circulating Bacteria Balances Rapid Restoration of Blood Sterility with Induction of Adaptive Immunity

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SUMMARY

Efficient clearance of bacteremia prevents life-threatening disease. Platelet binding to intravascular bacteria, a process involving platelet glycoprotein GPIb and bacterial opsonization with activated complement C3, influences blood clearance and anti-infective immunity. Using intravital microscopy of the bloodstream of mice infected with *Listeria monocytogenes*, we show that bacterial clearance is not a uniform process but a "dual-track" mechanism consisting of parallel "fast" and "slow" pathways. "Slow clearance" is regulated by time-dependent bacterial opsonization, stochastic platelet binding, and capture of bacteria-platelet-complexes via the complement receptor of the immunoglobulin superfamily, CRIg. The mechanism spares some bacteria from "fast clearance" and rapid destruction in the liver via Kupffer cell scavenger receptors, keeping them available for adaptive immunity induction by splenic CD8α^+ dendritic cells. We consistently find "fast" and "slow" clearance patterns for a broad panel of other Gram+ and Gram− bacteria. Thus, dual-track clearance balances rapid restoration of blood sterility with induction of specific antibacterial immunity.

INTRODUCTION

Under normal circumstances the bloodstream is sterile, but surgical interventions, intravenous (i.v.) catheter use, or disseminating infections may allow bacteria to reach the circulation, causing bacteremia. Bacteremia is connected to serious disease, including sepsis, infectious endocarditis (IE), and meningitis, and even with their incidence underestimated (Goto and Al-Hasan, 2013), bloodstream infections rank among the most common causes of death (Miniño et al., 2011). To prevent life-threatening situations from arising, it is an important task for the immune system to (1) quickly clear bacteria from the blood and (2) develop lasting adaptive immunity.

Phagocytes of the mononuclear phagocyte system (MPS) form an early line of innate defense that monitors and clears the circulation (van Furth et al., 1972). Liver-resident macrophages known as Kupffer cells (KCs) make up more than half the MPS, efficiently sequestering bacteria entering the circulation, including commensals from the portal vein (Jenne and Kubes, 2013; Balmer et al., 2014). The liver functions as a "firewall" that effectively removes bacteria from the circulation (Balmer et al., 2014), and indeed, liver cirrhosis patients show prolonged bacteremia persistence (Ashare et al., 2009). Blood clearance by liver KC is well-characterized, and detailed studies revealed how KC directly phagocytose and kill captured bacteria (Wardlaw and Howard, 1959) or serve as focal point for immune mechanisms by platelets (Wong et al., 2013) and neutrophils (Gregory et al., 1996).

For some bacterial infections to become fully controlled, however, early phagocytic restriction in the liver must be accompanied by pathogen-specific adaptive immunity. Full control of systemic infection with *Listeria monocytogenes* (LM), for instance, requires the induction of LM-specific cytotoxic T cell immunity (McGregor et al., 1970; Harty et al., 1996; Pamer, 2004). Such responses develop in the context of secondary lymphoid organs, induced by antigen-laden professional antigen-presenting cells, in the case of LM dendritic cells (DCs) (Jung et al., 2002) and splenic CD8α^+ DCs in particular (Belz et al., 2005; Reinicke et al., 2009; Verschoor et al., 2011). As the liver, the spleen sequesters bacteria during systemic infection, particularly in the marginal zone (MZ) (Aichele et al., 2003; Aoshi et al., 2009) where intravascular bacteria become localized

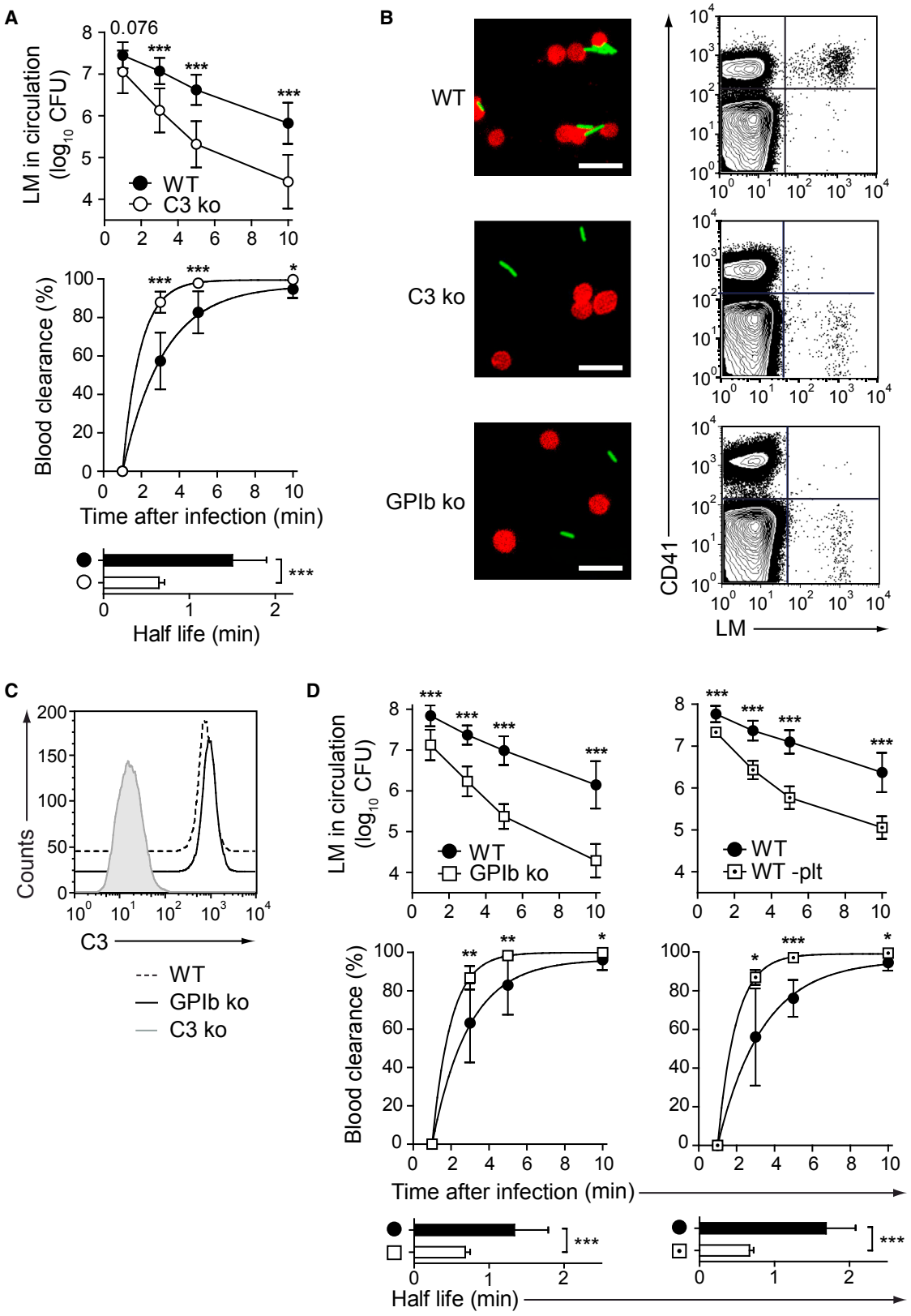


Figure 1. Complement-Enabled Platelet Binding Determines Blood Clearance Kinetics of LM

(A) Vascular clearance and half-lives of LM in min after i.v. injection into WT (black circles) and C3 ko (white circles) mice.

(B) Fluorescence microscopy (left) and flow cytometry (right) of blood samples obtained 1 min p.i. with CFSE-marked LM; platelets, red; LM, green. Scale bars, 5 μm.

(legend continued on next page)

to a network of MZ B cells, MZ macrophages, metallophillic macrophages, and DCs (Mebius and Kraal, 2005). In contrast to macrophages or granulocytes that rapidly destroy phagocytosed pathogens, DCs preserve antigenic information to induce antibacterial T cell immunity (Savina and Amigorena, 2007). Thus, to balance immediate with long-term protection, a finely tuned equilibrium must exist between phagocytes in liver and spleen that capture bacteria to rapidly destroy them and those preserving antigenic information to induce lasting adaptive immunity.

In the current study, we set out to define how the immune system orchestrates this balancing act in the well-characterized model of systemic LM infection in mice. By studying bacterial blood clearance profiles, organ and cellular targeting, bactericidal activity, and adaptive immunity induction under various experimental conditions, we find that collaboration between the complement system and blood platelets controls the early bio-distribution of blood-borne LM across specialized phagocyte populations of liver and spleen. Using intravital microscopy (IVM) of the blood circulation, a non-invasive and highly sensitive imaging application to study the circulatory behavior of intravascular bacteria, we find that blood clearance is not a uniform process. Instead, we find it to be the cumulative result of two distinct but parallel processes, namely (1) clearance of "bacteria-platelet complexes," requiring glycoprotein Ib (GPIb)-dependent binding of complement C3-opsonized bacteria to platelets, and (2) clearance of "free bacteria," which proceeds independent of complement and platelets. To reflect their characteristic kinetics, we refer to the individual routes of this "dual-track" clearance system as "slow clearance" and "fast clearance," respectively. Moreover, we identify the complement receptor (CR) of the immunoglobulin superfamily (CRIg) to mediate "slow" and scavenger receptors (SR) to mediate "fast" clearance.

Importantly, and building on previous work (Verschoor et al., 2011), we here describe that "dual-track" clearance critically controls the balance between rapid restoration of blood sterility and effective induction of adaptive immunity to intravascular bacterial infection. While SR-mediated "fast clearance" excels in the rapid capture and killing of free bacteria by KC, CRIg-mediated "slow clearance" allows a portion of platelet-bound bacteria to remain longer in the circulation and available to splenic CD8α^+ DCs to promote antibacterial cytotoxic T cell immunity. We show that this balance becomes especially relevant under conditions of limiting bacterial antigen, which is the norm during frequent forms of (transient) low-level bacteremia (Balmer et al., 2014; Ashare et al., 2009; Kellogg et al., 2000; Zhang et al., 2013). Our work therewith provides unprecedented insights in the molecular and cellular basis of blood clearance and shows how blood-borne systems (i.e., complement and platelets) establish a "division of labor" between specialized phagocyte populations of blood filtering organs, directing a balanced antibacterial immune response.

RESULTS

Complement, Platelets, and the Clearance of Systemic Bacteria

CRs form an integral part of the receptor palette enabling phagocytes to clear the circulation from unwanted substances (van Lookeren Campagne et al., 2007). Still, blood clearance rates may become accelerated, rather than delayed, under conditions of limited complement activity (Verschoor et al., 2011; Waxman et al., 1984; Davies et al., 1992). In conjunction with previous work (Verschoor et al., 2011), we show that this counterintuitive phenomenon is well-illustrated during systemic infection with LM: while complement C3-deficient (*C3* ko) mice clear >90% of bacteria from the circulation in <5 min, it takes complement-sufficient wild-type (WT) mice >10 min to reach this benchmark. Accelerated clearance in C3 ko animals is also reflected by their 2-fold shorter bacterial circulatory half-life compared to WT animals (Figure 1A), in agreement with previous observations (Verschoor et al., 2011).

Not only do CRs on phagocytes recognize C3-coated bacteria, but glycoprotein GPIb on platelets is critically involved in interaction with C3-opsonized bacteria, resulting in the formation of bacteria-platelet complexes (Figure 1B) (Verschoor et al., 2011). Strikingly, and in spite of intact complement opsonization (Figure 1C), blood clearance of GPIb ko mice (Figure 1D) is as accelerated as in C3 ko mice (Figure 1A). This indicates that platelet binding, rather than complement opsonization and recognition by CRs on phagocytes, dictates the blood clearance rate of systemic bacteria. Indeed, depletion of platelets in WT mice equally reduces their bacterial circulatory half-life by 2-fold (Figure 1D). Thus, complement slows bacterial blood clearance kinetics by promoting interaction between bacteria and blood platelets.

Distinct Clearance Rates for Platelet-Bound and Free Bacteria

To study how platelets affect the clearance of bacteria from the vasculature, we adopted IVM (Figure S1A). We first validated the method by recording vascular LM clearance in a pre-defined vascular segment at discrete time intervals in WT, C3 ko, and GPIb ko mice (Figure S1B; Movie S1); calculated the resulting clearance kinetics and circulatory half-lives (Figure S1C); and found them to closely match those previously determined through repeated blood sampling of the same mouse strains (Figures 1A, 1D, and S1D). Importantly, IVM opens the door to study the interplay between bacteria and platelets in the vasculature of a live animal in real time. Using fluorescently marked LM (white) and platelets (red), we observe that systemic bacteria do not form a homogenous population but rather coexist in platelet-bound and non-bound ("free") forms in the circulation of a WT mouse (Figure 2A; Movie S2). While overall LM clearance in WT mice remained as previously determined (Figures 1 and S1), IVM reveals that free LM clear drastically faster

(C) Flow cytometry of C3-deposition on LM in WT (solid line), C3 ko (gray line), and GPIb ko (broken line) plasma (exemplary histogram, representative of four independent experiments).

(D) Vascular clearance and half-lives of LM in min after i.v. injection into WT (black circles), GPIb ko (white squares) and platelet-depleted WT (white squares with dot) mice. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (unpaired, two-tailed Student's t test). Data in (A) and (D) represent two to four independent experiments (mean ± SD of five or more mice per strain). See also Figure S1 and Movie S1.

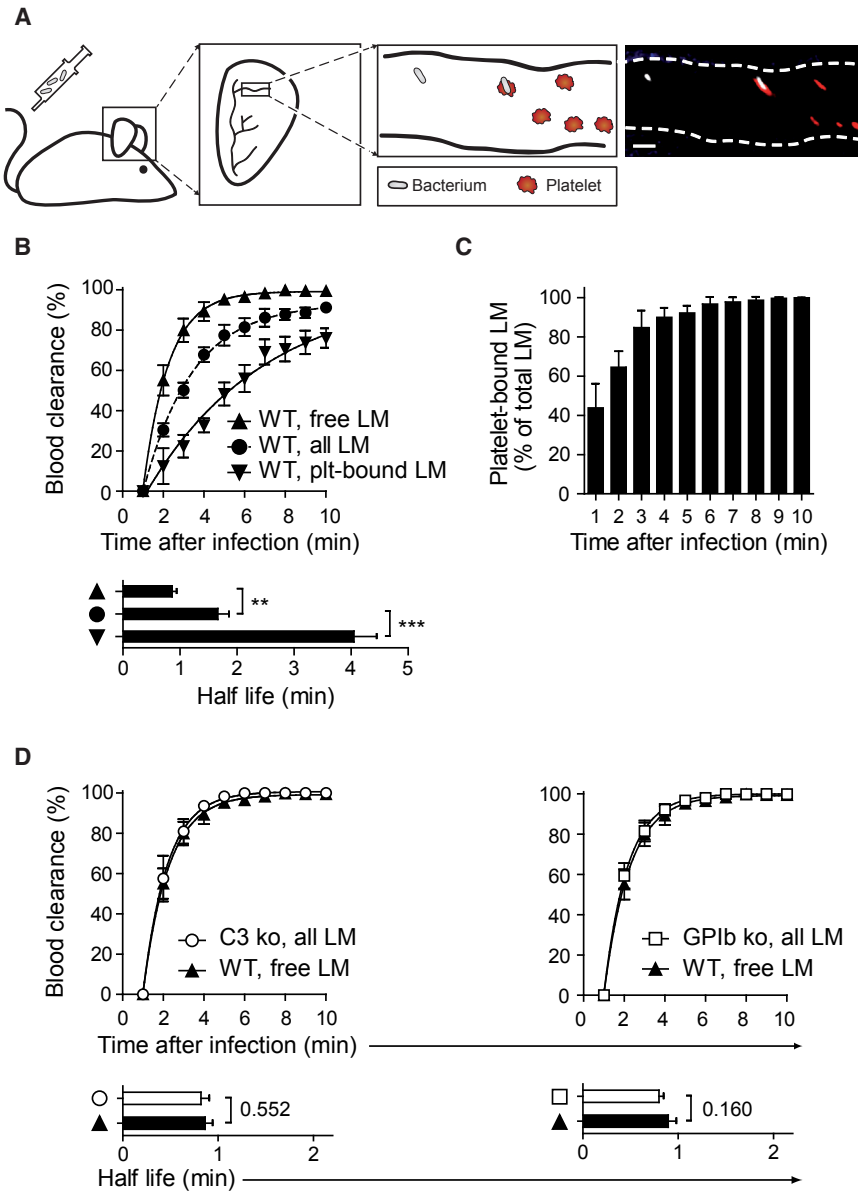


Figure 2. Platelet-Bound and Free LM Co-Exist in the Circulation of a Single Mouse and Clear with Distinct Kinetics

(A) Schematic illustration showing IVM of circulating free and platelet-bound LM; platelets, red; LM, white; collagen, blue (second harmonics); scale bar, 10 μm.

(B) Vascular clearance and half-lives of free LM (black upward-pointing triangles) and platelet-bound LM (black downward-pointing triangles) compared to overall LM (black circles) vascular clearance.

(C) Percentage platelet-bound LM (of all LM) in min p.i.

(D) Comparison of vascular clearance and half-lives between free LM in WT mice (black triangles) and all LM in C3 ko (white circles) or GPIb ko (white squares) mice, respectively. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (unpaired, two-tailed Student's t test). Data represent three to four independent experiments (mean ± SD of three to four mice per strain). See also Movie S2.

than their platelet-bound counterparts, with half-lives of ~1 min and ~4 min, respectively (Figure 2B). The result is that platelet-bound LM quickly dominate the circulation of WT mice, starting at ~40% after 1 min and reaching >90% within 5 min (Figure 2C). Intriguingly, the free LM fraction in WT mice clears with the same accelerated kinetics and reduced circulatory half-life as determined for LM in C3 ko and GPIb ko mice, where platelet binding cannot occur (Figure 2D). Collectively, our IVM approach to image bacterial blood clearance reveals, both qualitatively and quantitatively, that circulatory clearance of bacteria is not a uniform process. Instead, overall clearance proceeds via two parallel but distinct pathways in the circulation of a single infected host: "slow clearance" affecting platelet-bound bacteria and "fast clearance" of free bacteria. The slower clearance rate of platelet-bound LM causes such complexes to dominate in the circulation over time.

LM Are Efficiently Cleared by Liver KCs

Systemic bacteria are sequestered by phagocytes in liver and spleen (van Furth et al., 1972). To determine their relative contribution to LM clearance, we selectively depleted key liver- and/or spleen-resident phagocyte populations. Diphtheria toxin (DT) treatment of CD11c-DT receptor (CD11c-DTR) transgenic mice (Figure S2A) leaves liver KCs intact (Patsouris et al., 2008) but ablates the MZ (Probst et al., 2005), the microanatomical structure where systemic LM become initially trapped in the spleen (Aichele et al., 2003; Aoshi et al., 2009). MZ ablation did not measurably impair LM clearance compared to control DT-treated WT or PBS-treated CD11c-DTR mice (Figure 3A). In sharp contrast, depletion of professional phagocytes from spleen and liver with clodronate liposomes (Figure S2B) (Van Rooijen and Sanders, 1994) resulted in near-complete abrogation of LM clearance (Figure 3A). Given the negligible contribution of the spleen, our findings underline other studies assigning the liver and its KC a central role in clearing LM (Cousens and Wing, 2000) and other bacteria (Balmer et al., 2014; Jenne and Kubes, 2013) from the circulation.

Fluorescent immunohistochemical (FIHC) analysis of WT, C3 ko, and GPIb ko livers 15 min post-infection (p.i.)—a time where blood clearance is >99% complete—confirmed the predominant localization of fluorescently marked LM (green) to KC (red) (Figure 3B). Flow cytometric quantification of this localization shows that while LM^+ KC captured bacteria with comparable efficiency in all strains (comparable LM-associated MFI, Figure S2C), the percentage of LM^+ KC is higher in mice that do not support formation of bacteria complexes (~32% in C3 ko and GPIb ko strains) than in WT animals (~27%, Figure 3C). The data thus indicate that a higher

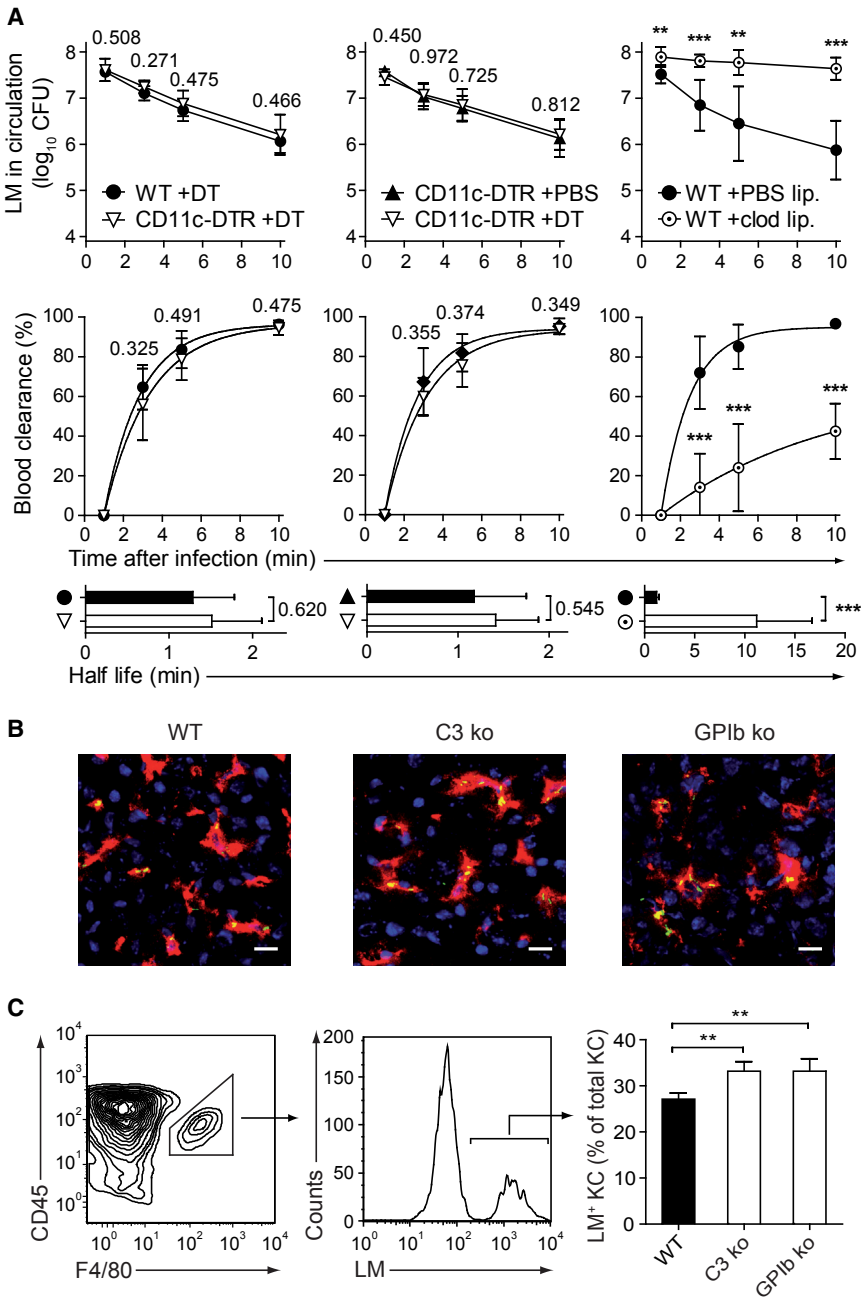


Figure 3. Liver KC Effectively Clear Intravascular LM

(A) Vascular clearance and half-lives of LM in min after i.v. injection into DT-treated WT and CD11c-DTR mice (left), DT- or PBS-treated CD11c-DTR mice (middle), and clodronate- or PBS-liposome-treated WT mice (right).

(B) FIHC of liver sections of WT, C3 ko, and GPIb ko mice; KC, red; LM, green; nuclei, blue; scale bar, 10 μm.

(C) Flow cytometry analysis of hepatic F4/80$^+$ KC from WT, C3 ko, and GPIb ko mice. Livers obtained 10 min p.i. and KC analyzed for LM uptake. Density plot is pregated on live cells. *p < 0.05, **p < 0.01, and ***p < 0.001 (unpaired, two-tailed Student's t test). Data represent two to four independent experiments per strain (mean ± SD of four to five mice per strain). See also Figure S2.

percentage of KC is able to participate in bacterial capture when platelets cannot bind LM, independent of the bacterium's complement opsonization status (compare C3 ko and GPIb ko, Figure 1C). Our results imply that complement-mediated binding of platelets, rather than complement opsonization per se, dictates the efficiency with which KC clear bacteria from the circulation.

Scavenger Receptors Mediate "Fast Clearance" of Free LM

If complement opsonization is not a prerequisite for efficient uptake of systemic bacteria by KC (Figure 3), actually slowing down overall clearance by promoting platelet-bacteria complexes (Figures 1 and 2), the question arises: what receptors, if not CR, are responsible for "fast clearance" of free bacteria? KCs express numerous pattern recognition receptors (PRRs) that recognize conserved pathogen associated molecular patterns. We pharmacologically inhibited distinct PRR classes in C3 ko mice to assay their effect on the clearance of non-opsonized, free LM. Of all tested substances, covering KC receptors spanning the C-type lectin, Toll-like receptor (TLR), and scavenger receptor (SR) families, only polyinosinic-acid (poly(I)) significantly inhibited the systemic clearance of LM, increasing their vascular half-life >10-fold (Figures 4A and 4B). In contrast, polycytidylic-acid (poly(C)), which unlike poly(I) does not inhibit SR (Terpstra et al., 2000; Rice et al., 2002), did not measurably impact LM clearance, thus identifying SR as responsible for "fast clearance" of free LM (Figure 4B).

CRIg Mediates "Slow Clearance" of Platelet-Bound LM

Although complement is dispensable for "fast clearance" of free LM via SR (Figure 4), it is required for the formation of the bacteria-platelet complexes (Figure 1), associated with "slow clearance" (Figure 2). Hence we probed if CR are involved in "slow clearance" using mice deficient for CR3, CRIg (both expressed on KC; van Lookeren Campagne et al., 2007), or CR4 (expressed on DCs and [activated] macrophage subsets) (Hume, 2008). While ablation of CR3 or CR4 had no noticeable effect on the systemic clearance of LM, absence of CRIg drastically delayed their clearance (Helmy et al., 2006), increasing their half-life to ~5 min, or ~3-fold longer than in WT controls (Figure 5A). Thus, while WT animals cleared nearly all LM by 10 min p.i., CRIg ko animals cleared only ~60% at the same time point.

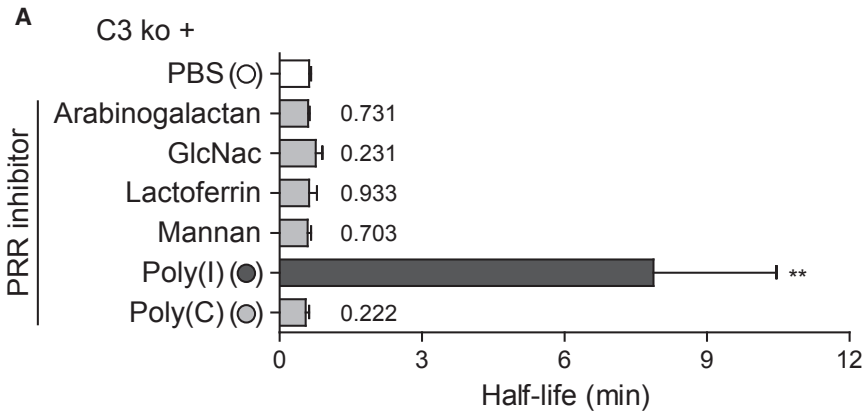
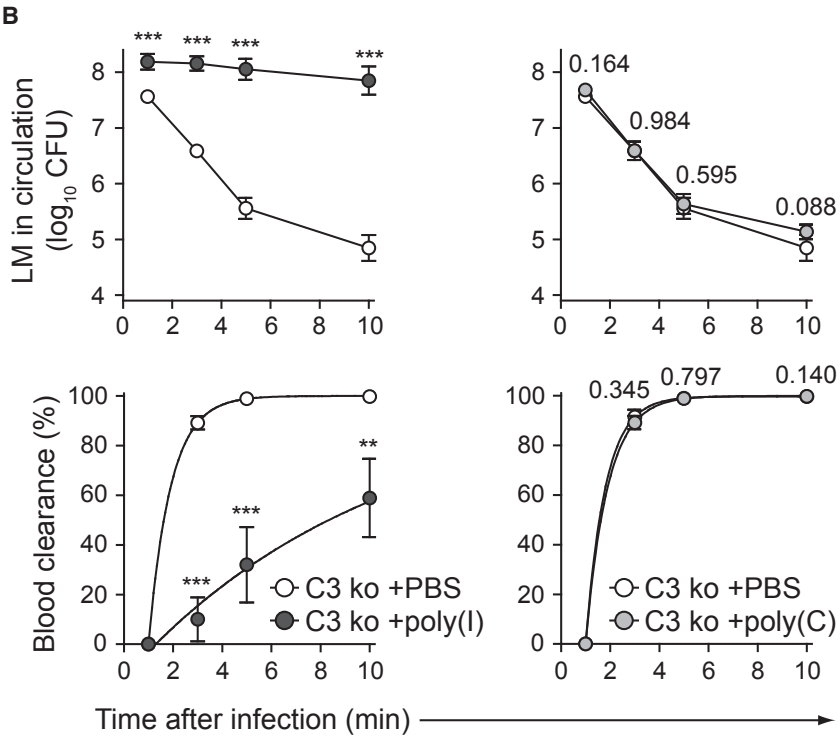


Figure 4. SR Mediates "Fast Clearance" of Free LM

(A) Circulatory half-lives of LM in C3 ko mice upon competitive pharmacological blockade of distinct PRR families with arabinogalactan, N-acetyl-D-glucosamine (GlcNac), lactoferrin, mannan, poly(I) or poly(C), with indicated P values versus PBS-treated control.

(B) Vascular clearance and half-lives of LM in min after i.v. treatment of C3 ko mice with 400 μg Poly(I) (left, dark gray cirlces), 400 μg Poly(C) (right, light gray circles), or 200 μl PBS (both, white circles). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (unpaired, two-tailed Student's t test). Data represent two to three independent experiments (mean ± SD of three to five mice per treatment).



We considered that, as in WT animals (Figure 2), overall blood clearance in CRIg ko mice is the cumulative result of "fast" and "slow" clearance of free and platelet-bound bacteria, respectively. Since complement opsonization is a rate-limiting step (taking ~1 min to reach full C3 coverage) (Figure S3), we hypothesized that while SR-mediated "fast clearance" of free LM may still be intact in CRIg ko mice, overall clearance could be delayed due to impaired "slow clearance" of platelet-bound bacteria. To test this, we sought to experimentally favor "slow" over "fast" clearance by promoting complex formation and reasoned that pre-opsonization should provide LM with a "head start" in binding platelets in vivo. Indeed, pre-opsonization increased the circulatory half-life of LM in C3 ko animals 3-fold over non-opsonized LM, namely from ~1 min to ~3 min, respectively (Figure 5B; Movie S3), confirming that pre-opsonization favors the "slow clearance" pathway. We next tested the contribution of CRIg to "slow clearance" and infused pre-opsonized LM into C3 ko animals that now also lacked CRIg (C3/CRIg dko). Strikingly, absence of CRIg led to a near complete abrogation of systemic clearance of pre-opsonized LM, showing an unprecedented half-life of nearly 150 min (Figure 5B; Movie S3). In sharp contrast, non-opsonized LM in the same mice retained their ~1 min half-life typical of "fast clearance" of free bacteria (Figure 5B; Movie S3). Thus, absence of CRIg does not compromise "fast clearance" of free LM, in line with our findings that rapid sequestration of this population depends on SR (Figure 4). Rather, the breakdown of clearance of pre-opsonized bacteria in the absence of CRIg reveals that this receptor is responsible for "slow clearance" of platelet-bacteria complexes.

To prove that indeed platelet complex-formation, and not the simple lack of recognition of pre-opsonized LM by CRIg is responsible for abrogated clearance in C3/CRIg dko mice (Figure 5B; Movie S3), we next prevented their formation by depleting platelets. In this setting, even pre-opsonized bacteria remain per definition free of platelets and thus available for "fast clearance." As predicted, depletion of platelets completely reversed the abolished clearance of pre-opsonized LM in C3/CRIg dko mice, rescuing their removal via "fast clearance" (Figure 5B, Movie S3). The results show that CRIg does not substantially contribute to "fast clearance" but rather mediates "slow clearance" of bacteria-platelet complexes.

Extending our observations with LM, we also observed "fast clearance" patterns in the absence of C3 using a broad panel of other Gram-positive and Gram-negative bacterial taxa, ranging from harmless laboratory strains to species associated with severe systemic infection (Figure S4, Gram

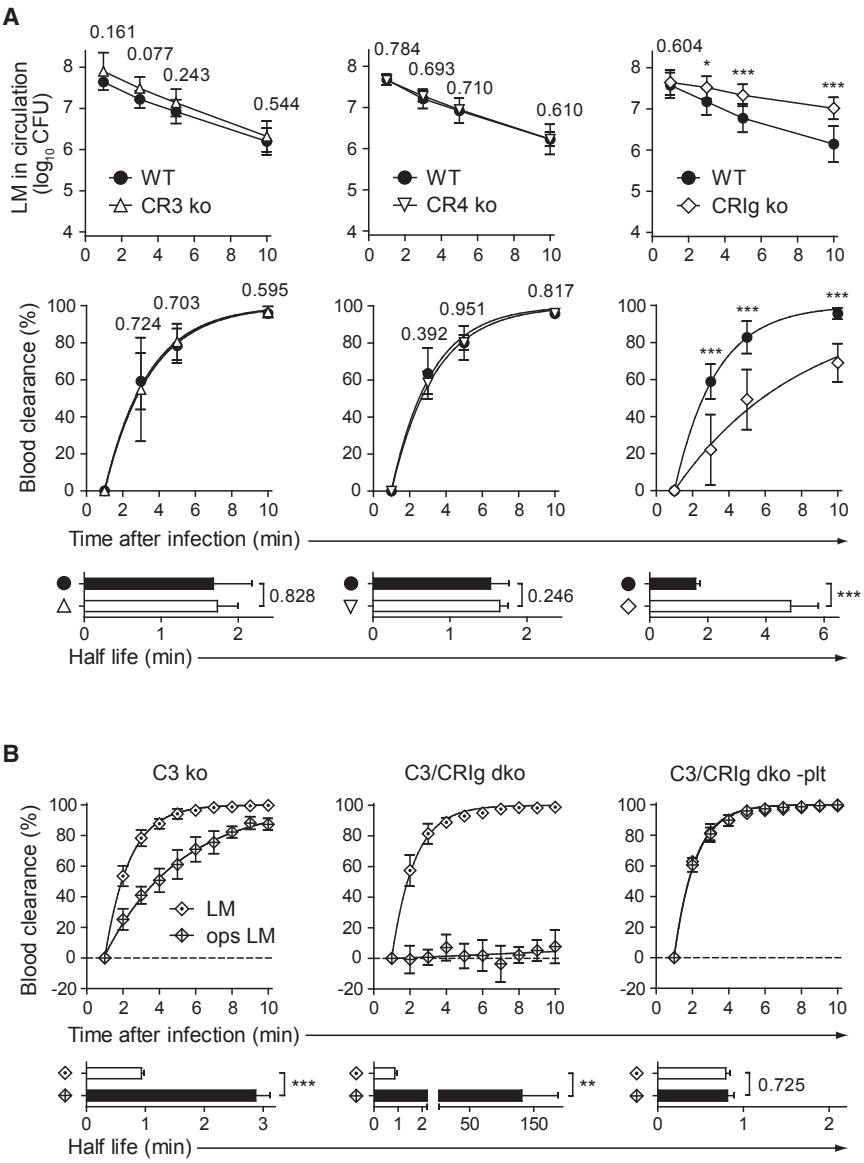


Figure 5. CRIg Mediates "Slow Clearance" of LM-Platelet Complexes

(A) Comparison of vascular clearance and half-lives of LM in min after i.v. injection into CD11b ko (left, white upward-pointing triangles), CD11c ko (middle, white downward-pointing triangles), and CRIg ko (right, white diamonds) mice as well as WT (black circles) control mice.

(B) Vascular clearance and half-lives (by IVM) of LM (white diamonds with dot) and pre-opsonized LM (white diamond with cross) in C3 ko (left) and C3/CRIg dko (middle) or platelet-depleted C3/CRIg dko (right) mice. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (unpaired, two-tailed Student's t test). Data represent three to four independent experiments (mean ± SD of three to four mice per strain and treatment). See also Figure S3 and Movie S3.

negative: *B.subtilis*, *E. faecalis*, and *S. epidermidis*; Gram negative: *E.coli*, *S. typhimurium*, and *K. pneumoniae*). Moreover, as observed for LM (Figure 5B), accelerated platelet binding through pre-opsonization of the bacteria shifts their clearance from a "fast" toward a CRIg-mediated "slow" patterns (Figure 6). Thus, complement controls the clearance-route and -rate of intravascular bacterial by allowing a portion of bacteria to bind platelets, rescuing them from "fast clearance" and shifting them into CRIg-mediated "slow clearance."

Balancing Innate and Adaptive Immunity

As liver KC mediate both "fast" and "slow" clearance mechanisms, the question arises: What biological purpose do two parallel clearance mechanisms by the same cell type serve? We showed that a greater portion of KC captures systemic LM in the absence of bacteria-platelet complexes (GPIb ko and C3 ko mice) than in their presence (WT mice) (Figure 3C). We next asked if this capture is accompanied by bactericidal activity and assessed the number of CFU arising from KC from these strains. Strikingly, absence of bacteria-platelet complexes in GPIb ko and C3 ko mice not only promotes "fast clearance" (Figure 1) through efficient capture of bacteria by KC (Figure 3) but also promotes efficient bacterial killing, with lower recoverable CFU than from WT counterparts (Figure 7A) that divert a portion of systemic bacteria into platelet complexes (Figure 2) that clear via CRIg (Figure 5). Combined, our data underscore that "fast clearance" of free LM via SR is followed by efficient bactericidal activity (Ishiguro et al., 2001).

So why would WT animals still force bacteria into platelet complexes? We again considered that the immune system must balance two crucial aims: (1) fast restoration of blood sterility and (2) induction of adaptive immunity. As the first aim is arguably covered by liver KC and their SR-dependent "fast clearance" and killing of free bacteria, we next assessed the influence of bacteria-platelet complexes on the induction of the adaptive antibacterial immune response.

A facultative intracellular pathogen, LM is ultimately controlled by infection-specific cytotoxic $CD8^+$ T cells that are induced from the naive T cell repertoire via priming by antigen-laden DCs (Pamer, 2004). We previously showed that shuttling of systemic bacteria to splenic $CD8\alpha^+$ DCs is reduced in C3 ko relative to WT mice (Verschoor et al., 2011) and now show that this is also the case in GPIb ko mice (Figure 7B). Thus, even though loss of platelet binding—irrespective of C3 opsonization (Figure 1C)—boosts bacterial clearance kinetics (Figures 1 and 2), enhances capture of bacteria by KC (Figure 3), and promotes their killing in the liver (Figure 7A), it does at the same time reduce bacterial capture by $CD8\alpha^+$ DCs in the spleen (Figure 7B). Conversely, reduced KC capture of LM-platelet complexes in CRIg ko mice

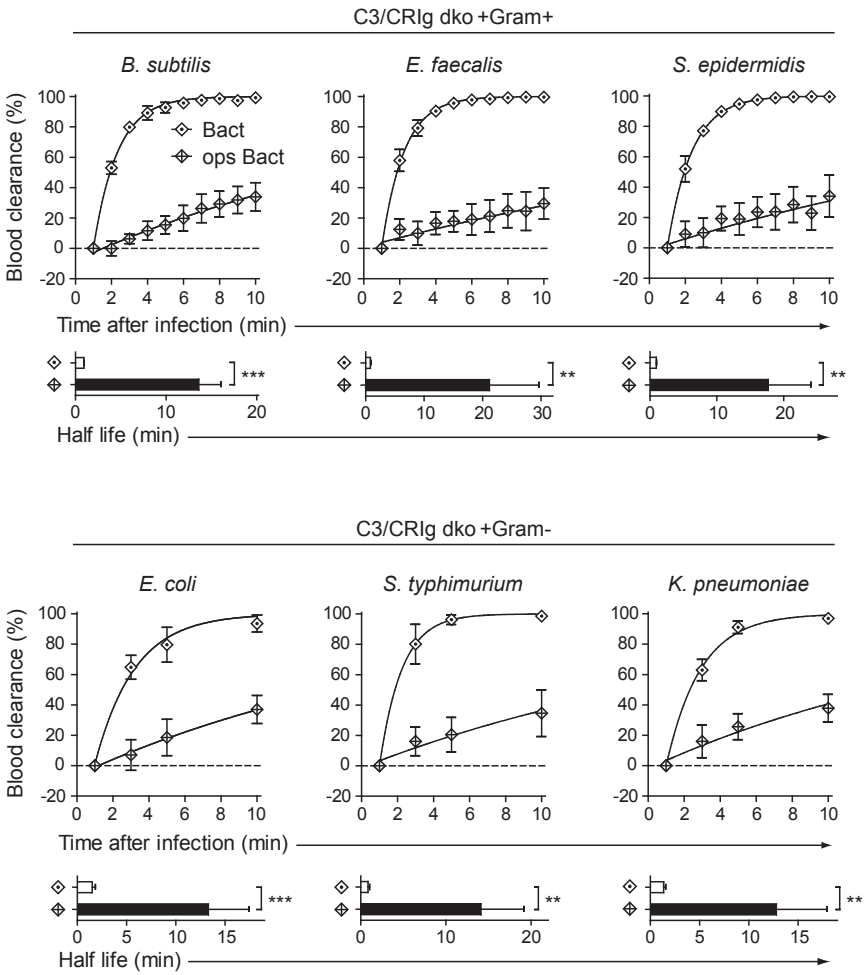


Figure 6. Platelet Binding Shifts Intravascular Bacteria from "Fast" into "Slow" Clearance Pathways

Vascular clearance and half-lives of selected Gram-positive (*Bacillus subtilis*, *Enterococcus faecalis*, and *Staphylococcus epidermidis* [by IVM] upper row) and Gram-negative (*Escherichia coli*, *Salmonella typhimurium*, and *Klebsiella Pneumonia*, lower row) bacteria, unopsonized (white diamond with dot) or pre-opsonized (white diamond with cross) in C3/CRIg dko mice. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (unpaired, two-tailed Student's t test). Data represent two to four independent experiments (mean ± SD of three or five mice per bacterial strain). See also Figure S4.

prolongs their presence in the circulation (Figure 5), augmenting their capture by splenic CD8α^+ DCs (Figure 7B).

As CD8α^+ DCs play a central role in priming LM-directed CD8$^+$ T cell responses (Belz et al., 2005; Reinicke et al., 2009; Jung et al., 2002), we tested if their ability to effectively acquire bacteria (Figure 7B) is mirrored in the antibacterial CD8$^+$ T cell response they prime. While a "high dose" infection with 10^5 CFU ovalbumin-expressing LM (LM-OVA) still induced WT-level OVA-specific CD8$^+$ T cell responses in all mouse strains, a 10-fold lower "intermediate dose" (10^4 CFU) already resulted in unsaturated responses in C3 ko and GPIb ko mice (Figure 7C). In sharp contrast, CRIg ko mice receiving this "intermediate dose" responded significantly better than WT animals, even maintaining the maximal response seen upon "high dose" infection. Also, a further 10-fold reduction to "low level" infection (10^3 CFU) still induced robust LM-directed CD8$^+$ T cell responses in CRIg ko mice, while WT, C3 ko and GPIb ko responses all dropped to marginally above-background.

Collectively, these data show that although free bacteria are efficiently captured and killed by liver KC via "fast clearance," the formation of "slow," CRIg-cleared bacteria-platelet complexes is crucial to efficiently drive adaptive immune responses, especially under conditions of limiting bacterial antigen.

DISCUSSION

The body maintains a wide variety of phagocytes, distributed across organs and tissues, collectively referred to as the MPS. During bacteremia, phagocyte populations in liver and spleen have simultaneous access to systemic bacteria, and both organs contribute to blood clearance. The role of each, however, is different: while the liver has been likened to a "firewall" whose KC effectively clear and destroy bacteria (Jenne and Kubes, 2013; Balmer et al., 2014), the spleen is a secondary lymphoid organ where specialized phagocytes promote adaptive antibacterial immunity (Mebius and Kraal, 2005). Both roles are crucial to guarantee immediate as well as long-term control of systemic bacteria. To serve these distinct biological and immunological functions, a finely tuned system of cellular specialization, receptor repertoires, and (micro)anatomical localization is required to effectively distribute systemic bacteria across phagocytes. In other words, to effectively monitor the circulation, a coordinated "division of labor" between phagocytes must exist.

Based on previous findings (Verschoor et al., 2011) and the ever-growing awareness of their immune-regulatory and antimicrobial capacities (Verschoor and Carroll, 2004; Yeaman, 2014; Verschoor and Langer, 2013), we hypothesized that platelets and complement may play a decisive role in this "division of labor," and we studied how they affect bacteria in the bloodstream. We developed a new application for IVM, using it as a form of "intravascular flow cytometry" to qualitatively and quantitatively study the behavior of bacteria in the bloodstream of live mice in real time. Importantly, and in contrast to traditional techniques, the method is comparatively non-invasive, eliminates repeated blood sampling, does not rely on pathogen survival for detection, and provides unprecedented temporal resolution. It allowed us to identify distinct bacterial (sub)populations in the circulation of a single mouse and to calculate their individual clearance kinetics. No prior technique to study blood clearance provided this level of fundamental insight.

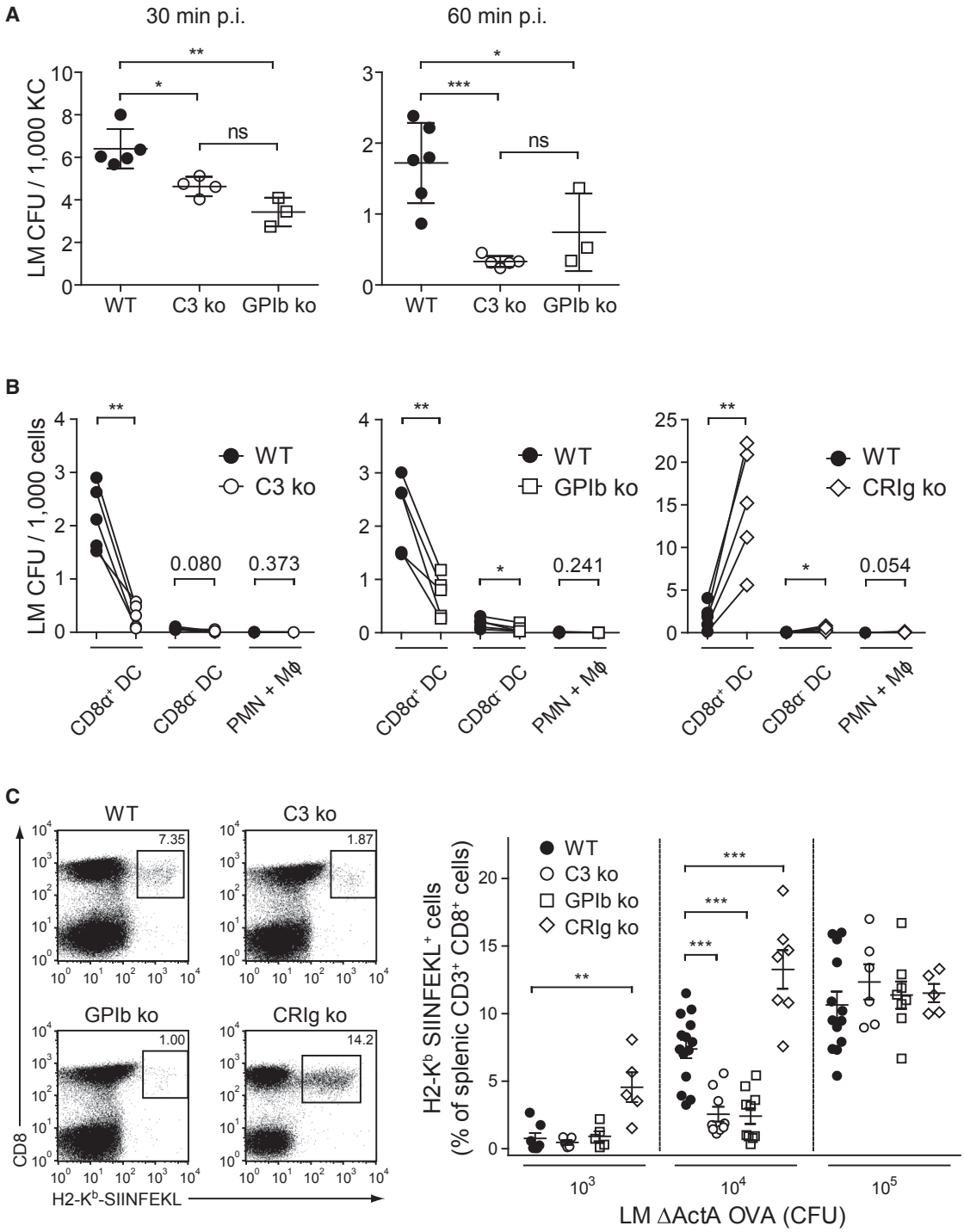


Figure 7. Bacteria-Platelet Complexes Balance Innate and Adaptive Immune Mechanisms

(A) Number of LM (CFU) arising from flow cytometry sorted F4/80$^+$ liver KC from C3 ko (white circle), GPIb ko (white square), and control WT (black circle) mice, 30 min (left) or 60 min (right) p.i.

(B) Number of LM (CFU) arising from flow-cytometry-sorted granulocytes and macrophages (PMN + Mϕ), CD8α$^+$ and CD8α$^-$ DCs, 1 hr p.i. from spleens of C3 ko (left, white circle), GPIb ko (middle, white square), CRIg ko (right, white diamond), and control WT (all, black circle) mice; knockout and corresponding control WT mouse analyzed in one experiment are indicated.

(C) Flow cytometry quantification of H2-K^b–SIINFEKL multimer-positive CD8$^+$ T cells (% of all CD8$^+$ T cells) in spleens of WT (black circle), C3 ko (white circle), GPIb ko (white square), and CRIg ko (white diamond) mice, 7 days p.i. with 10^3 (left), 10^4 (middle) or 10^5 (right) CFU spreading-deficient (ΔActA) LM-OVA. Dot plots are pre-gated on CD3$^+$ events. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ ([A], [C]: one-way ANOVA with Tukey's multiple comparison; [B]: paired, two-tailed Student's t test). Data represent three to five independent experiments (mean ± SD of three or more mice).

We find that intravascular bacteria are not a homogenous population but instead occur in "free" or "platelet-bound" forms that display disparate "fast" or "slow" blood clearance kinetics, respectively. It led us to interrogate the phagocyte-driven mechanisms behind these divergent clearance kinetics. We find that liver KC dominate overall elimination of systemic bacteria, using SR for "fast clearance" of "free" LM and CRIg for "slow clearance" of "platelet-bound" LM. Interaction between platelets and bacteria depends on (1) bacterial opsonization by complement C3 and (2) platelet glycoprotein GPIb (Verschoor et al., 2011). Our findings thus reveal how complement opsonization, platelet binding, and differential KC capture via CRIg and SR establish a "dual track" system of "fast" and "slow" clearance for bacteria from the circulation. More generally, our data provide a mechanistic explanation for the accelerated blood clearance phenotype various groups reported under hypocomplementic conditions (Verschoor et al., 2011; Waxman et al., 1984; Davies et al., 1992).

Importantly, the current study also explains how "dual-track" clearance ensures simultaneous and appropriate engagement of innate and adaptive immunity, by balancing bacterial distribution across specialized phagocyte populations in liver and spleen. This is important, as mice with impaired LM clearance by liver KC suffer from increased mortality (Ebe et al., 1999; Helmy et al., 2006; Gregory et al., 1996), while protection from reinfection requires the induction of LM-specific $CD8^+$ T cells by splenic $CD8\alpha^+$ DCs (Harty et al., 1996; Jung et al., 2002; Verschoor et al., 2011). Thus, uncontrolled bacterial capture by phagocytes in one organ bears the risk of creating a functional shortage in the other. Indeed, we find that $CD8\alpha^+$ DCs targeting and resultant LM-specific $CD8^+$ T cell proliferation are impaired if LM cannot bind platelets. Reversely, blocking CRIg-mediated uptake of bacteria-platelet complexes by liver KC shifts LM to $CD8\alpha^+$ DCs in the spleen, boosting LM-specific $CD8^+$ T cells. We show how under normal WT conditions only a minute fraction (~0.01%) of LM locates to $CD8\alpha^+$ DCs, not only illustrating the extraordinary efficiency with which adaptive immunity is induced in the spleen but also emphasizing the importance that this small fraction of bacteria is indeed rescued from rapid liver destruction by platelet binding. Our dose titration experiments stress that this becomes particularly vital for low numbers of bacteria. In that light, it is worth considering that low-level bacteremia is frequent and observed in >75% of healthy study participants after tooth brushing (Ashare et al., 2009).

Entry of systemic bacteria into "fast" or "slow" clearance routes depends on platelet binding. This, in turn, is controlled by the opsonization rate and stochasticity of platelet interaction. Our measurements show that maximal opsonization with complement C3 activation products requires ~1 min, a time at which ~40% of systemic LM bind platelets in vivo. This percentage quickly approaches 100% over the next minutes, reflecting cumulative platelet interactions, but also the efficient "fast clearance" of still free bacteria. Indeed, we show that the vascular half-life of "slow" cleared complexes is approximately four times longer than of "fast" cleared free bacteria, resulting in a relative accumulation of platelet-bacteria complexes in the circulation and their prolonged availability to $CD8\alpha^+$ DC. This makes opsonization time, intravascular sheer forces, chance interactions with platelets, and the divergent clearance speeds of CRIg and SR all parameters that influence the "division of labor" between phagocytes in liver and spleen.

How platelet binding rescues LM from "fast clearance," shifting them into the "slow clearance" route (a pattern we observe for all tested Gram-positive and Gram-negative bacteria), remains a topic for further study. Possibly, PRRs such as SR cannot effectively reach their ligands when platelets obscure the bacterial surface. Indeed, platelet binding, not opsonization itself, has this effect, as "fast clearance" via SR is unaltered by opsonization (compare C3 ko and GPIb ko mice). In contrast, our experiments with pre-opsonized bacteria in C3 ko mice confirm that CRIg interacts with C3 ligands on the bacterial surface, also after platelets bind. Indeed, as SR dominates clearance of free bacteria (even if opsonized), CRIg shows its strongest effect on clearance once bacteria-platelet complexes have formed. Our data therewith mechanistically extend the original findings describing CRIg as clearance receptor for C3b- and iC3b-opsonized pathogens (Helmy et al., 2006) by showing a dependence on the nature of the opsonized complex. Aside from receptor-ligand considerations, the sheer size of complexes, relative to a free bacterium, may favor capture in fine sinusoids, an aspect that may be particularly relevant for splenic $CD8\alpha^+$ DC. Indeed, several studies observed that trapping in the spleen improves with particle size (Moghimi et al., 1991; Liu et al., 1991), with particles >1 μm increasingly gravitating toward the spleen (Welch and Redvanly, 2003). Thus, even when disregarding the biological properties of platelets, the mere dimensional increase mouse platelets (0.5 μm) bring to a bacterium (0.4 by 1.5 μm for a typical LM rod) promotes their localization to the spleen.

In conclusion, a picture emerges in which complement opsonization and platelet binding alter the way phagocytes "see" a bloodstream infection, affecting innate blood clearance and the induction of adaptive antibacterial immunity.

EXPERIMENTAL PROCEDURES

Mice

C3 ko (*C3*$^{-/-}$, Wessels et al., 1995), GPIb ko (*GP1ba*$^{-/-}$, Kanaji et al., 2002), CD11c-DTR (*Itgax*-DTR^{tg}, Jung et al., 2002), CR3 ko (*Itgam*$^{-/-}$, Melo et al., 2000), CR4 ko (*Itgax*$^{-/-}$, Wu et al., 2004), CRIg ko (*Vsig4*$^{-/-}$, Helmy et al., 2006), C3/CRIg dko (*C3*$^{-/-}$*Vsig4*$^{-/-}$), C5 ko (*C5*$^{mut/mut}$, Nilsson and Müller-Eberhard, 1967), and WT mice, all on C57BL/6 genetic background, were bred and kept under specific pathogen-free conditions. Mice were analyzed at 8–14 weeks of age, and both sexes were included. Experiments were carried out according to the respective local veterinary laws and institutional guidelines.

Bacteria and Infections

LM WT strain 10403S and ΔActA-LM OVA were grown to exponential phase at 37°C in BHI broth, washed, and inocula prepared in PBS. LM was fluorescently labeled by incubation for 30 min at 37°C with 5 μM CSFE (carboxyfluorescein diacetate succinimidyl ester; Sigma) or 5 μM Cell Proliferation Dye eFluor 670 (eBioscience). For IVM, bacteria were heat-inactivated at 70°C for 2 hr, quantified by flow cytometry and aliquots of 10^8 LM administered i.v. Where indicated, LM were pre-opsonized for 5 min in fresh WT or C3 ko control mouse plasma at 37°C in presence of Lepirudin (50 IU/ml) and repeatedly washed in PBS.

Bacillus subtilis (ATCC 6051) was grown in BHI broth to exponential phase at 30°C, *Enterococcus faecalis* (ATCC 29212) and *Staphylococcus epidermidis* (ATCC 12228) at 37°C. Bacteria were washed, fluorescently labeled as described for LM, heat- inactivated for IVM in PBS at 56°C for 60 min for

B. subtilis or 90°C for 60 min for *E. faecalis* and *S. epidermidis* at densities of 10^{10} CFU/ml, and inocula quantified at 10^8 by flow cytometry. *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 13311), and *Klebsiella pneumonia* (ATCC 13883) were grown to exponential phase at 37°C in BHI broth, washed, and aliquoted. Pre-opsonization of Gram-positive strains was performed as described for LM, while Gram-negative strains were incubated in C5-deficient plasma to prevent complement-mediated lysis. Blood clearance kinetics of bacteria were determined by infusing 10^8 CFU i.v., followed by blood collection at 1, 3, 5, and 10 min p.i., serial plating on BHI agar, and colony counting. Splenic or hepatic cell sorting experiments were carried out with i.v. inocula of 10^6 and 10^7 CFU, respectively.

Treatments

Platelets were depleted with anti-CD42b according to the manufacturer's instruction (Emfret Analytics) or visualized by administration of 3 μg anti-GPIbβ-antibody (#X649, Emfret) as per manufacturer's instruction. MZ ablation was performed with DT (4 ng/g body weight i.p.; Sigma) in CD11c-DTR mice, while MPS ablation was achieved with clodronate liposomes i.v. (Van Rooijen and Sanders, 1994). Depletions were verified by FACS staining or FIHC. For pharmacological inhibition of PRR, 400 μg of either arabinogalactan, lactoferrin, mannan, N-acetyl-D-glucosamine (GlcNac), polycytidylic acid (poly(C)), or polyinosinic acid (poly(I)) (all from Sigma Aldrich) was given i.v. 2 min prior to infection.

IVM

IVM of the vasculature was performed with a TrimScope II (LaVision Biotech) connected to an upright microscope (Olympus) equipped with a Ti:Sa Chameleon Ultra II laser (Coherent) and a 16x 0.8NA water immersion objective (Nikon). Images were acquired at 800 nm excitation wavelength in a 200 × 200 μm frame with 512 × 512 pixels detected by PMTs (G6780-20, Hamamatsu). ImSpector Pro (LaVision Biotech) was used as acquisition software. An environmental box maintained a stable 37°C environment. Mice were anaesthetized and fixed on a custom-built stage to visualize the vasculature of the ear. The caudal vein was catheterized, blood flow verified with TRITC-Dextran (Invitrogen) and mice infused with a single population of 10^8 bacteria or a mixture of 5 × 10^7 pre-opsonized and 5 × 10^7 (differentially marked) non-opsonized bacteria. Images were acquired for 10 min, at a frame rate of 40/min, and analyzed with ImageJ (National Institute of Health). Clearance kinetics were determined by sequential enumeration of LM passing through a vessel segment of 100 μm length at discrete timeframes of 1 min (40 frames). Clearance kinetics were nonlinearly fit to exponential one phase decay curves and half-lives calculated with a 95% confidence interval with GraphPad Prism 5.0.4.

Flow Cytometry Analysis and Cell Sorting

Isolation of splenocytes for flow cytometry analysis and cell sorting was performed as described (Verschoor et al., 2011). Briefly, spleens were removed 1 hr (for cell sorting) or 7 days (for T cell analysis) p.i. For cell sorting, spleens were digested with DNase-collagenase (Sigma Aldrich) for 1 hr in the presence of 5 μg/ml gentamicin to limit extracellular LM. Cells were incubated with anti-CD16/32 (2.4G2; BD Biosciences). OVA-specific T cells were identified with H2-Kb-SIINFEKL multimers, anti-CD3 (17A2, BD Biosciences), and anti-CD8 (5H10, Invitrogen) and analyzed on a Cyan ADP Lx (Beckman Coulter). Propidium iodide (Invitrogen) was added in all experiments to exclude dead cells. For cell sorting, splenocytes were stained with anti-CD8α (53-6.7; Biolegend), anti-CD11c (N418; Biolegend), and anti-CD11b (M1/70; Biolegend). Cells were sorted on a MoFlo Legacy flow cytometer (Beckman Coulter) into PBS containing 25% FCS and 0.5% BSA. $CD8\alpha^+$ DCs were defined as $CD11c^+CD8\alpha^+$, $CD8\alpha^-$ DCs as $CD11c^+CD8\alpha^-$, and granulocytes (PMN) and macrophages (Mϕ) as $CD11c^-CD11b^+$. Purity (>95%) was verified on a Cyan ADP Lx (Beckman Coulter); splenocytes were lysed with Triton-X and plated on BHI for CFU quantification. Similar to spleen, livers were harvested at indicated time points p.i., digested for 30 min with DNase-collagenase, hepatocytes removed by centrifugation, and cells incubated with anti-CD45 (30F11; Biolegend) and anti-F4/80 (BM8; Biolegend). KC were defined as $CD45^+F4/80^+$.

Analysis of platelets was performed as described (Verschoor et al., 2011). Briefly, blood was immediately diluted 10-fold in PBS with 0.5% BSA containing lepirudin (50 IU/ml) and platelet-specific anti-CD41 (MWReg30; BD Biosciences) was added. Samples were acquired on a FACSCalibur (BD Biosciences) and analyzed with FlowJo software (TreeStar).

Fluorescent Immunohistochemistry

Livers were harvested 10 min p.i. with 10^8 CFSE-labeled LM, frozen in OCT compound (Leica), and cryosections prepared. Sections were acetone fixed at 4°C for 2 min and blocked for 15 min in PBS containing 4% BSA. KC were identified with anti-F4/80 (BM8), followed by secondary goat anti-rat IgG Dylight594 (Biolegend); sections were counter stained with Hoechst 33258 (Sigma), mounted with Roti-Mount FluorCare (Roth), and examined on a DRMB fluorescence microscope (Leica) equipped with a 100x 1.3NA oil immersion objective (Leica) and an AxioCam MRc with Axiovision software (Zeiss).

Statistical Analysis

Statistical analyses were done with assistance from GraphPad Prism 5.0.4 software. Student's t test and one-way ANOVA test were performed, as indicated. The results are expressed as mean ± SD unless stated otherwise. Group sizes, reproducibility, and p values for each experiment are stated in figure legends.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2016.05.023.

AUTHOR CONTRIBUTIONS

S.P.B., A.P., R.C., C.L., A.W., A.S., S.L., and A.V. did experiments; K.E., P.C.R., and M.L.C. assisted with data interpretation and supplied reagents and bacterial strains; S.P.B., A.P., and A.V. analyzed the data; S.M., D.H.B., and A.V. supervised the study; A.V. conceived the study and planned experiments; S.P.B. and A.V. wrote the manuscript.

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