**Cell Host & Microbe**

**CRIg Functions as a Macrophage Pattern Recognition Receptor to Directly Bind and Capture Blood-Borne Gram-Positive Bacteria**

**Highlights**
- Kupffer cells (KCs) capture circulating *S. aureus* in the liver
- Macrophage receptor CRIg, but not complement, is required for pathogen capture by KCs
- CRIg directly binds Gram-positive bacteria via lipoteichoic acid (LTA) recognition
- CRIg-LTA recognition is required for clearance of circulating bacteria

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**In Brief**
Liver macrophages called Kupffer cells (KCs) capture circulating pathogens. Using intravital imaging to visualize bacterial capture by KCs, Zeng et al. discover that CRIg functions as a pattern recognition receptor on KCs to directly bind Gram-positive bacteria via lipoteichoic acid (LTA). CRIg-LTA recognition is required for clearance of circulating bacteria.

Zeng et al., 2016, Cell Host & Microbe 20, 99–106
July 13, 2016 © 2016 Elsevier Inc.
http://dx.doi.org/10.1016/j.chom.2016.06.002
CRlg Functions as a Macrophage Pattern Recognition Receptor to Directly Bind and Capture Blood-Borne Gram-Positive Bacteria

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http://dx.doi.org/10.1016/j.chom.2016.06.002

SUMMARY

Kupffer cells (KCs), the vast pool of intravascular macrophages in the liver, help to clear blood-borne pathogens. The mechanisms by which KCs capture circulating pathogens remain unknown. Here we use intra-vital imaging of mice infected with Staphylococcus aureus to directly visualize the dynamic process of bacterial capture in the liver. Circulating S. aureus were captured by KCs in a manner dependent on the macrophage complement receptor CRlg, but the process was independent of complement. CRlg bound Staphylococcus aureus specifically through recognition of lipoteichoic acid (LTA), but not cell-wall-anchored surface proteins or peptidoglycan. Blocking the recognition between CRlg and LTA in vivo diminished the bacterial capture in liver and led to systemic bacterial dissemination. All tested Gram-positive, but not Gram-negative, bacteria bound CRlg in a complement-independent manner. These findings reveal a pattern recognition role for CRlg in the direct capture of circulating Gram-positive bacteria from the bloodstream.

INTRODUCTION

The liver has been well known as a blood-filtering organ. The unique portal venous blood inflow exposes the liver to a tremendous variety of food antigens, metabolic intermediates, and microbiota-derived products, all of which must be cleared by the liver to maintain tissue homeostasis (Crispe, 2009). Another key function of the liver is the clearance of blood-borne pathogens during infections to prevent bacterial dissemination and infection of more susceptible organs (e.g., brain, kidney) leading to sepsis and death (Jenne and Kubes, 2013). This bacterial filtration function would also make the liver an essential firewall eradicating gut-resident commensal bacteria that have translocated into blood (Balmer et al., 2014). Hence, understanding how the liver traps and clears circulating bacteria would be of broad therapeutic interest for the treatment of systemic bacteremia.

Kupffer cells are the largest population of tissue resident macrophages and are the key players for bacterial capture in the liver. These cells reside in liver sinusoids, forming an extensive scavenger network that actively and constitutively probes the slow-flowing sinusoidal blood, trapping and phagocytosing circulating bacteria (Hickey and Kubes, 2009). Sequestration of bacteria by Kupffer cells is necessary for an orchestrated intravascular immune response, including the rapid docking of platelets, recruitment of circulating neutrophils, attraction of patrolling invariant natural killer T cells, and activation of adaptive immune cells, which eventually all contribute to eliminating the infections (Wong et al., 2013; Lee et al., 2010; Egen et al., 2008). A key prerequisite to initiate this intravascular immunity is the efficient capture of bacteria from the bloodstream by Kupffer cells; however, the underlying mechanisms have not been fully elucidated. Despite the fact that numerous studies point to a role for opsonins, including antibodies and complement, which facilitate the uptake of bacteria by phagocytes under static conditions (Aderem and Underhill, 1999; Flannagan et al., 2012), bacterial interaction with host cells under flow conditions is largely affected by the shear forces (Isberg and Barnes, 2002). Therefore, whether these opsonins are involved in Kupffer-cell-mediated bacterial capture in vivo under flow conditions remains unclear.

Complement Receptor of Immunoglobulin superfamily (CRlg) has been identified as a receptor that recognizes the activated form of complement component C3, specifically C3b and iC3b, which opsonize pathogens, apoptotic cells, or foreign antigens (Holers, 2014). CRlg was found to be expressed on a restricted population of tissue-resident macrophages under steady state, notably on Kupffer cells (Helmy et al., 2006; Vogt et al., 2006). Mice genetically deficient in CRlg showed dramatically reduced capture of Staphylococcus aureus (S. aureus) and Listeria monocytogenes (L. monocytogenes) by Kupffer cells and exhibited much greater susceptibility to bacterial infection (Helmy et al., 2006). However, it should be noted that many
pathogens have evolved ways of subverting or evading the host completely susceptible to dissemination. In fact, it has been reported that depletion of complement has no impact on Kupffer-cell-mediated phagocytosis of *L. monocytogenes* (Gregory et al., 2002). These data raise the critical question whether complement is absolutely necessary for CRIg-mediated bacterial capture by Kupffer cells.

A major difficulty in studying bacterial capture by the liver is that in vitro isolation of Kupffer cells leads to rapid loss of cellular function, underscoring the need for in vivo visualization of bacterial capture. Therefore, in this study, spinning-disk confocal intravital microscopy was applied to directly visualize the very dynamic process of bacterial capture within liver sinusoids. This approach enabled identification of the key immune components needed for catching of blood-borne bacteria under flow conditions in vivo. We found that Kupffer-cell-mediated *S. aureus* capture largely relied on CRIg but was independent of opsonins, including complement or antibodies. Instead, CRIg directly bound the Gram-positive cell wall component lipoteichoic acid (LTA), and this interaction was involved in hepatic sequestration of circulating bacteria. Our study thus highlights a mechanism for bacterial catching in the liver that helps prevent systemic bacterial dissemination, which may have implications for treatment of Gram-positive bacteremia.

**RESULTS**

**Capture of Circulating *S. aureus* Is Dependent on Kupffer Cells and CRIg, but Not Complement**

*S. aureus* represents a leading cause of both community-acquired and nosocomial bacteremia. Using an intravascular *S. aureus* (USA-300, derived from a community-acquired methicillin-resistant strain) infection model, we found that the majority (60%–80%) of administrated bacteria were quickly trapped within the liver (Figure 1A), confirming the fundamental role of the liver in clearing blood-borne infections. To understand how the liver captured bacteria from the bloodstream, we took advantage of liver intravital imaging to directly visualize the dynamic interactions between circulating bacteria and liver cells. Time-lapse images showed that GFP-expressing *S. aureus* were caught immediately after they entered into liver sinusoids (Movie S1). Once caught, most of these bacteria colocalized with F4/80-labeled Kupffer cells but not with neutrophils, endothelium, or hepatocytes. Neutrophils were recruited into liver as early as 20 min post-infection, but essentially all bacteria were already sequestered in Kupffer cells (Figure 1B). 3D reconstruction with increasing transparency of Kupffer cells indicated that the trapped *S. aureus* were inside Kupffer cells (Figure 1C) (i.e., could not be seen in Figure 1Ci until the Kupffer cells were made more translucent [Figures 1Cii and 1Ciii]). Depletion of Kupffer cells with clodronate liposomes completely abrogated the capture of bacteria in the liver, whereas control liposomes had no effect (Figure 1D). Instead, one could see fluorescent bacteria freely streaking through the sinusoids (Movie S1) in Kupffer-cell-depleted mice. These data clearly demonstrate that Kupffer cells are the major cells that catch circulating bacteria in the liver via a mechanism not found on other immune cells such as neutrophils.

We next explored how Kupffer cells captured bacteria under shear conditions. While the dominant complement receptor for phagocytosis under static conditions on macrophage is CR3 (Mac-1) (Aderem and Underhill, 1999), CR3−/− Kupffer cells were able to catch bacteria equally well as wild-type (WT) Kupffer cells under shear conditions (Figure 1E). Previous work reported that a specific complement receptor—CRIg—on Kupffer cells was very effective at catching *S. aureus* and *L. monocytogenes* by Kupffer cells. We confirmed that CRIg co-stained with F4/80 in the liver of WT mice, and CRIg−/− mice completely lacked CRIg expression (Figure 1F). Furthermore, CRIg-deficient mice showed a 70% reduction in hepatic capture of *S. aureus* (Figures 1E and 1F; Movie S2) despite having a comparable amount of F4/80+ Kupffer cells as WT mice (Figure S1). As a result, in the absence of CRIg, there was rapid systemic bacterial dissemination, with fewer *S. aureus* in liver, but 5-fold more bacteria in lung and spleen and around 100-fold more bacteria in blood (Figure 1G).

Opsonins, including complement and antibodies, greatly enhance macrophage-mediated phagocytosis under static conditions. CRIg was originally identified as a receptor for C3b and iC3b (Helmy et al., 2006; Wiesmann et al., 2006). However, mice deficient in complement components C3, C4, or C5 were as efficient as WT mice in capturing *S. aureus* by the liver (Figure 1H; Movie S2). The catching ability also remained intact in antibody-deficient *Rag1*−/− mice (Figure 1I; Movie S2). These data indicated that complement and antibodies played dispensable roles in catching of circulating *S. aureus* by Kupffer cells under shear conditions. To confirm this, we further tested the catching of *S. aureus* in complement and Fc receptor double knockout mice (*C3*−/−/*FcγR−/−*, which are deficient for C3 and common γ chain of Fc receptors). These animals also retained the ability to capture *S. aureus* as efficiently as WT mice, further ruling out a role for complement and IgG (Figure 1J). Taken together, these data indicate that capture of circulating *S. aureus* by Kupffer cells is dependent on CRIg but independent of opsonins. This paradox prompted us to hypothesize that there is an alternative ligand interacting with CRIg to promote bacterial capture in the liver.

**CRIg Directly Binds Gram-Positive Bacteria**

The findings that opsonins (e.g., complement and antibody) were dispensable, but CRIg was indispensable, for the capture of circulating *S. aureus* implies that Kupffer cells may be able to catch unopsonized *S. aureus* via CRIg. To test this hypothesis, we determined if CRIg could directly bind to unopsonized *S. aureus*. Indeed, the recombinant extracellular domain of CR3 (CR3ex) bound *S. aureus* (Figures 2A and 2B; Movie S3). To ensure that this binding was CRIg-mediated and was not due to free dye leaching into cells, we used equal amounts of AF-647-labeled *S. aureus* (Figures 2A and 2B; Movie S3). Immunofluorescence confirmed binding of *S. aureus* to CRIg (Figure 2C).

Because *S. aureus* and *E. coli* are prototypical Gram-positive and Gram-negative bacteria, respectively, we tested several...
other Gram-positive and Gram-negative bacterial strains; CRlg bound directly to all of the Gram-positive strains tested, including *S. aureus* (MW2), *L. monocytogenes*, and *Bacillus cereus* (*B. cereus*). In contrast, CRlg did not bind directly to any of the Gram-negative strains that were tested, including *E. coli* (TOP10), *Salmonella typhimurium*, and *Pseudomonas aeruginosa* (Figure 2D). Taken together, these observations reveal an unexpected direct interaction between CRlg and Gram-positive bacteria.

**CRlg Does Not Recognize Bacterial Surface Proteins or Peptidoglycan**

The finding that CRlg selectively bound Gram-positive but not Gram-negative bacteria raised the possibility that a surface protein or a cell wall component of the Gram-positive bacteria was recognized by CRlg. Gram-positive bacteria display many proteins on their surface to facilitate the interaction with host cells, which prompted us to examine whether CRlg recognized surface proteins on Gram-positive bacteria. Sortase A has been...
identified as the critical transpeptidase supporting the anchor of secreted proteins to the peptidoglycan layer of *S. aureus* and many other Gram-positive bacteria (Mazmanian et al., 1999). In a Sortase A mutant *S. aureus* strain (*srtA*), the bacteria lost the ability to display surface proteins; however, CRIg bound this Sortase A mutant as efficiently as WT *S. aureus* (Figure 3A). In vivo, Kupffer cells captured similar amounts of *S. aureus* *srtA* as WT *S. aureus* (Figure 3B). These data indicated that surface proteins displayed on Gram-positive bacteria were not likely the ligands for CRIg. The Gram-positive cell wall is unique in having a thick, surface-exposed peptidoglycan layer along with incorporated teichoic acids. We chose to look if these evolutionarily conserved molecular patterns could bind CRIg. Purified peptidoglycan did not inhibit the binding of CRIg to *S. aureus*, as CRIg was able to efficiently bind *S. aureus* even in the presence of high concentrations of peptidoglycan (Figure 3C). In addition, no binding between FITC-labeled peptidoglycan and CRIg-expressing Jurkat T cells was detected (Figure 3D). These data ruled out the possibility of peptidoglycan as a ligand for CRIg.

**CRIg Recognizes Lipoteichoic Acid**

Teichoic acids are vastly present in the Gram-positive cell wall and are either connected to peptidoglycan (wall teichoic acids [WTAs]) or to the cytoplasmic membrane (LTAs). Both are produced by *S. aureus* and many other Gram-positive bacteria. The addition of purified LTA dose-dependently reduced the binding of CRIg to *S. aureus*. In fact, purified LTA at 10–40 μg/mL completely blocked the binding of both mouse and human CRIg to *S. aureus* (Figure 3E; data not shown). Addi-

**Figure 2. CRIg Can Directly Bind Gram-Positive Bacteria**

(A) Recombinant mouse (upper) or human (below) CRIg were labeled with AF-647. Their binding with *S. aureus* was detected by flow cytometry. AF-647-labeled α-toxin was used as a control. Blank means *S. aureus* only.

(B) *S. aureus* or *E. coli* were incubated with indicated concentrations of AF-647 labeled mCRIg. The binding was detected by flow cytometry.

(C) Representative fluorescence images of CRIg (AF647, blue) binding to *S. aureus* (GFP) or *E. coli* (RFP); scale bars: 10 μm.

(D) Quantification of the binding of AF647-labeled CRIg to various bacteria strains based on the geometric mean fluorescence intensity of AF 647 acquired by flow cytometry. Pooled results from three independent experiments were shown in (D), and data were expressed as mean ± SEM. **p < 0.01; N.S., no significance.”
the absence of LTA there were fewer bacteria caught in the liver leading to an order of magnitude more bacteria in kidney and blood.

In addition, we tested if LTA injection in vivo could block the CRIg binding to Gram-positive bacteria, resulting in decreased catching of \textit{S. aureus} by Kupffer cells. Indeed, mice saturated with LTA partially recapitulated the phenotype of CRIg \textsuperscript{−/−} mice, showing decreased numbers of trapped bacteria within Kupffer cells (Figure 4B) and increased numbers of circulating bacteria freely streaming along liver sinusoids (Figure 4C; Movie 1).

**Figure 3. CRIg Recognizes Lipoteichoic Acid**

(A) Binding of \textit{S. aureus}-GFP or \textit{S. aureus} \textit{dsrA}-GFP with AF647-labeled mCRIg.

(B) Mice were infected with \(5 \times 10^7\) CFU \textit{S. aureus}-GFP or \textit{S. aureus} \textit{dsrA}-GFP, and the captured bacteria within liver were counted over time. \(n = 3\) to 4 per group; six to seven FOV were counted per mouse.

(C) Binding of \textit{S. aureus} to AF647-labeled mCRIg in the absence or presence of purified \textit{S. aureus}-derived peptidoglycan (100 \(\mu\)g/ml).

(D) hCRIg-expressing Jurkat-T cells were incubated with indicated doses of FITC-labeled peptidoglycan. The binding was measured by flow cytometry.

(E and F) (E) Binding of \textit{S. aureus} with AF647-labeled mCRIg in the presence of different concentrations of \textit{S. aureus}-derived LTA or (F) in the presence of anti-LTA and ctrl Ig.

(G) The binding was quantified using geometric fluorescence intensity.

(H) Measurement of the binding of LTA to mCRIg- or BSA-coated plates by ELISA.

(I) LTA was pre-absorbed with or without soluble mCRIg; their binding to CRIg or BSA-coated plates was then measured by ELISA.

(J) AF-647-labeled CRIg binding to WT \textit{S. aureus} strain (WT-LAC), LTA-negative \textit{S. aureus} strains (\textit{ΔltaS-US3}, \textit{ΔltaS-UN2}), or WTA-negative \textit{S. aureus} strain (\textit{ΔtagO}).

(K) Quantification of the binding between AF-647-labeled CRIg and various LTA-deficient \textit{S. aureus} strains. Data were shown as mean ± SEM. Pooled results from at least three independent experiments in (G), or representative results of at least two experiments in (H)–(K) were shown. *\(p < 0.05\), **\(p < 0.01\), and ***\(p < 0.001\).
Moreover, we found that CRig−/− mice showed dramatically decreased ability to catch other Gram-positive bacterial strains we tested, including *S. aureus* (MW2), *B. cereus*, *L. monocytogenes*, *Enterococcus faecium* (*E. faecium*), *Enterococcus hirae* (*E. hirae*), and *Bacillus subtilis* (*B. subtilis*) (Figures 4D and S2; Movie S4). WT mice saturated with LTA from *E. hirae* and *B. subtilis* could also partially block their catching of *E. hirae* and *B. subtilis* (Figure S2). In addition, we found most of these Gram-positive bacteria, except *B. cereus*, are caught normally by Kupffer cells in CRig−/− mice (Figure S3). Taken together, these data strongly support a contribution of CRig-LTA recognition in Kupffer-cell-mediated capture of circulating Gram-positive bacteria.

**DISCUSSION**

The fundamental role of liver in capturing circulating bacteria was examined in this study using imaging technologies, which revealed that about 70% of total bacterial inoculums were quickly sequestrated by Kupffer cells within the first 5 min following i.v. infection. The liver-mediated bacterial capture plays a critical role in limiting the dissemination of bacteria into other susceptible organs during blood-borne infections, such as catheter-related bloodstream infections, which has rapidly become one of the most common nosocomial infections within intensive care units (Gahlot et al., 2014). Selective trapping of circulating bacteria by the liver rather than other organs may represent an evolutionary strategy to rapidly clear invading bacteria, not only because the liver contains the largest tissue resident macrophage pool, but also because the liver has an incredible capacity to regenerate after various insults. In addition, the immune tolerance properties of the liver may help to restrict inflammation following hepatic bacterial sequestration (Crispe, 2003). It is also noteworthy that although neutrophils were recruited to the liver, this only occurred after the capture of the pathogen by Kupffer cells. In fact, neutrophils lack CRig....
expression and the direct capacity to catch bacteria from the mainstream of blood. Instead, neutrophils have been reported to use neutrophil extracellular traps (NETs) to catch bacteria in blood (McDonald et al., 2012), and this still happens in CRIg−/− mice (data not shown).

Time-lapse intravital imaging revealed that CRIg was important for the capture of circulating S. aureus, confirming a previous report that measured CFUs to estimate the number of captured bacteria (Helmy et al., 2006). Paradoxically, we found that the previously identified CRIg ligand, C3, is dispensable for catching circulating S. aureus, suggesting that CRIg may support bacterial capture from bloodstream independent of C3b/iC3b recognition. Given the fact that Kupffer cells catch Gram-positive bacteria in less than one minute after they enter the bloodstream, it is very possible that the extent of complement opsonization in such a short time period is not sufficient to support CRIg-C3b-mediated capture of bacteria under shear conditions. In support of this, Gram-positive bacteria are notoriously difficult to opsonize: they have evolved elaborate immune evasion strategies to divert the effectiveness of complement activation on their surfaces. For example, S. aureus makes staphylococcal complement inhibitor, extracellular fibrinogen-binding protein, and extracellular complement-binding protein, all of which inhibit the deposition of C3b onto their surfaces (Jongerius et al., 2007; Rooyakkers et al., 2005). It should not be surprising that the host has evolved a way to counter this evasion mechanism. Our findings suggest that one such host-defense mechanism involved direct binding of LTA by CRIg. Given that LTA is an essential molecule for bacterial growth and cell division, loss of this molecule as a strategy for evading immunity is not likely and as such makes a good target for efficient capture of many Gram-positive bacteria.

In this work, we have identified LTA as a ligand for CRIg, suggesting that CRIg is a functional pattern recognition receptor that can recognize bacterial components directly. Interestingly, Kupffer cells expressed several other receptors that recognize LTA, including scavenger receptor (SR)-A1 and CD36 (reviewed in Canton et al., 2013). Whether they participate in assisting CRIg-mediated bacterial catching remains unknown. Previous work has reported that bacteria use catch bonds between receptor-ligand interactions to adhere under shear conditions. A characteristic of such receptors is the increasing affinity as shear forces increase (Sokurenko et al., 2008). This may therefore be the distinguishing feature of CRIg relative to other LTA receptors. It is also worth noting that LTA-recognizing receptors usually can act as signaling molecules leading to the initiation of inflammation. Whether CRIg is able to transduce similar cellular signaling to initiate inflammation is worthy of further investigation.

In conclusion, our study reveals that bacterial capture under shear conditions in vivo is a highly coordinated biological process, which does not absolutely require target opsonization but does require pattern recognition. We were able to directly visualize and characterize the mechanisms underlying the bacterial capture in vivo. Our finding that Kupffer cells efficiently capture circulating Gram-positive bacteria via CRIg-mediated recognition of LTA represents the discovery of a pattern recognition function.

EXPERIMENTAL PROCEDURES

Mice

CS7BL/6, C3−/−, C4−/−, C5−/−, Rag1−/−, and Mac-1−/− mice were obtained from The Jackson laboratory; FcγR−/− mice were obtained from Taconic Farms; CRIg−/− mice were from Dr. Menno Van Lookeren Campagne (Genetech Inc.). All mouse colonies were maintained in specific-pathogen-free facilities at the University of Calgary. The animal experiments were approved by the University of Calgary Animal Care Committee (Protocol#AC12-0162) and were performed in accordance with the guidelines established by the Canadian Council for Animal Care.

Bacteria

All Gram-positive bacteria were grown BHI media, and Gram-negative bacteria were grown in LB media. The cultures were grown overnight, followed by subculture until logarithmic phase (OD 660 nm = 1). When necessary, the following antibiotics were used: 10 μg/ml chloramphenicol for the pCM29 plasmid. Detailed information about bacterial strains can be found in the Supplemental Experimental Procedures.

Intravital Microscopy of the Bacterial Capture within Liver

Spinning-disk confocal intravital microscopy of mouse liver was performed as previously described (Lee et al., 2010; Wong et al., 2013). For in vivo imaging of bacterial capture, time-lapse images were captured from ~1 min to 20–30 min post-infection at a speed of two images per minute. A total of six to seven fields of view were recorded for each mouse under a 10× microscope objective. Images or movies were exported as .tiff or .avi, respectively, and processed and analyzed with a Velocity analysis software (v. 6.3 Improvise). The minimum threshold values were adjusted for each of the fluorescence channels to reduce background. The same threshold values were applied to images/movies from all treatment groups. The numbers of captured bacteria per field of view (FOV) were calculated by using the “Find object” function of Velocity software. Bacteria trapped within Kupffer cells for more than five time points (2.5 min) were considered as captured.

Flow Cytometry of CRIg Binding to Bacteria

Recombinant mouse or human extracellular domain of CRIg with a His-Tag (Creative Biomart) were labeled with AF 647 using a protein labeling kit according to the manufacturer’s instructions. AF 647-labeled recombinant CRIg protein (20 μg/ml or indicated concentrations) was incubated with 2 × 10⁶ CFU subcultured bacteria at room temperature for 2 to 3 hr with shaking. Bacterial cells were then washed three times, resuspended in fixation buffer (1% PFA), and subjected to flow cytometry. For competitive binding assays, recombinant CRIg was incubated with bacteria in the presence of purified peptidoglycan or LTA (from S. aureus, Sigma-Aldrich) at indicated concentration.

Statistical Analysis

Data were expressed as mean ± SEM and analyzed by an overall one-way ANOVA with Tukey’s test for multiple groups’ comparisons, or by unbiased student’s t test for comparison between two groups.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, four movies, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2016.06.002.

AUTHOR CONTRIBUTIONS

Z.Z. designed and performed the experiments, and wrote the manuscript; B.G.J.S. assisted in the experiments and contributed to writing the manuscript; C.H.Y.W. did some bacterial catching experiments; J.A.G. constructed the Sortase A and WTA mutant S. aureus; C.N.J. and P.K. supervised the study and wrote the manuscript.
ACKNOWLEDGMENTS

We thank Dr. Menno Van Lookeren Campagne (Genetech Inc.) for providing CRIg−/− mice and anti-CRIg antibodies; we thank Dr. Angelika Gründling (Imperial College London) for providing LTA-deficient S. aureus strains; we thank Dr. Bjorn Petri for helping with the whole-body imaging experiments. We thank Dara O’Halloran and Joana Carvalho for assistance with the construction of JαβA and JαβO mutants. We thank Trecia Nussbaumer for the mice breeding. P.K. is supported by Alberta Innovates Health Solutions, the Canadian Institutes of Health Research, and the Canada Research Chairs Program. B.G.J.S. is partially funded by Marie Curie actions FP7-PEOPLE-2013-IOF (grant no. 627575) and Alberta Innovates Health Solutions (AIHS). The authors declare no competing financial interests.

Received: January 28, 2016  
Revised: April 1, 2016  
Accepted: May 11, 2016  
Published: June 23, 2016

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