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Author manuscript

Nat Commun. Author manuscript; available in PMC 2015 May 11.

Published in final edited form as:

Nat Commun. ; 5: 5438. doi:10.1038/ncomms6438.

## The Staphylococcal Toxins $\gamma$ -Hemolysin AB and CB Differentially Target Phagocytes by Employing Specific Chemokine Receptors

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### Abstract

Evasion of the host phagocyte response by *Staphylococcus aureus* is crucial to successful infection with the pathogen.  $\gamma$ -Hemolysin AB and CB (HlgAB, HlgCB) are bicomponent pore-forming toxins present in almost all human *S. aureus* isolates. Cellular tropism and contribution of the toxins to *S. aureus* pathophysiology are poorly understood. Here, we identify the chemokine receptors CXCR1, CXCR2 and CCR2 as targets for HlgAB, and the complement receptors C5aR and C5L2 as targets for HlgCB. The receptor expression patterns allow the toxins to efficiently and differentially target phagocytic cells. Murine neutrophils are resistant to HlgAB and HlgCB. CCR2 is the sole murine receptor orthologue compatible with  $\gamma$ -Hemolysin. In a murine peritonitis model, HlgAB contributes to *S. aureus* bacteremia in a CCR2-dependent manner. HlgAB-mediated targeting of CCR2<sup>+</sup> cells highlights the involvement of inflammatory macrophages during *S. aureus* infection. Functional quantification identifies HlgAB and HlgCB as major secreted staphylococcal leukocidins.

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### AUTHOR CONTRIBUTIONS

J.S. and T.H. contributed equally to this work as senior authors. A.S., J.S. and T.H. wrote the manuscript. A.S., C.H., G.L., F.V., K.K. V.T., J.S. and T.H. designed experiments. A.S., M.V., P.W., C.H. and K.K. performed cellular assays. A.S. and T.H. performed *in vivo* experiments. C.B. produced recombinant proteins. T.R.-R. and Y.B. constructed bacterial mutants and complementations. C.D. and M.J. performed SPR analyses. E.O. performed qRT-PCRs.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

*Staphylococcus aureus* is one of the most prominent causes of bacterial infections in humans worldwide<sup>1</sup>. *S. aureus* causes a plethora of infections, ranging from superficial skin infections to severe invasive diseases<sup>2</sup>. Emergence of methicillin-resistant *S. aureus* (MRSA) strains has become a global challenge for first-line antibiotic treatment regimens of infections with the pathogen<sup>2</sup>. Failure of vaccine development programs poses an additional threat to public health and urges to a better understanding of the host-pathogen interaction<sup>3</sup>.

A key host defense mechanism against *S. aureus* is phagocyte-mediated killing<sup>4</sup>. The pathogen in return is well adapted to the human host, evading host immune responses at multiple levels using an arsenal of virulence factors<sup>5</sup>. A major mechanism deployed by *S. aureus* to target host phagocytes is production of cytolytic toxins<sup>6</sup>. Staphylococcal leukocidins comprise a class of bicomponent pore-forming toxins capable of damaging the host cell plasma membrane<sup>7,8</sup>. The leukocidins characterized from human *S. aureus* isolates include Pantan-Valentine Leukocidin LukSF (PVL)<sup>9</sup>,  $\gamma$ -Hemolysin (HlgAB and HlgCB)<sup>10,11</sup>, LukED<sup>12</sup>, and LukAB, also known as LukGH<sup>13,14</sup>. The genes encoding  $\gamma$ -Hemolysin are present in over 99.5% of human *S. aureus* isolates. In contrast, carriage of the genes encoding other leukocidins such as PVL and LukED is not as widely distributed<sup>15,16</sup>.  $\gamma$ -Hemolysin forms two functional bicomponent pore-forming toxins (HlgAB and HlgCB), which share the protein component HlgB<sup>10</sup>. In vitro, expression of  $\gamma$ -Hemolysin is highly up regulated during culture in blood<sup>17</sup> and during phagocytosis by neutrophils<sup>6</sup>.

Some leukocidins exert cytotoxicity in a species-specific manner<sup>18,19</sup>.  $\gamma$ -Hemolysin contributes partially to virulence during septic arthritis and systemic infection in mice<sup>17,20</sup> and endophthalmitis in rabbits<sup>21,22</sup>. However, to our knowledge, species-specific susceptibility of leukocytes to  $\gamma$ -Hemolysin has never been investigated. Although the ganglioside GM1 was found to inhibit  $\gamma$ -Hemolysin dependent killing<sup>23,24</sup>, identification of the membrane glycolipid GM1 as a  $\gamma$ -Hemolysin receptor cannot explain observed cellular tropism of the toxins<sup>7</sup>. Cell selectivity and species specificity of PVL are determined by the interaction of PVL with the human complement receptors C5aR and C5L2<sup>18</sup>. In addition, LukED targets CCR5, CXCR1 and CXCR2<sup>25,26</sup>. These receptors belong to the family of chemokine receptors, which play a crucial role in activation and migration of specific immune cells<sup>27,28</sup>.

Here, we report the identification of the human chemokine receptors CXCR1, CXCR2 and CCR2 as receptors for HlgAB and the human complement receptors C5aR and C5L2 as receptors for HlgCB. Toxin expression levels and functional pore forming activity of different clinical *S. aureus* isolates underscore HlgAB and HlgCB as major secreted staphylococcal leukocidins. The abundant expression of the receptors on phagocytes makes them primary targets and allows the toxins to differentially discriminate phagocytic cells from other cells. We show that murine neutrophils are resistant to both HlgAB and HlgCB, and identify CCR2 as the sole murine receptor orthologue compatible with  $\gamma$ -Hemolysin. Using a murine peritonitis model, we demonstrate that HlgAB contributes to *S. aureus* bacteremia in a CCR2-dependent manner. These findings highlight the involvement of inflammatory macrophages during *S. aureus* infection.

## RESULTS

### $\gamma$ -Hemolysin AB and CB target chemokine receptors

To evaluate host target cell tropism of  $\gamma$ -Hemolysin, we tested toxin-mediated cell permeability in freshly isolated human leukocytes using a membrane-impermeant fluorescent dye. Figure 1a confirms that HlgAB and HlgCB target human neutrophils and monocytes<sup>29</sup>. This pattern of cell specificity is similar to that of PVL<sup>18</sup>. In contrast to HlgCB, HlgAB shows limited cytotoxicity towards lymphocytes as well<sup>30</sup>. The extended cellular tropism of HlgAB compared to HlgCB implies the involvement of different host target cell receptors.

The molecular basis of cell selectivity of both HlgAB and HlgCB is not understood. Because PVL and HlgCB were predicted to share proteinaceous receptors<sup>29</sup>, we hypothesized that  $\gamma$ -Hemolysin targets chemokine receptors as well. As no specific predictions were available for HlgAB, we screened human embryonic kidney (HEK) cells transfected with a collection of human chemokine receptors for cytotoxic susceptibility to HlgAB and HlgCB. Untransfected cells were resistant to pore formation induced by both HlgAB and HlgCB (Fig. 1a, b).

Mirroring PVL specificity, HlgCB induced pore formation in cells transfected with the C5a receptors, C5aR or C5L2 (Fig. 1b). In addition, HlgCB induced pore formation in cells transfected with the complement receptor C3aR, though only at high toxin concentrations (Supplementary Fig. 1a). Assessment of half-maximal effective lytic concentrations ( $EC_{50}$ ) identified C5aR and C5L2 as the most efficient targets for HlgCB cytotoxicity, with  $\pm$  100 fold lower  $EC_{50}$  concentrations compared to C3aR (Supplementary Fig. 1b). HlgAB efficiently induced pore formation in cells transfected with the chemokine receptors CXCR1, CXCR2 and CCR2 (Fig. 1b). Furthermore, high concentrations of HlgAB induced pore formation in cells overexpressing the chemokine receptor CXCR4 (Supplementary Fig. 1a). Calculation of  $EC_{50}$  concentrations identified CXCR1, CXCR2 and CCR2 as the receptors most efficiently employed by HlgAB, with at least 30 fold lower  $EC_{50}$  concentrations compared to CXCR4 (Supplementary Fig. 1b). Importantly, the respective panel of HlgAB and HlgCB receptors did not overlap (Fig. 1b and Supplementary Fig. 1a), indicating that HlgA and HlgC control receptor specificity.

To obtain the affinity of the toxin-receptor interactions, Surface Plasmon Resonance (SPR) was performed using proteoliposomes containing C5aR, CXCR1, CXCR2 or CCR2. HlgC bound C5aR with a  $K_D$  of 5.64 ( $\pm$  1.53) nM. HlgA bound CXCR1, CXCR2, and CCR2 with a  $K_D$  of 5.69 ( $\pm$  1.94) nM, 27.20 ( $\pm$  5.54 nM), and 3.51 ( $\pm$  0.29) nM, respectively. Consistent with the cellular assays, no detectable binding of HlgC was observed on the HlgA receptors and *vice versa* (Table 1). The restricted receptor spectrum emphasizes HlgAB and HlgCB as two different and specific toxins.

In an attempt to better understand leukocyte tropism of  $\gamma$ -Hemolysin, we evaluated expression levels of the receptors most efficiently employed by HlgAB and HlgCB on the surface of freshly isolated human leukocyte populations. The receptors targeted by HlgCB are present on neutrophils and monocytes, but absent on lymphocytes (Fig. 1c). These

observations are in line with cell specificity of HlgCB. The receptors employed by HlgAB are abundantly present on neutrophils and monocytes (Fig. 1c). CCR2 is exclusively expressed on monocytes. Low-level expression of CXCR1 and CXCR2 on lymphocytes likely accounts for the limited cytotoxicity of HlgAB towards these cells.

In summary, these data suggest that cellular tropism of  $\gamma$ -Hemolysin is determined by the specific interaction of HlgA and HlgC with their respective protein receptors. The abundant expression of the efficient receptors on phagocytes allows the toxins to discriminate phagocytic cells from other cells.

### HlgAB and HlgCB lyse cells receptor-specifically

*S. aureus* isolates vary in both genetic contents and expression levels of leukocidins. Strain USA300 clone SF8300 is a minimally passed representative PVL-positive community-associated methicillin-resistant *S. aureus* (MRSA) isolate from the United States<sup>31</sup>. *S. aureus* Newman is a PVL-negative, methicillin-sensitive strain frequently used for *in vivo* studies<sup>32</sup>. We tested the interaction of native HlgAB and HlgCB with specific receptors using bacterial culture supernatants. CXCR2-mediated cytotoxicity of HlgAB was confirmed using supernatant of *S. aureus* USA300 SF8300 and its isogenic *hlgACB* mutant (*hlgACB* knockout) strain<sup>33</sup>. Induction of pore formation in U937 promyelocytic cells stably transfected with CXCR2 was lost using supernatant of the strain lacking HlgACB. Pore forming capacity of culture supernatant was restored by complementation with a plasmid encoding HlgAB, but not with a plasmid encoding HlgCB (Fig. 2a). Due to production of PVL, HlgCB cytotoxicity towards C5aR-transfected cells could not be investigated using a USA300 strain<sup>18</sup>. C5aR-mediated cytotoxicity of HlgCB was therefore validated using supernatants of strain Newman and its isogenic *hlgACB* mutant (*hlgACB* knockout) strain<sup>22</sup>. Culture filtrates of Newman wild type but not of the strain lacking HlgACB were lytic to C5aR-transfected U937 cells. Cytotoxicity was restored by complementation with a plasmid encoding HlgCB, but not HlgAB (Fig. 2b).

These data confirm the results obtained with recombinant toxins, and demonstrate that HlgAB and HlgCB produced by *S. aureus* specifically target their respective host receptors.

### HlgAB and HlgCB are major secreted *S. aureus* leukocidins

HlgCB shares receptors with PVL, while HlgAB partly shares receptors with LukED<sup>18,25,26</sup>. This finding suggests redundancy of the secreted toxins. To evaluate the relative importance of the different toxin-receptor pairs, we composed a panel of representative European clinical *S. aureus* strains isolated from blood cultures. As most of European *S. aureus* strains are PVL-negative<sup>16</sup>, we included the PVL-positive major MRSA-clone currently causing infections in the United States (USA300 LAC) as a control<sup>34</sup>. All the selected European clinical isolates were methicillin susceptible, and belonged to a variety of clonal complexes (Table 2).

We first determined which genes encoding secreted leukocidins are present in the different clinical isolates. Confirming expectations<sup>7,15,16</sup>, all clinical strains contained the genes encoding *hlgA* and *hlgC*, while none contained the *lukS-PV* gene. Following clonal

lineage<sup>35</sup>, half of the isolates encoded the *lukE* gene (Table 2). Next, we evaluated expression of the leukocidin S-component mRNAs by qRT-PCR. For PVL, culture broth dependent differential expression of the toxin has been described<sup>17,36,37</sup>. Strain USA300 LAC confirmed this broth dependent expression of *lukS-PV* (Supplementary Fig. 2 a, b). Quantities of *lukE* transcripts were broth dependent as well. In contrast, all clinical strains consistently expressed high levels of *hlgA* and *hlgC* mRNA in the two different culture broths tested (Table 2 and Supplementary Fig. 2a, b).

To investigate the relative contribution to pore formation of the respective secreted leukocidins, we subsequently tested supernatants of the different bacterial cultures on human neutrophils and HEK cells stably transfected with specific receptors. Untransfected HEK cells showed background pore formation of  $\pm 15\%$  on average (Supplementary Fig. 2b – d, f, g). Culture supernatants of all clinical isolates efficiently induced pore formation in HEK cells transfected with C5aR or CCR2 (Table 2 and Supplementary Fig. 2b, d), indicating that HlgCB and HlgAB are secreted in functional quantities. Although HEK cells transfected with CCR5 showed susceptibility to recombinant LukED as expected (Supplementary Fig. 2e)<sup>25</sup>, pore formation by culture supernatants in CCR5-expressing HEK cells was inefficient and not linked to *lukE* expression (Table 2 and Supplementary Fig. 2 a, b, f). However, culture supernatants of the clinical isolates induced efficient pore formation in HEK cells transfected with CXCR1 (Supplementary Fig. 2c). As CCR5-expressing HEK cells remained largely unaffected by the culture supernatants, it is likely that HlgAB mainly drives cytotoxicity towards CXCR1-transfected cells. Similar to cells expressing CCR5, pore formation in HEK cells co-transfected with CD11b/CD18 (CR3) was inefficient (Supplementary Fig. 2g). All culture supernatants showed high cytotoxic activity towards human neutrophils, which abundantly express the  $\gamma$ -Hemolysin receptors C5aR, CXCR1, and CXCR2 (Fig. 1c, Table 2 and Supplementary Fig. 2h).

Altogether, near-universal prevalence and consistent toxin expression discriminate  $\gamma$ -Hemolysin from other staphylococcal leukocidins, and identify HlgAB and HlgCB as highly significant secreted *S. aureus* toxins efficiently targeting phagocytic cells.

### HlgAB and HlgCB induce pro-inflammatory responses

In addition to inducing pore formation, staphylococcal leukocidins have pro-inflammatory properties<sup>33,38,39</sup>.

Sublytic concentrations of PVL prime neutrophil responses in a C5aR-dependent manner<sup>18</sup>. We thus tested if immune activating properties of HlgAB and HlgCB were receptor-mediated. HlgCB and HlgAB at low concentrations increased the N-formyl-methionine-leucine-phenylalanine (fMLP)-induced oxidative burst by neutrophils in a similar way as Tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), C5a, and Interleukin-8 (IL-8, CXCL8). Priming by HlgCB was fully antagonized after preincubation of cells with the specific C5aR-antagonist CHIPS<sub>31–121</sub> (Fig. 3a and Supplementary Fig. 3a)<sup>40</sup>. This finding demonstrates that the C5aR drives priming of neutrophils by sublytic concentrations of HlgCB. To our knowledge, no molecules are available that antagonize both human CXCR1 and CXCR2. Therefore, we tested the CXCR2-specific proteolytic enzyme Staphopain-A for interference

with HlgAB<sup>41</sup>. As expected based on the presence of CXCR1, preincubation of cells with Staphopain-A alone did not affect priming by HlgAB (Fig. 3b and Supplementary Fig. 3b).

In human macrophages, HlgCB activates inflammasome signaling<sup>33</sup>. CHIPS, a specific C5aR antagonist<sup>42</sup>, antagonized IL-1 $\beta$  release induced by HlgCB (Fig. 3c). This finding implies that the C5aR mediates the release of pro-inflammatory cytokine IL-1 $\beta$  in response to HlgCB.

Taken together, these findings indicate that the C5aR mediates the pro-inflammatory properties of HlgCB. CXCR1 and CXCR2 likely mediate priming of neutrophils by HlgAB.

### HlgA and HlgC are immunomodulatory molecules

Following activation with their natural ligands, signal transducing chemokine receptors provoke transient rises of intracellular calcium. Low concentrations of the single protein components HlgA and HlgC are not activating or cytotoxic<sup>43</sup>. Since both proteins bind chemokine receptors, we tested if HlgA and HlgC act as functional antagonists of the respective receptors by detection of intracellular calcium mobilization.

As HlgC binds the signal transducing C5aR, we tested if HlgC acts as a functional inhibitor of this receptor. Just as LukS-PV, HlgC antagonized activation induced by C5a on neutrophils (Fig. 3d)<sup>18</sup>. Because C5L2 is an atypical chemokine receptor and not coupled to a G-protein<sup>44</sup>, functional inhibition of this receptor by HlgC could not be tested. HlgA interacts with CXCR1, CXCR2, and CCR2. HlgA antagonized activation of neutrophils induced by IL-8 (a ligand of both CXCR1 and CXCR2) (Fig. 3e)<sup>45</sup>. In addition, HlgA inhibited activation of monocytes induced by the CCR2-specific ligand MCP-1 (CCL2) (Fig. 3f).

These data suggest that the binding proteins of  $\gamma$ -Hemolysin as single components have receptor-specific immunomodulatory properties.

### HlgAB and HlgCB feature receptor-selectivity in mice

*S. aureus* interacts with the host immune system in a highly human specific manner<sup>5</sup>. In order to explore murine susceptibility to  $\gamma$ -Hemolysin, we tested cytotoxicity of HlgAB and HlgCB towards murine bone marrow-derived leukocytes.

As observed for PVL, murine neutrophils and monocytes were resistant to HlgCB induced pore formation (Fig. 4a). In line with this result, HEK cells transfected with the murine C5aR or C5L2 were resistant to HlgCB (Fig. 4b).

HlgAB and LukED both target human CXCR1 and CXCR2 (Fig. 1b)<sup>26</sup>. However, murine neutrophils were resistant to HlgAB-induced pore formation (Fig. 4a). In mice, CXCR2 but not CXCR1 is expressed on neutrophils and monocytes<sup>46</sup>. Although HEK cells transfected with murine CXCR1 were susceptible to HlgAB-induced cytotoxicity, cells transfected with murine CXCR2 remained unaffected by HlgAB (Fig. 4b). The incompatibility of HlgAB with murine CXCR2 explains resistance of murine neutrophils to the toxin. In contrast,

LukED is compatible with murine CXCR2 (Supplementary Fig. 4a), accounting for susceptibility of murine neutrophils towards LukED (Fig. 4a).

Monocytes of wild type and *ccr5*<sup>-/-</sup> mice were susceptible to HlgAB induced pore formation, whereas monocytes of *ccr2*<sup>-/-</sup> mice were HlgAB-resistant (Fig. 4a and 4c). In agreement with this, HEK cells transfected with the murine CCR2 showed clear sensitivity to HlgAB (Fig. 4b). Murine bone marrow cells from *ccr2*<sup>-/-</sup> mice did not release IL-1 $\beta$  when exposed to HlgAB, in contrast to cells from wild type mice (Supplementary Fig. 4b). These results provide further evidence for a specific interaction of HlgAB with CCR2.

Collectively, our data show that  $\gamma$ -Hemolysin features distinct receptor-mediated specificity in mice and human. As a consequence, murine neutrophils are resistant to both HlgAB and HlgCB. Importantly, the results identify HlgAB and CCR2 as potential subjects for investigation in murine models of staphylococcal infection.

### HlgAB targeting of CCR2 enhances *S. aureus* pathogenesis

Although the genes encoding  $\gamma$ -Hemolysin are present in nearly all human *S. aureus* isolates, its contribution to staphylococcal pathophysiology is not fully understood. Since we discovered that HlgAB targets CCR2, we investigated the contribution of this interaction to *S. aureus* pathogenesis *in vivo* in a murine peritonitis model.

Inflammatory macrophages in the peritoneal cavity of mice, elicited by heat-inactivated *S. aureus*, express CCR2 (Fig. 5a)<sup>47</sup>.

To investigate  $\gamma$ -Hemolysin mediated targeting of CCR2, we monitored the presence of CCR2<sup>+</sup> inflammatory macrophages in the peritoneal cavity of mice infected with *S. aureus* USA300 clone SF8300 or its isogenic *hlgACB* mutant (*hlgACB* knockout) strain<sup>33,48</sup>. Mice infected with wild type *S. aureus* showed a significant decrease in the proportion of CCR2<sup>+</sup> inflammatory macrophages compared to mice infected with the *hlgACB* mutant strain (Fig. 5b). Notably, in the same mice, the fraction of CCR5<sup>+</sup> macrophages was equal between wild type and *hlgACB* mutant infected mice. In agreement with resistance of murine neutrophils to  $\gamma$ -Hemolysin, mice infected with wild type and *hlgACB* mutant had similar proportions of neutrophils (Fig. 5b). Using another USA300 clone, LAC<sup>34</sup>, and its isogenic *hlgA* mutant strain (*hlgA::bursa*), the observed modulation of the CCR2<sup>+</sup> inflammatory macrophage population was shown to be HlgA specific (Supplementary Fig. 5a). These data demonstrate that during infection, HlgAB is active and specifically targets CCR2<sup>+</sup> inflammatory macrophages in the peritoneal cavity.

Staphylococcal peritonitis is used as a model to study the spread of bacteria into the bloodstream<sup>49</sup>. We observed that the number of viable bacteria in the peripheral blood of mice is dependent on the presence of both HlgAB and CCR2. Twenty four hours after intraperitoneal infection, wild type mice infected with *S. aureus* USA300 clone SF8300 had  $\pm$  100 fold higher counts of viable bacteria in the peripheral blood compared to wild type mice infected with the *hlgACB* mutant strain (Fig. 5c). Using USA300 clone LAC and its isogenic *hlgA* mutant, the bacteremia was confirmed to be driven specifically by HlgA (Supplementary Fig. 5b). Cellular recruitment in response to sterile inflammatory stimuli is

impaired in *ccr2*<sup>-/-</sup> mice<sup>50</sup>. In contrast, infection with *S. aureus* induced similar influx of macrophages into the peritoneal cavity of wild type and *ccr2*<sup>-/-</sup> mice (Supplementary Fig. 5c). Compared to wild type mice, infection of *ccr2*<sup>-/-</sup> mice with *S. aureus* USA300 clone SF8300 resulted in  $\pm$  100 fold lower numbers of bacteria in the peripheral blood (Fig. 5c). The number of bacterial counts in the peripheral blood of *ccr2*<sup>-/-</sup> mice was independent of  $\gamma$ -Hemolysin. Moreover, no statistically significant differences were observed in the bacterial counts of the peritoneal cavity after twenty four hours in all infected mice (Fig. 5c and Supplementary Fig. 5b), representing comparable levels of bacteria as the seeding source. These results demonstrate that HlgAB contributes to *S. aureus* bacteremia in a CCR2-dependent manner. In addition, the interaction of HlgAB with CCR2<sup>+</sup> cells highlights the involvement of inflammatory macrophages during *S. aureus* infection.

## DISCUSSION

$\gamma$ -Hemolysin is highly prevalent in human *S. aureus* isolates<sup>15,16</sup>. Strikingly, its contribution to pathophysiology is poorly understood. As clinical epidemiology is inadequate in the context of high prevalence, a functional approach is needed to assess the role of  $\gamma$ -Hemolysin in staphylococcal virulence.

We show that HlgAB and HlgCB use chemokine receptors to induce pore formation in the target cell membrane. Comparison of half maximal effective concentrations needed to induce specific pore formation allows discrimination between efficient and inefficient receptors. While HlgAB efficiently targets CXCR1, CXCR2 and CCR2, the other toxin combination HlgCB efficiently targets C5aR and C5L2. Affinities of HlgA and HlgC for the respective receptors are in nanomolar range, a finding that is consistent with other leukocidins and their proteinaceous receptors<sup>25,51,52</sup>.

Based on studies with LukED<sup>26</sup>, it is likely that different domains of the toxins dictate receptor selectivity. The staphylococcal leukocidins HlgAB, HlgCB, PVL and LukED share considerable homology. LukAB however, is distinctive and more distantly related<sup>53</sup>. This divergence might account for the interaction of HlgAB, HlgCB, PVL and LukED with chemokine receptors, while LukAB strictly targets the CD11b subunit of the integrin Mac1<sup>52</sup>. The previously proposed cellular association of  $\gamma$ -Hemolysin with the ganglioside GM1 alone<sup>23,24</sup> failed to explain cell type specificities of the toxins<sup>7</sup>. Expression levels of the involved chemokine receptors on human leukocytes contribute to a better understanding of the cellular tropism of the toxins<sup>30</sup>. The abundant expression of the efficient receptors on phagocytes makes them major targets and allows the toxins to discriminate phagocytic cells from other cells.

In addition to cytotoxic capacities, HlgAB and HlgCB have pro-inflammatory and immunomodulatory properties as well. Just as for PVL, our data support the notion that both HlgAB and HlgCB engage the respective chemokine receptors directly and specifically during priming and inflammasome activation<sup>7,18</sup>. Simultaneous expression and secretion of the toxins as bicomponents – as observed here – however allows immediate formation of cytolytic pores on the target cells. Although binding of HlgA and HlgC to the receptors



results in antagonism of neutrophil and monocyte activation *in vitro*, it remains unclear if functional inhibition by single protein components contributes to pathogenesis of *S. aureus*.

The impact of  $\gamma$ -Hemolysin on virulence of *S. aureus* is incompletely understood. We suspect this is in part due to adaptation of *S. aureus* to the human host<sup>5</sup>. Indeed, our results show that current mouse models are not suitable to investigate the interaction of  $\gamma$ -Hemolysin with C5aR, C5L2, CXCR1 and CXCR2. As a result,  $\gamma$ -Hemolysin cytotoxicity towards neutrophils cannot be addressed in mice. Another consequence is that the apparent redundancy of LukED versus HlgAB, and PVL versus HlgCB cannot be studied *in vivo* currently. Since immunogenic resemblance limits the development of specific monoclonal antibodies for detection of leukocidin subunits, we developed an alternative approach to investigate the relative contribution to pore formation of the different leukocidins. Using cell lines expressing specific chemokine receptors and culture supernatants of a panel of diverse clinical *S. aureus* isolates, we demonstrate that HlgAB and HlgCB are functionally major secreted staphylococcal leukocidins employing phagocyte specific chemokine receptors. Since the clinical isolates tested were PVL-negative, the cytotoxicity towards C5aR-transfected cells is likely due to high expression of HlgCB as observed with mutant strains. As all clinical isolates show potent cytotoxic activity towards CCR2-transfected but not CCR5-transfected cells, expression and secretion of HlgAB most probably mediates CXCR1-specific toxicity as indicated with experiments using mutant strains. We demonstrate that expression and secretion of  $\gamma$ -Hemolysin AB and CB by various clinical *S. aureus* isolates in different conditions are consistent and functionally highly effective when compared to other members of the family of staphylococcal bicomponent pore-forming toxins. These observations are in line with reports investigating expression during culture in blood and after phagocytosis<sup>6,17</sup>, and indicate that HlgAB and HlgCB are prominent secreted toxins engaging receptors abundantly expressed on phagocytic cells.

High expression of both HlgCB and HlgAB allows the pathogen to differentially target phagocytes. The interaction of HlgAB with CCR2, a chemokine receptor most notably expressed on monocytes and inflammatory macrophages, is exclusive and non-redundant in relation to other staphylococcal leukocidins<sup>18,25,26,52</sup>. CCR2 is the only compatible murine  $\gamma$ -Hemolysin receptor orthologue. Using a murine peritonitis model, we validated the specific interaction of HlgAB with CCR2. *S. aureus* decreases the proportion of CCR2<sup>+</sup> inflammatory macrophages in a HlgAB dependent manner. In addition, we identified HlgAB as a virulence factor contributing to bacteremia by employing its host counterpart, CCR2. Importantly, numbers of macrophages in the infected peritoneal cavity of wild type and *ccr2*<sup>-/-</sup> mice were similar. Furthermore, *hlgACB* mutant bacteremia levels were not statistically significant between wild type and *ccr2*<sup>-/-</sup> mice. While we cannot exclude a contribution of CCL2/CCR2 signaling<sup>54,55</sup>, these observations strongly suggest that the reported phenotype in wild type versus *ccr2*<sup>-/-</sup> mice is due to a specific targeting of CCR2 by HlgAB and not due to a broad immune defect of *ccr2*<sup>-/-</sup> mice. Interestingly, a previous study described a mild impact on virulence of  $\gamma$ -Hemolysin in a murine intravenous infection model<sup>17</sup>. This contribution likely reflects the interaction between HlgAB and CCR2. Future research is needed to provide detailed insights into the mechanisms involved

in controlling bacteremia by HlgAB-mediated targeting of CCR2<sup>+</sup> cells in the peritoneal cavity.

The genes encoding  $\gamma$ -Hemolysin are present in nearly all human *S. aureus* isolates. The other leukocidin encoded by all *S. aureus* strains, LukAB, was previously reported to bind the bacterial surface and to promote survival by facilitating bacterial escape after phagocytosis<sup>13,14,56</sup>. Thus, LukAB has been proposed to act from within the phagocyte<sup>56</sup>. Pore formation induced by the different culture supernatants tested in HEK cells co-transfected with CD11b/CD18 was incomplete, indeed suggesting that the toxin is inefficiently secreted into the supernatant. In contrast, quantification of the expression levels and pore forming capacity of all tested clinical *S. aureus* isolates identify HlgAB and HlgCB as highly significant secreted *S. aureus* toxins efficiently targeting phagocytic cells. Altogether, these data highlight the differential and complementary mechanisms that *S. aureus* deploys to target phagocytes.

## METHODS

### Ethics Statement

Human leukocytes were isolated after informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. Approval was obtained from the medical ethics committee of the UMC Utrecht, The Netherlands. All experiments involving animals were reviewed and approved by the animal ethics committees of Lyon, France (CECCAPP, protocol number ENS2012\_033).

### Recombinant Protein Production and Purification

*S. aureus* strains used to amplify coding sequences of toxins are shown in Supplementary Table 1. HlgA, HlgB, HlgC, LukE, LukD, LukS-PV and LukF-PV were cloned and expressed as described elsewhere<sup>33</sup>. Briefly, the coding sequences omitting the regions predicted to encode the signal peptide were cloned and transformed into *E. coli* M15 or BL21 pLys. Protein expression was induced by adding isopropyl-b-d-thiogalactopyranoside during exponential growth of transformed *E. coli*. Polyhistidine-tagged proteins were isolated from supernatants of cell lysates on Ni-nitrilotriacetic acid columns (Qiagen). Contaminating LPS was eliminated using a Polymixin-B column (Thermo Scientific).

### Bacterial Strains and Culture

*S. aureus* strains used for this study are listed in Table 2 and Supplementary Table 2. Complementation of *hlgACB* mutants and generation of an erythromycin sensitive USA300 LAC *hlgA* strain is described in Supplementary Methods. Clinical *S. aureus* strains, isolated from blood cultures with a diverse infection focus and originating from patients admitted to the intensive care unit of the University Medical Center Utrecht, The Netherlands, were randomly chosen from a database created for tertiary hospital routine diagnostics. Standard microbiological diagnostic techniques were applied for microbial identification and antibiotic resistance profiling. All strains were cultured in casein hydrolysate and yeast extract (CCY)<sup>36,37</sup> or brain heart infusion (BHI), and overnight

supernatants filter sterilized. For *in vivo* experiments, mid-exponential subcultures were washed extensively in PBS.

### Typing of clinical *S. aureus* isolates

All clinical *S. aureus* isolates used in this study were typed by multi-locus sequence typing (MLST)<sup>57</sup>. Using the eBURST algorithm, isolates were assigned to a clonal complex<sup>58</sup>. Leukocidin S-component PCRs were performed on genomic DNA, which was isolated as previously described<sup>42</sup>. Primers were designed to be highly gene specific (Supplementary Table 3).

### *S. aureus* RNA Extraction and cDNA Synthesis

*S. aureus* RNA was isolated from samples using an RNeasy Plus Mini Kit (Qiagen) following instructions of the manufacturer. Bacteria were pre-cultured overnight and subsequently diluted in fresh medium for culture to early stationary phase (6,5 hrs; OD<sub>660</sub> ± 1,5). Bacteria were spun for 30 s at 13.000 rpm. Pellets were briefly kept in liquid nitrogen to stop bacterial metabolism, and resuspended in buffer RLT containing β-mercaptoethanol (Sigma). Resuspended bacteria were added to silica beads (Merlin), and disrupted using a mini-Beadbeater (BioSpec Products) for 1 min at 3.500 rpm. After cooling of the samples for 1 min on ice, an additional two cycles of disruption were applied. Contaminating chromosomal DNA was removed by consecutive treatment of samples with RNase Free DNase (Qiagen) and a RapidOut DNA Removal Kit (Thermo Scientific). RNA yield was measured using a NanoDrop ND1000 (NanoDrop Technologies), and quality was checked on agarose gel. Contamination with genomic DNA was excluded by a PCR specific for genomic DNA. Standardized quantities of total RNA were used immediately for synthesis of cDNA with a Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific).

### Quantitative Real-Time PCR

cDNA from the equivalent of 10 ng mRNA was used to perform qPCR for *hlgA*, *hlgC*, *lukE*, and *lukS-PV* using SYBR Green Master Mix (Qiagen) in a 7300 Real Time PCR System (Applied Biosystems) according to manufacturer's instructions. cDNA from the equivalent of 1 ng mRNA was used to amplify *I6S* as an endogenous control. Primers used to detect specific transcripts are listed in Supplementary Table 4. These primers are based on toxin sequences from strains Newman and USA300 (*lukS-PV*). Triplicate reactions for each primer set containing approximately 100 copies of *S. aureus* USA300 clone LAC genomic DNA were included to calibrate transcript abundance of each toxin. C<sub>T</sub> values were collected over 40 cycles of PCR, with primer annealing at 60°C, and data collection during the 30 second extension step at 72°C.

Analysis was performed using 7300 Real Time PCR System Software. Relative transcript abundance was calculated by the comparative C<sub>T</sub> (C<sub>T</sub>) method, using *I6S* as the endogenous control and USA300 clone LAC genomic DNA as the calibrator sample. Samples with an average C<sub>T</sub> value greater than 32 were considered background and were excluded from analysis. Toxin expression is indicated as the log<sub>10</sub> of the fold change in relative transcript abundance of each toxin compared to abundance from amplification from USA300 clone LAC genomic DNA.

## Cell Isolations

Human leukocytes, obtained from healthy volunteers, were isolated by Ficoll/Histopaque centrifugation<sup>42</sup>. Bone marrow of mice was harvested and immune cells collected as described<sup>18</sup>. Mice receiving intraperitoneal injections with heat-inactivated *S. aureus* USA300 SF8300 *hlgACB* ( $1 \times 10^8$  CFU, at 0 hr and 24 hr) were sacrificed after 48 hr. Immune cells were collected by peritoneal lavage with PBS.

All in vitro experiments with cells were performed with RPMI (Invitrogen) supplemented with 0.05% human serum albumin (Sanquin) unless specified otherwise, with cell concentrations adjusted to  $5 \times 10^6$  cell  $\text{mL}^{-1}$ .

## Quantification of Human Leukocyte Receptor Expression Levels

Freshly isolated human leukocytes were incubated in a total volume of 50  $\mu\text{L}$  at  $5 \times 10^6$  cell  $\text{mL}^{-1}$  on ice with 10  $\mu\text{g mL}^{-1}$  mouse anti-human chemokine receptor monoclonal antibodies for C5aR (clone S5/1, AbD SeroTec), C5L2 (clone 1D9-M12, Bio Legend), CXCR1 (clone 42705, R&D), CXCR2 (clone 48311, R&D), and CCR2 (clone 48607, R&D), followed by FITC-conjugated goat-anti-mouse antibody (1:50, Dako). Antibody binding was quantified by calibration to defined antibody binding capacity units using QIFIKIT (Dako) and corrected for isotype controls.

## Cell Lines and Transfections

U937 cells (a promyelocytic cell line) stably transfected with C5aR, CXCR2 or an empty expression vector were obtained from Eric R. Prossnitz (University of New Mexico, Albuquerque, NM, USA)<sup>59</sup>.

Human C5aR, C5L2, C3aR, CXCR1, CXCR2, CCR1, CCR2 and CCR5, and murine C5aR, C5L2, CXCR1, CXCR2, and CCR2 were cloned into a pcDNA3.1 vector (Invitrogen) as described previously<sup>18</sup>. All genes reside on a single exon. Amplification reactions were performed on QUICK-Clone cDNA of human bone marrow or mouse liver (BD Biosciences Clontech) using PfuTurbo DNA polymerase (Stratagene). Plasmids encoding human C5L2 and CXCR2 were purchased from UMR cDNA Resource Center. Plasmids encoding CXCR3, CXCR4, and CXCR6 were purchased from Thermo Fisher. Primer designs, restriction sites and accession numbers used in this study are displayed in Supplementary Table 5. HEK293T cells (a human embryonic kidney cell line) were transiently transfected according to the manufacturer's protocols with 4  $\mu\text{g}$  DNA and 5  $\mu\text{l}$  Lipofectamine 2000 (Life Technologies)<sup>18</sup>. After 24 hr, transfected cells were harvested with 0.05% Trypsin / 0.53 mM EDTA. Maximal pore formation of HEK293T cells transiently transfected with plasmids encoding chemokine receptors was defined by the percentage of receptor expressing cells as independently determined by flow cytometry.

For construction of HEK293T cells stably expressing human C5aR, CXCR1, CCR2, CCR5, or CR3 (CD11b/CD18), we cloned the complete coding regions of these receptors (Supplementary Table 6) into the bicistronic expression plasmid pIRESpuro3 (Clontech). CD18 was cloned into pIRESpuro3, whereas CD11b was cloned into pIRESHyg3 (Clontech). For stable expression, the constructs were transfected into HEK293T cells using

Lipofectamine 2000 (Life Technologies). One day after transfection, cells were put under selection pressure using  $1 \mu\text{g mL}^{-1}$  puromycin in DMEM with 10% FCS. A few weeks later, cells were subcloned and tested for stable receptor expression by flow cytometry.

Expression of human chemokine receptors on transfected cells was defined using  $10 \mu\text{g mL}^{-1}$  mouse anti-human chemokine receptor monoclonal antibodies for C5aR (clone S5/1, AbD SeroTec), C5L2 (clone 1D9-M12, Bio Legend), C3aR (clone 534625, R&D), CXCR1 (clone 42705, R&D), CXCR2 (clone 48311, R&D), CXCR3 (clone 49801, R&D), CXCR4 (clone 12G5, R&D), CXCR6 (clone MAB699, R&D), CCR1 (clone 53504, R&D), CCR2 (clone 48607, R&D), and CCR5 (clone 2D7, BD), followed by PE-labeled goat anti-mouse antibody (1:80, DAKO). For cells expressing CR3, cells were stained with an APC-labeled mouse monoclonal antibody directed towards CD18 (clone L130, 1:50, BD) and a FITC-labeled mouse monoclonal antibody detecting CD11b (clone ICRF44, 1:10, BD). Receptor expression levels were similar within each panel of transfected cells for all receptors (Supplementary Fig. 6a, b, c). As no monoclonal antibodies are available for all involved murine chemokine receptors, expression of murine receptors was defined by co-transfection with an IRES-GFP encoding pCDNA3 vector (Invitrogen)<sup>60</sup>. GFP expression was similar for all murine receptors investigated, and fully correlated with susceptibility of cells transfected with toxin-compatible receptors (Supplementary Fig. 6d).

### Cell Permeability Assays

Cells were exposed to recombinant proteins or overnight bacterial culture supernatant in a volume of 50–100  $\mu\text{L}$ . Human PBMC were pre-stained for CD14 expression (clone M5E2, 1:10, BD). Cells were incubated with toxin for 30 min at 37°C. Human leukocytes and cell lines were subsequently analyzed by flow cytometry and pore formation was defined as intracellular staining by propidium iodide (PI) or 4',6-diamidino-2-phenylindole (DAPI). After treatment with Fc $\gamma$ -receptor block, murine cell surfaces were stained with antibodies directed towards CD11b (clone M1/70, 1:400, BD), Ly6G (clone 1A8, 1:100, BD), Ly6C (clone AL-21, 1:200, BD) and F4/80 (clone BM8, 1:600, Bio Legend) antibodies. Following exposure to toxins, murine cells were incubated on ice with the fixable viability dye eFluor 780 (1:1000, eBiosciences) and cell viability was subsequently analyzed by flow cytometry in the presence of Flow-Count Fluorospheres (BD). Cell viability was defined as eFluor 780 negative cells in relation to buffer treated cells.

Analysis of pore formation in transiently transfected HEK293T cells was limited to receptor-positive cells. Half maximal effective lytic concentrations were calculated using nonlinear regression analyses.

As leukocidins are 2-component toxins, equimolar concentrations of polyhistidine-tagged proteins were used. To minimize bias due to production of the nonspecifically cytotoxic phenol soluble modulins (PSMs), experiments with culture supernatants were performed in the presence of 10% fetal calf serum<sup>61,62</sup>.

### Surface Plasmon Resonance Studies

Surface Plasmon Resonance of HlgA and HlgC and chemokine receptor proteoliposomes was performed using the Biacore T100 system and the series S L1 sensor chip using 10 mM

Tris 1 mM MgCl<sub>2</sub> and 1 mM CaCl<sub>2</sub> as the running buffer. Wheat-germ proteoliposomes of CXCR1, CXCR2, CCR2 and C5aR (Abnova) at a final protein concentration of 20 µg mL<sup>-1</sup> were immobilized onto test flow cells with a flow rate of 10 µL min<sup>-1</sup> for 10 min with a minimum capture level of 600RU. Empty, mock wheat-germ proteoliposomes (Abnova) were immobilized onto the reference flow cell using the same conditions with a minimum capture level of 300RU being obtained. Single cycle kinetics was initially used to establish concentration ranges for HlgA and HlgC for each chemokine receptor liposome with concentrations ranging from 0.01 to 20 µg mL<sup>-1</sup> with 5 µg mL<sup>-1</sup> used as the maximum concentration used in multiple cycle kinetics. A flow rate of 30 µL min<sup>-1</sup> was used for analysis with a contact time of 1 min and a disassociation time of 10 min for each concentration used. To ensure the chemokine receptors captured on the L1 chip were biologically relevant MCP-1 (Peprotech) was tested against CCR2. Concentrations of between 16 ng mL<sup>-1</sup> and 500 ng mL<sup>-1</sup> were tested. The affinity of MCP-1 with the captured CCR2 liposome was found to be 0.84 nM ± 014 nM on three replicate experiments. This was within the reported values found in the literature indicating that the proteoliposomes were in their native conformation<sup>63</sup>.

### Neutrophil Priming

Priming of human neutrophils has been described in detail recently<sup>18</sup>. Briefly, neutrophils were incubated for 5 min with 700 nM CHIPS<sub>31-121</sub> to block the C5aR, or for 15 min with 500 nM Staphopain-A to cleave the N-terminal CXCR2. Cells were primed with 10 ng mL<sup>-1</sup> TNFα, 1 nM C5a, 10 nM IL-8, or a range of sublytic concentrations of HlgCB or HlgAB for 30 min at 37°C. Subsequently, chemiluminescence was recorded in a luminometer. After 30 s of baseline recording, a final concentration of 1 µM fMLP was injected to stimulate the oxidative burst. For each sample, the area under the curve (AUC) after addition of fMLP was calculated. For comparison of experiments, AUC values were expressed relative to buffer-treated cells stimulated with fMLP.

### Interleukin-1β Production

Derivation of primary human macrophages from monocytes is described elsewhere<sup>33</sup>. To induce pro-IL-1β and prime the NLRP3-inflammasome, human macrophages were treated with heat-killed *S. aureus* at a multiplicity of 100:1 for 16 hr<sup>33</sup>. Cells were washed twice to remove heat-killed *S. aureus* and exposed to toxin during 1 hr at 37°C. Murine bone marrow cells were pretreated with 250 ng mL<sup>-1</sup> lipopolysaccharide for 3 hr and subsequently intoxicated with 6 nM HlgAB or 20 µM Nigericin during 30 min at 37°C. Release of human and murine IL-1β into the supernatant was quantified by enzyme linked immunosorbent assays (DuoSet ELISA kits, R&D Systems)<sup>33</sup>.

### Calcium Mobilization Assays

Activation of human neutrophils and monocytes was performed by real-time detection of calcium mobilization using flow cytometry<sup>18</sup>. Human monocytes were labeled with an anti-CD14 monoclonal antibody (BD). Cells, labeled with Fluo-3AM, were pre-incubated with HlgA or HlgC for 1 min at room temperature. Subsequently, baseline fluorescence was recorded in a flow cytometer. After 10 s of baseline recording, C5a (Sigma), IL-8, or MCP-1

(PeproTech) was added and fluorescence was recorded for an additional 50 s. Relative increase in fluorescence was expressed in comparison to uninhibited cells.

### Murine *In Vivo* Experiments

C57BL/6 wild type mice were obtained from Charles River Laboratories. *ccr2*<sup>-/-</sup> and *ccr5*<sup>-/-</sup> mice (Jackson Laboratories) were bred in house. 8 to 9 week old age and sex matched mice were infected intraperitoneally with 0.5 mL PBS containing 1×10<sup>8</sup> CFU of bacteria. At 24 hr postinfection, peripheral blood was obtained and mice were sacrificed in order to recover peritoneal lavage fluid. Peripheral blood and peritoneal lavage fluid were plated to evaluate the bacterial burden. After incubation on ice with the fixable viability dye eFluor 780, peritoneal cells were treated with Fcγ-receptor block and stained with monoclonal antibodies detecting CD11b, Ly6G, Ly6C, F4/80 and CCR2 (clone 475301, 1:100, R&D) or CCR5 (clone HMCCR5, 1:100, Bio Legend). Cells were subsequently analyzed by flow cytometry and viable cells were defined as eFluor 780 negative cells. In the model of staphylococcal peritonitis, mortality of mice was equal for all groups (± 20%).

### Graphical and Statistical Analyses

Flow cytometric analyses were performed with FlowJo (Tree Star Software). Statistical analyses were performed with Prism (GraphPad Software). Statistical significance was calculated using ANOVA and Student's t-tests where appropriate.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### ACKNOWLEDGEMENTS

The authors wish to thank Eric Prossnitz for donating cell lines, Binh Diep and Gilles Prévost for donating *S. aureus* strains, Thierry Walzer for donating mice, Jacqueline Marvel, Sophia Djebali, Peggy Parroche, Lisette Scheepmaker and Willemien van Rooijen for sharing expertise, and Bas Surewaard for reviewing the manuscript. We acknowledge the contribution of the PBES, flow cytometry, and AniRA-ImmOs Phenotyping Facility platforms of SFR Biosciences Gerland – Lyon Sud. The LAC *hlgA::bursa* parental strain was obtained from the Network of Antimicrobial Resistance in *S. aureus* (NARSA) program (NIAID/NIH contract HHSN272200700055C). This work was supported in part by grants from the EC (222718 to G.L., C.B., F.V.), the ANR (F.V., T.H, G.L.), foundation FINOVI (to T.H.), the NIAID/NIH (R01-AI105129 to V.T., T32-AI007180 to T.R.-R.), the NHMRC (565526 to M.J., C.D.), and the Smart Futures Fund Research Partnerships Program (M.J., C.D.).

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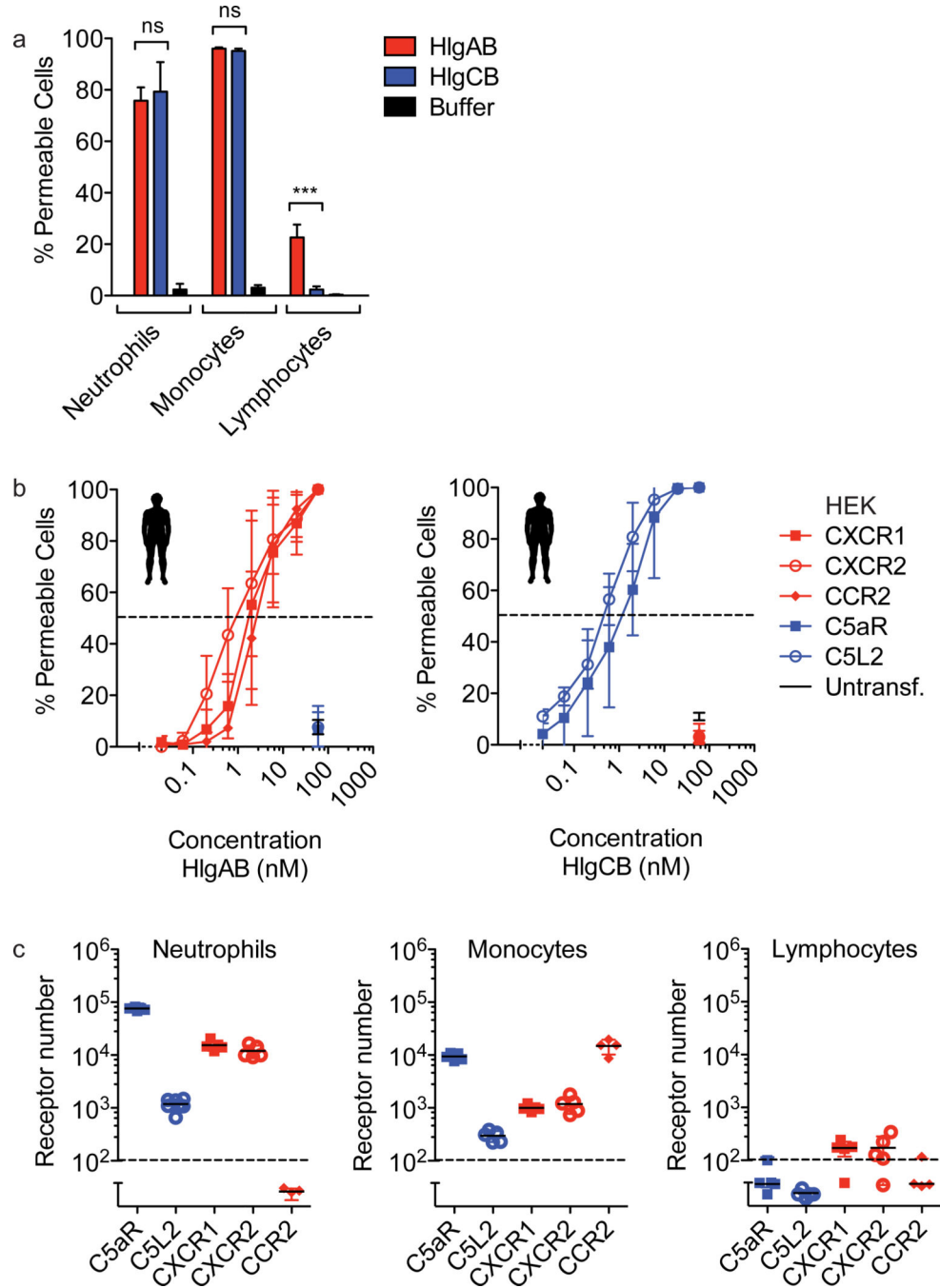
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**Figure 1. HlgAB and HlgCB target human phagocytes expressing specific chemokine receptors** (a) HlgAB and HlgCB (63 nM) efficiently induce pore formation in human neutrophils and monocytes. HlgAB shows limited cytotoxicity towards lymphocytes. (b) Pore formation following treatment with HlgAB or HlgCB was monitored in HEK cells transfected with plasmids encoding human chemokine receptors. The dashed line indicates 50% pore formation. (c) Expression of chemokine receptors efficiently targeted by HlgAB and HlgCB was quantified on human leukocytes, showing abundant expression on phagocytes. The dashed line indicates the detection threshold.

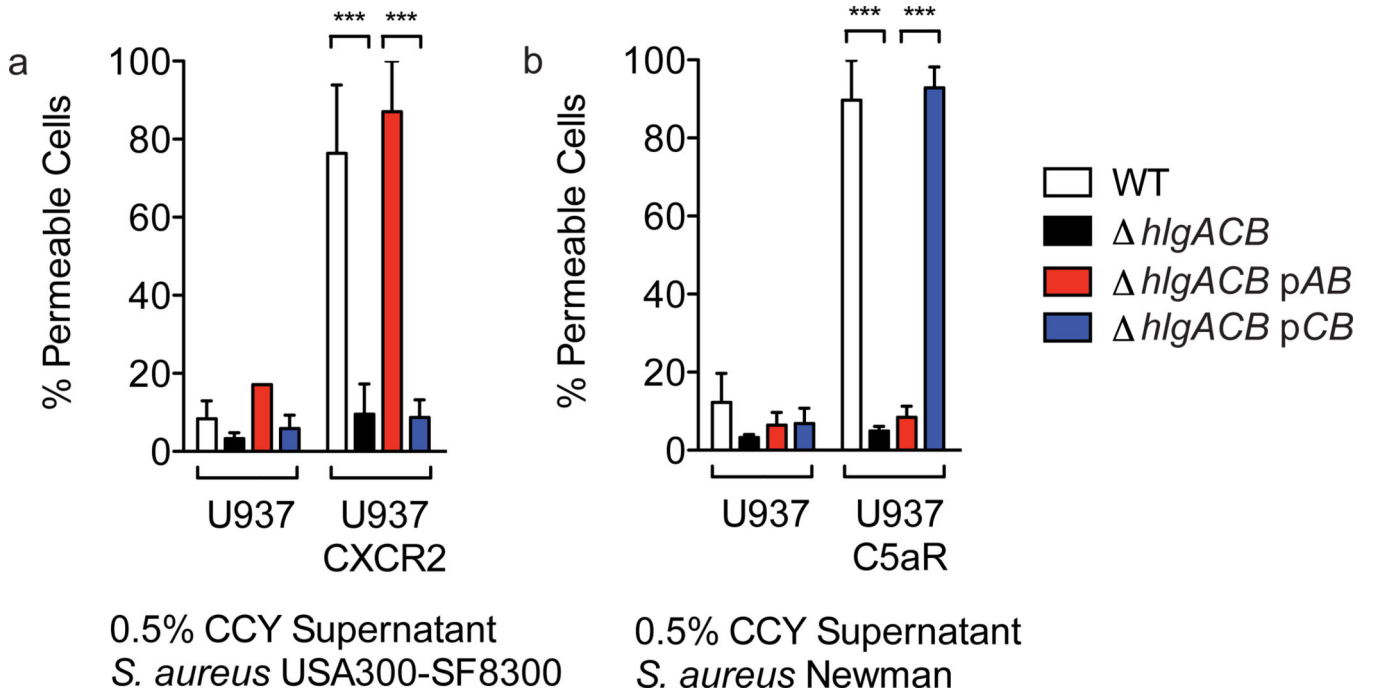
Permeable cells are PI or DAPI positive. Bars indicate SD, with  $n = 3-5$ . Statistical significance is displayed as  $***p < 0.005$  using Student's t-test. See also Supplementary Fig. 1.

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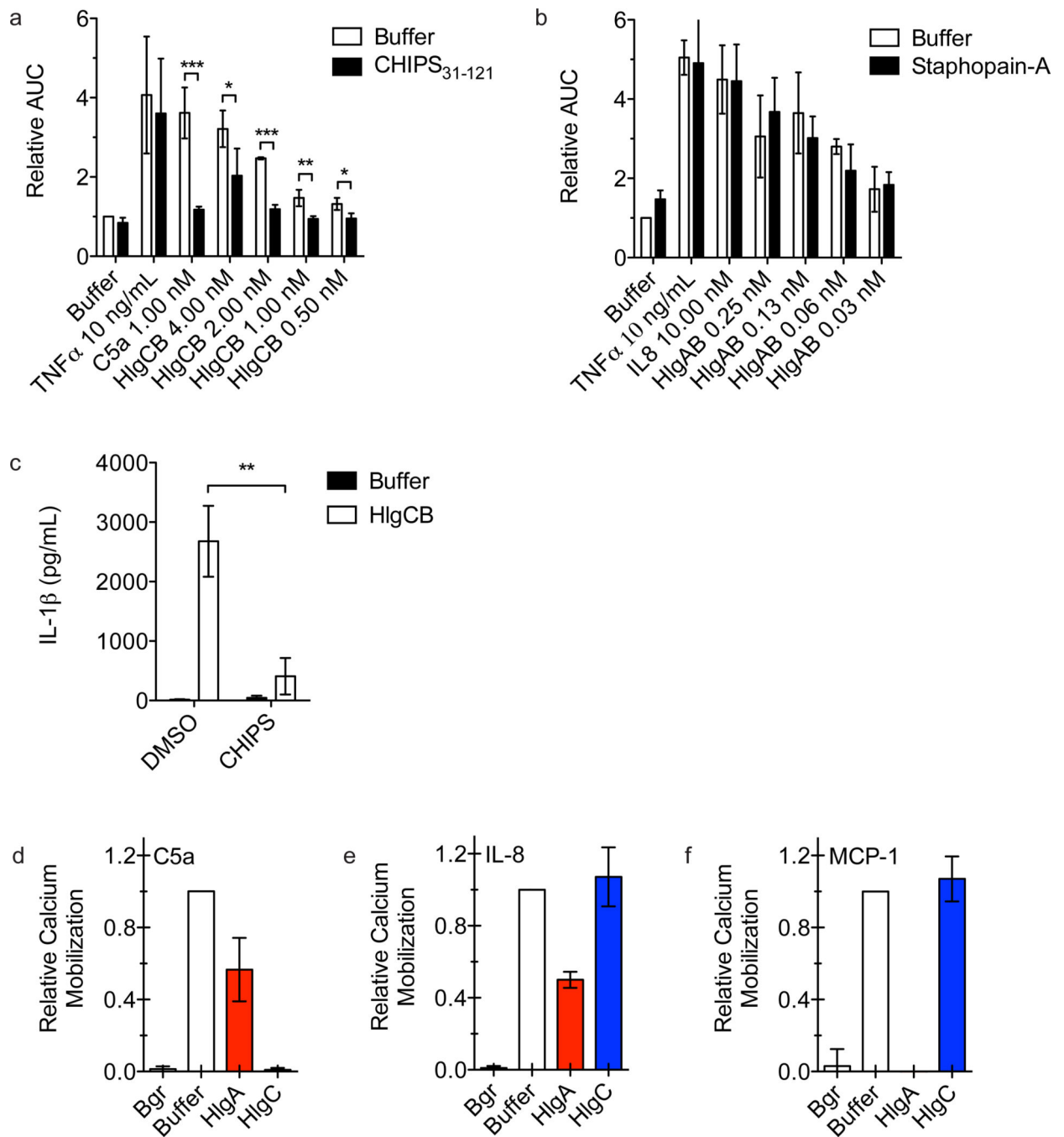
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**Figure 2. *S. aureus* secreted HlgAB and HlgCB lyse cells in a receptor-specific manner**  
 Pore formation in transfected U937 cells was assessed following 30 min exposure to 0.5% bacterial culture supernatant (16 hr culture in CCY medium). (a) HlgAB in supernatant of *S. aureus* strain USA300 clone SF830 induces CXCR2-specific lysis. (b) HlgCB in supernatant of *S. aureus* strain Newman induces C5aR-specific lysis. pAB: *hlgACB* mutant complemented with a plasmid encoding *hlgAB*. pCB: *hlgACB* mutant complemented with a plasmid encoding *hlgCB*  
 Permeable cells are PI or DAPI positive. Bars indicate SD, with n = 3–5. Statistical significance is displayed as \*\*\*p<0.005 using Student’s t-test.



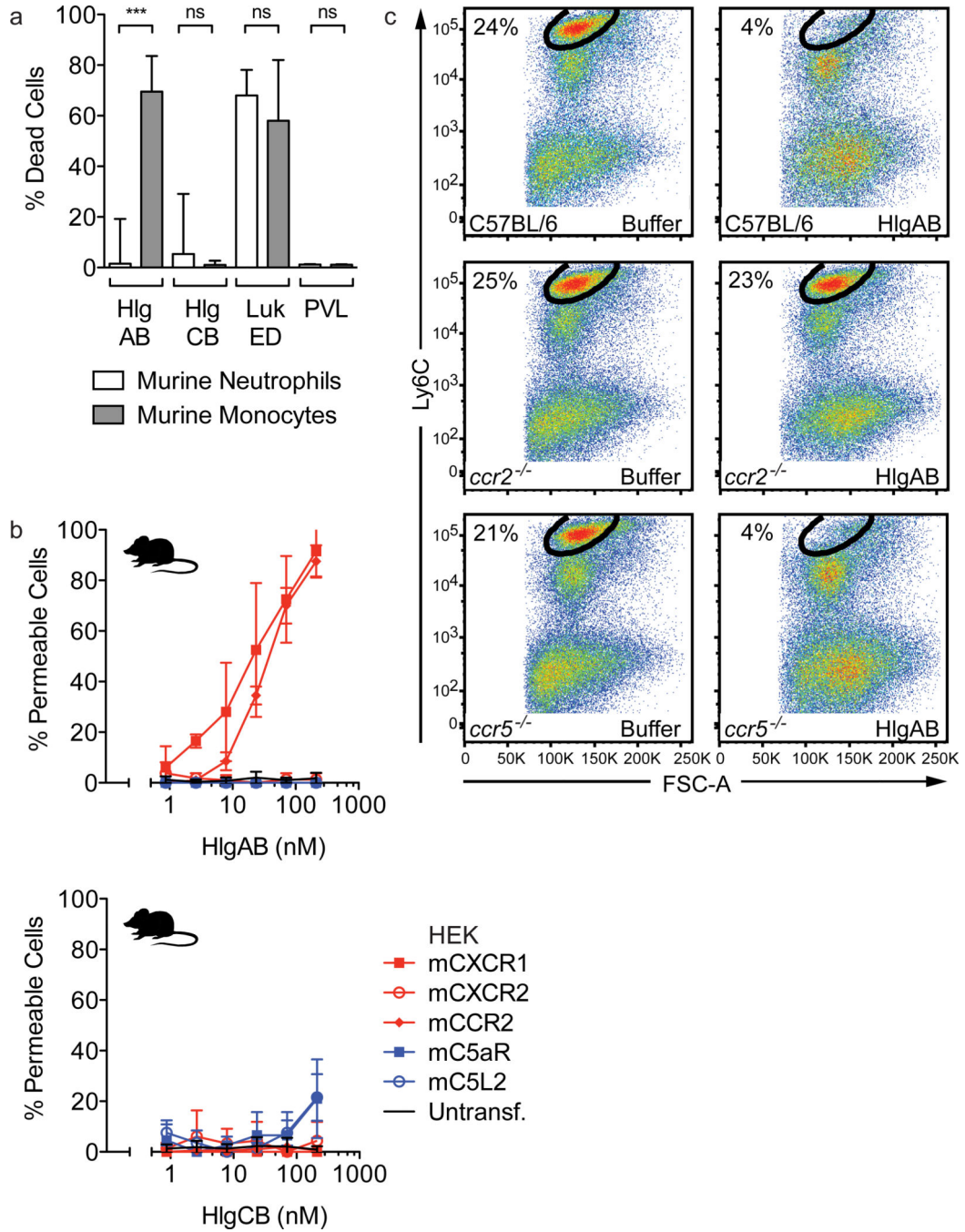
**Figure 3. HlgAB and HlgCB induce pro-inflammatory responses, and single components HlgA and HlgC are immunomodulatory molecules**

(a, b) Oxidative burst of human neutrophils induced by 1  $\mu$ M fMLP after priming with 10 ng mL<sup>-1</sup> TNF $\alpha$ , 1 nM C5a, 10 nM IL-8, and (a) 0.50 to 4.00 nM HlgCB or (b) 0.03 to 0.25 nM HlgAB. Cells were pretreated with (a) 700 nM of the specific C5aR antagonist CHIPS<sub>31-121</sub> or (b) 500 nM of the CXCR2-specific proteolytic enzyme Staphopain-A. Oxidative burst, detected by luminescence, is expressed as relative area under the curve (AUC). (c) IL-1 $\beta$  production in the supernatant of primary human monocyte-derived macrophages intoxicated with 20.00 nM HlgCB. Cells were pretreated with 700 nM CHIPS or DMSO. (d – f)

Calcium mobilization with (d) 0.1 nM C5a, (e) 0.5 nM IL-8 in human neutrophils and (f) 1.0 nM MCP-1 in human monocytes inhibited by 94.0 nM HIgA or HIgC.

Bars indicate SD, with  $n = 3-4$ . Statistical significance is displayed as \*\* $p < 0.01$  and \*\*\* $p < 0.005$  using Student's t-test.





**Figure 4. Murine phagocytes display selective sensitivity and resistance to HlgAB and HlgCB** (a) Pore formation in murine bone marrow-derived neutrophils (CD11b<sup>+</sup>, Ly6G<sup>+</sup>) and monocytes (CD11b<sup>+</sup>, Ly6G<sup>-</sup>, Ly6C<sup>high</sup>) following incubation with leukocidins (190 nM) identifies murine monocytes as the sole murine phagocytes susceptible to  $\gamma$ -Hemolysin. Dead cells are fixable viability dye positive. (b) Pore formation by HlgAB and HlgCB of HEK cells transfected with plasmids encoding murine chemokine receptors confirms susceptibility and resistance of primary murine phagocytes. Permeable cells are PI or DAPI positive. (c) Depletion of murine bone marrow-derived monocytes (CD11b<sup>+</sup>, Ly6G<sup>-</sup>,

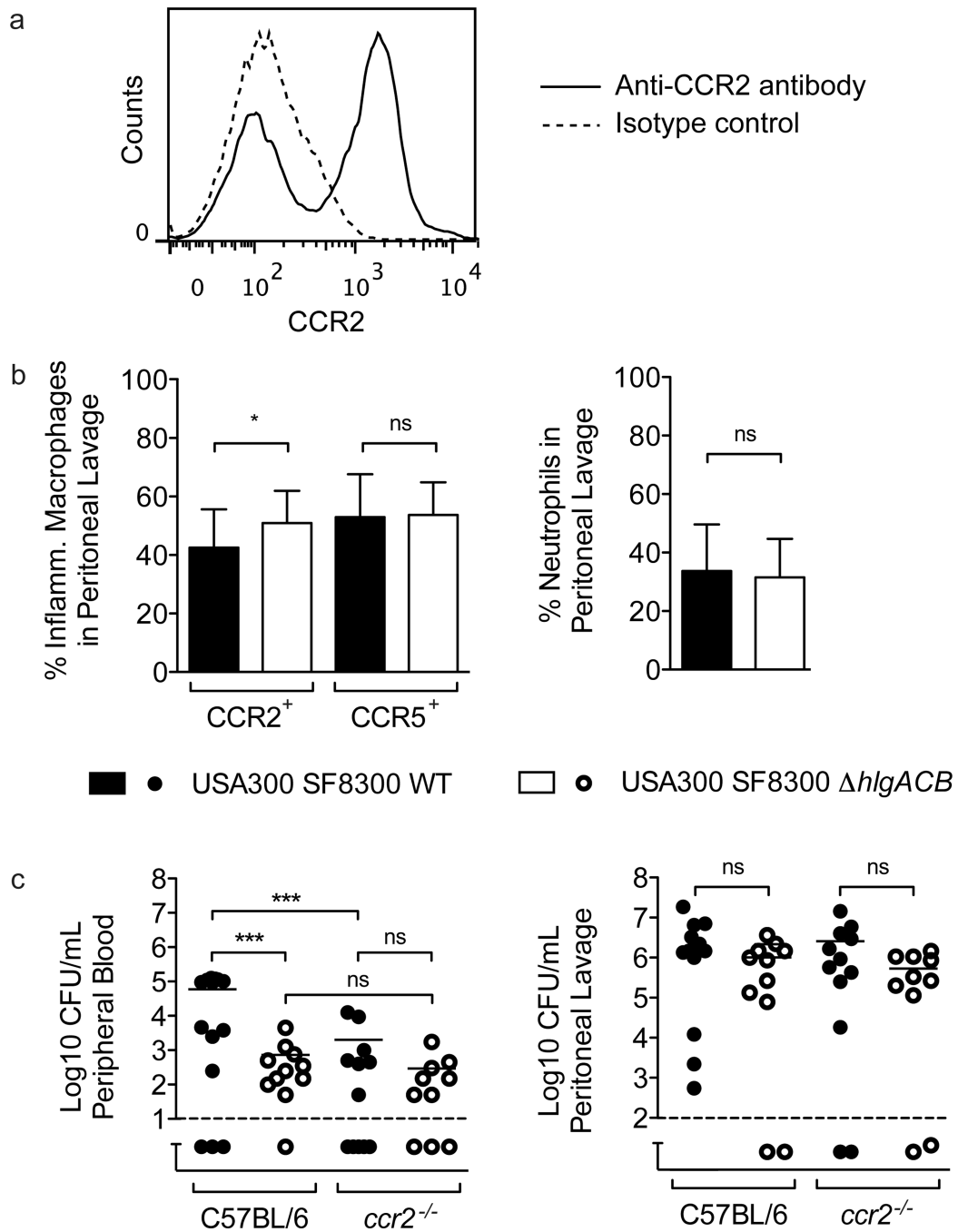
Ly6C<sup>high</sup>) by HlgAB (12,5 nM) is CCR2 dependent as shown as concatenates of 3 individual mice in each group of C75BL/6 wild type, *ccr2*<sup>-/-</sup> and *ccr5*<sup>-/-</sup> mice. Bars indicate SD, with n = 3. Statistical significance is displayed as \*\*\*p<0.005 using Student's t-test. See also Supplementary Fig. 4.

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**Figure 5. HlgAB targeting of CCR2 contributes to *S. aureus* pathogenesis**

(a) Murine peritoneal inflammatory macrophages (CD11b<sup>+</sup>, Ly6G<sup>-</sup>, F4/80<sup>+</sup>) elicited by heat-inactivated *S. aureus* express CCR2. Concatenates are shown, with n = 2–3. (b) Mice intraperitoneally infected with wild type *S. aureus* strain USA300 clone SF8300 show relatively less CCR2<sup>+</sup> but equal CCR5<sup>+</sup> peritoneal inflammatory macrophages compared to *hlgACB* bacteria infected mice at 24 hr post infection. Mice infected with wild type or *hlgACB* bacteria have similar proportions of neutrophils (CD11b<sup>+</sup>, Ly6G<sup>+</sup>) in the peritoneal cavity. (c) C57BL/6 wild type mice intraperitoneally infected with *hlgACB*

bacteria show lower amounts of bacteria in the peripheral blood at 24 hr post infection than mice infected with wild type bacteria. *ccr2*<sup>-/-</sup> mice infected with wild type bacteria show lower amounts of bacteria in the peripheral blood at 24 hr post infection than C57BL/6 wild type mice, independently of *hlgACB*. All mice show similar amounts of bacteria in the peritoneal lavage fluid.

Bars express mean with SD, with n = 10–15 per group of mice distributed over at least two independent experiments. Dashed lines indicate detection thresholds. Statistical significance is displayed as \*p<0.05 and \*\*\*p<0.005 using Student's t-test or two-way ANOVA with Bonferroni posttest correction for multiple comparison where appropriate. See also Supplementary Fig. 5.

**Table 1**

Apparent dissociation constants ( $K_D$ ) of HlgA and HlgC for chemokine receptors as determined by Surface Plasmon Resonance analyses.

Protein	Receptor	Affinities (nM)	
		$K_D$	SD
HlgA	CXCR1	5.69	± 1.94
	CXCR2	27.20	± 5.54
	CCR2	3.51	± 0.29
	C5aR	NI	
HlgC	CXCR1	NI	
	CXCR2	NI	
	CCR2	NI	
	C5aR	5.64	± 1.53

Results of three experimental replicates are shown. No interaction observed (NI) indicates that no binding was observed within the limits of detection. Average capture levels are as follows: CXCR1 683 RU ± 67; CXCR2 696 RU ± 58; CCR2 1056 RU ± 136; C5aR 713 RU ± 88. Average  $R_{max}$  (maximum response units at saturation) for interactions between HlgA and chemokine receptors are as follows: CXCR1 15.1 RU ± 1.9; CXCR2 20.5 ± 3.3; CCR2 21.4 RU ± 1.4. Average  $R_{max}$  for interaction between HlgC and C5aR is 16.3 RU ± 0.7.

**Table 2**

HlgAB and HlgCB are major secreted *S. aureus* leukocidins.

		Clinical isolates										
		USA300 LAC	Y0747	Y1092	Y1163	Y1238	Y1425	Y1694	Y1691	Y1731	Y2068	
		34	CRI	Phlebitis	Shunt	CRI	Arthritis	Endocarditis	Arthritis	CRI	Pneumonia	
Infection focus / Reference	R	S	S	S	S	S	S	S	S	S	S	
Methicillin susceptibility												
Typing	MLST	8	47	59	8	7	45	81	72	59	45	
	CC	5	45	49	5	7	45	5	5	59	45	
Gene content	<i>hlgA</i>	+	+	+	+	+	+	+	+	+	+	
	<i>hlgC</i>	+	+	+	+	+	+	+	+	+	+	
	<i>lukE</i>	+	-	-	+	+	-	+	+	-	-	
	<i>lukS-PV</i>	+	-	-	-	-	-	-	-	-	-	
Expression	<i>hlgA</i>	5.7	6.2	5.9	5.4	5.2	6.1	5.9	5.7	5.4	6.0	
	<i>hlgC</i>	6.0	6.9	7.1	5.4	6.8	6.9	6.8	6.5	6.2	6.5	
	<i>lukE</i>	5.1	n/d	n/d	4.3	7.0	n/d	7.0	5.3	n/d	n/d	
	<i>lukS-PV</i>	7.5	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	
	HEK C5aR	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Pore forming capacity	HEK CXCR1	+	+++	++	+	++	++	+++	+	+	++	
	HEK CCR2	++	++	+++	++	++	++	+++	+	++	+++	
	HEK CCR5	-	-	-	-	-	-	-	-	-	-	
	Neutrophils	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
	USA300 LAC	Y0747	Y1092	Y1163	Y1238	Y1425	Y1694	Y1691	Y1731	Y2068		

A panel of 9 representative European clinical *S. aureus* strains isolated from blood cultures, and strain USA300 clone LAC were tested for genomic content, leukocidin expression, and functional pore forming capacity. Isolates were typed by multi-locus sequence typing (MLST) and grouped in clonal complexes (CC). Genetic content was investigated using gene specific PCRs. Expression of *S.* component mRNA was quantified by qRT-PCR. For expression, mean relative fold change in transcript abundances calibrated to genomic DNA and normalized for *16S* as internal control at early stationary phase in CCY media (Supplementary Fig. 2) are shown. Data obtained from BHI based cultures show similar results (Supplementary Fig. 2). Pore formation of overnight bacterial culture supernatant was tested on stably transfected HEK cells and human neutrophils. For pore forming capacity, quantitative data from overnight CCY based cultures (Supplementary Fig. 2) were converted into qualitative symbols:



+++ indicates >50% pore formation by 1% culture supernatant in 30 min;

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indicates >50% pore formation by 1% culture supernatant in 60 min;



indicates >50% pore formation by 10% culture supernatant in 30 min;



indicates <50% pore formation by 10% culture supernatant in 60 min.

Results of three experimental replicates are shown. CRT: catheter related infection. R/S: Methicillin resistant/susceptible. +/-: Presence or absence of specific target in genomic DNA. n/d: Expression not determined as strain does not contain specific target. See also Supplementary Fig. 2.