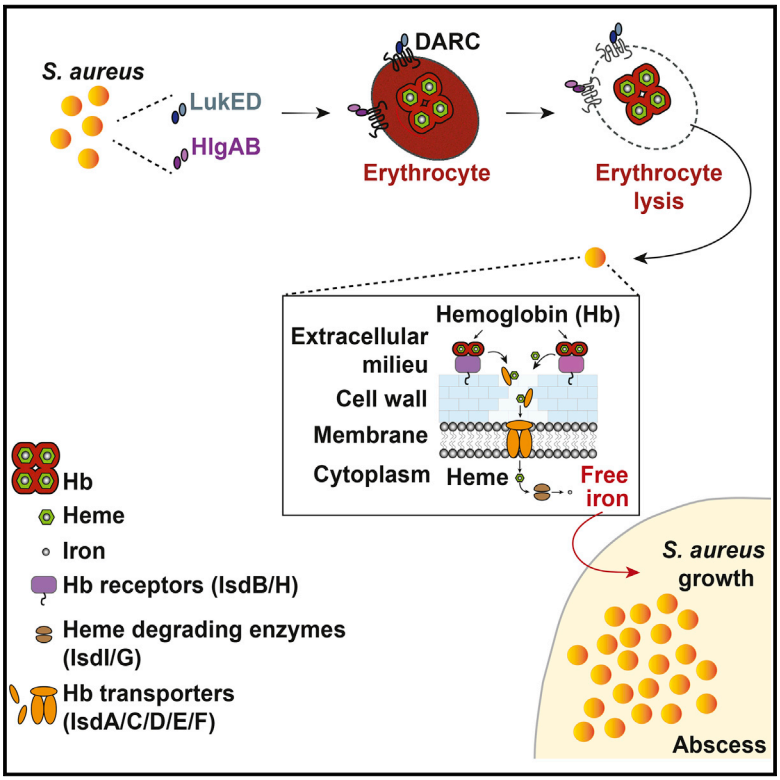
Short Article

Cell Host & Microbe

Staphylococcus aureus Targets the Duffy Antigen Receptor for Chemokines (DARC) to Lyse Erythrocytes

Graphical Abstract



Highlights

- The *S. aureus* leukocidins HlgAB and LukED target DARC to lyse erythrocytes
- HlgAB and LukED differentially interact with DARC
- DARC polymorphisms explain interindividual differences in hemolysis susceptibility
- DARC targeting by HlgAB and LukED contributes to *S. aureus* growth

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In Brief

Several *Staphylococcus aureus* bi-component leukocidins lyse erythrocytes, which is thought to provide iron for bacterial growth. Spaan and Reyes-Robles et al. demonstrate that the Duffy antigen receptor for chemokines (DARC) is the erythroid receptor for leukocidins and that targeting DARC promotes *S. aureus* growth in a hemoglobin acquisition-dependent manner.

Spaan et al., 2015, Cell Host & Microbe *18*, 363–370
September 9, 2015
http://dx.doi.org/10.1016/j.chom.2015.08.001

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Staphylococcus aureus Targets the Duffy Antigen Receptor for Chemokines (DARC) to Lyse Erythrocytes

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http://dx.doi.org/10.1016/j.chom.2015.08.001

SUMMARY

In order for *Staphylococcus aureus* to thrive inside the mammalian host, the bacterium has to overcome iron scarcity. *S. aureus* is thought to produce toxins that lyse erythrocytes, releasing hemoglobin, the most abundant iron source in mammals. Here we identify the Duffy antigen receptor for chemokines (DARC) as the receptor for the *S. aureus* hemolytic leukocidins LukED and HlgAB. By assessing human erythrocytes with *DARC* polymorphisms, we determined that HlgAB- and LukED-mediated lysis directly relates to DARC expression. DARC is required for *S. aureus*-mediated lysis of human erythrocytes, and DARC overexpression is sufficient to render cells susceptible to toxin-mediated lysis. HlgA and LukE bind directly to DARC through different regions, and by targeting DARC, HlgAB and LukED support *S. aureus* growth in a hemoglobin-acquisition-dependent manner. These findings elucidate how *S. aureus* targets and lyses erythrocytes to release one of the scarcest nutrients within the mammalian host.

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is an important human pathogen that plagues patients in hospitals worldwide, especially since the emergence of antibiotic resistance. *S. aureus* infections range from minor skin and soft tissue infections to more invasive and life-threating infections like sepsis, endocarditis, osteomyelitis, and pneumonia (Chambers and Deleo, 2009). The pathogenesis of this bacterium is multifactorial and is exacerbated by the combination of resistance to many antibiotics and the production of an arsenal of virulence factors (DeLeo et al., 2009).

To prevail in the harsh environments encountered in the bloodstream and deeper body tissues of the human host, pathogenic organisms must cope with the onslaught carried by the host innate immune system (Spaan et al., 2013b). *S. aureus* addresses this by producing a large number of immune-modulatory factors, including potent pore-forming toxins, that tamper with innate immunity (Spaan et al., 2013b; Vandenesch et al., 2012). Among these toxins, a single *S. aureus* clone associated with human infections can produce up to five bi-component leukocidins: LukSF-PV (or PVL), HlgAB and HlgCB (also known as gamma-toxins), LukED, and LukAB (also known as LukGH) (Alonzo and Torres, 2014), which target and eliminate specific innate and adaptive immune cell populations, contributing to *S. aureus* pathobiology.

Invading pathogens also have to overcome the barrier posed by nutrient limitation (Waldron and Robinson, 2009). As with many pathogens (Schaible and Kaufmann, 2004), iron is essential for proliferation and virulence of *S. aureus* (Cassat and Skaar, 2013). In the host, however, free iron is scarce, an innate immune defense strategy of the host known as "nutritional immunity" (Andrews et al., 2003; Schaible and Kaufmann, 2004; Weinberg, 1975). The main reservoir of iron in the host lies within erythrocytes, where iron is sequestered and bound to heme within hemoglobin. Heme iron is *S. aureus*'s preferred iron source during infection (Skaar et al., 2004). Although hemoglobin usage by *S. aureus* has been extensively investigated, little is known about the actual release of hemoglobin from erythrocytes. While it is presumed that *S. aureus* releases hemoglobin from erythrocytes by the action of hemolytic toxins, experimental data supporting this

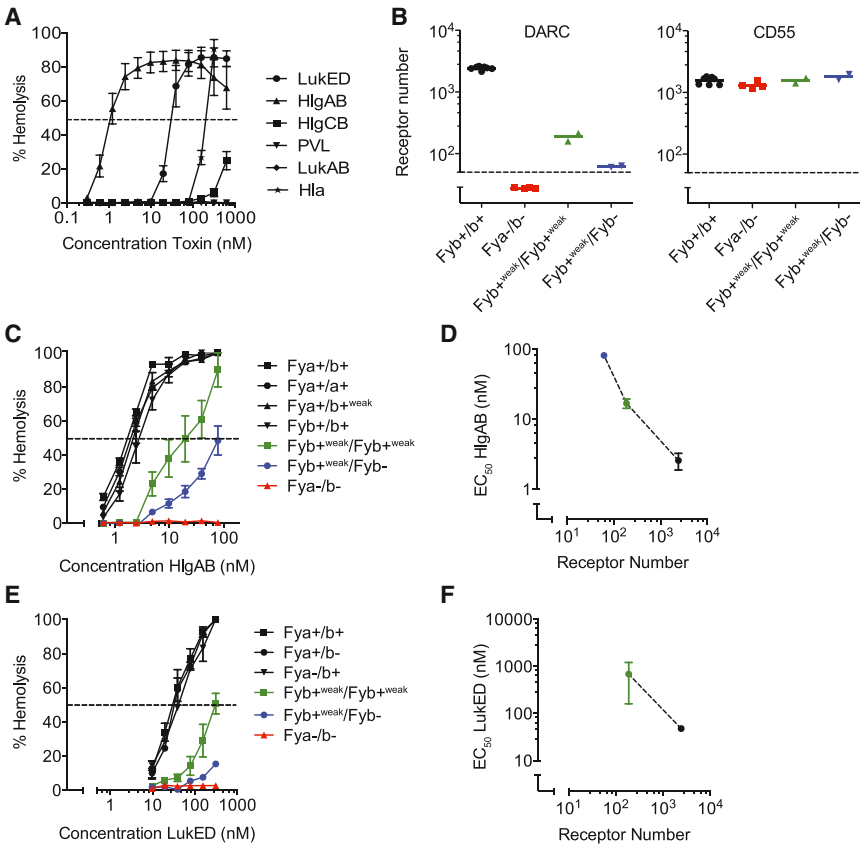

Figure 1. Hemolytic Activity of HlgAB and LukED Depends on DARC

(A) Susceptibility of human erythrocytes to *S. aureus* β-barrel pore-forming toxins. The dashed line indicates 50% hemolysis. $n = 6 \pm$ SEM.

(B) Levels of DARC and CD55 on erythrocytes of donors with different Fy phenotypes. The dashed line indicates the detection threshold. $n = 2–7 \pm$ SEM.

(C) Susceptibility of human erythrocytes with different Fy phenotypes to HlgAB. The dashed line indicates 50% hemolysis. $n = 2–7 \pm$ SEM.

(D) Correlation of half-maximal effective concentrations (EC_{50}) of HlgAB with the total number of receptors expressed on the erythrocyte surface. $n = 2–7 \pm$ SEM.

(E) Susceptibility of human erythrocytes with different Fy phenotypes to LukED. The dashed line indicates 50% hemolysis. $n = 2–7 \pm$ SEM.

(F) Correlation of half-maximal effective concentrations (EC_{50}) of LukED with the total number of receptors expressed on the erythrocyte surface. For Fyb+^{weak}/Fyb- donors, EC_{50} could not be calculated. $n = 2–7 \pm$ SEM.

notion are limited (Bernheimer et al., 1968; Skaar and Schneewind, 2004).

Staphylococcal toxins with hemolytic activity include α-toxin, β-hemolysin, and phenol-soluble modulins (PSMs). Although a potent hemolysin on mouse, rabbit, and sheep erythrocytes, α-toxin has limited hemolytic activity toward human erythrocytes (Hildebrand et al., 1991) due to the lack of its receptor, ADAM10 (Wilke and Bubeck Wardenburg, 2010). β-hemolysin (encoded by the *hlb* gene) is an enzyme with sphingomyelinase activity. Erythrocyte lysis by β-hemolysin is observed only after heat-cold shock incubation (Bernheimer et al., 1974), and in 90% of *S. aureus* isolates, the *hlb* gene is inactivated by integration of β-hemolysin-converting bacteriophages (van Wamel et al., 2006). PSMs, including δ-toxin, are small amphipathic peptides that perforate lipid bilayer membranes in a receptor-independent manner (Wang et al., 2007). Cytotoxic activity of PSMs is neutralized by serum lipoproteins (Surewaard et al., 2012). As such, the contribution of α-toxin, β-hemolysin, and PSMs to the release of iron from human erythrocytes seems unlikely.

The bi-component pore-forming leukocidins are considered a pathogenic strategy to evade the cellular host immune response (Alonzo and Torres, 2014; Spaan et al., 2013b). The respective immune cell receptor counterparts of these leukocidins have been identified (Alonzo et al., 2013; DuMont et al., 2013; Reyes-Robles et al., 2013; Spaan et al., 2013a, 2014). Interestingly, several of the leukocidins also exhibit hemolytic activity (Vandenesch et al., 2012). Despite this, the hemolytic activity of the toxins remains unexplained by the reported leukocyte receptors.

Here, we identify the Duffy antigen receptor for chemokines (DARC) as the erythroid receptor for both HlgAB and LukED, the main hemolytic leukocidins in *S. aureus*. Using DARC-positive and -negative erythrocytes, we show that hemolysis induced by *S. aureus* is strictly mediated by DARC and these leukocidins and that leukocidin-dependent hemolysis favors *S. aureus* growth.

RESULTS

Hemolytic Activity of HlgAB and LukED Depends on Erythroid Expression of DARC

Of the β-barrel pore-forming toxins produced by *S. aureus*, HlgAB and LukED are the most potent hemolytic leukocidins against human erythrocytes (Figure 1A). In contrast, HlgCB and α-toxin induce only limited hemolysis, while PVL and LukAB are inactive toward human erythrocytes (Figure 1A). The divergent activity of HlgAB and HlgCB, which share HlgB, indicates that HlgA drives erythroid specificity. The restricted hemolytic activity of the different but closely related bi-component toxins suggests targeting of specific cellular receptors.

While attempting to elucidate the molecular mechanisms of hemolytic activity induced by these toxins, we observed differential susceptibility of human donors to the toxins, which was reminiscent of the susceptibility of erythrocytes to the malarial parasites *Plasmodium vivax* and *Plasmodium knowlesi*, which target DARC for entry into erythrocytes (Miller et al., 1976; Miller et al., 1975). *P. vivax* uses its surface molecule *P. vivax* Duffy Binding Protein (PvDBP) to dock to the receptor (Choe et al., 2005; Tournamille et al., 2005). DARC is an atypical chemokine receptor and the antigen of the Duffy (Fy) blood group system (Tournamille

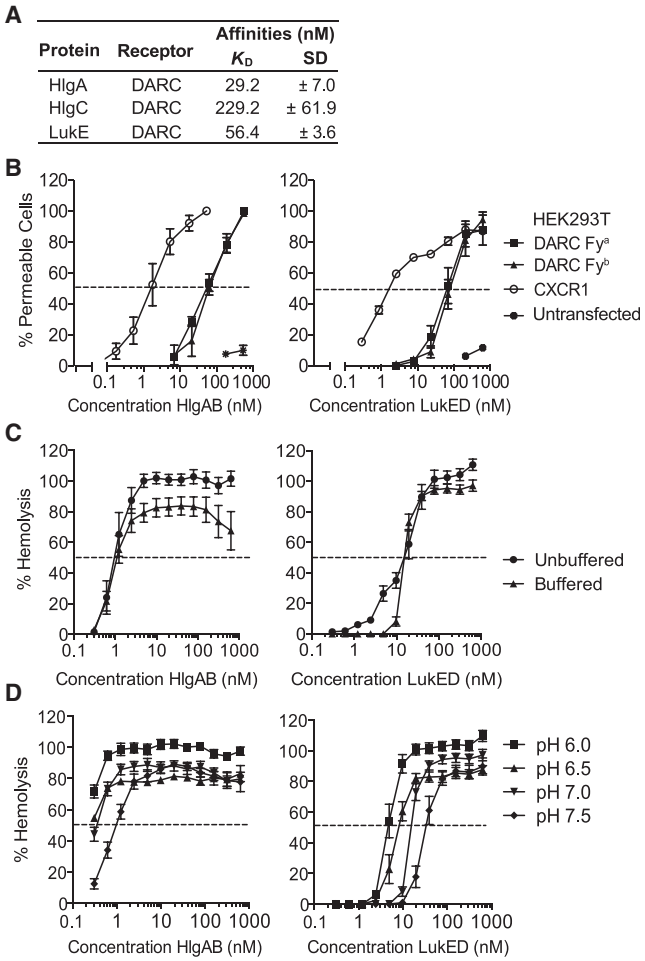

Figure 2. HlgAB and LukED Directly Target DARC

(A) Apparent dissociation constants (K_D) of HlgA, HlgC, and LukE for purified DARC as determined by SPR.

(B) Pore formation following HlgAB or LukED treatment in HEK293T cells transfected with plasmids encoding DARC Fy^a, DARC Fy^b, or CXCR1. The dashed line indicates 50% pore formation. n = 3–5 ± SEM.

(C) Hemolytic activity of HlgAB and LukED on human erythrocytes incubated in unbuffered or buffered media. The dashed line indicates 50% hemolysis. Bars indicate SEM, with n = 3 ± SEM.

(D) Hemolytic activity of HlgAB and LukED on human erythrocytes under different pH conditions. The dashed line indicates 50% hemolysis. Bars indicate SEM, with n = 6 ± SEM.

See also Figures S1 and S2.

et al., 1995, 1998; Wasniowska and Hadley, 1994). This system discriminates two antigens (Fy^a and Fy^b), based on a codon 42 polymorphism encoding a glycine in approximately half of human alleles (*FY*A*) and an aspartic acid in the other half (*FY*B*) (Iwamoto et al., 1995). A substantial part of the human population carries a homozygous mutation (FY*A^{ES} or FY*B^{ES} alleles for erythrocyte silent) in the erythroid-specific promoter of the *FY* gene, resulting in a lack of expression of the receptor on erythrocytes (Fya-/b- phenotype) but not in non-erythroid tissues (Tournamille et al., 1995; Zimmerman et al., 1999). This mutation is highly prevalent in individuals of African descent (Tournamille et al., 1995), and lack of erythroid DARC has been suggested to be an evolutionary adaptation to resist malaria in endemic areas (Miller et al., 1976; Miller et al., 1975). Other, yet rare, mutations result in low-level expression of DARC (allele FY*X resulting in Fyb+^{weak} phenotype) (Tournamille et al., 1998).

To directly investigate if hemolysis by HlgAB and LukED is mediated by DARC, we took advantage of erythrocyte samples from genotyped individuals from the French National Blood Transfusion Institute (INTS). In addition to genotyping, we evaluated DARC expression on erythrocyte surfaces (Figure 1B). Indeed, DARC-negative erythrocytes (phenotype Fya−/b− with genotype *FY*B^{ES}/FY*B^{ES}*) were fully resistant to HlgAB and LukED (Figures 1C and 1E). Compared to individuals expressing normal levels of DARC (phenotypes Fya+/b+, Fya+/b+^{weak}, Fya+/a+, and Fyb+/b+ with respective genotypes *FY*A/FY*B*, *FY*A/FY*X*, *FY*A/FY*A*, and *FY*B/FY*B*), individuals expressing intermediate (phenotype Fyb+^{weak}/b+^{weak} with genotype *FY*X/*X*) or very low levels of DARC (phenotype Fyb−/b+^{weak} with genotype *FY*B^{ES}/FY*X*) (Figure 1B) showed intermediate susceptibility to both HlgAB and LukED (Figures 1C and 1E). Half-maximal effective concentration (EC_{50}) of the toxins directly correlated with the total number of receptors on the erythrocyte surface (Figures 1D and 1F). The difference in susceptibility to HlgAB and LukED between donors was specifically related to erythroid expression levels of DARC, as levels of CD55 (also known as DAF) were equal in all donors (Figure 1B). Taken together, these data demonstrate that the hemolytic activity of HlgAB and LukED is dependent on DARC.

DARC Is the Receptor for HlgAB and LukED on Erythrocytes

To determine if HlgAB and LukED directly bind DARC, surface plasmon resonance (SPR) was performed using purified toxins and receptor. HlgA and LukE bound DARC with a K_D of 29.2 nM and 56.4 nM, respectively (Figure 2A). Consistent with the limited hemolytic activity of HlgCB, the K_D of HlgC for DARC was 229.0 nM (Figure 2A). The K_D value of HlgA for DARC is approximately 6-fold higher compared to CXCR1, which serves as a neutrophil receptor for HlgA (Spaan et al., 2014). To investigate if DARC was sufficient to render host cells susceptible to HlgAB and LukED, human embryonic kidney (HEK293T) cells, which are resistant to both HlgAB and LukED (Figure 2B), were transfected with DARC-Fy^a- or DARC-Fy^b-encoding plasmids (Figure S1). Consistent with the obtained affinities, at least a 10-fold higher EC_{50} was observed for both HlgAB and LukED for DARC when compared to CXCR1 (Figure 2B). DARC-Fy^a and -Fy^b conferred equal susceptibility to these toxins (Figure 2B).

Several of the *S. aureus* leukocidins exhibit species specificity, which render them highly active against human cells, but not murine cells (DuMont and Torres, 2014). Thus, we also evaluated if HlgAB and LukED can target murine DARC, which shares amino acid sequence homology of approximately 63% with human DARC (Luo et al., 1997). HEK293T cells were transfected with plasmids encoding either murine or human DARC, and the susceptibility to the toxins was evaluated. In contrast to human DARC, we observed that cells transfected with murine DARC were more susceptible to LukED than to HlgAB (Figures S2A and S2B). Consistent with the transfection data, primary murine

erythrocytes were susceptible to both HlgAB and LukED (Figure S2B).

It has been hypothesized that during skin infections and abscess formation, ischemia and necrosis cause a local acidification of the infected tissue, which leads to the conversion of ferric ions into ferrous iron, the preferred iron source for *S. aureus* (Skaar et al., 2004; Skaar and Schneewind, 2004). We observed that the hemolytic activity of both HlgAB and LukED was enhanced in saline (unbuffered) compared to buffer (Figure 2C). Similar results were observed with murine erythrocytes (Figure S2C). Additional studies showed increased hemolytic activity in low pH conditions (Figure 2D). Collectively, these data indicate that DARC is sufficient to render mammalian cells susceptible to HlgAB and LukED and that the hemolytic activity of these toxins is likely to be influenced by the local environment of infected tissues.

HlgAB and LukED Target Different DARC Regions for Cell Killing

To tease out the interaction of HlgAB and LukED with DARC, we screened HEK293T cells transfected with a collection of plasmids encoding DARC mutants (Tournamille et al., 2003). All receptors were expressed at similar levels on the cell surface of transfected cells (Figure S1). Mutation of N-terminal glycosylation sites (N16A, N27A, or N33A) resulted in moderately reduced susceptibility of cells toward HlgAB or LukED (Figures 3A and 3B). Mutation of the second N-terminal tyrosine (Y41A) but not the first (Y30A) resulted in resistance to both HlgAB and LukED (Figures 3A and 3B), which is similar to what has been observed for PvDBP (Choe et al., 2005; Tournamille et al., 2005). Mutation of a proline in close proximity to the first transmembrane region of the receptor (P50A) reduced susceptibility to both toxins (Figures 3A and 3B). While mutation of two adjacent aspartic acid residues (D58A+D59A) resulted in a partial resistance of cells to HlgAB, mutation of the same residues enhanced susceptibility to LukED (Figures 3A and 3B). Surprisingly, a disulfide bond-disrupting mutation (C51S) strongly affected susceptibility to LukED but not to HlgAB (Figures 3A and 3B). Lastly, mutations in the three extracellular loops (ECLs) differentially affected susceptibility to HlgAB and LukED. Mutation of a glutamic acid in ECL2 (E202A) slightly enhanced susceptibility of cells to HlgAB but reduced cytotoxicity of LukED. The inverse effect was observed with aspartic acid in ECL3 (D283A) (Figures 3A and 3B).

Next, we tested if DARC competition with monoclonal antibodies, chemokines, or PvDBP could interfere with hemolytic activity. Pre-treatment of erythrocytes with the DARC ligand CXCL8 (or IL-8) modestly shifted the EC_{50} for HlgAB (1.14 to 8.58 nM), while it completely antagonized hemolysis induced by LukED irrespective of buffer conditions (Figures 3C and S3A). In contrast to CXCL8, a commercially available monoclonal against DARC (Fy^a) did not confer protection to HlgAB or LukED (Figure S3B), nor did high concentrations of a nanobody targeting the DARC Fy^6-epitope. Similarly, PvDBP did not antagonize cytotoxicity of HlgAB, while it minimally protected against LukED (Figure S3B).

To directly evaluate if CXCL8 inhibits LukED hemolytic activity by blocking toxin-receptor interaction, we determined the effect of CXCL8 on the formation of LukE-DARC and HlgA-DARC complexes using SPR. CXCL8 did not affect HlgA binding to DARC (Figure 3D). In contrast, CXCL8 significantly inhibited LukE binding to DARC when the chemokine was present at 4-fold (1:0.25) or greater (1:0.125 and 1:0.0625) concentration than LukE (Figure 3D). Together, these data demonstrate that DARC targeting by these toxins is dictated by different domains of the receptor.

S. aureus Lyses Erythrocytes in a Leukocidin- and DARC-Dependent Manner

S. aureus presumably releases iron from erythrocytes by secreting various hemolytic toxins, promoting the bacterium's survival in the host (Bernheimer et al., 1968; Skaar and Schneewind, 2004; Torres et al., 2010). To address whether *S. aureus* lyses erythrocytes in a DARC-dependent manner, the CA-MRSA USA300 clone LAC was grown in the presence of DARC-positive or -negative erythrocytes and hemolysis was measured over time. We observed that *S. aureus* induced hemolysis of erythrocytes in a time-, bacterial-density-, and DARC-dependent manner (Figures 4A and S4).

We next defined the contribution of HlgAB and LukED to *S. aureus*-mediated lysis of DARC-positive erythrocytes. Growth of isogenic strains revealed that HlgAB is sufficient for the observed erythrocyte lysis in this ex vivo experiment (Figure 4B), and this was recapitulated with different *S. aureus* strains (Figure S4B). Notably, the lack of a LukED contribution is not surprising due to low production of this toxin in vitro (Alonzo et al., 2012; Gravet et al., 1998).

To directly evaluate if LukED and HlgAB promote bacterial replication as a result of erythrocyte lysis, *S. aureus* was grown in iron-starved medium supplemented with cell-free extracts of erythrocytes treated with saline, LukED, or HlgAB. We observed that HlgAB and LukED were each capable of promoting *S. aureus* growth (Figure 4C). To determine if this growth was dependent of hemoglobin acquisition, we used an isogenic strain lacking the *S. aureus* hemoglobin receptors IsdB and IsdH (Δ*isdBH*) (Pishchany et al., 2014; Torres et al., 2006). In contrast to wild-type (WT) *S. aureus*, the Δ*isdBH* strain was significantly growth impaired in medium supplemented with cell-free extracts from toxin-treated erythrocytes (Figure 4C).

To elucidate the potential role of toxin-mediated erythrocyte targeting in vivo, bacterial burden of mice infected systemically with *S. aureus* WT or isogenic mutants lacking *hlgACB* and *lukED* (Δ*hlg* Δ*lukED*), the gene encoding for α-toxin (*hla*; Δ*hla*), or *isdBH* (Δ*isdBH*) (Figure S4C) were evaluated. Inactivation of *lukED* and *hlgACB* phenocopied the deletion of the hemoglobin receptors, resulting in a 3-log and 4-log reduction in bacterial burden, respectively (Figure 4D). The bacterial burden reduction observed with the Δ*hlg* Δ*lukED* strain was specific, with no significant difference between the bacterial burden of WT and Δ*hla*-infected mice, even though α-toxin exhibits hemolytic activity in vitro toward murine erythrocytes (Figure S2C). Altogether, these data suggest that *S. aureus* targets DARC to lyse erythrocytes in a HlgAB- and LukED-dependent manner to release hemoglobin, promoting bacterial replication.

DISCUSSION

It is presumed that *S. aureus* releases hemoglobin from erythrocytes by secreting hemolytic toxins. Strikingly, however, the contribution of toxins to hemolysis-mediated bacterial growth

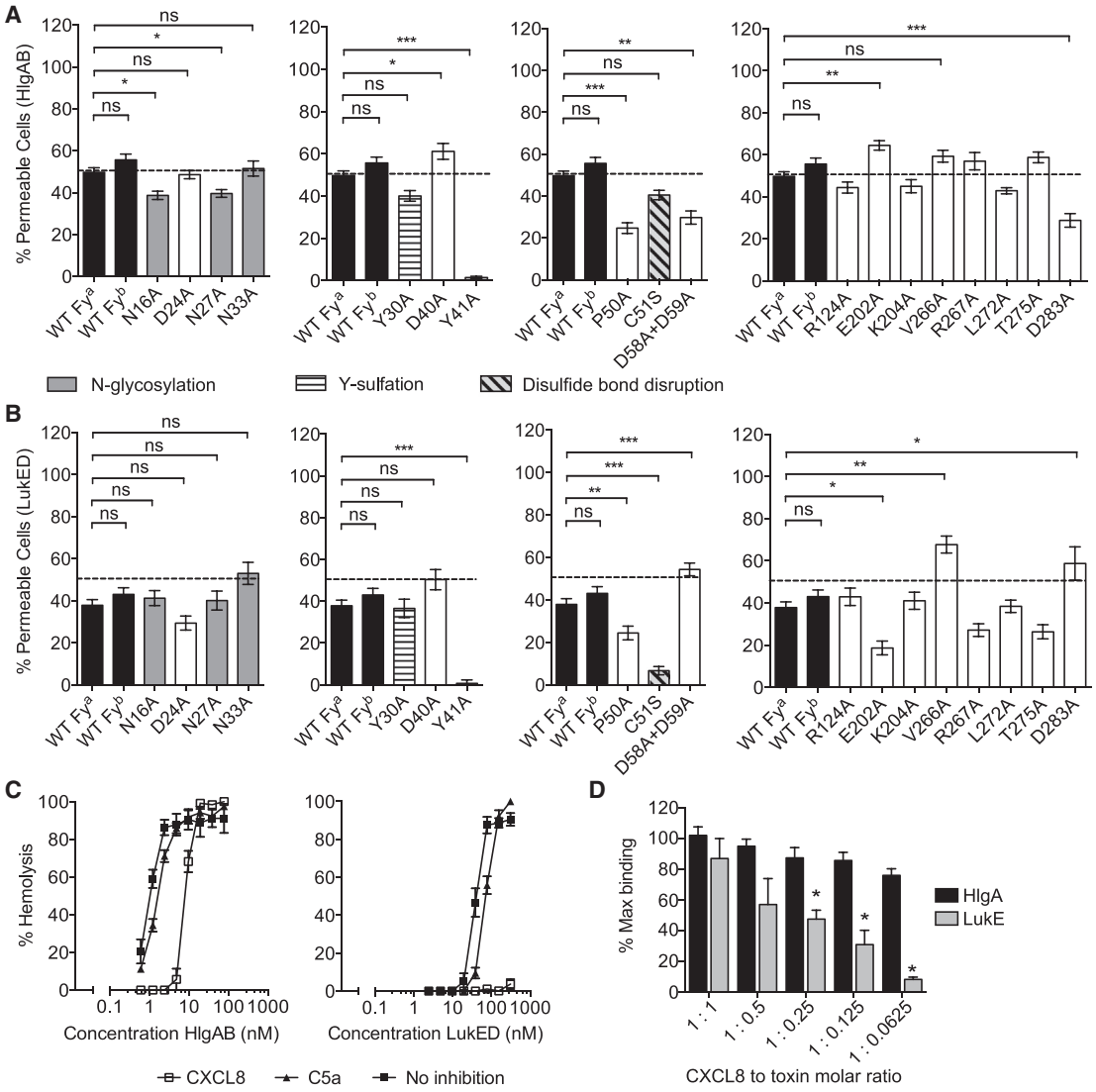

Figure 3. HlgAB and LukED Target Different DARC Regions for Cell Killing

(A) Pore formation following HlgAB treatment (70 nM) in HEK293T cells transfected with plasmids encoding DARC Fy^a, DARC Fy^b, or DARC alanine substitution mutants. The dashed line indicates 50% pore formation. $n \geq 18 \pm$ SEM. Statistical significance is displayed as ns (not significant), *$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$ using one-way ANOVA with Bonferroni post hoc test correction for multiple comparisons.

(B) Pore formation following LukED treatment (70 nM) in HEK293T cells transfected with plasmids encoding DARC Fy^a, DARC Fy^b, or DARC alanine substitution mutants. The dashed line indicates 50% pore formation. $n \geq 18 \pm$ SEM. Statistical significance is displayed as ns (not significant), *$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$ using one-way ANOVA with Bonferroni post hoc test correction for multiple comparisons.

(C) Effect of pretreatment with CXCL8 (10 μg/ml) during hemolysis induced by HlgAB and LukED. $n = 3 \pm$ SEM.

(D) Competition between CXCL8 and toxin subunits at varying molar ratios using SPR. $n = 3 \pm$ SEM. *Indicates significant difference to non-CXCL8-treated response units ($p < 0.01$) using t test.

See also Figure S3.

has never been proven empirically. By identifying DARC as the erythroid receptor for HlgAB and LukED, we show that these staphylococcal bi-component toxins play a central role in human erythrocyte lysis.

Our data show that the hemolytic activity of HlgAB and LukED is in the nanomolar range and is exclusively driven by DARC expression. The affinity of the binding components HlgA and LukE for DARC is lower than their myeloid receptors, CXCR1 and CXCR2 (Reyes-Robles et al., 2013; Spaan et al., 2014). High expression levels of DARC and possibly lack of cellular membrane repair mechanisms make erythrocytes highly susceptible to the toxins. Consistent with this, our data suggest that the actual number of receptors on the cell surface dictates susceptibility to these hemolytic toxins. Using alanine substitution mutants, we identified a tyrosine in the N terminus of DARC that is essential for the interaction with both HlgAB and LukED and has been described as sulfated (Choe et al., 2005). For PVL, sulfation of tyrosines in the N-terminal C5aR is critical

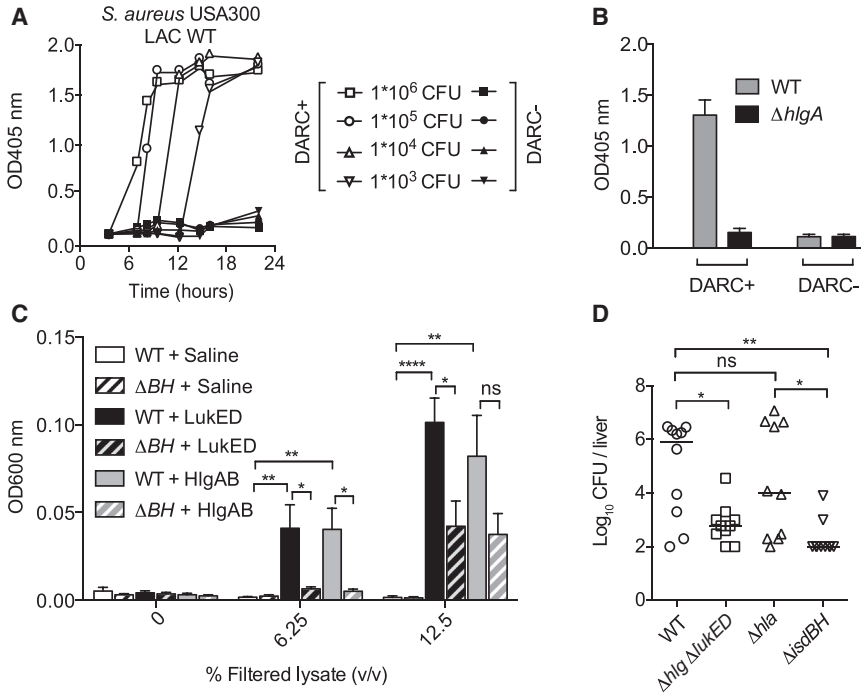

Figure 4. *S. aureus* Lyses Erythrocytes in a HlgAB-, LukED-, and DARC-Dependent Manner to Release Iron and Promote Growth

(A) *S. aureus* USA300 LAC grown in the presence of erythrocytes from donors with or without erythroid expression of DARC and hemolysis measured. Curves depict a representative sample.

(B) Hemolysis induced during overnight growth of *S. aureus* strain USA300 LAC and its *hlgA* mutant (*hlgA::bursa*) strain (infectious dose set at 1 × 10^6 CFU per sample). $n = 3 \pm$ SEM.

(C) Growth after 20 hr of *S. aureus* strains Newman WT or isogenic Δ*isdBH* as a result of erythrocyte lysis by LukED and HlgAB in iron-restricted medium. $n = 9 \pm$ SEM. Statistical significance is displayed as ns (not significant), *p < 0.05, **p < 0.01, and ****p < 0.0001 using one-way ANOVA with Tukey's post hoc test correction for multiple comparisons. Bacterial growth was measured at OD600 nm.

(D) Swiss-Webster female mice ($n = 10$ mice per group) infected systemically with *S. aureus* Newman isogenic strains: WT, Δ*hlg* Δ*lukED*, Δ*hla*, and Δ*isdBH* (~1 × 10^7 colony forming units, CFU). 96 hr post infection, mice were sacrificed and bacterial burden in the liver determined. Lines represent median log CFU. Statistical significance is displayed as ns (not significant), *p < 0.05, **p < 0.01, and ****p < 0.0001 using one-way ANOVA with Tukey's post hoc test correction for multiple comparisons.

See also Figure S4.

for initial binding of LukS-PV (Spaan et al., 2013a). Possibly, sulfated N-terminal tyrosines define a conserved host interaction site for the staphylococcal leukocidins. Otherwise, our data show that HlgAB and LukED interact differentially with DARC. An N-terminal cysteine (C51) identified as involved in the interaction of DARC with LukED is also involved in binding CXCL8 (Tournamille et al., 2003), supporting the notion that this chemokine directly blocks receptor binding by LukE.

The genes encoding HlgAB are present in over 99.5% of human *S. aureus* isolates (Prevost et al., 1995). Strictly following clonal lineage, approximately 80% of *S. aureus* strains carry the genes encoding LukED (McCarthy and Lindsay, 2013). The *S. aureus* strains investigated in this study all contain the genes encoding HlgAB and LukED, thus demonstrating that *S. aureus*-mediated hemolysis requires DARC and these leukocidins.

S. aureus is remarkably well adapted to the human host, thus multiple virulence factors of this bacterium are not compatible with non-human species frequently used during in vivo studies. One such factor is the staphylococcal hemoglobin receptor IsdB, which exhibits low affinity for murine hemoglobin as compared to human hemoglobin (Pishchany et al., 2010). Nevertheless, our in vivo studies revealed a remarkable similarity in the phenotypes of isogenic mutants lacking either the hemoglobin receptors or the hemolytic leukocidins, suggesting that these toxins contribute to nutrient acquisition during infection. However, to unequivocally demonstrate that the attenuated phenotype exhibited by the Δ*hlg* Δ*lukED* strain is due to impaired erythrocyte lysis, additional studies uncoupling the leukocidal and hemolytic activities of HlgAB and LukED are required.

The current epidemic of CA-MRSA in the United States and elsewhere disproportionally affects individuals of African descent with severe and invasive infections (Fridkin et al., 2005). Socio-economic factors and other underlying diseases likely contribute to this predisposition, precluding epidemiological assessment of the contribution of erythroid DARC expression to *S. aureus* infection. However, the resistance of DARC negative erythrocytes to the parasites *P. vivax* and *P. knowlesi*, together with our findings, further support the notion that this gene could undergo positive selection in response to different diseases caused by important human pathogens.

EXPERIMENTAL PROCEDURES

Ethics Statement

DARC blood samples were provided by the Centre National de Référence sur les Groupes Sanguins (CNRGS, Paris). Additional blood samples of consenting, healthy volunteers were obtained in accordance with the Declaration of Helsinki. Approval was obtained from the medical ethics committee of the UMC Utrecht, The Netherlands. Blood was also obtained from de-identified, consenting donors from the New York Blood Center.

All experiments involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of New York University and were performed according to NIH guidelines, the Animal Welfare Act, and US Federal law.

Hemolysis Assays with Recombinant Toxins

Erythrocytes were washed thrice in 0.9% saline, adjusted to 5 × 10^7 cells/ml, and then intoxicated at a final of 2.5 × 10^7 cells/ml per reaction with purified recombinant toxins for 30 min at 37°C + 5% CO_2 in a final volume of 160 μl. Equimolar concentrations of 6xHis-tagged proteins were used. Samples were centrifuged for 10 min at 1,780 × *g*, 4°C, and 100 μl of cell-free lysates were used to measure absorbance (OD405 nm). Hemolysis is expressed as the OD405 nm of cell-free lysates using an EnVision Plate Reader. The hemolysis experiments with recombinant proteins were performed using buffer

containing 30 mM Tris with 100 mM NaCl (pH 7.0) (buffered) or 0.9% saline (unbuffered) as indicated.

S. aureus Burden In Vivo

To evaluate bacterial burden in vivo, 4-week-old Swiss-Webster mice (Harlan) were anesthetized intraperitoneally with Avertin and infected retro-orbitally with 100 μl of Newman isogenic strains WT, Δ*hlg* Δ*lukED*, Δ*hla*, and Δ*isdBH* (1×10^7 CFU). 96 hr post-infection, mice were sacrificed, and the livers harvested, homogenized, and serially diluted.

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 with SEM where appropriate.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2015.08.001.

AUTHOR CONTRIBUTIONS

Conceptualization, A.S., T.R.-R., T.H., and V.J.T.; Methodology, A.S., T.R.-R., T.H., and V.J.T.; Investigation, A.S., T.R.-R., C.B., S.C., K.M.B., P.Y., C.J.C.d.H., and K.v.K; Resources, F.V., M.P.J., C.I.V.K., and Y.C.; Funding Acquisition, M.P.J., J.A.S., F.V., T.H., and V.J.T; Writing, A.S., T.R.-R., T.H., and V.J.T; Supervision, J.A.S., T.H., and V.J.T.

ACKNOWLEDGMENTS

We thank Manoj Duraising (Harvard School of Public Health) for the DARC-expressing plasmid, Olivier Bertrand and Stéphane Grangnard (Inserm U1134) for the nanobody targeting the DARC F6 epitope and PvDBP, Gérard Lina (Inserm U1111) for stimulating discussions, Raïhane Massulaha-Ahmed and Pauline Abrial (CIRI, Lyon) for technical assistance, Thierry Peyrard (CNRGS, Paris) for Fy-genotyped frozen blood samples, Eric Skaar (Vanderbilt University) for the Newman Δ*isdBH* strain, and Kayan Tam (NYU) for purified recombinant α-toxin.

This work was supported in part by grants from the French Agence Nationale de la Recherche (ANR-12-BSV3-0003 to F.V. and T.H.), the Finovi foundation to T.H., the NIH (T32 AI007180 and F31 AI112290 to T.R-R., T32 GM007308 to K.M.B., and R01 AI099394 and R01 AI105129 to V.J.T.), the NHMRC (565526 to M.P.J.), and the Smart Futures Fund Research Partnerships Program (M.P.J.). This work was performed within the framework of the LABEX ECOFECT (ANR-11-LABX-0048) of Université de Lyon, of the program "Investissements d'Avenir" (ANR-11-IDEX-0007) operated by the ANR. V.J.T. is a Burroughs Wellcome Fund Investigator in the Pathogenesis of Infectious Diseases.

Received: April 10, 2015
Revised: July 2, 2015
Accepted: August 3, 2015
Published: August 27, 2015

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