SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Recombinant toxins. Recombinant protein production and purification was done as described previously (Perret et al., 2012; Reyes-Robles et al., 2013). As leukocidins are two-component toxins, equimolar concentrations of polyhistidine-tagged proteins were used.

Bacterial strains. *S. aureus* strains used for this study are listed in Supplementary Table 1.

Hemolysis assays with recombinant toxins. Erythrocytes were washed thrice in 0.9% saline, adjusted to 5 x 10^7 cells.ml^{-1} then intoxicated at a final of 2.5 x 10^7 cells.ml^{-1} per reaction with a dose response of purified recombinant toxins for 30 minutes at 37°C + 5% CO_2 in a final volume of 0.160 ml. Samples were centrifuged for 10 min at 1,780 g, 4°C, and 0.1 ml 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 a buffer containing 30 mM Tris with 100 mM NaCl (pH 7.0) (buffered) or 0.9% saline (unbuffered).

For competition experiments, erythrocytes were pre-incubated for 15 min at 37°C with CXCL8 (Tebu Bio and Gemini Bio-Products) or C5a (Bachem), mouse anti-human DARC (clone 358307, R&D) or isotype control, an anti-DARC nanobody (VHH-CAIII) or anti-HIV p24 nanobody (A4) (Smolarek et al., 2010), the *P. vivax* Duffy Binding Protein (PvDBP) (Tournamille et al., 2005) or the staphylococcal protein CHIPS (de Haas et al., 2004). Following pre-incubation, erythrocytes were exposed to toxins as described above.

Hemolysis of murine erythrocytes. To collect murine erythrocytes, submandibular bleeding was performed using 4mm lancets (Goldenrod) and blood collected on heparin-coated tubes. Hemolysis assays were set up as described above (see Hemolysis assays with recombinant proteins).

Erythrocyte receptor quantification. For quantification of receptor expression levels, human erythrocytes were incubated in a total volume of 50 µL at 2 x 10^7 cells.mL^{-1} on ice with mouse

anti-human monoclonal antibodies for DARC (specificity: anti-Fy^a) (clone 358307, R&D), and CD55 (clone 278803, R&D), followed by FITC-conjugated goat-anti-mouse antibody (Dako). Antibody binding was quantified by calibration to defined antibody binding capacity units using QIFIKIT (Dako) and corrected for isotype controls.

Mammalian cell transfections. HEK293T cells (a human embryonic kidney cell line) were transiently transfected as described (Spaan et al., 2013). Cloning of plasmids encoding human DARC wild type and alanine substitution mutants has been described previously (Tournamille et al., 2003). Expression of receptors on transiently transfected HEK293T cells was verified by flow cytometry using mouse anti-human CXCR1 (clone 42705, R&D), and mouse anti-human DARC (clone 358307, R&D), followed by either PE-conjugated goat-anti-mouse antibody (Dako) or compared to PE-conjugated anti-mouse IgG2a isotype control (clone RMG2a-62, BioLegend) as indicated. To evaluate murine DARC surface levels, PE-conjugated anti-mouse/rat DARC was used (R&D) and compared to normal sheep IgG PE-conjugated isotype control (R&D).

For experiments involving human and murine DARC, HEK293T cells seeded at 3 x 10^6 in 9 ml of 1x RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gemini Bio-Products) and 1% l-glutamine (Gibco) were transiently transfected with GFP-tagged plasmids (OriGene) using Lipofectamine 2000 (Invitrogen) diluted in Opti-MEM media (Gibco) for 6-7 hours at 37°C + 5% CO_2. To evaluate susceptibility of human or murine DARC-transfected cells to LukED or HlgAB, cells were seeded at 5 x 10^4 cells 24 hours post transfection, then intoxicated the following day for 2 hours at 37°C + 5% CO_2 in a final volume of 100 µl. To measure viability of the cells, CellTiter (Promega) was added at a final concentration of 10% and incubated for 2 hours at 37°C + 5% CO_2. To calculate the percentage of dead cells, the absorbance was measured at OD492 nm using an EnVision Plate Reader. Transfection efficiency was also evaluated 48 hours post transfection by GFP positivity compared to empty-vector transfected cells using flow cytometry (BD LSRII with BD FACSDiva software).

Cell permeability assays. For comparison to other chemokine receptors, transiently transfected HEK293T cells were resuspended in a volume of 50-100 µL, then cells were exposed to recombinant proteins and incubated for 30 min at 37°C + 5% CO_2. 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). Experiments were performed with RPMI (Invitrogen) supplemented with 0.05% human serum albumin (Sanquin), with cell concentrations adjusted to 5 x 10^6 cells.mL^{-1}. Analysis of pore formation was corrected for transfection efficiency as described above. Half maximal effective lytic concentrations were calculated using nonlinear regression analyses.

For comparison of DARC alanine substitution mutant receptors, 70% confluent transiently transfected adherent cells were exposed to toxin in the presence of PI and incubated for 150 min at 37°C. Internalization of PI was measured using a monochromator-based microplate reader (TECAN and ClarioStar). Pore formation was defined as the relative fluorescence units related to maximal pore formation of cells transfected with plasmids encoding wild type receptors. Expression of receptors was verified by flow cytometry as described above.

Surface Plasmon Resonance studies. Surface Plasmon resonance (SPR) was run using the Biacore T100 system (GE) as described previously (DuMont and Torres, 2014; Spaan et al., 2014). Briefly, recombinant HlgA, HlgC or LukE were immobilized onto flow cell 2-4 of a series S sensor chip CM5 (GE) using the NHS capture kit and flow cell 1 was run as a blank immobilization. Recombinant Duffy/DARC purified from human HEK293T cells (OriGene) were run at concentrations ranging from 0.04-25 µg.mL^{-1} using single cycle kinetics with at least three experiments performed for each interaction. Contact time for each injection was 60 seconds while final disassociation was measured for 10 minutes. The affinities, on and off rates were calculated using the Biacore T100 evaluation software. The running buffer for all SPR experiments was 1x PBS at pH 7.2.

HlgA and LukE competition assays with CXCL8/IL-8 were performed at a constant CXCL8/IL-8 concentration (1.2 µM) and increasing concentrations of HlgA and LukE (0.5 nM –

1.2 µM). Response units at the concentrations were taken from three independent experiments and total binding was compared to normal binding for the tested concentration in the absence of CXCL8.

Hemolysis assay during growth of *S. aureus.* For hemolysis experiments during growth of *S. aureus*, bacterial cultures were prepared as described elsewhere (Torres et al., 2006). Briefly, *S. aureus* strains were grown overnight under iron-restricted conditions in RPMI supplemented with 1% Casamino Acids (CAS) and 200 µM 2,2'-dipyridyl. Bacterial cultures were subsequently diluted, grown to mid-exponential phase, and washed twice in NRPMI+ (Chelex-treated RPMI-CAS containing 25 µM $ZnCl_2$, 25 µM $MnCl_2$, 1 mM $MgCl_2$, 100 µM $CaCl_2$) supplemented with 500 µM 2,2'-dipyridyl and 10% Chelex-treated fetal calf serum (FCS). Bacterial concentrations were normalized by flow cytometric counting of viable bacteria using an Acccuri C6 system. Freshly obtained peripheral blood was washed twice in NRPMI+ supplemented with 500 µM 2,2'-dipyridyl and 10% Chelex-treated FCS, and erythrocyte concentrations were normalized. Bacteria (2×10^7 CFU.mL^{-1}) and erythrocytes (2×10^8 cells.mL^{-1}) were mixed 1:1 in a total volume of 100 µL and incubated for 16 hours at 37°C + 5% CO_2. After centrifugation, absorbance of the supernatant was measured at 405 nm.

For experiments measuring *S. aureus* growth in the presence of leukocidin-treated erythrocyte lysates, fresh human erythrocytes were adjusted to ~1.5×10^6 cells.ml^{-1} with 0.9% saline (Baxter), incubated in the presence of a LD_{90} of LukED or HlgAB (2-4 µg.ml^{-1}) for 30 minutes at 37°C + 5% CO_2 in a total volume of 1 mL, centrifuged for 5 minutes at 1500 RPM and 4°C, and the lysates filtered using a 0.2 µm filter. Lysates were added at a final concentration of 10% into 1x Roswell Park Memorial Institute (RPMI) 1640 without phenol red (Mediatech Cellgro) chelated using 2 µM ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid) (EDDHA) on polypropylene, round bottom tubes (Falcon). A single colony of *S. aureus* Newman wild type or an isogenic Δ*isdBH* strain was inoculated into 120 µl of the chelated media, then subcultured 1:400 into chelated media containing the lysates as described elsewhere (Pishchany et al., 2014). Growth curves were performed at 37°C with 180 RPM shaking. Absorbance at 600nm was used to evaluate *S. aureus* growth using an EnVision Plate Reader.

Immunoblot analyses of *S. aureus* Newman strains. To evaluate toxin production by *S. aureus* Newman isogenic strains WT, Δ*hlg* Δ*lukED*, Δ*hla*, and Δ*isdBH*, overnight cultures were done in Tryptic Soy Broth (TSB) at 37°C, 180 RPM, then subcultured 1:100 into TSB and grown for 5 hours at 37°C, 180 RPM. The cultures were centrifuged for 15 min at 4000 RPM, 4°C, and the supernatants filter-sterilized with a 0.22 µm filter, followed by a 30 minute incubation on ice. To precipitate proteins, 100% trichloroacetic acid (TCA) were added to 1.3 ml of filtered culture supernatants (final of 10% v/v) and incubated overnight at 4°C. The TCA-treated samples were centrifuged for 15 minutes at 4000 RPM, 4°C and the resulting protein pellets washed in 100% ethanol for 1 hour at 4°C, followed by a 15-minute centrifugation at 4000 RPM, 4°C and the final pellet allowed to dry overnight. The dried pellets were resuspended in 30 µl of 1x TCA-SDS sample buffer and boiled for 10 minutes. To perform immunoblot analyses, 5 µl were run on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane for 1 hour at 1 Amp. The membranes were blocked for 1 hour in 1x PBS-T (0.01% Tween-20) + 5% (w/v) powdered milk + 0.05% sodium azide), then incubated overnight at 4°C in blocking buffer containing the following polyclonal antibodies: anti-LukA (1:5,000), anti-Hla (1:5,000), anti-LukD (1:7,500), and anti-HlgC (1:5,000). The membranes were washed in 1x PBS-T (0.01% Tween-20) + 0.05% sodium azide, then incubated in Alexa Fluor-680 conjugated goat anti-rabbit IgG secondary antibody (Life Technologies, 1:25,000) for 1 hour at room temperature, washed thrice in 1x PBS-T (0.01% Tween-20) + 0.05% sodium azide and imaged using a Odyssey imager (Li-COR).

Evaluation of *S. aureus* bacterial load *in vivo*. To evaluate bacterial burden *in vivo*, 4-week old Swiss-Webster mice (Harlan) were anesthetized intraperitoneally with 300 µl Avertin (2,2-tribromoethanol dissolved in tert-amyl-alcohol and diluted to a final concentration of 2.5% v/v in sterile saline), then infected systemically via the retro-orbital venous plexus with 100 µl of Newman isogenic strains WT, Δ*hlg*Δ*lukED*, Δ*hla*, and Δ*isdBH* for a final of 1 x 10^7 colony forming units (CFU) in 1x PBS. 96-hours post infection, mice were sacrificed and the relevant organs harvested and homogenized in 1x PBS, serially diluted and incubated overnight at 37°C in Tryptic Soy Agar (TSA) plates.

Table S1: *S. aureus* strains used in this study (Related to Experimental Procedures).

***S. aureus* strains**	**Reference**
USA300 SF8300	(Diep et al., 2008)
USA300 SF8300 *Δ hlgACB*	(Perret et al., 2012)
USA300 LAC	(Diep et al., 2006)
USA300 LAC *Δ hlgA*	(Spaan et al., 2014)
Newman	(Duthie and Lorenz, 1952)
Newman *Δ hlgACB*	(Supersac et al., 1998)
Newman *Δ isdBH*	(Torres et al., 2006)
V8	(Prevost et al., 1995)
V8 *Δ hlgACB*	(Prevost et al., 1995)
Newman *Δ hlg Δ lukED*	(Reyes-Robles et al., 2013)
Newman *Δ hla*	(Torres et al., 2010)
Newman *Δ isdBH*	(Torres et al., 2006)

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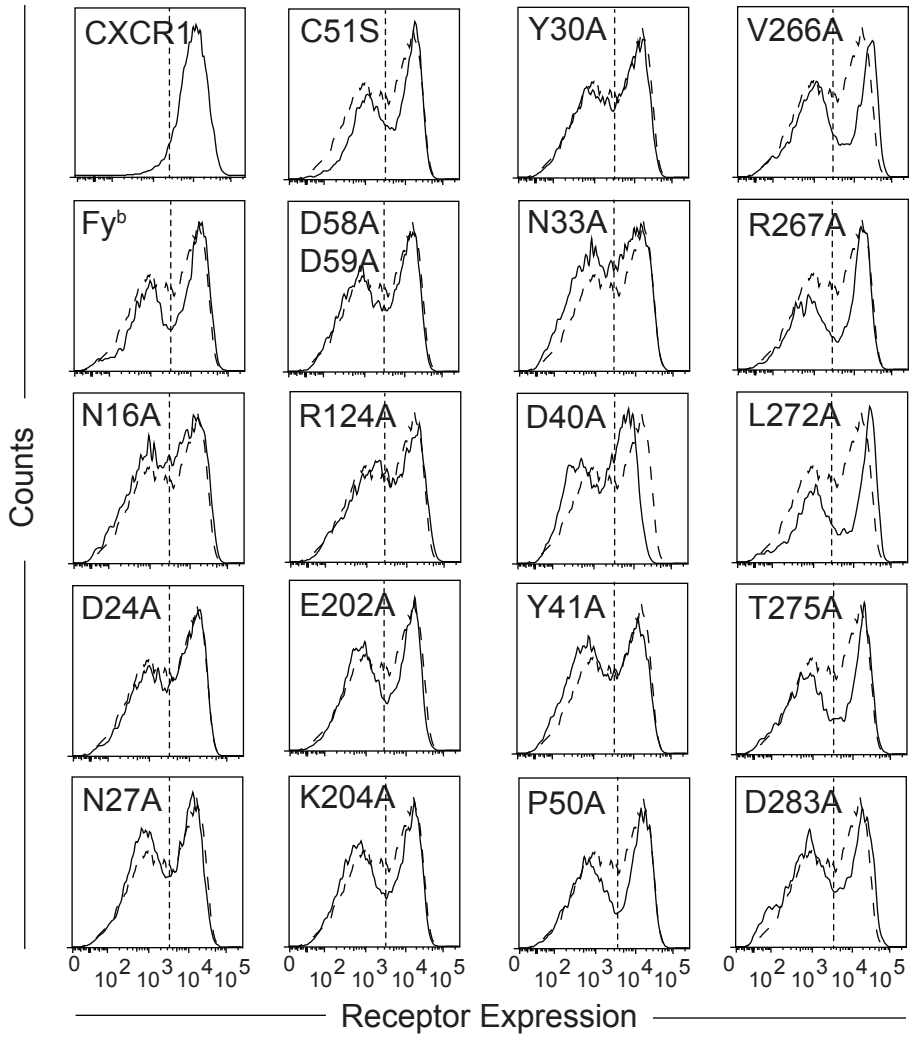

Figure S1 (Related to Methods and Figures 2 and 3). Transfected cell lines express similar levels of receptor on the surface.

Level of expression of CXCR1, DARC Fy^a, Fy^b, and various substitution mutant receptors on transiently transfected HEK293T cells, as detected using receptor specific monoclonal antibodies. Histograms depict a representative sample. Dashed lines indicate monoclonal antibody binding to cells transfected with a plasmid encoding DARC Fy^a. Solid lines indicate monoclonal antibody binding to cells transfected with plasmids encoding DARC mutants.

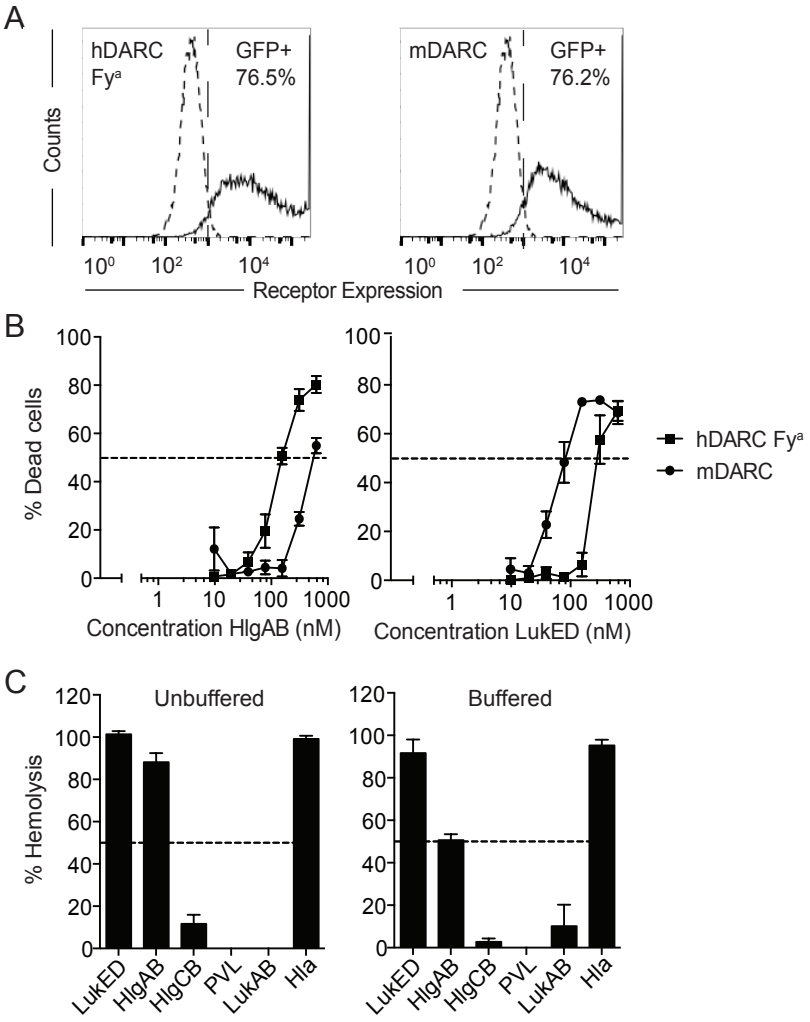

Figure S2 (Related to Figure 2). HlgAB and LukED target human and murine DARC.
(A) Level of expression of human DARC Fy^a or murine DARC on transiently transfected HEK293T cells, as detected by GFP positivity compared to empty vector-transfected cells. Histograms depict a representative sample. Dashed lines indicate empty vector-transfected cells. Solid lines depict receptor levels, as measured by flow cytometry.
(B) Viability of transiently transfected HEK293T cells with plasmids encoding human DARC Fy^a or murine DARC. Dashed lines indicate 50% pore formation. Bars indicate SEM, with n = 4.
(C) Hemolysis of murine erythrocytes in the presence of purified recombinant toxins (40 nM) under unbuffered or buffered conditions. Hemolysis is expressed as OD405 nm of the supernatant. Dashed lines indicate 50% hemolysis. Bars indicate SEM, with n = 3-6.

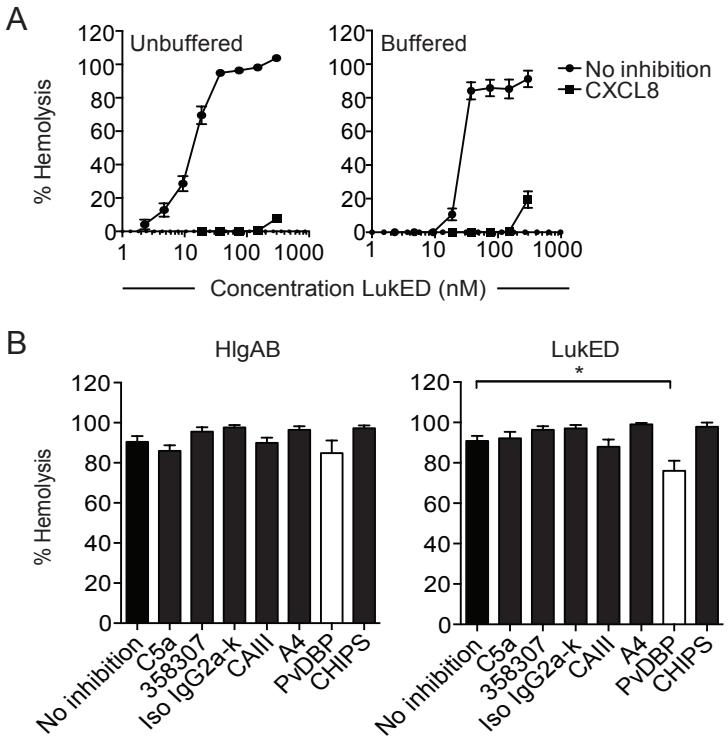

Figure S3 (Related to Figure 3). Lack of inhibition of HlgAB and LukED hemolysis by several DARC interacting proteins.

(A) Inhibition of LukED-mediated lysis of human erythrocytes by CXCL8 in unbuffered or buffered conditions. Bars indicate SEM, with n = 6.

(B) Hemolysis induced by HlgAB (4.9 nM) or LukED (156.2 nM) was tested on erythrocytes following pre-incubation with 10 $\mu g.mL^{-1}$ of C5a, with 10 $\mu g.mL^{-1}$ of a commercial DARC targeting monoclonal antibody (358307) or isotype control, with 10 $\mu g.mL^{-1}$ of a nanobody targeting the DARC Fy^6-epitope (CAIII) or human immunodeficiency virus (A4), and with 700 nM of the *P. vivax* Duffy Binding Protein (PvDBP) or the staphylococcal protein CHIPS. Bars indicate SEM, with n = 3-6. Statistical significance is displayed as *$p<0.05$ using One-Way ANOVA with Bonferroni post test correction for multiple comparison.

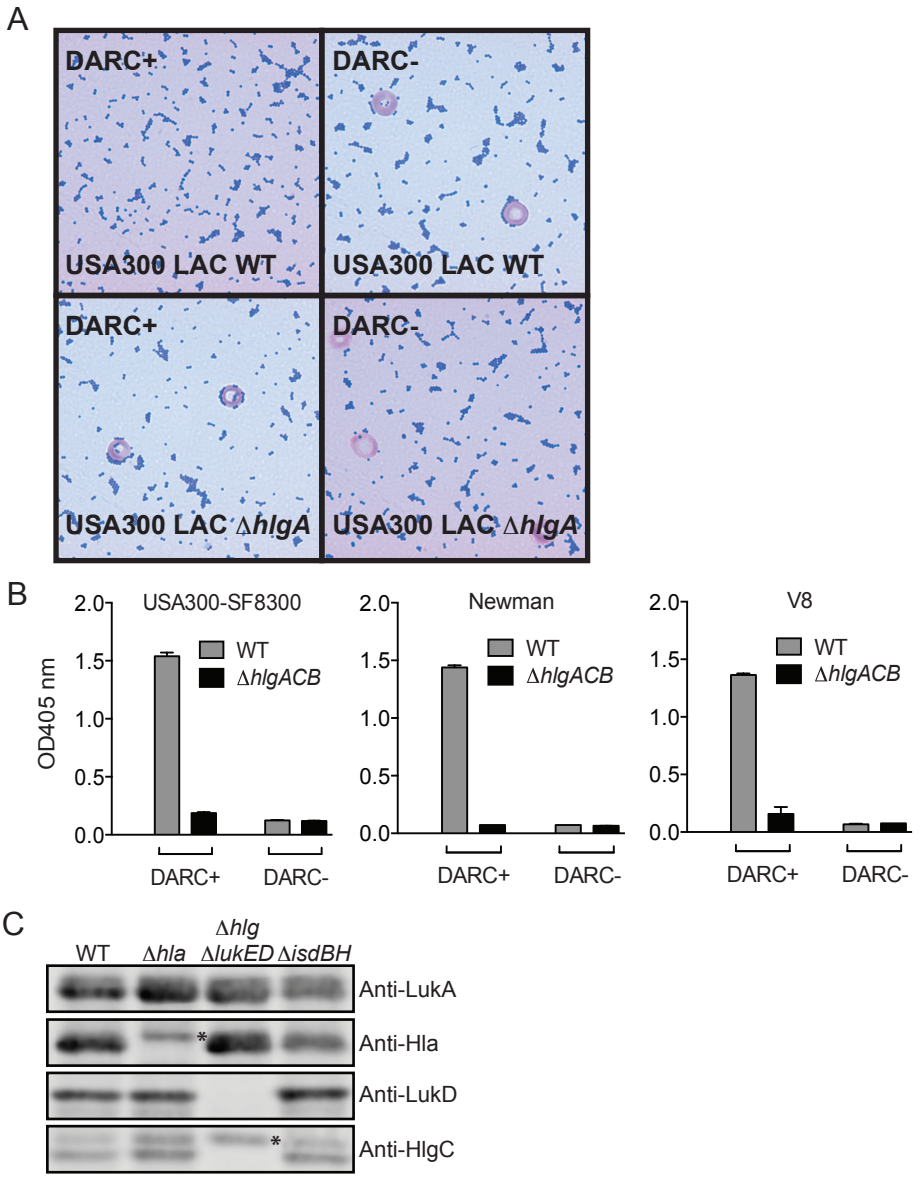

Figure S4 (Related to Figure 4). *S. aureus* lyses erythrocytes in a γ-hemolysin and DARC dependent manner.

(A) Bacteria grown in the presence of erythrocytes from donors with or without erythroid expression of DARC. Microscopic images of *S. aureus* strain USA300 clone LAC and its isogenic *hlgA* mutant strain (*hlgA::bursa*) grown overnight show depletion of erythrocytes in a DARC and *hlgA* dependent manner. Pictures show representative samples, with infectious dose set at 1 x 10^6 CFU per sample.

(B) Bacteria grown as in (A), and hemolysis induced overnight during growth of *S. aureus* strain USA300 clone SF8300, strain Newman, strain V8, and their respective *hlgACB* mutant isogenic strains (inoculation set at 1 x 10^6 CFU per sample). Hemolysis is expressed as OD405 nm of the supernatant. Bars indicate SEM, with n = 2.

(C) Immunoblot analyses of filtered culture supernatants of *S. aureus* strains Newman wild type (WT) and isogenic mutants Δ*hla*, Δ*hlg* Δ*lukED* and Δ*isdBH* against LukA, Hla, LukD, and HlgC antisera. Immunoblots are representative of two individual isolates per strain. Asterisks indicate cross reacting bands.