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CCR5 is a receptor for *Staphylococcus aureus* leukotoxin ED

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Abstract

Pore-forming toxins are critical virulence factors for many bacterial pathogens and are central to *Staphylococcus aureus*-mediated killing of host cells. *S. aureus* encodes pore-forming bi-component leukotoxins that are toxic toward neutrophils, but also specifically target other immune cells. Despite decades since the first description of *Staphylococcal* leukocidal activity, the host factors responsible for the selectivity of leukotoxins toward different immune cells remain unknown. Here we identified the HIV co-receptor, CCR5, as a cellular determinant required for cytotoxic targeting of subsets of myeloid cells and T lymphocytes by the *S. aureus* leukotoxin ED (LukED). We further demonstrate that LukED-dependent cell killing is blocked by CCR5 receptor antagonists, including the HIV drug maraviroc. Remarkably, CCR5-deficient mice are largely resistant to lethal *S. aureus* infection, highlighting the importance of CCR5 targeting in *S. aureus* pathogenesis. Thus, depletion of $CCR5^+$ leukocytes by LukED suggests a novel *S. aureus* immune evasion mechanism that can be therapeutically targeted.

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AUTHOR CONTRIBUTIONS: F.A. and V.J.T identified CCR5 as the LukED receptor. F.A., A.L.D., and T.R.R. purified the toxins. S.A.R. generated the CCR5 shRNA knockdown and CCR5 over-expressing cells. F.A., S.A.R., and A.L.D performed the cytotoxicity assays of cell lines. L.K. purified and sorted primary cells. D.U. designed the experiments for the effect of LukED on human cells. L.K. performed the experiments with primary human cells and S.A.R performed the HIV infection experiments. F.A. and T.R.R. conducted the biochemical and cell binding studies with LukED and GFP fusion proteins. F.A. and T.R.R conducted the animal studies. D.M performed the SPR experiments. N.R.L. provided cDNA plasmids and the Δ32 CCR5 primary cells. V.J.T. and D.U. coordinated and directed the project. All authors discussed the data and commented on the manuscript. F.A., D.U., and V.J.T interpreted the data and wrote the manuscript.

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Staphylococcus aureus is a bacterial pathogen that causes significant morbidity and mortality worldwide. The organism is responsible for a myriad of diseases, from skin and soft tissue infections, to more invasive diseases including necrotizing pneumonia and sepsis. *S. aureus* secretes a number of protein products that allow the organism to effectively subvert the host immune system. Such factors include super-antigens, antibody binding proteins, cytolytic peptides, and pore-forming cytotoxins [1].

Pore-forming toxins are secreted by a substantial number of pathogenic bacteria [2]. The toxins are secreted as water-soluble monomers that recognize host cell membranes, oligomerize, and insert α-helical or β-barrel pores into the lipid bilayer [2]. Pore-formation disrupts osmotic balance and membrane potential, ultmately leading to cell death [2]. *S. aureus* strains that infect humans produce up to four different β-barrel, bi-component, pore-forming toxins (HlgACB, LukED, LukSF-PV/PVL, and LukAB/HG) that exhibit a unique tropism for host immune cells and contribute to the greater virulence of *S. aureus* [1,3,4]. The precise repertoire of immune cells targeted by the pore-forming leukotoxins remains to be fully determined. Even now, more than a century since the first description of *Staphylococcal* leukocidal activity [5,6], our understanding of leukotoxin function *in vivo* is limited due to an absence of known host-derived specificity determinants.

CCR5 is required for LukED cytotoxicity

To identify potential leukotoxin receptors, we purified recombinant LukED, LukAB, and LukSF-PV and assessed their ability to kill a set of human cell lines [4,7]. Granulocyte-like human cells (PMN-HL60) were killed within 1 hour by LukAB and LukSF-PV, but not LukED (Fig. 1a). In contrast, LukED was cytotoxic to a human T cell line ectopically expressing CCR5 (HUT-R5); whereas another T cell line (Jurkat), which lacks detectable CCR5, was insensitive (Fig. 1a). This suggested that CCR5 was involved in LukED cytotoxicity towards HUT-R5 cells. Accordingly, when CCR5 levels were reduced in HUT-R5 cells using lentiviral *CCR5* shRNA, the cells were protected from LukED-mediated killing (Fig. 1b and S1a–b).

Complementary to these findings, ectopic expression of *CCR5* was sufficient to render Jurkat and H9 cells (Fig. S1c) susceptible to LukED cytotoxicity (Fig. 1c). As expected, based on the mode of action of the bi-component leukotoxins, CCR5-dependent LukED-mediated cytotoxicity required both LukE and LukD subunits (Fig. S2a–b). A human osteosarcoma cell line engineered to constitutively express CCR5 (GHOST.R5 cells) [8] was also sensitive to LukED, but not to LukAB or LukSF-PV (Fig. 1d). The sensitivity of GHOST cells to LukED was specific to CCR5 expression, as over-expression of additional T cell-specific chemokine receptors (CCR1, CCR2, CCR3, CXCR4, CCR8, and CXCR6) in these cells did not confer susceptibility to LukED (Fig. S2c).

CCR5 antagonists block LukED cell killing

CCR5 is a co-receptor required for HIV infection [9–11] and has been targeted with small molecule antagonists aimed at restricting HIV entry into host cells [11]. We found that one such clinically approved receptor antagonist, maraviroc, potently blocked LukED killing of $CCR5^+$ cells (Fig. 1e and S3a) at concentrations similar to those required to block HIV

infection (Fig. S3b). Similar inhibitory effects were observed with the CCR5 antagonists Vicriviroc and TAK-779, as well as chemokines that are natural ligands of CCR5 (Fig. S3a and S3c) [12,13]. We found that maraviroc resulted in complete blockade of LukED pore-formation, an essential process for cytotoxicity (Fig. 1f and S3d).

We next investigated whether *S. aureus* was able to kill CCR5$^+$ cells in a LukED-dependent manner. The expression level of *lukED* in *S. aureus* is inherently low during *in vitro* growth [7]. However, deletion of the transcription factor Rot, a potent repressor, results in the enhanced expression and production of LukED by *S. aureus* [7]. Thus, to assess *S. aureus* cytotoxicity towards CCR5$^+$ cells, Jurkat or Jurkat-R5 cells were infected with *S. aureus* Δ*rot* (*Sa* LukED$^+$) and *S. aureus* Δ*rot*Δ*lukED* (*Sa* LukED$^-$) mutants. Jurkat-R5 cells were killed by *S. aureus* in a LukED-dependent manner, while Jurkats lacking CCR5 were resistant to killing (Fig. 1g). Additionally, Jurkat-R5 killing by *S. aureus* was completely blocked by maraviroc (Fig. 1h).

LukE interacts directly with CCR5

To more precisely characterize the LukED-CCR5 interaction on target cells we first determined whether monoclonal antibodies specific towards extracellular regions of CCR5 [14] were sufficient to block toxin activity (Fig. 2a). Antibodies against extracellular loop 2 (ECL-2), but not the N-terminus of the receptor or CXCR4, significantly blocked toxin killing (Fig 2a) and prevented association of functional GFP-labeled toxin (Fig. S4) with the cell surface of sorted primary human CD4$^+$CCR5$^+$ T cells (Fig 2b). Furthermore, toxin association with the cell surface of CCR5$^+$ cells was also reduced in the presence of CCL5, and was completely blocked upon addition of maraviroc, similar to CD4$^+$CCR5$^-$ T cells (Fig. 2c). To determine if LukED interacts with CCR5, pull-down assays were conducted with purified toxin and solubilized CCR5. We found that CCR5 interacted with LukE but not LukD (Fig 2d). This interaction was significantly reduced in the presence of maraviroc, natural ligands of CCR5, as well as monoclonal antibody 45531 directed against ECL-2, but not 3A9 directed against the N-terminus of CCR5 (Fig. 2e and f). Additionally, incubation of LukE (75-fold molar excess) with CCR5$^+$ cells largely blunted native ligand-induced CCR5 signaling as measured via calcium mobilization (Fig. S5). Importantly, LukE itself does not appear to induce CCR5 signaling (Fig S6a–b). Surface plasmon resonance (SPR) studies with immobilized native CCR5 [15] and purified LukE or LukD subunits confirmed the pull-down studies and determined that LukE, but not LukD, binds to CCR5 in a time-dependent and saturable manner with an apparent K_D of 39.6+/−0.4nM (Fig. 2g and S7a–b). This interaction was specific as evidenced by an inability of LukE to bind native CXCR4 (Fig. 2g).

LukED kills CCR5$^+$ myeloid cells and T cells

We next sought to determine the subsets of primary human lymphoid and myeloid cells targeted by LukED. Treatment of blood lymphocytes with LukED resulted in specific depletion of CCR5$^+$ T cells, the majority of which were effector memory T lymphocytes (TEM) (Fig. 3a and S8). As with cell lines, the CCR5-dependent killing of primary cells was completely blocked by maraviroc (Fig. 3a and S8). A portion of individuals of Northern

European heritage harbors a 32 base-pair deletion in the CCR5 gene (Δ32 CCR5) resulting in a truncated protein that cannot be surface localized, thus rendering the $CD4^+$ T cells refractory to HIV infection [11,16,17]. Similarly, primary T cells expanded from a Δ32 CCR5 donor were also resistant to LukED cytotoxicity (Fig. 3b). In keeping with the notion that CCR5 is required for HIV-1 entry into $CD4^+$ T cells [9–11], selective depletion of $CCR5^+$ T cells by LukED suppressed HIV-1 spread (Fig. S9).

Memory T cells can be classified into functional subsets based on differential chemokine receptor profiles and cytokine production. Among T cell subsets, $CCR6^+CCR5^+$ subset produces more IL-17 and IFNγ than $CCR6^+$CCR5 T cells [18]. Consistent with this association, depletion of $CCR5^+CD4^+$ T cells with LukED greatly reduced the proportion of IFNγ- and IL-17-producing cells compared to purified $CD4^+$ T cell controls (Fig. 3c, Day 0). Incubation with the γc-cytokines IL-7 and IL-15 significantly enhances the proportion of $IL\text{-}17^+$ and $IL\text{-}17^+/IFN\gamma^+$ by $CCR6^+$ memory T cells [19]. We found that when human $CD4^+$ T cells were first treated with LukED, followed by 7-days of culture with IL-7 and IL-15, there was a substantial reduction in the induction of IFNγ and IL-17/IL-22-secreting $CCR6^+$ T cells (Fig. 3c); a finding that correlates well with depletion of the $CCR6^+CCR5^+$ memory progenitor subset (Fig. S10). In addition to Th1 and Th17 effector cells, LukED also killed macrophages and dendritic cells in a CCR5-dependent manner (Fig. 3d).

LukED targets $CCR5^+$ cells *in vivo*

Next we examined the contribution of CCR5 to *S. aureus* pathogenesis and determined the influence of LukED on the targeted killing of $CCR5^+$ cells *in vivo*. We found that murine CCR5 (mCCR5) renders transfected 293T cells fully susceptible to the toxin (Fig. S11a–b). Additionally, primary murine macrophages treated with high concentrations of maraviroc were partially protected from toxin-mediated killing, confirming that LukED is directly targeting mCCR5 (Fig. S11c). Because maraviroc is potent toward human CCR5 but not mCCR5 (Fig 1e and S11)[20] we chose to study WT and CCR5-deficient mice with the hypothesis that the latter would be resistant to LukED cytotoxicty. *S. aureus*-elicited lymphocytes and macrophages from WT mice were highly susceptible to purified LukED, while lymphocytes and macrophages isolated from $CCR5^{-/-}$ mice were markedly resistant (Fig. 4a–b). To further validate that *S. aureus* kills $CCR5^+$ leukocytes *in vivo*, we implemented a peritonitis model where WT and $CCR5^{-/-}$ mice were infected with *S. aureus*. CCR5 surface expression was not required for the initial influx of immune cells to the infection site, as the cells recovered and their profiles were identical among all mice (Fig. S12). However, lymphocytes and macrophages elicited *in vivo* in WT mice were more susceptible to *S. aureus* killing compared to those from the $CCR5^{-/-}$ mice (Fig. 4c–d). LukED is associated with *S. aureus* pathogenesis in a murine model of systemic infection[7]. Using this model, $CCR5^{-/-}$ mice infected with wildtype *S. aureus* exhibited significantly reduced bacterial burden in the kidneys compared to those of infected WT mice (Fig. 4e), a phenotype similar to that observed for mice infected with a *S. aureus* Δ*lukED* mutant[7]. After 96 hours, infected $CCR5^{-/-}$ mice also exhibited significantly reduced serum pro-inflammatory cytokines and chemokines and displayed a commensurate reduction in innate immune cells in the kidney compared to WT mice (Fig. 4f–g), signs consistent with infection resolution. Additionally, when WT mice were challenged systemically with WT or

a Δ*lukED* mutant we observed LukED-dependent killing of CCR5$^+$ macrophages within infected kidneys, consistent with our hypothesis that LukED is capable of targeting CCR5$^+$ leukocytes during infection (Fig. 4h). In support of the importance of CCR5 targeting *in vivo*, the mortality associated with *S. aureus* bloodstream infection was reduced for CCR5-deficient mice, a phenotype similar to that of mice challenged with strains of *S. aureus* lacking *lukED* (Fig. 4i).

Discussion

To our knowledge, CCR5 is the first described cellular receptor that is necessary and sufficient for the killing of mammalian cells by a *Staphylococcal* bi-component leukotoxin. Thus, in addition to HIV, *Toxoplasma gondii* and poxviruses (vaccinia and myxoma)[9,21–24], *S. aureus* can also exploit CCR5 to target immune cells. Interestingly, the Δ32 allele of *CCR5* is thought to have been acquired through selective pressure imparted by a deadly pathogen[25,26]. *Yersinia pestis* or variola virus were postulated as potential driving forces behind this selection, but these hypotheses have either been discounted or remain uncertain in favor of an older selection event incited by an immune cell-targeting pathogen[24,27]. Our findings put forth the possibility that resistance to *S. aureus* leukotoxins may have influenced the selection of the Δ32 allele.

The finding that LukED selectively kills CCR5$^+$ T cells, macrophages, and dendritic cells extends the repertoire of immune cells targeted by this leukotoxin and supports a role for these leukocytes in the resolution of *S. aureus* infection. The *lukED* gene is believed to be present in a large number of clinically relevant strains (>70%) including clones responsible for the majority of infections in the United States and Germany, though it is absent in a subset of strains causing hospital acquired infection (e.g. EMRSA15/16) in Great Britain[28–30]. The majority of isolates lacking *lukED* appear to be of clonal complex 30 (USA200/EMRSA16), which are known to produce low levels of cytotoxins[31]. Conceivably, the pathogenesis of these strains is influenced by the weakened immune status of hospitalized patients rather than toxic molecules. In contrast, we envisage that virulent clinical strains producing large amounts of LukED (e.g. clonal complex 8)[7] use the toxin to eliminate antigen presenting cells as well as *S. aureus*-specific CCR5$^+$ Th1/Th17 cells, which are induced by the bacterium[32] and are protective against infection[33,34]. In support of this hypothesis, we demonstrate that LukED kills CCR5$^+$ cells *in vivo* during systemic infection and that mice lacking CCR5 are protected from the mortality associated with acute *S. aureus* disease. Current systemic murine infection models are insufficient to reliably evaluate CCR5^{hi} T cell susceptibility to LukED (data not shown). However, our *in vitro* data and *in vivo* studies with CCR5$^+$ macrophages strongly support the notion that subsets of CCR5^{hi} T cells are also targeted *in vivo*.

Interestingly, LukED-mediated toxicity toward neutrophils and monocytes is not blocked by maraviroc (data not shown), suggesting LukED targets these cells via alternate and non-redundant mechanisms. This point also implies a role for CCR5$^+$ myeloid cells and T cells in resolving acute infection, one that extends beyond the initial control of infection imparted by neutrophils. Importantly, the finding that LukED toxicity toward CCR5$^+$ cells is potently neutralized by a clinically approved CCR5 antagonist (maraviroc) suggests that these types

of drugs could provide much-needed therapeutic alternatives in the treatment of *S. aureus* infections.

METHODS

Cell culture conditions and viruses

Mammalian cells were maintained at 37°C with 5% CO_2 in RPMI supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) and Penicillin (100 U/ml) and Streptomycin (0.1mg/ml) (P/S; Mediatech) unless stated otherwise. Lentivirus-based over-expression and knockdown of human CCR5, was conducted according to previously described transduction methods[19]. Virus stocks were produced by calcium phosphate-mediated DNA transfection as described[35]. CCR5 over-expressing and shRNA-encoding viruses, including non-coding shRNA or HSA (mCD24)-over-expressing controls, were used at a multiplicity of infection (MOI) of 1–3. HIV-R5 virus used for infection of primary T cells was used at an MOI of 0.3.

Isolation of human PBMC, T cell purification, and activation

Blood was obtained from de-identified, consenting healthy adult donors as Buffy coats (New York Blood Center) and from Δ32/Δ32 CCR5 donors. PBMCs were isolated from blood using a Ficoll-Paque PLUS (GE Amersham) gradient. Resting $CD4^+$ and $CD8^+$ human T cells were purified as previously described[19]. Briefly, $CD4^+$ and $CD8^+$ T cells were isolated from purified PBMC using Dynal $CD4^+$ or $CD8^+$ Isolation Kits (Life Technologies, Grand Island, NY) and were >99% pure. To purify naïve, central memory and effector memory subsets, isolated $CD4^+$ and $CD8^+$ cells were stained with CCR7 and CD45RO antibodies, and $CD45RO^-CCR7^+$ (TN), $CD45RO^+CCR7^+$ (TCM), $CCR7^-$ (TEM) subsets were sorted using a flow cytometer (FACSAria; BD Biosciences). In some experiments, total $CD45RO^+$ (T_M) cells were sorted into $CCR5^+$ and $CCR5^-$subsets. Sorted subsets were >98% pure. Primary human $CD4^+$ T cells for HIV-R5 infections were activated using anti-CD3/CD28 coated beads (Dynabeads, Invitrogen) and maintained in RPMI + Penicillin and Streptomycin + 10% FBS supplemented with 200 U/mL IL-2 and 2mM L-glutamine (Mediatech). In some experiments, $CD4^+$ T cells were cultured in 20 ng/ml IL-7 plus IL-15 (R&D systems) for 7 days. All experiments with primary PBMC from wildtype CCR5 donors were performed with cells from at least 3 independent donors. Experiments using Δ32 CCR5 PBMC were performed with cells from 2 donors.

Generation of Primary human Monocyte-derived macrophages, and dendritic cells

Monocyte derived macrophages and DCs from healthy donors were generated from $CD14^+$ cells as previously described[35]. Monocytes ($CD14^+$) cells were isolated from PBMC using anti-CD14 antibody coated bead based sorting using AutoMACS (Miltenyi Biotec) and were typically >99% pure. Monocyte-derived macrophages were generated from $CD14^+$ cells by supplementing the culture medium with human GMCSF (50 ng/ml)[36]. Monocyte-derived dendritic cells (DCs) were generated from $CD14^+$ cells by supplementing the culture medium with human GMCSF (50 ng/ml) + IL-4 (40 ng/ml)[37]. Cells were cultured for 5 days in the differentiation condition, folowed by addition of LukED as already described.

CCR5 ligands and Inhibitors

Maraviroc and TAK-779 were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Vicriviroc was purchased from Selleck Chemicals. Recombinant human Rantes (CCL-5) and Macrophage inflammatory protein-1β (MIP-1β, CCL-4) were obtained from R&D Systems. Macrophage inflammatory protein 1α (MIP-1α CCL-3) was obtained from Biolegend. Maraviroc was used at 100 ng/ml unless otherwise indicated.

FACS analysis

Cells were stained as previously described[35]. For intracellular staining, $CD4^+$ T cell cultures were stimulated for 5 hours at 37°C with PMA, Ionomycin and Golgistop (BD Biosciences). Stimulated cells were washed with PBS and stained with Fixable Viability Dye to gate on live cells. Cells were then fixed and permeabilized by a commercially available intracellular staining kit (eBioscience) as per the manufacturer's protocol. All FACS data were acquired on an LSRII flow cytometer (BD Biosciences) using FACSDiva software. Data analyses were done using Flowjo software (Treestar Inc.).

Antibodies and dyes

Antibodies used for surface and intracellular staining of primary human cells included—CD3-Percp Cy5.5 (clone UCHT1), CD4-Alexa700 (clone OKT4), CD8-Pacific Blue (clone RPA-T8), CXCR3-Percp Cy5.5 (clone G025H7), IL-17-Alexa488 (clone BL168), IFNγ-Alexa700 (clone 4S.B3) (Biolegend), CD45RO-PeCy7 (clone UCHL1), CCR6-biotin (clone 11A9), CCR4-PE (clone 1G1), CCR5-PE (clone 2D7) or CCR5-APC-Cy7 (clone 2D7), Streptavidin-APC, HSA-PE (clone M1/69) (BD Biosciences), and CCR7-FITC (clone 150503) (R&D systems), IL-22-PerCP-eFluor710 (clone 22URTI) (eBioscience).

Antibodies used for surface staining of primary murine cells included—CD3e-APC (clone 145-2C11), CD11b-PeCy7 (clone M1/70), CD11b-FITC (clone M1/70) Ly6G-FITC (clone 1A8), Ly6G-PE (clone 1A8), CCR5-biotin (C34-3448), CD16/CD32 Fc Block (clone 2.4G2)(BD Biosciences), F4/80-APC (clone BM8), F4/80-PeCy7 (clone BM8) Streptavidin-PerCP.Cy5.5, and B220-A700 (clone RA3-6B2) (Biolegend). Fixable viability dyes eFluor-450 and eFluor-780 were obtained from eBioscience.

Antibodies used for LukE-CCR5 interaction mapping included—CCR5 clones 45533, 45529, 45531, 45517, 45523, 45549, 3A9, 45502, and 45519[14] (R&D systems). The control CXCR4 antibody used in these studies was clone 44716 (R&D systems).

Leukotoxin treatments

Jurkat, H9, Hut-R5, and GHOST cell lines, primary human PBMCs and their sorted subsets, as well as primary murine peritoneal elicited cells were incubated with LukE, LukD, or LukED as previously described[7]. In all experiments cells were seeded into a 96-well plate (1×10^5–2×10^5 cells per well), treated for 1 hour at 37°C and evaluated for morphological changes and ethidium bromide (EtBr) uptake via microscopy, or viability using CellTiter

(Promega), CytotoxOne (Promega), cell scatter via FACS and staining with commercial viability dyes (eBioscience). CellTiter, CytotoxOne, and EtBr measurements were made using an EnVision 2103 Plate Reader (Perkin-Elmer). Intoxications were done in the presence of specific inhibitors (maraviroc, TAK779, and vicriviroc), chemokines (CCL3, CCL5, CCL5), or monoclonal CCR5 and CXCR4 antibodies where indicated in the text.

S. aureus in vitro infection experiments

S. aureus (Newman) Δ*rot*, and Δ*rot* Δ*lukED*[7] were subcultured for 5 hours in tryptic soy broth followed by washing in RPMI + 10% FBS and normalization to 1×10^9 CFU/ml in this same media. Normalized bacteria were then added to 2×10^5 Jurkat and Jurkat-R5 cells (multiplicity of infection 10:1) that had been prestained with α-CCR5-PE antibody (clone 2D7) and mixed at a ratio of 50/50. Staining of CCR5 with α-CCR5-PE antibody (clone 2D7) was previously determined to be stable for greater than six hours on the surface of Jurkat-R5 cells yet does not influence the killing of these cells by LukED (data not shown and Fig S13). Infected cells were incubated at 37 °C + 5% CO2 for four hours followed by the addition of lysostaphin to kill all bacteria. Samples were then analyzed on a BD LSRII flow cytometer. Depletion of $CCR5^+$ vs $CCR5^-$ cells was evaluated and displayed graphically as percent dead cells relative to no toxin controls. For studies with maraviroc, the inhibitor was added to cells 30 minutes prior to the addition of bacteria as described above. Experiments were conducted a total of three times in triplicate.

Generation of GFP fusion proteins

To generate recombinant N-terminal His_{6x}-GFP-tagged LukE and LukD, the mature protein coding sequences of LukE and LukD from *S. aureus* Newman genomic DNA were PCR-amplified using the following primers: *lukE*-F-SalI (5'-CCCC-GTCGAC-AATACTAATATTGAAAAT- 3'), *lukD*-F-SalI (5'-CCCCGTCGAC-GCTCAACATATCACA- 3'), *lukE*-R-NotI (5'-CCCC-GCGGCCGC-tta-ATTATGTCCTTTCACTTTAATTTCGTG-3 '), and *lukD*-R-NotI (5'-CCCCGCGGCCGC-tta-TACTCCAGGATTAGTTTCTTTAGAATC-3'). Amplified sequences were subcloned into pET-41b (Novagen) resulting in a fusion of His_{6x}-GFP with the N-terminus of mature LukE or LukD. Recombinant plasmids were transformed into *E.coli* DH5α and transformants selected by kanamycin resistance. Positive clones were transformed into *Escherichia coli* LysY/LacQ (New England BioLabs) for protein expression and purification.

Leukotoxin purification

LukE, LukD, GFP-LukE, GFP-LukD, LukS, LukF, LukA, and LukB were purified from *E. coli* LysY/LaqQ as previously described[4,7] followed by endotoxin removal with Detoxi-Gel Endotoxin Removal Gel (Thermo Scientific). The following alterations were made for purification of recombinant GFP-LukE and GFP-LukD: upon sonication of bacterial cell pellets, lysates were incubated with 1% Triton X-100 for 1 hour at room temperature. After incubation, lysates were centrifuged for 60 minutes at 12000 RPM and passed through a 0.22 μm filter prior to completing the purification protocol as described[7].

LukED membrane association studies

Association of LukED with the surface of $CCR5^+$ cells was measured as follows. A toxic dose of purified recombinant GFP-LukE or GFP-LukD with LukD or LukE respectively (final concentration 10 μg/ml) was incubated for 30 minutes on ice with sorted $CD4^+CCR5^+$ or $CD4^+CCR5^-$ T cells (5×10^4 cells/well) from three independent donors. Cells were gated as GFP positive compared to baseline fluorescence of untreated cells. A total of 50,000 events were collected in all conditions tested. Due to the high level of background fluorescence of GFP toxins with the membranes of transduced cell lines, we were unable to use these cells for membrane association assays (data not shown). As an alternative we used primary $CD4^+$ T cells for membrane association studies. To increase the abundance of CCR5 on these cells and foster reproducible measures of membrane association, $CD4^+CCR5^+$ cells were generated from $CD4^+$ cells infected with a lentivirus encoding CCR5 and sorted by FACS as $CCR5^+$ from the resulting $CD4^+$ population after surface staining for CCR5 using 2D7 clone (PE). $CD4^+CCR5^-$ cells were sorted from the same population as those cells with undetectable CCR5 surface expression. Importantly, CCR5 surface staining with 2D7 antibody does not influence toxin killing kinetics and therefore is unlikely to adversely influence membrane association, as the latter is required for the former (Fig. S13). Somewhat paradoxically, clone 2D7 also binds to ECL-2 of CCR5 similar to that of clone 45531, which blocks toxin activity. However, 2D7 and 45531 do bind to distinct portions of ECL-2 (the N-terminal portion and C-terminal portion, respecitvely) perhaps explaining this phenomenon[38]. Alternatively, our staining protocols may not sufficiently saturate all receptor sites, thereby allowing functional characterization of toxin in the presence of 2D7.

Experiments assessing maraviroc, natural ligand, or antibody inhibition of LukED membrane association were conducted in a similar fashion. However, in these instances cells were first preincubated for 30 minutes with maraviroc (100 ng/ml), CCL5 (5 μg/ml), 3A9, 45531, or CXCR4 monoclonal antibodies (25 μ-g/ml) or buffer prior to addition of a lethal concentration of LukE-GFP+LukD to the cells (5–10 μg/ml). After treatment, cells were washed, resuspended in fixation buffer (FACS buffer + 2% paraformaldehyde) for 15 minutes at room temperature, washed again, resuspended in FACS buffer, and fluorescence of bound toxin was monitored by flow cytometry. Cells are displayed as % GFP positive.

Surface plasmon resonance analysis of LukE and LukD binding to solubilized CCR5 and CXCR4

Binding kinetics of LukE and LukD to CCR5 and CXCR4 by Surface Plasmon resonance were measured as previously described[15,39–42]. This approach hasc also been employed to detect ligand interactions with CXCR1 and CXCR2[43,44]. A C9-tagged CCR5 was solubilized using 50 mM HEPES, pH 7.0, 150 mM NaCl, 0.1% DDM, 0.1% CHAPS, 0.02% CHS[15]. This solubilzation scheme is known to retain conformationally specific antibody binding to both CCR5 and CXCR4[15]. ~700 RU of the CCR5 receptor was captured onto a 1D4 antibody-bound CM5 chip[15,40,41]. Cells expressing a C9-tagged CXCR4 receptor were also solubilized as a control surface in the same buffer[41]. C9-CXCR4 was captured to ~1200 RU. LukE or LukD was diluted to 1.7 μM in running buffer containing 50 mM Hepes, pH 7.0, 150mM NaCl, 0.02% CHS, 0.1% DDM, and 0.1% Chaps and tested for binding in a 3-

fold dilution series at a flow rate of 50μl/min. Each concentration series was replicated two times as shown by the overlaid sensorgrams. All data were collected at 25 °C and conducted at least twice in duplicate.

Biochemical studies to detect interactions between LukED and CCR5

293T cells were transfected with a vector containing HA-tagged CCR5 (Missouri S&T cDNA Resource Center-www.cdna.org), followed by solubilization (~2.0×10^7 cells per condition) in PBS + 1% Brij010 + Complete EDTA-free protease inhibitor cocktail (Roche). Solubilized CCR5 was then added to 25μl of nickel resin containing no toxin or bound LukE, LukD, or LukED. For the maraviroc, natural ligand, and antibody inhibition experiments the solubilized CCR5 was pre-incubated for 30 minutes at room temperature with 5 μg/ml of maraviroc, 10 μg/ml of each chemokine, or 35 μg/ml of each antibody followed by incubation with nickel resin containing LukE. After incubation with cell lysates, the resin/protein complexes were fixed with 2 mM DTSSP (Pierce) for 30 minutes, quenched with 20 mM Tris pH 8.0 for 15 minutes, washed 4 times in PBS + 1% Brij010, and boiled in 4× SDS boiling buffer. All samples were run on a 10% SDS-PAGE gel at 80V, followed by transfer to nitrocellulose at 1 Amp for 1 hour. Membranes were blocked in PBS + 0.01% Tween + 5% milk for 1 hour and incubated overnight with either α-HA antibody for CCR5 (Covance) or α-His antibody (Cell Sciences) for LukE and LukD. The following day secondary goat α-mouse-HRP antibody (Bio-Rad) was added to the membranes for 1 hour followed by the addition of SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) for detection.

Measurement of CCR5 activation by calcium mobilization

Assessment of CCR5 activation by calcium mobilization in cell lines and primary cells was carried out using the commercial dye Fluo4-AM (Invitrogen). Cells were labeled for 30 minutes at room temperature with 3 μM Fluo4 in Hanks Balanced Salt Solution, followed by three washes in HBSS and incubation at 37° C for 30 minutes. Cells were analyzed on a flow cytometer over time and at 100 seconds, ligand (CCL3, CCL4, CCL5, 10ng/ml) or LukE (10–20 μg/ml) was added to the cells. Fluorescence was monitored thereafter by flow cytometry (500 events were collected per second) until the indicated completion of each experiment. For conditions in which inhibition of receptor activation was monitored cells were pre-incubated with either maraviroc (1 μg/ml) or LukE (10–20 μg/ml) during the 30 minute incubation at 37° C described above. Graphs show the mean fluorescence of all events collected in five second intervals.

Murine *in vitro* and *in vivo* experiments

in vitro assessment of peritoneal elicited immune cell killing by LukED was conducted as follows. Female age-matched (4–6 weeks) C57Bl6 WT or $CCR5^{-/-}$ mice (Taconic) were injected with 1×10^7 CFU heat killed *S. aureus* Newman Δ*lukED* intraperitoneally. 24 hours later mice were injected with an additional 1×10^7 CFU of the same strain. After another 24 hours, mice were sacrificed and peritoneal elicited immune cells were lavaged with 7ml of PBS followed by lysis of red blood cells in ACK lysing buffer and resuspension in RPMI + 10% FBS. LukED was then added to cells as described above and incubated for 1 hour at

37° C + 5% CO2. After incubation cells were washed in PBS and stained with viability dye followed by surface staining for B220, CD11b, F480, Ly6G, CD3, and CCR5. The percent dead cells shown are an average of cells isolated and intoxicated from three independent mice. Means and standard deviation are shown.

For experiments designed to measure *S. aureus* killing of $CCR5^{+}$ cells *in vivo*, female age-matched (4–6 weeks) C57Bl6 WT or $CCR5^{-/-}$ mice (Taconic) were injected on day 1 with 1×10^{7} heat-killed *S. aureus* to promote the recruitment of $CCR5^{+}$ macrophages and lymphocytes to the peritoneum. On day 2 mice were challenged with live *S. aureus* Δ*rot* followed by the isolation of peritoneal immune cells 16–20 hours later. Isolated cells were processed for FACS as described above and the viability of lymphocytes and macrophages was evaluated. The percent dead lymphocytes were averaged from 10 WT and 10 $CCR5^{-/-}$ animals and representative FACS plots are shown.

For murine systemic infections, female age-matched (4–6 weeks) C57Bl6 WT or $CCR5^{-/-}$ mice (Taconic) were infected with WT *S. aureus* Newman as previously described [7]. After 96 hours serum was collected and kidneys removed, homogenized, processed for FACS, and plated as previously described [7]. All survival curves were conducted as previously described using WT *S. aureus* Newman and an isogenic Δ*lukED* mutant [7]. For flow cytometry of immune cells from WT or Δ*lukED* infected kidneys, organs were removed after 96 hours and mechanically homogenized. Immune cells in homogenized tissues were enriched by performing a 40/80 Percoll (GE Healthcare) density gradient centrifugation. Cells were subsequently processed for surface and viability staining thereafter (see above).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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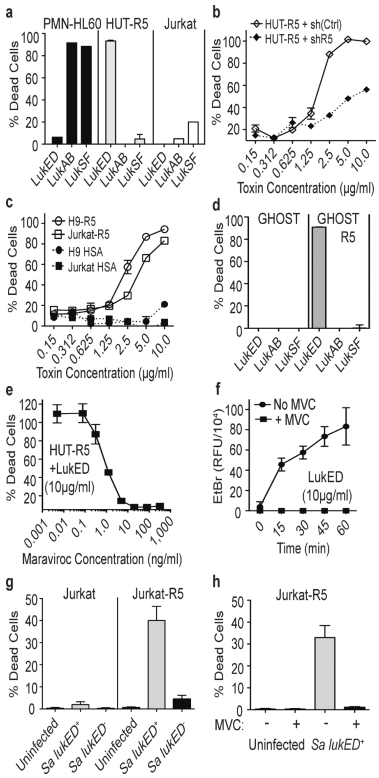

Figure 1. LukED requires CCR5 for cell killing

(a) Viability of cells exposed to different leukotoxins (10 μg/ml). (b) Viability of HUT-R5 cells transduced with control or *CCR5* shRNAs. (c) Viability of Jurkat and H9 cells transduced with CCR5 (-R5) or mouse CD24 (-HSA) followed by treatment with LukED. (d) Viability of GHOST cells over-expressing CCR5 and treated with indicated leukotoxins. (e) Viability of HUT-R5 pre-incubated with maraviroc and treated with LukED. (f) Pore-formation, as measured by ethidium bromide uptake, on Jurkat-R5 +/− maraviroc (MVC; 100 ng/ml) followed by incubation with LukED. (g–h) Viability of Jurkat or Jurkat-R5 cells infected with *S. aureus* (j) in the presence or absence of MVC (k). Means ± standard deviation (n = 3) are shown.

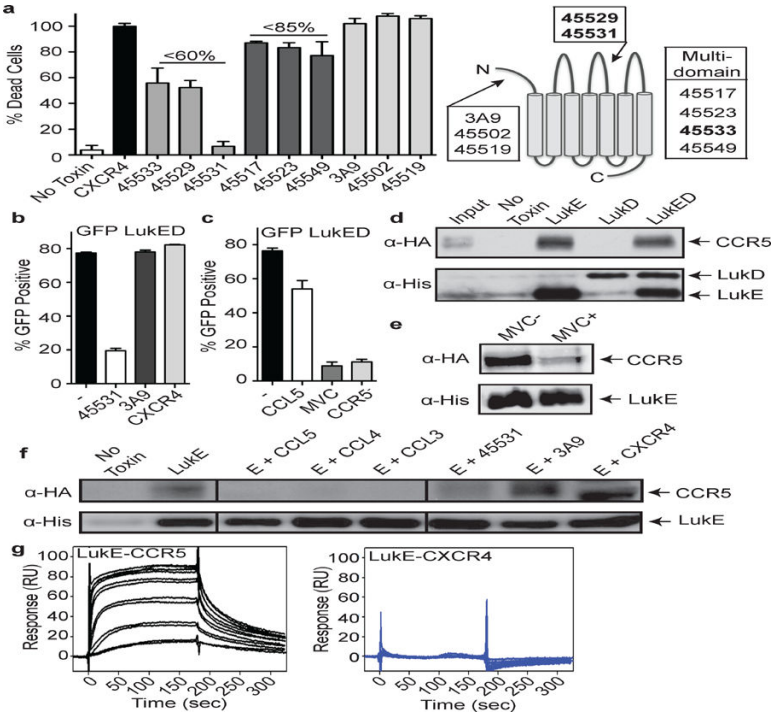

Figure 2. LukE directly interacts with CCR5

(a) Viability of cells treated with α-CCR5 monoclonal antibodies (35 μg/ml) followed by exposure to LukED (10 μg/ml). (b) Membrane association of GFP-LukED (10 μg/ml) to the surface of primary $CD4^+CCR5^+$ T cells +/− the indicated monoclonal antibodies (25 μg/ml) as determined by FACS. (c) Membrane association of GFP-LukED (10 μg/ml) on the surface of primary $CD4^+CCR5^+$ T cells +/− maraviroc (MVC) (100 ng/ml), CCL5 (5 μg/ml) or on $CD4^+CCR5^-$ T cells. (d–f) Evaluating the interaction between His-LukE, LukD, or LukED and HA-CCR5 (d) +/− MVC (5 μg/ml) (e), CCL5, CCL4, CCL3 (10 μg/ml) (f), and monoclonal antibodies 45531, 3A9, CXCR4 (35 μg/ml) (f). Immunoblots are representative of at least two independent experiments. (g) Measurement of the interaction of LukE with CCR5 and CXCR4 by SPR. Representative sensorgrams (h) of two experiments performed in duplicate are shown. Where relevant, means ± standard deviation (n = 3) are shown.

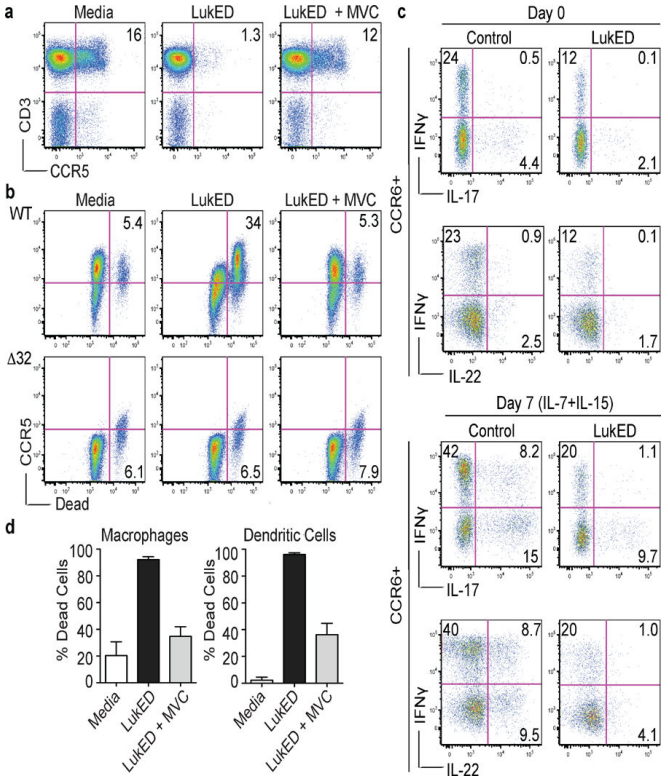

Figure 3. LukED kills CCR5$^+$ human memory T cells, macrophages and dendritic cells

(a) Analysis of total CCR5$^+$ primary human T cells (CD3$^+$/CCR5$^+$) incubated with media, LukED (2.5 μg/ml) or maraviroc (MVC; 100 ng/ml) followed by LukED treatment. (b) Susceptibility of T cells isolated from a Δ32-CCR5 or WT-CCR5 donor. Cell viability and CCR5 expression evaluated by flow cytometry as in (a). (c) Measurement of cytokine production of CD4$^+$ T-cells +/− LukED treatment (5 μg/ml) that were stimulated on day 0 with PMA and Ionomycin (P+I; top panel) or cultured in media supplemented with IL-7/IL-15 (20 ng/ml) for 7 days followed by stimulation with P+I (bottom panel). (d) Viability of monocyte-derived macrophages and dendritic cells incubated with LukED (3.0 μg/ml +/− MVC). For FACS plots (a–c) a representative from one of three independent donors is shown. Bar graphs show the mean ± standard deviation of results from three independent donors.

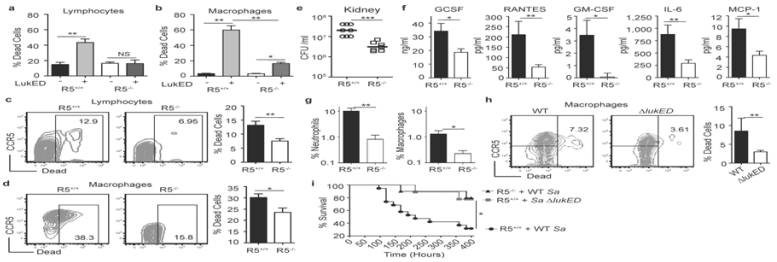

Figure 4. CCR5^{+} cell killing is important for *S. aureus* pathogenesis

(a–b) Viability of primary murine peritoneal elicited immune cells from R5$^{+/+}$ (n=3) or R5$^{-/-}$ (n=3) mice after incubation with LukED (10 μg/ml). (c–d) *in vivo* viability of recruited immune cells from R5$^{+/+}$ (n=10) or R5$^{-/-}$ (n=10) mice challenged with live *S. aureus* Δ*rot*. (e) Bacterial CFU recovered from the kidneys of R5$^{+/+}$ (n=8) or R5$^{-/-}$ (n=9) mice infected for 96 hours with WT *S. aureus*. (f) Measurement of serum cytokine and chemokine levels from animals in (e). (g) Quantification of neutrophils and macrophages recovered from infected kidneys 96 hours post-infection. (h) *in vivo* viability of recruited macrophages from R5$^{+/+}$ mice challenged with *S. aureus* WT (n=10) or δ*lukED* (n=10). (i) "Survival" of R5$^{+/+}$ mice infected with wildtype *S. aureus* (n=10) or a Δ*lukED* mutant (n=10) and R5$^{-/-}$ infected with wildtype *S. aureus* (n=20). FACS plots show a representative from 1 of 10 infected animals. *, $p < 0.05$; **, $p \leq 0.001$; *** $p \leq 0.0001$ by 1-way ANOVA (a–b), Student's T test (c–h), and Mantel Cox Test (i). Bar graphs show the mean ± standard deviation.