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CCR5 is a receptor for *Staphylococcus aureus* leukotoxin ED

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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 towards 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 towards different immune cells remain unknown. Here we identify the human immunodeficiency virus (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 new immune evasion mechanism of *S. aureus* that can be therapeutically targeted.

S. 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 several protein products that allow the organism to 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, ultimately 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 because of 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 in 1 h 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 amounts were reduced in HUT-R5 cells using lentiviral *CCR5* short hairpin RNA (shRNA), the cells were protected from LukED-mediated killing (Fig. 1b and Supplementary Fig. 1a, b).

Complementary to these findings, ectopic expression of *CCR5* was sufficient to render Jurkat and H9 cells (Supplementary Fig. 1c) susceptible to LukED cytotoxicity (Fig. 1c). As expected, on the basis of the mode of action of the bi-component leukotoxins, CCR5-dependent LukED-mediated cytotoxicity required both LukE and LukD subunits (Supplementary Fig. 2a, 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 overexpression of additional T-cell-specific chemokine receptors (CCR1, CCR2, CCR3, CXCR4, CCR8 and CXCR6) in these cells did not confer susceptibility to LukED (Supplementary Fig. 2c).

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 Supplementary Fig. 3a) at concentrations similar to those required to block HIV infection (Supplementary Fig. 3b). Similar inhibitory effects were observed with the CCR5 antagonists vicriviroc and TAK-779, as well as chemokines that are natural ligands of CCR5 (Supplementary Fig. 3a, c)[12,13]. We found that maraviroc resulted in complete blockade of LukED pore formation, an essential process for cytotoxicity (Fig. 1f and Supplementary Fig. 3d).

We next investigated whether *S. aureus* was able to kill CCR5$^+$ cells in a LukED-dependent manner. The expression 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*

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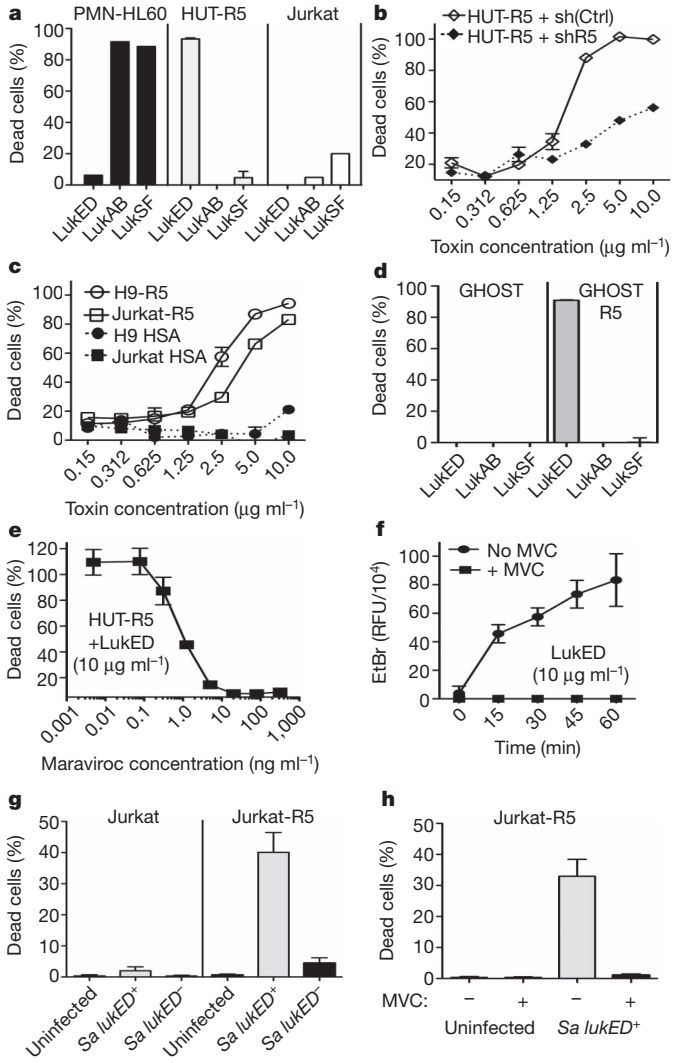

Figure 1 | LukED requires CCR5 for cell killing. **a**, Viability of cells exposed to different leukotoxins (10 μg ml^{-1}). **b**, Viability of HUT-R5 cells transduced with control (Ctrl) or *CCR5* shRNAs. **c**, Viability of Jurkat and H9 cells transduced with CCR5 (-R5) or mouse CD24 (-HSA) followed by treatment with LukED. **d**, Viability of GHOST cells overexpressing 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 with or without maraviroc (MVC; 100 ng ml^{-1}) followed by incubation with LukED. **g**, **h**, Viability of Jurkat or Jurkat-R5 cells infected with *S. aureus* (**g**), in the presence or absence of MVC (**h**). Means ± s.d. ($n = 3$) are shown.

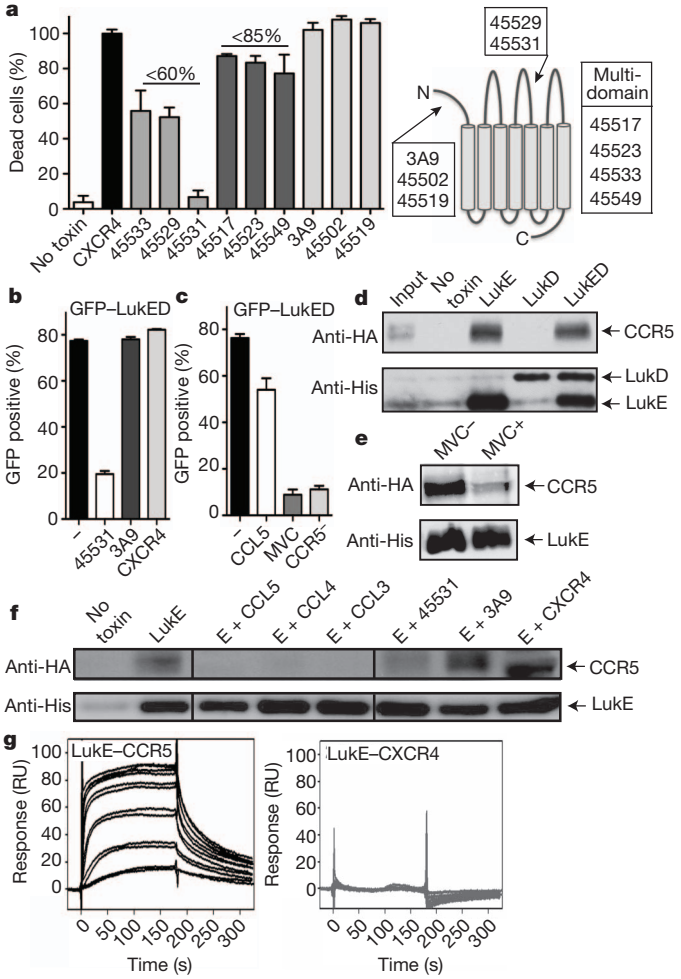

Figure 2 | LukE directly interacts with CCR5. **a**, Viability of cells treated with anti-CCR5 monoclonal antibodies (35 μg ml^{-1}) followed by exposure to LukED (10 μg ml^{-1}). **b**, Membrane association of GFP–LukED (10 μg ml^{-1}) to the surface of primary CD4$^+$CCR5$^+$ T cells with or without the indicated monoclonal antibodies (25 μg ml^{-1}) as determined by fluorescence-activated cell sorting (FACS). **c**, Membrane association of GFP–LukED (10 μg ml^{-1}) on the surface of primary CD4$^+$CCR5$^+$ T cells with or without maraviroc (MVC) (100 ng ml^{-1}), CCL5 (5 μg ml^{-1}) or on CD4$^+$CCR5$^-$ T cells. **d**–**f**, Interaction between His- LukE, LukD, or LukED and HA-CCR5 (**d**), with or without MVC (5 μg ml^{-1}) (**e**), CCL5, CCL4, CCL3 (10 μg ml^{-1}) (**f**) and monoclonal antibodies 45531, 3A9, CXCR4 (35 μg ml^{-1}) (**f**). In **f**, E stands for the LukE toxin subunit. Immunoblots are representative of at least two independent experiments. **g**, Interaction of LukE with CCR5 and CXCR4 by surface plasmon resonance. Representative sensorgrams (**g**) of two experiments performed in duplicate are shown. Where relevant, means ± s.d. ($n = 3$) are shown.

Δ*rot*Δ*lukED* (*Sa* LukED$^-$) mutants. Jurkat-R5 cells were killed by *S. aureus* in a LukED-dependent manner, whereas 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 characterize more precisely the LukED–CCR5 interaction on target cells, we first determined whether monoclonal antibodies specific towards extracellular regions of CCR5 (ref. 14) were sufficient to block toxin activity (Fig. 2a). Antibodies against extracellular loop 2 (ECL-2), but not the amino (N) terminus of the receptor or CXCR4, significantly blocked toxin killing (Fig. 2a) and prevented association of functional green fluorescent protein (GFP)-labelled toxin (Supplementary Fig. 4) 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 whether 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, f). Additionally, incubation of LukE (75-fold molar excess) with CCR5$^+$ cells largely blunted native ligand-induced CCR5 signalling as measured by calcium mobilization (Supplementary Fig. 5). LukE itself does not seem to induce CCR5 signalling (Supplementary Fig. 6a, b). Surface plasmon resonance studies with immobilized native CCR5 (ref. 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 dissociation constant (K_d) of 39.6 ± 0.4 nM (Fig. 2g and Supplementary Fig. 7a, 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, most of which were effector memory T lymphocytes (Fig. 3a and Supplementary Fig. 8). As with cell lines, the CCR5-dependent killing of primary cells was completely blocked by maraviroc (Fig. 3a and Supplementary Fig. 8). A proportion of individuals of Northern European heritage harbour 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 (Supplementary Fig. 9).

Memory T cells can be classified into functional subsets on the basis of differential chemokine receptor profiles and cytokine production. Among T-cell subsets, the CCR6$^+$CCR5$^+$ subset produces more interleukin (IL)-17 and interferon (IFN)-γ than CCR6$^+$CCR5$^-$ T cells[18]. Consistent with this association, depletion of CCR5$^+$CD4$^+$ T cells with LukED greatly reduced the proportion of IFN-γ- and IL-17-producing cells compared with 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-17$^+$ and IL-17$^+$/IFN-γ$^+$ 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 IFN-γ and IL-17/IL-22-secreting CCR6$^+$ T cells (Fig. 3c). This finding correlates well with depletion of the CCR6$^+$CCR5$^+$ memory progenitor subset (Supplementary Fig. 10). In addition to Th1 and Th17 effector cells, LukED also killed macrophages and dendritic cells in a CCR5-dependent manner (Fig. 3d).

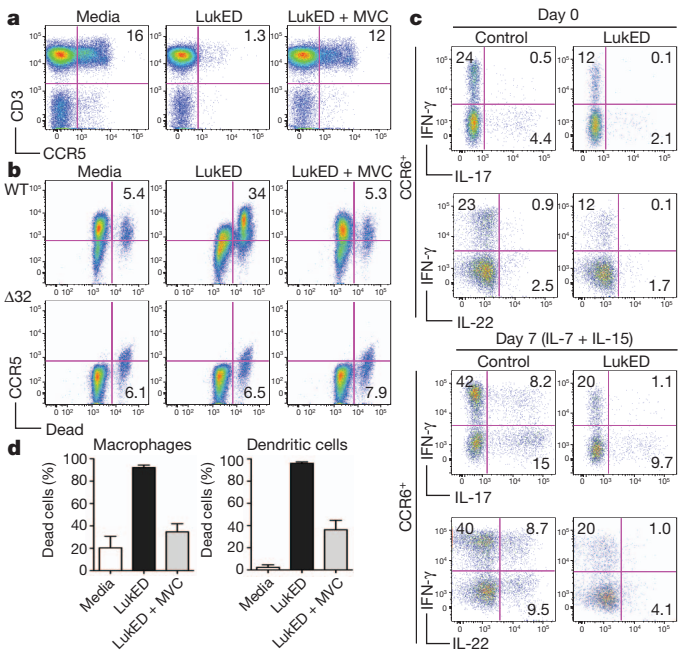

Figure 3 | LukED kills CCR5$^+$ human memory T cells, macrophages and dendritic cells. **a**, Total CCR5$^+$ primary human T cells (CD3$^+$/CCR5$^+$) incubated with media, LukED (2.5 μg ml^{-1}) or maraviroc (MVC; 100 ng ml^{-1}) followed by LukED treatment. **b**, Susceptibility of T cells isolated from a Δ32-CCR5 or WT-CCR5 donor. Cell viability and CCR5 expression evaluated by flow cytometry as in **a**. **c**, Cytokine production of CD4$^+$ T cells with or without LukED treatment (5 μg ml^{-1}) 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^{-1}) 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^{-1} with or without MVC). For FACS plots (**a**–**c**), a representative from one of three independent donors is shown. Bar graphs, mean ± s.d. of results from three independent donors.

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 (Supplementary Fig. 11a, b). Additionally, primary murine macrophages treated with high concentrations of maraviroc were partly protected from toxin-mediated killing, confirming that LukED is directly targeting mCCR5 (Supplementary Fig. 11c). Because maraviroc is potent towards human CCR5 but not mCCR5 (Fig. 1e and Supplementary Fig. 11)[20], we chose to study wild-type (WT) and CCR5-deficient mice with the hypothesis that the latter would be resistant to LukED cytotoxicity. *S. aureus*-elicited lymphocytes and macrophages from WT mice were highly susceptible to purified LukED, whereas lymphocytes and macrophages isolated from *CCR5*$^{-/-}$ mice were markedly resistant (Fig. 4a, b). To validate further that *S. aureus* kills CCR5$^+$ leukocytes *in vivo*, we implemented a peritonitis model in which 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 (Supplementary Fig. 12). However, lymphocytes and macrophages elicited *in vivo* in WT mice were more susceptible to *S. aureus* killing than 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 WT *S. aureus* exhibited significantly reduced bacterial burden in the kidneys than 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 h, infected *CCR5*$^{-/-}$ mice also exhibited significantly reduced serum pro-inflammatory cytokines and chemokines and showed a commensurate reduction in innate immune cells in the kidney compared with 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 in 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 favour 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.

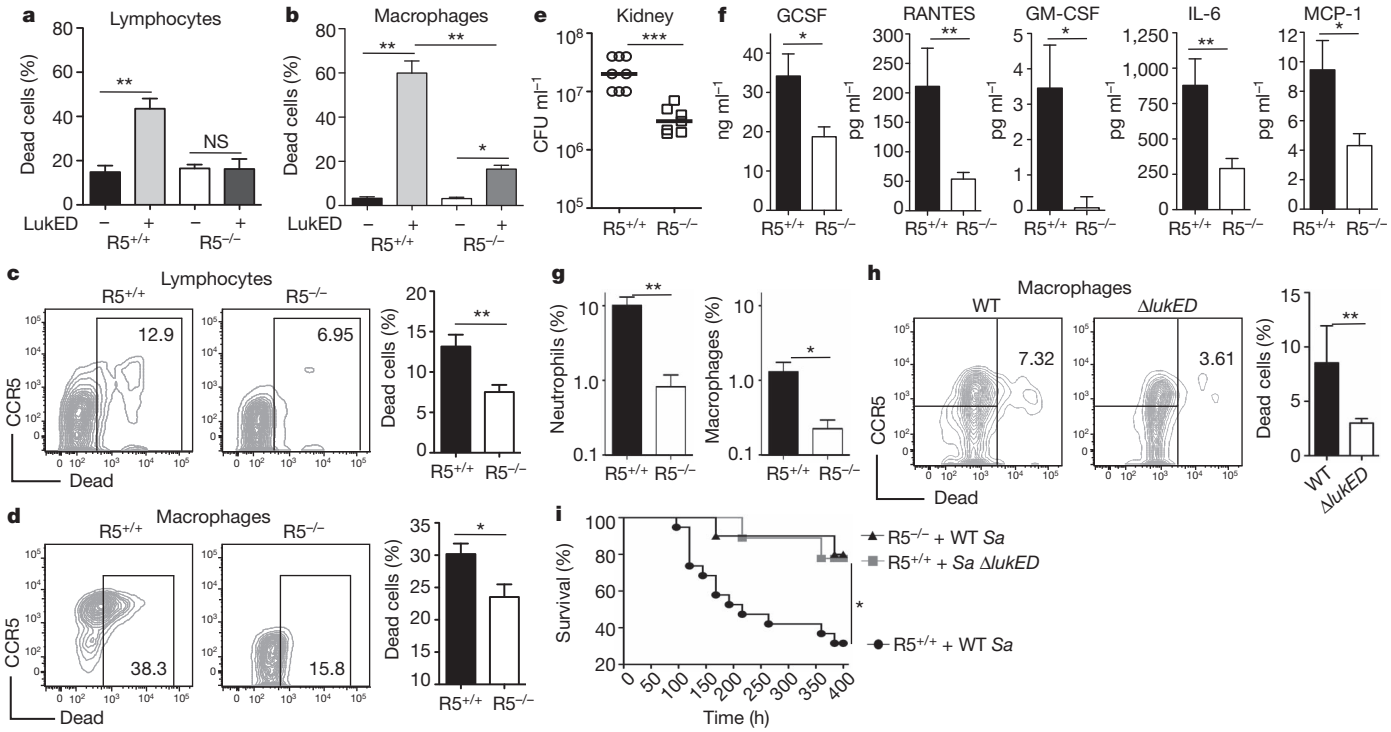

Figure 4 | CCR5$^+$ cell killing is important for *S. aureus* pathogenesis. **a**, **b**, Viability of primary murine peritoneal-elicited immune cells from R5$^{+/+}$ ($n = 3$) or R5$^{-/-}$ ($n = 3$) mice after incubation with LukED (10 μg ml^{-1}). **c**, **d**, *In vivo* viability of recruited immune cells from R5$^{+/+}$ ($n = 10$) or R5$^{-/-}$ ($n = 10$) mice challenged with live *S. aureus* Δ*rot*. **e**, Bacterial colony-forming units (CFU) recovered from the kidneys of R5$^{+/+}$ ($n = 8$) or R5$^{-/-}$ ($n = 9$) mice infected for 96 h with WT *S. aureus*. **f**, Serum cytokine and chemokine amounts from animals in **e**. **g**, Quantification of neutrophils and macrophages recovered from infected kidneys 96 h after 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 WT *S. aureus* ($n = 10$) or a Δ*lukED* mutant ($n = 10$) and R5$^{-/-}$ infected with WT *S. aureus* ($n = 20$). FACS plots show a representative from one of 10 infected animals. *$P < 0.05$; **$P \leq 0.001$; ***$P \leq 0.0001$ by one-way analysis of variance (**a**, **b**), Student's *t*-test (**c**–**h**) and Mantel–Cox test (**i**). Bar graphs, mean ± s.d.

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 many clinically relevant strains (>70%) including clones responsible for most infections in the USA and Germany, although it is absent in a subset of strains causing hospital-acquired infection (for example EMRSA15/16) in the UK[28–30]. Most isolates lacking *lukED* seem to be of clonal complex 30 (USA200/EMRSA16), which is known to produce low amounts 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 predict that virulent clinical strains producing large amounts of LukED (for example, 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 evaluate reliably 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 towards neutrophils and monocytes is not blocked by maraviroc (data not shown), suggesting LukED targets these cells through alternative 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. The finding that LukED toxicity towards CCR5$^+$ cells is potently neutralized by a clinically approved CCR5 antagonist (maraviroc) suggests that these types of drug could provide much-needed therapeutic alternatives in the treatment of *S. aureus* infections.

METHODS SUMMARY

Cell lines and primary human cells were maintained in RPMI plus 10% fetal bovine serum with penicillin and streptomycin, unless supplemented as otherwise indicated, and were incubated with LukE, LukD or LukED as previously described[7]. All blood samples were obtained from anonymous healthy donors as buffy coats (New York Blood Center). The New York Blood Center obtained written informed consent from all participants. Animal experiments were performed in accordance with the Institutional Animal Care and Use Committee at New York University School of Medicine. CCR5-overexpressing cell lines and *CCR5* shRNA knockdowns were generated by lentiviral-based tranduction as previously described[35]. Isolation of human peripheral blood mononuclear cells (PBMC) and their sorted subsets was performed as previously described[19].

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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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 surface plasmon resonance 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.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to V.J.T. (victor.torres@nyumc.org) or D.U. (derya.unutmaz@nyumc.org).

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^{-1}) and streptomycin (0.1 mg ml^{-1}) (Mediatech) unless stated otherwise. Lentivirus-based overexpression and knockdown of human CCR5 were conducted according to previously described transduction methods[19]. Virus stocks were produced by DNA transfection mediated by calcium phosphate as described[35]. CCR5 overexpressing and shRNA-encoding viruses, including non-coding shRNA or HSA (mCD24)-overexpressing controls, were used at a multiplicity of infection of 1–3. HIV-R5 virus used for infection of primary T cells was used at a multiplicity of infection 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. Human peripheral blood mononuclear cells (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 PBMCs using Dynal CD4$^+$ or CD8$^+$ Isolation Kits (Life Technologies) and were more than 99% pure. To purify naive, 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$^-$ (effector memory T lymphocyte) 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 more than 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^{-1} IL-2 and 2 mM L-glutamine (Mediatech). In some experiments, CD4$^+$ T cells were cultured in 20 ng ml^{-1} IL-7 plus IL-15 (R&D Systems) for 7 days. All experiments with primary PBMCs from WT CCR5 donors were performed with cells from at least three independent donors. Experiments using Δ32 CCR5 PBMCs were performed with cells from two donors.

Generation of primary human monocyte-derived macrophages, and dendritic cells. Monocyte-derived macrophages and dendritic cells from healthy donors were generated from CD14$^+$ cells as previously described[35]. Monocyte (CD14$^+$) cells were isolated from PBMCs using anti-CD14 antibody-coated bead-based sorting using AutoMACS (Miltenyi Biotec) and were typically more than 99% pure. Monocyte-derived macrophages were generated from CD14$^+$ cells by supplementing the culture medium with human granulocyte macrophage colony-stimulating factor (50 ng ml^{-1})[36]. Monocyte-derived dendritic cells were generated from CD14$^+$ cells by supplementing the culture medium with human granulocyte macrophage colony-stimulating factor (50 ng ml^{-1}) + IL-4 (40 ng ml^{-1})[37]. Cells were cultured for 5 days in the differentiation condition, followed 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^{-1} unless otherwise indicated.

FACS analysis. Cells were stained as previously described[35]. For intracellular staining, CD4$^+$ T-cell cultures were stimulated for 5 h 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) according to the manufacturer's protocol. All FACS data were acquired on an LSRII flow cytometer (BD Biosciences) using FACSDiva software. Data were analysed using Flowjo software (Treestar).

Antibodies and dyes. Antibodies used for surface and intracellular staining of primary human cells included the following: 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), IFN-γ-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 the following: CD3ε-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 the following: CCR5 clones 45533, 45529, 45531, 45517, 45523, 45549, 3A9, 45502 and 45519 (ref. 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 to 2×10^5 cells per well), treated for 1 h at 37 °C and evaluated for morphological changes and ethidium bromide (EtBr) uptake by microscopy, or viability using CellTiter (Promega), CytotoxOne (Promega), cell scatter by 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, TAK-779 and vicriviroc), chemokines (CCL3, CCL4, 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 h in tryptic soy broth followed by washing in RPMI plus 10% FBS and normalization to 1×10^9 CFU per millilitre 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 pre-stained 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 longer than 6 h on the surface of Jurkat-R5 cells yet did not influence the killing of these cells by LukED (data not shown and Supplementary Fig. 13). Infected cells were incubated at 37 °C + 5% CO_2 for 4 h followed by the addition of lysostaphin to kill all bacteria. Samples were then analysed on a BD LSRII flow cytometer. Depletion of CCR5$^+$ compared with CCR5$^-$ cells was evaluated and shown graphically as the percentage of dead cells relative to controls with no toxin. For studies with maraviroc, the inhibitor was added to cells 30 min before the addition of bacteria as described above. Experiments were conducted three times in triplicate.

Generation of GFP fusion proteins. To generate recombinant N-terminal His_6–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-AATACTAA TATTGAAAAT-3′), *lukD*-F-SalI (5′-CCCC-GTCGAC-GCTCAACATATCA CA-3′), *lukE*-R-NotI (5′-CCCC-GCGGCCGC-tta-ATTATGTCCTTTCACTT TAATTTCGTG-3′) and *lukD*-R-NotI (5′-CCCC-GCGGCCGC-tta-TACTCC AGGATTAGTTTCTTTAGAATC-3′). Amplified sequences were subcloned into pET-41b (Novagen), resulting in a fusion of His_6–GFP with the N terminus of mature LukE or LukD. Recombinant plasmids were transformed into *Escherichia coli* DH5α and transformants selected by kanamycin resistance. Positive clones were transformed into *E. 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 h at room temperature. After incubation, lysates were centrifuged for 60 min at 12,350*g* and passed through a 0.22 μm filter before 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^{-1}) was incubated for 30 min on ice with sorted CD4$^+$CCR5$^+$ or CD4$^+$CCR5$^-$ T cells (5×10^4 cells per well) from three independent donors. Cells were gated as GFP positive compared with baseline fluorescence of untreated cells. A total of 50,000 events were collected in all conditions tested. Owing to the high amount 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. 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 (Supplementary Fig. 13).

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 carboxy (C)-terminal portion, respectively) perhaps explaining this phenomenon[38]. Alternatively, our staining protocols may not have sufficiently saturated 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 pre-incubated for 30 min with maraviroc (100 ng ml^{-1}), CCL5 (5 μg ml^{-1}), 3A9, 45531 or CXCR4 monoclonal antibodies (25 μg ml^{-1}) or buffer before addition of a lethal concentration of LukE–GFP + LukD to the cells (5–10 μg ml^{-1}). After treatment, cells were washed, re-suspended in fixation buffer (FACS buffer + 2% paraformaldehyde) for 15 min at room temperature, washed again, re-suspended in FACS buffer, and the fluorescence of bound toxin was monitored by flow cytometry. Cells are shown as the percentage that were 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 has also been used to detect ligand interactions with CXCR1 and CXCR2 (refs 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 solubilization scheme is known to retain conformationally specific antibody binding to both CCR5 and CXCR4 (ref. 15). Approximately 700 relative units (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 approximately 1,200 RU. LukE or LukD was diluted to 1.7 μM in running buffer containing 50 mM HEPES, pH 7.0, 150 mM NaCl, 0.02% CHS, 0.1% DDM and 0.1% Chaps and tested for binding in a threefold dilution series at a flow rate of 50 μl min^{-1}. Each concentration series was replicated twice 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 (approximately 2.0×10^7 cells per condition) in PBS + 1% Brij010 + Complete EDTA-free protease inhibitor cocktail (Roche). Solubilized CCR5 was then added to 25 μl 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 min at room temperature with 5 μg ml^{-1} of maraviroc, 10 μg ml^{-1} of each chemokine or 35 μg ml^{-1} 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 min, quenched with 20 mM Tris pH 8.0 for 15 min, washed four times in PBS + 1% Brij010 and boiled in 4× SDS boiling buffer. All samples were run on a 10% SDS–PAGE gel at 80 V, followed by transfer to nitrocellulose at 1 A for 1 h. Membranes were blocked in PBS + 0.01% Tween + 5% milk for 1 h 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 h followed by the addition of SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) for detection.

Measurement of CCR5 activation by calcium mobilization. CCR5 activation by calcium mobilization in cell lines and primary cells was assessed using the commercial dye Fluo4-AM (Invitrogen). Cells were labelled for 30 min at room temperature with 3 μM Fluo4 in Hanks' balanced salt solution, followed by three washes in Hanks' balanced salt solution and incubation at 37 °C for 30 min. Cells were analysed on a flow cytometer over time and, at 100 s, ligand (CCL3, CCL4, CCL5, 10 ng ml^{-1}) or LukE (10–20 μg ml^{-1}) 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^{-1}) or LukE (10–20 μg ml^{-1}) during the 30 min incubation at 37 °C described above. Graphs show the mean fluorescence of all events collected in 5 s 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) C57BL/6 WT or CCR5$^{-/-}$ mice (Taconic) were injected with 1×10^7 CFU of heat-killed *S. aureus* Newman Δ*lukED* intraperitoneally. Twenty-four hours later, mice were injected with an additional 1×10^7 CFU of the same strain. After another 24 h, mice were killed and peritoneal-elicited immune cells were lavaged with 7 ml of PBS followed by lysis of red blood cells in ACK lysing buffer and re-suspension in RPMI + 10% FBS. LukED was then added to cells as described above and incubated for 1 h at 37 °C with 5% CO_2. 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 percentages of dead cells shown are an average of cells isolated and intoxicated from three independent mice. Means and standard deviations are shown.

For experiments designed to measure *S. aureus* killing of CCR5$^+$ cells *in vivo*, female age-matched (4–6 weeks) C57BL/6 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 h later. Isolated cells were processed for FACS as described above and the viability of lymphocytes and macrophages was evaluated. The percentages of dead lymphocytes were averaged from 10 WT and 10 CCR5$^{-/-}$ animals; representative FACS plots are shown.

For murine systemic infections, female age-matched (4–6 weeks) C57BL/6 WT or CCR5$^{-/-}$ mice (Taconic) were infected with WT *S. aureus* Newman as previously described[7]. After 96 h, 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 h 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).

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