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CXCR1 Regulates Pulmonary Anti-*Pseudomonas* Host Defense

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Key Words
CXCR1 · Chemokine receptors · *Pseudomonas* · Neutrophils · Cystic fibrosis · Toll-like receptor 5 · Reactive oxygen species

Abstract
Pseudomonas aeruginosa is a key opportunistic pathogen causing disease in cystic fibrosis (CF) and other lung diseases such as chronic obstructive pulmonary disease (COPD). However, the pulmonary host defense mechanisms regulating anti-*P. aeruginosa* immunity remain incompletely understood. Here we demonstrate, by studying an airway *P. aeruginosa* infection model, in vivo bioluminescence imaging, neutrophil effector responses and human airway samples, that the chemokine receptor CXCR1 regulates pulmonary host defense against *P. aeruginosa*. Mechanistically, CXCR1 regulates anti-*Pseudomonas* neutrophil responses through modulation of reactive oxygen species and interference with Toll-like receptor 5 expression. These studies define CXCR1 as a novel, noncanonical chemokine receptor that regulates pulmonary anti-*Pseudomonas* host defense with broad implications for CF, COPD and other infectious lung diseases.
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Introduction

Chemokines recruit immune cells to inflammatory sites through binding to cognate G-protein-coupled chemokine receptors [1, 2]. Chemokine receptor-mediated leukocyte migration is of particular relevance for infectious diseases to provide a cellular shield against invading pathogens. Neutrophils are the key effector cells of the innate immune system to combat bacterial and fungal in-

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fections, equipped with a variety of antimicrobial weapons [3–5]. Their physiological importance is exemplified in patients with neutropenia who suffer from life-threatening invasive bacterial infections.

While CC chemokines mainly act on mononuclear cells, neutrophils are primarily recruited by CXC chemokines to diseased microenvironments, particularly those binding CXCR1 and/or CXCR2, which are both highly expressed on the surface of neutrophils. Cxcr2 knock-out mice and small-molecule inhibitors have provided evidence that Cxcr2 mediates neutrophil migration to sites of inflammation and infection [6]. In contrast, the role of Cxcr1 remains enigmatic, owing to the lack of specific chemical inhibitors and the availability of *Cxcr1*$^{-/-}$ mice [7]. Solving the crystal structure of CXCR1 has recently paved the way for the development of CXCR1-specific targeting compounds [8]. Several investigations have suggested a distinct and nonredundant role for CXCR1: the dual inhibition of CXCR1 and CXCR2 demonstrates substantial differences compared to selective inhibition of CXCR2 in different disease models, including cancer [7, 9], indicating a specific role for CXCR1. HIV has been found to bind to CXCR1 [10] and variants in the *CXCR1* gene have been reported to modulate HIV disease activity [11] and cystic fibrosis (CF) [12], a fatal pulmonary disease characterized by neutrophilic airway inflammation and infections with the Gram-negative opportunistic pathogen *Pseudomonas aeruginosa* [13–17]. Besides its role in CF, *P. aeruginosa* colonizes airways from patients with chronic obstructive pulmonary disease (COPD) and causes severe nosocomial lung infections in immunocompromised individuals and ventilated patients in intensive care units. An efficient host defense against *P. aeruginosa* correlates with functional neutrophil responses in humans [18] and in mice [19], supporting the concept that neutrophils represent a major effector cell type in host-*Pseudomonas* interactions. We demonstrated previously that loss of human CXCR1 on neutrophils in airway fluids from CF patients was associated with an impaired anti-*Pseudomonas* host defense ex vivo [20], suggesting indirectly that CXCR1 regulates host defense against *P. aeruginosa*.

Inspired by these previous findings, we sought to decipher the function of Cxcr1 in vivo in the context of pulmonary *P. aeruginosa* infection in a comprehensive manner using newly generated *Cxcr1* knock-out mice. Here we demonstrate that Cxcr1 acts as a noncanonical chemokine receptor by regulating neutrophil-*Pseudomonas* interactions.

Materials and Methods

Mouse Models

Cxcr1$^{-/-}$ mice were generated by P. Murphy/NIH. Briefly, the *Cxcr1* gene was cloned from C57Bl/6 mouse genomic DNA. The targeting construct was created by replacement of the *Cxcr1* gene with the neomycin resistance gene, homologous recombinants of the targeted deletion were generated in mouse embryonic stem cell line R1 and chimeric mice were produced by microinjection of recombinant embryonic stem cells into C57Bl/6 blastocysts. The *Cxcr1*$^{-/-}$ mice used in the experiments have been backcrossed to C57Bl/6 mice for 11 generations. All animal studies were reviewed and approved by the Regierungspräsidium, Tübingen, Germany, and the Animal Care and Use Committee (ACUC) at NIAID, NIH, USA, and were carried out according to the guidelines of the German Law for the Protection of Animal Life. Mice were bred at the animal facility of the Institute of Pharmacology (Tübingen). Age- and sex-matched litter-mate controls were used for all experiments.

Isolation of Bone Marrow Cells

Negative selection of neutrophils from whole bone marrow cells was performed by Magnetic Cell Separation (MACS; Miltenyi Biotec) according to a previously published protocol, which allows isolation of highly purified primary untouched mouse neutrophils [21]. Briefly, bone marrow cells were flushed from the femur of mice and stained with the following anti-mouse antibodies (all biotinylated): CD5 (BD Biosciences), CD45R/B220 (BioLegend), CD49b/DX5, CD117, F4/80 (all eBioscience) and Ter 119 (BioLegend). After incubation, the unbound antibodies were washed away. Bone marrow cells were then incubated with magnetic beads (MACS, Miltenyi Biotec) labeled with streptavidin. Bead-coupled bone marrow cells were removed by immunomagnetic separation following the manufacturer's recommendation, resulting in highly purified neutrophils.

Acute P. aeruginosa Infection

The mouse model of acute pulmonary *P. aeruginosa* infection was performed as published recently by our group [22]. Mice were infected intranasally with the *P. aeruginosa* bacterial strain (PAO1) with doses ranging from 2×10^5 to 2×10^6 CFU, using established procedures [22]. Infections were carried out under antagonizable anesthesia. An inoculum of PAO1 was administered intranasally (50 µl/nostril). After infection, mortality and body weight were monitored once a day over 1 week. In vivo bioluminescence imaging was performed using a luciferase-expressing *P. aeruginosa* strain as published previously [23] and infecting mice with and 5×10^7 CFU intratracheally. Bioluminescence imaging was performed 24 h after infection using an IVIS spectrum preclinical in vivo imaging read-out system (Perkin Elmer, Rodgau-Jügesheim, Germany).

Flow Cytometry

The panel of antibodies used to stain mouse bronchoalveolar lavage fluid (BALF), blood or bone marrow cells included F4/80 Pacific-blue (clone BM8), CD19-PerCP/Cy5.5 (clone 6D5) and CD3 brilliant violet (clone 17A2) from BioLegend, Siglec-F-PE (clone E50-2440), CD11b-PE-Cy7 (clone M1/70) and CD11c-APC-Cy7 (clone HL3) from BD Biosciences and Ly6G/C-APC (clone RB6-8C5) from eBioscience. Flow cytometry was performed using BD FACS Canto II flow cytometers (BD Biosciences), and data were

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analyzed with FlowJo software. A panel of autofluorescence, CD11b, CD11c, CD15, CD16 and MHC-II (all BD Biosciences) was used to analyze human neutrophils and dendritic cells. Anti-mouse Toll-like receptor 5 (TLR5)-Alexa Fluor 647 was from BioLegend (clone ACT5) and anti-human TLR5-FITC was from Imgenex/Novus (clone 19D759.2). Anti-mouse TLR4-PE (clone UT41) and anti-human TLR4-PE were from eBioscience (clone HTA125). Anti-human CXCR1-PE was from BD Biosciences (clone 5A12).

Reactive Oxygen Species Production

For the determination of reactive oxygen species (ROS) production, luminol-dependent chemiluminescence was used, which is an established method to measure intracellular ROS production [24]. For this purpose, *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ neutrophils were isolated from whole bone marrow as described above. The cells were used at a concentration of 2.5×10^5/100 µl in Hank's balanced salt solution (HBSS) with $Ca2^+$/$Mg2^+$ and immediately stimulated with recombinant chemokines (100 ng), flagellin (100 ng), PMA (200 nM) or PAO1 (2×10^6/ml) diluted in HBSS buffer containing 0.5 mM luminol (Sigma) and 120 µg/ml HRP. Chemiluminescence was measured at 37°C with a luminometer (Fluoroskan Ascent FL, ThermoScientific, Ascent software v2.6) for 19 min.

Neutrophil Transmigration

We compared the chemotactic migration of neutrophils isolated from the bone marrow of age-matched *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ mice towards recombinant Cxcr1/2 chemokines and the putative CXCR1/2-ligand acetylated proline-glycine-proline using an established in vitro transwell migration system with a 3-µm pore size, as described previously in detail [25, 26].

Bacteria

P. aeruginosa wild-type strains (PAO1) were used as published previously by our group [22]. Strains of the culture collection were streaked on agar plates and incubated at 37°C overnight. Colonies were then inoculated into tryptic soy broth overnight. The next day, a 1:100 dilution in tryptic soy broth was performed and bacteria were cultured at 37°C for 4 h. The optical density was measured at 600 nm. A GFP-expressing *P. aeruginosa* strain was used for microscopical imaging studies. A luciferase-expressing *P. aeruginosa* strain was used for bioimaging studies as published previously [23].

Neutrophil-Pseudomonas Interactions

Bone marrow-isolated *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ neutrophils (5×10^5) were coincubated with opsonized PAO1 at MOI 50 for 7 or 60 min (RPMI 1640, 10% FCS) with shaking at 50 rpm. The contents of each well were centrifuged (500 *g*, 10 min) and then washed twice with HBSS (Gibco). To assess extracellular killing, supernatants were harvested and plated in triplicate onto *Pseudomonas* isolation agar plates. To assess intracellular killing, neutrophil pellets were treated with gentamicin (400 µg/ml) to ensure that extracellular and cell surface-associated bacteria were removed. Cells were washed twice in PBS, lysed with ice-cold water, and then plated in triplicate onto *Pseudomonas* isolation agar plates. For microscopical analyses, bacteria were stained using the LIVE/DEAD BacLight™ bacterial viability kit according to the manufacturer's instructions (Life Technologies, ThermoFisher) utilizing mixtures of SYTO 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide. SYTO 9 labels bacteria with intact membranes and those with damaged membranes, while propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. After fixation (in 4% formaldehyde for 10 min at room temperature), total bacteria (living and dead) were quantified using fluorescence microscopy. For image acquisition, a Leica DMRE microscope and an HCX PL APO ×100 (NA 1.35) oil objective was used.

Asthma Mouse Models

Ovalbumin and house dust mite murine asthma models were performed as published previously by our group [27].

Bronchoalveolar Lavage Fluid

BALF was obtained and processed as published previously by our group [27].

Colony-Forming Units

Lungs were removed and homogenized in 1 ml PBS. Samples were serially diluted and plated on agar media for CFU counts overnight.

Optical Imaging

We measured in vivo luciferase expression using the IVIS spectrum optical imaging system. Noninvasive in vivo optical imaging measurements were conducted 24 h after intratracheal infection of *Cxcr1*$^{-/-}$ and *Cxcr1*$^{+/+}$ mice with luciferase-expressing *P. aeruginosa* bacteria (TBCF10839 isogenic mutant D8A6, 5×10^7 CFU/mouse) as published previously [23]. During measurements, mice were anesthetized by inhalation of isoflurane-O_2 (1.5% Forane, Abbott GmbH, Wiesbaden, Germany) and body temperature was maintained at 37°C. Regions of interest (ROIs) were drawn on the right and left lung of *Cxcr1*$^{-/-}$ and *Cxcr1*$^{+/+}$ mice, which allows the semiquantitative analysis of the average radiance [p/s/cm^2/sr] of the bioluminescence to be performed. Image analyses were performed using Living Image software (Perkin Elmer).

Experimental Peritonitis

Peritonitis was initiated by injection of 0.7 ml of sterile aged 4% thioglycollate broth intraperitoneally 4 h before harvest. The peritoneum was lavaged with 10 ml of PBS and cells were counted by flow cytometry as described above.

Patients

BALF was analyzed from patients with non-CF chronic bronchitis (n = 5, mean age 22 years, with no *P. aeruginosa* infection) or from CF patients with chronic *P. aeruginosa* infections (n = 5, mean age 20 years) as described previously in detail [20, 22]. CXCR1 and TLR5 surface expression levels (mean fluorescence intensity, MFI) were quantified on airway/BALF dendritic cells ($CD11b^+$ autofluorescence^{low}CD11c^{high}MHC-II^{high}) or neutrophils ($CD11b^+CD15^+CD16^+$) from patients with bronchitis or CF and *P. aeruginosa* infections. The study was approved by the Institutional Review Board/Ethical Committee of the University of Tübingen and meets the standards of the Declaration of Helsinki. Informed consent was obtained from all study subjects.

Statistics

All calculations were performed using GraphPad Prism v6.0 software. Statistical significance ($p < 0.05$) was determined using the Mann-Whitney U test or by ANOVA.

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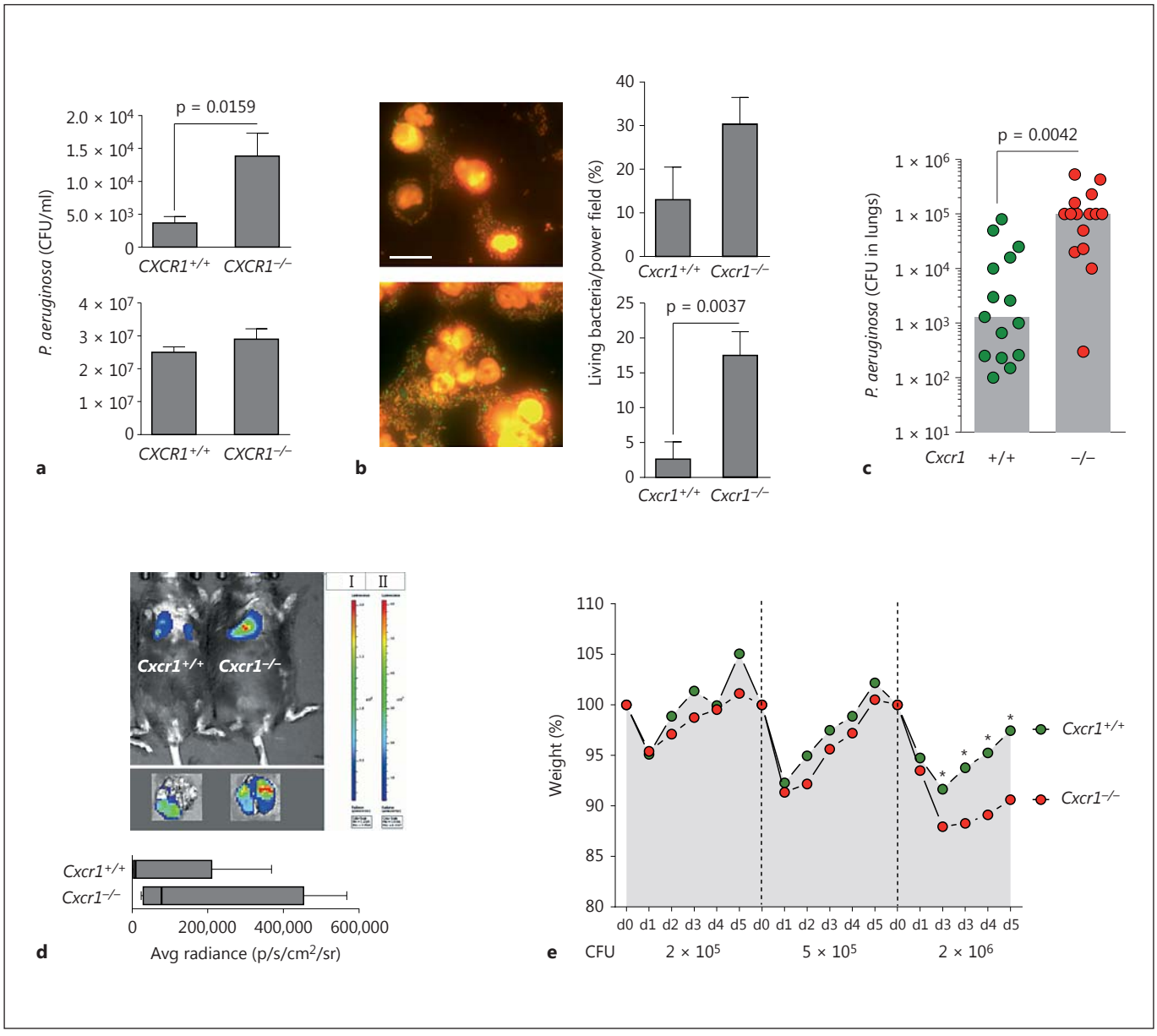

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Fig. 1. *P. aeruginosa* infections. **a** Intracellular killing (upper panel) and extracellular killing (lower panel). Bone marrow-isolated neutrophils from age- and sex-matched *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ mice were infected with PAO1 for 60 min at MOI 50, and CFU were counted. Data are from 5 independent experiments (means, SEMs). **b** GFP-PAO1 bacteria and bone marrow-isolated neutrophils from age- and sex-matched *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ mice were coincubated for 7 or 60 min at MOI 50. Bacteria were stained using a live/dead bacterial staining kit (left) and quantified microscopically (right). Representative images of *Cxcr1*$^{+/+}$ (upper left) and *Cxcr1*$^{-/-}$ (lower left) neutrophils 60 min after GFP-PAO1 infection are shown. Living bacteria are shown in green and dead bacteria in red. Scale bar: 10 µm. Bars show percentages of living bacteria at 7 min (upper right) and 60 min (lower right) at MOI 50. Data are from 5 independent experiments (means, SEMs). **c–e** Age- and sex-matched *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ mice were infected intranasally (**c**, **e**) or intratracheally (**d**) with PAO1 (except for **d**, luciferase-expressing TBCF10839 isogenic mutant D8A6). Lung CFU (**c**), bacterial lung in vivo clearance (**d**) and weight loss (**e**) were monitored. For these experiments, 2×10^6 CFU (**c**), 5×10^7 (**d**) or 2×10^5 to 2×10^6 CFU PAO1 (**e**) were inoculated. **c** Bars represent medians; CFU were quantified in lungs 12 h after infection. **d** Bioluminescence imaging was performed 24 h after the infection for 5 (*Cxcr1*$^{+/+}$) or 4 (*Cxcr1*$^{-/-}$) independent experiments; box and whiskers (range: min/max, quartiles, medians) are shown. **e** Mean weight points for days 0–5 (d0–d5) are shown. * $p < 0.05$ *Cxcr1*$^{+/+}$ versus *Cxcr1*$^{-/-}$ mice.

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Results

Cxcr1 Regulates Anti-Pseudomonas Host Defense

Since we observed in previous studies that CXCR1 is involved in the antibacterial host defense functions of human neutrophils in vitro and associated with *P. aeruginosa* infections in patients with CF ex vivo [12, 20], we systematically investigated the role of Cxcr1 by employing *P. aeruginosa* in vivo infection models. We started with quantifying the capacity of isolated *Cxcr1*$^{-/-}$ and *Cxcr1*$^{+/+}$ neutrophils to kill *P. aeruginosa* bacteria in vitro by using traditional CFU assays (fig. 1a) as well as by bacterial live/dead imaging methods (fig. 1b). These studies demonstrated that *Cxcr1*$^{-/-}$ neutrophils were impaired in intracellular, but not extracellular killing of *P. aeruginosa* bacteria compared to their wild-type counterparts (fig. 1a, b). Next, we interrogated this bacterial clearance defect in vivo and used a well-established *P. aeruginosa* lung infection model. These studies confirmed our in vitro findings and demonstrated that *Cxcr1*$^{-/-}$ mice were impaired in clearing *P. aeruginosa* from their lungs with an average of 100-fold higher bacterial loads in the lungs of *Cxcr1*$^{-/-}$ mice compared to age- and gender-matched *Cxcr1*$^{+/+}$ mice (fig. 1c). To visualize and quantify this impairment in bacterial clearance in a more refined spatiotemporal manner, we used a recently described in vivo bioluminescence imaging system (fig. 1d) [23]. These studies confirmed that *Cxcr1*$^{-/-}$ mice were impaired in clearing *P. aeruginosa* from their pulmonary compartment compared to age- and gender-matched *Cxcr1*$^{+/+}$ mice (fig. 1d). By inoculating different increasing doses of *P. aeruginosa* into the airways, we found that this clearance defect led to a dose-dependent higher morbidity (as quantified by weight loss) in *Cxcr1*$^{-/-}$ mice compared to age- and gender-matched *Cxcr1*$^{+/+}$ mice (fig. 1e), with significant differences for the *P. aeruginosa* CFU of 2×10^6, but without significant differences at lower CFU.

To exclude the possibility that the impaired pulmonary host-defense in *Cxcr1*$^{-/-}$ mice is a mere reflection of an underlying basal defect in neutrophil homeostasis, we quantified total cells and neutrophils in bone marrow and peripheral blood and studied their apoptosis and necrosis ex vivo. These studies demonstrated that neither total cell counts nor total neutrophil counts in bone marrow (online suppl. fig. S1A; for all online suppl. material, see www.karger.com/doi/10.1159/000444125) or peripheral blood (online suppl. fig. S1B) differed significantly between age-matched *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ mice. Percentages of neutrophils in bone marrow (online suppl. fig. S1A) were significantly increased in *Cxcr1*$^{-/-}$ compared to *Cxcr1*$^{+/+}$ mice, but there was no significant difference in neutrophil percentages in peripheral blood between *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ mice (online suppl. fig. S1B). Neutrophil survival studies demonstrated that neutrophil overall survival or neutrophil apoptosis did not differ between wild-type and knock-out animals, while there was a tendency towards less necrosis in *Cxcr1*$^{-/-}$ neutrophils compared to *Cxcr1*$^{+/+}$ neutrophils (online suppl. fig. S1C).

Cxcr1 Modulates ROS Production

Next, we sought to dissect the mechanisms by which Cxcr1 regulates pulmonary anti-*P. aeruginosa* host defenses. Since the production of ROS is a key mechanism by which neutrophils kill bacteria, we compared ROS production between *Cxcr1*$^{-/-}$ and *Cxcr1*$^{+/+}$ mice. These studies demonstrated that bone marrow cells isolated from *Cxcr1*$^{-/-}$ mice produced lower amounts of ROS upon stimulation with the recombinant chemokine CXCL8 compared to bone marrow cells from *Cxcr1*$^{+/+}$ mice (fig. 2a). Since *P. aeruginosa* is a flagellated bacterium and several previous studies have shown that flagellin represents a key pathogen-associated molecular pattern, which is essential for innate immune cell activation, we tested the effect of flagellin on ROS production by *Cxcr1*$^{-/-}$ and *Cxcr1*$^{+/+}$ bone marrow myeloid cells. These studies demonstrated that, in line with CXCL8, *Cxcr1*$^{-/-}$ myeloid cells also showed impaired ROS production upon stimulation with bacterial flagellin compared to bone marrow myeloid cells from *Cxcr1*$^{+/+}$ mice (fig. 2a). To further investigate whether these ROS changes in the myeloid compartment in *Cxcr1*$^{+/+}$ mice were specifically due to neutrophils, we isolated bone marrow neutrophils. These studies consistently demonstrated that highly purified isolated *Cxcr1*$^{-/-}$ neutrophils, similar to nonfractionated bone marrow cells, produced lower amounts of ROS compared to their *Cxcr1*$^{+/+}$ counterparts upon stimulation with the chemokine CXCL8 (fig. 2b), whole PAO1 or bacterial flagellin. We further extended these studies and included other Cxcr1/Cxcr2 chemokine receptor ligands into these assays. These studies demonstrated that *Cxcr1*$^{-/-}$ neutrophils showed a similar, albeit to a lesser extent than CXCL8, ROS impairment towards the putative Cxcr1 ligand Cxcl5 (LIX) as well as the recombinant chemokines Cxcl1 (KC) and Cxcl2 (MIP-2) (fig. 2b). Comparative analyses at the kinetic end point after neutrophil stimulation for all applied stimulants showed that ROS production by *Cxcr1*$^{-/-}$ neutrophils was significantly impaired towards CXCL8, Cxcl1, Cxcl5, flagellin and *P. aeruginosa*, but not Cxcl2 stimulation (fig. 2c, d). In

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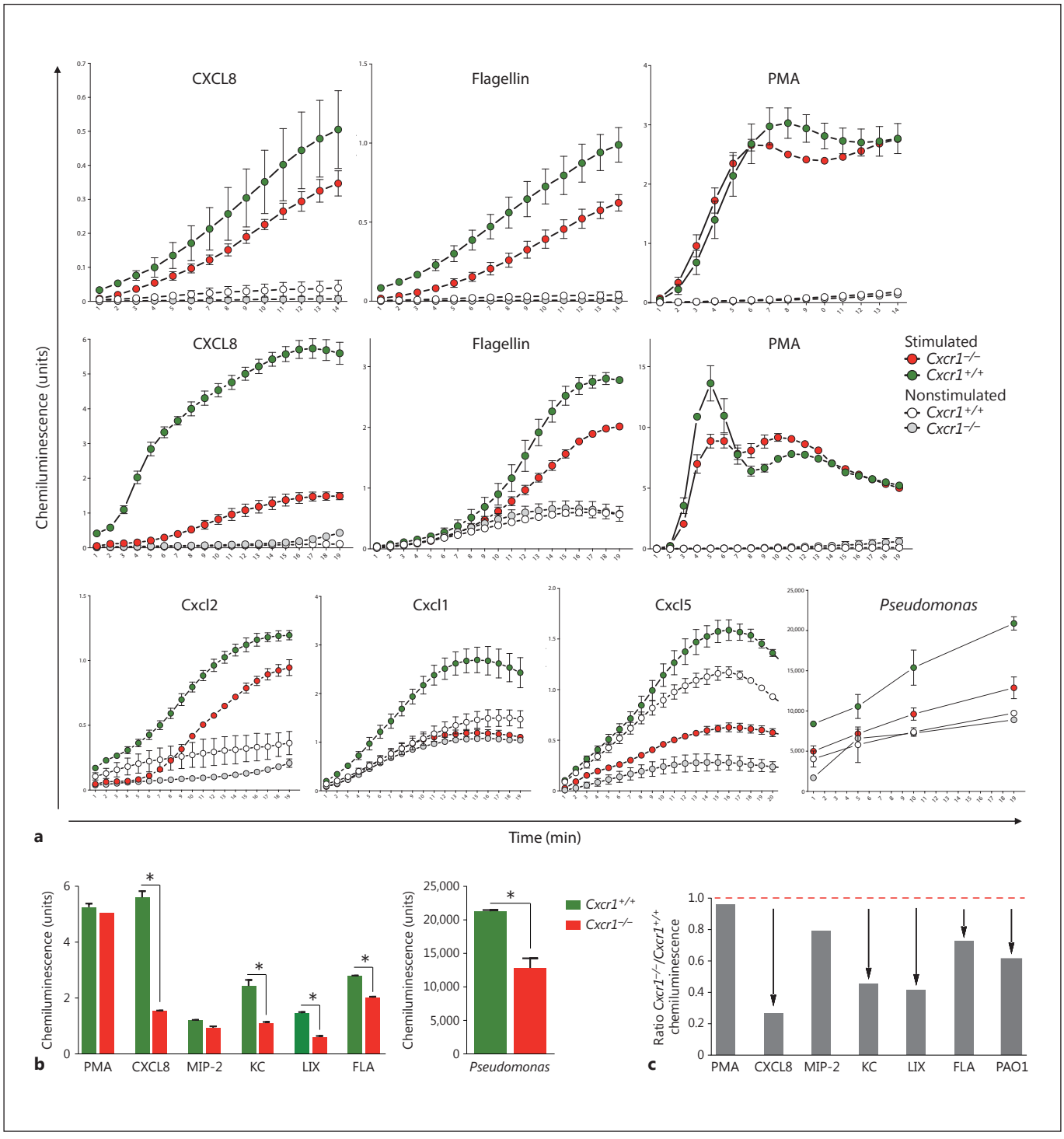

Fig. 2 ROS. Total bone marrow cells (**a**, top row) or neutrophils isolated from bone marrow (**a**, middle and bottom rows) of age- and sex-matched *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ mice were stimulated with recombinant chemokines (all at 100 ng), flagellin (100 ng), PMA (200 nM) or PAO1 bacteria (2×10^6/ml). ROS production was measured using chemiluminescence. Bar graph plots of stimulated *Cxcr1*$^{+/+}$ versus *Cxcr1*$^{-/-}$ neutrophils, as absolute chemiluminescence units (medians, IQRs, **b**) or ratio of *Cxcr1*$^{-/-}$ to *Cxcr1*$^{+/+}$ neutrophils (**c**). Asterisks (**b**) or arrows (**c**) denote $p < 0.05$, *Cxcr1*$^{+/+}$ versus *Cxcr1*$^{-/-}$ neutrophils. FLA = Flagellin; KC = Cxcl1; LIX = Cxcr1 ligand Cxcl5; MIP-2 = Cxcl2.

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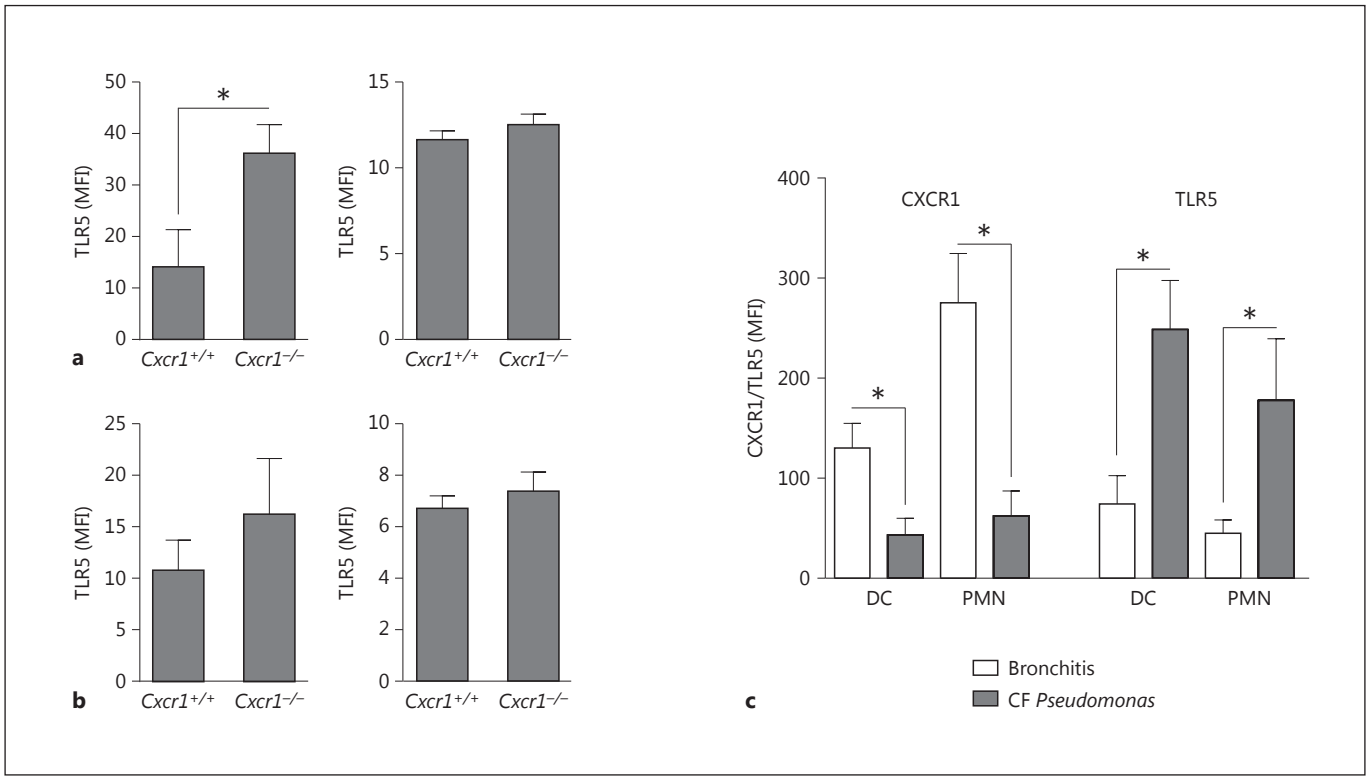

Fig. 3. TLR5 surface expression (MFI) was quantified on airway/BALF (**a**) or bone marrow (**b**) dendritic cells (left panel) or neutrophils (right panel) from age- and sex-matched *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ mice. * $p < 0.05$ *Cxcr1*$^{+/+}$ versus *Cxcr1*$^{-/-}$ neutrophils. **c** CXCR1 and TLR5 surface expression (MFI) was quantified on airway/BALF dendritic cells (DC) or neutrophils (PMN) from patients with bronchitis or CF and *P. aeruginosa* infections. * $p < 0.05$ bronchitis versus CF *Pseudomonas*.

contrast to specific stimulation with chemokines, *P. aeruginosa* or bacterial flagellin, nonspecific stimulation with the phorbol ester PMA did not elicit a differential ROS production in unfractionated bone marrow myeloid cells (fig. 2a) or isolated neutrophils from *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ mice (fig. 2b–d), suggesting that Cxcr1-mediated ROS production is chemokine- and *P. aeruginosa*-dependent, rather than reflecting a redundant and universal basal ROS impairment. Collectively, these studies demonstrate that Cxcr1 mediates anti-*P. aeruginosa* host defense in neutrophils through a mechanism involving ROS production.

Cxcr1 Modulates TLR5 Expression

Since generation of ROS is a universal antimicrobial host defense mechanism and not specifically related to *P. aeruginosa* infections, we next investigated the role of TLR5 as a key pattern recognition receptor in *P. aeruginosa*-associated lung infections, such as CF [28–32]. These studies demonstrated that genetic abrogation of *Cxcr1* led to an upregulation of Tlr5 surface expression on airway dendritic cells upon acute *P. aeruginosa* infection (fig. 3a), whereas no changes were found on airway neutrophils (fig. 3a) or bone marrow dendritic cells or neutrophils (fig. 3b). To assess the human disease relevance of these findings, we analyzed TLR5 expression on airway immune cells in patients with CF and *P. aeruginosa* infection, characterized by a loss of CXCR1 [20]. These investigations demonstrated that both dendritic cells and neutrophils (fig. 3c) in the airways of CF patients with *P. aeruginosa* infection showed a loss of CXCR1 paralleled by a concomitant upregulation of TLR5 surface expression on the respective cell types compared to non-CF bronchitis patients without *P. aeruginosa* infection. No significant difference in TLR4 expression levels was noted between *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ neutrophils ($p > 0.05$) or between CF patients with *P. aeruginosa* infection and non-CF bronchitis patients without *P. aeruginosa* infection ($p > 0.05$, data not shown). When viewed in combination, these studies in-

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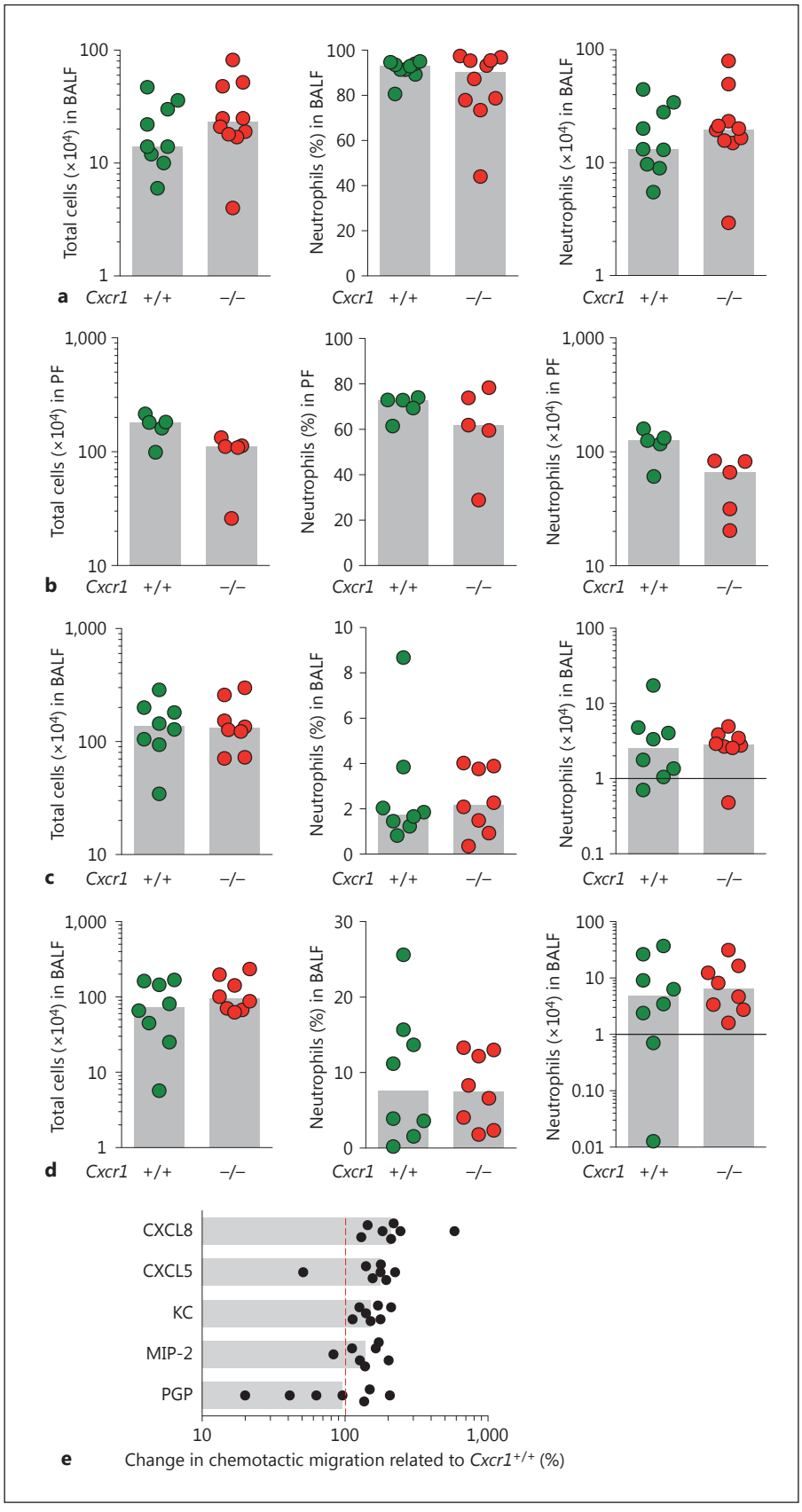

Fig. 4. Neutrophil recruitment was evaluated in vivo (**a–d**) and in vitro (**e**). **a–d** Total cells (left panel), percentage of neutrophils (middle panel) or total numbers of neutrophils (right panel) were quantified in age- and sex-matched *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ mice using murine models of PAO1 lung infection (**a**), thioglycollate-induced peritonitis (**b**), ovalbumin-induced allergic airway inflammation (**c**) and house dust mite-induced allergic airway inflammation (**d**). PF = Peritoneal fluid. **e** Neutrophils isolated from bone marrow of age- and sex-matched *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ mice were stimulated with the indicated chemokines (100 ng, acetylated proline-glycine-proline, 1 μM) and underwent a transwell-based chemotactic migration assay for 120 min. The relative migration of *Cxcr1*$^{-/-}$ compared to *Cxcr1*$^{+/+}$ neutrophils is shown (= 100%, red line). All bars represent medians. KC = Cxcl1; MIP-2 = Cxcl2; PGP = proline-glycine-proline.

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dicate that a loss of CXCR1 in mice or human CF patients is associated with an upregulation of TLR5 expression on immune cells in the airways.

Cxcr1 Is Dispensable for Neutrophil Migration

Since CXCR1 belongs to the family of chemokine receptors that primarily orchestrate leukocyte recruitment, we analyzed whether neutrophil migration is impaired in inflammatory settings in *Cxcr1*$^{-/-}$ mice in vivo and by using isolated *Cxcr1*$^{-/-}$ neutrophils in vitro. To assess neutrophil migration in an in vivo setting, we compared neutrophil transmigration into the bronchoalveolar space upon *P. aeruginosa* lung infection in *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ mice. These studies demonstrated that neutrophils from *Cxcr1*$^{-/-}$ mice were equally potent in bronchoalveolar infiltration compared to their wild-type counterparts (fig. 4a). To investigate whether this phenomenon is restricted to the pulmonary compartment or is common at inflammatory sites in general, we compared *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ neutrophil transmigration in a thioglycollate-induced peritonitis model. These studies demonstrated a tendency of *Cxcr1*$^{-/-}$ neutrophils towards a lower peritoneal infiltration compared to matched *Cxcr1*$^{+/+}$ neutrophils, which was, however, not statistically significant (fig. 4b). To further assess whether Cxcr1 modulates noninfectious neutrophil transmigration differentially, we compared *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ neutrophil bronchoalveolar recruitment in 2 different models of allergic airway inflammation, namely ovalbumin-induced (fig. 4c) and house dust mite-induced airway inflammation (fig. 4d). These studies consistently demonstrated that *Cxcr1*$^{-/-}$ neutrophils showed no impairment in migrating into the bronchoalveolar compartment upon noninfectious allergic airway inflammation.

To precisely investigate the migratory characteristics of *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ neutrophils in vitro, we compared the chemotactic migration of neutrophils isolated from bone marrow from age-matched *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ mice towards Cxcr1/2 chemokines using a transwell migration system [25, 26]. Consistent with our in vivo findings, these in vitro studies demonstrated that *Cxcr1*$^{-/-}$ neutrophils were not impaired in migrating to CXCR1/2 chemokines, but even rather showed a tendency towards a more efficient chemotactic migration, particularly towards recombinant CXCL8, which has been previously described to activate Cxcr1 [33] (fig. 4e). Taken together, these studies demonstrate that Cxcr1 is dispensable for neutrophil migration in infectious and noninfectious conditions in vitro and in vivo.

Discussion

While traditionally chemokines and their receptors have been limited to their role in leukocyte recruitment, a growing body of evidence suggests that these G-protein-coupled receptors have much broader roles in regulating physiological and pathophysiological processes at several levels [1, 34, 35]. Here, we demonstrate that the chemokine receptor Cxcr1 is dispensable for neutrophil transmigration under infectious and sterile inflammatory conditions, but regulates ROS production and TLR5 surface expression as critical bacterial sensing (TLR5) and killing (ROS) mechanisms. This cellular effector mechanism has disease relevance, as genetic deficiency of *Cxcr1* increases the susceptibility towards *P. aeruginosa* infections, which are a significant mortality factor in patients with CF or individuals with immunosuppression or on ventilation.

A variety of studies have analyzed the role of CXCR2 in vivo, supporting the concept that CXCR2 mediates neutrophil migration to sites of inflammation [6, 36]. In contrast, the role of Cxcr1 in vivo has not been defined so far due to a lack of a murine knock-out mouse model. Here, we provide evidence, inspired by our previous human data and mechanistic in vitro studies [12, 20, 37], that *Cxcr1*$^{-/-}$ mice show an impaired pulmonary neutrophilic host defense against *P. aeruginosa*, mediated through a ROS- and TLR5-mediated mechanism. Moreover, our studies demonstrate that neutrophil migration to the pulmonary site of inflammation and infection is not impaired in *Cxcr1*$^{-/-}$ mice. Therefore, in contrast to CXCR2, which is primarily involved in neutrophil recruitment, Cxcr1, based on our data, is dispensable for neutrophil recruitment but mediates neutrophil effector functions by a mechanism involving ROS and TLR5. The underlying subcellular pathways remain to be elucidated, but our studies highlight the notion that chemokine receptors play a broader role in leukocyte biology than mediating cell migration by regulating antimicrobial effector and bacterial sensing mechanisms.

Previous studies on human patients with CF who suffer from chronic *P. aeruginosa* infection showed that *CXCR1/2* polymorphisms modulate the disease outcome of these patients [12]. Furthermore, previous studies at the cellular and ex vivo level provided indirect evidence that human IL-8R1 (CXCR1), but not IL-8R2 (CXCR2), is involved in NADPH oxidase and phospholipase D activation [38] and anti-*P. aeruginosa* host defense functions [20]. Our in vivo studies confirm and extend these findings by demonstrating that Cxcr1 plays a novel and noncanonical role in host-pathogen interactions by regu-

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lating neutrophil ROS effector response without affecting neutrophil recruitment, homeostasis or survival. Since ROS are essential for host defense against a variety of bacterial pathogens beyond *P. aeruginosa*, these findings may have broad implications for infectious diseases. Moreover, ROS have been implicated in the proinflammatory harmful activities in chronic disease conditions, such as cardiovascular and chronic lung diseases [39, 40], suggesting that interfering with CXCR1 pharmacologically may have the potential to dampen oxidative stress in chronic diseases in vivo.

In contrast to CXCR2, which mediates neutrophil transmigration in vitro and in vivo, our studies strongly support the notion that Cxcr1 does not mediate neutrophil migration. Conversely, *Cxcr1*$^{-/-}$ neutrophils showed even a higher migratory potential in transwell assays in vitro, mainly towards the 2 putative Cxcr1 ligands Cxcl5 and CXCL8. While we have currently no explanation for this observation, we are tempted to speculate that, in line with previous findings in other CXC chemokine receptors, Cxcr1 may serve as a negative regulator of Cxcl5- and/or CXCL8-triggered leukocyte migration by acting as decoy receptor. An alternative hypothesis is that of CXCR1/CXCR2 heterodimerization [41], suggesting that the loss of Cxcr1 could have an impact on Cxcr2 receptor surface expression on neutrophils. While we observed no significant differences in Cxcr2 MFI surface expression levels between *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ neutrophils (online suppl. fig. 2A), we found moderately increased percentages of Cxcr2^{+} neutrophils in the bone marrow of *Cxcr1*$^{-/-}$ compared to *Cxcr1*$^{+/+}$ mice (online suppl. fig. 2B), suggesting that the observed tendency towards a higher migratory capacity of *Cxcr1*$^{-/-}$ neutrophils in vitro could be due to a relative increase of Cxcr2^{+} neutrophils. Moreover, the loss of Cxcr1 receptors probably also increases the availability of Cxcr1/Cxcr2 ligands for Cxcr2 binding. However, in-depth biochemical and pharmacological analyses would be required to dissect this interaction, which was beyond the scope of this study.

Beyond the involvement of ROS and Tlr5, the precise cellular mechanism(s) by which Cxcr1 is involved in anti-*P. aeruginosa* neutrophil activities remains elusive. Our in vitro read-out system provided evidence for an impaired intracellular, but not extracellular, killing capacity of *Cxcr1*$^{-/-}$ neutrophils. Based on this finding and the fact that we did not observe signs of neutrophil extracellular trap formation in our short-term in vitro assay systems, we speculate that the Cxcr1-mediated antibacterial mechanism in our experimental system mainly involved ROS-dependent intracellular phagocytic killing. Since our studies were, however, restricted to *P. aeruginosa*, we cannot exclude the possibility that Cxcr1 differentially regulates antimicrobial effector mechanisms depending on the type of pathogen.

We found that *Cxcr1*$^{-/-}$ neutrophils were impaired in generating ROS in response to *P. aeruginosa* or bacterial flagellin, whereas no difference was observed in response to nonbacterial generic ROS activation by stimulation with PMA. These findings inspired us to investigate the interaction of CXCR1 and flagellin-sensing pathways in mice and CF patients with *P. aeruginosa* infections. Our studies indicated that a loss of CXCR1 in mice or human CF patients leads to an upregulation of the flagellin receptor TLR5 on the surface of immune cells in the infected airways. Based on these findings, we speculate that in neutrophil-*P. aeruginosa* interactions, CXCR1 collaborates with TLR5 to efficiently combat *P. aeruginosa*. When CXCR1 is abrogated, this bacterial sensing (TLR5) and killing (ROS) mechanism is impaired, leading to uncontrolled *P. aeruginosa* infections. Previous studies provided evidence for a TLR-chemokine receptor cross-talk in lipid rafts in monocytes and macrophages in response to the Gram-negative bacterium *Porphyromonas gingivalis* [42]. The precise subcellular CXCR1/TLR5 interaction mechanisms in neutrophil-*P. aeruginosa* host defense remain to be dissected in future studies.

A clear limitation of our study, regarding the human disease translation, is the *P. aeruginosa* infection model that we used, which reflects acute pneumonia rather than chronic infective disease as is found in CF or COPD airways. However, our primary aim was to define the role of Cxcr1 in anti-*P. aeruginosa* host defense as a proof-of-principle in vivo using a well-established infection model, while chronic infection/colonization models were beyond our scope and have to be investigated in future studies. Some further aspects of our study remained poorly defined and should be investigated in the future. (1) What is the underlying reason for the difference in bone marrow neutrophils between *Cxcr1*$^{-/-}$ and *Cxcr1*$^{+/+}$ mice? (2) Are differential chemokines released by *Cxcr1*$^{-/-}$ and *Cxcr1*$^{+/+}$ neutrophils that could directly (as shown previously in the human system for the chemokine CXCL6 [43, 44]) or indirectly (through auto/paracrine effects) affect bacterial killing? (3) What is the underlying mechanism behind the upregulation of Tlr5 on *Cxcr1*$^{-/-}$ dendritic cells? Are TLR5-ROS interactions involved? [45] (4) What are the intracellular Cxcr1-downstream pathways regulating neutrophil effector responses?

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In summary, our studies demonstrate that CXCR1 regulates anti-*P. aeruginosa* host defense through a ROS- and TLR5-mediated mechanism. Beyond CF, these findings may have broader implications for other *P. aeruginosa*-associated pulmonary disease conditions, such as COPD or ventilator-associated pneumonia. Moreover, our results point towards caution in targeting chemokine receptors [46] without considering their so far underappreciated antimicrobial roles.

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Disclosure Statement

The authors have declared that no conflict of interest exists.

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