Author Manuscript

HHS Public Access
Author manuscript
J Innate Immun. Author manuscript; available in PMC 2017 March 08.

Published in final edited form as:
J Innate Immun. 2016 ; 8(4): 362–373. doi:10.1159/000444125.

CXCR1 regulates pulmonary anti-*Pseudomonas* host defense

M. Carevic[1], **H. Öz**[1], **K. Fuchs**[2], **J. Laval**[1], **C. Schroth**[1], **N. Frey**[1], **A. Hector**[1], **T. Bilich**[1], **M. Haug**[3], **A. Schmidt**[3], **S. E. Autenrieth**[4], **K. Bucher**[5], **S. Beer-Hammer**[5], **A. Gaggar**[6], **M. Kneilling**[2], **C. Benarafa**[7], **J. Gao**[8], **P. Murphy**[8], **S. Schwarz**[3], **B. Moepps**[9], and **D. Hartl**[1]

[1]Children's Hospital and Interdisciplinary Center for Infectious Diseases, University of Tübingen, Tübingen, Germany [2]Werner Siemens Imaging Center, Department of Preclinical Imaging and Radiopharmacy, Eberhard Karls University Tübingen, Röntgenweg 13, Tübingen, Germany [3]Department of Medical Microbiology, University of Tübingen, Tübingen, Germany [4]Department of Internal Medicine II, University of Tübingen, Tübingen, Germany [5]Department of Pharmacology and Experimental Therapy and Interfaculty Center of Pharmacogenomics and Drug Research, University of Tübingen, Tübingen, Germany [6]Department of Medicine and Division of Pulmonary, Allergy, and Critical Care Medicine, University of Alabama at Birmingham, Birmingham, Alabama, United States of America [7]Theodor Kocher Institute, University of Bern, Bern, Switzerland [8]Molecular Signaling Section, Laboratory of Molecular Immunology National institute of Allergic and Infectious Diseases, NIH, Bethesda, MD, USA [9]Institute of Pharmacology and Toxicology, Ulm University Medical Center, Ulm, Germany

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-*Pseudomonas aeruginosa* immunity remain incompletely understood. Here we demonstrate, by studying an airway *Pseudomonas aeruginosa* infection model, *in vivo* bioluminescence imaging, neutrophil effector responses and human airway samples, that the chemokine receptor CXCR1 regulates pulmonary host defense against *Pseudomonas aeruginosa*. Mechanistically, CXCR1 regulated 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 non-canonical chemokine receptor that regulates pulmonary anti-Pseudomonas host defense with broad implications for CF, COPD and other infectious lung diseases.

Correspondence: Prof. Dr. Dominik Hartl, Children's Hospital and Interdisciplinary Center for Infectious Diseases, University of Tübingen, Tübingen, Germany, ; Email: dominik.hartl@med.uni-tuebingen.de, Phone: +49707129-81460, Fax: +49707129-4449

D.H. and B.M. designed the study, supervised experiments and wrote the manuscript. M.C. and H.Ö. performed murine in vitro and in vivo studies. K.F. and M.K. performed and analyzed bioluminescence imaging experiments. J.L. performed in vitro neutrophil experiments. T.B. performed in vitro bacterial killing studies. C.S. performed in vitro immune experiments. J.J.-K. performed RT-PCR studies. N.F. performed imaging experiments. A.H. and C.B. co-supervised the study and wrote the paper. M.H., A.S. and S.S. contributed to in vitro bacterial killing studies. S.B.-H.., K.B. and S.A. contributed to in vivo studies. A.G. contributed to the PGP studies. J.G. and P.M. generated the *Cxcr1*$^{-/-}$ mice and contributed to the murine studies.

Conflict of interest: The authors have declared that no conflict of interest exists.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Keywords

CXCR1; chemokines; chemokine receptors; bacteria; Pseudomonas; neutrophil; lung; cystic fibrosis; TLR5; ROS

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 infections, equipped with a variety of anti-microbial 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] and several investigations have suggested a distinct and non-redundant role for CXCR1: The dual inhibition of CXCR1 and CXCR2 demonstrated substantial differences compared to selective inhibition of CXCR2 in different disease models, including cancer [7,9], indicating a specific role for CXCR1. HIV was 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) lung disease [12], a fatal pulmonary disease characterized by neutrophilic airway inflammation and infections with the Gram-negative opportunistic pathogen *Pseudomonas aeruginosa* [13–17]. Besides CF, *Pseudomonas 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 *Pseudomonas aeruginosa* correlates with functional neutrophil responses in man [18] and 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 impaired anti-*Pseudomonas* host defense *ex vivo* [20], suggesting indirectly that CXCR1 regulates host defense against *Pseudomonas aeruginosa*.

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

Author Manuscript

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 *Cxcr1* gene with neomycin resistance gene, homologous recombinants of the targeted deletion were generated in mouse embryonic stem (ES) cell line R1, and chimeric mice were produced by microinjection of recombinant ES 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 Animal Care and Use Committee (ACUC) at NIAID, NIH, USA, and were carried out according to the guidelines of the German law of protection of animal life. Mice were bred at the animal facility of the Institute of Pharmacology (Tübingen) under specific pathogen–free conditions. Age- and sex-matched littermate 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 Seperation (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 (eBiosciences), CD117 (eBiosciences), F4/80 (eBiosciences) and Ter 119 (Biolegend). After incubation the unbound antibodies were washed away. Bone marrow cells were then incubated with magnetic beads (MACS, Miltenyi Biotec, Germany) labeled with streptavidin. Bead coupled bone marrow cells were removed by immunomagnetic separation following the manufacturer's recommendation resulting in highly purified neutrophils.

Acute Pseudomonas aeruginosa infection

The mouse model of acute pulmonary *Pseudomonas aeruginosa* infection was performed as published recently by our group [22]. Mice were infected intranasally with *Pseudomonas aeruginosa* (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 *Pseudomonas aeruginosa* bacteria was administered intranasally (50μl/nostril). After infection, mortality and body weight were monitored once a day over one week. *In vivo* bioluminescence imaging was performed using a luciferase-expressing *Pseudomonas aeruginosa* strain as published previously [23] and infecting mice with and 5×10^7 CFU intratracheally. Bioluminescence imaging was performed 24h post infection using an IVIS spectrum preclinical *in vivo* imaging read-out system (Perkin Elmer).

Flow Cytometry

The panel of antibodies used to stain mouse 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) (all from Biolegend); Siglec-F-PE (clone E50-2440), CD11b-PE-Cy7 (clone M1/70) and CD11c-APC-Cy7 (clone HL3) (from BD Biosciences); Ly6G/C-APC (clone

Author Manuscript

RB6-8C5) from eBioscience. Flow cytometry was performed using BD FACS Canto II flow cytometers (BD Biosciences), and data were analysed 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 TLR5-Alexa Flour 647 was from Biolegend (clone ACT5), anti-human TLR5-FITC was from Imgenex/Novus (clone 19D759.2). Anti-mouse TLR4-PE was from eBioscience (clone UT41), anti-human TLR4-PE was from eBioscience (clone HTA125). Anti-human CXCR1-PE was from BD Biosciences (clone 5A12).

ROS Production

For determination of 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 /100μl in HBSS with $Ca2^+$/$Mg2^+$ and immediately stimulated with recombinant chemokines (100 ng), flagellin (100 ng), PMA (200 nM) or *Pseudomonas aeruginosa* (PAO1) bacteria (2×10^6/ml) diluted in HBSS buffer containing 0.5mM luminol (SIGMA) and 120 μg/ml HRP (Horseradish peroxidase). Chemiluminescence (CL) was measured at 37°C with a Luminometer (Fluoroskan Ascent FL, Thermo Scientific, Ascent Software Version 2.6) for 19 min.

Neutrophil transmigration

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

Bacteria

Pseudomonas 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 over night. 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 hours. The optical density was measured at 600 nm. A GFP-expressing *Pseudomonas aeruginosa* strain was used for microscopical imaging studies. A luciferase-expressing *Pseudomonas 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 co-incubated with opsonized *Pseudomonas aeruginosa* bacteria (PAO1) at MOI50 for 7 or 60 minutes (RPMI1640, 10% FCS) with shaking at 50 rpm. The contents of each well were centrifuged (500×g, 10 min) and then washed twice with Hank's Balanced Salt Solution (HBSS) (Gibco). To assess extracellular killing, supernatants were harvested and plated in triplicate onto *Pseudomonas* isolation agar plates. To assess intracellular killing, neutrophil pellets

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

were treated with gentamicin (400 μg/mL) to ensure that extracellular and cell surface-associated bacteria were removed. Then 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, Thermo Fisher) 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 (4% FA, 10 min RT), total bacteria (living and dead) were quantified using fluorescence microscopy. For image acquisition, a Leica DMRE microscope and a HCX PL APO 100x (NA 1.35) oil objective was used.

Asthma mouse models

OVA and HDM murine asthma models were performed as published previously by our group [27].

BALF

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

CFUs

Lungs were removed and homogenized in 1ml 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 (OI) system (Perkin Elmer, Rodgau-Jügesheim, Germany). Non-invasive *in vivo* OI measurements were conducted 24h after intratracheal infection of *Cxcr1*$^{-/-}$ and *Cxcr1*$^{+/+}$ mice with luciferase-expressing *Pseudomonas aeruginosa* bacteria (TBCF10839 isogenic mutant D8A6, 5×10^{7} CFU/mouse) as published previously [23]. During measurements, mice were anesthetized by inhalation of isoflurane-O2 (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 to perform semi-quantitative analysis of the average radiance [p/s/cm2/sr] of the bioluminescence. 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 (TG) broth intraperitoneally 4 hours before harvest. The peritoneum was lavaged with 10 mL of PBS and cells were counted by flow cytometry as described above.

Patients

BAL was analyzed from patients with non-CF chronic bronchitis (n=5, mean age: 22 years, no *Pseudomonas aeruginosa* infection) or CF patients and chronic *Pseudomonas aeruginosa*

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

infections (n=5, mean age: 20 years) as described previously in detail [20,22]. CXCR1 and TLR5 surface expression levels (MFI) were quantified on airway/BAL dendritic cells ($CD11b^{+}$ autofluorescence^{low}CD11c^{high}MHC-II^{high}) or neutrophils ($CD11b^{+}CD15^{+}CD16^{+}$) from patients with bronchitis or CF and *Pseudomonas aeruginosa* infections. This 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 Graph Pad Prism 6.0 software. Statistical significance ($P<0.05$) was determined using the Wilcoxon-Mann-Whitney *U* test or by ANOVA.

RESULTS

Cxcr1 regulates anti-Pseudomonas host defense

Since we observed in previous studies that CXCR1 is involved in anti-bacterial host defense functions of human neutrophils *in vitro* and was associated with *Pseudomonas aeruginosa* infections in patients with CF lung disease *ex vivo* [12,20], we systematically investigated the role of Cxcr1 by employing *Pseudomonas aeruginosa in vivo* infection models. We started with quantifying the capacity of isolated *Cxcr1*$^{-/-}$ and *Cxcr1*$^{+/+}$ neutrophils to kill *Pseudomonas aeruginosa* bacteria *in vitro* by using traditional CFU assays (Figure 1A) as well as by bacterial live/dead imaging methods (Figure 1B). These studies demonstrated that *Cxcr1*$^{-/-}$ neutrophils were impaired in intracellular, but not extracellular killing of *Pseudomonas aeruginosa* bacteria compared to their wild-type counterparts (Figure 1A and Figure 1B). Next, we interrogated this bacterial clearance defect *in vivo* and used a well-established *Pseudomonas aeruginosa* lung infection model. These studies confirmed our *in vitro* findings and demonstrated that *Cxcr1*$^{-/-}$ mice were impaired in clearing *Pseudomonas 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 (Figure 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 (Figure 1D) [23]. These studies confirmed that *Cxcr1*$^{-/-}$ mice were impaired in clearing *Pseudomonas aeruginosa* from their pulmonary compartment compared to age- and gender-matched *Cxcr1*$^{+/+}$ mice (Figure 1D). By inoculating different increasing doses of *Pseudomonas aeruginosa* into the airways, we found that this pulmonary *Pseudomonas aeruginosa* 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 (Figure 1E), with significant differences for *Pseudomonas aeruginosa* CFU of 2×10^{6}, but without significant differences at lower CFUs.

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 (Figure S1A) or peripheral blood (Figure S1B) differed

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

significantly between age-matched *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ mice. Percentages of neutrophils in bone marrow (Figure 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 (Figure 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 (Figure S1C).

Cxcr1 modulates ROS production

Next, we sought to dissect the mechanisms by which Cxcr1 regulates pulmonary anti-*Pseudomonas aeruginosa* host defenses. Since production of reactive oxygen species (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 (Figure 2A). Since *Pseudomonas aeruginosa* is a flagellated bacterium and several previous studies have shown that flagellin represents a key pathogen-associated molecular pattern (PAMP), 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 (Figure 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 non-fractionated bone-marrow cells, produced lower amounts of ROS compared to their *Cxcr1*$^{+/+}$ counterparts upon stimulation with the chemokine CXCL8 (Figure 2B), whole *Pseudomonas aeruginosa* bacteria (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) (Figure 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 *Pseudomonas aeruginosa*, but not Cxcl2 stimulation (Figure 2C and Figure 2D). In contrast to specific stimulation with chemokines, *Pseudomonas aeruginosa* or bacterial flagellin, non-specific stimulation with the phorbol ester PMA did not elicit a differential ROS production in unfractionated bone marrow myeloid cells (Figure 2A) or isolated neutrophils from *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ mice (Figures 2B–D), suggesting that Cxcr1-mediated ROS production is chemokine- and *Pseudomonas aeruginosa*-dependent, rather than reflecting a redundant and universal basal ROS impairment. Collectively, these studies demonstrate that Cxcr1 mediates anti-*Pseudomonas aeruginosa* host defense in neutrophils through a mechanism involving ROS production.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Cxcr1 modulates TLR5 expression

Since generation of ROS is an universal anti-microbial host defense mechanism and not specifically related to *Pseudomonas aeruginosa* infections, we next investigated the role of Toll-like receptor 5 (TLR5) as key pattern recognition receptor in *Pseudomonas aeruginosa*-associated lung infections, such as CF lung disease [28–32]. These studies demonstrated that genetic abrogation of *Cxcr1* led to an upregulation of Tlr5 surface expression on airway dendritic cells upon acute *Pseudomonas aeruginosa* infection (Figure 3A), whereas no changes were found on airway neutrophils (Figure 3A) or bone marrow dendritic cells or neutrophils (Figure 3B). To assess the human disease relevance of these findings, we analyzed TLR5 expression on airway immune cells in patients with CF lung disease and *Pseudomonas aeruginosa* infection, characterized by a loss of CXCR1 [20]. These investigations demonstrated that both dendritic cells and neutrophils (Figure 3C) in the airways of CF patients with *Pseudomonas 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 *Pseudomonas aeruginosa* infection. No significant difference in TLR4 expression levels was noted between *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ neutrophils (P>0.05) or between CF patients with *Pseudomonas aeruginosa* infection and non-CF bronchitis patients without *Pseudomonas aeruginosa* infection (P>0.05, data not shown). When viewed in combination, these studies indicate 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 *Pseudomonas 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 (Figure 4A). To investigate whether this phenomenon is restricted to the pulmonary compartment or common to 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 (Figure 4B). To further assess whether Cxcr1 modulates non-infectious neutrophil transmigration differentially, we compared *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ neutrophil bronchoalveolar recruitment in two different models of allergic airway inflammation, namely ovalbumin- (Figure 4C) and house-dust mite-induced airway inflammation (Figure 4D). These studies consistently demonstrated that *Cxcr1*$^{-/-}$ neutrophils showed no impairment in migrating into the bronchoalveolar compartment upon non-infectious 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

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

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 that has been previously described to unexpectedly activate Cxcr1 [33] (Figure 4E). Taken together, these studies demonstrate that Cxcr1 is dispensable for neutrophil migration in infectious and non-infectious 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 *Pseudomonas aeruginosa* infections, which are a significant mortality factor in patients with CF or individuals with immunosuppression or 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 *Pseudomonas aeruginosa*, mediated through a ROS- and TLR5-mediated mechanism. Moreover, our studies demonstrate that neutrophil migration to the pulmonary site of inflammation and infection was 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 dissected, but our studies highlight the notion that chemokine receptors play a broader role in leukocyte biology than mediating cell migration by regulating anti-microbial effector and bacterial sensing mechanisms.

Previous studies in human patients with CF who suffer from chronic *Pseudomonas aeruginosa* infections showed that *CXCR1/2* polymorphisms modulate disease outcome of those 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-*Pseudomonas aeruginosa* host defense functions [20]. Our *in vivo* studies confirm and extend these findings by demonstrating that Cxcr1 plays a novel and non-canonical role in host-pathogen interactions by regulating neutrophil ROS effector response without affecting neutrophil recruitment, homeostasis or survival. Since ROS are essential for host defense against a variety of

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

bacterial pathogens beyond *Pseudomonas aeruginosa*, these findings may have broad implications for infectious diseases. Moreover, ROS have been implicated into pro-inflammatory harmful activities in chronic disease conditions, such as cardiovascular diseases 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 two 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 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 (Supplementary Figure 2A), we found moderately increased percentages of Cxcr2^{+} neutrophils in the bone-marrow of *Cxcr1*$^{-/-}$ compared to *Cxcr1*$^{+/+}$ mice (Supplementary Figure 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 present study.

Beyond the involvement of ROS and Tlr5, the precise cellular mechanism(s) by which Cxcr1 is involved in anti-*Pseudomonas 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 (NET) formation in our short-term *in vitro* assay systems, we speculate that the Cxcr1-mediated anti-bacterial mechanism in our experimental system mainly involves ROS-dependent intracellular phagocytic killing. Since our studies were, however, restricted to *Pseudomonas aeruginosa*, we cannot exclude the possibility that Cxcr1 differentially regulates anti-microbial effector mechanisms depending on the type of pathogen.

We found that *Cxcr1*$^{-/-}$ neutrophils were impaired in generating ROS in response to *Pseudomonas aeruginosa* or bacterial flagellin, whereas no difference was observed in response to non-bacterial generic ROS activation by stimulation with protein kinase C using PMA. These findings inspired us to investigate the interaction of CXCR1 and flagellin sensing pathways in mice and CF patients with *Pseudomonas 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-*Pseudomonas aeruginosa* interactions CXCR1 collaborates with TLR5 to efficiently combat *Pseudomonas aeruginosa.*

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

When CXCR1 is abrogated, this bacterial sensing (TLR5) and killing (ROS) mechanism is impaired, leading to uncontrolled *Pseudomonas aeruginosa* infections. Previous studies provided evidence for a TLR-chemokine receptor crosstalk 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-*Pseudomonas aeruginosa* host defense remains to be dissected in future studies.

A clear limitation of our study, regarding the human disease translation, is the *Pseudomonas aeruginosa* infection model that we used, which reflects acute pneumonia rather than chronic infective disease as found in CF or COPD airways. However, our primary aim was to define the role of Cxcr1 in anti-*Pseudomonas aeruginosa* host defense as 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: (i) what is the underlying reason for the difference in bone marrow neutrophils between *Cxcr1*$^{-/-}$ and *Cxcr1*$^{+/+}$ mice? (ii) 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? (iii) what is the underlying mechanism behind the upregulation of Tlr5 on *Cxcr1*$^{-/-}$ dendritic cells? Are TLR5-ROS interactions involved [45]? (iv) what are the intracellular Cxcr1-downstream pathways regulating neutrophil effector responses?

In summary, our studies demonstrate that CXCR1 regulates anti-*Pseudomonas aeruginosa* host defense through a ROS- and TLR5-mediated mechanism. Beyond CF lung disease, these findings may have broader implications for other *Pseudomonas 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 anti-microbial roles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Antje Munder, MHH Hannover Germany, and Burkhard Tümmler, MHH Hannover Germany, for luciferase-expressing *Pseudomonas aeruginosa bacteria.* This work was supported by the German Research Foundation (DFG, Emmy Noether Programme HA 5274/3-1 to DH), the Fritz-Thyssen-Foundation (AH and DH) the CRC685 at Tübingen, and the Novartis Foundation (DH).

ABBREVIATIONS

BAL Bronchoalveolar lavage fluid

CF Cystic fibrosis

CFTR Cystic fibrosis transmembrane conductance regulator

COPD Chronic obstructive pulmonary disease

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

CXCR1	CXC chemokine receptor 1
ELISA	Enzyme-linked immunosorbent assay
PAMP	Pathogen-associated molecular patterns
ROS	Reactive oxygen species
TLR5	Toll-like receptor 5

References

1. Griffith JW, Sokol CL, Luster AD. Chemokines and chemokine receptors: Positioning cells for host defense and immunity. Annu Rev Immunol. 2014; 32:659–702. [PubMed: 24655300]
2. Moser B, Loetscher P. Lymphocyte traffic control by chemokines. Nat Immunol. 2001; 2:123–128. [PubMed: 11175804]
3. Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A. Neutrophil function: From mechanisms to disease. Annu Rev Immunol. 2012; 30:459–489. [PubMed: 22224774]
4. Nauseef WM, Borregaard N. Neutrophils at work. Nat Immunol. 2014; 15:602–611. [PubMed: 24940954]
5. Kruger P, Saffarzadeh M, Weber AN, Rieber N, Radsak M, von Bernuth H, Benarafa C, Roos D, Skokowa J, Hartl D. Neutrophils: Between host defence, immune modulation, and tissue injury. Plos Pathog. 2015; 11:e1004651. [PubMed: 25764063]
6. Reutershan J. Cxcr2--the receptor to hit? Drug News Perspect. 2006; 19:615–623. [PubMed: 17299604]
7. Russo RC, Garcia CC, Teixeira MM, Amaral FA. The cxcl8/il-8 chemokine family and its receptors in inflammatory diseases. Expert review of clinical immunology. 2014
8. Park SH, Das BB, Casagrande F, Tian Y, Nothnagel HJ, Chu M, Kiefer H, Maier K, De Angelis AA, Marassi FM, Opella SJ. Structure of the chemokine receptor cxcr1 in phospholipid bilayers. Nature. 2012; 491:779–783. [PubMed: 23086146]
9. Citro A, Cantarelli E, Maffi P, Nano R, Melzi R, Mercalli A, Dugnani E, Sordi V, Magistretti P, Daffonchio L, Ruffini PA, Allegretti M, Secchi A, Bonifacio E, Piemonti L. Cxcr1/2 inhibition enhances pancreatic islet survival after transplantation. J Clin Invest. 2012; 122:3647–3651. [PubMed: 22996693]
10. Giagulli C, Magiera AK, Bugatti A, Caccuri F, Marsico S, Rusnati M, Vermi W, Fiorentini S, Caruso A. Hiv-1 matrix protein p17 binds to the il-8 receptor cxcr1 and shows il-8-like chemokine activity on monocytes through rho/rock activation. Blood. 2012; 119:2274–2283. [PubMed: 22262769]
11. Vasilescu A, Terashima Y, Enomoto M, Heath S, Poonpiriya V, Gatanaga H, Do H, Diop G, Hirtzig T, Auewarakul P, Lauhakirti D, Sura T, Charneau P, Marullo S, Therwath A, Oka S, Kanegasaki S, Lathrop M, Matsushima K, Zagury JF, Matsuda F. A haplotype of the human cxcr1 gene protective against rapid disease progression in hiv-1+ patients. Proc Natl Acad Sci U S A. 2007; 104:3354–3359. [PubMed: 17360650]
12. Kormann MS, Hector A, Marcos V, Mays LE, Kappler M, Illig T, Klopp N, Zeilinger S, Carevic M, Rieber N, Eickmeier O, Zielen S, Gaggar A, Moepps B, Griese M, Hartl D. Cxcr1 and cxcr2 haplotypes synergistically modulate cystic fibrosis lung disease. Eur Respir J. 2012; 39:1385–1390. [PubMed: 22088968]
13. Lyczak JB, Cannon CL, Pier GB. Establishment of pseudomonas aeruginosa infection: Lessons from a versatile opportunist. Microbes Infect. 2000; 2:1051–1060. [PubMed: 10967285]
14. Lavoie EG, Wangdi T, Kazmierczak BI. Innate immune responses to pseudomonas aeruginosa infection. Microbes Infect. 2011; 13:1133–1145. [PubMed: 21839853]
15. Gomez MI, Prince A. Opportunistic infections in lung disease: Pseudomonas infections in cystic fibrosis. Curr Opin Pharmacol. 2007; 7:244–251. [PubMed: 17418640]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

16. Hoiby N. Recent advances in the treatment of pseudomonas aeruginosa infections in cystic fibrosis. BMC medicine. 2011; 9:32. [PubMed: 21463524]

17. Brugha RE, Davies JC. Pseudomonas aeruginosa in cystic fibrosis: Pathogenesis and new treatments. Br J Hosp Med (Lond). 2011; 72:614–619. [PubMed: 22083001]

18. Kragh KN, Alhede M, Jensen PO, Moser C, Scheike T, Jacobsen CS, Seier Poulsen S, Eickhardt-Sorensen SR, Trostrup H, Christoffersen L, Hougen HP, Rickelt LF, Kuhl M, Hoiby N, Bjarnsholt T. Polymorphonuclear leukocytes restrict growth of pseudomonas aeruginosa in the lungs of cystic fibrosis patients. Infect Immun. 2014; 82:4477–4486. [PubMed: 25114118]

19. De Simone M, Spagnuolo L, Lore NI, Rossi G, Cigana C, De Fino I, Iraqi FA, Bragonzi A. Host genetic background influences the response to the opportunistic pseudomonas aeruginosa infection altering cell-mediated immunity and bacterial replication. Plos One. 2014; 9:e106873. [PubMed: 25268734]

20. Hartl D, Latzin P, Hordijk P, Marcos V, Rudolph C, Woischnik M, Krauss-Etschmann S, Koller B, Reinhardt D, Roscher AA, Roos D, Griese M. Cleavage of cxcr1 on neutrophils disables bacterial killing in cystic fibrosis lung disease. Nat Med. 2007; 13:1423–1430. [PubMed: 18059279]

21. Hasenberg M, Kohler A, Bonifatius S, Borucki K, Riek-Burchardt M, Achilles J, Mann L, Baumgart K, Schraven B, Gunzer M. Rapid immunomagnetic negative enrichment of neutrophil granulocytes from murine bone marrow for functional studies in vitro and in vivo. Plos One. 2011; 6:e17314. [PubMed: 21383835]

22. Hector A, Schafer H, Poschel S, Fischer A, Fritzsching B, Ralhan A, Carevic M, Oz H, Zundel S, Hogardt M, Bakele M, Rieber N, Riethmueller J, Graepler-Mainka U, Stahl M, Bender A, Frick JS, Mall M, Hartl D. Regulatory t cell impairment in cystic fibrosis patients with chronic pseudomonas infection. Am J Respir Crit Care Med. 2015

23. Munder A, Wolbeling F, Klockgether J, Wiehlmann L, Tummler B. In vivo imaging of bioluminescent pseudomonas aeruginosa in an acute murine airway infection model. Pathog Dis. 2014; 72:74–77. [PubMed: 24833236]

24. Bylund J, Bjornsdottir H, Sundqvist M, Karlsson A, Dahlgren C. Measurement of respiratory burst products, released or retained, during activation of professional phagocytes. Methods Mol Biol. 2014; 1124:321–338. [PubMed: 24504962]

25. Bloes DA, Otto M, Peschel A, Kretschmer D. Enterococcus faecium stimulates human neutrophils via the formyl-peptide receptor 2. Plos One. 2012; 7:e39910. [PubMed: 22768166]

26. Kretschmer D, Gleske AK, Rautenberg M, Wang R, Koberle M, Bohn E, Schoneberg T, Rabiet MJ, Boulay F, Klebanoff SJ, van Kessel KA, van Strijp JA, Otto M, Peschel A. Human formyl peptide receptor 2 senses highly pathogenic staphylococcus aureus. Cell Host Microbe. 2010; 7:463–473. [PubMed: 20542250]

27. Mays LE, Ammon-Treiber S, Mothes B, Alkhaled M, Rottenberger J, Muller-Hermelink ES, Grimm M, Mezger M, Beer-Hammer S, von Stebut E, Rieber N, Nurnberg B, Schwab M, Handgretinger R, Idzko M, Hartl D, Kormann MS. Modified foxp3 mrna protects against asthma through an il-10-dependent mechanism. J Clin Invest. 2013; 123:1216–1228. [PubMed: 23391720]

28. Descamps D, Le Gars M, Balloy V, Barbier D, Maschalidi S, Tohme M, Chignard M, Ramphal R, Manoury B, Sallenave JM. Toll-like receptor 5 (tlr5), il-1 beta secretion, and asparagine endopeptidase are critical factors for alveolar macrophage phagocytosis and bacterial killing. P Natl Acad Sci USA. 2012; 109:1619–1624.

29. Morris AE, Liggitt HD, Hawn TR, Skerrett SJ. Role of toll-like receptor 5 in the innate immune response to acute p*Aeruginosa pneumonia*. Am J Physiol Lung Cell Mol Physiol. 2009; 297:L1112–1119. [PubMed: 19801452]

30. Koller B, Kappler M, Latzin P, Gaggar A, Schreiner M, Takyar S, Kormann M, Kabesch M, Roos D, Griese M, Hartl D. Tlr expression on neutrophils at the pulmonary site of infection: Tlr1/tlr2-mediated up-regulation of tlr5 expression in cystic fibrosis lung disease. J Immunol. 2008; 181:2753–2763. [PubMed: 18684966]

31. Blohmke CJ, Victor RE, Hirschfeld AF, Elias IM, Hancock DG, Lane CR, Wilcox PG, Smith KD, Overhage J, Hancock REW, Turvey SE. Innate immunity mediated by tlr5 as a novel antiinflammatory target for cystic fibrosis lung disease. J Immunol. 2008; 180:7764–7773. [PubMed: 18490781]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

32. Zhang Z, Louboutin JP, Weiner DJ, Goldberg JB, Wilson JM. Human airway epithelial cells sense pseudomonas aeruginosa infection via recognition of flagellin by toll-like receptor 5. Infect Immun. 2005; 73:7151–7160. [PubMed: 16239509]
33. Fan X, Patera AC, Pong-Kennedy A, Deno G, Gonsiorek W, Manfra DJ, Vassileva G, Zeng M, Jackson C, Sullivan L, Sharif-Rodriguez W, Opdenakker G, Van Damme J, Hedrick JA, Lundell D, Lira SA, Hipkin RW. Murine cxcr1 is a functional receptor for gcp-2/cxcl6 and interleukin-8/cxcl8. J Biol Chem. 2007; 282:11658–11666. [PubMed: 17197447]
34. Fricker SP, Metz M. Chemokine receptor modeling: An interdisciplinary approach to drug design. Future Med Chem. 2014; 6:91–114. [PubMed: 24358950]
35. Roy I, Evans DB, Dwinell MB. Chemokines and chemokine receptors: Update on utility and challenges for the clinician. Surgery. 2014; 155:961–973. [PubMed: 24856117]
36. Konrad FM, Reutershan J. Cxcr2 in acute lung injury. Mediators Inflamm. 2012; 2012:740987. [PubMed: 22719179]
37. Bakele M, Lotz-Havla AS, Jakowetz A, Carevic M, Marco V, Munta AC, Gerstin SW, Hartl D. An interactive network of elastase, secretases, and par-2 protein regulates cxcr1 receptor surface expression on neutrophils. J Biol Chem. 2014; 289:20516–20525. [PubMed: 24914212]
38. Jones SA, Wolf M, Qin S, Mackay CR, Baggiolini M. Different functions for the interleukin 8 receptors (il-8r) of human neutrophil leukocytes: Nadph oxidase and phospholipase d are activated through il-8r1 but not il-8r2. Proc Natl Acad Sci U S A. 1996; 93:6682–6686. [PubMed: 8692878]
39. Lambeth JD. Nox enzymes, ros, and chronic disease: An example of antagonistic pleiotropy. Free Radic Biol Med. 2007; 43:332–347. [PubMed: 17602948]
40. Langen RC, Korn SH, Wouters EF. Ros in the local and systemic pathogenesis of copd. Free Radic Biol Med. 2003; 35:226–235. [PubMed: 12885585]
41. Martinez Munoz L, Lucas P, Navarro G, Checa AI, Franco R, Martinez AC, Rodriguez-Frade JM, Mellado M. Dynamic regulation of cxcr1 and cxcr2 homo- and heterodimers. J Immunol. 2009; 183:7337–7346. [PubMed: 19890050]
42. Hajishengallis G, Wang M, Liang S, Triantafilou M, Triantafilou K. Pathogen induction of cxcr4/tlr2 cross-talk impairs host defense function. Proc Natl Acad Sci U S A. 2008; 105:13532–13537. [PubMed: 18765807]
43. Linge HM, Collin M, Nordenfelt P, Morgelin M, Malmsten M, Egesten A. The human cxc chemokine granulocyte chemotactic protein 2 (gcp-2)/cxcl6 possesses membrane-disrupting properties and is antibacterial. Antimicrob Agents Chemother. 2008; 52:2599–2607. [PubMed: 18443119]
44. Jovic S, Linge HM, Shikhagaie MM, Olin AI, Lannefors L, Erjefalt JS, Morgelin M, Egesten A. The neutrophil-recruiting chemokine gcp-2/cxcl6 is expressed in cystic fibrosis airways and retains its functional properties after binding to extracellular DNA. Mucosal immunology. 2015
45. Joo JH, Ryu JH, Kim CH, Kim HJ, Suh MS, Kim JO, Chung SY, Lee SN, Kim HM, Bae YS, Yoon JH. Dual oxidase 2 is essential for the toll-like receptor 5-mediated inflammatory response in airway mucosa. Antioxidants & redox signaling. 2012; 16:57–70. [PubMed: 21714724]
46. Owen C. Chemokine receptors in airway disease: Which receptors to target? Pulm PharmacolTher. 2001; 14:193–202.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

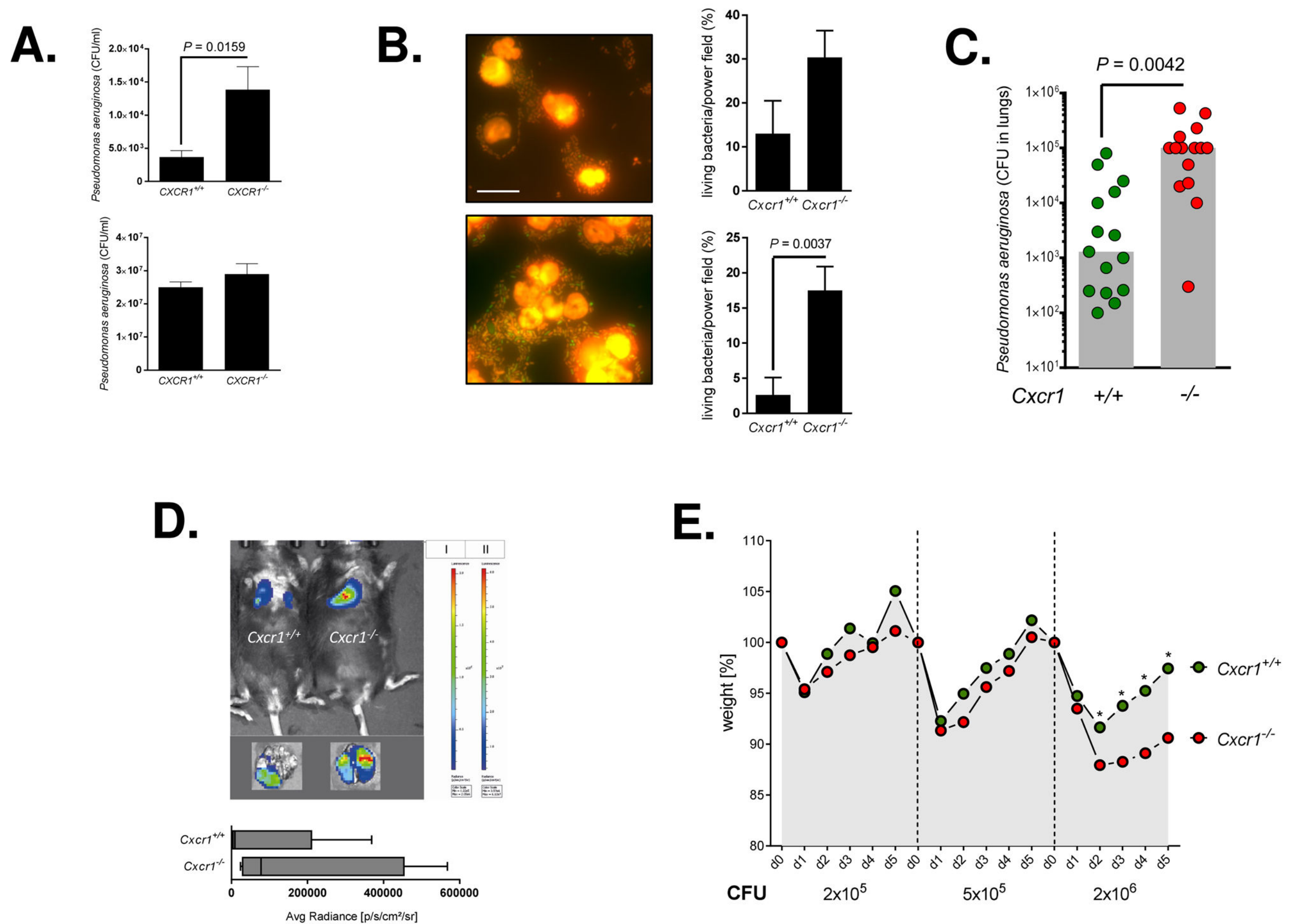

Fig. 1. ***Pseudomonas aeruginosa*** **infections**

(A) Upper panel: intracellular killing / Lower panel extracellular killing: Bone-marrow-isolated neutrophils from age- and sex-matched *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ mice were infected with *Pseudomonas aeruginosa* (PAO1) for 60 minutes at MOI50 and CFUs were counted. Data from n=5 independent experiments (means, SEMs) are shown.

(B) GFP-*Pseudomonas aeruginosa* (PAO1) bacteria and bone-marrow-isolated neutrophils from age- and sex-matched *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ mice were co-incubated for 7 or 60 minutes at MOI50. Bacteria were stained using a live/dead bacterial staining kit (left panel) and quantified microscopically (right panel). Representative images of *Cxcr1*$^{+/+}$ (upper left panel) and *Cxcr1*$^{-/-}$ (lower left panel) neutrophils 60 minutes after GFP-*Pseudomonas aeruginosa* (PAO1) infection (MOI50) are shown. Living bacteria are shown in green, dead bacteria in red. The scale/size bar represents 10µm. Bars show percentages of living bacteria at 7 minutes (upper right panel) and 60 minutes (lower right panel) at MOI50. Data from n=5 independent experiments (means, SEMs) are shown.

(C–E) Age- and sex-matched *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ mice were infected intranasally (**C, E**) or intratracheally (**D**) with *Pseudomonas aeruginosa* (PAO1 all except for **D**, luciferase-expressing TBCF10839 isogenic mutant D8A6) and 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 – 2×10^6 CFU PAO1 bacteria **(E)** were inoculated. **(C)** bars represent

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

medians; CFUs were quantified in lungs 12 hours after the infection; **(D)** Bioluminescence imaging was performed 24 hours after the infection for n=5 (*Cxcr1*$^{+/+}$) or n=4 (*Cxcr1*$^{-/-}$) independent experiments; box and whiskers (range: min/max, quartiles, medians) are shown; (**E**) mean weight points are shown. * $P < 0.05$ *Cxcr1*$^{+/+}$ versus *Cxcr1*$^{-/-}$ mice.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

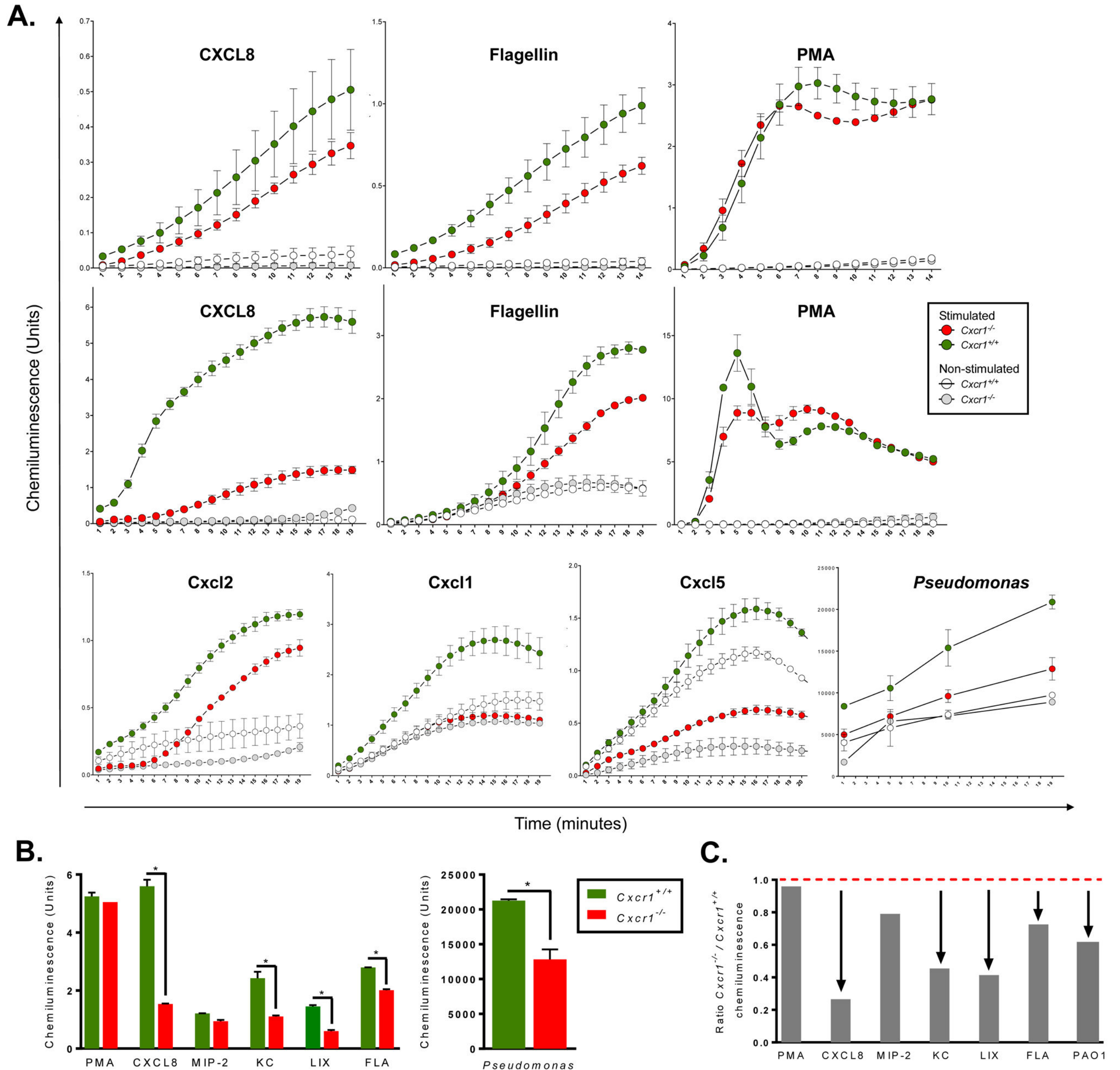

Fig. 2. Reactive oxygen species

Total bone marrow cells (**A**, upper row) or neutrophils isolated from bone marrow (**A**, middle and lower row) 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 *Pseudomonas aeruginosa* (PAO1) bacteria (2×10^6/ml). Reactive oxygen species (ROS) production was measured using chemiluminescence. Figure 2B and **C** show summarizing bar graph plots (medians with IQRs in **2B**, ratios in **2C**) of stimulated *Cxcr1*$^{+/+}$ versus *Cxcr1*$^{-/-}$ neutrophils, as absolute chemiluminescence units (**B**) or as ratio of *Cxcr1*$^{-/-}$ to *Cxcr1*$^{+/+}$ neutrophils (**C**). * (**B**) or arrows (**C**): $P < 0.05$ *Cxcr1*$^{+/+}$ versus *Cxcr1*$^{-/-}$ neutrophils

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

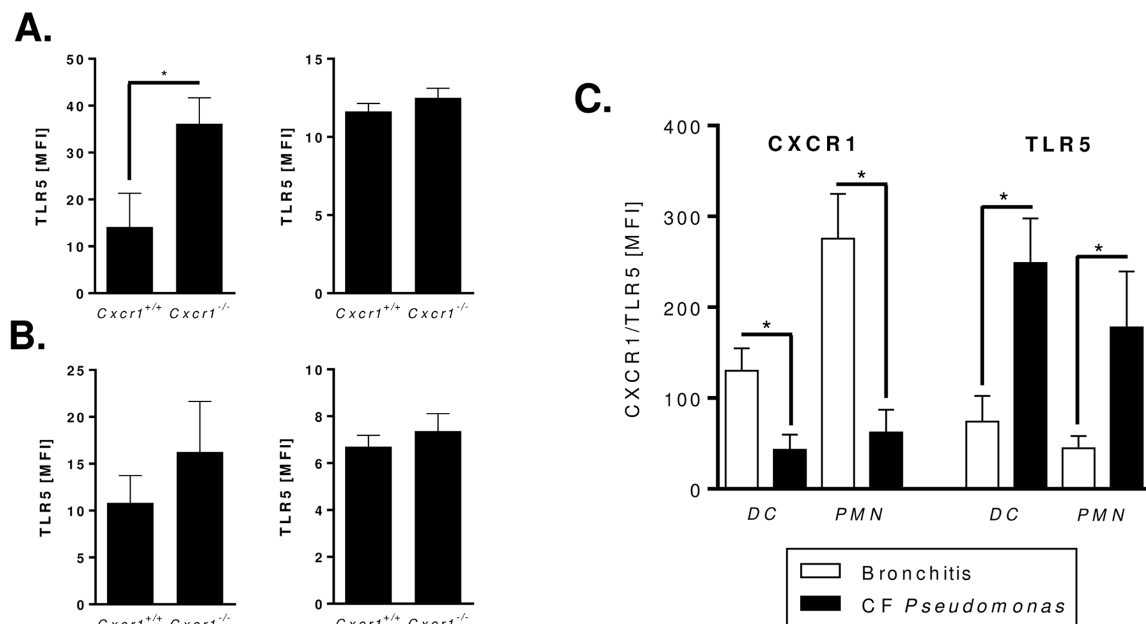

Fig. 3. Toll-like receptor 5

Toll-like receptor 5 (TLR5) surface expression (MFI) was quantified on airway/BAL **(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/BAL dendritic cells (DCs) or neutrophils (PMN) from patients with bronchitis or CF and *Pseudomonas aeruginosa* infections. * $P < 0.05$ bronchitis versus CF-*Pseudomonas*.

Author Manuscript

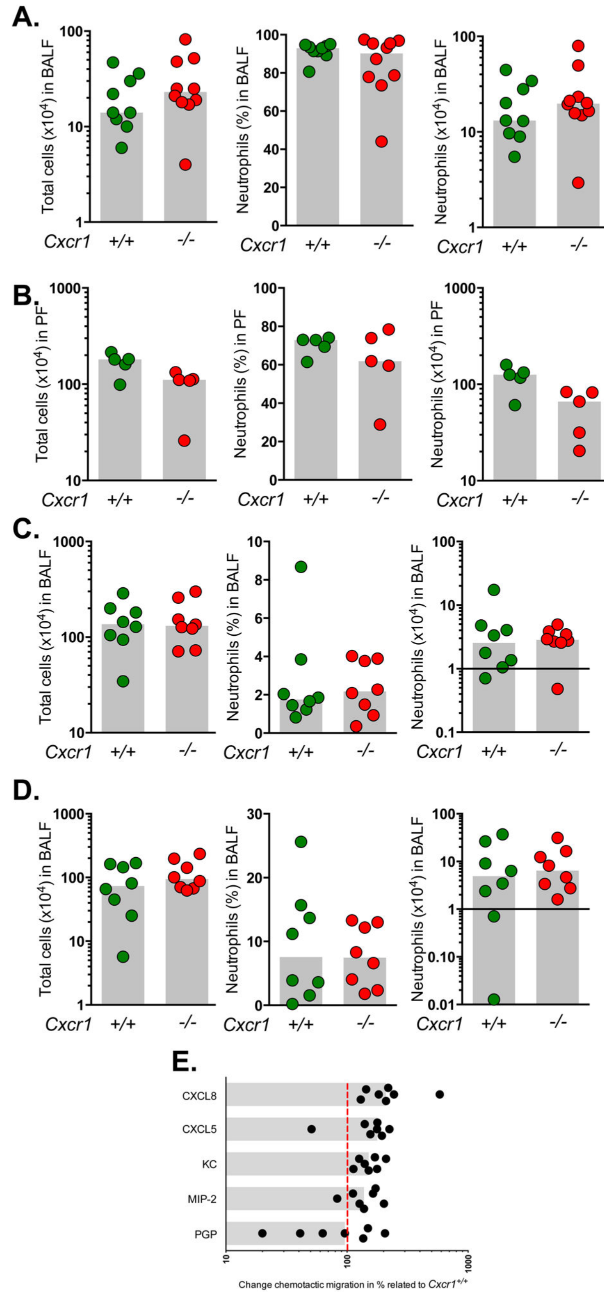

Fig. 4. Neutrophil recruitment

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 *Pseudomonas aeruginosa* (PAO1) lung infection **(A)**, Thioglycollate-induced peritonitis **(B)**, Ovalbumin-induced allergic airway inflammation **(C)** and house-dust mite-induced allergic airway inflammation **(D). (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, PGP 1μM) and

Author Manuscript

underwent a Transwell-based chemotactic migration assay for 120 minutes. Shown is the relative migration of *Cxcr1*$^{-/-}$ compared to *Cxcr1*$^{+/+}$ neutrophils (=100%, red line). All bars represent medians.