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], D. Hartl[1]

Online Data Supplement

Bacteria

Pseudomonas aeruginosa wild type strains (PAO1) were used as published previously by our group [1]. 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 luciferase-expressing *Pseudomonas aeruginosa* strain was used for bioimaging studies as published previously [2].

Neutrophil-Pseudomonas interactions

Bone marrow-isolated *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ neutrophils ($5x10^5$) were co-incubated with opsonized Pseudomonas aeruginosa PAO1 bacteria at MOI50 for 60 minutes (RPMI1640, 10% FCS) with shaking at 50 rpm. For CFU studies, cells were treated with gentamicin (200 µg/mL) to ensure that extracellular and surface-associated bacteria were killed. The contents of each well were centrifuged (500× g, 10 min), then washed twice with Hank's Balanced Salt Solution (HBSS) (Gibco). Cells were 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.

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, 5x10^{7} CFU/ mouse) as published previously [2]. 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).

Asthma mouse models

OVA and HDM murine asthma models were performed as published previously by our group [3]. Intratracheal (i.t.) and intranasal (i.n.) procedures were carried out under antagonisable anesthesia. Briefly, mice were sensitized with 50µg OVA s.c. (Sigma-Aldrich) and challenged with 1% aerosolized OVA for 30 min using an ultrasonic nebulizer (PariBoy-SX). HDM extracts (100µg) were delivered i.t.

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 [4]. 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.

BALF

Lung lavage was extracted through the trachea with 2 ml PBS. Total BAL cells were counted using trypan blue dye exclusion. After erythrocyte lysis with ACK lysis solution, cells were incubated with an Fc receptor block (1 µg/1 × 10^6 cells; BD Bioscience) to reduce nonspecific anti-body binding.

CFUs

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

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* infections (n=5, mean age: 20 years) as described previously in detail [1,5]. CXCR1 and TLR5 surface expression levels (MFI) were quantified on airway/BAL dendritic cells or neutrophils 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 5.0 software. Statistical significance ($P \leq 0.05$) was determined using the Wilcoxon-Mann-Whitney *U* test or by ANOVA.

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